Laminin-Induced Activation of Rac1 and JNKp46 Is Initiated by Src Family Kinases and Mimics the Effects of Skeletal Muscle Contraction†

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ABSTRACT: Binding of laminin to dystroglycan in the dystrophin glycoprotein complex causes signaling through dystroglycan-syntrophin-grb2-SOS1-Rac1-PAK1-JNK. Laminin binding also causes syntrophin tyrosine phosphorylation to initiate signaling. The kinase responsible was investigated here. PP2 and SU6656, specific inhibitors of Src family kinases, decreased the amount of phosphotyrosine syntrophin and decreased the level of active Rac1 in laminin-treated myoblasts, myotubes, or skeletal muscle microsomes. c-Src and c-Fyn both phosphorylate syntrophin, and inhibition of either with specific siRNAs diminishes the level of syntrophin phosphorylation. When the rat gastrocnemius was contracted, the level of Rac1 activation increased compared to that of the relaxed control muscle and Rac1 colocalized with β-dystroglycan. Similar results were obtained when the muscle was stretched. Contracted muscle also contained more activated c-Jun N-terminal kinase, JNKp46. E3, an expressed protein containing only laminin domains LG4 and LG5, increased the rate of proliferation of myoblasts, and PP2 prevented cell proliferation. In addition, Src family kinases colocalized with activated Rac1 and with laminin-Sepharose in solid-phase binding assays. Thus, contraction, stretching, or laminin binding causes recruitment of Src family kinase to the dystrophin glycoprotein complex, activating Rac1 and inducing downstream signaling. The DGC likely represents a mechanoreceptor in skeletal muscle-regulating muscle growth in response to muscle activity. Src family kinases play an initiating and critical role.

In skeletal muscle, dystrophin, dystroglycan, and syntrophins are found in the dystrophin glycoprotein complex, whose defects cause muscular dystrophies. Duchenne muscular dystrophy is the absence of dystrophin and the most common progressive muscle-wasting disease in humans (*1*). Congenital muscular dystrophy results from alterations in laminin−dystroglycan interaction (*2*). Either type of muscular dystrophy would disrupt the normal interaction of DGC[1] with laminin. We showed that laminin binding causes signaling through dystroglycan-syntrophin-grb2-SOS1-Rac1-PAK1-JNK that ultimately results in the phosphorylation of c-jun on Ser[63] (*3*). We have proposed that this or other cell signaling, which results from the DGC−laminin interaction, may serve a role in these pathologies. Although many activities of the DGC are known, its function is unclear.

Laminin is an $\alpha\beta\gamma$ heterotrimer. It binds to both dystroglycan and integrins in five globular domains (i.e., LG domains) of laminin's α-subunit. Laminin α2 chain LG modules 4 and 5 bind to the acidic polysaccharide chains of αDG (*4*); the integrin binding site in the LG1−5 region has not been mapped in detail (*5*). The binding site for αDG also is localized to the LG4 and LG5 modules of laminin α5; however, the binding site for α3β1 and α6β1 integrins localizes to LG1−3 (*6*). In laminin-1 (α1β1γ1), it is LG4 that binds αDG (*7*). In the studies presented here, the LG4−5 region of laminin α1 was expressed and is termed the E3 protein (*8*).

Laminin, or E3, binds to α-dystroglycan and initiates cell signaling cascades. Recently, E3 has been shown to substitute for laminin and cause tyrosine phosphorylation of syntrophin and alter binding of grb2 to initiate signaling (*9*). E3 or laminin binding also results in binding of heterotrimeric G-protein to the DGC (*10*). Binding of laminin to αDG also activates the PI3K/Akt pathway and inhibits apoptosis (*11*).

One essential problem with this laminin-induced signaling is that laminin is tightly bound by αDG in vivo, and it is unlikely that it ever dissociates. We hypothesize that it is not binding that normally activates signaling but rather stresses put on the laminin−dystroglycan interaction during muscle stretching or contraction that may initiate this signaling. Here, we test this hypothesis. We show that Rac1 colocalizes with β-dystroglycan on the sarcolemma of the rat gastrocnemius and Rac1 and JNK-p46 become active when muscles stretch or tension develops, showing that these stimuli can also initiate the same kinds of signaling that laminin binding does. Furthermore, the kinase which tyrosine

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[1] Abbreviations: DGC, dystrophin glycoprotein complex; JNKp46, 46 000 Da isoform of c-Jun N-terminal kinase; LG, laminin globular domains; E3, expressed protein containing only α1-laminin domains LG4 and LG5; αDG, α-dystroglycan; βDG, β-dystroglycan; αSG, α-sarcoglycan; Syn, syntrophin; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; PAK1, p21-activated kinase.

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phosphorylates syntrophin had not been identified, and experiments presented here show that Src family member kinases phosphorylate syntrophin. Src family tyrosine kinases also comprise a major group of cellular signals in various tissue types. These kinases regulate cellular functions, including mitogenesis, cell cycle progression, adhesion, and migration (*12*). The consequence of this signaling was also investigated. In C2C12 myoblasts, laminin E3 increased the rate of proliferation, and this was inhibited by inhibitors of the Src family kinases. Src family kinases also colocalize with activated Rac1 in solid-phase binding assays when laminin is present. PP2 or SU6656, each a specific inhibitor of Src family kinases, decreased the amount of activated Rac1 and inhibited activated Src (autophosphorylated on Tyr^{416}). These results indicate that laminin binding or muscle contraction or stretching causes recruitment of Src family members to the DGC and syntrophin phosphorylation and initiates Rac1 activation and downstream signaling. This may be an important contributor to the signals that maintain muscle mass, and the DGC may function as a mechanoreceptor.

MATERIALS AND METHODS

Materials. Antibodies against phospho-Tyr, phospho-Src (Tyr^{416}), c-Src, c-Fyn, and integrin β1 were from Santa Cruz Biotechnology. Rac1 and αDG antibodies were from Upstate Biotechnology. βDG was the generous gift of Tamara C. Petrucci (Laboratorio di Biologia Cellulare, Instituto Superiore di Sanita, Via le Regina Elena, Roma, Italy). Goat anti-mouse IgG (H+L)−horseradish peroxidase conjugate was from Sigma. Goat anti-mouse IgM (H+L)−horseradish peroxidase conjugate and goat anti-rabbit IgG were purchased from Southern Biotechnology Associates Inc. Mouse laminin-1 was obtained from Collaborative Biomedical Products. PP3, PP2, and SU6656 were from Calbiochem-Novabiochem. Purified, active c-Src was purchased from Millipore-Upstate Cell Signaling. The mouse C2C12 myogenic cell line was obtained from the American Type Culture Collection (Rockville, MD). All other chemicals were of the highest purity available commercially.

Merosin was purified from 15 g of rat skeletal muscle as described previously (*13*).

