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Research Article

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Role of c-fos and E2F in the Induction of Cyclin A Transcription and Vascular Smooth Muscle Cell Proliferation

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Abstract

Excessive proliferation of vascular smooth muscle cells (VSMCs) contributes to vessel renarrowing after angioplasty. Here we investigated the transcriptional regulation of the cyclin A gene, a key positive regulator of S phase that is induced after angioplasty. We show that Ras-dependent mitogenic signaling is essential for the normal stimulation of cyclin A promoter activity and DNA synthesis in VSMCs. Overexpression of the AP-1 transcription factor c-fos can circumvent this requirement via interaction with the cAMP-responsive element (CRE) in the cyclin A promoter. Moreover, c-fos overexpression in serum-starved VSMCs results in the induction of cyclin A promoter activity in a CRE-dependent manner, and increased binding of endogenous c-fos protein to the cyclin A CRE precedes the onset of DNA replication in VSMCs induced by serum in vitro and by angioplasty in vivo. We also show that E2F function is essential for both serum- and c-fos–dependent induction of cyclin A expression. Taken together, these findings suggest that c-fos and E2F are important components of the signaling cascade that link Ras activity to cyclin A transcription in VSMCs. These studies illustrate a novel link between the transcriptional and cell cycle machinery that may be relevant to the pathogenesis of vascular proliferative disorders. (*J. Clin. Invest.* 1998. 101:940–948.) Key words: vascular smooth muscle cell proliferation • cyclin A • Ras • c-fos • E2F

Introduction

A fully differentiated phenotype and low proliferative rate characterize vascular smooth muscle cells (VSMCs)[1] in adult animals. However, in response to different forms of insult to the vessel wall, VSMCs undergo abnormal proliferation and migration and organize into an atherosclerotic lesion that compromises normal blood flow (1–5). Excessive proliferation of VSMCs is also thought to contribute to restenosis, a complication that limits the long-term success of revascularization in $\sim$ 25–55% of patients undergoing percutaneous transluminal coronary angioplasty (6–8). Although previous studies using antisense oligonucleotides and arterial gene transfer strategies have implicated both positive and negative regulators of VSMC growth in lesion development after angioplasty (9–27), little is known about the transcriptional networks governing the expression of cell cycle control genes in VSMCs.

Progression through the mammalian cell cycle is regulated essentially by the balance between multiple cyclin-dependent kinase (CDK)/cyclin holoenzymes and growth suppressor proteins (28–33). Recent studies suggested that Ras-activated mitogenic signaling promotes cell proliferation via activation of the G1 CDK/cyclin/E2F pathway (34–40), and inhibition of cellular Ras prevents VSMC proliferation after vascular injury in vivo (41, 42). We have shown recently that injury-induced VSMC proliferation in rat and human arteries is associated with a temporally and spatially coordinated induction of CDK2 and its regulatory subunits, cyclin E and cyclin A (43). To elucidate molecular mechanisms underlying Ras-dependent induction of cell cycle control genes that may be relevant to the pathogenesis of vascular proliferative disorders, we have analyzed the transcriptional regulation of cyclin A gene expression in VSMCs. Our findings suggest that the interaction of c-fos with the cAMP-responsive element (CRE) site at position $-79/-72$ in the cyclin A promoter is an important component of the signaling cascade that links Ras activity to cyclin A expression and VSMC proliferation. We also show that both this CRE site and the E2F motif at $-37/-32$ are essential for the normal serum- and c-fos–dependent induction of cyclin A promoter activity in VSMCs.

Methods

Transient transfections and luciferase assays. The cloned PAC1 rat pulmonary arterial smooth muscle cell line was used in these studies, which has been shown to maintain through multiple subcultures many differentiated properties of intact VSMCs and can be transfected efficiently (44). Cells were seeded into 6-well dishes and maintained in

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; CMV, cytomegalovirus; CRE, cAMP-responsive element; CREB, CRE-binding protein; EMSA, electrophoretic mobility shift assay; RSV, Rous sarcoma virus; VSMC, vascular smooth muscle cell.

M199 medium supplemented with 20% FBS as described previously (9). The next day, cells (~ 60–80% confluence) were transiently transfected with Lipofectamine reagent (GIBCO BRL, Gaithersburg, MD) and the indicated reporter constructs and expression vectors (1:3 DNA/Lipofectamine ratio). To determine the efficiency of transfection, PAC1 cells were transfected with 1–2.5 μg of pHook-3*LacZ* expression vector (Invitrogen Corp., Carlsbad, CA) which expresses β-galactosidase driven by the cytomegalovirus (CMV) promoter. 3 d after transfection, cells were fixed with 2% paraformaldehyde/PBS followed by colorimetric assay for β-galactosidase using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside substrate (0.5 mg/ml; Promega Corp., Madison, WI). Microscopic examination disclosed lack of staining in mock-transfected cells and 6.1% of stained cells for both 1 and 2.5 μg of transfected pHook-3*LacZ* plasmid (total number of cells scored = 2,002 and 2,756, respectively).

Luciferase reporter plasmids driven by the human cyclin A promoter region from −924 to +245 (wild-type and CRE mutant) (45, 46) were provided by J. Sobczak-Thépot (INSERM, Paris). The human cyclin A promoter regions from −79 to +100 (wild-type and E2F mutant) and from −54 to +100 were generated by PCR and subcloned into pGL2-Basic (Promega Corp.). The −79/+100 E2F mutant contained the sequence AATTCG at position −37 to −32, which disrupts the E2F site that confers serum responsiveness of the human cyclin A promoter (47). PCR-generated plasmids were verified by sequence analysis. p-356wt/fos luciferase reporter construct containing the murine c-fos promoter region from −356 to +109 (48) and pEMSV-c-fos expression vector containing the full-length cDNA for rat c-fos were provided by M. Simonson (Case Western Reserve University, Cleveland, OH). Expression vector pCMV5-Kip1 containing the full-length cDNA for murine $p27^{Kip1}$ (49) was provided by J. Massagué (Memorial Sloan-Kettering Cancer Center, New York). Final DNA concentration in experiments involving cotransfection of expression vectors was equalized by adding empty expression vector. To correct for differences in transfection efficiency, luciferase activity was normalized relative to the level of alkaline phosphatase activity produced from cotransfected pSVAPAP plasmid (0.5 μg), which contains the reporter gene under the control of the simian virus 40 enhancer-promoter (50). Cells were incubated with transfection mixtures for 90 min and then were fed M199 medium supplemented with FBS as indicated. Luciferase and alkaline phosphatase activity were measured as described previously (51). Results represent the mean±SEM of three independent transfections.

