Inhibition of Transforming Growth Factor β-enhanced Serum Response Factor-dependent Transcription by SMAD7*

Received for publication, March 23, 2006. Published, JBC Papers in Press, May 10, 2006, DOI 10.1074/jbc.M602748200

Blanca Camoretti-Mercado†, Darren J. Fernandes†, Samantha Dewundara‡, Jason Churchill†, Lan Ma†, Paul C. Kogut†, John F. McConville‡, Michael S. Parmacek§, and Julian Solway‡

From the †Department of Medicine, University of Chicago, Chicago, Illinois 60637 and the ‡Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Transforming growth factor (TGF)-β is present in large amounts in the airways of patients with asthma and with other diseases of the lung. We show here that TGFβ treatment increased transcriptional activation of SM22α, a smooth muscle-specific promoter, in airway smooth muscle cells, and we demonstrate that this effect stems in part from TGFβ-induced enhancement of serum response factor (SRF) DNA binding and transcription promoting activity. Overexpression of Smad7 inhibited TGFβ-induced stimulation of SRF-dependent promoter function, and chromatin immunoprecipitation as well as co-immunoprecipitation assays established that endogenous or recombinant SRF interacts with Smad7 within the nucleus. The SRF binding domain of Smad7 mapped to the C-terminal half of the Smad7 molecule. TGFβ treatment weakened Smad7 association with SRF, and conversely the Smad7-SRF interaction was increased by inhibition of the TGFβ pathway through overexpression of a dominant negative mutant of TGFβ receptor I or of Smad3 phosphorylation-deficient mutant. Our findings thus reveal that SRF-Smad7 interactions in part mediate TGFβ regulation of gene transcription in airway smooth muscle. This offers potential targets for interventions in treating lung inflammation and asthma.

TGFβ3 is overexpressed in the airways of asthmatics, where it is thought to play a key role in the airway remodeling characteristic of their disease, by regulating an array of cellular functions (1) that includes gene expression, cell proliferation, differentiation (2), apoptosis (3), and migration (4) and remodeling of the extracellular matrix (5). To accomplish these diverse effects, TGFβ binds to two unique cell surface receptors, designated TGFβ type I (TβRI) and type II (TβRII), which are present in almost every cell type and are directly involved in signal transduction through their serine/threonine kinase activities (6). Binding of TGFβ to TβRII induces phosphorylation and activation of TβRI, which in turn phosphorylates Smad proteins, the major transducers of TGFβ signals (7). Once phosphorylated at their C terminus (the MH2 domain), these receptor-activated Smads or “R-Smads” (Smad2 and Smad3) associate with the common mediator Smad4 (“Co-Smad”); R-Smad-Co-Smad complexes then translocate into the nucleus, where they bind to regulatory regions of target genes (at the Smad-binding element [SBE]) and interact with other transcription factors, co-activators, and co-repressors to regulate expression of TGFβ-responsive genes.

In several cell types, the inhibitory Smads, Smad6 and Smad7 (“I-Smads”), negatively regulate TGFβ signaling (8). After TGFβ stimulation, Smad7 translocates to the plasma membrane where it binds to TβRI and inhibits further signaling. Two mechanisms of suppression of TGFβ signaling involving Smad7 are presently known, and both occur outside the nucleus. (i) Smad7 is able to interfere with the association between the R-Smads and activated receptors (9, 10), (ii) A complex containing Smad7 and the E3 ubiquitin ligase Smurf1 or Smurf2 translocates to the plasma membrane and induces ubiquitination and consequent degradation of TGFβ receptors (10).

Several genes with expression restricted to the smooth muscle (SM) lineages have been identified (11), and many of them are responsive to TGFβ (12). In vascular smooth muscle cells, TGFβ induces expression of α-sm-actin, an abundant SM protein whose transcriptional regulation is under at least partial control of a TGFβ control element (TCE) located within the promoter region (13). A similar TCE, to which Kruppel-like transcription factors bind (14), is required for in vivo expression of SM22α, one of the earliest and most widely expressed smooth muscle cell markers identified (11). Recently, TGFβ was reported to up-regulate SM22α expression in fibroblasts through binding of Smad3 to two Smad-binding motifs located within the first exon (15, 16). Furthermore, signaling through Smad2 and Smad3 plays an important role in the development of SM cells from totipotent embryonic stem cells (17). Analysis of the regulatory regions of SM-specific genes reveals that the vast majority of them (including SM22α) contain CArG
sequences, the binding site for the MADS-box transcription factor serum response factor (SRF) (18). Besides its essential role driving SM- and non-SM muscle-specific gene expression, SRF is also critical for regulation of cell proliferation and differentiation (19). The transcription promoting activity of SRF is modulated by its subcellular localization (20–22) and through its association with a variety of other transcription factors, activators, and co-repressors (mostly via its MADS domain). Indeed, the competition for binding of various cofactors (e.g., myocardin versus Elk-1) to a common docking site on SRF may underlie the molecular mechanism of SM plasticity, its ability to switch between differentiated and proliferative phenotypes in response to extracellular cues (23).

Beyond its influence at the TCE, TGFβ can also promote smooth muscle-specific gene transcription through effects on SRF. First, immunoprecipitation studies show that Smad3 can form complexes with SRF within the nucleus, and such complexes formed on the SM22α promoter (where Smad3 also binds to an SBE in the first exon) appear to enhance SM22α transcription (15). Second, artificial overexpression of inhibitory Smads 6 and 7 partially blocks SRF-VP16-mediated activation of the SM22α promoter (15), through an undetermined mechanism. Third, TGFβ can stimulate the accumulation of the SRF protein (24). Together, these findings point to SRF as an important mediator of TGFβ effects on smooth muscle-specific gene transcription.

