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Advances in Brief

Transforming Growth Factor β-induced Activation of Cyclin E-cdk2 Kinase and Down-Regulation of p27^{Kip1} in C3H 10T½ Mouse Fibroblasts[1,2]

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Abstract

Transforming growth factor (TGF-β)-stimulated induction of DNA synthesis is preceded by the activation of cyclin E/cyclin-dependent kinase (cdk)2 kinase in late G_1 in C3H 10T½ mouse fibroblasts. TGF-β has no effect on the steady-state level of cdk4, while having only a modest inductive effect on cyclin D1 expression. TGF-β stimulation does, however, lead to the striking down-regulation of p27^{Kip1} expression during G_1 in a manner consistent with the timing of cyclin E-cdk2 activation. Coimmunoprecipitation analysis reveals that the amount of p27^{Kip1} in complexes with the cdk2 catalytic subunit is drastically reduced at the time in late G_1 when cyclin E-cdk2 activity is maximal. These data indicate that cyclin E-cdk2 is inhibited by p27^{Kip1} in the growth-arrested state and that TGF-β relieves this inhibition by down-regulating the steady-state level of the p27^{Kip1} inhibitor protein, thus reducing the level of inhibitor present in complexes with cdk2.

Introduction

Cell cycle progression through the G_1-S transition has been shown to be controlled by the activity of the cdk[4] family member p33^{cdk2} under the regulation of the cyclin E subunit (1, 2). TGF-β1 inhibits the growth of Mv1Lu epithelial cells in late G_1 by preventing the formation of active cyclin E-cdk2 complexes (3–4). By contrast, TGF-β stimulates growth in established cell lines of mesenchymal origin.

The formation of active cdk2 requires, in addition to the presence of the appropriate cyclin subunit, various posttranslational modifications (5). In addition, recent work has revealed the existence of p27^{Kip1}, which can tightly bind to and inhibit the activity of cyclin E-cdk2 (6–8). This inhibitor, if present, must be somehow inactivated or removed before the cyclin E-cdk2 complex can become active. One possible mode is by the sequestration of the p27^{Kip1} inhibitor by p34^{cdk4}, a cyclin D-dependent cdk family member that becomes activated in mid-G_1 prior to cdk2 activation. In Mv1Lu cells, TGF-β prevents the sequestration of p27^{Kip1} by the cyclin D2-cdk4 holoenzyme via down-regulation of p34^{cdk4} expression (6).

Because TGF-β stimulates growth of C3H 10T½ mouse fibroblasts, we set out to determine whether the cyclin E-cdk2 kinase is responsible for TGF-β-induced transit through late-G_1 in this cell type. These mesenchymal cells can be synchronized by growth factor depletion, making them ideal candidates for studies of the cell cycle. We find that the stimulation of cyclin E-cdk2 kinase activity by TGF-β in this cell type occurs in late-G_1, prior to S phase entry. We were interested in the possibility that p27^{Kip1} might be sequestered from cyclin E-cdk2 by cyclin D1-cdk4 upon growth stimulation by TGF-β. Therefore, we examined the influence of TGF-β on the expression of these proteins by immunoblotting. The lack of a profound induction of either cdk4 or cyclin D1 suggested the possibility that TGF-β might be able to regulate the expression of p27^{Kip1} itself. We find that in C3H 10T½ mouse fibroblasts, induction of cell proliferation responses by TGF-β is accompanied by a striking down-regulation of the p27^{Kip1} protein. Coimmunoprecipitation analysis reveals that the amount of p27^{Kip1} in complexes with the cdk2 catalytic subunit is drastically reduced at the time in late G_1 when cyclin E-cdk2 activity is maximal. These results indicate that TGF-β relieves inhibition of cyclin E-cdk2, at least in part, by down-regulating the steady-state level of the p27^{Kip1} inhibitor, thereby reducing the amount of inhibitor present in complexes with cdk2.

