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Inhibition of G1 Phase Cyclin Dependent Kinases by Transforming Growth Factor β1

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Abstract Transforming growth factor β1 (TGFβ1) inhibits epithelial cell proliferation late in the G1 phase of the cell cycle. We examined the effect of TGFβ1 on known late G1 cell cycle regulators in an attempt to determine the molecular mechanism of growth inhibition by this physiological inhibitor. The results demonstrate that TGFβ1 inhibits the late G1 and S phase specific histone H1 kinase activity of $p33^{cdk2}$. This inhibition is not due to TGFβ1's effect on $p33^{cdk2}$ synthesis, but rather due to its negative effects on the late G1 phosphorylation of $p33^{cdk2}$. It is also shown that TGFβ1 inhibits both late G1 cyclin A and cyclin E associated histone H1 kinase activities. The inhibitor has no effects on the synthesis of cyclin E but is shown to inhibit the synthesis of cyclin A protein in a cell cycle dependent manner. If TGFβ1 is added to cells which have progressed further than 8 hours into G1, then it is without inhibitory effect on cyclin A synthesis. These effects of TGFβ1 on late G1 cell cycle regulators correlate well with its inhibitory effects on cellular growth and suggest that these G1 cyclin dependent kinases might serve as targets for TGFβ1-mediated growth arrest.

Key words: epithelial cell proliferation, histone H1 kinase, cyclin E, cyclin A, fetal bovine serum, epidermal growth factor

Transforming growth factor β1 (TGFβ1) plays an important role in the regulation of cell proliferation, differentiation, and extracellular matrix production [for reviews see Massague, 1990; Moses et al., 1990]. A potent growth inhibitor for cells of non-mesenchymal origin, TGFβ1 is postulated to maintain homeostasis by suppressing uncontrolled cell proliferation. Loss of a growth inhibitory response to TGFβ1 has been implicated in cellular transformation [Shipley et al., 1986], atherosclerosis [Battegay et al., 1990], and lymphoproliferative diseases [Shull et al., 1992; Kulkarni et al., 1993].

Three cell surface receptors (types I, II, and III) for TGFβ have been characterized as mediating the action of TGFβ in most cell types [Wang et al., 1991; Lin et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Franzen et al., 1993]. Both the type I and II receptors are predicted to encode for transmembrane serine-threonine kinases [Lin et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Franzen et al., 1993], and signaling is postulated to require the formation of a heteromeric complex between the various TGFβ receptor species [Wrana et al., 1992; Lopez-Casillas et al., 1993]. It has also been proposed that each receptor type is responsible for mediating the various activities of TGFβ [Boyd and Massague, 1989; Howe et al., 1990; Chen et al., 1993]. The type I receptor is responsible for the induction of extracellular matrix type genes, while the type II receptor is postulated to mediate TGFβ's effects on the activity of the retinoblastoma gene product, Rb, and cellular proliferation [Chen et al., 1993].

The exact mechanism by which TGFβ1 inhibits its cellular proliferation remains unclear. Its inhibitory activity, however, has been shown to be cell cycle dependent [Howe et al., 1991; Thoresen et al., 1992]. When quiescent cultures of epithelial cells are stimulated to re-enter the cell cycle they are sensitive to the growth inhibitory effects of TGFβ1 during the first 9 to 10 h of the 12 h prereplicative G1 phase. Once these cultures have committed to DNA synthesis they become insensitive to growth inhibition by TGFβ1. It has also been shown that inhibition by TGFβ1 occurs at a distinct cell cycle point

Abbreviations used: cdk(s), cyclin dependent kinase(s); EGF, epidermal growth factor; FBS, fetal bovine serum; TGFβ1, transforming growth factor β1.

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late in G1, approximately 2–3 h prior to S phase [Howe et al., 1991]. This TGFβ1 arrest point late in G1 correlates temporally with the previously described R and/or W arrest points [Pledger et al., 1978; Campisi et al., 1982].

