Myostatin Induces Cyclin D1 Degradation to Cause Cell Cycle 
Arrest through a Phosphatidylinositol 3-Kinase/AKT/GSK-3β 
Pathway and Is Antagonized by Insulin-like Growth Factor 1*

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Myostatin is a transforming growth factor β superfamily member and is known as an inhibitor of skeletal muscle cell proliferation and differentiation. Exposure to myostatin induces G1 phase cell cycle arrest. In this study, we demonstrated that myostatin down-regulates Cdk4 activity via promotion of cyclin D1 degradation. Overexpression of cyclin D1 significantly blocked myostatin-induced proliferation inhibition. We further showed that phosphorylation at threonine 286 by GSK-3 was required for myostatin-stimulated cyclin D1 nuclear export and degradation. This process is dependent upon the activin receptor IIB and the phosphatidylinositol 3-kinase/Akt pathway but not Smad3. Insulin-like growth factor 1 (IGF-1) treatment or Akt activation attenuated the myostatin-stimulated cyclin D1 degradation as well as the associated cell proliferation repression. In contrast, attenuation of IGF-1 signaling caused C2C12 cells to undergo apoptosis in response to myostatin treatment. The observation that IGF-1 treatment increases myostatin expression through a phosphatidylinositol 3-kinase pathway suggests a possible feedback regulation between IGF-1 and myostatin. These findings uncover a novel role for myostatin in the regulation of cell growth and cell death in concert with IGF-1.

Myostatin, also known as growth differentiation factor 8, or GDF-8, is a transforming growth factor-β (TGF-β) superfamily member and is known to be involved in regulation of skeletal muscle mass (1). Unlike most TGF-β/GDF family members, which are expressed nonspecifically in many tissue types, myostatin is highly expressed in developing and adult skeletal muscle cells, where it plays an essential role in limiting skeletal muscle growth (1, 2). In addition, as low levels of myostatin expression have been observed in cardiac muscle, mammary gland, adipose, and preadipose tissues, it may also serve a broader biological function (3–5). Indeed, myostatin null mice exhibited a phenotype that was characterized by a marked hypertrophy and hyperplasia of skeletal muscle and loss of fat mass (1). Similarly, enlarged skeletal muscle mass is evident in naturally occurring mutations of myostatin in humans and cattle (6, 7). Recent evidence suggests that myostatin expression and activity may be involved in the development of metabolic disorders, such as muscular dystrophy, obesity, and type II diabetes mellitus (8). With the aim of utilizing the inhibitory effects of myostatin upon multiple physiological processes and ultimately achieving an improved quality of life for obese and diabetic patients, various strategies for manipulating the biological activities of myostatin are under development (9).

Cell fate determination and proliferation of myogenic cells are critical processes in skeletal muscle formation during early embryo development. Studies have demonstrated that myostatin can inhibit the proliferation of myoblasts, satellite cells, and rhabdomyosarcoma cells. Myostatin treatment prevented myoblast progression from the G1 phase to the S phase of the cell cycle by up-regulating expression of the Cdk (cyclin-dependent kinase) inhibitor p21Waf1,Cip1 and decreasing the levels and activity of Cdk2. Also, the retinoblastoma (Rb) protein was found to be present predominantly in the hypophosphorylated form in myostatin-stimulated myoblasts (10). Myostatin has also been implicated in the regulation of satellite cell activation, proliferation, and self-renewal that accompanies the up-regulation of p21Waf1,Cip1 (11). However, work by Langley et al. (12) showed that myostatin treatment inhibited rhabdomyosarcoma cell proliferation through an Rb-independent mechanism. Therefore, the antiproliferative effects of myostatin could be achieved by utilization of multisignal pathways.

Experiments in vitro and in vivo have implicated activin receptor IIB, TGF-β receptor I (ALK5), and activin receptor IB (ALK4) as receptors in the mediation of myostatin signals (13). The binding of myostatin to heteromeric receptor complexes typically induces phosphorylation and activation of the associated intracellular signal transducers (Smad2/3), which then translocate into the nucleus and together with co-Smad (Smad4) regulate the expression of target genes (14). In addition, it has been suggested that p38 and Akt pathways involved...
in myostatin signal transduction (3, 15). Recently, we have also found that the Erk1/2 mitogen-activated protein kinase pathway participated in the regulation of muscle cell growth and differentiation by myostatin (16).

Cyclins are essential components of the cell cycle machinery; each binds and activates specific Cdk partner proteins. Normal progression through the G1 phase of the cell cycle requires both D- and E-type cyclins. The cyclin-Cdk complexes formed during G1 phase initiate phosphorylation of the Rb family of tumor suppressor proteins (pRb, p107, and p130) and thereby abrogate their inhibitory activity (17). The D-type cyclins primarily suppressor proteins (pRb, p107, and p130) and thereby abrogate their inhibitory activity (17). The D-type cyclins primarily activate Cdk4 and -6, whereas cyclin E activates Cdk2. Given the crucial role that D-type cyclins play in the progression through G1 phase of the cell cycle, it is not surprising that their expression is frequently down-regulated in cells stimulated with anti-proliferative cytokines, such as TGF-β family members. This down-regulation generally manifests both at the level of mRNA transcription and protein stabilization. Diehl et al. (18) showed that the cyclin D1 proteolysis was accelerated via the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK-3β pathway. GSK-3β phosphorylates cyclin D1 at threonine 286, which triggers its nuclear export, ubiquitination, and subsequent degradation. In contrast, mitogens, such as insulin-like growth factor 1 (IGF-1), inhibit GSK-3β kinase activity and stabilize the cyclin D1 protein by activating the PI3K/Akt pathway (19).

