c-myc Is a Downstream Target of the Smad Pathway*

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The transforming growth factor-β (TGF-β)1 superfamily is a group of multifunctional cytokines that affect cell growth, cell death, differentiation, and morphogenesis. It consists of more than 40 family members including TGF-βs, activins, and bone morphogenetic proteins. TGF-β transduces signals via hetero-meric complex formation of cognate type II and type I serine/threonine kinase receptors. TGF-β type II receptor kinase phosphorlates serine and threonine residues in the GS domain of TGF-β type I receptor, which results in activation of the type I receptor kinase. Then, activated type I receptor transduces signals into cytoplasm through phosphorylation of receptor-regulated Smads (R-Smad). Smad2 and Smad3 are the R-Smads activated by the TGF-β isoforms and is frequently impaired in cancer cells. We determined a Smad-responsive element in the c-myc promoter. This element is a complex of the TGF-β1 inhibitory element (TIE) originally identified in the transin/stromelysin promoter and an E2F site responsible for transcriptional activation of the c-myc promoter. Smad3 and E2F-4 directly bound to the element (TIE/E2F), and substitution of two nucleotides in TIE/E2F impaired binding of both Smad3 and E2F-4 as well as serum-induced activation and TGF-β-induced suppression of the c-myc promoter activity. Smad3 bound TIE/E2F within 1 h after stimulation with TGF-β, before the suppression of c-myc transcription, whereas binding of p130 to TIE/E2F became augmented later than 12 h. TGF-β1 signaling did not compete with E2F-4 for binding to TIE/E2F, but reduced p300 co-immunoprecipitating with E2F-4. Therefore, TGF-β1 signaling may suppress c-myc promoter activity by dissociating p300 from E2F-4.

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TGF-β1 inhibits the growth of many divergent cell types, and loss of TGF-β sensitivity has been implicated in tumorigenesis (4–6). Some tumors acquire TGF-β resistance following inactivation of TGF-β receptors (7, 8) and others by mutations in Smad genes (9–12). However, such alterations cannot account for many cancers in which TGF-β responsiveness is lost. Identification of more components essential for the TGF-β signaling pathway leading to growth arrest might therefore be required to identify genetic alterations responsible for TGF-β resistance in cancer cells.

c-myc was discovered as the cellular homologue of the retroviral v-myc oncogene, and the c-myc proto-oncogene was subsequently found to be activated in various animal and human tumors. Amplification of the c-myc gene has been described in ~15% of all human tumors (13). TGF-β1 down-regulates expression of c-myc in human endothelial cells, breast carcinoma cell lines, a Balb/MK mouse keratinocyte cell line, and HaCaT cells (14–17), and overexpression of c-myc abrogates the growth inhibition of keratinocytes induced by TGF-β1 (18, 19). Genetic screening also showed that c-myc as well as NF-IX-1 sustained proliferation of Mv1Lu cells in the presence of TGF-β (20). Loss of TGF-β1-induced suppression of c-myc correlated well with TGF-β resistance in colon cancer (21), thyroid cancer (22), and human squamous cell carcinoma cell lines (23, 24).

TGF-β1 was shown to induce G1 arrest through its effects on the Rb/E2F pathway (25–27), and the c-myc promoter is also a target of the Rb/E2F pathway. However, dephosphorylation of Rb family proteins requires more than several hours, whereas TGF-β1 rapidly down-regulates levels of c-myc mRNA beginning within 1 h of its administration (28). Furthermore, Myc down-regulation was shown to be required for activation of p15INK4b (29, 30) and p21cip1 (19), which precedes inactivation of G1 cyclin-dependent kinases and dephosphorylation of Rb family proteins. There thus must be a pathway through which TGF-β1 down-regulates c-myc early after TGF-β1-stimulation other than that mediated by Rb family proteins.

To identify this pathway, we analyzed TGF-β1-responsive elements in the human c-myc promoter and found that Smad proteins directly bound to an element in the c-myc promoter and suppressed c-myc promoter activity.
A Smad-responsive Element in the c-myc Promoter

EXPERIMENTAL PROCEDURES

Cells—HaCaT cells and the R mutant of Mv1Lu cells (clone 4-2) were provided by N. E. Fusenig (DKFZ, Heidelberg) and J. Massagué (Sloan-Kettering Cancer Center, New York), respectively. Mv1Lu cells and COS-7 cells were obtained from the American Type Culture Collection. These cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 10 μg/ml gentamicin. Subclones of HaCaT cells were maintained in MCDB153 medium (Sigma) supplemented with 0.1 mM calcium chloride, 10 ng/ml epidermal growth factor, 10 μg/ml gentamicin, and 5% dialyzed fetal bovine serum. These subclones were used for experiments including DNA transfection. HaCaT cells stably expressing FLAG-Smad3D407E were previously described (31).