TGFβ1 has also been shown to affect the activity of several cellular products which play key roles as cell cycle regulators [Laiho et al., 1990; Pietenpol et al., 1990; Howe et al., 1991; Koff et al., 1993]. Prominent among these are the retinoblastoma gene product, Rb, and two members of the cyclin dependent kinases, $p34^{cdc2}$ and $p33^{cdk2}$ kinases. The activity of these kinases is regulated by a cell cycle dependent association with a cyclin regulatory subunit, as well as by specific phosphorylations [for review see Sherr, 1993]. While $p34^{cdc2}$ has been demonstrated to regulate both the G1/S and G2/M phase transitions in yeast [Hartwell, 1974; Nurse and Bissett, 1981], $p33^{cdk2}$ is more restricted to G1/S [Tsai et al., 1991; Elledge et al., 1992]. It is fairly well accepted that the cyclin B-$p34^{cdc2}$ complex represents the mitotic form of the kinase; however, determining all the possible combinatorial cell cycle dependent associations of the various catalytic subunits with distinct cyclins will require further investigation. For instance, it is postulated that cyclin E-$p33^{cdk2}$ governs the G1/S phase transition, yet cyclin E can also complex with $p34^{cdc2}$ [Dulic et al., 1992; Koff et al., 1992]. Cyclin A has been shown to complex both with $p33^{cdk2}$ and $p34^{cdc2}$, and cyclin D1, a cyclin which is induced earlier than cyclin E, may not associate with either $p33^{cdk2}$ or $p34^{cdc2}$, but with $p36^{cdk4}$ [for review see Sherr, 1993]. Clearly, more work is required before categorically ascribing the regulation of a particular cell cycle transition to a distinct or individual cyclin dependent kinase complex.

We have previously shown that TGFβ1 inhibits the phosphorylation and activation of $p34^{cdc2}$ kinase in a cell cycle dependent manner [Howe et al., 1991]. This inhibitory effect on $p34^{cdc2}$ correlates well with TGFβ's ability to maintain Rb in a hypophosphorylated state and to suppress cellular proliferation [Laiho et al., 1990; Zentella et al., 1991]. More recently, others have shown that in TGFβ treated cells formation of a stable cyclin E-$p33^{cdk2}$ complex is impaired [Koff et al., 1993], thereby rendering kinase activity inactive. It was postulated that cyclin E-$p33^{cdk2}$ complexes were forming, but that TGFβ inhibited the ensuing stabilizing phosphorylation of the $p33^{cdk2}$ subunit [Koff et al., 1993]. In this manuscript, we examine the effect of TGFβ1 on cyclin A and cyclin E dependent kinase activity in mink lung epithelial cells. We show that while TGFβ1 inhibits both cyclin A and cyclin E dependent histone kinase activity in a cell cycle specific manner, it specifically blocks the accumulation of cyclin A protein.

METHODS

Cell Culture

Mink lung epithelial cells (ATCC CCL64) were cultured in DMEM supplemented with 10% calf serum. After 3 to 5 days of growth at 37°C, the cells were serum starved for 48 h. These density-arrested, quiescent cultures were used for subsequent experimentation.

Immunoprecipitations and Immunoblotting

Quiescent cells were stimulated with 10% fetal bovine serum (FBS) and epidermal growth factor (EGF) (20 ng/ml) in the presence or absence of TGFβ1 (5 ng/ml). At the indicated times, cell lysates were prepared and immunoprecipitated by using either anti-$p33^{cdk2}$, anti-cyclin E (both purchased from U.B.I.), anti-cyclin A (generated against a Gst fusion protein and provided by Dr. Robert Abraham, Mayo Clinic), or anti-$p34^{cdc2}$ (C-terminus antisera provided by Dr. E.B. Leof, Mayo Clinic). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography. For immunoblotting, cell lysates were prepared by extraction with 50 mM Tris-HCl (pH 8.0), containing 150 mM NaCl, 2 mM $MgCl_2$ and 1% NP40. Equal amounts (~125 μg) of proteins were electrophoresed on polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with the various antibodies, followed by ^{125}I-protein A. For the phosphorylation analysis of Figure 1C, cells were stimulated ± TGFβ1 in the presence of 250 μCi/ml of $^{32}PO_4^{3-}$ in phosphate free medium, and at the times indicated immunoprecipitations were performed as described above.