Given the importance of TGFβ in asthmatic airway remodeling, and in airway smooth muscle hypertrophy in particular (25), we have further explored how TGFβ modulates SRF-dependent gene transcription, by testing the hypothesis that inhibitory Smad7 associates with SRF, thereby reducing its transcription-promoting activity. Our experiments demonstrate for the first time that this association does indeed occur in both myogenic and nonmyogenic cells and that TGFβ stimulation reduces this interaction, thereby restoring the transcription-promoting potential of SRF.

**MATERIALS AND METHODS**

**Cell Culture**—Canine tracheal smooth muscle cells (CTSMC) were grown in 24-well uncoated plastic dishes. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (1:1) plus 10% fetal bovine serum, 0.1 mM non-essential amino acids, 50 units/ml penicillin, and 50 μg/ml streptomycin. All reagents were from Invitrogen. COS and HEK293 cells were obtained from ATCC (Manassas, VA).

**Plasmids**—We used several SRF-dependent reporter plasmids in our studies. In pSM22luc, luciferase expression is driven by bp −445 to +41 of the mouse SM22α gene (26). SME plasmids containing CARG sequence mutations that prevent SRF binding to the 5′-CARG box, the 3′-CARG box, or both sites were described previously (27). The artificial p5xCARGluc (Stratagene, La Jolla, CA) contains five identical sites (CCATATTAGG) separated by 4 bases and a minimal TATA box. pSRE.Lluc (gift from Dr. N. Dulin, University of Chicago) contains two identical CARG sites (CCATATTAGG) separated by 23 bases. pSM-MHC.luc contains 3.3 kb of 5′-flanking DNA upstream of the human SM-myosin heavy chain gene (28) driving luciferase expression. pMSV.luc contains a strong viral promoter that is constitutively active and has been described previously (29). pSE8x2tkluc, which contains the thymidine kinase promoter plus two copies of the GTCT sequence (Smad/ski-binding site) which confer responsiveness to activated Smad pathway, was provided by Dr. E. Medrano, Baylor College of Medicine. pGRELuc is an artificial promoter that harbors a glucocorticoid-responsive element and TATA box (Clontech). In pMSVβgal, the murine sarcoma virus promoter drives β-galactosidase. pCMV5 and the expression plasmids pCMVSmad7-HA and pSmad3(3A)FLAG, which contain serine to alanine mutations in the Smad3 phosphorylation sites, were gifts from Dr. L. Attisano (University of Toronto). Plasmids encoding FLAG tag fused to amino acid residues 2–259 of human Smad7 (FN-FLAG-Smad7) or 206–426 (FC-Smad7-FLAG) were provided by Dr. C-H. Heldin (Uppsala University, Sweden). FLAG-TβRII-ΔN plasmid, which expresses a kinase domain mutant of TβRI, was a gift from Dr. P. ten Dijke (The Netherlands Cancer Institute). EGFP-SRF fusion proteins were generated by cloning cDNAs encoding full-length or corresponding mutants SRF into pEGFP-C1 (Clontech) that encodes enhanced green fluorescent protein. In plasmid EGFP-mNLS-EGFP, amino acids 95 and 96 within the nuclear localization signal 95RRGLKR100 were mutated to EE. In plasmid pEGFP-mDM-SRF amino acid residues 183VLLL187 within the dimerization domain were replaced with AAAAA.

**Transfection**—To measure the activity of SRE.L, 5xCARG, GRE, and SBE promoters, passage 2 CTSMC of 70–80% confluence in 24-well plates was transiently transfected the day after plating with 0.1 μg of promoter luciferase reporter plasmid, 0.1 μg of pMSVβgal, and 25 ng of expression plasmid DNA mixture with 1.5 μl of Plus Reagent (Invitrogen). Liposomes were formed by adding 2 μg of Lipofectamine (Invitrogen). After 4 h of transfection, the liposome suspension was removed, and cells were fed with DMEM/F-12 plus 10% fetal bovine serum for 5 h. After that, cells were serum-deprived overnight in DMEM/F-12 supplemented with 0.1% bovine serum albumin (Sigma) plus antibiotics. The following day, cells were treated with or without TGFβ1 (150 pM, R & D Systems, Minneapolis, MN) for 8 h in serum-free medium and then harvested. In some cases, canine airway myocytes were long term serum-deprived (4–7 days) and, where indicated, transfected as described previously (20) prior to TGFβ treatment. For transfection using SM22α and SME promoter constructs, cells were seeded in 6-well plates and co-transfected the following day with 1.8 μg of indicated luciferase reporter, 600 ng of MSVβgal plasmid, and 100 ng of empty or Smad7 expression vector with 12 μg of Lipofectamine. After 6 h, the medium was changed to DMEM/F-12 plus insulin, transferring selenium mixture as described previously (20) with or without 150 pM TGFβ. Cells were harvested 48 h after transfection. In all cases, cells were lysed with M-PER detergent (Pierce) and frozen once, and luciferase activity was measured using a commercially available kit (Promega, Madison, WI). β-Galactosidase assays were performed as internal control to correct for differences in transfection efficiency. Promoter function was determined as normalized luciferase activity over β-galactosidase activity; TGFβ effect was expressed as a ratio of normalized
luciferase activity in TGFβ-treated cells relative to control untreated cells. Results are expressed as average ± S.D. from at least three independent experiments, each performed in triplicate wells, and were analyzed by analysis of variance and Student’s t test. A p value of 0.05 or less was considered to be significant.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA analyses were performed as described (20). Probes were end-labeled oligonucleotides corresponding to either the 5’-CArG of SM22α promoter (CTGCCCATAAAAAGTTTTTTC, SRF-binding site underlined), the SM22α-TCE (TGGAGTGAGGGGCACCCGG, TGFβ-responsive site underlined), or a PAI-1 probe (TCGAGAGCCGCAAGGAGGGAGCAAGGAGCCAGACAC, TGFβ-responsive sites underlined). Between 5 and 20 μg of nuclear protein was used per reaction, in a total volume of 15 μl. For supershift studies, 1 μl (1–2 μg) of antibody was added after the 15-min incubation period, and the reactions were incubated for an additional 20 min.

