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# Transforming Growth Factor β1 (TGFβ1) Reduces Cellular Levels of p34$^{cdc2}$, and This Effect is Abrogated by Adenovirus Independently of the E1A-Associated pRB Binding Activity

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We have used E1A probes to study the roles of the p34$^{cdc2}$ kinase and the retinoblastoma tumor susceptibility gene product (pRB) in transforming growth factor β1(TGFβ1)-mediated growth suppression in mink lung epithelial (Mv1Lu) cells. In agreement with previous reports, we see a decline in p34$^{cdc2}$ kinase activity and a loss of pRB phosphorylation after TGFβ1 treatment. We report here that TGFβ1 induces not only a change in p34$^{cdc2}$ kinase activity but a strong repression of p34$^{cdc2}$ synthesis. Loss of p34$^{cdc2}$ kinase activity is not seen until the steady-state level of p34$^{cdc2}$ declines, suggesting that the intra-cellular signals induced by TGFβ1 affect p34$^{cdc2}$ at the level of expression, rather than by altering the posttranslational modifications of p34$^{cdc2}$ that regulate its kinase activity. Infection with adenovirus expressing either wild-type E1A or a mutant E1A (pm928) defective for pRB binding alleviated TGFβ1-mediated suppression of DNA synthesis, indicating that E1A does not need to bind pRB physically to keep cell growth-suppressing functions from being activated by TGFβ1. The E1A.928 mutant virus is able to maintain p34$^{cdc2}$ expression and kinase activity, as well as pRB phosphorylation in the presence of TGFβ1, which may account for its ability to maintain cell cycle activity without directly sequestering pRB. Overall our results suggest that TGFβ1 acts by signaling changes at the level of control of G1 gene expression, not at the level of posttranslational modification of p34$^{cdc2}$ or its substrates.

## INTRODUCTION

Transforming growth factor β1 (TGFβ1) is a potent growth inhibitor for most epithelial cells and induces cells to arrest in the mid-to-late G1 phase of the cell cycle (reviewed in Roberts *et al.*, 1988; Barnard *et al.*, 1990; Massague, 1990; Moses *et al.*, 1990). The mechanism by which TGFβ1 mediates growth inhibition is not completely understood, although TGFβ1 treatment can affect several cellular products thought to play key roles in cell growth control. Prominent among these are the product of the retinoblastoma tumor susceptibility gene, pRB (Laiho *et al.*, 1990) and the cell cycle regulating kinase, p34$^{cdc2}$ (Howe *et al.*, 1991).

The retinoblastoma (RB) gene product (reviewed in Weinberg, 1990) is an apparent suppressor of cell growth, which may be active only when the protein is underphosphorylated, inasmuch as pRB is hyperphosphorylated in the S, G2, and early M phases of cycling cells (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Ludlow *et al.*, 1989, 1990; Mihara *et al.*, 1989; Furukawa *et al.*, 1990a). In mink lung epithelial (Mv1Lu) cells, which are strongly inhibited by TGFβ1 (Tucker *et al.*, 1984; Like and Massague, 1986), TGFβ1 suppresses phosphorylation of pRB (Laiho *et al.*, 1990).

The activity of the cdc2 kinase family of proteins has also been strongly implicated in the control mechanisms governing cell growth (reviewed in Draetta, 1990; Nurse, 1990; Pines and Hunter, 1990; Maller, 1991). Recently, Howe *et al.* (1991) showed that p34$^{cdc2}$ kinase activity is inhibited in Mv1Lu cells by TGFβ1 treatment and suggested that this might be due to alterations in posttranslational modifications of p34$^{cdc2}$, which are

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known to affect the specific activity of the enzyme. There are likely to be multiple $p34^{cdc2}$ kinase substrates critical for cell growth, and these may include pRB. Indeed recent evidence suggests strongly that $p34^{cdc2}$ and/or a closely related kinase, designated $p33^{cdk2}$, is the cellular pRB kinase (Taya *et al.*, 1989; Furukawa *et al.*, 1990b; Lin *et al.*, 1991; Tsai *et al.*, 1991).

The early region 1A (E1A) transforming gene product of Adenovirus type 5 (Ad5) can activate cell growth control pathways that apparently overlap with those affected by TGFβ1 (Pietenpol *et al.*, 1990; Missero *et al.*, 1991). The E1A protein products contain three independent active sites that can affect expression of host cell products (reviewed in Boulanger and Blair, 1991). One of the E1A functional domains interacts directly with the RB gene product, physically binding it, and presumably inactivating its growth suppressive effects (Whyte *et al.*, 1988; 1989). Recent evidence suggests that this interaction permits activation of the cellular E2F transcription factor (Chellapan *et al.*, 1991), although it is not yet known what specific cellular products are transactivated as a result of this process. The E1A products can also induce $p34^{cdc2}$ expression and kinase activity in quiescent primary cells (Draetta *et al.*, 1988a; Wang *et al.*, 1991). This ability does not require the pRB binding function of the E1A products; it is apparently encoded redundantly in E1A (Wang *et al.*, 1991).

In the present study, we used E1A as a genetic probe to study the roles of the cdc2 kinase and the retinoblastoma gene product in TGFβ1-mediated growth suppression in Mv1Lu cells. In agreement with previous reports, we see a decline in $p34^{cdc2}$ kinase activity and a loss of pRB phosphorylation after TGFβ1 treatment. We report here that TGFβ1 induces not only a change in $p34^{cdc2}$ kinase activity but a strong repression of $p34^{cdc2}$ synthesis. Loss of $p34^{cdc2}$ kinase activity is not seen until the steady-state level of $p34^{cdc2}$ declines, suggesting that the intracellular signals induced by TGFβ1 affect $p34^{cdc2}$ at the level of expression, rather than by altering the posttranslational modifications of $p34^{cdc2}$ that regulate its kinase activity. Infection with adenovirus expressing either wild-type E1A or a mutant E1A (pm928) defective for pRB binding counteracts TGFβ1-mediated suppression of DNA synthesis, demonstrating that the E1A products do not need to bind pRB physically to prevent cell growth-suppressing functions from being activated by TGFβ1. The pm928 mutant is able to maintain $p34^{cdc2}$ expression and kinase activity, as well as pRB phosphorylation in the presence of TGFβ1, which may account for its ability to maintain cell cycle activity without directly sequestering pRB.

