SOCS-3 Induces Myoblast Differentiation*

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Myoblast differentiation is characterized by a sequence of events that includes an increase in insulin-like growth factor (IGF)-I and contractile gene expression. The increase in IGF-I expression activates cell signaling mechanisms that participate in the differentiation process. One potential contributor is the SOCS-3 (suppressor of cytokine signaling-3) gene, which regulates signaling mechanisms and may be sensitive to changes in IGF-I concentrations. For the first time, the role of SOCS-3 is investigated in myoblast differentiation. SOCS-3 mRNA levels and SOCS-3 transcriptional activity increase during myoblast differentiation. SOCS-3 gene expression is induced, at least in part, by activation of the IGF-I receptor during myoblast differentiation. Overexpression of SOCS-3 cDNA significantly increased transcriptional activation of the 2.0-kb skeletal α-actin promoter in differentiating C2C12 myoblasts. In addition, overexpression of SOCS-3 specifically increased serum response factor-driven transcriptional activity but had no effect on nuclear-factor-activated T cell-driven transcriptional activity. SOCS-3 overexpression induced skeletal α-actin transcription in a myoblast cell line that cannot respond to endogenous IGF-I, indicating that SOCS-3 can contribute to the myoblast differentiation process in the absence of IGF-I. These data suggest that IGF-I induces myoblast differentiation, in part, by increasing SOCS-3 expression.

Myogenesis is a highly regulated process in striated muscle that is controlled by a complex interaction of numerous intracellular signaling mechanisms (1). Myogenesis is composed of multiple fundamentally distinct stages, with myoblast differentiation allowing for the increase in contractile gene expression and cell fusion inducing the appearance of multinucleated myotubes (2). Myoblasts utilize this process to ultimately form mature muscle during development or to repair damaged muscle fibers.

In culture, myoblast differentiation occurs in what is defined as growth factor withdrawal; although the terminology is widely used, it suggests that little to no growth factors are exposed to the myoblasts during the differentiation process. However, differentiating myoblasts increase autocrine/paracrine production of insulin-like growth factor (IGF)1 proteins (3). Indeed, during the differentiation process myoblasts increase the expression of IGF-I mRNA by 6–10-fold and IGF-II mRNA by 25-fold (3, 4). The increased concentration of IGF-I is thought to induce specific signaling mechanisms that are necessary for the induction of the differentiation process. Although the production of IGF-I is much lower by the myoblasts than IGF-II, the role of IGF-I still appears to be critical for the differentiation process (5). Unfortunately, the mechanisms by which IGF-I induce myoblast differentiation remain undefined.

A recently described family of proteins, the suppressor of cytokine signaling (SOCS) family, acts in a negative feedback loop to regulate cytokine or growth factor signaling (6, 7). The SOCS family consists of eight family members, with each member having a SH2 domain and a conserved homologous C-terminal region designated as a SOCS box (7). The SH2 domain allows for interactions with key regulatory tyrosine regions in the activation loop of other signaling proteins (7). For example, SOCS-3 has been implicated as having the ability to regulate insulin signaling in cultured adipocytes (8). Because insulin and IGF-I share many signaling properties, it is possible that SOCS-3 may regulate IGF-I signaling as well. Indeed, Dey et al. (9) described an interaction between SOCS-3 and the IGF-I receptor in embryonic kidney 293 cells, and because the activation of the IGF-I receptor is critical for the induction of myoblast differentiation (10), it is possible that SOCS-3 is critical for myoblast differentiation. SOCS-3 is expressed in skeletal muscle (11, 12); however, a mechanistic role for the expression of SOCS-3 in skeletal muscle has yet to be defined.

Therefore, the purpose of this investigation was to determine whether SOCS-3 expression contributed to myoblast differentiation. The following hypotheses were tested: 1) IGF-I production by the differentiating myoblasts will increase SOCS-3 expression and 2) SOCS-3 expression will alter the transcriptional activity of various contractile proteins during myoblast differentiation. For the first time, a mechanistic role for SOCS-3 has been defined in skeletal muscle with respect to myoblast differentiation.

