MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 2030–2037 Vol. 17, No. 4
0270-7306/97/$04.00+0

The Viral Oncoprotein E1A Blocks Transforming Growth Factor β-Mediated Induction of p21/WAF1/Cip1 and p15/INK4B

MICHAEL B. DATTO,[1] PATRICK PEI-CHIH HU,[1] TIMOTHY F. KOWALIK,[2]†
JONATHAN YINGLING,[1] AND XIAO-FAN WANG[1]*

Departments of Pharmacology[1] and Genetics,[2] Duke University Medical Center, Durham, North Carolina 27710

Received 25 September 1996/Returned for modification 12 November 1996/Accepted 17 January 1997

The adenovirus early gene product E1A is a potent stimulator of cellular proliferation, which when overexpressed can overcome the growth-inhibitory effects of the polypeptide hormone transforming growth factor β (TGF-β). The ability of TGF-β to arrest cell growth in G_1 correlates with the transcriptional induction of the cyclin-dependent kinase inhibitors, p15/INK4B and p21/WAF1/Cip1; an inhibition of the G_1 cyclin-Cdk complexes; and a maintenance of the retinoblastoma susceptibility gene product, Rb, in a hypophosphorylated state. The ability of E1A to overcome TGF-β-mediated growth inhibition derives, in part, from its ability to sequester Rb and Rb family members. We report here that E1A also acts upstream of Rb by blocking the TGF-β-mediated induction of p15 and p21. Consistent with these findings, E1A expression also blocks the ability of TGF-β to inhibit Cdk2 kinase activity, as well as its ability to hold Rb in a hypophosphorylated state. The effect of E1A on the induction of p15 and p21 is independent of E1A's Rb binding activity. The E1A-mediated decrease in p15 levels is primarily the result of a block at the level of transcriptional activation by TGF-β. This effect is dependent on E1A's ability to bind p300, one of E1A's target proteins. Thus, the ability of E1A to affect p15 and p21 expression represents an additional possible mechanism by which E1A can circumvent the negative regulation of cell cycle progression.

The adenoviral oncoprotein E1A is an early gene product from the adenovirus genome that acts as a potent stimulator of cell cycle progression. In quiescent cells, E1A can force inappropriate S-phase progression, which, in the context of the viral life cycle, is necessary for replication of the viral genome (reviewed in reference 38). This proliferative effect of E1A is also at the root of its actions as a potent oncoprotein and is thought to stem from its ability to bind to and inactivate a group of proteins which includes the tumor suppressor gene product Rb (54), the Rb family members p107 (16) and p130 (17, 30), and the CBP-related protein, p300 (2, 12, 34–36, 55).

Perhaps the best understood of these E1A-binding proteins is Rb. Rb is a classic tumor suppressor whose regulation through phosphorylation is intimately linked to cell cycle progression (5, 7, 10, 32). In G_0 and early G_1, Rb exists in a hypophosphorylated state, enabling it to bind to a family of transcription factors known as E2Fs which regulate the expression of many genes that are necessary for S-phase progression (3, 6, 11, 37). By binding to the E2Fs, Rb and Rb family proteins prevent the transcription of these S-phase genes, both by blocking the transactivating capabilities of E2F and by acting as an active transcriptional repressor in the form of Rb-E2F complexes when bound at certain promoters (4, 52; also reviewed in references 37 and 51). As cells progress through G_1 and enter S phase, Rb becomes hyperphosphorylated by the G_1 cyclin-dependent kinases, cyclin D-Cdk4/Cdk6 and cyclin E-Cdk2 (15, 39, 46; also reviewed in references 21 and 48). In their hyperphosphorylated state, Rb and Rb family proteins no longer bind to E2F family members, and these repressive effects of Rb are lost, resulting in the transcription of S-phase-specific genes and S-phase progression (reviewed in reference 37).

In this conceptual context, a possible mechanism through which E1A causes S-phase progression becomes apparent. By binding to Rb family proteins and preventing their association with the E2Fs, E1A can increase the level of active E2F and relieve the transcriptional repression of S-phase-specific genes to force S-phase progression (3, 22, 45).

Several studies of the role of E1A in overcoming the growth-inhibitory effects of transforming growth factor β (TGF-β) have suggested that E1A may exhibit multiple actions leading to the inactivation of Rb function (1, 50). TGF-β is a potent growth-inhibitory protein hormone which causes a G_1 cell cycle arrest in many cell types. This arrest is associated with a decrease in G_1 cyclin-Cdk complex kinase activity (23); an activation of the cyclin-dependent kinase inhibitors, p21/WAF1/Cip1 (8, 13, 28), p15/INK4B (17), and p27/KIP1 (43, 44, 47); an association of these inhibitors with their G_1 cyclin-Cdk targets; and a maintenance of Rb in a hypophosphorylated state (25; reviewed in references 21, 41, and 57). By keeping Rb hypophosphorylated, TGF-β prevents the E2F-mediated activation of S-phase-specific genes. Expression of E1A prevents TGF-β-mediated cell cycle arrest, due in part to the ability of E1A to bind to and inactivate Rb (33, 42).

Interestingly, E1A also stimulates Rb phosphorylation in TGF-β-treated cells (50) and blocks the TGF-β-mediated decrease in the activity of cyclin-Cdc2 complexes. These effects of E1A are independent of Rb binding, since mutant E1A with attenuated Rb binding ability functions like wild-type E1A (1, 31). These studies led to the hypothesis that E1A works not only by binding to Rb but also by acting upstream of Rb to cause its phosphorylation through the activation of cyclin-dependent kinases (50). Given recent work demonstrating the TGF-β-mediated regulation of the cyclin-dependent kinase complexes by p15 and p21, these two cyclin-dependent kinase inhibitors become likely candidates as targets for E1A's action. To test this hypothesis, we examined the ability of E1A to

* Corresponding author. Mailing address: Department of Pharmacology, Box 3813, Duke University Medical Center, Durham, NC 27707. Phone: (919) 681-4861. Fax: (919) 681-7152.

† Present address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, Worcester, MA 01617.

block the TGF-β-mediated transcriptional induction of p15 and p21.

MATERIALS AND METHODS

Cell culture and viral reagents. HaCaT cells were the generous gift of N. E. Fusenig and P. Baukamp. HaCaT cells were maintained in α-minimum essential medium with the addition of 10% fetal bovine serum (FBS), glutamine, penicillin, and streptomycin (Gibco BRL). Purified wild-type E1A-expressing adenovirus with a deletion in the E1B locus, E1A-928 mutant adenovirus also with a deletion in the E1B locus, and the adenovirus control vector with deletions for both E1A and E1B were the generous gift of J. Nevins. The E1A wild-type and 928 mutant viruses were created by E. Moran and first described in reference 36. The relative multiplicity of infection (MOI) values for the different viruses were determined by a standard viral titer assay using the highly infectible 293 cells.

