p21<sup>CIP1</sup>-mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene

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gax, a diverged homeobox gene expressed in vascular smooth muscle cells (VSMCs), is down-regulated in vitro by mitogen stimulation and in vivo in response to vascular injury that leads to cellular proliferation. Recombinant Gax protein microinjected into VSMCs and fibroblasts inhibited the mitogen-induced entry into S-phase when introduced either during quiescence or early stages of G<sub>1</sub>. Overexpression of gax with a replication-defective adenovirus vector resulted in G<sub>0</sub>/G<sub>1</sub> cell cycle arrest of VSMCs and fibroblasts. The gax-induced growth inhibition correlated with a p53-independent up-regulation of the cyclin-dependent kinase inhibitor p21. Gax overexpression also led to an association of p21 with cdk2 complexes and a decrease in cdk2 activity. Fibroblasts deficient in p21 were not susceptible to a reduction in cdk2 activity or growth inhibition by gax overexpression. Localized delivery of the virus to denuded rat carotid arteries significantly reduced neointima formation and luminal narrowing. These data indicate that gax overexpression can inhibit cell proliferation in a p21-dependent manner and can modulate injury-induced changes in vessel wall morphology that result from excessive cellular proliferation.

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Homeobox transcription factors are key regulators of cell differentiation and migration, and the deregulated expression of some of these factors is associated with abnormal cell growth. Overexpression of the homeobox genes HB24, mxx1, and Hoxb8 has been shown to transform cells and enable them to form tumors in nude mice [Aberdam et al. 1991; Song et al. 1992; Deguchi and Kehrl 1993], and expression of the POU factor GHF1 is required for the proliferation of somatotrophic cell lines [Castrillo et al. 1991]. However, much less is known about the growth-suppressive properties that may be conferred by this class of regulatory molecules. Expression of the gax homeobox gene is largely confined to adult cardiovascular tissues, including the vascular smooth muscle cell (VSMC) layer of the aorta wall [Gorski et al. 1993]. VSMCs are capable of responding to mitogens and re-entering the cell cycle, in the process changing their state of differentiation to resemble that of their more primitive, embryonic precursors [Campbell et al. 1988]. These features distinguish vascular myocytes from skeletal and cardiac muscle cells, which are terminally differentiated and incapable of re-entering the cell cycle in response to mitogen stimulation. The abnormal proliferation of VSMCs is of particular interest because it is a key feature of the acute reaction to injury following the balloon angioplasty of occluded vessels and in the pathology of atherosclerosis [Ross 1993]. An unusual feature of gax expression in the vessel wall is its rapid down-regulation following an injury that leads to VSMC proliferation [Weir et al. 1995]. In cultured VSMCs gax expression is also down-regulated during the G<sub>0</sub> to G<sub>1</sub> transition of the cell cycle following mitogen activation, and the extent of down-regulation correlates with the mitogen's ability to stimulate DNA synthesis [Gorski et al. 1993]. This pattern of gax expression in VSMCs is similar to that of growth arrest-specific (gas) genes [Schneider et al. 1988] and growth arrest and DNA damage-inducible (gadd) genes [Fornace et al. 1992], some of which appear to function as negative regulators of the cell cycle [Del Sal et al. 1992; Barone et al. 1994; Zhan et al. 1994].
Here we examine the growth regulatory properties of the Gax homeoprotein using several methods of overexpression. First, gax was expressed as a fusion protein in bacteria, and the recombinant protein was microinjected into quiescent VSMCs and fibroblasts to determine its effect on serum-induced proliferation in vitro. Growth inhibition occurred when Gax was microinjected in G0 or early to mid-G1 phases of the cell cycle. Second, a replication-defective adenovirus vector encoding the gax gene inhibited the proliferation of both rat and human VSMCs. Overexpression of gax resulted in up-regulation of the cdk (cyclin-dependent kinase) inhibitor p21, leading to a marked reduction in cdk2 activity. In contrast, overexpression of gax in fibroblasts lacking functional p21 did not lead to growth inhibition or down-regulation of cdk2 activity. Recombinant adenovirus was also used to assess the effect of gax gene overexpression on the proliferative response of VSMCs to balloon injury in rat carotid arteries. In this model of vessel wall stenosis gax inhibited the neointimal formation and luminal narrowing that is characteristic of proliferative vascular disorders. These data indicate that homeodomain proteins can be used to modulate vascular lesion formation, perhaps through their ability to regulate cell growth.

Results

Microinjected Gax protein inhibits cell proliferation

Quiescent rat VSMCs were microinjected with buffer or recombinant protein, and the percentage of growth inhibition was determined (Fig. 1). Every VSMC within a region of a gridded coverslip was injected with recombinant protein, and all cells within this region were then scored for their ability to incorporate 5-bromo-2'-deoxyuridine (BrdU) in their nuclei in the presence of serum (Fig. 1A–C). The microinjection of a 0.5 mg/ml solution of glutathione S-transferase (GST)-Gax decreased the extent of mitogen-stimulated S-phase entry with inhibition values in separate experiments ranging from 29% to 67%, with a mean inhibition of 50 ± 3% [±S.E.M.]. [Fig. 1D]. The inhibition by microinjection of GST-Gax was comparable to that observed with microinjected GST-MyoD fusion protein (2 mg/ml), a basic helix-loop-helix factor that activates the skeletal muscle lineage in mesodermally derived cells [Davis et al. 1987] and functions in cell cycle withdrawal [Crescenzi et al. 1990; Sorentino et al. 1990]. However, GST–Gax was less effective than a neutralizing antibody to ras [8 mg/ml], which has been shown previously to be effective at inhibiting NIH–3T3 cell proliferation [Mulcay et al. 1985]. We note that our method of quantifying growth inhibition will tend to underestimate the inhibitory effect because of limitations in the efficiency of microinjection and serum deprivation. When microinjection efficiency is corrected for [80%–90% in these experiments as determined by the microinjection of a dye conjugated to dextran], inhibition of VSMC entry into S phase by microinjection of Gax, MyoD, and the anti-Ras antibody approach 63%, 57%, and 76%, respectively. These data with anti-Ras antibody are similar to results obtained by microinjection of quiescent NIH–3T3 cells where the antibody inhibited the mitogen-stimulated S-phase entry by 86% [Mulcay et al. 1985]. It was also comparable to the level of inhibition reported for the microinjection of bacterially produced GADD153/CHOP protein in fibroblasts [60% inhibition] [Barone et al. 1994]. The inhibition of VSMC proliferation was specific to the Gax homeoprotein, as no inhibition was observed when MHox, another
homeodomain protein (Cserjesi et al. 1992), was microinjected into VSMCs (Fig. 1D). Furthermore, neither the zinc finger transcription factor YY1 fused to GST (Shi et al. 1991; Gualberto et al. 1992) nor GST alone significantly inhibited mitogen-stimulated S-phase entry in VSMCs (Fig. 1D). GST–Gax was also shown to inhibit the mitogen-induced proliferation of the fibroblast cell line BALB/c 3T3 by 43.8% suggesting that Gax protein action is not cell type-specific (Table 1). Similar to the results with VSMCs, the proliferation of these fibroblasts was also inhibited by the microinjection of recombinant MyoD and anti-Ras antibody, but not by recombinant MHOX protein or the GST protein alone. We have also observed that recombinant rat Gax protein is able to inhibit the mitogen-induced S-phase entry of human VSMCs while GST alone has no detectable effect (data not shown), demonstrating a cross-species reactivity.

