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Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism

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ABSTRACT **The transforming growth factor βs (TGF-βs) are a group of multifunctional growth factors which inhibit cell cycle progression in many cell types. The TGF-β-induced cell cycle arrest has been partially attributed to the regulatory effects of TGF-β on both the levels and the activities of the G_1 cyclins and their kinase partners. The activities of these kinases are negatively regulated by a number of small proteins, p21 (WAF1, Cip1), $p27^{Kip1}$, p16, and $p15^{INK4B}$, that physically associate with cyclins, cyclin-dependent kinases, or cyclin–Cdk complexes. p21 has been previously shown to be transcriptionally induced by DNA damage through p53 as a mediator. We demonstrate that TGF-β also causes a rapid transcriptional induction of p21, suggesting that p21 can respond to both intracellular and extracellular signals for cell cycle arrest. In contrast to DNA damage, however, induction of p21 by TGF-β is not dependent on wild-type p53. The cell line studied in these experiments, HaCaT, contains two mutant alleles of p53, which are unable to activate transcription from the p21 promoter when overexpressed. In addition, TGF-β and p53 act through distinct elements in the p21 promoter. Taken together, these findings suggest that TGF-β can induce p21 through a p53-independent pathway. Previous findings have implicated $p27^{Kip1}$ and $p15^{INK2B}$ as effectors mediating the TGF-β growth inhibitory effect. These results demonstrate that a single extracellular antiproliferative signal, TGF-β, can act through multiple signaling pathways to elicit a growth arrest response.**

The transforming growth factor βs (TGF-βs), a group of protein hormones which regulate many cellular functions, inhibit cell proliferation by causing growth arrest in the G_1 phase of the cell cycle (1–4). Progression through G_1 is dependent on the sequential formation, activation, and subsequent inactivation of the G_1 cyclin–Cdk complexes, primarily cyclin D–Cdk4 and cyclin E–Cdk2 complexes (5, 6). The TGF-β-induced G_1 cell cycle arrest has been partially attributed to the regulatory effects of TGF-β on both the levels and activities of these G_1 cyclins and Cdks (7–9). Recently, a family of low molecular weight cyclin-dependent kinase inhibitors (CdkIs) have been shown to play essential roles in arresting cell cycle progression. These CdkIs, which include p21 (WAF1, Cip1), $p27^{Kip1}$, p16, and $p15^{INK4B}$, physically associate with their target cyclin–Cdk complexes to inhibit their activities (10–19).

p21 was first identified as a component of the quaternary complex composed of cyclin D, Cdk4, and proliferating cell nuclear antigen (PCNA) (20). Subsequent cloning and characterization indicated that p21 is under the transcriptional control of p53 (11), is transcriptionally induced by γ-irradiation (10, 21), acts as an inhibitor of multiple cyclin–Cdk complexes (11, 13, 16), and controls DNA replication by interaction with PCNA (22). Because of these characteristics, p21 is thought to be an effector for p53-mediated suppression of cell proliferation in response to DNA damage (10, 11, 16, 21–23). In comparison to p21, p27 has been shown to be induced only by extracellular signals for growth arrest. p27 was initially identified as an inhibitor of cyclin E-Cdk2 complex activity in TGF-β treated or contact inhibited mink lung epithelial cells (14). p27 has since been cloned and shown to cause a G_1 cell cycle arrest when overexpressed (24, 25). The mechanism by which p27 is activated by TGF-β is still unresolved.

On the basis of these observations, it has been postulated that these two inhibitor molecules may mediate growth arrest signals from two distinct sources: p21 responds to intracellular signals initiated by DNA damage with p53 as the mediator, whereas p27 responds to extracellular signals, such as TGF-β and cell contact (23). The fact that p21 is a potent inhibitor of cell cycle progression prompted us to test if it can also be regulated by TGF-β and consequently serve as an effector in TGF-β-mediated growth inhibition.

EXPERIMENTAL PROCEDURES

Cell Culture. HaCaT was a gift of P. Boukamp and N. Fusenig (26). SW480 cells were obtained from the American Type Culture Collection. Both types of cells were maintained in α-MEM (GIBCO/BRL) containing 10% fetal bovine serum.

