TGF-β inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3

Dong Liu,[1] Brian L. Black,[2] and Rik Derynck[1,3]

[1]Departments of Growth and Development and Anatomy, Programs in Cell Biology and Developmental Biology, and [2]Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143, USA

Transforming growth factor-β (TGF-β) is a potent inhibitor of skeletal muscle differentiation, but the molecular mechanism and signaling events that lead to this inhibition are poorly characterized. Here we show that the TGF-β intracellular effector Smad3, but not Smad2, mediates the inhibition of myogenic differentiation in MyoD-expressing C3H10T1/2 cells and C2C12 myoblasts by repressing the activity of the MyoD family of transcriptional factors. The Smad3-mediated repression was directed at the E-box sequence motif within muscle gene enhancers and the bHLH region of MyoD, the domain required for its association with E-protein partners such as E12 and E47. The repression could be overcome by supplying an excess of E12, and covalent tethering of E47 to MyoD rendered the E-box-dependent transcriptional activity refractory to the effects of Smad3 and TGF-β. Smad3 physically interacted with the HLH domain of MyoD, and this interaction correlated with the ability of Smad3 to interfere with MyoD/E protein heterodimerization and binding of MyoD complexes to oligomerized E-box sites. Together, these results reveal a model for how TGF-β, through Smad3-mediated transcriptional repression, inhibits myogenic differentiation.

[*Key Words*: TGF-β; Smads; MyoD; transcription; myogenesis]

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Transforming growth factor-β (TGF-β) profoundly influences the differentiation of many cell types of mesenchymal origin, including preadipocytes (Ignotz and Massagué 1985; Choy et al. 2000), osteoblasts (Centrella et al. 1994), and myoblasts (Olson 1992). In cultured myoblasts, TGF-β inhibits the induction of muscle-specific gene expression and myotube formation without affecting cell proliferation (Massagué et al. 1986; Olson et al. 1986). The ability of TGF-β to suppress muscle differentiation and silence the expression of a wide array of muscle-specific genes suggests that it may target one or more master regulator(s) of myogenesis.

Central to the induction of myogenic differentiation is the function of the MyoD family of basic helix-loop-helix (bHLH) transcription factors, which include MyoD, myogenin, Myf5, and MRF4 (Emerson 1993; Weintraub 1993; Olson and Klein 1994) and are collectively referred to as myogenic regulatory factors or MRFs. Efficient activation of muscle-specific genes by the MRFs occurs through their dimerization with a distinct class of ubiquitously expressed bHLH proteins in the E-protein family, including the two alternatively spliced E2A gene products, E12 and E47. The MRF/E protein heterodimers then bind to a conserved DNA sequence, CANNTG, also known as the E-box, which is located in regulatory regions of many muscle-specific genes (Murre et al. 1989; Davis et al. 1990; Lassar et al. 1991). MyoD and Myf5 are expressed in proliferating myoblast cells during embryonic development or in culture systems well before activation of myogenic differentiation, and initiation of myoblast differentiation in culture depends on the depletion of mitogenic stimuli (Davis et al. 1987; Braun et al. 1989; Sassoon et al. 1989; Ott et al. 1991). These observations reflect the existence of regulatory mechanisms that restrict the activity of the myogenic factors in response to extracellular conditions. Such a notion is also shown by the ability of peptide growth factors, such as TGF-β, to block differentiation in the presence of constitutively overexpressed myogenin or MyoD (Vaidya et al. 1989; Brennan et al. 1991). Despite the recognition of TGF-β as a prominent inhibitor of MRF functions, the molecular mechanism that underlies this inhibition has remained obscure.

Research during the last few years has resulted in the characterization of the TGF-β receptors and the Smads, a class of intracellular effectors of TGF-β signaling (Derynck et al. 1998; Massagué and Wotton 2000). The

[3]Corresponding author.
E-MAIL derynck@itsa.ucsf.edu; FAX (415) 476-1499.
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current model for the TGF-β signal transduction pathway maintains that TGF-β acts through a heteromeric complex of serine/threonine kinase receptors. Ligand binding to the receptor complex results in C-terminal phosphorylation of Smad2 and Smad3. Each of the two receptor-activated Smads (R-Smads) forms heteromeric complexes with Smad4, a common partner for R-Smads. The Smad complexes then translocate into the nucleus and activate gene expression. Whereas Smads bind to the promoters of certain TGF-β-responsive genes (Denissova et al. 2000), efficient activation of transcription in response to TGF-β occurs most commonly through the ability of Smads to associate and cooperate with other transcription factors and cofactors (Derynck et al. 1998; Massagué and Wotton 2000).

Most studies to date have documented the role of Smads as transcriptional activators, although TGF-β signaling often results in downregulation of gene expression. Several corepressors, such as Ski/SnoN (Luo et al. 1999; Stroschein et al. 1999; Xu et al. 2000), Evi-1 (Kurokawa et al. 1998), TGIF (Wotton et al. 1999), and SNIP1 (Kim et al. 2000), have been shown to suppress Smad-activated gene expression. However, it is not known whether Smads in conjunction with these corepressors directly participate in repressing gene expression. Recently, Smad3 has been implicated in the inhibition of transcription activated by the androgen receptor (Hayes et al. 2001) and osteoblast transcription factor CBFA1 (Alliston et al. 2001). However, the mechanisms of transcriptional repression were largely unexplored in these reports.

In this study we investigated the mechanism through which TGF-β inhibits myogenic differentiation. We show that Smad3, but not Smad2, acts downstream of TGF-β to repress the function of the MyoD family of bHLH factors, and that altered Smad3 signaling affects the differentiation of cultured myoblasts and their response to TGF-β in this process. We present evidence that Smad3 physically and functionally interacts with the bHLH domain of MyoD, and that TGF-β signaling through Smad3 interferes with the formation of an active MyoD/E protein complex and its subsequent binding to multimerized E-box sequences.

Results

Smad3 inhibits MyoD-induced myogenic conversion

Previous studies have shown that TGF-β inhibits myogenic differentiation in the presence of constitutive levels of MyoD or myogenin (Vaidya et al. 1989; Brennan et al. 1991). Therefore, we investigated the role of TGF-β-activated Smads in inhibiting the function of myogenic bHLH transcription factors. Specifically, we evaluated the ability of Smad2 or Smad3 to influence the myogenic differentiation induced by ectopically expressed MyoD in C3H10T1/2 fibroblast cells. Transfection of the multipotential 10T1/2 cells with a MyoD expression vector followed by incubation in low mitogen differentiation medium resulted in myogenic conversion, as evidenced by the expression of myosin heavy chain (MHC) and the formation of myotubes (Fig. 1A,B). In agreement with a

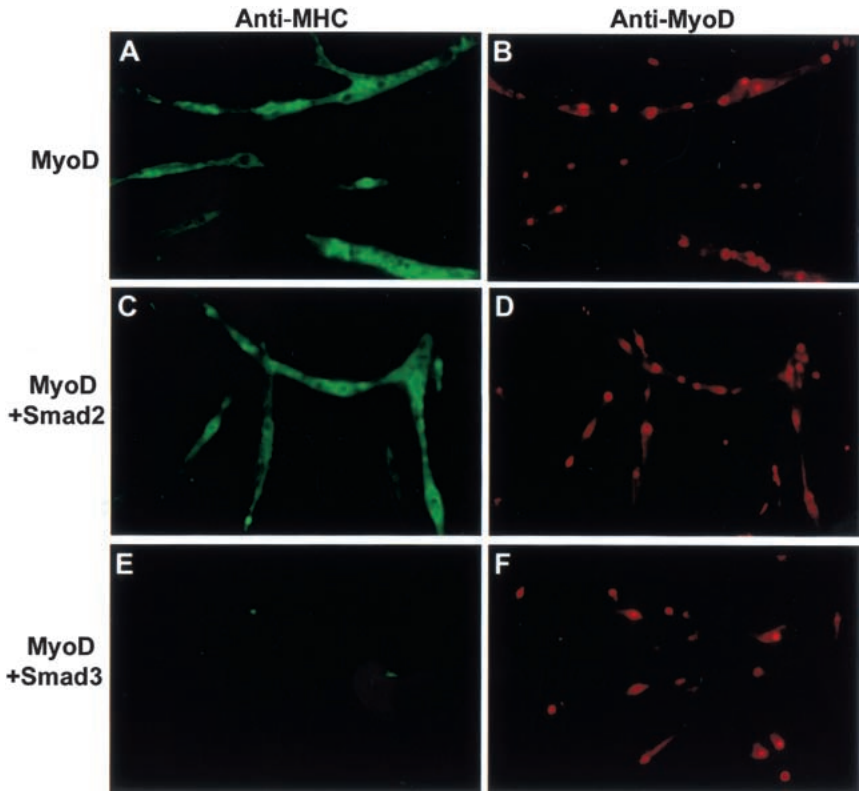

Figure 1. Smad3, but not Smad2, inhibits MyoD-induced myogenic differentiation of C3H10T1/2 fibroblasts. 10T1/2 cells were transiently transfected with an expression plasmid for MyoD alone (*A*,*B*) or together with an expression plasmid for Smad2 (*C*,*D*) or Smad3 (*E*,*F*), respectively. At day 1 post transfection, cells were shifted to differentiation medium for an additional 2 d. Cells were fixed and myofiber formation was assessed by immunostaining with an anti-MHC monoclonal antibody (green). MyoD expression in transfected cells was visualized by immunofluorescence using an anti-MyoD antibody, revealing predominantly nuclear staining (red).

previous report (Martin et al. 1992), addition of 1-ng/mL TGF-β to the differentiation medium completely blocked the MyoD-dependent myogenic conversion (data not shown).

Coexpression of Smad2 with MyoD had only a marginal effect on myofiber formation and MHC expression (Fig. 1C,D). In contrast, Smad3, expressed at a similar level as Smad2 (data not shown), resulted in significant inhibition of myogenic conversion of MyoD-transfected cells despite the normal nuclear localization of MyoD in these cells (Fig. 1E,F). Overall, coexpression of Smad3 with MyoD reduced the number of MHC-positive cells by > 90%, when compared with cells expressing MyoD alone (Table 1). These results suggest that Smad3, but not Smad2, can mediate TGF-β signaling to inhibit the myogenic potential of muscle-specific bHLH proteins.