EXPERIMENTAL PROCEDURES

C2C12 Cell Culture Conditions

All of the cell culture experiments were performed using C2C12 mouse myoblasts (an immortalized mouse myoblast cell line; ATCC, Rockville, MD) that were maintained at a subconfluent density at 37 °C in 10% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 20% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin-antibiotic (Invitrogen). Differentiation was induced by transferring the myoblasts to Dulbecco’s modified Eagle’s medium containing 2% horse serum and 1% penicillin-streptomycin antibiotic.

C2BP5 Cell Culture Conditions

C2BP5 cells are C2 cells that are stably expressing mouse IGFBP5 cDNA, rendering the cells nonresponsive to endogenous IGF-I (13). The protein; PBS, phosphate-buffered saline; MCK, muscle creatine kinase; GFP, green fluorescent protein; EF, elongation factor; SRE, serum response element; P13-K, phosphatidylinositol 3-kinase; RT, reverse transcription.
cells were cultured according to previously described conditions (13) that mimicked the conditions described above except for the inclusion of G418 (400 μg/ml). C2B25 cells were induced to differentiate as described above; however, as previously described by James et al. (13), the cells will undergo minimal differentiation without the inclusion of exogenous R3-IGF-I. R3-IGF-I is an IGF-I analog that mimics the actions of IGF-I, but lacks the IGFBP binding region, and therefore can induce the C2B25 myoblasts to differentiate (13). Therefore, when it was necessary to induce differentiation, 2 nM R3-IGF-I was included in the differentiation medium as previously described (13).

**Transient Transfection of Myotubes**

Transient transfections were performed with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s directions and previously described methods (14). The mouse SOCS-3 promoter (15), pEF-driven SOCS-3 DNA expression construct (16), human skeletal α-actin promoter construct (17), −99 skeletal α-actin promoter (18), and mouse myosin heavy chain Ix and Ib promoters (19) were utilized as previously described. In addition, the SRF and NFAT “sensor” constructs were employed as previously described (14, 20). All of the SRF and NFAT constructs contain four multimerized consensus cis-binding elements for either the SRF or NFAT transcription factors, respectively. The pGL3 and pGL2 plasmids were purchased from Promega (Madison, WI). Briefly, transfections were carried out on 80% confluent myoblasts in 24-well tissue culture plates with a total of 0.5 μg of DNA (in 100 μl of serum-free Eagle’s medium). All of the assays were performed using equimolar ratios of DNA, with the total amount of DNA-transfection adjusted using the parent vector of SOCS-3 to ensure that each well received 0.5 μg of cDNA as previously described (14, 20). After completion of the transfection, the culture medium was changed to differentiation medium. Each experimental condition was performed three or four times in three independent experiments.

**Cell Lysis and Luciferase Measurements**

Cell lysis and luciferase measurements were performed utilizing the luciferase assay kit (Promega, Madison, WI) as previously described (20). At the prescribed time point, the cells were gently washed twice with PBS, cell lysis buffer was then added, and the plates were gently rocked for 15 min at 4 °C. The homogenates were then transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for 1 min. The supernatant was retained and stored at −80 °C. All of the luciferase measurements were normalized as previously described (21). The data are presented as the fold increase in promoter activity over the “promoterless” control plasmids transfected under the identical conditions. For all conditions at least three independent experiments were performed, each consisting of three or four separate measurements.

**MCK-SOCS-3-GFP Construct**

To determine whether SOCS-3 was enhancing the differentiation process, the SOCS-3 DNA was subcloned into a vector containing the skeletal muscle-specific MCK promoter (22). More specifically, the SOCS-3 gene was ligated out of the pEF plasmid using XbaI and subsequently cloned into the XbaI site of the previously described MCK-green fluorescent protein (GFP) plasmid (23). Using an inverse PCR strategy, primers were generated to allow for removal of the stop codon on the GFP sequence, which allowed for the creation of a N-terminal SOCS-3-GFP fusion construct. Briefly, the primers (forward, 5′-CGA AGA TCT GTG ACC ACC ACG AAG-3′; reverse, 5′-TAG AGA TTC GAT TAG TTC ATC-3′) were designed to remove the sequence containing the stop codon and ensure that the GFP and SOCS-3 sequence were in frame. In the primers, the allowed for the introduction of a BglII site (underlined letters). PCR amplification using 5 units of Pfu DNA polymerase (Invitrogen), 50 ng of template cDNA, 0.5 mM dNTPs (Invitrogen), and 3 mM MgCl2. Amplifications were performed in an Eppendorf Mastercycler with an initial denaturing step of 94 °C for 2 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 58 °C, and 2 min at 72 °C as previously described (27). The final cycle ended with 10 min at 72 °C. For IGF-I amplification, an initial denaturing step was performed at 94 °C for 2 min, followed by 35 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C as previously described (28). The final cycle ended with 7 min at 72 °C. For myogenin or skeletal α-actin amplifications, an initial denaturing step was performed at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The final cycle ended with 10 min at 72 °C. The PCR products were then separated on a 2% agarose gel and stained with ethidium bromide. The gels were scanned, and signal quantification was performed with ImageQuant software. The signal determined for each target was subsequently normalized to the signal for the 18 S target.