Northern Blot Analysis. Approximately 5×10^6 HaCaT cells in 15-cm culture dishes were incubated in the presence or absence of 100 pM TGF-β1 for various times. Total RNA was isolated and Northern blot analysis was performed, using a 2-kb full-length human p21 cDNA or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as previously described (27). Fold induction of p21 mRNA was calculated by normalizing the levels of p21 mRNA to the level of GAPDH mRNA and comparing the amount of p21 mRNA to the amount of p21 in untreated HaCaT cells.

Immunoprecipitation and Western Blot Analysis. Approximately 1×10^6 HaCaT cells in 10-cm culture dishes were incubated in the presence or absence of 100 pM TGF-β1 for 20 hr. During the final 4 hr of treatment, cells were incubated in methionine-free medium to which 0.25 mCi (1 mCi = 37 MBq) of [^{35}S]methionine per ml (Tran^{35}S label, ICN) had been added. Cells were then lysed in 1 ml of a Nonidet P-40 lysis buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.5% Nonidet P-40) in the presence of proteinase inhibitors. Immunoprecipitations were done with 0.5 μg of polyclonal antibodies to p21 (27), Cdk2 (17), and cyclin D1 (17) from equal amounts of lysate as previously described (17). For samples in which a competing p21 peptide antigen was used, anti-p21 antibody was preincubated with 0.5 μg of a peptide containing the 15 amino-terminal amino acids of p21. For Western blot analysis,

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Abbreviations: TGF-β, transforming growth factor β; CdkI, cyclin-dependent kinase inhibitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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equal protein amounts of total cell lysate or immunoprecipitates from equal amounts of cell lysate were resolved on an SDS/15% polyacrylamide gel. Proteins were transferred onto Immobilon transfer paper (Millipore) and probed with a polyclonal anti-p21 antibody (PharMingen) as previously described (21). The blots were developed by using the ECL (enhanced chemiluminescence) detection method (Amersham).

Cdk2 Histone H1 Kinase Assay. HaCaT cells were treated as described for Cdk2 immunoprecipitation assays. Immunoprecipitated Cdk2 was washed once with kinase reaction buffer (50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$, 1 mM dithiothreitol). Kinase assays were then performed in 50 μl of the kinase buffer, 5 μg of histone H1 (Boehringer Mannheim), and 10 μCi of [γ-^{32}P]ATP at 30°C for 30 min as previously described (7). Samples were resolved on a 15% polyacrylamide gel and autoradiography was performed.

Growth Inhibition Assay. HaCaT cells were plated into 12-well plates at a density of 20,000 cells per well and treated with 100 pM TGF-β1 for various times. The cells were labeled for the last 2 hr with 4 μCi of [^{3}H]thymidine, fixed in 10% trichloroacetic acid, and lysed in 0.2 M NaOH. [^{3}H]thymidine incorporation into the DNA was measured with a scintilation counter.

Creation of p21 Promoter Deletion Constructs. The p21 promoter construct, WWP-luc, was a gift of B. Vogelstein (11). The 2.4-kb *Hin*dIII fragment of WWP-luc, containing the p21 promoter region, was inserted into a luciferase reporter construct, pGL2-basic (Promega), to create p21P. The 5′ end of the promoter was digested with the restriction enzymes *Bgl* II and *Kpn* I and a progressive deletion of the p21 promoter was performed by using the exonuclease III system, Erase-a-Base (Promega). p21PΔ1.1 was determined to have undergone a deletion of approximately 1.1 kb from the 5′ end of the p21 promoter. p21P-SmaΔ1 was created by *Sma* I digestion and religation of p21P.

Creation of a HaCaT cDNA Library and Isolation of the HaCaT Mutant Alleles of p53. Total RNA was isolated by the method of RNAzol (Biotecx Laboratories, Houston) from HaCaT cells, and poly(A)-containing RNA was further isolated by using a protocol provided by Promega. The construction, packaging, and screening of the HaCaTλ-ZAP II cDNA library were done according to the protocols of Stratagene. The probe used in the screening was a 1.2-kb p53 cDNA. A total of 20 positive clones were isolated from initial screening. Four of these clones were found to represent distinct cDNA clones containing the full-length coding region of the p53 gene with differences in the 5′ and 3′ nontranslated region. Further sequence analysis indicated that two of the clones contain a mutation at codon 282 resulting in an arginine-to-tryptophan substitution, and two clones contain a mutation at codon 179 with a histidine-to-tyrosine substitution. These results are consistent with the previously reported mutations in the HaCaT p53 alleles (29). All four cDNAs were cloned in the expression vector pCMV5 for transfection assays.

