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Nuclear Function of Smad7 Promotes Myogenesis[▽]

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In the "canonical" view of transforming growth factor β (TGF-β) signaling, Smad7 plays an inhibitory role. While Smad7 represses Smad3 activation by TGF-β, it does not reverse the inhibitory effect of TGF-β on myogenesis, suggesting a different function in myogenic cells. We previously reported a promyogenic role of Smad7 mediated by an interaction with MyoD. Based on this association, we hypothesized a possible nuclear function of Smad7 independent of its role at the level of the receptor. We therefore engineered a chimera of Smad7 with a nuclear localization signal (NLS), which serves to prevent and therefore bypass binding to the TGF-β receptor while concomitantly constitutively localizing Smad7 to the nucleus. This Smad7-NLS did not repress Smad3 activation by TGF-β but did retain its ability to enhance myogenic gene activation and phenotypic myogenesis, indicating that the nuclear, receptor-independent function of Smad7 is sufficient to promote myogenesis. Furthermore, Smad7 physically interacts with MyoD and antagonizes the repressive effects of active MEK on MyoD. Reporter and myogenic conversion assays indicate a pivotal regulation of MyoD transcriptional properties by the balance between Smad7 and active MEK. Thus, Smad7 has a nuclear coactivator function that is independent of TGF-β signaling and necessary to promote myogenic differentiation.

Skeletal muscle differentiation results from a highly orchestrated program of gene expression. Extensive biochemical and genetic evidence has implicated a family of DNA binding transcriptional regulatory proteins encoded by the *myogenic regulatory factor* (*MRF*) genes *myf5*, *myod*, *myogenin* (*myog*), and *mrf4* in this process. In conjunction with the proteins encoded by the *myocyte enhancer factor two* (MEF2A-D) gene family, the MRFs activate an evolutionarily conserved program of gene expression, which leads to the generation of terminally differentiated multinucleated myotubes from mononucleated precursor cells (10, 16, 22, 24, 40, 41, 45, 51). The transcriptional activation properties of the MRF and MEF2 complexes are potently regulated by diverse protein-protein interactions (4, 6, 25, 26, 30, 31, 35, 37, 43, 47, 49, 61) and myriad posttranslational modifications (7, 9, 13, 23, 46, 54, 59, 60). This integrated network of protein complexes specifies a unique code for the establishment of myogenic lineage commitment and differentiation. The dynamic nature of these transcriptional regulatory complexes is acquired by an exquisite responsiveness to the milieu of cytokines and growth factors that regulate the myogenic cascade (1, 14, 19, 21, 38, 44, 50, 52, 55). Among a plethora of secreted soluble growth factors affecting muscle differentiation, transforming growth factor β (TGF-β) and myostatin have been implicated as potent repressors of the myogenic gene expression program.

The TGF-β superfamily of cytokines has been shown to function through a "canonical" pathway in which the receptor-regulated Smads (R-Smads) transduce signals to the nucleus to modulate gene expression in response to ligand-receptor interactions. An interesting feature of this signal transduction cascade is the existence of inhibitory Smads (I-Smads; Smad6 and Smad7) which serve to repress receptor-mediated signaling in an autoregulatory feedback loop. Smad7 is characterized primarily as a negative regulator of the TGF-β–Smad2/3 pathway (17). The "canonical" view is that Smad7 prevents Smad2/3 from being phosphorylated by the TGF-β type I receptor (ALK5) by physical interaction with the cytoplasmic tail of the receptor complex; as a result, Smad7 inhibits Smad2/3 and Smad4 complex formation and subsequent nuclear accumulation of this complex (42, 56). TGF-β and myostatin repress myogenesis *in vitro* and *in vivo*, respectively (2, 3, 5, 15, 36, 57). These pathways converge on Smad2/3 through the formation of activated receptor complexes with type I (ALK5) and II TGF-β receptors for TGF-β (18, 33, 34, 53, 58) and type I (ALK7) and IIB activin receptors for myostatin (27, 28). Despite the commonality of this effector system, we previously found that exogenous Smad7, which functions as an inhibitory Smad, reverses the inhibitory effect of myostatin but not TGF-β on muscle differentiation, suggesting that some aspects of the downstream signaling are divergent (26). In these studies, it was documented that Smad7 fulfills an essential and enhancing role for muscle differentiation. Preliminary evidence suggested that Smad7 might cooperate with a nuclear transcription factor in order to enhance muscle differentiation through potentiation of the transcriptional properties of MyoD (26).

In this report, we systematically dissect the role of Smad7 in the nucleus in myogenic cells. Constitutive nuclear localization of Smad7 was engineered by fusion of a nuclear localization signal (NLS) to Smad7 (Smad7-NLS). Smad7-NLS accumulates in the nucleus, bypassing its "canonical" ability to inhibit Smad3 activation by TGF-β. The nuclear Smad7 retains its capacity to enhance MyoD's transcriptional properties and myogenic differentiation independent of its ability to abrogate Smad3 activation at the level of the receptor. In addition, we

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found that Smad7 antagonizes the repressive effects of mitogen-activated protein (MAP) kinase kinase (MEK) on MyoD function. Collectively, these observations support a promyogenic role of nuclear Smad7 independent of its role in antagonizing TGF-β signaling.

MATERIALS AND METHODS

Plasmids. Smad7 and Smad7T (a deletion mutant which lacks the last 18 amino acids [aa] [aa 409 to 426]) expression vectors were described previously (26). The open reading frame (ORF) of enhanced green fluorescent protein (EGFP) without a stop codon was inserted at the HindIII site of the pcDNA3-Smad7 or pcDNA3-Smad7T expression vector for expression of the fusion peptide, and the EGFP ORF with a stop codon was inserted at the HindIII site of the pcDNA3 empty vector (Invitrogen). The NLS of simian virus 40 (SV40) (5′TC GAG GGT GGA GGT CC ACCT AAA AAG AAG CGG AAA GTG GGT GGA GGT T 3′ and 5′CT AGA ACC TCC ACC CAC TTT CCG CTT CTT TTT AGG TGG ACC TCC ACC C 3′) was inserted at the XhoI/XbaI site in frame to generate an EGFP-Smad7-NLS construct. MRF expression plasmids were constructed using pEMSV as described elsewhere (8). MyoD deletion constructs were a kind gift from S. Tapscott (Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA). An activated (ΔN3 S218D/S222E) human MEK1 expression construct was a gift from A. Natalie (32). The reporter construct pMCK-EGFP was provided by A. Ferrer-Martinez (Universitat de Barcelona, Spain). 3TP-Lux and expression vectors for DN-ActIIR (the dominant negative form of an activin type II receptor) and DN-TβIIR (the dominant negative form of a TGF-β type II receptor) were from J. Wrana (University of Toronto and Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). The expression vector for DN-TβIIR was from J. Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY). Smad7 promoter-luciferase-reporter constructs were provided by S. Dooley (Molecular Alcohol Research in Gastroenterology, II, Medical Clinic, Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany). The (CAGA)X10-Luc reporter construct was generated by insertion of a 10×CAGA sequence from the *pai-I* promoter (11) followed by a c-*fos* minimal promoter in the pGL3-basic (Promega) luciferase reporter vector. Transcription reporter constructs, pMCK-luc (12), and pCMV–β-galactosidase (β-Gal) were described elsewhere (26). The myogenin promoter region was excised from pMyoG-luc by SacI/BglII digestion. The resultant 1,152-bp fragment was inserted at the SacI/BglII sites of the pGL4-10 vector (Promega). The dsRed2-N1 expression vector was purchased from Clontech Laboratories. All constructs used in this study were verified by DNA sequencing (York University Molecular Core Facility).

Antibodies. The primary antibodies used in this study were MyoD (sc-304), GFP (sc-5385), actin (sc-1616), c-Jun (sc1694), and Myf5 (sc-302) antibodies from Santa Cruz Biotechnology; MEK1/2 (9122) and phospho-MEK1/2 (Ser217/221) (9121) antibodies from Cell Signaling Technology; Myogenin (F5D) and Myc (9E10) antibodies from the Developmental Studies Hybridoma Bank; the MyoD1 antibody (clone 5.8A; M3512) from DakoCytomation; and the Smad7 antibody (MAB2029) from R&D Systems. Normal mouse IgG (sc-2025) was from Santa Cruz Biotechnology.

Cell culture. C2C12 myoblasts and C3H10T1/2 cells were obtained from the American Type Culture Collection and cultured in growth medium (GM) consisting of 10% fetal bovine serum (FBS) (HyClone) in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO_2. Myotube formation was induced by replacing GM with differentiation medium (DM), which consisted of 2% horse serum (Atlanta Biologicals) in DMEM supplemented with 1% penicillin-streptomycin. For TGF-β or CT-1 treatment, recombinant human TGF-β (240-B; R&D Systems) or CT-1 (438-CT; R&D Systems) was resuspended with solvent (4 mM HCl, 0.1% bovine serum albumin [BSA]) and added to the media. For myotube formation assays, DM with TGF-β (1 ng/ml) or CT-1 (10 ng/ml) was replenished every 2 days.

