Skeletal muscle LIM protein 1 (SLIM1/FHL1) induces α5β1-integrin-dependent myocyte elongation

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Submitted 20 May 2003; accepted in final form 10 August 2003

Skeletal muscle LIM protein 1 (SLIM1/FHL1) induces α5β1-integrin-dependent myocyte elongation. Am J Physiol Cell Physiol 285: C1513–C1526, 2003. First published August 13, 2003; 10.1152/ajpcell.00207.2003.—Skeletal muscle LIM protein 1 (SLIM1/FHL1) contains four and a half LIM domains and is highly expressed in skeletal and cardiac muscle. Elevated SLIM1 mRNA expression has been associated with postnatal skeletal muscle growth and stretch-induced muscle hypertrophy in mice. Conversely, SLIM1 mRNA levels decrease during muscle atrophy. Together, these observations suggest a link between skeletal muscle growth and increased SLIM1 expression. However, the precise function of SLIM1 during skeletal muscle differentiation is not known. This study investigated the effect of increased SLIM1 expression during skeletal muscle differentiation. Western blot analysis showed an initial decrease followed by an increase in SLIM1 expression during differentiation. Overexpression of SLIM1 in C2C12 skeletal muscle cell lines, at levels observed during hypertrophy, induced distinct effects in differentiating myocytes and undifferentiated reserve cells, which were distinguished by differential staining for two markers of differentiation, MyoD and myogenin. In differentiating skeletal myocytes, SLIM1 overexpression induced hyperelongation, which, by either plating cells on poly-L-lysine or using a series of peptide blockade experiments, was shown to be specifically dependent on ligand binding to the α5β1-integrin, whereas in reserve cells, SLIM1 overexpression induced the formation of multiple cytoplasmic protrusions (branching), which was also integrin mediated. These results suggest that SLIM1 may play an important role during the early stages of skeletal muscle differentiation, specifically in α5β1-integrin-mediated signaling pathways.

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This study demonstrates that SLIM1 overexpression in both the Sol8 and C2C12 skeletal muscle cell lines, at levels previously observed during physiological hypertrophy, induces distinct phenotypes in differentiating myocytes compared with reserve cells. The results presented here demonstrate that SLIM1 induces hyperelongation of differentiating Sol8 and C2C12 skeletal muscle myocytes downstream of α5β1-integrin signaling pathways. In addition, SLIM1 overexpression induces branching of skeletal reserve cells. These results suggest that SLIM1 contributes to integrin-mediated signaling pathways in skeletal muscle, particularly those that may be important during differentiation.

MATERIALS AND METHODS

Antibodies and Peptides

Antibodies. The polyclonal, affinity-purified anti-SLIM1 antibody was generated against amino acids 261NKRVFHVQEQQYTV272 from the fourth LIM domain of human SLIM1 (8). The mouse monoclonal myogenin and MyoD antibodies and the goat polyclonal actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-hemagglutinin (HA) antibody was from AMRAD (Victoria, Australia). Rabbit polyclonal antibodies against the phosphoinositide (PI) 3-kinase p85 subunit were purchased from Upstate (Temecula, CA). The rat monoclonal α5β1-integrin antibody was obtained from Chemicon International (Temecula, CA). The anti-rabbit horseradish peroxidase (HRP)- and anti-rabbit and anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG secondary antibodies were from Silenus (AMRAD). The anti-rabbit tetramethylrhodamine isothiocyanate (TRITC)-conjugated IgG secondary antibodies were from Molecular Probes (Eugene, OR).

Peptides and extracellular matrices. Fibronectin, collagen, and polyl-lysine were purchased from Sigma. Laminin was from Life Technologies, and growth factor reduced Matrigel was from Collaborative Biomedical Products (Becton Dickinson). The peptide GRGDNP (RGD) was purchased from Life Technologies, and the DELPQLVTLPHPNLHGPEILDVPST (EILDV) peptide was from Sigma.

Characterization of SLIM1 Antibody

To assess the specificity of the SLIM1 antibody, Triton-soluble lysates prepared from HA-SLIM1- and HA-empty vector-transfected COS-1 cells and from mature skeletal muscle were loaded in parallel with 35S-labeled SLIM1 protein and a negative reticulolysate control on a 12.5% SDS-PAGE. After Western transfer, the lanes containing the 35S-labeled protein were removed from the membrane and visualized by fluorography. The remaining membrane was immunoblotted with the polyclonal, affinity-purified anti-SLIM1 antibody. Immunoblots were developed using anti-rabbit HRP-conjugated secondary antibody (1:20,000) and enhanced chemiluminescence (ECL).

The SLIM1 cDNA was previously cloned into the eukaryotic expression vector pSVTf (9). The pSVTf.SLIM1 construct was linearized with the restriction enzyme XbaI and translated in the presence of [35S]methionine by using the TNT coupled wheat germ extract system (Promega) according to the manufacturer’s protocol. A negative control was also performed in which no DNA was added to the reticulolysate mixture.

Skeletal Muscle Lysate Preparation

Mouse skeletal muscle was homogenized on ice for 3×20 s (Labsonic 1510) in 5× (vol/wt) Triton lysis buffer (30 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Triton X-100, 250 μg/ml PMSF, 400 μM leupeptin, 0.83 mM benzamidine, 5 mM β-mercaptoethanol, and 400 μM aprotinin) and extracted overnight at 4°C. The homogenate was centrifuged at 100,000 g for 1 h at 4°C.

