Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism

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Abstract

As a member of the TGF-β superfamily, myostatin is a specific negative regulator of skeletal muscle mass. To identify the downstream components in the myostatin signal transduction pathway, we used a luciferase reporter assay to elucidate myostatin-induced activity. The myostatin-induced transcription requires the participation of regulatory Smads (Smad2/3) and Co-Smads (Smad4). Conversely, inhibitory Smad7, but not Smad6, dramatically reduces the myostatin-induced transcription. This Smad7 inhibition is enhanced by co-expression of Smurf1. We have also shown that Smad7 expression is stimulated by myostatin via the interaction between Smad2, Smad3, Smad4 and the SBE (Smad binding element) in the Smad7 promoter. These results suggest that the myostatin signal transduction pathway is regulated by Smad7 through a negative feedback mechanism.
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1. Introduction

Myostatin is a growth and differentiation factor belonging to the TGF-β superfamily, which acts as a negative regulator of skeletal muscle mass [1–3]. Deletion of myostatin in mice causes a dramatic and widespread increase in skeletal muscle mass [1,4,5]. This “double-muscled” phenomenon appears in certain breeds of cattle, such as Belgian Blue and Piedmontese. Mutations have been identified in the myostatin coding region in these breeds [6–8]. Myostatin shares many characteristics in common with other members of the TGF-β superfamily, including a signal peptide, an N-terminal propeptide domain, and a conserved pattern of cysteine residues in the C-terminal mature region [9,10]. Proteolytic processing between the propeptide domain and the C-terminal domain releases the propeptide and mature myostatin [11,12]. Like TGF-β, the mature myostatin dimer and two molecules of propeptide remain non-covalently associated after cleavage, producing a latent complex. The myostatin ligand is incapable of binding to its receptor when associated with propeptide in the latent complex [13,14]. Upon dissociation, mature myostatin binds to the ActRIIB receptor to trigger signal transduction [11,15].

The signal transduction pathway for TGF-β has been well studied in the past several years [16–19]. TGF-β and related proteins initiate cellular responses by binding to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptor is activated by type II receptor upon ligand binding, and initiates specific intracellular signals by Smad proteins [20–22]. Smad proteins are a group of molecules that function as intracellular signal transducers downstream
of the receptors of the TGF-β superfamily. Eight different Smad proteins have been identified in mammals, divided into three subfamilies based upon their function: receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads) [23–27]. R-Smads are activated by the type I receptor serine kinase through phosphorylation. This family consists of Smad1, Smad2, Smad3, Smad5, and Smad8. Smad1, Smad5, and Smad8 mediate signaling for bone morphogenetic proteins (BMP) and anti-Müllerian hormone pathways [28,29], while Smad2 and Smad3 act in the TGF-β and activin pathways [22–25]. Smad4 is the Co-Smad, which positively regulates all the above pathways [26,27]. In contrast to R-Smads and Co-Smad, I-Smads, including Smad6 and Smad7, bind to the intracellular domain of type I receptors. They compete with R-Smads for activation by the type I receptors, resulting in inhibition of TGF-β superfamily signaling [30–32]. Smad6 inhibits BMP signaling [33,34], while Smad7 inhibits TGF-β and activin signaling [35–37].

For TGF-β and activin signaling, phosphorylation and activation by the active type II and type I receptor complex causes Smad2 and Smad3 to form hetero-oligomers with Smad4 and translocate the entire complex into the nucleus. There they interact with different cellular partners, bind to DNA, and regulate transcription of various downstream response genes. Smad7 also inhibits this signaling by forming a complex with Smad2/3 and thereby interfering with the complex formation between Smad2/3 and Smad4 [35–37], and preventing further signal propagation. Transcription of Smad7 is initiated by both TGF-β and activin stimulation, providing a regulatory feedback mechanism to terminate signaling through the activated receptors [35–41].

