Cell Cycle-mediated Regulation of Smooth Muscle α-Actin Gene Transcription in Fibroblasts and Vascular Smooth Muscle Cells Involves Multiple Adenovirus E1A-interacting Cofactors*

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Expression of smooth muscle α-actin in growth factor-induced myofibroblasts and in differentiated vascular smooth muscle cells is transcriptionally controlled by multiple positive or negative trans-acting factors interacting with distinct cis-elements in the 5′-flanking region of the gene. Because none of the transcriptional regulators reported to date is smooth muscle cell- or myofibroblast-specific per se, the dynamic interplay among many factors interacting at specific sites along the promoter appears to be a signature feature of smooth muscle α-actin gene regulation in these cell types. Herein, the ability of the adenovirus E1A 12 S protein to bind and functionally inactivate specific cell regulatory factors has been exploited to identify several previously unknown coactivators of the mouse smooth muscle α-actin promoter in rodent fibroblasts and vascular smooth muscle cells. In transient cotransfection assays, ectopic expression of wild type E1A suppressed promoter activity in a dose-and cis-element-dependent manner. In asynchronous cells, N-terminal E1A mutants defective in CREB-binding protein (CBP) and p300 binding capacity exhibited markedly reduced inhibitory activity toward a smooth muscle α-actin promoter driven by a composite TEF-1-, SRF-, and Sp1/3-regulated enhancer. In synchronized cells, however, a more complex mutant E1A inhibitory pattern indicated that collaboration between CBP/p300 and the retinoblastoma family of pocket proteins was required to produce a fully functional enhancer. Cotransfection experiments conducted with Rb−/− fibroblasts demonstrated the necessity of pRB in augmenting smooth muscle α-actin enhancer/promoter activity. Physical interaction studies with the use of purified wild type and mutant E1A proteins confirmed that CBP, p300, and pRB were targets of E1A binding in nuclear extracts of vascular smooth muscle cells and/or fibroblasts. Collectively, these results suggest that a repertoire of E1A-interacting proteins, namely CBP/p300 and pRB, serve to integrate the activities of multiple trans-acting factors to control smooth muscle α-actin gene transcription in a cell type- and cell cycle-dependent manner.

Actin is the most abundant intracellular protein in the eukaryotic cell and accounts for ~1–5% of the total cell protein in non-muscle cells and ~10% in muscle cells (1). As a core component of the cytoskeleton, actin plays a vital role in regulation of cell locomotion, control of cell shape and division, organelle transport, and muscle contraction. The vertebrate actin family of cytoskeletal proteins is highly conserved and consists of six isoforms, which include four muscle-specific actins and two other non-muscle actins (2). The non-muscle β- and γ-actins are expressed in all cells and constitute the primary structural proteins of microfilaments. Genes encoding the non-muscle actins are members of the “immediate early” gene family, and their activation coincides with cell growth and proliferation. In the adult mammal, expression of the muscle-specific actins is much more restricted, and genes encoding the α-actin, α-cardiac, α-vascular smooth muscle, and γ-otic smooth muscle isoforms are generally activated upon terminal differentiation in cells of myogenic lineage (3–5). Thus, the vertebrate actin genes serve as convenient models for elucidating molecular mechanisms that promote what is generally considered to be mutually exclusive events, cell proliferation and cell differentiation.

Our specific interest in smooth muscle α-actin (SMαA) gene regulation stems from the fact that, unlike the other muscle-specific actins, expression of this particular isoform is modulated in both myogenic and non-myogetic cell types in response to pathophysiologic de- or trans-differentiation signals. Such signals are produced during the progression of atherosclerosis and restenosis where inflammatory mediators, released at the site of arterial injury, induce the phenotypic conversion of quiescent contractile vascular smooth muscle cells (VSMCs) into migratory growth-activated fibroblast-like cells that promote neointimal hyperplasia (6, 7). Importantly, recent evidence suggests that this process, commonly referred to as phenotypic modulation, is not entirely restricted to medial VSMCs and may involve reprogramming of resident adventitial fibroblasts as well (8, 9). This is a particularly intriguing notion because SMαA-expressing myofibroblasts have long been recognized as critical players in tissue repair in the context of wound healing (10–12). Thus, although arterial remodeling in

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§ The abbreviations used are: SMαA, smooth muscle α-actin; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; CMV, cytomegalovirus; CREB, cAMP-response element-binding protein; E1A, adenovirus early region 1A; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; β-gal, β-galactosidase; MEF, mouse embryo fibroblast; Ni-NTA, nickel-nitrilotriacetic acid; pRB, retinoblastoma tumor suppressor protein; SRF, serum response factor; TEF-1, transcription enhancer factor 1; TF, transcription factor; TGF-β1, transforming growth factor β1; VSMC, vascular smooth muscle cell; wt, wild type.
response to atherogenic stimuli was once considered entirely pathogenic, this process may represent a physiological adaptation to injury, which may influence the overall vulnerability of an atherosclerotic plaque to lethal rupture (13).