Smad3 mediates the inhibition of myogenic differentiation by TGF-β in myoblasts

To further assess the role of Smad3 in the regulation of myogenesis, we investigated the effect of altered Smad signaling on the differentiation of myoblast cells. C2C12 myoblasts in culture differentiate into myotubes on serum withdrawal, and this process is inhibited by TGF-β (Massagué et al. 1986; Olson et al. 1986). To modify the activity of Smad3, we stably infected C2C12 cells with retroviral vectors that constitutively overexpress one of two versions of epitope-tagged Smad3 proteins. Smad3 with an N-terminal Flag tag (Smad3NF) is fully functional and has identical activity as wild-type Smad3 in transcription assays. In contrast, attachment of a Flag tag to the C-terminus of Smad3 interferes with receptor-activated phosphorylation, such that overexpression of this protein (Smad3CF) dominant-negatively inhibits TGF-β-induced gene expression and growth inhibition (Liu et al. 1997). As shown in Figure 2A, Smad3NF and Smad3CF were expressed at high levels in the infected cells.

To verify that overexpression of Smad3NF and Smad3CF in the retrovirus-transduced C2C12 cell lines affected TGF-β signaling, we evaluated the Smad-dependent transcriptional response of these cell lines after transfecting the cells with a reporter construct, 3TP-lux. This reporter expresses luciferase under the control of TGF-β responsive sequences from the collagenase I and plasminogen activator inhibitor-1 (PAI-1) promoters and is commonly used to assay the transcriptional response

Table 1. *Smad3 inhibits MyoD-induced myogenic conversion of 10T1/2 cells*

Expression plasmid	Nuclei of MHC-staining cells (% of control)[a]
MyoD	217 (100)
MyoD + Smad2	183 (84)
MyoD + Smad3	12 (6)

[a]The number of nuclei within MHC-staining cells in 10 randomly selected microscopic fields was counted. The results shown are representative of three independent experiments.

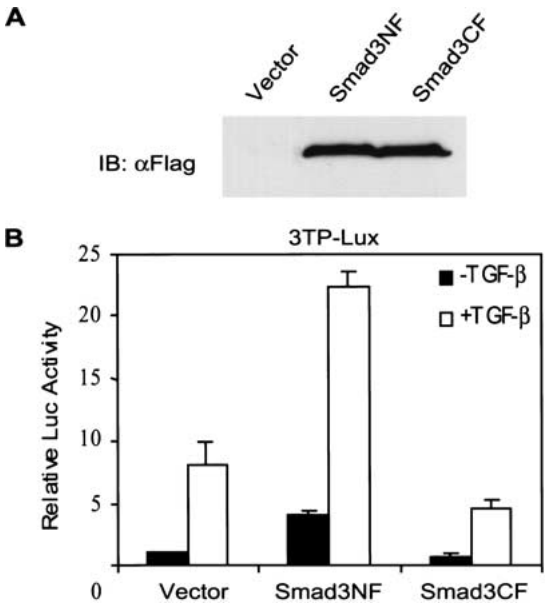

Figure 2. Stable expression of wild-type and dominant-negative Smad3 in C2C12 myoblast cells. (*A*) Immunoblot analysis (IB) of Smad3 expression in lysates of C2C12 cells stably infected with a viral vector for either an N-terminally (Smad3NF) or a C-terminally Flag-tagged (Smad3CF) Smad3. Lysates from cells infected with the empty vector were used as control. (*B*) TGF-β-induced transcription from the 3TP-Lux reporter plasmid in transiently transfected C2C12 cells that stably express Smad3NF or Smad3CF, or vector control cells. Luciferase expression values, normalized for transfection efficiency, are shown as fold induction relative to the basal promoter activity.

to TGF-β/Smad signaling (Cárcamo et al. 1995). Enhanced Smad3 activity in Smad3NF-expressing myoblast cells resulted in an increase of basal and TGF-β-induced reporter activities. Conversely, these activities were decreased in Smad3CF-expressing myoblasts (Fig. 2B). The latter result indicates that when overexpressed in C2C12 myoblasts, Smad3CF functions as a dominant-negative mutant in TGF-β signaling.

We next evaluated the ability of Smad3NF- and Smad3CF-expressing C2C12 cells to undergo myogenic differentiation in the absence or presence of TGF-β. Incubation of control cells in differentiation medium induced the expression of muscle-specific genes and cell fusion into multinucleated myotubes (Fig. 3A). In contrast, stable expression of Smad3NF inhibited myotube formation. Consequently, a majority of these cells maintained the mononucleated, nondifferentiated phenotype, and the number of cells expressing MHC was reduced in comparison to vector-infected control cells (Fig. 3C). As expected, exposure to 1-ng/mL TGF-β effectively blocked myotube formation and MHC expression in both control and Smad3NF-expressing cells (Fig. 3B,D). In the absence of TGF-β the differentiation characteristics of Smad3CF cells appeared similar to control cells. Then differentiation, however, was partially resistant to the antagonistic effect of TGF-β, because significant numbers of multinucleated myotubes and MHC staining were still apparent in the presence of 1-ng/mL TGF-β (Fig. 3E,F).

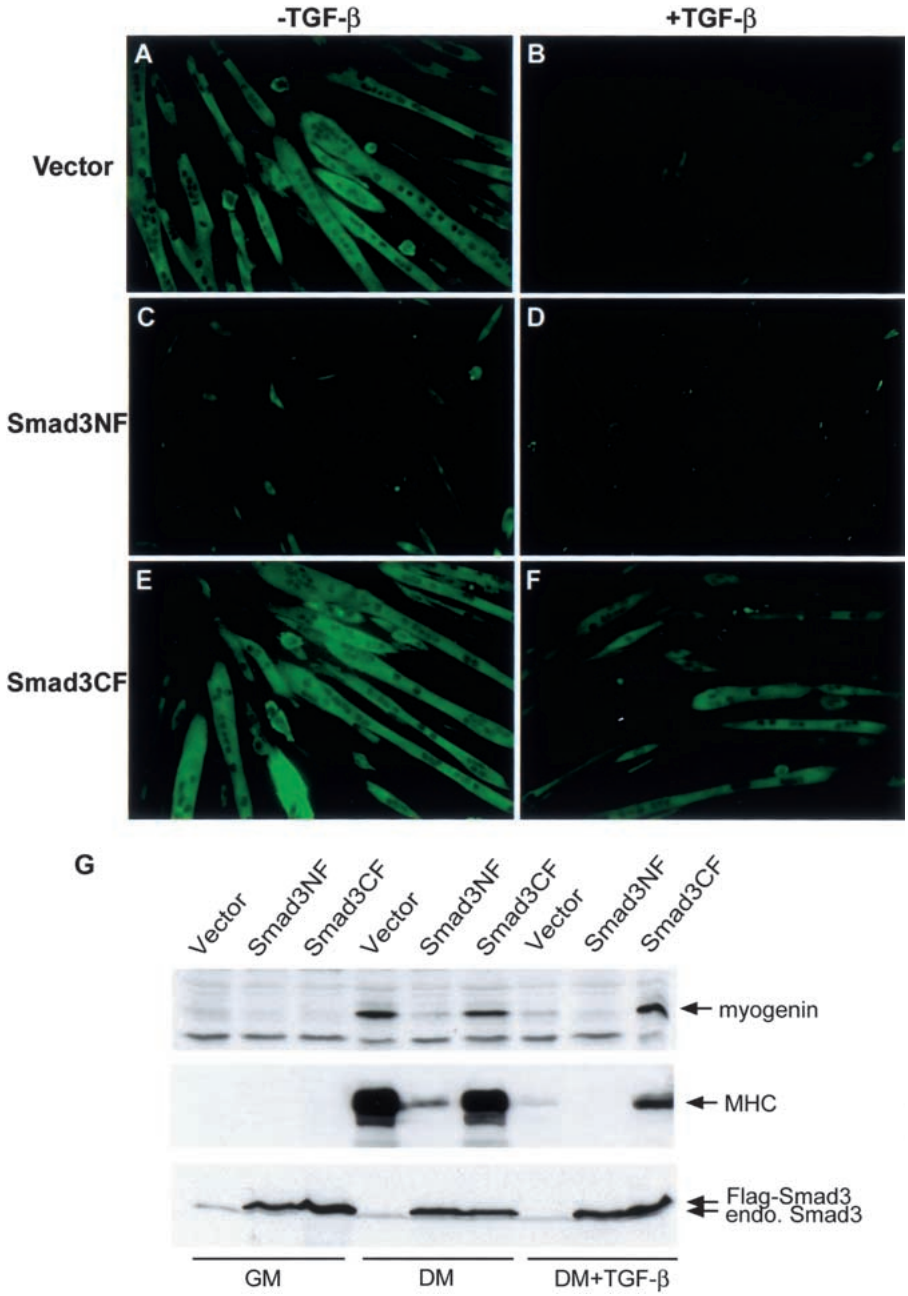

Figure 3. Smad3 regulates myoblast differentiation and the inhibitory response to TGF-β. C2C12 cells stably infected with control viral vector (*A*,*B*) or those harboring coding-sequences of Smad3NF (*C*,*D*) or Smad3CF (*E*,*F*) were grown to near confluence and then shifted to differentiation medium without or with 1-ng/mL TGF-β, as indicated, for 4 d. Terminal differentiation and myotube formation were assessed by immunofluorescence with an anti-MHC antibody. (*G*) Expression of myogenin and MHC, as assessed by Western blotting, in control-infected or Smad3NF- or Smad3CF-expressing C2C12 cells. Cells were cultured in growth medium (GM) and shifted to differentiation medium (DM) as in *A–F*. The *lower* panel shows expression of Smad3 detected by an anti-Smad3 antibody. Flag-tagged Smad3 was expressed at considerably higher levels than endogenous Smad3 represented by the slightly faster migrating band.

