

# Smad proteins regulate transcriptional induction of the SM22 $\alpha$ gene by TGF- $\beta$

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## ABSTRACT

**Smad proteins transduce signals from transforming growth factor- $\beta$  (TGF- $\beta$ ) receptors and regulate transcription of target genes. TGF- $\beta$  is implicated in the regulation of the smooth muscle cell specific gene SM22 $\alpha$ , but little is known about how Smads are involved in SM22 $\alpha$  gene transcription. In this report, we demonstrate that TGF- $\beta$  activation of the SM22 $\alpha$  promoter is Smad dependent in C3H10T1/2 cells, BALB 3T3 cells and neural crest Monc-1 cells. We find that the promoter region from -162 to +41 is sufficient to up-regulate the reporter gene upon TGF- $\beta$  induction. Smad3, Smad1 and Smad4 are found in TGF- $\beta$  inducible complexes that bind to a region containing a Smad binding site (SBS) and a medea box. Both the SBS and medea box are necessary for complex formation and are functionally important. Smad4 is limiting for TGF- $\beta$  induction, and Smad3, but not Smad1, significantly contributes to maximal activation. Time course luciferase assays and time course gel mobility shift assays reveal that the Smad3/4 complex is largely responsible for the immediate response of the SM22 $\alpha$  promoter to TGF- $\beta$  induction, and also contributes to the maximal promoter activity. We further demonstrate that AP-1 elements contribute to induction of the SM22 $\alpha$  promoter by TGF- $\beta$ .**

## INTRODUCTION

During development and growth of the vasculature, recruitment and differentiation of smooth muscle cells (SMCs) is a critical process. SMCs provide hemostatic control and protect new endothelium-lined vessels against rupture or regression. SMCs also assist endothelial cells in acquiring specialized functions in different vascular beds and maintain vascular tone and integrity in the adult (1). The mechanisms of SMC differentiation from various precursors remain obscure, but transforming growth factor- $\beta$  (TGF- $\beta$ ) family ligands and downstream signaling intermediates have been shown to play roles in SMC differentiation in a variety of systems. TGF- $\beta$ 1, TGF- $\beta$  receptors (including endoglin, T $\beta$ R-II, T $\beta$ R-I and Alk1) and signaling intermediates have all been identified by

homologous recombination in the mouse as critical for embryonic angiogenesis. Loss of any of these components leads to defects in smooth muscle cell recruitment and/or differentiation during angiogenesis in the developing embryo and extraembryonic structures (2). *In vitro* studies have shown that TGF- $\beta$ 1 can induce differentiation of C3H10T1/2 cells to a smooth muscle phenotype directly (3). Likewise, TGF- $\beta$  can induce neural crest stem cells to express SMC differentiation markers (4), suggesting that the role of TGF- $\beta$  in regulating SMC differentiation is fairly broad.

To understand the molecular mechanism of SMC differentiation, the expression of SMC specific genes such as  $\alpha$ -SM actin, SM myosin heavy chain and SM22 $\alpha$  has been studied by several groups (5,6). Among these, SM22 $\alpha$  is one of the earliest and most widely expressed SMC markers identified (7–10). SM22 $\alpha$  is a 22-kDa calponin-related protein that interacts with other contraction-associated proteins such as tropomyosin and F-actin. SM22 $\alpha$  expression is specific for smooth muscle, and the 5' regulatory regions necessary for tissue specific expression have been well characterized. TGF- $\beta$  can induce expression of SM22 $\alpha$ , and this transcriptional regulation is under at least partial control of a TGF- $\beta$  control element (TCE) in the 5' regulatory region. Although the TCE had been shown to bind to gut-enriched Kruppel-like factor (GKLF), GKLF repressed the TGF- $\beta$ -dependent increases of SM22 $\alpha$  promoter activity, and it is unclear what factors bind the TCE under physiological conditions (11). Other elements in the promoter responsive to TGF- $\beta$ , including CArG elements, have been identified (12), but the mechanism whereby TGF- $\beta$  regulates these elements has not been fully elucidated.

TGF- $\beta$  signaling is initiated by ligand binding to the transmembrane receptor serine-threonine kinases, T $\beta$ R-I and T $\beta$ R-II. Signaling from the receptors to the nucleus occurs predominantly by phosphorylation of receptor-associated Smad proteins (R-Smads) (13,14), which combine with the common Smad, Smad4, and translocate into the nucleus where they function as transcription factors alone or in association with other DNA binding factors. Much work clearly demonstrates, however, that other pathways including those activated by MAP and JNK kinases and ras also play a role (15). The aim of this study was to investigate the function of Smad proteins in TGF- $\beta$ -induced SM22 $\alpha$  gene transcription. Using reporter gene assays with the SM22 $\alpha$  promoter and mutational analysis, we find that the SM22 $\alpha$  promoter is Smad-dependent. We further demonstrate that Smad proteins bind to a

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bipartite site in the first exon and 5' flanking sequence of the SM22 $\alpha$  gene. We provide evidence that Smad proteins are responsible for the immediate SM22 $\alpha$  gene activation upon TGF- $\beta$  stimulation. We further show that AP-1 proteins may contribute to Smad-dependent activation of the SM22 $\alpha$  promoter.

