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The MEK Pathway Is Required for Stimulation of $p21^{WAF1/CIP1}$ by Transforming Growth Factor-β*

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Patrick Pei-chih Hu‡, Xing Shen‡, David Huang, Yueyi Liu, Christopher Counter, and Xiao-Fan Wang§

From the Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Transforming growth factor-β (TGF-β)can induce the cyclin-dependent kinase inhibitors p21 and p15 in a variety of cell types. We have shown previously that Smad3 is required for the growth inhibitory activity of TGF-β, whereas overexpression of Smads is not sufficient to activate the expression of p21 in HaCaT cells. These data suggest that an additional signaling pathway may be involved in stimulating p21 in HaCaT cells. Given the recent finding that the mitogen-activated protein kinase (MAPK) pathway can cause p21 induction and arrest cells, we examined the involvement of this pathway for p21 and p15 induction by TGF-β. We found that TGF-β can regulate the MAPK pathway, leading to the increased transactivation ability of transcription factor Elk. Constitutively active components in the MAPK pathway activate p21 expression, and inhibitors or dominant negative constructs for the MAPK pathway significantly decrease p21 induction by TGF-β. Both constitutively active MEK and inhibitors for MEK have no effect on Smad activity, including DNA binding, localization, and interaction with coactivator p300/CBP. These findings suggest that the MAPK pathway may be an independent pathway that is involved in p21 and p15 induction by TGF-β.

Transforming growth factor-β is a cytokine that causes growth arrest in late G_1 of the cell cycle (reviewed in Refs. 1 and 2). In the human keratinocyte cell line HaCaT, growth inhibition is thought to depend on the ability of TGF-β[1] to increase synthesis of the cyclin-dependent kinase inhibitors p21 and p15. Increased p21 leads to an increase in its association with cyclin D-CDK4 and cyclin E-CDK2 and a decrease in cyclin-CDK complex activity. Increased p15 expression leads to an increase in association and sequestration of CDK4 and CDK6 from the regulatory cyclin partners.

On the cell membrane, TGF-β ligand binds to the type II receptor, which recruits the type I receptor into the complex. The type I receptor is phosphorylated by the type II receptor kinase and, when activated, phosphorylates Smad2 and Smad3, allowing them to heteromerize with Smad4 and translocate into the nucleus. In the nucleus, Smad3 can bind to DNA with the consensus sequence GTCTAGAC that is found in the promoters of many TGF-β-regulated genes (3–8), as well as to other proteins such as the transcriptional coactivators p300 and CBP (9–12) in a phosphorylation-dependent manner. Smad3 is also required for TGF-β-mediated growth inhibition because murine embryonic fibroblasts deficient in Smad3 have lost their ability to become growth inhibited by TGF-β (13).

Although Smads are required for TGF-β-mediated growth arrest, the mechanism underlying their action is still unknown. In HaCaT cells, TGF-β induction of the cyclin-dependent kinase inhibitors p21 and p15 correlates with both Smad nuclear translocation as well as growth arrest, but when Smad3 and Smad4 are overexpressed, they are unable to activate p21 and p15 transcription. In contrast, overexpression of Smad3 and Smad4 is sufficient to activate transcription of plasminogen activator inhibitor (14), which contains Smad-binding elements in its promoter region (4, 15). This suggests that even though Smads may be required for growth inhibition by TGF-β, they are not sufficient to drive the transcription of p21 and p15, indicating that other pathways may also be needed for the transcriptional induction of p21 and p15 upon TGF-β treatment.

Contrary to their role as the mediators of many mitogens, the MAPKs have been recently implicated in cellular growth arrest and senescence. Overexpression of Raf leads to growth arrest by impinging directly on the cell cycle through increasing p21 expression, leading to its association and concomitant inhibition of CDKs (16, 17). In addition, altering the cellular ratio of existing Ras and Rho proteins can also lead to stimulation of p21 (18). TGF-β can also increase GTP-bound Ras, and activate MAPK pathway in other cell types (19). Because activation of the Ras-Raf-MEK-MAPK pathway can induce transcription of p21, in this study we asked whether this pathway was involved in the ability of TGF-β to stimulate p21 and p15 expression.

Here, we show that overexpression of constitutively active forms of different components in the MAPK cascade can substitute for the ability of TGF-β to stimulate p15 and p21 reporter activity. Stable lines overexpressing a constitutively active form of MEK, MEK Q56P (20), also have increased expression of p21. Furthermore, inhibitors of this pathway prevent TGF-β induction of p21 and reduce TGF-β-mediated growth arrest in parental HaCaT cells. In contrast, TGF-β activation of Smad signaling is not affected by these inhibitors. Taken together, these data suggest that the MAPK pathway is involved in p21 up-regulation by TGF-β.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Culture—The human p21 and p15 minimal promoter constructs, p21P93S and p15P113S, have been described previously (21). GAL4-Sp1 and GAL4-Sp3 full-length, GAL4-Sp1, and Sp3 B domain constructs have been were described previously (22). GAL4-Elk,

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‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed: Dept. of Pharmacology and Cancer Biology, Box 3813, Duke University Medical Center, Durham, NC 27710. Tel.: 919-681-4861; Fax: 919-681-7152; E-mail: wang@galactose.mc.duke.edu.

