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TGFβ-induced Growth Inhibition in Primary Fibroblasts Requires the Retinoblastoma Protein

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Transforming growth factor β (TGFβ) inhibits cell proliferation by inducing a G_1 cell-cycle arrest. Cyclin/CDK complexes have been implicated in this arrest, because TGFβ treatment leads to inhibition of cyclin/CDK activity. We have investigated the role of the retinoblastoma protein (pRb) in TGFβ-induced growth arrest by using $RB^{+/+}$ and $RB^{-/-}$ primary mouse embryo fibroblasts. In both of these cell types, TGFβ inhibits CDK4-associated kinase activity. However, whereas CDK2-associated kinase activity was completely inhibited by TGFβ in the wild-type cells, it was reduced only slightly in the *RB* mutant cells. In addition, at high-cell density the growth-inhibitory effects of TGFβ are no longer observed in the $RB^{-/-}$ cells; on the contrary, TGFβ treatment promotes the growth of these mutant fibroblasts. Thus, under certain cellular growth conditions, elimination of pRb transforms the growth-inhibitory effects of TGFβ into growth-stimulatory effects. These observations could help to explain why TGFβ is often found to enhance tumorigenicity in vivo and why inactivation of the *RB* gene leads to tumorigenesis.

INTRODUCTION

The retinoblastoma gene (*RB*) is inactivated in a wide variety of human tumors (Weinberg, 1995), indicating that its role as a growth suppressor is not confined to a specific cell or tissue type. Retinoblastoma protein (pRb), the protein encoded by the *RB* gene, displays properties of a cell-cycle regulatory protein. During early to middle G_1, pRb is in an underphosphorylated state. However, several hours before the end of G_1, pRb undergoes phosphorylation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; De Caprio *et al.*, 1989; Geng and Weinberg, 1993). This phosphorylation converts pRb from an actively growth-suppressing form into a form that seems to be functionally inactive. Moreover, this phosphorylation alters the biochemistry of pRb in that it can no longer bind various cellular transcription factors.

Various types of experiments indicate that the phosphorylation of pRb is performed by certain cyclin-dependent kinases (CDKs) in association with their regulatory cyclin subunits (Hinds *et al.*, 1992; Ewen *et al.*, 1993a; Kato *et al.*, 1993). Of particular interest are cyclin D/CDK4,6 complexes and cyclin E/CDK2 complexes. These are active in G_1 at the time of pRb phosphorylation and have been shown to phosphorylate pRb in vitro. In addition, ectopic expression of these cyclins causes pRb phosphorylation in living cells.

Besides serving as a substrate for cyclin E/CDK2 complexes, pRb also inhibits expression of cyclin E (Geng *et al.*, 1996; Herrera *et al.*, 1996), the activity of which is necessary for progression into S phase (Tsai *et al.*, 1993). This inhibition is achieved through transcriptional repression of the cyclin E promoter, most likely by pRb binding to E2F and inhibiting its transactivation potential. This control of cyclin E expression may be the reason that inactivation of pRb leads to a shortened G_1 phase in primary mouse embryo fibroblasts (Lukas *et al.*, 1995; Herrera *et al.*, 1996).

Treatment of various cell types with transforming growth factor β (TGFβ) results in a wide range of biological effects. These include growth inhibition, growth promotion, escape from contact inhibition, and induction of growth factor and extracellular matrix production (Massagué *et al.*, 1992). Cells that are growth inhibited by TGFβ are found in the G1 phase

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of the cell cycle. Such cells express pRb in its underphosphorylated, growth-suppressing form (Laiho *et al.*, 1990; Furukawa *et al.*, 1992; Geng and Weinberg, 1993). Thus, it has been suggested that TGFβ blocks progress through the G1 phase by preventing phosphorylation and functional inactivation of pRb (Derynck, 1994). In addition, it has been shown that pRb is necessary for TGFβ inhibition of N-*myc* and c-*myc* transcription (Moses, 1992; Serra and Moses, 1995).

Recently, this inhibition of pRb phosphorylation has been explained at the biochemical level through the observation that TGFβ causes the synthesis or activation of several low-molecular-weight CDK inhibitors (CDKIs) (Hannon and Beach, 1994; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Datto *et al.*, 1995; Li *et al.*, 1995; Reynisdóttir *et al.*, 1995). Taken together, these various lines of research suggest a chain of events in which TGFβ induces or activates CDKIs that proceed to block pRb phosphorylation; pRb, in turn, continues to sequester a series of transcription factors, the activity of which is required for advance into the late G1 and S phases. To determine whether TGFβ arrest is indeed mediated by pRb, we used primary mouse embryo fibroblasts derived from *RB*-deficient embryos (Jacks *et al.*, 1992). These cells are genetically identical with cells derived from wild-type littermates, with the exception of the introduced mutation in the *RB* gene.