Cell Culture and Differentiation of Sol8 and C2C12 Skeletal Myoblast Cell Lines

The Sol8 (CRL-2174) and C2C12 (CRL-1772) mouse skeletal myoblast cell lines (American Type Tissue Culture, Manassas, VA) were grown at low confluence (<50%) in growth medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1% streptomycin). To induce differentiation, cells were grown to confluence and switched to differentiation medium (DMEM supplemented with 5% horse serum, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1% streptomycin) for a further 24–144 h.

Detection of Endogenous SLIM1 in Differentiating C2C12 Myoblasts

Dishes (100 mm) were coated with 5 μg/ml fibronectin for 24 h and blocked for 1 h with 1% bovine serum albumin (BSA). C2C12 myoblasts were plated at a density of 4.5×104 cells/cm2 and grown for 24 h in growth medium. Myoblasts were differentiated in differentiation medium for 144 h. Cells were washed in phosphate-buffered saline (PBS), and whole cell lysates were prepared by sonication for 5 min (Unisonic) in 500 μl of PBS containing 250 μg/ml PMSF, 400 μM leupeptin, 0.83 mM benzamidine, 5 mM β-mercaptoethanol, and 400 μM aprotinin. Lysates were cleared by centrifugation at 13,000 g for 10 min at 4°C, and the protein concentration of the supernatant was measured, using a Bio-Rad DC protein assay. For Western blot analysis, 20–100 μg of each lysate were separated by SDS-PAGE and immunoblotted with anti-SLIM1, anti-myogenin (1:200; differentiation marker), or anti-PI3-kinase p85 subunit (1:2,000; protein loading control) antibodies. The immunoblots from three separate experiments were analyzed by densitometry, using the Gel Pro Analyzer V3.1 program (Media Cybernetics), and the amount of SLIM1 relative to p85 at each time point was determined. The results are presented as the means of three separate experiments.

Transient Transfection of Sol8 and C2C12 Myoblasts with HA-SLIM1

All peptide blocking experiments were modified from Dasnik and Rando (16). Coverslips were coated with one of either 5 μg/ml fibronectin, 5 μg/ml laminin, 0.3% bovine tendon collagen plus 0.1% growth factor-reduced Matrigel, or 20 μg/ml RGD or EILDV peptides for 24 h at room temperature. Alternatively, coverslips were coated with 1 mg/ml poly-l-lysine for 5 min at room temperature. Before plating, coverslips were blocked for 1 h with 1% BSA, and Sol8 or C2C12 myoblasts were then plated at a density of 4.5×103 cells/cm2 in growth medium. Where indicated, cells were preincubated with 20 μg/ml soluble RGD or EILDV peptides or 10 μg/ml α5β1-integrin-blocking antibody for 30 min before being plated on fibronectin in the presence of additional soluble peptide or antibody, which was replaced every 24 h. The cells were transfected using the pCGN vector (49). pCGN contains a CMV promoter. The open-reading frame of SLIM1
was cloned into the XbaI site, resulting in a recombinant protein with an NH2-terminal HA tag. For all cultures, after 24-h incubation myoblasts were transiently transfected with HA-SLIM1, HA-β-galactosidase (β-gal), or HA-empty vector by using Lipofectamine according to the manufacturer’s protocol (Life Technologies) and grown for an additional 24 h in growth medium. To induce differentiation to myotubes, cells were switched to differentiation medium for 24–96 h. The transfection efficiency varied between 30 and 60%.

To quantitate the level of SLIM1 overexpression, C2C12 cells were lysed with Triton lysis buffer, and to ensure equal protein loading, the protein concentration was measured using a Bio-Rad DC protein assay. Lysates were separated by SDS-PAGE and immunoblotted with the anti-SLIM1, anti-HA (1:5,000), or anti-actin (1:1,000) antibodies. The immunoblots were analyzed by densitometry, and the amount of SLIM1 relative to the amount of actin (protein loading control) was determined for each lysate. The results represent the means of three separate experiments.

**Immunofluorescence of Sol8 and C2C12 Cells**

Cells were gently washed with PBS, fixed and permeabilized with 3% formaldehyde plus 2% Triton X-100 in PBS for 10 min, washed in PBS, and blocked in 1% BSA for 10 min. All antibodies were diluted in 1% BSA in PBS, and each was incubated 1 h at room temperature. Recombinant HA-tagged protein was detected by using mouse anti-HA antibody (1:1,000), followed by incubation with FITC-conjugated anti-mouse IgG secondary antibody (1:400). As indicated, cells were co-stained with either rabbit anti-MyoD (1:200) or anti-myogenin (1:200) antibodies, followed by incubation with TRITC-conjugated anti-rabbit IgG secondary antibody (1:400). Cells were viewed using a Leica TCS NT laser scanning confocal microscope (Monash Micro Imaging, Monash University). The percentage of transfected Sol8 cells demonstrating branching was determined by counting the number of transfected cells with four or more major cytoplasmic projections. The length of Sol8 transfected cells was measured by using Leica TCS NT confocal software, and the average cell length was calculated. Data presented on the average cell length and the percentage of cells demonstrating branching represent the means of at least three separate transfection experiments in which >50 cells were counted. The statistical significance of results was determined by using a Student’s t-test.