DNA Constructs—c-myc cDNA was excised from pBS0/1 Myc (provided by R. Blackwood and R. N. Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA) and subcloned into pcDNA3 mammalian expression vector (Invitrogen). cDNA for E2F-1 and E2F-4 was provided by K. Helin (European Institute of Oncology, Milan, Italy) and C. Sardet (Institut de Genetique Moleulaire, Montpellier, France), respectively. AR5-lux was provided by L. Attisano (University of Toronto, Toronto, Canada). pHX1Luc, a luciferase reporter construct containing AR3-lux was provided by L. Attisano (University of Toronto, Toronto, Canada) and subcloned into pcDNA3 mammalian expression vector. FLAG-Smad3D407E in FLAG-Smad3D407E-transfected HaCaT cell lines (H3.3, H3.4, and H3.9) is shown in the upper panel (anti-FLAG). These cells were stimulated with TGF-β (100 ng/ml, 6 h) as indicated and subjected to Northern blot analysis for c-myc and GAPDH and immunoblot analysis for c-myc, as indicated. GI (%), percentage reduction of [3H]thymidine incorporation of each clone after incubation with TGF-β (100 ng/ml, 24 h).

Northern Blot Analysis—Total cellular RNA was extracted using Isogen (Nippogene, Tokyo, Japan) following the manufacturer’s recommendations. Twenty-μg aliquots were electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon membrane (Biondyne A; Pall BioSupport Co.). The membranes were hybridized at 42 °C overnight with random primed DNA probes labeled with [α-32P]dCTP in a hybridization buffer containing 5× SSC, 25% formamide, 1% SDS, 5× Denhardt’s solution, and 0.2 mg/ml denatured salmon testis DNA. The membranes were washed to a final stringency of 1× SSC, 0.1% SDS at 65 °C, and analyzed using a Fuji BAS 2500 Bio-Image Analyzer (Fuji Photo Film, Tokyo, Japan) and autoradiography.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as previously described (34). Cells were solubilized in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After clearing with centrifugation, total cell lysates or immunoprecipitates obtained using anti-FLAG (12CA5, Roche Molecular Biochemicals) or anti-p300 (Upstate Biotechnology, Inc., Lake Placid, NY) were subjected to SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membranes (ProBlott; Applied Biosystems) and subjected to immunoblotting. Anti-Myc (9E10; Calbiochem), anti-FLAG (M2; Sigma), anti-Smad2/3 (clone 18, Transduction Laboratories, Lexington, KY), anti-E2F-1 (KH95; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-E2F-4 (C-20, Santa Cruz), anti-p107 (C-10; Santa Cruz Biotechnology), anti-p107 (BD PharMingen), anti-Rb (G3-245; BD PharMingen), anti-hemagglutinin (HA) (3F10; Roche Molecular Biochemicals), and anti-p300 (Upstate Biotechnology) antibodies were used as first antibodies for immunoblotting. Reacted antibodies were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For reblotting, the membranes were stripped following the manufacturer’s protocol.

Growth Inhibition Assay—Cells were seeded in 24-well plates at a density of 5 × 10^4 cells/well and cultured for 24 h in the growth medium for each cell line. Then the culture fluids were changed to growth medium containing various concentrations of human TGF-β. After

FIG. 1. c-myc is an immediate early responsive gene downstream of the Smad pathway. A, Northern blot analysis for c-myc. Exponentially growing HaCaT cells were incubated with 100 µM TGF-β for the indicated periods in the absence or presence of cycloheximide (CXH). Relative levels of c-myc expression were determined by densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. B, TGF-β does not down-regulate c-Myc in the presence of a dominant-negative Smad3. Immunoblot (Blot) analysis for FLAG-Smad3D407E in FLAG-Smad3D407E-transfected HaCaT cell lines (H3.3, H3.4, and H3.9) is shown in the upper panel (anti-FLAG). These cells were stimulated with TGF-β (100 ng/ml, 6 h) as indicated and subjected to Northern blot analysis for c-myc and GAPDH and immunoblot analysis for c-myc, as indicated. GI (%), percentage reduction of [3H]thymidine incorporation of each clone after incubation with TGF-β (100 ng/ml, 24 h).