E3 was purified from the hLNa1-E3 293 (HEK293) cell line transfected with the LG4−5 domains of human laminin α1, which was generated as described previously (*8*). Briefly, the cultured medium was harvested at 24 and 48 h. Diluted medium was loaded onto a 25 mL DEAE-Sepharose column whose outlet was connected to a 5 mL Hi-trap (Pharmacia) heparin column. The eluted protein was detected by absorption at 280 nm or by gel electrophoresis (*14*). The concentration of E3 was determined (*15*) using bovine serum albumin as a standard.

Syntrophin and Other Fusion Proteins. Syntrophin and syntrophin A were produced as His-tag fusion proteins and purified as previously described (*16*). Additionally, an αSG fusion protein was produced. Mouse RNA (1 μg) was used with random hexamers to produce a crude cDNA template for polymerase chain reaction (PCR) using the Invitrogen DNA cycle kit and AMV reverse transcriptase. This was then used as a template for PCR using 10 pmol each of 5′-ATGGCGGCGGCCGCG (αSG 255−269) and 5′-cggaatTCAGTGCTGGTCCAGGATG (αSG 1400−1418) as upstream and downstream primers, respectively. The bold sequence shows an EcoRI restriction site engineered for directional cloning. This was then first cloned into pMALc and then subcloned into pET28a and expressed in *Escherichia coli* strain BL21, and positive clones were selected by miniprep and restriction mapping. Positive clones were confirmed by DNA sequencing, and the protein was expressed and purified as described for the syntrophin fusion proteins.

siRNA Transfection. C2C12 cells ($2-4 \times 10^5$ per well) on a six-well plate were transiently transfected with 10 μM control siRNA or with siRNAs specific for c-Src, c-Fyn, or mixed c-Src and c-Fyn siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA). The transfection was according to the protocol provided by the manufacturer. Cells were harvested 48 h after incubation and washed with PBS. Cells were then lysed in RIPA (PBS, 1% SDS, 0.5% DOC, and 1% NP-40) buffer, and this was used for several experiments.

In Vitro c-Src Assay. Catalytically active c-Src from Upstate Biotechnology was used for in vitro phosphorylation following the procedure of Dowler et al. (*17*), except that 25 μM Na_4VO_3 and 2.5 mM β-glycerophosphate were included as phosphatase inhibitors and 25 μM ATP was used. The final assay contained 20 000 cpm of [γ-^{32}P]ATP, 5 μg of either syntrophin A or α-sarcoglycan, and 1 unit of c-Src. Assays (20 μL) were incubated for 10 min at 30 °C. Samples were mixed with an equal volume of twice-concentrated SDS−PAGE sample buffer, and after electrophoresis, the gel was dried and used to expose photographic film for autoradiography.

Cell Culture and Cell Proliferation Assay. Mouse C2C12 cells were grown and maintained as myoblasts in DMEM with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. For cell growth experiments, cells were plated in six-well plates and grown in medium containg E3 for 24 or 48 h. Cells were counted after trypsinization using trypan blue. Propidium iodide was incorporated into nuclei after using 70% methanol to fix the cells. Samples were analyzed by flow cytometry. For experiments with myotubes, myoblasts were cultured as described above to 80% confluence, and then the medium was changed to DMEM containing 1% FBS and grown for a further 5 days to allow fusion to myotubes. Myotubes or myoblasts were washed with PBS and harvested by adding trypsin/EDTA solution [0.05% trypsin, 0.02% EDTA in Hank's balanced salt solution (HBSS)] for 5 min at 37 °C. The cells were then immediately suspended in DMEM containing 10% FBS and removed from the plate. This treatment has been previously shown to also deplete the cells of laminin but not DGC components (*9*).

Preparation of Contracted Muscle. Sprague-Dawley rats (200−300 g) were used. Animals were housed in light- and temperature-controlled quarters where they received food and water *ad libitum*. Animals were anesthetized with isofluorane for surgery and tissue removal. Muscle was contracted by electric stimulation of the sciatic nerve (10 V, 10 Hz, 1 min) in one hindleg and either immediately frozen in liquid nitrogen or fixed and embedded in Tissue-Tek O.C.T. compound at −20 °C. Samples were stored at −80 °C until they were used. In other experiments, one Achilles tendon was severed and the muscle was stretched to approximately

125% of its relaxed length by pulling on the tendon with hemostats for 1 min. In all experiments, the contralateral muscle was treated the same except that it was not stimulated. All animal experiments were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center.

Preparations of Microsomes from Skeletal Muscle and C2C12 Cells. Frozen rabbit (3 g) or frozen rat skeletal muscle (0.5 g) was cut into very fine slices using a razor blade and then homogenized in 7 volumes of pyrophosphate buffer [20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.303 M sucrose, and 0.5 mM EDTA (pH 7.0)] in the presence of a cocktail of protease inhibitor as described previously (*3*). The homogenate was centrifuged at 13000*g* for 15 min at 4 °C. The supernatant was centrifuged for 30 min at 32500*g* to pellet muscle microsomes. For making C2C12 cell membranes, 10^6-10^7 cells were suspended in 100−500 μL of pyrophosphate buffer in the presence of a cocktail of protease inhibitor and homogenized using a Dounce homogenizer with a type B pestle. The sample was centrifuged at 13000*g* for 15 min at 4 °C. The supernatant was centrifuged at 400000*g* for 30 min to pellet microsomes. The pellets were suspended in buffer K [20 mM Hepes (pH 7.5), 10 mM $MgCl_2$, and 100 mM KCl] or 50 mM Tris (pH 7.5) and 100 mM NaCl if they were to be treated with heparin-Sepharose and then suspended in buffer K.

Skeletal muscle membranes were also depleted of laminin by using heparin-Sepharose (*3*) for some experiments. The microsomes were divided into two portions. The larger portion was incubated with heparin-Sepharose and the smaller portion with Sepharose 4B as a negative control for 1 h at 4 °C on a wheel type tube rotator providing gentle mixing. After incubation, the beads were removed by slow speed centrifugation (2000 rpm in a microfuge). For cell culture, the cells were either scraped from the plates, to retain endogenous laminin, or removed with trypsin/EDTA solution (0.05% trypsin and 0.02% EDTA in HBSS) for 10 min at 37 °C, which depletes laminin (*9*).

Preparation of Laminin or E3-Sepharose. Preactivated CNBr Sepharose (Sigma) was swollen by suspension in ice-cold 1 mM HCl for 15 min and washed with 1 mM HCl on a sintered glass filter; 100 μg of laminin or E3 in coupling buffer [0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 8.3)] was mixed with 0.5 mL of the swollen, activated Sepharose in a screwcap plastic tube on a wheel rotator overnight at 4 °C. The laminin- or E3-Sepharose was washed with blocking buffer [0.1 M Tris-HCl and 0.5 M NaCl (pH 8.0)] and again mixed overnight. Control-Sepharose was prepared in the same way except without any added protein.