Protein Kinase Assays

At the indicated times after stimulation, cells were lysed in kinase lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM NaF, 0.1 mM NaVanadate, 50 μg/ml PMSF, 25 μg/ml of aprotinin, and 1 μg/ml of leupeptin) and immunoprecipitated as described above. Immunoprecipitates were washed twice

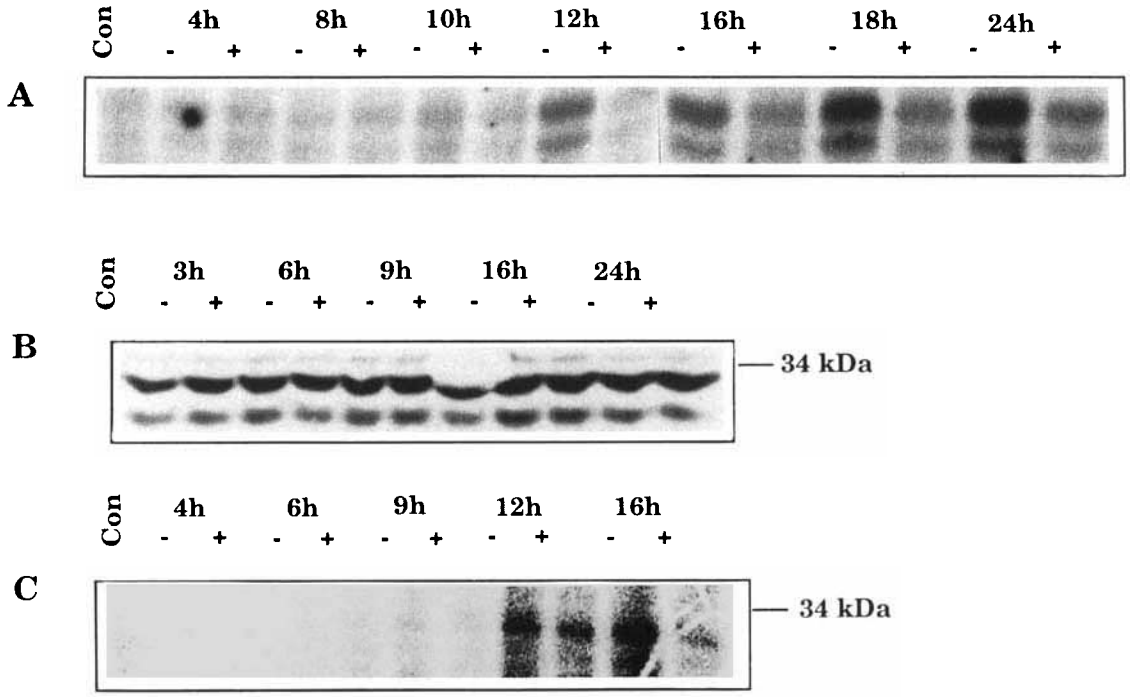

Fig. 1. Effect of TGFβ1 on $p33^{cdk2}$ kinase. **A:** Quiescent density arrested CCL64 cells were stimulated with 10% FBS and 20 ng/ml EGF in the absence (−) or presence (+) of TGFβ1 (5 ng/ml). At the indicated times, cells were harvested and immunoprecipitated with 2.5 μg/ml anti-$p33^{cdk2}$ antibody, and in vitro histone H1 activity in the immunocomplexes determined as described in Materials and Methods. **B:** CCL64 cells were stimulated as above, and at the indicated times cell lysates (equal amounts of protein ~125 μg) were subjected to gel electrophoresis, and immunoblotting was performed using anti-$p33^{cdk2}$ antibodies and ^{125}I-protein A. **C:** Quiescent CCL64 cells were stimulated as in panel A, except that phosphate-free media containing 250 μCi/ml of $^{32}PO_4^{3-}$ was employed. At the indicated times after stimulation, immunoprecipitations with anti-$p33^{cdk2}$ were performed as described.

with kinase lysis buffer and once in kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol), and then suspended in 50 μl of kinase buffer containing 1 μM ATP, 5 μCi of [^{32}Pγ]-ATP, and 0.83 mg/ml of histone H1 (Boehringer-Mannheim). Reactions were incubated for 5 min at 30°C, stopped with 50 μl of sample buffer, and analyzed by SDS-PAGE.