Microscopy and fluorescence. Fluorescence and phase-contrast photomicrographs were obtained using an epifluorescence microscope (Axiovert 35; Carl Zeiss MicroImaging), with appropriate phase and filter settings, and either 4× 0.10-numerical-aperture (NA) or 10× 0.25-NA Achrostigmat objective lenses. Images were recorded with a digital camera (EOS D60; Canon).

Nuclear protein extraction. Nuclear proteins were extracted from the cells by use of an NE-PER kit (Pierce) according to the manufacturer's protocol.

Western blot analysis. Total cellular protein extracts were prepared in NP-40 lysis buffer (0.1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], supplemented with a protease inhibitor cocktail [P-8340; Sigma]). Protein concentrations were determined by a standard Bradford assay (Bio-Rad). Equivalent amounts of protein were resolved by using sodium dodecyl sulfate (SDS)-polyacrylamide gels, followed by electrophoretic transfer to an Immobilon-P membrane (Millipore) as directed by the manufacturer. Blots were incubated with a primary antibody in 5% milk in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS)-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) or 5% BSA in TBS-T, according to the manufacturer's protocol, at 4°C overnight with gentle agitation. After a brief wash, the blots were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies in 5% milk in PBS or TBS-T at room temperature according to the manufacturer's protocols (Santa Cruz Biotechnology and Cell Signaling Technology). After three washes with 1× PBS or 1× TBS (depending on the primary antibody) at room temperature, the blots were treated with enhanced chemiluminescence reagent (Amersham) to detect immunoreactive proteins. The blots were exposed to Biomax film (Kodak) for visual representation.

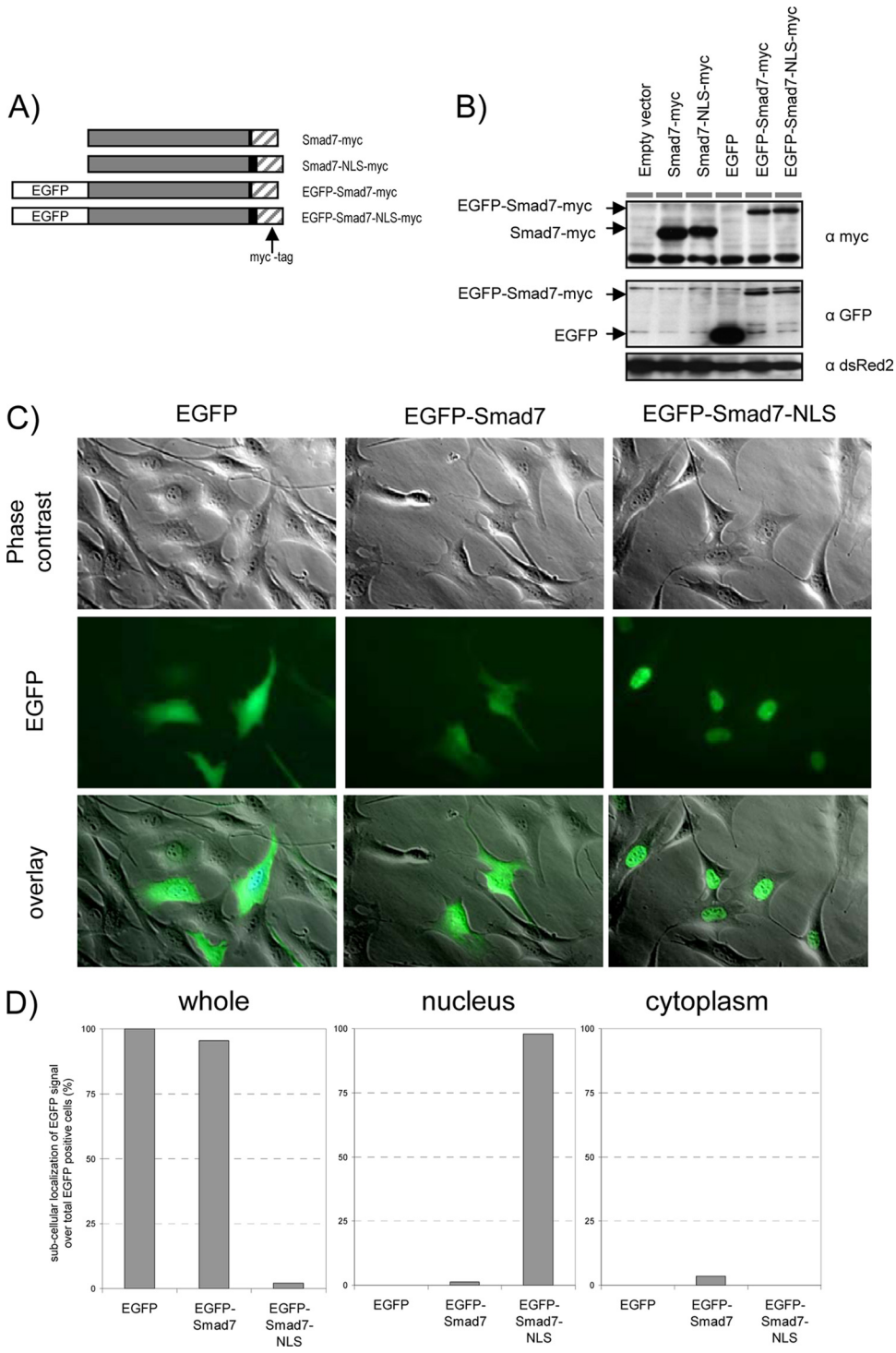
Transcription reporter gene assays. C2C12 myoblasts were transfected by use of a standard calcium phosphate-DNA precipitation method with the reporter gene and expression constructs and pCMV–β-galactosidase to monitor transfection efficiency. After transfection, the cells were washed with PBS and maintained in GM and then treated as indicated in the figure legends. Total cellular protein was extracted with luciferase lysis buffer (20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100). Luciferase and β-galactosidase enzyme assays were performed according to the manufacturer's protocol (Promega). Luciferase activity was quantified using a luminometer (Berthold Lumat 9501) and standardized according to β-galactosidase activity. Relative luciferase units (RLU) normalized for β-galactosidase activity were determined and are plotted as averages of triplicate determinations, and error bars represent standard deviations of the triplicate values.

Coimmunoprecipitation analysis. Equal amounts of total cellular protein (250 μg) were diluted with NP-40 lysis buffer to a final concentration of 1 μg/μl. Protein complexes were immunoprecipitated with antibody and 25 μl of protein G-Plus Sepharose beads (50% slurry) (Santa Cruz Biotechnology) by incubation at 4°C overnight on a rotating platform. The beads were washed with three changes of NETN wash buffer (0.1% NP-40, 150 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 8.0). Beads were boiled in SDS sample buffer, and protein complexes were resolved by SDS-PAGE and immunoblotted as described above.

RESULTS

Constitutive nuclear localization of Smad7 bypasses its inhibitory role at the TGF-β receptor complex. Since our preliminary studies suggested a possible dual role for Smad7 at the level of the TGF-β receptor and also in the nucleus, we aimed to dissect these potentially independent facets of Smad7 activity in a systematic manner. Initially, we sought to engineer a nuclear localized Smad7 that was independent of receptor-mediated events in order to test whether this property of Smad7 could recapitulate the promyogenic effect that we previously documented for the wild-type Smad7 molecule (26). One strategy was to add a nuclear localization signal (NLS) to Smad7, the rationale being that such a modification of Smad7 would abrogate its ability to interfere with R-Smad activation by the receptor while concomitantly localizing it to the nuclear compartment, where, based on our previous observations, we predicted it might still function in the control of myogenic gene expression.

Therefore, we fused the NLS of SV40 at the C terminus of Smad7. In addition, to track the subcellular localization of the Smad7-NLS fusion protein in living cells, we generated a fusion in which we added EGFP at the N terminus of Smad7 (Fig. 1A). The fusion proteins were expressed in transfected C2C12 cells at the expected molecular weights. Addition of the NLS to Smad7 had no apparent effect on the expression levels of the Smad7 proteins (whether conjugated with EGFP or not) in myogenic cells (Fig. 1B).