Growth inhibition assays. HaCaT cells were plated in 12-well culture plates at a density of 20,000 cells per well. Cells were then incubated in 0.2 ml of serum-free medium with the indicated MOI of virus for 4 h. A total of 0.8 ml of medium containing 10% FBS was then added to the cells. After 8 h, 100 pM TGF-β was added to the TGF-β-treated cells, and cells were incubated for an additional 14 h. Four microcuries of [^{3}H]thymidine was then added to each well, and after 2 h, cells were washed with phosphate-buffered saline, fixed for 1 h in 10% trichloroacetic acid, and lysed in 0.2 N NaOH. Incorporation of [^{3}H]thymidine into DNA was measured with a scintillation counter. Percent growth inhibition is the percent reduction of [^{3}H]thymidine incorporation in TGF-β-treated cells versus non-TGF-β-treated controls.

Western blot analysis. HaCaT cells were plated in 10-cm culture plates at a density of 10^6 cells per plate. Cells were then incubated in 2 ml of serum-free medium with the indicated MOI of virus for 4 h. Eight milliliters of medium containing 10% FBS was then added to each plate. After 8 h, 100 pM TGF-β was added to the TGF-β-treated cells, and cells were incubated for an additional 16 h. Cell lysates were prepared by lysing cells in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. Protein concentrations were determined, and equal protein amounts of each sample were resolved on either an 8% acrylamide–0.05% bisacrylamide–sodium dodecyl sulfate gel for Rb or a 15% acrylamide–0.5% bisacrylamide–sodium dodecyl sulfate gel for all others. Proteins were then transferred to Immobilon P transfer membrane (Millipore). Blocking and antibody incubations were performed in 4% dried milk and 0.1% Tween 20 in phosphate-buffered saline for 1 h. Antibodies used for Western blots were as follows: anti-p-21 (α-p21) (Santa Cruz C-19), α-p15 (Santa Cruz C-20), α-E1A (Pharmingen M58), α-Cdk2 (Santa Cruz M2), α-cyclin E (Santa Cruz HE111), α-Rb (monoclonal mouse antibody XZ091), horseradish peroxidase-conjugated goat anti-rabbit, and goat anti-mouse (Bio-Rad). Monoclonal anti-Rb antibodies were the generous gift of Ed Harlow (20). Western blots were developed with the ECL reagent (Amersham).

Cdk2 histone H1 kinase assays. HaCaT cells were plated, infected, and treated with TGF-β as described for Western blotting. CDK2 histone H1 kinase assays were performed by first immunoprecipitating Cdk2 with a polyclonal Cdk2 antibody (Upstate Biotechnology Inc. 06-148) and then assaying the ability of the immunoprecipitates to phosphorylate histone H1 as previously described (8).

Luciferase assays. The DNA constructs used in luciferase assays were as follows: the reporter vector p15P113-Luc as described in reference 29; the cytomegalovirus (CMV)-driven E1A expression vectors CMV-E1A, CMV-E1A 928, and CMV-E1A Δ2-36, which were the generous gift of J. Nevins (24); the CMV-driven p300 expression vectors CMV-p300 and CMV-p300dl10, which were the generous gift of Y. Shi (26); and an empty CMV expression vector. HaCaT cells, at a density of 200,000 cells per well in six-well culture plates, were transfected with the appropriate DNA mixes by using a DEAE-dextran transfection protocol which has been previously described (9). In each transfection, 0.25 μg of a CMV-driven β-galactosidase expression vector was included. Twelve hours after transfection, cells were cultured either in the presence or in the absence of TGF-β for 16 h. Cell lysates were then prepared, and luciferase activity and β-galactosidase activity were measured as previously described (9). Transfection efficiency and nonspecific effects of E1A were controlled for by cotransfection of a β-galactosidase reporter construct, and luciferase activity was normalized to β-galactosidase activity.

RNase protection assay. The p15 cDNA was a generous gift of Y. Xiong. An *Sph*I-to-*Sma*I fragment containing the entire p15 cDNA was subcloned into pGEM-7zf(+) (Promega) to generate p15Sph-Sma. p15Sph-Sma was digested with *Sma*I and used as template to synthesize the p15 riboprobe which contains the entire p15 coding region. Riboprobe was synthesized with T7 RNA polymerase in the presence of 650 μM (each) ATP, CTP, and GTP and 5 μl of [α-^{32}P]UTP (800 Ci/mmol). Riboprobe was purified by 5% polyacrylamide–7 M urea gel electrophoresis. HaCaT cells were plated at a density of 2×10^6 cells per 10-cm culture plate and infected with virus as described above. Cells were then treated with 100 pM TGF-β1, and cytoplasmic RNA was prepared at 6 h post-treatment by a standard protocol. RNase protection assays were carried out with an Ambion RPAII kit (Ambion, Austin, Tex.) according to the manufacturer's protocol. The protected fragment was resolved on a 5% polyacrylamide–7 M urea gel and exposed to X-ray film overnight at room temperature.

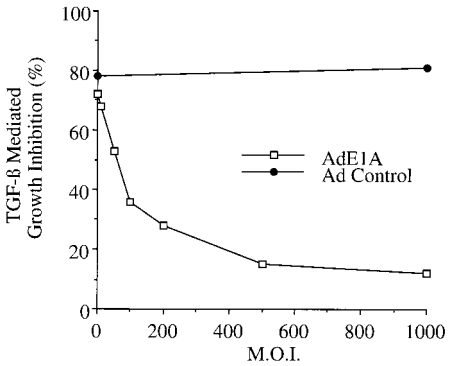

FIG. 1. E1A antagonizes the growth-inhibitory effects of TGF-β. HaCaT cells were infected with an increasing titer of 12S E1A-expressing, E1B$^-$ adenovirus or the control E1A$^-$ E1B$^-$ adenovirus and cultured either in the presence or in the absence of TGF-β. The growth-inhibitory effects of TGF-β were assayed by [^{3}H]thymidine incorporation. Percent growth inhibition represents the percent decrease in thymidine incorporation on TGF-β treatment.

RESULTS

E1A expression blocks the TGF-β-mediated induction of p15 and p21. To examine the effects of E1A on the TGF-β-mediated induction of the cyclin-dependent kinase inhibitors, p15 and p21, we used the HaCaT human keratinocyte model system. HaCaT is a spontaneously immortalized human keratinocyte cell line whose proliferation is inhibited by TGF-β. This inhibition is associated with a dramatic increase in both the RNA and the protein levels of p15 and p21, a decrease in G_1 cyclin-dependent kinase activity, and a maintenance of Rb in a hypophosphorylated state (8, 18, 47). To study the effects of E1A on TGF-β-mediated growth inhibition in this system, we first assayed the ability of E1A to block TGF-β-mediated growth arrest. E1A was introduced into these cells by infection with an adenovirus construct which contains only the 12S form of the E1A gene with a deletion in the E1B locus. This adenoviral construct produces only the 12S form of E1A (36). Since the 13S transactivating form of E1A is not produced, no other viral genes should be expressed nor should viral DNA replication occur in infected cells.