RESULTS

Effects of TGFβ1 on Cyclin Dependent Kinase Activity

Numerous studies have implicated a role for $p33^{cdk2}$ during the G1/S phase transition of eukaryotic cells [for review see Sherr, 1993]. Its kinase activity is first evident during the middle of G1, as is its association with cyclin E [Dulic et al., 1992; Koff et al., 1992], while its association with cyclin A is thought to be formed at the onset of S phase [Tsai et al., 1991; Elledge et al., 1992]. Thus, it is proposed that $p33^{cdk2}$-cyclin E may function to control progression through G1, and $p33^{cdk2}$-cyclin A may function in regulating entry and the start of S phase. Since TGFβ1 arrests cell cycle progression in late G1 [Howe et al., 1991; Thoresen et al., 1992], a transition that the $p33^{cdk2}$-cyclin complex is postulated to regulate, we wished to determine whether TGFβ1 might mediate its inhibitory response through the $p33^{cdk2}$-cyclin complex. To study the profile of $p33^{cdk2}$ histone H1 kinase activity during the cell cycle, density arrested mink lung epithelial cells (Mv1Lu; CCL64) were serum starved for 48 h. These quiescent, G0-arrested cells, were then stimulated to re-enter the cell cycle by the addition of FBS and EGF, and subsequently harvested at successive time intervals up to 24 h and subjected to immunoprecipitation and in vitro histone H1 kinase assays. As shown in Figure 1A, when $p33^{cdk2}$ immunoprecipitates were analyzed, kinase activity became apparent in lysates prepared from cells 12 h after stimulation and reached maximal levels at 24 h. Addition of TGFβ1 at the time of stimulation inhibited the cell cycle dependent activation of $p33^{cdk2}$ kinase activity (Fig. 1A). TGFβ1 inhibited $p33^{cdk2}$ kinase activity by 30–40% between 18 and 24 h after serum stimulation. To get an

insight into the mechanism of regulation of $p33^{cdk2}$ kinase activity by TGFβ1, immunoblot and immunoprecipitation analyses were performed. Immunoblot analysis reveals that $p33^{cdk2}$ levels are relatively constant throughout the cell cycle and do not fluctuate. Furthermore, addition of TGFβ1 to these restimulated, quiescent cultures does not have any noticeable effect on $p33^{cdk2}$ levels (Fig. 1B). Since $p33^{cdk2}$ is thought to be regulated by phosphorylation in a cell cycle dependent manner [Gu et al., 1992], we next examined the effects of TGFβ1 on the phosphorylation of $p33^{cdk2}$. As shown in Figure 1C, phosphorylation of $p33^{cdk2}$ begins approximately 9 h after growth factor stimulation and continues to increase until 16 h. Addition of TGFβ1 inhibited $p33^{cdk2}$ phosphorylation approximately 50–70%.

We next examined the effect of TGFβ1 on cyclin E and cyclin A dependent kinase activity by measuring histone H1 kinase activity in anti-cyclin E and A immunoprecipitates. The results (Fig. 2) show that cyclin E (Fig. 2A) associated kinase activity appears about 9 h after stimulation, is maximal at 12 h, and goes down by 16 h. Addition of TGFβ1 at the time of restimulation inhibits this cyclin E dependent kinase activity. Cyclin A associated kinase activity (Fig. 2B) is also regulated in a cell cycle dependent manner. It becomes evident at about 9 h and peaks at approximately 16 h after growth factor stimulation. Similar to cyclin E associated activity, it is also sensitive to negative regulation by TGFβ1 (75% inhibition at 16 h). When these kinetic data are replotted (Fig. 2C), the results show that cyclin E associated kinase activity precedes cyclin A dependent kinase activity. Cyclin E activity is maximal during mid and late G1, while that of cyclin A is maximal during S.