Luciferase Assays. HaCaT cells were plated into six-well plates at a density of 200,000 cells per well and grown overnight. Cells were transfected with 6 μg of p21P, p21PΔ1.1, or p21P-SmaΔ1, using DEAE-dextran as described (28). Six hours after transfection cells were incubated in the presence or absence of 100 pM TGF-β1 for 20 hr. Cells were lysed and the amount of luciferase activity in the lysates was assayed by integrating total light emission over 30 sec, using a Berthold luminometer (28).

SW480 cells were plated similarly and cotransfected with 2 μg of reporter construct (p21P, p21PΔ1.1, or p21P-SmaΔ1) and 0.5 μg of the wild-type p53 construct, p11-4 (30), 0.5 μg of the HaCaT mutant p53 constructs, or 0.5 μg of the vector, pCMV5; 0.5 μg of a β-galactosidase reporter construct was included to normalize luciferase activity to transfection efficiency. Transfections were performed by using the standard $Ca_2(PO_4)_3$ DNA precipitation method. Luciferase activity was assayed 24 hr after transfection. Fold induction was calculated by normalizing luciferase activity to β-galactosidase activity and comparing the luciferase activity in SW480 cells cotransfected with either the wild-type or the mutant p53 constructs with those cotransfected with the vector.

RESULTS

p21 mRNA and Protein Levels Increase upon TGF-β Treatment of HaCaT Cells. Previous studies have shown that p21 is transcriptionally activated by DNA damage, and an accumulation of p21 mRNA ultimately leads to inhibition of the activity of the G_1 cyclin–Cdk complexes (11, 13, 16). To investigate whether TGF-β also affects the steady-state level of p21 mRNA, we studied the effects of TGF-β on p21 in HaCaT (26), a cell line of spontaneously immortalized human keratinocytes which can be growth arrested by TGF-β1 in G_1 (8). An asynchronous population of HaCaT cells was treated with TGF-β1 for 20 hr, and Northern blot analysis was performed. TGF-β treatment resulted in an approximately 6- to 7-fold increase in the steady-state level of p21 mRNA as compared

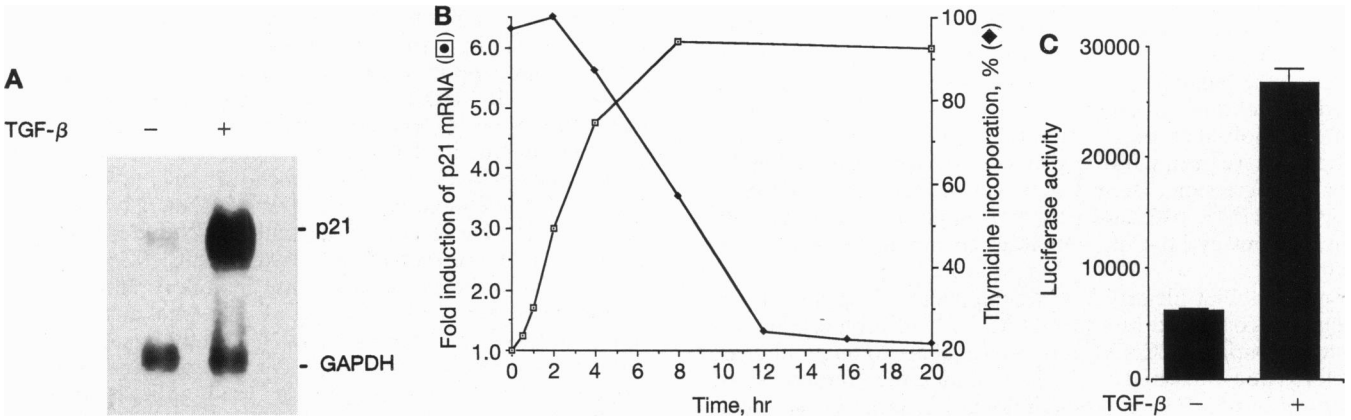

FIG. 1. Induction of p21 expression by TGF-β. (*A*) Effect of TGF-β on the steady-state level of p21 mRNA. Northern blot analysis was performed on RNA samples derived from TGF-β-treated and -untreated cells. Equal loading was confirmed by ethidium bromide staining and hybridization with a rat GAPDH mRNA probe. (*B*) Time course of p21 mRNA induction by TGF-β. RNA samples were isolated from asynchronously growing HaCaT cells at different times after treatment with TGF-β1 and Northern blot analysis for p21 was performed. [^{3}H]Thymidine incorporation was measured on similarly treated cells; incorporation is relative to cells not treated with TGF-β. (*C*) Transcriptional activation of the p21 promoter by TGF-β. Cells were transiently transfected with 6 μg of WWP-Luc (11) and TGF-β-induced luciferase activity was measured. Error bars represent the average deviation of two separate measurements of two transfections in a single experiment.