To study the effect of Ras inactivation on various promoters, PAC1 cells were cotransfected with the indicated amounts of luciferase reporter genes and pMT-Ras^{Asn17} (52) (a gift from G.M. Cooper, Dana-Farber Cancer Institute, Boston, MA). Transfection of this construct results in high levels of basal expression of dominant-negative human Ras^{Asn17} mutant driven by the mouse metallothionein-I gene promoter (52). To rescue the inhibitory effect of Ras^{Asn17}, cells were cotransfected with pEMSV-c-fos (0.7 μg). Transfected cells were maintained in 0.5% FBS for 2–3 d and then stimulated with 20% FBS for 16–18 h.

Bromodeoxyuridine (BrdU) incorporation in transfected cell populations. PAC1 cells were seeded into 60-mm culture dishes containing glass coverslips. The next day, cells were cotransfected with pCMV-CD20 (0.125 μg) (a gift from S. van den Heuvel, Massachusetts General Hospital, Boston, MA), pMT-Ras^{Asn17} (0.5 μg), and pEMSV-c-fos (1.5 μg) as indicated. Final DNA concentration in transfection solutions was equalized by adding empty pMT-Bam expression vector. Cells were maintained in 20% FBS for 36 h and then were incubated for an additional 13 h in fresh medium containing 10 μM BrdU (Amersham Corp., Arlington Heights, IL). BrdU incorporation in transfected cell populations was analyzed by indirect immunofluorescence using mouse mAbs to the CD20 cell-surface marker and BrdU. Cells were incubated for 1 h at room temperature with FITC-conjugated anti-CD20 antibody (1:10 dilution; DAKO Corp., Carpinteria, CA) and then fixed with 100% methanol. BrdU immunofluorescent detection was done as described previously (53) using a biotinylated anti-BrdU antibody (1:60 dilution; Zymed Laboratories, Inc., South San Francisco, CA) and streptavidin-R-phycoerythrin (1:400 dilution; Caltag Laboratories, Inc., South San Francisco, CA). Nuclei were counterstained with 4′, 6-diamidine-2′-phenylindole dihydrochloride (0.5 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN).

Electrophoretic mobility shift assay (EMSA). Arterial and PAC1 extracts were prepared as described previously (9). EMSA was carried out in buffer containing 10 mM Tris-HCl (pH 7.5), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 0.05 mg/ml poly(dI–dC)/poly(dI–dC). After 10 min preincubation on ice, extracts were incubated for 30 min with a ^{32}P-labeled double-stranded oligonucleotide probe spanning the human cyclin A promoter region from −84 to −63 (5′-TTGAATGACGTCAAGGCCGCG-3′; CRE underlined). The mutated CRE oligonucleotide contains the sequence from −84 to −63 in the −924/+245 cyclin A CRE mutant reporter construct (5′-TTAAATGAATTCAAGGCCGCG-3′) (46). Unlabeled oligonucleotide was added to the preincubation mixture for competition assays (25–50-fold molar excess). For supershift experiments, 1 μl of polyclonal antibodies to c-fos (sc-253x) or CRE-binding protein (CREB)-1 (sc-186x) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added for an additional 30 min after incubation with the probe. Binding reactions were separated at 4°C in nondenaturing 4% acrylamide gels in 0.5× TBE running buffer (22.5 mM Tris-borate, 0.5 mM EDTA).

Flow cytometry analysis. PAC1 cells were maintained for 3 d in 0.5% FBS/M199 and then stimulated with 20% FBS/M199 for different periods of time. Cells were trypsinized, fixed in 100% methanol, and stained with a solution containing 50 μg/ml each of propidium iodide and ribonuclease A (Boehringer Mannheim Biochemicals). Samples were analyzed in triplicate with a FACScan® using CellFIT cell-cycle analysis software (Becton Dickinson, San Jose, CA).

Balloon angioplasty, in vivo BrdU labeling, and immunohistochemistry. Balloon denudation of the left common carotid artery in male Sprague-Dawley rats was performed as described (9, 54). For in vivo BrdU labeling, rats received two intraperitoneal infusions of BrdU 24 and 12 h before death (30 mg/kg each dose). Control and balloon-injured arteries were fixed in situ with 100% methanol, embedded in paraffin, and cut in 5-μm sections. Immunohistochemistry was done with an mAb to BrdU (1:50 dilution; DAKO Corp.) and a biotin/streptavidin-peroxidase detection system (Signet Laboratories, Inc., Dedham, MA). Peroxidase activity was detected with 0.05% (wt/vol) 3,3′-diaminobenzidine tetrahydrochloride dihydrate substrate (Vector Laboratories, Inc., Burlingame, CA). For c-fos immunohistochemistry, specimens were treated with antigen retrieval solution (DAKO Corp.) and biotin/avidin blocking kit (Vector Laboratories, Inc.) before incubation with a polyclonal antipeptide antibody to c-fos (sc-052, 1:300 dilution; Santa Cruz Biotechnology Inc.). Peroxidase activity in c-fos–containing immunocomplexes was detected using AEC substrate (BioGenex Labs, San Ramon, CA).

