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Transforming Growth Factor β1 Increases the Stability of p21/WAF1/CIP1 Protein and Inhibits CDK2 Kinase Activity in Human Colon Carcinoma FET Cells[1]

JianGen Gong,[2] Sudhakar Ammanamanchi,[2] Tien C. Ko, and Michael G. Brattain[3]

Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263 [S. A., M. G. B.]; Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43614 [J. G.]; and Department of Surgery, The University of Texas Medical Branch, Galveston, Texas 77555 [T. C. K.]

ABSTRACT

We examined transforming growth factor-β1 (TGF-β1) effects on cell cycle progression of human colon carcinoma FET cells. TGF-β1 inhibited DNA synthesis and cyclin-dependent kinase (CDK) activity after release from growth arrest in association with induction of the p21 CDK inhibitor, whereas cyclins, CDKs, and p27 protein levels remained relatively unchanged. The decrease in CDK2 kinase activity was the result of increased p21 association with cyclin A-CDK2 and cyclin E-CDK2. TGF-β1 treatment in late G_1 showed reduced induction of p21 protein levels in association with increased DNA synthesis. Consequently, p21 induction in early G_1 is critical for TGF-β1 inhibition of CDK2 kinase activity. Although TGF-β1 treatments in late G_1 failed to induce p21 protein, p21 mRNA induction was observed in late G_1 and in S phase. Further analysis showed that TGF-β1 treatment in early G_1 increases p21 protein stability throughout the G_1 and S phases of the cell cycle. Our results demonstrate that TGF-β1 stimulation of p21 is regulated at the posttranscriptional and transcriptional levels. This is a novel mechanism of TGF-β1 inhibition requiring early G_1 induction and stabilization of p21 protein, which binds to and inhibits cyclin E-CDK2 and cyclin A-CDK2 kinase activity rather than direct modulation of cyclin or CDK protein levels as seen in other systems.

INTRODUCTION

TGF[4]-β belongs to a large family of multifunctional polypeptides that are involved in the regulation of cell growth, adhesion, and differentiation (1). TGF-β inhibit proliferation of most epithelial cells and some cancer cells (1). Treatment of cells with TGF-β1 results in decreased cell proliferation and arrest of the cell cycle before S-phase entry (2, 3). The current model of cell cycle control holds that the transitions between different cell cycle phases are regulated at specific check points (4). These check points include the restriction point in G_1 after which the cell is committed to DNA replication, the transition from G_1 to S phase and the transition from G_2 to M phase.

Progression of cells through the cell cycle requires sequential assembly and activation of CDK complexes that are composed of a regulatory subunit (cyclin) and a catalytic subunit (CDK; Ref. 5). In mammalian cells, cyclin D-CDK4/CDK6, cyclin E-CDK2, and cyclin A-CDK2 act primarily in middle-to-late G_1 phase, the G_1-S-phase boundary, and S phase, respectively (5). CDK activity can be regulated by changes in cyclin or CDK levels and by posttranslational modification of the CDK subunit (5). A set of proteins called CKIs block CDK activity through stoichiometric mechanisms by complex formation (6). These inhibitors include p21/WAF1/CIP1 and p27/KIP1, which can inhibit a wide range of CDKs, as well as p15 and p16 from the INK family, which specifically act on the cyclin D-CDK4/CDK6 complexes (6).

The p21 protein was identified by its ability to bind to CDK2-cyclin complexes and function as a CKI to inhibit kinase activity (7). Later, it was found to be a universal inhibitor of cyclin-CDK complexes (6), and overexpression of p21 inhibits the proliferation of various tumor cells in culture (8). Several lines of evidence suggest that p21 is a mediator of p53-induced cell cycle arrest (9, 10). Although the expression of p21 is induced by irradiation in cells having wild-type p53 (11), regulation of p21 can also be independent of p53 during cellular differentiation (12, 13) and TGF-β1 treatment (14–17). It was also shown that members of the Smad protein family cooperate with Sp1 in TGF-β1-induced p21 expression in hepatic cells (18). The involvement of the mitogen-activated protein/extracellular signal-regulated kinase (MEK) pathway has been reported in the TGF-β1-mediated p21 induction in HaCaT cells (19). Autocrine TGF-β1 activity has been reported to induce radiation-mediated p21 expression in the p53-mutant pancreatic cancer cells (20).

Previous studies have shown that TGF-β1 is able to inhibit cell growth when it is added to cell culture in early or late G_1 phase (21, 3). TGF-β1 inhibits CDK4 or CDK6 kinase activity by down-regulation of cyclin D1 and CDK4 proteins in some cell types (22, 23), by the stimulation of p15, which specifically binds to CDK4 and CDK6 complexes (24). Inhibition of cyclin E- and cyclin A-associated kinase activity by TGF-β1 may result from the down-regulation of cyclin E and cyclin A, respectively (21, 25, 26). CKIs, such as p21 and p27, can negatively regulate G_1 phase progression in response to TGF-β1. TGF-β1 stimulates the expression of p21 in certain cell types (14–16) and increases levels of cyclin E-CDK2 complexes associated with p27 (15, 6). Thus, there is a wide range of mechanisms by which TGF-β1 inhibits CDKs.

