Phosphorylation of the Cyclic AMP Response Element Binding Protein Mediates Transforming Growth Factor β -Induced Downregulation of Cyclin A in Vascular Smooth Muscle Cells[∇]

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Transforming growth factor β (TGF β), a multifunctional cytokine associated with vascular injury, is a potent inhibitor of cell proliferation. The current results demonstrate that the TGF β -induced growth arrest of vascular smooth muscle cells (VSMCs) is associated with cyclin A downregulation. TGF β represses the cyclin A gene through a cyclic AMP (cAMP) response element, which complexes with the cAMP response element binding protein (CREB). The CREB-cyclin A promoter interaction is hindered by TGF β , preceded by a TGF β receptor-dependent CREB phosphorylation. Induction of CREB phosphorylation with forskolin or 6bnz-cAMP mimics TGF β 's inhibitory effect on cyclin A expression. Conversely, inhibition of CREB phosphorylation with a CREB mutant in which the phosphorylation site at serine 133 was changed to alanine (CREB-S133A) upregulated cyclin A gene expression. Furthermore, the CREB-S133A mutant abolished TGF β -induced CREB phosphorylation, cyclin A downregulation, and growth inhibition. Since we have previously shown that the novel PKC isoform protein kinase C delta (PKC δ) is activated by TGF β in VSMCs, we tested the role of this kinase in CREB phosphorylation and cyclin A downregulation. Inhibition of PKC δ by a dominant-negative mutant or by targeted gene deletion blocked TGF β -induced CREB phosphorylation and cyclin A downregulation. Taken together, our data indicate that phosphorylation of CREB stimulated by TGF β is a critical step leading to the inhibition of cyclin A expression and, thus, VSMC proliferation.

The stimulation of vascular smooth muscle cells (VSMCs) to proliferate following vascular injury is critical to the pathogenesis of both atherosclerosis and re-stenosis. Without such a stimulus, VSMCs are quiescent (nonproliferative) and transdifferentiated, evident by the high expression of contractile proteins. Following vascular injury or reconstruction, the vessel wall is exposed to chemical and mechanical stimuli, which lead to an active and dedifferentiated VSMC phenotype (23). Through mechanisms not fully understood, these activated VSMCs re-enter the cell cycle and engage in excessive cell division. This abnormal proliferation of VSMCs, along with enhanced migration and matrix protein production, leads to the formation of a highly cellular lesion (intimal hyperplasia) in atherosclerotic or re-stenotic vessels (23).

In many ways, the pathogenesis of atherosclerosis and re-stenosis is similar to that of tumorigenesis; both involve excessive mitogenic responses and/or diminished growth inhibition. Among the known inhibitory growth factors, transforming growth factor β (TGF β), a member of a large family of a multipotent cytokines, induces cell cycle arrest in many cell types, including VSMCs (20, 21). Furthermore, the loss of TGF β -induced growth inhibition has been implicated in tu-

morigenesis (20). The acquisition of TGF β resistance found in several types of tumor cells is due to the inactivation of TGF β receptors or Smad genes (20). Despite the fact that TGF β is among the key cytokines implicated in the response to vascular injury, the molecular components of the TGF β signaling pathway leading to the inhibition of VSMC proliferation have yet to be defined. The purpose of the current study is to define the TGF β signaling pathway in order to understand the full significance of TGF β -induced growth arrest of VSMCs in the pathophysiology of vascular disease.

Since the discovery of Smad proteins, substantial knowledge has been uncovered regarding how the TGF β signal is transduced from the cell membrane to the nucleus, as summarized by several recent reviews (5, 30, 33a). TGF β initiates signaling by binding to a heterotetrameric complex consisting of the transmembrane serine/threonine kinases, known as the type I and type II TGF β receptors. Ligand binding allows the type II receptor to phosphorylate the type I receptor kinase domain, which then propagates the signal via Smad proteins. There are three functional classes of Smad proteins: the receptor-regulated Smad (R-Smad), the co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads (Smad2 and Smad3 for TGF β ligands and Smad1, Smad5, and Smad8 for BMP ligands) are directly phosphorylated and activated by the type I receptor kinases. Following phosphorylation, they become associated with the Co-Smad class Smad4 protein, forming a Smad complex, which then translocates to the nucleus where it regulates the expression of TGF β target genes. The I-Smads (Smad6 and Smad7) regulate TGF β signaling by competing

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with R-Smads for receptors and for Co-Smad interactions while also targeting the receptor for degradation.