## MATERIALS AND METHODS

### Plasmids

Smad expression vectors have been described (16,17). SM22 $\alpha$  promoter luciferase constructs (a generous gift from Dr Julian Solway, University of Chicago, Chicago, IL) have been described (8). Promoter mutants were generated using the Quikchange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. The Smad binding site (SBS) sequence GTCT (from nucleotide -157 to -154) was substituted to GAGA for p-162SM22m4, CAGAC (from nucleotide -95 to -91) to CACTG for p-162SM22m5, GTCT (from nucleotide +6 to +9) to CAGT for p-162SM22m6, CAGAC (from nucleotide +19 to +22) to CACTG for p-162SM22m7 and CAGA (from nucleotide -58 to -55) to CACT for p-162SM22m8. For TCE mutant construct p-162TCEm, the wild type sequence 5'-GGAGTGAGTGGGGCGGCCCG-3' (from nucleotide -132 to -112) was substituted by 5'-GGAGTtA<sub>t</sub>TGttGCGGCCG-3'. Medea box mutant (p-162-MBm) was created by converting the sequence GCCGGCG (from nucleotide -11 to -5) to GCCttCG (18,19). Mutant plasmid p-162-SM-dm at both SBS and medea box were generated according to the individually mutated sequences. All plasmids were sequenced prior to use.

### Cell culture and transfections

C3H10T1/2 (10T1/2) and Balb 3T3 cells (American Type Culture Collection, Manassas, VA), and Smad3 knockout fibroblast cells [S3KO, a gift from Dr Ester Piek (National Cancer Institute, Bethesda, MD) (20)] were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (Invitrogen, Gaithersburg, MD). Prior to transfection, cells were plated at  $6 \times 10^4$  cells/well in six-well plates and incubated until they reached 70% confluency. Monc-1 cells were a gift from Dr David Anderson (California Institute of Technology, Pasadena, CA) and were grown as described (21). Before transfection, Monc-1 cells were grown in DMEM containing 10% FBS for 2 days to induce smooth muscle differentiation. Cells were transiently transfected (in triplicate) with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations. Each well received luciferase reporter construct, Renilla luciferase and other expression plasmids as indicated. The total transfected DNA was adjusted with pBSK+ DNA and/or pCDNA3.0. After transfection, the medium was replaced with fresh medium and incubated for 24 h before being treated for 16 h or the indicated time with TGF- $\beta$  (5 ng/ml) (R and D Systems, Minneapolis, MN) or vehicle alone in media containing 0.2% FBS. As indicated in some experiments, a constitutively active TGF- $\beta$  receptor construct was used instead of TGF- $\beta$ . Luciferase activity of

cell lysates was determined using the Dual-Luciferase Assay System from Promega (Madison, WI). The relative luciferase activity values were normalized to Renilla activity. All measurements represent the mean of duplicate corrected luciferase measurements from triplicate wells  $\pm$  SEM. Results were analyzed using ANOVA with pairwise comparisons between relevant groups. All experiments were performed at least twice with representative results shown.

### Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

10T1/2 cells were plated at a density of  $3 \times 10^6$ /15 cm dish, grown to 70% confluency as above, and then starved in 0.2% serum medium overnight and treated with or without 5 ng/ml TGF- $\beta$  for the times indicated. Nuclear extracts were prepared as described (22). Protein concentrations were measured using BCA Protein Assay Reagent (Pierce). Oligonucleotide probes were labelled and EMSAs were performed as described (22). In competitive or antibody supershift assays, competitor oligonucleotides or Smad antibodies (Dr Peter ten Dijke, The Netherlands Cancer Institute, Amsterdam, The Netherlands) were added simultaneously to the reaction buffer with labeled probes and incubated on ice for 45 min.

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

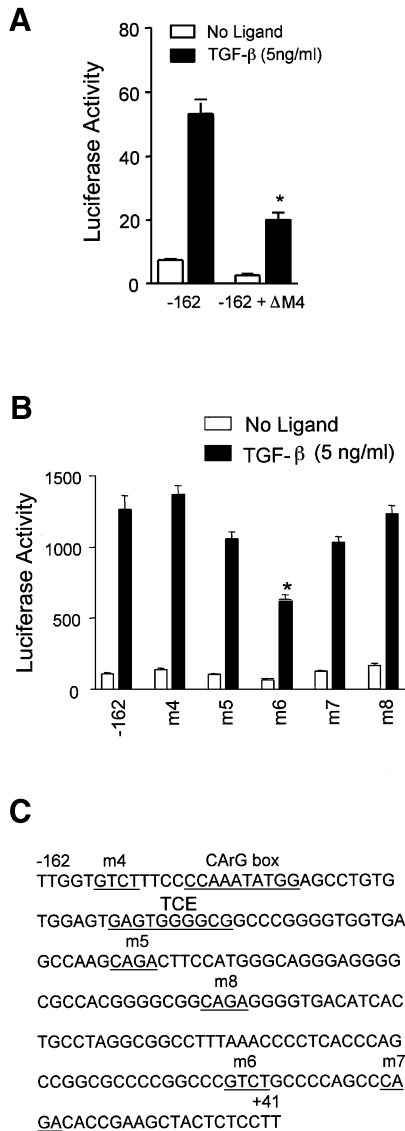
Cells were treated with TGF- $\beta$  for the time indicated in DMEM containing 0.2% FBS. Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions. RT-PCR was performed using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) and primers specific to SM22 $\alpha$  or cyclophilin (as a loading control). Primer sequences are available upon request.