**Immunoblotting**

To determine STAT3 content and phosphorylation status, cells were plated to obtain ~75–80% confluency. To obtain protein from cells in the myoblast state, the medium was aspirated 24 h after the initial plating, and the cells were then washed twice with sterile PBS and lysed as described below. To obtain cellular protein from differentiating myoblasts (48 h) or myotubes (96 h), the medium was replaced with 2% horse serum and allowed to incubate until designated time points when the plates were removed, the medium was aspirated, and the cells were

**RNA Isolation**

RNA was isolated according to previously described methodology (25, 26). Briefly, the cells were grown to 80% confluent on 35-mm plates. The cells were induced to differentiate by washing cells twice with sterile PBS and subsequently exposed to 2% horse serum. At the prescribed time point, the cells were gently washed twice with PBS. One ml of TRIzol was added to the plate, and RNA was isolated accordingly. RNA concentrations were determined by measuring the optical density at 260 nm. The quality of the RNA was examined visually by ethidium bromide staining of 18 and 28 S staining of gel electrophoresed RNA. The RNA was subsequently stored at −80 °C for later use.

**RT-PCR**

**Reverse Transcription—One microgram of total RNA was reverse transcribed.** Briefly, the RNA was incubated with Super Script II reverse transcriptase (Invitrogen), mixed oligo(dT), and random decamers (Ambion, Austin, TX) in a 25-μl reaction at 42 °C for 50 min. The reaction was inactivated by incubation at 70 °C for 15 min. The samples were subsequently stored for 4 °C for later use.

**PCR—All methods have been previously described.** Briefly, a semi-quantitative form of PCR was employed using 18 S as a standard (Ambion) for each reaction. The following primer sequences were utilized (5′ → 3′): SOCS-3 sense (GGA CGA GGC CCA CTG CTT CAC), SOCS-3 antisense (TAC TGG TCC AGG AAC TCC CGA), IGF-I sense (ACA TCT ACC ACT TCC GTT CAT TTC CTG TG), IGF-I antisense (CCG TCT ACT TGG CTT CAA ATG TAC TCT C), myogenin sense (ACT CTT TTA CGT CCA TCG TG), myogenin antisense (GAC GAG ACC ACT TAA AA), skeletal α-actin sense (GCC CAA GTA CTC AGT GTG GA), and skeletal α-actin antisense (CAC GAT TGT CGA TTT GCG TG) as previously described (27, 28). All of the primers were purchased from Invitrogen. The SOCS-3 primers produced a product size of 220 bp and were designed based on GenBank™ accession number U83328. The IGF-I primers produced a product size of 514 bp and were designed based on GenBank™ accession number M14156. The myogenin primers produced a product size of 175 bp and were designed based on GenBank™ accession number NM_031189. The skeletal α-actin primers produced a product size of 184 bp and were designed based on GenBank™ accession number M12233.

**Microinjection of pCMV-MCK-GFP and pCMV-MCK-SOCS-3-GFP Transfection Reaction**

All microinjections of either pCMV-MCK-GFP or pCMV-MCK-SOCS-3-GFP transfection reactions were mixed with 12.5 μl of AccuPrime Super Mix II (Invitrogen), 0.5 μM 18 S primer/competimer mix, and 0.2 μM target primer mix in final 25-μl volume. However, for some reactions 18 S amplifications were done as separate but parallel reactions using the identical cycling conditions. SOCS-3 amplifications were performed in an Eppendorf Mastercycler with an initial denaturing step of 94 °C for 2 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 58 °C, and 2 min at 72 °C as previously described (27). The final cycle ended with 10 min at 72 °C. For IGF-I amplification, an initial denaturing step was performed at 94 °C for 2 min, followed by 35 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C as previously described (28). The final cycle ended with 7 min at 72 °C. For myogenin or skeletal α-actin amplifications, an initial denaturing step was performed at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The final cycle ended with 10 min at 72 °C. The PCR products were then separated on a 2% agarose gel and stained with ethidium bromide. The gels were scanned, and signal quantification was performed with ImageQuant software. The signal determined for each target was subsequently normalized to the signal for the 18 S target.
SOCS-3 Increases SRF Transcriptional Activity