[1] The abbreviations used are: TGF-β, transforming growth factor-β; MAPK, mitogen-activated protein kinase; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

Ras 17A, Raf-N4, Raf-CAAX, MEK S218/222D, Rac 17N, cdc42 17N, MKK4/SEK-1, and SEK-AL constructs were the generous gift of Dr. Channing Der. The MEK Q56P pBABE-puro construct was the generous gift of Dr. Scott Lowe. HaCaT cells were the gift of Dr. Baukamp and Dr. Fusenig. HaCaT were maintained in 10% fetal bovine serum (Life Technologies, Inc) in α-minimum essential medium (Life Technologies, Inc.).

Luciferase Assays—For luciferase assays, cells were lysed, and luciferase activity in the lysates were assayed by integrating total light emission over 30 s using a Berthold luminometer. The luciferase activities were normalized based on the amount of expressed β-galactosidase activity expressed from co-transfected pCMV-β-galactosidase.

Western Blot—Cells were lysed in universal lysis buffer (ULB) as described previously (10). Equal protein amounts of each sample were resolved on a 12% acrylamide-0.5% bisacrylamide sodium dodecyl sulfate gel. Proteins were transferred to Immobilon P transfer membrane (Millipore Corp.). Blocking and antibody incubations were performed in 5% dried milk and 0.1% Tween 20 in phosphate-buffered saline for 1 h at room temperature. Antibodies used were: anti-p21 (Santa Cruz Biotechnology, C-19), p15 (Santa Cruz Biotechnology, C-20), anti-Smad-3 (Santa Cruz Biotechnology, H-2), and horseradish peroxidase-conjugated goat-anti-rabbit (Bio-Rad). Western blots were developed with ECL reagent (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay—Complementary oligonucleotides corresponding to two copies of the Smad3-binding consensus element (GTCTAGAC) were synthesized. Electrophoretic mobility shift assays and nuclear extracts preparation were performed as described previously (8).

MEK Q56P Retroviral Infection—The MEK Q56P DNA construct inserted into the retroviral vector pBABE puro was a generous gift from Scott Lowe. Amphotrophic pBabe-puro-MEK Q56P retrovirus was then created using standard techniques (23). A control retrovirus was also generated. HaCaT cells were infected with the retroviruses and grown 10% fetal bovine serum α-minimum essential medium supplemented with 0.6 μg/ml puromycin for (pBabe-puro-MEK Q56P) or 300 μg/ml hygromycin (control) for several days.

Immunostaining—Cells were plated on coverslips and grown overnight. The next day, cells were treated with TGF-β for 30 min, fixed in paraformaldehyde for 10 min at room temperature, and permeabilized in 0.5% Triton X-100 for 5 min. Slips were then incubated in primary (anti-Smad-3, Santa Cruz Biotechnology, H-2) and secondary antibody for 1 h at 37 °C with frequent washes. Slips were then mounted and photographs taken on a Zeiss confocal microscope.

Immunoprecipitation and GST Pull-down—Approximately 200 μg of proteins from cells lysed in ULB were incubated with protein A (Amersham Pharmacia Biotech) and α-Smad3 (Santa Cruz Biotechnology) for immunoprecipitation experiments or glutathione-Sepharose bound GST-p300C (20 μg) for GST pull-down experiments. Binding reactions were for 2 h at 4 °C with continual rotation. The beads were collected and washed three times (3 min/wash) with ULB. Bound proteins were eluted by boiling in 1× Laemmli sample buffer, separated by SDS-PAGE, and subjected to immunoblot analysis.

RESULTS

TGF-β Stimulates Elk-dependent Transcription—Activation of the MAPK pathway leads to phosphorylation of transcription factor substrates such as Elk-1, c-Jun, and c-Myc and subsequently increases their transactivation potential (reviewed in Refs. 24 and 25). Elk-1 appears to be a good substrate for ERK1/2 *in vitro*, and its phosphorylation kinetics correlate with MAPK activation. To investigate whether MAPK pathway is activated upon TGF-β treatment, a GAL4-protein fused to Elk-1 and a reporter containing five concatemerized GAL4 sites (5× GAL4) in its promoter were transfected into HaCaT cell. As shown in Fig. 1, TGF-β treatment of transfected cells leads to increased luciferase expression from the 5× GAL4 reporter. This result suggests that TGF-β is potentially able to regulate and activate the MAPK pathway in HaCaT cells.

