Ankyrin repeat and SOCS box protein 15 regulates protein synthesis in skeletal muscle

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McDanel, T. G., K. Hannon, and D. E. Moody. Ankyrin repeat and SOCS box protein 15 regulates protein synthesis in skeletal muscle. Am J Physiol Regul Integr Comp Physiol 290: R1672–R1682, 2006.—Ankyrin repeat and SOCS box protein 15 (ASB15) is an Asb family member expressed predominantly in skeletal muscle. We have previously reported that ASB15 mRNA abundance decreases after administration of β-adrenergic receptor agonists. Because β-adrenergic receptor agonists are known to stimulate muscle hypertrophy, the objective of this study was to determine whether ASB15 regulates cellular processes that contribute to muscle growth. Stable myoblast C2C12 cells expressing full-length ASB15 (ASB15-FL) and ASB15 lacking the ankyrin repeat (ASB15-Ank) or SOCS box (ASB15-SOCS) motifs were evaluated for changes in proliferation, differentiation, protein synthesis, and protein degradation. Expression of ASB15-FL caused a delay in differentiation, followed by an increase in protein synthesis of ∼34% (P < 0.05). A consistent effect of ASB15 overexpression was observed in vivo, where ectopic expression of ASB15 increased skeletal muscle fiber area (P < 0.0001) after 9 days. Expression of ASB15-SOCS altered differentiation of myoblasts, resulting in detachment of cells from culture plates. Expression of ASB15-Ank increased protein degradation by 84 h of differentiation (P < 0.05), and in vivo ectopic expression of an ASB15 construct lacking both the ankyrin repeat and SOCS box motifs decreased skeletal muscle fiber area (P < 0.0001). Together, these results suggest ASB15 participates in the regulation of protein turnover and muscle cell development by stimulating protein synthesis and regulating differentiation of muscle cells. This is the first study to demonstrate a role for an Asb family member in skeletal muscle growth.

β-adrenergic receptor agonist; protein turnover

THE REGULATION OF MUSCLE GROWTH is a complex process, and many specific mechanisms involved are not fully defined. It is well documented that muscle growth is enhanced by β-adrenergic receptor agonists (BA) (3, 19). Although specific mechanisms underlying the effect of BA on muscle growth are poorly understood (3, 19), they clearly enhance net protein accretion, in part through increased expression of myofibrillar proteins (1, 5, 11, 20). In an effort to identify additional genes that are differentially expressed in response to BA, we completed a differential display experiment to compare bovine skeletal muscle gene expression before and after BA administration (16). ASB15 was significantly downregulated at the mRNA level 24 h after BA administration.

It has been proposed that Asb proteins participate in ubiquitination and proteasomal degradation of targeted cellular proteins in some cell types (9, 24). However, the function of Asb proteins in skeletal muscle has not been investigated. Our initial hypothesis was that ASB15 functions as a negative regulator of muscle growth such that the downregulation of ASB15 by BA facilitates the hypertrophic effects of BA on skeletal muscle. The objective of this research was to determine whether ASB15 regulates cellular processes that contribute to muscle growth. We performed cell culture experiments to determine the effect of ASB15 on muscle cell proliferation, differentiation, and protein turnover and utilized an in vivo model to investigate the impact of ectopic ASB15 expression on muscle fiber area.

MATERIALS AND METHODS

ASB15 constructs. Four expression constructs were utilized for the cell culture and in vivo experiments. An expression construct containing bovine ASB15 (ASB15-FL) under control of the cytomegalovirus (CMV) promoter in the pcDNA3.1 expression vector was generated by amplifying the full-length cDNA sequence (GenBank accession no. NM_174687) from an existing construct (Fig. 1A) and incorporating a 3′ FLAG tag through the reverse PCR primer (Pr1R; Table 1). Bovine ASB15 has been annotated in GenBank to include a series of four ankyrin repeats and a single SOCS box motif. Modified constructs were created by PCR as shown in Fig. 1, using primers listed in Table 1. Modified constructs included ASB15-Ank, ASB15-SOCS, and ASB15-DM, which contained deletions of a single ankyrin repeat, the SOCS box motif, and both a single ankyrin repeat and SOCS box motif, respectively. The integrity of all clones was verified by sequencing. A LacZ construct also was utilized in the in vivo electroporation experiment. Expression of LacZ was driven by a CMV promoter, and the vector was supplied by Center Commercial de Gros.