In addition to the Smad pathway, several classical signaling molecules, such as mitogen-activated protein kinases, protein kinase C, and nonreceptor tyrosine kinase (4, 19, 28, 35, 38), can also be activated by TGF β . Whether TGF β regulates its target genes through a Smad or through these additional non-Smad pathways appears to be dependent on the nature of the target gene as well as the cell type under scrutiny.

Using a rat aortic VSMC line (A10) and a mouse primary aortic VSMC culture, we examined the expression of the p21, p27, and cyclin A genes, all of which have been identified as TGF β target genes in non-smooth muscle cells (10, 14, 25). Our results demonstrate that TGF β selectively inhibits cyclin A expression without significantly upregulating the protein levels of cyclin-dependent kinase (CDK) inhibitor p21 or p27. Therefore, we sought to investigate the detailed molecular mechanism underlying TGF β 's inhibitory regulation of cyclin A gene expression.

MATERIALS AND METHODS

Cell culture. Rat aortic A10 line VSMCs, obtained from ATCC, were grown as recommended at 37°C in 5% CO₂ in Dulbecco's minimal essential medium (DMEM) modified to contain 4 mM L-glutamine, 4.5 g/liter glucose, 1 mM sodium pyruvate, 1.5 g/liter sodium bicarbonate supplemented with 10% fetal bovine serum (FBS; Gemini, Woodland, CA), and antibiotics. Generation of the protein kinase C delta (PKC δ) target deletion has been described elsewhere (22). Mouse aortic VSMCs were isolated from the thoracic aortas of PKC δ -deficient or wild-type mice, based on a protocol described by Clowes et al. (3), and maintained in DMEM containing 10% FBS at 37°C with 5% CO₂. Cells between passages 4 and 8 were used for experiments.

Immunoblot analysis. VSMCs (80% confluent) were made quiescent by incubation in medium containing 0.5% FBS for 48 h and then treated with TGF β . A10 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred as described previously (17). The membrane was incubated with rabbit polyclonal antibodies to p27, p21, cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-CREB-specific (Ser133) (Upstate, Chicago, IL), total CREB (Cell Signaling, Beverly, MA), or β -actin (Sigma-Aldrich, St. Louis, MO). Labeled proteins were visualized with an enhanced chemiluminescence system (Perkin Elmer, Boston, MA).

Proliferation assay. Proliferation was assayed by measuring DNA synthesis as previously described (16). VSMCs were seeded onto 24-well plates (10,000 cells/well) in 10% FBS medium overnight and then starved in 0.5% serum for 48 h, followed by incubation for 24 h with TGF β as indicated. During the final 5 h of the assay, 2 μ Ci of [*methyl*-³H]thymidine was added to each well. Incorporated [³H]thymidine was precipitated with 10% trichloroacetic acid and measured with a liquid scintillation counter.

Northern blotting. Total RNA was extracted using an RNAqueous kit (Ambion, Austin, TX). Equal amounts of total RNA (10 to 20 μ g) were resolved, transferred to Hybond-N membranes, and hybridized as previously described (13). Restriction enzyme fragments of human cyclin A cDNA (kindly provided by Cyrus Vaziri [11]) or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA (Sigma Chemical Co., St. Louis, MO) were labeled with ³²P using a Prime-a-Gene labeling system from Promega (Madison, WI).

Transient transfection and luciferase assay. A luciferase construct containing a human cyclin A promoter (−516 to +245) was obtained from Rik Derynck (10) and used to generate restriction enzyme digestion fragments that were subcloned into pGL3-basic vector (Promega). The mutant cyclic AMP (cAMP) response element (Mt-CRE) construct was generated by replacing the −79/+100 fragment with the corresponding fragment from the CRE mutant construct provided by V. Andres (33). Transient transfection and luciferase assays were carried out as previously described (13). After transfection, cells were incubated in medium containing 0.5% FBS overnight and then treated or not treated with TGF β (5 ng/ml) for 18 h.