In other experiments, GST–Gax was microinjected into asynchronously cycling subconfluent VSMCs, after which the cells were labeled with BrdU for 4 hr. No significant growth inhibition was observed, with BrdU labeling indices of 0.31 ± 0.05, 0.27 ± 0.06, and 0.27 ± 0.05 for uninjected cells, buffer-injected cells, or GST–Gax-injected cells, respectively. These results, similar to those observed when recombinant retinoblastoma (Rb) protein was injected into asynchronously cycling Saos-2 cells (Goodrich et al. 1991), indicate that VSMCs are sensitive to growth arrest by GST-Gax only during a short period within the cell cycle. To define the period of gax action in the cell cycle, GST–Gax was microinjected into VSMCs at different times following the addition of serum. S-phase onset was found to occur at 16–18 hr following mitogen stimulation as determined by assay of serum-stimulated [3H]thymidine incorporation ([3H]thymidine for 1 hr at different time points after stimulation (Fig. 2). To determine when Gax is effective in inhibiting cell proliferation relative to the onset of S phase, quiescent VSMCs were microinjected with GST-Gax (0.5 mg/ml) at different times after stimulation with 10% FBS (Fig. 2). The percent inhibition relative to buffer-injected cells was determined. Each data point represents the mean ± s.e. of three experiments.

**Table 1. Effect of microinjected proteins on the serum-induced proliferation of BALB/c 3T3 cells**

| Protein injected | Number of experiments | Total number of cells injected | Mean percent inhibition of FBS-stimulated growth |
|------------------|-----------------------|-------------------------------|-------------------------------------------------|
| Anti-Ras antibody | 2                     | 268                           | 56.5 ± 21.3                                     |
| GST–Gax          | 4                     | 464                           | 43.8 ± 10.9                                     |
| GST–MyoD         | 4                     | 432                           | 43.5 ± 7.6                                      |
| MHOX             | 4                     | 400                           | -3.0 ± 15.1                                     |
| GST              | 2                     | 222                           | -0.2 ± 1.2                                      |

Quiescent subconfluent cultures of BALB/c 3T3 cells were microinjected with recombinant protein and then immediately stimulated with 10% FBS for 24 hr in medium containing BrdU. Afterward the cells were fixed and the percentage inhibition of BrdU labeling was determined. The numbers are reported as the mean ± s.e.

**Figure 2.** Growth inhibition of vascular myocytes by microinjected GST–Gax occurs during the middle and late phases of G1. The time of S-phase onset in rat VSMCs was determined by stimulating quiescent VSMCs with 10% FBS and pulsing with [3H]thymidine for 1 hr at different time points after stimulation (Fig. 2). To determine when Gax is effective in inhibiting cell proliferation relative to the onset of S phase, quiescent VSMCs were microinjected with GST–Gax (0.5 mg/ml) at different times after stimulation with 10% FBS (Fig. 2). The percent inhibition relative to buffer-injected cells was determined. Each data point represents the mean ± s.e. of three experiments.

Critical step late in G1, perhaps at or around the restriction point when cells committed to DNA replication are no longer sensitive to serum withdrawal. The time-dependent action of Gax also provides evidence that the growth inhibition is not the result of toxicity of the recombinant protein.

**Inhibition of cell proliferation by a replication-defective adenovirus encoding gax**

The microinjection data provided preliminary evidence that the Gax homeodomain protein might function as an endogenous regulator of VSMC proliferation. To further explore the growth regulatory properties of gax, a replication-defective adenovirus encoding the full-length rat gax cDNA was constructed (Fig. 3A). The resulting adenovirus, referred to as Ad-Gax, contains the amino-terminal peptide sequence of the influenza hemagglutinin epitope [HA] fused to gax, and its expression was under control of the cytomegalovirus (CMV) promoter. The regulatory properties of this construct were compared to those of a control virus, Ad-β-Gal, which expresses the gene for β-galactosidase under the control of the Rous sarcoma virus (RSV) long terminal repeat promoter unless otherwise noted.

The Ad-Gax construct was used to examine the effect of gax on primary human and rat VSMC cultures, as shown in Figure 3, B and C. We observed a dose-dependent inhibition of DNA synthesis, as determined by [3H]thymidine incorporation, when the cells were transduced with Ad-Gax at multiplicities of infection (m.o.i.s) between 100 and 1000 plaque-forming units.
gax overexpression inhibits cell growth

Figure 3. The effect of adenoviral constructs on cell proliferation in vitro. (A) Structure of the adenovirus encoding the rat gax cDNA. Primary cultures of human [B] or rat [C] VSMCs were made quiescent in low mitogen media (0.5% FBS) for 72 hr prior to addition of virus. Cells were transduced with adenovirus at the indicated m.o.i. for 24 hr. Following transduction, growth medium (15% FBS) was added for 24 hr. [3H]thymidine was added for 6 hr to measure DNA synthesis at 24 hr postserum stimulation, and incorporated label was determined by TCA precipitation. The graphs depict the effect of adenoviral constructs on the serum-stimulated growth of VSMCs by Ad-Gax (•), Ad-RSV-β-Gal (△), and Ad-CMV-β-Gal (○). Viral transduction is shown as the percent of Ad-Gax transduced cells that stained positive for the presence of the HA epitope [●].

The inhibition of cell growth by Ad-Gax was also observed by flow cytometry (Fig. 4). Quiescent rat VSMCs, transduced with adenovirus constructs, were stimulated for 24 hr postinfection with 15% fetal bovine serum (FBS). This resulted in 79 ± 3% of the Ad-Gax-transduced cells being arrested in the G0/G1 phase of the cell cycle, comparable to the uninfected quiescent cells. In contrast, serum-stimulated cells, transduced with Ad-β-Gal or untransduced control cells had only 35 ± 5% or 39 ± 9% cells in the G0/G1 phase, respectively. Similarly, there was a corresponding decrease in the proportion of cells in the S and G2/M phases in Ad-Gax-transduced cells when compared with Ad-β-Gal and mock-transduced cells. Collectively, these data corroborate the microinjection experiments and provide further evidence to indicate that Gax induces arrest in G1, the same phase of the cell cycle in which gax is down-regulated.
gax induces p21 expression in a p53-independent manner

The cdk inhibitor p21 is induced early during the skeletal myogenesis program and regulates the decision between cell cycle withdrawal, leading to differentiation, and cell death [Andrés et al. 1996; Wang et al. 1996]. The putative involvement of p21 and Gax in skeletal muscle development (Skopicki et al. 1997) led us to examine the possibility of p21 involvement in gax-induced cell cycle arrest in VSMCs. Growth and differentiation is influenced by matrix composition [Pauly et al. 1992; Koyama et al. 1996], and analysis of Gax and p21 expression in cultured VSMCs revealed their coregulation by matrices that modulate cell growth. The expression of Gax and p21 was higher in quiescent VSMCs plated on a basement membrane preparation, which promotes differentiation and inhibits proliferation [Li et al. 1994], than in VSMCs plated on a gelatin matrix (Fig. 5). With either matrix composition, both Gax and p21 were down-regulated when cells were exposed to medium containing serum, although the expression of both proteins was more robust in cells plated on basement membrane. Therefore, the coexpression of Gax and p21 under conditions of VSMC quiescence, and their down-regulation by conditions favoring proliferation indicated that p21 could mediate gax-induced growth arrest.