FIG. 2. Down-regulation of c-Myc is required for inhibition of growth of HaCaT cells by TGF-β signaling. A, establishment of HaCaT cells stably expressing c-Myc. Immunoblot analysis for c-Myc in the absence or presence of 100 ng/ml TGF-β in c-myc-transfected HaCaT cell lines is shown. HaCaT, HaCaT cells; Mock, a mock-transfected clone of HaCaT cells; HaCaT-c-myc clone 1 and clone 2, c-myc-transfected clones of HaCaT cells. B, stable expression of c-myc confers resistance to TGF-β-induced growth inhibition. Effects of TGF-β on [3H]thymidine incorporation by c-myc-transfected HaCaT cell lines (A) are shown. ○, HaCaT; ●, HaCaT-Mock; □, HaCaT-c-myc clone 1; ■, HaCaT-c-myc clone 2.
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RESULTS

c-myc Is an Early TGF-β-responsive Gene and Must Be Decreased for Growth Inhibition of HaCaT Cells—Levels of c-myc mRNA in exponentially growing HaCaT cells were suppressed rapidly after TGF-β-stimulation. c-myc mRNA began to decrease within 1 h and reached less than 30% of the control level within 6 h. The decrease was not affected by the presence of cycloheximide, indicating that this response did not require new protein synthesis (Fig. 1A). To elucidate whether c-myc is suppressed through the Smad pathway, we used HaCaT cells expressing a dominant negative mutant of Smad3 (31). As shown in Fig. 1B, Smad3D407E blocked down-regulation of c-Myc in HaCaT cells both in mRNA and protein levels, suggesting that c-myc is a downstream target of the Smad pathway. In addition to dysregulation of c-myc, these cell lines lost growth-inhibitory response to TGF-β as previously reported (31). Furthermore, we established HaCaT cells stably express-
ing exogenous c-myc. Levels of c-Myc in these transfectants were not suppressed by TGF-β (Fig. 2A). Growth of these transfected cells was not affected by TGF-β (Fig. 2B), as previously shown for Balb/MK (18), Mv1Lu (20), and HaCaT cells (19).

**Identification of Smad3-binding Elements in the c-myc Promoter**—We next mapped TGF-β-responsive region(s) in the human c-myc promoter (Fig. 3A). Within the −2329/+510 human c-myc promoter, the proximal 150 bp (−142/+16) was sufficient for serum-induced activation and TGF-β-dependent suppression of c-myc promoter activity. The −367/+16 promoter stronger serum-induced activation and repression by TGF-β much stronger than the −2329/+510 reporter, suggesting the presence of negative regulatory element(s) in −2329/−367 or +16/+510. We further examined whether Smad3 directly bound the c-myc promoter. As shown in Fig. 3B, purified GST-Smad3ΔMH2 (Smad3 lacking the MH2 domain) (35) directly bound the c-myc promoter including −142/+16. The shift band was diminished by the presence of an excess amount of a cold probe. Smad binding elements in this DNA fragment were then identified by DNase I footprinting analyses. There were three Smad3 binding elements in this region: a GTCT element upstream of the P1 promoter, a GC-rich element, and TIE overlapping with the E2F element (Fig. 3C, a, b, and c, respectively).

**TIE/E2F Element Is Responsible for Repression by TGF-β**—We next mutated or deleted each Smad3-binding element in the −367/+16 c-myc promoter as shown in Fig. 4A. Loss of Smad binding to these mutated elements was confirmed by DNase I footprinting analyses (data not shown). Mutations in the GTCT element and deletion of the GC-rich element affected neither control promoter activity nor TGF-β-responsiveness in growing Mv1Lu cells. Concerning the TIE/E2F element, we created a TIE mutant (m-TIE) that did not change the original E2F element (36) and a mutant in the E2F element (m-E2F) that kept the intact TIE sequence (37) (Fig. 4A). Both of these mutants considerably reduced serum-induced activation of the c-myc promoter and completely abrogated TGF-β-dependent repression (Fig. 4, B and C). TIE/E2F was indispensable for TGF-β-induced repression of the c-myc promoter activity, whereas serum-responsive elements should exist besides TIE/E2F because m-TIE still responded to serum (Fig. 4C). We next constructed a reporter consisting of three tandem repeats of the TIE/E2F (3× TIE/E2F) connected to the core P2 promoter (−40/+16) of c-myc. Serum stimulation clearly enhanced transcriptional activity from the element, and this enhancement was totally blocked by TGF-β. These responses were completely lost with a TIE mutation (Fig. 4C). Therefore, the TIE/E2F element is involved in both positive and negative regulation of c-myc promoter activity.

