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Bax-mediated cell death by the Gax homeoprotein requires mitogen activation but is independent of cell cycle activity

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Tissues with the highest rates of proliferation typically exhibit the highest frequencies of apoptosis, but the mechanisms that coordinate these processes are largely unknown. The homeodomain protein Gax is down-regulated when quiescent cells are stimulated to proliferate, and constitutive Gax expression inhibits cell proliferation in a $p21^{WAF/CIP}$-dependent manner. To understand how mitogen-induced proliferation influences the apoptotic process, we investigated the effects of deregulated Gax expression on cell viability. Forced Gax expression induced apoptosis in mitogen-activated cultures, but quiescent cultures were resistant to cell death. Though mitogen activation was required for apoptosis, neither the cdk inhibitor $p21^{WAF/CIP}$ nor the tumor suppressor p53 was required for Gax-induced cell death. Arrest in G_1 or S phases of the cell cycle with chemical inhibitors also did not affect apoptosis, further suggesting that Gax-mediated cell death is independent of cell cycle activity. Forced Gax expression led to Bcl-2 down-regulation and Bax up-regulation in mitogen-activated, but not quiescent cultures. Mouse embryonic fibroblasts homozygous null for the Bax gene were refractive to Gax-induced apoptosis, demonstrating the functional significance of this regulation. These data suggest that the homeostatic balance between cell growth and death can be controlled by mitogen-dependent pathways that circumvent the cell cycle to alter Bcl-2 family protein expression.

Keywords: apoptosis/Bax/cell cycle/homeobox gene/mitogen-regulated transcription factor

Introduction

Homeostasis is achieved through a balance of cell growth and cell death. Many recent studies have shown that the proper regulation of cell cycle activity is crucial for cell viability. Deregulated expression of immediate early genes (Evan *et al*., 1992), cell cycle regulators (Hoang *et al*., 1994; Shi *et al*., 1994) or cell cycle-dependent transcription factors (Qin *et al*., 1994; Kowalik *et al*., 1995; Liu and Kitsis, 1996) can initiate an abortive cell cycle followed by apoptosis. In addition, the withdrawal of growth factors *in vitro* (Batistatou and Greene, 1993; Wang and Walsh, 1996) or *in vivo* (Colombel *et al*., 1992) from differentiated cells also induces abortive cell cycle progression and apoptosis. These data suggest that cell cycle activity can markedly influence the susceptibility of cells to apoptosis and that aberrant cell cycle progression can induce apoptosis. However, the mechanisms by which cell proliferation and cell death are coordinately regulated are largely unknown. The Bcl-2 family of proteins are apoptotic regulators that function as molecular rheostats to control cellular survival (Yang and Korsmeyer, 1996). Expression of the apoptotic antagonists, Bcl-2 or Bcl-x_L, protects cells against various apoptotic stimuli (Hockenbery *et al*., 1990; Hockenbery, 1995), while overexpression of apoptotic accelerators, such as Bax, Bad or Bcl-x_S, induces apoptosis (Oltvai *et al*., 1993; Yang and Korsmeyer, 1996). However, the effects of cell proliferation on Bcl-2 family protein expression remains to be elucidated.

The smooth muscle cells that comprise the vessel wall can undergo high rates of proliferation (Clowes *et al*., 1983; Kearney *et al*., 1997; Wei *et al*., 1997) and apoptosis (Bochaton-Piallat *et al*., 1995; Han *et al*., 1995; Isner *et al*., 1995; Perlman *et al*., 1997) in a number of vascular disorders. Mitogen-regulated transcription factors function as regulators of proliferation of vascular smooth muscle cells (VSMCs) in response to vessel injury (Walsh and Perlman, 1996). One such transcription factor, the homeoprotein Gax (growth arrest-specific homeobox), was isolated from adult rat aorta cDNA (Gorski *et al*., 1994). Gax is expressed in quiescent VSMCs and is down-regulated rapidly by conditions which promote proliferation both *in vitro* and *in vivo* (Gorski *et al.*, 1993; Weir *et al.*, 1995; Yamashita *et al.*, 1997).

The regulation of *gax* by mitogens resembles the *gas* (growth arrest-specific) and *gadd* (growth arrest DNA-damaging) family of genes (Hoffman and Liebermann, 1994). *gas* and *gadd* genes are down-regulated rapidly by mitogens and up-regulated by conditions that promote growth arrest. Presumably the down-regulation of *gas* and *gadd* genes is required for proper cell cycle progression in response to mitogen activation. Indeed, the constitutive expression of *gas1*, *gas3*, *gadd45* or *gadd153* inhibits S-phase entry (Del Sal *et al*., 1992; Zhan *et al*., 1994; Zoidl *et al*., 1995). Analyses of Gax action *in vitro* have revealed that its overexpression inhibits proliferation in the G_1 phase of the cell cycle (Smith *et al*., 1997). This growth inhibition is associated with the p53-independent up-regulation of the cyclin-dependent kinase inhibitor $p21^{WAF/CIP}$. Fibroblasts homozygous null for the *p21*$^{WAF/CIP}$

gene are not susceptible to Gax-mediated growth inhibition, demonstrating that $p21^{WAF/CIP}$ is essential for Gax-induced growth arrest (Smith *et al*., 1997). Furthermore, *gax* overexpression inhibits the formation of injury-induced vascular lesions that are comprised of proliferating VSMCs (Maillard *et al*., 1997; Smith *et al*., 1997).

Based on previous investigations demonstrating interrelationships between cell proliferation and apoptosis, we examined the effects of *gax* expression on cell viability. *gax*-transduced cultures were viable under conditions of quiescence, but underwent apoptosis between 24 and 48 h following serum stimulation. In contrast to Gax-induced cell cycle arrest, Gax-induced apoptosis was independent of the cdk inhibitor $p21^{WAF/CIP}$, demonstrating that its effects on cell cycle and apoptosis are mediated by separate pathways. Gax-induced cell death was associated with mitogen-dependent up-regulation of Bax, and fibroblasts homozygous null for *bax* were refractory to Gax-induced cell death. These data suggest that mitogen-regulated transcription factors can coordinate cell proliferation and death through their ability to modulate Bcl-2 family protein expression independent of cell cycle activity.

Results

Gax expression reduces viability of mitogen-activated VSMCs

We previously demonstrated that Gax arrests VSMCs in the G_1 phase of the cell cycle following the stimulation of quiescent cells with serum (Smith *et al*., 1997). However, the effect of Gax expression on cell viability was not addressed previously. Quiescent VSMC cultures were infected with a replication-deficient adenovirus expressing the hemagglutinin-tagged Gax cDNA (Ad-Gax) (Smith *et al*., 1997) and incubated with serum. At 48 h post-serum stimulation, the Ad-Gax-infected cells displayed a morphology that differed markedly from that of control cells. Light microscopic analyses revealed cytoplasmic shrinkage and detachment from the plate surface in the Ad-Gax-infected cultures (Figure 1A) that overexpress the epitope-tagged Gax protein (Figure 1B). Cultures treated with a replication-deficient adenovirus containing the β-galactosidase cDNA (Ad-β-gal) remained viable and attached to the plate surface under these conditions. A cell viability assay using trypan blue exclusion (Raffo *et al*., 1995) demonstrated a marked decrease in cell viability at 48 h post-serum stimulation in the Ad-Gax-infected VSMCs compared with mock- or Ad-β-gal-transduced cells (Figure 1C). In contrast, quiescent cells transduced with Ad-Gax remained viable over this time course.