Induction of p21 occurred in p53−/− fibroblasts transiently transfected with the expression plasmid pCGN-Gax, which encodes full-length wild-type gax (Fig. 6A). Gax protein and p21 colocalized in the nucleus, consistent with the known cdk and proliferating cell nuclear antigen (PCNA) inhibitory properties of p21 [Harper et al. 1993; Waga et al. 1994; Guo et al. 1995]. In contrast, up-regulation of p21 expression was not detected when cells were transfected with pCGN-GaxΔHD, which contains a gax construct in which the homeodomain region was deleted (Fig. 6A). Transfection with the wild-type gax construct also had a strong inhibitory effect on DNA synthesis (74% inhibition), as measured by BrdU incorporation, whereas the AHD construct did not affect proliferation. Increased protein levels of this inhibitor were also observed in human, rat, and rabbit VSMCs transduced with the adenovirus construct encoding the gax gene but not in cells transduced with Ad-β-Gal. This induction was notable in rat VSMCs when examined by immunocytochemical methods (Fig. 6B). An increase in p21 mRNA levels was also observed when Ad-Gax-infected cultures of VSMCs were examined by Northern blot analysis, as shown in Figure 6C.

To test whether gax could function to transactivate the p21 promoter, a 2.4-kbp p21 promoter–luciferase reporter construct was cotransfected with wild-type and mutant gax expression plasmids. Cotransfection with pCGN–Gax increased luciferase expression from the p21 promoter by 3.4-fold over levels observed with the empty vector in quiescent cultures of p53−/− mouse embryonic fibroblasts (MEFs) (Fig. 6D). This transactivation of the p21 reporter construct appeared dependent on the ability of Gax to act as a transcriptional regulator, as a gax construct lacking the homeodomain region of the protein pCGN–GaxΔHD was unable to up-regulate luciferase expression compared to control. Taken together, these data suggest a correspondence between inhibition of DNA synthesis and up-regulation of the cdk inhibitor p21, which is consistent with the ability of gax overexpression to induce G1 cell cycle arrest.

gax overexpression inhibits cdk2 activity

Cell cycle progression requires the kinase activity of cyclin-dependent kinase-2 (cdk2) which, in tandem with cyclins A or E regulatory subunits, modulates the function of a number of cell cycle regulatory proteins, including Rb, which are essential for proper cell cycle progression (van den Heuvel and Harlow 1993; Krek et al. 1995; Ohtsubo et al. 1995; Weinberg 1995). Ad-Gax substantially inhibited cdk2 activity in serum-stimulated human and rat VSMCs compared to the Ad-β-Gal-infected control cells (Fig. 7A and B, respectively). However, no decrease in cdk2 kinase activity by Ad–Gax transduction was observed in p21−/− fibroblasts (Fig. 7C) in accordance with the lack of growth inhibitory effect on these cells. In contrast, cdk2 activity was markedly inhibited in p53−/− fibroblasts infected with Ad–Gax but not in cells infected with Ad–β-Gal (Fig. 7D).

To determine the basis for the reduction in cdk2 activity, whole-cell extracts of Ad–Gax and Ad–β-Gal-transduced p53−/− cells (Fig. 8) and VSMCs (data not shown) were examined for levels of various cell cycle components. Despite the marked decrease of cdk2 activity in Ad–Gax-transduced cells, the levels of this kinase were similar in Ad–Gax, Ad–β-Gal and mock-infected cultures (Fig. 8A). However, as can be seen from the anti-p21 immunoblot in Figure 8B, p21 was highly induced in Ad–Gax-transduced cells, whereas no p21 was detected in quiescent, serum-stimulated, or Ad–β-Gal-transduced cells. The p21 in the Ad–Gax-transduced cells was shown to be associated with cdk2 complexes by anti-p21

Figure 5. Endogenous Gax and p21 are expressed in quiescent human VSMCs plated on a basement membrane matrix and are down-regulated in proliferating cells. Primary human VSMC cultures were plated on dishes coated with either gelatin or a basement membrane preparation (Matrigel) in D-Stim containing 5% FBS overnight. Cultures were incubated for 72 hr in either serum-free D-Stim medium to obtain quiescent (Q) cultures or maintained in serum-containing medium to obtain proliferating (P) cultures. Whole-cell extracts (50 μg) were prepared from the cultures, subjected to SDS-PAGE on polyacrylamide gels, and transferred to Immobilon P for immunoblot analysis. Endogenous protein expression was examined with rabbit polyclonal antibodies directed against either Gax or p21.
Figure 6. Gax overexpression induces p21 expression. [A] Gax overexpression in transfected MEFs colocalizes with increased p21 levels. Quiescent p53−/− MEFs were transfected with either pCGN-Gax, expressing the wild-type gax fused to the HA epitope, or pCGN-GaxΔHD, expressing a Gax/HA fusion from which the homeodomain has been deleted, using lipofectamine in low serum medium. Following transfection and 24 hr incubation to allow expression of the transfected gene, cultures were transferred to growth medium for 24 hr. Cultures were then fixed and stained with anti-HA and anti-p21 antibodies. Following treatment with rhodamine-conjugated [red, anti-HA] or fluorescein-conjugated [green, anti-p21] secondary antibodies, cultures were stained for DNA content with Hoechst. [B] Ad-Gax-transduced rat VSMC cultures exhibit increased p21 levels. Quiescent rat VSMC cultures were transduced with either Ad-Gax or Ad-β-Gal at an m.o.i. of 750 PFU/cell for 12 hr. Following infection, incubation was continued for an additional 12 hr in low mitogen media after which cultures were transferred to growth medium for 24 hr. Cells were then fixed and stained with a rabbit anti-p21 primary antibody followed by a rhodamine-conjugated anti-rabbit secondary and stained for DNA with Hoechst. Shown are mock-infected [control], Ad-β-Gal, and Ad-Gax-transduced cells. [C] Ad-Gax transduction of human VSMCs up-regulates p21 expression. Northern blot analysis of p21 expression in adenovirus-infected cultures. Quiescent human VSMCs were transduced with either Ad-Gax, Ad-β-Gal, or uninfected [control], at an m.o.i. of 500 for 1 hr in low mitogen medium. Following infection, the virus was removed and growth medium was added for 24 hr. Total RNA preparations from these cultures were fractionated on agarose gels containing formaldehyde and transferred to nitrocellulose. The filter was hybridized with probes to human p21 cDNA and GAPDH. [D] Gax transactivates expression from the p21 promoter. Quiescent p53−/− MEFs were transfected with a combination of pWWP-Luc, a p21 promoter–luciferase reporter construct, pSV2-AP, which expresses the bacterial alkaline phosphatase gene, and either pCGN-Gax, expressing wild-type gax, pCGN-GaxΔHD, expressing the gax homeodomain deletion, or none. Following transfection, cultures were incubated for an additional 24 hr in fresh medium. Luciferase and alkaline phosphatase activities were measured in whole-cell extracts prepared from the transfected cultures, and luciferase activity was reported as relative light units based on the ratio of luciferase to alkaline phosphatase activity. Each bar represents the mean ± s.e. of three determinations from a representative experiment.