Materials and Methods

Materials. Polyclonal rabbit antibodies directed against the carboxy terminus of human p33^{cdk2} were a gift of Dr. M. Pagano (Mitotix, Inc., Boston, MA; Ref. 9). Polyclonal antibodies to human cyclin E were a gift of Dr. A. Koff (Memorial Sloan-Kettering Cancer Center, New York, NY; Ref. 10). A polyclonal antibody directed against murine cdk4 was a gift of Dr. C. Sherr (St. Judes Childrens Hospital, Memphis, TN; Ref. 11). The rabbit polyclonal antibodies to murine p27^{Kip1} were a gift of Dr. J. M. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 7), as was the recombinant p27^{Kip1} protein. Rabbit polyclonal antibodies directed against human cyclin D1 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Horseradish peroxidase-conjugated sheep anti-rabbit IgG was purchased from Amersham Corp. (Arlington Heights, IL). TGF-β1 was a gift of Dr. K. Leister (Bristol-Myers-Squibb, Syracuse, NY).

Cell Culture. Mouse embryonic fibroblasts (C3H 10T1/2; American Type Culture Collection) were plated in 24-well plates for the analysis of DNA synthesis and in 150-mm dishes for whole cell and nuclear extracts. Cells were grown in DMEM (GIBCO) supplemented with 10% FBS (GIBCO and UBI) and 0.001% gentamicin (GIBCO) as an antimicrobial agent. Growth medium was renewed every 4 to 5 days, and cells reached confluency 8–10 days postplating.

Analysis of DNA Synthesis. [^{3}H]Thymidine (1 μCi of 50 Ci/mmol; ICN Radiochemicals) was introduced for 2-h pulse incorporations to measure DNA synthesis. [^{3}H]Thymidine incorporation into acid-insoluble fraction was assayed as described in Cutry *et al.* (12).

Whole Cell and Nuclear Extracts. Whole cell extracts were prepared according to the method used in Kim *et al.* (13). Nuclear extracts were prepared according to the method of Dignam *et al.* (14).

Immunoprecipitations and Histone H1 Kinase Assay. Immunoprecipitation of cdk2 used 350 μg of whole cell extract and 1 μl of anti-cdk2 antibody and was performed according to the procedure outlined in Kim *et al.* (13). Kinase activity was assayed according to the procedure of Pagano *et al.* (9) using 180 μg of nuclear extracts and 1 μl of anti-cyclin E antisera for the immunoprecipitations. Materials used were as in Kim *et al.* (13).

Western Blotting Analysis. For Western blots, 90–150 μg of whole cell extracts were subjected to SDS-PAGE on 12% gels after boiling 4 min in SDS sample buffer and transferred to nitrocellulose (Bio-Rad Trans-Blot) using a Bio-Rad Trans-blot semidry transfer cell. After transfer, the blots were blocked

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[4] The abbreviations used are: cdk, cyclin-dependent kinase; TGF-β, transforming growth factor β; PAGE, polyacrylamide gel electrophoresis.

in buffer [25 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 5% nonfat dry milk (Carnation) and 0.05% Tween-20 (Sigma Chemical Co.). Following probing with primary and secondary antibody (horseradish peroxidase-conjugated IgG from Amersham), the blots were visualized by Enhanced Chemiluminescence (ECL; Amersham) and recorded with Kodak X-Omat AR film.

Results

Growth Stimulation by TGF-β in C3H 10T½ Mouse Fibroblasts. To achieve synchronous cell populations, postconfluent cells were used 4 days postfeeding with DMEM containing 10% FBS. At 24 h prior to $t = 0$, the cells were further growth factor deprived by changing to a medium with low serum (0.1% FBS). TGF-β1 (5 ng/ml) administered at $t = 0$ stimulates [^{3}H]thymidine incorporation in these cells (Fig. 1). TGF-β stimulates DNA synthesis by 18 h, with peak [^{3}H]thymidine incorporation occurring by 24 h, where it is stimulated 9-fold over the control value. Previous results from our laboratory demonstrate similar kinetics of [^{3}H]thymidine incorporation when the media change is carried out at $t = 0$ immediately prior to growth factor addition (13).

Induction of Cyclin E-cdk2 Kinase Activity by TGF-β. We were interested to learn if cyclin E was responsible for cdk2 activation during late G_1 in C3H 10T½ fibroblasts. We used nuclear extracts in assays of cyclin E-associated histone H1 kinase activity because they should contain exclusively that subpopulation of cyclin E-cdk2 complexes that are localized to the nucleus and whose state of activation is, therefore, representative of its nuclear function. Anti-cyclin E immunoprecipitates of nuclear extracts were subject to the histone H1 kinase assay, and histone bands were excised and counted (Fig. 2). TGF-β (5 ng/ml) induces cyclin E-associated kinase activity by 12 h of stimulation and the activity peaks by 18 h poststimulation. Furthermore, by 24 h the activity of the kinase associated with cyclin E drops significantly. We repeated the experiment of Fig. 2 using whole cell extracts in place of the nuclear extracts and obtained similar results (data not shown). These results demonstrate that the activity of cyclin E-cdk2 is stimulated by TGF-β during late G_1 in C3H 10T½ mouse fibroblasts.