## MATERIALS AND METHODS

### *Cells and Viruses*

Mv1Lu cells (CCL-64, freshly obtained from the American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 μg/ml of penicillin. For the growth-arrest experiment, cells were plated at a density of $2 \times 10^6$ cells/cm$^2$. After 7 d of incubation, the quiescent cells were stimulated with either 10% FBS or 10% FBS plus epidermal growth factor (EGF; 20 ng/ml) in the presence or absence of TGFβ1 as described by Howe *et al.* (1991).

Ad5dl309, Ad5dl312 (Jones and Shenk, 1979), and E1A.928 (Moran *et al.*, 1986) viruses were propagated on 293 cells (Graham *et al.*, 1977), which express E1A constitutively.

### *Infections*

In all experiments, growing Mv1Lu cells were infected at a multiplicity of infection (moi) of 10 plaque-forming units (pfu) per cell. The viruses were added and allowed to adsorb at 37°C for 1 h, after which time the medium was removed and replaced with fresh DMEM supplemented with 10% FBS (and TGFβ1, if appropriate).

### *TGFβ1 Treatment*

TGFβ1 from porcine platelets (R&D Systems, Minneapolis, MN) dissolved in 4 mM HCl was added to cells at a concentration of 3.2 ng/ml, at 1 h post-virus infection, for the times indicated in the figures. An equivalent volume of the buffer (4 mM HCl) was added to the untreated cells.

### *DNA Synthesis Assay*

Cells were labeled with [$^3$H]thymidine (ICN) (20 μCi per ml of medium) and assayed for TCA precipitable counts, as described previously (Zerler *et al.*, 1987).

### *Immunoprecipitations*

Mv1Lu cells were metabolically labeled for the time periods indicated in the text with 0.2 mCi of Tran[$^{35}$S] label (NEN, Wilmington, DE) in 8 ml of DMEM minus methionine or 1 mCi of $^{32}P_i$ (ICN, Irvine, CA) in 3 ml of phosphorus-free DMEM. Fresh TGFβ1 was included in the labeling medium as appropriate to maintain TGFβ1 treatment through the entire incubation period. Cells were lysed in 0.7 ml of lysis buffer containing 50 mM tris(hydroxymethyl)aminomethane(Tris), pH 7.5, 250 mM NaCl, 0.1% Triton-X 100, 5mM EDTA, 50 mM NaF, 0.1 mM $Na_3VO_4$ supplemented with 1 μg/ml each of aprotinin, pepstatin, leupeptin, and 375 μg/ml phenylmethylsulfonyl fluoride (United States Biochemicals, Cleveland, OH). Extracts were precleared and normalized to total cell counts, as described previously, (Yaciuk *et al.*, 1991), before adding antibodies. Proteins were immunoprecipitated with the appropriate antibody by rotating at 4°C for 1 h, followed by the addition of protein A sepharose beads (Pharmacia, Piscataway, NJ) and rotation at 4°C for 1 h. In the case of pRB antibody, 1 μg of rabbit-anti-mouse serum [(RAM) Cappel, Durham, NC] was added along with the protein A sepharose beads. Immune complexes were purified by five washes of the beads with 0.5 ml of lysis buffer and by centrifuging at $100 \times g$. Samples were resuspended in 30 μl of 2× Laemmli buffer, boiled for 5 min, and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970). $p34^{cdc2}$ specific rabbit anti-carboxy-terminal-peptide serum (G6) has been described in Draetta and Beach (1988). E1A-specific mouse monoclonal antibodies, M73 (Harlow *et al.*, 1985), and pRB-specific mouse monoclonal antibodies, XZ-133 (Hu *et al.*, 1991), have been described previously and were provided by Qianjin Hu and Ed Harlow. G6 antipeptide serum was provided by Giulio Draetta, Jim Bischoff, and David Beach. Densitometric readings were recorded on a Molecular Dynamics (Sunnyvale, CA) series 100A densitometer.

### *Kinase Assay*

p34cdc2 histone H1 kinase activity was assayed by affinity precipitating p34cdc2 protein with P13 beads from unlabeled cell lysates followed by reaction with histone H1 substrate in the presence of [$\gamma^{32}$P]ATP as described previously (Draetta *et al.*, 1988; Giordano *et al.*, 1989). The protein concentration of the lysates was measured in a Coomassie Brilliant Blue dye binding assay (Bradford 1976). Total cellular protein level was normalized to 1 mg per immunoprecipitation reaction. The immunoprecipitation procedure was the same as that described in the previous paragraph. p13-Sepharose beads were prepared according to the procedure of Brizuela *et al.* (1987) and used essentially the same way as described by Giordano *et al.* (1989) and Blow and Nurse (1990). Immune complexes were incubated for 5 min at 30°C in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml histone H1 (Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 μCi of [$\gamma^{32}$P] ATP (ICN), and 1 μM cold ATP. Reactions were stopped by the addition of 30 μl of 2× Laemmli buffer (Laemmli, 1970), boiled for 5 min, and loaded on a 15% SDS-polyacrylamide gel. Gels were stained with Coomassie Blue, dried, and autoradiographed.

## RESULTS

### *Effect of TGFβ1 on DNA synthesis*

TGFβ1 reversibly inhibits DNA synthesis and proliferation of Mv1Lu cells (Tucker *et al.*, 1984). Adenovirus E1A can protect DNA synthesis and cell growth from the repressive effect of TGFβ1 in human and mouse keratinocytes (Pietenpol *et al.*, 1990; Missero *et al.*, 1991). To verify that adenovirus has a similar protective effect in the cell line studied here, we assayed the effect of TGFβ1 on the growth of Mv1Lu cells that had been infected with Ad5dl309 virus expressing wild-type E1A or Ad5dl312, a virus encoding a near total deletion of the E1A region. Duplicate sets of exponentially growing

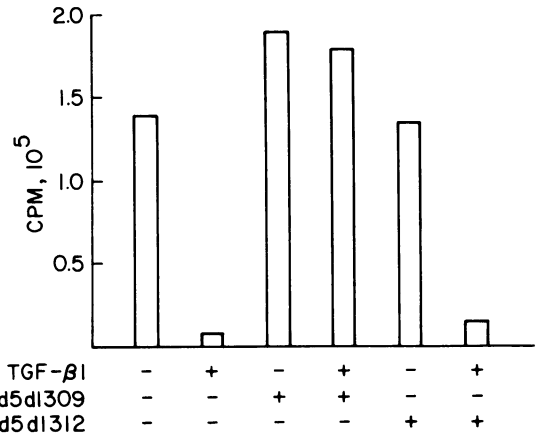


**Figure 1.** Effect of TGFβ1 on DNA synthesis. Identical sets of exponentially growing Mv1Lu cells were left uninfected or were infected with Ad5dl309 or Ad5dl312 and incubated in either the absence or presence of TGFβ1 for 24 h. Cells were labeled with [$^3$H]-thymidine from 22 to 24 h after TGFβ1 addition. DNA synthesis was measured by [$^3$H]-thymidine incorporation and is plotted on the Y-axis as TCA precipitable counts per minute (cpm). The numbers shown are an average of three separate experiments each done in duplicate.