Cell culture experiment. Proliferating C2C12 myoblasts were maintained in growth medium (GM) consisting of Dulbecco’s modified Eagle’s medium (DMEM) adjusted to contain 10% fetal bovine serum (Life Technologies, Bethesda, MD) and selected antibiotics. Differentiation was induced in all experiments by replacing fetal bovine serum with 2% horse serum in confluent monolayers of myoblasts. Culture conditions were maintained at 37°C and 5% CO2. Stable lines of C2C12 cells were created containing the ASB15-FL, ASB15-Ank, and ASB15-SOCS constructs, as well as the empty pcDNA3.1 expression vector. Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) at ∼50% confluency. Transfection medium was replaced with GM after 5 h, and G418 (400 μg/ml) was added for selection of stable transfectants. Individual colonies were selected after 21 days and expanded into cell lines. Expression of ASB15 and related constructs in stable cell lines was confirmed by Western blot analysis using an anti-FLAG antibody (Sigma, St. Louis, MO) (Fig. 2). Endogenous and exogenous (expressed from the transfected construct) ASB15 mRNA were quantified in cell lines before stimulation of differentiation (0 h; 90% confluent) and 24 and 72 h after stimulation of differentiation by quantitative RT-PCR (QRT-PCR). Primers

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were cultured in 96-well plates at a starting density of 2
cells/well. When cells reached a confluence of 60%, GM supple-
mented with bromodeoxyuridine (BrdU) was added. Proliferation
was measured as BrdU incorporation in a total of 18 wells for each
construct 2, 24, and 36 h after addition of the BrdU-supplemented
GM. Because of a significant time by construct interaction (P < 0.05),
data were analyzed separately for each time point. Data were analyzed
using analysis of variance to evaluate the effect of construct, and
contrasts were defined to describe differences between each construct
and the pcDNA3.1 control line.

Differentiation was evaluated qualitatively by visual evaluation.
Images of cells before stimulation of differentiation (0 h; 90% con-
fluence) and 24 and 96 h after stimulation of differentiation were
captured. Differentiation was evaluated quantitatively by measuring
creatine kinase activity as previously described by Florini et al. (6) in
90% confluent proliferating cells (0 h) and 48 and 96 h after stimu-
lation of differentiation. For each time point, creatine kinase activity
was measured in three wells of a six-well plate for each construct.
Total protein was measured by bicinchoninic acid (BCA) assay
(Molecular Probes, Eugene, OR), and RNA quality was
evaluated by gel electrophoresis. One microgram of total RNA was
to mouse (forward, 5′-ATCGTCCGGCTGCTTCTCT-3′; re-
verse, 5′-GCAGCTCAGCATGATCTCA-3′) and bovine (forward,
5′-GGCTAACCACAAATTCACTCAG-3′; reverse, 5′-CCGAC-
CACAAAAACGAGGTTCCTCAA-3′) ASB15 sequences were used to
distinguish between endogenous and exogenous ASB15 mRNA ex-
pression, respectively. Total RNA was extracted from three wells of
cells representing each construct by using TRIzol reagent following
the manufacturer’s recommended protocol (Invitrogen). Contaminat-
ing DNA was removed by digestion with DNase (DNA-FREE;
Ambion, Austin, TX). Concentration of RNA was determined using
RiboGreen (Molecular Probes, Eugene, OR), and RNA quality was
evaluated by gel electrophoresis. One microgram of total RNA was
reverse transcribed to cDNA using the iScript reagent (Bio-Rad,
Hercules, CA). All QRT-PCR assays were carried out in the Bio-Rad
iCycler in a 25-μl final reaction volume with the use of iQ SYBR
Green Supermix (Bio-Rad). Duplicate reactions were carried out for
each experimental sample (n = 3 per cell line) as well as for standard
controls created from log dilutions (108 to 102) of the ASB15 PCR
product generated from purified plasmid DNA containing each
region; Table 1) to introduce the KpnI restriction enzyme recognition site. D:
ASB15 lacking both the ankyrin repeat and SOCS box sequence motifs (ASB15-DM) was generated from the
ASB15-SOCS construct by using PCR primers Pr2F and
Pr4R (5′ region) and Pr4F and Pr2R (3′ region; Table 1) to intro-
duce the KpnI restriction enzyme recognition site.

Table 1. Sequences of PCR primers used for construction of ASB15 expression construct

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Pr1R</td>
<td>5′-ccgtagatt1ctgtgctgccggtagctgctcctgctgccctagct-3′</td>
</tr>
<tr>
<td>Pr1F</td>
<td>5′-taagctaac1ttaatccctctagccatgctatagccgacctg-3′</td>
</tr>
<tr>
<td>Pr2F</td>
<td>5′-tcatggacacgctccagctgtag-3′</td>
</tr>
<tr>
<td>Pr2R</td>
<td>5′-catgaagccacicagcttagctgtag-3′</td>
</tr>
<tr>
<td>Pr3F</td>
<td>5′-cagctaccgcggagctgactgctgctgccctagcggagct-3′</td>
</tr>
<tr>
<td>Pr3R</td>
<td>5′-gtcgcgcgcggcagtctccttctttagcttgcggagg-3′</td>
</tr>
<tr>
<td>Pr4F</td>
<td>5′-cagctaccgcggagctgactgctgctgccctagcggagcta-3′</td>
</tr>
<tr>
<td>Pr4R</td>
<td>5′-cagctaccgcggagctgactgctgctgccctagcggagcta-3′</td>
</tr>
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See Fig. 1 for structure of ankyrin repeat and SOCS box protein 15 (ASB15) construct. Underlined regions indicate restriction enzyme recognition sites. Bold region indicates sequence of the FLAG epitope tag, R, reverse; F, forward.

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 Plates were rinsed twice with prewarmed DMEM to remove excess differentiation, the tritiated differentiation medium was removed and stimulated to differentiate. Forty-eight hours later, differentiation was measured as disintegrations per minute per plate, normalized for DNA content, but with similar results (data not shown).