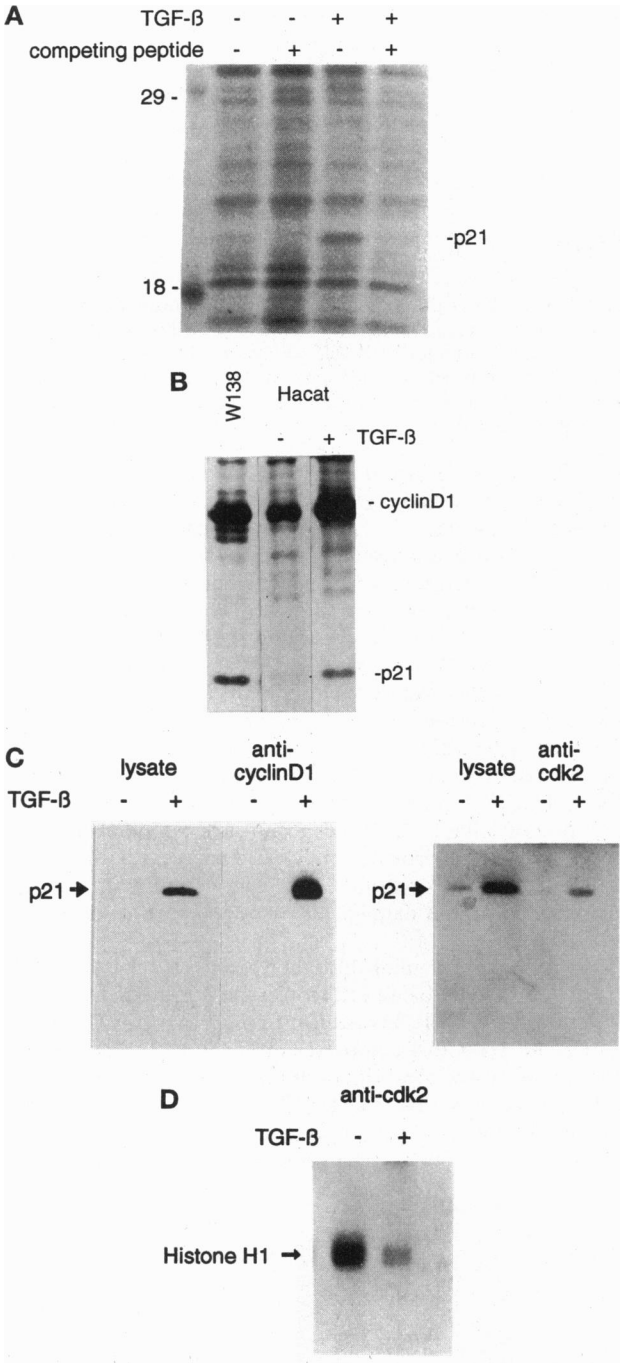

FIG. 2. Effect of TGF-β on the level of p21 protein. (*A*) TGF-β treatment increases the level of p21 protein. Asynchronously growing HaCaT cells were incubated in presence or absence of TGF-β1 for 20 hr, metabolically labeled with [^{35}S]methionine, and immunoprecipitated with antisera against human p21 in the presence or absence of a competing p21 peptide antigen. Positions of 18- and 29-kDa markers are indicated on the left. (*B*) Association of p21 with cyclin D1 upon TGF-β treatment. HaCaT cells were synchronized to a quiescent state by growth to confluency followed by 3 days of serum starvation in 0.2% fetal bovine serum (FBS) in α-MEM. Cells were then split to 10% FBS in α-MEM in the presence or absence of 100 pM TGF-β1. After 16 hr, cells were metabolically labeled and immunoprecipitations were performed with antisera specific to human cyclin D1. Asynchronously growing human diploid fibroblast W138 cells were analyzed in parallel as a control (16). Similar results were obtained when asynchronously growing HaCaT cells were used in the analysis. (*C*) Western blot analysis of p21 protein levels on whole cell lysate and immunoprecipitations with antibodies to cyclin D1 and Cdk2 in TGF-β-untreated and -treated HaCaT cells. (*D*) Histone H1 kinase activity of Cdk2 immunoprecipitates in TGF-β-treated and -untreated HaCaT cells.