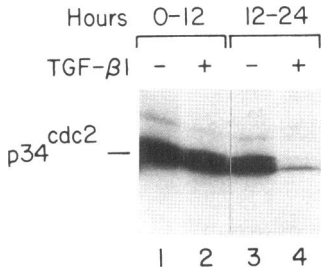


**Figure 2.** TGFβ1 reduces the steady-state level of p34cdc2 protein. Exponentially growing Mv1Lu cells were incubated in the absence or presence of TGFβ1 and labeled with tran[$^{35}$S] label for 12 h-intervals. At 12 and 24 h after TGFβ1 treatment, cell lysates were immunoprecipitated with anti-p34cdc2 serum as described in MATERIALS AND METHODS.

Mv1Lu cells were left uninfected or were infected with Ad5dl309 or Ad5dl312 and incubated in the presence or absence of TGFβ1 for 24 h. DNA synthesis was assayed by pulse labeling the cells with [$^3$H]thymidine 22–24 h after TGFβ1 treatment (Figure 1). TGFβ1 treatment reduced DNA synthesis in uninfected cells by ~90%, as reported previously (Boyd and Massague, 1989; Laiho *et al.*, 1990; Howe *et al.*, 1991); however, no inhibition of [$^3$H]-thymidine incorporation was seen in cells infected with Ad5dl309. Ad5dl312 shows no protective effect on DNA synthesis in these conditions. Thus adenovirus infection is capable of overcoming TGFβ1-mediated growth inhibition in Mv1Lu cells, and this effect is dependent on E1A expression.

### *TGFβ1 Gradually Reduces the Steady-State Level of p34cdc2 Protein*

The activity of the cdc2-kinase family of proteins has been directly implicated in the control mechanisms governing cell growth. In addition, the ability of E1A to mediate cell cycle activation is linked with the ability of E1A to stimulate p34cdc2 expression (Draetta *et al.*, 1988a; Wang *et al.*, 1991). We therefore examined the effect of TGFβ1 on the expression of p34cdc2 and determined whether adenovirus alters TGFβ1-mediated effects. We first monitored the level of p34cdc2 expression in cycling cells by steady-state labeling with tran[$^{35}$S] label for 12-h intervals in the presence of TGFβ1 (Figure 2). p34cdc2 protein was assayed by immunoprecipitating labeled cell lysates with p34cdc2-specific antipeptide serum, G6 (Draetta and Beach, 1988). We detected only a moderate decrease in the steady-state level of p34cdc2 within the first 12 h after TGFβ1 addition (lane 2 versus lane 1); densitometric tracings indicate that the steady-state level of p34cdc2 up to 12 h after exposure to TGFβ1 is still ~67% of the level in untreated cells. In contrast, a very marked reduction, to <15% of the level in untreated cells, is observed within 12–24 h after treatment (lane 4 versus lane 3). Thus TGFβ1 treatment appears

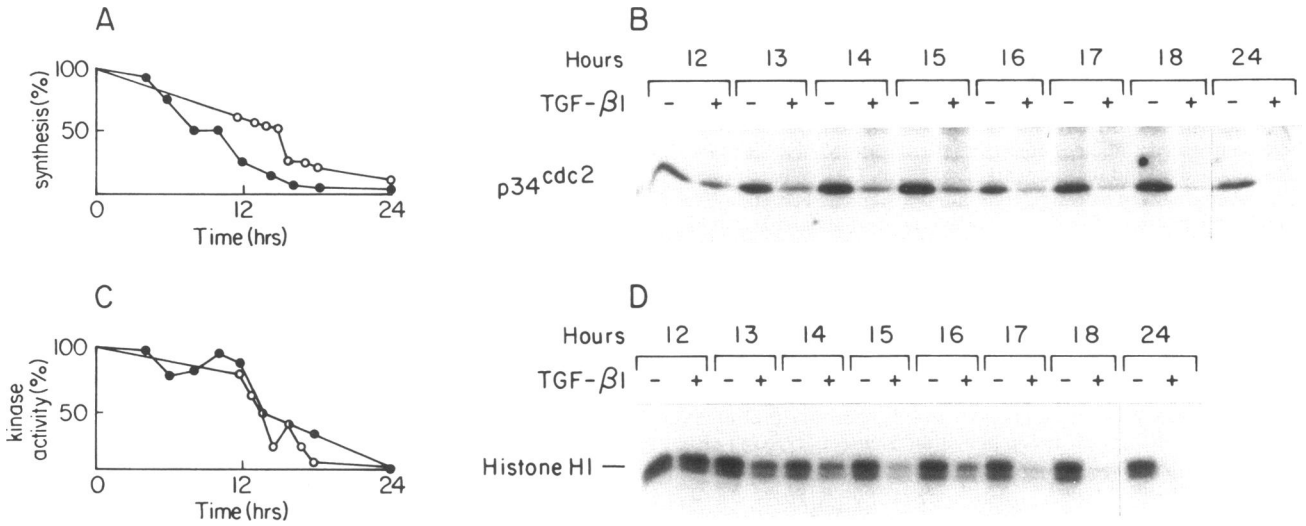


**Figure 3.** Kinetics of TGFβ1-mediated inhibition of $p34^{cdc2}$ kinase activity and synthesis. (A) Quantitative densitometric readings measuring percentage of $p34^{cdc2}$ synthesis in the presence of TGFβ1 as compared with untreated cells at successive time intervals from two independent experiments. Exponentially growing Mv1Lu cells were incubated in the absence or presence of TGFβ1. The cells were labeled for the last hour of their respective incubation period with tran[$^{35}$S] label. At the indicated times, cells were harvested and lysates were immunoprecipitated with anti-$p34^{cdc2}$ G6 serum as described in MATERIALS AND METHODS. (B) Autoradiogram of the experiment represented by open circles in A. (C) Quantitative densitometric readings measuring percentage of $p34^{cdc2}$ histone H1 kinase activity in the presence of TGFβ1 compared with untreated cells at successive time intervals from two independent experiments. At the indicated times, cells were harvested and $p34^{cdc2}$-specific histone H1 kinase activity was assayed as described in MATERIALS AND METHODS. (D) Autoradiogram of the experiment represented by open circles in C.

to reduce the steady-state level of $p34^{cdc2}$ dramatically, but this is not an immediate effect.