Results

Serum stimulation and overexpression of c-fos results in the induction of cyclin A promoter activity dependently of the CRE and E2F sites in the cyclin A promoter. We showed previously that VSMC proliferation in response to mechanical acute injury in rat and human arteries correlates with the induction of CDK2, cyclin E, and cyclin A (43). Since members of the AP-1 family of transcription factors have been implicated in the control of cellular growth (55), and induction of their expression precedes the onset of injury-induced VSMC proliferation (56, 57), we explored a potential transcriptional link between the AP-1 transcription factor c-fos and cyclin A gene expression. To this end, VSMCs were transiently transfected with luciferase reporter constructs driven by the human cyclin A pro-

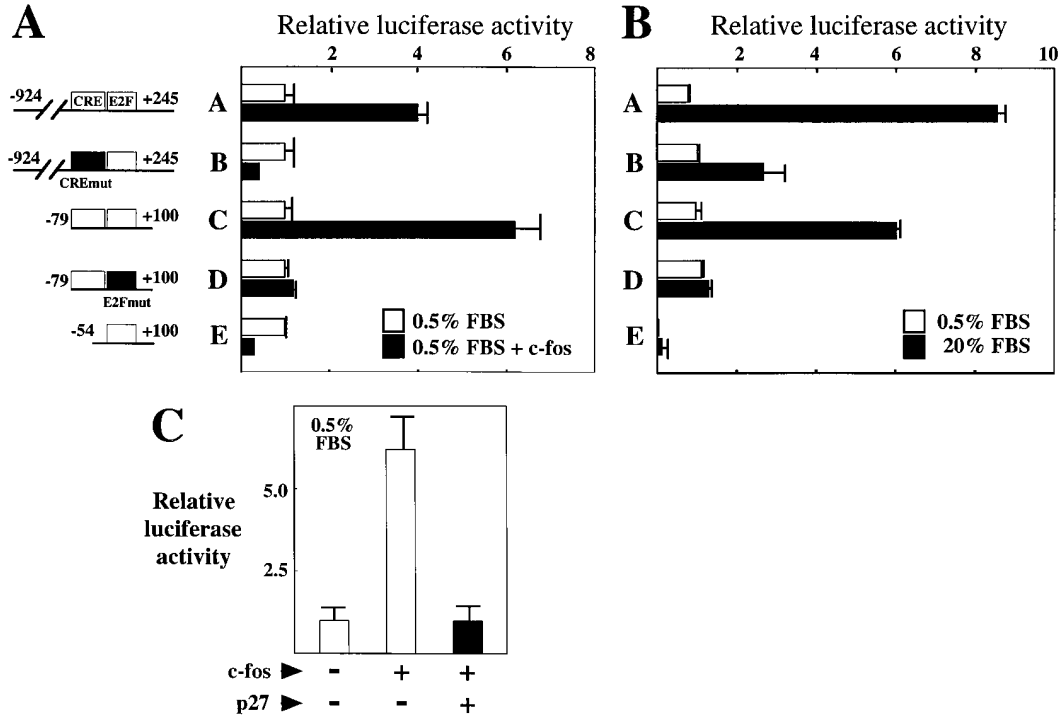

Figure 1. Both the CRE and E2F sites in the cyclin A promoter are required for c-fos– and serum-dependent induction of cyclin A expression. (*A*) PAC1 cells were transiently transfected with luciferase reporter genes containing the indicated promoter fragments from the human cyclin A gene (5 μg per transfection). −924/+245 CRE mut and −79/+100 E2F mut refer to reporter constructs containing point mutations that affected the CRE at position −79 to −72 and the E2F motif at position −37 to −32, respectively. Cells were maintained in 0.5% FBS for 3 d after transfection. *White bars*, Control transfections. *Black bars*, Cells cotransfected with pEMSV-c-fos (1 μg). Activity of each reporter gene in cells cotransfected with pEMSV-c-fos is normalized relative to its basal activity in the absence of pEMSV-c-fos (= 1). Except for the −54/+100 construct, activity of cyclin A promoter–dependent reporter genes was nearly identical in serum-starved cells (see *B* and Table I). (*B*) PAC1 cells transfected as indicated in *A* were maintained in M199 medium supplemented with 0.5% FBS (*white bars*) or restimulated with 20% FBS (*black bars*). Activity of each reporter gene is normalized relative to the activity of the −79/+100 construct in serum-starved cells (= 1). (*C*) PAC1 cells were transfected with 5 μg of −79/+100 cyclin A–luciferase construct and were maintained in M199 medium supplemented with 0.5% FBS. Cells were cotransfected with 1 μg of c-fos and $p27^{Kip1}$ expression vectors as indicated. Results are normalized relative to the basal activity in control cells (first bar = 1).

moter. Cotransfection of c-fos expression vector in serum-starved VSMCs resulted in a four- to sixfold induction of cyclin A promoter–dependent reporter activity (Fig. 1 *A*, constructs *A* and *C*). Transcription from the cyclin A promoter was also induced 6–10-fold by serum refeeding of starvation-synchronized VSMCs (Fig. 1 *B*, constructs *A* and *C*; see also Fig. 4, *A* and *B*), in agreement with previous studies in fibroblasts (45, 47, 58–60). Likewise, serum restimulation induced 6–12-fold cyclin A promoter activity in transfected 10T1/2 and NIH 3T3 fibroblasts, and c-fos stimulated 4–8-fold cyclin A promoter activity in serum-starved 10T1/2 cells (data not shown).

Mutations affecting the CRE at position −79 to −72 relative to the major transcription initiation site in the cyclin A promoter reduced the normal serum-dependent induction of cyclin A promoter activity (Fig. 1 *B*, construct *B*; see also Fig. 4 *B*), consistent with previous studies in NIH 3T3 fibroblasts showing that maximal cyclin A promoter activity after serum restimulation requires the integrity of the CRE (60). As shown in Fig. 1 *A*, mutation of the CRE also abrogated c-fos–dependent inducible transcription from the cyclin A promoter in VSMCs (construct *B*). Thus, c-fos overexpression in serum-starved VSMCs is sufficient to induce cyclin A promoter activity, and both c-fos– and maximal serum-dependent inducible transcription from the cyclin A promoter require the integrity of the CRE at position −79 to −72.