As shown in Fig. 1, the responsiveness of HaCaT cells to the TGF-β growth-inhibitory signal was gradually decreased upon infection with an increasing MOI of the E1A 12S-expressing adenovirus, as assayed by tritiated thymidine incorporation. In contrast, HaCaT cultures infected with a control virus vector with deletions of both E1B and E1A are inhibited in growth normally by TGF-β, suggesting that the inhibition of TGF-β-mediated growth arrest is due to E1A expression. As shown in later figures, the stimulatory effect of E1A on tritiated thymidine incorporation is paralleled by an increase in Cdk2 kinase activity and an increase in Rb phosphorylation. These effects, therefore, likely represent an inappropriate progression of E1A-expressing cells into S phase. The apparent relatively high MOIs needed to observe the maximal effect of E1A on TGF-β-mediated growth inhibition are most likely due to major differences in the efficiency of infection between HaCaT cells and 293 cells, in which the virus titers were determined. As seen below, this assumption is supported by the evidence that the expression of E1A is barely detectable in infected cells when an MOI of 10 is used, suggesting that the productive or

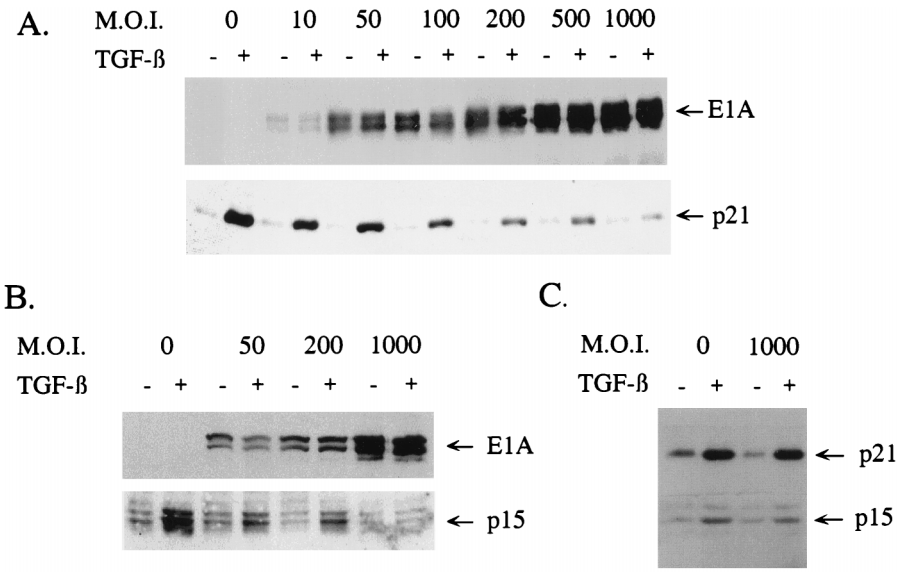

FIG. 2. E1A antagonizes the TGF-β-mediated induction of p21 and p15. (A) HaCaT cells were infected with an increasing viral titer of the E1A-expressing adenovirus and cultured either in the presence or in the absence of TGF-β. Total cell lysates were prepared from these cells, and Western blot analyses of E1A and p21 were performed. (B) HaCaT cells were treated as for panel A. Western blot analyses of E1A and p15 were performed. (C) HaCaT cells were infected with an $E1A^-$ $E1B^-$ control adenovirus at an MOI of either 0 or 1,000× and cultured either in the presence or in the absence of TGF-β. Western blot analyses of p21 and p15 were then performed.

true MOIs of the viruses are 10 times lower than the apparent values.

We next assayed the induction of p15 and p21 protein levels upon TGF-β treatment in E1A virus-infected HaCaT cells. As shown in Fig. 2, the level of E1A expression increases proportionally with an increasing MOI of E1A-expressing adenovirus. This increase in E1A expression leads to a graded decrease in the induction of p15 and p21 by TGF-β (Fig. 2A and B). At an MOI of 10, very little E1A expression can be detected, indicating a low efficiency of infection in HaCaT cells. In the p15 Western blot, two closely migrating bands are detected, bands which probably represent p15 and an antigenically related protein which is also induced upon TGF-β treatment. At the highest level of E1A expression in this assay, only a slight induction of p21 can be detected, and the induction of p15 is completely eliminated. No significant effect of E1A expression is seen on the basal levels of p15 and p21 proteins, suggesting that E1A specifically blocks the TGF-β-induced expression of p21 and p15 without affecting the cellular machinery which dictates their basal expression levels. Control experiments were performed with the control adenovirus with deletions in E1A and E1B, which shows no effect on the TGF-β-mediated induction of p21 and p15 (Fig. 2C).

Consequences of E1A expression on cyclin-dependent kinase activity and Rb phosphorylation. The postulated role of p15 and p21 in TGF-β-mediated growth arrest is to inhibit G_1 cyclin-dependent kinase complexes, resulting in an inhibition of phosphorylation of critical substrates in G_1, including Rb (14, 18, 19, 56). Therefore, by blocking the ability of TGF-β to induce p15 and p21, E1A should prevent the TGF-β-mediated reduction of Cdk activity and block the ability of TGF-β to maintain Rb in a hypophosphorylated state. Consistent with this prediction, TGF-β no longer inhibits Cdk2-associated kinase activity in the presence of E1A, as assayed by the phosphorylation of an exogenous substrate, histone H1 (Fig. 3A). Since the Cdk2-cyclin E complex is a demonstrated binding target of p21 following TGF-β treatment (8), these findings are consistent with the inability of TGF-β to upregulate p21 in the presence of E1A. No change in the level of Cdk2 is observed on infection with E1A (Fig. 3A), although the cyclin E levels increase with E1A virus infection, likely as a result of transcriptional activation of the cyclin E gene by E2F (11) (Fig. 3A).

We have also assayed the state of Rb phosphorylation as an in vivo measurement of these effects of E1A on the TGF-β-mediated inhibition of G_1 cyclin-dependent kinase activity. As shown in Fig. 3B, TGF-β treatment results in the accumulation of hypophosphorylated Rb. In contrast, the majority of Rb is in

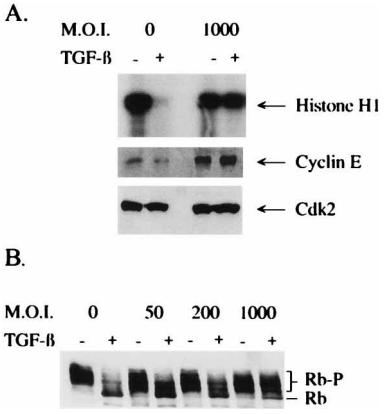

FIG. 3. (A) E1A blocks the TGF-β-mediated inhibition of Cdk2 kinase activity. HaCaT cells were infected with the E1A-expressing adenovirus at an MOI of either 0 or 1,000× and cultured either in the presence or in the absence of TGF-β. Total cell lysates were prepared, and Cdk2 was immunoprecipitated. Kinase activity was determined by assaying the ability of the precipitated complexes to phosphorylate the exogenous substrate histone H1. Western blot analysis of cyclin E and Cdk2 was also performed. (B) E1A antagonizes the ability of TGF-β to hold Rb in a hypophosphorylated state. HaCaT cells were infected and treated as for Fig. 2A. Total cell lysates were prepared, and Western blot analysis of Rb was performed.

