Down-regulation of the Cyclin A Promoter by Transforming Growth Factor-\(\beta\)1 Is Associated with a Reduction in Phosphorylated Activating Transcription Factor-1 and Cyclic AMP-responsive Element-binding Protein* 

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Transforming growth factor (TGF)-\(\beta\)1 prevents cell cycle progression by inhibiting several regulators, including cyclin A. To study the mechanisms by which TGF-\(\beta\)1 down-regulates cyclin A gene expression, we transfected reporter plasmids driven by the cyclin A promoter into mink lung epithelial cells in the absence and presence of TGF-\(\beta\)1. The TGF-\(\beta\)1-induced down-regulation of cyclin A promoter activity appeared to be mediated via the activating transcription factor (ATF) site, because mutation of this site abolished down-regulation. Surprisingly, although TGF-\(\beta\)1 treatment for 24 h markedly decreased cyclin A promoter activity, it did not decrease the abundance of the ATF-binding proteins ATF-1 and cyclic AMP-responsive binding protein (CREB). However, we detected 90 and 78% reductions (by Western analysis) in phosphorylated CREB and ATF-1, respectively, in mink lung epithelial cells treated with TGF-\(\beta\)1. TGF-\(\beta\)1-induced down-regulation of cyclin A promoter activity was reversed by okadaic acid (a phosphatase inhibitor) and by cotransfection with plasmids expressing the cAMP-dependent protein kinase catalytic subunit or the simian virus small tumor antigen (Sm-t, an inhibitor of PP2A). These data indicate that TGF-\(\beta\)1 may down-regulate cyclin A promoter activity by decreasing phosphorylation of CREB and ATF-1.

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) inhibits proliferation of most normal cell types in culture and in vivo (1, 2). Because TGF-\(\beta\) arrests progression of the cell cycle in mink lung epithelial (Mv1Lu) cells when added both early and late in the G1 period (3), the inhibitory effect of TGF-\(\beta\) could be mediated by more than one mechanism. The progress of the cell cycle is regulated by the sequential expression of cyclins, followed by the activation of their associated cyclin-dependent kinases (Cdks) (4). Inhibitors of the Cdks block cell cycle progression (5–8). The many cell cycle regulators that TGF-\(\beta\) has been shown to affect include Cdk2, Cdk4, p15\(^{INK4a}\), p21\(^{Waf1/Cip1}\), p27\(^{Kip1}\), and cyclin A (9–17).

Cyclin A associates with Cdk2 in the S phase of the cell cycle and with Cdc2 in the G2/M phase, and it is required for DNA replication in the S phase (4, 18). Cyclin A can also affect the G1/S transition, as overexpression of cyclin A but not cyclin D1 or E overcomes the G1/S block induced by loss of cell adhesion (19). The human and mouse cyclin A genes have been cloned recently and their promoters analyzed (20–24), and the CDE/CHR consensus sequence has been implicated as a negative regulator of cyclin A promoter activity during the cell cycle (20, 23). Although the transcription factors that bind to the CDE/CHR site have not been cloned, these factors bind to the site only during the G1 and the early G1 phases of the cell cycle.

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The cyclin A ATF site is bound by ATF-1 and the cyclic AMP-responsive binding protein (CREB), which function as positive regulators of the cyclin A promoter (21). The many cell cycle regulators that TGF-\(\beta\) has been shown to affect include Cdk2, Cdk4, p15\(^{INK4a}\), p21\(^{Waf1/Cip1}\), p27\(^{Kip1}\), and cyclin A (9–17).

We describe in this report mechanisms by which TGF-\(\beta\)1 down-regulates cyclin A promoter activity in mink lung epithelial cells. A TGF-\(\beta\)1-induced down-regulation of cyclin A promoter activity was mediated through the ATF site. Twenty-four hours of TGF-\(\beta\)1 treatment decreased cyclin A mRNA and promoter activity but not the abundance of the ATF-binding proteins ATP-1 and CREB. Using antibodies specific for phosphorylated ATF-1 and CREB, we detected a significant reduction in phosphorylated ATF-1 and CREB only in TGF-\(\beta\)1-treated cells. The TGF-\(\beta\)1-induced down-regulation of cyclin A promoter activity was reversed by cotransfection with expression plasmids harboring cAMP-dependent protein kinase (PKA) or simian virus 40 small tumor antigen (Sm-t).