**RESULTS**

Effect of SLIM1 Overexpression on Myoblast Differentiation

Skeletal myoblasts induced to differentiate withdraw from the cell cycle and fuse to form myotubes. In the initial stages of differentiation, mononucleated myoblasts form elongated, bipolar myocytes, which fuse to form primary multinucleated myotubes (32, 46). The expression of SLIM1 protein in skeletal muscle myoblasts and differentiating myocytes was determined by using an affinity-purified SLIM1 antibody, described previously, directed against a unique amino acid sequence located in the fourth LIM domain and not found in the alternatively spliced SLIM1 isoforms KyoT2 and SLIMMER, which are translated in an alternate open-reading frame (8). The anti-SLIM1 antibody immunoblotted a 36-kDa polypeptide in HA-

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**Fig. 1.** Skeletal muscle LIM protein 1 (SLIM1) is expressed in skeletal myoblasts and differentiating myocytes. A: characterization of the anti-SLIM1 antibody. Triton-soluble lysates prepared from hemagglutinin (HA)-SLIM1- or HA-empty vector (HA-vector)-transfected COS-1 cells and mature murine skeletal muscle (Sk M) were separated on 12.5% SDS-PAGE in parallel with 35S-labeled in vitro translated SLIM1 and reticulolysate negative control (−ve), in which no DNA was included in the transcription-translation reaction. After Western blot transfer, the lanes containing 35S-labeled protein were removed and visualized by fluorography. The remaining membrane was immunoblotted with the polyclonal anti-SLIM1 antibody. Molecular mass markers are indicated at left. B: whole cell lysates were prepared from undifferentiated C2C12 myoblasts (0 h) and myoblasts induced to differentiate to myotubes for the indicated times. Each lysate was immunoblotted with antibodies for SLIM1, myogenin (differentiation marker), and phosphoinositide (PI) 3-kinase p85 subunit (loading control) as indicated. Migration of the molecular mass markers is shown at left. C: relative expression level of SLIM1. Densitometry was performed on SLIM1 and p85 immunoblots from 3 separate differentiation experiments. The ratio of SLIM1 to p85 expression at each time point (0–144 h) was determined and expressed relative to the ratio at time 0, which was given a value of 1.0. Results represent means ± SD of 3 separate experiments. *P < 0.1, **P < 0.005.
SLIM1-transfected COS-1 lysates consistent with the size of recombinant tagged HA-SLIM1 (molecular mass of HA + molecular mass of SLIM1 = 4 + 32 kDa), which was not detected in HA-empty vector-transfected COS-1 lysates (Fig. 1A). The anti-SLIM1 antibody detected a single 32-kDa polypeptide in Triton-soluble lysates prepared from mature murine skeletal muscle. This is consistent with the predicted molecular mass of SLIM1, and the polypeptide migrated at the same position as 35S-labeled in vitro-translated SLIM1.

Immunoblot analysis using the anti-SLIM1 antibody demonstrated SLIM1 protein expression in undifferentiated C2C12 mouse skeletal myoblasts before the onset of differentiation (0 h) (Fig. 1B). In the first 24–48 h in differentiation medium, there was an initial decline in the level of SLIM1 protein, which then increased as differentiation to myotubes progressed (96–144 h). The differentiation of myoblasts to myotubes was confirmed by evidence of myogenin expression, a specific marker of skeletal muscle differentiation (Fig. 1B) (7, 37). The amount of SLIM1 at each time point was determined in three separate differentiation experiments by densitometry and expressed relative to the amount of the p85 subunit of the PI 3-kinase (p85), the protein loading control (10). An initial decline of 40% in the level of SLIM1 expression over the first 24–48 h in differentiation medium was detected, which then increased up to 144 h (Fig. 1C).

The effect of SLIM1 overexpression was examined in two muscle cell lines, C2C12 and Sol8, both derived from mouse skeletal muscle, to confirm that any phenotypic effect was not limited to one cell line. C2C12 cells rapidly align and fuse to form multinucleated myotubes, which undergo further rounds of fusion to form secondary myotubes and express the contractile apparatus (6, 15). Sol8 cells also align and fuse; however, although they show some signs of multinucleation, this is less evident than in C2C12 cells, and the progression of differentiation is slower and incomplete, allowing for examination of early prefusion muscle differentiation (5, 15, 28).

Sol8 mouse skeletal myoblasts, plated on fibronectin, were transiently transfected with HA-SLIM1, HA-β-gal, or HA-empty vector and induced to differentiate by serum withdrawal for up to 96 h. The level of SLIM1 overexpression in the transfected cells was determined by immunoblot analysis of nontransfected vs. HA-SLIM1-transfected Sol8 cell lysates. Immunoblotting with the anti-SLIM1 antibody demonstrated increased SLIM1 expression in the transfected cells (Fig. 2A). The anti-HA blot demonstrated recombinant HA-SLIM1 expression only in the transfected cell lysates. Equal protein loading was confirmed by immunoblotting for actin. Densitometry of SLIM1 immunoblots from three separate experiments confirmed an average 3.5-fold overexpression of SLIM1 relative to nontransfected cells (Fig. 2A, graph).

To confirm that HA-SLIM1 and HA-β-gal were expressed throughout the differentiation experiment, transfected lysates at time points from 0 to 96 h were immunoblotted with the HA antibody to detect recombinant protein expression. This demonstrated that HA-SLIM1 and HA-β-gal proteins were expressed constantly over the time course of the experiment with minimal proteolysis (Fig. 2B).