Adenovirus infection. Recombinant adenovirus vectors expressing a phosphorylation-resistant mutant cAMP response element binding protein (CREB) in which serine 133 is mutated to alanine (Ad-CREB-S133A) or a PKC δ kinase

FIG. 1. TGF β decreases cyclin A expression in VSMCs. (A) Quiescent A10 cells or mouse aortic VSMCs were treated with 5 ng/ml of TGF β for 24 h. [³H]thymidine (2 μ Ci/ml) was added to VSMCs during the final 5 h of the TGF β treatment, and incorporated [³H]thymidine in the cells was isolated by trichloroacetic acid precipitation. (B) Western blots of cell lysates isolated from A10 cells and primary mouse VSMCs stimulated with 5 ng/ml of TGF β for 18 h. (C) A10 VSMCs were incubated with 5 ng/ml of TGF β or solvent for 6, 12, 18, and 24 h. Cell lysates were blotted with antibodies specific to cyclin A, p27, p21, or β -actin. Values are expressed as means $\pm$ standard errors of the means (**, $P < 0.01$; *, $P < 0.05$, compared to solvent-treated control).

dead mutant were provided by A. J. Zeleznik (31) and T. J. Biden (2). Adenovirus preparation and infection were carried out as previously described (29).

Gel shift assay. Following a 60-min TGF β treatment (5 ng/ml), nuclear extracts were prepared and a gel shift assay was performed as previously described

FIG. 2. The CRE located between positions -79 and -54 of the human cyclin A promoter mediates TGFβ's downregulation of cyclin A. (A) A10 VSMCs were incubated with 5 ng/ml of TGFβ or solvent for 12, 18, or 24 h. The level of cyclin A mRNA was determined with Northern blotting ($n = 3$; $P < 0.05$, compared to solvent-treated control). (B) A diagram of luciferase reporters with various 5' deletions of the cyclin A promoter. A10 cells were transfected with human cyclin A/luciferase reporter. Following transfection, cells were stimulated for 18 h with 5 ng/ml of TGFβ. Reporter activities were expressed as ratios of firefly luciferase to *Renilla* luciferase ($n = 3$; $P < 0.05$, compared to solvent-treated control). (C) A diagram of luciferase reporters with wild-type (Wt) or Mt-CRE cyclin A promoters. A10 cells were transfected with Wt or mutant cyclin A/luciferase reporters. TGFβ treatment and luciferase assays were carried out as described in the legend to panel A ($n = 3$; $P < 0.05$, compared to solvent-treated control).

(15). A ^{32}P -labeled double-stranded oligonucleotide probe spanning the human cyclin A promoter region from -84 to -63 (5'-TTGAATGACGTCAAGGCC GCG-3' [the CRE is underlined]) was used. The mutated CRE oligonucleotide was synthesized as 5'-TTAAATGAATTCAAGGCCGCG-3' (33). Unlabeled oligonucleotide was added to the preincubation mixture for competition assays (50-fold molar excess).

Statistical analysis. Values were expressed as means $\pm$ standard error of the means. The unpaired Student *t* test was used to evaluate the statistical differences between control and treated groups. Values of P of <0.05 were considered significant. All experiments were repeated at least three times.

RESULTS

TGFβ suppresses cyclin A expression in VSMCs. We used mouse aortic VSMCs and a line of nondifferentiated rat aortic VSMCs (A10), which bear significant resemblance to cells of intimal lesions (26). Cells were treated with 5 ng/ml of TGFβ for 24 h, and cell proliferation was determined by [^3H]thymidine incorporation. TGFβ inhibited DNA synthesis in both A10 and mouse VSMCs (Fig. 1A) in a fashion similar to that

described in our previous report with human saphenous vein VSMCs (21). Next, we evaluated the expression of three cell cycle regulators known to be affected by TGFβ in non-VSMCs. TGFβ decreased the level of cyclin A protein at 24 h (Fig. 1B). In contrast, levels of the CDK inhibitors p21 and p27 were not altered (Fig. 1B). A time course analysis indicated that the TGFβ effect on cyclin A was evident at 12 h but more prominent at 18 h. Interestingly, TGFβ also elicited a transient decrease in the level of p27 without affecting that of p21 (Fig. 1C). We next examined the steady-state level of cyclin A mRNA following 12, 18, and 24 h of TGFβ treatment by Northern blotting. TGFβ significantly reduced the level of cyclin A mRNA at all time points (Fig. 2A).