### *Kinetics of TGFβ1-Mediated Inhibition of $p34^{cdc2}$ Kinase Activity and Synthesis*

Howe *et al.* (1991) showed that $p34^{cdc2}$ histone H1 kinase activity is inhibited by TGFβ1 and suggested that this might be due to alterations in posttranslational modifications of $p34^{cdc2}$, which are known to affect the intrinsic activity of the enzyme. Such a mechanism for inhibiting $p34^{cdc2}$ activity would not require an effect at the level of $p34^{cdc2}$ expression. However given the results shown above, it is possible the decrease in $p34^{cdc2}$ kinase activity results from decreased protein levels. It is conceivable though, that a decrease in kinase activity modulated directly through steps affecting posttranslational modifications of $p34^{cdc2}$ protein might eventually, as a result of a consequent shut-off of cell cycle activity, lead indirectly to decreased expression of $p34^{cdc2}$ protein. To delineate the steps in the TGFβ1-mediated pathway, it is important to distinguish these possibilities. Therefore we compared the kinetics of TGFβ1-mediated inhibition of $p34^{cdc2}$ protein synthesis and histone H1 kinase activity in growing cells. Proliferating Mv1Lu cells were incubated in the presence or absence of TGFβ1, and $p34^{cdc2}$ protein synthesis and kinase activity were assayed as described in MATERIALS AND METHODS.

Quantitative densitometric readings from two independent experiments measuring $p34^{cdc2}$ synthesis at successive time intervals after exposure to TGFβ1 are plotted in Figure 3A. The results from one of these experiments (represented by the open circles in Figure 3A) are shown in the form of an autoradiogram in Figure 3B. Kinase activity was measured in parallel samples in each of these experiments. The corresponding kinase activity at each point is shown quantitatively for both experiments in Figure 3C and visually for the experiment represented by the open circles in Figure 3D. The curves derived in Figure 3A indicate that $p34^{cdc2}$ synthesis is strongly repressed by TGFβ1. Repression of de novo synthesis was detectable within a few hours after treatment; inhibition of synthesis was nearly maximal by 18 h after treatment. In contrast, there appears to be a significant lag, relative to suppression of de novo synthesis, before kinase activity declines appreciably. Kinase activity remained fairly stable until ~12 h after treatment, after which it declined sharply (Figures 3, C and D); inhibition was nearly complete by 18 h after treatment.

In combination with the assay of the steady-state level of $p34^{cdc2}$ shown in Figure 2, these results suggest that while an effect of TGFβ1 on de novo synthesis of $p34^{cdc2}$ is apparent soon after treatment, the protein is sufficiently stable that it can be maintained at nearly normal levels until ~12 h after treatment. After this time, as shown in Figure 2, steady-state levels of $p34^{cdc2}$ decline

precipitously. Likewise it is at about this point that p34cdc2 kinase activity begins to decline sharply, as seen in Figures 3,C and D. There is no evidence that loss of kinase activity occurs earlier in time than the shut-off of p34cdc2 protein synthesis. Thus it is most likely that reduction of p34cdc2 kinase activity is mediated at the level of expression of p34cdc2, not at the level of altering intrinsic kinase activity.

### *TGFβ1 Inhibits Induction of p34cdc2 Expression in Growth-Arrested Cells*

The preceding experiments assessed the effect of TGFβ1 on actively growing cells. Because TGFβ1 also blocks growth factor-mediated stimulation of cell cycle activity in growth-arrested cells, we determined the effects of TGFβ1 on expression and kinase activity of p34cdc2 in these conditions. Quiescent Mv1Lu cells were stimulated with either 10% FBS or 10% FBS plus EGF. To confirm that the cells were indeed quiescent and that cell cycle activity was stimulated by treatment with serum, or serum plus EGF, we monitored $^3$H-thymidine incorporation (Figure 4A). $^3$H-thymidine was incorporated at only very low levels in the unstimulated cells. TGFβ1 did not further repress incorporation in the arrested cells. Addition of 10% FBS stimulated $^3$H-thymidine incorporation ~8-fold, and treatment with EGF in addition to FBS resulted in even greater stimulation, ~14-fold. Stimulation by FBS and EGF was blocked completely if TGFβ1 was present during treatment.

Having confirmed that the cells were responding as expected, we measured the level of p34cdc2 histone H1 kinase activity in parallel sets of cells. The kinase activity (Figure 4B) correlated closely with the growth activity of the cells determined by $^3$H-thymidine incorporation. Kinase activity was barely detectable in growth-arrested cells (lane 1). It was induced by 10% FBS (lane 3) and induced further by EGF in addition to FBS (lane 5). However serum and EGF-mediated induction of p34cdc2-specific kinase activity were blocked completely if TGFβ1 was present during treatment (lanes 4 and 6).

The repressive effect of TGFβ1 on p34cdc2 was clearly evident at the level of p34cdc2 synthesis (Figure 4C). Synthesis of p34cdc2 was barely detectable in growth-arrested cells (lane 1) and was stimulated sharply by treatment with 10% FBS (lane 3) or FBS plus EGF (lane 5). Growth factor-mediated stimulation of p34cdc2 synthesis was blocked severely when TGFβ1 was present during treatment (lanes 4 and 6). Thus in synchronously stimulated cells, as well as in asynchronously growing cells, it appears that the repressive effect of TGFβ1 on p34cdc2 kinase activity is exerted at the level of expression of p34cdc2.