Chromatin Immunoprecipitation (ChIP) Assay—Eight million HEK293 cells were treated with and without TGFβ for 24 h and cross-linked with formaldehyde. Total lysates were prepared, sonicated, and divided into five tubes for ChIP experiments, using a commercially available kit (Upstate, Charlottesville, VA). One percent lysate was set aside to isolate control input DNA. The manufacturer’s protocol was followed with two modifications. To reduce nonspecific background, after sonicated lysates were pre-cleaned with salmon sperm DNA/protein G-agarose and incubated with protein A bound-agarose beads that were previously blocked with 1% bovine serum albumin were added. In addition, immunoprecipitated chromatin was washed twice (with half of the recommended volume each time) with high salt buffer. One μg of total antibody was used per ChIP reaction. Anti-SRF (G-20) and anti-Smad7 (N-19 and H-79 used in equal amounts) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-RNA polymerase II antibody and normal IgG were from the Upstate kit (Upstate, Charlottesville, VA). Two percent of purified DNA (1 μl), including saved input control DNA, was used as template for 32 cycles in PCR. Primers pairs employed to amplify the human SM22α promoter that harbors the CArG and TCE were as follows: forward, TCCATCTCAAAAGCGTACG, and reverse, CCCCTCGT-TAGAGGAAGCC, which map to bp −224 and +18, respectively. For ChIP-positive control reaction, primers that amplify the human GAPDH promoter provided by the Upstate kit were used.

Knockdown of Smad7 by siRNA Interference—HEK293 cells were seeded in 24-well plates with antibiotic-free media the day before transfection and used at 80–90% confluence the next day. Four wells were transiently transfected with a mixture that contains 100 ng of pSM22lac, 100 ng of pMSVβgal, 1.5 μl of Plus Reagent, and 1 μl of Lipofectamine in Opti-MEM medium along with siRNA duplex targeting either Smad7 (purchased from Santa Cruz Biotechnology) or Foxm1 (as control, a gift from Dr. V. Kalinichenko, University of Chicago). siRNAs were mixed with RNAiFect reagent (Qiagen, Valencia, CA) in a 1:6 ratio. In two experiments, a nonsilencing RNA duplex from Qiagen was used as an additional control. After 6 h, cells were fed with complete medium and harvested 54 h later. Luciferase and β-galactosidase activities were measured in three replicate wells, and total RNA was isolated from the fourth replicate well. The average of normalized luciferase activity in siRNA

FIGURE 1. An SRF-dependent mechanism mediates TGFβ1 enhancement of the SM22α promoter activity. A, TGFβ stimulates SM22α promoter function in a dose-dependent manner. Long term serum-deprived CTSMC were incubated with the indicated amounts of TGFβ1 (black bars) or left untreated (white bar). TGFβ1 effect was determined as normalized promoter activity relative to untreated cells. B, TGF isoforms β-1, β-2, and β-3 but not TGFα increased SM22α promoter function. Subconfluent serum-fed CTSMC were transfected with wild type SM22α promoter and treated with 100 pm of indicated TGF isoform (black bars) or left untreated (white bar). The effect of TGF isoforms on promoter SM22α activity was determined as in A. C, SRF-binding sites are necessary for stimulation of SM22α promoter activity by TFG-β1. CTSMC were transfected with wild type (white bars) or the indicated SM22α isoforms harboring point mutations in either the 5'-CArG sites (SME4, black bars) or both (dmSME, black bars). Cells were treated with 300 pm TGFβ1 (right bars) or left untreated (left bars). Mutation of CArG sequences that abolish SRF binding reduced but did not ablate TGFβ induction of SM22α promoter activity (p < 0.01 between none and TGFβ-treated cells, except for SME4 (p = 0.06)).
Smad7-SRF Interactions

![Image](50x313 to 407x733)

**FIGURE 2. TGFβ increases SRF-DNA binding capacity**. A, NE from long term serum-deprived CTSM cells exposed to 150 pm TGFβ1 for the indicated times were incubated with a probe containing the SM22α-CArG site. TGFβ1 administration elevated SRF DNA binding activity (arrow) with increasing exposure times. B, cross-linked chromatin-containing lysates were prepared from untreated (−) or TGFβ1-treated (+) HEK293 cells. CHIP was performed with anti-SRF, anti-Smad7, or anti-RNA polymerase II (RNAP) antibodies as indicated, along with no added antibody as negative control (none). Chromatin that undergoes no IP serves as positive control (input). Amplification of SM22α promoter sequences that contain the CArG plus TCE sites (top) or of GAPDH promoter sequences (bottom) was performed by PCR from purified DNA immunoprecipitated from chromatin. Binding of SRF to the SM22α promoter increased in cells treated with TGFβ (compare intensities of lane 1 versus lane 2). IP with anti-Smad7 antibodies was able to retrieve DNA containing the SRF-binding site (lane 7), although a fainter signal was obtained from TGFβ-treated cells (lane 8). Similar levels of binding to the GAPDH promoter, a gene reported to be SRF-independent, were achieved in chromatin treated with anti-RNAP II (lanes 13 and 14) but not with anti-SRF antibody (lanes 17 and 18). Negligible background signals were observed when no antibody (lanes 3, 4, 11, and 12) or DNA (lanes 9 and 19) was added. C, total cell lysates were prepared from control CTSM or cells treated with 150 pm TGFβ for 24 h. The membrane was probed first with anti-SRF antibody and then reprobed with anti-β actin antibody. D, NE were prepared from serum-fed CTSM exposed to 150 pm TGFβ for 24 h and incubated with either the SM22α-CArG or the PAI-I probe that contains a highly TGFβ-responsive element. E, formation of DNA-protein complexes on the SM22α-TCE. NE were prepared after exposure of serum-fed CTSM to 100 pm TGFβ for the indicated times. Probe contains the TCE from the SM22α promoter. TGFβ induced appearance of two TCE binding activities (arrows) in a time-dependent fashion.