## RESULTS

### SM22 $\alpha$ gene expression is Smad dependent

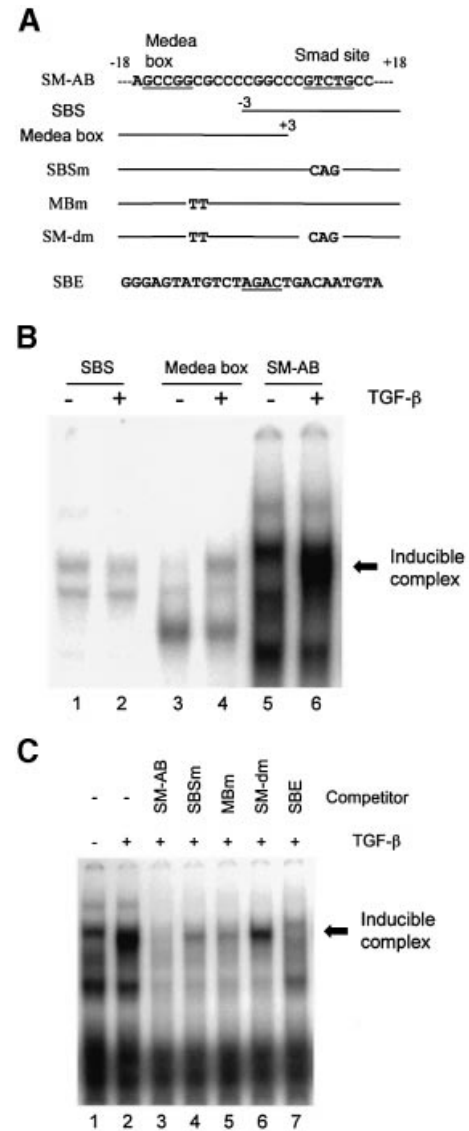
To determine the role of Smad proteins in regulating TGF- $\beta$ -induced SM22 $\alpha$  gene transcription, we first identified the minimum promoter region necessary for TGF- $\beta$  induction in 10T1/2 cells. The SM22 $\alpha$  promoter region from -441 to +41 bp has been shown to correctly direct arterial expression of SM22 $\alpha$  during development (23). We found that either the regions from -162 to +41 bp or from -441 to +41 bp gave identical fold-activation to TGF- $\beta$  induction in 10T1/2 cells (data not shown). The -162 to +41 bp construct was used in all subsequent studies. To determine if TGF- $\beta$  induction of this construct was dependent on Smad proteins, we used a well-characterized dominant interfering Smad4 construct (Smad4 $\Delta$ M4) (24). Smad4 $\Delta$ M4 was cotransfected with the reporter plasmid into 10T1/2 cells and luciferase activity determined. Smad4 $\Delta$ M4 significantly reduced the level of TGF- $\beta$  induced luciferase activity (Fig. 1A), which demonstrated that Smad proteins play an essential role in full activation of the SM22 $\alpha$  gene by TGF- $\beta$ .

Smads bind to the consensus sequences CAGA or GTCT (Smad binding sites or SBS) which confer TGF- $\beta$  responsiveness (25). Sequence analysis of the SM22 $\alpha$  5' regulatory region identified five potential Smad binding sites located at



**Figure 1.** The SM22 $\alpha$  promoter is dependent on Smads. (A) Effect of a Smad4 mutant on SM22 $\alpha$  promoter activity. p-162SM22luc plasmid was transfected with pcDNA3.0 or Smad4 mutant expression vector (Smad4 $\Delta$ M4) into 10T1/2 cells and luciferase activity determined with or without TGF- $\beta$ . (B) Activity of the SM22 $\alpha$  promoter with mutations at potential Smad binding sites. The wild type or mutant plasmids were transfected into 10T1/2 cells and luciferase activity determined as described following treatment as indicated. (C) The sequence of SM22 $\alpha$  promoter region from -162 to +41 bp. Numbers correspond to the transcription start site. The potential Smad binding, TCE and CArG sites are underlined and named as indicated. \* $P < 0.01$  for comparison to TGF- $\beta$  treated control.

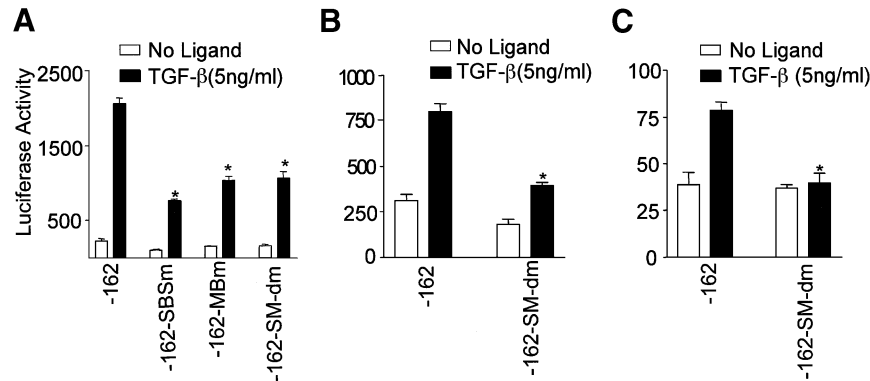
-157, -95, -58, +6 and +19 (Fig. 1C). To determine whether these sites function in TGF- $\beta$  induction, each SBS was individually mutated and the mutant reporters used in transient transfection experiments. The SBS mutation, -162m6, decreased TGF- $\beta$  induction by >50% compared to the wild type (Fig. 1B). However, the other SBS mutations -162m4, -162m5, -162m7 or -162m8 only had a slight effect on promoter response to TGF- $\beta$ . These results suggest that Smad proteins may selectively activate SM22 $\alpha$  transcription in response to TGF- $\beta$  through the SBS at position +6.



