Human colon carcinoma cells HCT116 that lack transforming growth factor β (TGF-β) type II receptor (RII) demonstrated restoration of autocrine TGF-β activity upon reexpression of RII without restoring inhibitory responses to exogenous TGF-β treatment. RII transfectants (designated RII Cl 37) had a longer lag phase relative to NEO-transfected control cells (designated NEO pool) before entering exponential growth in tissue culture. The prolonged growth arrest of RII Cl 37 cells was associated with markedly reduced cyclin-dependent kinase (CDK)/2 activity. Our results demonstrate that p21 induction by autocrine TGF-β is responsible for reduced CDK2 activity, which at least partially contributes to prolonged growth arrest and reduced cell proliferation in RII Cl 37 cells. In contrast to RII transfectants, HCT116 cells transfected with chromosome 3 (designated HCT116Ch3), which bears the RII gene, restored the response to exogenous TGF-β as well as autocrine TGF-β activity. Autocrine TGF-β activity in HCT116Ch3 cells induced p21 expression as seen in RII Cl 37 cells; however, in addition to autocrine activity, HCT116Ch3 cells responded to exogenous TGF-β as decreased CDK4 expression and reduced pRb phosphorylation mediated a TGF-β inhibitory response in these cells. These results indicate that autocrine TGF-β regulates the cell cycle through a pathway different from exogenous TGF-β in the sense that p21 is a more sensitive effector of the TGF-β signaling pathway, which can be induced and saturated by autocrine TGF-β, whereas CDK4 inhibition is a less sensitive effector, which can only be activated by high levels of exogenous TGF-β.

Cell cycle progression is mediated by sequential assembly, activation, and subsequent inactivation of a series of serine/threonine protein kinases that consist of a catalytic cyclin-dependent kinase (CDK)1 subunit and a regulatory cyclin subunit (1). The progression from G1/S to S phase is regulated by G1/S cyclins and their partner CDKs. The mammalian cyclins responsible for progression through the G1 restriction point are the D-type cyclins (D1, D2, and D3) (2–4), whereas cyclin E and cyclin A control S phase entry and progression (5–7). Cyclin-CDK activity is regulated by phosphorylation and dephosphorylation of the CDK subunit and programmed degradation of the cyclin regulatory subunit (8). In addition, CDK inhibitory proteins (CKIs) have been shown to bind to the cyclin-CDK complexes and inhibit their activities (9, 10). Although CDK inhibitors p14/p15 (11, 12), p16 (12, 13), p18 (12, 14), and p19 (14, 15) bind specifically to cyclin D-CDK4/6 complexes and inhibit their activity, p21 (16–20), p27 (21, 22), and p57 (23) have been shown to be potent inhibitors of a variety of cyclin-CDKs.

The transforming growth factor βs (TGF-βs) are a group of multifunctional polypeptides that regulate a number of cellular processes through binding to TGF-β receptors. Three major types of TGF-β receptor, type I (RI), type II (RII), and type III (RIII), have been identified in most cells (24, 25). Both RI and RII are transmembrane serine/threonine kinases indispensable for TGF-β signaling (26–29). After TGF-β binds to a heteromeric complex of RI and RII, RI is transphosphorylated by RII. The activated RI phosphorylates selected Smads, and these receptor-activated Smads then form a complex with a common Smad (Smad4). Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes (30, 31). TGF-β has been shown to inhibit cell proliferation by inducing cell cycle arrest (24, 32, 34, 35). There are two mechanisms that have been proposed to be involved in cell cycle arrest induced by exogenous TGF-β treatment. One is that exogenous TGF-β down-regulates the levels and/or the activities of G1/S cyclins and CDKs (33, 35–40). The other is that exogenous TGF-β up-regulates the expression of CKIs, p15 (11, 41, 42), p21 (43–46), and p27 (21, 47, 48).