In C2C12 cells, exogenously expressed EGFP-Smad7 localized to the nucleus and cytoplasm, while EGFP-Smad7-NLS was localized almost exclusively to the nucleus, as indicated by EGFP signal localization (Fig. 1C). To quantitate these observations, we randomly chose 10 fields and scored subcellular localization of the EGFP signals. The results, summarized in Fig. 1D, document that, in contrast to EGFP-Smad7, EGFP-Smad7-NLS localizes essentially to the nucleus. We further determined the subcellular localization of the Smad7 fusion proteins by biochemical fractionation of cytoplasmic and nuclear extracts. Western blot analysis of the fractionated samples for positive markers of the cytoplasmic (MEK1/2) and nuclear (c-Jun) fractions revealed a very good level of enrichment in the respective fractions (Fig. 1E). Subsequent analysis

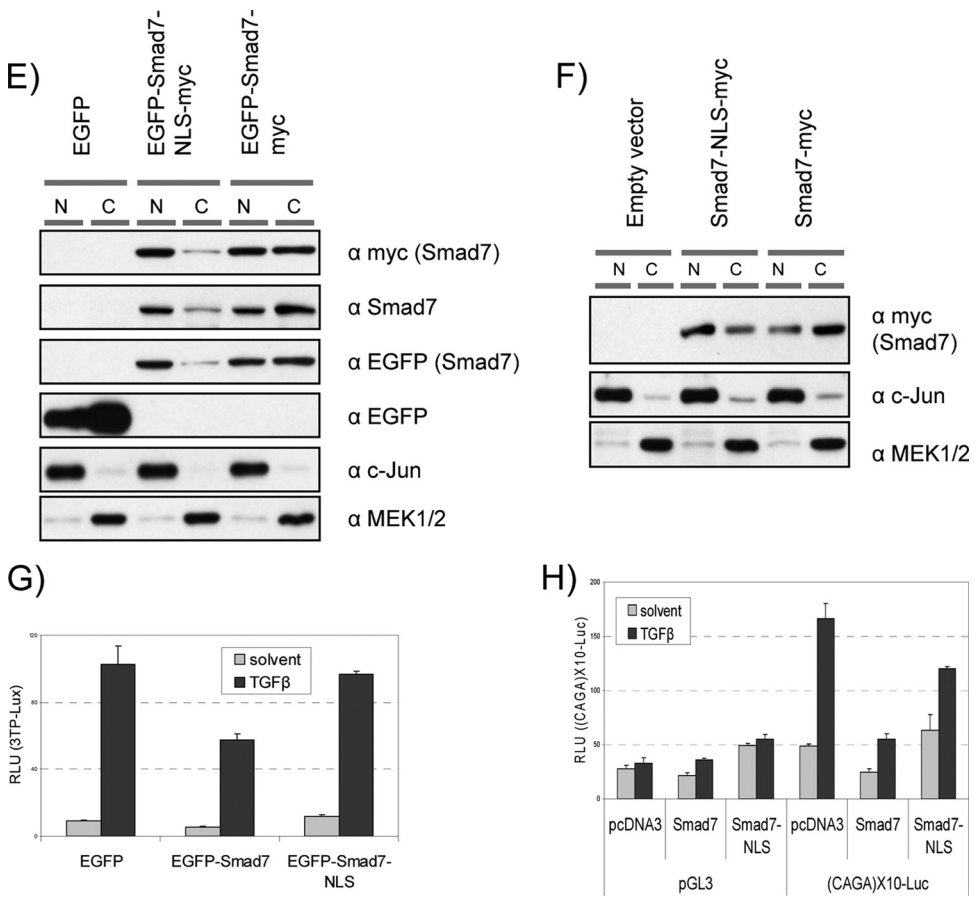


FIG. 1. Constitutive nuclear localization of Smad7 bypasses its inhibitory role at the TGF-β receptor complex. (A) Schematic presentation of Smad7 proteins. NLS, SV40 large-T nuclear localization sequence (black box). (B) C2C12 cells were transfected with the indicated constructs with pCMV-dsRed2, and expression was verified by Western blot analysis with the indicated antibodies. The dsRed2 blot was included as an indicator of transfection efficiency. (C) C2C12 cells were transfected with the indicated EGFP constructs (1 μg) to determine expression of EGFP fusion proteins. The transfected cells were maintained in DM for 16 h. The cell morphology was recorded by phase-contrast microscopy. The subcellular localization of EGFP, EGFP-Smad7, or EGFP-Smad7-NLS was monitored by the green fluorescence protein signal. Overlay images were generated from the phase-contrast and EGFP micrographs. (D) Five fields were randomly chosen for each condition, and the subcellular localization of the indicated EGFP proteins was scored as "whole," "nucleus," or "cytoplasm." Graphs indicating the percentage of the subcellular localization of the EGFP signal over the total number of EGFP-positive cells were generated. (E and F) C2C12 cells were transfected with the indicated constructs and subjected to extraction of nuclear/cytoplasmic proteins by use of an NE-PER kit. Nuclear (N) and cytoplasmic (C) protein samples were analyzed by Western blotting techniques. MEK1/2 and c-Jun were markers for cytoplasmic and nuclear proteins, respectively. (G) C2C12 cells were transfected with the 3TP-luciferase reporter gene construct (3TP-Lux) (0.5 μg) and an expression vector of EGFP, EGFP-Smad7, or EGFP-Smad7-NLS (1 μg). In addition, to monitor transfection efficiency, the pCMV–β-Gal construct (0.3 μg) was included for each condition. The transfected cells were maintained for 16 h in TGF-β (1 ng/ml) or its solvent in DM. Total protein samples were harvested with a luciferase lysis buffer. The luciferase activity under each condition was measured independently and normalized according to the β-galactosidase activity to calculate the RLU. The bars represent the average RLU for the three individually transfected samples (±standard deviations). (H) C2C12 cells were transfected with the pGL3-basic (pGL3) or the (CAGA)X10-luciferase reporter gene construct (0.5 μg) and the expression vector for Smad7 or Smad7-NLS or an empty expression vector (pcDNA3) (1.0 μg) as a control. In addition, to monitor transfection efficiency, the pCMV–β-Gal construct (0.3 μg) was included under each condition. The transfected cells were maintained for 16 h in TGF-β (1 ng/ml) or its solvent in DM. Total protein samples were analyzed as stated above. The bars represent the means from triplicate samples (±standard deviations). α, anti.

of the different Smad7 proteins showed that EGFP-Smad7 was detected in both the nuclear and the cytoplasmic fraction, whereas, as predicted, EGFP-Smad7-NLS was localized essentially in the nuclear fraction, as assessed by use of three different primary antibodies (recognizing myc, Smad7, and GFP proteins) (Fig. 1E and F). Thus, we concluded that adding the NLS to Smad7 effects a very efficient relocalization of Smad7 in the nucleus and largely eliminates the cytoplasmic accumulation which is characteristic of the wild-type protein. Thus, based on the biochemical and fluorescence data we concluded that we have effectively engineered a variant of SMAD7 that is localized in the nucleus and should be incapable of functioning at the level of the TGF-β receptor.

We next assessed whether the engineered changes in localization of Smad7 resulted in functional alterations in its properties. First, TGF-β potently activated TGF-β/Smad3-dependent reporter gene (3TP-Lux) activity, and TGF-β-induced 3TP-Lux activity was reduced in the presence of EGFP-Smad7 (Fig. 1G), consistent with the known function of wild-type Smad7. However, as we predicted, ectopic expression of

EGFP-Smad7-NLS did not interfere with 3TP-Lux activation by TGF-β, as was seen with wild-type Smad7 (Fig. 1G). However, we noted that 3TP-Lux consists of a Smad binding element (SBE) and also 3 copies of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE), which recognizes the activator protein 1 (AP-1) transcription factor. Because we have recently observed that TGF-β can activate AP-1 in this system (data not shown), we also constructed a multimerized Smad3 binding site (CAGA)-driven luciferase reporter gene [(CAGA)X10-luc] to test whether this effect was primarily dependent on the SBE and independent of the TREs in 3TP-Lux (11). In agreement with the results observed for 3TP-Lux, Smad7 inhibited TGF-β-induced (CAGA)X10-luc activity, while Smad7-NLS expression had essentially no effect on reporter gene activation by TGF-β (Fig. 1H). These results indicate that constitutive Smad7 localization to the nucleus bypasses repression of TGF-β signaling at the level of the receptor. Thus, the cytoplasmic localization of Smad7 is required for Smad7's "canonical" inhibitory effect on the TGF-β/Smad3 pathway.

Nuclear Smad7 enhances MyoD's transcriptional activation properties independent of inhibiting the TGF-β/Smad3 pathway. Having successfully engineered Smad7-NLS, which is localized to the nucleus and is unable to interfere with TGF-β receptor-mediated Smad3 activation, we tested whether this molecule could still function to enhance myogenesis. Since we previously observed that Smad7 can physically associate with MyoD and enhances MyoD's transcriptional properties (26), we sought to determine whether Smad7-NLS retains the ability to potentiate MyoD's transcriptional properties. First, we determined the effect of exogenous expression of Smad7 on nuclear MyoD protein levels with or without the addition of TGF-β (1 ng/ml). Exogenously expressed Smad7 and Smad7-NLS were detected in the nuclei, and in agreement with the above-described results, Smad7-NLS accumulated in the nucleus in considerably larger amounts than did wild-type Smad7 (Fig. 2A). Although it was previously reported that MyoD protein levels are downregulated when cells are exposed to TGF-β (26), there was no effect of exogenous Smad7 on endogenous MyoD protein levels in the presence or absence of TGF-β. In addition, ectopic expression of Smad7-myc and Smad7-NLS-myc had no effect on endogenous expression of MyoD, Myf5, or c-Jun, and transfection efficiency was not affected by the expression of Smad7 fusion proteins, as assessed by cotransfected dsRed2 expression (Fig. 2B).

To further examine whether Smad7 enhances myogenesis independent of Smad7's "canonical function" of inhibiting R-Smad activation mediated by the activated TGF-β receptor complex, we silenced TGF-β and myostatin signaling by expression of dominant negative (DN) forms of the corresponding receptors (Fig. 2C). The activity of a Smad3-dependent reporter gene (3TP-luc) was repressed by expressing the DN form of an activin type II receptor (DN-ActIIR), of a TGF-β type II receptor (DN-TbIIR), or a combination of both. Under these conditions of complete receptor blockade, Smad7 still activated MyoG promoter-reporter gene activity, suggesting that Smad7 enhances MyoD's transcriptional properties in a manner independent of TGF-β–Smad3 signaling.