**EXPERIMENTAL PROCEDURES**

Cell Culture—Mink lung epithelial cells (Mv1Lu, CCL-64) were obtained from the American Type Culture Collection and cultured in Dulbeco’s modified Eagle’s medium supplemented with 10% fetal calf
serum (HyClone, Logan, UT) and antibiotics as described (21). The activity of the cyclin A promoter was very sensitive to confluence in Mv1Lu cells, as it is in vascular endothelial cells (21, 27). Cells were plated at a density of about 30,000 cells/cm² on a 10-cm dish (surface area, 55 cm²) for 72 h. To keep them from becoming confluent, the cells were trypsinized, transferred from the 10-cm dish to a 15-cm dish to avoid contact inhibition (which down-regulates cyclin A promoter activity), treated with or without 50 pM TGF-β1 for an additional 36 h, and harvested as described (34). For each construct, the plasmid pCMV-βGal was cotransfected to correct for differences in transfection efficiency, and relative luciferase units were obtained by dividing luciferase activity by β-galactosidase activity. Shown for each plasmid is the ratio (percent) of relative luciferase activity in TGF-β1-treated cells to that in untreated cells (mean ± S.E.).

For the cotransfection study, a catalytic subunit (mut-PKA) into the same vector. The expression plasmids were transfected into Mv1Lu cells by the calcium phosphate method (21, 51). Sixteen to twenty hours after transfection, Mv1Lu cells were trypsinized, transferred from a 10-cm plate to a 15-cm plate to avoid contact inhibition (which down-regulates cyclin A promoter activity), treated with or without 50 pM TGF-β1 for an additional 36 h, and harvested as described (34). For each construct, the plasmid pCMV-βGal was cotransfected to correct for differences in transfection efficiency, and relative luciferase units were obtained by dividing luciferase activity by β-galactosidase activity. Shown for each plasmid is the ratio (percent) of relative luciferase activity in TGF-β1-treated cells to that in untreated cells (mean ± S.E.).

Transfection and Luciferase Assays—Reporter constructs containing fragments of the human cyclin A 5′-flanking region were inserted into the promoterless luciferase reporter plasmid pGL2-Basic (Promega, Madison, WI) as described (21). Cyclin A plasmids contained a 3500-bp Smal-SmoI restriction fragment (bp – 3200 to + 245), a 761-bp ScaI-SmoI fragment (bp – 516 to + 245), and fragments of 471 bp (bp – 266 to + 205), 338 bp (bp – 132/133+205), and 132 bp (bp – 133/–2). The ATF consensus sequence (TGACGTCA) in the plasmids –266/205 and –133/205 was mutated to TGCCCCCCA by polymerase chain reaction to generate the plasmids mut –266/205 and mut –133/205 as described elsewhere (21).

Mv1Lu cells were transfected with 15 µg of luciferase construct by the calcium phosphate method as described (28). To correct for variability in transfection efficiency, we cotransfected 2 µg of pCMV-βGal (containing the potent cytomegalovirus enhancer and promoter driving the β-galactosidase gene) in all experiments. The luciferase assay and β-galactosidase assay were performed as described (21), and the ratio of luciferase activity to β-galactosidase activity in each sample was used as a measure of normalized luciferase activity. Each construct was transfected at least two times, and each transfection was done in quadruplicate. Data for each construct are presented as the mean ± S.E.