Previous work in our laboratory has indicated that autocrine TGF-β activity rather than responsiveness to exogenous TGF-β is a key factor in negative growth regulation and tumor suppression (49–52). Increased autocrine negative TGF-β activity led to decreased cell proliferation and reduced tumorigenicity in human colon carcinoma cells (49, 50). However, the mechanism(s) by which autocrine TGF-β functions to inhibit cell growth and suppress tumor progression has not been addressed.

We utilized a unique experimental system, HCT116 cells, TGF-β RII-transfected cells (designated RII Cl 37), and cho-
mosome 3-transfected cells (designated HCT116Ch3) to elucidate the mechanisms of cell cycle regulation by autocrine and exogenous TGF-β. As reported previously, HCT116 cells had no autocrine TGF-β activity and no inhibitory response to exogenous TGF-β as a result of mutational inactivation of TGF-β RII, whereas RII Cl 37 cells exhibited autocrine negative TGF-β activity after expression of wild type TGF-β RII cDNA (49, 53). However, responsiveness to growth inhibition by exogenous TGF-β was not restored in RII Cl 37 cells. The RII-transfected cells showed reduced tumorigenicity relative to NEO-transfected control cells (49). In contrast, HCT116Ch3 cells that had regained wild type TGF-β RII from chromosome 3 showed restored responsiveness to both autocrine and exogenous TGF-β (54). In this report, we show that autocrine TGF-β induced p21 expression, which resulted in markedly reduced CDK2 activity and prolonged growth arrest in RII Cl 37 cells in contrast to HCT116Ch3 cells in which autocrine TGF-β increased p21 expression, whereas exogenous TGF-β treatment led to decreased CDK4 expression and reduced pRb phosphorylation in association with growth inhibition. These results indicate that autocrine TGF-β regulates the cell cycle through a pathway different from exogenous TGF-β in the sense that autocrine TGF-β saturates the more sensitive p21 pathway at a relatively low level of receptor occupation. Although this saturation could occur through exogenous TGF-β treatment if autocrine TGF-β were absent, the occupation of additional receptors by exogenous TGF-β leads to the induction of a second, less sensitive pathway involving the inhibition of CDK4 expression and Rb phosphorylation.

MATERIALS AND METHODS

Cell Culture—The RII Cl 37 cells were obtained by transfection of wild type TGF-RII cDNA into HCT116 human colon carcinoma cells, whereas NEO pool cells were generated by transfection of a control plasmid as described previously (49). The HCT116Ch3 and HCT116Ch2 cells were obtained by transferring normal human chromosomes 3 and 2 into HCT116 cells, respectively, as described by Koi et al. (55).

Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in McCoy's 5A 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 (56).

Cell Proliferation and Flow Cytometry—Growth curves were generated by plating NEO pool and RII Cl 37 cells at a sparse density (5 × 104 cells/well) in 6-well cell culture plates and measuring DNA synthesis every day after initiation of culture, as described previously (50). Briefly, the cells were labeled with 51 Ci/mmol [3H]thymidine (American Biosciences) for 1 h. DNA was then precipitated with 10% trichloroacetic acid and solubilized in 0.2 M NaOH. The amount of [3H]thymidine incorporated into DNA was analyzed by liquid scintillation counting in a Beckman LS6000 scintillation counter.

To study the effects of TGF-β1-neutralizing antibody on cell proliferation, NEO pool and RII Cl 37 cells were plated at a low density (5 × 102 cells/well) in 24-well tissue culture plates. TGF-β1-neutralizing antibody or normal chicken IgG (R&D system) was added to the medium at a concentration of 50 μg/ml. [3H]Thymidine incorporation was measured on days 4 and 5 as described above. Inhibition of [3H]thymidine incorporation in HCT116Ch3 cells by exogenous TGF-β was determined as described previously (51, 52) using a standard DNA synthesis assay.