TGFβ inhibits cyclin A promoter activity through a *cis* element that contains a CRE. To test whether the TGFβ signal acts on the cyclin A promoter to reduce transcription, we transfected A10 cells with a luciferase reporter plasmid that contains the proximal region of the human cyclin A promoter

FIG. 3. TGF β reduces CRE-protein complex formation. (A) Nuclear extract was isolated from control or TGF β -treated (5 ng/ml, 60 min) A10 VSMCs. A gel shift assay was performed with a double-stranded 32 P-labeled oligonucleotide containing the CRE region of the cyclin A promoter. Cold oligonucleotides were added at 50-fold excess where indicated. (B) The gel shift assay was carried out as described in the legend to panel A. Before addition of the probe, nuclear extracts were incubated with antibody to CREB or Smad2/3. (C) Western blots of cell lysates isolated from A10 cells stimulated with or without 5 ng/ml of TGF β for 60 min. Wt, wild type; Mt, mutant; IgG, immunoglobulin G.

(-516 to +100) (Fig. 2B). Treatment with TGF β for 18 h reduced the reporter activity by ~40%. A 5' deletion up to -54 bp completely eliminated the TGF β response, while deletion up to -79 bp led to a small reduction, suggesting that the major TGF β -responsive element is located in a region between positions -79 and -54 of the human cyclin A promoter (Fig. 2B). Since this potential TGF β -responsive element contains a CRE motif, previously demonstrated to be essential for cyclin A expression (6), we tested whether this CRE motif plays a role in TGF β 's regulation of cyclin A expression. To this end, we transfected A10 cells with a cyclin A reporter that contained a mutation in the CRE sequence (Fig. 2C). This CRE mutation markedly diminished the ability of TGF β to decrease cyclin A transcription (Fig. 2C). Consistent with previous reports, the basal level of cyclin A promoter activity in VSMCs was also reduced by the CRE mutation (data not shown).

TGF β inhibits the protein-CRE interaction. We next characterized the binding between nuclear proteins and the CRE

FIG. 4. TGF β treatment leads to a rapid increase in the level of phosphorylated CREB (Phospho-CREB) via the TGF β receptor kinase. (A) A10 cells were treated with TGF β (5 ng/ml) for the indicated durations. Phosphorylation of CREB was evaluated using antibodies specific for phosphoserine 133 ($n = 3$; **, $P < 0.01$, and *, $P < 0.05$, compared to solvent-treated control). (B and C) A10 cells were pre-incubated in 5 μ M SB-431542 (type I TGF β receptor inhibitor) for 1 h and treated with TGF β (5 ng/ml) for 15 min (B) or forskolin (25 μ M) for 30 min (C). Phospho-CREB levels were measured by Western blotting.

motif of the cyclin A promoter by using a gel shift assay. A double-stranded oligonucleotide (oligo) DNA fragment corresponding to the -84-to-+63 region of the human cyclin A promoter was 32 P labeled and used as a probe. When it was incubated with nuclear extract isolated from control A10 cells, the labeled probe formed a DNA-protein complex, which showed as a retarded or shifted band (Fig. 3A). To confirm the specificity of this DNA-protein complex, we performed the gel shift assay in the presence of an excess amount of unlabeled (cold) oligo. The presence of the cold oligo completely eliminated the band shift. In contrast, a cold oligo that bore the CRE mutation had no effect (Fig. 3A). Once the specificity of the DNA-protein complex was confirmed, we then tested how TGF β affected the formation of this complex. A10 VSMCs were treated with TGF β (5 ng/ml) for 60 min, and the gel shift assay was performed as described above. As shown in Fig. 3A, TGF β markedly inhibited protein-CRE binding. Since CREB is among the transcription factors known to interact with the CRE, we next tested whether CREB is the protein factor or one of the factors that binds to the cyclin A promoter. To this end, we performed the gel shift assay in the presence of an

FIG. 5. Stimulation of CREB phosphorylation decreases cyclin A expression. (A) A10 cells were treated with forskolin (25 μ M) or 6bnz-cAMP (100 μ M) for 30 min. Activation of CREB was evaluated using antibodies specific for phosphorylated CREB (Phospho-CREB). (B) A10 cells were transfected with the cyclin A/luciferase reporter. Following transfection, cells were stimulated for 18 h with forskolin or 6bnz-cAMP at the indicated concentrations. Reporter activities were expressed as ratios of firefly luciferase to *Renilla* luciferase. ($n = 3$; *, $P < 0.05$, compared to solvent-treated control). (C) Cells were treated with forskolin (25 μ M) for 30 min. Nuclear protein was analyzed by gel shift assay using a 32 P-labeled probe containing the cAMP responsive element of cyclin A. (D) Western blots show cell lysates isolated from A10 cells stimulated with forskolin for 18 h.

antibody specific to CREB. The CREB antibody shifted the DNA-protein complex further (Fig. 3B), indicating the presence of CREB in the protein-CRE complex. In contrast, an antibody specific to Smad2/Smad3 did not alter the band shift (Fig. 3B). TGF β treatment resulted in a significant reduction in both shifted and super-shifted bands (Fig. 3B). Western blotting analysis using the same Smad2/Smad3 antibody confirmed that A10 VSMCs express both TGF β -dependent Smad proteins (Fig. 3C).