Although different models have been proposed to explain how myostatin causes cell cycle arrest, most studies are based on correlative results or overexpression. The mechanism underlying the function of myostatin remains unclear. In this study, we have demonstrated that myostatin augments cyclin D1 protein degradation. The PI3K/Akt/GSK-3β signaling cascade participates in myostatin-regulated cyclin D1 degradation and cell proliferation inhibition through a proteasome-dependent pathway. We report biochemical and cellular evidence indicating that these effects of myostatin on cyclin D1 expression and cell proliferation can be blocked by treatment with IGF-1, a survival and proliferation promotion factor. Most significantly, we found that blocking the IGF-1/PI3K/Akt survival pathway caused C2C12 cells to undergo apoptosis in response to myostatin. Finally, our data indicate that myostatin expression in C2C12 cells is induced by IGF-1 treatment through the PI3K/Akt pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following antibodies and reagents were used in this study: anti-cyclin D1, anti-cyclin E, anti-Cdk2, anti-p27, anti-p21, anti-Cdk4, anti-β-actin, anti-tubulin, anti-c-Myc, and anti-hemagglutinin antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Smad3, anti-Akt, anti-phospho-Akt (Ser473), anti-GSK-3β, and anti-phospho-GSK-3β (Ser9) antibodies and wortmannin from Cell Signaling (Beverly, MA); LiCl and MG132 from Sigma; recombinant IGF-1 and antibody against IGF-1 receptor from R&D (Minneapolis, MN). Recombinant myostatin and monoclonal antibody against myostatin were prepared as previously reported (20, 21).

**DNA Constructs**—The expression vector for GST-Rb-(773–928) was generously provided by Dr. Fang Liu (State University of New Jersey). The wild type and T286A mutant cyclin D1 were constructed by using pHIT-Myc3 retroviral vector with the following primers (5′-CGG AAT TCA TGG AAC ACC AGC TTC TGT G-3′ (upper) and 5′-CCC AAG CTT TCA GAT GTT CA GTC ATC GCC GAC GCC GTT GGT GAG TCA AGC-3′ (lower) for the wild type; 5′-CGG AAT TCA TGG AAC ACC AGC TTC TGT G-3′ (upper) and 5′-CCC AAG CTT TCA GAT GTT CA GTC ATC GCC GAC GCC GTT GGT GAG TCA AGC-3′ (lower) for the T286A mutant).

**Cell Culture and Transfection**—Mouse C2C12 cells were from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/liter glucose, 4 mm l-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT), and penicillin/streptomycin at 37 °C in a 5% CO2 atmosphere. Primary fibroblasts isolated from Smad3 knock-out or wild type mice were provided by Dr. Xiao Yang. The inducible Akt overexpression C2C12 cell line was generously provided by Dr. Zhenguo Wu. During exponential growth, cells were treated with myostatin at a final concentration of 500 ng/ml for the indicated time, and cells used as control were treated with the same amount of phosphate-buffered saline (PBS). For electroporation transfection, cells were rinsed with PBS and trypsinized with 1 ml of trypsin/EDTA buffer for 1–2 min at 37 °C. After centrifugation, the cells were resuspended in a 600-µl culture medium (Dulbecco’s modified Eagle’s medium) and incubated with plasmid DNA for 10 min at 4 °C. The cell suspension was then transferred into a 4-mm gap electroporation cuvette and electroporated at 350 V, 16 ms, 1 pulse using a Bio-Rad electroporator.

**Retrovirus Production and Infection**—The retrovirus was produced by transient transfection of retroviral constructs into the Phoenix helper-free retrovirus producer cell line (a gift from Dr. Gary Nolan, Stanford University) using the calcium phosphate method according to the standard protocol. For the infection of C2C12 myoblasts, the retroviral supernatant (48 h after transfection) was filtered and added into each C2C12 plate, with the addition of 3 µg/ml polybrene, and the cells were incubated overnight at 37 °C for infection.

**Immunoblotting**—For cell lysate preparation, monolayer cells on 100-mm plates were lysed with 1.2 ml of lysis buffer (50 mm Tris–HCl, pH 7.5, 150 mm NaCl, 0.5% Nonidet P-40, 50 mm NaF, 1 mm Na3VO4, 5 mm β-glycerophosphate, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride). The lysate was clarified by centrifuging at 14,000 × g for 20 min. Boiled samples with 2× SDS loading buffer were loaded onto a 10–12% polyacrylamide gel, and, after electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (PALL, East Hills, NY). The resulting blots were blocked with 1% bovine serum albumin for phosphoprotein antibodies and 10% milk for nonphosphoprotein antibodies for 1 h and then incubated with the primary antibody overnight at 4 °C. The secondary antibody used in the immunoblot was a 1:3000 dilution of horseradish peroxidase-linked anti-IgG. The ECL reagent (Amersham Biosciences) was used as the substrate for detection, and the membrane was exposed to an x-ray film for visualization.