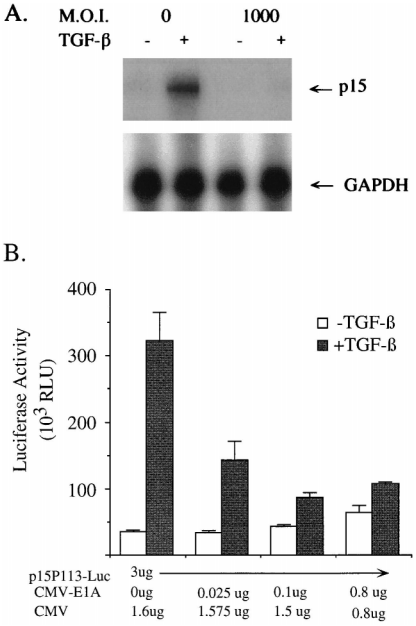

FIG. 4. E1A inhibits the ability of TGF-β to activate the p15 promoter. (A) RNase protection analysis was performed for p15 with RNA purified from both infected and uninfected HaCaT cells which were either treated or not treated with TGF-β for 6 h. RNase protection for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. (B) HaCaT cells were transiently cotransfected with the p15 promoter luciferase reporter construct, p15P113-Luc, and an increasing amount of an E1A expression vector, pCMV-E1A. The total concentration of transfected DNA was held constant with an empty vector, pCMV. A total of 0.25 μg of a CMV promoter-driven β-galactosidase expression vector was included in each transfection to control for transfection efficiency. Values represent luciferase activity normalized to the β-galactosidase activity in both TGF-β-treated and nontreated cells. Error bars represent the standard deviations between duplicate transfections in a single experiment. RLU, relative light units.

the hyperphosphorylated state in both the TGF-β-treated and untreated cells at the highest MOI of the E1A-expressing adenovirus (Fig. 3B). Because the cyclin D-Cdk4 and cyclin D-Cdk6 complexes are primarily responsible for Rb phosphorylation in G_1 and are targets for the TGF-β-induced inhibitors (14, 18, 19, 56), these results are consistent with an E1A-mediated inhibition of p15 and p21 expression, leading to an accumulation of hyperphosphorylated Rb.

E1A blocks the TGF-β-mediated transcriptional activation of the p15 promoter. Having shown that E1A blocks the TGF-β-mediated induction of p15 and p21 proteins, we next focused on defining the level at which this block occurs. RNase protection analysis was performed with RNA samples isolated from either E1A-infected or uninfected cells which were treated or not treated with TGF-β. As shown in Fig. 4A, E1A expression completely eliminates the TGF-β-induced accumulation of p15 mRNA. Interestingly, the effect of E1A on p21 RNA is less pronounced than the effect seen on p21 protein, although RNA levels decrease by approximately 50% in E1A-infected cells (data not shown). For this reason, we focused further studies on understanding the mechanism through which E1A blocks the induction of p15 mRNA accumulation. The TGF-β-responsive element in the p15 promoter has been defined to contain a consensus binding site for the Sp1 family of transcription factors which is critical for TGF-β-mediated activation of transcription (29). Using a reporter construct which contains the TGF-β-responsive element of the p15 promoter, p15P113-Luc (29), we next determined if E1A directly affects the transcriptional activation of the p15 promoter by TGF-β. As shown in Fig. 4B, a 16-fold activation of luciferase activity from this p15 promoter construct is observed upon TGF-β treatment. When HaCaT cells are cotransfected with the reporter construct and increasing amounts of an E1A expression plasmid, a graded decrease in the TGF-β-induced activation of the p15 promoter is observed (Fig. 4B). At the highest level of E1A expression, a greater than fivefold reduction in this activation of luciferase activity by TGF-β is observed. No effect of E1A is seen on the basal activity of the p15 promoter, suggesting that the effect of E1A is specific to TGF-β-induced transcriptional activation.

The effects of E1A on p15 and p21 expression are not dependent on Rb binding. The above results suggest that E1A may act through at least two different mechanisms to overcome the growth-inhibitory effects of TGF-β: direct association and sequestration of Rb and inhibition of the TGF-β-mediated induction of the cyclin-dependent kinase inhibitors, p15 and p21. To determine if these effects are mediated by separable functions of E1A, we assayed the ability of an E1A mutant protein, which is severely compromised in its ability to bind to Rb, to block TGF-β-mediated p15 and p21 induction. This mutant E1A contains an amino acid substitution of base 928, changing the cysteine at codon 124 to a glycine (1, 36, 50). Confirming previous findings, no association between the E1A 928 mutant protein and Rb can be detected in HaCaT cells, whereas a significant amount of Rb can be seen in association with the wild-type E1A (data not shown).

Expression of the E1A 928 mutant blocks the TGF-β-mediated induction of p15 and p21 protein in a dose-dependent fashion similar to that of the wild-type E1A (Fig. 5A and B). Again, this inhibition is specific for the TGF-β-induced levels of p15 and p21, since the basal levels of the two proteins remain unchanged by E1A 928 expression. Similar to the effect of wild-type E1A, the 928 mutant blocks the TGF-β-mediated down regulation of Cdk2-associated kinase activity (Fig. 5C) and attenuates the ability of TGF-β to hold Rb in a hypophosphorylated state. At the highest concentration of E1A 928, a significant proportion of Rb is still hyperphosphorylated even in the presence of TGF-β (Fig. 5D).

Like wild-type E1A, the 928 mutant of E1A blocks the TGF-β-mediated induction of p15 mRNA and transcriptional activation of the p15 promoter (Fig. 6). The identical results for wild type and the 928 mutant of E1A strongly suggest that the ability of E1A to block the TGF-β-mediated induction of p15 and p21 is independent of its ability to bind to Rb.

The ability of E1A to bind p300 is required for its effects on p15 expression. In addition to its ability to bind Rb and Rb family members, E1A also binds to the transcriptional coactivator, p300 (2, 12). Since the effects of E1A on p15 and p21 are independent of Rb binding, we next tested whether these effects of E1A depend on its ability to sequester p300 by using an E1A mutant (Δ2-36) which harbors a deletion in the amino terminus of E1A, eliminating its p300 binding ability (24). This p300 binding mutant of E1A was cotransfected into HaCaT cells with the p15 promoter reporter construct, and TGF-β-activated luciferase activity was measured. In contrast to the effects of wild-type E1A and the E1A 928 mutant, which completely block TGF-β-mediated activation of the p15 promoter, E1A Δ2-36 is not able to significantly inhibit the ability of TGF-β to induce transcription from the p15 promoter (Fig. 7A). An increase in both the basal and the TGF-β-stimulated promoter activity is also seen on E1A Δ2-36 cotransfection. This increase is specific to the p15 promoter, as all transfections are internally controlled by cotransfection of a pCMV-β-galactosidase reporter construct. Despite this increase in