It has been reported that ActRIIB is the type II receptor for myostatin [11,12,15]. The binding between myostatin and the ActRIIB receptor is specific. Transgenic mice over-expressing a dominant negative form of ActRIIB show increased skeletal muscle mass [12]. Binding of myostatin to the ActRIIB receptor can be inhibited by the activin-binding protein follistatin and by the myostatin propeptide [13,14], blocking the binding of myostatin to the receptor. The components involved in the downstream signal transduction of myostatin have not been defined. In the present study, we demonstrate that the signal transduction of myostatin requires the participation of Smad2/3 and Smad4, whereas Smad7 negatively regulates myostatin signaling. Conversely, our endogenous and exogenous Smad7 gene expression studies demonstrated that Smad7 expression is induced by myostatin through Smad2, Smad3, and Smad4. These results suggest that Smad7 functions as a negative feedback inhibitor for the myostatin signal pathway, similar to reports in the TGF-β signal pathway [22–25,35–41].

2. Results

2.1. p(CAGA)12 reporter construct can be induced by myostatin

The p(CAGA)12-MLP-Luc reporter construct, containing 12 copies of the consensus SBE sequence CAGA, has been widely used as a TGF-β inducible promoter system [13,14,24]. Here we used this common sequence motif to study myostatin signaling. p(CAGA)12-MLP-Lucwas transiently transfected into A204 (human rhabdomyosarcoma) cells. The transcription-based luciferase assays were performed in the presence or absence of myostatin induction. Human myostatin maximally activated the p(CAGA)12 promoter activity 12 fold over basal, with an ED50 of 10 ng/ml myostatin (Fig. 1A). The maximal induction level of myostatin is similar to that of activin A, while the ED50 for activin A is around 5 ng/ml. No TGF-β induction was observed in transfected A204 cells. These results are consistent with reports that the initiation of myostatin signaling is through ActRIIB (like activin A), while TGF-β binds to TGF-βRII because A204 is a TGF-βRII deficient cell line [11,38,39]. Fig. 1B shows that the myostatin induction of p(CAGA)12-MLP-Luc can be inhibited by the propeptide of myostatin, follistatin [12], and the soluble ActRIIBFc (the extracellular fragment of ActRIIB fused with an Fc fragment of human IgG1) [12]. Propeptide inhibits myostatin with an IC50 ~ 1 nM, almost the same as that for follistatin. The inhibition of ActRIIFc on myostatin is slightly weaker than that of follistatin, with an IC50 ~ 3 nM.

2.2. Smad proteins participate in the myostatin-induced p(CAGA)12 promoter activity

Since myostatin signaling shares the same type II receptor (ActRIIB) as activin, we investigated whether myostatin signal transduction also involves similar downstream components as activin signaling. p(CAGA)12-MLP-Luc plasmid was transiently co-transfected into A204 cells with R-Smads (Smad1, Smad2, Smad3, or Smad5) or/and the common Smad4 for the luciferase assays. As shown in Fig. 2A, Smad2, Smad3, and Smad4 can enhance the myostatin-induced luciferase activity of the reporter construct, while both Smad1 and Smad5 show no effect on the myostatin-induced promoter activity. These results are consistent with previous reports describing TGF-β and activin signaling [23–27]. In the absence of myostatin, cells co-transfected with Smad3 give two to three fold higher basal level of luciferase activity compared with the control cells with p(CAGA)12-MLP-Luc and pcDNA3 co-transfection. Smad2 or Smad4 only enhance the basal promoter activities slightly. Co-transfection of Smad2/Smad3, Smad2/Smad4, Smad3/Smad4, or Smad2/
Smad3/Smad4, all result in an increased luciferase induction compared to each respective Smad alone. This synergistic effect observed with the Smads has been previously reported [24]. Smad2 and Smad3 synergized, but less than the combinations of Smad2 and Smad4, or Smad3 and Smad4. The highest luciferase activity was observed from the co-transfection of Smad2, Smad3, and Smad4. However, when Smad1 or Smad5 was co-transfected with Smad4, no such synergistic effect was observed. It should be noted that the total amount of transfected DNA in each well was the same, compensated by vector DNA. When a truncated Smad3 mutation (a deletion causing the loss of 39 amino acids in the C-terminal region of Smad3) was co-transfected with p(CAGA)$_{12}$-MLP-Luc, the cells did not demonstrate the higher myostatin-induced luciferase activity showed by the wild-type Smad3 (Fig. 2B). The deletion construct reduces the myostatin-induced luciferase activity to 50% of the control cells with p(CAGA)$_{12}$-MLP-Luc and pcDNA3 co-transfection. The truncated Smad3 also inhibits the synergy of Smad2 and Smad4 in p(CAGA)$_{12}$ promoter activation.