Nuclear Smad7 enhances muscle differentiation. Based on the above-described observations for the *myog* promoter, we next tested the hypothesis that the promyogenic role of Smad7 could be recapitulated by the nuclear localized Smad7. To address this question, we first examined the effect of Smad7-NLS and Smad7 on MyoD's transcriptional activity. As indicated by a *myog* promoter-luciferase reporter gene (pMyoG-luc), Smad7-NLS enhanced MyoD-driven *myog* reporter gene activity to an extent similar to that for wild-type Smad7 (Fig. 3A) without affecting MyoD protein levels (Fig. 3B). Next, Smad7-NLS was expressed in C2C12 myoblasts in which a muscle differentiation marker gene (*muscle creatine kinase* [*mck*]) promoter EGFP reporter construct (pMCK-EGFP) was included to monitor myogenesis. Transfected cells were marked by cotransfection of pCMV-dsRed2. The progression of muscle differentiation was monitored by documenting EGFP expression driven by the *mck* promoter. After 48 h under differentiation conditions, the cells transfected with the empty expression vector (pcDNA3) started forming multinucleated myotubes, and *mck*-driven EGFP signals were observed in these myotubes (Fig. 3C). In agreement with our previous studies (26), Smad7-transfected cells generated larger-caliber myotubes and stronger MCK-EGFP signals than did the control cells. Moreover, Smad7-NLS-expressing cells showed an enhancement of myogenesis to similar that observed with wild-type Smad7, and these observed effects were restricted to transfected cells (dsRed2-positive cells) with Smad7 or Smad7-NLS expression, in contrast to empty-vector-transfected cells (Fig. 3C).

Therefore, the Smad7-NLS chimera preserves the nuclear function of Smad7 such that it enhances MyoD's transactivation properties and promotes myogenesis while completely losing its capacity to inhibit the TGF-β–Smad signaling pathway. These results indicate that Smad7 nuclear localization, independent of its ability to inhibit receptor-regulated Smad activation, is sufficient for Smad7's promyogenic effect.

The C terminus (aa 409 to 426) of Smad7 is required for nuclear accumulation and promyogenic activity. We previously documented that Smad7 promoted myogenesis and potently inhibited TGF-β-induced Smad3 activity in C2C12 cells (26) (Fig. 1G and H and Fig. 3D). In contrast, Smad7T, a deletion mutant which lacks the last 18 aa (aa 409 to 426) (Fig. 4A), was not able to repress activation of Smad3 by TGF-β (Fig. 4B, left). In addition, we observed that Smad7T failed to reverse the inhibition of myogenesis by myostatin (26) and was incapable of enhancing the activity of myogenic reporter genes, such as the *myog* gene (Fig. 4B, right). Next, we assessed the possibility that Smad7T might be dysfunctional because of its localization in the cell. To investigate this, expression vectors for EGFP-Smad7 and EGFP-Smad7T were generated (Fig. 4A), and their subcellular localization was documented (Fig. 4C and D). We found that EGFP-Smad7 localized to both the nucleus and the cytoplasm in C2C12 cells, as was previously documented (20, 26). However, EGFP-Smad7T was essentially excluded from the nucleus (Fig. 4C and D), indicating that the 18 amino acids at the C terminus of Smad7 are required for its proper nuclear localization (20) and, importantly, that nuclear accumulation is required for the promyogenic function of

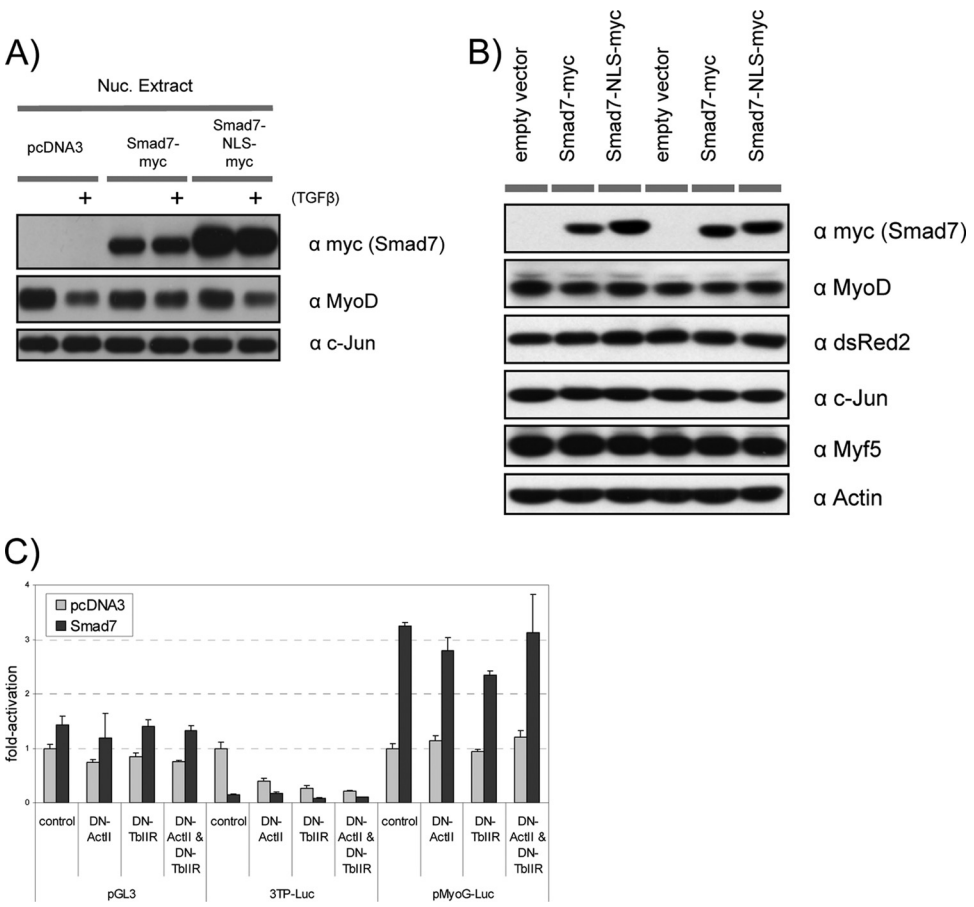


FIG. 2. Nuclear Smad7 enhances MyoD's transcriptional activation properties independent of inhibiting the TGF-β/Smad3 pathway. (A) C2C12 cells were transfected with the expression vector for Smad7-myc, Smad7-NLS-myc, or the control (pcDNA3) (1.0 μg). The transfected cells were maintained for 16 h in TGF-β (1 ng/ml) or its solvent in DM. Nuclear protein was extracted by use of an NE-PER kit. The amount of the indicated nuclear protein was visualized with a standard Western blotting technique. Equal protein loading was monitored by c-Jun immunoblotting. Nuclear Smad7 with a myc epitope tag was identified by use of an anti-myc antibody. (B) C2C12 cells were transfected with the indicated constructs. Expression levels of the indicated proteins were assessed by Western blot analysis. An actin blot indicated equal loading of the protein samples, and a dsRed2 blot showed similar transfection efficiencies. (C) C2C12 cells were transfected with the 3TP-Luc, pMyoG-Luc, or pGL3-basic reporter gene construct (0.3 μg) and an expression vector for the dominant negative form of the type IIB activin receptor (DN-ActII), the dominant negative form of the type II TGF-β receptor (DN-TbIIR), or a combination of both (1.0 μg each, total of 2.0 μg). An expression vector for either Smad7 or the empty control (pcDNA3) (1.0 μg) was added for each condition. The pCMV–β-Gal construct (0.3 μg) was included under each condition to monitor transfection efficiency. The transfected cells were maintained for 16 h in DM. Total protein samples were analyzed as stated above to calculate the RLU. Fold activation was calculated with respect to the level for the control. The bars represent the average fold activation levels for the three individually transfected samples (±standard deviations). α, anti.

Smad7. This observation lends further support to our idea that the promyogenic role of Smad7 resides in its ability to function in the nucleus, although Smad7T does also seem to be deficient in its ability to abrogate canonical TGF-β signaling (Fig. 4B).

Smad7 can promote muscle differentiation by antagonizing the inhibitory effect of activated MEK on MyoD's transcriptional activation properties. We previously demonstrated that Smad7 physically interacts with MyoD and that reduction of Smad7 expression by small interfering RNA (siRNA) technology severely represses myogenesis, suggesting that cooperation between MyoD and Smad7 is required for myogenesis (26). We and others have reported that the activity of MyoD is repressed by its interaction with MEK (39, 47). The MEK-MyoD interaction may be a nodal point for myogenic repression, since a number of cytokines, such as Cardiotrophin-1 (CT-1), inhibit myogenesis by promoting the association of MyoD with MEK (39). Based on these observations, we postulated that one nuclear function of Smad7 might be to antagonize the repressive function of MEK on MyoD's transactivation properties.