SOCS-3 mRNA expression and SOCS-3 promoter activity increases as the C2C12 myoblast progresses through the differentiation process. A and B, SOCS-3 mRNA is increased after 72 h of differentiation when compared with levels found in the myoblast. Total RNA was isolated from C2C12 myoblasts and after 24–72 h of differentiation on 35-mm plates. RT-PCR was performed to detect SOCS-3, myogenin or 18S transcripts. These results were achieved in three independent experiments and quantified in B. * indicates a value statistically different from the myoblast group (p < 0.05). C, 2.0-kb SOCS-3 promoter activity increases during myoblast differentiation. C2C12 myoblasts were transiently transfected with the 2.0-kb mouse SOCS-3 promoter, and the cells were either maintained in the myoblast state or induced to differentiate for either 24, 48, or 72 h. At the defined time point, the luciferase activity was subsequently quantified as previously described (21). The data are expressed as the fold increases in promoter activity over the promoterless control plasmid (pGL3). These results are from three independent experiments. * and # indicate values statistically different from the myoblast group (p < 0.05).

Statistics

All of the data are expressed as the means ± S.E. Statistical significance was determined using a one-way analysis of variance for multiple comparisons followed by a Tukey’s post hoc test. A p value of <0.05 was considered significant.

RESULTS

SOCS-3 mRNA expression significantly increased after 72 h of differentiation compared with myoblasts that were maintained in a proliferative state (Fig. 1, A and B). Proper activation of the differentiation process was confirmed by increases in myogenin mRNA (Fig. 1A). This increase in SOCS-3 mRNA expression was coupled with a significant increase in 2.0-kb SOCS-3 promoter activity after 48 and 72 h of differentiation when compared with nondifferentiated myoblasts (Fig. 1C). Interestingly, SOCS-3 promoter activity decreased after the first 24 h of differentiation when compared with the proliferating myoblast condition.

The signal for the up-regulation of SOCS-3 expression during myoblast differentiation remains unclear. Both growth hormone and insulin can induce SOCS-3 mRNA expression in muscle (29, 30), although neither growth hormone or insulin are necessary for myoblast differentiation. However, IGF-I is necessary for differentiation (10), and myoblast production of IGF-I increases during the differentiation process (3); therefore IGF-I could induce SOCS-3 expression. IGF-I mRNA expression increases in C2C12 myoblasts after 48 h of differentiation (Fig. 2A). To determine whether IGF-I affects SOCS-3 expression, the 2.0-kb SOCS-3 promoter was transiently transfected into the C2C12 myoblasts, and the myoblasts were induced to differentiate with and without recombinant IGF-I (Fig. 2B). The 2.0-kb SOCS-3 promoter was significantly increased after exposure to 15 and 30 ng/ml of recombinant IGF-I when compared with the myoblasts not treated with exogenous IGF-I, respectively. To determine whether IGF-I was necessary for induction of transcriptional activity of the SOCS-3 promoter, C2C12 myoblasts were induced to differentiate in the presence of a specific IGF-I receptor antibody (αIR3, 3 μg/ml), which prevents the activation of the IGF-I receptor during myoblast differentiation (10). Inclusion of the antibody significantly reduced SOCS-3 promoter activity after 72 h of differentiation; however receptor inhibition did not completely reduce activity to baseline levels (dashed line) (Fig. 2C). Similarly, SOCS-3 transcriptional activity was also reduced in C2BP5 cells, a cell line that is stably transfected with IGFBP5 cDNA and cannot respond to endogenously produced IGF-I (Fig. 3). More specifically, after the transfected C2BP5 myoblasts were exposed to differentiation medium for 72 h without R3-IGF-I (analog form of IGF-I, which is not buffered by the IGFBPs), there was still
a moderate increase in SOCS-3 promoter activity compared with the myoblast condition. However, when R3-IGF-I was added to the medium for 72 h there was a significant increase in SOCS-3 promoter activity when compared with either the myoblast or the 72-h non-IGF-I-treated condition. Currently, it is unclear why inhibition of the IGF-I receptor or use of C2BP5 cell line did not completely abolish SOCS-3 transcription; however, it is possible that another unidentified factor is stimulating SOCS-3 transcription in parallel to IGF-I. These findings suggest that autocrine/paracrine production of IGF-I, at least in part, contributes to the increase in SOCS-3 expression during differentiation.