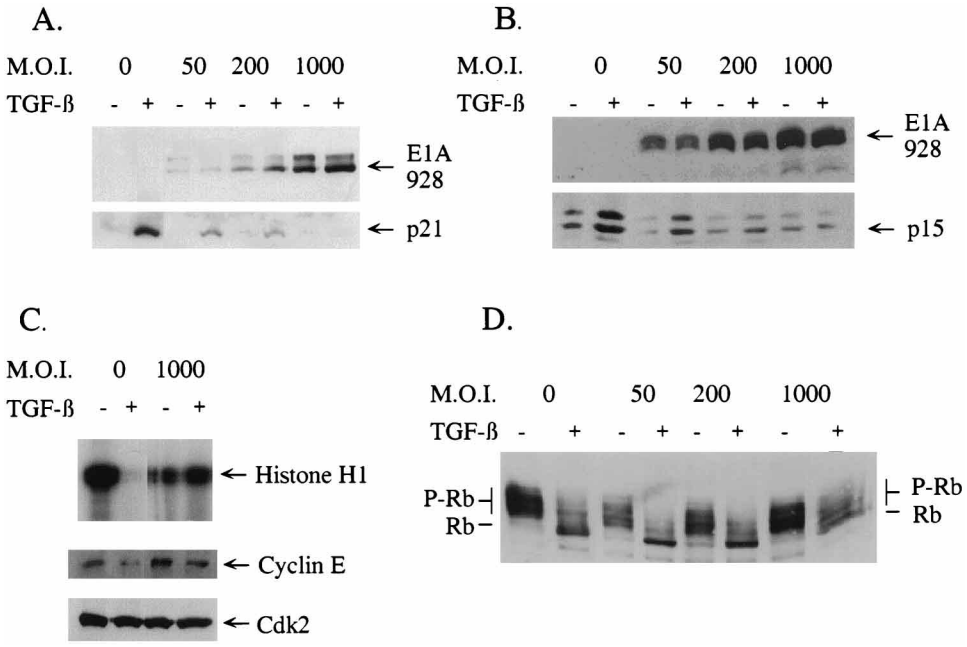

FIG. 5. E1A 928 mutant blocks the TGF-β-mediated p21 and p15 induction as effectively as wild-type E1A. (A) HaCaT cells were infected with a mutant E1A 928-expressing, E1B$^-$ adenovirus at various MOIs and cultured in either the presence or the absence of TGF-β. p21 and E1A Western blot analyses were performed as for Fig. 2A. (B) HaCaT cells were treated as for panel A. Western blot analyses of E1A and p15 were performed. (C) HaCaT cells were infected with the E1A 928-expressing adenovirus at an MOI of either 0 or 1,000× and cultured in the presence or the absence of TGF-β. Histone H1 kinase assays and cyclin E and Cdk2 Western blot analyses were performed as for Fig. 3A. (D) HaCaT cells were infected and treated as for panel A. Total cell lysates were prepared, and Western blot analysis of Rb was performed.

basal promoter activity, a significant amount of induction by TGF-β is still seen.

Although the E1A Δ2-36 mutation eliminates p300 binding, this mutation may also interfere with other functions of E1A. To further establish that p300 binding activity of E1A is linked to its ability to block transactivation of the p15 promoter by TGF-β, we performed cotransfection experiments in which wild-type E1A, the p15 promoter reporter construct, and different amounts of p300 cDNA were introduced into HaCaT cells. We used both wild-type p300 and a mutant form of p300 deficient in E1A binding, p300dl10 (12, 26), to test if overexpression of p300 could rescue the TGF-β-mediated promoter transactivation in the presence of E1A. As shown in Fig. 7B, cotransfection of E1A with the p15 promoter abolishes TGF-β-induced luciferase activity. Cotransfection of increasing amounts of p300dl10, however, leads to an incremental restoration of the ability of TGF-β to activate the p15 promoter. Indeed, the expression of p300dl10 markedly increases both the basal and TGF-β-stimulated p15 promoter activity. Cotransfection of increasing amounts of wild-type p300 also has a less marked effect in restoring TGF-β-mediated induction of the p15 promoter. The dampened rescuing effect of the wild-type p300 is expected in comparison to that of p300dl10, since the wild-type p300 is sequestered by the overexpressed E1A. These findings are consistent with our hypothesis that p300 is required for the TGF-β-mediated induction of p15 and that E1A blocks this induction through its binding and sequestration of p300.

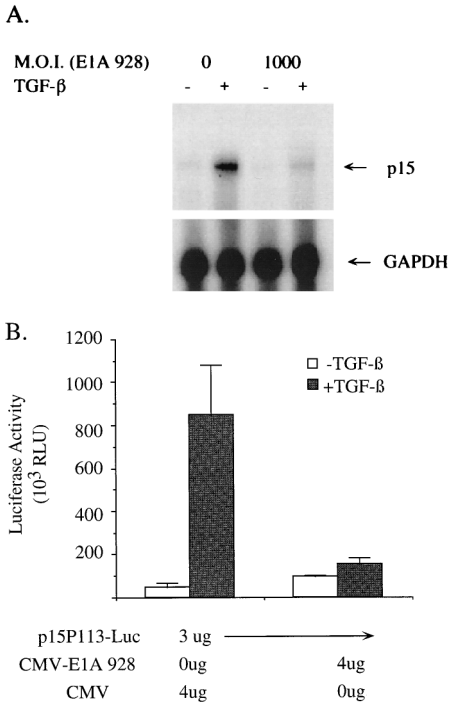

FIG. 6. E1A 928 mutant inhibits the ability of TGF-β to activate the p15 promoter. (A) RNase protection analysis was performed for p15 with RNA purified from HaCaT cells either infected or not with E1A 928 virus and either treated or not treated with TGF-β for 6 h. RNase protection for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. (B) HaCaT cells were transiently cotransfected with the p15 promoter luciferase reporter construct, p15P113-Luc, and the E1A 928 expression vector, pCMV-E1A 928. Luciferase assays were performed as for Fig. 4A. RLU, relative light units.