Fusion Protein PAK1 and Preparation of PAK1-Sepharose. A chimeric fusion of GST and the p21-binding domain of PAK1 was expressed in *E. coli* strain BL21 and purified by affinity chromatography on glutathione−agarose beads (Sigma) as described elsewhere (*18*). After being washed, some of the beads were used for pull-down experiments without elution of the GST−PAK1 conjugate. For other uses, the protein was eluted with glutathione, and its purity was determined by 12% SDS−PAGE using the method of Laemmli (*14*). The protein concentration was determined (*15*) using bovine serum albumin as the standard.

Pull-Down Assays. Untreated or PP3-, PP2-, and SU6656-pretreated membranes from rabbit, rat, and C2C12 cells were incubated with control-Sepharose, laminin- or E3-Sepharose, or GST−PAK1-Sepharose in buffer K [20 mM Hepes (pH 7.5), 100 mM KCl, and 10 mM $MgCl_2$] containing 1 mM GTPγS, 1 mM ATP, and 1 mM $CaCl_2$ with or without laminin for 1 h at 4 °C with gentle mixing and then solubilized via addition of 2× Dig [50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 0.5 M sucrose, and 2% digitonin]. The samples were centrifuged at 2000 rpm in a microfuge at room temperature for 1 min. The resins were washed three times with buffer K containing 1× Dig. The bound protein was eluted using 60 μL of twice-concentrated Laemmli SDS−PAGE sample buffer. Samples were heated for 5 min at 95 °C and centrifuged for 5 min at room temperature to remove the resin. The supernatants were subjected to electrophoresis on a 4 to 20% Bio-Rad SDS−PAGE ready gel and electroblotted to nitrocellulose. The blots were blocked with 5% skim milk in TTBS [0.2% Tween 20, 20 mM Tris, and 0.5 M NaCl (pH 7.5)]. The blot was incubated with anti-Rac1 (Upstate Biotechnology, 1:1000), anti-phospho-Src (Tyr^{416}, Upstate Biotechnology, 1:1000), anti-Src and anti-Fyn (Santa Cruz Biotechnology, 1:1000), or anti-αDG (Upstate Biotechnology, 1:1000) at room temperature for 1 h. Goat anti-rabbit IgG−horseradish conjugate (1:10000) or goat anti-mouse IgG or IgM (H+L)−horseradish perixodase conjugate (1:3000) was used as a secondary antibody as required by the experiment. The blots were then developed using the enhanced chemiluminescence method (*3*).

Loading Control. For loading control, the blots were stained with ponceau red S (0.2% ponceau in 3% TCA) before being blocked with 5% skim milk to confirm that the same amount of protein was loaded into each well. In other experiments, the blot was stripped and reprobed with a different antibody, typically against β-actin or syntrophin. In all cases, equal loads were confirmed.

Inhibitor Blockade. C2C12 cells were cultured with PP3 (5−10 μM), PP2 (2.5−10 μM), or SU6656 (10 μM) for various times (from 6 to 12 h) as specified in the figure legends at 37 °C in six-well plates. The cells were collected using trypsin/EDTA solution and were washed three times with PBS; 10^6 cells in 100 μL of PBS containing protease inhibitor cocktail were incubated with or without 3 μg of laminin at 37 °C for an additional 1 h.

Immunocytochemistry. Muscle cross sections (10 μm) were prepared with a cryostat and collected on gelatin-coated glass slides. To minimize autofluorescence, sections were sliced, stained, and viewed on the same day. Sections were fixed in tissue fixative (Histochoice) for 5 min at room temperature. The slides were washed three times with Krebs-Henseleit bicarbonate buffer. PAK−GST (10 μg) fusion protein in 100 μL of PBS was overlaid on the slides of sections for 15 min at room temperature, and then the slides were washed three times with KH buffer. The rabbit polyclonal antibodies against GST (1:500) or βDG (1:100) and the mouse monoclonal antibody against Rac1 (1:50) were diluted in KH buffer with 3% BSA and were added to the slides. The slides were incubated for 30 min at room temperature in a humidified container and then washed with KH buffer. The goat anti-rabbit IgG (H+L)−Alexa Fluor 488 and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Fc) secondary antibodies (1:100) were added. Washed slides were mounted with 50% glycerol mounting