To gain greater insight into the molecular mechanisms underlying phenotypic modulation in the vessel wall, our laboratories have focused on identifying the repertoire of cis-elements and trans-acting factors responsible for mediating both constitutive and inducible mouse SMαA gene expression in cultured fibroblasts and VSMCs. This effort was inspired by seminal studies demonstrating that the mouse SMαA gene was subject to differential regulation in myogenic versus non-myogenic cell types via functional interplay among multiple positive and negative cis-elements in concert with an assortment of ubiquitous and tissue-restricted transcription factors including the CARG motif-binding protein, serum response factor (SRF), the MCAT element-binding protein, transcription enhancer factor 1 (TEF-1), and several putative single-stranded DNA-binding repressors (14–17). Subsequent identification and characterization of these 5′-polyuridine-polypyrimidine tract-binding repressors as members of the Pur Y box families of single-stranded DNA/RNA-binding proteins lent credence to the concept of a negatively regulated enhancer (18, 19) and led to further promoter mutagenesis and biochemical experiments confirming the participation of Purα, Purβ, and MSY1 in cryptic MCAT enhancer regulation in both fibroblasts and VSMCs (20, 21). In keeping with a putative role for Pur proteins in preventing overproduction or inappropriately timed expression of SMαA during developmental and injury-induced cardiovascular remodeling in vivo (22), more recent in vitro studies suggest that Purα and Purβ may differentially antagonize certain SMαA-associated activators such as TEF-1, SRF, or Sp1 in specific cell types and in a manner regulated by transforming growth factor β (TGF-β1) signaling (23, 24).

The preceding work is consistent with findings of other investigators who have espoused a similar level of combinatorial complexity when SMαA gene regulation was evaluated in primary VSMCs (25) or in the developing mouse (26–28). Although it remains a generally accepted paradigm that SMαA expression by quiescent VSMCs within the medial layer of the blood vessel wall is constitutive, transcriptional reprogramming of SMαA and other markers of smooth muscle differentiation during injury-induced arterial remodeling in vivo (29) suggests that VSMCs retain an adaptive flexibility that is similar to the myofibroblast during wound healing (30). Thus, plasticity of SMαA expression appears to be a requisite feature of certain cells types that are molecularly tailored to respond to environmental signals promoting either migration and growth (low SMαA, synthetic/activated phenotype (6)) or immobilization and differentiation (high SMαA, contractile phenotype). As a consequence, it follows logically that regulation of cell cycle progression and SMαA gene expression must in some way be linked at the molecular level to permit cells to shift, at the most physiologically opportune time, between activated and contractile phenotypes during wound repair and vascular remodeling.

In this report, we test the hypothesis that cell cycle progression and SMαA gene expression are coupled at the molecular level by evaluating the effect of the adenovirus early region 1A (E1A) 12 S protein, and selected mutants thereof, on core SMαA enhancer/promoter activity in asynchronous versus synchronized fibroblasts or VSMCs in vitro. The rationale for adopting this loss-of-function strategy is that the E1A 12 S protein contains multiple independent domains that serve as binding sites for certain well characterized transcription cofactors and cell cycle regulatory proteins implicated in modulating mammalian cell growth and differentiation (31–33). As such, ectopically expressed E1A, by virtue of sequestration and functional inactivation of specific intracellular targets, can selectively alter transcription/expression of particular host cell genes critical to maintenance of cell phenotype. Importantly, E1A-dependent alteration of gene expression has been reported to produce dramatic changes in the phenotypic properties of certain myogenic and metastatic tumor cell types both in vitro and in vivo (34, 35 and references therein). Herein, we have applied the E1A 12 S protein as a biochemical tool to physically and functionally expose the participation of members of the CBP/p300 family of histone acetyltransferases along with the retinoblastoma tumor suppressor protein (pRB) in modulating SMαA gene transcription under specific cell culture conditions. Our findings suggest a plausible regulatory connection between smooth muscle-like differentiation status and cell cycle progression and may offer new mechanistic insight into the process of phenotypic modulation of SMαA-expressing cell types.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—All cell lines were cultured in a humidified incubator at 37 °C and 5% CO2 in specific growth medium containing heat-inactivated fetal bovine serum (FBS) (HyClone). Mouse embryo-derived AKR-2B fibroblasts (15) were maintained in McCoy’s 5A medium (Invitrogen) plus 5% v/v FBS. Rat A7r5 VSMCs were maintained in high n-glucose (4.5 g/liter) Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% v/v FBS. RB−/− mouse embryo fibroblasts (MEFs) were cultured in high glucose Dulbecco’s modified Eagle’s medium with 3.7 g/liter sodium bicarbonate and 10% v/v FBS. A7r5 cells were obtained from ATCC Bioresources Center, and RB−/− MEFs were graciously provided by Tyler Jacks (Massachusetts Institute of Technology). Experiments were performed with cell lines at passage 3–15.

**SMαA Promoter:Reporter and E1A Expression Plasmids**—Construction of mouse SMαA promoter/chloramphenicol acetyltransferase (CAT) reporter constructs (15, 36) and expression plasmids encoding wild type or mutant E1A (37) have been described previously. Expression vector encoding pRB was kindly provided by William G. Kaelin, Jr. (Dana-Farber Cancer Institute). Expression plasmids encoding p107 and p130 were kindly provided by Brian Dynlacht (New York University School of Medicine). All plasmids used in transient transfection experiments were purified by double cesium chloride gradient centrifugation.