TGF β induces rapid CREB phosphorylation. We next sought to understand how TGF β inhibits the interaction between CREB and the cyclin A promoter. We evaluated the levels of phosphorylated CREB in the control and in TGF β -treated A10 VSMCs by using an antibody specific to phosphoserine 133. TGF β treatment elicited a rapid increase in the level of phosphorylated CREB without affecting the level of total CREB (Fig. 4A). This induction of CREB phosphorylation was dependent on the activation of the TGF β receptor. Pretreatment of A10 cells with SB-431542, a specific inhibitor

of the type I TGF β receptor, completely blocked the induction of CREB phosphorylation by TGF β without affecting CREB phosphorylation caused by forskolin (Fig. 4B and C).

Stimulation of CREB phosphorylation represses cyclin A promoter activity. Since the induction of CREB phosphorylation precedes the inhibition of the CREB-cyclin A promoter interaction, we sought to test whether TGF β -induced CREB phosphorylation underlies cyclin A downregulation. We started by stimulating CREB phosphorylation with forskolin or with the cAMP analog 6bnz-cAMP. Indeed, treating A10 VSMCs with forskolin or 6bnz-cAMP increased the level of phosphorylated CREB (Fig. 5A). Moreover, both forskolin and 6bnz-cAMP inhibited cyclin A reporter activity (Fig. 5B). To test whether enhanced CREB phosphorylation affected its ability to bind to the cyclin A promoter, we performed a gel shift assay using the wild-type cyclin A promoter oligo as a probe. Increasing CREB phosphorylation, via forskolin treatment, inhibited the CREB-cyclin A promoter interaction (Fig. 5C). Furthermore, Western blotting demonstrated that the level of

FIG. 6. Inhibition of CREB phosphorylation by expression of a CREB phosphorylation mutant stimulates cyclin A expression. (A) A10 cells were infected with an adenovirus-expressing mutant of CREB in which the phosphorylation site at Ser133 was changed to alanine (AdCREB-S133A). Forty-eight hours after infection, cell lysates were analyzed for phosphorylated CREB (Phospho-CREB) and total CREB. AdNull, adenovirus null construct. (B) A10 cells were infected with adenoviruses, followed by transfection with the cyclin A reporter ($-516/+100$). Luciferase activity was evaluated as described in Materials and Methods ($n = 3$; *, $P < 0.05$, compared to AdNull). (C) Cellular extract was isolated 48 h after viral infection. DNA-protein interaction was evaluated by means of a gel shift assay using the CRE region of the cyclin A promoter, and (D) cyclin A expression was measured by Western blotting. A band shift is visible in lane 2 after a longer exposure.

cyclin A protein in forskolin-treated cells was also markedly diminished (Fig. 5D). These data suggest that stimulation of CREB phosphorylation by the cAMP pathway can simulate the effect of TGF β on cyclin A gene expression.

Inhibition of CREB phosphorylation stimulates cyclin A expression. We next employed a CREB mutant in which serine 133, the major phosphorylation site of CREB, is changed to alanine (CREB-S133A). Infection of A10 cells with AdCREB-S133A led to an increase in CREB abundance, as the same antibody recognizes both the wild-type and the mutant CREB. Of note, expression of the mutant CREB-S133A also reduced the level of phosphorylation of endogenous CREB (Fig. 6A). To test whether the reduction in CREB phosphorylation affected its ability to activate the cyclin A promoter, we carried out the promoter analysis with cells expressing mutant CREB-S133A. Expression of CREB-S133A markedly stimulated cyclin A reporter activity (Fig. 6B). Additionally, gel shift analysis indicated that the CREB-S133A mutant dramatically increased the amount of nuclear proteins bound to the cyclin A promoter (Fig. 6C). Furthermore, the level of cyclin A protein was also markedly elevated in CREB-S133A-expressing cells (Fig. 6D). These data suggest that inhibition of CREB phosphorylation facilitates CREB-cyclin A promoter interaction, which in turn stimulates cyclin A expression.