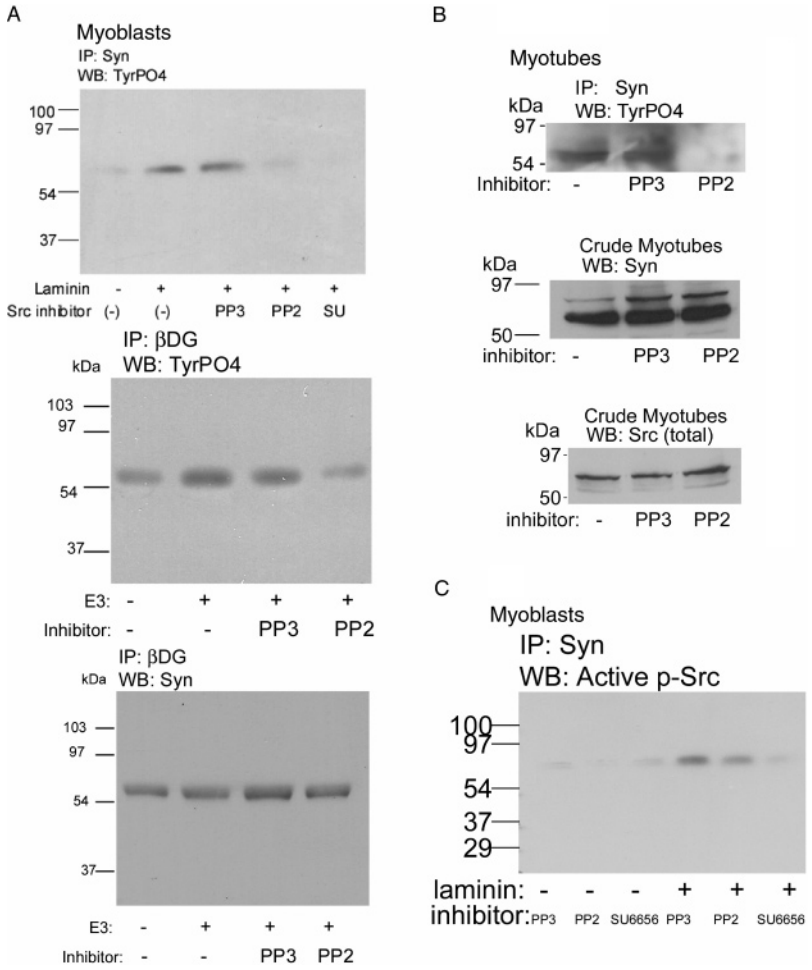

FIGURE 1: Inhibitors of Src family kinases inhibit laminin-induced signaling. (A) C2C12 myoblasts were cultured with SU6656, PP2, or PP3 (10 μM each) at 37 °C for 12 h. Microsomes were immunoprecipitated by syntrophin antibody and protein G-Sepharose in buffer K containing 1 mM $CaCl_2$, 1 mM GTPγS, 1 mM ATP, and 1% digitonin overnight at 4 °C. After the cells had been washed, SDS−PAGE buffer was added to each sample. Samples, after electrophoresis and electroblotting, were probed with antibody against the phosphotyrosine antibody ($TyrPO_4$). Similarly, immunoprecipitation was also performed with a βDG antibody after myoblasts were treated with E3 (1 μg/mL) and inhibitors (5 μM PP3 or 2.5 μM PP2) and Western blotted with the $TyrPO_4$ antibody (middle panel), or the same blot was stripped and then Western blotted with an antibody against syntrophin (Syn). (B) Experiments similar to those in panel A were also performed on C2C12 myotubes. In the top panel, immunoprecipitation with the syntrophin antibody was performed after the myotubes had been treated with PP3 (5 μM) or PP2 (2.5 μM), and the blots were probed with the $TyrPO_4$ antibody. To show that the inhibitors were not affecting protein expression, in the middle and bottom panels, myotubes without immunoprecipitation were separated by SDS−PAGE, blotted, and probed with a syntrophin antibody, and the blot was then stripped and reprobed with the Src antibody. (C) C2C12 myoblasts were harvested by using trypsin/EDTA solution to deplete laminin and were treated with PP3 (5 μM) or PP2 (2.5 μM) for 3 h in PBS at 37 °C. Additional laminin (3 μg/100 μL) was added where shown, and incubation was continued for an additional 1 h. Samples were washed with PBS twice. Cell lysate was made by adding RIPA buffer and precleared with protein G-Sepharose, and the supernatant was incubated with syntrophin antibody. The immune complexes were incubated with protein G-Sepharose. After samples had been washed, the bound protein was eluted with SDS−PAGE sample buffer. After electrophoresis and electroblotting, the blots were detected with anti-Src (p-416) antibody and visualized by ECL.

medium. The immunofluorescence in the muscle sections was observed with a Zeiss LSM 5 confocal microscope using Ar and HeNe lasers.

RESULTS

Src Family Kinase Inhibitors Also Inhibit Laminin-Induced Tyrosine Phosphorylation of Syntrophin and Rac1 Activation. Previously, we have shown that when laminin binds to αDG, syntrophin is phosphorylated on tyrosine and this results in downstream activation of Rac1 by way of Grb2 and SOS1 (*9*). In Figure 1A, treatment of C2C12 myoblasts with two specific Src family kinase inhibitors, 10 μM PP2 and SU6656, inhibited laminin binding-induced syntrophin phosphorylation on tyrosine, whereas the inactive analogue, PP3, had no effect (top panel). This phosphorylated syntrophin is in a complex with βDG (two bottom panels) showing that this is DGC syntrophin. Figure 1B shows that in myotubes, syntrophin is also phosphorylated, and this is inhibited by 2.5 μM PP2 (top panel). The bottom panels of Figure 1B show that inhibitor treatment is not affecting the total amounts of syntrophin or c-Src. Finally, Figure 1C shows that as laminin is added to trypsinized C2C12 myoblasts, c-Src is bound to syntrophin and becomes activated. c-Src activation occurs by its autophosphorylation on Tyr^{416}, which is detected by this antibody. Ponceau red staining, prior to the Western blot, shows that each well contained equal loads of protein (Supporting Information, Figure S1). Thus, two different Src family kinase inhibitors, in cultured myotubes or myoblasts, prevent syntrophin phosphorylation, while c-Src activation occurs in response to laminin binding.

Furthermore, c-Src colocalizes along with syntrophin, in the DGC.

Laminin Binding and Src Family Kinases Also Activate Rac1 in Skeletal Muscle. Previously, we had shown that laminin binding or the binding of laminin E3 results in syntrophin phosporylation and Rac1 activation (GTP binding) and initiates downstream signaling through c-jun N-terminal kinases. Furthermore, syntrophin binds Grb2 and Sos1 to cause Rac1 activation, and syntrophin phosphorylation on tyrosine results in the Grb2 binding by its SH2 domain (*3*, *9*). Figure 2A shows that even 2.5 μM PP2 was sufficient to inhibit the downstream activation of Rac1 in skeletal muscle microsomes whereas PP3 was ineffective, even at a higher concentration. Rac1 binds to only PAK1 when Rac1 is in the GTP-bound active form, and so the Rac1 bound by the PAK1-Sepharose in this experiment is activated Rac1. Only a portion of the total Rac1 is activated by laminin binding. Thus, the activation of Rac1 requires syntrophin phosphorylation by a Src kinase family member. Figure 2B shows that the activation of Rac1 occurs when even the small LG4−5 region of the laminin α-chain is present. Figure 2C shows that c-Fyn is also associated with Rac1, and thus also with syntrophin, in a laminin-dependent manner. We conclude that a Src kinase family member, probably c-Src or c-Fyn, phosphorylates syntrophin in a laminin-dependent manner which results in Rac1 activation.

Muscle Microsomes or Purified c-Src and c-Fyn Phosphorylate Syntrophin Fusion Proteins on Tyrosine and the Inhibition by siRNAs. The results in Figures 1 and 2 were obtained with the endogenous proteins present in muscle microsomes or mouse muscle cultured cells. In panels A and B of Figure 3, we used bacterially expressed fusion proteins to confirm that syntrophin is indeed a substrate for c-Src. In Figure 3A, skeletal muscle microsomes were mixed with ATP and syntrophin or syntrophin A (mouse syntrophin protein residues 2−274) fusion proteins and incubated to allow phosphorylation. Recovering the tyrosine-phosphorylated proteins and staining with an antibody for the T7 antigen, present in these fusion proteins, show that the microsomes contained a protein tyrosine kinase which utilizes syntrophin or the N-terminal half of syntrophin (syntrophin A) as the substrate (left panel). The right panel shows that the result is specific for the T7 antigen and is not observed with a negative control antibody, in this case one against maltose-binding protein. In Figure 3B, commercially available active c-Src was used to perform in vitro phosphorylation assays with [γ-^{32}P]ATP. Because c-Src autophosphorylates, it is also observed in these autoradiograms. An α-sarcoglycan fusion protein expressed from the same expression system was used as a negative control to eliminate the possibility that phosphorylation was of the fused sequences rather than syntrophin. Syntrophin A, rather than syntrophin, was used because it has a molecular weight very different from that of c-Src and can be easily distinguished. Syntrophin A, but not the α-sarcoglycan fusion protein, is a substrate of c-Src. In panels C and D of Figure 3, we used siRNA transfection in C2C12 cell to investigate the c-Src family kinases involved. Levels of both c-Src and c-Fyn protein expression were reduced after transfection with their respective siRNA, while neither affects syntrophin expression (Figure 3C). c-Src has been used to phosphorylate syntrophin proteins (Figure 3B). In Figure 3D, the phosphorylation of syntrophin also was significantly blocked by c-Src and c-Fyn siRNA, but the effect is only partial, perhaps because neither siRNA completely blocks its corresponding kinase (left panel). However, the mixture of both c-Src and c-Fyn siRNA eliminates syntrophin phosphorylation (right panel). We conclude that either c-Src or c-Fyn can phosphorylate syntrophin. In other experiments (data not shown), c-Yes was not found to be associated with DGC syntrophin and was thus excluded.