The participation of Smad2 and Smad3 in the myostatin-induced p(CAGA)$_{12}$ promoter activation was further investigated by immunoblot analysis. Human hepatoma, HepG2 cells have been shown to respond to TGF-β through a Smad signaling system. We have observed that HepG2 cells can also respond to myostatin as well as activin and TGF-β. When a cell lysate from HepG2 cells was immunoblotted using an antibody against phosphorylated Smad2/3, a faint signal was detected. However, the lysate from the HepG2 cells treated with myostatin 1 h before harvest gave two prominent bands migrating around 60 kDa (Fig. 2C). These two bands correspond to the size of phosphorylated Smad2 and Smad3, in agreement with previous studies of TGF-β signal transduction [40]. The above results indicate that both phosphorylated Smad2 and phosphorylated Smad3 are produced in response by myostatin.

To further test the role of Smad4 in the myostatin-induced p(CAGA)$_{12}$ promoter activity, MDA-MB-468, a human epithelial cell line derived from a breast cancer and shown to be deficient for endogenous Smad4, was used [39]. When p(CAGA)$_{12}$-MLP-Luc was transiently transfected into MDA-MB-468 cells, no promoter activity was detected in the presence or absence of myostatin (Fig. 2D). However, co-transfection of Smad4 with p(CAGA)$_{12}$-MLP-Luc in MDA-MB-468 restored the activity of myostatin on p(CAGA)$_{12}$ promoter activation, further demonstrating that Smad4 is necessary for myostatin-induced p(CAGA)$_{12}$ promoter activity.

2.3. Smad7, but not Smad6, inhibits the myostatin-induced p(CAGA)$_{12}$ promoter activity

To determine whether the myostatin-induced p(CAGA)$_{12}$ promoter activity is regulated by the I-Smads as other members of the TGF-β superfamily, plasmids encoding either Smad6 or Smad7 were co-transfected with p(CAGA)$_{12}$-MLP-Luc into A204 cells. As shown in Fig. 3, Smad7 inhibited the myostatin-induced luciferase activity of the p(CAGA)$_{12}$ promoter dramatically. Only slight inhibition was observed in A204 cells co-transfected with Smad6 and p(CAGA)$_{12}$-MLP-Luc. Additionally, a Smad7 mutation Smad7-408stop (Smad7M, a deletion of 19 amino acids in the C-terminal end) abolished the inhibition of the myostatin-induced activity. This is consistent with previous reports in TGF-β signaling that Smad7 can interact with type I receptors, blocking the phosphorylation and activation of receptor-restricted...
Smads, and preventing further signal propagation \[35,36\]. This Smad7 inhibition activity is enhanced in the presence of Smad Ubiquitin Regulatory Factor 1 (Smurf1) \[42,43\]. When the Smurf1 expression vector was included into the co-transfection of Smad7 and p(CAGA)\textsubscript{12}-MLP-Luc, the combined action of both Smad7 and Smurf1 completely inhibited the myostatin-induced p(CAGA)\textsubscript{12} luciferase activity.

2.4. Smad7 expression is activated by myostatin-induced signal transduction

The signal transduction of both TGF-\(\beta\) and activins is inhibited by Smad7; however, the transcription of Smad7 is stimulated by both TGF-\(\beta\) and activins, providing a regulatory feedback mechanism to terminate signaling through the activated receptors \[22,23\]. To find out whether the expression of Smad7 is also regulated by myostatin, we subcloned the mouse Smad7 promoter region (from \(-613\) to \(+112\)), containing all required elements for the maximal expression of Smad7 according to Brodin et al. \[41\]. The promoter was subcloned into pGL3-basic vector and fused with a luciferase reporter gene. The resulting plasmid was named pGL3S7P.