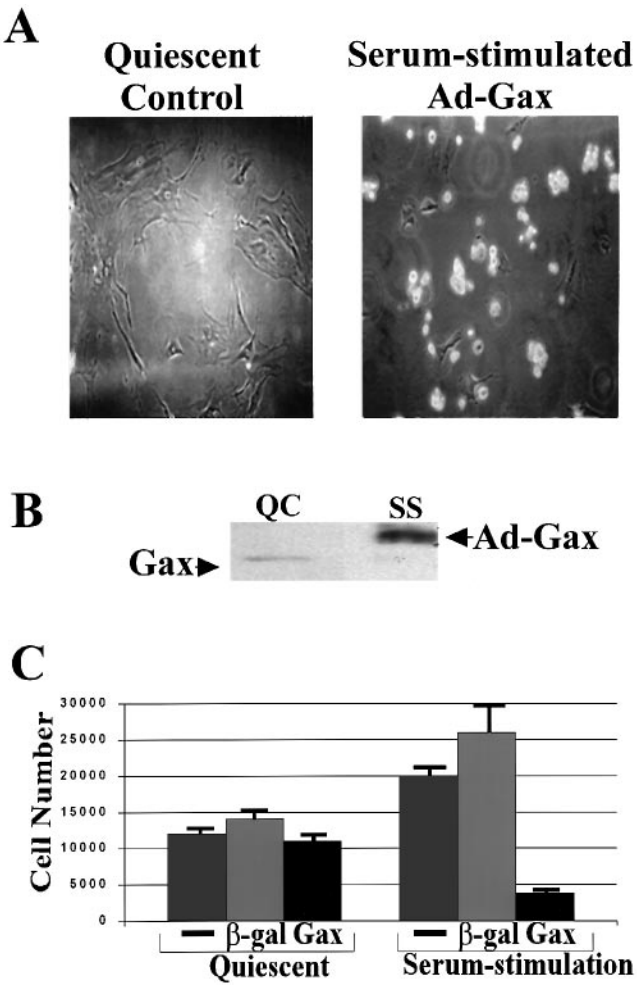

Fig. 1. Adenovirus-mediated Gax expression reduces viability of serum-stimulated but not quiescent cells. Primary cultures of rat VSMCs were made quiescent for 72 h in low mitogen medium (0.5% FBS). Cells were mock-transduced or transduced at an m.o.i. of 750 p.f.u./cell with either Ad-β-gal or Ad-Gax for 12 h, after which the virus was removed and cultures were returned to low mitogen medium for an additional 12 h. High serum medium was then added for 48 h to stimulate proliferation. For analysis of quiescent cells, parallel cultures were retained in low mitogen media and harvested 72 h after infection. (**A**) Representative phase-contrast micrographs of quiescent control cells and Ad-Gax-transduced cells that were stimulated with mitogens for 48 h. (**B**) Immunoblot analysis of control quiescent (CQ) cells, that express endogenous Gax, and serum-stimulated (SS) cells transduced with Ad-Gax. VSMC whole-cell extract (50 μg) was subjected to SDS–PAGE, and immunoblot analysis was performed with antibodies directed against the Gax protein. The adenovirus-encoded Gax protein is tagged with the hemagglutinin epitope leading to an increase in molecular weight. (**C**) Quantitative analysis of VSMC viability under mitogen deprivation and mitogen activation (48 h). Cells treated as described above were harvested by trypsinization, fixed and counted with a hemacytometer using the trypan blue exclusion method. Each time point represents the mean ± standard error of three determinations from a representative experiment.

Gax induces the apoptotic phenotype in mitogen-activated cells

Apoptosis is characterized by membrane blebbing, cellular and cytoplasmic shrinkage, chromosomal condensation, and activation of endonuclease-dependent chromosomal fragmentation (Yang and Korsmeyer, 1996). To determine whether the reduction in cell number in Ad-Gax-transduced VSMC cultures can be attributed to apoptosis, transmission electron microscopic analysis was performed to identify morphological features that are characteristic of apoptosis. The Ad-Gax-infected VSMCs displayed cytoplasmic blebbing and chromatin condensation while retaining membrane-bound organelles (Figure 2A). These features were not detected in control cells.

DNA fragmentation was analyzed in individual cells using the TdT-mediated dUTP-fluorescein nick-end labeling (TUNEL) analysis. Ad-Gax-treated VSMCs displayed a high frequency of TUNEL-positive labeling, while Ad-β-gal-treated cultures displayed little or no

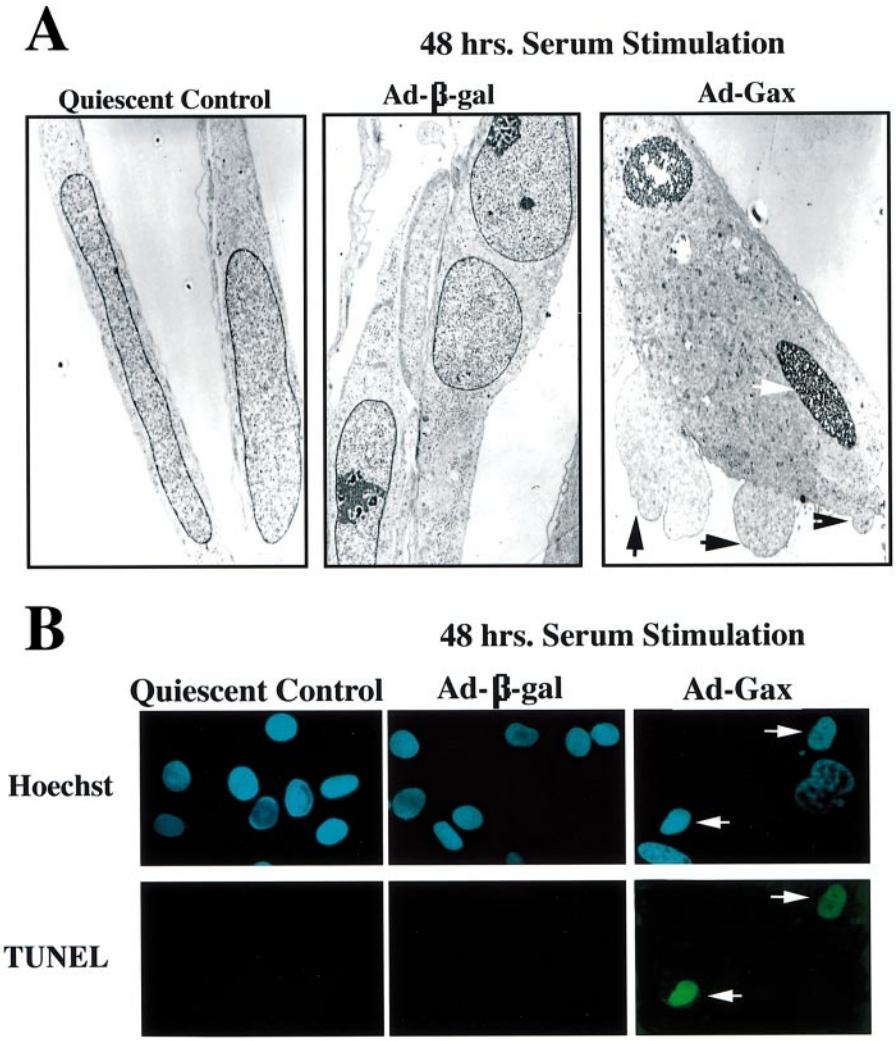

Fig. 2. Ad-Gax-infected cells exhibit apoptotic features following mitogen activation. (**A**) Transmission electron microscopic analysis of uninfected quiescent VSMCs and VSMCs were transduced with either Ad-β-gal or Ad-Gax at an m.o.i. of 750 p.f.u./cell and stimulated with serum for 48 h. The quiescent (5200×) and Ad-β-gal-transduced (2950×) VSMCs displayed normal chromatin structure, membrane-bound organelles and visible nucleoli in the plane of focus. The Ad-Gax-transduced VSMCs (3900×) display cytoplasmic blebbing (black arrow) and chromatin condensation (white arrow). (**B**) Gax-transduced VSMCs are TUNEL positive. Uninfected quiescent VSMCs and mitogen-stimulated VSMCs that had been transduced with the indicated adenovirus construct were stained with TUNEL and Hoechst 33258.