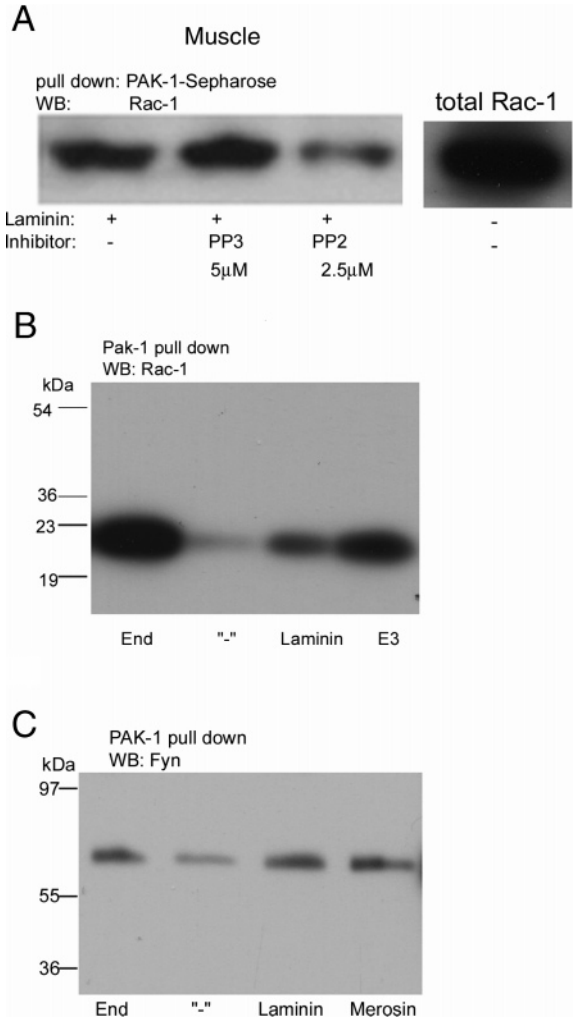

FIGURE 2: c-Fyn association and activation of Rac1 are laminin-dependent and processes which PP2 inhibits. (A) Rabbit skeletal muscle membranes were treated with heparin-Sepharose for 1 h at 4 °C. The membranes were incubated with PP2 (2.5 μM) and PP3 (5 μM) at 4 °C for 3 h, and laminin was added and incubation continued for an additional 1 h in buffer K containing 1 mM $CaCl_2$, 1 mM GTPγS, and 1 mM ATP mixed with PAK-1 bound to glutathione-Sepharose. Bound proteins were eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibody against Rac1. The small panel to the right shows the same amount of microsomes not treated with PAK-1-Sepharose but also Western blotted with Rac1 antibody in a parallel experiment to show the total amount of Rac1 present. (B and C) Muscle microsomes were either treated with Sepharose to retain endogenous (End) laminin or extrated with heparin-Sepharose to deplete laminin (−), and laminin, E3, or merosin as indicated was added to depleted microsomes. Panel B shows that relative to depleted microsomes, endogenous laminin, exogenous laminin, or E3 results in greater activation of Rac1. Panel C shows the same basic experiment but probed with the c-Fyn antibody, showing a laminin-dependent association of c-Fyn with this protein complex, which is shown for c-Src in Figure 1B.