For the cotransfection study, a catalytic subunit of PKA, obtained from Dr. R. A. Maurer (Oregon Health Sciences University, Portland, OR) (29) was cloned into pcDNA3 (Invitrogen, San Diego, CA). As a negative control, we cloned an inactivated form of the PKA catalytic subunit (mut-PKA) into the same vector. The expression plasmids pCEP4 Sm-t and pCB6-t-1, encoding full-length Sm-t and a constitutively active form of -1, respectively, were provided by Drs. A. Alberts (Imperial Cancer Research Fund, London, United Kingdom) and S. Shenolikar (Duke University, Durham, NC).

RNA Extraction and Northern Analysis—Total RNA was prepared from Mv1Lu cells by guanidinium isothiocyanate extraction and centrifugation through cesium chloride (30, 31). The human cyclin A cDNA fragment was amplified by the reverse transcription-polymerase chain reaction as described (21). The human ATF-1 plasmid (32) and a human CREB plasmid (33) were obtained from Drs. M. R. Green (University of Massachusetts, Amherst, MA) and M. R. Montminy (Harvard Medical School, Boston).

Total RNA was fractionated on 1.3% formaldehyde-agarose gels and transferred to nitrocellulose filters. The filters were hybridized with a randomly primed, 32P-labeled cyclin A cDNA probe. The hybridized filters were washed in 30 mM NaCl, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate at 40 °C and autoradiographed on x-ray film or stored on phosphor screens for 8–10 h (6). To correct for differences in RNA loading, the filters were washed in a 50% formamide solution at 80 °C and rehybridized with a radiolabeled 18 S rRNA oligonucleotide probe (34). The filters were scanned, and radioactivity was measured on a PhosphorImager running the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Gel Mobility Shift Assay—Nuclear extracts from Mv1Lu cells were prepared as described (21). Protein concentrations in nuclear extracts were measured by the Bio-Rad protein assay system, which is based on the Bradford method (35). Double-stranded oligonucleotide probes synthesized according to the sequence of the human cyclin A ATF site (bp – 84 to –63, 5′ TGAATGACGTCAAGGCCGCGAG 3′) were radiolabeled as described (34). A typical binding reaction mixture contained DNA probe at 20,000 cpm, 1 µg of poly(dI-dC)·poly(dI-dC), 25 mM HEPES (pH 7.9), 40 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 3 µg of nuclear extract in a final volume of 25 µl. The reaction mixture was incubated at room temperature for 20 min and analyzed by 0.5% native polyacrylamide gel electrophoresis in 0.25 × TBE buffer (22 mM Tris base, 22 mM boric acid, and 0.5 mM EDTA). To characterize specific DNA-binding proteins, we incubated nuclear extracts with various antibodies for 12 h at 4 °C before addition of the probe. The polyclonal antibodies were raised against human ATF-1 and ATF-2 (Upstate Biotechnology, Inc., Lake Placid, NY) and CREB (New England Biolabs, Beverly, MA).

Western Analysis—To examine the phosphorylation of CREB and ATF-1, we separated 30 µg of nuclear extract (prepared from Mv1Lu cells treated with or without TGF-β1 for 24 h) on a 10% SDS-polyacrylamide gel and immunoblotted the extract with phospho-CREB-specific (Ser-133) antibody according to the protocol provided by the manufacturer (PhosphoPlus CREB [Ser-133] antibody kit, New England Biolabs). This antibody specifically recognizes the phosphorylated forms of CREB and ATF-1. The same blot was then incubated with stripping buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, and 100 mM 2-mercaptoethanol) for 30 min at 50 °C and reblotted with anti-ATF-1 (C41–5.1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which does not cross-react with other members of the ATF/CREB family, and then with anti-CREB (C-21) antibody (Santa Cruz Biotechnology), which is specific for CREB1.