detectable TUNEL-positive cells (Figure 2B). Furthermore, DNA extracted from VSMCs at 48 h post-serum stimulation displayed a characteristic 180 bp increment DNA ladder in the Ad-Gax-treated cultures, but not in mock- or Ad-β-gal-infected cultures (not shown).

Flow-cytometric analysis was also employed to detect cell populations containing hypodiploid DNA content (<2N) which is indicative of DNA fragmentation. At 24 h post-serum stimulation, the Ad-Gax-treated VSMCs predominately displayed G_0/G_1 growth arrest (Figure 3A), consistent with our previous report (Smith *et al.*, 1997). However, by 48 h post-serum stimulation, the Ad-Gax-treated VSMCs displayed a marked increase in hypodiploid DNA, while parallel cultures of saline- or Ad-β-gal-treated cells showed normal DNA content. A population of cells containing hypodiploid DNA was not detected in serum-stimulated VSMCs transduced with an adenoviral vector expressing the general cdk inhibitor $p21^{WAF/CIP}$, though G_0/G_1 cell cycle arrest was evident (Figure 3A).

Quiescent VSMCs express endogenous Gax protein (Gorski *et al.*, 1993), yet they do not undergo apoptosis. Quiescent VSMCs were also refractory to adenovirus-mediated Gax overexpression (Figure 3B). In contrast, VSMCs infected with a replication-defective adenovirus expressing Fas ligand (Ad-FasL), a known apoptosis inducer (Sata *et al.*, 1998), displayed hypodiploid DNA content under both quiescent and mitogen-activated conditions.

G_1- and S-phase inhibitors do not block Gax-induced cell death

Chemical inhibitors of the cell cycle can prevent abortive cell cycle and apoptosis (Farinelli and Greene, 1996). Therefore, the effects of cell cycle inhibition at different phases was examined in VSMCs transduced with either Ad-β-gal or Ad-Gax. Addition of the G_1-phase inhibitor rapamycin (Figure 4A), which blocks Rb phosphorylation (Marx *et al.*, 1995), arrested Ad-β-gal-transduced VSMCs in G_1 and had no effect on viability. However, parallel cultures of Ad-Gax-transduced VSMCs underwent similar

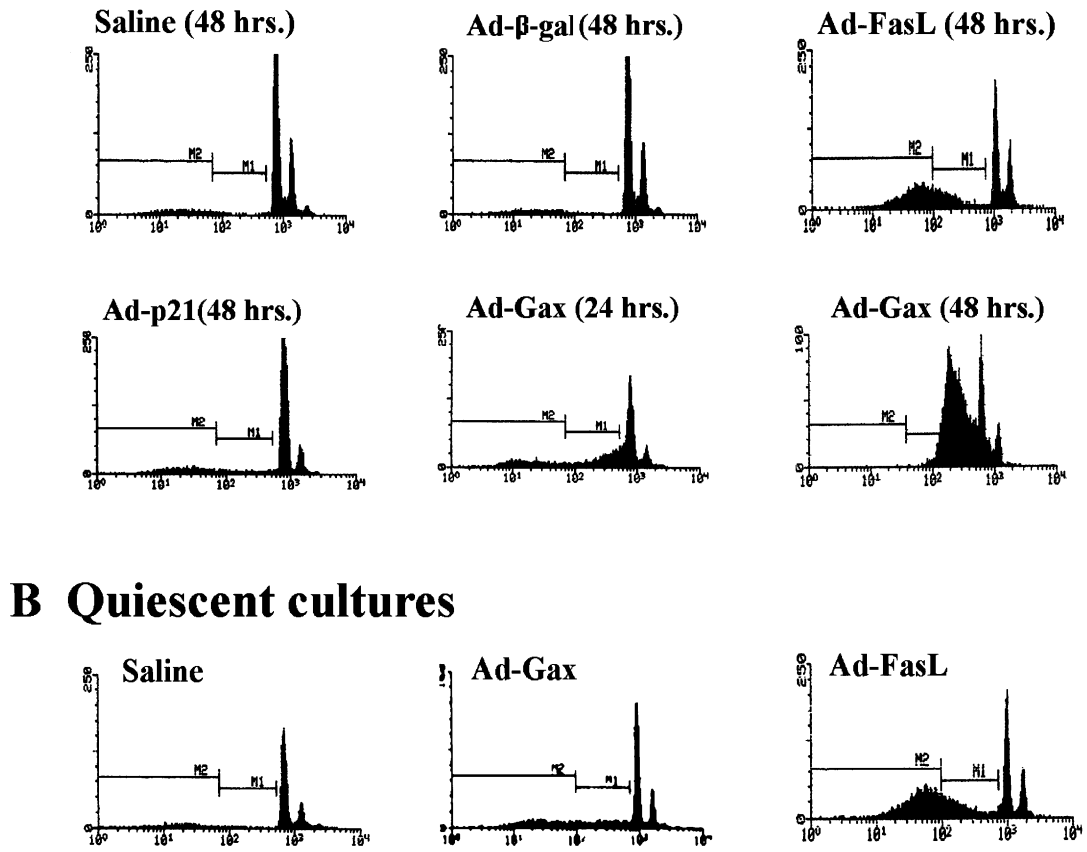

Fig. 3. Gax-induced DNA fragmentation requires mitogen activation. (**A**) FACS analysis in serum-stimulated VSMC cultures. Quiescent VSMC cultures were infected by the indicated adenoviral construct at an m.o.i. of 750 p.f.u./cell for 12 h after which the virus was removed and cultures were returned to low mitogen medium for an additional 12 h. Cultures were transferred to high serum medium for the indicated period of time prior to analysis by flow cytometry. (**B**) FACS analysis of quiescent cultures. Quiescent VSMC cultures were infected with the indicated adenoviral construct at an m.o.i. of 750 p.f.u./cell for 12 h and then transferred to low serum for 60 h prior to analysis by flow cytometry to determine DNA content.

frequencies of apoptosis in the presence or absence of either rapamycin (Figure 4B) or mimosine (not shown). In addition, the late G_1/S inhibitor hydroxyurea or the S-phase inhibitor aphidicolin (Farinelli and Greene, 1996) also did not affect the frequency of cell death induced by the combination of mitogen activation and Gax expression.