It has been reported elsewhere that growth arrest in the conditions described here does not reduce de novo synthesis of p34cdc2 significantly in Mv1Lu cells (Howe

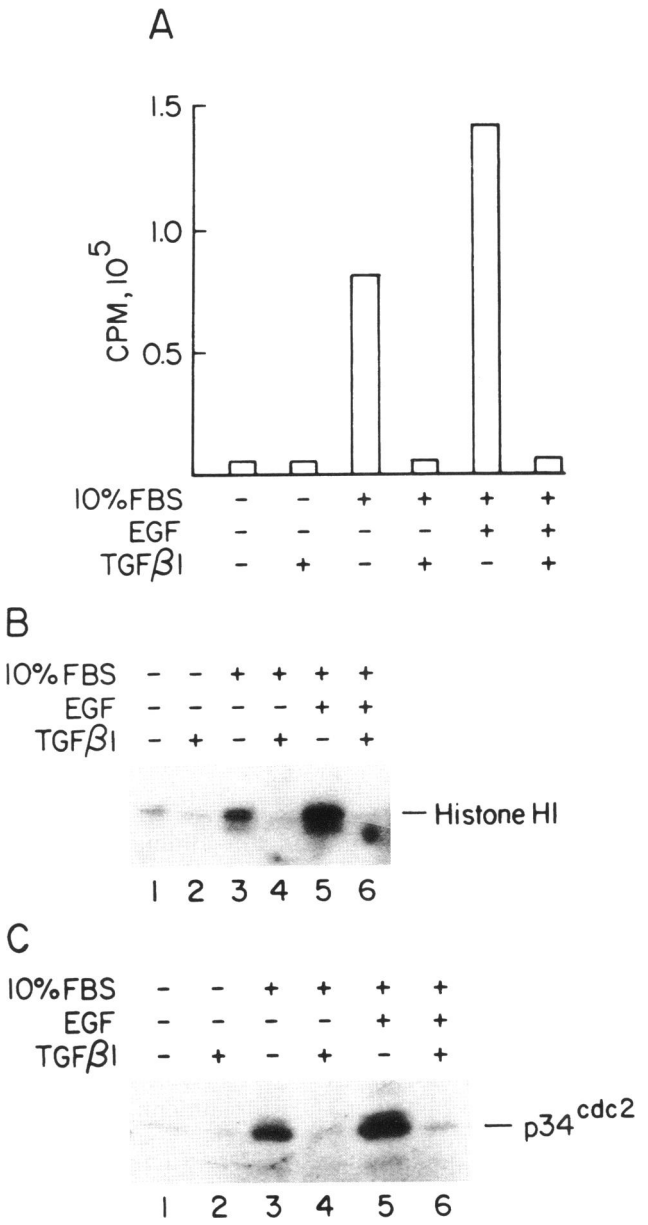


**Figure 4.** TGFβ1 inhibits p34cdc2 expression and kinase activity in growth-arrested cells. Quiescent Mv1Lu cells were left untreated or were stimulated with either 10% FBS or 10% FBS plus EGF, in the absence or presence of TGFβ1. (A) DNA synthesis measured by $^3$H-thymidine incorporation from 22 to 24 h after stimulation as described in MATERIALS AND METHODS. (B) p34cdc2 kinase activity. At 24 h after stimulation p34cdc2 specific histone H1 kinase activity was assayed as described in MATERIALS AND METHODS. (C) p34cdc2 expression. Cells were labeled with tran[$^{35}$S] label from 22 to 24 h after stimulation. At 24 h cell lysates were immunoprecipitated with G6 serum as described in MATERIALS AND METHODS.

*et al.*, 1991). In that report therefore, no stimulation of p34cdc2 synthesis was observed when the cells were stimulated, and no effect of TGFβ1 was seen at this

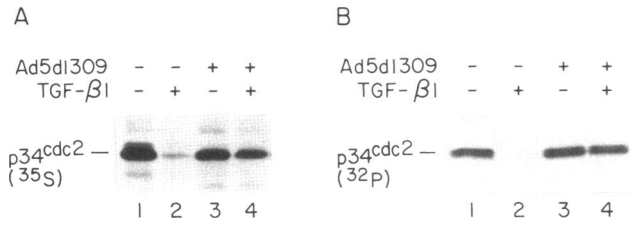


**Figure 5.** Adenovirus infection maintains synthesis and phosphorylation of $p34^{cdc2}$ in the presence of TGFβ1. Exponentially growing Mv1Lu cells were left uninfected or were infected with Ad5dl309 and incubated in the absence or presence of TGFβ1. Cells were labeled with either tran[$^{35}$S] label (A) or [$^{32}P_i$] (B), from 22 to 24 h after TGFβ1 addition. At 24 h cell lysates were immunoprecipitated with anti-$p34^{cdc2}$-specific G6 serum as described in MATERIALS AND METHODS.

level. It is not clear why we observe repression of $p34^{cdc2}$ synthesis in conditions where another research group has not, but this effect is invariable in our hands and suggests, at the least, that in conditions where changes in $p34^{cdc2}$ expression are apparent, TGFβ1 has a strong effect at the level of expression.

### *Adenovirus Maintains Synthesis and Phosphorylation of $p34^{cdc2}$ Protein in the Presence of TGFβ1*

From the above results, it appears that TGFβ1 inhibits $p34^{cdc2}$ histone H1 kinase activity by inhibiting the synthesis of the protein itself. To determine whether adenovirus is capable of overcoming this inhibition by TGFβ1, we assayed the effect of TGFβ1 on the synthesis of $p34^{cdc2}$ protein in infected cells. Proliferating Mv1Lu cells were left uninfected or were infected with Ad5dl309 and grown in the absence or presence of TGFβ1 for 24 h. Synthesis of $p34^{cdc2}$ protein (Figure 5A) was assayed by labeling the cells in vivo with tran[$^{35}$S] label from 22 to 24 h after TGFβ1 treatment. Cell lysates were immunoprecipitated with $p34^{cdc2}$-specific antibody. TGFβ1 inhibited the synthesis of $p34^{cdc2}$ protein in uninfected cells (lane 2 versus lane 1) whereas $p34^{cdc2}$ protein synthesis was maintained in cells infected with Ad5dl309 even in the presence of TGFβ1 (lane 4). Thus TGFβ1 inhibits the expression of the $p34^{cdc2}$ protein, and infection with an adenovirus expressing E1A can block the effect of TGFβ1 at this level. The ability of Ad5dl309 to maintain $p34^{cdc2}$ synthesis in the presence of TGFβ1 is consistent with the ability of adenovirus to induce $p34^{cdc2}$ expression in quiescent cells (Draetta *et al.*, 1988). Adenovirus does not induce $p34^{cdc2}$ synthesis above normal levels in actively cycling cells. Indeed, infection with Ad5dl309 consistently causes a slight reduction in $p34^{cdc2}$ synthesis in Mv1Lu cells, which can be seen in Figure 5A (lane 3 versus lane 1). This effect is specific for the E1A-expressing virus, as infection with Ad5dl312 has no discernable effect.