**Figure 2.** Smad proteins interact with the SM22 $\alpha$  promoter sequence. (A) Sequence scheme of DNA fragments used in the EMSAs. Numbers correspond to distance from the transcription start site of the SM22 $\alpha$  gene. The SBS and medea box are underlined. Substituted nucleotides for the SBS and medea box are shown. (B) EMSA with SBS and medea box. Nuclear extracts from 10T1/2 cells treated with vehicle (-) or TGF- $\beta$ 1 (5 ng/ml) (+) for 30 min were incubated with  $^{32}$ P-labeled probes for SBS, medea box, or both sites (SM-AB) and resultant complexes resolved as described. The TGF- $\beta$  induced complexes in lanes 4 and 6 are indicated by arrows. (C) EMSAs with the competitive oligos were performed using labeled SM-AB and indicated cold competitor oligonucleotides.

### Both Smad binding site and the upstream medea box are necessary for formation of a TGF- $\beta$ inducible complex

Examination of the sequence surrounding the SBS at +6 uncovered an upstream GCCGGCG sequence (Fig. 2A). This GC-rich stretch matches the binding sequence for the *Drosophila* Smad4 homolog, medea (26) and resembles Smad responsive elements in the Id1 (27), Smad6 (28) and Col7A (29) promoters. To determine whether the SBS and medea box could bind Smad proteins following TGF- $\beta$



**Figure 3.** (A) Effect of mutations at SBS and medea box on SM22 $\alpha$  promoter activity. Wild type (-162) or plasmids with mutation at SBS (-162-Sm), medea box (-162-MBm) or both sites (-162-SM-dm) were transfected into 10T1/2 cells and luciferase activity determined. (B and C) Wild type or mutant reporter plasmids as indicated were transfected into Balb 3T3 (B) or Monc-1 (C) cells and luciferase activity determined. \* $P < 0.01$  for comparison to TGF- $\beta$  treated -162 construct.

stimulation, EMSAs were performed with nuclear extracts from 10T1/2 cells and DNA probes containing both the SBS and medea box (SM-AB) or individual sites alone (SBS or medea box). As shown in Figure 2B, the TGF- $\beta$  induced complex bound to the DNA sequence containing both the SBS and medea box. The medea box alone also formed an inducible complex of similar size but lower intensity with the nuclear extracts from TGF- $\beta$  treated cells, while SBS alone was not sufficient for the formation of an inducible complex. We also saw a complex of about the same size as the induced complex that was present in extracts from uninduced cells that disappeared following TGF- $\beta$  treatment (Fig. 2B, lane 5). This complex did not appear to contain Smad proteins and loss of binding had no effect on promoter activity (data not shown).

To determine further the role of the medea box and SBS in binding TGF- $\beta$  inducible proteins, the SBS and medea box were mutated separately and jointly as shown in Figure 2A. Competitive EMSA (Fig. 2C) showed that although the wild type sequence could completely compete for binding, the inducible interaction was reduced, although not completely, by the individual mutations at either SBS or medea box (lanes 4 and 5). The double mutation was unable to significantly compete for complex formation (lane 6). Together these data suggest that both SBS and medea box are necessary for the formation of the TGF- $\beta$ -inducible complex. Additionally, the previously characterized Smad binding element (SBE) (30) was able to efficiently compete the formation of an inducible complex (lane 7), further demonstrating the involvement of Smad protein(s) in forming a TGF- $\beta$  inducible complex.

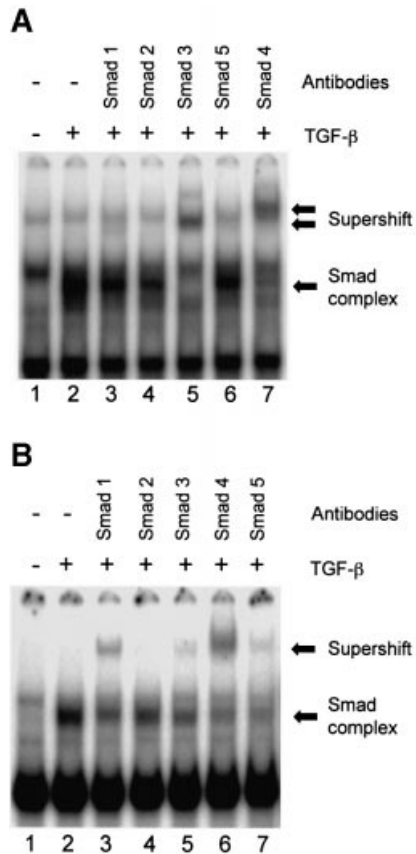
#### Both the SBS and medea box contribute to SM22 $\alpha$ gene activation

In order to determine the functional importance of the medea box and SBS, individual and joint mutations were created at the SBS and medea box in the p-162SM22luc, and luciferase activity determined following transient transfection. As shown in Figure 3A, the individual mutation at either the SBS or medea box significantly reduced promoter activity. The double mutation, however, did not further decrease reporter gene expression, suggesting that the SBS and medea box do not have a synergistic effect in response to TGF- $\beta$  induction. In order to determine whether the effects we observed were

unique to 10T1/2 cells, we performed similar transfection experiments in two other cell lines. In Balb 3T3 fibroblasts, introduction of the double mutation had a similar effect, lowering the induction of luciferase activity (Fig. 3B), but not eliminating it completely. As a second model of smooth muscle differentiation, we used the neural crest stem cell line Monc-1. In Monc-1 cells, TGF- $\beta$  induced an ~2-fold activation of SM22 $\alpha$  promoter activity, and in contrast to 10T1/2 and Balb 3T3 cells, this induction was completely abolished by combined mutations in the Smad and medea sites (Fig. 3C). Similar experiments performed using the longer -441 promoter demonstrated that it, too, was inhibited to the same degree by the double mutations (data not shown). This demonstrates that Smad sites play critical roles in regulating SM22 $\alpha$  promoter activity in multiple cell types, and are absolutely required in at least one.