MATERIALS AND METHODS

Cell Culture

Early passage (<8) primary fibroblasts isolated from *RB* mutant and wild-type embryos at 12.5 d of gestation (Jacks *et al.*, 1992) were used in all experiments. The cells were maintained in DMEM/15% inactivated fetal calf serum (FCS) plus antibiotics. Cells were grown to a density of at least 7800 cells/cm^2 in all experiments. For serum starvation, the cells were maintained in DMEM lacking serum for 3 d. Such treatment leads to equal synchrony in both the $RB^{+/+}$ and $RB^{-/-}$ cells (Herrera *et al.*, 1996). Readdition of inactivated FCS led to entry into G_1 in at least 70% of both cell types. TGFβ$_1$ (a gift of P. Segarini, Celtrix Pharmaceuticals, Santa Clara, CA) was added to 100 pM unless otherwise noted in the figure legends.

For the analysis of Figure 3, cells were plated at the indicated amounts per well of a 24-well plate. After 12 h, TGFβ was added to 100 pM for 24 h. The cells were then exposed to a dose of 2775 RAD of γ-irradiation to induce G_1 arrest. Onto these arrested cells 5×10^4 $RB^{-/-}$ cells were added. After 24 h, the cells were labeled with ^{3}H-thymidine as described below. The γ-irradiated feeder cells displayed only low levels of ^{3}H-thymidine incorporation. These low amounts of background radioactivity were subtracted from the total radioactivity incorporated by cocultured living cells.

^{3}H-Thymidine Labeling of Cells

For ^{3}H-thymidine incorporation experiments, cells were labeled with 1 mCi/ml ^{3}H-thymidine in DMEM minus serum for 1 h. The cells were then treated with ice-cold 5% trichloroacetic acid for 30 min before extensive washing with H_2O, lysis with 0.5 M NaOH, and scintillation counting.

Immunoprecipitations and Kinase Assays

Kinase assays were performed as described for CDK2 (Mäkelä *et al.*, 1995) and CDK4 (Matsushime *et al.*, 1994) by using equal amounts of protein for each immunoprecipitation. Antibodies to CDK2 and CDK4 were from Santa Cruz Biotechnology (Tebu, France).

Immunoblot and RNA Analyses

Immunoblot analyses and Northern blots were performed according to standard procedures (Ausubel *et al.*, 1993) and as described (Herrera *et al.*, 1996). RNA amounts were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reprobing. Cyclin E mRNA was detected by a radiolabeled murine cyclin E cDNA (a gift of P. Steiner and M. Eilers, Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany). Protein amounts were normalized by spectrophotometric methods and Ponceau S staining of the nitrocellulose filters. Antibodies to cyclin E were a gift from J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA), whereas those to CDK2, CDK4, p21, and p27 were obtained from Santa Cruz Biotechnology. Specificity of the antibodies was confirmed by detection of recombinant cyclin proteins and migration in SDS-PAGE.

RESULTS

Effects of TGFβ on Mouse Embryo Fibroblasts

TGFβ was added to primary fibroblasts prepared from wild-type embryos or embryos homozygous for an inactivated allele of *RB*. At 24 h after the addition of TGFβ to various concentrations, the rate of DNA synthesis was measured by ^{3}H-thymidine incorporation. As shown in Figure 1, TGFβ inhibited DNA synthesis in the wild-type cells at concentrations as low as 10 pM and reached a maximal level of inhibition at 40 pM. The $RB^{-/-}$ cells, in contrast, displayed no inhibition but instead showed increased ^{3}H-thymidine incorporation at all concentrations of TGFβ tested.

The increase in ^{3}H-thymidine incorporation in the $RB^{-/-}$ fibroblasts reflected their continued prolifera-

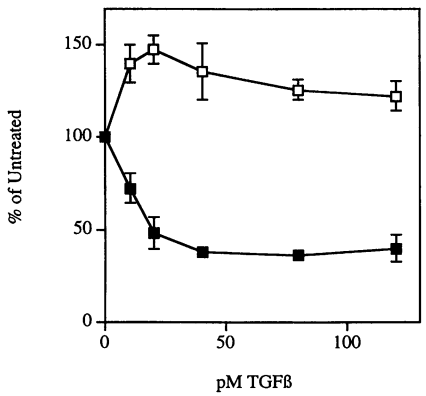

Figure 1. TGFβ titration on primary $RB^{+/+}$ and $RB^{-/-}$ fibroblasts. TGFβ was added at the indicated concentrations to confluent cultures of $RB^{+/+}$ and $RB^{-/-}$ fibroblasts. After 24 h, the cells were labeled with ^{3}H-thymidine for 1 h before lysis and counting. The Y-axis is expressed as the percentage cpm of samples that were not treated with TGFβ.