Samd7-transfected cells was expressed relative to that obtained in control siRNA-transfected cells. Samd7 expression was analyzed by reverse transcription-PCR using cDNA prepared from 1 μg of total RNA and a set of commercially available Samd7 primers from Santa Cruz Biotechnology. A 521-bp amplicon was seen following the manufacturer’s instructions. Expression of Samd7 protein in the same lysates used for luciferase and β-galactosidase measures was analyzed by Western blot using the anti-Samd7 antibody N-19 from Santa Cruz Biotechnology, and was visualized by ECL from Pierce. siRNA experiments were performed four times with similar results.

**RESULTS**

**TGFβ1 Stimulates Transcriptional Activation of Smooth Muscle-specific Genes in Myocytes from the Airway through an**
changes in promoter function were observed with TGFβ and Smad-insensitive promoter plasmid plus empty CMV or Smad7 expression vectors as above. No significant effect on promoter activity by expression of Smad7. *, p < 0.02. C, cells were transfected with the artificial Smad-sensitive p5BEX2.tk promoter as above. TGFβ stimulation enhanced more than 2-fold SBE promoter function (black bars) compared with control (white bars). This increase was abolished by expression of Smad7. *, p < 0.01. D, cells were co-transfected with GRE.luc, a glucocorticoid-dependent but SRF-independent reporter plasmid plus empty CMV or Smad7 expression vectors as above. No significant changes in promoter function were observed with TGFβ stimulation (black bars) either with or without Smad7 overexpression relative to control (white bars).

FIGURE 3. TGFβ increases SRF-dependent transcriptional activity. CTSMC were co-transfected with two different SRF-dependent luciferase reporter plasmids 5’CArG(A) and SREL(B) plus empty CMV (left) or Smad7 (right) expression vectors. TGFβ (100 μM) was added to selected wells (black bars), and controls were left untreated (white bars). Activity of both promoters increased more than 2-fold with TGFβ treatment relative to untreated cells, and overexpression of Smad7 diminished basal as well as TGFβ-induced promoter function (#, p < 0.03, *, p < 0.01). C, cells were transfected with the artificial Smad-sensitive p5BEX2.tk promoter as above. TGFβ stimulation enhanced more than 3-fold SBE promoter function (black bars) compared with control (white bars). This increase was abolished by expression of Smad7. *, p < 0.02. D, cells were co-transfected with GRE.luc, a glucocorticoid-dependent but SRF- and Smad-insensitive promoter plasmid plus empty CMV or Smad7 expression vectors as above. No significant changes in promoter function were observed with TGFβ stimulation (black bars) either with or without Smad7 overexpression relative to control (white bars).

SRF-dependent Pathway—We first tested whether TGFβ affects promoter function of SM-specific genes in myocytes from the airway. Fig. 1A shows that TGFβ treatment enhanced transcription from the SM22α promoter after transient transfection into long term serum-deprived myocytes by 3–9-fold in a dose-dependent manner. Comparable stimulation was observed for the human smooth muscle myosin heavy chain promoter, but not with the viral murine sarcoma virus long terminal repeat promoter under the same conditions (data not shown). This indicates that TGFβ action on SM-specific promoters was selective. Transcriptional activation of SM22α promoter was also inducible by TGFβ in subconfluent serum-fed cells, although to a lesser extent, and all three TGFβ isoforms (but not TGFα) increased SM22α promoter activity to a similar degree (Fig. 1B).

To delineate the sequence(s) responsible for TGFβ responsiveness, we investigated the potential role of the CArG elements present in the SM22α promoter by inactivating them through site-directed mutagenesis. Point mutations that abolished SRF binding at either the 5’-CArG site at –275 bp or the 3’ CArG site at –150 bp reduced basal SM22α promoter activity (Fig. 1C, left bars) and also decreased the TGFβ-dependent enhancing effect (Fig. 1C, right bars). Moreover, mutation of both SRF-binding sites inhibited even further the TGFβ-induced augmentation of SM22α promoter function. Interestingly, the TGFβ effect was not completely abolished, supporting the notion that an SRF-independent mechanism also operate in airway myocytes.

TGFβ Increases SRF-DNA Binding Activity—The mutagenesis studies described above prompted us to investigate whether TGFβ influenced SRF DNA binding activity. To test this, we performed EMSAs using nuclear extracts prepared from subconfluent airway smooth muscle cell cultures and a probe containing the SM22α-5’-CArG site (Fig. 2A). Compared with untreated controls, TGFβ exposure increased the intensity of the DNA–protein complex in a time-dependent manner, up to 3 h of treatment. By competition and supershift analyses with anti-SRF antibody (not shown here but see Fig. 5D), we confirmed the presence of SRF within this complex.