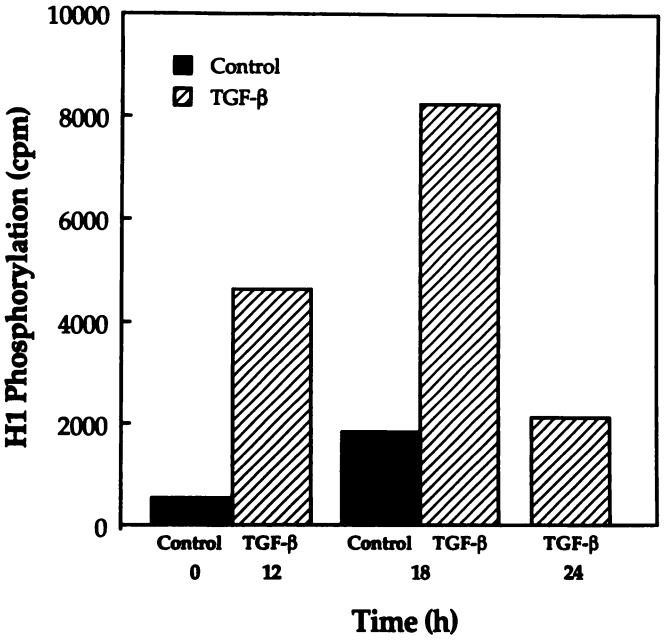

Fig. 2. Induction of cyclin E-associated histone H1 kinase activity by TGF-β in nuclear extracts. Postconfluent, quiescent C3H 10T½ cells were switched to media containing 0.1% FBS at $t = 0$ when TGF-β (5 ng/ml) was added (*TGF-β*) or not added (*Control*), and nuclear extracts were prepared at the indicated times. Immunoprecipitates were prepared using a polyclonal antibody for cyclin E bound to protein A-Sepharose, and the beads were subjected to a kinase assay using [γ-^{32}P]ATP and histone H1 as substrates. Samples were separated on 15% SDS-PAGE gels, and the bands were excised and counted. Counts represent ^{32}P incorporated into histone H1 after subtraction of nonspecific binding of labeled ATP in a control reaction without extract and containing histone.

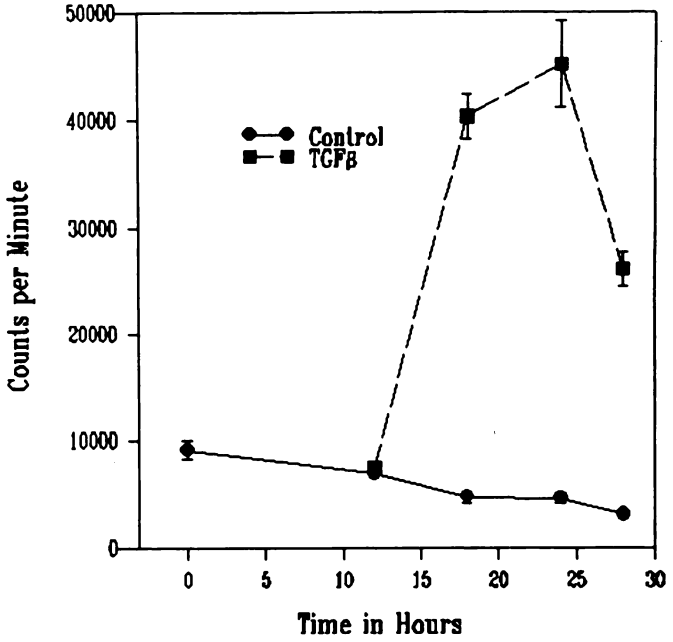

Fig. 1. The effect of TGF-β on DNA synthesis. Postconfluent, quiescent cells were switched to DMEM containing 0.1% FBS 24 h prior to $t = 0$ when TGF-β (5 ng/ml) was added. DNA synthesis was measured by 2-h pulse [^{3}H]thymidine incorporation at the indicated times.