tion after TGFβ treatment (Figure 2). Thus, when TGFβ and fresh serum were added to confluent cultures of $RB^{+/+}$ and $RB^{-/-}$ fibroblasts daily for 3 d, the number of wild-type cells declined slightly, while the $RB^{-/-}$ cells continued to multiply. In contrast, addition of serum alone had no effect on either cell type. This suggests that TGFβ treatment allows the $RB^{-/-}$ cells to overcome contact inhibition. Indeed, visual observation suggested that the addition of TGFβ to the mutant cells enabled them to grow in multilayers (our unpublished observations).

Density Dependence of TGFβ Effects

Others have reported that the effects of TGFβ on cellular proliferation are highly dependent on cell density in vitro (Smith, 1994; Morton and Barrack, 1995). In these studies, it was found that TGFβ was able to inhibit proliferation of rat prostate adenocarcinoma cells (Morton and Barrack, 1995) and rat intestinal epithelial cells (Smith, 1994) when treated at low density. However, TGFβ lost its antiproliferative properties when added to dense cultures of these cells.

These reports caused us to determine whether the effects of TGFβ observed above were cell density-dependent. $RB^{+/+}$ and $RB^{-/-}$ cells were plated at various densities ranging from ~50 to 100% confluency. TGFβ was then added to the cells for 24 h before labeling with ^{3}H-thymidine. Growth inhibition of the wild-type cells by TGFβ was observed at all densities tested (Figure 3A). In the absence of TGFβ, the mutant cells showed slightly decreased levels of thymidine incorporation when plated at higher densities (Figure 3B). Addition of TGFβ to these mutant cells at low density inhibited cell growth, whereas the growth-stimulatory effects of TGFβ became apparent at high densities (Figure 3B). Hence, the effect of TGFβ treatment on the mutant cells at higher densities was to reverse the decreased growth rate of these cells.

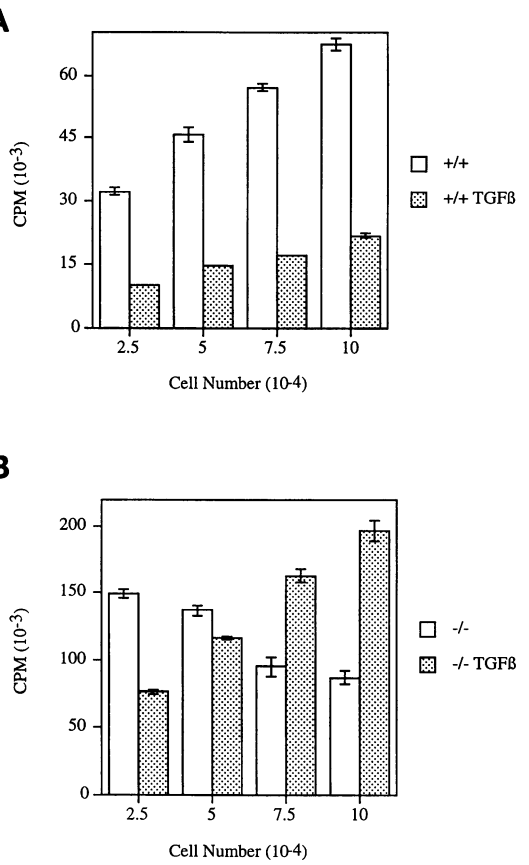

Figure 3. Importance of cell density on TGFβ effects in $RB^{+/+}$ and $RB^{-/-}$ fibroblasts. $RB^{+/+}$ (A) and $RB^{-/-}$ (B) cells were plated at the indicated cell number per well of a 24-well plate. Twelve hours later, 100 pM TGFβ was added. Labeling with ^{3}H-thymidine was done 24 h after TGFβ addition. The Y-axis represents cpm × 10^3. The higher number of counts in the $RB^{-/-}$ cultures is reflective of the greater percentage of the mutant cell present in S phase (Herrera *et al.*, 1996).