DISCUSSION

Over the past 10 years, studies of the E1A viral oncoprotein have provided insight into the mechanisms through which cell cycle progression is regulated (reviewed in reference 38). From

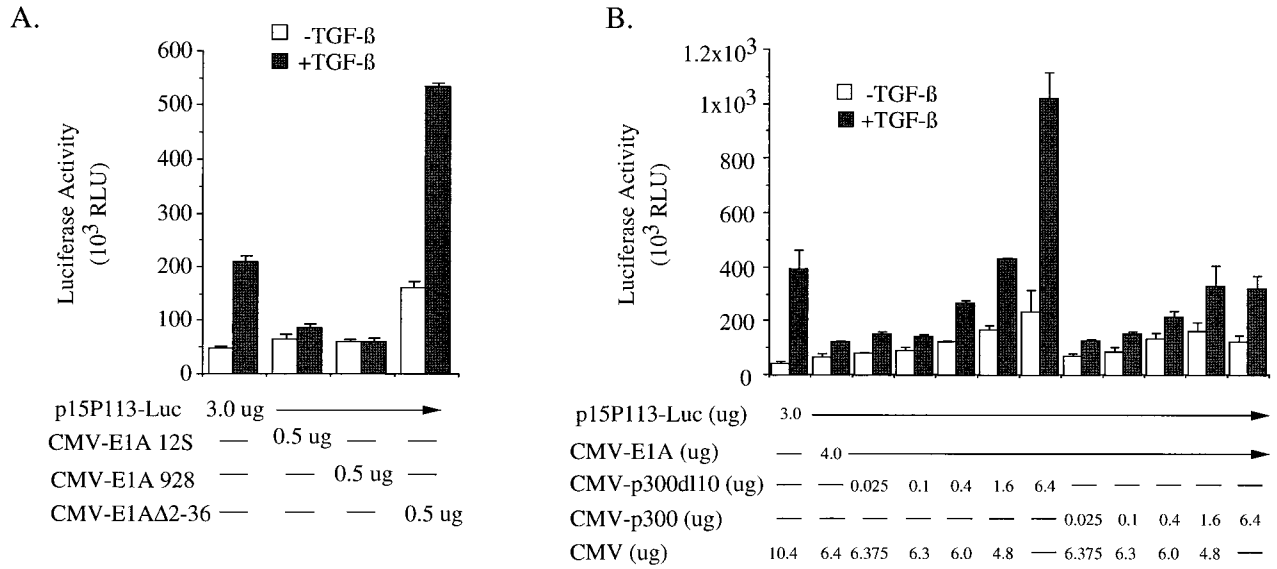

FIG. 7. A p300 binding mutant of E1A cannot block TGF-β-mediated activation of the p15 promoter. (A) HaCaT cells were transiently cotransfected with the p15 promoter luciferase reporter construct, p15P113-Luc, and either an E1A wild-type, E1A 928, or E1A Δ2-36 expression vector. Luciferase assays were performed as for Fig. 4A. (B) HaCaT cells were transiently cotransfected with the p15 promoter reporter construct, p15P113-luc, a wild-type E1A expression vector, and an increasing amount of either a wild-type p300 or an E1A binding mutant p300 expression vector, p300dl10. Luciferase assays were performed as for Fig. 4A. RLU, relative light units.

these studies, a picture has emerged in which E1A functions, in part, by binding to and inactivating a number of critical targets of the cyclin-dependent kinases including Rb (54) and the Rb family members p107 (16) and p130 (17, 30, 34–36, 55). These proteins act to negatively regulate cell growth, and their activity is normally controlled through phosphorylation by the G_1 cyclin-dependent kinases. Thus, the action of E1A can be viewed as the nonphysiological equivalent of the phosphorylation of these proteins. Since Rb is believed to be an important target of the TGF-β-mediated growth-inhibitory signal, E1A's Rb binding activity probably plays an important role in the ability of E1A to overcome the growth-inhibitory effects of TGF-β (33, 42).

In this report, we describe a novel and distinct function for E1A in its ability to block TGF-β-mediated inhibition of cell cycle progression. Specifically, we find that E1A can inhibit the TGF-β-mediated induction of the cyclin-dependent kinase inhibitors, p21/WAF1/Cip1 and p15/INK4B. This novel function of E1A provides a molecular basis for the previous observation that E1A blocks the ability of TGF-β to down-regulate Cdc2 kinase activity and to hold Rb in a hypophosphorylated state (1, 50). In our system, we show that E1A prevents both the TGF-β-mediated inhibition of Cdk2 kinase activity and the maintenance of Rb in a hypophosphorylated state. These effects of E1A appear to be independent of its ability to bind to Rb, since an E1A mutant which is severely compromised in its Rb binding ability behaves like wild-type E1A. Those results provide support for the hypothesis that the effects of E1A on p15 and p21 represent an alternative potential mechanism through which E1A can circumvent the negative regulation of cell cycle progression, other than through its physical interaction with Rb.

The effect of E1A on the induction of p15 expression by TGF-β occurs primarily at the level of the p15 mRNA accumulation. In addition, we have shown that E1A blocks the induction of the p15 promoter by TGF-β in a p300 binding-dependent fashion. Interestingly, the effect of E1A on p21 could not be solely attributed to a reduction in p21 mRNA accumulation. E1A caused a more marked decrease in p21 protein than the steady-state level of p21 mRNA, suggesting an additional level of regulation of p21 protein levels by E1A other than regulation of transcription. Analysis of the p21 promoter, however, did indicate that E1A could suppress the TGF-β-mediated activation from this TGF-β-responsive promoter. We are currently investigating the relative contribution of E1A-mediated inhibition of transcription of p21 and the other potential E1A regulatory effects on the p21 protein itself.

The inhibitory effects of E1A on the activation of p15 and p21 may cooperate with the ability of E1A to directly sequester Rb through physical association in overcoming TGF-β-mediated growth inhibition. Although the precise mechanism by which TGF-β causes cell cycle arrest remains to be firmly established, the dramatic induction of these two Cdk inhibitors by TGF-β would certainly contribute to the inhibition of the G_1 cyclin-Cdk complexes. By inhibiting this induction, E1A may retain the G_1 Cdk complexes in an active state even in the presence of TGF-β. These complexes can then proceed to phosphorylate their normal G_1 substrates, including Rb. Thus, through two distinct mechanisms, by physically sequestering Rb and by promoting the functional inactivation of Rb through its phosphorylation, E1A can act to ensure that Rb is completely inactivated and that cell cycle progression proceeds (Fig. 8).

Alternatively, the ability of E1A to block the TGF-β-mediated induction of p15 and p21 may serve a separate function in driving cell cycle progression, in addition to ensuring inactivation of E1A binding targets, such as Rb. Recent studies suggest the existence of two rate-limiting steps during G_1-to-S phase progression (46). The first step is dictated by the ability of cyclin D1-Cdk4 to phosphorylate in vivo substrates, including Rb. The second step is dictated by the ability of cyclin E-Cdk2 to phosphorylate a much broader range of in vivo targets, which may include proteins in addition to Rb and Rb family members. In the presence of TGF-β, an elevation of p21 and p15 would result in the inhibition of phosphorylation of all of these Cdk targets. In this context, the activation of the G_1

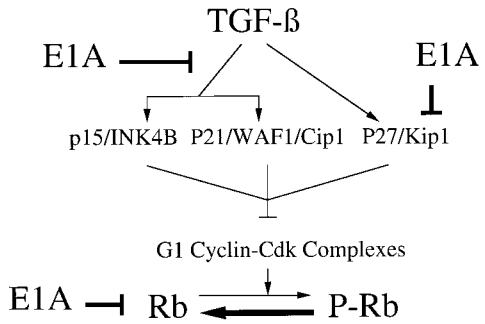

FIG. 8. Model for the multiple effects of E1A in overcoming TGF-β-mediated inhibition of cellular proliferation.

cyclin kinase complexes by E1A plays a crucial role in causing the phosphorylation of these substrates, which are not direct E1A binding targets. This event, coupled with the physical binding of Rb and Rb family members by E1A, would lead to S-phase progression. Consistent with this notion, microinjection of anti-cyclin E antibodies into either $Rb^{+/+}$ or $Rb^{-/-}$ cells causes the same block in S-phase progression, suggesting that cyclin E-Cdk2 complexes may have other targets besides Rb, probably components involved in the initiation of DNA replication (40). Taken together, the evidence supports a model in which the down regulation of p15 and p21 by E1A constitutes an indispensable step for the activation of the G_1 cyclin-dependent kinase complexes and subsequent phosphorylation of their targets, which is a necessary event in cell cycle progression.