p34^{cdk4} Is Constitutively Expressed in C3H 10T½ Cells. Because the level of p34^{cdk4} protein is down-regulated by TGF-β in epithelial cells, we examined C3H 10T½ cells to see if TGF-β had any effect on its expression when acting as a mitogen in this mesenchymal cell line. Western blotting analysis performed using anti-cdk4 antibody as a probe revealed the presence of a band migrating at M_r 34,000 (Fig. 3*a*). The level of protein expression remains constant, both in control samples and in TGF-β-treated samples harvested at various times. This indicates that in C3H 10T½ mouse fibroblasts, p34^{cdk4} is expressed throughout G_1, even in the absence of stimulation by TGF-β.

TGF-β Regulation of Cyclin D1. It is known that the p27^{Kip1} inhibitor has a greater affinity for the cyclin D-cdk4 holoenzyme than for either subunit by itself (6). Thus, we considered the possibility that D cyclins could be regulated by TGF-β in our cell type, in lieu of regulation of cdk4 by TGF-β. Western blotting analysis using an anti-cyclin D1 polyclonal antibody revealed a band migrating at M_r 36,000 which is present throughout G_1 and into S, regardless of whether the cells were treated with TGF-β (Fig. 3*b*). There is a modest increase in the expression of the protein in this band between 6 and 18 h poststimulation with TGF-β. The control at $t = 18$ appears slightly elevated. This is probably due to the input of fresh media components at $t = 0$, which has a slight stimulatory effect in these cells.

TGF-β Down-Regulates Expression of p27^{Kip1} in C3H 10T½ Cells. Because the level of p34^{cdk4} is not influenced by TGF-β in our cell type and TGF-β does not have a profound effect on cyclin D1 expression, it seemed likely that another mechanism must operate to liberate cyclin E-cdk2 from p27^{Kip1} inhibition, other than sequestration by cyclin D1- cdk4. We first checked to see if and at what times

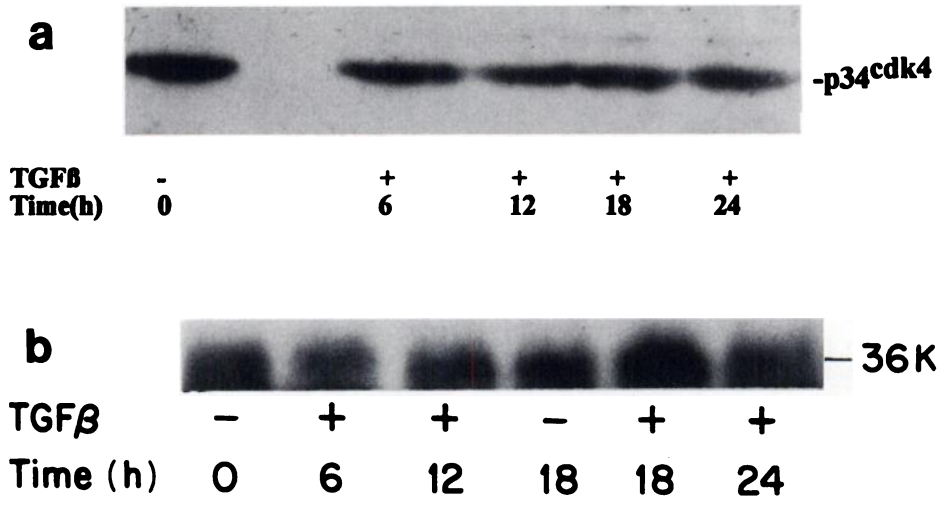

Fig. 3. TGF-β regulation of cdk4 and cyclin D1. Postconfluent, quiescent C3H 10T½ cells were switched to media containing 0.1% FBS 24 h prior to $t = 0$ when TGF-β (5 ng/ml) was added (+) or not added (−). At the indicated times, whole cell extracts were prepared, subjected to SDS-PAGE on 12% gels, and transferred to nitrocellulose. *a*, blots were probed with a polyclonal antibody to cdk4. *b*, blots were probed with a polyclonal antibody to cyclin D1. Detection was by ECL.

this inhibitor is present in cycling or noncycling cells. Control samples contained high levels of a protein migrating at M_r 27,000 (Fig. 4*a*). In lighter exposures, we observed that this protein has the character of a doublet (data not shown). Application of TGF-β results in the diminution of this band by 12 h poststimulation, and this effect is maximal by 24 h. The fact that the sample prepared at 18 h appears slightly more intense than that at 12 h is due to a slight overloading of the 18-h sample, as seen on the stained gel. Recombinant p27 protein was included in the last lane of Fig. 4*a* as a specific marker for $p27^{Kip1}$. The recombinant protein migrates slightly above the position of the M_r 27,000 band, which can be attributed to the presence of a tag containing 4–5 histidine residues attached to the COOH-terminus of the protein.