**Immunoprecipitation-coupled Western Blot**—C2C12 cells with various treatments were lysed in a buffer containing 50 mm Tris, pH 7.5, 100 mm NaCl, 2 mm EDTA, 1% Nonidet P-40, 10
mm iodoacetamide, and protease inhibitors. This was followed by centrifugation at 14,000 × g for 10 min. The clarified lysates were immunoprecipitated with either the anti-Cdk4 antibody or the anti-β-actin antibody as negative control. Protein A/G plus agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and washed with the lysis buffer. Samples were separated by 12% SDS-PAGE and immunoblotted with anti-cyclin D1, anti-p27, and anti-Cdk4 antibodies.

Small Interfering RNA (siRNA)—The target sequences of double-stranded nucleotides used for siRNA knockdown are GCC TCA GCT CAT GAA CGA C for ActRlIlb (Ambion, Austin, TX), GAA CGG AGA GCT CCA GAT C for GSK-3β (number 1) (RIBOBIO, Guangzhou, Guangdong, China), GTA ACC CCC CTC TGG CCA C for GSK-3β (number 2) (RIBOBIO), GGA CAT TGG AGG AGA GC C for IGF-1 receptor (number 1) (RIBOBIO), TCT CAT CTG AGG AGA GGA G for IGF-1 receptor (number 2) (RIBOBIO), and ATCCAGTGACCCGTCA for GAPDH (RIBOBIO). Cells cultured in a 6-well plate (2 × 10^5 cells/well) were transfected with 70 μM siRNA with Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were treated with myostatin or PBS for 2 h. The total protein extracts from the cells were used for Western blot analysis.

Proliferation Assay—About 2 × 10^5 cells were seeded in 6-well plates and maintained in a Dulbecco’s modified Eagle’s medium culture medium for retrovirus infection and myostatin stimulation. During the last 12 h of each treatment, 5 μCi of [3H]thymidine was added to the culture medium. The cells were washed three times with PBS and trypsinized for radioactivity measurement in scintillation vials. The assay was performed in three replicates and repeated three times for statistic analysis.

In Vitro Kinase Assay—C2C12 cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol and protease and phosphatase inhibitors. Antibodies against Cdk4 and β-actin (negative control) were used for immunoprecipitations. The kinase assay was carried out for 1 h in a 30-μl reaction containing 50 mM HEPES, pH 7.4, 15 mM MgCl₂, 1 mM EGTA, 0.1% Tween 20, 1 mM dithiothreitol, 50 mM ATP, 5 μCi of γ-32P]ATP (3000 Ci mmol), and affinity-purified GST-Rb-(773–928) as substrates at 30 °C. The reaction mixtures were then separated in a 12% polyacrylamide gel, and proteins phosphorylated by the immunoprecipitant were visualized by autoradiography.

Reverse Transcription-PCR Analysis—Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Semiquantitative reverse transcription-PCR was performed according to standard protocol to measure the expression of p110 with the primers p110-U (5′-CCC CCA CGA ATC CTA GTG GAA TGT TTA C-3′) and p110-L (5′-TGA AAA AGC CGA AGG TCA CAA AGT CGT C-3′).

Immunofluorescence—The cells were fixed with 4% (w/v) paraformaldehyde in PBS, washed three times with a permeabilization buffer (0.3% Triton X-100 in PBS) for 10 min each time, and then blocked with 3% (w/v) bovine serum albumin (Calbiochem) in PBS. The samples were incubated for 2 h at room temperature with either the anti-GSK-3β (1:100) antibody or the anti-cyclin D1 antibody and for 1 h with fluorescein isothiocyanate- or TRITC-conjugated secondary antibodies against mouse IgG (Santa Cruz Biotechnology). Finally, the cells were washed with PBS and mounted onto microscope glass slides. 4′,6-Diamidino-2-phenylindole staining was performed simultaneously to show the position of the nuclei.