The potential importance of E1A-induced cyclin-Cdk complex activation is further illustrated by a recent finding that E1A is capable of physically associating with p27/Kip1, a molecule structurally related to p21. By binding to p27, E1A was shown to relieve the p27-mediated repression of cyclin E-Cdk2 kinase complexes (31). Thus, in addition to blocking the transactivation of p15 and possibly p21 genes, E1A can inactivate p27 through a direct physical interaction. E1A, therefore, has evolved at least two separate mechanisms to ensure the activation of the G_1 cyclin complexes (Fig. 8). This new evidence lends weight to the hypothesis that activation of the G_1 cyclin-Cdk complexes is an important and necessary step in E1A-induced cell cycle progression. Interestingly, no binding of p21 to E1A could be detected. This may be due to the relatively low levels of p21 in E1A-infected cells, making detection of p21-E1A complexes difficult.

The inhibition of TGF-β-mediated induction of p21 by E1A may have yet another functional consequence in the control of cell cycle progression. In addition to its role as a cyclin-dependent kinase inhibitor, p21 has been shown to bind to the DNA polymerase complex protein proliferating cell nuclear antigen. By associating with proliferating cell nuclear antigen, p21 has been shown to block in vitro DNA replication (49). In the presence of TGF-β, up-regulated p21 may lead to an inhibition of DNA replication. E1A may force the initiation of DNA replication and S-phase entry by down-regulating p21, thus circumventing the negative control of DNA replication by TGF-β.

The mechanism by which E1A acts as a potent stimulator of cell cycle progression has largely been defined by the ability of E1A to bind to and functionally inactivate the Rb family members. In contrast, the biological effect of E1A binding to p300 has not been extensively investigated. Through the use of an E1A mutant which does not interact with p300, we have determined that the p300 binding domain of E1A is necessary for its ability to block the TGF-β-mediated induction of the p15 promoter. Furthermore, we showed that the expression of a p300 mutant with a deficiency in E1A binding restores the ability of TGF-β to activate the p15 gene. These two pieces of evidence suggest that the ability of E1A to block the TGF-β-mediated induction of p15 stems from its ability to bind to p300. As such, this data also suggests that p300 probably plays an essential role in TGF-β-induced expression of the p15 gene, which is substantiated by the ability of p300dl10 to superactivate the p15 promoter. Thus, by targeting p300, E1A may allow the virus to achieve the same goal as binding to the Rb family members in circumventing the normal negative regulation of cell growth and consequently forcing an inappropriate S-phase progression in supporting replication of the viral genome.

p300 has the characteristics of a transcriptional coactivator and has been shown in several contexts to stimulate transcription from certain promoters in concert with specific transcription factors (references 2, 12, 26, and 27 and references therein). Previous studies have shown that the TGF-β-responsive elements in both the p21 and the p15 promoters bind to the Sp1 family of transcription factors (9, 29). Recently, we have determined that the TGF-β signal is mediated specifically by the B domain of Sp1, by using chimeric proteins containing different portions of the transactivation domain of both Sp1 and Sp3 fused to the GAL4 DNA binding domain. In addition, this TGF-β-induced transactivation through the B domain of Sp1 is inhibited by E1A (8a). Although this is yet to be determined, p300 may be interacting with Sp1 in a TGF-β-dependent fashion. By changing the phosphorylation state of Sp1 and/or p300, TGF-β may promote a productive interaction between these two transcription factors to lead to the activation of p21 and p15 transcription. Additional studies on the role of p300 in the induction of p21 and p15 by TGF-β will provide further insight into the mechanisms by which TGF-β regulates cell cycle progression.

ACKNOWLEDGMENTS

We are grateful to Amgen Inc. for providing TGF-β1; J. Nevins, G. Leone, and J. DeGregori for providing the E1A, E1A928, and control adenoviruses and the E1A, E1A 928, and E1AΔ2-36 CMV expression vectors; and R. Eckner for providing the p300 cDNA. We also thank Y. Yu, J.-M. Li, H. Symonds, and M. Zhang for technical assistance and C. Bassing, J.-M. Li, H. Symonds, P.-C. Hu, J. Nevins, J. DeGregori, and C.-H. Huang for helpful discussions of the manuscript.

This work was supported by grants from the National Institutes of Health (DK45746) and the Council for Tobacco Research (3613). M.B.D. is supported by a Medical Scientist Training Program grant from the National Institutes of Health. P.P.H. is supported by a predoctoral fellowship from the National Science Foundation. T.F.K. is a Leukemia Society Special Fellow. X.-F.W. is a Leukemia Society Scholar.

The first two authors contributed equally to this work.

REFERENCES

1. **Abraham, S. E., M. C. Carter, and E. Moran.** 1992. Transforming growth factor β1 (TGFβ1) reduces cellular levels of p34cdc2 and this effect is abrogated by adenovirus independently of the E1A-associated pRB binding activity. Mol. Biol. Cell **3:**655–665.
2. **Arany, Z., W. R. Sellers, D. M. Livingston, and R. Eckner.** 1994. E1A associated p300 and CREB-associated CBP belong to a conserved family of coactivators. Cell **77:**799–800.
3. **Bandara, L. R., and N. B. LaThangue.** 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature **351:**494–497.
4. **Bremner, R., B. L. Cohen, M. Sopta, P. A. Hamel, C. J. Ingles, B. L. Gallie, and R. A. Phillips.** 1995. Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol. Cell. Biol. **15:**3256–3265.
5. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.

6. **Chellappan, S. P., S. Hiebert, M. Mudryj, M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65:**1053–1061.
7. **Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee.** 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1193–1198.
8. **Datto, M. B., Y. Li, J. F. Panus, D. J. Howe, Y. Xiong, and X.-F. Wang.** 1995. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. USA **92:**5545–5549.

8a. **Datto, M. B., and X.-F. Wang.** Unpublished data.