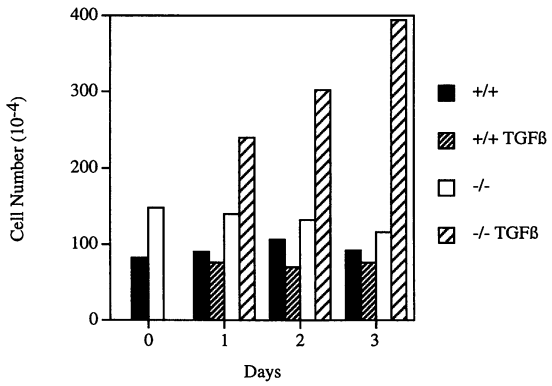

Figure 2. Growth profile of $RB^{+/+}$ and $RB^{-/-}$ fibroblasts grown in the absence or presence of TGFβ, as indicated. Growth media or growth media plus 100 pM TGFβ were added to confluent cultures of $RB^{+/+}$ and $RB^{-/-}$ fibroblasts grown on 35-mm plates every day for the indicated number of days before cell number determination.

Release of Growth-stimulatory Substances by $RB^{-/-}$ Fibroblasts

The above results indicate that TGFβ has paradoxical effects on cells. In cells expressing pRb, growth is inhibited. In those lacking pRb, growth is stimulated at high-cell densities. This density-dependent growth stimulation suggested the involvement of some factor or substance that is released from cells after TGFβ treatment and succeeds in stimulating cell growth in an autocrine and/or paracrine manner.

To determine whether the $RB^{+/+}$ or $RB^{-/-}$ cells released some growth-stimulatory substance in response to TGFβ treatment, the experiment of Figure 4 was performed. $RB^{-/-}$ and $RB^{+/\pm}$ cells were plated at different densities, as shown in Figure 3. TGFβ was

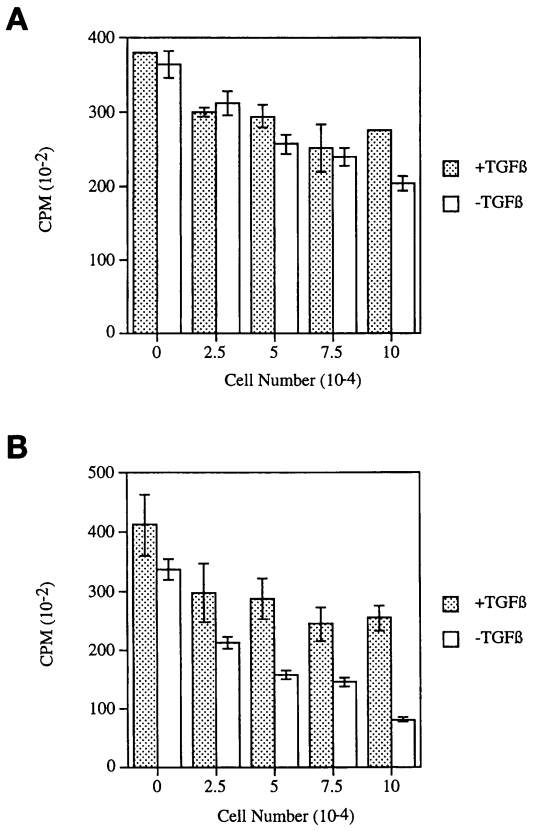

Figure 4. Stimulatory effect of TGFβ-treated $RB^{-/-}$ feeder cells. $RB^{+/+}$ (A) or $RB^{-/-}$ (B) fibroblasts were plated at the indicated amounts per well of a 24-well plate. Twelve hours later, TGFβ was added to 100 pM for 24 h. The cells were then γ-irradiated, as described in MATERIALS AND METHODS to induce growth arrest. Onto these cells 5×10^4 $RB^{-/-}$ cells were plated. After 24 h, the cells were labeled with ^{3}H-thymidine, as described in MATERIALS AND METHODS.

then added for 24 h, after which time the cells were γ-irradiated to arrest their growth. *RB* mutant cells (5×10^4) were then plated onto these arrested cells. After 24 h, the cultures were labeled with ^{3}H-thymidine as a measure of proliferation. The γ-irradiated cells alone incorporated only background levels of ^{3}H-thymidine, confirming their arrest (our unpublished observations).

Figure 4A shows that addition of mutant cells to γ-irradiated and TGFβ-treated wild-type cells does not affect their proliferation relative to non-TGFβ–treated controls at all cell densities tested. Therefore, the $RB^{+/+}$ cells do not release a factor that is growth stimulatory for the mutant cells after TGFβ treatment.