Cells for flow cytometry analysis were prepared from cultures in 6-well plates as described above and harvested on days 3–5. The cells were trypsin treated, washed, and stained for DNA by resuspending them in stain solution I (50 μg/ml propidium iodide (Sigma), 3% polyethylene glycol, 0.1% Triton X-100, and 4 μg/ml sodium citrate) with 20 μg/ml RNase A, followed by incubation for 30 min at 37 °C. Equal volumes of stain solution II (50 μg/ml propidium iodide, 3% polyethylene glycol, 0.1% Triton X-100, and 400 μg/ml sodium chloride) were added to the above solution, and the samples were then stored on ice for at least 4 °C for at least 4 h. The samples were analyzed on a FACScan flow cytometer (BD Biosciences). Cell cycle compartments were analyzed using a ModFit LT program (Verity Software House, Inc.).

Histone H1 Kinase Assay—Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 μM dithiothreitol, 25 μg/ml aprotinin, 25 μg/ml trypsin inhibitor, and 25 μg/ml leupeptin) at 4 °C. The supernatants were cleared by centrifugation. 100 μg of fresh total cell lysates was immunoprecipitated for 2 h at 4 °C with rabbit polyclonal antibodies against CDK2 (M2, Santa Cruz Biotechnology), cyclin A (H-432, Santa Cruz Biotechnology), cyclin E (C-19, Santa Cruz Biotechnology) and p21 (C-19, Santa Cruz Biotechnology), followed by incubation with immobilized protein A-agarose beads (Invitrogen) for another 2 h at 4 °C with rotation. The beads were then washed three times with Nonidet P-40 lysis buffer and another three times with kinase buffer (20 mM Tris-HCl, pH 7.5, and 4 mM MgCl2). Phosphorylation of histone H1 was measured by incubating the beads with 10 μl of reaction mixture containing 10 μCi of [γ-32P]ATP (3,000 Ci/mmol, PerkinElmer Life Sciences) and 2.4 μg of histone H1 (Sigma) in kinase buffer for 30 min at 37 °C. The reaction was stopped by placing the samples on ice. The samples were then boiled in ×2 sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 20 mM glycerol, 0.04% bromophenol blue, and 4% β-mercaptoethanol) for 5 min and resolved by 10% SDS-PAGE. The gel was dried and subjected to autoradiography.

Immunoprecipitation and Western blot analysis—Cells were lysed in Nonidet P-40 buffer as described above. The supernatants were cleared by centrifugation. Equal amounts of total cell lysates were boiled in ×2 sample buffer, resolved by 12 or 15% SDS-PAGE, and transferred to nitrocellulose membranes (Amersham Biosciences) for Western blot analysis. The blots were probed with various primary antibodies at a concentration of 1 μg/ml anti-p21, anti-CDK2, anti-cyclin A, anti-p27 (C-19, Santa Cruz Biotechnology), anti-CDK4 (H-22, Santa Cruz Biotechnology), anti-cyclin E (HE12, Santa Cruz Biotechnology) and anti-phospho-pRB (ser 780), which was then followed by incubation with 0.2 μg/ml horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Jackson ImmunoResearch). The proteins were then detected by the enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation followed by Western blot analysis was performed to detect protein levels in the complexes. Equal amounts of total cell lysates were immunoprecipitated overnight at 4 °C with the various antibodies (anti-p21 and anti-CDK2 (M2, Santa Cruz Biotechnology)) on days 4 and 5 as described above. The blots were probed with various primary antibodies at a concentration of 1 μg/ml anti-p21, anti-CDK2, anti-cyclin A, anti-cyclin E, and anti-phospho-pRB. The proteins were then detected by the enhanced chemiluminescence system (Amersham Biosciences).

RNA Analysis—RNase protection assays were performed as described previously (52) to compare mRNA expression of p21 in NEO pool and RII Cl 37 cells. The cells were plated at a low density (3.5 × 102 cells/dish) in 100-mm tissue culture dishes. Total RNA was isolated on days 3–5. The p21 antisense riboprobe plasmid was constructed by inserting a 321-bp EcoRI-ApaI fragment of the 5′-region of the human p21 gene (inserted into pBSK+). The riboprobe was synthesized in vitro using T7 RNA polymerase. The probe protects a 196-base fragment of p21 mRNA. The 32P-labeled probes were 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 protected fragments were resolved on 6% urea-polyacrylamide gel electrophoresis and visualized by autoradiography. Actin mRNA protected by an actin riboprobe was used to normalize sample loading as described previously (52).