Gax induces apoptosis independent of p21$^{WAF/CIP}$ and p53

It was of interest to determine whether forced Gax expression could induce apoptosis in mouse embryonic fibroblasts null homozygous for both *p21*$^{WAF/CIP}$ alleles (p21–/– MEFs) which previously have been shown to be refractory to Ad-Gax-induced growth arrest (Smith *et al.*, 1997). Flow-cytometric analysis was not utilized since p21–/– MEFs are polyploid (Deng *et al.*, 1995; H.Perlman, unpublished observations), thereby confounding the analysis of hypodiploid DNA content. Instead, viability was assessed by directly counting cells using trypan blue exclusion (Raffo *et al.*, 1995). p21–/– MEF cultures transduced with Ad-Gax displayed a 79% decrease in cell viability compared with the quiescent control cultures (Figure 5). In contrast, Ad-β-gal-infected cultures increased in cell number during this time course, as did mock-infected cultures (not shown). TUNEL analyses of saline-, Ad-β-gal- or Ad-Gax-infected p21–/– MEF cultures revealed that a large portion of the Ad-Gax-transduced cells were TUNEL positive and displayed chromatin condensation, while the control cultures were TUNEL negative (not shown).

To determine if Gax-induced apoptosis requires p53, spontaneously immortalized MEFs containing deletions of both *p53* alleles (10.1 MEFs) (Harvey and Levine, 1991) were either treated with saline or infected with the adenoviral constructs and subjected to flow-cytometric analyses at 48 h post-serum stimulation. Ad-Gax-infected cells displayed hypodiploid DNA content while uninfected or Ad-β-gal-infected cells displayed normal DNA profiles (Figure 6A). In addition, Ad-Gax-infected cultures exhibited nuclei with condensed chromatin that stained positive for TUNEL (not shown).

Since the adenovirus E4 region can influence apoptosis (Marcellus *et al.*, 1996), it was necessary to determine whether the apoptosis induced by the Gax transgene was modulated by adenoviral genes. An established cell death assay (Miura *et al.*, 1993; Boyd *et al.*, 1995; Chittenden *et al.*, 1995) was performed using expression plasmids encoding Gax or other test genes transfected in combin-

A

		Go/G1	S	G2/M
Ad-β-gal	Saline	64	18	18
	Rapamycin	74	7	19
	Hydroxyurea	80	12	8
	Aphidicolin	55	35	10
Ad-Gax	Saline	92	3	5
	Rapamycin	92	5	3
	Hydroxyurea	76	20	4
	Aphidicolin	94	4	2

B

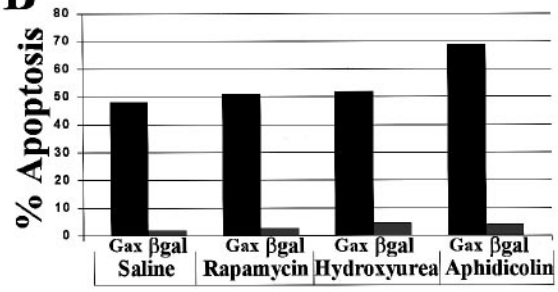

Fig. 4. G_1- and S-phase inhibitors do not affect Gax-induced cell death. Quiescent VSMC cultures were infected with the indicated adenoviral construct at an m.o.i. of 750 p.f.u./cell for 12 h. After virus removal, cultures were incubated in low mitogen medium for an additional 12 h and then transferred to high serum medium in the absence (saline) or presence of the cell cycle inhibitors, rapamycin, hydroxyurea or aphidicolin for 48 h prior to analysis by flow cytometry. (**A**) Percentage of cells with ⩾2N DNA content in G_1, S and G_2/M phases following infection with Ad-β-gal or Ad-Gax and treatment with the indicated cell cycle inhibitor. (**B**) Percentage of total cells with <2N DNA content.

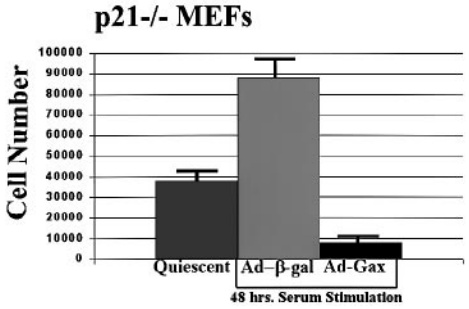

Fig. 5. Gax overexpression induces apoptosis independently of $p21^{WAF/CIP}$. Quiescent p21–/– MEFs were infected either with Ad-β-gal or Ad-Gax at an m.o.i. of 750 p.f.u./cell for 12 h. Virus was removed and cultures were returned to low mitogen medium for an additional 12 h prior to incubation in high serum media for 48 h. Parallel quiescent cultures were incubated for 72 h in low mitogen media without viral transduction. Cultures were harvested by trypsinization, fixed and cell number was determined by the trypan blue exclusion method (Raffo *et al.*, 1995). Each time point represents the mean ± standard error of three determinations from a representative experiment.

ation with a β-galactosidase expression plasmid. This assay allows assessment of changes in cell viability as determined by variations in the number of β-galactosidase-positive cells. Expression vectors were utilized that encoded either wild-type Gax or a mutant form of Gax lacking the homeodomain (ΔHD-Gax), which is ineffective at inducing cell cycle arrest and transactivating the $p21^{WAF/CIP}$ promoter (Smith *et al.*, 1997). ΔHD-Gax- and empty vector-transfected 10.1 MEFs (Figure 6B, D and E), p21–/– MEFs (not shown) and A7r5 VSMCs (not shown) displayed numerous β-galactosidase-positive cells compared with the cultures transfected with wild-type Gax expression plasmid. These data suggest that the homeodomain is essential for Gax-induced cell death. Apoptosis induced by the Gax expression plasmid was also indicated by the presence of Gax-positive pyknotic nuclei (Figure 6C). Incubation of Gax-transfected cultures with 50 μM *N*-acetyl-cysteine (NAC), an antioxidant that inhibits apoptosis (Hockenbery *et al.*, 1993; Fabbretti *et al.*, 1995), significantly increased the number of β-galactosidase-positive cells (Figure 6B and D). Furthermore, co-transfection of equal amounts of pCDNA-Bcl-2 expression plasmid (Figure 6E) with pCGN-Gax expression plasmid also increased the number of β-galactosidase-positive MEFs, suggesting that Bcl-2 overexpression can rescue cells from Gax-induced cell death ($P < 0.05$).

Constitutive Gax expression modulates Bcl-2 and Bax proteins

The expression levels of the Bcl-2 family of proteins were determined by Western blot analyses on extracts prepared from quiescent and serum-stimulated cultures treated with either saline (mock-infected), Ad-β-gal or Ad-Gax. Relative to control cultures, serum-stimulated cultures infected with Ad-Gax displayed elevated Bax levels and decreased Bcl-2 levels (Figure 7A). Bad levels also increased under these conditions (not shown), but no changes were observed in levels of the Bcl-2 family proteins Bcl-x_L, Bak or Bag, while Bcl-x_S was not detected in any samples (not shown). In contrast, Ad-Gax infection had little or no effect on Bax and Bcl-2 expression when VSMC cultures were retained in low mitogen media (Figure 7B).

Infection with Ad-Gax markedly up-regulated Bax and down-regulated Bcl-2 in 10.1 MEFs, that lack p53, and in p21–/– MEFs that are sensitive to Gax-induced cell death (Figure 7C and D). RNase protection analysis using a multi-probe template set revealed that Gax overexpression had little or no effect on *bax* or other *bcl-2* gene family transcript levels in VSMCs (Figure 7E), indicating that Bax up-regulation occurs at a post-transcriptional level. Expression of Bax was evaluated further in individual cells by immunofluorescence (Figure 7F). Control VSMCs displayed diffuse staining of Bax protein, while Ad-Gax-infected cultures displayed an intense staining pattern. Bcl-2 expression was detected in the control cells, but not in the Ad-Gax-transduced cultures (not shown). Collectively, these data demonstrate that the up-regulation of Bax and down-regulation of Bcl-2 consistently occurs in cell types that are susceptible to Gax-induced cell death.