SOCS-3 gene expression is mediated at least in part by activation of the transcription factor STAT3 (15). To confirm this finding in the muscle culture system, C2C12 myoblasts were co-transfected with a dominant negative STAT3 expression vector and the 2.0-kb SOCS-3 reporter construct. The cells were induced to differentiate with and without IGF-I. After 48 h, IGF-I (15 ng/ml) significantly enhanced SOCS-3 promoter activation, whereas the dominant negative STAT3 blocked the IGF-I-induced transcriptional activation of the SOCS-3 promoter (Fig. 4A). Endogenous STAT3 activation was measured using an antibody specific to an active form of STAT3. STAT3 activation is regulated by phosphorylation of tyrosine residue 705 (31), which was detected at very low levels in proliferating myoblasts; however as the myoblasts were induced to differentiate, there was a large increase in the phosphorylation levels of STAT3 (Fig. 4B). Further, STAT3 activation was reduced by inhibition of the IGF-I receptor using the same previously described antibody inhibition strategy during differentiation (Fig. 4C).

Increases in skeletal α-actin mRNA and promoter activity are known markers of C2C12 myoblast differentiation (17, 32). To determine the role of SOCS-3 expression in differentiating myoblasts, the SOCS-3 cDNA expression vector driven by the elongation factor (EF) promoter was co-transfected with the 2.0-kb skeletal α-actin promoter. Overexpression of SOCS-3 increased skeletal α-actin promoter activity by ~2-fold when compared with the differentiating myoblasts transfected with the vector lacking the SOCS-3 cDNA (Fig. 5B). These data indicate that SOCS-3 overexpression increases the transcriptional activity of contractile apparatus genes normally associated with myoblast differentiation.

To further demonstrate the role of SOCS-3 in myoblast differentiation, a SOCS-3-GFP fusion construct was generated and driven by the muscle creatine kinase promoter (22). To ensure proper generation of the fusion protein, Western blotting procedures were performed for GFP expression. GFP or SOCS-3-GFP expression was measured in myoblasts or after 24–72 h of myoblast differentiation (Fig. 6A). Expression of GFP or the SOCS-3-GFP fusion protein was detectable as early as 48 h after the induction of the differentiation process (Fig. 6A). In addition, expression continued to increase after 72 h of differentiation. GFP expression from the GFP only
increased SRF transcriptional activity by moterless control plasmid (pGL3). These results are from three independent experiments. * indicates a value statistically different from the myoblast and 24-h group (p < 0.05). # indicates statistically different from the 72-h non-IGF-I-treated group (p < 0.05).

vector was detected at the predicted molecular mass (~30 kDa), whereas SOCS-3-GFP fusion protein expression was detected at the predicted combined molecular mass of GFP and SOCS-3 (~51 kDa). These data suggested that proper formation of the fusion protein had occurred. The control construct (GFP only) and the GFP-SOCS-3 construct demonstrated detectable fluorescent GFP signal as early as 36 h (examples presented after 48 h; see Fig. 6B) as detected by fluorescence microscopy. To determine the effects of SOCS-3 on myoblast differentiation, the constructs were transiently transfected into C2C12 myoblasts. Total RNA was subsequently isolated from the transfected myoblasts or from transfected myoblasts induced to differentiate for various lengths of time (24–72 h). Myogenin and skeletal α-actin mRNA expression was subsequently measured as a marker of myoblast differentiation. The forced expression of SOCS-3 induced the expression of myogenin mRNA earlier and appeared to enhance the expression of myogenin and skeletal α-actin mRNA in the differentiating myoblasts.