**Transient Transfection and Reporter Gene Assays**—Confluent cell monolayers in 175-mm flasks were trypsinized, and cells were suspended in growth medium for counting. Cells were seeded on 60-mm tissue culture dishes at a plating density of 2.0 × 105 for A7r5 and 1.2 × 106 A7r5 cells for VSMCs or 1.0 × 106 AKR-2B fibroblasts were typically at 40–50% confluence after overnight incubation. Adherent cells were transfected with a fixed amount of DNA in 1 ml of serum-free medium with the use of GenePORTER transfection reagent (Gene Therapy Systems) at a ratio of 2 μg/μg DNA. Cells were routinely exposed to lipid/DNA mixtures consisting of 4.8 μg of VSPM-CAT reporter, 0.2 μg of pCMVβ (control β-galactosidase (β-gal) reporter), and selected amounts of pCI or pCI-E1A expression plasmid up to 2 μg. Empty pCI vector was added to keep the total amount of DNA transfected constant. After 3 h, cells were fed with complete growth medium and incubated for an additional 45 h. In some experiments, after a 16–18-h recovery period in complete growth medium, transfected cells were serum starved for 48 h in MCDB-402 (fibroblasts) or Dulbecco’s modified Eagle’s medium (VSMCs) then restimulated for 6 h with fresh medium plus 20% v/v FBS. Transfected cell monolayers were washed with ice-cold phosphate-buffered saline, and whole cell lysates were prepared by application of 0.5 ml of 1 × CAT lysis buffer (Roche Applied Science) supplemented with protease inhibitors for 30 min. After centrifugation for 10 min at 14,000 rpm, cleared cell lysates were assayed for total protein content by BCA assay (Sigma) with the use of bovine serum albumin (BSA) as a standard. CAT and β-gal reporter proteins were measured with the use of commercial immunoassay kits (Roche Applied Science). Reporter values were normalized for total cell protein. Transfections were routinely performed in triplicate or quadruplicate and repeated at least three times to ensure reproducibility. Data sets were subjected to analysis of variance to assess statistical significance at p < 0.05. Expression of E1A was verified by Western blotting of transfected cell lysates with the use of a monoclonal E1A antibody.
Preparation of Nuclear Extracts—Mouse AKR-2B fibroblasts or rat A7r5 VSMCs were cultured in complete growth medium in 175-mm flasks until the cells reached confluence. Cell monolayers were washed once with Versene 1.5000 (Invitrogen), and adherent cells were detached by brief exposure to 0.05% trypsin-EDTA (Invitrogen). Cells were then suspended in complete growth medium containing 5 or 10% FBS, transferred to 50-ml polypeylene tubes, and centrifuged at 450 \( \times g \) for 10 min at 4 °C. Pelleted cells were gently resuspended with 40 ml of cold phosphate-buffered saline containing 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 450 \( \times g \) for 10 min at 4 °C. This wash procedure was repeated two more times. After estimating the packed cell volume, cells were gently resuspended with a 10-fold volume of cold sucrose extraction buffer consisting of 10 mM HEPES, pH 8.0, 0.25 \% sucrose, 25 mM KCl, 5 mM MgCl\(_2\), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 \( \mu \)g/ml each leupeptin, pepstatin A, aprotinin, and 0.2\% v/v Triton X-100. Cells were rocked gently for 15 min at 4 °C. Lysed cells were then centrifuged at 3,220 \( \times g \) for 10 min at 4 °C. The supernatant (cytosolic extract) was removed, and pelleted nuclei were extracted by addition of 5 \( \times \) the packed nuclear volume of cold sucrose buffer consisting of 20 mM Hepes, pH 8.0, 25 \% glycerol, 25 mM KCl, 5 mM MgCl\(_2\), 0.5 mM dithiothreitol, 0.5 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 \( \mu \)g/ml each leupeptin, pepstatin A, aprotinin, and nuclease. Nuclei were mixed vigorously with the use of a vortex shaker for 3 \( \times \) 10 \( s \). The extract was transferred to polypeylene tubes and centrifuged at 20,000 \( \times g \) for 20 min at 4 °C. Cytosolic and nuclear extracts were stored at −80 °C. The protein concentration of cell extracts was determined by a Bio-Rad (Sigma) assay with BSA as a standard. For pull-down assays, extracts of AKR-2B or A7r5 cells cultured either in complete growth medium or under serum-free conditions for 2 days were prepared as described previously (38).

Construction and Purification of His\(_6\)-tagged E1A—Bacterial expression constructs encoding C-terminal histidine-tagged versions of wild type E1A 12 S protein and selected mutants were constructed by subcloning derived from pCAH-EB vector to mammalian expression plasmid pQE70 (Qiagen). His-tagged E1A proteins were expressed in Escherichia coli strain M15 and purified with the use of Ni-NTA-agarose under denaturing conditions as directed by the manufacturer (Qiagen). Proteins were dialyzed extensively against renaturation buffer consisting of 100 mM NaH\(_2\)PO\(_4\), 10 mM Tris, pH 8.0, and quantified by BCA protein assay. His-tagged E1A protein was >95\% pure as judged by SDS-PAGE and staining with Coomassie Blue R-250. Molecular concentrations of E1A were calculated based upon amino acid composition-derived molecular masses of wild type or mutant proteins.