#### Smad3, Smad4 and Smad1 are present in the nuclear complexes that bind to SM22 $\alpha$ promoter CAGA box

In the above gel shift experiments, we observed that TGF- $\beta$  induced complexes were formed with both SM-AB, which contains both SBS and medea box, and medea box alone probes. The formation of either of these two complexes was completely eliminated by a consensus Smad binding element (compare with Fig. 2C; data for medea box alone is not shown). To determine which Smads are present in these TGF- $\beta$ -inducible complexes, the nuclear extracts were incubated with well-characterized antibodies specific to Smads 1, 2, 3, 4 or 5 (31). We detected an obvious supershift of TGF- $\beta$  dependent binding complex with anti-Smad3 and anti-Smad4 IgG using the SM-AB probe (Fig. 4A, lanes 5 and 7). A faint supershift band could also be observed with anti-Smad1 IgG (Fig. 4A, lane 3), and a slight reduction in complex intensity was observed with anti-Smad2 IgG. As demonstrated in panel B, anti-Smad1 IgG could supershift more than half of the complex formed by medea box (Fig. 4B, lane 3). Smad4 antibody showed the same degree of supershift to the complex formed by medea box alone as that by the larger SM-AB fragment containing both medea box and SBS (Fig. 4B, lane 6). Similar to the SM-AB probe, a small decrease in band intensity was seen in the complex formed with the medea box probe and anti-Smad2 IgG. Unlike SM-AB, however,

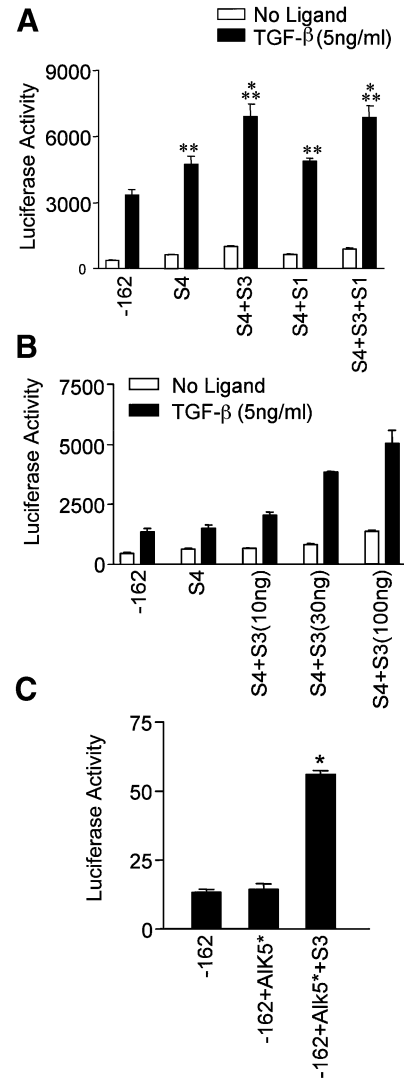


**Figure 4.** Supershift assays of proteins binding to the SBS and medea box. Nuclear extracts from 10T1/2 cells treated with vehicle (-) or TGF-β1 (+) for 30 min were incubated with radiolabeled SM-AB (A) or medea box (B). Anti-Smad antibodies were simultaneously added to the reaction. Smad complexes and antibody supershifted complexes are indicated.

anti-Smad5 IgG also caused a decrease in band intensity. We conclude that the TGF-β-inducible complex that binds SM-AB contains predominantly Smad3 and Smad4, with a small amount of Smad1, while the complex that binds the medea box contains predominantly Smad1 and Smad4 with a small contribution of Smad5 and Smad3. The presence of Smad2 in either complex cannot be completely ruled out. It is unlikely that the differences in binding seen are due to differences in expression of the various Smads, as expression levels as measured by semi-quantitative RT-PCR were roughly equal (data not shown).

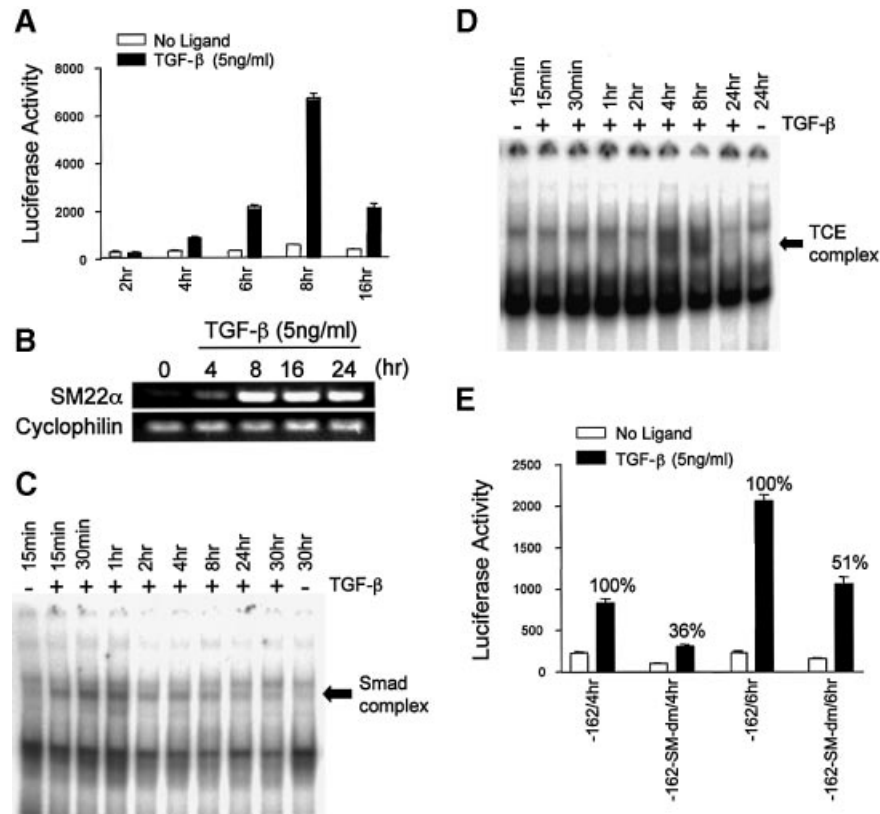
### Smad3 is critical to the TGF-β induction of SM22α gene promoter activity