Inhibition of CREB phosphorylation blocks TGF β 's effects on cyclin A expression. To test the hypothesis that TGF β

downregulates cyclin A gene expression by phosphorylating CREB, we expressed the CREB phosphorylation mutant, CREB-S133A, in A10 cells prior to TGF β treatment. Western blotting confirmed that the CREB-S133A mutant blocked CREB phosphorylation in response to TGF β (Fig. 7A). Corresponding with diminished CREB phosphorylation, TGF β 's effect on cyclin A promoter activity was also eliminated (Fig. 7B). Moreover, in CREB-S133A-expressing A10 cells, TGF β failed to downregulate cyclin A expression (Fig. 7C). Finally, we tested whether CREB phosphorylation was involved in TGF β 's regulation of cell proliferation. As shown in Fig. 7D, AdCREB-S133A diminished the ability of TGF β to inhibit DNA synthesis. Taken together, our data demonstrate that TGF β -induced CREB phosphorylation is essential for this cytokine to regulate cyclin A gene expression as well as for VSMC proliferation.

The TGF β -induced CREB phosphorylation and cyclin A downregulation involves PKC δ . We have previously shown, using the same VSMCs, that PKC δ is activated by TGF β and plays a critical role in the regulation of fibronectin expression by this cytokine. To test whether PKC δ plays any role in CREB phosphorylation and cyclin A downregulation, we inhibited endogenous PKC δ activity via a dominant-negative mutant. Forty-eight hours following infection with an adenovirus that expresses the PKC δ mutant (Ad δ KD) or an empty vector, cells were stimulated with TGF β (5 ng/ml) for 15 min. As shown

FIG. 7. Inhibition of CREB phosphorylation abolishes the inhibitory effect of TGFβ on cyclin A. (A) A10 cells were infected with an adenovirus null construct (AdNull) or Ad-CREB-S133A. Forty-eight hours after infection, cells were treated with solvent or TGFβ for 15 min. Cell lysates were analyzed for phosphorylated CREB (Phospho-CREB) or total CREB ($n = 4$; *, $P < 0.05$, compared to solvent-treated control). (B) A10 cells, which were infected with AdNull or Ad-CREB-S133A, were transfected with a cyclin A/luciferase reporter. After transfection, cells were treated with solvent or TGFβ for 18 h. Reporter activities were expressed as ratios of firefly luciferase to *Renilla* luciferase ($n = 3$; $P < 0.05$, compared to solvent-treated control). (C) A10 cells were infected with AdNull or Ad-CREB-S133A. Forty-eight hours after infection, cells were treated with solvent or TGFβ. The level of cyclin A in the cell lysates was analyzed by Western blotting ($n = 3$; $P < 0.05$, compared to solvent-treated control). (D) A10 cells were infected with AdNull or Ad-CREB-S133A. Eighteen hours after infection, cells were reseeded into 24-well plates and incubated in medium containing 0.5% FBS for 48 h. DNA proliferation, measured by [³H]thymidine incorporation, was performed in the presence or absence of TGFβ as described in Materials and Methods ($n = 3$; *, $P < 0.05$, compared to solvent-treated control).

above, TGFβ elicited an increase in phosphorylated CREB, while expression of the AdδKD mutant completely blocked this induction (Fig. 8A). Similar inhibition was also observed with the selective PKCδ chemical inhibitor rottlerin (data not shown). Interestingly, ectopic expression of PKCδ in VSMCs resulted in enhanced CREB phosphorylation in the absence of the TGFβ ligand (Fig. 8A). These data suggest that PKCδ is one of the signaling components that mediates CREB phosphorylation in response to TGFβ.

Next, we examined whether inhibition of PKCδ affects the ability of TGFβ to regulate cyclin A expression. Following viral infection, cells were treated with TGFβ (5 ng/ml) for 18 h. As shown earlier, TGFβ treatment led to a reduction of cyclin A protein. However, inhibition of PKCδ activity by the dominant-negative mutant (AdδKD) completely eliminated TGFβ-induced cyclin A downregulation (Fig. 8B). Conversely, activation of PKCδ alone through overexpression was sufficient to suppress the cyclin A expression (Fig. 8B).