Protein-Protein Interaction Assays—Pull-down assays were conducted as follows. AKR-2B or A7r5 nuclear protein was exchanged into sodium phosphate-buffered saline containing 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 450 \( \times g \) for 10 min at 4 °C. This wash procedure was repeated two more times. After overnight 18-h incubation at 4 °C, coating solution was removed by aspiration, and adherent cells were detached by brief exposure to 0.05% trypsin-EDTA and centrifuged at 450 \( \times g \) for 10 min at 4 °C. The supernatant (cytosolic extract) was removed, and pelleted nuclei were extracted by addition of 5 \( \times \) the packed nuclear volume of cold sucrose buffer consisting of 20 mM Hepes, pH 8.0, 0.25 \% sucrose, 25 mM KCl, 5 mM MgCl\(_2\), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 \( \mu \)g/ml each leupeptin, pepstatin A, aprotinin, and nuclease. Nuclei were mixed vigorously with the use of a vortex shaker for 3 \( \times \) 10 \( s \). The extract was transferred to polypeylene tubes and centrifuged at 20,000 \( \times g \) for 20 min at 4 °C. Cytosolic and nuclear extracts were stored at −80 °C. The protein concentration of cell extracts was determined by a Bio-Rad (Sigma) assay with BSA as a standard. For pull-down assays, extracts of AKR-2B or A7r5 cells cultured either in complete growth medium or under serum-free conditions for 2 days were prepared as described previously (38).

RESULTS

Mapping of cis-Elements Required for E1A-mediated Repression of SM\(_{A}\) Promoter Activity in Fibroblasts and VSMCs—Previous reports have established that the E1A 12 S protein is capable of repressing skeletal and cardiac \( \alpha \)-actin gene transcription and myogenic differentiation in vitro (39). To ascertain whether E1A would similarly affect SM\(_{A}\) gene transcription in cell types relevant to wound repair and arterial remodeling, transient cotransfection assays were performed with the use of a full-length mouse SM\(_{A}\) promoter-reporter construct or specific deletion mutants in the presence or absence of E1A 12 S expression plasmid in both A7r5 VSMCs and AKR-2B fibroblasts cultured under exponential growth conditions (Fig. 1). The SM\(_{A}\) promoter-reporter construct known as VSPM8 contains an ~3.6-kbp contiguous region of the mouse SM\(_{A}\) gene extending from −1 kbp upstream of the start site of transcription through the first intron fused to the CAT reporter gene (Fig. 1A). This segment of the SM\(_{A}\) gene has been shown previously to promote robust smooth muscle-specific transgene expression in multiple transgenic mouse lines (36, 40, 41). Deletion mutants VSPM4, VSPM5, and VSPM7 all lack intron 1 but possess differentially truncated 5'-flanking regions (Fig. 1A). As reported previously (20), VSPM4 demonstrates elevated transcriptional activity in asynchronous A7r5 and AKR-2B cells because of the absence of several 5'-nucleotides essential for Pur/MSY1 repressor-protein binding to the cryptic MCAT enhancer (Fig. 1, B and C). However, VSPM5 and VSPM7 show markedly reduced activity relative to VSPM4 because of the removal of the unrepressed MCAT enhancer, the THR element, and two downstream CAR\(_{G}\) boxes, which in native context appear to facilitate functional interaction among DNA-bound TBE1, SRF, and Sp1/3 activator proteins (20, 21). Cotransfection of an E1A expression plasmid in A7r5 VSMCs resulted in potent inhibition of VSPM8 and VSPM4 but did not affect the basal activity of VSPM5 and VSPM7 (Fig. 1B). In contrast, all SM\(_{A}\) promoter constructs tested were subject to inhibition by E1A in AKR-2B fibroblasts (Fig. 1C). These results indicate that the presence of the segment spanning −191 to −150 is absolutely required for E1A-mediated inhibition of SM\(_{A}\) promoter activity in A7r5 VSMCs, but in AKR-2B fibroblasts, E1A appears to target multiple SM\(_{A}\) cis-elements within the −191 to +46 core enhancer region independently.
Delineation of E1A Domains Responsible for Repression of SMaA Enhancer:Promoter Activity in Proliferating VSMCs—

To assess the putative involvement of E1A-interacting cofactors in SMaA promoter regulation in VSMCs, cotransfection studies were performed with a repertoire of expression plasmids encoding adenovirus E1A 12 S mutants known to be defective in binding to histone acetyltransferases, CBP and p300, or to the retinoblastoma family of pocket proteins, pRB, p107, or p130 (Table I). In these studies, the truncated SMaA reporter, VSMP4, was utilized because this construct exhibits core enhancer activity resulting from the composite affect of multiple cis-elements and associated DNA-binding proteins working in a cooperative manner (20, 21) and, as such, is very sensitive to the presence of ectopically expressed repressors (23, 24). To identify the minimum amount of E1A required for maximal inhibition, selected quantities of wild type E1A expression vector ranging from 25 ng to 2 μg were tested in A7r5 cells cotransfected with VSMP4 and a control β-gal reporter, pCMVβ. Specific and dose-dependent suppression of VSMP4 activity was observed with maximal inhibition achieved at 0.5 μg of pCI-E1Awt expression plasmid (Fig. 2A). The activity of the viral promoter-driven β-gal reporter was not affected under these conditions. To identify the region within E1A responsible for repression, plasmids encoding specific E1A mutants were evaluated relative to wild type E1A in transfected A7r5 cells. As shown in the inset of Fig. 2A, N-terminal E1A mutants d2–36, dR2G, and d2–36/C124G, which have been shown previously to be defective in CBP/p300 binding in both cultured fibroblasts and melanoma cells (35, 37), were much less efficient inhibitors of VSMP4 than either wild type E1A or the pocket protein-binding mutants dCR2, C124G, and Y47H/C124G. Western blotting of lysed A7r5 transfectants indicated comparable expression of E1A mutants relative to wild type E1A (Fig. 2B).