The identical experiment was then performed with $RB^{-/-}$ cells to form the irradiated cell mat onto which live mutant cells were subsequently plated (Figure 4B). The $RB^{-/-}$ cells forming the mat were either exposed to TGFβ or grown without TGFβ before irradiation. As seen in Figure 4B, when 5×10^4 $RB^{-/-}$ cells were plated onto a mat of $RB^{-/-}$ cells that had been grown without TGFβ before irradiation, they displayed progressively reduced ^{3}H-thymidine incorporation in inverse correlation with the density of the cellular mat. This result was reminiscent of what was observed when $RB^{-/-}$ cells were plated directly at high density (Figure 3).

However, when the $RB^{-/-}$ cells were plated onto an irradiated mat of $RB^{-/-}$ cells that had been treated with TGFβ before irradiation, the growth of the overlayed cells was markedly stimulated (Figure 4B). This effect was observed at all cell densities tested but became more pronounced at higher plating densities. Hence, the effect of TGFβ treatment was to abolish and reverse the growth-inhibitory effects released by the γ-irradiated cells present in the cell mat. This mimics the effect seen when TGFβ is directly added to $RB^{-/-}$ cells (Figure 3B). These data confirm that the irradiated mutant cells produce and release some factor or substance that is growth stimulatory.

Differential Effects of TGFβ on Cyclin/CDK Kinase Activity in the $RB^{+/+}$ and $RB^{-/-}$ Fibroblasts

As mentioned, TGFβ treatment of various cell types is known to inhibit G_1 cyclin/CDK activity through the induction or activation of CDKIs (Ewen *et al.*, 1993b; Geng and Weinberg, 1993; Koff *et al.*, 1993; Hannon and Beach, 1994). Therefore, we investigated the effects of TGFβ on CDK kinase activity in both the $RB^{+/+}$ and $RB^{-/-}$ fibroblasts. CDK2 or CDK4 complexes were immunoprecipitated from cell lysates prepared from wild-type and mutant embryo fibroblasts after 36 h of TGFβ treatment. These immunoprecipitates were then assayed for kinase activity by using either GST-pRb or histone H1 as substrates for CDK4 and CDK2, respectively.

TGFβ treatment led to complete inhibition of CDK4-associated kinase activity in both cell types (Figure 5A). In contrast, CDK2-associated kinase activity was completely inhibited by TGFβ in the wild-type cells but only slightly reduced in the mutant cells (Figure 5B). Thus, the TGFβ-mediated inhibition of CDK2 kinase activity is largely dependent on the presence of pRb, whereas the inhibition of CDK4 kinase activity is not.

These results demonstrate that CDK4 functions upstream of pRb in a TGFβ regulatory pathway that is active in G_1. pRb, in turn, is upstream of CDK2. These findings also support a G1 progression model, according to which the activation of cyclin D-associated CDK4/6 kinase activity is followed temporally by the phosphorylation and inactivation of pRb, which in turn results in the activation of cyclin E/CDK2 kinase activity (Resnitzky and Reed, 1995).

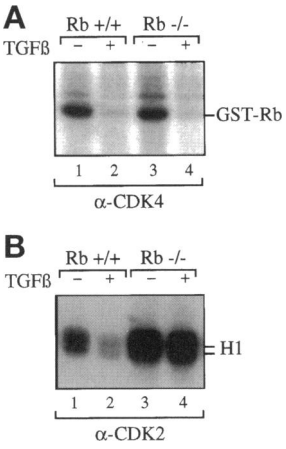

Figure 5. Effects of TGFβ on CDK4 and CDK2 kinase activities in $RB^{+/+}$ and $RB^{-/-}$ fibroblasts. (A) GST-pRb kinase activity of anti-CDK4 immunoprecipitates prepared after the addition of media or media plus 100 pM TGFβ to confluent $RB^{+/+}$ and $RB^{-/-}$ fibroblasts for 36 h. (B) Histone H1 kinase activity of anti-CDK2 immunoprecipitates prepared after the addition of media (DMEM/15% FCS) or media plus 100 pM TGFβ to confluent $RB^{+/+}$ and $RB^{-/-}$ fibroblasts for 36 h.

Effects of TGFβ on the Levels of Cyclin E, CDK2, CDK4, p21, and p27

We further explored the mechanism of TGFβ-induced inhibition of CDK4 and CDK2 activity by examining other cell-cycle regulatory factors. Immunoblot analysis showed that TGFβ had no effect on the levels of CDK2, cyclin E, or CDK4 when added to asynchronous cultures of either the $RB^{+/+}$ or $RB^{-/-}$ fibroblasts (Figure 6A). These results suggested that the observed TGFβ inhibition of CDK kinase activities was mediated by another mechanism, such as activation of the low-molecular-weight inhibitors $p15^{INK4B}$, p21, or p27, all of which have been shown to be induced by TGFβ (Hannon and Beach, 1994; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Datto *et al.*, 1995; Li *et al.*, 1995; Reynisdóttir *et al.*, 1995).