We next investigated to see if the $p27^{Kip1}$ inhibitor is present in complexes with cdk2, to which it is known to bind tightly. We chose to perform the assay at 18 h poststimulation with TGF-β because this is when the activity of cyclin E-cdk2 is maximal in our cells. Probing of anti-cdk2 immunoprecipitates with antibody to $p27^{Kip1}$ reveals a band migrating at M_r 27,000, again somewhat below the histidine-tagged recombinant protein (Fig. 4*b*). While $p27^{Kip1}$ is clearly present in the control sample at 18 h, application of TGF-β to the cells leads to a sharp reduction in the amount of $p27^{Kip1}$ that coimmunoprecipitates with cdk2. In one lane, antibody specific to cdk2 was omitted from the immunoprecipitation, and in this case no $p27^{Kip1}$ can be detected. The results here demonstrate that mitogenic stimulation by TGF-β results in the down-regulation of $p27^{Kip1}$ protein expression in C3H 10T½ mouse fibroblasts and thereby reduces the level of this inhibitor present in complexes with cdk2.

Discussion

TGF-β Regulation of Cyclin E-cdk2 Activity and Cyclin D-cdk4 Expression. The addition of TGF-β to C3H 10T½ cells stimulates cyclin E-associated histone H1 kinase activity beginning in mid-G_1 phase, prior to the onset of DNA synthesis. This activity peaks in late G_1 and drops significantly by mid-S phase. Western blots of $p34^{cdk4}$ indicate that, in C3H 10T½ cells, TGF-β has no effect on the expression of this protein. This result is in contrast to that found in epithelial cells, where TGF-β exerts growth-inhibitory effects and leads to the down-regulation of cdk4 expression. Western blotting analysis for cyclin D1 reveals a M_r 36,000 band, which corresponds to the known molecular weight of cyclin D1 and shows only a modest induction when cells are stimulated with TGF-β.