RESULTS

Myostatin Promotes Proteasome-dependent Cyclin D1 Degradation—Myostatin has been known as an inhibitor of skeletal muscle cell proliferation. To examine the possible mechanism of myostatin-induced cell cycle arrest during the G1 phase, the level of G1 regulators was assessed in C2C12 myoblasts exposed to myostatin (Fig. 1a). The results revealed that myostatin stimulation significantly reduced the steady level of cyclin D1 protein in a time-dependent manner. Meanwhile, the level of cyclin E was slightly down-regulated by myostatin. Consistent with previous reports, myostatin also increased the level of p21 in C2C12 cells, and the rapid decrease of cyclin D1 protein suggests that the protein stability may be regulated by myostatin. Blockade of the protein degradation pathway with the proteasome inhibitor MG132

FIGURE 1. Myostatin inhibits Cdk4 activity and Rb phosphorylation by promoting proteasome-dependent cyclin D1 degradation. a, proliferating C2C12 cells were treated with 500 ng/ml myostatin (Mstrn) or the same volume of PBS for the indicated time. Cell lysates were used for Western blot detection of G1 cell cycle regulators cyclin D1, cyclin E, Cdk4, Cdk2, and p21. Tubulin served as the sample equal loading control. b, proliferating C2C12 cells were cultured with 500 ng/ml myostatin or the same volume of PBS in the absence or presence of the proteasome inhibitor MG-132 for 3 h. Levels of cyclin D1 and tubulin were detected by Western blot assay. c, top, immunoprecipitated Cdk4 kinase assay in vitro. C2C12 cells were cultured in the absence or presence of myostatin for 12 h. Cdk4 immunoprecipitated from C2C12 cell lysate with 1.2 μg of Cdk4 antibody was used in a kinase assay with GST-Rb-(773–928) as the substrate. The antibody against β-actin was used as a nonrelated IgG for the negative control. Bottom, equal amounts of GST-Rb were visualized by Coomassie Brilliant Blue staining. d, myostatin treatment affects the abundance of cyclin D1-Cdk4 complexes. Protein lysates from C2C12 cells with or without myostatin treatment were immunoprecipitated with anti-Cdk4 antibody and anti-β-actin antibody as a negative control. Western blots were performed with anti-cyclin D1 antibody. The same blot was reprobed with Cdk4 antibody to confirm that relatively equal amounts of immunoprecipitated Cdk4 proteins were present. The input control was performed using the total cell lysates.
almost completely inhibited the cyclin D1 protein degradation induced by myostatin (Fig. 1b). These results suggest that the proteasome-dependent pathway mediates myostatin-stimulated cyclin D1 degradation and that the down-regulation of cyclin D1 may play a very important role during myostatin-induced proliferation suppression.

**Myostatin Inhibits Cdk4 Activity and Rb Phosphorylation through Down-regulation of Cyclin D1**—To test whether myostatin-induced cyclin D1 protein degradation alters Cdk4 function, we assayed Cdk4 kinase activity in immunoprecipitates from C2C12 cells in the presence or absence of myostatin. Specifically, phosphorylation of exogenous GST-Rb-(773–928) was measured, and the immunoprecipitate from a nonrelated IgG was used as a negative control. Cdk4 kinase activity was readily detected in PBS-treated cells, whereas only negligible Cdk4 kinase activity was detected in myostatin-treated cells (Fig. 1c). The myostatin-induced loss of Cdk4 kinase activity due to the accelerated cyclin D1 protein degradation was further confirmed by immunoprecipitation-coupled Western blot analysis. As shown in Fig. 1d, cyclin D1 was co-immunoprecipitated with Cdk4 from the extracts of PBS-treated cells. However, in myostatin-stimulated C2C12 cells, no association of Cdk4/cyclin D1 could be detected. To verify the expression of these CDK4 and cyclin D1 proteins, input control of total cell lysate was blotted with antibodies against Cdk4 and cyclin D1, respectively (Fig. 1d). These findings provide experimental evidence indicating that myostatin inhibits myoblast proliferation by inhibiting Cdk4 kinase activity via promotion of proteasome-dependent cyclin D1 degradation.

**Attenuation of Myostatin-induced Growth Inhibition by Overexpression of Cyclin D1**—To directly test whether cyclin D1 down-regulation is a determining factor in the growth inhibitory effects of myostatin, we assayed cyclin D1 expression and cell proliferation in C2C12 cells infected with retroviruses carrying a mock vector or cyclin D1 gene to measure the effects of myostatin on cyclin D1 expression and cell proliferation. As shown in Fig. 3, a similar effect was achieved when cells were transfected with synthetic siRNAs before myostatin treatment (Fig. 3d). Compared with the GAPDH siRNA control, myostatin failed to promote cyclin D1 protein degradation in the absence of endogenous GSK-3β (Fig. 3d).

The phosphorylation of cyclin D1 at threonine 286 by GSK-3β is required for its degradation (18, 20). To further confirm the requirement of GSK-3β in cyclin D1 degradation induced by myostatin, either the Myc-tagged wild type or T286A mutant cyclin D1 was introduced into C2C12 cells using retrovirus-mediated gene transfer. We found that down-regulation of cyclin D1 induced by myostatin was threonine 286-dependent (Fig. 3e); these data provide direct biochemical evidence that GSK-3β plays a functional role in myostatin-mediated cyclin D1 protein degradation.