To address this, we utilized the pMyoG-luc reporter gene to quantify MyoD's transcriptional activity in response to perturbations in MEK and Smad7 activity. Exogenously expressed, activated MEK1 (Act.MEK1) repressed MyoD-driven pMyoG reporter gene activity in a dose-dependent manner, as previously reported (47), without affecting protein levels of Smad7 and MyoD (Fig. 5A and B). Also, Smad7 partially reversed the inhibitory effect of Act.MEK1 on MyoD (Fig. 5A), suggesting that Smad7 and MEK signaling converge on MyoD in a reciprocal manner to regulate muscle differentiation. We tested this idea further with a myogenic conversion assay, which takes advantage of

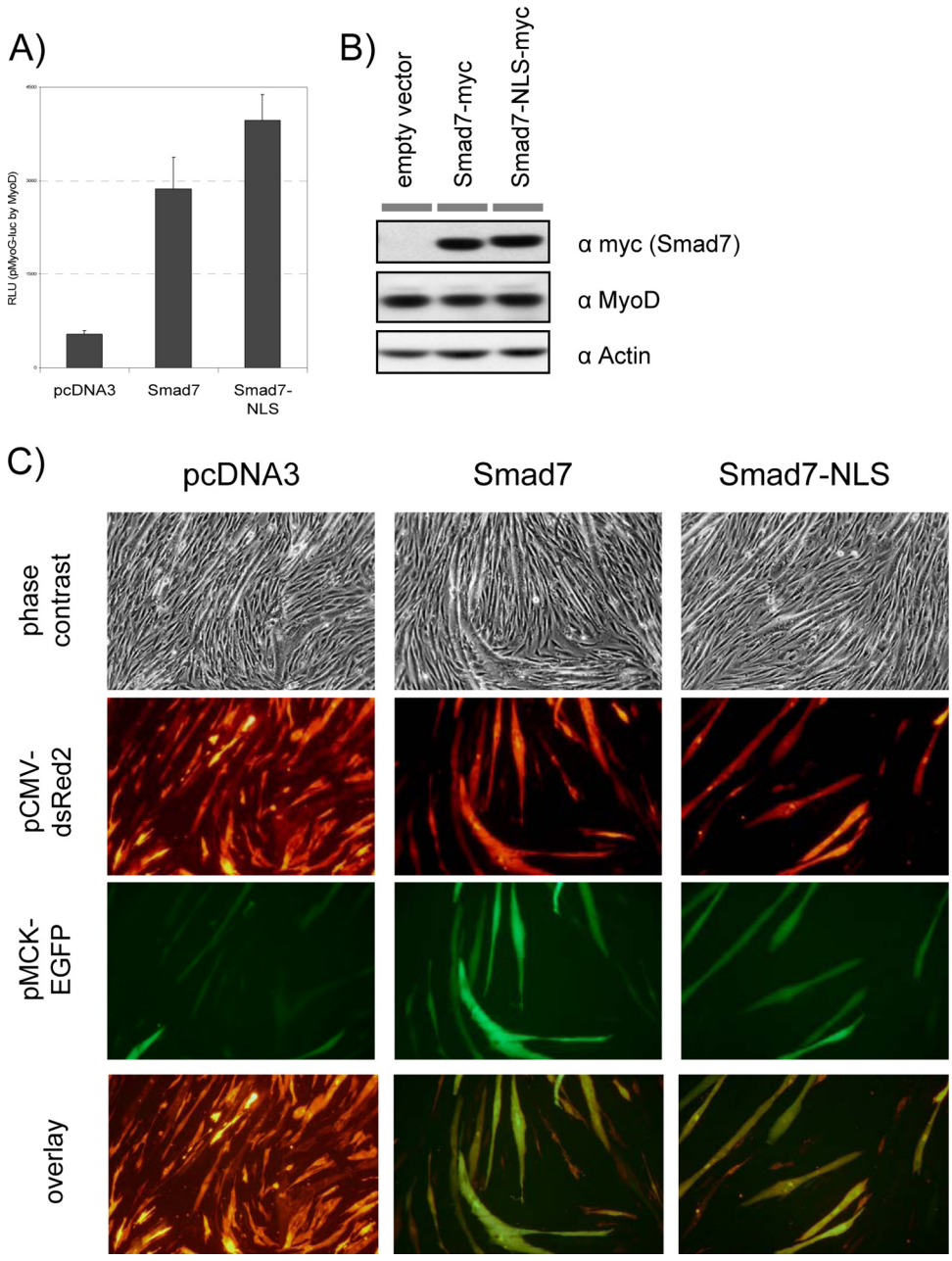


FIG. 3. Nuclear Smad7 enhances muscle differentiation. (A) C2C12 cells were transfected with a pMyoG-luciferase reporter gene construct (pMyoG-luc) (0.5 μg) with the MyoD expression vector (pEMSV-MyoD) (1.0 μg). In addition, the expression vector for Smad7 or Smad7-NLS or an empty expression vector (pcDNA3) (1.0 μg) was included as a control. The pCMV–β-Gal construct (0.3 μg) was included under each condition to monitor transfection efficiency. The transfected cells were maintained for 16 h in DM. The bars represent the average fold activation levels for the three individually transfected samples (±standard deviations). (B) C2C12 cells were transfected with the indicated constructs, and the transfected cells were subjected to Western blot analysis to determine expression of the indicated proteins. α, anti. (C) C2C12 cells were plated at equal densities and transfected with pCMV-dsRed2 (0.5 μg) and pMCK-EGFP (1.0 μg). The transfected cells were maintained in DM for 48 h to induce myotube formation. The cell morphology was recorded by phase-contrast microscopy, and transfected cells were monitored by the dsRed2 signal. MCK promoter activity was assessed by the EGFP signal. Overlay images were generated from the phase-contrast and EGFP micrographs.

MyoD's capacity to induce myogenesis in the 10T1/2 fibroblast line. The results of these studies were striking in that ectopic expression of an active form of MEK1 prevents induction of MyoG by MyoD in this assay (Fig. 5C) and Smad7 and Smad7-NLS enhance MyoD-driven myogenesis (Fig. 5D). However, ectopic expression of Smad7 as well as Smad7-NLS reverses this antagonism of MyoG induction by MEK1 (Fig. 5E and F). It is worth mentioning that these reciprocal effects of Smad7 and MEK1 on target genes expressed in muscle appear to be dependent on MyoD, since another MEK-