Protein synthesis data also were analyzed after being normalized for DNA content. These data also were analyzed after being normalized for DNA content, but with similar results (data not shown).

In vivo experiment. Growing (average body weight: 23 g) and adult (average body weight: 39 g) male NIH Swiss mice were purchased from Harlan (Indianapolis, IN) for in vivo experiments 1 and 2, respectively. All animals were maintained in cages containing three animals at 25°C with a 12:12-h light-dark cycle and had ad libitum access to water and feed (Rodent Laboratory Chow; Ralston Purina, St. Louis, MO) throughout the experiment. All animals were handled in accordance with the protocol approved by the Purdue Animal Care and Use Committee.

Mice were anesthetized via intraperitoneal injection of a mixture of ketamine and xylazine. In both experiments, the left gastrocnemius/soleus muscles were injected with a mixture of 25 μg of LacZ plus 25 μg of pcDNA3.1 constructs. In experiment 1, the contralateral right gastrocnemius/soleus muscles received 25 μg of ASB15-FL plus 25 μg of LacZ. Five minutes after DNA injection, muscles were electrophoresed as previously described (18). Mice were allowed to recover from the electroporation procedure for 2 days before additional treatments were administered. Mice were then divided into two treatment groups for administration of the BA clenbuterol (n = 8) or a vehicle control (n = 5). Clenbuterol (Sigma) was prepared by dissolving in ethyl alcohol and diluting to 0.25 mg/ml with 1:1 PEG-200:PBS. Treatments were administered daily for 7 days via intraperitoneal injections at a dosage of 1 mg/kg body wt. In experiment 2, six mice received injections of 25 μg of ASB15-FL plus 25 μg of LacZ constructs in the contralateral right gastrocnemius/soleus muscles, whereas a second set of six mice were injected with a mixture of 25 μg of ASB15-DM plus 25 μg of LacZ. Five minutes after DNA injection, muscles were electrophoresed as previously described (18). No additional treatments were administered in experiment 2.

For both experiments, mice were killed and gastrocnemius/soleus muscles removed 9 days after electroporation. Muscles were weighed, embedded in Tissue-Tek (Fisher Scientific, Itasca, IL), and stored at −80°C until cryosectioning with a Shandon-Lipshaw cryostat at a thickness of 14 μm. Sections were dried and stored at −20°C. Muscle sections were fixed as previously described (2). Muscle fibers that took up expression constructs were identified by staining for β-galactosidase activity, indicating ectopic expression from the LacZ construct. After staining, sections were coverslipped with Permount (Fisher Scientific), and images from stained sections were captured using a Leaf Micro-Lumina scanning digital camera (Scitex, Tel-Aviv, Israel). Images were imported into Photoshop (Microsoft; experiment 1) or ImageJ (NIH; experiment 2) to quantify muscle fiber area.

We analyzed 6–25 sections from each muscle for each mouse, and 10–20 muscle fibers that stained positive for LacZ were measured for each section.

For experiment 1, body weight gain, muscle weight, and muscle fiber area were analyzed using the Proc Mixed procedure of SAS (22) with fixed effects of construct (ASB15-FL vs. pcDNA), treatment (control vs. clenbuterol), and their interaction. Mice within treatment

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**Fig. 2. Expression of ASB15 constructs in C2C12 myoblasts. A: expression of ASB15-FL was confirmed by Western blot analysis using a mouse anti-FLAG antibody in multiple cell lines (lanes 1–3). Nontransfected C2C12 myoblasts were used as a negative control (lane 4). The cell line represented in lane 1 was used for subsequent experiments. B: expression of ASB15-Ank (lanes 1 and 2) and ASB15-SOCS (lanes 3 and 4) was confirmed in multiple cell lines by Western blot analysis using a mouse anti-FLAG antibody. Cell lines represented in lanes 2 and 4 were used in subsequent experiments.**

(72 h after stimulation of differentiation). For 0 h, cells from ASB15-FL, ASB15-Ank, and pcDNA3.1 control lines were grown to 90% confluency in 60-mm cell culture dishes. Differentiation was stimulated with differentiation medium supplemented with 0.25 μCi/ml tritiated amino acids, and protein synthesis was measured after 2, 12, and 24 h. Similar methods were followed for the 72-h time point, where cells were incubated in differentiation medium for 3 days before addition of differentiation medium supplemented with 0.25 μCi/ml tritiated amino acids. Protein synthesis was measured subsequently at 74, 84, and 96 h after stimulation of differentiation. All plates were washed three times with ice-cold PBS, and cells were scraped into 2 ml of 3 x myosin solubilization buffer (0.9 M KCl, 3 M KH2PO4, 0.15 M K2HPO4, and 0.12 M EDTA, pH 6.5) and lysed by freeze-thawing at −80°C. Total protein concentration was measured by BCA assay (Sigma), and total protein synthesis was measured by precipitating 0.75 ml of cell extract with 0.75 ml of 20% TCA at 4°C for 4 h. Samples were then centrifuged (10,000 g for 10 min), and the pellet was resuspended in 500 μl of tissue solubilizer (Amersham Biosciences, Piscataway, NJ) for counting in 5 ml of scintillation cocktail. The myofibrillar protein fraction was separated by centrifugation of 0.75 ml of cell lysate (1,600 g for 10 min), and the supernatant was precipitated with 0.75 ml of 20% TCA at 4°C for 4 h and centrifuged (10,000 g for 10 min). The resulting pellet was resuspended in 500 μl of tissue solubilizer (Amersham Biosciences) and dispersed in 5 ml of scintillation cocktail. Total DNA content of each plate was quantified using RiboGreen (Molecular Probes). Three plates per construct were evaluated at each time point, and the complete experiment was repeated across two independent replicates. Measurements of disintegrations per minute per plate were log transformed and regressed on time of analysis to determine protein degradation rate as the slope of the regression line. Effective half-life was estimated as previously described (23). Rates of protein degradation and effective half-lives were evaluated using analysis of variance as described for protein synthesis data. These data also were analyzed after being normalized for DNA content, but with similar results (data not shown).