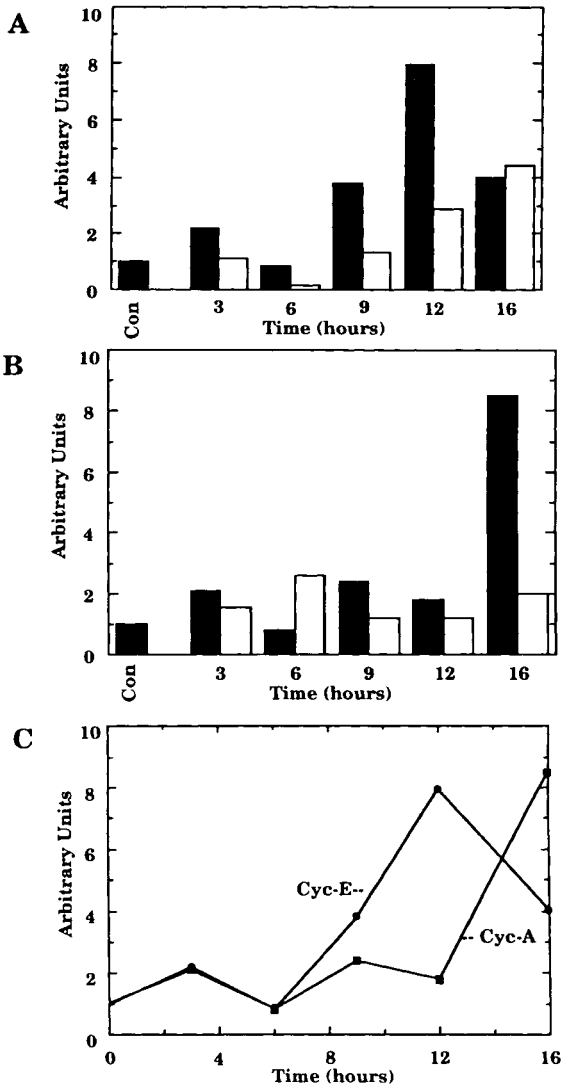

Fig. 2. Effect of TGFβ1 on cyclin associated histone kinase activity. Quiescent CCL64 cells were stimulated with FBS and EGF in the absence (−) or presence (+) of TGFβ1 (5 ng/ml). At the indicated times, cell lysates were subjected to immunoprecipitation using either anti-cyclin E (**A**), or cyclin A (**B**), and in vitro histone H1 phosphorylation was carried out as described in Materials and Methods. The bands corresponding to the phosphorylated histone H1 were quantitated by Phospholmager and results expressed as arbitrary units. Open bars represent the presence of TGFβ1. **C:** The histone kinase activity of cyclin E and cyclin A at various times (obtained from A and B) is plotted as a function of time.

Effects of TGFβ on Cyclin A and Cyclin E Protein

Cyclin A levels fluctuated in a cell cycle dependent manner. Low levels of cyclin A protein are present in quiescent cells but as the cells approach S phase cyclin A levels become maximal. At 16 h after growth factor addition there is a 3-fold induction in cyclin A level as compared to control, quiescent cells. TGFβ1 significantly reduced cyclin A protein levels by 50% measured at 16 h post growth factor stimulation (Fig. 3A,B). In contrast to cyclin A, cyclin E protein levels are not affected in TGFβ1 treated cells (data not shown; Koff et al. [1993]).

We have previously shown that TGFβ1 reversibly inhibits CCL64 cells late in the G1 phase of the cell cycle, and that it could be added to the cells late in G1 and inhibit entry into S phase [Howe et al., 1991]. Its presence was therefore not required during the first half of G1 to maintain cells out of S phase. We chose to analyze the kinetics of the inhibitory effects of TGFβ1 on the expression of cyclin A to determine whether they paralleled those observed for growth inhibition. Since the results of Figure 3A and B dem-

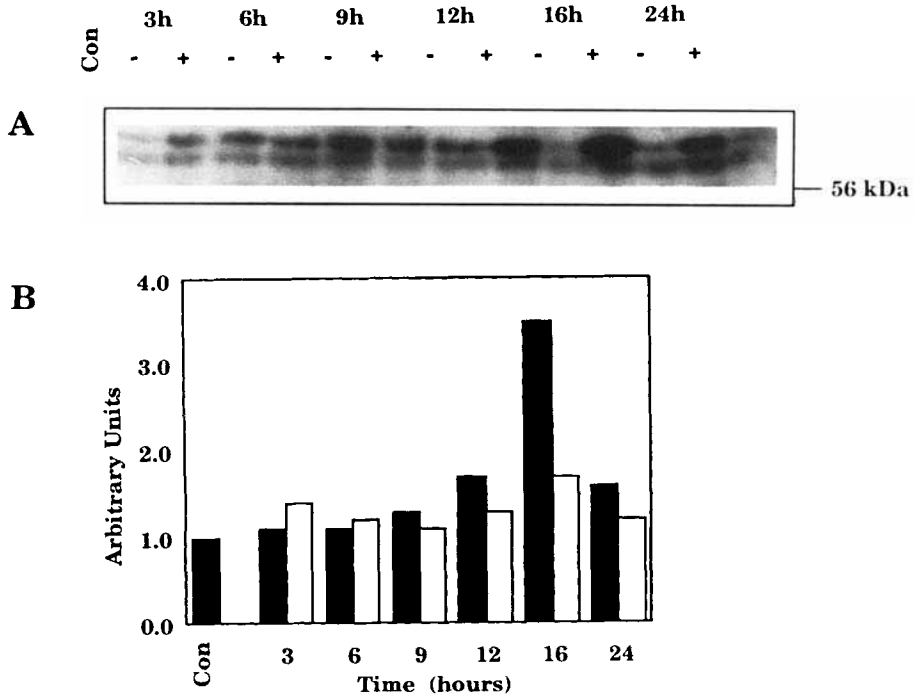

Fig. 3. Effect of TGFβ1 on cyclin A protein. Quiescent, density-arrested CCL64 cells were stimulated with FBS and EGF in the absence (−) or presence (+) of 5 ng/ml TGFβ1. At the indicated times, cell lysates (equal amounts of protein ~125 μg) were subjected to gel electrophoresis and immunoblotting was performed with anti-cyclin A (**A**) antibodies and ^{125}I-protein A. **B:** Band corresponding to cyclin A was quantitated by PhosphoImager and plotted as a function of time. Open bars represent the presence of TGFβ1.