Because cell cycle checkpoints are often deregulated in oncogenesis, a better understanding of how TGF-β1 modulates cell cycle progression of cancer cells may ultimately have therapeutic implications. We have investigated the molecular mechanisms of cell cycle inhibition by TGF-β1 in human colon carcinoma FET cells. We found that TGF-β1 dramatically increases p21 protein levels, leading to an increased association with CDK2, but had no effect on the cyclin E-CDK2 bound p27 levels as is typically observed in $p15^{ink}$ TGF-β mediated inhibition (25). In contrast to previously reported inhibition of cyclin A levels by TGF-β1 (21, 26, 27), cyclin A expression is not affected by TGF-β1 in these cells. However, the increased p21 resulting from TGF-β1 treatment targeted cyclin A-CDK2 complexes and inhibited this type of kinase activity as well. TGF-β1 can inhibit the ability of specific cell types to enter S phase when it is added to cultures at early G_1 (21), late G_1 (2), or throughout the G_1 phase (28). The mechanisms underlying TGF-β1 inhibition of cell cycle progression in these studies involved down-regulation of cyclin E and cyclin A, decreased phosphorylation of CDK2, and down-regulation of c-myc, respectively. We found that the inhibition of DNA synthesis and the stimulation of p21 by TGF-β1 in colon cancer cells was most effective in the early G_1 phase. Our studies show that the loss of p21 protein occurs very close in time to the loss of responsiveness of the cell cycle to TGF-β1 during delayed treatment of TGF-β1. These

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[2] J. G. and S. A. contributed equally to the manuscript.

[3] To whom requests for reprints should be addressed, at Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-3557; Fax: (716) 845-4437; E-mail: Michael.Brattain@roswellpark.org.

[4] The abbreviations used are: TGF, transforming growth factor(s); CDK, cyclin-dependent kinase; CKI, CDK inhibitor; CHX, cycloheximide; SM, McCoy's medium without growth factor supplementation; β-gal, β-galactosidase.

results indicated that induction of p21 by TGF-β1 in early G_1 is important for the inhibition of CDK2 kinase activity and cell cycle transit in human colon carcinoma FET cells. Furthermore, we confirmed earlier results that TGF-β1 stimulation of p21 mRNA is a consequence of transcriptional activation of the *p21* gene (14), and then, we expanded this observation. We found that TGF-β1 gradually lost its ability to induce p21 protein when treatment was delayed during G_1 progression; however, p21 mRNA was still induced when TGF-β1 was added in late G_1 phase as well as in the S phase. More importantly, we have shown for the first time that TGF-β1 was capable of increasing the stability of p21 protein. This indicated that posttranslational mechanisms are involved in the control of TGF-β1 induction of p21. Hence, our studies provide new insights into how TGF-β1 stimulates p21 expression and inhibits cell cycle progression.

MATERIALS AND METHODS

Cell Culture and Reagents. FET colon carcinoma cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in McCoy's serum-free medium (Sigma) supplemented with pyruvate, vitamins, amino acids, antibiotics, 10 ng/ml epidermal growth factor, 20 μg/ml insulin, and 4 μg/ml transferrin. Confluent cells were maintained in SM for 4–6 days to render them quiescent, as described previously (29). Release from quiescence was achieved by changing to fresh SM. Cyclin A (sc-751), cyclin E (sc-198), p21 (sc-469), p27 (sc-528), and CDK2 (sc-163) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). All of the protease inhibitors and CHX were from Sigma.

[^{3}H]Thymidine Incorporation Assay. Cells were plated in 24-well plates at a density of 3×10^4 cells/well, grown to confluence, and rendered quiescent. Fresh SM was used to release cells from different time periods as indicated in the appropriate experiments. [^{3}H]thymidine (7 μCi; Amersham) was added to triplicate wells and was pulsed for 1 h; DNA was then precipitated with 10% trichloroacetic acid and [3H]thymidine incorporation was determined.

Immunoprecipitation and Western Blot Analysis. Cells were lysed for 30 min at 4°C with lysis buffer [150 mM NaC1, 0.5% NP-40, 50 mM Tris (pH 8)] containing 25 μg/ml leupeptin, 25 μg/ml aprotinin, 5 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation at 15,000 rpm for 20 min at 4°C, and the amount of protein was quantitated by a colorimetric assay (Bio-Rad). Fifty μg of total protein was resolved by 12% SDS-PAGE. After electrophoresis, samples were transferred to nitrocellulose filters by electroblot transfer for 1 h, filters were incubated in TRIS buffered saline blocking solution [20 mM Tris (pH 7.5) and 0.5 M NaC1, containing 5% nonfat dried milk and 0.05% Tween 20] for 1 h at room temperature, and then was washed with TRIS buffered saline and double-distilled H_2O. The nitrocellulose filter (Amersham) was incubated overnight with the primary antibody at 1:1000 dilution in blocking solution followed by 2 h of incubation with a horseradish peroxidase-conjugated goat antimouse immunoglobulin at 1:2000 dilution (sc-2005). Protein was detected using an enhanced chemiluminescence method (ECL) according to the manufacturer's instructions (Amersham). For immunoprecipitation studies, 200 μg of total protein were precipitated by anti-CDK2, anti-cyclin A, or anti-cyclin E antibody. Immunocomplexes were bound to either protein A agarose or protein G agarose, resolved by 12% SDS-PAGE and then blotted with various antibodies, as described in the Results section (Fig. 3*B* and Fig. 3*C*).

Protein Kinase Assay. Cell lysates were prepared as described above, and 50 μg of total protein were incubated with anti-CDK2, anti-cyclin A, or anti-cyclin E antibodies for 2–3 h with agitation, followed by incubation with protein A or G agarose for 1–2 h. Beads were then washed three times with lysis buffer and three times with kinase buffer [20 mM Tris (pH 7.5) and 4 mM $MgC1_2$] and resuspended in 10 μl of reaction buffer containing 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear), 1.6 μg of histone H1 (Sigma), and 2 μl of 2× kinase buffer. The reaction mixture was then incubated at 37°C for 30 min. The reaction was stopped by the addition of 12 μl of 2× loading buffer [62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol], was resolved by 10% SDS-PAGE, and was visualized by autoradiography.