We performed ChIP experiments to examine whether there is elevation of binding of endogenous SRF to the CArG sites within the SM22α promoter upon TGFβ stimulation in vivo. Fig. 2B shows that anti-SRF antibody was able to immunoprecipitate cross-linked chromatin that contains DNA sequences harboring the CArG site from the SM22α promoter (lane 1). A stronger binding was observed from cells stimulated with TGFβ (Fig. 2B, lane 2). These results indicate that SRF occupies the CArG site within the chromatin, and SRF binding to DNA is increased upon TGFβ stimulation. Furthermore, Fig. 2B also demonstrates that anti-Smad7 antibody was capable of retrieving chromatin harboring SM22α promoter sequences that possess SRF-binding sites (lane 7). If cells were treated with TGFβ, the efficiency of recovering CArG sites-containing DNA was less evident (Fig. 2B, lane 8). As controls, TGFβ induced no change in the signal for GAPDH promoter sequences that are precipitated using anti-RNA polymerase II antibody (Fig. 2B, lanes 13 and 14). Retrieval of sequences from the SM22α (Fig. 2B, lanes 3 and 4) or GAPDH (lanes 11 and 12) promoters were negligible in chromatin treated with no antibody. No GAPDH promoter sequences could be amplified from chromatin precipitated using anti-SRF antibody (Fig. 2B, lanes 17 and 18). Altogether, our data indicate that Smad7 associates in vivo with SRF over the CArG element of the...
**Smad7-SRF Interactions**

SM2α promoter, and TGFβ stimulates SRF-DNA binding activity to the SM22α promoter.

Hautmann et al. (24) reported that increased SRF-DNA binding capacity was associated with augmented SRF content in TGFβ-treated vascular myocytes, but no such increase in SRF abundance was present in aorta endothelial cells. Western analysis of total cell lysates demonstrated that enhanced SRF-DNA binding activity in our system occurs with only minimal increase in SRF abundance (Fig. 2C). Fig. 2D shows that the level of stimulation of SRF SM22α–5′CArG complex formation by TGFβ administration was comparable with that observed by using a probe from the plasminogen activator inhibitor (PAI)-1 promoter, which is highly inducible by TGFβ and contains Smad3/Smad4-binding sites. Finally, we demonstrated DNA-protein interactions using nuclear extracts from airway smooth muscle cells incubated with a probe containing the TCE from the SM22α promoter region (Fig. 2E). This sequence, which spans bp −132 to −111, is devoid of CArG sites. Formation of TCE binding activities from serum-fed airway smooth muscle cells was evident as early as 15 min of TGFβ treatment.

**Smad7 Is a Negative Regulator of SRF-dependent Transcription Activity—**Previous studies have shown that Smad sites within the first exon of SM22α gene mediate TGFβ responsiveness of SM22α and have identified a putative TCE in the 5′-flanking region. Thus, to establish whether TGFβ induces smooth muscle gene transcription through activation of SRF transcription-promoting function independently of these sites, we performed transient transfection studies using two promoter reporter constructs solely responsive to SRF. In p5xCArGluc, five tandem copies of the CArG site and a TATA box drive expression of luciferase reporter; in pSRE.LLuc, the Ets-binding site in the c-fos promoter serum-response element is inactivated leaving only the SRF-binding sequences intact. Because TGFβ action is mediated by Smad members in many cell types, we further hypothesized that overexpression of the inhibitor Smad7 would decrease SRF-dependent transcriptional activity induced by TGFβ in airway myocytes. Fig. 3, A and B, shows that TGFβ increased transcription from 5xCArG and SRE.L promoters by 2-fold, relative to untreated control cells. Moreover, overexpression of Smad7 markedly reduced transcription from these promoters, in the presence of TGFβ and even in non-TGFβ-treated cells. The latter finding probably reflects autocrine secretion of TGFβ by our airway myocytes, as addition of anti-TGFβ antibodies to culture medium also reduces transcription from the SM22α promoter in non-TGFβ-treated cells.3 We found parallel results for transcription from pSBEx2.tk.luc, which harbors a promoter sensitive to Smad activation. Relative to untreated controls, TGFβ increased reporter transcription more than 3-fold (Fig. 3C, left bars), and co-expression of Smad7 reduced this enhancement to basal levels (Fig. 3C, black bars). Smad7 also diminished basal SBE promoter function in the absence of added TGFβ, again suggesting the presence of a TGFβ autocrine pathway. As a negative control, TGFβ treatment did not increase transcription from a glucocorticoid-sensitive (GRE) promoter, which contains neither Smad sites nor CArG boxes (Fig. 3D, left bars). Likewise, overexpression of Smad7 did not decrease GRE promoter function in treated or nontreated cells. Altogether, these results indicate that Smad7 specifically inhibits TGFβ-stimulated activation of SRF-dependent transcription in airway myocytes.

**Silencing of Smad7 Stimulates SRF-dependent Promoter Function—**To test the participation of endogenous Smad7 in regulating SRF-dependent promoter transcription, we performed promoter reporter assays in transiently transfected cells in which endogenous Smad7 was depleted via siRNA interference. Fig. 4 shows that knockdown of endogenous Smad7 in transiently transfected HEK293 cells is sufficient to more than double the promoter function of the SM22α promoter compared with cells treated with control siRNA. Thus, although overexpression of Smad7 is capable of inhibiting basal function of SRF-dependent promoters (Fig. 3, A and B and Fig. 7), blockade of Smad7 expression, conversely, results in exaggerated SRF-dependent transcriptional activity.