**GSK-3β Is Required for Myostatin-induced Cyclin D1 Degradation**—To examine the possibility that GSK-3β may be involved in myostatin-mediated inhibition of cell proliferation, GSK-3β activity in C2C12 cells was inhibited with the chemical inhibitor lithium chloride (LiCl) in the presence or absence of myostatin. Inhibition of GSK-3β kinase activity by LiCl attenuated myostatin-induced cell proliferation suppression (Fig. 3, a and b). We then tested whether myostatin-mediated cyclin D1 degradation is also regulated by GSK-3β with the use of LiCl and synthetic siRNAs. Pretreatment of C2C12 cells with LiCl suppressed the myostatin-induced cyclin D1 degradation (Fig. 3c). A similar effect was achieved when cells were transfected with synthetic siRNAs before myostatin treatment (Fig. 3d). Compared with the GAPDH siRNA control, myostatin failed to promote cyclin D1 protein degradation in the absence of endogenous GSK-3β (Fig. 3d).

GSK-3β has been shown to remain in the cytoplasm in an inactivated form. However, once activated, it is translocated into the nucleus to phosphorylate cyclin D1 at threonine 286, and then the phosphorylated cyclin D1 is in turn translocated from the nucleus to the cytoplasm, where the phosphorylated cyclin D1 is degraded through a proteasome-mediated pathway (18). We assayed the intracellular translocation of GSK-3β and cyclin D1 proteins with immunofluorescent labeling of C2C12 cells treated with myostatin. As shown in Fig. 3f, GSK-3β was most abundant in the cytoplasm of PBS-treated control C2C12 cells but was then translocated into the nucleus after myostatin treatment. In contrast, cyclin D1 could hardly be detected in myostatin-treated C2C12 cells, presumably due to its reduced stability. Blockade of the proteasome-mediated degradation pathway with MG132 revealed that myostatin induced cyclin D1 translocation from the nuclei to the cytoplasm in C2C12
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Myostatin Activates GSK-3β by Inhibiting the PI3K/Akt Pathway—Ser9 of GSK-3β can be phosphorylated by multiple kinases, principally from the PI3K/Akt pathway (22). Therefore, to elucidate the involvement of GSK-3β upstream molecules in myostatin-regulated cyclin D1 degradation, we assessed the phosphorylation status of Akt and GSK-3β following myostatin treatment and the correlation between cyclin D1 protein levels and Akt/GSK-3β phosphorylation. Results showed that myostatin significantly inhibited Akt phosphorylation at Ser473 and GSK-3β phosphorylation at Ser9 while down-regulating cyclin D1 (Fig. 4a). During the preparation of this paper, Akt phosphorylation was reported to be down-regulated by myostatin in hypertrophic cardiomyocytes (3).

Because of the correlation between Akt/GSK-3β dephosphorylation and cyclin D1 deregulation in C2C12 cells in response to myostatin, we used wortmannin, an inhibitor of PI3K, to mimic the inhibitory influence of myostatin on the PI3K/Akt/GSK-3β pathway. Wortmannin treatment suppressed the phosphorylation of Akt and GSK-3β and destabilized cyclin D1 in a dose- and time-dependent manner (Fig. 4b and data not shown). To further test whether the PI3K/Akt/GSK-3β pathway participates in myostatin-regulated cyclin D1 degradation, a hemagglutinin-tagged constitutively active Akt protein was overexpressed in C2C12 cells, and the levels of cyclin D1 and phosphorylated GSK-3β were measured. Transfection with the constitutively active Akt resulted in a marked increase in cyclin D1 levels and blocked myostatin-induced cyclin D1 degradation (Fig. 4c). PI3K consists of the regulatory subunit p85 and the catalytic subunit p110, which cooperatively regulate the activity of downstream Akt. The expression levels of the p85 regulatory and p110 catalytic subunits of PI3K were determined by immunoblotting and semiquantitative reverse transcription-PCR. There was no apparent effect of myostatin on p85 levels, but the observed decrease in p110 expression may partially explain the mechanism underlying myostatin-induced PI3K/Akt pathway inhibition (Fig. 4, d and e).

ActRIib but Not Smad3 Is Required for Myostatin-mediated Cyclin D1 Degradation—ActRIib is specific and crucial for myostatin binding and signal transduction (14). To determine
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Myostatin activates GSK-3β by inhibiting the PI3K/Akt pathway. a, C2C12 cells were treated with 500 ng/ml myostatin for 5 min, 15 min, 30 min, 1 h, 2 h, or 3 h, and Western blots were performed using antibodies against cyclin D1, phospho-Akt (Ser473), total Akt, phospho-GSK-3β (Ser9), and total GSK-3β. b, C2C12 cells were treated with the PI3K inhibitor wortmannin (0, 10, 50, or 100 nM) for 6 h. The endogenous levels of cyclin D1, phospho-Akt (Ser473), total Akt, phospho-GSK-3β (Ser9), and total GSK-3β were measured by immunoblotting. c, wild type C2C12 cells and C2C12 cells overexpressing hemagglutinin-tagged constitutively active Akt were treated with myostatin or PBS for 12 h. The levels of cyclin D1, phospho-GSK-3β, and total GSK-3β were assessed by Western blots. d, C2C12 cells were treated with different concentrations of myostatin for 12 h. Western blots were performed to detect the expression of the PI3K p85α and p110 subunits and cyclin D1. Tubulin protein served as the loading control. e, semiquantitative PCR was carried out for amplification of the PI3K p110 subunit and GAPDH mRNAs in myostatin-stimulated C2C12 cells.