Biochemical Analysis Implicates Multiple E1A-interacting Factors in SMaA Gene Regulation—To confirm that mutation of the N-terminal domain eliminated CBP and p300 binding in a VSMC context, protein-protein interaction assays were conducted by incubating a fixed quantity of nuclear protein harvested from either A7r5 cells or AKR-2B fibroblasts with microtiter wells precoated with differing amounts, 400–6.25 ng, of either His-tagged wild type E1A or the d2–36 mutant. After incubation for 3 h, wells were washed and then probed by ELISA to detect E1A-bound CBP or p300. Absorbance data were normalized for modest differences in coating efficiency between the wild type and mutant E1A proteins as described under “Experimental Procedures.” Fig. 3, A and B, illustrates that both CBP and p300 derived from either AKR-2B or A7r5 nuclear extract interacted specifically with wild type E1A, although the overall colorimetric signal generated with A7r5 extract was somewhat less than that obtained with AKR-2B extract. This observation could be explained by a cell type difference in the endogenous level of these cofactors or by a species difference in the relative affinity of the C-terminal-specific rabbit polyclonal antibodies for mouse AKR-2B- versus rat A7r5-derived CBP/p300. Irrespective of this distinction, the d2–36 mutant consistently demonstrated a deficit in CBP/p300 binding capacity relative to wild type E1A when compared within a given cell type. It is important to note that control experiments were conducted to ensure that the immobilized d2–36 mutant retained expected pocket protein binding capacity (data not shown).

More comprehensive screening was also conducted to assess qualitatively the spectrum of transcription factor (TF) binding by wild type E1A and the d2–36 mutant with the use of this in vitro assay system. The analysis was limited only to those TFs either known or suspected to be relevant to SMaA gene regulation and also detectable by Western blotting of AKR-2B and A7r5 cell extracts (data not shown). In these experiments, differential CBP/p300 binding to wild type E1A versus the d2–36 mutant served as a positive control. Wells coated with BSA alone served as a negative control for nonspecific TF and/or antibody binding. Each primary antibody was also
tested for nonspecific interaction with immobilized E1A to account for false positive signal resulting from cross-reactivity between TF antibody and E1A. Corrected absorbance values were calculated by subtracting absorbance caused by nonspecific binding from the total signal generated in E1A-coated wells exposed to nuclear extract. As indicated in Fig. 3, C and D, only CBP and p300 exhibited a striking difference in binding to wild E1A versus the d2–36 mutant. Antibodies against the reported TEF-1-interacting coactivator, poly(ADP-ribose) polymerase (42) and the SRF-binding cofactor myocardin (43) generated relatively weak signals, which were not significantly above background. Similar results were obtained for TEF-1, SRF, and Sp1, again indicating that E1A is remarkably specific for CBP and p300 at least in the context of nuclear extracts from AKR-2B fibroblasts and A7r5 VSMCs (Fig. 3, C and D). Interestingly, association of Sp3 with wild type E1A was detectable, but, unlike CBP and p300, this apparent interaction was not abolished by removal of the 2–36 domain. This is an intriguing observation because the –191 to –150 region of the core SmoA enhancer has been reported previously to contain a binding site (THR) for Sp1/3 (Fig. 1 and Ref. 21). Sequestration (35, 37).

TABLE I

| Designation       | Mutationa | E1A-protein interaction affectedb |
|-------------------|-----------|----------------------------------|
| pCI-E1Aw        | None      | None                             |
| pCI-E1Ad2–36    | Delete aa 2–36 | CBP, p300                        |
| pCI-E1AR2G      | Delete aa 121–139 | CBP, p300, pRB, p107, p130       |
| pCI-E1AdCR2     | Change Cys to Gly at aa 124 | pRB, p107, p130                  |
| pCI-E1AC124G    | Change Cys to Gly at aa 124 | pRB, p107, p130                  |
| pCI-E1AY747/C124G | Change Cys to Gly at aa 124 | pRB, p107, p130                  |
| pCI-E1Ad2–36/C124G | Delete aa 2–36 and change Cys to Gly at aa 124 | CBP, p300, pRB                  |

a Expression plasmids were constructed as described previously (37). aa, amino acid(s).

b Based on qualitative coimmunoprecipitation assays conducted with whole cell extracts from E1A-transfected cells as described previously (35, 37).

To test this notion further, an E1A d2–36/C124G double mutant, previously reported to be defective in binding to CBP, p300, and pRB in fibroblasts and melanoma cells (Table I), was functionally evaluated in synchronized A7r5 cells and found to be completely devoid of inhibitory activity toward VSMP4 (Fig. 5A). Western blotting confirmed comparable dose-dependent expression of this E1A mutant relative to wild type E1A in synchronized A7r5 cells (Fig. 5B). Taken together, these results implicate pRB in addition to CBP/p300 in regulation of SmoA gene transcription in VSMCs albeit under conditions restricted to early phases of the cell cycle.