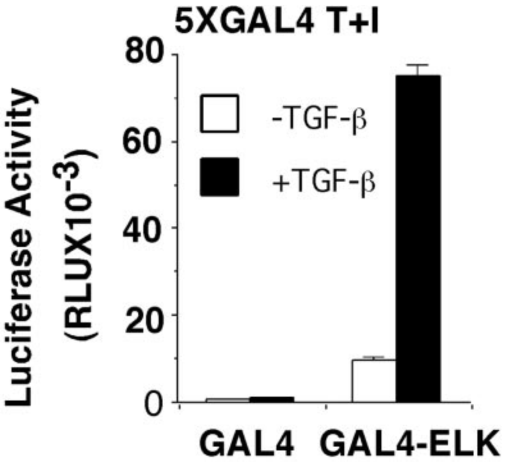

FIG. 1. **GAL4-Elk transactivation increased upon TGF-β treatment.** Duplicate experiments were performed in HaCaT cells by co-transfecting a 5× GAL4 minimal promoter attached to a luciferase reporter (*5XGAL4*) with GAL4 vector alone or GAL4-Elk using DEAE-dextran as described under "Experimental Procedures." 12 h after glycerol shock, the cells were left untreated or treated with TGF-β for 20–24 h and harvested. Luciferase and β-galactosidase activity were measured from lysates.

Constitutively Active Components of MAPK Pathway Can Substitute for TGF-β to Induce p21 and p15—Although overexpression of Smad3 and Smad4 was not sufficient to induce p21 reporter activity, the recent discovery that activation of the Ras pathway could stimulate p21 expression prompted us to test this observation in HaCaT cells, our model system for studying TGF-β signaling. To ascertain whether the MAPK pathway was sufficient to induce p21 reporter activity, we overexpressed a constitutively activated form of MEK (S218/222E) in HaCaT cells and treated the transfected cells with TGF-β. As shown in Fig. 2*A*, overexpression of the activated MEK1 not only dramatically increases basal p21 and p15 reporter activity in the absence of TGF-β but also almost completely substitutes for the TGF-β effect, suggesting that TGF-β signaling may overlap with this pathway to stimulate p21 transcription. Because the responsive element in the p21 promoter has been mapped to a GC-rich, Sp1-binding element (21) and GAL4-Sp1 but not GAL4-Sp3 (22) can mediate TGF-β-dependent transcription when using the 5× GAL4 reporter, we tested whether MEK could stimulate GAL4-Sp1 or GAL4-Sp3-mediated transcription. As shown in Fig. 2*B*, GAL4-Sp1 but not GAL4-Sp3 mediated transcription activation with expression of the constitutively active MEK (S218/222E). This response parallels the effect of TGF-β.

To determine whether overexpression of other members of the Ras-Raf-MEK-MAPK pathway were also able to sufficiently induce p15 and p21 promoter activities, we performed similar experiments with a constitutively membrane-bound form of Raf (Raf-CAAX) and Ras (Ras61L). For both p21 and GAL4-Sp1-dependent transcription, the constitutively active form of Raf activated transcription robustly (Fig. 2). Ras61L also activated transcription from p21 promoter, although the effect was not as strong as that of Raf-CAAX (data not shown). This difference in the ability of Ras and Raf-1 mutants to activate the p21 promoter may be the result of a difference in either the expression levels of those proteins or their intrinsic activity. In contrast, overexpression of MKK4/SEK1 and MKK4 dominant negatives (SEKAL) did not influence basal or TGF-β-dependent p21 reporter activity (Fig. 2).

To determine whether overexpression of MEK could increase endogenous expression of p21, we used a retroviral construct containing a MEK point mutant Q56P that is constitutively active (20). Stable mass populations of infected human fibroblasts with this retrovirus after selection with puromycin express higher levels of p21 (20). Similar results were obtained in our stable mass populations of HaCaT cells with an increase in endogenous p21 protein levels (Fig. 3*A*). Interestingly, although the p21 levels are highly abundant in these cells, they