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(72 h after stimulation of differentiation). For 0 h, cells from ASB15-FL, ASB15-Ank, and pcDNA3.1 control lines were grown to 90% confluency in 60-mm cell culture dishes. Differentiation was stimulated with differentiation medium supplemented with 0.25 μCi/ml tritiated amino acids, and protein synthesis was measured after 2, 12, and 24 h. Similar methods were followed for the 72-h time point, where cells were incubated in differentiation medium for 3 days before addition of differentiation medium supplemented with 0.25 μCi/ml tritiated amino acids. Protein synthesis was measured subsequently at 74, 84, and 96 h after stimulation of differentiation. All plates were washed three times with ice-cold PBS, and cells were scraped into 2 ml of 3 x myosin solubilization buffer (0.9 M KCl, 3 M KH2PO4, 0.15 M K2HPO4, and 0.12 M EDTA, pH 6.5) and lysed by freeze-thawing at −80°C. Total protein concentration was measured by BCA assay (Sigma), and total protein synthesis was measured by precipitating 0.75 ml of cell extract with 0.75 ml of 20% TCA at 4°C for 4 h. Samples were then centrifuged (10,000 g for 10 min), and the pellet was resuspended in 500 μl of tissue solubilizer (Amersham Biosciences, Piscataway, NJ) for counting in 5 ml of scintillation cocktail. The myofibrillar protein fraction was separated by centrifugation of 0.75 ml of cell lysate (1,600 g for 10 min), and the supernatant was precipitated with 0.75 ml of 20% TCA at 4°C for 4 h and centrifuged (10,000 g for 10 min). The resulting pellet was resuspended in 500 μl of tissue solubilizer (Amersham Biosciences) and dispersed in 5 ml of scintillation cocktail. Total DNA content of each plate was quantified using RiboGreen (Molecular Probes). Three plates per construct were evaluated at each time point, and the complete experiment was repeated across two independent replicates. Protein synthesis data, measured as disintegrations per minute per plate, were evaluated using analysis of variance to determine the effect of ASB15 constructs. Protein synthesis data also were analyzed after being normalized for DNA content, but with similar results (data not shown).

Protein degradation was measured after 72 h of differentiation in cells from the ASB15-FL, ASB15-Ank, and pcDNA3.1 control lines. Cells were grown to 90% confluency in 60-mm cell culture dishes and stimulated to differentiate. Forty-eight hours later, differentiation medium was replaced with differentiation medium supplemented with 0.25 μCi/ml tritiated amino acids. After 24 h of incubation (72 h of differentiation), the tritiated differentiation medium was removed and plates were rinsed twice with prewarmed DMEM to remove excess tritiated amino acids. Cells were then incubated in fresh differentiation medium with nonlabeled amino acids consisting of 1 mM lysine, 1 mM phenylalanine, 1 mM tyrosine, 1 mM leucine, and 1 mM proline. The differentiation medium was changed every 12 h, and myotubes were harvested at 72, 74, 84, 96, 108, 120, and 132 h after stimulation of differentiation. Cell lysates were harvested as previously described, and the amount of tritiated amino acids remaining in the cells was quantified. Total DNA content of each plate was quantified using RiboGreen (Molecular Probes). Three plates per construct were evaluated for protein degradation at each time point, and the complete experiment was repeated across two independent replicates. Measurements of disintegrations per minute per plate were log transformed and regressed on time of analysis to determine protein degradation rate as the slope of the regression line. Effective half-life was estimated as previously described (23). Rates of protein degradation and effective half-lives were evaluated using analysis of variance as described for protein synthesis data. These data also were analyzed after being normalized for DNA content, but with similar results (data not shown).
were included as a random effect for the analysis of fiber area. Muscle fiber area in experiment 2 was analyzed in a similar manner, with the data from mice expressing different constructs analyzed separately.