immunoblot analysis of cdk2 immunoprecipitates [Fig. 8C]. This considerable increase in cdk2-associated p21 was not the result of differences in immunoprecipitation efficiency, as can be seen by the comparable levels of material detected by anti-cdk2 immunoblot analysis [Fig. 8D]. Furthermore, the inhibition of cell cycle progression by Ad-Gax does not appear to result from an alteration in cyclin E expression, as can be seen by cyclin E immunoblot analysis [Fig. 8E]. However, consistent with the inability of Ad-Gax-transduced cells to progress through S phase, anti-cyclin A immunoblot analysis indicated that cyclin A expression was substantially diminished in extracts of Ad-Gax-transduced cells, whereas cyclin A levels in the Ad-β-Gal transduced cells were induced above the levels observed in serum-stimulated mock-infected cells [Fig. 8F].

p21 is essential for gax-induced growth arrest

A MEF cell line with a homozygous disruption of the p21
with growth medium (P, Gax, and β-Gal) for 12 hr. Whole-cell DNA synthesis did not result from the inability of adenoviral constructs to transduce p21+/− fibroblasts because immunocytochemical analysis for the HA epitope indicated that the level of gax expression was similar for both cell types. Ad-Gax also inhibited the proliferation of a p53+/− fibroblast reaching 86% inhibition at an m.o.i. of 300 [Ad-Gax (Gax) or Ad-β-Gal (β-Gal)] or uninfected [quiescent (Q) or proliferating (P)] for 12 hr in low mitogen media. Virus was then removed and cells were incubated with low mitogen media for an additional 12 hr before serum stimulation with growth medium (P, Gax, and β-Gal) for 12 hr. Whole-cell extracts [5 μg] were incubated with anti-cdk2 antibody, and immunoprecipitates were assayed for histone H1 kinase activity for 20 min at room temperature. (A) Human VSMCs, (B) rat VSMCs, (C) p21−/− MEFs, (D) p53+/− MEFs.

Localized infection of Ad-Gax inhibits luminal narrowing and neointimal formation in balloon-injured rat carotid arteries

In light of the antiproliferative effects of gax demonstrated in vitro, it was of interest to test whether Ad-Gax could inhibit the proliferative response that occurs upon vessel wall injury using the rat carotid artery model of balloon denudation (Clowes et al. 1983). In this model VSMCs dedifferentiate, proliferate, and migrate to form a neointima that can partially occlude the artery within 2 weeks of the injury. That replication-defective adenovirus vectors can transduce the vessel wall at a high frequency following balloon deendothelialization has been well established [Lee et al. 1993; French et al. 1994; Willard et al. 1994; Chang et al. 1995; Simari et al. 1996]. Rat carotid arteries were denuded with a balloon catheter and exposed immediately to 1 x 10⁶ PFU of Ad-Gax or Ad-β-Gal virus in a solution containing 15% polyoxamer 407, shown previously to facilitate adenovirus infectivity in vitro (March et al. 1995) and in vivo (C. Pastore, L.J. Feldman, M. Perricadet, and P.G. Steg, unpubl.). Following a 20-min incubation, the virus solution was withdrawn and the ligatures were removed to restore circulation. Rats were sacrificed 2 weeks later and quantitative morphometric analyses were performed on cross sections of the treated vessels (Fig. 10).

All nine Ad-β-Gal-transfected carotid arteries developed considerable neointimal thickening: The area of the neointima was 0.186 ± 0.02 mm² with a range of 0.10–0.28 mm² [Fig. 10A]. Luminal patency was correspondingly narrowed by 40 ± 4% (range of 21%–63%). The intima/media ratio was 1.51 ± 0.1 (range of 0.87–2.17). The results are similar to those for saline-treated control vessels reported previously by our laboratory [Pastore et al. 1995]. In contrast, Ad-Gax treatment markedly reduced the hyperproliferative response to balloon injury. For the Ad-Gax-treated vessels the mean area of the neointimal lesions was 0.076 ± 0.02 mm² (range of 0.0–0.19 mm²),

Figure 7. Inhibition of cdk2 kinase activity by Ad-Gax is mediated by p21. Cells were infected with adenovirus at an m.o.i. of 300 [Ad-Gax (Gax) or Ad-β-Gal (β-Gal)] or uninfected [quiescent (Q) or proliferating (P)] for 12 hr in low mitogen media. Virus was then removed and cells were incubated with low mitogen media for an additional 12 hr before serum stimulation with growth medium (P, Gax, and β-Gal) for 12 hr. Whole-cell extracts [5 μg] were incubated with anti-cdk2 antibody, and immunoprecipitates were assayed for histone H1 kinase activity for 20 min at room temperature. (A) Human VSMCs, (B) rat VSMCs, (C) p21−/− MEFs, (D) p53+/− MEFs.

Figure 8. Immunoblot analyses of cyclins, cdk2, and p21 in p53+/− MEFs. p53+/− MEFs were infected with adenovirus at an m.o.i. of 300 [Ad-Gax (Gax) or Ad-β-Gal (β-Gal)] or uninfected [quiescent (Q) or proliferating (P)] for 12 hr in low mitogen media. Virus was removed and cells were incubated with low mitogen media for an additional 12 hr before serum stimulation with growth medium (P, Gax, and β-Gal) for 12 hr. Whole-cell extracts [50 μg] were prepared from the cultures, subjected to SDS-PAGE on 15% polyacrylamide gels, and transferred to Immunobond-P for immunoblot analysis with antibodies against the indicated protein (C,D). (A) Anti-p21; (B) anti-cdk2; (C) anti-p21 of cdk2 IP; (D) anti-cdk2 of cdk2 IP; (E) anti-cyclin E; (F) anti-cyclin A.
gax overexpression inhibits cell growth

Figure 9. Inhibition of \[^{3}H\text{thymidine incorporation in p21}^{+/+}\text{ and p53}^{--/} \text{ but not p21}^{--/} \text{ MEFs by Ad-Gax transduction. MEFs were made quiescent in low mitogen medium (0.5% FBS) for 72 hr. Cultures were transduced with adenovirus at the indicated m.o.i. for 24 hr in low mitogen medium. Following transduction, growth medium (10% FBS) was added and the cells were allowed to progress into the cell cycle. \[^{3}H\text{thymidine was added to measure DNA synthesis at 14 hr postserum stimulation and incorporated label was determined by TCA precipitation. Cultures were transduced with either Ad-Gax or Ad-β-Gal. Viral transduction is shown as the percent of Ad-Gax-transduced cells that stained positive for the presence of the [HA] epitope.}\] Each point represents the mean ± s.e. of three determinations from a representative experiment.

Discussion

Deregulated homeobox gene expression can lead to enhanced cell growth and tumorigenicity (Aberdam et al. 1991; Song et al. 1992; Deguchi and Kehrl 1993). Homeobox factors may also function as inhibitors of cell growth consistent with their roles in cellular differentiation. In cultured VSMCs, expression of the gax homeobox gene is rapidly down-regulated by mitogens and is gradually up-regulated by conditions that lead to growth arrest (Gorski et al. 1993). As a homeobox gene, gax is a unique member of the gas and gadd gene families (Schneider et al. 1988; Fornace et al. 1989) whose expression in vitro is repressed by mitogens. Here we demonstrate an inhibition of cell growth when cells are either microinjected with recombinant Gax homeodomain protein or when they are transduced with a replication-defective adenovirus that encodes the gax gene. Recombinant Gax protein inhibited mitogen-stimulated S-phase entry in VSMCs when microinjected into cells either immediately prior to mitogen stimulation or early in the G1 phase of the cell cycle when the endogenous gax gene is down-regulated. In addition, a replication-defective adenovirus expressing the rat gax gene exhibited a dose-dependent inhibition of VSMC proliferation and arrested the cells in G1.