Degradation of p21 Protein. Quiescent FET cells were released with fresh medium in the presence of TGF-β1 for different times as indicated in the Figs. 6*A*–6*D* and then were changed to medium containing 10 μg/ml CHX in the presence or absence of TGF-β1 for 0.5, 1, 2, 4, and 6 h. Cell lysates were immunoblotted for p21 protein. To compare the stability of p21 protein in cells treated with TGF-β1 in early G_1 with that of cells treated with TGF-β1 at later times in G_1, cells were treated with TGF-β1 at 0 h or at 9 h after release from quiescence and then were changed to medium containing CHX at 12 h after release. Cell lysates were prepared at different times after the addition of CHX and were examined for p21 by Western blot analysis.

RNA Analysis. Total RNA was extracted by guanidine thiocyanate homogenization, as described previously (30). The p21 antisense riboprobe plasmid was constructed by inserting a 321-bp *Eco*RI-*Apa*I fragment of the 5′ region of the human p21 cDNA into pBSK(−). After linearizing with *Stu*I, the probe was synthesized *in vitro* using T7 RNA polymerase. The probe protects a 196-base fragment of p21 mRNA. RNase protection assay was performed, as described previously (30). Briefly, the ^{32}P-labled p21 probe was hybridized with 40 μg of total RNA. The hybridization mixture was then digested with RNase A and RNase T1 followed by proteinase K treatment. The RNase-resistant fragment of the probe was analyzed by Urea-PAGE and visualized by autoradiography. Protected actin mRNA was used to normalize sample loading.

Luciferase Activity Assay. The p21 promoter-luciferase reporter constructs p21P, p21PΔ1.1, and p21PsmaΔ1, were generously provided by Dr. Xiao-Fan Wang (Department of Pharmacology, Duke University Medical Center, Durham, NC) (14). Confluent cells were cotransfected with 30 μg of p21P, p21PΔ1, or p21PsmaΔ1, and with 10 μg of β-gal plasmid (pCH110) by electroporation (Bio-Rad Gene Pulser at 250 V and 960 μF). The electroporated cells were plated into 6-cm culture dishes in serum-free medium (SM supplemented with 20 μg/ml insulin, 4 μg/ml transferrin, and 10 ng/ml epidermal growth factor) for 24 h and were then treated with 10 ng/ml TGF-β1 for 22 h. Cells were lysed with luciferase assay buffer (Promega) according to the manufacturer's instructions, and the amount of luciferase activity in the lysates was measured by using a Berthold luminometer. β-gal activity in the cell extracts was used to normalize luciferase activity to the transfection efficiency. The fold induction of p21 promoter activity was calculated by normalizing luciferase activity to β-gal activity.

RESULTS

Kinetics of TGF-β1-Mediated Inhibition of DNA Synthesis and Stimulation of p21 Expression in FET Cells. FET cells were grown to confluence and were rendered quiescent by nutrient and growth factor deprivation, as described previously (29). Release from growth arrest was obtained by replenishing the cells with fresh medium in the presence or absence of 10 ng/ml TGF-β1 for 8, 12, 18, and 22 h, respectively. [^{3}H]thymidine incorporation into cellular DNA was increased by medium replenishment, and TGF-β1 significantly inhibited this DNA synthesis (Fig. 1*A*). Cell cycle analysis of cells released with fresh medium indicated that cells started to enter S phase at 12 h after release (data not shown). We then determined the effects of TGF-β1 on the expression of CDKs, cyclins, and CKIs in FET cells. FET cells were released from growth arrest in the presence and absence of 10 ng/ml TGF-β1 and were harvested at the time points indicated in Fig. 1*B*. The p21 protein level in quiescent cells was very low. Stimulation of cells by medium replenishment induced p21 protein at 3 h, but p21 levels quickly dropped back to basal levels. In contrast, p21 protein levels were increased within 2 h of TGF-β1 treatment and remained elevated for at least 24 h after TGF-β1 treatment. Previous studies have shown that TGF-β1 up-regulates p27 protein in WM35 cells (15) and down-regulates cyclin A levels in certain cell types (26, 27). In contrast, p27 and cyclin A protein levels were unaffected by TGF-β1 in FET cells (Fig. 1*B*). CDK2 and cyclin E remained unchanged after TGF-β1 treatment (data not shown).

TGF-β1 Inhibits Cyclin A-CDK2 and Cyclin E-CDK2 Kinase Activity. Because p21 protein expression was induced by TGF-β1, we next determined whether the induction of p21 affected CDK2