Levels of ASB15 mRNA expression were evaluated in skeletal muscles after injection and electroporation to determine how exogenous levels of the expression construct compared with endogenous levels of ASB15. A total of 12 mice received a mixture of 25 μg of LacZ plus 25 μg of pcDNA3.1 constructs into the left gastrocnemius/soleus muscles. The contralateral right gastrocnemius/soleus muscles of six mice received 25 μg of ASB15-FL plus 25 μg of LacZ, whereas the remaining six mice received 25 μg of ASB15-DM plus 25 μg of LacZ. Five minutes after DNA injection, muscles were electroporated as previously described (18). Mice were killed 5 or 9 days after electroporation, and gastrocnemius/soleus muscles were removed. Total RNA from skeletal muscle was extracted using TRIzol reagent following the manufacturer’s recommended protocol (Invitrogen), and contaminating DNA was removed by digestion with DNase (DNA-FREE). The RNA was evaluated and QRT-PCR assay completed as previously described.

RESULTS

Cell culture proliferation and differentiation. Potential effects of ASB15 on muscle cell proliferation and differentiation were evaluated using C2C12 myoblast cells. Proliferation

Fig. 3. Proliferation of ASB15 stable cell lines was measured as bromodeoxyuridine (BrdU) incorporation (absorbance at 450 nm) after 12, 24, and 36 h of proliferation. Stable cell lines expressed ASB15-FL, ASB15-SOCS, and ASB15-Ank. The control line was stably transfected with the empty pcDNA3.1 expression vector. Abs, absorbance. *P < 0.05, significant difference in BrdU incorporation compared with control.

Fig. 4. Abundance of ASB15 mRNA (A) and images of differentiating cells (B) from 4 C2C12 cell lines stably transfected with ASB15 constructs. Stable cell lines expressed ASB15-FL, ASB15-SOCS, and ASB15-Ank. The control line was stably transfected with the empty pcDNA3.1 expression vector. mRNA abundance of ASB15 expressed from the endogenous gene and transfected constructs (exogenous ASB15) was measured using quantitative PCR in proliferating (0 h) and differentiating cells (24 and 72 h after initiation of differentiation). Results are reported as the log starting copy number (LSCN) of ASB15 mRNA. Exogenous ASB15 was not detected (ND) in cells transfected with the pcDNA3.1 expression vector. Images of proliferating myoblasts (0 h) and differentiating cells (24 and 96 h after initiation of differentiation) were obtained from each stably transfected line.
measured as incorporation of BrdU (Fig. 3). Proliferation of ASB15-Ank cells was greater than that of pcDNA3.1 cells at the 12- and 24-h time points (Fig. 3), but there were no differences among other lines at those times. Incorporation of BrdU at 36 h was similar for all lines (Fig. 3).

Expression of endogenous Asb15 was low (<10 copies per μg of total RNA) in proliferating myoblasts (0 h; Fig. 4A) but increased upon stimulation of differentiation. After 72 h of differentiation, Asb15 mRNA levels were ~100-fold greater than in proliferating myoblasts (Fig. 4A). In contrast, expression of exogenous ASB15-FL, ASB15-Ank, and ASB15-SOCS driven by the CMV promoter was consistent across proliferating myoblasts and throughout differentiation. The amount of exogenous ASB15 mRNA driven by the transfected constructs was similar to that of endogenous ASB15 observed after 72 h of differentiation.

No differences in cell morphology, DNA content, or creatine kinase activity were observed among 90% confluent myoblast cells (0 h of differentiation; Figs. 4B and 5). Creatine kinase activity significantly increased upon stimulation of differentiation medium for all lines (Fig. 5D). However, a delay in differentiation due to overexpression of ASB15-FL was apparent based on visual inspection of cells at 24 h of differentiation (Fig. 4B). This observation was confirmed by decreased levels of creatine kinase activity at 48 h of differentiation (Fig. 5D). Despite this initial delay in differentiation, the ASB15-FL cells appeared to ultimately undergo normal differentiation and had similar normalized creatine kinase activity compared with control cells 96 h after stimulation of differentiation (Fig. 5D). No difference in differentiation between the ASB15-Ank and control cells was apparent upon visual evaluation of the cells (Fig. 4B). However, both creatine kinase activity and total protein in the ASB15-Ank line decreased from 48 to 96 h after stimulation of differentiation (Fig. 5, A and B), leading to lower normalized creatine kinase activity at 96 h of differentiation (Fig. 5D). Over the same time period, DNA content of the ASB15-Ank cells remained constant (Fig. 5C). The ASB15-SOCS construct had important effects on differentiation throughout the time period evaluated (Fig. 4B). Visual evaluation of cultures indicated rapid differentiation of ASB15-SOCS cells at 24 h and detachment of cells from the culture plates as early as 48 h after stimulation of differentiation. By 96 h of differentiation, the number of ASB15-SOCS cells on culture plates was significantly less than in other lines (Fig. 4B), but those remaining cells appeared highly differentiated. Total and normalized creatine kinase activity was similar at 48 and 96 h of differentiation in ASB15-SOCS cells but significantly less than that of control cells (Fig. 5, A and D). Similarly, total protein remained similar throughout the time points evaluated, but the ASB15-SOCS cells had less total protein compared with control cells at 48 and 96 h of differentiation (Fig. 5B). Despite the relatively constant levels of creatine kinase activity and total protein, DNA content of the ASB15-SOCS cells decreased dramatically at 96 h, indicating a significant loss in cell numbers between 48 and 96 h of differentiation (Fig. 5C). For this reason, experiments to investigate the effect of ASB15-SOCS on protein synthesis and degradation were terminated.