In order to determine which Smads that bind to the SM22α cis elements contribute to promoter activation, we performed co-expression studies in 10T1/2 cells using Smad expression plasmids and the p-162SM22luc reporter. Smad1, Smad3 or Smad4 expression plasmids were co-transfected individually or in combination with p-162SM22luc plasmid and luciferase activities assayed. Preliminary experiments indicated that co-transfection of Smad1 or Smad3 in the absence of additional Smad4 had little effect on promoter activity (data not shown), suggesting that Smad4 is limiting in this assay. Co-transfection of Smad4 had a small but significant effect on promoter



**Figure 5.** SM22α promoter activity is Smad3 dependent. (A) p-162SM22luc plasmid was transfected into 10T1/2 cells with indicated expression constructs and luciferase activity determined following TGF-β treatment. \* $P < 0.01$  for comparison to TGF-β treated S4 or S4+S1. \*\* $P < 0.01$  for comparison to TGF-β treated -162. (B) Smad3 activates SM22α promoter activity in a dose dependent manner. Increasing amounts of Smad3 expression construct were co-transfected with Smad4 and -162luc and luciferase activity determined following TGF-β stimulation. (C) Smad3 restores the SM22α promoter activity in Smad3 null fibroblasts. p-162SM22luc plasmid was transfected alone (-162) or with activated TβR-I (-162+Alk5\*) or activated TβR-I plus Smad3 (-162+Alk5\*+S3) into Smad3 null fibroblasts and luciferase activity determined. \* $P < 0.01$  for the comparison -162+Alk5\*+S3 to -162 or -162+Alk5\*.

activity, and promoter activity was further increased by the addition of Smad3 and Smad4 together (Fig. 5A). Co-expression of Smad1 and Smad4 had no effect above that of Smad4 alone on p162SM22luc activity. In separate experiments in 293 cells, immunoblot analysis showed that all three constructs were expressed at roughly equivalent levels following transient transfection (data not shown). Similarly, Smad2, which did not convincingly demonstrate significant DNA binding in the supershift assay (Fig. 4) did not have any effect on promoter activity (data not shown). The co-transfection of Smad3, Smad1 and Smad4 did not significantly



**Figure 6.** Smad proteins are responsible for the immediate response of SM22 $\alpha$  promoter activity to TGF- $\beta$  induction. (A) Time course of SM22 $\alpha$  promoter activity. 10T1/2 cells were transfected with p-162SM22luc plasmid and treated with vehicle or TGF- $\beta$ 1 (5 ng/ml) for the indicated times and luciferase activity determined.  $P < 0.01$  for comparison of untreated to TGF- $\beta$ -treated for all time points except 2 h. (B) RT-PCR demonstrating SM22 $\alpha$  mRNA expression. 10T1/2 cells were treated with TGF- $\beta$  at the concentration and time indicated or left untreated. Total RNA was extracted and subjected to RT-PCR using primers specific to SM22 $\alpha$  or cyclophilin (as a loading control) as indicated. (C and D) Time course EMSA of Smads and TCE complex. Nuclear extracts untreated or treated with TGF- $\beta$  for the indicated times were incubated with SM-AB (SBS and medea box) (C) or TCE DNA oligo (D) and resolved as described. Complexes are indicated by arrows. (E) The effect of Smad binding on the early induction of SM22 $\alpha$  promoter activity. Wild type (-162) and mutant plasmid (-162-SM-dm) were transfected into 10T1/2 cells and luciferase activity assayed at the indicated times.  $P < 0.01$  for the difference in percent activation from 4 to 6 h.

alter promoter activity beyond that obtained in the presence of Smad3 and Smad4 together. These results suggest that Smad3 is the major R-Smad contributing to activation of the SM22 $\alpha$  promoter induced by TGF- $\beta$ . To confirm the importance of Smad3 in regulating SM22 $\alpha$  promoter activity, we performed a dose response experiment using increasing amounts of transfected Smad3. As shown in Figure 5B, the effect of Smad3 was dose dependent, further suggesting an important role for Smad3 in the positive regulation of SM22 $\alpha$  activity. To determine whether Smad3 was absolutely required for SM22 $\alpha$  promoter activity, we examined p-162SM22luc reporter activity in Smad3 null fibroblast cells. We used an activated TGF- $\beta$  receptor construct to induce reporter expression since these late passage fibroblasts appeared to lose responsiveness to exogenous ligand (data not shown), perhaps as a result of down regulation or loss of cell surface receptors. As seen in Figure 5C, constitutively active T $\beta$ R-I did not have any effect on promoter activity in Smad3 knockout cells. When co-transfected with Smad3, however, the promoter response was recovered and showed approximately a 4-fold increase in reporter activity. Taken together, these data

demonstrate that Smad3 plays a significant role in regulating SM22 $\alpha$  promoter activity, and that the effect is specific. Although Smad1 can apparently bind to the involved promoter elements, it does not appear to contribute to transcriptional activation of the promoter, nor does overexpression of Smad1 inhibit promoter activity.