Because $p34^{cdc2}$ histone H1 kinase activity is associated with cell cycle dependent phosphorylation and dephosphorylation of the $p34^{cdc2}$ protein itself (Draetta and Beach, 1988; Draetta *et al.*, 1988b; Lee *et al.*, 1988), we asked whether adenovirus infection can maintain phosphorylation as well as synthesis of $p34^{cdc2}$ in the presence of TGFβ1. Phosphorylation of $p34^{cdc2}$ was assayed by labeling proliferating, uninfected or Ad5dl309-infected, Mv1Lu cells in vivo with $^{32}P_i$ from 22 to 24 h after TGFβ1 treatment. The presence of phosphoproteins (Figure 5B) was detected by immunoprecipitation as described above. Consistent with the repression of $p34^{cdc2}$ synthesis in the presence of TGFβ1, no phosphorylated forms of $p34^{cdc2}$ are apparent in lane 2. However in infected cells, the appearance of phosphorylated $p34^{cdc2}$ protein was maintained in the presence of TGFβ1 (lane 4 versus lane 2).

### *Adenovirus Maintains $p34^{cdc2}$ Histone H1 Kinase Activity in the Presence of TGFβ1*

To determine whether adenovirus also protects $p34^{cdc2}$ kinase activity from the effect of TGFβ1, proliferating Mv1Lu cells were infected with Ad5dl309 or Ad5dl312 and incubated in the absence or presence of TGFβ1 for 24 h. Kinase activity (Figure 6) was assayed as described above. TGFβ1 as expected inhibited $p34^{cdc2}$ kinase activity in uninfected cells (lane 2 versus lane 1) whereas kinase activity was not inhibited by TGFβ1 in cells expressing E1A as a consequence of infection with Ad5dl309 (lane 4 versus lane 3). The protective effect is dependent on E1A expression because the E1A deletion mutant, Ad5dl312, gives no protection (lane 6). Thus E1A expression serves directly or indirectly to maintain $p34^{cdc2}$ histone H1 kinase activity in the presence of TGFβ1; this function of E1A may contribute to its ability to protect cells from TGFβ1-mediated growth inhibition.

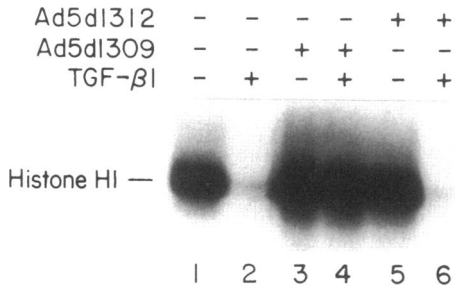


**Figure 6.** Adenovirus infection maintains $p34^{cdc2}$ histone H1 kinase activity in the presence of TGFβ1. Exponentially growing Mv1Lu cells were left uninfected or were infected with Ad5dl309 or Ad5dl312 and incubated in the absence or presence of TGFβ1. $p34^{cdc2}$ histone H1 kinase activity was assayed 24 h after TGFβ1 treatment as described in MATERIALS AND METHODS.

### *Adenovirus can Maintain p34$^{cdc2}$ Expression and Kinase Activity in the Presence of TGFβ1 Independently of the E1A-Associated pRB Binding Activity*

The retinoblastoma gene product (pRB) is implicated in the pathway by which TGFβ1 signals repression of epithelial cell growth (Pietenpol *et al.*, 1990). In addition, the ability of the E1A products to bind pRB appears to be a mechanistic step in one of the pathways by which E1A can stimulate cell cycle activity (Howe *et al.*, 1990; Wang *et al.*, 1991; Raychaudhuri *et al.*, 1991; Chellapan *et al.*, 1991). However, while E1A-mediated protection from specific effects of TGFβ1 such as the repression of cellular *myc* gene expression may be dependent on the pRB binding function (Pietenpol *et al.*, 1990), it is possible that the demonstrated ability of E1A to activate multiple pathways of cell cycle stimulation (Howe *et al.*, 1990; Wang *et al.*, 1991) could give adenovirus the ability to protect cell growth functions from the repressive effects of TGFβ1, even in the absence of a pRB binding function. This possibility was also suggested in a recent report showing that E1A mutants defective for pRB binding can protect keratinocyte DNA synthesis activity from the repressive effects of TGFβ1 (Missero *et al.*, 1991). To explore the mechanisms underlying TGFβ1-mediated effects and the steps at which TGFβ1-mediated signals can be counteracted, we asked whether the ability of adenovirus to maintain p34$^{cdc2}$ expression and kinase activity in TGFβ1 treated cells is dependent on the E1A pRB binding function.

An E1A point mutation product (pm.928), which encodes a single amino acid substitution in the pRB binding region of the E1A proteins, is devoid of the E1A-mediated immortalization function (Moran *et al.*, 1986) and does not bind pRB detectably (Moran, 1988; Wang *et al.*, 1991). We have shown previously that expression of the pm.928 products is very similar to the E1A.WT products in HeLa and baby rat kidney (BRK) cells (Moran *et al.*, 1986; Moran and Zerler, 1988; Wang *et al.*, 1991). When we did similar controls in Mv1Lu cells, however, we found that expression of the pm.928 products in E1A.928-infected cells lags behind that of the wild-type E1A products. Therefore, to obtain similar levels of expression, infection with the E1A.928 virus was started 12 h before TGFβ1 addition, whereas infection with the wild-type virus, Ad5dl309, was started 1 h before TGFβ1 addition, and all assays were done 24 h after addition. The synthesis of the wild-type E1A and E1A.928 products was compared at this point by immunoprecipitating labeled cell lysates with E1A-specific monoclonal antibody (series M73; Harlow *et al.*, 1985). The results show that the synthesis rates of wild-type and mutant E1A products at this point are similar and are not affected by the presence of TGFβ1 (Figure 7A). DNA synthesis activity was protected from TGFβ1-mediated inhibition in cells expressing E1A.928 in a pattern similar to that observed with wild-type E1A (Figure 7B). E1A.928 infection also protected p34$^{cdc2}$ histone H1 kinase activity from TGFβ1-mediated in-