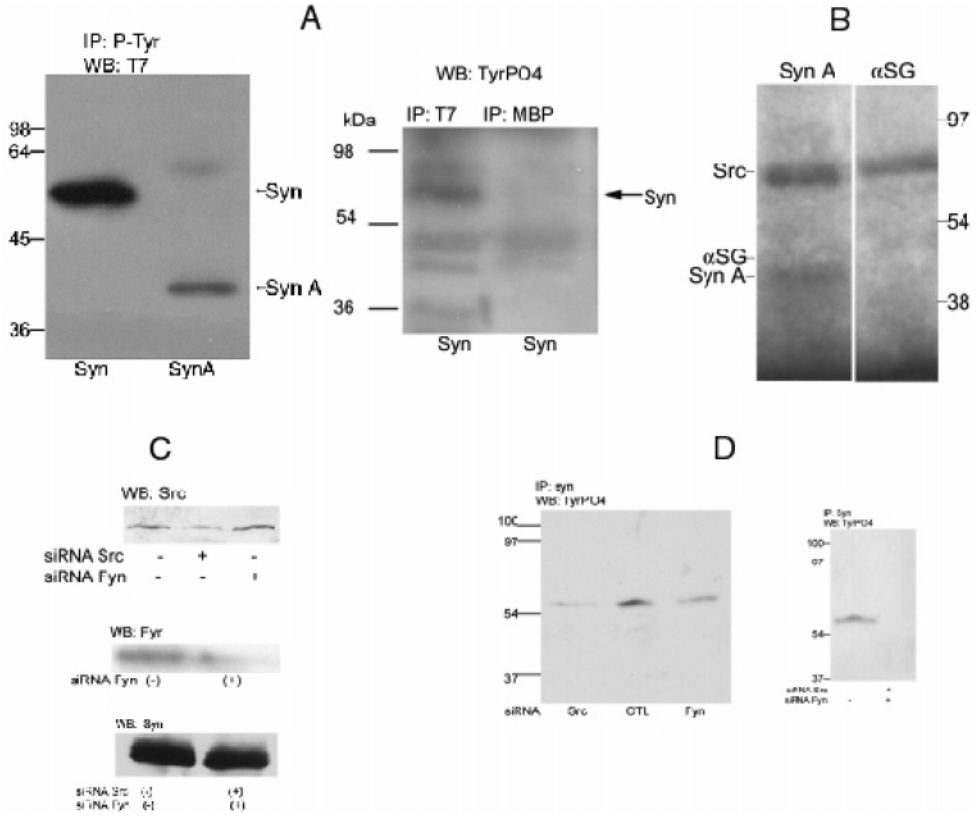

FIGURE 3: Muscle microsomes or purified c-Src phosphorylates syntrophin fusion proteins while siRNA inhibits. (A) Bacterially expressed syntrophin (Syn) or syntrophin A (Syn A) His-tag fusion proteins also have the T7 antigen at their N-terminus. The proteins (5 μg) were incubated with 100 μL of rabbit skeletal muscle microsomes in buffer K containing 1 mM $CaCl_2$, 1 mM GTPγS, 1 mM ATP, and 1% digitonin for 1 h at 37 °C. After cells had been precleared with protein G-Sepharose, 3 μL of phosphotyrosine (p-Tyr, left panel), T7, or maltose binding protein (MBP, an irrelevant control) antibody was added, incubated for 1 h on ice, and then with protein G-Sepharose for an additional 1 h. After samples had been washed, the proteins were eluted with SDS−PAGE sample buffer. After electrophoresis and electroblotting, the fusion proteins were detected with the T7 (left panel) or $TyrPO_4$ (right panel) antibodies and ECL. (B) Syntrophin A or α-sarcoglycan (αSG) fusion proteins were phosphorylated in vitro using commercially available active c-Src under conditions described in Materials and Methods using 20 000 cpm/μL of [γ-^{32}P]ATP. After incubation, the assay was mixed with an equal volume of twice-concentrated SDS−PAGE sample buffer, and after electrophoresis, the gel was dried and placed on photographic film for autoradiography. The expected sizes of the fusion proteins are indicated by the arrows. (C and D) C2C12 myotubes were transfected with siRNA against c-Src, Fyn, or both c-Src and c-Fyn for 48 h as described above. Proteins were eluted with SDS−PAGE sample buffer (C). In panel D, proteins were immunoprecipitated with syntrophin antibody. After SDS−PAGE and electroblotting, blots were probed with the antibodies against c-Src, c-Fyn, and syntrophin (C) and $TyrPO_4$ (D).

Laminin Binds a Complex Containing both c-Src and c-Fyn. Figure 4A shows that laminin-Sepharose binds a complex containing both c-Src and c-Fyn. In addition, an appreciable fraction of the c-Src in this complex was phosphorylated and thus active. While Sepharose did bind a small amount of these kinases under these conditions, clearly laminin-Sepharose bound a much greater amount. Previously, we have used blocking antibodies to show that laminin binds to αDG to cause syntrophin phosphorylation and Rac1 activation (*3*, *9*). Thus, laminin-Sepharose is most likely binding αDG, and the DGC and both c-Src and c-Fyn are associated with this complex. The results in Figure 4B further strengthen this argument. Under these same conditions, we found that laminin-Sepharose bound αDG and not to an appreciable amount of integrins. All muscle integrins are β1-integrins, yet laminin-Sepharose does not bind detectable amounts of this subunit. We emphasize that this is likely true only under these conditions since it is clear from other investigations that integrins do bind laminin. However, under the conditions used here, laminin-Sepharose binds a complex of proteins that contains αDG, c-Src, and c-Fyn, and previous figures show that this complex contains phosphorylated syntrophin and activated Rac1.

Contraction or Stretching Causes Similar Signaling. Because laminin is tightly bound to the exterior of the sarcolemma, it is unlikely that it is the presence or absence of laminin that regulates this signaling. However, contraction or stretching of muscle could stress the laminin−αDG linkage, and this could provide an alternate way in which signaling could be initiated. This was tested in Figures 5 and 6. In Figure 5A, in both contracted (stimulated) and relaxed (unstimulated) muscle, Rac1 colocalized with DGC β-dystroglycan on the sarcolemma. This shows that a major portion of the Rac1 in muscle is colocalized with the DGC at this resolution. The images are not identical in that both β-dystroglycan and Rac1 stain less intensely in the uncontracted muscle. In Figure 5B, the activation state of Rac1 was investigated by overlaying the sections with PAK1, which binds only active Rac1. Contraction of muscle leads to a much higher level of activation of Rac1. Results virtually identical to those shown in Figure 5 when muscle was contracted were also observed when muscle was stretched

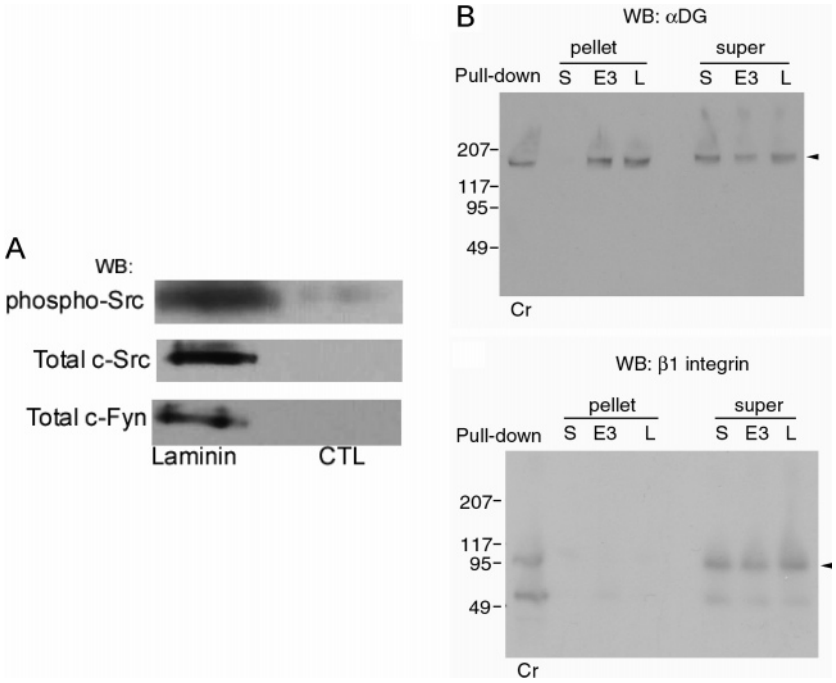

FIGURE 4: Laminin-Sepharose binds a complex containing Src family members and α-dystroglycan. (A) Laminin-Sepharose (Laminin) and control-Sepharose (CTL) were incubated with rabbit skeletal muscle membranes in buffer K containing 1 mM $CaCl_2$, 1 mM GTPγS, and 1 mM ATP. Digitonin was added to the microsomes, and incubation was continued for 1 h. After the samples had been washed, bound protein was eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies against phospho-Src (Tyr^{416}), c-Src, and c-Fyn. (B) Rabbit skeletal muscle microsomes were treated as described for panel A except that either laminin-Sepharose (L), E3-Sepharose, or control-Sepharose (S) was used and the supernatants and pelleted resins were washed, eluted, subjected to SDS−PAGE, and electroblotted. The blots were then probed with either the IIH6 αDG antibody or a commercially available antibody against integrin β1 subunit. The crude microsomes (Cr) are also included for comparison.

(Supporting Information, Figure S2). Thus, it is stress on the laminin−DGC interaction which initiates signaling.

One shortcoming of the experiment in Figure 5B is that PAK1 can bind to either active Rac1 or active cdc42, another p21 G-protein. That Rac1 is indeed activated is addressed in Figure 6A. When muscle samples are contracted, the PAK1-bound protein is shown to stain with the Rac1 antibody and contracted muscle contained much more activated Rac1 than the relaxed muscle. Thus, at least two characteristics of laminin-dependent signaling, the association of Rac1 with the DGC and the activation of Rac1, are also stimulated by muscle contraction. In a previous report, it was shown that downstream of active Rac1 is the activation of JNKp46 (*3*). Thus, the same contracted and relaxed muscle samples were also investigated for this downstream signaling. Figure 6B shows that muscle contraction also leads to activation of JNKp46 relative to the relaxed muscle. JNK is a family of three different kinases, JNK1, -2, and -3, each with multiple isoforms (*19*). In Figure 6C, the blot shown in Figure 6B was stripped and stained for JNK1. These data (Figure 6C) show that the active JNKp46 is a JNK1 isoform. In addition, Figure 6C also provides a loading control showing that it is the activation of JNK1 that changed while the total amount of JNK1 was similar in the two samples.