FIGURE 5. Myostatin-regulated cyclin D1 reduction is mediated through activin receptor IIb but is Smad3-independent. a, C2C12 cells were transfected with ActRIIb-specific siRNA or an siRNA complementary to GAPDH as a control. Forty-eight hours after transfection, the cells were incubated with or without myostatin (500 ng/ml) for 3 h. Levels of cyclin D1, phospho-Akt (Ser473), total Akt, phospho-GSK-3β, total GSK-3β, and ActRIIb were assessed by Western blots. Tubulin protein served as the loading control. b, fibroblasts isolated from Smad3 knock-out or wild type mice were cultured in the presence or absence of 500 ng/ml myostatin. The protein levels of cyclin D1 and Smad3 were evaluated by Western blots. c, embryonic fibroblasts isolated from Smad3−/− mice were pretreated with 20 mM LiCl for 30 min and then incubated with PBS or myostatin for 2 h. Western blots were performed to detect cyclin D1 levels. Tubulin protein served as the loading control.

whether myostatin-stimulated down-regulation of cyclin D1 and Akt was achieved via the ActRIIb receptor, synthesized siRNA duplexes complementary to mouse ActRIIb mRNA were generated in vitro and transfected into C2C12 cells to suppress ActRIIb expression. siRNA duplexes complementary to mouse GAPDH mRNA was used as a control for specificity in these experiments. As shown in Fig. 5a, ActRIIb protein levels were diminished dramatically by the ActRIIb, but not the GAPDH, siRNA. Note that cyclin D1 expression and Akt and GSK-3β phosphorylation were not down-regulated by myostatin when ActRIIb expression was blocked. These findings provide molecular evidence indicating that the type II receptor is required for myostatin-mediated down-regulation of cyclin D1 through the Akt/GSK-3β pathway.

To test whether the Smad3 intracellular signal transducer for myostatin participates in myostatin-induced cyclin D1 down-regulation through ActRIIb receptors, we proceeded to measure cyclin D1 levels in mouse embryonic fibroblasts isolated from Smad3 knock-out mice and compared the findings with those in wild type mice in the presence or absence of myostatin. The loss of Smad3 did not affect cyclin D1 down-regulation stimulated by myostatin (Fig. 5b), suggesting that pathways other than Smad3 regulate cyclin D1 degradation. Evidence has been shown in this report that GSK-3β activity is required for myostatin-induced cyclin D1 degradation. Therefore, in Smad3 null fibroblasts, the cyclin D1 level was measured in response to myostatin in the presence or absence of LiCl. Consistent with the results from the C2C12 myoblasts (Fig. 3c), we found that myostatin-induced cyclin D1 down-regulation was dependent on GSK-3β, but not Smad3 (Fig. 5c), suggesting that myostatin transduces signaling through an unanticipated ActRIIb/PI3K/Akt/GSK-3β cascade.

IGF-1 Rescues Myostatin-induced Proliferation Inhibition—IGF-1 has pleiotropic physiological effects on cell proliferation, survival, skeletal muscle hypertrophy, and regeneration (23). The PI3K/Akt pathway is the major intracellular pathway activated by IGF-1 stimulation during myogenesis (24). It has been reported that cyclin D1 expression was increased by IGF-1 in myoblasts (25). Together with previous studies, our results suggest that IGF-1 functions as an antagonist to attenuate myostatin-regulated cyclin D1 degradation and proliferation inhibition through the PI3K/Akt/GSK-3β pathway. To test this hypothesis, C2C12 cells were pretreated with IGF-1 for 30 min before being subjected to myostatin treatment. The IGF-1 pretreatment blocked myostatin-induced inhibition of cell proliferation (Fig. 6A) by stabilizing cyclin D1 protein and increasing Akt and GSK-3β phosphorylation (Fig. 6B). These findings are consistent with the possibility that IGF-1 may antagonize the inhibitory effects of myostatin via the PI3K/Akt/GSK-3β pathway.

IGF-1 is a very important growth factor for cell survival, and myostatin only causes cell cycle arrest and does not induce apoptosis. Our observations suggest that IGF-1 might play a predominant role in protecting cells from death mediated by myostatin. Thus, in the presence of IGF-1, cells would be arrested at
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**DISCUSSION**

We observed that myostatin-stimulated cyclin D1 reduction in C2C12 myoblasts suppressed cell proliferation. The processes of myostatin-induced down-regulation of the PI3K/Akt pathway and activation of GSK-3β appear to be responsible for cyclin D1 phosphorylation and degradation. Moreover, the skeletal muscle growth-promoting factor IGF-1 has a potent ability to antagonize the antiproliferative effects of myostatin through the common intracellular Akt signaling pathway.