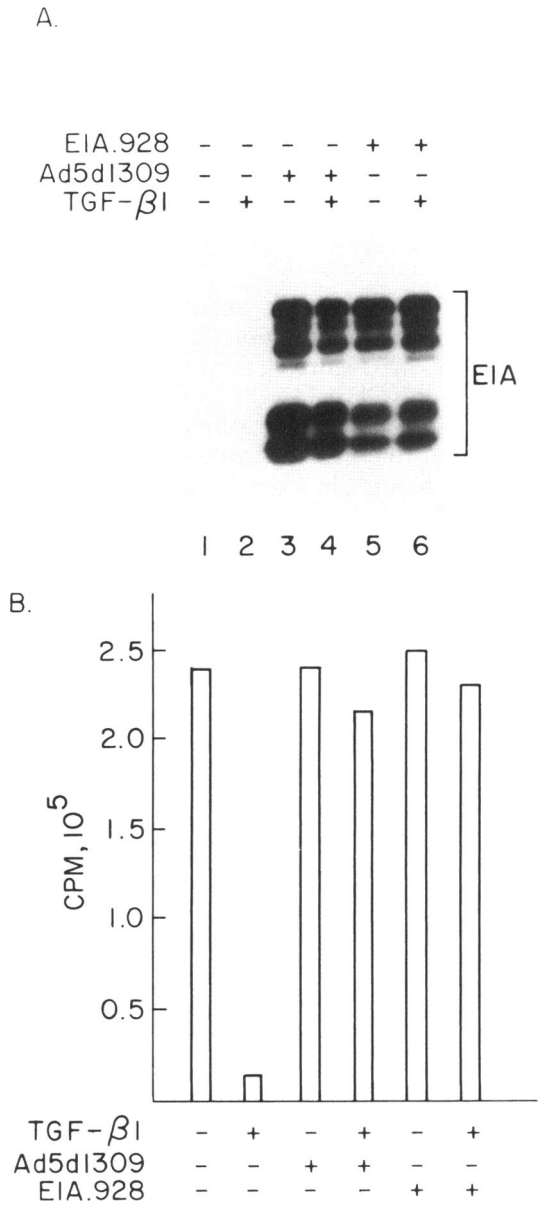


**Figure 7.** Adenovirus effects in the absence of pRB binding. (A) Expression of E1A. Exponentially growing Mv1Lu cells were left uninfected or were infected with Ad5dl309 or E1A.928 and incubated in the absence or presence of TGFβ1. The cells were labeled with trans[$^{35}$S] label from 22 to 24 h after treatment. Cells were harvested at 24 h and lysates were immunoprecipitated with E1A-specific M73 monoclonal antibody as described in MATERIALS AND METHODS. (B) DNA synthesis was assayed by [$^3$H]thymidine incorporation at 24 h, as described in Figure 1, in growing Mv1Lu cells that were left uninfected or were infected with Ad5dl309 or E1A.928 and incubated in the absence or presence of TGFβ1.

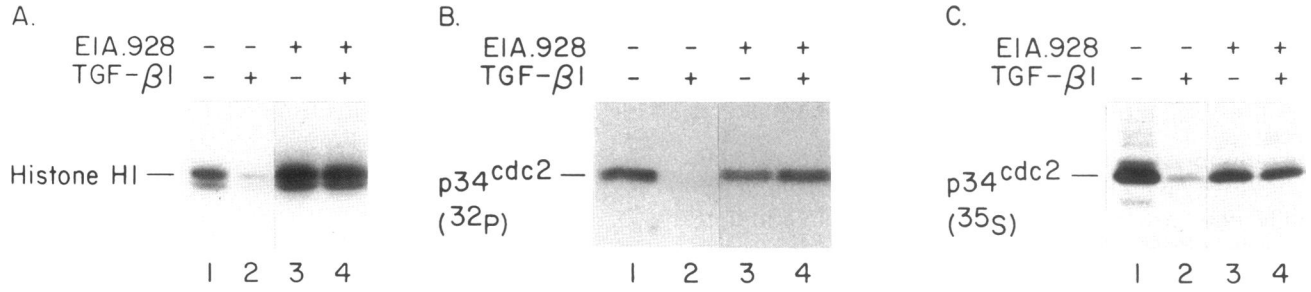


**Figure 8.** Adenovirus can maintain $p34^{cdc2}$ expression and kinase activity in the presence of TGFβ1 independently of pRB binding. Exponentially growing Mv1Lu cells were left uninfected or were infected with E1A.928 and incubated in the absence or presence of TGFβ1. (A) $p34^{cdc2}$ histone H1 kinase activity was assayed at 24 h as described in Figure 3B. (B) Phosphorylation of $p34^{cdc2}$ protein was assayed at 24 h as described in Figure 4B. (C) $p34^{cdc2}$ protein expression was assayed at 24 h as described in Figure 4A.

hibition (Figure 8A, lane 4 versus lane 2). Likewise, $p34^{cdc2}$ protein phosphorylation (Figure 8B) and $p34^{cdc2}$ protein expression (Figure 8C) were both maintained in the presence of TGFβ1 in cells infected with E1A.928 (lane 4 versus lane 2 in each panel). Thus the ability of adenovirus to protect DNA synthesis activity independently of pRB binding in the presence of TGFβ1 extends to the level of protection of $p34^{cdc2}$ expression and kinase activity.