When pGL3S7P was transiently transfected into A204 cells, a two to three fold increase in luciferase activity was observed in response to myostatin or activin A (Fig. 4A). No stimulation was observed with TGF-\(\beta\) because A204 cells lack the TGF-\(\beta\)RII receptor. The level of induction of the Smad7 promoter by myostatin or activin A was somewhat lower in comparison with similar studies for the Smad7 promoter in other systems \[38\textendash}41\]. One possible reason is that a relatively higher basal level of Smad7 transcription was observed in A204 cells (Fig. 4A). The assays were then performed in HepG2 cells, a cell line regularly used for TGF-\(\beta\)-induced transcription studies \[38\textendash}41\]. A much higher (5\textendash}6 fold) level of luciferase activity (Fig. 4B) was observed in HepG2 cells in response to myostatin,
activin A, and TGF-β, consistent with previous studies [39].

To further demonstrate that Smad7 expression is activated by myostatin-induced signaling, the transcription profile of endogenous Smad7 in myostatin-treated HepG2 cells was examined using Northern blot analysis. Shown in Fig. 5, a basal level of Smad7 transcripts was detected in untreated HepG2 cells. Myostatin induction (20 ng/ml) increased Smad7 transcripts, peaking around 1.5 h post-induction, then decreasing to basal levels at 8 h post-induction.

2.5. Mutation of the SBE in the Smad7 promoter completely abolished myostatin-induced luciferase activity

Previous reports have indicated that a consensus sequence in the Smad7 promoter region, the Smad binding element (SBE), is required for Smad2/3 initiated transcription in TGF-β signaling [39–41]. To determine whether this is also the case in myostatin-induced Smad7 promoter activity, the SBE site was mutated by substitution of two nucleotides as shown in Fig. 6A. The mutated promoter (pGL3S7SBE) was not able to respond to TGF-β or myostatin in HepG2 cells (Fig. 6B). In addition, the SBE mutant Smad7 promoter showed higher basal level than that of the wild-type Smad7 promoter. This indicates that the SBE is required for the myostatin-induced transcription of Smad7. Co-transfection of pGL3S7P with Smad2, Smad3, or Smad4 increased the level of Smad7 transcription, while Smad1 and Smad5 did not affect the Smad7 promoter activity (data not shown).

2.6. Characterization of the interaction between the Smads and the SBE in the Smad7 promoter

To examine the myostatin-induced SBE binding activity in the presence or absence of myostatin, a biotinylated double-stranded SBE DNA fragment was used as a probe in EMSA (Fig. 7). A myostatin-inducible complex was observed 30 min after the addition of myostatin (Fig. 7, lane 3) similar to the TGF-β induction studies [40], this complex was also visible in cells without induction (Fig. 7, lane 2) at a lower level.
The short interval suggests preexisting factors. This myostatin-inducible complex was competed out in the presence of excess unlabeled double-stranded SBE oligonucleotides, but was not competed out by the unlabeled mutant SBE oligonucleotides. To examine the composition of the complex, nuclear extracts were incubated with specific antiserum against phosphorylated Smad2/Smad3 (Fig. 7, lane 7), or phosphorylated Smad1 (Fig. 7, lane 6) as indicated. Supershift bands were detected with the antiserum against phosphorylated Smad2/Smad3, but not the antiserum against phosphorylated Smad1. These results show the physical interaction between the SBE and the Smad proteins. An additional band was observed in the EMSAs, which appears unaltered in the presence or absence of myostatin. It was not competed by excess unlabeled double-stranded SBE oligonucleotides, nor was it supershifted by the antiserum against phosphorylated Smad2/3. Therefore, we conclude that it is nonspecific binding (NS) from the nuclear extracts. The gel shift data complements the reporter gene assay, demonstrating that the Smad7 promoter is regulated by myostatin through the Smad2 and Smad3 proteins. The fact that the antiserum against phosphorylated Smad2/Smad3 is able to supershift the complex indicates that the phosphorylation of Smad proteins is required for myostatin signaling. This is consistent with previous reports for both TGF-β and activin [32,39,40].