with untreated cells (Fig. 1*A*). p21 mRNA induction is rapid; an increase in p21 mRNA was observed as early as 1 hr after TGF-β treatment. The steady-state level of p21 mRNA continues to increase for several hours after TGF-β addition and reaches a plateau 8 hr later. The time course of p21 induction parallels an inhibition of DNA synthesis in these cells upon TGF-β treatment as assayed by [^{3}H]thymidine incorporation (Fig. 1*B*).

To determine the mechanism by which TGF-β induces the accumulation of p21 mRNA, a plasmid construct harboring a luciferase reporter gene under the transcriptional control of the p21 promoter, WWP-Luc (11), was transfected into HaCaT, and TGF-β-induced luciferase activity was measured. TGF-β treatment of transfected cells resulted in a 4- to 5-fold increase in luciferase activity (Fig. 1*C*). This result suggests that TGF-β-induced accumulation of p21 mRNA is in part a consequence of transcriptional activation of the p21 gene.

We next examined if the observed transcriptional activation of the p21 gene leads to an increase in the p21 protein level. Immunoprecipitation of ^{35}S metabolically labeled HaCaT cell lysates with a polyclonal anti-p21 antibody revealed a significant increase of p21 protein in cells treated with TGF-β (Fig. 2*A*). Western blot analysis of p21 further confirmed this increase in p21 protein upon TGF-β treatment (Fig. 2*C*). HaCaT cell lysates were next immunoprecipitated with a variety of cyclin and Cdk antibodies to determine if the TGF-β-induced increase in p21 protein leads to association of p21 with its cyclin or Cdk targets. When [^{35}S]methionine metabolically labeled cell lysates were immunoprecipitated with antibodies against a number of G_1 cyclins and Cdks, we found that the level of p21 associated with cyclin D1 was significantly increased in TGF-β-treated cells in comparison with untreated cells (Fig. 2*B*). On Western blot analysis for p21, we also observed that TGF-β treatment results in an increased association of p21 with both cyclin D1 and Cdk2 (Fig. 2*C*).

Since p21 has been previously shown to inhibit the kinase activity of cyclin E–Cdk2 complexes, we analyzed the activity of Cdk-2 upon TGF-β treatment. Cdk2 was immunoprecipitated from both TGF-β-treated and -untreated HaCaT cells, and the activity of Cdk2 was assayed by measuring its ability to phosphorylate an exogenous substrate, histone H1. As shown in Fig. 2*D*, TGF-β treatment resulted in a marked decrease in Cdk2 kinase activity. This observed decrease in Cdk2 kinase activity on TGF-β treatment is consistent with the possibility that it is a result of p21 association. It is also possible, however, that this decrease in Cdk2 kinase activity is a result of the activation of multiple CdkIs, including p21, p15, and p27, which may all be capable of inhibiting Cdk2 activity (14, 19, 24, 25).

TGF-β Induces p21 Through a p53-Independent Mechanism. Similar to the observed TGF-β induction of p21, DNA damage has been previously shown to cause a transcriptional activation of p21. This induction is dependent on the presence of a functional p53 (10, 21). To determine the role of p53 in TGF-β-mediated induction of p21, we studied the regions of the p21 promoter responsible for its activation both by p53 and by TGF-β. As described in *Experimental Procedures*, a series of 5′ deletion constructs of the p21 promoter were created. One of these constructs (p21PΔ1.1) has undergone a 1.1-kb deletion from the 5′ region of the 2.4-kb p21 promoter, removing the previously described consensus p53-responsive element (11). A second deletion construct of the p21 promoter (p21P-SmaΔ1) contains only 60 bp of DNA proximal to the previously defined transcription start site (11).