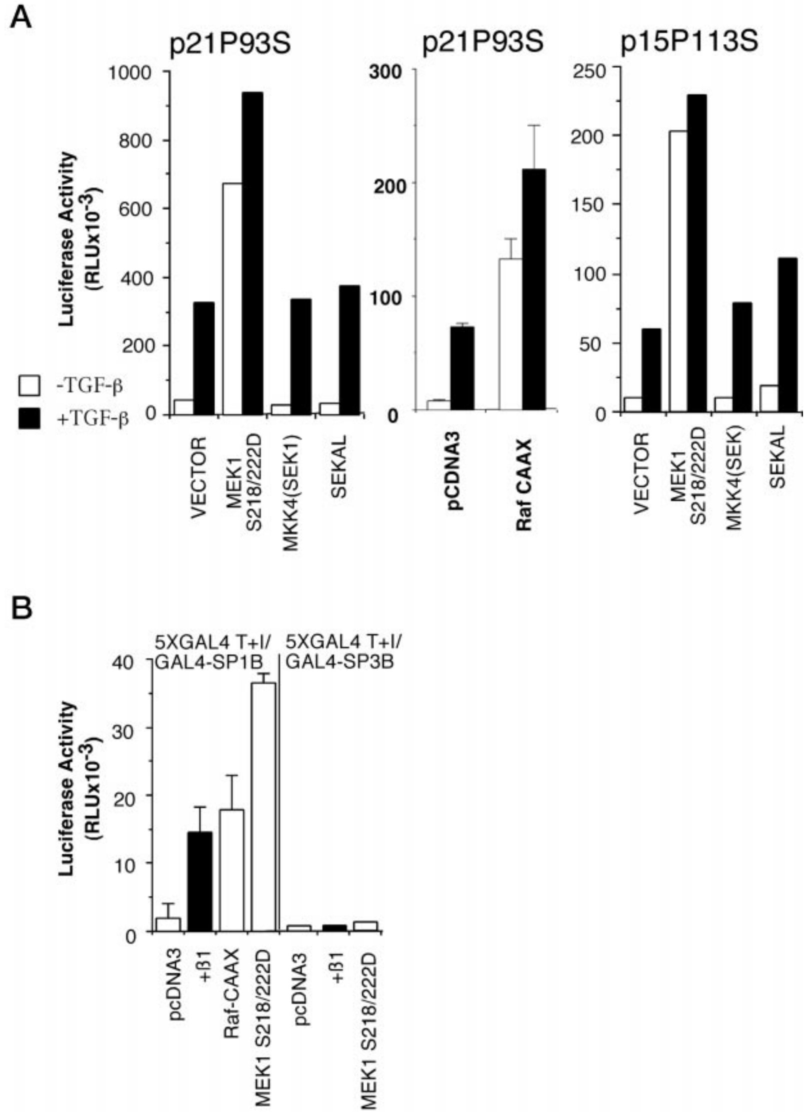

FIG. 2. **Constitutively active Raf and MEK stimulate p21 and p15 reporter activity as well as Sp1 transactivation.** *A*, duplicate transfections were performed as described in Fig. 1, co-transfecting either constitutively active MEK1 (S218/222D), Raf-CAAX, MKK4 (SEK1), or SEK-AL with either the p21 or p15 reporter constructs. 12 h after glycerol shock, cells were treated with TGF-β and harvested. Luciferase and β-galactosidase activity was then measured. *B*, duplicate experiments were performed as in *A*, except that the GAL4-Sp1B domain was also co-transfected with the 5× GAL4 reporter.

remain viable and appear to divide as rapidly as parental HaCaT cells. However, morphologically, these cells are larger, and there seems to be an increase in extracellular matrix deposition (data not shown).

Recent reports suggest that TGF-β and other ligands such as epidermal growth factor and hepatocyte growth factor can induce Smad-2 phosphorylation, translocation, and nuclear function through the MAPKs (26). Others suggest that activation of the MAPKs can lead to a Smad phosphorylation event that prevents its nuclear localization (27). With our MEK Q56P-infected HaCaT cells, we were able to investigate whether constitutively active MEK could affect Smad signaling and therefore to determine whether the MAPK pathway lay upstream of Smad signaling or could cross-talk and inhibit Smad function. We found that HaCaT cells infected with MEK Q56P behave normally in regard to Smad activity. Upon TGF-β treatment, an inducible complex is still observed in the electrophoretic mobility shift assay with the Smad-binding element as a probe (Fig. 3*B*). In addition, the overexpression of MEK Q56P in those cells does not influence the ability of Smads to translocate into the nucleus upon TGF-β treatment (Fig. 3*C*). To determine whether TGF-β could exert growth inhibitory effect on those cells, the amount of [^{3}H]thymidine incorporated during a 2-h period was measured after 18 h TGF-β treatment. With Smad signaling not affected by MEK Q56P overexpression, it is not surprising that TGF-β can still potently inhibit the proliferation of those cells (Fig. 3*D*).