9. **Datto, M. B., Y. Yu, and X.-F. Wang.** 1995. Functional analysis of the transforming growth factor β responsive elements in the WAF1/Cip1/p21 promoter. J. Biol. Chem. **270:**28623–28628.
10. **DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C. M. Huang, and D. M. Livingston.** 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell **58:**1085–1095.
11. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
12. **Eckner, R., M. E. Ewen, D. Newsom, M. Gerdes, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. **8:**869–884.
13. **Elbendary, A., A. Berchuck, P. Davis, L. Havrilesky, R. C. Bast, J. D. Iglehart, and J. R. Marks.** 1994. Transforming growth factor beta 1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. Cell Growth Differ. **5:**1301–1307.
14. **El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
15. **Ewen, M. E., H. K. Slus, C. J. Sherr, H. Matsushime, J.-Y. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73:**293–302.
16. **Ewen, M. E., Y. G. Wing, J. B. Lawrence, and D. M. Livingston.** 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66:**1155–1164.
17. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev. **7:**2378–2391.
18. **Hannon, G. J., and D. Beach.** 1994. P15INK4B is a potential effector of TGF-β-induced cell cycle arrest. Nature **371:**257–260.
19. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.
20. **Hu, Q., C. Bautista, G. M. Edwards, D. Defeo-Jones, R. E. Jones, and E. Harlow.** 1991. Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. Mol. Cell. Biol. **11:**5792–5799.
21. **Hunter, T., and J. Pines.** 1994. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell **79:**573–582.
22. **Ikeda, M.-A., and J. R. Nevins.** 1993. Identification of distinct roles for separate E1A domains in disruption of E2F complexes. Mol. Cell. Biol. **13:**7029–7035.
23. **Koff, A., M. Ohtsuki, K. Polyak, J. M. Roberts, and J. Massague.** 1993. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-β. Science **260:**536–539.
24. **Kraus, V. B., E. Moran, and J. R. Nevins.** 1992. Promoter-specific *trans*-activation by the adenovirus $E1A_{12S}$ product involves separate E1A domains. Mol. Cell. Biol. **12:**4391–4399.
25. **Laiho, M., J. A. DeCaprio, J. W. Ludlow, D. M. Livingston, and J. Massague.** 1990. Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell **62:**175–185.
26. **Lee, J.-S., R. H. See, T. Deng, and Y. Shi.** 1996. Adenovirus E1A downregulates cJun- and JunB-mediated transcription by targeting their coactivator p300. Mol. Cell. Biol. **16:**4312–4326.
27. **Lee, J.-S., S. Zhang, and Y. Shi.** 1996. Differential interactions of the CREB/ATF family of transcription factors with p300 and adenovirus E1A. J. Biol. Chem. **271:**17666–17674.
28. **Li, C.-Y., L. Suardet, and J. B. Little.** 1995. Potential role of WAF1/Cip1/p21 as a mediator of TGF-β cytoinhibitory effect. J. Biol. Chem. **270:**4971–4974.
29. **Li, J.-M., M. A. Nichols, S. Chandrasekharan, Y. Xiong, and X.-F. Wang.** 1995. Transforming growth factor β activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site. J. Biol. Chem. **270:**26750–26753.
30. **Li, Y., C. Graham, S. Lacy, A. M. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev. **7:**2366–2377.
31. **Mal, A., R. Y. C. Poon, P. H. Howe, H. Toyoshima, T. Hunter, and M. L. Harter.** 1996. Inactivation of p27Kip1 by the viral E1A oncoprotein in TGF-β treated cells. Nature **380:**262–265.
32. **Mihara, K., X.-R. Cau, A. Yen, S. Chandler, B. Driscoll, A. L. Murphee, A. T'Ang, and Y.-K. T. Fung.** 1989. Cell cycle dependent regulation of phosphorylation of the human retinoblastoma gene product. Science **246:**1300–1303.
33. **Missero, C., E. Filvaroff, and G. P. Dotto.** 1991. Induction of transforming growth factor β1 resistance by the E1A oncogene requires binding to a specific set of cellular proteins. Proc. Natl. Acad. Sci. USA **88:**3489–3493.
34. **Moran, E., and M. B. Mathews.** 1987. Multiple functional domains in the adenovirus E1A gene. Cell **48:**177–178.
35. **Moran, E., and B. Zerler.** 1988. Interactions between cell growth regulating domains in the products of the adenovirus E1A oncogene. Mol. Cell. Biol. **8:**1756–1764.
36. **Moran, E., B. Zerler, T. M. Harrison, and M. B. Mathews.** 1986. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol. Cell. Biol. **6:**3470–3480.
37. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–428.
38. **Nevins, J. R.** 1995. Adenovirus E1A: transcription regulation and alteration of cell growth control. Curr. Top. Microbiol. Immunol. **199:**25–32.
39. **Ohtsubo, M., and J. M. Roberts.** 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science **259:**1908–1912.
40. **Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano.** 1995. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol. **15:**2612–2624.
41. **Peter, M., and I. Herskowitz.** 1994. Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. Cell **79:**181–184.
42. **Pietenpol, J. A., R. W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R. M. Lyons, M. R. Pittelkow, K. Munger, P. M. Howley, and H. L. Moses.** 1990. TGF-β1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell **61:**777–785.
43. **Polyak, K., J.-Y. Kato, M. J. Solomon, C. J. Sherr, J. Massague, J. M. Roberts, and A. Koff.** 1994. p27Kip1, a cyclin-cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev. **8:**9–12.
44. **Polyak, K., M.-H. Lee, H. Erdjument-Bromage, A. Koff, J. M. Roberts, P. Tempst, and J. Massague.** 1994. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell **78:**59–66.
45. **Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins.** 1991. Domains of the adenovirus E1A protein that are required for oncogenic activity are required for dissociation of cellular transcription factor complexes. Genes Dev. **5:**1200–1211.
46. **Resnitzky, D., and S. I. Reed.** 1995. Different roles for cyclins D1 and E in regulation of the G_1-to-S transition. Mol. Cell. Biol. **15:**3463–3469.
47. **Reynisdottir, I., K. Polyak, A. Iavarone, and J. Massague.** 1995. Kip/Cip and Ink4 inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Devel. **9:**1831–1845.
48. **Sherr, C. J.** 1993. Mammalian G1 cyclins. Cell **73:**1059–1065.
49. **Waga, S., G. Hannon, D. Beach, and B. Stillman.** 1994. The p21 inhibitor of cyclin dependent kinases controls DNA replication by interaction with PCNA. Nature **369:**574–578.
50. **Wang, H.-G. H., G. Draetta, and E. Moran.** 1991. E1A induces phosphorylation of the retinoblastoma protein independently of direct physical association between the E1A and retinoblastoma products. Mol. Cell. Biol. **11:**4253–4265.
51. **Weinberg, R. A.** 1996. E2F and cell proliferation: a world turned upside down. Cell **85:**457–459.
52. **Weintraub, S. J., K. N. B. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature **375:**812–815.
53. **Whyte, P. K.** 1995. The retinoblastoma protein and its relatives. Semin. Cancer Biol. **6:**83–90.
54. **Whyte, P. K., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow.** 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature **334:**124–129.
55. **Whyte, P. K., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by adenovirus E1A proteins. Cell **56:**67–75.
56. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. P21 is a universal inhibitor of cyclin kinases. Nature **366:**701–704.
57. **Yingling, J. M., X.-F. Wang, and C. H. Bassing.** 1995. Signaling by the transforming growth factor-β receptors. Biochim. Biophys. Acta **1242:**115–136.