HaCaT cells were transfected with these two reporter constructs and TGF-β-induced luciferase activity was measured. As shown in Fig. 3, the full-length p21 promoter construct (p21P) and the 1.1-kb deletion construct (p21PΔ1.1) showed identical extents of induction by TGF-β. This is in contrast to the minimal promoter construct (p21P-SmaΔ1)

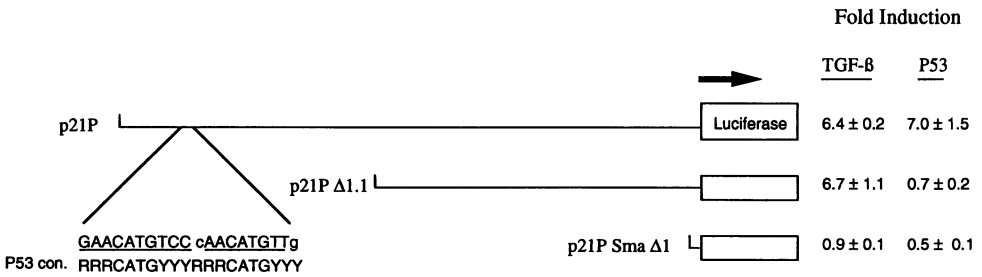

FIG. 3. TGF-β-inducible and p53-inducible elements in the p21 promoter are distinct. Transcriptional activation of the p21 promoter constructs by TGF-β was determined by transiently transfecting HaCaT cells with 6 μg of WWP-Luc and measuring TGF-β-induced luciferase activity. Fold induction was determined by comparing luciferase activity of TGF-β-treated cells to that of untreated cells and was averaged among four separate transfections in two different experiments. Activation of the p21 promoter constructs by p53 was determined by cotransfecting 2 μg of the promoter constructs with 0.5 μg of p11-4 into SW480 cells and measuring luciferase activity. Fold induction was determined by comparing luciferase activity in cells transfected with p11-4 to cells transfected with vector and averaging among four separate transfections in two different experiments.

which showed no induction by TGF-β (Fig. 3). We next assayed the ability of these constructs to be induced by overexpression of p53 in SW480 cells, a colon carcinoma cell line which has been used to assess the activity of p53 (11). The full-length p21 promoter construct (p21P) was activated 7-fold over vector control by overexpression of the wild-type p53. This is in contrast to the two deletion constructs, which are not induced by p53 overexpression (Fig. 3). From these results, we concluded that the sites responsible for p53-mediated induction of the 2.4-kb p21 promoter region are in the 1.1-kb region deleted in p21PΔ1.1. The sites responsible for activation by TGF-β, however, are in the 3′ 1.3-kb half of this promoter. Thus, the regions of the promoter responsible for the induction by TGF-β and p53 overexpression are distinct.

To further investigate the role of p53 in TGF-β-mediated induction of p21, we examined the functional nature of the HaCaT endogenous p53 alleles. It has been reported that HaCaT possesses two mutant alleles of p53, each containing a single amino acid substitution (29). Both of these residues could play important structural and functional roles (31), as supported by the observation that they are mutated at a significantly increased frequency in various types of tumor cells (32, 33). To confirm that HaCaT expresses these mutant alleles of p53, we created a HaCaT cDNA library and isolated both mutant alleles of p53. By sequence analysis, these mutant p53 cDNAs were determined to represent individual clones which contain the entire coding region for p53, as well as the two specific point mutations, as previously described (29). To study the functional consequences of these mutations, the cDNAs were inserted into the expression vector pCMV5 and their function was studied by cotransfection with the full-length p21 promoter construct in SW480 cells (11). As shown in Fig. 4, wild-type p53 activated the p21 promoter by approximately 6-fold. In contrast, the two HaCaT mutant alleles of p53 failed to activate the p21 promoter when overexpressed. Identical results were obtained when an additional independent clone for each mutant allele of p53 was used. These results suggest that both alleles of p53 expressed in HaCaT cells are incapable of transcriptionally activating the p21 promoter and support a hypothesis that TGF-β activates the p21 promoter through a p53-independent pathway.

DISCUSSION

It has been proposed that TGF-β causes a G_1 cell cycle arrest by regulating the activity of the G_1 cyclin–Cdk complexes, primarily cyclin D–Cdk4 and cyclin E–Cdk2 (7–9). We present a possible mechanism through which TGF-β may act to inhibit the activities of these complexes. Our results demonstrate that TGF-β causes a rapid and significant induction of a cyclin–Cdk inhibitor, p21. This induction of p21 leads to its increased association with at least two of its G_1 targets, cyclin D1 and Cdk2. It has been previously reported that p21 can bind to and inhibit the kinase activity of both cyclin D–Cdk4 and cyclin E–Cdk2 complexes (11, 13, 16, 18). Thus, the observed increase in p21 level and its association with cyclin D1 and Cdk2 upon TGF-β treatment may be sufficient to inhibit the activities of these complexes. In support of this, a decrease in the kinase activity of Cdk2 was also observed upon TGF-β treatment. Our results also demonstrate that the induction of p21 by TGF-β is concurrent with an inhibition of cell entry into S phase. These results, taken together with previous findings which implicate a similar involvement of p21 in DNA damage-induced cell cycle arrest (10, 21), suggest that the induction of p21 by TGF-β may play a causative role in TGF-β-mediated inhibition of cell growth. These results also support a model in which an individual Cdk inhibitor, p21, can serve as an effector for diverse signals for cell cycle arrest, including intracellular signals such as DNA damage and extracellular signals, such as TGF-β.