onstrate that the synthesis of cyclin A is maximal at 16 h, we chose this time point to analyze TGFβ1 effects on cyclin A synthesis. Quiescent, G0-arrested CCL64 cells were stimulated with FBS and EGF, and at the indicated times, TGFβ1 was added (Fig. 4). After 16 h, the cells were harvested and anti-cyclin A immunoblots were performed. The results demonstrate that TGFβ1 has to be added within the first 8 h after exit from G0 for it to exert its inhibitory effect on cyclin A synthesis. If TGFβ1 is added following the first 8 h of G1, then it is without inhibitory effect (Fig. 4).

Effects of TGFβ1 on Cyclin Dependent Kinases in *ras*-Transfected CCL64 Cells

Our most recent results implicate a role of $p21^{ras}$ in TGFβ1 signaling by demonstrating 1) that CCL64 cells transfected with oncogenic forms of $p21^{ras}$ loose their inhibitory response to TGFβ1 [Longstreet et al., 1992], and 2) that microinjection of oncogenic $p21^{ras}$ into late G1 TGFβ1-blocked CCL64 cells can overcome growth inhibition and drive cells into S phase [Howe et al., 1993]. It was of interest, therefore, to study the regulation of $p33^{cdk2}$ and the various cyclin associated kinase activities by TGFβ1 in these *ras* transfected CCL 64 cells, CCL64-R1 cells. Since CCL64-R1 cells could not be quiesced, we analyzed the various cyclin associated kinases in rapidly growing cells. As shown in Figure 5A, TGFβ1 inhibits the histone H1 kinase activity of $p33^{cdk2}$, $p34^{cdc2}$, cyclin A, and cyclin E in rapidly growing CCL64 cells, similarly to that observed in quiescent restimulated cells. In the *ras*-transfected CCL64-R1 clone, however, such a negative regulation of kinase activity by TGFβ1 is not seen. Both $p34^{cdc2}$ and $p33^{cdk2}$, as well as cyclin A and E associated kinase activities are insensitive to regulation by TGFβ1. Further, in Figure 5B, it is shown that the inhibition of cyclin A synthesis by TGFβ1 is not observed in the *ras*-transfected cells as it is in the parental CCL64 cell line.

DISCUSSION

Eukaryotic cell proliferation is primarily regulated by molecular events which occur during the G1 phase of the cell cycle. Traverse of G1 and entry into S phase commits a cell to com-

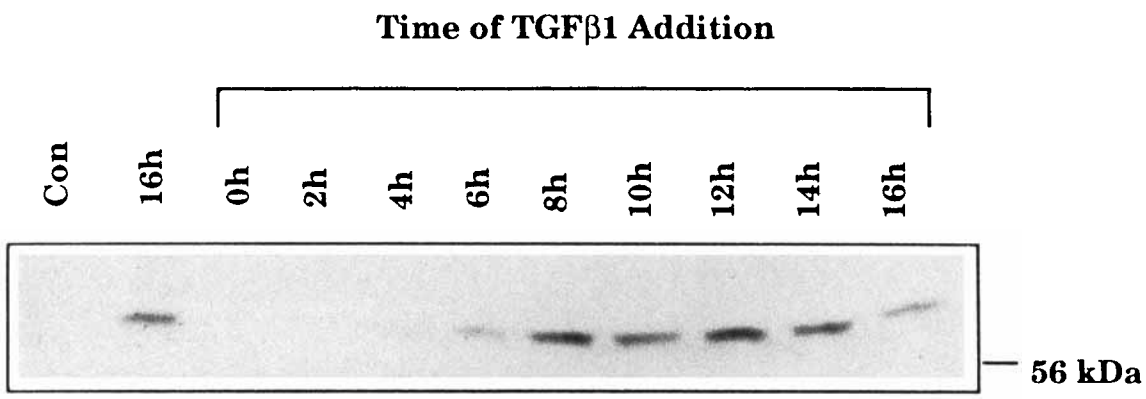

Fig. 4. Time course of TGFβ1's effect on cyclin A protein. Quiescent, density-arrested CCL64 cells were stimulated with FBS and EGF, and at the indicated times post-stimulation TGFβ1 was added. All cultures were harvested after a total incubation period of 16 h and equal amount of cell lysate (~125 μg) was immunoblotted using anti-cyclin A antibodies and ^{125}I-protein A.