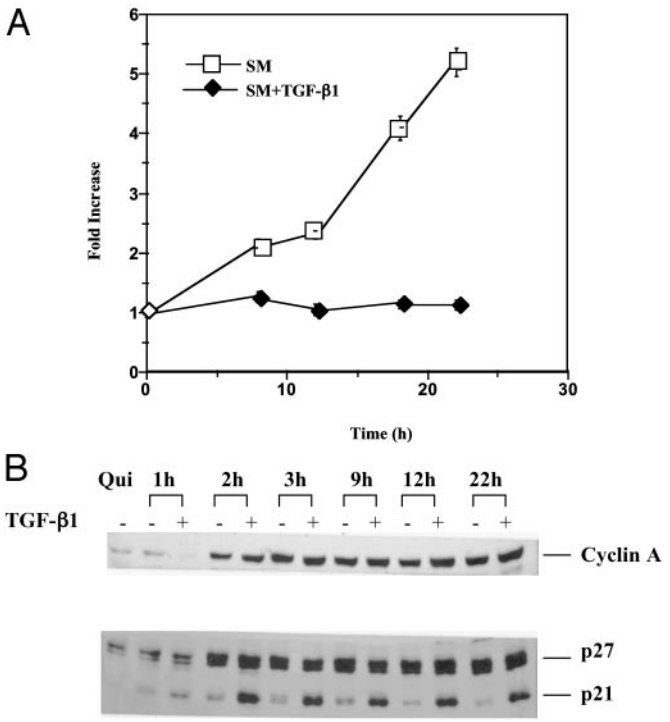

Fig. 1. Effect of TGF-β1 on cell proliferation and expression of p21. *A*, human colon carcinoma FET cells were rendered quiescent (*Qui*) by growth factor and nutrient deprivation as described in "Materials and Methods" and were stimulated to re-enter the cell cycle with fresh medium (SM) in the presence or absence of 10 ng/ml TGF-β1. [3H]thymidine (7 μCi) was added to triplicate wells for 1 h. [3H]thymidine incorporation into cells was measured at the indicated times after stimulation, as described in "Materials and Methods." Each value is a mean ± SE derived from three wells. *B*, quiescent FET cells were released by fresh medium (SM) treatment with or without 10 ng/ml TGF-β1, as described above. Cell lysates were prepared at the indicated times, and 50 μg of total protein were resolved by 12% SDS-PAGE. p21, p27, and cyclin A were examined by Western blot analysis, as described in "Materials and Methods."

kinase activity. Quiescent cells were released with fresh medium in the presence or absence of 10 ng/ml TGF-β1 for different times as indicated. Cyclin A- and cyclin E-associated complexes were assayed for histone H1 kinase activities after immunoprecipitation with anti-cyclin A and anti-cyclin E antibodies, respectively. Both cyclin A- and cyclin E-associated kinase activity were increased after release from growth arrest by medium replenishment. However, both kinase activities were inhibited by TGF-β1 treatment at 6, 9, 12, and 16 h after release from quiescence (Fig. 2*A*). Fig. 2*B* shows that quiescent cells had very low CDK2 kinase activity, whereas cells at 20 h after release from quiescence by fresh medium (peak DNA synthesis) showed increased CDK2 kinase activity. CDK2 kinase activity was dramatically inhibited at 20 h after TGF-β1 treatment. As expected, because of its different function with respect to cell cycle progression, cyclin A-associated kinase activity was much higher than cyclin E-associated kinase activity at 20 h (S phase) after release from quiescence.

Effect of TGF-β1 Treatment on CDK2 Complex Formation. Cell lysates prepared from TGF-β1-treated or -untreated cells were immunoprecipitated with anti-CDK2, cyclin A, or cyclin E antibodies, and the resulting immunocomplexes were subjected to Western analysis to further determine whether the reduction of CDK2 kinase activity was associated with increased binding of p21 to cyclin E-CDK2 and cyclin A-CDK2 levels. Kinetics of immunoprecipitation with CDK2 antibody after release from quiescence demonstrated that CDK2-associated p21 protein levels were increased within 2 h after TGF-β1 treatment and remained at an elevated level for 20 h (Fig. 3*A*). Fig. 3*B* shows that CDK2 and CDK2-associated p27 levels remained unchanged at 20 h after TGF-β1 treatment; however, CDK2-associated p21 protein levels were significantly increased after TGF-β1 treatment. Fig. 3*C* shows that cyclin A- and cyclin E-associated p21 levels were increased by TGF-β1 treatment at 20 h after release. It has been shown that TGF-β1 dissociates the p27 from cyclin D-CDK4 complexes and leads to an increase or stabilization of the association of p27 with cyclin E-CDK2 complexes by up-regulation of either p15 or p21 levels (15, 17, 25). In contrast, we found that TGF-β1 had no effect on the cyclin A- and cyclin E-associated p27 (Fig. 3*C*). These results indicated that the inhibition of cyclin A- and cyclin E-associated kinase activity seemed to be attributable to the increased levels of p21 associated with cyclin E- and cyclin A-CDK2 complexes in FET cells.

TGF-β1 Induction of p21 mRNA through Activation of p21 Promoter Activity. Cells treated with TGF-β1 were assayed for p21 mRNA expression by RNase protection assay. TGF-β1 treatment significantly increased steady-state p21 mRNA levels in FET cells at 1, 3, and 22 h after release from quiescence (Fig. 4*B*). To determine whether induction of p21 mRNA is regulated at the transcriptional level, p21 promoter-luciferase chimera constructs were transiently transfected into FET cells. These constructs included p21P containing both p53 and TGF-β1 responsive-elements, p21PΔ1.1 containing only the TGF-β1 responsive element, and p21PsmaΔ1 with the entire p21 promoter region deleted (14). As shown in Fig. 4*A*, TGF-β1 had no effect on the luciferase activity in p21PsmaΔ1 transfected cells but increased the luciferase activity about 4.3-fold and 4-fold in p21PΔ1.1 and p21P-transfected cells, respectively. Cells transfected with p21P and p21PΔ1.1 had similar responses to TGF-β1, indicating that induction of p21 promoter activity by TGF-β1 is independent of p53 in FET cells.

Inhibition Requires the Presence of TGF-β1 in Early G_1 Phase. Cells were rendered quiescent, as described above. TGF-β1 was then added to cells at various times after release from quiescence to determine how long after stimulation with medium replenishment TGF-β1 treatment could be delayed before the ability to block cell commitment to DNA synthesis was lost. When TGF-β1 was added at