Given the pivotal role of E2F transcription factors in G1 to S phase progression (61–65), we next investigated the role of E2F on the regulation of cyclin A transcription in VSMCs. Point mutations that disrupted the E2F motif present at position −37/−32 in the cyclin A promoter abrogated serum-dependent induction of the −79/+100 reporter gene in VSMCs (Fig. 1 *B*, construct *D*), in agreement with previous findings in NIH 3T3 fibroblasts demonstrating a critical role of this E2F motif on serum-mediated induction of cyclin A promoter activity (47). However, both the basal activity of the −54/+100 cyclin A promoter region in serum-starved VSMCs and its activity in serum-restimulated VSMCs was decreased markedly (Fig. 1 *B*, construct *E*, and Table I). Thus, the E2F motif at position −37/−32 in the human cyclin A gene promoter is necessary but not sufficient for serum-induced activation of cyclin A transcription.

Notably, c-fos–dependent inducible transcription from the cyclin A promoter also required the integrity of the −37/−32 E2F site (Fig. 1 *A*, construct *D*). Moreover, induction of cyclin A promoter activity by c-fos was blocked by overexpression of $p27^{Kip1}$ (Fig. 1 *C*), an inhibitor of VSMC proliferation (9, 66) that has been shown to repress cyclin A transcription through its E2F binding site at −37 to −32 (67). Collectively, the results of these transfection assays indicate that both the CRE at position −79/−72 and the E2F motif at position −37/−32 in the cyclin A promoter region are required for the normal serum- and c-fos–dependent induction of cyclin A gene expression.

VSMC proliferation induced by serum in vitro and by an-

Table I. Reduced Basal Activity and Lack of Serum Responsiveness of the −54/+100 Cyclin A Promoter Region in Transfected VSMCs

Reporter gene	Low serum	High serum	Ratio low/high
pGL2-Basic	1±0.1	1.1±0.1	1
−79/+100 cyclin A–Luc	29.6±2.1	167.1±33.6	5.6
−54/+100 cyclin A–Luc	2.3±0.2	1.4±0.5	0.6

PAC1 cells were cotransfected with the indicated luciferase reporter genes and pSVAPAP plasmid. Cells were maintained for 2 d in 0.5% FBS/M199 (*Low serum*) or serum-restimulated with 20% FBS/M199 after serum starvation (*High serum*). Luciferase/alkaline phosphatase activity ratios are normalized relative to the activity of the promoterless pGL2-Basic reporter in serum-starved cells (= 1). Data are expressed as mean±SEM of three independent transfections.

gioplasty in vivo is associated with increased binding of c-fos to the cyclin A CRE. Previous studies have demonstrated the involvement of AP-1/CREB heterodimers in the regulation of CRE site–dependent transcriptional regulation (55). To test whether c-fos and CREB-1 factors interact with the cyclin A CRE, we performed EMSAs using an oligonucleotide probe bearing this element and extracts prepared at different time points after serum restimulation of starvation-synchronized VSMCs. Serum-restimulated cells disclosed a distinct nucleoprotein complex whose intensity increased transiently during G1 phase and preceded both the induction of cyclin A promoter activity and DNA synthesis (Fig. 2, *A* and *C*). This nucleoprotein complex required the CRE sequence for its assembly (Fig. 2 *A*) and contained c-fos and CREB-1 proteins (Fig. 2 *B*).

Taken together with the results of our transfection assays, these findings suggest that binding of c-*fos* and CREB-1 to the cyclin A CRE contributes to maximal cyclin A transcription at G1/S. Evidence that these findings may be relevant in vivo was provided using the well-characterized rat carotid artery model of balloon angioplasty, in which increased cyclin A expression correlates with VSMC proliferation and neointimal accumulation (43). VSMC proliferation after angioplasty in the rat carotid artery peaks between 33 and 48 h and declines thereafter to return to baseline levels within 2–3 wk (54, 68). Consistent with these findings, BrdU incorporation into VSMCs was negligible in uninjured arteries and 1 d after angioplasty, and increased 3 d after injury (Fig. 3 *A*). Expression of c-fos was markedly induced exclusively in VSMCs before the onset of injury-induced proliferation and coincident with the proliferative wave (Fig. 3 *B*). In contrast, c-fos protein was not detected in uninjured, quiescent arteries, or 18 d after injury, when the proliferative index has returned to basal levels (Fig. 3 *B*) (54, 68).