Luciferase Assays—The reporter constructs of p21 promoter (p21P, p21PΔ1.1, and p21P-SmaΔ11) have been described previously (44). Briefly, p21P is a full-length p21 promoter, whereas p21PΔ1.1 is the promoter with the minimal enhancer region deleted. The p21P-SmaΔ11 is the control construct with the entire p21 promoter region deleted. Each of these constructs was fused to a luciferase cDNA to generate a promoter-reporter chimera. HCT116 NEO pool and RII Cl 37 cells were transiently transfected with 30 μg of one of the three reporter constructs using electroporation as described previously (49, 50). A 1.1-kb Gaα2/3 lucasidase reporter construct was cotransfected to normalize luciferase activity to transfection efficiency. After transfection, the cells were plated on 100-mm dishes at a low density (3.5 × 104 cells/dish) and grown for 48 h. The cells were then harvested, and luciferase activity was assayed using the Luciferase Assay Systems (Promega). Luciferase activity was normalized to β-galactosidase activity.

RESULTS

Effects of Autocrine TGF-β Activity on Cell Proliferation and Growth Arrest—Growth curves of sparsely plated cells were
increased [3H]thymidine incorporation (43%) relative to normal chicken IgG were added separately at a concentration of 30 
pg/ml. DNA synthesis assays were performed on days 4 and 5 by measuring [3H]thymidine incorporation as described under “Materials and Methods.” Values are the means ± S.E. of three replicates.

generated to compare the growth rate of NEO pool and RII CI 37 cells and to define operationally the lag phase of growth for each cell type. The lag phase is a period of adaptation following subculture and reseeding during which the cells build up growth promoting factors to a level to support maximal reentry into the cell cycle and consequently, exponential growth. NEO pool and RII CI 37 cells were plated at equal clonal densities. As shown in Fig. 1, growth rates in the exponential growth phase were essentially identical for NEO pool and RII CI 37 cells, but RII CI 37 cells had a longer lag phase than NEO pool cells before entry into exponential phase. Treatment of RII CI 37 cells in the lag phase by TGF-β1-neutralizing antibody led to increased [3H]thymidine incorporation (43%) relative to normal IgG-treated cells, whereas NEO pool cells did not show any response to the antibody (Fig. 2). These results indicate that autocrine negative TGF-β activity in RII CI 37 cells antagonized the cumulative effects of positive growth factors in the lag phase, thus preventing RII CI 37 cells from reentering the cell cycle as quickly as NEO pool cells. Flow cytometric analysis of DNA content in NEO pool and RII CI 37 cells was then performed to confirm this observation. FACS analysis revealed that there was a lower percentage of S phase and a higher percentage of G0/G1 phase cells in RII CI 37 than in NEO pool (Table I). On day 5, there was a 35% increase of NEO pool cells entering S phase, but there was no significant increase of the S phase cell population of RII CI 37 over days 3 and 4. Thus, the percentage of S phase cells in NEO pool is about 1.6-fold higher than that in RII CI 37. This indicated that RII CI 37 cells took a longer time to reenter the cell cycle than NEO pool cells after plating at sparse densities.

Inhibition of CDK2 Activity Accompanies Autocrine TGF-β-induced Growth Arrest—Because CDK2/4-associated kinase activity is rate-limiting for progression from G1 into S phase (1), we compared the CDK2 and CDK4 activities in NEO pool and RII CI 37 cells by histone H1 phosphorylation assays and CDK4 kinase assays, respectively. The results revealed that RII CI 37 cells had markedly reduced CDK2-associated kinase activity relative to NEO pool cells (Fig. 3), whereas there was no difference of CDK4-associated kinase activity in both cell types (data not shown). This indicated that the failure of RII CI 37 cells to reenter the cell cycle as quickly as NEO pool cells is at least partially the result of reduced CDK2 activity. Western blot and immunoprecipitation analyses indicated that the suppression of CDK2 activity in RII CI 37 cells was not caused by reductions of the CDK2, cyclin A, or cyclin E level (Fig. 4A) or by disruption of CDK2-cyclin A or CDK2-cyclin E complexes (Fig. 4B).