The possible involvement of p27 or p21 in CDK4 inhibition was analyzed in Figure 6B. TGFβ was added to both cell types for various times before lysis. Although lower levels of p21 were observed in the wild-type cells, immunoblot analyses showed that TGFβ treatment had no effects on the levels of p27 or p21 in either the $RB^{+/+}$ or $RB^{-/-}$ cells. In addition, immunoprecipitation with either CDK2 or CDK4 antibodies followed by immunoblotting with p21 or p27 antibodies showed that TGFβ treatment did not affect their associations with CDK2 and CDK4 (our unpublished observations). This is in contrast to studies that used human keratinocytes and mink lung epithelial cells (Reynisdóttir *et al.*, 1995), in which changes in the associations of p21 and p27 with CDK4 and CDK2 were observed after TGFβ treatment.

These results indicated that the observed TGFβ-induced inhibition of CDK4 activity was regulated by another mechanism. An attractive candidate is $p15^{INK4B}$, which others have shown is induced in TGFβ-treated mink lung cells (Hannon and Beach, 1994). However, Northern analysis showed no change in the steady-state levels of p15 mRNA after TGFβ treatment of either $Rb^{-/-}$ or $Rb^{+/+}$ cells (our unpublished observations), and because of the lack of appropriate antibodies, we have been unable to ascertain the effect of TGFβ on p15 protein levels.

TGFβ Effects on Cyclin E Transcription

We have shown previously (Herrera *et al.*, 1996) that, in the primary embryo fibroblasts used here, cyclin E and CDK2 protein levels do not change significantly during mid/late G_1, whereas CDK2 activity and cyclin E mRNA levels do increase substantially. These results indicated that CDK2-associated kinase activity, through unknown mechanisms, is uncoupled from the total protein levels of cyclin E or CDK2 but is correlated with the transcriptional induction of cyclin E. For

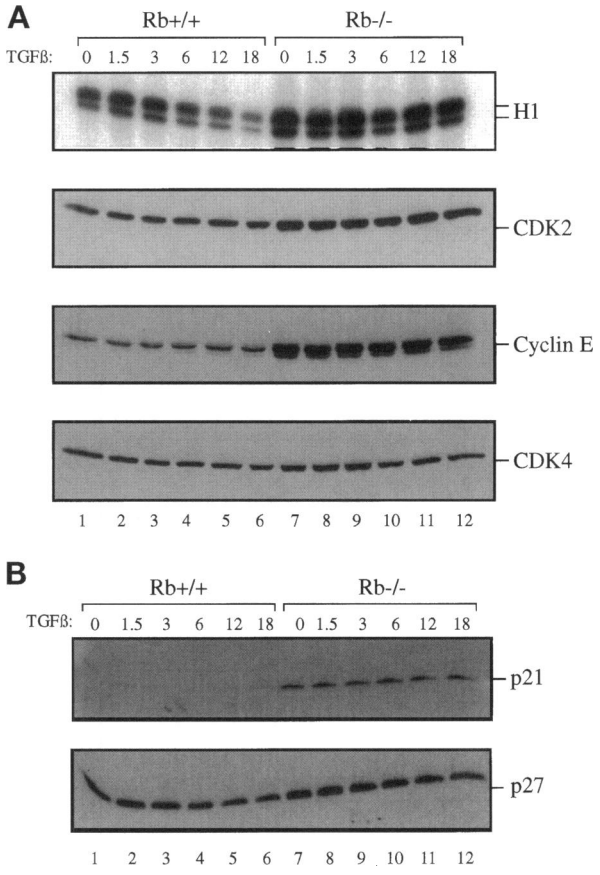

Figure 6. Molecular effects of TGFβ in $RB^{+/+}$ and $RB^{-/-}$ fibroblasts. (A) TGFβ was added to confluent cells maintained in DMEM/15% FCS for the indicated number of hours before lysis. Then anti-CDK2 immunoprecipitates were prepared and used in histone H1 kinase assays (top panel). Alternatively, the lysates were used directly for immunoblot analyses with indicated antibodies. The higher cyclin E levels in the $RB^{-/-}$ fibroblasts is due to deregulation of cyclin E in these cells (Herrera *et al.*, 1996). (B) Immunoblot analysis of lysates was prepared as above, except that p21 and p27 antibodies were used.

these reasons, it was of interest to determine the effects of TGFβ on cyclin E transcription and CDK2-associated activity in G_1.