In addition to morphological and immunofluorescent examinations, the effect of modulating Smad3 function on myoblast differentiation was also apparent in immunoblot analyses of muscle-specific proteins (Fig. 3G). The induction of expression of myogenin, an early marker for myogenic differentiation, and MHC, a late differentiation marker, was diminished in Smad3NF cells. In contrast, Smad3CF-expressing cells showed myogenin and MHC expression after incubation in differentiation medium, even in the presence of TGF-β, suggesting that impaired Smad3 signaling suppressed the ability of TGF-β to repress myogenic differentiation. In both types

of stable C2C12 cell lines, the ectopic expression of Flag-tagged Smad3 was considerably higher than that of endogenous Smad3 and did not change with the culture conditions (Fig. 3G, bottom). Thus, altering Smad3 activity by a combination of overexpression, ligand-induced activation, or dominant-negative inhibition has profound effects on myogenic differentiation. These results strongly suggest that Smad3 mediates TGF-β signaling to inhibit the terminal differentiation of myoblast cells.

In parallel, we generated C2C12 cell lines that expressed wild-type or dominant-negative forms of Smad2. These cells differentiated similarly to the control cell line (data not shown). Although such results are consistent with the lack of an effect imposed by Smad2 in MyoD-induced 10T1/2 myogenic conversion (Fig. 1), the lower levels of exogenous Smad2 expression, when compared to Smad3, precluded us from deriving definitive conclusions concerning the role of Smad2 using these experiments.

TGF-β and Smad3 inhibit the activity of muscle-specific bHLH transcription factors

The results outlined above suggest that TGF-β, through Smad3, directly represses the activity of MyoD-family of muscle-specific bHLH factors, and that such repression may be the basis for TGF-β inhibition of myogenesis. To test this hypothesis, we performed transfection reporter assays using a reporter plasmid that contains the luciferase gene under the control of the muscle creatine kinase promoter and enhancer (MCK-Luc) (Sternberg et al. 1988). 10T1/2 cells were cotransfected with expression plasmids for MyoD and Smads, along with the MCK-Luc reporter plasmid. Ectopic MyoD expression strongly transactivated the reporter expression, and this activity was repressed by TGF-β (Fig. 4A). Cotransfection of Smad3 repressed MyoD-activated transcription by up to 80%, and this repression was further enhanced by TGF-β. In addition, cotransfection of Smad4 with Smad3 caused an even greater level of repression. Smad2, in contrast, had only a modest effect on MCK reporter activation by MyoD. Transcriptional repression by TGF-β and Smad3 was also observed for myogenin (Fig. 4B), suggesting that other myogenic bHLH factors may be subjected to similar regulation by the TGF-β pathway as well.

The MCK promoter/enhancer contains a variety of regulatory elements, including E-boxes that bind to MyoD or related MRFs. To determine whether the repression of MyoD and myogenin by TGF-β/Smad3 was directed at the E-box sites, we performed reporter assays using 4R-tk-Luc. This reporter contains the luciferase gene under the control of four tandem repeats of an E-box site from the MCK enhancer, upstream of the thymidine kinase basal promoter (Weintraub et al. 1990). As shown in Figures 4C and 4D, the transcriptional activation of the E-box-based reporter by MyoD or myogenin was strongly repressed by TGF-β or Smad3 overexpression. The Smad3-mediated repression was enhanced by treating the cells with TGF-β or by Smad4 coexpression. Unlike Smad3, Smad2 only weakly repressed the MyoD or myogenin activity at the E-box-based promoter.

To further establish Smad3 as the TGF-β effector in inhibiting MyoD activity, we assessed the effect of dominant-negative Smad3 mutants on MyoD-dependent transcription. Smad3ΔC, a Smad3 mutant with a C-terminal truncation of 39 amino acids, and Smad3SA, in which the last three serines are mutated to alanines, cannot be phosphorylated following TGF-β receptor activation. As a result, these mutants interfere with Smad3 signaling in a dominant-negative manner (Macias-Silva et al. 1996; Zhang et al. 1996; Liu et al. 1997). Expression of both Smad3 mutants counteracted the repression of MyoD activity at the E-box sites by TGF-β (Fig. 4E) or an activated type-I TGF-β receptor (data not shown). The dominant-negative Smad3 mutants also enhanced the reporter transcription without exogenously added TGF-β, consistent with the existence of autocrine TGF-β signaling in myogenic cells (Lafyatis et al. 1991). We also evaluated the effect of two truncated Smad3 mutants on MyoD transactivation of E-box sites. Coexpression of Smad3NL, encompassing the N-terminal MH1 domain and linker region, did not affect MyoD activity, whereas the C-terminal MH2 domain of Smad3 alone (Smad3C) effectively repressed MyoD activity. Consistent with the reporter assays, Smad3C, but not Smad3NL, inhibited MyoD-dependent myogenic conversion of 10T1/2 cells (data not shown). Finally, we tested whether TGF-β can influence MyoD-dependent transcription in Smad3$^{-/-}$ fibroblasts. In the absence of Smad3, TGF-β did not significantly repress the transcriptional activity of MyoD (Fig. 4F). In contrast, reconstituting Smad3 expression in the Smad3-deficient cells from a viral promoter restored the TGF-β-mediated repression. We conclude that TGF-β represses MyoD transactivation of E-box sites by signaling through Smad3, and that the MH2 domain of Smad3 is essential for its repressor function.

The inhibition of MyoD activity by Smad3 is targeted to its bHLH domain

MyoD and related MRFs form obligate dimers with class-A bHLH proteins in the E-protein family to achieve efficient DNA binding to E-box sequences and transcriptional activation of muscle-specific genes. To determine whether TGF-β/Smad3 signaling affects the transcriptional activity of the nonmyogenic E proteins, we used an E12/E47-responsive reporter plasmid, $(E2\text{-}5)_4$-TATA-CAT, which contains four E47 binding sites from the immunoglobulin heavy chain enhancer linked to TATA box minimal promoter (Henthorn et al. 1990). As shown in Fig. 5A, neither Smad2 nor Smad3, in conjunction with TGF-β, repressed the transcriptional activity of E47. Thus, the repression of MyoD activity by Smad3 is specifically targeted to myogenic bHLH factors.

To investigate whether Smad3 signaling inhibits MyoD function by repressing its transcriptional potential independent of DNA binding, we evaluated the transactivation of a Gal4-responsive promoter by MyoD fused to the Gal4 DNA-binding domain (Gal4-DBD) in

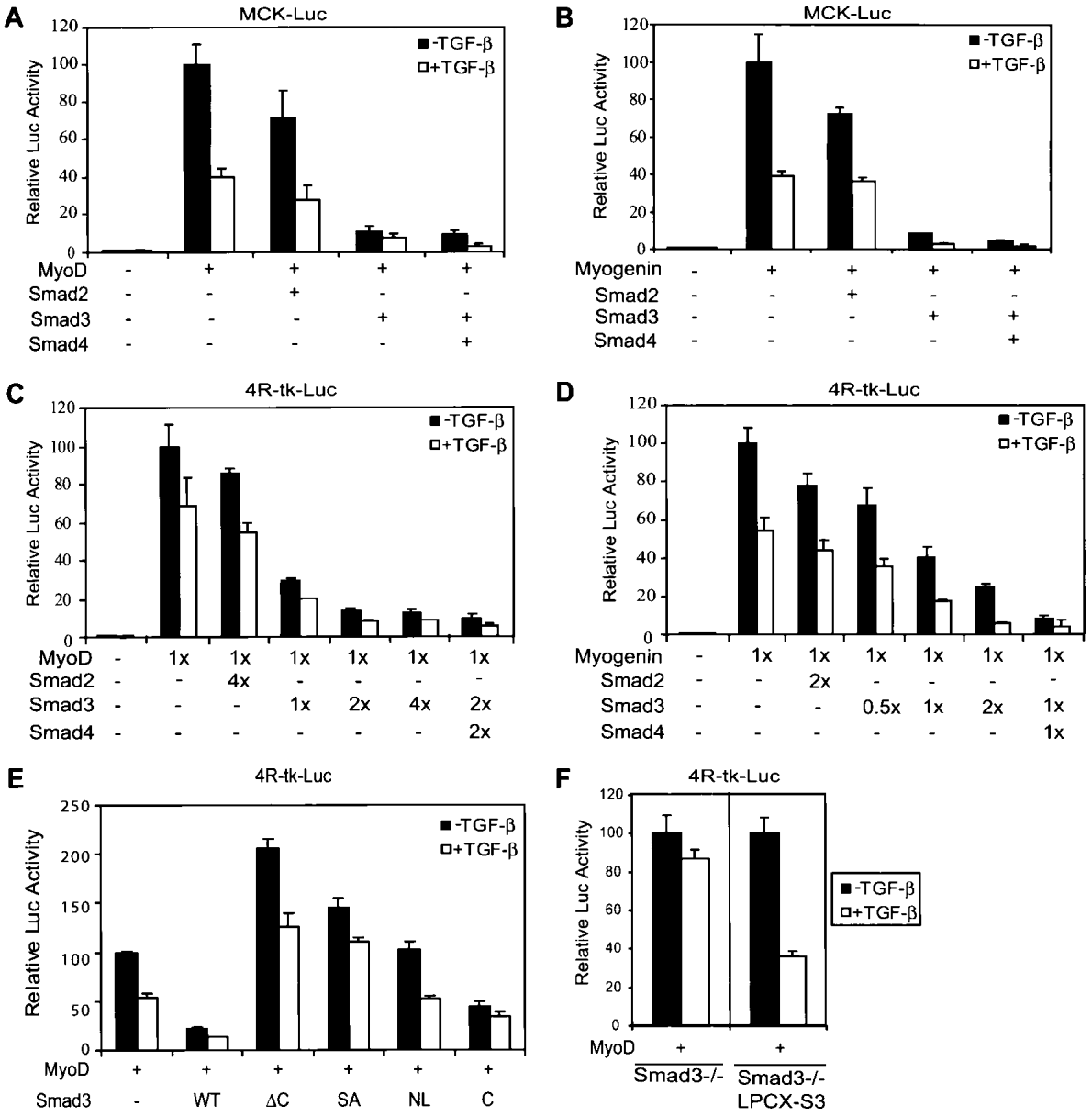

Figure 4. TGF-β signaling through Smad3 inhibits the transcriptional activation of muscle-specific enhancers by myogenic bHLH factors. (*A*,*B*) Effects of TGF-β, Smad2, or Smad3 on MyoD- or myogenin-directed transcription from the MCK promoter. 10T1/2 cells were cotransfected with the MCK-Luc reporter plasmid and the indicated expression plasmids. Transfected cells were cultured with or without 1-ng/mL TGF-β before measuring luciferase activities. (*C*,*D*) Similar transcriptional assays were performed using the 4R-tk-Luc reporter. Increasing quantities of a Smad3 expression plasmid were cotransfected with MyoD or myogenin to show the dosage dependence. (*E*) Effects of wild-type and mutant forms of Smad3 on MyoD-directed transcription from the 4R-tk promoter. 10T1/2 cells were cotransfected with 4R-tk-Luc, expression plasmids for MyoD and wild-type Smad3 (WT), Smad3 dominant-negative mutants SA, and ΔC or truncation mutants NL and C. (*F*) Effect of TGF-β on MyoD transactivation of the 4R-tk-Luc reporter in Smad3$^{-/-}$ mouse embryonic fibroblasts and in Smad3$^{-/-}$ cells engineered to constitutively express wild-type Smad3. All luciferase values, normalized for transfection efficiency, are denoted as a percentage of the activity in cells expressing MyoD or myogenin alone in the absence of TGF-β.