Induced Expression of the p21 CDK Inhibitor by Autocrine TGF-β Activity—Next we explored the possible roles of CDK2 inhibitors p21 and p27 in the suppression of CDK2 activity in RII CI 37 cells. The expression levels of p21 and p27 were...
examined in NEO pool and RII Cl 37 cells by Western blot assays. Expression of p21 was 3–4-fold higher in RII Cl 37 cells than in NEO pool cells, whereas the p27 expression levels were similar in both cell types (Fig. 5A). Western blot analysis following immunoprecipitation with CDK2 antibody indicated that there was 3–4-fold more p21 complexed with CDK2 in RII Cl 37 cells than in NEO pool cells, whereas the amount of p27 complexed with CDK2 was the same in both cell types (Fig. 5B). These results suggested that the increased expression and association of p21 with CDK2 are responsible for the reduced CDK2 activity in RII Cl 37 cells. However, recent studies suggest that p21-containing complexes exist in both catalytically active and inactive forms in vitro and in vivo (58, 59). To confirm that p21 is inhibitory for the CDK2 complexes, p21-associated CDK2 activity was determined by histone H1 kinase assay. Analysis of NEO pool and RII Cl 37 cell lysates immunoprecipitated with p21 antibody showed that the p21-associated CDK2 complexes were catalytically inactive (Fig. 5C). These results indicate that p21 is inhibitory for the CDK2 complexes because increased p21 and its association with CDK2 resulted in reduced CDK2 activity, in turn leading to prolonged growth arrest in RII Cl 37 cells.

Direct evidence of p21 induction by autocrine TGF-β was obtained by treatment of RII Cl 37 cells with TGF-β1-neutralizing antibody to suppress autocrine TGF-β activity as described previously (49, 50). The expression level of p21 was decreased in RII Cl 37 cells after TGF-β1-neutralizing antibody treatment, whereas it remained unchanged in NEO pool cells (Fig. 6A). TGF-β1-neutralizing antibody also reduced the p21 level associated with CDK2 in RII Cl 37 but not in NEO pool cells (Fig. 6B). These results demonstrated that induction of p21 is critical to the mechanism of autocrine TGF-β-mediated cell cycle regulation.

It has been shown that exogenous TGF-β up-regulates the expression of CDK4 inhibitor p15 (11, 41, 42). To determine whether p15 expression is also affected by autocrine TGF-β, mRNA and protein expression of p15 were determined in NEO pool and RII Cl 37 cells. RNase protection and Western blot assays revealed that both cell types expressed similar levels of p15 (data not shown). Moreover, there was no difference in pRb phosphorylation between these two cell types (data not shown). Taken together with CDK4 activity in those cells, these results indicated that p15 and p15-mediated CDK4 activity are not involved in the mechanism of autocrine TGF-β in RII Cl 37 cells.

Transcriptional Control of p21 by Autocrine TGF-β Independent of Wild Type p53—We then compared mRNA levels of p21 in NEO pool and RII Cl 37 cells by an RNase protection assay to determine the mechanism(s) of p21 induction by autocrine TGF-β. The expression level of p21 mRNA was 2–3-fold higher in RII Cl 37 cells than in NEO pool cells (Fig. 7A). Three reporter constructs of the p21 promoter were transiently transfected into NEO pool and RII Cl 37 cells to determine p21 transcriptional activity. As shown in Fig. 7B, the full-length p21 promoter construct (p21P) showed 2–3-fold higher induction of luciferase activity in RII Cl 37 cells than in NEO pool cells, thus accounting for the difference in p21 mRNA levels between the two cell types. HCT116 cells have wild type p53 that has been shown to transcriptionally regulate p21 expression in a number of other cell types (16, 17, 60). However, in our study, the full-length p21 promoter construct (p21P) and the p53-responsive element deleted construct (p21Δ1, 1) showed identical levels of induction by autocrine TGF-β in RII Cl 37 cells. This indicated that induction of p21 promoter activity by autocrine TGF-β is independent of wild type p53 in RII Cl 37 cells.