Inhibitors of Ras, Raf, and MEK-1 Block p21 and p15 Induction by TGF-β—To determine whether the Ras-Raf-MEK-MAPK cascade was required for the ability of TGF-β to induce p21, p15, as well as GAL4-Sp1-mediated transcription, dominant negative Ras (Ras 17A) or Raf (Raf-N4) were co-expressed in HaCaT cells with the p21 minimal promoter reporter (p21P93S), the p15 minimal promoter reporter (p15P113S), or GAL4-Sp1B with the 5× GAL4 minimal reporter and treated with TGF-β for 20–24 h. As shown in Fig. 4*A*, the ability of TGF-β to stimulate p21 and p15 promoters was abrogated by co-expression of the dominant negative Ras. Raf-1 dominant negative has a similar effect on the p15 promoter, but to p21 promoter the effect is not as dramatic as that of the Ras dominant negative (Fig. 4*A*). Again, this difference in the ability of Ras and Raf-1 dominant negative mutants to block the TGF-β-mediated induction of those promoters may be the result of a difference in either the expression levels of those proteins or their intrinsic activity. Nevertheless, these results suggest that both Ras and Raf are potentially required for TGF-β to stimulate promoter activities of p15 and p21. As controls, the same experiments were performed with a Cdc42 17N dominant negative as well as a Rac 17N dominant negative; in both cases the ability of TGF-β to induce the p21