Bax is essential for Gax-induced cell death

MEFs with homozygous disruptions in both *bax* alleles (Bax–/– MEFs) (Knudson *et al.*, 1995) were analyzed to determine whether this Bcl-2 family protein is essential for death signal transmission induced by forced Gax

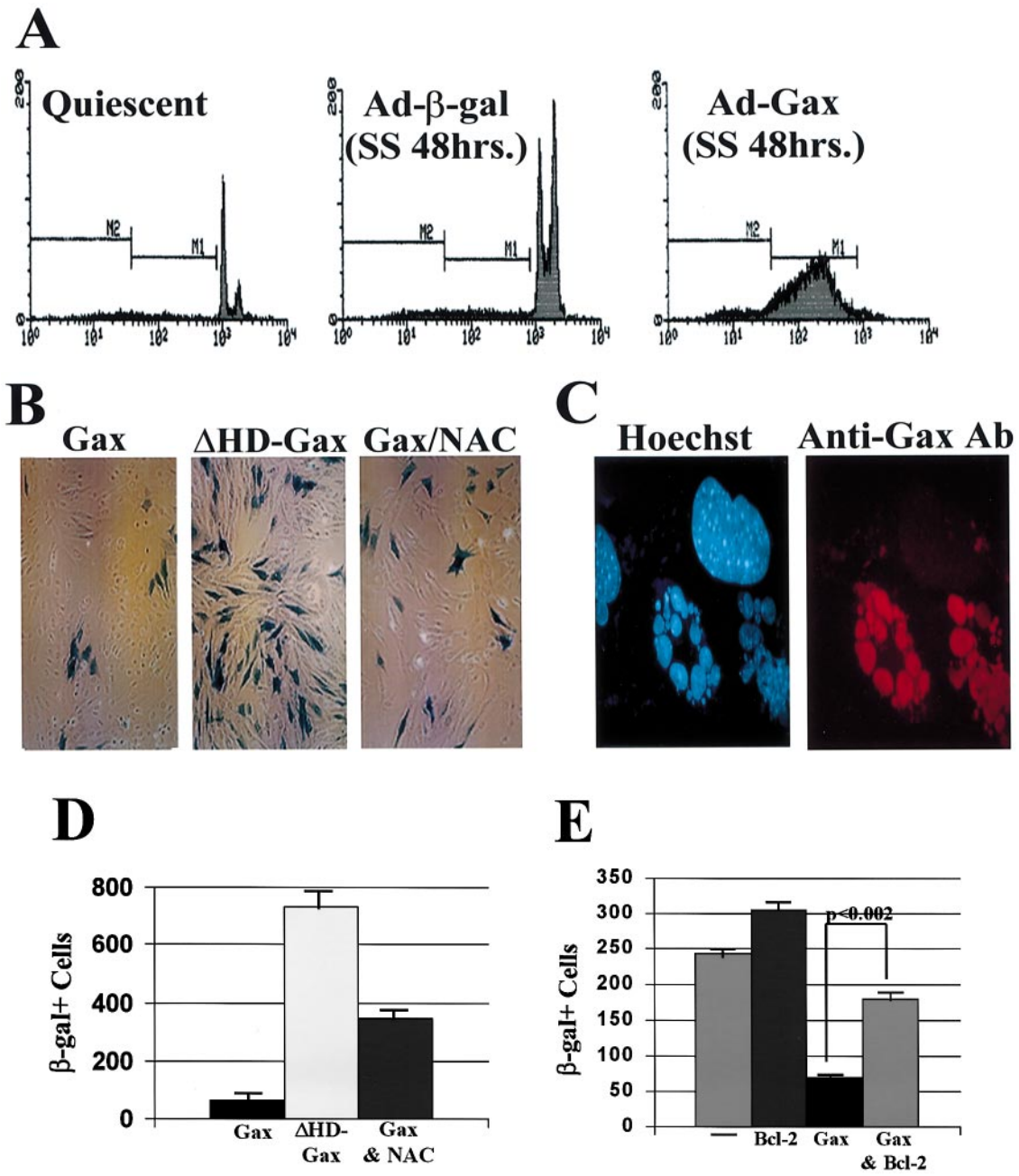

Fig. 6. Gax overexpression induces apoptosis independently of p53. (**A**) Quiescent 10.1 MEFs that are null for p53 were infected with Ad-β-gal or Ad-Gax at an m.o.i. of 750 p.f.u./cell for 12 h. The virus was removed, cultures were returned to low mitogen medium for an additional 12 h and then transferred to growth medium for 48 h. DNA content was analyzed by FACS analysis. An uninfected quiescent culture of 10.1 MEFs is shown for comparison. (**B**) 10.1 MEFs co-transfected with β-galactosidase and wild-type Gax expression plasmids displayed reduced number of β-galactosidase-positive cells. Shown is a representative micrograph of 10.1 MEFs co-transfected with pCMV-β-galactosidase and plasmids that express wild-type Gax or a mutant Gax lacking the homeodomain. Quiescent 10.1 MEFs transfected with 1 μg of the β-galactosidase (pCMV-β-gal) and 4 μg of either pCGN-Gax (Gax) or pCGN-ΔHD-Gax (ΔHD-Gax) expression vectors using LipofectAmine (Gibco-BRL). Following transfection, cultures were transferred to high serum for 48 h and then fixed and stained with X-gal. Inclusion of 50 μM NAC increased the number of β-galactosidase-positive cells that were co-transfected with pCGN-Gax. (**C**) Representative micrograph demonstrating co-localization of pyknotic nuclei and Gax protein from the pCGN-Gax expression plasmid. Transfected 10.1 MEFs were fixed and stained with anti-Gax antibody, followed by treatment with a rhodamine (red)-conjugated secondary antibody. Cellular DNA was stained with Hoechst 33258. (**D**) Quantitative analysis of 10.1 MEFs transfected with pCMV-β-gal and either pCGN-Gax (Gax) or pCGN-ΔHD-Gax (ΔHD-Gax) expression plasmids as described above in the presence or absence of 50 μM NAC. Cultures were fixed and stained with X-gal and the number of β-galactosidase-positive cells was determined. Each time point represents the mean ± standard error of three determinations from a representative experiment. (**E**) Bcl-2 overexpression rescues 10.1 MEFs transfected with the Gax expression plasmid. 10.1 MEFs were transfected with 1 μg of pCMV-β-gal and either 2 μg of pCDNA-Bcl-2 (Bcl-2), 2 μg of empty vector (–), 2 μg of pCGN-Gax (Gax) or 2 μg of pCDNA-Bcl-2 and 2 μg of pCGN-Gax (Gax & Bcl-2). Some mixes contained additional pCGN empty vector such that all transfections were performed with 5 μg of plasmid DNA. Values represent the mean ± standard error and were compared for statistical significance by ANOVA and student *t*-test analysis ($P < 0.05$).