Previous literature has indicated that the transcription factors SRF and NFAT play integral roles in myoblast differentiation and in myoblast fusion, respectively (33–35). SRF or NFAT transcriptional activity was measured in differentiating C2C12 cells using reporter constructs that contains four consensus serum response elements (SREs) or four consensus NFAT cis-elements upstream of the luciferase reporter construct. The SRE cis element is the known binding element for SRF (18) (Fig. 5A). After 48 h of differentiation, there were significant increases in SRF and NFAT transcriptional activity (Fig. 7). Interestingly, the increase in SRF and NFAT transcriptional activity coincides with the increase in SOCS-3 transcriptional activity during myoblast differentiation. To determine whether SOCS-3 expression alters SRF or NFAT transcriptional activity, the SOCS-3 expression plasmid driven by the EF promoter was co-transfected with either the SRF sensor or NFAT sensor construct. SOCS-3 (0.1 μg) significantly increased SRF transcriptional activity by ~2-fold when compared with the differentiating cells transfected with the empty vector (Fig. 8A); however, SOCS-3 expression had no effect on NFAT transcriptional activity (Fig. 8B).

Previously, SRF has been implicated as a key regulator of the skeletal α-actin promoter, in that in the proximal region of the promoter there is a SRE (Fig. 5A), which is bound by SRF (18). To confirm the findings of the SRF sensor construct, a skeletal α-actin reporter construct containing only the proximal region (~99) was co-transfected with the SOCS-3 expression construct driven by the EF promoter (Fig. 9A). The proximal region contains a single SRE that has been shown to be sufficient to maintain promoter activity in differentiated myoblasts (36). Overexpression of SOCS-3 significantly increased the transcriptional activity of the minimal skeletal α-actin promoter construct by ~2-fold when compared with the differentiating cells transfected with the empty vector (Fig. 9B).

To determine whether SOCS-3 can induce myoblast differentiation independent of endogenous production of IGF-I by the differentiating myoblasts, the C2BP5 cells were co-transfected with the SOCS-3 expression plasmid driven by the EF promoter and either the skeletal α-actin, myosin heavy chain IIx, or myosin heavy chain IIb promoter construct. After 72 h, overexpression of SOCS-3 without R3-IGF-I significantly increased the transcriptional activity of the skeletal α-actin promoter (Fig. 10) but failed to have any effect on either myosin heavy chain promoter constructs (data not shown). Inclusion of the R3 analog of IGF-I in the C2BP5 medium, which is insen-
sitive to IGFBP5, increased myosin heavy chain IIx and IIb transcriptional activity by 14- and 6-fold (data not shown), respectively, but only marginally increased skeletal /H9251-actin transcriptional activity (Fig. 10).

DISCUSSION

Myoblast differentiation is characterized by an exit from the proliferative state by the myoblast and a subsequent increase in contractile gene expression (37). Differentiation of the myoblast contributes to the building or rebuilding of muscle fibers that occurs during muscle development or muscle regeneration, respectively (2). The data presented here demonstrate for the first time that endogenous production of IGF-I by the myoblast increases SOCS-3 expression, which contributes to mechanisms that activate myoblast differentiation (Fig. 11). The data also indicate that overexpression of SOCS-3 increases the transcriptional activity of the skeletal α-actin promoter. Further, SOCS-3 also specifically increases SRF transcriptional activation, suggesting that the enhanced differentiation may be mediated by SRF, because SRF has been implicated as a key player in the myoblast differentiation process (34, 35). Finally, SOCS-3 cannot totally induce myoblast differentiation independent of IGF-I, suggesting that IGF-I may induce the activation of a signaling protein with which SOCS-3 interacts to mediate SRF transcriptional activity (Fig. 11). Another possible interpretation is that IGF-I induces myoblast differentiation through an alternative SRF-independent pathway in which SOCS-3 does not directly interact but enhances through parallel activation of SRF-driven myogenesis.