the presence or absence of TGF-β and/or coexpressed Smad3. As shown in Figure 5B, TGF-β and Smad3 had no apparent effect on the transcriptional activity of Gal4-MyoD. The HLH domain of MyoD mediates its association with other proteins, including E12/E47, and may also serve as part of an internal mask for the transcriptional activity of MyoD (Weintraub et al. 1991). Accordingly, deletion of the HLH domain from Gal4-MyoD, which leaves the N-terminal transcriptional activation domain (TAD) of MyoD intact, resulted in enhanced transcriptional activity. However, this fusion protein, Gal4-MyoDΔHLH, was also not responsive to TGF-β

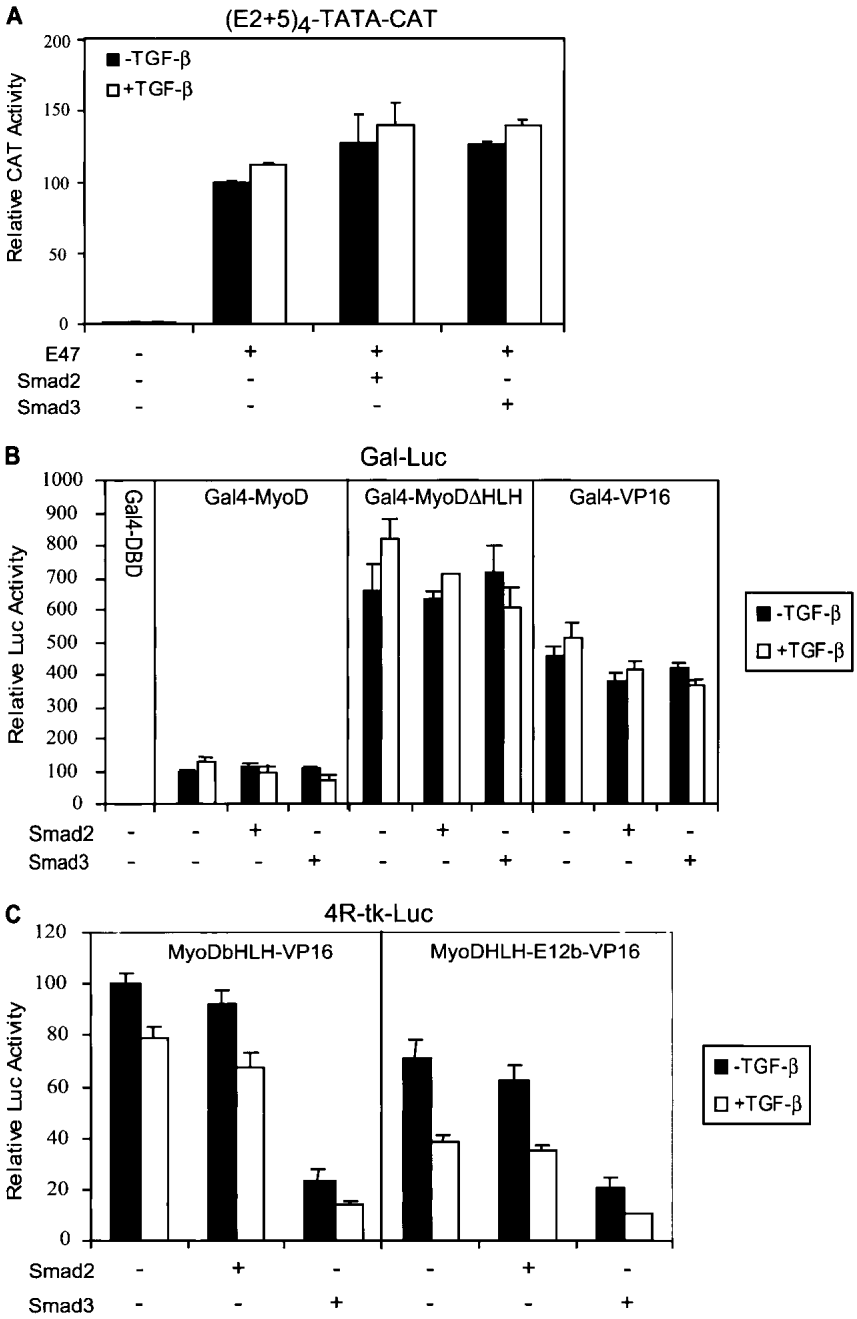

Figure 5. Transcriptional repression by TGF-β/Smad3 signaling is directed at the bHLH domain of MyoD. (*A*) TGF-β/Smad signaling does not affect the transcriptional activity of E47. 10T1/2 cells were transfected with the reporter plasmid $(E2\text{-}5)_4$-TATA-CAT and expression plasmids for E47, Smad2, or Smad3, as indicated. Transfected cells were treated with or without TGF-β as in Fig. 4, and CAT activities were measured. (*B*) TGF-β/Smad3 signaling does not affect the transcriptional potential of MyoD. 10T1/2 cells were transfected with a plasmid encoding either Gal4 DNA-binding domain (Gal4-DBD) or the fusion of Gal4-DBD with MyoD (Gal4-MyoD) or a similar fusion with deletion of the HLH domain (Gal4-MyoDΔHLH), or Gal4-DBD fused to the VP16 transcriptional activation domain (Gal4-VP16). Cells were cotransfected with reporter construct Gal-Luc, containing five copies of Gal4-binding sites driving luciferase expression, as well as Smad2 or Smad3 expression plasmids. (*C*) TGF-β/Smad3 signaling regulates the function of the HLH domain of MyoD. 4R-tk-Luc reporter assays were performed in 10T1/2 cells expressing the bHLH domain of MyoD fused to the VP16 TAD (MyoDbHLH-VP16) or a similar fusion protein, in which the basic domain of MyoD was replaced by that of E12 (MyoDHLH-E12b-VP16). In all assays, values for reporter activities are represented as percentage of the activities of each bHLH transcription factor obtained in the absence of TGF-β and coexpressed Smads.

and/or Smad3. These data strongly suggest that the repression of MyoD activity by TGF-β signaling via Smad3 is not directed at its TAD. In addition, TGF-β and/or Smad3 did not repress the transcriptional activity of the Gal4-fused VP16 activation domain (Fig. 5B, last three lanes), indicating that the effect of Smad3 on transcription by MyoD was not because of general transcriptional repression or squelching.

It has been shown previously that fusing the VP16 activation domain to the bHLH domain of MyoD or myogenin created a fully functional myogenic factor (MyoDbHLH-VP16) (Molkentin et al. 1995). Transcription assays using this fusion protein can, therefore, allow us to evaluate specifically the role of the bHLH domain of MyoD in TGF-β/Smad3-mediated repression. As shown in Figure 5C (left panel), TGF-β and Smad3 repressed the E-box-dependent transcription activated by MyoDbHLH-VP16. The conserved residues within the basic domain of MyoD family proteins have been shown to be required for its E-box-dependent transcriptional activity, whereas linking MyoD to the VP16 activation domain could bypass this requirement (Weintraub et al. 1991; Davis and Weintraub 1992). Replacement of the MyoD basic domain in MyoDbHLH-VP16 with the corresponding sequence from E12 did not abolish its repression by TGF-β/Smad3 signaling (Fig. 5C, right panel).

Taken together, these results show that, at least in the context of the VP16 activation domain, the HLH segment alone is necessary and sufficient to confer sensitivity to Smad3-mediated transcriptional repression.

Smad3 physically interacts with MyoD

The ability of Smad3 to repress MyoD-dependent transcription raised the possibility that the two proteins physically interact. To explore this possibility, we coexpressed Smad3 or Smad2 with MyoD and tested the MyoD/Smad interactions by coimmunoprecipitation analysis. MyoD was detected in association with Smad3 but barely detectable in Smad2 immunoprecipitations (Fig. 6A). Moreover, the C-terminal MH2 domain of Smad3 (Smad3C), but not the NL segment (Smad3NL), coimmunoprecipitated with MyoD, which correlated well with the distinct ability of the two Smad3 mutants to inhibit MyoD function (Fig. 4E). A direct MyoD/Smad3 interaction was also observed in GST absorption analyses, in which in vitro translated MyoD was specifically retained by a GST fusion of full-length Smad3 immobilized to glutathione-sepharose beads (Fig. 6B). We also tested the interaction of other Smad proteins with MyoD in this assay. Despite the overall similarity of Smad1–4, Smad2 and Smad4 showed minimal affinity for MyoD. Smad1, however, also bound to MyoD, which might imply a functional interaction that mediates the antagonistic effect of BMP signaling on myogenesis (Yamamoto et al. 1997). Surprisingly, the GST-adsorption assays revealed an affinity of MyoD for the MH1 domain instead of the MH2 domain of Smad3. This discrepancy suggests that the affinity of the MH1 domain of Smad3 for MyoD is insufficient to allow for coimmunoprecipitation, and that the interaction of the MH2 domain of Smad3 with MyoD in vivo involves additional cellular factor(s) in a multiprotein complex.