E3 Causes the Proliferation of the C2C12 Muscle Cell Line Myoblasts. C2C12 cells were incubated for 48 h with E3 (the LG4−5 region of the laminin α1 chain containing the αDG binding sequences). Propidium iodide was incorporated into the fixed cells to stain DNA, and the cell cycle was analyzed by flow cytometry. For this experiment (Figure 7A), more than 40 000 cells were counted for each condition. The number of cells in the G2-S phase (shown as the M2 bar in the figure) significantly increased from 8 to 14% with addition of E3. Thus, E3 or laminin (data not shown) causes cells to enter the synthesis phase, and mitosis was initiated.

In Figure 7B, the effect of PP2 Src family kinase inhibitor and E3 is shown. In other experiments, we determined that 10 μM PP3 had no effect on cell proliferation (data not shown) and that E3 increased the rate of cell proliferation (see the Supporting Information, Figure S3). In the experiment shown here, E3 also caused cell proliferation. Initially, the plates were seeded with 1×10^4 cells, and after they had grown for 24 h, this number increased to 4.3×10^4 cells, a number similar to that observed for PP3 in Figure 7B. On the other hand, 10 μM PP2 inhibited cell proliferation in the presence or absence of E3. An increasing E3 concentration increased the cell number, showing that E3 stimulated proliferation, but clearly PP2, the Src family kinase inhibitor, prevented most of this cell proliferation. At the highest concentration of E3, the cell number increased approximately 2-fold in the presence of PP2, while the cell number increased more than 9-fold in the presence of inactive PP3.

In other experiments, we have observed a similar proliferative effect of laminin (data not shown), in agreement with others (*2*, *20*). Thus, the proliferative effect of laminin results, at least in part, from the binding of a relatively small region of the laminin α-chain, the LG4−5 modules, which is also the region of laminin-1 that binds to αDG. This same E3 region of laminin also stimulates syntrophin tyrosine phosphorylation and Rac1 activation as well as other signaling through the DGC (*3*, *9*, *10*). One consequence of laminin- or E3-induced signaling is cell proliferation in myoblasts, and this proliferative effect is inhibited by Src family kinase inhibition.

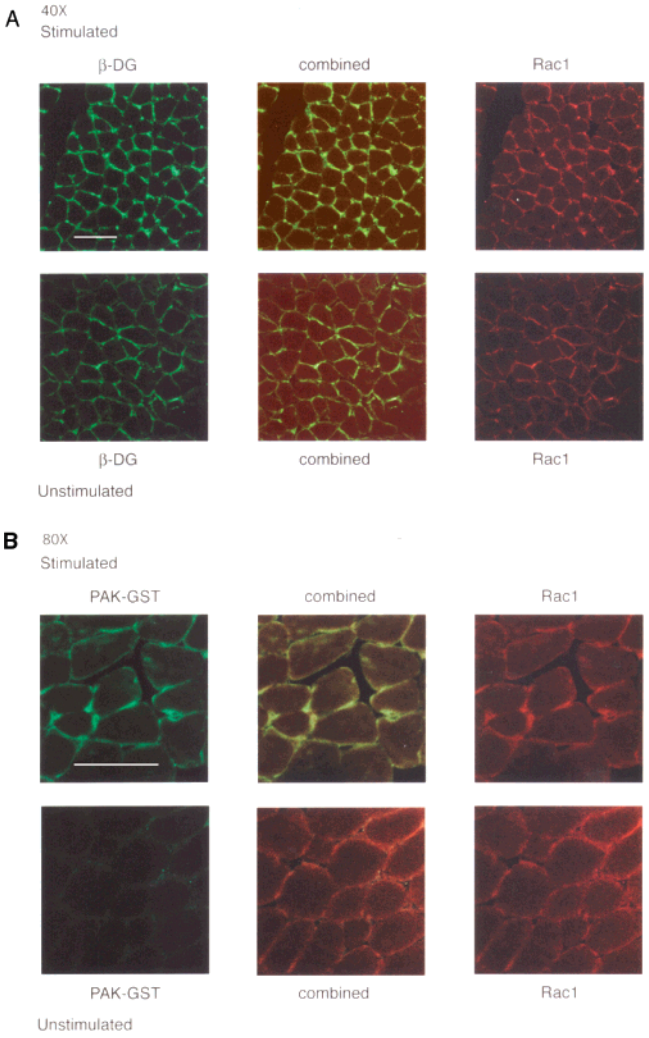

FIGURE 5: Contraction increases the level of association of Rac1 with the DGC and causes Rac1 activation. β-Dystroglycan and Rac1 colocalize over the sarcolemma, and the level of Rac1 activation was increased by electric stimulation of the sciatic nerve. The gastrocnemius muscle in one rat hindlimb was contracted by electric stimulation of the sciatic nerve; the contralateral muscle was treated similarly except with no electrical stimulation. Panel A shows staining of the same section of rat muscle with βDG (green) and Rac1 (red) antibodies. The combined (merged) image shows yellow where the two colocalize. Panel B shows the amount of PAK−GST fusion protein (green) was greater in stimulated muscle compared with unstimulated muscle. Combined is the merged image showing yellow wherever PAK1 and Rac1 colocalize. The white bar is 50 μm.

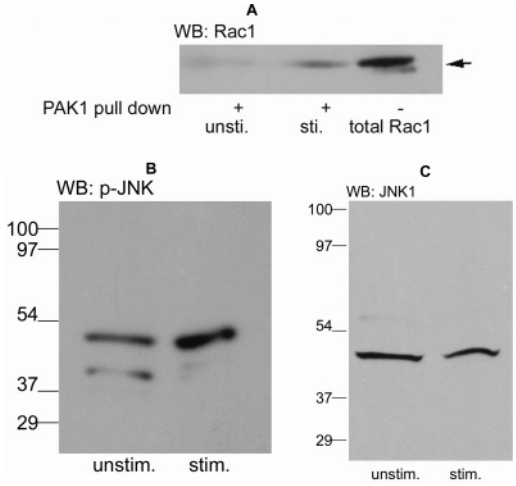

FIGURE 6: Contraction causes Rac1 and JNKp46 activation. (A) Samples were prepared as described in the legend of Figure 5 from either contracted (stim.) or relaxed (unstim.) gastrocnemius muscle. Microsomes were prepared from each and incubated with PAK1-Sepharose in buffer K containing 1 mM $CaCl_2$, 1 mM GTPγS, and 1 mM ATP. Digitonin was added to the microsomes, followed by incubation for 1 h. Then, bound protein was eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies against Rac1 (A). Because only activated (GTP-bound) Rac1 binds to PAK1, staining shows the amount of active Rac1. For comparison, the crude microsomes from stimulated muscle were also included to show the total amount of Rac1 present. The same microsomes as in panel A were probed (B) with antibodies against phosphorylated (active) JNK or (C) with antibodies against total JNK1.