**TIE/E2F Binds both Smads and E2F-4**—To examine whether TGF-β-mediated suppression of c-myc depends on two-Smad interaction, we tested whether Smad3 bound TIE/E2F in a TGF-β-dependent manner, COS-7 cells were transfected with FLAG-Smad3 in the absence or presence of constitutively active form of TGF-β type I receptor. Total cellular lysates were subjected to DNAP analyses using a biotinylated TIE/E2F oligonucleotide as a probe. Smad3 but not a dominant-negative mutant of Smad3 bound TIE/E2F oligonucleotides in a TGF-β signaling-dependent manner (Fig. 5A). This binding was specific to the sequence, since two-nucleotide substitution in the TIE element (m-TIE/E2F) totally abrogated Smad binding to the DNA. E2F-1 and E2F-4 were also highly expressed in COS-7 cells upon transient DNA transfection. In this condition, E2F-4, but not E2F-1, bound TIE/E2F (Fig. 5B).

Binding of endogenous Smad proteins and E2F in HaCaT cells was further examined. Both of the TGF-β receptor-regulated Smads, Smad2 and Smad3, bound the TIE/E2F element at 1 h after TGF-β stimulation (Fig. 5C). Since Smad2 does not bind DNA directly, it may bind through Smad3 or Smad4. Mutations either in the TIE element or in the E2F element impaired Smad binding to this DNA fragment (Fig. 5C), while a CAGA mutation did not affect the binding, corresponding to the results of functional assay shown in Fig. 4.

Endogenous E2F-4 bound TIE/E2F regardless of the presence of TGF-β signaling. In addition, the E2F-4 binding and the Smad binding were impaired by either the TIE mutation or the E2F mutation (Fig. 5C). Since TGF-β-regulated Smads and
E2F-4 bound the same TIE/E2F element, we further examined whether binding of these two was mutually exclusive. However, binding of Smad did not affect E2F-4 binding to TIE/E2F, as shown in Fig. 5C (see also Fig. 6A). TGF-β signaling thus is not likely to repress c-myc promoter activity by competing with E2F-4 for DNA binding.

Since E2F mutation as well as TIE mutation abrogated Smad binding to TIE/E2F, we confirmed whether GST-Smad3MH2 directly bound TIE/E2F in EMSA. GST-Smad3MH2 directly bound TIE/E2F in the absence of E2F-4 in a sequence-specific manner (Fig. 5D). Complexes of FLAG-Smad3 and TIE/E2F in COS-7 cells were also analyzed by EMSA. E2F-4 made detectable complexes with TIE/E2F only in the presence of DP-1. Anti-E2F-4 supershifted complexes of E2F-4 and TIE/E2F. In contrast, shift bands containing FLAG-Smad3 were not affected so much by the presence of anti-E2F-4 both in the absence and in the presence of anti-FLAG antibody. These results suggest that Smad3 and E2F-4 independently bind TIE/E2F and that only a part of TIE/E2F binds both Smad3 and E2F-4 (Fig. 5E).

**Time Course of Binding of E2F, Smads, and Rb Family Proteins to TIE/E2F after TGF-β Stimulation**—We next examined the time course of binding of E2F-4, Smads, and Rb family proteins to TIE/E2F after administration of TGF-β (Fig. 6A). HaCaT cells were stimulated with TGF-β for the indicated times, and total cell lysates were subjected to DNAP analyses using 3× TIE/E2F as a probe. Binding of E2F-4 and pRb was not affected by TGF-β. Smad2/3 was induced to bind TIE/E2F within 2 h and kept binding up to 24 h (Fig. 6A). Binding of p107 decreased after 6 h, inversely proportional to p130 (Fig. 6A).

**TGF-β Signaling dissociates p300 from E2F-4**—We tried to detect endogenous p300 in the precipitates after DNAP with 3× TIE/E2F, but it was under detectable levels. Therefore, the effect of TGF-β signaling on the complex formation between
E2F-4 and p300 was analyzed using overexpressed proteins in COS-7 cells. E2F-4 and p300 decreased inverse proportionally with the increase of p300-bound Smad3 in the presence of TGF-β signaling (Fig. 6C). The effects of TGF-β on the cooperation between E2F-4 and p300 cooperatively enhanced the promoter activity. This cooperative effect of p300 with E2F-4 was again blocked by TGF-β signaling (Fig. 6C).

**DISCUSSION**

c-Myc is an essential regulator of cell cycle progression that is required for activation of cyclin-dependent kinase complexes (19, 29, 30, 38). Overexpression of c-myc overcomes TGF-β-induced cell cycle arrest, and c-myc is frequently highly expressed in cancer cells. c-myc was also identified as a target of the APC pathway (39) and of tumor-derived p53 mutants (40). The Ras/mitogen-activated protein kinase pathway was shown to stabilize Myc protein (41). Furthermore, c-Myc was reported to activate telomerase (42). All of these recent findings confirmed that c-Myc plays essential roles in carcinogenesis.