To identify the domain of MyoD required for its interaction with Smad3, we analyzed the ability of several MyoD deletion mutants to coimmunoprecipitate with Smad3 (Fig. 6C). Full-length MyoD, as well as MyoD truncated at the N-terminus immediately before the bHLH domain (ΔN1), were able to interact with Smad3. Further truncation encompassing the bHLH domain (ΔN2) of MyoD abolished its interaction with Smad3. Conversely, a MyoD mutant missing the basic domain (ΔB) was still able to bind Smad3, albeit with reduced affinity. These data indicate that MyoD interacts with Smad3 through its HLH domain.

Smad3 inhibits MyoD activity by interfering with the functional MyoD/E protein heterodimer formation

Heterodimerization of MyoD with one of the ubiquitous E proteins such as E12 and E47 is a prerequisite for its ability to activate muscle-specific gene expression. Because Smad3 interacts with the HLH domain of MyoD, the domain that mediates the association of MyoD with E12/E47, we postulated that Smad3 perturbs the dimerization of MyoD with E proteins and that this may account for TGF-β/Smad-mediated repression. We tested this hypothesis in a series of experiments.

If Smad3 represses MyoD activity by displacing a limited cellular pool of E12/E47 from interacting with MyoD, then the repression would be overcome by titration with an excess of E12/E47. We tested this hypothesis using an N-terminally truncated version of E12, which lacks part of the transcriptional activation domain and, consequently, has minimal E-box-transactivating potential by itself (Murre et al. 1989). As expected, cotransfection of the truncated E12 reversed TGF-β/Smad3 inhibition of MyoD activity at the 4R-tk promoter (Fig. 7A). Coexpression of the E12 mutant also incrementally rescued the Smad3-mediated repression of MCK-Luc reporter activation by MyoD, although the efficiency of such rescue was less robust in comparison with results obtained using the E-box-based reporter (Fig. 7B).

As a further demonstration that Smad3-mediated repression was targeted at the step of MyoD/E dimer formation, we examined the activity of MyoD~E47, a fusion protein in which MyoD and E47 were tethered by a flexible peptide linker (Neuhold and Wold 1993). As shown in Fig. 7C, MyoD~E47 showed strong transactivation of the 4R-tk promoter, but unlike the untethered wild-type MyoD, its activity was refractory to the repression by Smad3/TGF-β signaling. This result suggests that the forced dimerization with E47 renders MyoD no longer susceptible to the physical hindrance from Smad3. Interestingly, the transactivation of MCK-Luc reporter by MyoD~E47 remained mildly repressed by Smad3 in TGF-β-stimulated cells (Fig. 7D), although the extent of this repression was significantly lower compared with Smad3-mediated repression of untethered MyoD (Fig. 4A). This residual sensitivity of MyoD~E47-activated MCK reporter expression to Smad3 bears resemblance to the inhibitory effect of the HLH protein Id. Id lacks a DNA-binding domain and inhibits the activity of the myogenic bHLH factors by forming transcriptionally inactive heterodimers with MRFs or by sequestering E proteins (Benezra et al. 1990; Jen et al. 1992). Whereas transactivation of the 4R-tk promoter by MyoD~E47 is resistant to high levels of Id expression, activation of MCK enhancer/promoter by the same fusion protein is still partially inhibited by Id (Neuhold and Wold 1993). The difference between the response of MyoD~E47 in the two reporter systems presumably reflects the fact that unlike the simple concatemerized E-box sites, maximum activation of the MCK transcriptional unit requires the cooperative function of Smad3-sensitive regulators in addition to MyoD complexes, including secondary factors such as myogenin.

We also tested whether Smad3 was able to disrupt the MyoD/E12 association in mammalian two-hybrid assays. In this experiment, expression plasmids for E12 fused to Gal4-DBD and MyobHLH-VP16 (see Fig. 5C) were transfected into 10T1/2 cells together with a Gal4-responsive reporter. The reporter gene activation quan-

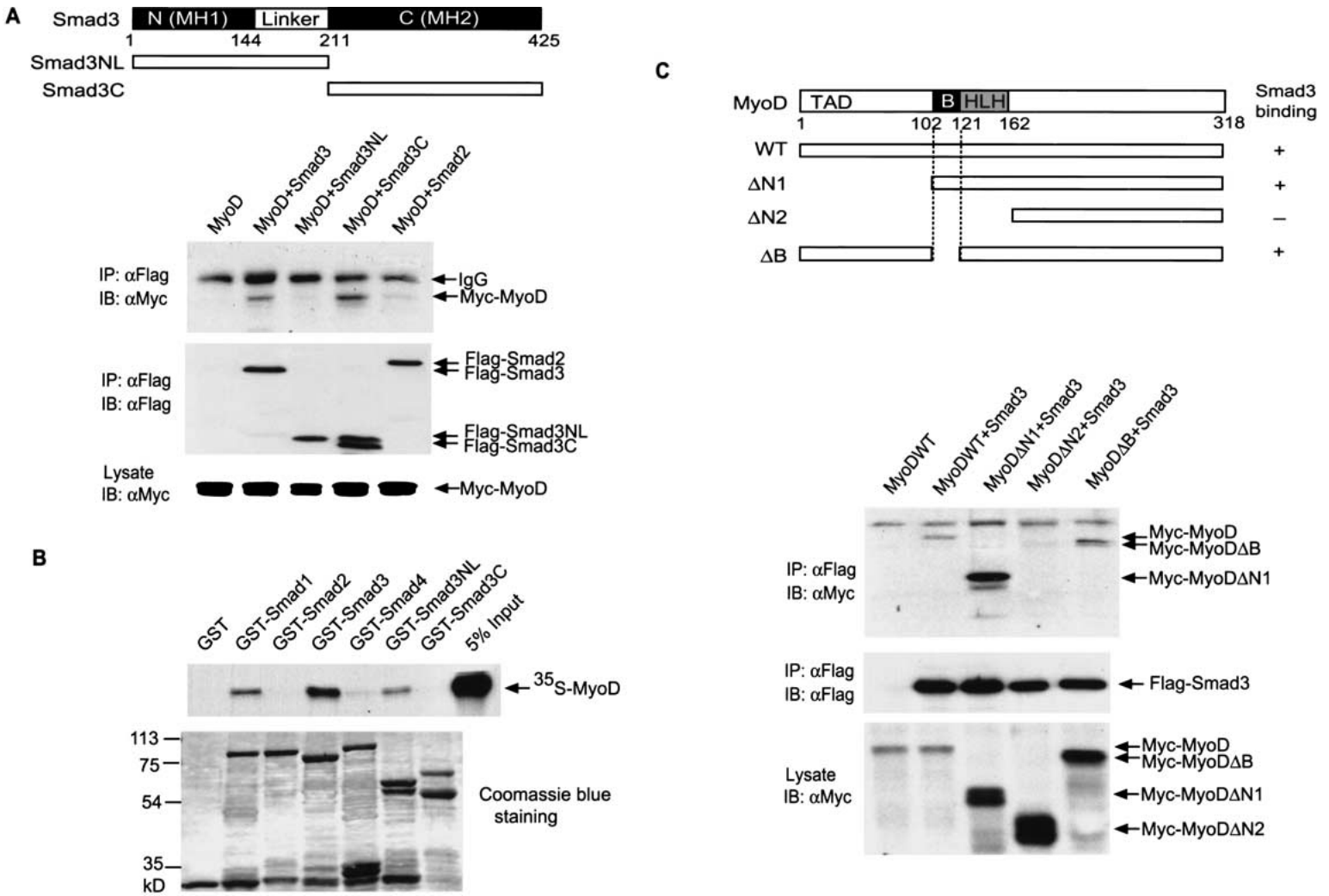

Figure 6. Smad3 interacts with MyoD in vivo and in vitro. (*A*) Association of Smad3 with MyoD in transfected cells. COS cells were transfected with expression plasmids for Myc-tagged MyoD Flag-tagged full-length Smad2 or Smad3 or Smad3 fragments (Smad3NL and Smad3C). Cell lysates were subjected to Flag immunoprecipitation followed by immunoblotting using anti-Myc tag antibody to detect the coprecipitated MyoD, or anti-Flag antibody to detect the Smad proteins. Expression of Myc-MyoD was assessed by direct immunoblotting of a portion of the cell lysates. (*B*) Interaction of ^{35}S-labeled in vitro-translated MyoD with GST-Smad fusion proteins. The *upper* panel shows MyoD protein retained by the indicated GST-Smad beads. The Coomassie Blue-stained gel in the *lower* panel shows the integrity and equal loading of the GST-fusion proteins. (*C*) Mapping of the MyoD domain that interacts with Smad3 in vivo. (*Top*) Schematic diagram shows the location of the MyoD deletion mutants. (*Bottom*) Myc-tagged full-size MyoD or its deletion mutants were coexpressed with Flag-Smad3 in transfected COS cells. Flag immunoprecipitates were subjected to immunoblotting using anti-Myc antibody as in *A*. The expression levels of epitope-tagged proteins were analyzed by immunoblotting (*middle* and *lower* panel).