Serum-starved $RB^{+/+}$ and $RB^{-/-}$ fibroblasts were restimulated with serum in the presence or absence of TGFβ. In agreement with earlier results, total cyclin E protein levels remained unchanged in both cell types throughout G1, whether or not TGFβ was added (Figure 7A). However, TGFβ completely inhibited the transcriptional induction of the cyclin E gene in an pRb-dependent manner (Figure 7B). Similarly, the complete inhibition by TGFβ of serum-induced CDK2-associated kinase activity was seen only in the $RB^{+/+}$ cells (Figure 7C). Taken together, these results once again indicate a tight coupling between control of cyclin E transcription and of CDK2 activity. In addition, TGFβ effects on cyclin E transcription and CDK2 kinase activity are shown to be pRb dependent. These data also reinforce the notion that cyclin E is downstream of pRb, in particular that, in normal cells, the inactivation of pRb is necessary for cyclin E transcriptional induction and the activation of cyclin E-associated CDK2 kinase activity.

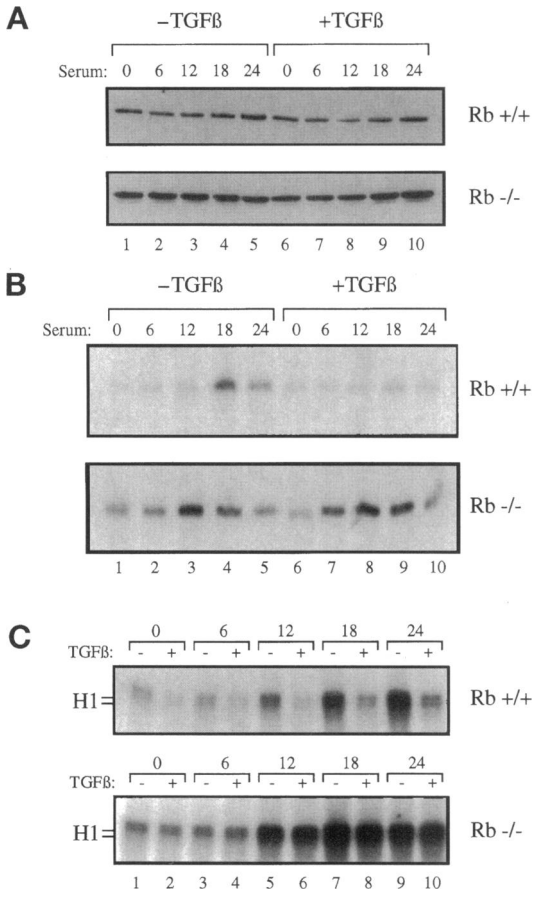

Figure 7. Effects of TGFβ on serum induction of cyclin E transcription, protein levels, and CDK2 activity. (A) Cyclin E immunoblot analysis with lysates from $RB^{+/+}$ and $RB^{-/-}$ fibroblasts that had been serum starved for 48 h before the readdition of serum with or without TGFβ for the indicated number of hours. (B) As in A, except that mRNA was isolated instead of protein and subjected to Northern analysis by using a murine cyclin E cDNA probe. (C) As in A and B, except that anti-CDK2 immunoprecipitates were prepared from the protein lysates and used in histone H1 kinase assays.

DISCUSSION

The present results indicate that primary mouse fibroblasts lacking functional pRb are no longer inhibited by TGFβ when grown at high density; on the contrary, their proliferation is promoted. These conditions of high density mimic the situation in vivo in which cells exist in close association. The effect of induced proliferation seems to be due to an ability of the $RB^{-/-}$ cells to overcome contact inhibition in the presence of TGFβ.

This density-dependent growth stimulation suggests the involvement of some substance released from cells after TGFβ treatment that stimulates cell growth in an autocrine and/or paracrine manner. We speculate that this substance is the extracellular matrix (ECM), the production of which is known to be stimulated by TGFβ treatment (Roberts *et al.*, 1992; Newman, 1993). In addition, Morton and Barrack (1995) found that ECM also blunted the growth-inhibitory potential of TGFβ on prostate adenocarcinoma cells. Production of proteins such as fibronectin and TIMP-1 might result in the formation of an ECM that serves as a substrate conducive to cell proliferation (Roberts *et al.*, 1992; Newman, 1993). TGFβ-induced morphological changes that we observed in the $Rb^{-/-}$ cultures were also compatible with effects of the ECM. TGFβ is known to induce the release of mitogenic growth factors from treated cells. We discount their role in the present experiments, because all cultures described here were grown in the presence of 15% fetal bovine serum, which provides very high concentrations of these factors including, notably, platelet-derived growth factor (PDGF).