Evidence for Involvement of pRB in SmoA Gene Transcription Extends to Fibroblasts—To ascertain whether the preceding observations were unique to constitutive SmoA-expressing VSMCs, analogous experiments were conducted in AKR-2B fibroblasts, a cell line shown previously to assume a myofibroblast phenotype after stimulation with serum or TGF-β1 (21, 24, 45). As shown in Fig. 6, A and B, ectopic E1A expression inhibited VSMP4 transcriptional activity in a specific and dose-dependent manner in synchronized AKR-2B cells. To identify regions within E1A required for inhibition, selected mutants were tested for repressor activity toward VSMP4 in both synchronous and asynchronous cultures of AKR-2B fibroblasts. Similar to results obtained with A7r5 cells, the N-terminal region of E1A appeared to play the dominant inhibitory role in asynchronous AKR-2B cells, whereas the contribution of the pocket protein binding region was greater in synchronized AKR-2B cells as evidenced by the diminished repressor activity of the dCR2 and d2–36/C124G mutants (Fig. 6C).

To validate biochemically the ability of E1A to bind and sequester pRB in a nuclear milieu, pull-down experiments were performed with purified His-tagged E1A proteins and nuclear extracts prepared from either exponentially growing or serum-starved AKR-2B fibroblasts. This approach differs from previously described qualitative coimmunoprecipitation experiments conducted with whole cell lysates from E1A-transfected cells (35, 37) in that an equimolar amount of wild type or mutant E1A was combined with a fixed amount of nuclear protein thereby permitting comparative assessment of pRB binding capacity. As shown in Fig. 7A, Western blotting of protein complexes released from Ni-NTA beads revealed that wild type E1A and the d2–36 mutant were each capable of interacting with nuclear derived pRB, but mutants lacking residues required for high affinity pocket protein binding (dCR2 and Y47H/C124G) showed reduced pRB binding capacity (compare lanes 3–6 with lanes 7–10). Reprobing of the pRB blot with a monoclonal E1A antibody verified that these differences were not caused by unequal gel loading or degradation of
FIG. 2. Repression of core SMoA enhancer activity by E1A is specific and requires the N-terminal CBP/p300 binding region in asynchronous VSMCs. A, A7r5 cells were transiently transfected with 4.8 μg of VSMP4, 0.2 μg of pCMVβ, and selected amounts of pCI-E1Awt expression plasmid. The total amount of DNA transfected was fixed at 7 μg with empty pCI vector. Lysates of asynchronous cells were prepared 48 h after transfection and assayed for total cell protein, CAT, and β-gal reporters. Symbols show the mean CAT (closed circles) or β-gal (open circles) values normalized for total cell protein ± S.D. (inset). A7r5 cells were cotransfected with 4.8 μg of VSMP4, 0.2 μg of pCMVβ, and 0.5 μg of wild type or mutant E1A expression plasmids. Lysates were harvested and analyzed as above. Bars show the mean normalized VSMP4 activity ± S.D. relative to the no E1A control defined as 1. B, lysed cell protein (50 μg/lane) was separated by 10% SDS-PAGE and analyzed by Western blotting with a mouse monoclonal E1A antibody to confirm expression of wild type E1A (lane 2) and the indicated E1A mutant proteins (lanes 3–11). Lane 1 contained protein from control pCI-only transfected cells. The blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody as a loading control. Numbers on the left designate marker proteins in kDa.

mutant E1A proteins during the incubation period with nuclear extract (Fig. 7B). It is noteworthy that the electrophoretic mobility of E1A-bound pRB was identical in nuclear extract prepared from either growth-arrested or proliferating cells (Fig. 7A, compare lanes 3 and 5 with lanes 4 and 6). The size of the pRB band is consistent with the faster migrating hypophosphorylated form of pRB, which is the predominant species in G1-arrested cells through 6 h of serum stimulation (Fig. 7C).

Although results implicating CBP/p300 in SMoA gene transcription were not completely surprising given the precedent for involvement of these factors in coactivating SRF-dependent expression of other markers of smooth muscle differentiation (46), the apparent necessity of pRB, and perhaps other pocket proteins, seemed to warrant experimental corroboration. To test further whether pRB participates as a cofactor in SMoA enhancer/promoter regulation, transient transfection assays were conducted with pRB-deficient MEFs. As shown in Fig. 8, cotransfection of a pRB expression plasmid significantly increased the level of VSMP4 transcriptional activity in R6−/− MEFs. The trend was similar when expression plasmids encoding p107 or p130 were evaluated. However, pRB consistently demonstrated the most potent level of coactivator activity when tested at different doses of expression plasmid. These findings validate the results of mutant E1A inhibition and protein interaction studies and lend further support to the contention that pRB is likely a key player in SMoA gene regulation.