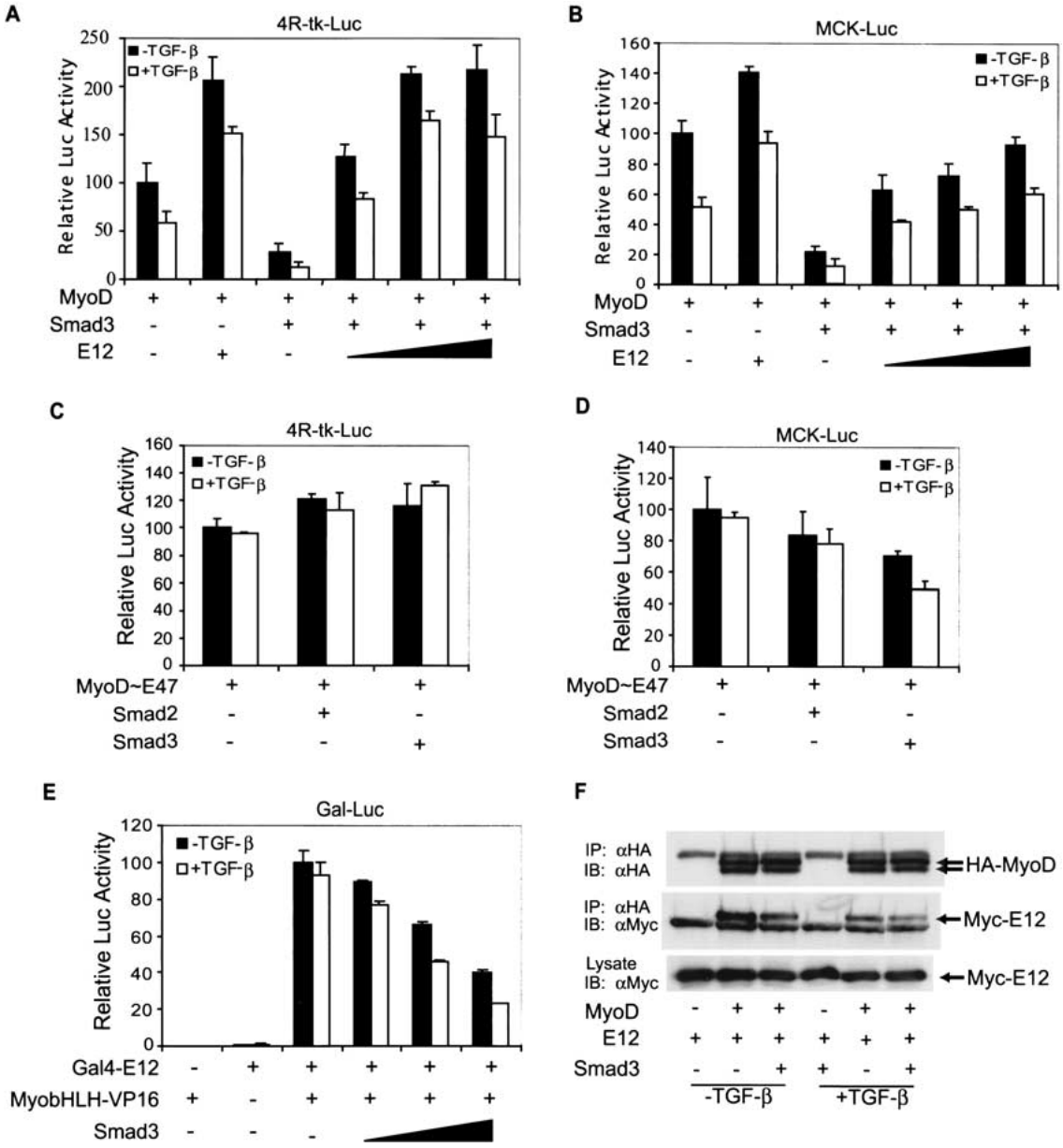

Figure 7. TGF-β and Smad3 repress the transcriptional activity of MyoD by limiting its access to the E2A class of bHLH protein partners. (*A*,*B*) Increased E12 expression counteracts Smad3-mediated repression of transcription by MyoD. 10T1/2 cells were transfected with the 4R-tk-Luc (*A*) or MCK-Luc (*B*) reporter plasmid and expression plasmids for MyoD and Smad3, as well as increasing amounts of an E12 expression plasmid. Luciferase expression levels in the absence or presence of TGF-β were quantitated as in Fig. 4. (*C*,*D*) The activity of a covalently tethered MyoD~E47 dimer is resistant to repression by TGF-β/Smad3 signaling. 10T1/2 cells were transfected with the 4R-tk-Luc (*C*) or MCK-Luc (*D*) reporter, together with expression plasmids for MyoD~E47 and Smad2 or Smad3. Luciferase activities were scored relative to the value of MyoD~E47 alone in the absence of TGF-β. Cotransfection of equivalent quantities of Smad3 and MyoD expression plasmid resulted in a repression of the MyoD activity by > 80% (see Fig. 4). (*E*) Mammalian two-hybrid analyses of the association between MyoD and E12. 10T1/2 cells were transfected with the indicated expression plasmids and a Gal4-responsive reporter Gal-Luc in the presence of increasing amounts of Smad3 expression plasmids. The normalized luciferase activities, as a consequence of the interaction between Gal4-E12 and MyoDbHLH-VP16 fusion proteins, are shown. (*F*) Evaluating the MyoD/E12 interaction by coimmunoprecipitation. HA-tagged MyoD and Myc-tagged E12 were expressed in 10T1/2 cells in the presence or absence of 2ng/mL TGF-β and/or cotransfected Smad3. Cell lysates were subjected to anti-HA immunoprecipitation, and MyoD and coprecipitated E12 in the protein complexes were detected by immunoblotting, using anti-HA (*top*) or anti-Myc antibodies (*middle*), respectively. The expression of E12 in the cell lysates was monitored by immunoblotting using anti-Myc antibody (*bottom*).

titatively reflects the extent of physical interaction between the Gal4 and VP16 fusion proteins. As shown in Figure 7E, in the absence of TGF-β and Smad3, the interaction between E12 and MyoD bHLH domain resulted in a strong transcriptional activation from the Gal4-responsive promoter. Coexpression of Smad3 inhibited the interaction, as indicated by reduced luciferase expression. TGF-β treatment provided a further yet modest repression, which was presumably limited by the low levels of cell surface receptors relative to that of exogenous Smad3. In agreement with the results from the two-hybrid assays, the association of ectopically expressed MyoD and E12 in 10T1/2 cells was also detected by coimmunoprecipitation analyses (Fig. 7F). Treatment of the cells with TGF-β and/or Smad3 coexpression reduced the relative amount of E12 in complex with MyoD. Together, these results suggest that Smad3-mediated signaling interferes with the ability of MyoD to form functional heterodimers with E2A gene products, and that this interference leads to transcriptional repression.

Smad3 interferes with the cooperative binding of MyoD to E-box sequences

Dimerization of MyoD with E proteins is required for efficient binding of MyoD to its target DNA sequences. Association of MyoD with E12/E47 has also been shown to facilitate cooperative DNA binding and synergistic activation of muscle-specific enhancers with multiple E-box binding sites (Bengal et al. 1994). Because Smad3 affected the MyoD/E dimer formation, we examined the effect of Smad3 on the binding of MyoD to an oligonucleotide sequence with two juxtaposed E-box sites (2xMEF1) in electrophoretic mobility shift analysis (EMSA). Nuclear extracts prepared from MyoD-transfected 10T1/2 cells gave rise to a specific DNA–protein complex that was absent in control-transfected cells (Fig. 8A, lanes 1 and 2). The identity of this complex was further confirmed by a supershifted pattern following incubation of the extract with anti-Myc antibody, which recognized the Myc-tagged MyoD (lane 4). TGF-β or Smad3 coexpression decreased the levels of this complex (Fig. 8A, cf. lanes 2, 3, and 6), which was further diminished when the Smad3-expressing cells were exposed to TGF-β (Fig. 8A, cf. lanes 6 and 7).

As an alternative approach to verify these observations, we used a DNA absorption assay to assess the binding of MyoD to a biotinylated 2xMEF2 oligonucleotide immobilized to streptavidin beads (Fig. 8B). Consistent with the results from EMSA, TGF-β and Smad3 diminished the affinity of MyoD-containing protein complexes to the biotynylated probe (Fig. 8B).

Nuclear extract from cells transfected with MyoD~E47 also gave rise to a specific band shift in EMSA using the 2xMEF1 probe, and this complex was supershifted by an anti-HA antibody directed at the epitope tag sequence within the fusion protein (Fig. 8C). In contrast to the results obtained with wild-type MyoD, the gel-shift patterns were not significantly changed by TGF-β and coexpressed Smad3. Taken together, these results suggest that TGF-β signaling through Smad3 inhibits the cooperative binding of MyoD/E protein complexes to E-box sequences.

Discussion

Consistent with the key role of the MyoD family of MRFs in initiating the myogenic program, the potent inhibition exerted by TGF-β on myoblast differentiation can be attributed to its inhibitory effects on the expression and function of these MRFs. Whereas TGF-β treatment causes downregulation of MyoD mRNA in myoblast cultures, the ability of TGF-β to inhibit differentiation of cells with constitutive expression and normal nuclear localization of MyoD or myogenin (Vaidya et al. 1989; Brennan et al. 1991) suggests a posttranscriptional repression of MRF activity by TGF-β. The reduced MyoD expression in the presence of TGF-β could then indirectly result from a diminished ability of MyoD to maintain its own expression through an autoregulatory loop (Thayer et al. 1989).

In this report, we showed that Smad3 acts as the downstream effector of TGF-β to block MyoD-induced myogenic differentiation. Smad3 interacts physically with MyoD both in vitro and in vivo, and this interaction may underlie the ability of Smad3 to inhibit the transactivation of E-box-containing muscle enhancers by MyoD. The Smad3-mediated transcriptional repression is specific to myogenic bHLH transcription factors and appears to result from an interference with the efficient heterodimerization of MyoD with E12/47. Our results also indicate that Smad3-mediated repression is required for TGF-β to inhibit the terminal differentiation of myoblast cells, because overexpression of a dominant-negative Smad3 can partially override the TGF-β action.

Among the two receptor-activated Smads that respond to TGF-β, i.e. Smad2 and Smad3, only Smad3 potently represses the transcription of genes associated with muscle differentiation, whereas Smad2 shows only minimal effects (Figs. 1, 4). The prominent repressor role for Smad3 correlates with its higher affinity for MyoD in coimmunoprecipitation analyses. Despite the overall amino acid sequence similarity (83%) of the two proteins, Smad2 and Smad3 apparently mediate different aspects of the cellular response to TGF-β. This is also illustrated by studies using Smad2- and Smad3-deficient fibroblasts, in which the TGF-β-induced activation of gene expression was selectively dependent on Smad2 or Smad3 (Piek et al. 2001). In addition to their differential roles in myogenesis, Smad2 and Smad3 vary in their ability to regulate other mesenchymal differentiation programs. Smad3, but not Smad2, mediates repression of the differentiation of adipocytes (Choy et al. 2000) and osteoblasts (Alliston et al. 2001). The structural and functional features that distinguish the two TGF-β-activated Smads remain to be characterized.