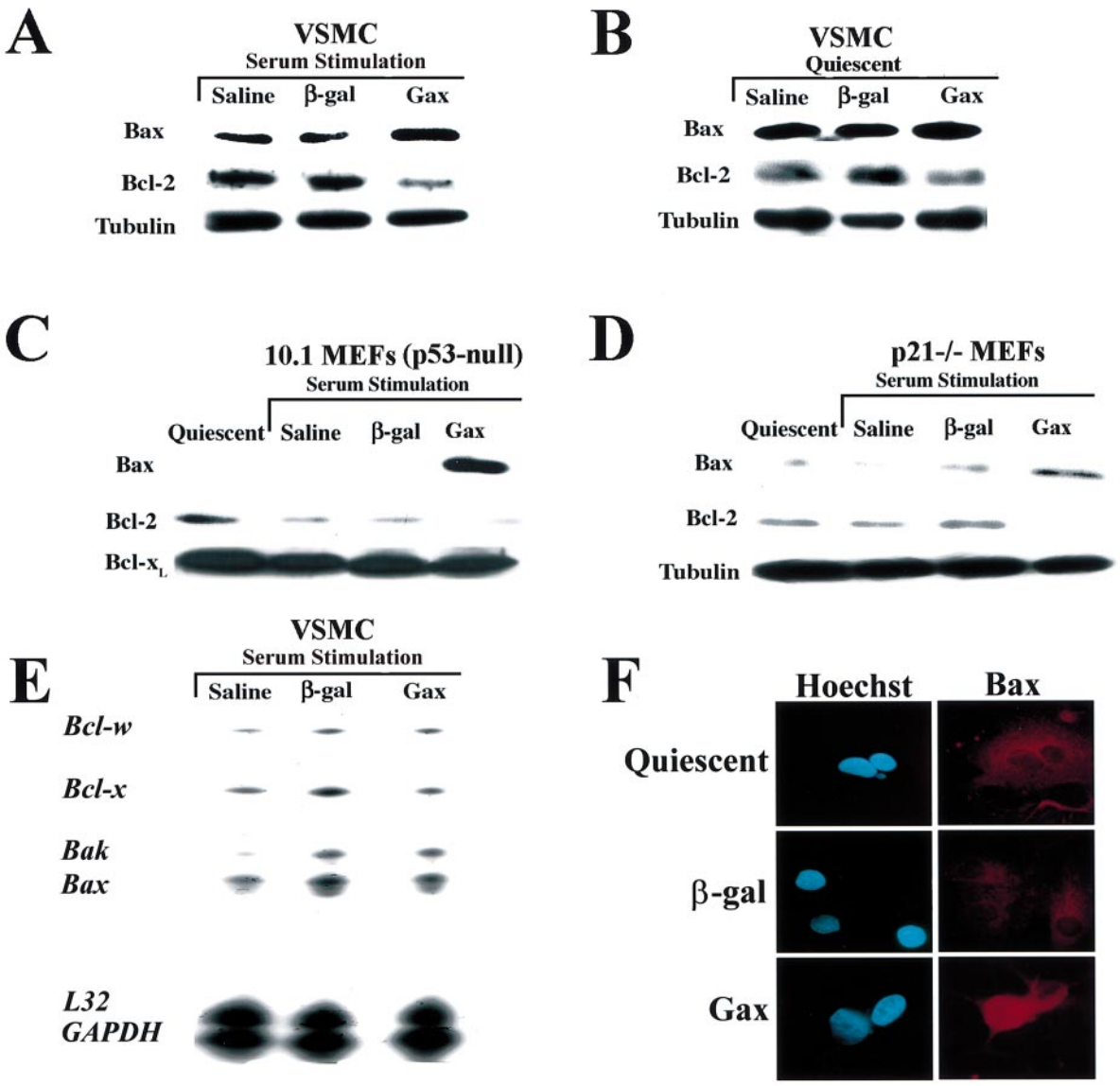

Fig. 7. Mitogen-dependent modulation of Bcl-2 family proteins by the Gax homeoprotein. (**A**–**D**) Immunoblot analyses of Bcl-2 family proteins in VSMCs, 10.1 MEFs and p21–/– MEFs. Whole-cell extracts (50 μg) were prepared from the indicated quiescent and serum-stimulated cultures that were either mock-infected (saline) or infected with Ad β-gal or Ad-Gax. Immunoblot analysis was performed with antibodies directed against the indicated proteins. Tubulin or Bcl-x_L immunoblots indicate equal loading of protein. Antibody specificity was demonstrated by loss of signal when a molar excess of immunogenic peptide was pre-incubated with antibody (not shown). (**E**) RNase protection assay of *bcl-2* family transcripts. Quiescent cultures of VSMCs were infected with Ad-β-gal, Ad-Gax or mock-infected (saline) and serum-stimulated for 48 h. Total RNA was prepared and hybridized to a multi-probe template set and fractionated by acrylamide gel electrophoresis. (**F**) Immunohistochemical analysis of Bax. Quiescent VSMCs or VSMCs stimulated with serum after infection with Ad-β-gal or Ad-Gax were fixed and stained with an anti-Bax primary antibody. The signal was detected with rhodamine-conjugated anti-rabbit secondary antibody and DNA was stained with Hoechst 33258.

expression. Serum-stimulated Bax–/– MEFs transduced with Ad-Gax did not undergo apoptosis since normal DNA profiles, devoid of hypodiploid DNA, were observed by flow-cytometric analysis (Figure 8A). In contrast, isogenic wild-type MEFs transduced with Ad-Gax exhibited decreased cell viability (not shown) and 40% hypodiploid DNA content (Figure 8B). Bax–/– and wild-type MEFs transduced with Ad-β-gal displayed normal DNA profiles that were similar to saline-treated cultures. These data demonstrate that Gax-induced apoptosis is dependent on the function of Bax.

Discussion

Homeobox genes encode a class of transcription factors that are key regulators of body plan and organogenesis (Sharkey *et al*., 1997). At a cellular level, they control differentiation, migration and growth, and have also been implicated in promoting cell survival during embryogenesis. Examples of homeobox genes necessary for survival include *Hox11* for spleen development (Dear *et al*., 1995), *repo* for neurons in the optic lobe (Xiong and Montell, 1995) and *cux-1* for mouse embryonic kidney cells (Quaggin *et al*., 1997). In all of these cases, the

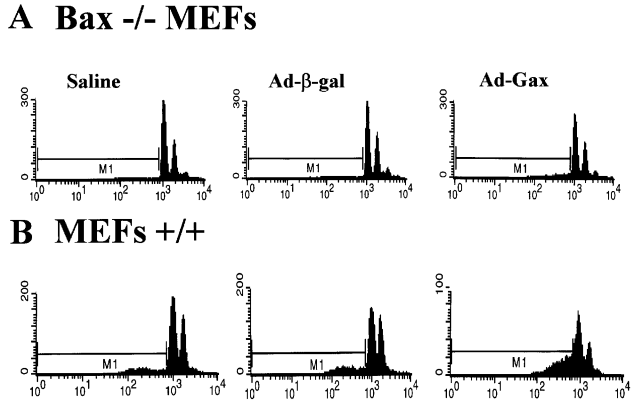

Fig. 8. Bax is essential for Gax-induced apoptosis. Wild-type MEF and isogenic Bax–/– MEF cultures were mock-infected (saline) or infected with Ad-β-gal and Ad-Gax at an m.o.i. of 750 p.f.u./cell for 12 h. The virus was then removed and cultures were returned to low mitogen media for an additional 12 h followed by incubation in high mitogen media for 48 h prior to analysis by flow cytometry. (**A**) DNA content of Bax–/– MEFs. (**B**) DNA content of isogenic wild-type MEFs.

homeodomain protein appears to function as a positive regulator of cell viability. Recently, it was shown that overexpression of *msx2* enhances apoptosis in aggregates of P19 carcinoma cells but not in single monolayer cells (Marazzi *et al*., 1997). Therefore, homeodomain proteins may function as either positive or negative regulators of cell viability, but the apoptosis signaling pathways affected by these factors have not been delineated.