3. Discussion

Myostatin binds to the type II receptor ActRIIB to initiate signaling. The signal transduction of myostatin can be blocked by its own propeptide, follistatin and the soluble ActRIIB receptor as shown in Fig. 1B and previous reports [12–14]. As a member of the TGF-β superfamily, we speculated that myostatin shares the same signaling pathway as other members in this family. To investigate this possibility, we used a cell based reporter assay system to identify the downstream components involved in the signal transduction. When p(CAGA)12 promoter construct was co-transfected with the four different R-Smads, only Smad2 and Smad3 enhanced the myostatin-induced transcription activity, while Smad1 and Smad5 did not affect myostatin-induced signaling. These results are consistent with previous reports [38–40], in which Smad2 and Smad3 are involved in TGF-β and activin signaling, whereas Smad1 and Smad5 participate in the BMP signaling. Since myostatin binds the same type II receptor as activins [12], it is reasonable to consider that myostatin utilizes the same Smad proteins as activin A. When Smad2 or Smad3 was co-transfected with Smad4, synergistic effects were observed as reported previously. The highest synergy was observed with the co-transfection of Smad2, Smad3, and Smad4. To examine whether Smad3 is essential for the myostatin-induced transcription activity, a truncated Smad3, reported to inhibit TGF-β signal transduction, was constructed. When the truncated Smad3 was co-transfected with p(CAGA)12-MPL-Luc, the myostatin-induced transcription activity was reduced by 50%, indicating a dominant

![HepG2](image)

Fig. 5. The endogenous Smad7 gene expression in HepG2 cells is regulated by myostatin. Total RNA (10 µg) from HepG2 cells stimulated with myostatin (20 ng/ml) for various times as indicated was probed with HRP-labeled Smad7 and GAPDH.

![Sequence of the wild-type and mutant SBE](image)

A. Sequence of the wild-type and mutant SBE

Wild type: ACAGGGTGTCTAGACCACG
Mutant: ACAGGGTGTCTAAACACG

B. Fold over basal

![Graph](image)

Fig. 6. The SBE in the Smad7 promoter region is required for the myostatin-mediated transcription. (A) The wild-type and mutant SBE sequence. (B) HepG2 cells were transiently transfected with 1 µg/well of the wild-type Smad7 reporter construct (pGL3S7P) or a mutant Smad7 reporter construct (pGL3S7SBE). Twenty-four hours post-transfection, cells were induced by myostatin (20 ng/ml), activin A (10 ng/ml) or TGF-β (5 ng/ml). The results are from at least three independent experiments, each carried out in triplicate.
negative effect for the mutant Smad3. This indicates that Smad3 plays a key role in myostatin-induced promoter activity. Phosphorylated Smad2 and Smad3 proteins are up-regulated in response to myostatin stimulation in immunoblot analysis. This is the direct evidence showing myostatin signal transduction mediated through activation of Smad2 and Smad3. When p(CAGA)12-MLP-Luc was transiently transfected into MDA-MB-468 cells, a human epithelial cell line deficient for Smad4, no myostatin-induced p(CAGA)12 promoter activity was observed. However, co-transfection of Smad4 with p(CAGA)12-MLP-Luc into MDA-MB-468 cells restored the activity of myostatin on the promoter activity. This indicates that Smad4 is necessary for the myostatin-induced promoter activity.

Inhibitory Smad6 and Smad7 are able to block TGF-β superfamily signaling [30–33]. To explore whether these I-Smads are able to block the myostatin signaling, Smad6 or Smad7 expression vectors were co-transfected with p(CAGA)12-MLP-Luc into A204 cells for luciferase assays. The results indicate that Smad7, but not Smad6, can reduce the myostatin-induced transcription activity dramatically. This inhibition required full length Smad7 protein. A truncated Smad7 with a deletion of 19 amino acids in its C-terminus abolished its activity. When Smad7 was co-transfected with Smurf1, the combined function of Smad7 and Smurf1 inhibited the myostatin-induced p(CAGA)12 promoter activity completely. This result is consistent with reports in TGF-β signal transduction, indicating that Smurf1 cooperates with Smad7 for negative regulation of TGF-β signaling.