RESULTS

The ATF Site Mediates Down-regulation of Cyclin A Promoter Activity in TGF-β1-treated Mv1Lu Cells—To identify sequences that mediate down-regulation of cyclin A promoter activity by TGF-β1, we transfected a series of luciferase re-

**FIG. 1.** ATF site mediates down-regulation of the cyclin A promoter by TGF-β1. A, plasmids containing various lengths of the 5′-flanking sequence of the cyclin A gene and a luciferase reporter (Lac) gene are depicted. The transcription start site and cis-acting elements are indicated by an arrow and boxes, respectively (21, 24). Mutation of the ATF site is denoted by an X. Each plasmid (15 µg) was transfected into Mv1Lu cells by the calcium phosphate method (21, 51). Sixteen to twenty hours after transfection, Mv1Lu cells were trypsinized, transferred from a 10-cm plate to a 15-cm plate to avoid contact inhibition (which down-regulates cyclin A promoter activity), treated with or without 50 pM TGF-β1 for an additional 36 h, and harvested as described (34). For each construct, the plasmid pCMV-βGal was cotransfected to correct for differences in transfection efficiency, and relative luciferase units were obtained by dividing luciferase activity by β-galactosidase activity. Shown for each plasmid is the ratio (percent) of relative luciferase activity in TGF-β1-treated cells to that in untreated cells (mean ± S.E.).
porter gene plasmids containing various lengths of the human cyclin A 5' flanking sequence into Mv1Lu cells treated with or without TGF-β1 for 48 h. The ratio of luciferase activity on the luciferase activity of pGL2-Control (driven by the potent SV40 enhancer and promoter), it decreased luciferase activity by 80% in all but two plasmids containing the cyclin A promoter (Fig. 1). In these two plasmids (mut −266/+205 and mut −133/+205), the ATF sequence had been mutated. These data indicate that the ATF site mediates down-regulation of cyclin A promoter activity by TGF-β1 in Mv1Lu cells. We also determined the time course of the TGF-β1-induced down-regulation of cyclin A promoter activity. TGF-β1 decreased luciferase activity by 25% at 12 h and by 70% at 24 h (Fig. 2A). Considering the half-life of the luciferase protein (~6 h), inhibition of cyclin A promoter activity must have occurred before 12 h. This possibility was further supported by the finding that TGF-β1 markedly decreased cyclin A mRNA abundance at 12 h (Fig. 2B).

**TGF-β1 Treatment for 24 h Has No Effect on the Abundance of ATF-binding Proteins**—To determine the effect of TGF-β1 on ATF-binding proteins, we performed gel mobility shift analysis with a 22-bp probe encoding the ATF consensus sequence (21) and nuclear extracts prepared from Mv1Lu cells treated with TGF-β1 for 12, 24, 36, and 48 h. Incubation of nuclear extract from untreated Mv1Lu cells with the 22-bp probe resulted in three specific DNA-protein complexes, X, Y, and Z (Fig. 2C), as we have observed in nuclear extract from vascular endothelial cells (21). The three complexes were competed away by an identical unlabeled oligonucleotide but not by an oligonucleotide containing a mutated ATF site (data not shown). To our surprise, we found that TGF-β1 treatment for up to 24 h had no effect on the abundance of complexes X, Y, and Z. This lack of effect is in sharp contrast to the significant reduction in cyclin A mRNA abundance and promoter activity observed 24 h after TGF-β1 treatment. TGF-β1 eventually decreased the abundance of all three complexes of ATF-binding proteins after 36 and 48 h (Fig. 2C). This reduction in ATF-binding proteins may have played an important role in the complete down-regulation of cyclin A transcription at 36 and 48 h (Fig. 2A).

To identify proteins that bind to the ATF-site, we performed gel mobility shift analysis with antibodies. Before addition of the probe, Mv1Lu cell extracts were incubated with or without antibody to ATF-1, CREB, or ATF-2 (Fig. 3). The faint X complex visible in Fig. 2C disappeared in Fig. 3, even in nuclear extracts preincubated without an antibody. Preincubation of nuclear extracts with antibody specific to ATF-1 or CREB, but not ATF-2, markedly decreased the density of the DNA-protein complexes (indicated by an asterisk). These data identify ATF-1 and CREB as the major binding proteins for the cyclin A ATF site in Mv1Lu cells, as we have observed previously in vascular endothelial cells (21).