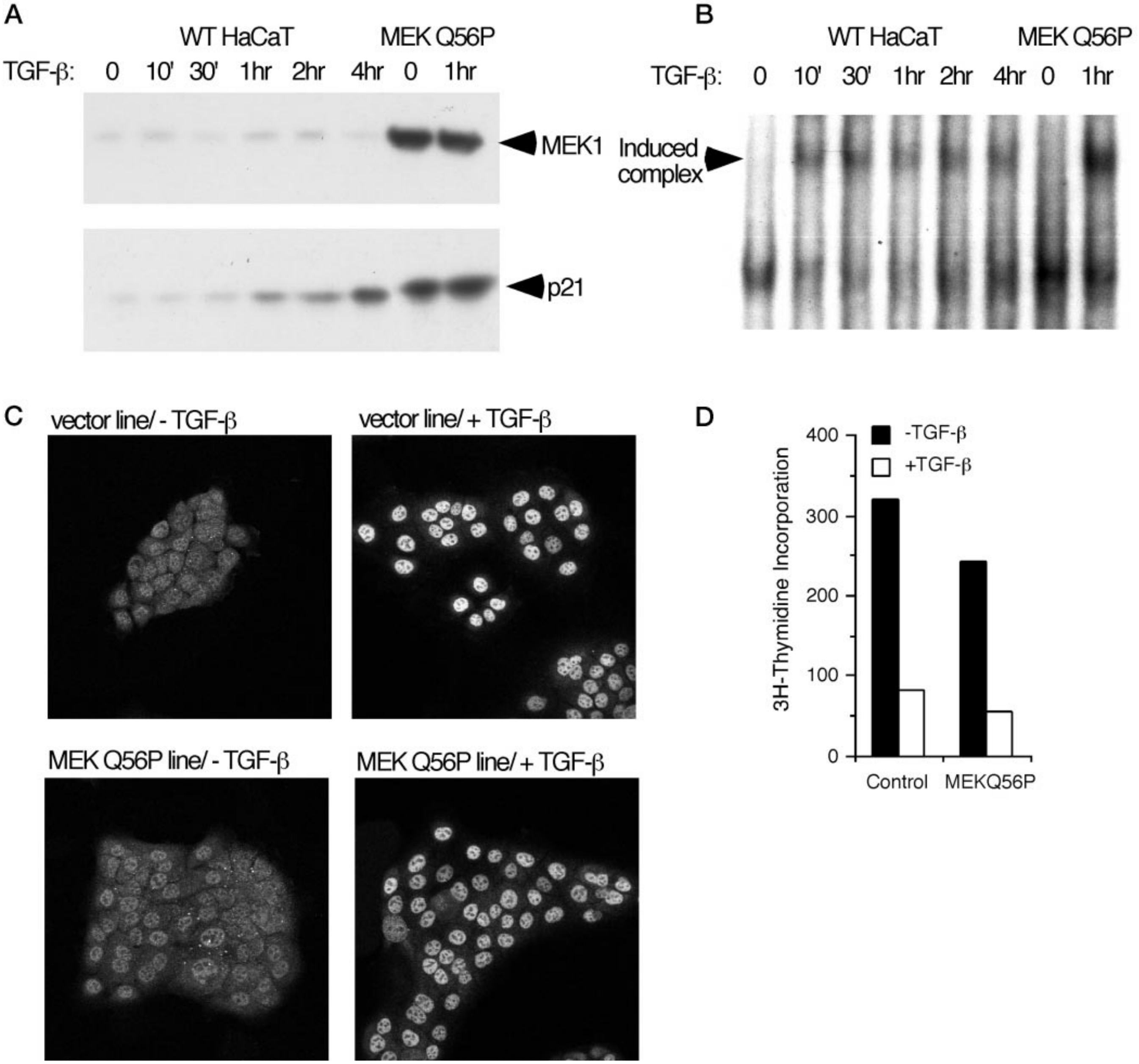

FIG. 3. **MEK Q56P-infected HaCaT cells increase p21 expression but does not influence Smad activity.** *A*, amphotrophic retrovirus was generated containing MEK Q56P and infected into parental HaCaT cells. After selection in 0.6 μg/ml puromycin for several days, the cells were lysed and compared with wild type HaCaT cells by Western blot to ascertain endogenous MEK and p21 levels. *B*, the same lysates as in *A* were incubated with radiolabeled Smad-binding elements in an electrophoretic mobility shift assay analysis, and after gel-electrophoresis, the gel was dried and autoradiographed. The induced complex corresponds to previously established Smad-containing DNA complexes. *C*, MEK Q56P-infected cells as well as control infected cells were treated with TGF-β or left untreated for 30 min. After fixing in paraformaldehyde and permeabilization in 0.5% Triton X-100, coverslips were incubated with α-Smad3 to determine Smad3 nuclear localization. *D*, experiments were performed in triplicate with cells from MEK Q56P infection as well as control cells infected with vector alone that were treated with TGF-β or left untreated for 18 h. During the last 2 h, the cells were incubated in [^{3}H]thymidine. Cells were then washed twice in phosphate-buffered saline and incubated in 10% trichloracetic acid before lysing in 0.2 N NaOH. Samples were then mixed with 4 ml of SafetySolve, and [^{3}H]thymidine incorporation was measured.

reporter was not affected (Fig. 4*B*). This is similar to a previously reported study where a dominant negative Ras (17N) can block the ability of TGF-β3 to induce p27 and p21 in epithelial cells (28).

Next, we asked whether MEK was required for TGF-β to stimulate the expression of endogenous p21 and p15. A new MEK inhibitor, U0126 (Promega), was recently developed and found to be more potent than the MEK inhibitor, PD98059, at specifically blocking MEK and ERK activity, without affecting the activity of other ERK family members, p38 and JNK. The availability of this compound allowed us to test the *in vivo* significance of MEK activity in TGF-β stimulation of p21 and p15 expression. Cells were treated with U0126 at concentrations of 10 or 70 μM for 30 min before treatment with TGF-β for 12 h. As shown in Fig. 4*C*, the ability of TGF-β to induce p21 and p15 protein expression was dramatically reduced in the presence of the inhibitor. In contrast, CDK2 levels and basal levels of p21 and p15 remain relatively unchanged. Other specific experimental controls were performed. The ability of Smad3 to bind to Smad4 upon TGF-β treatment (Fig. 4*E*) and the ability of Smad3 to bind to GST-p300C (Fig. 4*E*) were not affected by incubation with 70 μM U0126, strongly implying