To explore whether the inhibition of myostatin signaling by Smad7 is through a negative feedback loop, a 725 base pair of mouse Smad7 promoter was cloned and fused with luciferase reporter gene. This Smad7 promoter region (from −C255 to +613) contains all the required elements for the maximal expression of Smad7 [41]. As expected, the mouse Smad7 promoter is activated by myostatin. The activation of myostatin on the transcription of the Smad7 promoter is enhanced by co-transfection of Smad2/3 and Smad4 as indicated in the transcription of p(CAGA)12 promoter, but not by Smad1 and Smad5. This is further confirmed by the rapid increase in endogenous Smad7 mRNA level, peaking at 1.5 h post-induction, and the decrease to basal level 8 h post-induction. There exists an SBE site
in the Smad7 promoter, which is recognized as a Smad binding site for TGF-β signaling [39–41]. To demonstrate whether the SBE in the Smad7 promoter is required for the myostatin-induced transcription, we mutated this SBE site by site-directed mutagenesis. The mutant Smad7 promoter lost its response to myostatin stimulation, although it gave a slightly higher basal promoter activity. The higher basal promoter activity of the mutated Smad7 promoter is not surprising, since the Smad7 promoter is activated by many other transcription factors, such as AP-1 and Sp1 [40,41].

The interaction between the SBE of the Smad7 promoter and Smad2/3 was showed by EMSAs. The SBE in the Smad7 promoter forms a complex with A204 nuclear extracts; this complex was increased with induction by myostatin. The interaction could be competed by an excess of unlabeled wild-type SBE oligonucleotides but not by mutant SBE oligonucleotides. This is the direct physical interaction between the Smad7 promoter and the Smad2/Smad3. Furthermore, the complex was “supershifted” by a specific antiserum against phosphorylated Smad2/3, but not by an antiserum against phosphorylated Smad1. These results indicate that the complexes are formed by the phosphorylated Smad2/3 specifically. It indicates that the role of the phosphorylation of Smad proteins in myostatin signaling is a common event in the signal transduction pathway for the members in the TGF-β superfamily.

Our results demonstrate that the same Smad proteins are involved in myostatin signal transduction as that of TGF-β and activin. This indicates the close relationship between myostatin, TGF-β, and activin. Our data demonstrating the functional components of a signaling pathway similar to that employed for activin also explains previous reports involving known activin inhibitors [11,12]. Over-expression of the known activin antagonists result in a dramatic increase in skeletal muscle mass, which would be predicted based upon the signal transduction described in this work. These findings allow us to utilize the information from both the TGF-β and activin signal transduction pathways for understanding myostatin signaling. It remains to be addressed how myostatin functions specifically in skeletal muscle. It is highly likely that there are specific regulator(s) to direct the muscle specific response to myostatin.

In summary, our data indicate that myostatin signaling is through the common Smad signal pathway. Two different R-Smads (Smad2 and Smad3) and the Co-Smad (Smad4) are responsible for myostatin signaling. The signal transduction pathway of myostatin is inhibited by Smad7 and Smurf1. Conversely, the expression of Smad7 is induced by myostatin. We show that Smad3 and Smad2 are involved in myostatin-induced Smad7 promoter binding activity. All combined data suggest that Smad7 provides a negative regulatory feedback loop mechanism to terminate myostatin signaling.

4. Materials and methods

4.1. Cells and culture conditions

The human rhabdomyosarcoma cell line, A204 (ATCC HTB-82), was cultured in McCoys 5A medium (Invitrogen) supplemented with 2 mM glutamine, and 10% fetal calf serum. The human hepatoma cell line, HepG2, was cultured in Dulbecco’s modified Eagle’s medium/F-12 medium with 4 mM L-glutamine, including 10% fetal calf serum. The human breast adenocarcinoma cell line, MDA-MB-468 (HTB132), was grown in Leibovitz’s L-15 medium with 2 mM L-glutamine and 10% fetal calf serum. All three cell lines were maintained at 37 °C, 5% CO2 in a humidified atmosphere.