**SRF Interacts with Smad7—**The ChIP studies described above strongly indicate that the inhibitory effect of Smad7 on SRF-dependent transcription stems from an association of Smad7 with SRF. To confirm this observation, we performed co-immunoprecipitation assays using nuclear (NE) and cytosolic (CE) extracts from COS or HEK293 cells that were co-transfected with plasmids encoding myc-SRF or Myc alone along with Smad7-HA expression plasmids or empty vector. Fig. 5A shows that Smad7 was pulled down from NE only when SRF was co-expressed (lane 8), indicating that SRF interacts with

---

3. B. Camoretti-Mercado and J. Solway, unpublished observations.
Smad7 within the nucleus. We are also able to demonstrate interaction of Smad7-HA or 6×myc-Smad7 with EGF-SRF or GST-SRF in COS, CTSM, and HEK293 cells as well. Interestingly, association of Smad7 to SRF in the cytosolic fraction was undetectable in all three cell types (see Fig. 6D, and data not shown). To corroborate this interaction between the endogenous molecules as well, we prepared NE and CE from nontransfected HEK293 and COS cells and immunoprecipitated Smad7 with a polyclonal anti-Smad7 antibody in the presence of conjugated IgA/IgG-agarose beads. Pulled down proteins were size-fractionated by PAGE, blotted onto nitrocellulose membrane, and probed with anti-SRF antibody. Fig. 5B shows that endogenous Smad7 associates with endogenous SRF inside the nuclei of both cells but not in the cytosol of HEK293 cells (note that in this preparation, SRF expression in COS cells was exclusively nuclear (lane 6 versus lane 8)). These results rule out the possibility that interaction of Smad7 and SRF occurs only with overexpressed and chimeric recombinant proteins.

Smad members participate in protein-protein interactions via their MH2 domain localized within the C terminus of the molecule. To determine the domain of Smad7 that associates with SRF, we performed pull-down assays with extracts from cells co-transfected with myc-SRF and FN-Smad7-FLAG or FC-Smad7-FLAG, in which the first 259 residues or amino acids 206–426 of Smad7, respectively, are fused to the FLAG tag. Fig. 5C shows that in HEK293 cells, SRF interacts with the C-terminal portion of Smad7 in the nucleus (lane 4), but binding is negligible in the cytoplasm (lane 8). On the other hand, virtually no FN-Smad7-FLAG could be pulled down by SRF in the nucleus (Fig. 5C, lane 2) under identical conditions. This indicates that structural features that enable Smad7 to associate with SRF reside in the second half of the Smad7 molecule. Moreover, the Smad7-SRF interaction appears to be maintained even when SRF is bound to DNA, which is consistent with our ChIP results. Indeed, Fig. 5D shows that complexes formed by airway smooth muscle nuclear proteins on the 3′-CArG-SM22α probe contains SRF (as anti-SRF antibody supershifts the bands; lane 2) and Smad7 (as anti-C Smad7 antibody prevents complex formation).

SRF contains a functional nuclear localization signal (NLS) within its N terminus, and the MAD5 domain of SRF participates in a variety of functional protein-protein interactions. We tested whether these two features were involved in the ability of SRF to associate with Smad7. Co-immunoprecipitation assay shown in Fig. 5E demonstrates that the EGF-SRF fusion protein harboring mutations within either the nuclear localization signal (mNLS-SRF) or the dimerization domain (mDM-SRF)
**Smad7-SRF Interactions**

**FIGURE 6.** The TGFβ signaling pathway modulates SRF-Smad7 interaction. A, COS cells were co-transfected with Myc-SRF and Smad7-HA expression vectors. After transfection, cells were fed with serum-containing medium for 18 h and the next day treated with TGFβ or left untreated for 3 h. Nuclear extracts were prepared and IP performed after extracts were preincubated with normal IgG-coupled beads, using anti-Myc antibody-bound beads. Western blot was done with anti-HA antibody to detect Smad7. P, pellet; S, supernatant after IgG preincubation. TGFβ stimulation decreased the interaction of Smad7 with SRF. B, COS cells were co-transfected with Myc-SRF and Smad7-HA expression plasmids plus either a vector encoding the phosphorylation-deficient Smad3(3A) mutant (lanes 1 and 2), wild type Smad3 (lanes 3 and 4), or empty vector CMV5 (lanes 5 and 6). IP and Western analysis were performed with NE pretreated with normal IgG beads, as above. Overexpression of signaling-defective Smad3 mutant increased Smad7 binding to SRF (lane 2 versus 6). C, IP was performed with COS cells NE co-transfected with Myc-SRF and Smad7-HA vectors along with the empty (lanes 1–3) or kinase-defective TβRI (TβRI-DN, lanes 4–6) expression plasmids. Smad7 bound to SRF was detected by using anti-Myc antibody-conjugated beads followed by anti-HA Western blot of inputs (I), pulldown (P), and supernatants (S), as described above. The results demonstrated that overexpression of SRF by overexpression of a dominant negative TβRI mutant augmented Smad7 interaction to SRF (lanes 2 versus 5). D, NE and CE were prepared from HEK293 cells co-transfected with Myc-SRF and Smad7-HA. After transfection, cells were treated with IL-6 (50 ng/ml) or IL-13 (50 ng/ml) as indicated, for 18 h. IP was performed with anti-Myc antibody beads. Bound Smad7 was detected by anti-HA Western blot. Binding of Smad7 to SRF in the nuclei is weakened in the presence of these cytokines, compared with untreated cells (lanes 2 and 3 versus 6). Interestingly, SRF and Smad7 became competent to associate in the cytosol of IL-treated cells (lanes 8 and 10 versus 12).

exhibited a decreased ability to associate with Smad7 compared with wild type SRF.