#### Smad proteins are critical in the early response of SM22 $\alpha$ promoter to TGF- $\beta$ induction

Many studies have indicated that Smad proteins accumulate in the nucleus immediately following the stimulation of TGF- $\beta$ , but reporter gene analyses are typically performed after 16–48 h of induction with ligand. To analyze the dynamic regulation of the SM22 $\alpha$  gene promoter upon TGF- $\beta$  induction, we transfected the p-162SM22luc plasmid into 10T1/2 cells and performed luciferase assays at 2, 4, 6, 8 and 16 h after TGF- $\beta$  treatment. As shown in Figure 6A, luciferase activity was significantly increased as early as 4 h after TGF- $\beta$  addition. The maximal activity was achieved at 8 h of induction. After 16 h of stimulation, the promoter activity retained only 30% of the maximum. Similar results were obtained by examining

expression of induction of endogenous transcripts, although there was a more sustained expression of SM22 $\alpha$  mRNA as measured by RT-PCR in 10T1/2 cells (Fig. 6B).

In order to determine what proteins are responsible for the early response of the SM22 $\alpha$  promoter to TGF- $\beta$ , EMSAs were performed at various times following TGF- $\beta$  treatment to detect the dynamic interactions of Smads and TCE binding proteins with sequences in the SM22 $\alpha$  promoter. TCE binding proteins have been reported to be important to TGF- $\beta$  induced SM22 $\alpha$  promoter activity, but this activity was observed at 48 h of induction and the TGF- $\beta$  inducible TCE complex was only detected at 4 h after TGF- $\beta$  induction (11). As shown in Figure 6C, the Smad complex was formed as early as 15 min after TGF- $\beta$  treatment, reached the highest level at 30 min, and started to decrease after 2 h. The TCE complex, however, was not present until 4 h of induction (Fig. 6D). This suggests that *de novo* protein synthesis is not necessary for Smad activity and that the pre-existing Smads are rapidly translocated into the nucleus. Given the time course of the binding, however, the inducible protein or proteins that bind to the TCE, likely requires synthesis after TGF- $\beta$  induction.

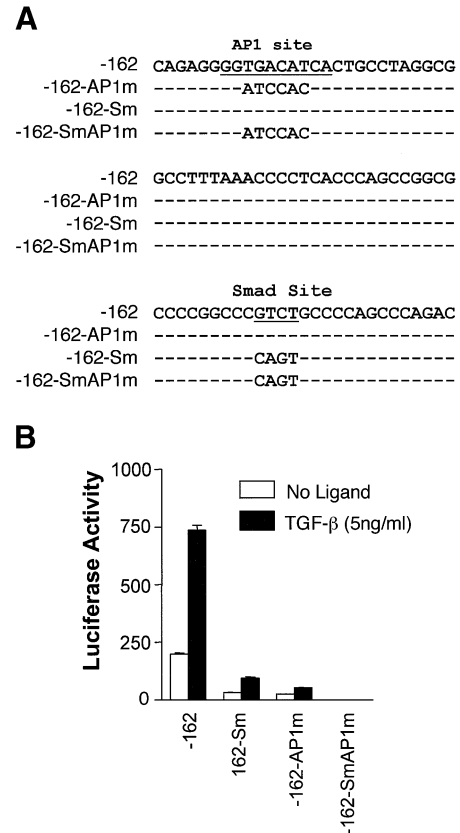
To determine further the role of Smad proteins in the early response of SM22 $\alpha$  promoter to TGF- $\beta$ , plasmid p-162SM22luc or the plasmid with double mutations at the SBS and medea box were transfected into 10T1/2 cells and luciferase activity determined after 4 or 6 h of TGF- $\beta$  induction (Fig. 6E). We found that the double mutations, which eliminated the binding of Smads to the SM22 $\alpha$  promoter, decreased the TGF- $\beta$  induced activity to only 36% of control at 4 h. After 6 h of treatment, promoter activity was reduced to 51% of control values, suggesting that the Smad complex is responsible for the initial response of SM22 $\alpha$  promoter to stimulation by TGF- $\beta$ .

#### AP-1 cis-elements contribute to SM22 $\alpha$ promoter activation by TGF- $\beta$

To determine the additional factor involved in TGF- $\beta$  induced SM22 $\alpha$  promoter activity, we examined the promoter sequence and found that a consensus AP-1 site is located ~40 bp upstream of the Smad binding sites (Fig. 7A). AP-1 components have been shown to interact with Smads (32), and can contribute to responsiveness of transcriptional induction by TGF- $\beta$  (33). In order to determine if the potential AP-1 binding site in the SM22 $\alpha$  promoter contributes to induction by TGF- $\beta$ , we mutated this site and performed reporter assays as for the Smad mutants. As shown in Figure 7B, mutation of the AP-1 consensus sequence caused a significant inhibition of activation by TGF- $\beta$ . When mutations in the AP-1 site were combined with the the Smad site, promoter activity was completely lost, suggesting a synergistic interaction between these two sites in regulating SM22 $\alpha$  promoter activity.