Cell culture protein synthesis and degradation. Subsequent experiments were done to define the effect of exogenous expression of ASB15 and related constructs on rates of protein synthesis and degradation in differentiating myoblasts. The ASB15-FL line had significantly less total protein compared with the control line 12 and 24 h after stimulation of differentiation (Fig. 6A). However, total protein in ASB15-FL cells

Fig. 5. Creatine kinase activity (A), total protein content (B), total DNA content (C), and creatine kinase activity corrected for total protein (D) were measured in ASB15 stable cell lines. Stable cell lines expressed ASB15-FL, ASB15-SOCS, and ASB15-Ank. The control line was stably transfected with the empty pcDNA3.1 expression vector. All parameters were measured in 90% confluent proliferating myoblasts (0 h) and differentiating cells after 48 and 96 h of differentiation. *P < 0.05, significant difference compared with control.
Fig. 6. Total protein after 0 (A) and 72 h of differentiation (B) was measured in ASB15 stable cell lines. Stable cell lines expressed ASB15-FL and ASB15-Ank. The control line was stably transfected with the empty pcDNA3.1 expression vector. Total protein was measured in 90% confluent proliferating myoblasts (0 h) and differentiating cells after 72 h of differentiation. *P < 0.05, significant difference compared with control.

Fig. 7. Total protein synthesis at 0 (A) and 72 h of differentiation (B) and myofibrillar protein synthesis at 0 (C) and 72 h of differentiation (D) were measured in ASB15 stable cell lines. Stable cell lines expressed ASB15-FL and ASB15-Ank. The control line was stably transfected with the empty pcDNA3.1 expression vector. Differentiation medium (DM) supplemented with 3H-labeled amino acids (3H AA) was added to cells at 0 and 72 h of differentiation. Incorporation of 3H AA was measured at 2, 12, 24, 74, 84, and 96 h after stimulation of differentiation. *P < 0.05, significant difference compared with control.
was significantly greater than in control cells 84 and 96 h after stimulation of differentiation (Fig. 6B). Investigations of rates of protein synthesis showed that differences in total protein between ASB15-FL and control lines could be attributed to differences in the rate of protein synthesis. The rate of protein synthesis in both total protein and myofibrillar protein fractions measured at 24 h was significantly less in ASB15-FL compared with control cells (Fig. 7, A and C), although a transient increase in protein synthesis rate of ASB15-FL cells was observed in the myofibrillar protein fraction at 12 h. In contrast, protein synthesis rates were significantly greater in the ASB15-FL line compared with the control line after 74 h of differentiation (Figs. 6B and 7, B and D). This result was consistent in both the myofibrillar and total protein fractions measured at 84 and 96 h after stimulation of differentiation. No significant difference in total protein or protein synthesis rates was observed between the ASB15-Ank and control lines for the time points of differentiation evaluated, with the exception of a transient increase in protein synthesis in the myofibrillar protein fraction at 12 h of differentiation (Fig. 7C).

Rates of protein degradation did not differ between the ASB15-FL and control lines in either the total or myofibrillar protein fraction (Fig. 8). However, protein degradation rates in the total and myofibrillar protein fractions of the ASB15-Ank line were significantly greater than in the control line between 72 and 132 h after stimulation of differentiation (Fig. 8), accounting for a shorter protein half-life in ASB15-Ank relative to control and ASB15-FL cells (Fig. 8, C and D). The increased rates of protein synthesis and degradation of the ASB15-FL and ASB15-Ank lines, respectively, are reflected in differences in the amount of total protein measured throughout the protein degradation experiment (Fig. 9). Results from all cell culture experiments are summarized in Table 2.

In vivo model body weight gain and muscle weight. An initial experiment was designed to evaluate the interaction between ectopic expression of ASB15-FL and the BA clenbuterol. In this experiment, body weight increased over a 7-day period by 2.52 and 4.25 g for control and clenbuterol-treated mice, respectively \( (P < 0.001) \). In addition, administration of clenbuterol led to a significant increase (0.02 g, \( P < 0.001 \)) in weight of the gastrocnemius/soleus muscles relative to control.
mice. Transfection of expression constructs was successful in vivo, as indicated by positive staining for β-galactosidase activity in muscle fibers (Fig. 10A). Exogenous and endogenous levels of ASB15 mRNA expression were similar within electroporated muscles (Fig. 10B). However, it is important to note that exogenous ASB15 was only expressed by muscle fibers or cells that took up the electroporated expression constructs, whereas all fibers and cells had the potential to express endogenous ASB15. Therefore, muscle fibers that took up the exogenous ASB15 constructs were likely expressing ASB15 at significantly greater levels than normal nontransfected fibers. There was no significant effect of expression vector construct on overall body weight gain or muscle weight for either experiment 1 or experiment 2 (P > 0.05).