The overexpression of HA-SLIM1 in differentiating Sol8 cells induced two distinct phenotypes. First, a significant proportion of SLIM1-expressing cells demonstrated a highly “branched” phenotype with four or more major cytoplasmic protrusions from the cell body (Fig. 2C, e and f, arrowhead; see high-power image in g). The degree of branching in SLIM1-transfected cells appeared more extensive at 96 h but at 48 h (Fig. 2C, e–g). To determine the relative frequency of the branched cell phenotype, the percentage of branched HA-SLIM1- vs. HA-empty vector-transfected cells were compared. By 48 h, almost a quarter of the HA-SLIM1-transfected cells had developed the branched phenotype, and by 96 h, this number had increased to ~50% of the HA-SLIM1-transfected Sol8 cells (Fig. 2D). In contrast, fewer than 5% of the HA-empty vector-transfected cells demonstrated cell branching at any time up to 96 h (Fig. 2D).

In addition, a significant percentage of HA-SLIM1-transfected cells comprised mononuclear, spindleshaped, hyperelongated cells with extremely long bipo-

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**Fig. 2.** Overexpression of SLIM1 induces branching and hyperelongation in differentiating Sol8 skeletal myoblasts plated on fibronectin. A: lysates from nontransfected (UT) and HA-SLIM1-transfected Sol8 cells were analyzed by SDS-PAGE and immunoblotted with anti-SLIM1, anti-HA, and anti-actin (protein loading control) antibodies (top). WB, Western blot. Densitometry was performed on the SLIM1 and actin immunoblots from three separate experiments and represent the level of SLIM1 expression in transfected cells relative to the level in nontransfected cells. B: Sol8 skeletal myoblasts plated on fibronectin were transiently transfected with HA-SLIM1, HA-β-galactosidase (HA-β-gal) or HA-empty vector controls and induced to differentiate in low serum for up to 144 h. Lysates of HA-β-gal- and HA-SLIM1-transfected Sol8 cells differentiated for 0–144 h were analyzed by SDS-PAGE and immunoblotted with HA antibodies. C: HA-β-gal (α–c) or HA-SLIM1-transfected cells (d–g) were plated on fibronectin and grown in differentiation medium for 0, 48, or 96 h as indicated. HA-transfected cells were stained using an anti-HA antibody and visualized using laser scanning confocal microscopy. Arrowheads indicate “branched cells,” and arrows and asterisks indicate hyperelongated myocytes. Image in g is a high-power magnification of cells showing “branched” phenotype. Scale bars: 100 μm (a–f), 150 μm (g). D: percentage of HA-SLIM1 (solid bars, n = 205)- and HA-empty vector-transfected cells (open bars, n = 140) demonstrating a branched phenotype (4 or more cytoplasmic protrusions/cell) was calculated by counting >50 cells in three separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are means ± SE. *P < 0.05. E: average cell length of HA-SLIM1 (n = 285)- and HA-empty vector-transfected cells (n = 170) was determined from three separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are means ± SE. *P < 0.05.
SLIM1-induced Cell Branching and Hyperelongation are Dependent on Ligand Binding to Integrins

The branched and elongated cell shape changes observed in SLIM1-overexpressing Sol8 cells suggested that overexpression of SLIM1 resulted in reorganization of the cytoskeleton after the onset of differentiation. Integrin signaling pathways are important regulators of the cytoskeleton; therefore, we investigated the role of integrin-ligand binding in the development of these phenotypes. HA-SLIM1-transfected myoblasts were plated on poly-L-lysine, which blocks integrin activation, and maintained in differentiation medium for up to 96 h (4). On poly-L-lysine, <5% of the HA-SLIM1-expressing cells were branched and significant elongation was not observed up to 96 h (Fig. 3, Ad, B, and C). The average length of HA-SLIM1-expressing cells after 96 h in differentiation medium was only 70 μm on poly-L-lysine vs. 180 μm on fibronectin (Fig. 3C). These results suggest that SLIM1-induced branching and hyperelongation are dependent on extracellular matrix ligand binding to integrin receptors.

To determine whether SLIM1-induced Sol8 cell branching and elongation were dependent on activation of a specific integrin, Sol8 myoblasts were plated on collagen or laminin, which activate αβ1, α6β1, α5β1, and α7β1, α7β1, α8β1, α6β1 integrins, respectively, distinct from α5β1, α6β1, αβ1, activated by fibronectin (25). By 96 h of differentiation, significant cell branching was observed in HA-SLIM1-expressing cells on fibronectin (52%), collagen (44%), and laminin (71%), compared with poly-L-lysine (5%) (Fig. 3, A, arrowheads, and B). In contrast, hyperelongation of HA-SLIM1-expressing Sol8 cells was observed only on fibronectin (185 μm), not on collagen (98 μm) or laminin (100 μm) (Fig. 3, A, arrows, and C). These results suggest that SLIM1-induced Sol8 hyperelongation is dependent on fibronectin-activated integrins but not collagen- or laminin-activated integrins. Although SLIM1-induced branching was also integrin dependent, it was observed on a variety of extracellular matrixes, including fibronectin, collagen, and laminin.

In control experiments, the length of HA-β-gal-transfected Sol8 cells plated on fibronectin, poly-L-lysine, collagen, or laminin was compared. The average length of HA-β-gal-transfected Sol8 cells increased with differentiation and was the same on fibronectin, collagen, laminin, and poly-L-lysine at each time point (Fig. 3D). The length of HA-β-gal-transfected Sol8 cells plated on collagen and laminin was not significantly different from the length of HA-SLIM1-transfected cells plated on these ligands (Fig. 3, C vs. D). Significantly increased elongation of HA-SLIM1-overexpressing cells compared with HA-β-gal control cells was only observed when the cells were plated on fibronectin (Fig. 3, C and D). Reserve cell branching was observed in <5% of HA-β-gal-transfected cells on fibronectin, laminin, collagen, or poly-L-lysine (results not shown), again demonstrating that this phenotype is dependent on SLIM1 overexpression.