Effects of Exogenous TGF-β on Expression of p21 and CDK4 in Chromosome 3-Transfected HCT116 Cells—HCT116 cells transfected with a normal chromosome 3 that contains TGF-β RII gene (designated HCT116Ch3 cells) restored sensitivity to exogenous TGF-β, whereas the cells transfected with irrelevant chromosome 2 (designated HCT116Ch2 cells) remained insensitive to TGF-β (Fig. 8). The response to exogenous TGF-β by HCT116Ch3 cells allowed us to compare exogenous TGF-β effects on cell growth and cell cycle with those induced by autocrine TGF-β. The expression level of p21 was examined in HCT116Ch3 and HCT116Ch2 cells. Western blot analysis revealed that p21 expression was higher in HCT116Ch3 cells than in HCT116Ch2 cells (Fig. 9A). However, treatment of HCT116Ch3 cells with exogenous TGF-β did not result in further induction of p21 levels over those arising from restoration of autocrine TGF-β by chromosome 3 transfer (Fig. 9B). Further experiments showed that CDK4 expression was decreased in HCT116Ch3 cells after exogenous TGF-β treatment, whereas there was no change of CDK4 levels in HCT116Ch2 cells (Fig. 10A). The increased CDK4 protein levels suggested that exogenous TGF-β might affect pRb phosphorylation in HCT116Ch3 cells. An antibody specific for phospho-pRb was then used to determine changes in pRb phosphorylation after exogenous TGF-β treatment. Fig. 10B
shows that exogenous TGF-β reduced pRb phosphorylation in HCT116Ch3 cells but had no effect in HCT116Ch2 cells. These results indicate that the differences in the sensitivity of the p21 induction pathway and the CDK4 inhibition pathway allow for the p21 pathway to be induced at low receptor occupation generated by autocrine production of TGF-β in RII Cl37 cells. However, the receptor saturation generated by exogenous TGF-β recruits the less sensitive CDK4 pathway as well in HCT116Ch3 cells.

**DISCUSSION**

TGF-β has been shown to be an autocrine-negative growth factor, as evidenced by stimulation of growth of several cell lines treated with TGF-β-neutralizing antibody (49–52, 61, 62). Previous work in our laboratory showed that in colon carcinoma cells, suppression of autocrine TGF-β activity by constitutively repressing endogenous TGF-β expression without eliminating the ability to respond to exogenous TGF-β led to a more progressed malignant phenotype (51, 52). Restoration of autocrine negative TGF-β activity by replacing mutationally inactivated TGF-β RII in microsatellite unstable cells with wild type RII resulted in reduced cell proliferation and diminished tumorigenicity. However, responsiveness to growth inhibition by exogenous TGF-β was not reestablished (49, 50). These results indicated that autocrine negative TGF-β activity rather than responsiveness to exogenous TGF-β is an important deterrent to malignant progression in human colon carcinoma cells. Therefore, exploration of the mechanisms by which autocrine TGF-β functions is important for our understanding of the carcinogenic process and malignant progression. Many studies have shown that exogenous TGF-β inhibits cell proliferation by inducing cell cycle arrest. This inhibition can be induced through a variety of mechanisms leading to modulation of the levels and/or activities of cyclins, CDKs, and CKIs (24, 32–34, 37, 39, 45, 46). However, there are no reports characterizing the mechanistic basis for the inhibitory effects of autocrine TGF-β versus that of exogenous TGF-β in isogenic model systems.