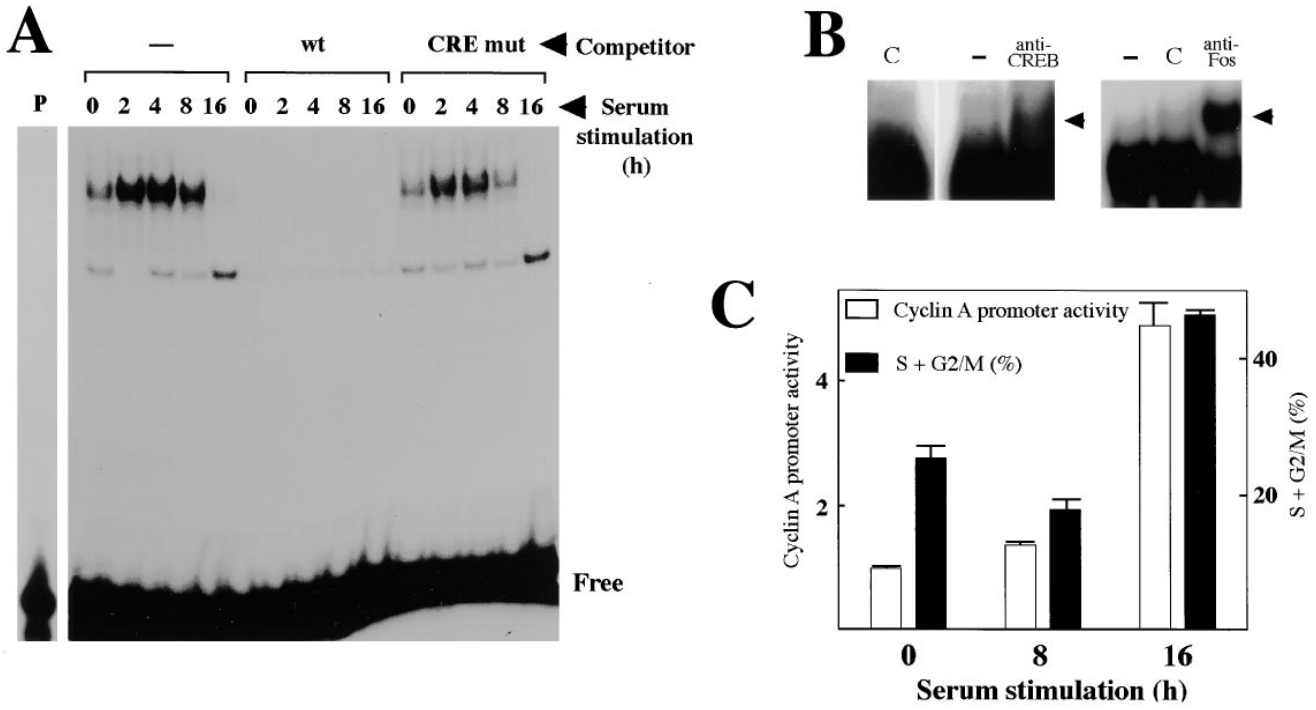

Figure 2. Serum restimulation of VSMCs induces c-fos and CREB-1 binding to the cyclin A CRE before the stimulation of cyclin A promoter activity and DNA synthesis. PAC1 cells were maintained in 0.5% FBS for 3 d and then stimulated with 20% FBS for the indicated periods of time. (*A*) EMSA was performed using a radiolabeled probe containing the cyclin A CRE (−84 to −63 cyclin A promoter region). Competition experiments were done with a 25-fold excess of −84/−63 oligonucleotide. *wt*, Wild-type. *CRE mut*, Mutated CRE. Lane *P*, Binding reaction with probe and no extract. (*B*) Polyclonal antibodies to CREB-1 and c-fos supershifted the nucleoprotein complex in cells stimulated with serum for 2 h. *Arrowheads*, The supershifted bands. *C*, Control unrelated antibody (anti-MEF2). The c-fos and CREB-1 antibodies also gave rise to a supershift in cells stimulated for 8 h (not shown). (*C*) *White bars*, Reporter gene activity in cells transfected with the −79/+100 cyclin A–luciferase construct (2.5 μg). Results are normalized relative to the activity in serum-starved cells (first bar = 1). *Black bars*, Percentage of cells in S and G2/M phase was determined by flow cytometry analysis of cells stained with propidium iodide.