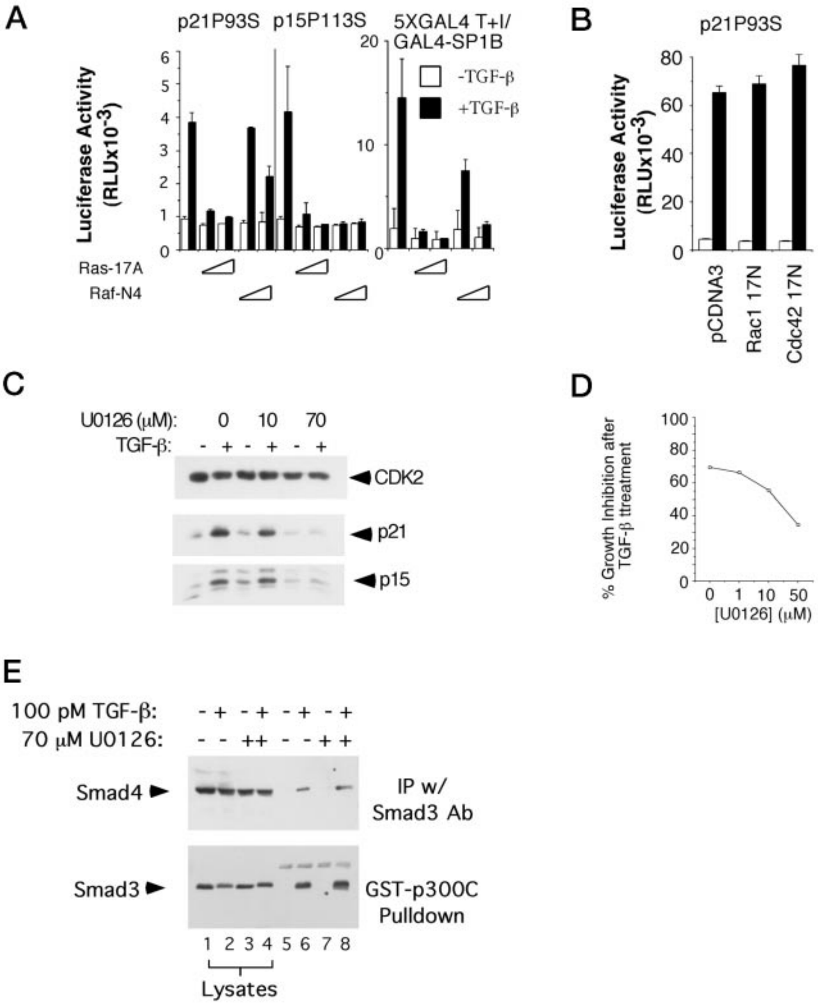

FIG. 4. **Inhibitors of Ras, Raf, and MEK prevent TGF-β mediated p21 and p15 induction.** *A*, duplicate experiments were performed as in Fig. 1 by co-transfecting different concentrations of dominant negative Ras 17A, Raf-N4, or vector with the p21, p15 or 5× GAL4/GAL4-Sp1 construct. As before, cells were treated with TGF-β for 20–24 h before harvesting to determine luciferase and β-galactosidase activity. In *B*, dominant negatives Rac1 17N or Cdc42 17N were transfected with the p21 reporter and treated with TGF-β and luciferase, and β-galactosidase activity was determined. *C*, the MEK inhibitor U0126 (Promega) was incubated in exponentially growing HaCaT cells for 1 h before treating with TGF-β for 12 h. After lysis and protein equilibration, Western blots were performed using α-p21 and α-p15. α-CDK2 was used to show equal protein loading. *D*, as in Fig. 3*D*, [^{3}H]thymidine incorporation was measured in cells incubated with increasing dosage of U0126 and TGF-β treated for 18 h to determine whether the MEK inhibitor could block TGF-β-dependent growth inhibition. *E*, cells were incubated with or without inhibitor and treated with TGF-β for 2 h or left untreated before harvesting and lysing in ULB. Lysate was either incubated with protein A and α-Smad3 before performing SDS-PAGE and a Western blot for Smad4 association or incubated with glutathione bead-bound GST-p300C before performing SDS-PAGE and a Western blot for Smad3 association.

that a nonspecific blockage of TGF-β type I receptor kinase activity does not occur at the concentration of 70 μM for the inhibitor U0126. In addition, basal phospho-p38 levels do not change with incubation of 70 μM U0126 (data not shown), suggesting that this inhibitor acts as a specific MEK/MAPK inhibitor.

To determine the consequences of the inability of TGF-β to induce p21 and p15 expression, we measured the ability of TGF-β to cause growth inhibition in the presence of the inhibitor using [^{3}H]thymidine labeling. Exponentially growing cells were treated with TGF-β in the presence of increasing concentrations of U0126 for 18 h and pulse-labeled with [^{3}H]thymidine for the final 2 h. As shown in Fig. 4*D*, the ability of TGF-β to inhibit growth is reduced with increasing concentrations of U0126. In addition, the cells treated with U0126 do not develop the morphological changes normally associated with TGF-β treatment (data not shown).

Our results and those of others suggest that Ras activation may be a part of the TGF-β signaling pathway required for p21 induction. The molecular link between Ras activation and the TGF-β receptor complex, however, remains unknown. Because one mechanism for the activation of Ras by many mitogenic signals often involves the activation of tyrosine kinases, we explored this possibility by using herbimycin A, an inhibitor of tyrosine kinase activity (29). As shown in Fig. 5, the ability of TGF-β to increase p21 protein expression is significantly reduced upon incubation of HaCaT cells with 1 μM herbimycin A, suggesting that a tyrosine kinase may be involved in mediating the TGF-β signal.

DISCUSSION

A paradigm is emerging whereby multiple signals use the Ras/Raf/MEK signaling cascade to induce p21 and cause growth arrest. In mammalian cells, signals such as TGF-β, myogenesis, epidermal growth factor, nerve growth factor, high levels of extracellular calcium, a histone deacetylase inhibitor, the mycotoxin fumonsin B1, UV radiation, and the geranylgeranyltransferase I inhibitor GGTI-298 (21, 30–36) have all been shown to induce p21 expression as well as growth arrest. In addition, most of these signals appear to signal