DISCUSSION

The data presented here clearly show that a c-Src family kinase phosphorylates syntrophin to initiate downstream signaling through Rac1 and JNKp46. Laminin binding and muscle tension also induce this signaling. c-Src phosphorylates syntrophin somewhere in its N-terminal half (present in syntrophin A), and skeletal muscle microsomes contain this same activity or a similar one. This region of syntrophin has four tyrosine residues, including two that could provide a site of phosphorylation, as we previously speculated (*9*). The precise residue(s) phosphorylated is a topic of current investigation.

Whether it is c-Src itself or another member of the c-Src family that causes the phosphorylation is unknown. This family of similar kinases has overlapping regulation, inhibition by PP2 and SU6656, and substrate specificity. At least one other member, c-Fyn, is also present in the laminin-bound complex (Figure 4) and is associated with active Rac1 (Figure 2C), and decreasing its level with its siRNA also slows syntrophin phosphorylation (Figure 3D). Thus, whether it is c-Src or c-Fyn which normally initiates this signaling is unclear.

Laminin binding induces this signal cascade, and here we show that muscle stimulation by either contraction or stretching induces the same signal cascade. This is what one would expect if binding of laminin to the DGC constitutes a mechanoreceptor, signaling muscle functional activity. Interestingly, a different laminin receptor, the elastin laminin receptor, has also been proposed as a mechanoreceptor in vascular smooth muscle (*21*). Interestingly, stretching of diaphragm muscle activates Akt, and the mdx mouse has stronger Akt activation than control animals (*22*). It has also been shown that binding of laminin to αDG results in Akt activation and a decreased level of apoptosis (*11*). Thus, contraction or stretching initiates a variety of signaling pathways originating at the DGC. These pathways are also affected by mutations within this complex, and these affect muscle cell survival. Contraction or stretching in muscle inhibits atrophy and induces hypertrophy, but how this is signaled is currently unknown. We propose that the laminin−DGC interaction participates in this signaling in muscle. The precise details of how this signaling affects muscle are only now beginning to emerge.

Another important aspect of this study and our previous studies (*3*, *9*, *10*) is that this signaling pathway is functioning throughout muscle development, in myoblasts, myotubes, and skeletal muscle. Myoblasts express much smaller amounts

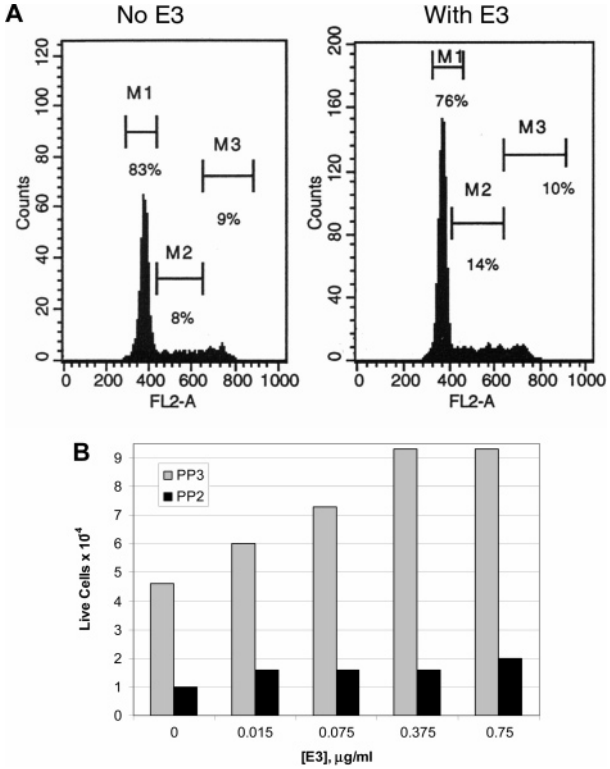

FIGURE 7: Laminin LG4 and LG5 domains cause an increased level of cell division in myoblasts, and this is inhibited by a Src family kinase inhibitor. (A) C2C12 myoblasts were incubated with E3 medium (containing 0.15 μg/mL E3) for 48 h. Propidium iodide was incorporated after cells were fixed using 70% methanol. Samples were analyzed by flow cytometry: M1, G_0/G_1 phase; M2, S phase; and M3, G_2-M phase. (B) C2C12 cells (1×10^4) were cultured for 24 h in 24-well plates in DMEM containing 10% FCS. With no additions, they grew to $(4.2 \pm 1) \times 10^4$ cells ($n = 6$). When present, PP3 or PP2 was added to a final concentration of 10 μM in the medium, and the concentrations of E3 shown were added. The number of live cells, which exclude trypan blue, was counted. Each bar represents determination on triplicate cultures.

of DGC proteins (*9*, *23*), and the DGC is much less well characterized in myoblasts than it is in skeletal muscle and may be somewhat different. However, a complex containing αDG, βDG, and syntrophin has been shown to cause laminin-dependent signaling through Rac1, which ultimately leads to c-jun phosphorylation by JNK, and which also requires Src family kinase phosphorylation of syntrophin and is initiated by the same LG4−5 E3 region of laminin (Figures 1−3 and refs *3*, *9*, and *10*). Regardless of the exact composition of the DGC complex in myoblast, the complex that is there is functional in cell signaling and acts similar to the one in myotubes and skeletal muscle.

Muscle can increase mass by fusing myoblasts with the syncytial myotubes and increasing the level of expression from existing myonuclei. Here we show that laminin E3 induces proliferation of myoblasts. Because c-Src family kinase inhibitors prevent this proliferation, myoblast proliferation presumably also requires member(s) of this kinase family. In myoblasts, c-Src inhibitors also inhibit syntrophin phosphorylation (Figure 1A), and most aspects of the syntrophin-Rac1-JNKp46 pathway have also been shown to exist in myoblasts (*3*, *9*, *10*). Thus, laminin binding initiates this signaling throughout the life of the muscle cell and may, by increasing the rate of myoblast proliferation, provide a means for hypertrophy in muscle. Because this signaling is also induced by contraction or stretching, either the laminin-αDG-syntrophin-Rac1-JNKp46 pathway we have studied here or the laminin-αDG-PI3K-Akt pathway described by others (*11*, *22*) may be different functional aspects of the same mechanoreceptor. Defects in this mechanoreceptor may help explain the severe pathologies of muscular dystrophy when the laminin−DGC mechanoreceptor is altered by genetic mutation.

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SUPPORTING INFORMATION AVAILABLE

C2C12 myoblasts that were harvested by using trypsin-EDTA to deplete laminin and were treated with PP3, PP2, or SU6656 (Figure S1), muscle that was stretched, sectioned, and stained (Figure S2), and C2C12 myoblasts grown in the presence of the concentration of E3 but in the absence of any inhibitors (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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