A number of lines of evidence suggest that the cdk inhibitor p21 mediates the growth inhibitory actions of gax. Ectopic gax expression, with either plasmid or adenoviral vectors, led to substantial up-regulation of p21 protein expression in VSMCs as well as in fibroblasts that lack p53. The induction of p21 was also detected at the level of mRNA, and the ability of gax to transactivate the p21 promoter by a factor of 3 suggests a transcriptional link between gax and this cyclin-dependent kinase inhibitor that can partially account for this up-regulation. Others have also reported a three- to fivefold stimulation of the p21 promoter by CCAAT/enhancer-binding protein α (C/EBPα) Timchenko et al. 1996), MyoD (Halevy et al. 1995), or the vitamin D3 receptor (Liu et al. 1996). The regulation of p21 by gax is remarkably similar to that reported for C/EBPα, which induces p21 mRNA and promoter activity 3-fold but increases p21 protein 12- to 20-fold.

gax overexpression was also found to inhibit cdk2 activity in VSMCs and p53^-/- MEFS. This reduction in kinase activity correlated with a greatly enhanced association of p21 with cdk2. The assertion that p21 fulfills an essential function in gax-mediated growth arrest was further supported by the finding that p21^-/- MEFS were not susceptible to growth inhibition upon transduction with Ad-Gax. In contrast, isogenic wild-type MEFS or p53^-/- MEFS were readily growth arrested by gax overexpression. The p53-independent up-regulation of p21
Figure 10. Ad-Gax inhibits neointimal formation and luminal narrowing in the rat carotid model of restenosis. (A) Cross sections of rat carotid arteries reveal Ad-Gax-specific inhibition of neointimal formation and luminal narrowing. The common carotid arteries of male Sprague-Dawley rats were deendothelialized by distention with a balloon catheter to induce the hyperproliferation of vascular myocytes. The de-endothelialized region was then treated with 1 x 10⁹ PFU of adenovirus in a solution of 15% poloxamer 407 for 20 min. The rats were sacrificed at 14 days following treatment, and the initially de-endothelialized region of each artery was recovered at necropsy. Tissue was fixed in 100% methanol prior to paraffin embedding and staining. Hematoxylin-eosin-stained cross sections from the arteries of three rats treated with Ad-β-Gal or 9 rats treated with Ad-Gax are shown. Arrows indicate the internal elastic lamina.

(B) Histological images from the cross sections of arteries treated with either Ad-β-Gal or Ad-Gax were projected on a digitizing board and the luminal, intimal, and medial areas were measured by quantitative morphometric analysis using a computer sketching program. From these measurements the percent luminal narrowing and the intima to media ratio were calculated. Values represent the mean ± s.e. and were compared for statistical significance by an unpaired two-tailed Student’s t-test: P < 0.003 (∗); P < 0.001 (∗∗); P < 0.0001 (∗∗∗).

has also been implicated in the withdrawal of skeletal myocytes from the cell cycle in the process of terminal differentiation (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995; Andrés and Walsh 1996). Thus, the requirement for p21 in gax-induced growth arrest indicates similarities in the regulatory controls of VSMC and skeletal muscle proliferation.

Ectopic expression of several inhibitory proteins by adenovirus can prevent the hyperproliferative response of VSMCs in both the rat carotid artery (Guzman et al. 1994; Chang et al. 1995a; Rade et al. 1996) and the porcine femoral artery (Ohno et al. 1994) systems, and the utility of this vector is presumed to be attributable to the high efficiency of adenoviral infection, reported to be as much as 70% (Chang et al. 1995; Simari et al. 1996). Recently, we found that gax is down-regulated in the rat carotid artery in response to a balloon injury that exposes medial VSMCs to growth stimulatory signals (Weir et al. 1995). When the Ad-Gax virus was tested for its effects on lesion formation following denudation of the rat carotid artery endothelium by balloon angioplasty we found that the intimal area was reduced by 59% and the neointimal-to-medial ratio by 69% [P < 0.0001 and P < 0.001, respectively, using a two-tailed unpaired t-test]. Luminal narrowing was also inhibited by 56% [P < 0.003]. The fact that gax was able to potentiate inhibit neointima formation strengthens the proposition that it can function to modulate the high proliferative potential of VSMCs.

These findings with gax compare favorably with results reported by others using adenovirus-encoded genes for a mutant of Rb (Chang et al. 1995), the herpes simplex virus thymidine kinase gene (HSV-tk) (Guzman et al. 1994), p21 (Chang et al. 1995a), and hirudin (Rade et al. 1996) in the injured rat carotid model. Furthermore, correlative evidence suggests that p21 may function in the cessation of neointima formation in a porcine model of stenosis (Yang et al. 1996). Overall, the inhibition of
the intima-to-media ratio by these agents ranged from 35%-46%. The finding that gax up-regulates p21 expression indicates that the inhibitory effect of gax on neo-intima formation may be exerted, at least in part, by this cyclin kinase inhibitor. Consistent with this hypothesis, we find a smaller percentage of PCNA-positive cells in the Ad-Gax-treated vessels than in the control group [R.C. Smith, unpubl.]. gax overexpression may have other effects on the vessel wall in addition to its anti-proliferative activity. Homeobox transcription factors may also function to promote differentiation, modulate matrix-adhesion molecule interactions [Edelman and Jones 1993], or affect cell migration [Salser and Kenyon 1992; Niehrs et al. 1993]. Because these factors may have pleiotropic regulatory functions they may prove to be effective molecular targets for the management of postinterventional restenosis [Walsh and Perelman 1996]. Therefore, further analyses of gax-transduced cells may reveal mechanisms that coordinate VSMC cell cycle with other phenotypic manifestations of mitogen activation.

Although growth factors and signaling cascades have been studied extensively in VSMCs [Nilsson 1986; Ross et al. 1990; Ross 1993], little is known about the nuclear targets of the signaling pathways that influence the proliferation and differentiation of these cells. The down-regulation of gax expression in VSMCs in response to either mitogen stimulation or vessel wall injury suggests that this homeobox transcription factor might serve as a control point through which signals to proliferate and differentiate are coordinated. The data presented here show that overexpression of gax, an endogenous VSMC protein, can function to negatively regulate cell proliferation, and as such it may prove to be a valuable tool for further studies on the mechanisms of smooth muscle dedifferentiation and proliferation that is a characteristic pathological feature of a number of vascular diseases, including the restenosis that frequently occurs following balloon angioplasty of occluded arteries.