**DISCUSSION**

Although conventionally viewed as a mere marker of smooth muscle differentiation status, SMoA plays a critical role in maintaining vessel wall homeostasis and in promoting stromal cell acquisition of transient fibrocontractile properties after tissue injury (11, 30). As a consequence, misregulation of SMoA gene expression in VSMCs and fibroblasts is associated with certain chronic pathologic conditions. In terms of elevated or inducible SMoA expression in stromal fibroblasts, although transient myofibroblast activation represents a normal physiological response to injury, sustained accumulation of SMoA-expressing myofibroblasts can lead to hypertrophic scarring and fibrosis of the lung, liver, and kidney (12). Other clues to the significance of aberrant changes in SMoA expression can be gleaned from in vitro studies of cell transformation and motility. In particular, a seminal report by Leavitt and co-workers (47) revealed that down-regulation of SMoA was correlated with microfilament rearrangement, changes in cell shape, and elevation of tumorigenic potential in several spontaneously and virus-transformed rodent fibroblast cell lines. This example is in keeping with later work from other investigators suggesting that the SMoA isoform is not only necessary for contraction but may also serve other important biological roles such as imped-
with immobilization and differentiation may aid in the development of treatment strategies aimed at tipping the balance from dysfunctional to perhaps therapeutic arterial remodeling.

In this study, we have taken advantage of the well documented nuclear protein binding properties of the adenovirus E1A 12 S protein to uncover a role for several specific E1A-interacting transcription cofactors and cell cycle regulatory proteins in mediating SM\(\alpha\)/H9251A gene transcription in either proliferating or growth-restricted fibroblasts and VSMCs. The logic for adopting an E1A-dependent loss-of-function approach is based on the hypothesis that regulation of proliferation and differentiation of SM\(\alpha\)/H9251A-expressing cell types must be linked for cells to have the necessary phenotypic plasticity to respond efficiently to local environment cues promoting vascular repair and remodeling. Furthermore, use of E1A as a surrogate trigger of myogenic dedifferentiation has proven to be a successful strategy in elucidating transcriptional mechanisms governing the expression of cardiac and skeletal muscle specific genes, including the striated muscle \(\alpha\)-actins (32, 39). Consistent with these previous findings, our results indicate that wild type E1A protein functions as a potent repressor of mouse SM\(\alpha\)A enhancer/promoter activity in rat A7r5 VSMCs (Figs. 1, 2, 4, and 5) and mouse AKR-2B fibroblasts (Figs. 1 and 6). These cell lines were chosen for analysis because of their established utility as models for studying SM\(\alpha\)A gene expression in a constitutive and growth factor-inducible context (20, 21, 45). In keeping with the notion of cell type specificity in SM\(\alpha\)A gene regulation, promoter mapping studies revealed that E1A targeted a different repertoire of essential cis-regulatory elements found within a core enhancer module located between \(-191\) and \(+46\) relative to the start site of transcription in fibroblasts versus VSMCs (Fig. 1). In AKR-2B fibroblasts, E1A appeared to mediate inhibition through multiple independently repressible elements. Notable among these elements is a region spanning \(-150\) to \(-60\), which contains two highly conserved CArG boxes. Interestingly, the importance of CArG elements as a conduit for E1A inhibition has been documented previously for the skeletal and cardiac \(\alpha\)-actin promoters in rodent myoblasts (39), suggesting that E1A may block the activity of certain transcriptional regulatory proteins shared among the \(\alpha\)-actin gene promoters.
We have reported previously that mouse SM\(\alpha\)A CARG2 (−120 to −111) serves as a high affinity binding site for SRF and is required for basal and serum-inducible promoter activity in A7r5 VSMCs and AKR-2B fibroblasts, respectively (20). Moreover, the essential role of CARG elements, and by extension, SRF, in the tissue-specific expression of genes encoding SM\(\alpha\)A, smooth muscle myosin heavy chain, and SM22\(\alpha\) has been codified by a number of independent \textit{in vivo} studies in transgenic mice (26, 58–61). However, because of its relative ubiquity and ability to regulate growth-promoting genes as well, SRF requires the assistance of tissue-restricted coactivators to fulfill its obligate role in mediating expression of genes involved in smooth muscle differentiation (43). Among the growing list of muscle-associated SRF-interacting coactivators, the CBP/p300 family of histone acetyltransferases represents the only well characterized targets of E1A binding and inhibition that have also been implicated in myogenic differentiation (62–65). Thus, it is significant that N-terminal E1A mutants defective in CBP/p300 binding were found to be impaired in their ability to inhibit the core SM\(\alpha\)A enhancer in both A7r5 and AKR-2B cells irrespective of cell synchrony (Figs. 4–6).