4.2. Construction of expression vectors encoding different Smads and Smurf1 expression vectors

The expression vectors of Smad1 and Smad5, pCMVSmad1 and pcDNASmad5, were kind gifts from Dr. Di Chen in University of Texas at San Antonio. The expression vectors for Smad6 was a gift from Dr. Masa Kawabata at National Sanatorium, Minamikyusyu Hospital in Japan and that for Smad7 was a gift from Dr. Serhiy Souchelnytskyi in the Ludwig Institute for Cancer Research at Uppsala. The expression vector for Smurf1, pcDNASmurf1, was a kind gift from Dr. Kohei Miyazono of the Cancer Institute of the Japanese Foundation for Cancer Research.

Three pairs of primers were designed and obtained from Integrated DNA Technologies, Inc. (Corvalle, IA 52241) for subcloning of Smad2, Smad3, and Smad4. They are 5’ TTCTCGAAGGAGCTTACGCGATGTGTC3’ and 5’ TTCTCGAGCTTACATGTGCGGATTGTTACCTTTTGG3’ and 5’ TTCTCGAGCAGTTAAGACGGAAGTCTT3’ for Smad2, 5’ TTCTCCAGAAGGCAAGCCATCCTGCTCACC3’ and 5’ TTCTCGAGTCAGGTCCTCCGATACACC3’ for Smad3, and 5’ AGGTCATCTCGTTCACCCAGTGC3’ for Smad4. Four-week-old mice were obtained from Charles River Lab. (Maryland). Total RNA from both muscle and livers of mice were isolated by the TRIzol reagent (Invitrogen, Carlsbad, CA) and purified by the RNase kit from Qiagen. cDNAs were synthesized using the SuperScript kit from Invitrogen. RT-PCR was performed according to the protocol provided by the manufacturer. The PCR products with the expected sizes (1471 bp for Smad2, 1669 bp for Smad3, and 2121 bp for Smad4) were obtained and subcloned into the expression vector pcDNA3; resulting plasmids were confirmed by DNA sequence analysis and were named as pcDNASmad2, pcDNASmad3, and pcDNASmad4, respectively. The expression vectors for both the truncated Smad3 (deletion of last 39 amino acids) and the truncated Smad7 (deletion of last 19 amino acids) were generated by utilizing the QuickChange™ site-directed
mutagenesis kit from Stratagene (La Jolla, CA 92037). The resulting plasmids were confirmed by DNA sequence analysis, and were named as pcDNASmad3M and pcDNASmad7M, respectively.

4.3. Isolation of the mouse Smad7 promoter

Amplification of the mouse Smad7 promoter region generated a 725 nucleotide fragment from nucleotide –613 to +112 of the BamHI–XhoI fragment according to Brodin et al. [41]. The primers used are 5′TTTGATCCGCCGCCAGGCCCTTA3′ and 5′AAACTCGAGTCTTCTTGTGCCGGCCT3′ containing BamHI and XhoI restriction sites, respectively. Mouse genomic DNA was isolated from four-week-old mice using the Wizard genomic DNA purification kit from Promega (Madison, WI 53711). The desired PCR products were obtained with a standard PCR method (Invitrogen, Carlsbad, CA) and 20 ng of genomic DNA as template. The cycling conditions were as follows: 94 °C, 2 min, 1 cycle; 94 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 2 min, 35 cycles. The resulting 725 nucleotide PCR products were subcloned into BamHI and XhoI sites of pGL3-Basic vector resulting in pGL3S7P, confirmed by DNA sequence analysis. The SBE of the pGL3S7P was mutated by a PCR-based mega primer site-directed mutagenesis method [44] using primers 5′ GTACACGGGCCCTTATAGACACCCTGTCGC3′ and 5′AAACTCGAGTCTTCTTGTGCCGGCCT3′ containing BamHI and XhoI restriction sites, respectively. Human myostatin was produced from CHO cells suspended in 400 μl/dish of ice-cold buffer A (20 mM HEPES–KOH, pH 7.9, 0.5 mM dithiothreitol (DTT), 1.5 mM MgCl2, 0.8 M KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were allowed to swell on ice for 10 min and then lysed by 15 strokes of a Dounce all-glass homogenizer. Nuclei were pelleted by centrifugation and resuspended in 50 μl/dish of ice-cold buffer C (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, and 1 μg/ml