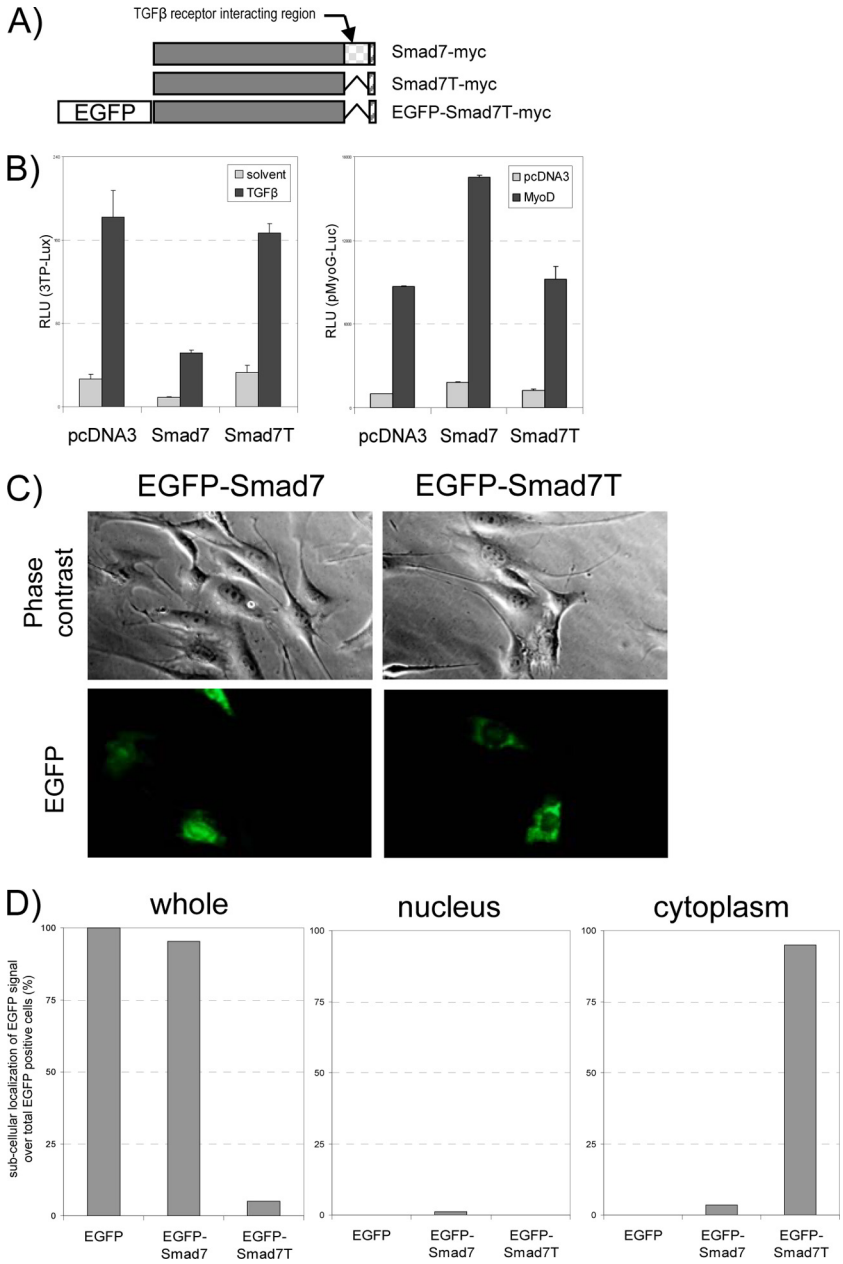


FIG. 4. The C terminus (amino acids 409 to 426) of Smad7 is required for nuclear accumulation and promyogenic activity. (A) Schematic presentation of Smad7 proteins. To create Smad7T, the TGF-β receptor interaction region was deleted from Smad7. (B) C2C12 cells were transfected with 3TP-Lux (left) or pMyoG-Luc (right) (0.5 μg) and the expression vector of Smad7, Smad7T, or the empty control (pcDNA3) (1.0 μg). The MyoD expression vector (pEMSV-MyoD) or the empty vector (pEMSV) (1.0 μg) was also included (right). In addition, to monitor transfection efficiency, the pCMV–β-Gal construct (0.3 μg) was included under each condition. The transfected cells were maintained for 16 h in TGF-β (1 ng/ml) or its solvent in DM (left) or in DM (right). The luciferase activity under each condition was measured independently and normalized according to the β-galactosidase activity to calculate the RLU. The bars represent the average RLU for the three individually transfected cellular samples (±standard deviations). (C) C2C12 cells were transfected with the indicated EGFP constructs (1.0 μg) for expression of EGFP fusion peptides. The cell morphology was recorded by phase-contrast microscopy. The subcellular localization of EGFP-Smad7 or EGFP-Smad7T was monitored by the EGFP signal. (D) Five fields were randomly chosen for each condition, and the subcellular localization of the indicated EGFP proteins was scored as "whole," "nucleus," or "cytoplasm." Graphs indicating the percentage of the subcellular localization of the EGFP signal over the total number of EGFP-positive cells were generated.

inducible gene, *sprr1a* (48), which is not targeted by MyoD but is expressed in muscle cells, was not repressed by exogenous expression of Smad7 (data not shown). We further observed precocious upregulation of MyoG, which is a key target of MyoD, by ectopic expression of Smad7-myc and Smad7-NLS-myc in differentiating myoblasts (Fig. 5G).

To further characterize the MyoD-Smad7 interaction, we investigated the transcriptional activities of a number of MyoD

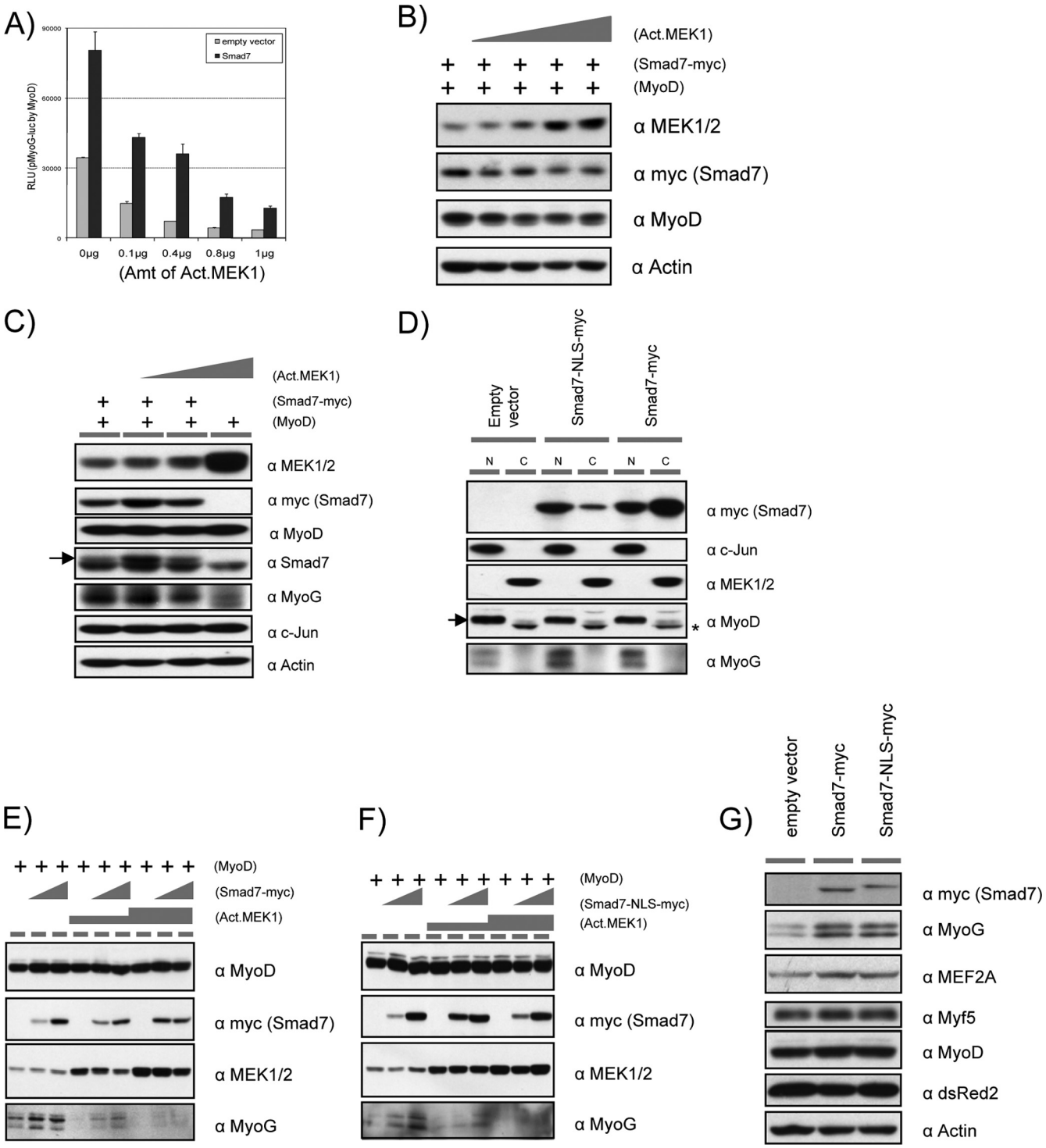


FIG. 5. Smad7 can promote muscle differentiation by antagonizing the inhibitory effect of activated MEK on MyoD's transcriptional properties. (A) C2C12 cells were transfected with pMyoG-Luc (0.5 μg), a MyoD expression vector (1.0 μg), pCMV–β-Gal (0.3 μg), and also increasing amounts (0, 0.1, 0.4, 0.8, and 1.0 μg) of an expression vector for the activated form of MEK1 (Act.MEK1) with a combination of the empty vector as a control (3.0, 2.9, 2.6, 2.2, and 2.0 μg) (total of 3.0 μg for each condition). Finally, the expression vector for Smad7 (1.0 μg) or the empty control was added. The transfected cells were maintained in DM for 16 h. The cells were harvested and subjected to a luciferase assay and a β-Gal assay. The luciferase activity was normalized according to the β-galactosidase activity to calculate the RLU (±standard deviations). (B) C2C12 cells were transfected with the indicated constructs (5 μg of Smad7-myc and MyoD expression vectors under all conditions), with increasing amounts (0, 0.5, 2, 2.5, and 5 μg) of Act.MEK1. The transfected cells were maintained in DM for 16 h. The expression levels of the indicated proteins were assessed by Western blot analysis. An actin blot indicated equal loading of the protein samples. (C) C3H10T1/2 cells were transfected with the indicated constructs (5 μg of MyoD expression vector under all conditions) with or without Smad7-myc (5 μg) and increasing amounts (0, 0.5, 1, and 5 μg) of Act.MEK1. The transfected cells were maintained in DM for 16 h. The expression levels of the indicated proteins were assessed by Western

deletion mutants (Fig. 6A and B) in the presence of either Act.MEK1 or Smad7. Western blot analysis showed that MyoD and its deletion mutants are appropriately expressed in transfected cells (Fig. 6B). As previously reported (47), Act. MEK1 repressed pMyoG reporter gene activity driven by MyoD or a series of deletion mutants, except for one lacking aa 3 to 56 (MyoDΔ3–56) (Fig. 6C). Cardiotrophin-1 (CT-1), which inhibits myogenesis by activation of the MEK/extracellular signal-regulated kinase (ERK) pathway (39), also repressed this reporter gene activity (Fig. 6D). Smad7 enhanced MyoD's transcriptional activity, but this enhancement was diminished by deletion of the N-terminal part of MyoD (aa 3 to 56 or aa 63 to 99) (Fig. 6E). These data indicate that MEK1 and Smad7 may functionally interact with an overlapping region of MyoD, suggesting that their interaction might be mutually exclusive. To explore this possibility, we exogenously expressed MyoD and Act.MEK1 with or without a myc epitope-tagged Smad7 to assess the amount of MEK1 in the MyoD-containing immunocomplex. We postulated that if MEK1 and Smad7 associate with the MyoD protein in a competitive manner, the amount of MEK1 interacting with MyoD should be reduced in the presence of Smad7. Smad7 and MEK1 were detected in the immunocomplex precipitated by a MyoD antibody (Fig. 6F). However, in the presence of enhanced Smad7 expression, there was no apparent effect on the amount of MEK1 in the MyoD immunocomplex, suggesting that Smad7 and MEK1 associate with the MyoD protein in a noncompetitive manner.