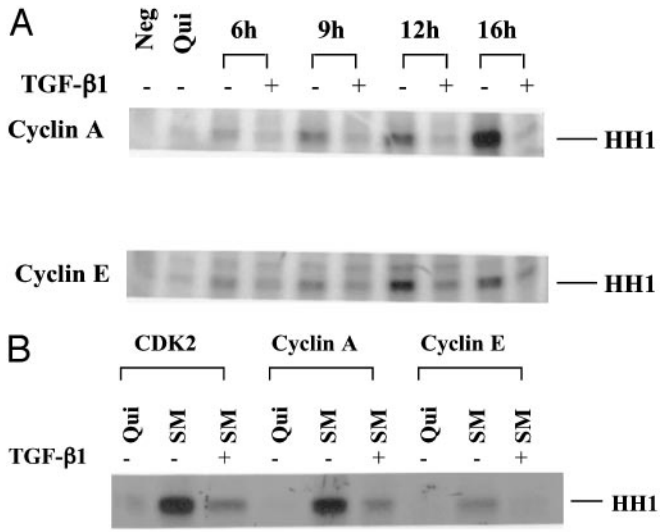

Fig. 2. TGF-β1 inhibition of cyclin A-CDK2 and cyclin E-CDK2 kinase activity. FET cells were released from quiescence in the presence or absence of 10 ng/ml TGF-β1 for different times as indicated. Total protein (50 μg) was immunoprecipitated with anti-CDK2, anti-cyclin A, or anti-cyclin E antibodies. The resultant immunocomplexes were then assayed for histone H1 (*HH1*) kinase activity, as described in "Materials and Methods." *A*, kinetics of induction of cyclin E- and -A-associated kinase activity. *B*, assay for CDK2-, cyclin A-, and cyclin E-associated kinase activity in quiescent (*Qui*) cells and at 20 h after release with growth medium (SM) with (+) or without (−) TGF-β1. *Neg*, negative control.

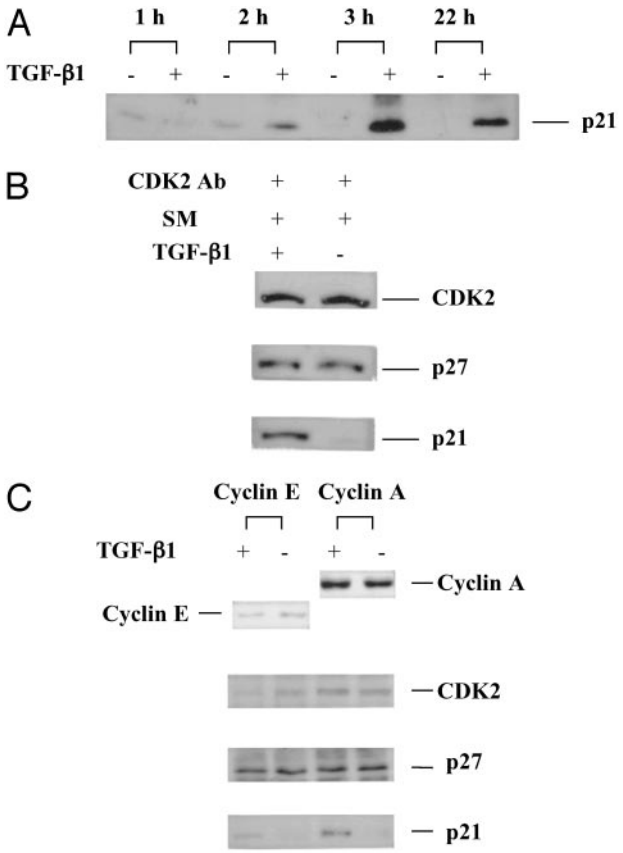

Fig. 3. Effect of TGF-β1 on CDK2 complex formation. FET cells were rendered quiescent, as described in Fig. 1 and were subsequently released by SM treatment with or without 10 ng/ml TGF-β1 for 1, 2, 3, and 22 h (*A*) or 22 h (*B*). Immunoprecipitation was performed with anti-CDK2 antibody using 200 μg of total protein. Immunocomplexes were resolved by 10% SDS-PAGE and immunoblotted; the blot was probed with anti-CDK2, anti-p21 or anti-p27 antibodies. *C*, TGF-β1 stimulation of cyclin A- and cyclin E-associated p21 protein levels. Anti-cyclin A and anti-cyclin E antibodies were used to precipitate cyclin A and cyclin E from 200 μg of protein lysates prepared from cells treated with TGF-β1, as described in *B*. Immunocomplexes were separated by 12% SDS-PAGE and immunoblotted with anti-p21 or anti-CDK2 antibodies. *Ab*, antibody.

2–8 h after release, [3H]thymidine incorporation into cells was reduced from ~80 to 50% relative to that of untreated cells. When added at 12 h after quiescent release, TGF-β1 had significantly less effect on DNA synthesis (Fig. 5*A*). Thus, the ability of TGF-β1 to block DNA synthesis deteriorated rapidly with progression of G_1 phase in FET cells. Next, we determined whether TGF-β1 inhibits DNA synthesis and stimulates p21 protein expression with similar kinetics. Maximum p21 stimulation occurred when TGF-β1 was added to cells at 0~2 h after release (Fig. 5*B*). Thereafter, TGF-β1 began to lose its ability to stimulate p21 protein expression. Thus, induction of p21 protein by TGF-β1 also deteriorates rapidly with G_1 progression in FET cells. RNase protection assay was performed to determine p21 mRNA expression from cells treated by TGF-β1, as described above. In contrast to protein levels, the addition of TGF-β1 to cells at different time points after quiescent release stimulated p21 mRNA to similar elevated levels throughout G_1 progression (Fig. 5*C*). This result indicates that TGF-β1-induced p21 protein may be degraded more rapidly when TGF-β1 addition is delayed until after cell cycle transit has begun. Thus, translational and/or posttranslational mechanisms are involved in the control of TGF-β1 induction of p21 in addition to transcriptional control.

TGF-β1 Treatment Increases the Stability of p21 Protein. If TGF-β1 increases the stability of p21 protein at late G_1, then removal of TGF-β1 should allow for more rapid p21 degradation. When cells were treated with TGF-β1 for 3 h after release and then were changed to the medium containing CHX alone or CHX plus TGF-β1, p21 protein was stable over the next 6 h under both of these conditions (Fig. 6*A*). However, when the cell cycle was allowed to progress 10 h after release with fresh medium in the presence of TGF-β1, subsequent treatment with CHX in the absence of TGF-β1 resulted in rapid degradation of p21. The half-life of p21 protein under these conditions was about 2 h. Treatment with CHX in the presence of TGF-β1 increased the half-life of p21 protein to ~4 h (Fig. 6*B*). These results indicate that p21 protein became more labile as the G_1 phase of the cell cycle progressed. Because TGF-β1 increased the stability of p21 protein, we conclude that posttranslational mechanisms are involved in the control of TGF-β1 induction of p21. Interestingly, TGF-β1 has been shown to increase p15 half-life ~2 h in another model (25).