SLIM1-Induced Hyperelongation is Dependent on α5β1-Integrin-Ligand Binding

In myoblasts, the main fibronectin receptors are the α5β1- and α6β1-integrins (16). The α5β1-integrin is activated by the RGD peptide of fibronectin, whereas the α6β1-integrin, is activated by the EILDV region of fibronectin (44, 53). Activation of α5β1 by fibronectin leads to protein kinase C-mediated activation of α5β1 via “inside-out signaling” (16). We investigated the role of α5β1-integrin-ligand binding in the development of SLIM1-induced elongation and branching by using either the RGD peptide or an α5β1-blocking antibody. Immobilized RGD in solid phase activates the α5β1-integrin, whereas soluble free RGD in solution blocks α5β1 activation (44, 53). HA-SLIM1-transfected myoblasts plated on immobilized RGD developed both branched (Fig. 4Ad, arrowhead) and elongated phenotypes (Fig. 4Ad, arrows), identical to the phenotype observed for SLIM1-overexpressing cells plated on fibronectin (Fig. 4Ab). The average length of HA-SLIM1-expressing cells after 96 h of differentiation was 190 μm for cells plated on both fibronectin and immobilized RGD (Fig. 4B). This indicates that ligand binding to the α5β1-integrin in HA-SLIM1-expressing cells is sufficient for induction of both the hyperelongated and branched cell phenotypes. Furthermore, HA-SLIM1-mediated hyperelongation was blocked by incubation with either free blocking RGD (Fig. 4Ah) or α5β1-blocking antibody treatment (Fig. 4Aj) before plating on fibronectin. The average length of the HA-SLIM1-

lar extensions often extending outside the visual field (Fig. 2C, e and f, arrows). Some of the elongated myo-
cytes were also thickened in appearance but remained mononucleated (Fig. 2Cf, asterisk). The hyperelon-
gated phenotype of the HA-SLIM1-transfected cells was evident by 48 h but was most marked at 96 h, by which time ~50% of HA-SLIM1-expressing cells had developed significant hyperelongation (Fig. 2C, e and f, arrow and asterisk).

The average length of HA-SLIM1- vs. HA-empty vector-transfected cells was also determined (Fig. 2E).
Although the HA-SLIM1-transfected cells appeared slightly longer at time 0, this result was not statisti-
cally significant. The cells had been transfected for 24 h and maintained in high-serum growth medium before this time 0 point. It is possible that SLIM1 overexpression itself, before culture in differentiation medium, may have promoted some cell elongation, but this was not statistically significant. After 48 h in differentia-
tion medium, the average cell length of HA-SLIM1-
expressing cells was significantly greater than that of HA-empty vector-transfected cells. By 96 h, the aver-
age length of HA-SLIM1-transfected cells plated on fibronectin was more than double that of the HA-empty vector-transfected cells (190 vs. 90 μm) (Fig. 2E).

Neither the branched nor hyperelongated cell types were observed in HA-β-gal-transfected cells (Fig. 2C, b and c) or in HA-empty vector-transfected cells (images not shown).
Fig. 3. SLIM1-induced hyperelongation and branching is dependent on integrin-ligand interactions. A: HA-SLIM1-transfected Sol8 myoblasts were plated on fibronectin (a, b), poly-L-lysine (c, d), collagen (e, f), or laminin (g, h) and induced to differentiate for 0–96 h as indicated. HA-SLIM1-transfected cells were detected using an anti-HA antibody and viewed using laser scanning confocal microscopy. Arrows indicate SLIM1-induced hyperelongated myocytes; arrowheads indicate branched cells. Scale bar: 100 μm. B: percentage of HA-SLIM1-transfected cells with a branched phenotype (minimum 4 cytoplasmic protrusions/cell) was compared on different surfaces: fibronectin (solid bars, n = 205), collagen (open bars, n = 190), laminin (hatched bars, n = 180), and poly-L-lysine (shaded bars, n = 160). The percentage was calculated by counting >50 cells on each surface in 3 separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are means ± SE. C: average cell length of HA-SLIM1-transfected cells plated on fibronectin (●, n = 285), collagen (○, n = 365), laminin (■, n = 360), and poly-L-lysine (▲, n = 280) was determined from 3 separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are means ± SE. *P < 0.05. D: average cell length of HA-βgal-transfected Sol8 myoblasts plated on fibronectin (●), collagen (○), laminin (■), or poly-L-lysine(▲) was determined from 3 separate transfection experiments at 0, 48, and 96 h in differentiation medium. Values are means ± SE.
Fig. 4. SLIM1-mediated myocyte hyperelongation is dependent on αβ1-integrin-ligand binding. A: HA-SLIM1-transfected Sol8 myoblasts were plated on fibronectin (a, b) or immobilized RGD peptide (c, d) and induced to differentiate for 0–96 h. Where indicated, cells were pretreated with blocking, soluble RGD peptide (g, h) or αβ1 integrin-blocking antibody (i, j) in addition to plating on fibronectin. HA-SLIM1-transfected cells were detected using an anti-HA antibody and viewed using laser scanning confocal microscopy. Arrows indicate SLIM1-induced hyperelongated myocytes; arrowheads indicate branched cells. Scale bars: 100 μm. B: average cell length of HA-SLIM1-transfected cells plated on fibronectin (●, n = 285), immobilized RGD (○, n = 200), fibronectin with soluble RGD (◆, n = 230), or fibronectin with blocking αβ1 antibody (○, n = 210) was calculated from 3 separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are ± SE. *P < 0.05. C: average cell length of HA-βgal-transfected cells plated on fibronectin (●), immobilized RGD (○), or fibronectin with soluble RGD (◆) was calculated from 3 separate transfection experiments at 0, 48, and 96 h in differentiation medium. Values are ± SE. D: percentage of HA-SLIM1-transfected cells plated on fibronectin (solid bars, n = 205), fibronectin with soluble RGD (open bars, n = 210), or fibronectin with blocking αβ1 antibody (hatched bars, n = 255) demonstrating a branched phenotype (minimum 4 cytoplasmic protrusions/cell). Data represent means ± SE of 3 separate transfection experiments in which >50 cells were counted.
expressing cells in the presence of free blocking RGD (82 μm) or the α5β1-blocking antibody (78 μm) was significantly less than the average cell length detected on fibronectin alone (190 μm) (Fig. 4B). In control experiments, RGD peptide in solution did not block the elongation of HA-β-gal-transfected Sol8 cells plated on fibronectin (Fig. 4C), demonstrating that myocyte elongation during differentiation is not specifically dependent on α5β1 integrin.