plete the remainder of its cell division cycle. One of the key mediators for G1/S traverse is p33^{cdk2} [Tsai et al., 1991, 1993]. Human p33^{cdk2} can bind both cyclin A and E, as well as be found in complexes with the Rb-related p107 and with the E2F transcription factor. The periodicity of cyclin A and E synthesis during G1 and the role of p107 and E2F during G1 further implicate p33^{cdk2} as a G1 regulator [for review see Sherr, 1993]. Since TGFβ1 inhibits cell cycle progression at a specific arrest point also in G1 [Howe et al., 1991], we wished to determine whether p33^{cdk2} might serve as a target for TGFβ1 mediated growth inhibition. We show in this report that the kinase activity of p33^{cdk2} increases as the cells exit out of quiescence and traverse G1. At the G1/S phase border and in S phase p33^{cdk2} kinase activity is maximal. TGFβ1 inhibits this cell cycle dependent increase in p33^{cdk2} kinase activity (Fig. 1A). This inhibition is not due to negative effects of the inhibitor on p33^{cdk2} protein levels, since p33^{cdk2} is constant throughout the cell cycle in the absence or presence of TGFβ1 (Fig. 1B). These results on p33^{cdk2} demonstrating its activation during late G1/S, its constant level during the cell cycle, and regulation of its kinase activity by TGFβ1 are identical to the results we previously showed for p34^{cdc2} [Howe et al., 1991].

We further show that p33^{cdk2} becomes increasingly phosphorylated at the onset of S phase and during DNA synthesis, and that TGFβ1 inhibits this phosphorylation (Fig. 1C). Phosphorylation of specific threonine and tyrosine residues on p33^{cdk2} is thought to occur subsequent to cyclin binding and promotes complex stability [Gu et al., 1992]. It has previously been shown that TGFβ1 inhibits the activation of cyclin E-p33^{cdk2} complex, not by inhibiting complex formation but presumably through its inhibition of the phosphorylating event on p33^{cdk2} required for stabilization [Koff et al., 1993].

We next wished to examine whether TGFβ1 inhibition of p33^{cdk2} kinase activity was specific for either the cyclin A and/or cyclin E complex. We were unable, however, to show that TGFβ1 preferentially inhibited either cyclin A or E associated kinase activity since it was able to inhibit both activities. Our results do show that cyclin E associated kinase activity precedes that of cyclin A by about 4 h (Fig. 2C), confirming studies in WI38 fibroblasts where E synthesis precedes A by about 5 to 6 h [Tsai et al., 1993]. We attempted to immunodeplete cyclin A or E from cell lysates to address the specificity of TGFβ1 for either one of the cyclin associated kinase activities but were unsuccessful in our efforts. In this regard, it has been shown in vitro that TGFβ1 can inhibit not only stable complex formation between cyclin E and p33^{cdk2} and p34^{cdc2} but also between cyclin A and both these catalytic subunits. These authors postulate that TGFβ1 may be a general inhibitor of cyclin-cdk interactions during G1 [Koff et al., 1993].

We further demonstrate that the inhibitory effects of TGFβ1 are specific to the synthesis of cyclin A (Fig. 3) and have no effects on the synthesis of cyclin E (data not shown, Koff et al. [1993]). Negative effects of TGFβ1 on the synthesis of cyclin A protein have been observed in CCL39 hamster lung fibroblasts cells where it was determined to be due to a transcriptional