To further confirm the role of PKCδ in the regulation of CREB phosphorylation and cyclin A expression, we isolated

aortic VSMCs from PKCδ-deficient mice and their wild-type littermates. Both PKCδ null cells and wild-type VSMCs were treated with TGFβ and examined for CREB phosphorylation and cyclin A expression, as described above. As observed in A10 VSMCs, the wild-type mouse VSMCs responded to TGFβ stimulation with a rapid induction of CREB phosphorylation, followed by a reduction in cyclin A protein expression (Fig. 8C and D). These effects of TGFβ, however, were markedly blunted in PKCδ-deficient VSMCs (Fig. 8C and D).

DISCUSSION

TGFβ inhibits proliferation of most normal cell types by halting cell cycle progression (20). Possible cell cycle-related TGFβ target genes include those encoding p21, p27, cyclin A, cyclin D, *c-myc* and others, depending on the specific cell type (20). Data from the present studies demonstrate that TGFβ negatively regulates cyclin A in VSMCs, which at least in part explains its inhibition of cell proliferation.

Cyclin A plays a critical role in the cell cycle G₁-to-S-phase

FIG. 8. Inhibition of PKC δ blocks TGF β -induced CREB phosphorylation and cyclin A downregulation. (A and B) A10 cells were infected with AdPKC δ , AdPKC δ KD, or an adenovirus null construct (AdNull). Forty-eight hours after infection, cells were treated with solvent or TGF β (5 ng/ml) for 15 min (A) ($n = 3$) or for 18 h (B) ($n = 3$; *, $P < 0.05$, compared to solvent-treated control). Cell lysates were blotted for phosphorylated CREB (Phospho-CREB) (A) or cyclin A (B). (C and D) Aortic VSMCs, isolated from PKC δ -deficient ($-/-$) mice or their wild-type ($+/+$) littermates, were stimulated with TGF β as described in panel A or B legends. Cell lysates were blotted for Phospho-CREB (C) ($n = 4$) or cyclin A (D) ($n = 3$; *, $P < 0.05$, compared to solvent-treated control).

transition by forming complexes with and regulating the activities of CDC2 and CDK2 (36). Expression of cyclin A is tightly regulated through control of gene transcription. Within its promoter, several DNA *cis* elements including the CRE, SP1, and E2F have been identified; in particular, the CRE site is required to achieve maximal levels of cyclin A transcription starting in the late G₁ phase (7). Our results also establish that the CRE site in the cyclin A promoter mediates the TGF β response in VSMCs. Moreover, disruption of the CRE site by deletion or site-directed mutagenesis reduced basal activity of the cyclin A promoter and eliminated its response to TGF β . In addition to our finding in VSMCs, the importance of the CRE site in the regulation of cyclin A by TGF β has also been confirmed in mink lung epithelial cells and lung fibroblasts (37) (1).

The best-characterized transcription factors that bind to the CRE site are CREB, ATF1, and ATF2, members of the ATF/CREB family. Using a gel shift assay, we identified that CREB is a component of the protein-DNA complex that forms on the -79-to--54 region of the cyclin A gene. This binding is sig-

nificantly inhibited by TGF β , suggesting that this cytokine suppresses cyclin A transcription by preventing the CREB family of transcription factors from binding to the promoter. Similarly, Djaborbkhel et al. reported that in lymphoma cells, TGF β impedes CREB1 and ATF2 from binding to the CRE site of the cyclin A promoter (9). However, in mink lung epithelial cells (Mv1Lu), the CREB-CRE interaction was found to be unaffected by TGF β (37). Although the exact cause of this discrepancy is unknown, one apparent difference between the present study and the one by Yoshizumi et al. is the duration of TGF β treatment. We chose to examine the CREB-CRE interaction following 60 min of TGF β treatment, based on the fact that during this window of time, nuclear accumulation of R-Smad is readily detectable (18). In contrast, Yoshizumi and colleagues performed their analyses at much later time points (12 or 24 h), at which time TGF β 's reduction of cyclin A mRNA and protein has already reached the maximum. The differences in TGF β treatment may also help to explain the seemingly discordant reports concerning CREB phosphorylation. We demonstrated that treatment of VSMCs with TGF β

produced a rapid, receptor-dependent induction of CREB phosphorylation. This induction is consistent with the rapid effect of TGF β on cyclin A promoter-nuclear protein interaction. In the Mv1Lu study, however, TGF β was found to decrease phosphorylation of CREB and ATF-1 after 24 h of TGF β treatment (37). Similar inhibition of CREB phosphorylation and other CRE binding proteins was also reported for lymphoma cells (9). When examining CREB phosphorylation following prolonged treatment with TGF β (>12 h), we also observed a reduction of CREB phosphorylation (K. Sakakibara and B. Liu, unpublished observation). Therefore, our data are not entirely inconsistent with the previous reports. However, we believe that the early induction of CREB phosphorylation rather than the late inhibition is more relevant to the regulation of cyclin A expression, at least in VSMCs.