When cultured at low density, the growth of the $RB^{-/-}$ cells was weakly inhibited by TGFβ. Although this remains unexplained, it is possible that TGFβ inhibition of phosphorylation of other CDK4/6 substrates, including the pRb-related proteins p107 and p130, results in a weak inhibition at low cell densities. We note that the effects of TGFβ on both CDK2 and CDK4 kinase activities are independent of cell density (our unpublished observations).

TGFβ inhibits CDK4 activity equally in the $RB^{+/+}$ and $RB^{-/-}$ fibroblasts, whereas CDK2 is efficiently inhibited only in the wild-type cells. This suggests the regulatory pathway depicted in Figure 8. During progression through G_1, D-type cyclins, in association with CDKs 4 and 6, are at least partially responsible

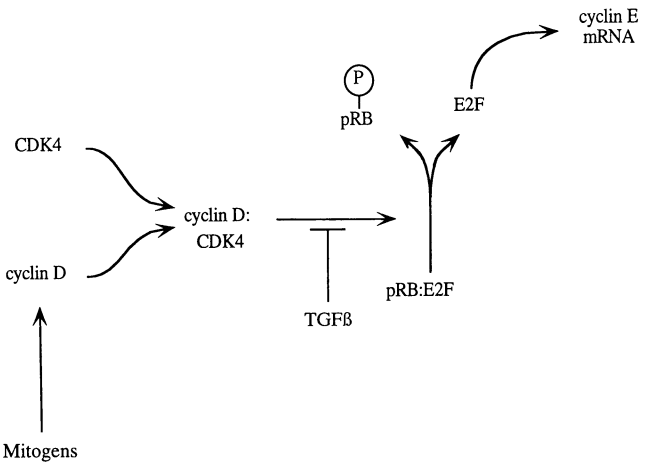

Figure 8. Cyclin D/CDK4,6 regulation of pRb phosphorylation and its inhibition by TGFβ. Further described in text.

for pRb phosphorylation. The resulting functional inactivation of pRb influences the activation of the cyclin E promoter via newly released E2F.

For unknown reasons, the increased accumulation of cyclin E protein normally observed in the G_1 phases of other cell types is not observed in the primary mouse embryo fibroblasts studied here. However, cyclin E message and CDK2 activity do increase in these cells. Therefore, cyclin E mRNA levels closely reflect CDK2 activity. In the wild-type cells, inhibition of CDK4 activity by TGFβ results in an absence of pRb phosphorylation in G_1 and therefore a lack of cyclin E transcriptional induction via E2F. In the mutant cells, the inhibition of CDK4 activity has no negative effect on cell proliferation, because E2F is no longer controlled by the phosphorylation state of pRb. This results in no decrease in cyclin E mRNA levels and CDK2 activity after TGFβ treatment. Although we have obtained evidence that pRb controls cyclin E levels via E2F (Geng *et al.*, 1996), we do not understand how pRb modulates cyclin E/CDK2 activity. Thus, the mechanism underlying the concomitant increases of cyclin E mRNA and cyclin E/CDK2 activity remains obscure.

Regardless of the identity of these TGFβ-induced stimulatory substances, it is clear that only the $RB^{-/-}$ cells can take advantage of their growth-stimulatory effects. In the wild-type cells, any stimulatory effects evoked by these substances are overridden by the TGFβ-imposed block of the cell-cycle clock in mid/late G1. Taken together, these results suggest that loss of pRb function during tumorigenesis may frequently underly the observed nonresponsiveness of transformed cells to inhibition by TGFβ and may actually lead to paradoxical stimulatory effects elicited by signals that are normally growth inhibitory (Fynan and Reiss, 1993). Indeed, TGFβ has been found to increase tumorigenicity of several tumor cell types both in vivo (Arrick *et al.*, 1992; Steiner and Barrack, 1992; Ueki *et al.*, 1992; Arteaga *et al.*, 1993a,b; Chang *et al.*, 1993; Fitzpatrick *et al.*, 1994) and in vitro (Huggett *et al.*, 1991; Arrick *et al.*, 1992; Suardet *et al.*, 1992; Fitzpatrick *et al.*, 1994; Park *et al.*, 1994; Rodeck *et al.*, 1994).

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