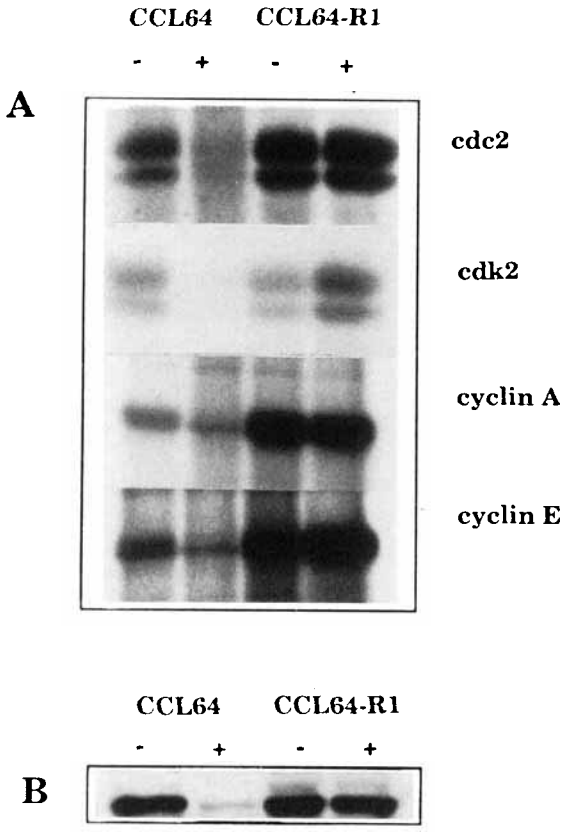

Fig. 5. Differential regulation of the cyclin dependent kinases in CCL64 and CCL64-R1 cells by TGFβ1. **A:** Rapidly growing (60% confluent) CCL64 and CCL64-R1 cells were treated in the absence (−) or presence (+) of 5 ng/ml TGFβ1 for 24 h at 37°C. Cells were harvested and immunoprecipitations performed using antibodies to $p34^{cdc2}$, $p33^{cdk2}$, cyclin E, or cyclin A, and in vitro histone H1 kinase activity in the immunocomplexes was determined as described in Materials and Methods. **B:** Cell cultures were treated as described in panel A in the absence (−) or presence (+) of TGFβ1 and cell lysates (~125 μg) were subjected to immunoblot analysis using anti-cyclin A antibodies and ^{125}I-protein A.

inhibition [Barlat et al., 1993]. Perhaps, the cyclin E-cdk2 complex is involved in the regulation of cyclin A synthesis; the kinetics shown here would support such a hypothesis, and that the effects of TGFβ1 on cyclin A synthesis are secondary to its direct effects on a cyclin E-cdk transcriptional complex. We also show that in order for TGFβ1 to inhibit the synthesis of cyclin A it must be added within the first 8 h of G1. If it is added later than 8 h after the cells have exited G0 then TGFβ1 is without inhibitory effect. This window of sensitivity to the inhibitory effects of TGFβ1 on cyclin A synthesis during G1 is similar to the effects we previously observed on cell growth [Howe et al., 1991]. If cells progress beyond the TGFβ1 arrest point in late G1, then they become refractile to its growth inhibitory properties.

As a control for these studies on the effects of TGFβ1 on cell cycle regulators we have used a *ras*-transfected epithelial cell line (CCL64-R1) which overexpresses an activated Ha-*ras* protein [Longstreet et al., 1992; Howe et al., 1993]. The CCL64-R1 clone contains normal amounts of the known TGFβ receptors but is insensitive to growth arrest by TGFβ1. In this clone we demonstrate that negative regulation of the cyclin dependent kinases by TGFβ1 is uncoupled by overexpression of oncogenic $p21^{ras}$. TGFβ1 is unable to inhibit the kinase activity of either $p34^{cdc2}$, $p33^{cdk2}$, cyclin A, or cyclin E in this clone (Fig. 5A). These results are similar to those which demonstrate that TGFβ1 regulation of growth inhibition and key cell cycle mediators in CCL39 fibroblasts is uncoupled by transformation with polyoma virus [Barlat et al., 1993].

These data indicate that G1 cyclin dependent kinases are targets for the growth inhibitory effects of TGFβ1. Whether this effect is specific for one cdk-cyclin complex which regulates other downstream cdk-cyclin complexes or is general for all G1 cyclin dependent kinases is not clear. Recent results would favor the latter hypothesis since the activation in vitro of either cyclin E and cyclin A associated kinase is inhibited by a TGFβ1-treated cellular lysate [Koff et al., 1993]. Alternatively, TGFβ's inhibitory target(s) could be an upstream regulator of the G1 cyclin dependent kinase and the observed effects could be secondary to that regulation. Ewen et al. [1993] have recently shown that TGFβ1 inhibits the synthesis of cdk4, which is an upstream kinase and appears earlier than cdk2 in cell cycle. Overexpression of cdk4 and not cdk2 overcomes growth inhibition by TGFβ1. It is clear, however, that growth inhibitory factors such as TGFβ1 are functioning by interfering with the progression of normal positive proliferative signals.

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