Cardiotrophin-1-mediated MEK activation is antagonized by Smad7. Recently, we identified that CT-1 is a physiological regulator of skeletal muscle differentiation and that CT-1 inhibits muscle differentiation through activation of MEK, which antagonizes MyoD activation (39). Therefore, we used CT-1 as a biological regulator of muscle differentiation and as an activator of MEK for these experiments. In agreement with the above-described results, CT-1 inhibited *myog* promoter-reporter gene activation by MyoD in a dose-dependent manner, and exogenously expressed Smad7 reversed CT-1's inhibitory effect on the *myog* promoter (Fig. 7A). Collectively, these results suggest that activation of the MEK/ERK pathway prevents premature differentiation of myoblasts by repressing MyoD's transcriptional activation properties (39). Moreover, Smad7 antagonizes MEK's inhibitory effect on MyoD to induce transcription of the *myog* gene (Fig. 5A), which is an essential step for myogenesis. Because of these observations, we hypothesized that altering the levels of Smad7 may cause precocious differentiation even in the presence of CT-1. We previously reported that exogenously expressed Smad7 accelerated the formation of myotubes and upregulated pMCK-EGFP activity compared to results with control cells (pcDNA3) (26) (Fig. 3C and 7B). As previously observed (39), CT-1-treated C2C12 cells in DM were repressed from differentiation, as indicated by the prevalence of the mononucleated myoblast phenotype and also the lack of pMyoG-luc activation after 48 h in DM. Importantly, these inhibitory effects of CT-1 on myogenesis were essentially reversed by the exogenous expression of Smad7 (Fig. 7B), suggesting that the balance between activated MEK and Smad7 regulates the initiation of muscle differentiation (Fig. 8).

DISCUSSION

In this study, we document that nuclear Smad7 enhances MyoD's transcriptional activation properties. This is the first report to identify a clear biological function of nuclear Smad7. We found that Smad7 in the nucleus interacts with MyoD and enhances MyoD's ability to induce muscle differentiation. Our previous study showed that reduced expression of Smad7 by siRNA technology antagonized myogenic differentiation, suggesting that Smad7 is an essential component of the program of myogenic differentiation (26). It is well established that MyoD requires activation to induce myogenesis, since myogenic lineage-determining factors MyoD and Myf5 are detected in undifferentiated myoblasts, where they are inactive as initiators of the myogenic gene expression program. The exact molecular mechanism by which the MRFs shift from a repressed to an active state to induce the myogenic program of gene expression is not completely understood, although a number of studies have found that MyoD activity is regulated extensively by protein-protein interactions. The data presented here implicate Smad7 as an essential cofactor in the myogenic cascade. Independent of the well-known function of Smad7 in the cytoplasm, nuclear Smad7 physically interacts with MyoD and potentiates MyoD's transcriptional activation properties.

Since MRF activity is sensitive to extracellular secreted factors, it is reasonable that downstream signaling molecules associated with these signaling pathways play an important role in the regulation of MRF activity. Accumulating evidence indicates that the MEK/ERK pathway also plays a fundamental role in MyoD's transcriptional activation properties. Interest-

blot analysis. Endogenous MyoG and c-Jun protein levels were also assessed. An actin blot indicated equal loading of the protein samples. (D) C3H10T1/2 cells were transfected with Smad7-NLS-myc, Smad7-myc, or the control empty vector (8 μg) and a constant amount of the MyoD expression vector (5 μg). The transfected cells were maintained in DM for 16 h to induce MyoG and subjected to extraction of nuclear/cytoplasmic proteins by use of an NE-PER kit. Nuclear and cytoplasmic protein samples were analyzed by Western blotting techniques. Endogenous MEK1/2 and c-Jun were markers for cytoplasmic (C) and nuclear (N) proteins, respectively. The expression levels of the indicated proteins in each cell compartment were assessed by Western blot analysis. For the MyoD blot, the arrow indicates the MyoD protein and the asterisk indicates a nonspecific cytoplasmic cross-reactant. (E and F) C3H10T1/2 cells were transfected with Smad7-myc (E), Smad7-NLS-myc (F), or a control empty vector (0, 2, or 8 μg) and Act.MEK1 (0, 2, or 8 μg), with a constant amount (4 μg) of the MyoD expression vector. Total protein samples were extracted and analyzed by Western blotting techniques. Endogenous MEK1/2 and c-Jun were markers for cytoplasmic and nuclear proteins, respectively. The expression levels of the indicated proteins were analyzed with a Western blotting technique. (G) C2C12 cells were transfected with Smad7-myc, Smad7-NLS-myc, or an empty vector by use of Lipofectamine (Invitrogen). The transfected cells were maintained in DM for 48 h after transfection to induce differentiation, total protein was extracted, and expression levels of the indicated proteins were analyzed by Western blotting. Actin was used as a loading control and dsRed2 as a marker for transfection efficiency. α, anti.

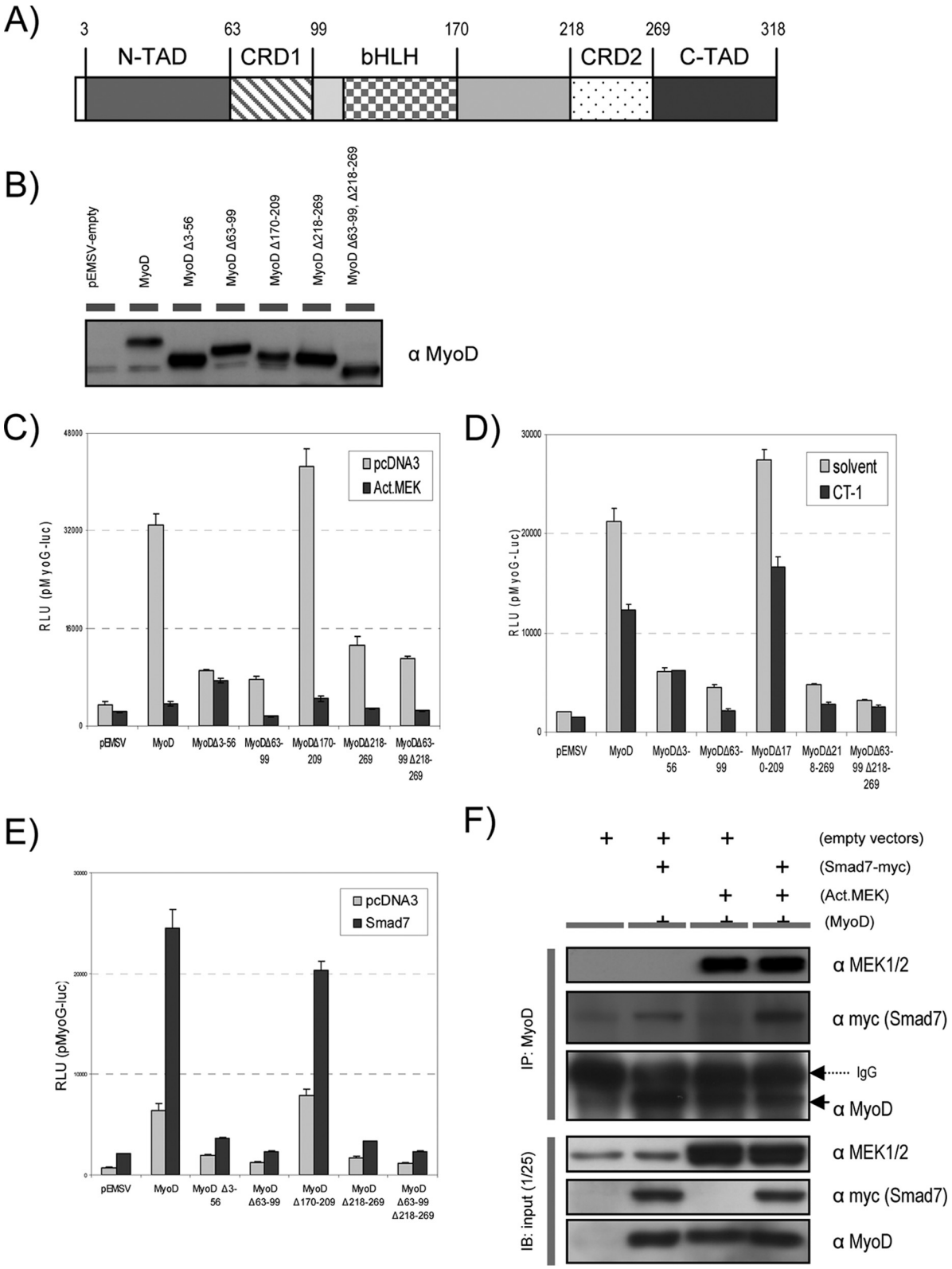


FIG. 6. Smad7 and MEK1 interact with MyoD. (A) Schematic presentation of the MyoD protein. The numbers correspond to amino acid coordinates. (B) Western blot analysis of expression of deletion mutants of MyoD proteins. C3H10T1/2 cells were transfected with the indicated expression vectors (1.0 μg) to determine expression of the indicated MyoD deletion mutant, wild-type MyoD protein, or empty control. The transfected cells were maintained in DM for 16 h and harvested for total protein samples. The extracted total proteins were subjected to Western blot analysis as described in Materials and Methods to confirm expression of the corresponding MyoD protein. (C to E) C3H10T1/2 cells were transfected with pMyoG-Luc (0.5 μg) and the indicated wild type or deletion mutant forms of MyoD or the empty expression vector (1.0 μg), as