Activation of signaling pathways by growth factors is limited in both magnitude and duration (38). Abnormal activation of signaling pathways can lead to uncontrolled tissue expansion through increased cellular growth or proliferation; therefore cells contain proteins capable of acting as modulators of signaling pathways (38). One such family, the SOCS family of proteins, contains a SH2 domain that allows for interaction and regulation of other signaling proteins (7). Recently, SOCS-3 has been implicated as having the ability to regulate insulin signaling (8, 11, 30, 39), and therefore SOCS-3 could potentially alter IGF-I signaling simply based on the homology that insulin and IGF-I share. Unfortunately, deletion of SOCS-3 is lethal in mice, so traditional knock-out technology cannot be employed (7). As of late, data have come forth indicating that SOCS proteins do not always act in a negative fashion and are capable of potentiating a signal that alters a biological mechanism of the cell. For example, insulin can induce SOCS-6 expression resulting in improved glucose metabolism (40). In addition, SOCS-2 overexpression promotes neurite outgrowth (41) and enhances growth hormone signaling (42). Finally, SOCS-3 also appears to be able to enhance cell survival by sustaining mitogen-activated protein kinase activation (43). Although the original descriptions initially indicated that SOCS family acted in an inhibitory fashion, recent data coupled with these data suggest that under the given conditions the SOCS family of proteins can also induce various biological mechanisms.

Production of IGF-I by the differentiating myoblast has long been known to contribute to the differentiation process (44) through activation of specific signaling proteins (45). The importance of endogenously produced IGF-I is echoed by myoblasts that stably express IGFBP5 cDNA, in that they fail to differentiate properly because of enhanced buffering of the endogenously produced IGF-I (13). A variety of genes are
known to be sensitive to IGF-I during myoblast differentiation, such as skeletal α-actin (14), myosin heavy chain (13), and myogenin (46), and therefore it is thought that IGF-I induces myoblast differentiation by activating signaling mechanisms that induce transcription of these genes. Based on the results found here, a model (Fig. 11) is presented for the role SOCS-3 may play in the myoblast differentiation process. SOCS-3 does not appear to have the ability to totally induce myoblast differentiation independent of IGF-I, in that overexpression of SOCS-3 did not induce increased myosin IIx or IIb transcription (data not shown) in cells that are not responsive to endogenously produced IGF-I. However, SOCS-3 expression did

FIG. 6. Overexpression of SOCS-3 enhanced myogenin and skeletal α-actin mRNA expression during C2C12 myoblast differentiation. A, cDNA plasmids (1 μg) expressing a SOCS-3-GFP fusion protein or only the GFP protein both driven by the MCK promoter were transiently transfected into C2C12 myoblasts on 35-mm plates. The transfected myoblasts were either maintained in the myoblast state (Mb) or induced to differentiate for defined periods of time (24, 48, or 72 h). Cellular protein was isolated at the defined time point, and GFP protein expression was measured through Western blotting techniques. GFP expression was detected in the GFP only vector at ~30 kDa, whereas SOCS-3-GFP protein expression was found at ~51 kDa, which is approximately the predicted molecular mass for a successful SOCS-3-GFP fusion protein. These data suggest that a successful SOCS-3 fusion protein was generated. B, GFP fluorescence was detected in differentiating myoblasts after transfection of either the MCK-GFP or MCK-SOCS-3-GFP expression vectors. C2C12 myoblasts were transiently transfected as described above (in Fig. 6A) and induced to differentiate for 48 h. Using fluorescence microscopy, GFP expression or SOCS-3-GFP expression was detected in differentiating C2C12 myoblasts after 48 h of differentiation. C, myogenin and skeletal α-actin mRNA expression was enhanced in the differentiating myoblasts after transfection of the MCK-SOCS-3-GFP expression plasmid. C2C12 myoblasts were transiently transfected with either the MCK-GFP or MCK-SOCS-3-GFP plasmid as described above (Fig. 6A). The transfected myoblasts were either maintained in the myoblast state (Mb) or induced to differentiate for a defined period of time (24, 48, 72 h). Total RNA was subsequently isolated at the described time point, and myogenin, skeletal α-actin, or 18 S mRNA was measured using RT-PCR.