Although the preceding results are consistent with the precedent established for functional interaction between CBP/p300 and SRF in SM22\(\alpha\) promoter activation in VSMCs and fibroblasts (46), CARG elements alone do not fully account for E1A-mediated inhibition of SM\(\alpha\)A promoter activity. In A7r5 cells, an upstream sequence element spanning −191 to −150 was absolutely required for E1A to exert its dominant inhibitory effect. This region contains a consensus TEF-1-interacting MCAT element reported previously to function as a cryptic and SRF/CARG-collaborating enhancer in both VSMCs and fibroblasts (20). Importantly, additional biochemical and functional characterization of the core mouse SM\(\alpha\)A enhancer spanning −191 to +46 has revealed that the spectrum of transcription factors required for elaboration of basal enhancer activity in AKR-2B fibroblasts also includes members of the Sp family of GC box-binding proteins (21). Curiously, one of the reported Sp1/3 binding sites (THR) is located in the −191 to −150 region adjacent to the
MCAT element, whereas another functionally relevant site (TCE) is positioned just upstream of the TATA box (Fig. 1). Moreover, recent findings suggest that TGF-β1 activation of the core TEF-1-, SRF-, and Sp1/3-regulated SmαA enhancer during myofibroblast differentiation is accompanied by dynamic changes in nucleoprotein interactions among Sp1/3, Smad2/3 coactivators, and Puro/β repressor proteins (24). Of these proteins, Smad2 and Smad3 have also been reported to interact directly with E1A in synchronized AKR-2B cells. Numbers on the left designate marker proteins in kDa. C, AKR-2B cells were cotransfected with 4.8 μg of VSMP4, 0.2 μg of PCMVβ, and 0.5 μg of wild type or mutant E1A expression plasmids. After overnight recovery in medium containing 10% FBS, some transfectants were synchronized by serum starvation for 48 h and then treated with 20% FBS-containing medium for 6 h. These culture conditions were selected to maximize core SmαA enhancer induction (15) before AKR-2B cells resumed DNA synthesis (75). Lysates were assayed for total cell protein, CAT, and β-gal reporters. Bars show the mean CAT or β-gal values normalized for total cell protein ± S.E. B, Western blotting indicated dose-dependent expression of wild type E1A in synchronized AKR-2B cells. Numbers on the left designate marker proteins in kDa.

Fig. 6. The core SmαA enhancer exhibits differential responsiveness to selected E1A mutants as a function of cell synchrony in fibroblasts. A, AKR-2B cells were transiently transfected with 4.8 μg of VSMP4, 0.2 μg of PCMVβ, and selected amounts of pCIE1Awt expression plasmid. The total amount of DNA transfected was fixed at 6 μg with empty pCI vector. After overnight recovery in medium containing 10% FBS, transfectants were synchronized by serum starvation for 48 h and then treated with 20% FBS-containing medium for 6 h. These culture conditions were selected to maximize core SmαA enhancer induction (15) before AKR-2B cells resumed DNA synthesis (75). Lysates were assayed for total cell protein, CAT, and β-gal reporters. Bars show the mean CAT or β-gal values normalized for total cell protein ± S.E. B, Western blotting indicated dose-dependent expression of wild type E1A in synchronized AKR-2B cells. Numbers on the left designate marker proteins in kDa. C, AKR-2B cells were cotransfected with 4.8 μg of VSMP4, 0.2 μg of PCMVβ, and 0.5 μg of wild type or mutant E1A expression plasmids. After overnight recovery in medium containing 10% FBS, some transfectants were synchronized by serum starvation for 48 h. Lysates were harvested 6 h after treatment of quiescent cells with 20% FBS-containing medium. Lysates from asynchronous cultures were prepared 48 h after transfection. Bars show the mean normalized VSMP4 activity ± S.E. relative to the no E1A control defined as 1.

Although the putative TF-binding partners of pRB within the SmαA promoter are presently unknown, precedent for physical and functional interaction between pRB and Sp1 particularly during S phase progression and skeletal muscle differentiation (67, 68) suggests a potential candidate in the form of Sp1. In this respect, it is important to reiterate that deletion of the −191 to −150 element containing the Sp1/3 THR binding site eliminated E1A responsiveness in A7r5 cells and reduced it in AKR-2B cells. Irrespective of the exact pRB target, these results suggest a direct regulatory link between differentiation...
and growth of SmoA-expressing cell types and, as such, offer a promising avenue of investigation which might enhance our conceptual understanding of mechanism(s) governing phenotypic modulation in myofibroblasts and VSMCs. In this regard, several recent reports from other laboratories have provided evidence for coordinated, cell cycle-dependent regulation of VSMC proliferation, migration, and gene expression (69, 70).

So it is not unreasonable to speculate that signals inducing pRB hyperphosphorylation and resultant cell cycle progression may simultaneously promote SmoA gene deactivation by altering protein-protein interaction among pRB and putative binding partners such as Sp1/3 or the Pura/β repressor proteins (20, 71). Such changes could have a ripple effect leading to destabilization of higher order nucleoprotein complexes containing TEF-1, Sp1/3, SRF, and associated coactivators such as CBP/p300 and Smad2/3 along the length of the SmoA promoter.

In summary, the results of E1A inhibitor studies point to the involvement of the CBP/p300 family of histone acetyltransferases and the retinoblastoma family of tumor suppressor proteins in SmoA gene regulation. Both families have been implicated in facilitating myogenic differentiation in cultured cells (63, 72–74) or during mouse development (64, 65), but this is the first study to provide evidence for participation of pRB in cell cycle-dependent transcriptional regulation of a marker of smooth muscle differentiation. At the molecular level, sequestration of CBP/p300 and pRB by E1A presumably disrupts certain protein-protein interactions and/or interferes with essential TF or histone acetylation necessary for transcriptional activation of the SmoA promoter. Because dynamic interplay among multiple transcriptional activators and repressors appears to be a key mechanism by which expression of the SmoA gene is modulated in both injury-activated VSMCs and fibroblasts, future studies will focus on 1) identifying the TFs targeted for modification and/or interaction by CBP/p300 and pRB along the SmoA promoter as a function of cell cycle progression, and 2) characterizing the effect of E1A and selected mutants thereof on endogenous gene expression and cell phenotype with the use of stable E1A-expressing cell lines and transgenic mouse models.

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