The *gax* homeobox gene is expressed in quiescent VSMCs and down-regulated under conditions that promote proliferation *in vitro* (Gorski *et al*., 1993) and *in vivo* (Weir *et al.*, 1995). Here, we have demonstrated that apoptotic cell death is induced by sustained Gax expression in mitogen-activated VSMCs and fibroblasts. Apoptosis was demonstrated by multiple lines of evidence including the observation of hypodiploid DNA by flow-cytometric analysis, TUNEL-positive staining and detection of morphological changes as visualized by transmission electron microscopy. Furthermore, cells transfected with a *gax* expression plasmid exhibited pyknotic nuclei and decreased cell number, indicating that the induction of apoptosis is independent of proteins encoded by the adenoviral genome.

Surprisingly, Gax overexpression induces apoptosis in mitogen-activated cells, but cells remain viable under conditions of quiescence. Based upon these observations, we propose that Gax down-regulation by mitogens is essential for cell viability under conditions that promote proliferation, such as following vascular injury (Walsh and Perlman, 1996). Previous investigations have examined the effects of aberrant cell cycle activity on apoptosis in quiescent cells by overexpression of c-Myc (Evan *et al*., 1992), cyclin A (Hoang *et al*., 1994), E1A (Liu and Kitsis, 1996) or E2F (Qin *et al*., 1994; Kowalik *et al*., 1995). Here, we approached the issue of aberrant cell cycle activity and apoptosis from the opposite perspective as we focused on the effects of a negative cell cycle regulator, Gax, and its effect on apoptosis under mitogen activation conditions. Multiple lines of evidence suggest that a signaling conflict between growth (mitogen stimulation) and growth arrest (Gax expression) is unlikely to account

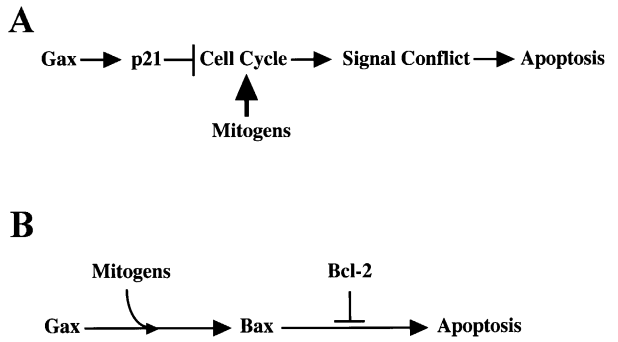

Fig. 9. Schematic diagram of two potential models of mitogen-dependent apoptosis induced by Gax expression. (**A**) Gax induces apoptosis through conflicting signals of $p21^{WAF/CIP}$-dependent growth arrest and mitogen stimulation of the cell cycle. (**B**) Gax induces apoptosis via mitogen-dependent pathways that circumvent the cell cycle to up-regulate Bax expression.

for Gax-mediated cell death (Figure 9A). First, overexpression of the cdk inhibitor $p21^{WAF/CIP}$, a downstream target of Gax, induces cell cycle arrest under mitogen activation conditions, but has no effect on cell viability. Secondly, p21–/– MEFs are refractive to Gax-induced cell cycle arrest (Smith *et al*., 1997), but are susceptible to Gax-mediated apoptosis. Finally, cell cycle arrest in G_1 or S phase with chemical inhibitors had no effect on mitogen-dependent apoptosis induced by Gax. Based on these data, we conclude that the cell death pathway invoked by Gax requires mitogen activation, but is independent of cell cycle activity. The dual capacity of Gax to inhibit cell growth and induce apoptosis through separable pathways is similar to the tumor suppressor protein p53, which has also been shown to induce cell cycle arrest or apoptosis depending on environmental cues and the genotype of the cell (Ko and Prives, 1996).

Under mitogen activation conditions, Gax induces the pro-apoptotic protein Bax and represses the apoptosis inhibitory protein Bcl-2 in both VSMCs and fibroblasts. In contrast, Gax overexpression in quiescent cells, that are refractory to Gax-induced apoptosis, had little or no effect on Bax or Bcl-2 levels relative to control cultures. Conditional modulation of Bax and Bcl-2 expression by Gax is reminiscent of p53 action, as p53 transcriptionally activates *bax* and represses *bcl-2* (Miyashita *et al.*, 1994a,b; Miyashita and Reed, 1995). However, Gax differs from p53 in that the up-regulation of Bax occurs at a post-transcriptional level. It is of note that Gax-induced apoptosis, as well as Gax regulation of Bax and Bcl-2, occurred in cells that are null for p53.

The functional significance of Bax up-regulation was demonstrated by the finding that MEFs homozygous null for both *bax* alleles are refractive to Gax-induced cell death under mitogen activation conditions. Recently, Bax has been shown to be required for transmitting apoptotic signals under a variety of conditions including neuronal cell death induced by trophic factor deprivation (Deckwerth *et al*., 1996) and p53-dependent apoptosis in cells that express E1A (McCurrach *et al*., 1997). In the present study, Bax was found to mediate apoptosis induced by the conflict between mitogen activation and sustained expression of a growth-inhibitory transcription factor that is normally down-regulated under these conditions (Figure 9B). As noted previously, this apoptotic program is inde-

pendent of the effects of Gax on cell cycle activity. Therefore, our data suggest that the balance between cell proliferation and death can be controlled by mitogen-dependent pathways that circumvent the cell cycle.

Material and methods

Cell culture

Cells were incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Primary cultures of rat smooth muscle cells were prepared from thoracic aortas of adult male Sprague–Dawley rats according to Mader *et al.* (1992). MEFs with a homozygous deletion of the p53 alleles designated 10.1 MEFs were a generous gift from Dr Arnold Levine (Harvey and Levine, 1991). MEFs that contain a homozygous disruption of the p21 alleles were a generous gift from Dr Philip Leder (Deng *et al*., 1995). MEFs that contain a homozygous disruption of the Bax alleles and isogenic wild-type MEFs were a generous gift from Dr Stanley J.Korsmeyer (Knudson *et al*., 1995).

Adenoviral infections in culture (Smith *et al*., 1997) and adenoviral constructs have been described previously: Ad-β-gal (Stratford-Perricaudet *et al*., 1992), Ad-Gax, (Smith *et al*., 1997) Ad-p21 (Clayman *et al*., 1996; Wang and Walsh, 1996) and Ad-FasL (Sata *et al*., 1998). For infections, cells were plated in growth medium (10% FBS) and allowed to attach before being transferred to low mitogen medium. Rat VSMCs, wild-type MEFs, 10.1 MEFs and Bax–/– MEFs were induced to the quiescent state by serum starvation for 2–3 days in 0.5% FBS/DMEM, while p21–/– MEFs cells were serum starved in 0.2% FBS/DMEM for 4 days. Cells were then counted and cultures were incubated with the indicated adenovirus for 12 h in low mitogen medium. At the end of the infection period, the virus was removed by washing with phosphate-buffered saline (PBS) and returned to low mitogen medium for an additional 12 h. The cultures were then stimulated for 24–48 h by the addition of growth (10% FBS) medium.