**Myostatin Inhibits Cell Growth by Decreasing Cyclin D1**—Increasing evidence indicates that regulation of cell proliferation is a distinctive feature of myostatin in both embryonic and postnatal myogenesis (1, 11, 26). Also, satellite cell activation, proliferation, and fusion to existing muscle fibers are negatively regulated by myostatin, ultimately influencing postnatal fiber size control and regeneration (11, 27). Therefore, it is very important to elucidate the molecular mechanism of myostatin action in cell cycle regulation.

It has been previously reported that p21 up-regulation and the reduction of Cdk2 levels results in a loss of cyclin E/Cdk2 activity and subsequently an alteration in Rb protein activity, which is involved in the antiproliferative action of myostatin (10). In this study, we did observe p21 up-regulation in response to myostatin stimulation. More interestingly, our data also showed that cyclin D1 levels and Cdk4 activity were significantly down-regulated by myostatin treatment in C2C12 myoblasts and that this reduction was critical for myostatin-induced proliferation inhibition, because overexpressing cyclin D1 could rescue the myostatin-induced cell proliferation inhibition. During myogenesis, D-type cyclins are required for cell proliferation but are redundant for myogenic differentiation (28). Consistent with our data, Langley et al. (12) demonstrated...

G1/S and not undergo apoptosis in response to myostatin. Indeed, we found that blockade of the IGF-1/PI3K/Akt survival pathway by knocking down the IGF-1 receptor with synthetic siRNA did result in apoptosis of C2C12 cells in response to myostatin (Fig. 6, C–E). These results further suggest that these two factors, myostatin and IGF-1, may regulate each other with a negative feedback mechanism that acts to maintain physiological homeostasis between cell growth and cell death during normal development. This means that augmented IGF-1 growth signal may require more myostatin-inhibitory function to reach the balance between cell growth and cell death and *vice versa*. When C2C12 cells were treated with IGF-1 (Fig. 6, F and G), myostatin expression was induced in a dose-dependent manner. IGF-1-induced myostatin expression was blocked by the PI3K inhibitor wortmannin. Conversely, we did not observe increased expression of IGF-1 in response to myostatin treatment (data not shown). Taken together, our findings suggest that there is a negative feedback mechanism involving the action of the growth-promoting factor IGF-1 whose effect is antagonistic to that of the growth-suppressing factor myostatin and that these factors function in opposition to maintain homeostasis between cell growth and cell death by cross-talk through the common PI3K/Akt/GSK-3β pathway.
that myostatin could inhibit rhabdomyosarcoma cell proliferation through a slight reduction of cyclin D1 expression and down-regulation of cyclin E and Cdk2 protein levels. Myostatin and cyclin D1 levels have been found to be inversely correlated during skeletal muscle resistance loading (29). In addition, TGF-β causes growth inhibition in many cell types, usually via the down-regulation of D-type cyclins (30–32). Meanwhile, the amplification and overexpression of cyclin D1 have been detected in cells that show resistance to TGF-β (33). These results demonstrate that, as an important component of the cell cycle machinery, cyclin D1 is a major intracellular target for myostatin during myostatin-induced G1 cell cycle arrest and proliferation inhibition.

**The PI3K/Akt/GSK-3β Pathway Participates in Myostatin-induced Cyclin D1 Degradation and Proliferation Inhibition**—A highly complex network of interacting pathways precisely controls G1 cell cycle progression. Mitogen deprivation or anti-proliferative factors accelerate cyclin D1 proteolysis via the PI3K/Akt/GSK-3β pathway (34, 35). We reported here that myostatin inhibited the phosphorylation level of Akt at Ser473 and GSK-3β at Ser9 and that the pattern of cyclin D1 down-regulation by myostatin was consistent with the dephosphorylation of Akt and GSK-3β. Our data support the notion that myostatin is able to stimulate Akt inhibition, GSK-3β activation, and cyclin D1 destabilization. Morissette et al. (3) recently demonstrated that myostatin was dynamically regulated in the heart and acted to modulate cardiomyocyte growth in a stimulus-specific manner through down-regulation of the Akt signaling, which provides additional evidence to support the findings from this report. PI3K/Akt function in skeletal muscle is essential for both myogenesis and proper maintenance of muscle mass, whereas myostatin negatively regulates skeletal muscle development and maintenance. A recent study showed that Akt phosphorylation becomes elevated with a high fat diet in transgenic mice with a loss of myostatin function mutation (36). Here, we present direct biochemical evidence indicating that myostatin inhibits Akt activity through PI3K in a Samd3-independent manner. However, the kinetics of myostatin-induced cyclin D1 protein degradation mediated by the PI3K/Akt/GSK3β pathway is an interesting question and is currently under investigation.