**Materials and methods**

**Cell lines**

Human VSMC lines were either purchased from Clonetics Corporation [San Diego, CA] and grown according to the vendor’s instructions or derived as described previously [Pickering et al. 1992] from unused saphenous vein segments excised at the time of coronary bypass surgery at St. Elizabeth’s Medical Center. Human VSMC cultures obtained from saphenous vein were used before passage 10 and were maintained in high glucose Dulbecco’s modified Eagle medium [DMEM] (GIBCO BRL, Gaithersburg, MD) containing 100 U/ml of penicillin G, 100 U/ml of streptomycin sulfate [GIBCO BRL], and 15% FBS [GIBCO BRL]. Rat primary VSMCs were obtained by enzymatic digestion of the media from the thoracic aorta of male Sprague-Dawley rats according to the method of Mader (1992) and used before passage 10 in the same media as for human VSMCs. Prior to use, primary smooth muscle cell cultures were stained with a monoclonal antibody to smooth muscle α-actin (Sigma Chemical Co., St. Louis, MO) to verify purity. MEFs with either wild-type p21 allele (+/+) or a homozygous disruption [-/-] [Deng et al. 1995] were maintained in DMEM with penicillin/streptomycin and 10% FBS. MEFs with a homozygous deletion of the p53 allele designated [10/1] [Harvey and Levin 1991] were obtained from Arnold J. Levine and maintained in DMEM with penicillin/streptomycin and 10% FBS. BALB/c 3T3 cells were obtained from the American Type Culture Collection and were maintained in DMEM with penicillin/streptomycin and 10% FBS. Synchronous populations of cells were obtained by placing cultures in media containing 0.5% FBS for 48-72 hr. Viral transduction was conducted using mitogen-deprived quiescent cultures in fresh low mitogen medium containing 0.5% FBS.

Plates for studies involving matrix-dependent properties were coated with either 2% gelatin [Sigma, St. Louis, MO] or 100 μg/ml of growth factor-reduced Matrigel [Taub et al. 1990] [Becton Dickinson Labware, Bedford MA] in PBS for 1 hr at 37°C, and human VSMCs were plated at 10%-20% confluency in D-Stim (Becton Dickinson Labware) containing 5% FBS and allowed to attach overnight. Quiescent cultures were incubated for 72 hr in D-Stim basal medium.

**Production of recombinant proteins**

To produce recombinant proteins for microinjection, the cDNA coding regions for myoD [Lassar et al. 1989], gax [Genoki et al. 1993], and YY1 [Park and Atkinson 1991; Shi et al. 1991] were fused in-frame to the pGEX-2T expression vector, expressed in Escherichia coli, and the resultant GST fusion proteins were purified by affinity chromatography on glutathione–agarose beads [Smith and Johnson 1988]. Protein purity was >90% as estimated by SDS-polyacrylamide gels stained with Coomassie blue. To produce recombinant MHox [Cserjesi et al. 1992], its cDNA was fused in-frame to the pQE-9 vector, expressed in bacteria, and purified by adsorption to a nickel column [Hochuli et al. 1988]. Gel mobility shift assays were performed using DNA probes containing the binding site for each factor: for MHox, the A/T-rich site in the mouse muscle creatine kinase enhancer (MCK) was used as a probe [Cserjesi et al. 1992]; for GST–YY1, the muscle regulatory element of the chicken skeletal actin promoter, –100 to –75 nucleotides from the start of transcription [Gualberto et al. 1992]; for GST–MYOD, the right E-box of the mouse MCK enhancer [Lassar et al. 1989]. Because Gax also binds to the MHox A/T-rich site in the MCK enhancer (not shown) and because its optimal binding site has not been reported yet, activity of recombinant GST–Gax was checked by gel shift using this site. For microinjection, proteins were concentrated in a buffer consisting of 20 mM Tris, 40 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, and 2% glycerol using Centricon-30 [Amicon, Beverly, MA] microconcentrators. Concentrated proteins were stored in this buffer in aliquots at –80°C.

**Microinjections and cell proliferation assays**

Prior to being microinjected, VSMCs were plated on gridded coverslips, allowed to grow to ~50%-75% confluence, then rendered quiescent by incubating in medium containing 0.5% calf serum for 3-4 days. In each experiment, using a semiautomated microinjection apparatus [Eppendorf Inc., Madison, WI], 100-250 cells per experimental group in defined areas of a coverslip were injected with either microinjection buffer alone or with protein. Concentrations of injected proteins: Y13-259 anti-ras antibody and mouse anti-human IgG, 8 mg/ml; GST–Gax, 0.5 mg/ml; MHox, 1.6 mg/ml; GST–MYOD, 2 mg/ml; GST–YY1, 1.2 mg/ml; and GST alone, 2 mg/ml. Following the completion of the injections the cells were stimulated with 10% FBS supplemented with BrdU for 24 hr [Ansorge et al. 1988; Pep...
were then fixed in ice-cold acid-ethanol and BrdU-labeled nuclei were detected using a cell proliferation assay kit (Amer- sham, Arlington Heights, IL), which utilizes a monoclonal anti- body to BrdU and a secondary anti-IgG antibody conjugated to horseradish peroxidase (HRP), according to the instructions of the kit’s manufacturer. The anti-ras antibody Y13-259 (Mulcahy et al. 1985) was the kind gift of D. Stacey (Cleveland Clinic Foundation, OH).

Percentage inhibition was calculated by the following equa- tion:

\[
\% \text{Inhibition} = \left( \frac{CL}{IT} - \frac{IL}{IT} \right) \times 100
\]

where, for each experiment, IL represents the number of cells injected with recombinant protein that labeled positive for BrdU; IT, the total number of cells injected with a control protein [GST alone] labeling with BrdU; and CT, the total number of cells injected with a control protein [GST]. Negative numbers represent a stimulation of cell growth, using this formula. To determine when S-phase onset occurs in VSMCs, quiescent cells were stimulated with 10% FBS and pulse-labeled with [3H]thymidine (5 μCi/ml) for 1 hr at different times after stimulation. Then, quiescent VSMCs were microinjected both immediately prior to and at different times after stimulation with 10% FBS followed by labeling with BrdU beginning at 10 hr after stimulation. At 24 hr after stimulation the cells were fixed and the percent inhibition determined.

**Construction of Gax plasmids**

The rat gax open reading frame (ORF) was obtained as an Xbal–BamHI fragment by PCR amplification of a plasmid derived from a K ZAP cDNA clone described previously (Gorski et al. 1993). This Xbal–BamHI fragment was inserted between the Xbal and BamHI sites of the pCGN vector (Tanaka and Herr 1990), resulting in an in-frame fusion of the gax gene, starting at codon 2 of the putative ORF (Gorski et al. 1993), to the amino-terminal part of the influenza virus HA epitope (Field et al. 1988). This plasmid, designated pCGN–Gax carries the CMV early promoter upstream of the tk gene 5'-untranslated region (UTR), containing untranslated leader sequences, the HA–Gax fusion protein, and the rabbit β-globin 3' UTR containing splicing and poly[A] signal addition sequences.

A homeodomain deletion of gax lacking amino acids 188–245 was derived from pCGN–Gax by digestion of that plasmid with Psrl and isolation of the two restriction fragments of 5520 and 430 bp. The 430-bp fragment containing internal sequences from the gax ORF was further digested with AvrII and the 230-bp fragment was isolated. The Psrl–AvrII fragment was ligated to synthetic oligonucleotides (5'-CTAGGGGAGGACACACAAAGGAGCTGCA-3' and 5'-GCTCCTTGTCTTGGGCTCCCC-3') and the product was ligated to the 5520-bp Psrl fragment of pCGN–Gax to generate pCGN–Gax3HD containing a deletion of the homeobox domain and an in-frame fusion of the carboxyl and amino termini.