In addition, neither RGD in solution nor the α5β1-blocking antibody inhibited the formation of branched HA-SLIM1-expressing cells (Fig. 4A, h and j, arrowheads). By 96 h, the proportion of HA-SLIM1-expressing cells demonstrating branching was not significantly different in cells plated on fibronectin alone (52%) or on fibronectin with specific blocking of the α5β1-integrin by free RGD peptide (42%) or α5β1-blocking antibody (54%) (Fig. 4D). These studies indicate that SLIM1-induced hyperelongation of differentiating Sol8 cells requires α5β1-integrin-ligand interactions. In contrast, the HA-SLIM1-induced branched phenotype, while dependent on integrin-ligand binding, does not appear to be dependent specifically on the α5β1-integrin.

**SLIM1-Induced Hyperelongation Is Not Dependent on α5β1-Integrin-Ligand Binding**

The importance of the α5β1-integrin in the SLIM1-induced phenotypes was investigated using solid-phase and soluble EILDV peptide (53). By 96 h in differentiation medium, the average length of HA-SLIM1-expressing cells plated on immobilized EILDV was 117 μm (Fig. 5, Ad and B), significantly less than the 180 μm observed on fibronectin (Fig. 5, Ab, arrow, and B). This indicates that extracellular ligand binding to the α5β1 alone is not sufficient to mediate the hyperelongated phenotype in SLIM1-overexpressing cells. Furthermore, treatment of HA-SLIM1-expressing cells with soluble blocking EILDV, before plating on fibronectin (170 μm), did not impair SLIM1-induced hyperelongation (180 μm) (Fig. 5, Af, arrow, and B). The elongation of control HA-β-gal-transfected cells was similar when plated on solid-phase EILDV and fibronectin (89 vs. 90 μm) (Fig. 5C). Soluble blocking EILDV peptide did not significantly alter the length of differentiating HA-β-gal-transfected cells (Fig. 5C). Similarly, soluble blocking EILDV peptide did not significantly inhibit the percentage of HA-SLIM1-expressing myoblasts developing the branched phenotype (data not shown).

Collectively, these results demonstrate that SLIM1 overexpression in differentiating Sol8 myoblasts results in two distinct phenotypes: branched cells and hyperelongation. The branched cell phenotype was dependent on integrin-matrix interactions, given that it was inhibited on poly-L-lysine, but not ligand binding to a specific integrin. In contrast, SLIM1-induced hyperelongation was dependent on ligand binding to the α5β1-integrin.

**SLIM1 Induces Branching in Reserve Cells and Hyperelongation in Differentiating Myocytes**

The HA-SLIM1 overexpression studies were repeated in the C2C12 skeletal muscle cell line, which differentiates more rapidly and completely into multinucleated myotubes, with Z-line assembly (5, 6, 15, 28). In the early stages of differentiation, up to 48 h in differentiation medium, HA-SLIM1 overexpression induced phenotypic changes in the C2C12 cell line identical to those observed in the Sol8 muscle cell line (Fig. 6Ad). HA-SLIM1-overexpressing C2C12 cells became branched (Fig. 6Ad, arrowhead; see high-power image in Ae) or hyperelongated (Fig. 6Ad, arrows). Neither of these phenotypes was observed in control HA-β-gal-transfected C2C12 cells (Fig. 6Ab). We performed peptide blocking experiments in C2C12 cells as described for the Sol8 cell line above and confirmed that the SLIM1-induced hyperelongated phenotype was also dependent on α5β1-integrin-ligand interactions (data not shown).