Previous work in mink lung epithelial cells has suggested that p27 may serve as an effector in TGF-β-mediated growth inhibition (14, 24). It has been proposed that p27 is released from cyclin D1–Cdk4 complexes upon TGF-β treatment and subsequently associates with cyclin E-Cdk2 complexes to block its kinase activity (14, 23, 24). Recently, $p15^{INK4B}$, another inhibitor of the G1 cyclin-Cdk complexes, was found to be induced by TGF-β to associate with Cdk4 and Cdk6 complexes

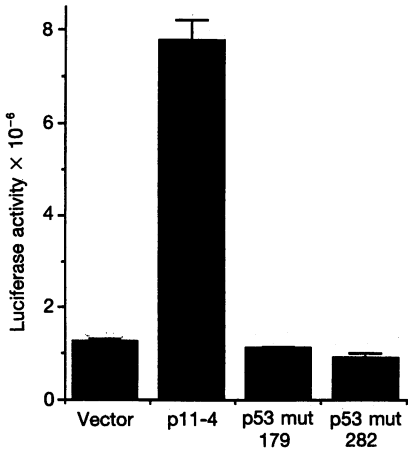

FIG. 4. Functional analysis of the HaCaT alleles of p53. Activation of the full-length p21 promoter construct by p53 and by the two mutant HaCaT alleles of p53 (His-179 → Tyr and Arg-282 → Trp) was determined by cotransfecting 2 μg of the promoter constructs with 0.5 μg of the p53 expression constructs into SW480 and measuring luciferase activity. Results shown are of a representative experiment. Error bars represent the average deviation of two measurements of two separate transfections in a single experiment.

in HaCaT cells (19). These results, together with our findings, clearly support the idea that TGF-β may affect cell cycle progression by inducing the activities of multiple CdkIs through at least two different mechanisms. These inhibitors, p21, p27, and p15, may serve both unique and overlapping functions in inhibiting the activities or the activation of the G_1 cyclin–Cdk complexes in response to TGF-β. Specific inhibitors may function preferentially to inhibit specific complexes. Thus, the combined action of multiple inhibitors may be necessary to completely inhibit the progression of cells into S phase. Conversely, each inhibitor alone may be sufficient to block the activity of G_1 cyclin–Cdk complexes and cause cell cycle arrest. In this case, simultaneous induction of several inhibitors by TGF-β may have evolved to serve as a functional safeguard to ensure a proper response to a growth-inhibitory signal. It is also possible that different individual inhibitors act in different cell types or under different growth conditions to mediate the growth-inhibitory effects of TGF-β.

The TGF-β-mediated induction of p21 in HaCaT cells reported here is not a unique phenomenon. We have recently observed a similar induction of p21 expression by TGF-β in MCF-7 breast epithelial carcinoma cells stably expressing the TGF-β type II receptor (ref. 34; M.B.D. and X.-F.W., unpublished results). In addition, the TGF-β-mediated p21 induction has been reported in TGF-β-responsive ovarian cancer cell lines (35).

In assessing the potential role of p53 in TGF-β-mediated induction of p21, we studied the specific regions in the p21 promoter which are responsible for the transcriptional activation of p21 by TGF-β and by p53. We have found that the promoter elements necessary for the induction of p21 by TGF-β and by p53 are physically unlinked. We have since determined that the TGF-β-responsive element is harbored in a 30-bp DNA fragment near the transcription initiation site of the p21 promoter (M.B.D. and X.-F.W., unpublished results). These results suggest that TGF-β transcriptionally activates p21 through a p53-independent mechanism. This tentative conclusion is further supported by our confirmation that both alleles of p53 in HaCaT cells encode mutant p53 proteins which are incapable of transcriptionally activating its target genes.

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