The nucleotide sequence of inserts was verified by the cycle- sequencing method using fluorescent dideoxy terminator nucleotides on an Applied Biosystems 373A automated DNA sequencer. Analyses of derived sequences were conducted using MacVector and Sequence Navigator software on Macintosh Quadra computers.

**Replication-defective adenovirus construction and preparation**

An XmnI–SfiI fragment containing the hybrid gax expression cassette from pCGN–Gax was inserted at the EcoRV site of the pCOI vector containing the Ad5 adenoviral sequences required for homologous recombination. The resulting plasmid was linearized by XmnI and cotransfected in 293 cells with the large Clcl fragment of the Ad5 d1324 viral DNA (Stratford-Perricau det et al. 1993). The resulting replication-defective recombinant adenoviruses were purified from isolated plaques and viral DNA prepared. Recombinant adenoviruses containing the gax cDNA were identified by restriction fragment analysis and amplified in 293 cells. At the early steps of amplification, the transduction efficiency of the recombinant adenoviruses was also assessed in primary smooth muscle cells by immunofluorescence with anti-HA antibodies as described below. The viral preparations used for both in vitro and in vivo studies were purified by two CsCl gradient centrifugations, dialyzed against buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM MgCl2, and 135 mM NaCl and stored at -80°C in 10% glycerol. Viral titer was determined by plaque assay on 293 cells as described previously (Graham and von der Eb 1973) and expressed as PFU/ml. The construction of the control Ad-β-Gal used in both the in vitro and in vivo studies has been described previously (Stratford-Perricaut et al. 1993). This E1-deleted recombinant adenovirus expresses the β-galactosidase gene containing a nuclear localization signal, under the transcriptional control of the RSV long terminal repeat promoter.

**Adenovirus infection in vitro and analyses of cell proliferation**

Cells were plated at ~10% confluence in six-well tissue culture dishes in DMEM containing 15% FBS overnight. Cultures were made quiescent by incubation for 3 days in DMEM containing 0.5% FBS. Cells were harvested by trypsinization and the cell number per well was determined by counting with a hemacytometer. Viral dilutions were prepared from purified viral stocks in DMEM containing 0.5% FBS, and infections were conducted in 0.5 ml/well for 24 hr. At the end of the infection period the virus-containing medium was removed and the cells were washed once with PBS. The culture medium was changed to growth medium, and the infection continued (14 hr for rat or mouse cells, 24 hr for human cells) to allow cultures to enter S phase. Cell proliferation was determined by [3H]thymidine incorporation during S phase in medium containing 3 μCi/ml of [3H]thymidine (6.7 Ci/m mole, DuPont NEN, Boston, MA) for 4–6 hr. Wells were washed twice with PBS, and cold 10% TCA was added. After 1 hr the TCA was removed, the wells were rinsed twice with water, and the precipitated material was solubi- lized with 0.25 m sodium hydroxide. Tritium content of the sodium hydroxide solution was determined by liquid scintilla- tion counting in Scintiverse II (Fisher Scientific, Pittsburgh, PA) utilizing a Beckman LS 5000TD scintillation counter.

The infection rate was determined by staining for HA expression in the Ad-Gax-treated cultures. Cells were fixed with for 5 min with HC tissue fixative MB (Amresco, Solon, OH), permeabilized for 10 min with 0.25% NP-40, and treated with 0.03% sodium azide solution for 10 min. Nonspecific binding was blocked with normal goat serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) followed by incubation of the cul-
ture with 0.10 μg/ml of solution of a mouse monoclonal anti-HA antibody (Boehringer Mannheim, Indianapolis, IN) in PBS containing 1% BSA overnight at 4°C. Primary antibody was detected by incubation with a biotin-conjugated goat anti-mouse antibody (Kirkegaard & Perry) for 1 hr at room temperature followed by streptavidin-conjugated HRP (Kirkegaard & Perry) for 20 min. The complex was visualized using TrueBlue peroxidase substrate (Kirkegaard & Perry), and the number of labeled cells determined from photographs of microscopic fields.

**Flow cytometric analysis of transduced VSMC cultures**

Primary rat VSMCs were plated in six-well dishes in growth medium (15% FBS) and allowed to attach before being transferred to low mitogen (0.5% FBS) medium for 72 hr. Cell number was determined and cultures were incubated with either Ad-Gax or Ad-β-Gal at an m.o.i. of 750 for 1 hr 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 hr. The cultures were then stimulated to enter the cell cycle for 24 hr by the addition of growth (15% FBS) medium. Cells were harvested by trypsinization, fixed overnight in 70% ethanol, and stained for DNA content in propidium iodide staining reagent overnight. The cell cycle profile was determined 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).

**Immunocytochemical analysis**

Rat VSMCs or p53−/− MEFs were plated at <20% confluent in growth media on acid-washed glass coverslips. Cultures were transferred to low mitogen (0.5% FBS) medium for 48 hr before viral infection. Adenovirus infection was carried out in low serum medium for 12 (p53−/− or 24 (rat VSMCs) hr after which the virus solution was removed and replaced with growth medium containing 10 μM BrdU. At 24 hr after serum stimulation the cells were fixed for 10 min with 4% neutral buffered formalin and permeabilized with 0.5% NP-40 for 5 min. When BrdU staining was utilized, coverslips were treated sequentially with 2 N HCl for 30 min at 37°C, 0.1 M sodium tetraborate (pH 8.5), twice for 10 min each and then for 30 min at 37°C with 0.2% pepsin, 0.01 M HCl. All coverslips were incubated for 30 min with blocking solution [2% goat serum, 0.1% NP-40, 0.01% sodium azide in PBS] prior to addition of primary antibody. Primary antibody(ies) was incubated overnight at 4°C in blocking solution. A mouse monoclonal anti-HA antibody (cat. no. 1583816, Boehringer Mannheim) was used at 4 μg/ml and a rabbit anti-p21 antibody (cat. no. SC-397, Santa Cruz Biotechnology, Inc., CA) was used at 0.16 μg/ml for Figure 7A. FITC-conjugated mouse monoclonal anti-BrdU (cat. no. 1202693 Boehringer Mannheim) was used at 0.4 μg/ml. Antibodies were removed by washes with PBS and coverslips were incubated with secondary antibody(ies) for 45 min. A rhodamine-conjugated goat anti-mouse secondary antibody (cat. no. 03-18-06, Kirkegaard & Perry) and a FITC-conjugated goat anti-rabbit antibody (cat. no. 02-15-06, Kirkegaard & Perry) were used at 2.5 μg/ml for Figure 7A. A rhodamine-conjugated goat anti-rabbit secondary antibody (cat. no. 03-15-06, Kirkegaard & Perry) was used at 2.5 μg/ml in PBS for Figure 6B. Following antibody treatments coverslips were treated with 0.01% Triton X-100 in PBS and stained for 2 min with a 0.1 μg/ml of Hoechst no. 33528 (Boehringer Mannheim) to visualize nuclear DNA. Coverslips were mounted with fluorescence-mounting media (Kirkegaard & Perry) and visualized by fluorescence microscopy utilizing a Nikon Diaphot microscope equipped with a Nikon Epi-Fluorescence attachment and a high-pressure mercury light source. DNA content was visualized under UV illumination with a Chroma UV-2A filter, rhodamine with a Nikon DMS10 filter, and fluorescein with a Nikon DMS80 filter.