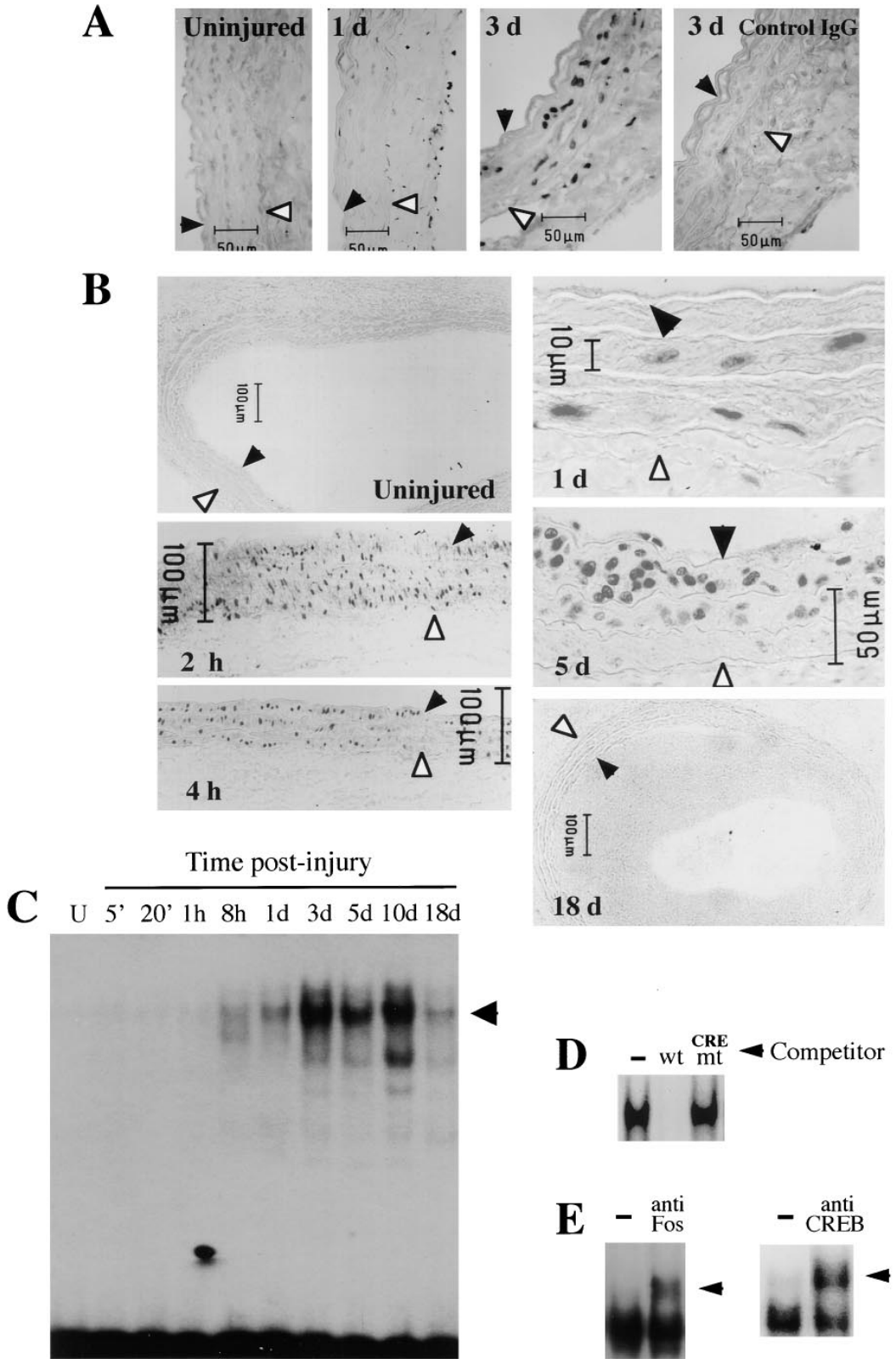

Figure 3. Expression and activity of c-fos after balloon angioplasty in the rat carotid artery. BrdU incorporation (*A*) and c-fos expression (*B*) in uninjured arteries and at the indicated time points after balloon angioplasty were analyzed by immunohistochemistry. *Black* and *white arrowheads*, The internal and external elastic lamina, respectively. Arteries were sectioned longitudinally (2- and 4-h time points) or transversally (rest). Specimens incubated with the anti-BrdU antibody were counterstained with hematoxylin. As negative control for BrdU immunostaining, specimens were incubated with nonimmune mouse IgG (*Control IgG*). Control experiments in which the c-fos antibody was preincubated with a 10-fold mass excess of immunogenic peptide revealed no signal (not shown). (*C–E*) EMSA was performed using the cyclin A −84/−63 probe which contains the CRE site, and arterial extracts prepared at the indicated time points after angioplasty. Lane *U*, Uninjured. *Arrowhead* (*C*) The retarded band shown in *D* and *E*. Competition was done with 5-d postinjury extracts and a 50-fold excess of the −84/−63 cyclin A oligonucleotide. *wt*, Wild-type. *CRE mt*, Mutated CRE. Similar results were obtained with 3- and 10-d postinjury extracts (not shown). Supershift assays were done using 3-d postinjury extract and the indicated antibodies. *Arrowhead*, The supershifted band.

We next examined the binding activity associated with the cyclin A CRE in response to balloon angioplasty. EMSA demonstrated an injury-induced nucleoprotein complex whose assembly required the cyclin A CRE (Fig. 3, *C* and *D*) and contained c-fos and CREB-1 proteins (Fig. 3 *E*). This injury-induced nucleoprotein complex was first detected 8 h after angioplasty, peaked between 3 and 10 d, and then declined (Fig. 3 *C*). Thus, in agreement with the above findings in cultured VSMCs, c-fos expression and binding to the cyclin A CRE are spatially and temporally consistent with a role for this factor in

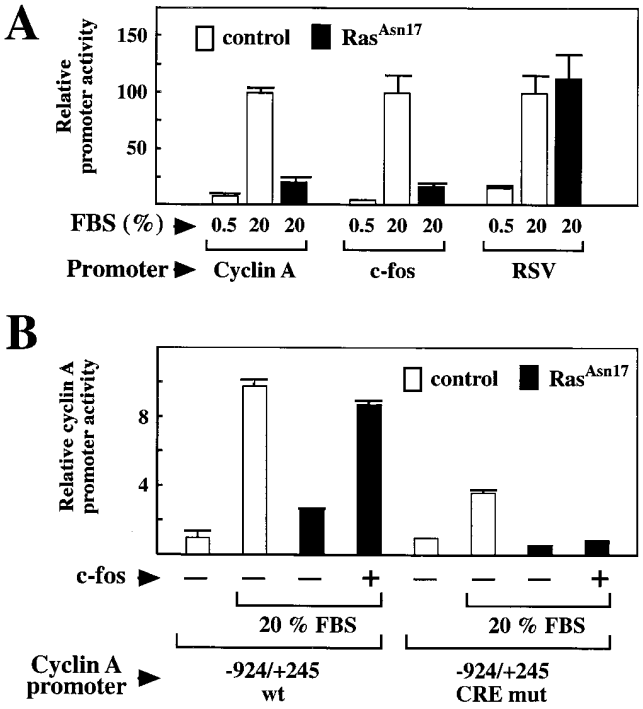

Figure 4. Overexpression of c-fos circumvents the inhibitory effect of Ras inactivation on cyclin A promoter activity. (*A*) PAC1 cells were transfected with luciferase reporter constructs driven by the following promoter fragments: cyclin A (−79/+100; 2.5 μg), c-fos (−356/+109; 1 μg), and RSV enhancer-promoter (1 μg). *White bars*, Control transfections. *Black bars*, Cells cotransfected with pMT-Ras^{Asn17} (1.5 μg), which expresses dominant inhibitory Ras^{Asn17} mutant (52). After transfection, cells were maintained for 3 d in 0.5% FBS. Bars show the activity of serum-starved cells (0.5% FBS), or cells restimulated overnight after serum starvation (20% FBS). Activity in control serum-stimulated cells is defined as 100%. (*B*) PAC1 cells were transfected with 2.5 μg of −924/+245 cyclin A–luciferase construct. *wt*, Wild-type. *CRE mut*, Mutated CRE. *White bars*, Control transfections. *Black bars*, Cells cotransfected with pMT-Ras^{Asn17} (0.25 μg). +, Cotransfection of pEMSV-c-fos expression vector (*c-fos*; 0.7 μg). Transfected cells were serum-starved for 2 d and then stimulated overnight with 20% FBS. Activity of each reporter construct is normalized relative to basal activity in serum-starved cells (first bar = 1).

injury-induced stimulation of cyclin A expression and VSMC proliferation in vivo.