4.4. Luciferase assays

The reporter vector, p(CAGA)12-MLP-Luc was a gift from Dr. JM Gauthier at Laboratoire Glaxo Wellcome in France. Cells for assays were split into 24 well plates the day before transfection with about 95% confluence in suitable media. Cells were transiently transfected by either the reporter plasmid alone or together with expression vector(s) using the Lipofectamine™ 2000 (Invitrogen). Total amount of transfected DNA was the same in each experiment. Following the transfection, cells were incubated in 5% CO2 at 37 °C, the desired stimulators or inhibitors were added 24 h post-transfection. Activin A, TGF-β, and soluble ActRIIB were purchased form R and D Systems (Minneapolis, MN). Human myostatin was produced from CHO cells and purified by HPLC. Cell lysis and luciferase assays were carried out 6 h after induction using the luciferase kit (PE Applied Biosystem) as described in the manufacturer’s instructions. All transient expression experiments were done in triplicate. Luciferase activity values were normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector (pCR3lacZ; Invitrogen).

4.5. Northern blot analysis

Total RNA was extracted from the HepG2 cells using an RNaseay Mini Kit (Qiagen). Ten micrograms of total RNA per sample was electrophoresed in a formaldehyde-agarose gel and blotted onto a nylon membrane. Smad7 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) DNA fragments were isolated as previously reported [35], and labeled using North2South Direct HRP Labeling (Pierce). Hybridization and washing were performed according to the manufacturer’s instruction.

4.6. Immunoblot analysis

HepG2 cells were grown to confluence, followed by serum starvation for 24 h. Myostatin induction was for 1 h (20 ng/ml), and cell lysates from induced and control cells were prepared. Ten micrograms of each cell lysate was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA for 2 h and then probed with antibodies against phosphorylated Smad2/3 (Goat polyclonal IgG; Santa Cruz Biotecno) for 1 h at room temperature. Blots were washed three times with wash buffer (PBS, 0.05% Tween 20 (Sigma)). The membranes were incubated with the horseradish peroxidase-labeled rabbit anti-goat secondary antibody (1:5000; Pierce) for 1 h at room temperature, followed by four washes of the above wash buffer. Blots were developed with the Amersham ECL detection kit.

4.7. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from control and myostatin-treated A204 cells. The medium was changed from 10–0.5% FCS overnight, and subsequently the cells treated with 20 ng/ml myostatin. Cells were harvested 30 min after treatment and processed according to the protocol of Dennler et al. [24] with minor modifications. Briefly, confluent cells from four dishes (100 mm2) were washed with phosphate-buffered saline (PBS) and scraped. After another washing, cells were suspended in 400 μl/dish of ice-cold buffer A (20 mM HEPES–KOH, pH 7.9, 0.5 mM dithiothreitol (DTT), 1.5 mM MgCl2, 0.8 M KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were allowed to swell on ice for 10 min and then lysed by 15 strokes of a Dounce all-glass homogenizer. Nuclei were pelleted by centrifugation and resuspended in 50 μl/dish of ice-cold buffer C (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, and 1 μg/ml
peptatin). The nuclear membranes were lysed by 15 strokes of a Dounce all-glass homogenizer. The resulting suspension was stirred for 30 min at 4 °C and pelleted by centrifugation at 12,000 rpm at 4 °C for 30 min. The clear supernatant was aliquoted and frozen at −80 °C. Biotinylated oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA 52241). LightShift™ chemiluminescent EMSA kit from PIERCE (Rockford, IL 61105) was used for the binding reactions. Binding reactions containing 10 µg of nuclear extracts and 2 fmol of labeled oligonucleotides and were performed for 20 min at room temperature in 20 µl of binding buffer as supplied by the manufacturer. Protein–DNA complexes were resolved in 6% polyacrylamide gels containing 0.5× TBE. For supershift analysis, Smad-specific antisera against phosphorylated Smad2/3, and phosphorylated Smad1 (Santa Cruz Biotechnology, CA), were added to the nuclear extracts in binding buffer and incubated for 10 min at 4 °C, before addition of the biotinylated oligonucleotides.

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References

[33] Smad7 and Smad6 cooperate with Smad1 to inhibit transforming growth factor beta-induced DNA sequence and expression in normal and transformed cells. Nature 1995;316:701–5.