**Analysis of p21 mRNA expression in adenovirus-transduced human VSMCs**

Quiescent human vascular smooth muscle cells were infected with adenovirus at an m.o.i. of 500 for 1 hr in low serum medium (0.5% FBS) before being transferred to growth medium (5% FBS). Cultures were harvested for RNA preparation as described previously (Chomzynski and Sacchi 1987) after 24 hr of serum stimulation. Northern blot analysis was performed using 20 μg of RNA fractionated by formaldehyde-denaturing agarose gel electrophoresis and transferred to nitrocellulose. Integrity of the RNA preparation was ascertained by examination of the 18S and 28S rRNA bands. Hybridization was for 24 hr at 42°C in 5x SSPE, 5x Denhardt's reagent, 0.5% formamide, 0.5% SDS, 10% dextran, and 0.5 mg/ml of sonicated salmon sperm DNA using a full-length p21 [WAFl] probe prepared by random priming (Rediprime, Amersham) according to the manufacturer's instructions. The filter was stripped in water at 65°C for 45 min before being probed for GAPDH.

**Transactivation of the p21 promoter**

Subconfluent p53−/− MEFs were plated in six-well plates and made quiescent in low serum medium (0.5% FBS) by incubation for 48 hr. Transfections utilized 21 μg of lipoctepe (GIBCO BRL), 1 μg of a p21 promoter–luciferase reporter construct, pWWP-Luc (E1-Deiry et al. 1993), 0.6 μg of pSV2-AP (Henthorn et al. 1988) containing the bacterial alkaline phosphatase gene under control of the SV40 promoter/enhancer, to provide an internal control for transfection frequency, and 2 μg of either pCGN–Gax, pCGN–GaxAHD, or none. Transfections were carried out for 4 hr in low serum medium after which the cultures were incubated for an additional 24 hr in fresh low-serum medium. Whole-cell extracts were prepared as described previously (Andrés et al. 1995). Alkaline phosphatase activity of transfected cells was measured using CSPD chemiluminescent substrate (Tropix, Bedford, MA), and luciferase activity was measured using the Luciferase Assay System from Promega (Madison, WI). Measurements were made for 5 sec on a LB 9501 Lumat luminometer [EG & G Berthold], and activity was reported as relative light units based on the ratio of luciferase to alkaline phosphatase activity.

**Preparation of anti-Gax antibody**

The carboxy-terminal peptide from the published gax sequence, amino acids 282–302 (Gorski et al. 1993), was cross-linked to rabbit serum albumin. The cross-linked peptide (500 μg) was prepared in Freund's complete adjuvant and inoculated via intramuscular injection into male New Zealand white rabbits. The rabbits were given five booster inoculations at 2-week intervals with 500 μg of cross-linked peptide in incomplete Freund's adjuvant. Bleeds were collected from the ear vein, and IgG was affinity purified from serum utilizing peptide linked to agarose [SulfoLink, Peirce, Rockford, IL]. Antibody was titered by ELISA utilizing the antigenic peptide coated on 96-well plates.
cdk2 kinase assay of adenovirus-transduced cells

Cells were plated at subconfluent levels in growth media and growth arrested in low mitogen media for 48 hr prior to viral transduction. Cultures were infected at an m.o.i. of 300 for 12 hr in low mitogen medium. Cell extracts were prepared and cdk activity was measured as reported previously (Guo et al. 1995). cdk2 assays were conducted using 5 μg of cell extract.

Immunoprecipitations and immunoblot analysis

Cells were plated at subconfluent levels in growth media (15% FBS). The next day the cultures were transferred to low mitogen (0.5% FBS) medium for 48–72 hr. Cell number was determined by harvesting with trypsinization and cells were counted on a hemacytometer. The m.o.i. was calculated, and cultures were incubated with the appropriate amount of virus (m.o.i. of 300) for 12 hr in low mitogen medium. Cultures were then washed free of virus, fresh low-mitogen medium was added, and the incubations were continued for an additional 12 hr. Following infection, the cultures were transferred to growth medium for 12 hr and the cultures were used to prepare whole cell extracts as reported previously (Andrés et al. 1995) and stored at -80°C.

Immunoprecipitations were conducted with 125 μg of the appropriate whole-cell extract in RIPA buffer containing 0.3 trypsin inhibitor units/ml of aprotinin (cat. no. A6279, Sigma), 100 μg/ml of phenylmethylsulfonyl fluoride (cat. no. P7626, Sigma). Extracts were precleared with 1 μg of rabbit IgG (cat. no. 1-1000, Vector Laboratories, Burlingame, CA) and 20 μl of protein A-agarose (cat. no. 1719408, Boehringer Mannheim) for 1 hr at 4°C. The gel was removed by centrifugation for 5 min at 1500 rpm, and the supernatant was incubated with 1 μg of a rabbit anti-cdk2 antibody (cat. no. sc-505, Santa Cruz Biotechnology) for 1 hr on ice. Following this incubation, 20 μl of protein A-agarose was added, and the immunoprecipitation was continued overnight at 4°C on a rotating platform. The immunoprecipitate was harvested by centrifugation and washed four times with RIPA buffer containing protease inhibitors. The washed gel was resuspended in 20 μl of 1.5× SDS sample buffer and denatured for 3 min in a boiling water bath. Twenty microliters of each supernatant was analyzed by SDS-PAGE on a 8.5–16% polyacrylamide minigel under reducing conditions (β-mercaptoethanol).

Protein transfer to Immobilon-P was conducted by the semidyry method on a Hoefer apparatus at 1.3 mAMPS/cm² for 60 min. The resulting blots were incubated in 2% milk/TBS-T containing the appropriate antibody overnight at 4°C with agitation. Blots were rinsed briefly in TBS-T, and washed in TBS-T for 15 min and in two additional 5-min washes. The blots were incubated with an appropriate HRP-conjugated goat secondary antibody (Amersham) in 2% milk/TBS-T at room temperature for 1 hr and washed. The antigen–antibody complex was visualized by incubation for 1 min with the Amersham ECL reagent at room temperature and exposed to Kodak XAR-5 film.

Other immunoblot analyses were conducted using 50 μg of each extract denatured under reducing conditions. SDS-PAGE, transfer to Immobilon-P, and the immunodetection of proteins were as described above. Immunoblot analyses were conducted using anti-Gax at 0.9 μg/ml, anti-p21 (cat. nos. sc-397 and sc-397-G, Santa Cruz), rabbit anti-cdk2 (cat. no. sc-163, Santa Cruz), and rabbit anti-cyclin E (cat. no. sc-481, Santa Cruz) antibodies at 0.5 μg/ml. Immunoblot analysis was conducted using the rabbit anti-cyclin A antibody (cat. no. sc-751, Santa Cruz) at 0.05 μg/ml.

Statistical analysis

Results were expressed as the mean ± S.E.M. Differences between groups were analyzed using an unpaired two-tailed Student’s t-test. Statistical significance was assumed when the probability of a null hypothesis was <0.05.

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