**Modulation of Smad7-SRF Association by TGFβ**—Because Smad7 has a pivotal role in controlling TGFβ actions, and TGFβ itself regulates Smad7 function, we hypothesized that SRF-Smad7 interaction might be modulated by activation of the TGFβ cascade. To test this possibility, we performed pulldown assays using NE from cells treated with and without TGFβ. Fig. 6A shows that TGFβ-stimulated cells exhibited a weaker SRF-Smad7 interaction than untreated cells. Conversely, when TGFβ action was interrupted by interfering with the propagation of the signal via expression of a defective Smad3 mutant (Fig. 6B) or at the receptor level by co-expression of a dominant negative TβRI mutant (TβRI-DN) deficient in its kinase activity (Fig. 6C), a stronger interaction of Smad7 and SRF was elicited.

These results prompted us to investigate whether other interventions also modulated Smad7-SRF association. Fig. 6D shows that interleukin (IL)-13 or IL-6 treatment weak-

**DISCUSSION**

In this study, we demonstrate that modulation of SRF-Smad7 interactions in part mediates TGFβ-stimulated transcription of smooth muscle-specific genes in airway myocytes. We found that TGFβ stimulates transcriptional activity of SM-specific promoters through an SRF-dependent pathway and that overexpression of Smad7 reduces TGFβ-induced stimulation of purely SRF-dependent promoters. We demonstrated that SRF associates with Smad7 in cell nuclei and that this association is reduced by TGFβ treatment. Thus, we propose a novel mechanism by which TGFβ influences SRF-dependent transcription. 1) Smad7 interacts with SRF, thereby reducing its transcription promoting activity. 2) TGFβ treatment reduces SRF-Smad7 interaction, thereby restoring transcription promoting activity of SRF.

Fig. 8 illustrates possible ways by which Smad7 could inhibit SRF-dependent transcriptional activity. First, as is the case in other cell types such as lung epithelial cells (8), Smad7 may
translocate into the cytoplasm upon binding of TGFβ to TβRII and prevent phosphorylation of R-Smads, propagation of TGFβ signaling, and subsequent stimulation of SRF-dependent genes. Second, Smad7 may interfere with formation of transcriptionally competent active R-Smad-SRF complexes. In this regard, Qiu et al. (15) demonstrated that SRF could bind Smad3 in fibroblasts. In a third scenario elucidated here, Smad7 in the nucleus interacts with SRF, thereby reducing SRF function. The latter notion is consistent with previously known features of Smad7 as follows: (i) Smad7 localizes within the nucleus of some cells in the absence of TGFβ (30); (ii) Smad7 possesses a repressing function in transcription assays, even independently of TGFβ (Ref. 31 and our observations); (iii) Smad7 can interact with co-activators and transcription factors in the nucleus (32); and (iv) physiologically relevant regulation of Smad7 export from the nucleus has been reported (33). To our knowledge, we are the first to demonstrate an association of Smad7 with SRF within the nucleus and to postulate that Smad7 may perturb the capability of SRF to enhance transcriptional functions. Based on our IP results, we believe that this inhibitory activity does not likely involve gross changes in the subcellular localization of Smad7 or SRF.

Hautmann et al. (24) reported that a marked augmentation in SRF-DNA binding in aortic SM cells correlated with substantially higher levels of SRF expression in TGFβ-treated cells compared with control cells. Interestingly, we observed in airway smooth muscle that the SRF binding to SM22α-CARG sites increased after TGFβ treatment, but this occurred with only minor increase in SRF abundance. Whether this difference from the study of Hautmann et al. (24) stems from distinct experimental conditions or diverse properties of airway and vascular myocytes is not yet known.

Our results reveal that the C terminus of Smad7 is necessary for binding to SRF, which is consistent with previous reports that established that this region is involved in Smad7-protein interactions, including binding to TβRI. As expected, import of SRF to the nucleus was important for SRF association with Smad7; in addition, the MADS domain of SRF was required for nuclear SRF-Smad7 interaction.

Recent studies reveal a prominent role of TGFβ- and SRF-dependent pathways in lung homeostasis. Smad7 adds to the list of proteins that regulate SRF function. That Smad7 associates with and inhibits SRF and that this functionally significant association is modulated by the external cell milieu may have important biological consequences. TGFβ is abundant in asthmatic airways, in which airway smooth muscle hypertrophy is also a prominent feature. Expression of TGFβ is also up-regulated in patients with chronic obstructive pulmonary disease (34) or with lymphangioleiomyomatosis, a disease of abnormal proliferation of smooth muscle cells within the lung (35). In our studies we demonstrated that TGFβ, IL-13, and IL-6 were able to disrupt SRF-Smad7 interaction, which may lead to alleviation of the inhibitory effect of Smad7 on SRF function. It is reasonable to speculate that overactivation of SRF by persistent TGFβ stimulation might contribute to smooth muscle hypertrophy in inflammatory lung diseases.

Acknowledgments—We thank Drs. L. Attisano, N. Dulin, V. Kalinichenko, E. Medrano, C.-H. Heldin, and P. ten Dijke for the gifts of reagents and Drs. A. Halayko, S. Forsythe, and H.-W. Liu for assistance in the initial phase of this study.