In both experiments, ectopic expression of ASB15-FL significantly increased skeletal muscle fiber area (26.6 and 15.3% in experiments 1 and 2, respectively; Fig. 11, A and B). In addition, muscles that expressed the ASB15-DM construct displayed a significant decrease in skeletal muscle fiber area (9.7%) compared with control fibers in experiment 2 (Fig. 11B). Although a significant effect of clenbuterol was observed on body weight gain and muscle weight in experiment 1, clenbuterol did not have a significant effect on muscle fiber area or alter the effects of ectopic expression of ASB15 on skeletal muscle fiber area.

DISCUSSION

The data reported provide evidence that ASB15 promotes muscle growth. This conclusion is based on consistent results from both in vivo and cell culture experiments that demonstrate increased protein synthesis and greater myofiber area associated with ASB15 overexpression. In addition, cell culture experiments revealed a delay in differentiation of myoblasts

![Fig. 10. Expression from electroporated constructs was confirmed by LacZ staining (A) and measurement of ASB15 mRNA abundance (B). A: muscle sections of the gastrocnemius and soleus muscles 9 days after injection and electroporation of constructs were stained in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl2 in PBS for β-galactosidase activity. The blue color was used to identify fibers expressing LacZ from the electroporated construct. B: abundance of ASB15 mRNA expressed from the endogenous gene and from electroporated constructs (exogenous ASB15) was measured using quantitative PCR 5 and 9 days after electroporation. Endogenous and exogenous ASB15 mRNA was measured in mice that received either the empty pcDNA3.1 control construct (pcDNA) or a construct containing ASB15-FL in contralateral limbs, as well as mice that received either pcDNA or a construct containing ASB15-DM in contralateral limbs. Results are presented as the LSCN of ASB15 mRNA. Exogenous ASB15 was not detected in limbs injected with the pcDNA construct.]

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<tr>
<th>Construct</th>
<th>Proliferation</th>
<th>Differentiation</th>
<th>Protein Synthesis</th>
<th>Protein Degradation</th>
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<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>36 h</td>
<td>0h</td>
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<tr>
<td>ASB15-FL</td>
<td>NC</td>
<td>NC</td>
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<td>ASB15-Ank</td>
<td>U</td>
<td>U</td>
<td>NC</td>
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<td>ASB15-SOCS</td>
<td>NC</td>
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Data are summarized by construct and study. An increase, decrease, and no change in data point of interest for each construct is represented by U, D, and NC, respectively, compared with the control line (pcDNA3.1). Differentiation is determined by an increase in creatine kinase levels. Protein synthesis and degradation are represented by the total protein fraction. Protein synthesis data are reported for the 24- and 96-h time points after addition of differentiation medium. Protein degradation data are reported for 132 h after addition of differentiation medium. Data for the ASB15-SOCS line are only presented for the proliferation and differentiation studies.
due to ASB15 overexpression, potentially contributing to muscle growth by providing an opportunity for additional proliferation of myogenic precursor cells. Although other Asb family members have been shown to function in biological processes relating to cell growth (4, 7, 14), tissue development (4, 7, 13, 14, 15), and insulin signaling (24) via interaction with the ubiquitin and proteasomal degradation pathways (12, 26), this is the first research to demonstrate a direct role for an Asb family member in skeletal muscle growth.

The most significant effect of ASB15-FL was that of increased protein synthesis in differentiated muscle cells; significant differences in total protein were consistently observed beginning ~84 h after the stimulation of differentiation. The 35–85% increase in protein synthesis due to ASB15-FL is within the range of that reported for other well-characterized mitogenic proteins (8, 17). Thus we propose that an important function of ASB15 in differentiated muscle cells is the regulation of protein synthesis. Current literature suggests that members of the Asb family regulate cellular functions via interactions between their SOCS box motif and the Elongin B/C complex to initiate ubiquitination and proteasomal degradation of targeted proteins that interact with the ankyrin repeat motif (9, 24). To date, the SOCS box motifs of two Asb family members, ASB8 and ASB6, have been shown to interact with the Elongin B/C complex (9, 24). In addition, Wilcox et al. (24) demonstrated that the ankyrin repeat region of ASB6 interacts with the APS adaptor protein to regulate insulin signaling in 3T3-L1 adipocytes. In light of this model and data from our current experiments, we propose that the ankyrin repeat region of ASB15 interacts with negative regulators of protein synthesis. These interacting proteins are then targeted for ubiquitination and proteasomal degradation via interaction between the SOCS box motif of ASB15 and the Elongin B/C protein complex. In this way, additional ASB15 leads to a reduction of inhibitors of protein synthesis, ultimately resulting in increased rates of protein synthesis. Although our experiments were not designed to define the specific function of the SOCS box and ankyrin repeat motifs of ASB15, expression of the ASB15-Ank mutant showed that the complete ankyrin repeat motif is required for increased protein synthesis rates associated with ASB15, whereas the SOCS box motif is necessary to maintain viable cells throughout differentiation.

No direct effect of ASB15 on protein degradation was demonstrated. However, the increase in protein degradation associated with the ASB15-Ank construct suggests a potential role for ASB15 in protein degradation. It is possible that ASB15-Ank interacted with inhibitors of protein degradation normally targeted by ASB15, but in such a way that they were not ubiquitinated and destined for proteasomal degradation. Alternatively, ASB15-Ank may have altered the interaction of ASB15 with its target proteins such that ubiquitin-proteasomal degradation was enhanced, thereby increasing overall protein degradation.