When TGF-β1 was added in late G_1 or S phase, expression of p21 protein was reduced relative to cells treated in early G_1. This suggested that the factor(s) that mediates the stability of p21 protein may be induced in early G_1 phase and that one function of TGF-β1 may involve the inhibition of this component of cell cycle control. To determine this possibility, we compared stability of p21 in cells treated with TGF-β1 at 0 h after release from quiescence with the stability in cells that were not treated with TGF-β1 until 9 h after release. Fig. 6*E* shows a diagram depicting the design of this experiment. Cells treated with TGF-β1 at 0 h were changed to fresh medium containing CHX (without TGF-β1) at 12 h after release. The

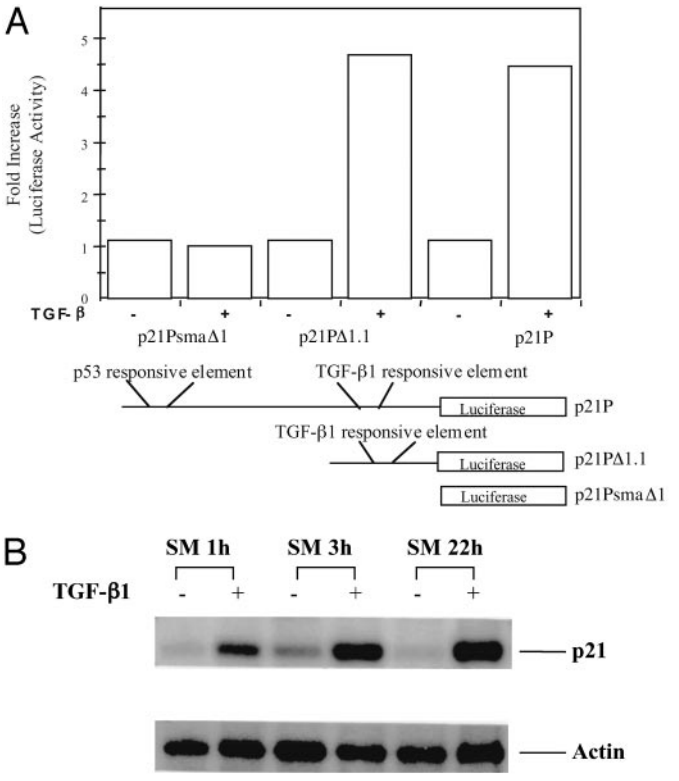

Fig. 4. Induction of p21 mRNA expression by TGF-β1. *A*, TGF-β1 stimulation of p21 promoter activity. FET cells were transiently transfected with 30 μg of p21 promoter DNA driving the luciferase reporter gene and 10 μg of β-gal plasmid-pCH110. Cells were then treated with 10 ng/ml TGF-β1 for 22 h at 24 h after transfection. Luciferase activity was then measured in TGF-β1-treated and -untreated cells. Induction was determined by comparing luciferase activity in TGF-β1 treated cells with untreated cells and averaging two different experiments. *B*, quiescent FET cells were released with fresh medium in the presence or absence of TGF-β1 for the indicated times. Total RNA was prepared, and the RNase protection assay for p21 was performed. The human p21 cDNA probe was hybridized with 40 μg to total RNA. The RNase-resistant fragment of the probe was resolved by Urea-PAGE and was visualized by autoradiography. Actin mRNA was used to normalize sample loading.