The existence of cross-talk between the IGF-1 and TGF-β signaling pathways has gained broad recognition in terms of its physiological and pathological significance during development. It has been reported that TGF-β signals cross-talk at multiple levels with various components of the IGF-1 signaling pathway, and this cross-talk may play an important role in regulating myoblast differentiation and muscle growth. Akt activation is required for TGF-β-induced transformation of mesenchymal cells into smooth muscle cells and for fibroblast responsiveness (37). For example, during the progression of carcinomas, the function of TGF-β deteriorates from tumor suppressor to tumor promoter (38). Akt activation is required for TGF-β-induced transformation of mesenchymal cells into smooth muscle cells and for fibroblast responsiveness (37). TGF-β can activate the PI3K/Akt pathway via association of TβRII with the p85 regulatory subunit of PI3K, transcriptional down-regulation of PTEN, and activation of PDK1 or integrin-linked kinase 1 (40). However, it has also been reported that inhibition of the PI3K/Akt/mTOR pathway can enhance growth inhibition of TGF-β in microglial cells and prostate cancer cells (41, 42). In this report, we present compelling data indicating that inhibition of the PI3K/Akt pathway and activation of GSK-3β are required for myostatin-induced cyclin D1 degradation and repression of cell proliferation. PI3K possesses an Src homology 2 domain that binds specifically to phosphorylated tyrosine residues in receptors, adaptor proteins, and insulin receptor substrate. Whereas PTEN and SHIP have been implicated in this Src homology 2 binding interaction during PI3K/Akt pathway activation, the detailed mechanism linking the TGF-β superfamily member receptors with PI3K upstream adaptor proteins is not well characterized (43).

In this study, myostatin treatment did not affect the level of phosphorylation in insulin receptor substrate-1 (data not shown), which is usually phosphorylated in response to insulin and IGF-1 stimulation and functions as an adaptor protein to transduce the signal to downstream molecules. Intriguingly, however, myostatin did decrease gene expression of the p110 subunit of PI3K at both transcriptional and translational levels, suggesting that myostatin inhibition of PI3K/Akt signaling may be mediated via regulation of this PI3K subunit. Thus, a better understanding of how the p110 subunit of PI3K is mutually regulated by both IGF-1 and myostatin is likely to unveil a target molecule that is linked to both IGF-1 and TGF-β.

**IGF-1 and Akt Activation Antagonize the Cytostatic Action of Myostatin**—Sufficient IGF-1 signaling is critical for cell survival as well as skeletal muscle development and maintenance. IGF-1-induced inhibition of TGF-β signaling is achieved by blocking activation of Smad3 via its association with Akt (44). Thus, it is possible that abnormal activation of the PI3K/Akt pathway may block the growth-inhibitory effects of TGF-β in some cancers. We provided evidence herein indicating that IGF-1 treatment or constitutively active Akt could block myostatin-stimulated cyclin D1 degradation and proliferation inhibition. We also demonstrated that although IGF-1 and myostatin have opposite functions in controlling cell growth, they use the same PI3K/Akt/GSK-3β signaling pathway. Therefore, under normal conditions and during disease development, the local concentrations and activities of Akt-boosting factors (such as IGF-1) and Akt-inactivating factors (such as myostatin) play critical roles in maintaining physiological homeostasis between cell growth and cell death. Based on our experimental data, a schematic model is proposed (Fig. 7) to demonstrate a novel role for myostatin in the regulation of cell growth and cell death in concert with IGF-1 through PI3K/Akt/GSK-3β. In point of fact, IGF-1 is frequently used to improve dystrophic muscle in muscular dystrophy therapy. Therefore, it is conceivable that the benefits of this IGF-1 treatment may be realized by its ability to antagonize myostatin-inhibitory function through the PI3K/Akt/GSK-3β pathway.

As a strong cell survival cascade, the PI3K/Akt pathway is predominantly activated by factors such as insulin and IGFs. IGF-1 has previously been shown to block TGF-β-induced apoptosis via a PI3K/Akt-dependent pathway (45). Akt is a critical mediator downstream of IGF-1 protection of TGF-β-induced apoptosis, inactivating a number of key proapoptotic proteins, such as caspase-9, Bad, FKHR, and GSK-3β (46). In this study,
we report for the first time that myostatin-treated C2C12 cells undergo apoptosis rather than cell cycle arrest when IGF-1 signaling is blocked, suggesting a principal protective role of IGF-1 in cells exposed to myostatin. These results may partially explain why myostatin causes cell cycle arrest but not apoptosis under normal growth conditions both in vitro and in vivo.

It has been reported that muscle-specific inactivation of the IGF-I receptor induces an initial period of impaired postnatal growth followed by a period of compensatory hyperplasia in skeletal muscles (47). This suggests that the IGF-1 signal plays a crucial role in maintaining homeostasis during muscle development (48). We have shown herein that IGF-1 has the potential to induce myostatin expression via the PI3K/Akt pathway; however, myostatin did not induce IGF-1 expression (data not shown). It is well known that IGF-1 functions as an extrinsic growth factor for promoting muscle development. However, as organ size is tightly controlled, intrinsic cell type-specific mitotic inhibitors, also known as "chalones," are expected (49). Our current findings suggest that myostatin may be an IGF-1-induced chalone produced locally by skeletal muscle cells that function to limit the skeletal muscle growth stimulated by growth-promoting factors, such as IGF-1. Skeletal muscle growth appears to be controlled by multiple signaling pathways. The specificity of each pathway and the cross-talk between these pathways during normal development and disease processes, such as cachexia, remain to be determined.

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