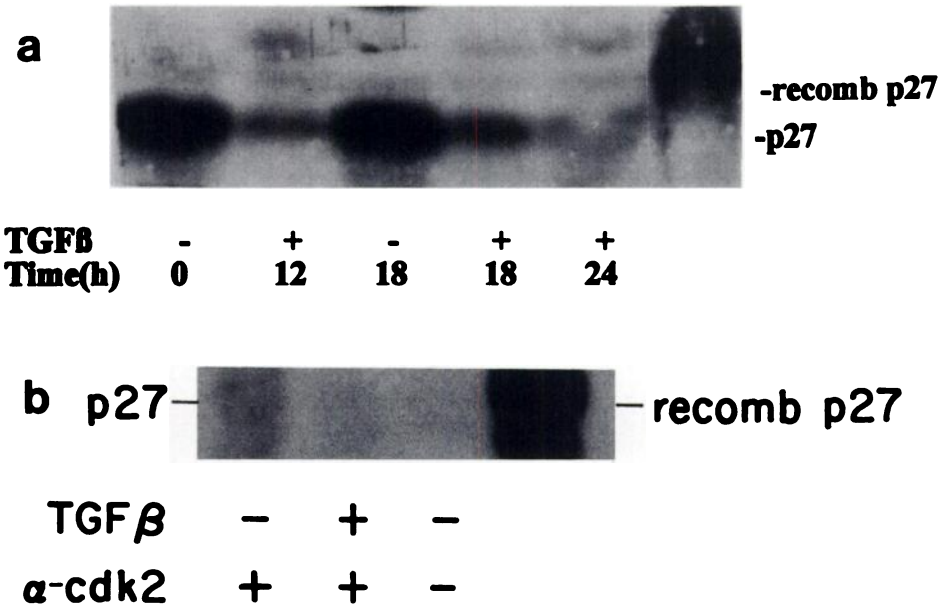

Fig. 4. TGF-β down-regulates expression of $p27^{Kip1}$ in C3H 10T½ cells. Postconfluent, quiescent C3H 10T½ cells were switched to media containing 0.1% FBS 24 h prior to $t = 0$ when TGF-β (5 ng/ml) was added (+) or not added (−). *a*, whole-cell extracts prepared at the indicated times were subjected to SDS-PAGE on 12% gels. After transfer to nitrocellulose, the blots were probed with polyclonal antibody to $p27^{Kip1}$. Detection was by ECL. *b*, whole-cell extracts prepared 18 h post-addition of TGF-β were first immunoprecipitated with antibody to cdk2 bound to protein A-Sepharose prior to SDS-PAGE and probing with anti-$p27^{Kip1}$ as in (*a*). *Recomb p27*, recombinant $p27^{Kip1}$ protein with COOH-terminal tag of 4–5 histidine residues.

Down-Regulation of $p27^{Kip1}$ by TGF-β. Given that cdk4 expression is constitutive, our finding that cyclin D1 is not strongly up-regulated leads to the question as to how inhibition of cyclin E-cdk2 by the $p27^{Kip1}$ inhibitor can be alleviated upon TGF-β-induced activation of this kinase. While $p27^{Kip1}$ is present at high levels in unstimulated control samples, stimulation of cells with TGF-β leads to the diminution of $p27^{Kip1}$ by 12 h, and it nearly disappears by 24 h poststimulation. This demonstrates that TGF-β stimulation of cell cycle progression in C3H 10T½ fibroblasts leads to down-regulation of $p27^{Kip1}$. We also observed down-regulation of $p27^{Kip1}$ when our cells are stimulated with epidermal growth factor (data not shown). Coimmunoprecipitation analysis reveals that the amount of $p27^{Kip1}$ in complexes with the cdk2 catalytic subunit is drastically reduced at the time in late G_1 when cyclin E-cdk2 activity is maximal. Thus, we find that stimulation with TGF-β leads to the down-regulation of $p27^{Kip1}$, resulting in its disappearance from complexes with cdk2. Such a finding with TGF-β is in sharp contrast to the results of Polyak *et al.* (6), who found that, in Mv1Lu cells, $p27^{Kip1}$ is present at the same level in untreated as in TGF-β treated cells, albeit in a sequestered form. This difference could be related to cell type. However, a similar result to that of Polyak *et al.* (6) is obtained in serum-stimulated Swiss 3T3 mouse fibroblasts (15). On the other hand, $p27^{Kip1}$ down-regulation could represent an alternative pathway for TGF-β induction of cyclin E-cdk2 kinase activity in C3H 10T½ mouse fibroblasts. Factors such as cyclin D1 may be induced to greater or lesser degrees, depending on the length of time that cells remain in a quiescent state prior to growth factor stimulation. Therefore, the down-regulation of $p27^{Kip1}$ may function in place of the sequestration of $p27^{Kip1}$ by cyclin D-cdk4 or to augment it, thus ensuring activation of cyclin E-cdk2 in late G_1, even in the absence of a profound accumulation of cyclin D-cdk4.

Potential Regulatory Mechanisms in TGF-β-induced $p27^{Kip1}$ Down-Regulation. Several possibilities exist concerning the mechanism of down-regulation of $p27^{Kip1}$ in C3H 10T½ mouse fibroblasts: (*a*) TGF-β could be inducing proteolytic enzymes or causing $p27^{Kip1}$ to be ubiquinated, leading to degradation of the p27 protein itself; (*b*) TGF-β could be having an effect on *Kip1* message stability, or on the translation of the message itself. TGF-β could potentially induce factors that bind to the single-stranded RNA message, thus inhibiting its translation from polyribosomes; and (*c*) TGF-β could be affecting the transcription of the *Kip1* gene.

In summary, TGF-β-stimulated entry of C3H 10T½ mouse fibroblasts into S phase follows the induction of cyclin E-cdk2 kinase activity, and this activation is correlated with the down-regulation of the inhibitor $p27^{Kip1}$ and a loss of $p27^{Kip1}$ from complexes with cdk2. These results demonstrate that, in C3H 10T½ mouse fibroblasts, the down-regulation of $p27^{Kip1}$ expression represents a unique pathway leading to cyclin E-cdk2 activation different from that involving sequestration of the inhibitor by a cyclin D-cdk4 holoenzyme.

Acknowledgments

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