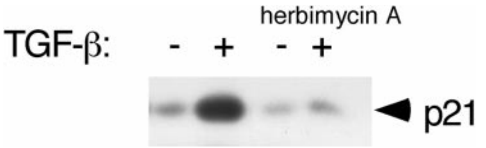

FIG. 5. **A tyrosine kinase activity may be required for TGF-β induction of p21.** Cells were incubated with herbimycin A (1 μM) for 1 h before treatment with TGF-β for 3 h. Cells were then harvested and lysed in ULB. Equivalent proteins per lane were loaded and subjected to SDS-PAGE, and a Western blot was performed for p21.

through Sp1- and Sp3-binding response elements in the p21 promoter. Activation of the MAPK pathway could be a common pathway these signals may utilize to stimulate p21 expression. Interestingly, in budding yeast an analogous situation occurs during pheromone-induced response, where a MAPK-like (STE 20, STE11, STE 7, Fus3/Kss1) pathway is activated, resulting in the phosphorylation of the transcription factor STE12, a subsequent increase in transcription of Far1, the yeast equivalent of a cyclin-dependent kinase inhibitor, and cell cycle arrest (37). Like STE12, phosphorylation of Sp1 by the MAPKs may be one mechanism by which transcription of p21 is increased in these cells. TGF-β can activate different MAPK members to stimulate transcriptional events. In a recent report by Hocevar *et al.* (38), TGF-β was shown to stimulate JNK activity to increase fibronectin synthesis in a Smad4-independent manner. Increases in SAPK/JNK activity have been reported elsewhere (39), as have increases in ERK (40, 41). We have taken advantage of the available phospho-specific antibodies for JNK, p38, and ERK and performed time-dependent Western blots to ascertain which MAPK family member is regulated by TGF-β in our system. Although GAL4-Elk-mediated transcription is indeed increased upon TGF-β treatment, in HaCaT cells these three MAPK family members do not appear to be significantly regulated by TGF-β (data not shown). This result suggests that some other MAPK-like family proteins may be regulated by TGF-β. Although TGF-β-activated kinase, a MAPK kinase kinase homolog responsive to TGF-β, is one such candidate (42), expression of a dominant negative of TGF-β-activated kinase does not block the ability of TGF-β to stimulate p21 expression (data not shown).

It is intriguing to speculate on how TGF-β turns on the MAPK pathway. Multiple signals can activate MAPK signaling through different pathways. In tyrosine kinase receptor signaling, upon addition of ligand such as platelet-derived growth factor, the receptor protein tyrosine kinase is autophosphorylated and recruits adaptor molecules such as Grb2 and SOS. SOS can, in turn, associate with Ras and which results in Ras becoming GTP-bound. In budding yeast, the serpentine receptors become activated during mating, causing dissociation of Gα from Gβγ, which can then activate a MAPK-like (STE 20, STE11, STE 7, Fus3/Kss1) cascade (37). The ability of tyrosine kinase inhibitor, herbimycin A, to prevent TGF-β induction of p21 suggests that a tyrosine kinase activity is required for its effect. Although specific tyrosine residues on the TGF-β type II receptor may be phosphorylated (Tyr^{259}, Tyr^{336}, and Tyr^{424}) (reviewed in Ref. 43), it remains to be discovered whether these residues contribute to TGF-β signaling function.

Smad proteins play a pivotal role in TGF-β signaling pathway, and here we show that MAPK pathway is also essential for p21 induction by TGF-β. How Smads functionally relate to the MAPK pathway and potentiate growth arrest activity for TGF-β is still unclear. Previous experiments suggest that Smads are required for TGF-β-mediated growth arrest but on the other hand, overexpression of Smads may not be sufficient for the p21 induction. Our data indicate that the MAPK pathway does not function upstream of Smads because cells stably overexpressing constitutively activated MEK Q56P do not show an effect on TGF-β-induced Smad localization or DNA binding. Therefore, the MAPK pathway could be a distinct, parallel pathway from Smad signaling. Alternatively, it could play a role as a downstream effector of Smad signaling, although if the latter scenario is the case, at least another signal input in addition to Smads is probably needed to activate the MAPK/p21 pathway. In certain cells, this other signal may already be constitutively activated, as is likely the case in hepatic cells where overexpressed Smads alone can stimulate p21 promoter activity (44). Because in HaCaT cells activation of the MAPK pathway alone, but not Smad3/Smad4 overexpression, is sufficient to stimulate p21 expression and in hepatic cells overexpression of Smads alone can also stimulate p21, it is more likely that the MAPK pathway acts downstream of Smads. It is possible that Smad3 can also act as a required intermediate cytoplasmic protein, in addition to its role in the nucleus that mediates the association between the TGF-β receptors and Ras. In supporting this possibility, a recent report showed that Smad3 may associate with other novel cytoplasmic adaptor proteins such as SARA to modulate appropriate TGF-β signaling (45).

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