We have shown that autocrine TGF-β affects the regulation of cell cycle reentry in the lag phase of tissue culture but has no effect on doubling time. TGF-β affects cell cycle progression by inducing the expression and/or the activities of multiple CKIs. Specific inhibitors have their own specific CDK targets. However, they may also function collaboratively and/or concomitantly. TGF-β has been shown to inhibit cell proliferation through up-regulation of p15 (11, 41, 42), p21 (43–46) and p27 (21, 47, 48) in various cells. In our study, autocrine TGF-β induces p21 mRNA and protein expression, leading to decreased CDK2 activity and prolonged growth arrest, whereas...
expression of p27 and p15 is not affected. Thus, our data indicated that p21 is a downstream effector of autocrine TGF-β-mediated growth regulation in HCT116 cells. Other studies have shown that overexpression of p21 markedly suppressed in vivo tumorigenicity in colon and prostate carcinoma cell lines (63), and delivery of the p21 gene by an adenoviral vector significantly suppressed prostate tumor growth (64). These lines of evidence, together with our findings, suggest the potential for p21 tumor suppressive activity. However, it has been shown that exogenous TGF-β up-regulates the expression of other CKIs such as p15 (13, 41, 42) and p27 (21, 47, 48) in other cell types.

Several studies have shown that p21 expression is under transcriptional control of the tumor suppressor p53 in response to DNA damage (16, 17, 60) as well as a p53-independent mechanism (30, 44). The p53-independent induction of p21 pathway has been found to be preserved in tumor cells regardless of the status of p53 (wild type or mutant) (63). Our studies indicated that although HCT116 cells have wild type p53, p21 expression is regulated at the transcriptional level by autocrine TGF-β in a p53-independent manner.

Several mechanisms that have been suggested for CKIs to inhibit CDK activity. First, CKIs may inhibit or disrupt the cyclin-CDK complex formation (48). Second, they may block the phosphorylation of CDKs by the CDK-activating kinase (65, 66). Finally, CKIs may associate with cyclin-CDK complexes and inhibit their catalytic activity (48). In our study, CDR2-associated cyclin A and cyclin E levels are the same in NEO pool and RII Cl 37 cells, although there is a higher level of p21 in RII Cl 37 cells (Fig. 4B). This indicates that p21 neither prevents nor disrupts CDR2 complex formation. Moreover, p21 was associated with both the phosphorylated and unphospho-
Cells were harvested at the indicated time points, and Western blot assays were performed using anti-p21 (A) or anti-phosphorylated pRb (B).

**Fig. 9. p21 expression in HCT116Ch3 and Ch2 cells.** Cells were plated at 5 x 10^4 cells/well in 6-well plates. 100 μg of cell lysates was analyzed by Western blot assays using anti-p21 (A). Cells were rendered quiescent (Q) and then stimulated to reenter the cell cycle by adding fresh medium containing growth factors (TIE) in the presence or absence of 5 ng/ml TGF-β1. 5 ng/ml TGF-β1 was added 2 h before the cells were harvested. Cells were harvested at the indicated time points, and Western blot assays were performed using anti-p21 (B).

**Fig. 10. Effects of exogenous TGF-β on CDK4 expression and pRb phosphorylation in HCT116Ch3 and Ch2 cells.** Cells were plated at 5 x 10^4 cells/well in 6-well plates, rendered quiescent (Q), and then stimulated to reenter the cell cycle by adding fresh medium containing growth factors (TIE) in the presence or absence of 5 ng/ml TGF-β1. 5 ng/ml TGF-β1 was added 2 h before the cells were harvested. Cells were harvested at the indicated time points, and Western blot assays were performed using anti-CDK4 (A) or anti-phosphorylated pRb (B).

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Autocrine and Exogenous Transforming Growth Factor β Control Cell Cycle Inhibition through Pathways with Different Sensitivity
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J. Biol. Chem. 2004, 279:40237-40244.
doi: 10.1074/jbc.M401665200 originally published online July 22, 2004

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