Previous studies have shown that ~50% of myoblasts differentiating in cell culture conditions become bipolar myocytes, which then fuse to form multinucleated myotubes (56). The other 50% of cells remain mononucleated, undifferentiated “reserve cells,” which represent the in vitro equivalent of in vivo satellite cells. Satellite cells mediate muscle repair and regeneration (21). We hypothesized that SLIM1 overexpression induced branching in the reserve cells and that hyperelongation occurred in differentiating myocytes. Reserve cells and differentiating myocytes may be distinguished by their differential staining for the skeletal muscle-specific transcription factors MyoD and myogenin (56). Differentiating myocytes stain positive for MyoD and myogenin. In contrast, reserve cells express neither MyoD nor myogenin. (56). HA-SLIM1-expressing C2C12 cells (green) differentiated for 48 h were costained for MyoD (Fig. 6B, a and b) or myogenin (Fig. 6B, c and d) with specific antibodies. MyoD and myogenin stain the nuclei red in these images. Branched HA-SLIM1-expressing C2C12 cells did not stain for MyoD (Fig. 6Ba) or myogenin expression (Fig. 6Bc), confirming their identity as reserve cells. In contrast, the hyperelongated HA-SLIM1-expressing cells stained positively for MyoD, indicated by red/yellow nuclei due to colocalization of HA-SLIM1 (green) with MyoD (red) staining in the nucleus (Fig. 6Bb). Furthermore, the hyperelongated, HA-SLIM1-expressing cells also stained positive for myogenin (Fig. 6Bd). Therefore, in differentiation medium, overexpression of SLIM1 induced cell branching in reserve C2C12 cells and hyperelongation in differentiating myocytes.

**DISCUSSION**

SLIM1 is highly expressed in striated muscle, and increased SLIM1 levels have been associated with both skeletal and cardiac muscle hypertrophy (23, 24, 31, 33, 39, 40). However, the function of SLIM1 is unknown. In this study we demonstrated that SLIM1 is expressed in undifferentiated myoblasts and declines...
in the first 48 h of differentiation, before then again increasing upon differentiation into myotubes. The focus of this report was to characterize the cellular effect of increased levels of SLIM1, which are increased 2- to 4-fold in stretch-induced skeletal muscle hypertrophy (33) and up to 10- to 15-fold in postnatal skeletal muscle hypertrophy (38), in differentiating skeletal myocytes.

Fig. 5. SLIM1-mediated myocyte hyperelongation is not dependent on αβ1-integrin-ligand binding. A: HA-SLIM1-transfected Sol8 myoblasts were plated on fibronectin (a, b) or immobilized EILDV peptide (c, d) or treated with soluble, blocking EILDV peptide while plated on fibronectin (e, f) and induced to differentiate for 0–96 h as indicated. HA-SLIM1-transfected cells were detected using an anti-HA antibody and viewed using laser scanning confocal microscopy. Arrows indicate SLIM1-induced hyperelongated myocytes; arrowheads indicate branched cells. Scale bars: 100 μm. B: average length of HA-SLIM1-transfected cells plated on fibronectin (●, n = 285), immobilized EILDV (○, n = 200), or fibronectin with soluble EILDV (▲, n = 200) was determined from 3 separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are means ± SE. *P < 0.05. C: average length of HA-gal-transfected cells plated on fibronectin (●), immobilized EILDV (○), or fibronectin with soluble EILDV (▲) was determined from 3 separate transfection experiments at time points from 0 to 96 h in differentiation medium. Values are means ± SE.

Fig. 6. SLIM1-induced hyperelongation in MyoD- and myogenin-positive myocytes and cell branching in MyoD- and myogenin-negative reserve cells. A: HA-gal (a, b)- or HA-SLIM1-transfected C2C12 skeletal muscle myoblasts (c–e) were plated on fibronectin and induced to differentiate in low serum for 0–48 h as indicated. HA-transfected cells were detected using an anti-HA antibody. Cells were viewed using laser scanning confocal microscopy. Arrows indicate SLIM1-induced hyperelongated cells; arrowheads indicate SLIM1-induced branched cells. e: high-power image of SLIM1-induced branched cells. Scale bars: 150 μm. B: HA-SLIM1-expressing C2C12 skeletal myoblasts were plated on fibronectin and maintained in differentiation medium for 48 h. Branched HA-SLIM1-expressing cells (a, c) and hyperelongated HA-SLIM1-expressing cells (b, d) were detected with an anti-HA antibody (green) and counterstained with MyoD (a, b) or myogenin (c, d) antibodies (red). Cells were viewed using laser scanning confocal microscopy. Coexpression of HA-SLIM1 and MyoD or myogenin is observed as yellow or red staining of nuclei in merged images. Scale bars: 100 μm.
During muscle differentiation, reorganization of the cytoskeleton results in myoblast elongation and formation of myocytes, which align and fuse to form multinucleated myotubes (32, 46). A subpopulation of cells, termed reserve cells, which are the tissue culture equivalent of in vivo satellite cells, remain undifferentiated and mononucleated (56). Overexpression of SLIM1 promoted branching in undifferentiated reserve cells, and α5β1-integrin mediated hyper-elongation of differentiating myocytes. Several studies have implicated LIM proteins in the regulation of skeletal muscle differentiation. Overexpression of MLP promotes myogenesis by enhancing the activity of the muscle-specific transcription factor MyoD (1, 26). Similarly, overexpression of FHL2 promotes differentiation by binding to β-catenin (35). However, the appearance of hyperelongated myocytes or branched reserve cells was not reported after the overexpression of either MLP or FHL2. In addition, overexpression of another four-and-a-half LIM protein related to SLIM1, FHL3, did not induce hyperelongation in differentiating myocytes (data not shown). Branching or “arborization,” however, has been reported previously (14, 22), when replicating, precursor myogenic cells or postmitotic myoblasts are treated with cytochalasin B, which inhibits actin polymerization. These arborized cells did not stain for myosin and did not demonstrate ordered hexagonal arrays of thick and thin filaments.