**TGF-β1 Decreases Phosphorylation of CREB and ATF-1**—At 24 h, TGF-β1 decreased cyclin A mRNA levels and promoter activity (Fig. 2). Surprisingly, it had no effect on the abundance of ATF-binding proteins that regulate cyclin A promoter activity. To investigate this paradox, we examined CREB and ATF-1 phosphorylation, because it has been shown that only the phosphorylated forms of CREB and ATF-1 are active (26, 36–38). We performed Western analysis with 30 μg of nuclear extract from Mv1Lu cells treated with or without TGF-β1 for 24 h and antibodies that recognized phosphorylated CREB, total CREB, phosphorylated ATF-1, and total ATF-1. Although TGF-β1 had
FIG. 4. TGF-β1 treatment for 24-h decreases phosphorylated but not total nuclear CREB and ATF-1 protein. Nuclear extract (30 μg) prepared from Mv1Lu cells treated with 50 pM TGF-β1 for 24 h was separated on SDS-polyacrylamide gels and immunoblotted with an antibody specific for phosphorylated CREB that recognizes the phosphorylated forms of CREB (p-CREB) and ATF-1 (p-ATF-1). The same blot was stripped and then reblifted with an anti-ATF-1 antibody, which recognizes total ATF-1 but not other members of the ATF/CREB family, and an antibody that recognizes total CREB.

little effect on the total amount of CREB and ATF-1, it decreased the phosphorylated forms of CREB and ATF-1 by 90 and 78%, respectively (Fig. 4), as measured on a PhosphorImager.

Reversal of TGF-β1-induced Down-regulation of Cyclin A Promoter Activity by PKA, Okadaic Acid, and Sm-t—If TGF-β1 inhibits cyclin A promoter activity by decreasing phosphorylation of ATF-1 and CREB, agents that increase the phosphorylation of ATF-1 and CREB should counteract this inhibitory effect. To test this hypothesis we cotransfected Mv1Lu cells 15 μg of cyclin A reporter plasmid –266/+205 and various amounts of a PKA catalytic subunit expression plasmid. This form of PKA has been shown to phosphorylate and activate ATF-1 and CREB (24). Cotransfection of the PKA expression plasmid reversed TGF-β1-induced inhibition of cyclin A promoter activity in a dose-dependent manner (Fig. 5). Twenty micrograms of PKA expression plasmid completely suppressed down-regulation of cyclin A promoter activity by TGF-β1. As a negative control, we also cotransfected a mutant PKA expression plasmid that does not phosphorylate CREB. The mutant PKA expression plasmid did not prevent TGF-β1-induced inhibition of cyclin A promoter activity (data not shown).

In contrast with PKA, protein Ser/Thr phosphatases type 1 (PP1) and 2A (PP2A) dephosphorylate CREB (39–41). Inhibitors of CREB/ATF-1 phosphatases should therefore increase phosphorylation of CREB/ATF-1. We first examined the effect of okadaic acid (42), an inhibitor of PP1 and PP2A, on TGF-β1-induced down-regulation of cyclin A promoter activity. Although low doses of okadaic acid had no effect, 20 nM okadaic acid almost completely reversed the inhibitory effect of TGF-β1 on cyclin A promoter activity (Fig. 6A). To determine whether 20 nM okadaic acid affected TGF-β1-induced decreases in ATF-1 and CREB phosphorylation, we treated Mv1Lu cells with or without 20 nM okadaic acid for 30 min before adding TGF-β1. Okadaic acid prevented the down-regulation of ATF-1 and CREB induced by TGF-β1 (Fig. 6B). These data suggest that the phosphorylation status of ATF-1 and CREB may be responsible for TGF-β1-mediated down-regulation of cyclin A transcription. To test further whether inhibition of PP1 or PP2A prevented TGF-β1-induced down-regulation of cyclin A promoter activity, we cotransfected 7.5 μg of cyclin A reporter plasmid –266/+205 with 15 μg of expression plasmid encoding a constitutively active form of I-1 or full-length Sm-t, which inhibit PP1 and PP2A, respectively (39–41, 43, 44). Cotransfection with the Sm-t, but not the I-1, expression plasmid abolished the inhibitory effect of TGF-β1 (Fig. 7A). Furthermore, the Sm-t expression plasmid reversed the inhibitory effect of TGF-β1 in a dose-dependent manner (Fig. 7B). As little as 3.5 μg of Sm-t expression plasmid almost completely abolished inhibition by TGF-β1.