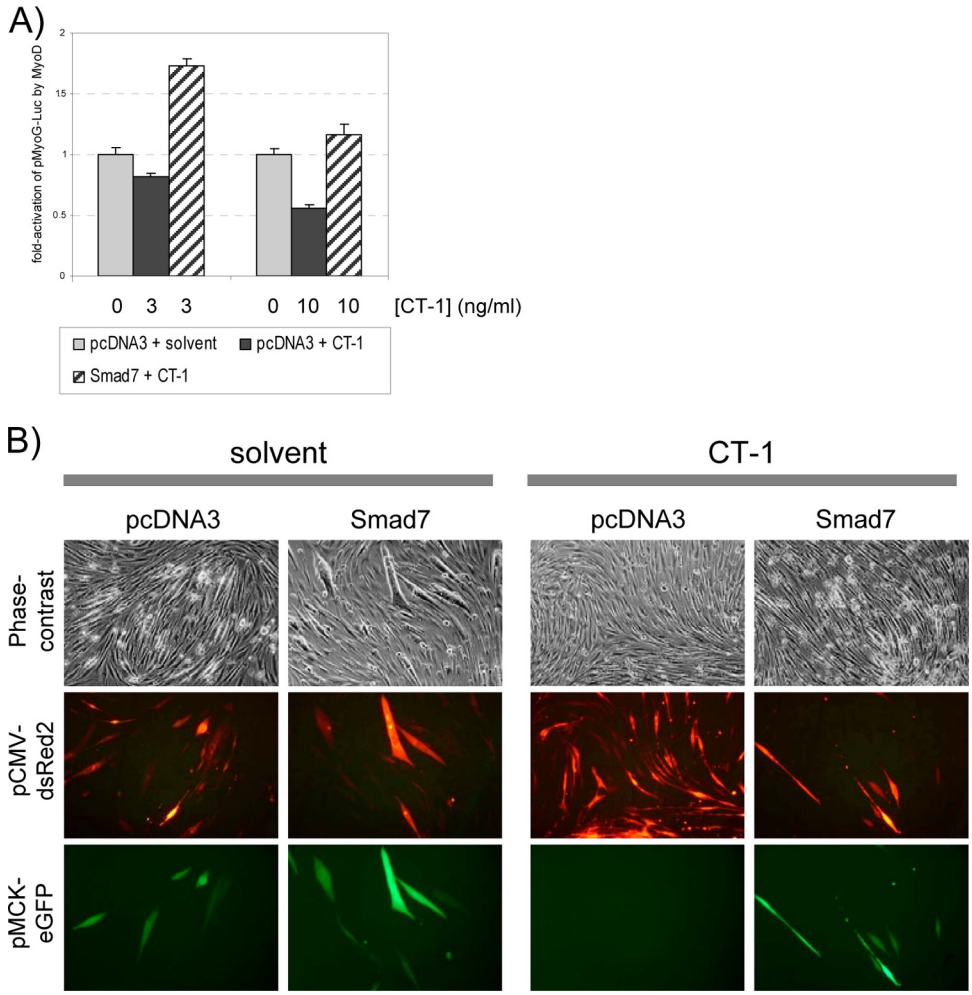


FIG. 7. Cardiotrophin-1-mediated MEK activation is antagonized by Smad7. (A) C2C12 cells were transfected with a pMyoG-Luc (0.5 μg) reporter gene construct. pCMV–β-Gal (0.3 μg) was also added under each condition for monitoring transfection efficiency. The transfected cells were maintained in DM containing either CT-1 (3 ng/ml or 10 ng/ml) or solvent for 16 h. The cells were harvested and subjected to a luciferase assay and a β-Gal assay. The luciferase activity was normalized according to the β-galactosidase activity to calculate the RLU. The bars represent the average RLU for three individually transfected samples (±standard deviations). (B) C2C12 cells were plated at equal densities and transfected with pCMV-deRed2 (0.5 μg), pMCK-EGFP (1.0 μg), and the expression vector for either Smad7 or the empty control (pcDNA3) (1.0 μg). The transfected cells were maintained in CT-1 (10 ng/ml) or solvent containing DM for 48 h to induce myotube formation. The cell morphology was recorded by phase-contrast microscopy, and transfected cells were monitored by the red fluorescence signal. The MCK promoter activity was assessed by the green fluorescence signal.

ingly, a previous report showed that activated MEK physically interacts with MyoD and inhibits MyoD activity (47). In addition, we have recently documented that CT-1 represses myogenesis through the inactivation of MyoD by activation of the MEK/ERK pathway (39). In this study, we demonstrate that Smad7 can reverse CT-1's inhibitory effect on myogenesis. This effect is not confined to CT-1, since Smad7 is also capable of reversing the inhibitory effect of MEK on MyoD. Since MEK and Smad7 can form complexes with MyoD, MEK signaling and Smad7 converge on MyoD. Interestingly, Smad7 expression is regulated by MyoD at the transcriptional level through the E box in the *smad7* promoter. Therefore, Smad7 and

well as pCMV–β-Gal (0.3 μg). In addition, the expression vector (1.0 μg) for the active form of MEK1 or an empty control (C) or Smad7 or an empty control (E) was included. The transfected cells were maintained in DM for 16 h (C and E) or in DM containing CT-1 (10 ng/ml) or solvent (D). The cells were harvested and subjected to a luciferase assay and a β-Gal assay. The luciferase activity was normalized according to the β-galactosidase activity to calculate the RLU (±standard deviations). (F) C3H10T1/2 cells were transfected with combinations of the indicated constructs. Total protein samples were extracted from the cells maintained in DM. The exogenous expression of MyoD, an activated form of MEK1, or Smad7-myc was detected by immunoblotting (IB) (10 μg loading) with the specific antibodies. A coimmunoprecipitation (IP) analysis was performed with the total protein extract (250 μg) with MyoD antibody (mouse) and protein G-conjugated beads. Precipitated immunocomplexes were eluted off of the protein G beads and subjected to immunoblotting with MEK antibody or myc antibody. α, anti.

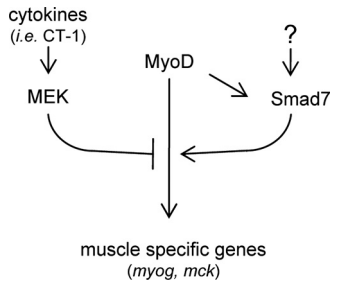


FIG. 8. Model of nuclear Smad7 function in myogenesis. The depicted schematic indicates the hierarchal relationship between MyoD, MEK, and Smad7 in the control of muscle-specific genes, based on the data presented here.

MyoD may constitute a mutually reinforcing feed-forward loop to initiate myogenesis. Thus, the downregulation of *smad7* mediated by MEK activation could be a common mechanism to inhibit myogenesis utilized by a number of different growth factors and cytokines, such as CT-1, FGF, EGF, and PDGF (Fig. 7). Interestingly, although both myostatin (a member of the TGF-β family)- and CT-1-mediated repression of myogenesis was "rescued" by exogenous Smad7 expression, Smad7 could not reverse TGF-β's inhibitory phenotype (26). However, TGF-β's inhibitory effect is partially reversed by MEK inhibitors (T. Miyake, unpublished observation). Therefore, activation of MyoD's transcriptional activation properties, either by upregulation of an activator (for example, Smad7) or by downregulation of an inhibitor (for example, active MEK), may constitute a key nodal point for the regulation of MyoD activity and subsequently the decision to initiate differentiation.

Interestingly, Levy and Hill identified Smad4-dependent and -independent TGF-β target genes by reducing Smad4 expression by siRNA technology (29), indicating that there are divergent groups of TGF-β-responsive target genes regulated by different facets of the signaling pathway. Smad7-NLS could also prove to be a useful tool in dissecting noncanonical aspects of the TGF-β signaling pathway. A similar approach of dissecting the TGF-β-independent target genes of the nuclear form of Smad7 has the potential to identify a unique group of myogenic genes that are regulated by MyoD and Smad7.

In summary, we document a novel function of nuclear Smad7 in myogenic cells (Fig. 7). Nuclear Smad7 antagonizes the inhibitory effect of MEK on MyoD's transcriptional activation properties and, importantly, enhances myogenesis independent of its inhibitory role in TGF-β–Smad3 signaling. These observations document a novel "noncanonical" nuclear role for Smad7 in modulating the properties of MyoD and potentiating myogenic differentiation.

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