### *Influence of E1A Expression on TGFβ1-Mediated Inhibition of pRB Phosphorylation*

The growth-suppressing activity of the RB product is associated with the underphosphorylated forms of pRB (reviewed in Weinberg, 1990), and TGFβ1 treatment reduces the extent of pRB phosphorylation in Mv1Lu cells (Laiho *et al.*, 1990). Although E1A can mediate aspects of cell cycle activity independently of pRB binding, it is not clear whether E1A acting in this manner bypasses the role of pRB entirely or perhaps mediates cellular signals that converge on pRB by some means other than direct pRB binding. The ability of non-RB-binding E1A mutants to stimulate pRB phosphorylation in quiescent primary cells (Wang *et al.*, 1991) suggests strongly that the latter possibility is the more likely. To explore these questions, we examined the ability of viruses expressing wild-type E1A or the E1A.928 product to alter the effect of TGFβ1 on the phosphorylation state of pRB in Mv1Lu cells. Exponentially growing Mv1Lu cells were left uninfected or were infected with Ad5dl309 or E1A.928 as described above and were incubated in the presence of TGFβ1 for 24 h. Phosphorylation of pRB was assayed by pulse labeling the cells with $^{32}Pi$ from 22 to 24 h after TGFβ1 treatment. Cell lysates were immunoprecipitated with pRB-specific antibody (series XZ-133; Hu *et al.*, 1991) (Figure 9). As shown previously (Laiho *et al.*, 1990), pRB phosphorylation was almost completely suppressed by TGFβ1 treatment (lane 2 versus lane 1). As was also reported previously (Laiho *et al.*, 1990), pRB continued to be expressed in TGFβ1 treated cells, although at a somewhat reduced rate from that seen in exponentially growing cells. pRB phosphorylation was still mostly suppressed by TGFβ1 in cells infected with Ad5dl309 (lane 4), although Ad5dl309 infection even in the absence of TGFβ1 had a suppressive effect on the degree of pRB phosphorylation (lane 3 versus lane 1). In cells infected with E1A.928, pRB remained largely in the phosphorylated state (lane 5) even in the presence of TGFβ1 (lane 6). These results demonstrate that in the absence of a pRB binding function, adenovirus can still counteract the signals by which TGFβ1 induces the suppression of pRB phosphorylation. The observation that the wild-type E1A products have an intermediate effect on pRB phosphorylation is not unexpected and is considered further in the discussion.

## DISCUSSION

The results of this study indicate that TGFβ1 treatment induces not only a change in the $p34^{cdc2}$ phosphorylation state and kinase activity, it causes a strong repression of $p34^{cdc2}$ protein synthesis. The loss of $p34^{cdc2}$ kinase activity does not occur until $p34^{cdc2}$ synthesis declines, which suggests that TGFβ1 affects $p34^{cdc2}$ at the level of expression, rather than at the level of posttranslational modification. In addition, our results suggest that the ability of the E1A products to activate $p34^{cdc2}$ expression and kinase activity, and to maintain these cellular functions in the presence of TGFβ1, is intrinsic to their ability to counteract the effects of TGFβ1.

There is plausible evidence to suggest that TGFβ1 signals activation of a pRB-mediated growth repression function dependent on reducing the extent of pRB phosphorylation (Laiho *et al.*, 1990; Pietenpol *et al.*, 1990). It is also likely that the ability of the E1A products to form stable complexes with pRB can serve to prevent pRB-mediated inhibition of cell cycling. However, the results presented here demonstrate that adenovirus can counteract the effect of TGFβ1 independently of the E1A-associated pRB binding function. The ability of the

E1A.928 mutant virus to maintain cellular DNA synthesis in the presence of TGFβ1 correlates with its ability to maintain expression and activity of the cell cycle control kinase, p34$^{cdc2}$. In addition, the E1A.928 mutant virus is capable of maintaining pRB in a phosphorylated state even in the presence of TGFβ1. These results suggest that the multiple gene activation functions of E1A can act through more than one mechanism to maintain cell cycle activity despite TGFβ1-mediated repression signals. The E1A products appear to function either directly by sequestering pRB, or by intervening at an alternative point in the cellular pathway by which TGFβ1 signals suppression. The implication that TGFβ1 affects p34$^{cdc2}$ at the level of expression supports the suggestion that the ability of E1A to activate G1 gene expression through multiple pathways (Howe *et al.*, 1990; Wang *et al.*, 1991) is the basis for the resistance to TGFβ1-mediated growth suppression observed in cells expressing E1A. It is also possible that the gene-regulating activities of E1A act indirectly to stimulate expression of other viral products, which contribute to the protective effect. We are continuing to investigate this possibility. However, the results of the present study make it clear that the pRB binding function of E1A is not required for the protective effect of adenovirus in TGFβ1 treated cells.

An intriguing result presented here is the observation that TGFβ1-mediated suppression of pRB phosphorylation is counteracted in the presence of the mutant E1A.928 but not in the presence of wild-type E1A. This is actually consistent with the model that wild-type E1A blocks TGFβ1 by directly sequestering pRB and is also consistent with the observation of Laiho *et al.* (1990) that pRB phosphorylation continues to be suppressed in response to TGFβ1 treatment in the presence of another pRB-binding DNA tumor virus oncogene product, SV40 T antigen. These authors suggested that the binding of T antigen to pRB interferes with pRB phosphorylation, while also making it unnecessary for continued cell growth. The results with E1A.928 add further support to this model. Wild-type E1A products might sterically block pRB phosphorylation even while stimulating cellular mechanisms that would normally lead to pRB phosphorylation: the ability of the E1A.928 products to maintain cell cycle activity without physically interacting with pRB could leave pRB open for phosphorylation. The link between maintenance of pRB phosphorylation and resistance to the inhibitory effect of TGFβ1 on DNA synthesis is also strengthened by a comparison between non-RB binding mutants of E1A and T antigen. The ability to induce G1- and S-phase events remains largely intact in the absence of the pRB-binding function in E1A, to a much greater extent than in T antigen, apparently because of multiple independent cellular gene-regulating regions in the E1A products (Moran and Mathews, 1987; Moran and Zerler, 1988; Howe *et al.*, 1990; Stein *et al.*, 1990; Wang *et al.*, 1991). Thus while E1A.928 protects both pRB phosphorylation and DNA synthesis, an analogous mutant of T antigen, designated K1, protects neither (Laiho *et al.*, 1990). This co-segregation of the ability to protect DNA synthesis with the ability to protect pRB phosphorylation increases the likelihood that these effects of TGFβ1 are related.

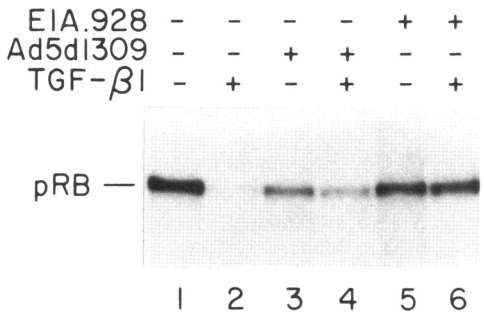


**Figure 9.** Influence of adenovirus on TGFβ1-mediated inhibition of pRB phosphorylation. Exponentially growing Mv1Lu cells were left uninfected or were infected with Ad5dl309 or E1A.928 and incubated in the absence or presence of TGFβ1. Cells were labeled with $^{32}P_i$, from 22 to 24 h after TGFβ1 treatment. Cells were harvested at 24 h and lysates were immunoprecipitated with pRB-specific monoclonal antibody, XZ-133 as described in MATERIALS AND METHODS.

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