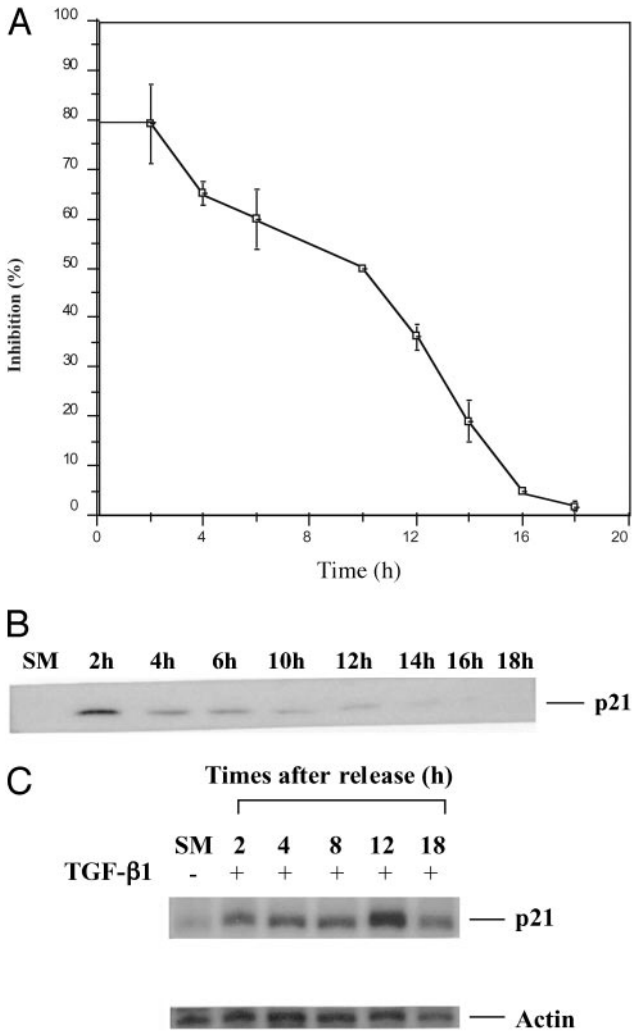

Fig. 5. Effect of delayed addition of TGF-β1 after quiescent release on DNA synthesis and p21 expression. *A*, effect of delayed TGF-β1 addition on [3H]thymidine incorporation. FET cells were rendered quiescent and then stimulated to re-enter the cell cycle with fresh medium. TGF-β1 was added at each indicated time after release from quiescence. [3H]thymidine incorporation was then measured for all of the time points at 22 h after quiescent release, as described in "Materials and Methods." Each value represents a mean of three replicates. *B*, cells were treated with TGF-β1, as described in *A*. Cell lysates were prepared at 22 h after release from quiescence. p21 expression was determined by Western blot analysis with anti-p21 antibody. *C*, cells were treated with TGF-β1 at different times after release from quiescence as indicated. RNase protection assays were performed as described in "Materials and Methods" to determine expression of p21 mRNA. Actin mRNA was used for normalization of loading.

resulting half-life of p21 under these conditions was ~2 h (Fig. 6*C*). However, when cells were treated with TGF-β1 at 9 h after release and then were maintained in fresh medium containing CHX (without TGF-β1) at 12 h after release from quiescence, the half-life of p21 declined to 0.5 h (Fig. 6*D*). These results indicated that the addition of TGF-β1 in early G_1 was necessary to protect p21 protein from degradation, suggesting that the early addition of TGF-β1 was able to inhibit the factor(s) that mediates the degradation of p21.

DISCUSSION

We have investigated the mechanisms by which TGF-β1 inhibits CDK activity in FET colon carcinoma cells. TGF-β1 dramatically inhibited cyclin E- and cyclin A-associated kinase activity in FET cells. It has been shown that cell cycle arrest induced by TGF-β1 may occur through disruption of activation of the cyclin E- or cyclin A-associated kinase complexes (15, 31). Inactivation of cyclin E-CDK2 kinase activity results from down-regulation of cyclin E in HaCat cells (21) and increased levels of p27 and/or p21 in other cell types (14–17, 25, 28). Reynisdottir *et al.* (17) demonstrated that, in Mv1Lu cells and HaCat keratinocytes, TGF-β1 causes the induction of p15 with a concomitant redistribution of p27 from CDK4-cyclin D complexes to cyclin E-CDK2 complexes. The increased cyclin E-CDK2 bound p27 leads to inhibition of its kinase activity. However, Florenes *et al.* (15) reported that this is not a universal mechanism for inhibition of cyclin E-CDK2 kinase activity by TGF-β1. These investigators demonstrated that, in WM35 cells (which lack p15) up-regulation of p21 may fulfill a function similar to that of p15 and that