Overexpression of c-fos overcomes the inhibitory effect of Ras inactivation on cyclin A promoter activity and VSMC proliferation. The Ras proteins are essential for mitogen-stimulated progression through the G1 and S phase of the mammalian cell cycle (69–73) and for injury-induced VSMC proliferation in vivo (41, 42). Therefore, we sought to investigate a potential role for Ras on the regulation of cyclin A gene transcription and VSMC proliferation. As shown in Fig. 4 *A*, expression of dominant inhibitory Ras^{Asn17} mutant (52) blocked the normal induction of c-fos and cyclin A promoter–dependent reporter gene activity in serum-stimulated VSMCs. In contrast, Ras^{Asn17} did not affect reporter activity driven by the Rous sarcoma virus (RSV) enhancer-promoter (Fig. 4 *A*), thus excluding the possibility that inactivation of Ras might inhibit essential components of the transcriptional machinery. We next examined the effect of Ras inactivation on DNA synthesis in VSMCs. To this end, cells were cotransfected with expression vectors for Ras^{Asn17} and CD20 cell-surface marker. BrdU incorporation in transfected cell populations was analyzed by indirect immunofluorescence using antibodies to CD20 and BrdU. As shown in Fig. 5, Ras^{Asn17} blocked de novo DNA synthesis in serum-stimulated VSMCs.

The above results link Ras-dependent mitogenic signaling to inducible c-fos and cyclin A promoter activity and DNA synthesis in VSMCs, and suggest that inactivation of c-fos may contribute to the inhibitory effects of Ras^{Asn17}. Indeed, overexpression of c-fos circumvented efficiently the inhibition of serum-dependent inducible cyclin A promoter activity (Fig. 4 *B*) and DNA synthesis (Fig. 5) due to Ras inactivation. As expected, mutations within the cyclin A CRE that impaired binding of c-fos abrogated its ability to overcome the inhibitory effect of Ras^{Asn17} (Fig. 4 *B*). Together, these findings suggest that c-fos, through its interaction with the cyclin A CRE, acts in a signaling cascade that links Ras activity to cyclin A transcription and VSMC proliferation.

Discussion

Although several positive and negative regulators of cell cycle progression have been involved in neointimal lesion development after balloon angioplasty (9–27), the molecular mechanisms regulating the expression of cell cycle control genes and governing VSMC proliferation are poorly understood. In this study, we investigated the transcriptional regulation of the cyclin A gene, a key positive regulator of S phase that is induced after angioplasty (43). Our results suggest that the interaction of c-fos/CREB-1 heterodimers with the CRE in the cyclin A promoter plays an important role in the induction of cyclin A gene expression and VSMC growth in vitro and in vivo. First, c-fos overexpression could override the requirement for Ras-dependent signaling on S phase entry and cyclin A gene transcription in serum-stimulated VSMCs, and this latter effect required the integrity of the cyclin A CRE site. Second, overexpression of c-fos in serum-starved VSMCs, which normally display low cyclin A promoter activity, stimulated transcription from the cyclin A promoter in a CRE-dependent manner. Third, VSMC proliferation induced by both serum in vitro and balloon angioplasty in vivo was associated with binding of c-fos and CREB-1 to the cyclin A CRE site. Finally, expression of c-fos in VSMCs after balloon angioplasty was correlated temporally and spatially with the kinetics of VSMC proliferation. Previous studies demonstrated the rapid induction of c-myc and AP-1 factors after balloon angioplasty in the rat aorta, suggesting that early protooncogene expression contributes to the initiation of VSMC proliferation in response to acute mechanical injury (56, 57). Our EMSA and immunohistochemistry analysis in injured arteries extend these previous findings by demonstrating the sustained DNA-binding activity and expression of c-fos during the proliferative wave and subsequent downregulation at later time points coincident with the reestablishment of the quiescent phenotype. Maximal CRE-associated binding activity preceded the peak of cyclin A promoter–dependent reporter (luciferase) gene activity in transfected cells. One potential explanation for this apparent discrepancy is a delay in translation into luciferase protein after transcription of the transfected cyclin A–luciferase reporter and/or the