REFERENCES
1. Piek, E., Heldin, C.-H., and ten Dijke, P. (1999) FASEB J. 13, 2105–2124
2. Moustakas, A., Pardali, K., Gaal, A., and Heldin, C.-H. (2002) Immunology Lett. 82, 85–91
3. Schuster, N., and Krieglstein, K. (2002) Cell Tissue Res. 307, 1–14
4. Holgate, S. (2000) Clin. Exp. Allergy 30, 37–41
Smad7-SRF Interactions

5. Bartram, U., and Speer, C. P. (2004) *Chest* **125**, 754–765
6. Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H., and Miyazono, K. (1993) *Cell* **75**, 681–692
7. Massague, J. (2000) *Nat. Rev. Mol. Cell Biol.* **1**, 169–178
8. Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C.-H., Heldin, N.-E., and ten Dijke, P. (1998) *J. Biol. Chem.* **273**, 29195–29201
9. Hanyu, A., Ishidou, Y., Ebisawa, T., Shimannuki, T., Imamura, T., and Miyazono, K. (2001) *J. Cell Biol.* **155**, 1017–1028
10. Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) *Mol. Cell* **6**, 1365–1375
11. Solway, J., Forsythe, S. M., Halayko, A. J., Vieira, J. E., Hershenson, M. B., and Camoretti-Mercado, B. (1998) *Am. J. Respir. Cell Mol. Biol.* **31**, S100–S108
12. Parmacek, M. (2001) *Curr. Top. Dev. Biol.* **51**, 69–89
13. Hautmann, M. B., Madsen, C. S., and Owens, G. K. (1997) *J. Biol. Chem.* **272**, 10948–10956
14. Adam, P. J., Christopher, R., Hautmann, M. B., and Owens, G. K. (2000) *J. Biol. Chem.* **275**, 37798–37806
15. Qiu, P., Feng, X.-H., and Li, L. (2003) *J. Mol. Cell. Cardiol.* **35**, 1407–1420
16. Chen, S., Kulik, M., and Lechleider, R. J. (2003) *Nucleic Acids Res.* **31**, 1302–1310
17. Solway, J., Forsythe, S. M., Halayko, A. J., Vieira, J. E., Hershenson, M. B., and Camoretti-Mercado, B. (1998) *Am. J. Respir. Crit. Care Med.* **158**, S100–S108
18. Camoretti-Mercado, B., Dulin, N., and Solway, J. (2003) *Respir. Physiol. Neurobiol.* **137**, 223–235
19. Camoretti-Mercado, B., Liu, H.-W., Halayko, A. J., Forsythe, S. M., Kyle, J. W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J. E., Patel, N. M., Hershenson, M. B., Fuchs, E., Sinha, S., Miano, J. M., Parmacek, M. S., Burkhardt, J. K., and Solway, J. (2000) *J. Biol. Chem.* **275**, 30387–30393
20. Liu, H. W., Halayko, A. J., Fernandes, D. J., Harmon, G. S., McConville, J., Fu, Y., Forsythe, S. M., Kogut, P., Bellam, S., Dowell, M., Churchill, J., Lesso, H., Kassiri, K., Mitchell, R. W., Hershenson, M. B., Camoretti-Mercado, B., and Solway, J. (2003) *Am. J. Respir. Cell Mol. Biol.* **29**, 39–47
21. Ding, W., Gao, S., and Scott, R. (2001) *J. Cell Sci.* **114**, 1011–1018
22. Wang, Z., Wang, D.-Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E. N. (2004) *Cell* **124**, 185–189
23. Hautmann, M. B., Adam, P. J., and Owens, G. K. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19**, 2049–2058
24. Halayko, A. J., Kartha, S., Stelmack, G. L., McConville, J., Tam, J., Camoretti-Mercado, B., Forsythe, S. M., Hershenson, M. B., and Solway, J. (2004) *Am. J. Respir. Cell Mol. Biol.* **31**, 266–275
25. Solway, J., Seltzer, J., Samaha, F. F., Kim, S., Alger, L. E., Niu, Q., Morrisey, E. E., Ip, H. S., and Parmacek, M. S. (1995) *J. Biol. Chem.* **270**, 13460–13469
26. Kim, S., Ip, H., Lu, M., Clendenin, C., and Parkmez, M. (1997) *Mol. Cell. Biol.* **17**, 2266–2278
27. Salesky, S. M., Kogut, P. C., McConville, J. F., Fu, Y., McCauley, I. A., Halayko, A. J., Liu, H. W., Kao, A., Fernandes, D. J., Bellam, S., Fuchs, E., Sinha, S., Bell, G. I., Camoretti-Mercado, B., and Solway, J. (2002) *Am. J. Respir. Cell Mol. Biol.* **26**, 298–305
28. Camoretti-Mercado, B., Forsythe, S. M., LeBeau, M. M., Espinosa, L., Rafael, Vieira, J. E., Halayko, A. J., Willadsen, S., Kurtz, B., and Ober, C. (1998) *Genomics* **49**, 452–457
29. Bai, S., and Cao, X. (2002) *J. Biol. Chem.* **277**, 4176–4182
30. Pulaski, L., Landstrom, M., Heldin, C.-H., and Souchelyntskyi, S. (2001) *J. Biol. Chem.* **276**, 14344–14349
31. Gronroos, E., Hellman, U., Heldin, C.-H., and Ericsson, J. (2002) *Mol. Cell* **10**, 483–493
32. Reguly, T., and Wrana, J. L. (2003) *Trends Cell Biol.* **13**, 216–220
33. Silverman, E. K., Mosley, J. D., Palmer, L. J., Barth, M., Senter, J. M., Brown, A., Drazen, J. M., Kwiatkowski, D. J., Chapman, H. A., Campbell, E. J., Province, M. A., Rao, D. C., Reilly, J. L., Ginn, L. C., Speizer, F. E., and Weiss, S. T. (2002) *Hum. Mol. Genet.* **11**, 623–632
34. Evans, S. E., Colby, T. V., Ryu, J. H., and Limper, A. H. (2004) *Chest* **125**, 1063–1070