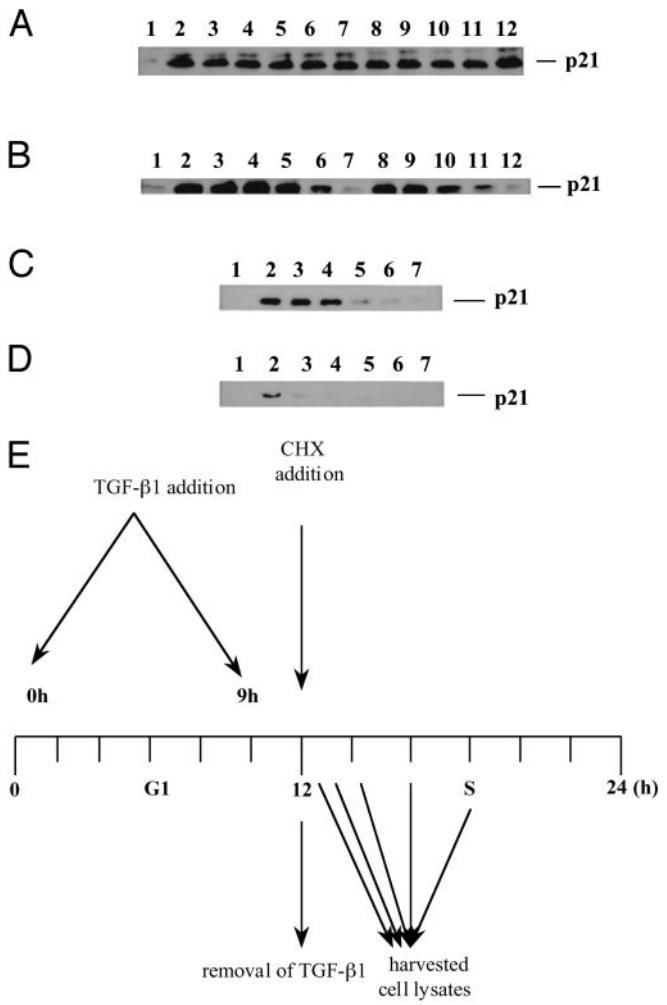

Fig. 6. Effect of TGF-β1 treatment on the stability of p21 protein. Quiescent FET cells were exposed to TGF-β1 for 3 h (*A*) or 10 h (*B*) after release, and then were changed to fresh medium containing 10 μg/ml CHX alone (*Lanes 8–12*) or 10 μg/ml CHX plus TGF-β1 (*Lanes 3–7*). At 0.5 (*Lanes 3* and *8*), 1 (*Lanes 4* and *9*), 2 (*Lanes 5* and *10*), 4 (*Lanes 6* and *11*), and 6 h (*Lanes 7* and *12*) after addition of CHX, cell lysates were immunoblotted for p21, as described in "Materials and Methods." *Lane 1*, cells released with fresh medium alone; *Lane 2*, cells released with fresh medium in the presence of TGF-β1. Cells were treated with TGF-β1 at 0 h (*C*) or 9 h (*D*) after release from quiescence and then were changed to fresh medium containing 10 μg/ml CHX (without TGF-β1) at 12 h after release from quiescence. At 0.5 (*Lane 3*), 1 (*Lane 4*), 2 (*Lane 5*), 4 (*Lane 6*), and 6 h (*Lane 7*) after addition of CHX, cell lysates were immunoblotted for p21, as described in "Materials and Methods." *Lane 1* = cells released with fresh medium alone; *Lane 2* – cells treated with TGF-β1. *E*, diagram of the design of the experiment comparing the stability of p21 in cells treated with TGF-β1 in early G_1 with that of cells treated with TGF-β1 in late G_1. FET cells were starved by growth factor and nutrient deprivation and were released with fresh medium. Cells enter S phase at 12 h after release. Cells were treated with TGF-β1 at 0 h or at 9 h after release from quiescence. Cells were washed three times with fresh medium at 12 h after quiescence release to remove TGF-β1 and were maintained in fresh medium containing 10 μg/ml CHX. Cell lysates were harvested at 0.5, 1, 2, 4, and 6 h after the addition of CHX, and p21 was determined by Western blot analysis.

both p21 and p27 contribute to the inhibition of cyclin E-CDK2 kinase activity by TGF-β1. We found that TGF-β1 up-regulates p21 but had no effect on p27. Immunoprecipitation with CDK2 or cyclin E followed by Western blot analysis indicated that TGF-β1 increased cyclin E-CDK2-bound p21 but did not affect cyclin E-CDK2-associated p27 levels (Fig. 3). These results suggest that p21, but not p27, contributes to cyclin E-CDK2 inactivation. TGF-β1 prevents the induction of cyclin A in several cell types (15, 21, 31); however, TGF-β1 did not prevent the accumulation of cyclin A in FET cells (Fig. 1*B*). Inactivation of cyclin A kinase activity was caused by an increase in the association of p21 with the cyclin A-CDK2 complexes (Fig. 3).

It has been reported that TGF-β1 can inhibit cell proliferation when added to cultures in either early or late G_1 phase (2, 21, 31). Evidence presented in this study demonstrated that TGF-β1 was a more effective inhibitor of FET cells when it was added in early G_1 phase (Fig. 5*A*). It is likely that TGF-β1 may target different cell cycle components when it is added in early, as opposed to later, G_1. Inhibition of one of these components may be responsible for increased p21 stability during peak DNA synthesis.

Several studies have shown that TGF-β1 is able to induce p21 expression (14–16, 25); however, these studies did not determine whether induction of p21 is associated with a specific stage of the cell cycle. We found that the induction of p21 expression was most effective when TGF-β1 was added in early G_1 phase (Fig. 5*B*). However, with increasing delay between stimulation of reentry into the cell cycle and TGF-β1 addition, the ability to induce p21 protein expression, but not p21 mRNA, was lost. TGF-β1 inhibits DNA synthesis and stimulates p21 protein expression with similar kinetics. These data indicate that p21 induction in early G_1 is critical for TGF-β1-inhibitory effects in FET cells. Immunoprecipitation at various times after release from quiescence demonstrated that cyclin A- and cyclin E-associated p21 levels were increased within 2 h by TGF-β1 treatment. Therefore, increased p21 binding to cyclin A and cyclin E complexes resulting from early-G_1 TGF-β1 treatment may ultimately be responsible for inhibition of CDK2 activity and, consequently, for inhibition of DNA synthesis.

Results from RNase protection and promoter activity assays demonstrated that the induction of p21 mRNA by TGF-β1 was at least partially attributable to the stimulation of p21 promoter activity through a p53-independent pathway (Fig. 4). This is consistent with a previous study (14). Interestingly, as noted above, p21 mRNA was maintained at an elevated level throughout G_1 (Fig. 5*C*), whereas p21 protein levels were decreased in late G_1 (Fig. 5*B*) when TGF-β1 treatment was delayed to various time points after stimulation to re-enter the cell cycle. Additional experiments demonstrated that TGF-β1 treatment in early G_1 increased the stability of p21 protein relative to TGF-β1 treatments, which were delayed as described above (Fig. 6). This indicates that a posttranslational mechanism is involved in controlling the induction of p21 by TGF-β1. When quiescent cells were released with fresh medium in the absence of TGF-β1, p21 protein increased during the early stages of the cell cycle but quickly dropped back to basal levels. Taken together, the results indicate that after release from quiescence, cells synthesize a protease(s), which degrades p21. When TGF-β1 was added to cells immediately after release, p21 protein was stimulated and maintained at an elevated level (Fig. 1*B*). These results suggest that TGF-β1 treatment indirectly protects p21 from degradation, perhaps through complex formation to cyclin A, cyclin E, and/or CDK2 in early G_1 or through reduction of protease levels. For example, Lovastatin, another G_1 blocker, was shown to inhibit ubiquitin-mediated proteolysis of p21 in breast cancer cells (32). TGF-β1 has been shown to stabilize p15 protein levels in another model system (25).

In conclusion, our study demonstrated a novel mechanism by which TGF-β1 treatment increased cyclin A-associated p21 levels, leading to the inhibition of its kinase activity rather than modulation of cyclin A levels. Induction of p21 by TGF-β1 in early G_1 is critical for the inhibition of CDK2 kinase activity and cell cycle transit. Moreover, stimulation of p21 by TGF-β1 is controlled at the transcriptional level through a p53-independent pathway as well as at the posttranslational level. Posttranslational control is dependent on treatment with TGF-β1 in early G_1, whereas TGF-β1-mediated transcriptional control does not appear to be a function of the time of TGF-β1 treatment during cell cycle progression.

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Transforming Growth Factor β1 Increases the Stability of p21/WAF1/CIP1 Protein and Inhibits CDK2 Kinase Activity in Human Colon Carcinoma FET Cells

JianGen Gong, Sudhakar Ammanamanchi, Tien C. Ko, et al.

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