## DISCUSSION

SM22 $\alpha$  is one of several SMC markers and serves as an excellent model for studies of developmentally regulated, lineage-specific gene expression in SMCs. Determining the mechanism of the transcriptional regulation of these marker genes is of obvious importance in understanding the molecular mechanism of SMC differentiation. TGF- $\beta$  has been shown to up-regulate the expression of SMC differentiation markers



**Figure 7.** Effect of AP1 site mutation on SM22 $\alpha$  promoter activity. (A) Partial nucleotide sequence from SM22 $\alpha$  promoter. AP1 site and Smad sites are underlined and mutant sequences of the various constructs are shown below the wild type sequence. (B) Loss of activity with AP1 site mutation. p-162SM22luc wild type (-162) and plasmids with mutations at Smad site (-162-Sm), AP1 site (-162-AP1m), or both (-162-SmAP1m) were transfected into 10T1/2 cells. Luciferase activity was assayed 16 h after TGF- $\beta$  treatment.

through a TCE, serum response factor (SRF) box and other DNA elements (11,12,34,35). To date, the direct involvement of Smad proteins, the primary signaling intermediates downstream of the TGF- $\beta$  receptors, has not been demonstrated.

In the present study, we have found that Smads play an essential role in TGF- $\beta$  induced SM22 $\alpha$  promoter activity. Four lines of evidence support our conclusion. First, we found that SM22 $\alpha$  promoter activity is dependent on Smad signaling. Co-transfection of a dominant inhibitory Smad4 significantly reduced promoter activity induced by TGF- $\beta$ . Second, disruption of one of the five potential Smad binding sites in the SM22 $\alpha$  promoter significantly reduced TGF- $\beta$ -induced promoter activity. Third, Smad proteins physically interact with SM22 $\alpha$  promoter sequences. Smad3, Smad1 and Smad4 form a complex or complexes (perhaps with additional factors) that binds to a region containing a Smad binding site and an upstream medea box. Finally, Smad3 is necessary for SM22 $\alpha$  promoter activity since the promoter is non-functional in Smad3 null fibroblast cells and co-transfection of Smad3 restores reporter gene expression. Taken together, these data demonstrate that Smad proteins are essential for full activation of the SM22 $\alpha$  gene following TGF- $\beta$  stimulation.

It is also clear from our data that in some cells, Smad proteins do not act alone to regulate SM22 $\alpha$  expression. Although mutation of the SBS or medea box significantly reduces SM22 $\alpha$  activity, it does not eliminate it. Mutation of the upstream AP-1 site completely abolishes SM22 $\alpha$  promoter activity at early time points, and greatly diminishes it at later times (data not shown), while combined mutations at both Smad and AP-1 sites completely abrogates promoter activity. Interestingly, treatment of the cells with bone morphogenic protein 2 (BMP2) did not induce promoter activity (data not shown) even though Smad1 can bind to sites within the promoter. These results suggest that both Smads and AP-1 are important for SM22 $\alpha$  promoter regulation, and that the interaction may be specific for Smad3. The extent of Smad interaction with proteins that can bind to AP-1 consensus sites has been well demonstrated. Although Smad proteins can clearly bind to fos and jun family members (32,36,37), the exact location and role of this binding remains unclear. In some systems AP-1 and Smads can act synergistically to activate transcription (32,36,38), while in others such interactions are clearly inhibitory (18,37,39,40). Similarly, it is not clear whether Smad and AP-1 proteins physically interact on or off DNA (18). Current data suggests that both interactions are possible, and may depend in part on the nature of the DNA sequences and AP-1 family members involved (18). One explanation for our results is that Smads can still interact with AP-1 in the absence of direct Smad-DNA binding, which would allow partial activation of the promoter in the Smad/medea mutants, and that AP-1 is an essential component of the activation complex and must bind to DNA or cooperatively facilitates Smad binding. This would explain the loss of activity of the AP-1 mutant promoter constructs.

Our results further suggest that Smad proteins are important for the early activation of SM22 $\alpha$  promoter activity in response to TGF- $\beta$ . Interestingly, the Smad site is located in the first exon, just downstream from the transcriptional start site. Regulatory elements located in the 5' untranslated region (UTR) are thought to be important in early transcriptional activation (41,42). Smad proteins have been shown to bind in the 5'UTR of the c-jun promoter, which is rapidly induced following TGF- $\beta$  stimulation (33). SM22 $\alpha$  promoter activity can be detected at 4 h of TGF- $\beta$  induction and reached a maximal level at 8 h of TGF- $\beta$  stimulation. Smad proteins mediate at least part of this early induction since the Smad complex was observed as early as 15 min after TGF- $\beta$  addition, and reached a maximal level at 30 min, with a decrease after 1–2 h of induction. Functional significance of this finding is demonstrated when mutation of the Smad binding region of SM22 $\alpha$  promoter eliminates most of the early response to TGF- $\beta$  induction. We propose a model whereby Smad activation and DNA binding is responsible for immediate induction of SM22 $\alpha$  promoter activity. Continued expression relies on induction and maintenance by other cellular factors. In our hands AP-1 sites make an important contribution to this early effect, as evidenced by the incomplete loss of signaling in 10T1/2 and Balb 3T3 cells. In some cell types, for example in *Monc-1* neural crest cells and Smad3 knockout mouse embryonic fibroblasts (KO MEFs), however, loss of Smad activity completely eliminated transcriptional activation. Other factors such as SRF and YY1 have been shown to regulate SM22 $\alpha$  gene expression, and these may

contribute to sustained activity (11,34,43). The molecular basis for these differences awaits further study.

Examination of the promoter sequences of other SMC-specific genes, including SM  $\alpha$ -actin, SM myosin heavy chain and calponin, identified several potential Smad binding sites present in the 5'-flanking regions (data not shown). Similar to the SM22 $\alpha$  gene, one or two CAGA boxes are located in the 5'UTR (35,44,45). It is therefore possible that other genes involved in SMC differentiation share a similar mechanism of Smad initiation followed by maintenance by other factors during TGF- $\beta$  induced transcription.

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