The branching of reserve cells induced by SLIM1 overexpression suggests that SLIM1 promotes cytoskeletal reorganization in these cells, which is dependent on integrin-ligand binding. There is little published literature on cytoskeletal changes and regulation in either reserve cells or their in vivo equivalent, satellite cells. Activated satellite cells use filopodia-like projections, “branching” to enable migration through the sarcolemma, to regenerate damaged muscle (34). Whether this branching also occurs in activated satellite cells in skeletal muscle hypertrophy, is unknown. Therefore, the branching of reserve cells possibly indicates cytoskeletal rearrangement necessary for fusion of activated satellite cells with existing muscle fibers, resulting in muscle regeneration and hypertrophy.

Overexpression of the α5-integrin subunit in quail myoblasts promotes proliferation and inhibits differentiation under high-serum conditions, whereas differentiation is not affected in low-serum conditions (47). However, neither hyperelongation of myocytes nor branching of reserve cells resulted from overexpression of the α5-integrin, suggesting that SLIM1 overexpression may not directly activate the α5β1-integrin but, rather, may modify specific pathways downstream of this integrin.

Integrins are important regulators of the actin cytoskeleton and perform diverse roles during muscle differentiation, including regulation of proliferation, differentiation, and hypertrophic signaling (3, 5, 11, 27, 43). Skeletal muscle-specific integrins are differentially expressed throughout muscle differentiation (20). The α5β1-integrin is expressed in myoblasts and during primary myogenesis but is downregulated in mature muscle. The α5β1-integrin is critical during the early stages of muscle development (51, 55). Mice deficient in the α5-integrin subunit die early in embryogenesis, and posterior somite development is impaired. Furthermore, mice chimeric for expression of the α5-integrin develop a form of muscular dystrophy (51). In view of our finding that SLIM1-induced elongation is dependent on ligand binding specifically to the α5β1-integrin, which binds fibronectin, it is noteworthy that extracellular fibronectin is increased in skeletal and cardiac muscle hypertrophy (18, 20, 29, 48). Similarly, elevated SLIM1 mRNA expression has been associated with postnatal skeletal muscle growth and stretch-induced muscle hypertrophy (33, 39, 40).

SLIM1 induction of hyperelongation in myocytes and branching in reserve cells indicates that SLIM1 may participate in integrin signaling pathways that regulate the cytoskeleton, a process that is necessary to facilitate differentiation. The participation of SLIM1 in integrin signaling is consistent with its integrin-dependent localization to focal adhesions in skeletal muscle myoblasts (45). Furthermore, SLIM1 has been shown to regulate integrin-mediated functions in myoblasts, including cell adhesion, spreading, and migration (44). However, although SLIM1 contains four LIM domains, which predict for protein-protein interactions, no SLIM1 binding partners have been identified to date. This is in contrast to the related family members FHL2 and FHL3, which are also highly expressed in striated muscle (19, 40). FHL2 and FHL3 have recently been shown to regulate Rho-specific signals to the nucleus (42). Rho is a potent regulator of actin cytoskeleton stress fibers. However, unlike FHL2 and FHL3, SLIM1 does not activate Rho-mediated transcription. FHL2 also binds to various integrin α- and β-subunits, including the muscle-specific integrin subunits α7A, β1A and β1D (52). Recently, we demonstrated that both FHL2 and FHL3 and not SLIM1 bind to skeletal actin (13). Furthermore, overexpression of FHL3 inhibited α-actinin-mediated actin bundling and promoted cell spreading. This suggests that SLIM1 regulation of the cytoskeleton is via pathways distinct from those of FHL2 and FHL3.

In an earlier study we demonstrated that the localization of SLIM1 in Sol8 myoblasts is regulated by integrins, specifically α5β1. α5β1 ligand binding promotes SLIM1 localization to both the nucleus and focal adhesions in undifferentiated myoblasts. Furthermore, SLIM1 overexpression promotes spreading and migration of undifferentiated myoblasts on fibronectin (45). These myoblasts were maintained in high serum and were proliferating, unlike the differentiating conditions used in the current study. The results indicate an important functional link between SLIM1 and the α5β1-integrin, which regulates SLIM1 localization in myoblasts and also mediates the cytoskeletal effects of overexpression in undifferentiated myoblasts, differentiating myocytes, and reserve cells.

SLIM1 is highly expressed in skeletal and cardiac muscle and has been implicated in skeletal muscle hypertrophy. This current study demonstrates distinct
phenotypes specific for the effect of SLIM1 overexpression in myoblasts vs. reserve cells stimulated to differentiate. The results presented here demonstrate that SLIM1 induces elongation in differentiating Sol8 and C2C12 skeletal muscle myoblasts, downstream of αβ-integrin signaling pathways. Previous studies have shown that the αβ-integrin plays an important role during early muscle development and during hypertrophy. In addition, SLIM1 overexpression induces branching of skeletal muscle reserve cells. Taken together, these findings suggest that the FHL family of LIM proteins may perform important roles in integrin signaling and the regulation of cytoskeletal dynamics during myogenic differentiation.

We acknowledge Dr. Mark Prescott and Denny Cottle (Department of Biochemistry and Molecular Biology, Monash University) for providing the plasmid of HA-β-gal.

DISCLOSURES

This study was supported by National Health and Medical Research Council of Australia Grant 143541. M. McGrath is a recipient of a National Heart Foundation Fellowship. S. Brown is a recipient of a National Heart Foundation Postgraduate Scholarship. M. McGrath is a recipient of a National Heart Foundation Fellowship.

REFERENCES

29. Loughna PT, Mason P, Bayol S, and Brownson C. The LIM-domain protein FHL1 (SLIM1) exhibits functional regulati