We have shown in the present study that c-myc is an early responsive gene required for TGF-β-induced growth inhibition of HaCaT cells. It was controlled downstream of the Smad pathway, and the TIE/E2F element was responsible for both serum-induced activation and TGF-β-induced suppression of the c-myc promoter activity. This element was previously reported as the cell cycle-dependent element, including an E2F site (43).

**FIG. 6. Effects of TGF-β signaling on the binding of E2F, Smads, and Rb family proteins to the TIE/E2F element.** A, binding of p130 to TIE/E2F requires more than several hours after stimulation with TGF-β, whereas binding of Smad is a quick event. HaCaT cells were stimulated with TGF-β (100 pm) for indicated periods, lysed, and subjected to DNAP using biotinylated TIE/E2F as a probe. After SDS-PAGE and transfer, the membranes were blotted with antibodies against E2F-4, Smad2/3, p130, p107, or pRb, as indicated. B, TGF-β dissociates p300 from E2F-4. COS-7 cells were transfected with the indicated expression plasmids, and cell lysates were subjected to immunoprecipitation against p300 followed by immunoblotting for E2F-4 (top panel). Aliquots of the cell lysates were directly subjected to immunoblotting using anti-E2F-4, anti-FLAG, and anti-HA antibodies to detect levels of E2F-4, Smad3 and TβR-I(TD), and p300, as indicated. C, dissociation of p300 from E2F-4 depends on Smad3. COS-7 cells were transfected with indicated expression plasmids, and cell lysates were subjected to immunoprecipitation against p300 followed by immunoblotting for E2F-4 or Smad3, as indicated (upper panels). Aliquots of the cell lysates were directly subjected to immunoblotting using anti-E2F-4, anti-HA, and anti-FLAG antibodies to detect levels of E2F-4, p300, Smad3, and TβR-I(TD), as indicated. D, transcriptional activation by E2F-4 and p300 via the TIE/E2F element is blocked by TGF-β signaling. Mv1Lu cells were transfected with 3× TIE/E2F or 3× m-TIE/E2F reporter constructs together with E2F-4 and p300 expression vectors as indicated and cultured for 4 h. Then, cells were incubated with TGF-β (100 pm) for another 24 h and subjected to luciferase analysis.
and E2F1 (49, 50), are repressed through an E2F-mediated mechanism in G2/M and derepressed in late G1. In such cases, mutations in the E2F site up-regulate transcription of these genes in quiescent cells (46, 47, 51). However, in the case of the c-myc promoter, abrogation of E2F binding to this element decreased transcriptional activity of the promoter (Fig. 4, B and C), suggesting that the E2F site in the human c-myc promoter acts essentially as a positive regulatory element. In case of the genes suppressed by the E2F element, the E2F site cooperates with a contiguous corepressor element, termed the cell cycle genes homology region (CHR). The CHR sequence was first identified in the B-myb, cyclin A, cdc2, and cdc25C genes (51).

Identical CHR sequence was reported also in the c-myc gene in quiescent cells (46, 47, 51). However, in the case of the genes suppressed by the E2F site up-regulate transcription of these genes. However, in the case of the genes suppressed by the E2F site up-regulate transcription of these genes suppressed by the E2F site cooperates with a contiguous corepressor element, termed the cell cycle genes homology region (CHR). The CHR sequence was first identified in the B-myb, cyclin A, cdc2, and cdc25C genes (51).

A similar sequence was also identified as a TGF-β-responsive element in the mouse tissue transglutaminase gene (54). A similar sequence was also identified as a TGF-β-responsive element in the mouse tissue transglutaminase gene (54).

We found that transcriptional regulation via TIE/E2F of the c-myc promoter was selectively impaired in cancer cell lines. We do not know the mechanism of this selectivity. Recently, MCF-10A cells double-transfected with c-Ha-ras (G12V) and c-erbB2 were reported to selectively lose TIE-mediated suppression of c-myc by TGF-β signaling, suggesting that defective regulation of c-myc via the TIE element might be a rather common phenomenon in carcinogenesis (61). Since c-myc plays essential roles in carcinogenesis, further studies to clarify TGF-β-induced transcriptional regulation via the TIE/E2F element should yield important findings for the understanding of dysregulated expression of c-myc and possibly for the autonomous growth of cancer cells.

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