Cell culture experiments demonstrated important effects of ASB15 on the differentiation of myoblast cells. Evaluation of ASB15 RNA abundance showed that ASB15 is expressed at low levels in proliferating myoblasts but increases dramatically upon differentiation. Confirming ASB15 mRNA expression in cell culture suggests that ASB15 is expressed in cells in vivo, which we have previously reported for whole skeletal muscle samples. This pattern of expression in cell culture is consistent with a potential role for ASB15 in stimulating or permitting differentiation to take place in muscle cells. However, constitutive expression of ASB15-FL resulted in an initial delay in differentiation, suggesting that ASB15 may function as a negative regulator of differentiation. Results from the ASB15-SOCS construct support this hypothesis. The SOCS box motif of the ASB15 gene was removed, but the ankyrin repeat region was intact for this construct. Therefore, it is likely that ASB15-SOCS interacted with appropriate target proteins through the ankyrin repeat region but was unable to target them for ubiquitination and proteasomal degradation. Because ASB15-SOCS was expressed at significantly greater levels than endogenous ASB15 at initial stages of differentiation, this mechanism would permit ASB15-SOCS to have dominant negative activity and block the normal function of endogenous ASB15. Cells expressing the ASB15-SOCS construct differentiated more rapidly than control cells until they ultimately released from the cell culture plates. This result is consistent with unregulated differentiation of cells, culminating in contraction and release of cells from culture plates.

Cells expressing the ASB15-Ank construct appeared phenotypically similar to control cells throughout differentiation. However, unusual results were observed for an important indicator of differentiation in that creatine kinase activity decreased dramatically from 48 to 96 h of differentiation while DNA content remained constant. Mammalian myotubes are not generally thought to undergo dedifferentiation, but this process has been described in C2C12 cells that were genetically modified (21). Although the biological relevance of decreased creatine kinase activity is unclear in our experiment, the
possibility that this is indicative of some type of dedifferentiation event should not be ignored. The increase in protein degradation observed in the ASB15-Ank cells also is consistent with dedifferentiation previously reported for C2C12 cells (21).

Consistent effects of ASB15-FL were observed in cell culture and in vivo experiments as increased rates of protein synthesis in cell culture translated to increased muscle fiber area in vivo. The 15.3–26.6% increase in muscle fiber area attributed to expression of ASB15-FL is less than the 41–51% increase previously reported for IGF-I using the same experimental model (2). However, ASB15-FL consistently stimulated increased muscle fiber area in mice representing two distinct phases of growth: young animals that were rapidly gaining body weight and muscle mass and mature animals that had reached a plateau in muscle growth. In addition, the lack of increase in muscle fiber size from the ASB15-DM construct confirmed that the results observed for ASB15-FL were not simply due to expression of a nonfunctional protein driven by the CMV promoter. The ASB15 construct lacking both the ankyrin repeat and SOCS box motif also produced results in vivo that were consistent with those observed for the ASB15-Ank construct in cell culture in that increased protein degradation in cell culture was seen as decreased muscle fiber area in vivo.

Clenbuterol was administered in the first in vivo experiment to investigate the interaction between clenbuterol and ASB15. Although a significant anabolic response to clenbuterol was confirmed by a difference in body weight change due to clenbuterol administration, clenbuterol did not have a significant effect on muscle fiber area. In this experiment, muscle fibers from the gastrocnemius and soleus muscles were measured, with fibers from the soleus representing the majority of the data. Administration of clenbuterol has been reported to have a greater effect on type II relative to type I muscle fibers (25). Therefore, the lack of a significant effect of clenbuterol on muscle fiber area may be due to the measurement of primarily type I fibers, which are predominant in soleus muscle. Thus, even though no evidence for interaction between ASB15 and clenbuterol was found in this experiment, additional research in this area is warranted.

We initially reported that ASB15 is downregulated upon administration of BA compounds that stimulate muscle growth, and we hypothesized ASB15 to be a negative regulator of muscle growth. In this way, downregulation of ASB15 by BA would facilitate the hypertrophic response of skeletal muscle to BA. In contrast to this initial hypothesis, the data reported provide strong evidence that ASB15 acts as a positive regulator of muscle growth. Although the role of ASB15 as a positive regulator of muscle growth appears inconsistent with its downregulation in response to BA, we propose that this downregulation of ASB15 occurs as part of a feedback mechanism to protect cells against situations of extreme protein synthesis that might occur if both the BA and ASB15 pathways were stimulated simultaneously.

In summary, this research implicates ASB15 as a positive regulator of muscle growth via two potential mechanisms. First, ASB15 acts as a negative regulator of differentiation of proliferating muscle cells. This may permit the additional proliferation of myogenic precursor cells before their recruitment into muscle fibers in vivo. Second, once myoblasts undergo differentiation, ASB15 functions to increase their rate of protein synthesis. Further research is needed to define specific protein targets with which ASB15 interacts in muscle cells and to more clearly describe the relationship between ASB15 and BA-stimulated muscle hypertrophy.

GRANTS

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