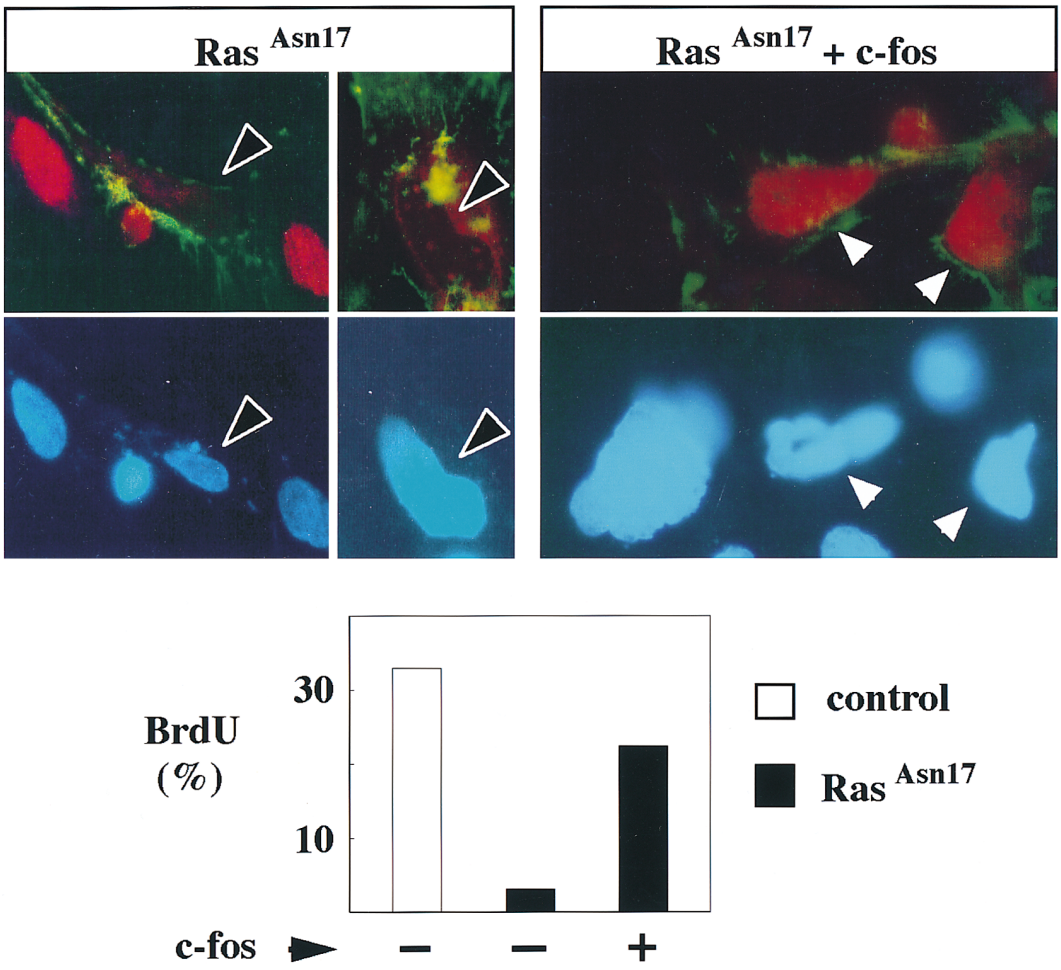

Figure 5. Overexpression of c-fos overcomes the inhibitory effect of Ras inactivation on DNA synthesis. PAC1 cells were cotransfected with pMT-Ras^{Asn17} (0.5 μg) and pCMV-CD20 (0.125 μg) expression vectors. To rescue the inhibitory effect of Ras^{Asn17}, cells were cotransfected with pEMSV-c-fos (*c-fos*; 1.5 μg). Cells were maintained in 20% FBS and labeled with BrdU for indirect immunofluorescence analysis. Incorporation of BrdU in transfected cell populations was analyzed using FITC-conjugated anti-CD20 antibody (*green*) and biotin-conjugated anti-BrdU antibody. BrdU-containing immunocomplexes were detected using streptavidin-R-phycoerythrin (*red*). 4′, 6-diamidine-2′-phenylindole dihydrochloride nuclear counterstaining of each field is also shown (*blue*). *Black* and *white arrowheads*, CD20-positive/BrdU-negative and CD20-positive/BrdU-positive cells, respectively. (*Bottom*) Approximately 250 CD20-positive cells were scored in each experiment.

requirement of additional factors at later time points after serum restimulation (see below).

It has been shown previously that the E2F motif at position −37/−32 in the cyclin A promoter region is necessary for serum responsiveness of cyclin A expression in NIH 3T3 fibroblasts (47). While our results with the −79/+100 E2F mutant in transfected VSMCs are consistent with this notion, the results with the −54/+100 reporter indicate that the E2F site at position −37/−32 is not sufficient to confer the normal serum-dependent induction of cyclin A promoter activity. Likewise, the CRE site at position −79/−72 appears to be necessary for maximal cyclin A promoter activity in serum-stimulated NIH 3T3 cells (60) and VSMCs (Fig. 1 *B* and Fig. 4 *B*). Collectively, these results suggest that both the −79/−72 CRE and the −37/−32 E2F sites in the cyclin A promoter region are required for the normal serum-dependent induction of cyclin A expression. Further evidence supporting this notion was provided by analyzing the role of E2F on c-fos–dependent induction of cyclin A transcription. Indeed, the ability of c-fos to stimulate cyclin A promoter activity in transfected VSMCs was abrogated when E2F function was disrupted either by point mutations of its binding site in the cyclin A promoter or by overexpression of p27^{Kip1}, an inhibitor of VSMC proliferation (9, 66) that represses cyclin A promoter activity through its E2F motif (67).

While Ras-dependent signaling is essential for injury-induced VSMC proliferation in vivo (41, 42) and growth factor–dependent activation of the G1 CDK/cyclin/E2F pathway in vitro (34–40), the molecular basis of these events is not well understood. Previous studies in fibroblasts have implicated Ras-dependent signaling and c-jun in the activation of the cyclin D1 promoter (74–76), a key regulator of G1 phase. Our results demonstrate that Ras activity is essential for the normal induction of c-fos and cyclin A promoter activity, as well as DNA replication in serum-stimulated VSMCs. Ectopically expressed c-fos, through its interaction with the CRE, circumvented the inhibitory effect of Ras inactivation on cyclin A promoter activity and was effective at restoring mitogen-induced DNA synthesis in the absence of Ras activity. Thus, the AP-1 transcription factors appear to play an important role in linking the Ras and cell cycle regulatory pathways in different cell types. Of note, repression of cyclin A promoter activity by Ras^{Asn17} in the presence of serum was approximately four-

fold for either the wild-type or the CRE mutant reporter constructs. When taken together with recent studies demonstrating that Ras inactivation blocks the normal serum-dependent accumulation of E2F and prevents the induction of cyclin A mRNA levels (36), these results suggest that suppression of E2F function may contribute to the inhibitory effect of Ras^{Asn17} on the CRE mutant construct.

On the basis of our results and recent studies (34–40, 74–76), it is clear that the molecular mechanisms coupling Ras-mediated signaling to the cell cycle machinery are complex. We propose that c-fos and E2F transcription factors are downstream effectors of Ras which stimulate cyclin A gene expression and VSMC proliferation. When taken together with our in vivo data demonstrating the localized expression of c-fos in VSMCs and its interaction with the cyclin A CRE in response to balloon denudation, these findings illustrate a new transcriptional network that may be relevant to the development of vascular proliferative lesions.

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