The transcriptional control by Smads depends on a variety of transcription factors or cofactors that associate with Smads in the transcription complexes. These inter-

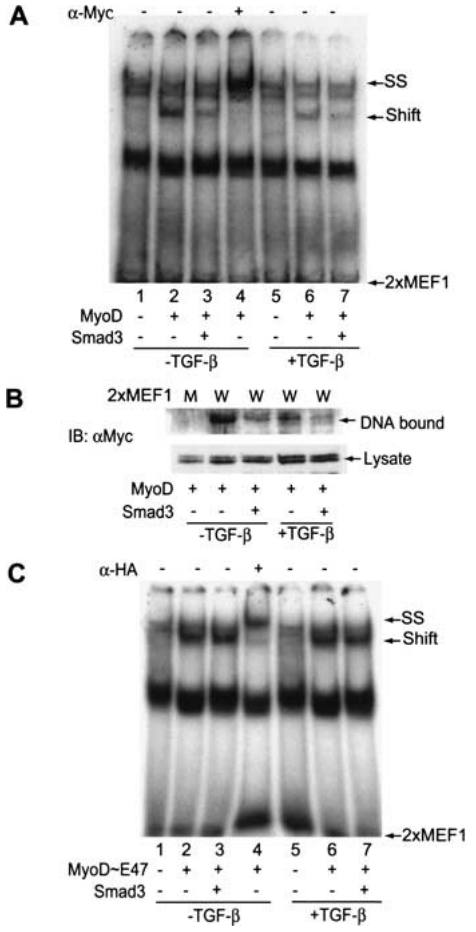

Figure 8. TGF-β/Smad3 signaling diminishes the binding of MyoD protein complexes to E-box DNA sequences. (*A*) Electrophoretic mobility shift assay (EMSA) using nuclear extracts from 10T1/2 cells transfected with Myc-tagged MyoD and Flag-tagged Smad3, as indicated, in the presence or absence of TGF-β. The oligonucleotide probe contains two direct repeats of an E-box sequence from the MCK enhancer (2xMEF1). In lane *4*, anti-Myc antibody, which recognizes Myc-tagged MyoD, was added to the binding reaction. The MyoD-containing DNA-protein complex (Shift) was identified by comparing the gel-shift patterns from extracts of vector-transfected control and MyoD-transfected cells, and by the appearance of a supershifted (SS) tertiary complex in the presence of anti-Myc antibody. The composition of the lower mobility complexes, present in all samples, including control reactions, is not clear; however, these bands were absent when the oligonucleotide contained only one E-box sequence (data not shown). (*B*) Binding of MyoD to the MEF1 sites was analyzed using biotin-labeled oligonucleotides. The biotinylated wild-type 2xMEF1 (W) or a mutant MEF1 (M) oligonucleotide immobilized on streptavidin beads were incubated with lysates of the transfected 10T1/2 cells as in *A*, and the DNA-bound MyoD complexes were analyzed by gel electrophoresis followed by immunoblotting using anti-Myc antibody. The expression of MyoD or Smad3 in the cells was detected by immunoblotting of a fraction of the lysates. (*C*) EMSA was also performed for nuclear extracts of cells transfected with HA-tagged MyoD–E47 fusion protein. Binding of this protein to the 2xMEF2 site, as identified by the supershifted band following incubation with anti-HA antibody, was not significantly affected by TGF-β treatment and Smad3 coexpression.

actions could explain the diversity and specificity among cellular responses to TGF-β in different cell types and contexts (Massagué and Wotton 2000). Several Smad-interacting proteins, including TGIF (Wotton et al. 1999), Ski/SnoN (Liu et al. 2001), and Evi-1 (Kurokawa et al. 1998; Izutsu et al. 2001), can interact with Smad2 or Smad3 and attenuate Smad-activated transcription by recruiting histone deacetylases (HDACs) or C-terminal binding protein (CtBP). None of these corepressors, however, has been implicated in transcriptional repression in response to TGF-β/Smad signaling. Transcriptional corepressors are also involved in the negative regulation of MyoD activity. MyoD associates with an HDAC either directly or through the nuclear receptor corepressor (N-CoR) adaptor protein in undifferentiated myoblast cells, and these interactions silence MyoD-dependent induction of muscle-specific genes (Bailey et al. 1999; Mal et al. 2001). Our results argue against a role for Smad3 in recruiting corepressors or in promoting corepressor associations with MyoD, because the transcriptional activities of MyoD or MyoDΔHLH linked to Gal4-DBD are not repressed by TGF-β/Smad3 when assayed using a Gal4-dependent promoter (Fig. 5).

An alternative possibility could be that TGF-β signaling via Smad3 disrupts the function of a MyoD coactivator, which is required for its transactivation of E-box-containing muscle promoters. The histone acetyltransferases, CBP/p300 and PCAF, have been shown to interact with MyoD and are required for MyoD-dependent activation of muscle differentiation (Eckner et al. 1996; Yuan et al. 1996; Puri et al. 1997). CBP and p300 also serve as essential coactivators of Smad3-activated transcription (Feng et al. 1998). Furthermore, competitive recruitment of coactivators has recently been suggested to account for Smad3-mediated repression of macrophage gene activation (Werner et al. 2000), as well as CEBP/β- and STAT3-induced activation of the haptoglobin promoter in response to IL-6 (Zauberman et al. 2001). However, our preliminary results indicate that overexpression of p300 or CBP did not restore MyoD activity in the presence of TGF-β/Smad3 (data not shown). Therefore, these coactivators are unlikely to represent major targets for Smad3 in its repression of MyoD activity.

The MEF2 family of transcription factors acts as coactivator for the MRFs through combinatorial association and transcriptional cooperation (Black and Olson 1998). Previous studies have shown that inhibition of the Myf5 and myogenin activities by Notch or Raf signaling is targeted at MEF2 (Wilson-Rawls et al. 1999; Winter and Arnold 2000). Although TGF-β reportedly restricts the function of MEF2 by regulating its nuclear localization (De Angelis et al. 1998), TGF-β/Smad signaling enhances MEF2-dependent transcription (Quinn et al. 2001; data not shown). Furthermore, Smad3 does not inhibit the transcriptional cooperativity between MEF2 and MyoD (data not shown), suggesting that the Smad3-mediated repression of MRFs is independent of MEF2 function.

Instead, multiple lines of evidence suggest that Smad3 represses the activity of MyoD by interfering with its heterodimerization with E12/E47 and with its acquisi-

tion of a transcriptionally competent state. First, the repression by Smad3 targets the bHLH domain of MyoD (Fig. 5C), the interface that accommodates the interaction of MyoD with E12/E47. Second, Smad3 physically interacts with the MyoD HLH domain (Fig. 6C). Third, Smad3 interferes with the MyoD/E12 dimer formation (Fig. 7E,F). Fourth, increased expression of E12 counteracts Smad3-mediated repression, which is most easily explained by competitive displacement (Fig. 7A,B). Finally, the repression at the E-box sites can be abolished by covalent tethering of E47 to MyoD (Fig. 7C). Other proteins have been shown to repress myogenic transcription by disrupting the functional cooperation of myogenic bHLH proteins with E proteins. Perhaps the best-known example is the serum-induced Id family of HLH proteins. Like Id (Neuhold and Wold 1993), Smad3 was unable to block the transcriptional activity of the MyoD~E47-forced dimer. Although TGF-β stimulation of myoblasts does not induce Id1 expression (Brennan et al. 1991), we cannot exclude the possibility that the repression of MyoD is in part mediated by posttranslational regulation of Id or depends on other Id-family or Id-like myogenic inhibitors that target MyoD/E dimerization, such as MyoR (Lu et al. 1999) and Mist1 (Lemercier et al. 1998).

We also found that TGF-β/Smad3 signaling reduced the binding of MyoD protein complexes to two adjacent E-box sites (Fig. 8). The upstream regulatory sequences of many muscle-specific genes, including MLC1/3 (Wentworth et al. 1991), acetylcholine receptor α (Piette et al. 1990), MCK (Buskin and Hauschka 1989), and MyoD (Goldhamer et al. 1995), contain multiple E-box sites. In general, at least two E-box sites are required for the activation of these genes by the MRFs. This requirement may be attributable to a limited affinity of MRF complexes to a single E-box and the ability of these complexes to cooperatively bind to multiple E-box sites (Weintraub et al. 1990; Bengal et al. 1994). It is conceivable that the collaboration among multiple MyoD/E dimers that occupy neighboring E-box sites stabilizes the active transcriptional complexes formed on muscle promoters. Dimerization with E-protein partners has been suggested to result in conformational changes in MyoD that enhance its DNA-binding and transcriptional potential (Bengal et al. 1994). Therefore, by interfering with the MyoD/E protein interaction, Smad3 may prevent efficient binding of MyoD to complex target-DNA sites. Although we (data not shown) and others (Kong et al. 1995) have not observed an overt effect of TGF-β or Smad3 on the equilibrium binding of MyoD to a single E-box sequence, gel-shift analysis using a single binding site may not be sufficiently sensitive to detect a dynamic change in the retention and dissociation rates of MyoD protein complex.

Our study, nonetheless, does not rule out the existence of additional mechanisms that potentially contribute to the inhibition of myogenesis by TGF-β. For example, changes in the cell adhesion induced by TGF-β (Heino and Massagué 1990), and crosstalk between TGF-β signaling and the MEK/ERK mediated receptor tyrosine kinase cascade (Peña et al. 2000), have been suggested to be involved in the suppression of some aspects of myoblast differentiation. In muscle development, multiple growth factor or oncogenic signals may provide multiple reinforcing levels of control on the myogenic machinery to ensure that differentiation only proceeds when appropriate environmental cues are received.