DISCUSSION

Although it has been shown that a functional TGF-β receptor complex (including both types I and II) is required for TGF-β1-induced down-regulation of cyclin A promoter activity (45), the downstream inhibitory pathway has not been completely elucidated. In this report we show that the ATF site mediates inhibition of the cyclin A promoter by TGF-β1 in mink lung epithelial cells (Fig. 1). Treatment with TGF-β1 for 24 h markedly decreased cyclin A mRNA levels but had no effect on the abundance of ATF-binding proteins (Fig. 2).

Phosphorylation of ATF-1 and CREB is crucial to their ability to trans-activate target genes (26). Phosphorylation of
Thus, TGF-β resulted in a marked reduction in phosphorylated ATF-1 and CREB, overcame the inhibitory effect of TGF-β1’s catalytic subunit, which increases phosphorylation of ATF-1 (36, 37, 49). PKA and Ca²⁺ (46–48), and phosphorylation at serine 63 is important to the binding protein) and activate transcription of its target genes CREB at serine 133 is required for it to bind to CBP (CREB-binding protein) and activate transcription of its target genes CREB/ATF-1 phosphorylation to regulate the activity of CREB/ATF-1 (26). Thus, the total activity of ATF-1 PP1 and PP2A have been shown to dephosphorylate CREB and of these transcription factors. Also, the Ser/Thr phosphatases point of CREB/ATF-1 phosphorylation to regulate the activity (50). It appears that multiple signal pathways converge at the pathways) has also been shown to function as a CREB kinase (26). Thus, Sm-t reverses the inhibitory effect of TGF-β1 on cyclin A promoter activity (Figs. 5 and 6).

Although we did not examine the effect of TGF-β1 on individual kinases and phosphatases, TGF-β1 treatment for 24 h resulted in a marked reduction in phosphorylated ATF-1 and CREB but had little effect on total ATF-1 and CREB (Fig. 4). Thus, TGF-β1 may suppress cyclin A activity by reducing ATF-1 and CREB phosphorylation. This conclusion is further supported by our observation that okadaic acid and the PKA catalytic subunit, which increases phosphorylation of ATF-1 and CREB, overcame the inhibitory effect of TGF-β1 on cyclin A promoter activity (Figs. 5 and 6).

The suppression of the inhibitory effect of TGF-β1 on cyclin A promoter activity by okadaic acid in Mv1Lu cells implies the presence of an okadaic acid-sensitive CREB/ATF-1 phosphatase in this cell type. PP1 is the principal CREB phosphatase in fibroblasts and thyroid follicular cells (39, 40), whereas PP2A is the principal CREB phosphatase in hepatocytes (41). This discrepancy may be due to differences in protein expression among cell types or to differences in protein phosphatase preparations (41). The relative abundance of these phosphatases in Mv1Lu cells has not been estimated. Our finding that cotransfection of Sm-t, but not I-1 (Fig. 7), abolished TGF-β1’s inhibitory effect indicates that PP2A may play a role in TGF-β1-induced dephosphorylation of CREB/ATF-1 in Mv1Lu cells, but certainly does not exclude a role for PP1-mediated dephosphorylation of CREB/ATF-1 in this cell type.

Our data indicate that TGF-β1 may inhibit cyclin A promoter activity by decreasing the phosphorylation and activity of CREB and ATF-1 but not their abundance. These data illustrate one of the biochemical pathways by which TGF-β inhibits cyclin A transcription and progression of cell cycle.

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