FIG. 7. SRF and NFAT transcriptional activity increased during myoblast differentiation. A, SRF transcriptional activity increased after 48 h of C2C12 myoblast differentiation. The SRF activity sensor plasmid was transiently transfected in C2C12 myoblasts. After the transfection was completed, the myoblasts were induced to differentiate. At the defined time point, the luciferase activity was subsequently quantified. The data are expressed as the fold increases in promoter activity over the promoterless control plasmid. These results are from three independent experiments. * indicates a value statistically different from the 24-h group ($p < 0.05$). B, NFAT transcriptional activity increased after 48 h of myoblast differentiation. The NFAT activity sensor plasmid was transfected in C2C12 myoblasts. After the transfection was completed, the myoblasts were induced to differentiate. At the defined time point, the luciferase activity was subsequently quantified. The data are expressed as the fold increase in promoter activity over the promoterless control plasmid. These results are from three independent experiments. * indicates a value statistically different from the 24-h group ($p < 0.05$).
increase skeletal α-actin transcription in this same cell line, so it is possible that SOCS-3 can enhance genes that are directly activated by the transcription factor SRF independent of IGF-I.

This is plausible because the myosin heavy chain IIX and IIB promoters are not thought to be activated by SRF (19); however, SRF is a potent activator of skeletal α-actin transcription (17, 47). These data suggest that another signaling pathway that is “IGF-I-sensitive” may act in concert with SOCS-3 or in parallel with SOCS-3 to induce myoblast differentiation. Previously, a number of studies have demonstrated that the PI3-K-AKT pathway, which is very responsive to IGF-I, is critical for the initiation of the myoblast differentiation response (45, 48). However, Wilson et al. (49) elegantly demonstrated that PI3-K acted independently of AKT and mTOR to regulate late stages of differentiation; however, the authors were unable to determine through which signaling component PI3-K was acting. Recent evidence has indicated that SOCS proteins can functionally interact with the p85 subunit of the PI3-K complex to alter PI3-K activity (40); therefore it is possible that SOCS-3 could be enhancing differentiation by interacting with PI3-K. In further support of this possibility, PI3-K is capable of in-
Fig. 11. A schematic model depicting that the endogenous production of IGF-I by myoblast contributes to the increase in SOCS-3 expression during myoblast differentiation. Increases in SOCS-3 expression can increase SRF-driven transcriptional activity possibly through interactions with a yet defined signaling protein, which may lead to myoblast differentiation.

Increasing SRF transcriptional activity resulting in increased skeletal α-actin promoter activity (50). The possibility that SOCS-3 is interacting with PI3-K during myoblast differentiation is currently being tested in my laboratory.

If SOCS-3 contributes to the development of myoblast differentiation, then it is expected that SOCS-3 expression would be elevated under conditions where active myogenesis is occurring. SOCS-3 mRNA expression is increased during cardiotoxin-induced muscle regeneration in the tibialis anterior muscle, and SOCS-3 protein expression is elevated during early stages of plantaris muscle hypertrophy induced by the synergist ablation model. 2

In support of these findings Paoni et al. (51) found that during ischemia-induced muscle regeneration there was a significant up-regulation of SOCS-3 mRNA expression as measured by microarray analysis. This suggests that in the whole animal the up-regulation of SOCS-3 expression in skeletal muscle may contribute to the myogenesis process during the recovery from muscle damage or increased loading of the muscle, and the up-regulation of SOCS-3 expression may be induced by IGF-I. IGF-I production is elevated during both muscle regeneration (52) and muscle hypertrophy induced by synergist ablation (53).

Previously, Kataoka et al. (54) found that STAT3 could inhibit MyoD activity and maintain cells in a proliferative state. Although it appears that these data would conflict with those presented here, it in fact alludes to the complexity of the role for STAT3 activity in skeletal muscle. Activation of STAT3 has been shown to induce both proliferation (55) and differentiation (56) in various cells. It appears that STAT3 is capable of inducing various responses in different cells depending upon which growth factor or cytokine induced the activation of the transcription factor. Kataoka et al. (54) found that LIF, which activates STAT3, maintains the cells in a proliferative state. These findings agree with previous results indicating that LIF could maintain various types of stem cells in a proliferative state (57). The data presented here suggest that the induction of STAT3 by IGF-I may contribute to myoblast differentiation, suggesting that functional roles of STAT3 may differ depending upon the background of the tissue and ligand that induces it (54).

In summary, these data demonstrate that SOCS-3 expression can increase transcriptional activation of key contractile proteins during myoblast differentiation. In addition, the data also suggest that the up-regulation of SOCS-3 expression is dependent upon autocrine and/or paracrine production of IGF-I. Further studies are being conducted to determine the upstream signaling proteins with which SOCS-3 interacts to induce myoblast differentiation.

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