Materials and methods

Expression plasmids

Coding sequences for N-terminally Myc- or HA-tagged MyoD and myogenin or their defined fragments were generated by oligonucleotide- or PCR-based techniques and subcloned at the *Eco*RI–*Xba*I sites of pRK5 (Graycar et al. 1989). Gal4 DNA-binding domain fusion proteins of MyoD or E12 were generated by inserting the coding sequences into the *Eco*RI–*Xba*I sites of pXF1Gal vector (Feng et al. 1998), which were derived from pRK5. Detailed descriptions of the construction of these plasmids are available on request. Expression plasmids pcDNA1-MyoDbHLH-VP16, encoding a fusion protein of the MyoD bHLH domain with the VP16 activation domain, and pcDNA1–MyoDHLH–E12b–VP16, in which the basic domain of MyoD was replaced by the corresponding region of E12, were described previously (Molkentin et al. 1995). The cDNA of a partially truncated E12 with a reconstituted translation start site (Murre et al. 1989), full-length E47, and the MyoD~E47 fusion tethered through a polypeptide bridge (Neuhold and Wold 1993), were subcloned into pRK5 for transient expression experiments. N-terminally Flag-tagged versions of Smads and their derivatives were expressed using pRK5 vector as described (Zhang et al. 1998). The construction of pLNCX or pLPCX retroviral vectors, expressing Smad2 and Smad3 and their dominant-negative mutants, has been described previously (Choy et al. 2000).

Cell culture and transfections

C3H101/2 mouse embryonic fibroblast cells (ATCC) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). C2C12 myoblasts (ATCC) were maintained in growth medium (GM), consisting of high-glucose DMEM with 15% FBS. Cells were grown in subconfluent conditions before the initiation of myogenesis, which occurred when cells were transferred to differentiation medium (DM) containing 2% horse serum. COS cells, Phoenix E retrovirus packaging cells (obtained from G. Nolan, Stanford University), Smad3$^{-/-}$ mouse embryonic fibroblasts (Datto et al. 1999) (obtained from X.F. Wang, Duke University) or a derivative cell line, which stably expressed wild-type Smad3 through viral vector transduction, were grown in DMEM with 10% FBS.

Transient transfections of 10T1/2 or Smad3$^{-/-}$ fibroblast cells were performed using Effectene reagents (QIAGEN), and COS cells were transfected using Lipofectamine (Life Technologies), according to the manufacturers' instructions. In all transfections, the total quantities of transfected DNA were kept constant by adding an appropriate amount of empty vector pRK5 as needed.

Generation of stable cell lines by retroviral transduction

Retroviral vectors were used to establish stable cell populations overexpressing Smad2 or Smad3 or their derivatives. Infectious retroviral particles, containing the pLNCX- or pLPCX-based expression vectors, were generated following transfection of Phoe-

nix E packaging cells, as described previously (Choy et al. 2000). C2C12 cells were plated in six-well dishes at a density of 2×10^5/well 1 d before infection. Cells were overlaid with 1.5 mL of conditioned medium containing recombinant viruses in the presence of 4 μg/mL polybrene, and the infection was facilitated by two rounds of low-speed centrifugation at room temperature for 45 min each. Selection of the infected cells was initiated 48 h after infection in the presence of 2 μg/mL puromycin (for pLPCX vectors) or 1-mg/mL G418 (for pLNCX vectors), and proceeded for 1 to 2 wk, during which time cells were subcultured before reaching confluence.

Induction of myogenic differentiation and immunofluorescence detection

10T1/2 cells transfected with a MyoD expression alone or in combination with Smad2/3 expression plasmids were grown in DM for 2 d, by which time the MyoD-induced myogenic conversion was evident from the appearance of myotubes. Cells were fixed and permeabilized in PBS with 4% formaldehyde and 0.2% Triton X-100 for 20 min. After a blocking step in PBS containing 1% goat serum for 20 min, the cells were stained with an anti-MHC monoclonal antibody MF20 (Developmental Studies Hybridoma Bank, IA) or MY32 (Sigma), together with an anti-MyoD polyclonal antibody M318 (Santa Cruz). The slides were then washed three times in PBS, and the primary complexes were detected using Oregon Green-conjugated goat anti-mouse antibody for MHC or Texas Red-conjugated goat anti-rabbit antibody for MyoD (both from Molecular Probes), and examined using a Zeiss Axioskop microscope. Nuclei were identified by staining with Hoechst 33258 (Sigma). Differentiation of C2C12 myoblast cells was initiated by shifting subconfluent monolayer cells grown in GM to DM in the presence or absence of 1-ng/mL TGF-β. The medium was replaced every day and after 3 to 4 d in DM, cells were processed for MHC immunostaining using the MF20 monoclonal antibody as outlined above.

Transcription reporter assays

The TGF-β responsive, Smad-activated reporter 3TP-lux has been described (Cárcamo et al. 1995). To assay the activities of muscle-specific bHLH transcription factors, we used the reporter plasmid MCK-Luc, which contains the promoter/enhancer region of the MCK gene driving luciferase expression (Lu et al. 1999), or the 4R-tk-Luc reporter plasmid, which contains four tandem repeats of the high affinity right E-box site from the MCK enhancer linked to the thymidine kinase (tk) basal promoter and the luciferase gene (Lassar et al. 1991). The reporter plasmid used to assess the activities of E2A gene products, $(E2\text{-}5)_4$-TATA-CAT, contains the chloramphenicol acetyl transferase (CAT) gene downstream of four copies of E47-binding sites from the IgH enhancer and TATA box (Henthorn et al. 1990). Transactivation by Gal4-fused transcription factors was determined using a Gal4-responsive luciferase reporter pFR-Luc (Stratagene). One day after transfection with the reporter and expression plasmids, cells were transferred to culture medium containing 0.3% FBS with or without 2-ng/mL TGF-β. Cells were harvested 24 to 48 h later and lysed in Reporter Lysis Buffer (Promega) before luciferase assay using a detection kit (Pharmingen) and luminometric measurements. Liquid scintillation CAT assays were performed as described previously (Newmann et al. 1987). The luciferase and CAT activity obtained were normalized to the β-galactosidase activity from the cotransfected expression plasmid pRK5-βgal (Feng et al. 1998). Each assay was performed in replicate and was representative of at least three experiments.

Immunoprecipitations and Western blotting

COS or 10T1/2 cells transfected with expression plasmids for epitope-tagged MyoD, E12, and full-length Smads or defined fragments, were harvested 24 to 48 h post transfection and lysed by brief sonication in LSLD buffer (Wotton et al. 1999) (50-mM HEPES at pH 7.4, 50-mM NaCl, 0.1% Tween-20, 10% glycerol, 50-mM NaF, 1-mM NaV_2O_4, and protease inhibitor cocktails). The lysates were then subjected to immunoprecipitation using antibody-coated protein A-Sepharose. The Sepharose beads were washed extensively in wash buffer (50-mM Tris-HCl at pH 7.5, 200-mM NaCl, 0.5% NP-40) and the absorbed protein complexes were analyzed by SDS-PAGE and Western blotting. The antibodies included anti-Flag M2 monoclonal antibody (Sigma) for Flag-tagged Smads, or anti-HA and anti-Myc tag monoclonal antibodies (Covance) for HA- or Myc-tagged MyoD and E12. A fraction of the cell lysates was subjected to direct immunoblotting to control for expression of the protein of interest and, if needed, the amount of lysates used for immunoprecipitation was adjusted accordingly. The antibodies used in the immunoblot analysis of protein expression during C2C12 myogenic differentiation included rabbit polyclonal antimyogenin (Santa Cruz), mouse monoclonal anti-MHC (Sigma), and rabbit polyclonal anti-Smad3 (Zymed).

GST protein interaction assays

GST fusion proteins of full-length Smads and Smad3 fragments (Zhang et al. 1998) were expressed in *Escherichia coli* and purified by absorption to glutathione-Sepharose 4B (Amersham Pharmacia). ^{35}S-labeled MyoD was generated by in vitro transcription and translation using the TNT kit (Promega). The radiolabeled translation mixture was precleared by incubation with recombinant GST protein bound to glutathione-Sepharose beads before incubation with 5 μg each of the GST-Smad fusion protein bound to the beads in binding buffer (50-mM Tris-HCl at pH 7.5, 200-mM NaCl, and 0.5% Triton X-100). The beads were washed extensively in the same buffer, and the absorbed proteins were separated on SDS-PAGE and visualized by autoradiography.

Electrophoretic mobility shift (EMSA) and biotinylated oligonucleatide-binding assays

For EMSA, double-stranded oligonucleotides, corresponding to two tandem copies of the high-affinity MEF1 (2xMEF1) MyoD-binding sites from the MCK enhancer (Weintraub et al. 1990), were labeled with [γ-^{32}P]dCTP using T4 polynucleotide kinase. The upper strand of 2xMEF1 has the sequence 5′-CCCCAA CACCTGCTGCCTGACCAACACCTGCTGCCTGA-3′, with the E-box site underlined. Nuclear extracts of 10T1/2 cells transiently transfected with expression constructs encoding Smad3, and either Myc-tagged MyoD or HA-tagged MyoD~E47 in the presence of absence of TGF-β, were prepared essentially as described (Kong et al. 1995). The labeled oligonucleotide probes were incubated with 15 μg of nuclear proteins in reaction buffer containing 20-mM Tris-HCl (pH 7.6), 50-mM NaCl, 0.5-mM EDTA, 1-mM $MgCl_2$, 0.5-mM DTT, 5% glycerol, and 0.05-mg/mL poly(dI-dC). For supershift analyses, anti-HA or anti-Myc tag antibodies (1 μg) were preincubated with 10T1/2 cell nuclear extracts in reaction buffer before the addition of probes. The DNA–protein complexes were resolved on 5% polyacrylamide gels in 0.5× TBE buffer and visualized by autoradiography.

For biotinylated oligonucleotide binding experiments, a double-stranded 2xMEF1 oligonucleotide or a mutant MEF1 oligonucleotide (5′-CCCAACACGGTAACCCTGAG-3′) with 5′-biotin modification, was coupled to streptavidin magnetic beads (Promega). The beads were incubated with 200 μL of total cell lysates from 10T1/2 cells transfected with MyoD and Smad3 expression plasmids and 5 μg of poly(dI-dC) in binding reaction buffer containing final concentrations of 20-mM Tris-HCl (pH 7.5), 100-mM NaCl, 0.5-mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 5% glycerol, and 0.1% Triton X-100 for 1 h at 4°C. The beads were washed extensively using a magnetic stand in 20-mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5-mM EDTA, 1 mM $MgCl_2$, 0.5 mM DTT, and 1% glycerol. Proteins coprecipitated with the immobilized DNA probes were analyzed by SDS-PAGE followed by Western blotting.

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