For transient transfections, cells were cultured on 60 mm plates containing 1.5% gelatin-coated glass coverslips and were serum starved for 3 days in 0.5% FBS/DMEM. Four μg of test plasmids were co-transfected with 1 μg of cytomegalovirus (CMV)-β-gal expression plasmid using the LipofectAmine procedure (Gibco-BRL) in a 1:6 ratio for 2–4 h. Cultures were serum stimulated for 48 h and fixed in 4% neutral buffered formalin for 10 min. Where indicated, NAC (50 μM; Sigma) was added to the medium following serum stimulation. Fixed cultures were either stained with X-gal or stained for Gax expression. The expression plasmids pCGN-Gax and pCGN-ΔHD-Gax were previously described by Smith *et al*. (1997), and pCDNA-Bcl-2 expression plasmid was described by Chittenden *et al*. (1995). pCMV-β-gal expression vector was purchased from Clontech.

TUNEL labeling and nuclear condensation

Cultured cells were plated on 60 mm plates containing 1.5% gelatin-coated glass coverslips. Cells were serum starved for 3 days in 0.5% FBS/DMEM. Following infection, cultures were incubated in growth media for 48 h. Cultures were fixed in 4% neutral buffered formalin for 5 min and subjected to multiple washes in PBS. Individual coverslips were treated with 0.5% NP-40 for 5 min followed by two PBS washes. TdT enzyme and a cocktail containing dUTP conjugated to fluorescein were added to the coverslips according to the manufacturer's specifications (Boehringer Mannheim *in-situ* death detection kit). Nuclei were counterstained with Hoechst 33258 (Sigma), and mounted for examination using mounting media for fluorescence (Kirkegaard & Perry Laboratories, Inc.). Specimens were examined and photographed on a Diaphot microscope (Nikon Inc.) equipped with phase-contrast and epifluorescence optics.

Flow-cytometric analysis of transduced VSMC cultures

Primary rat VSMCs, wild-type MEFs, Bax–/– MEFs or 10.1 MEFs cells were plated in 6-well dishes in growth medium (10% FBS) and allowed to attach before being transferred to low mitogen (0.5% FBS) medium for 72 h. Cells were counted and cultures were incubated with either Ad-Gax, Ad-FasL, Ad-p21 or Ad-β-gal at an m.o.i. of 750 for 12 h in low mitogen medium. At the end of the infection period, the virus was removed by washing with PBS and returned to low mitogen medium for 12 h. The cultures were then stimulated for 24–48 h by the addition of growth (10% FBS) medium. Following serum stimulation, cell cycle inhibitors were added at a concentration of 400 μm mimosine (Sigma), 10 μm rapamycin (Calbiochem), 10 μm aphidicolin (Sigma) or 3 mM hydroxyurea (Calbiochem). Cells were harvested by trypsinization, fixed overnight in 70% ethanol and stained for DNA content with a propidium iodide cocktail. The apoptotic profile was determined by FACS utilizing a Beckton Dickinson Vantage flow cytometer and Lysis II cell cycle analysis software. Flow cytometry was conducted at the Core Flow Cytometry Facility of the Dana-Farber Cancer Institute, Boston, MA.

Transmission electron microscopy

Primary rat VSMCs were cultured under growth media conditions in 6-well plates at 40% confluency. The 72 h quiescent control, saline-, Ad-β-gal- and Ad- Gax-transduced cells were fixed in 2.5% glutaraldehyde buffered by 0.1 M sodium cacodylate for 12 h at 4°C. Cells were washed three times in 0.1 M cacodylate buffer for 10 min. Afterwards, cells were post-fixed with 1% osmium tetroxide buffered by 0.1 M sodium cacodylate at 4°C overnight. Cells were then dehydrated in increasing amounts of ethanol for 10 min each and infiltrated with propylene oxide for 15 min. Cells were infiltrated with 1:1 propylene:epon for 1 h followed by 1:2 propylene:epon for 1 h, and finally infiltrated with 100% epon for 2 h. Cells were embedded in fresh epon into molds and oven dried for 24 h. Thin sections were cut for less contrast and placed on 3 mm grids for conventional staining with 3% uranyl acetate in water and SATO's lead stain. Grids were examined using a Philips CM-10 electron microscope and photographed.

Western blot analysis

Whole-cell extracts were prepared from VSMCs, p21–/– MEFs and 10.1 MEFs. Aliquots (50 μg) of the extracts were analyzed by SDS–PAGE on a 12.5% polyacrylamide gel, and transferred to Immobilon-P (Millipore) by semi-dry blotting. Filters were blocked for 1 h at room temperature in PBS/0.2%Tween-20/5% non-fat dry milk. The filters were then incubated with either mouse anti-tubulin, anti-Bak (Calbiochem), anti-Bad, anti-Bcl-2 or anti-Bcl-x antibodies (Transduction Laboratories) at a concentration of 0.25–0.4 μg/ml. Rabbit anti-Bax, anti-Bag, anti-Bad, anti-Bcl-2, anti-Bcl-x (Santa Cruz Biotechnology, Inc.), rabbit IgG (Sigma) and anti-Gax antibodies (Smith *et al*., 1997) were used at a concentration of 0.25–0.4 μg/ml overnight at 4°C in PBS/0.2% Tween-20/2% non-fat dry milk. Filters were washed in PBS/0.2%Tween-20/2% non-fat dry milk, and incubated with donkey anti-rabbit or anti-mouse secondary antibody (1:2000 dilution) conjugated to horseradish peroxidase (Amersham). Visualization of the immunocomplex was carried out as recommended by the manufacturer (Enhanced Chemiluminescence kit; Amersham).

Immunocytochemical analysis

Rat VSMCs or 10.1 MEFs were plated at 40% confluence in growth media on 1.5% gelatin-coated glass coverslips. Cultures were transferred to low mitogen (0.5% FBS) medium for 48 h before viral infection. Adenovirus infection (750 p.f.u./cell) was carried out in low serum medium for 12 h, after which the virus solution was removed and replaced with fresh low serum media for 12 h. At 48 h after serum stimulation, the cells were fixed for 10 min with 4% neutral buffered formalin and permeabilized with 0.1% NP-40 for 5 min. All coverslips were incubated for 1 h in blocking solution (5% goat serum, 0.01% NP-40, 0.01% sodium azide in PBS) prior to addition of primary antibody. Primary antibody(ies) were incubated overnight at 4°C in blocking solution. Rabbit anti-Bax, anti-Bcl-2 (Santa Cruz Biotechnology, Inc.), rabbit IgG (Sigma) and anti-Gax antibodies (Smith *et al*., 1997) were used at a concentration of 2.5–4 μg/ml. Antibodies were removed by multiple washes with PBS and coverslips were incubated with secondary antibody(ies) for 45 min. A rhodamine-conjugated goat anti-rabbit secondary antibody (Kirkegaard & Perry) was used at a concentration of 2.5 μg/ml. Following antibody treatments, coverslips were rinsed with PBS three times and stained for 2 min with 0.1 μg/ml Hoechst 33528 (Sigma) to visualize DNA. Coverslips were mounted with fluorescence mounting media (Kirkegaard & Perry) and visualized by fluorescent microscopy.

RNase protection assay

Quiescent rat VSMCs were infected with adenovirus at an m.o.i. of 750 for 12 h in low serum medium (0.5% FBS) before being transferred to growth medium (10% FBS). Cultures were harvested for RNA preparation as previously described by Chomczynski and Sacchi (1987) after 24 h of serum stimulation. The RNase protection assay was performed according to the manufacturer's specifications (PharMingen).

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