We postulate that CREB phosphorylation is a critical step in TGF β 's downregulation of cyclin A transcription, based on several experiments using both molecular and chemical approaches. First, inhibition of TGF β phosphorylation led to enhanced protein-DNA interaction, cyclin A transcription, and cyclin A protein expression. In contrast, the opposite effects were observed with reagents that stimulated CREB phosphorylation. More importantly, blocking TGF β -induced phosphorylation of CREB eliminated the effect of TGF β on cyclin A expression and VSMC proliferation. It is not clear how TGF β -induced CREB phosphorylation (at Ser133) could prevent the binding of this transcription factor to the cyclin A promoter. One possibility is that TGF β stimulation leads to phosphorylation of CREB at additional sites, which could be inhibitory to CREB function. For example, calcium/calmodulin-dependent protein kinase II promotes phosphorylation of CREB at Ser133 and Ser142 (32). It is thought that the concurrent phosphorylation at Ser142 blocks the interaction of CREB with its coactivator CBP (24). Alternatively, TGF β -phosphorylated CREB may have a higher affinity for a CRE located in a promoter other than that of cyclin A. The observation that the ectopic expression of the S133A CREB mutation in VSMCs led to enhanced binding to the cyclin A promoter supports this hypothesis. However, we cannot exclude the possibility that the increased gel shift signal shown in Fig. 6C is due to the presence of a large amount of CREB mutant in Ad-CREB-S133A-infected cells. Finally, TGF β could also affect CREB's function through other mechanisms, such as affecting its interaction with other nuclear proteins.

Our finding that treatment of VSMCs with a cAMP analog or forskolin leads to the inhibition of cyclin A transcription is consistent with the proquiescent roles of cAMP and CREB in the vessel wall. It has been demonstrated both in vivo and in vitro that the CREB content correlates negatively with the proliferation of VSMCs (12). Moreover, forced expression of active CREB decreases mitogen-stimulated proliferation and migration (12). More recently, adenovirus-mediated gene transfer of a CREB-DNA binding mutant was reported to promote VSMC apoptosis and inhibit intimal lesions following balloon angioplasty (34). It is postulated that in the healthy vessel wall, transient exposure to agonists result in phosphorylation of CREB, which in turn promotes VSMC differentiation and survival; however, in the diseased vessel, chronic stimulation causes downregulation of CREB phosphorylation, which leads to cell death (27). Given the frequent presence of the

CRE in the genome and the fact that CREB can be activated and influenced by multiple kinases and signals, it is no surprise that the role of CREB in the vessel wall is complex and multifunctional.

While it appears that Smad2 and/or Smad3 is not directly involved in the regulation of cyclin A transcription, Smad2/3 could potentially influence CREB activity and, thus, cyclin A expression, since the CREB coactivators CBP and P300 have been shown to interact with the Smads (30). Furthermore, Smad3 has been recently found to first bind and then activate protein kinase A (39), the kinase downstream of cAMP. However, in Chinese hamster lung fibroblasts, TGF β downregulates cyclin A gene expression in a PKA-independent mechanism, although activation of the cAMP/PKA also potently inhibits cyclin A expression, suggesting that TGF β and cAMP/PKA attenuate cyclin A expression through separate pathways (1). We speculate that TGF β regulates cyclin A in VSMCs through a PKC δ -dependent pathway, since inhibition of PKC δ blocks both TGF β -induced CREB phosphorylation and cyclin A downregulation. Similar non-Smad-mediated pathways in TGF β signaling have been increasingly appreciated (5). Using VSMCs, we have recently shown that PKC δ can be activated rapidly by TGF β and such activation is critical for another TGF β function in smooth muscle cells, i.e., to stimulate the synthesis of the matrix protein fibronectin (28).

In conclusion, the present study demonstrates that TGF β represses the expression of an important cell cycle regulator through CREB, which, at least in part, leads to inhibition of VSMC proliferation. Despite being known as a major profibrotic factor, TGF β is also a potent inhibitor of VSMC growth. Identifying signaling intermediates and transcriptional mediators activated by TGF β will allow us to isolate mutations or alterations in the pathway that lead to the loss of growth repression observed during tumorigenesis and the pathogenesis of atherosclerosis.

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