Binding of Laminin α1-Chain LG4−5 Domain to α-Dystroglycan Causes Tyrosine Phosphorylation of Syntrophin to Initiate Rac1 Signaling†

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ABSTRACT: Previously, a signaling pathway was described [Oak, Zhou, and Jarrett (2003) *J. Biol. Chem. 278*, 39287−39295] that links matrix laminin binding on the outside of the sarcolemma to Grb2 binding to syntrophin on the inside surface of the sarcolemma and by way of Grb2-Sos1-Rac1-PAK1-JNK ultimately results in the phosphorylation of c-jun on Ser^{65}. How this signaling is initiated was investigated. Grb2-binding to syntrophin is increased by the addition of either laminin-1 or the isolated laminin α1 globular domain modules LG4−5, a protein referred to as E3. This identifies the LG4−5 sequences as the region of laminin responsible for signaling. Since laminin α1 LG4 is known to bind α-dystroglycan, this directly implicates α-dystroglycan as the laminin-signaling receptor. E3 or laminin-1 increase Grb2-binding and Rac1 activation. In the presence of E3 or laminin-1, syntrophin is phosphorylated on a tyrosine residue, and this increases and alters Grb2 binding. The α-dystroglycan antibody, IIH6, which blocks binding of laminins to α-dystroglycan, blocks both the laminin-induced Sos1/2 recruitment and syntrophin phosphorylation, showing that it is α-dystroglycan binding the LG4−5 region of laminin that is responsible. The C-terminal SH3 domain of Grb2 (C-SH3) binds only to nonphosphorylated syntrophin, and phosphorylation causes the Grb2 SH2 domain to bind and prevents SH3 binding. Syntrophin, tyrosine phosphate, β-dystroglycan, and Rac1 all co-localize to the sarcolemma of rat muscle sections. A model for how this phosphorylation may initiate downstream events in laminin signaling is presented.

The gene lesion causing Duchenne muscular dystrophy is traced to a gene encoding the protein dystrophin (*1*). Dystrophin is found in a complex with other proteins and glycoproteins, termed the dystrophin glycoprotein complex (DGC)[1] (*2*). The dystrophin glycoprotein complex binds extracellular matrix laminin by way of α-dystroglycan (α-DG) and spans the sarcolemma by way of proteins including βDG, α-, β-, γ-, and δ-sarcoglycan (δ-SG), and sarcospan (SSPN). On the inside of the cell, dystrophin and syntrophin bind to other proteins including actin; these interactions thus provide a mechanical link between the extracellular matrix and the cytoskeleton (*3*−*5*). Laminin binding can also initiate cell signaling. Binding of laminin activates the PI3K/Akt pathway and inhibits apoptosis (*6*), modulates syntrophin binding to heterotrimeric Gs via Gβγ, and decreases Ca^{2+}-entry via the L-type Ca^{2+}-channels (*7*). Laminin binding also initiates signaling through syntrophin-Grb2-Sos1-Rac1-Pak1-JNK, resulting in c-jun phosphorylation on Ser^{65} (*8*). Although the latter signaling most likely arose from laminin-binding to αDG in the DGC, this has not been directly demonstrated.

Muscular dystrophies include defects in DGC dystrophin, sarcoglycans, and sarcospan. Congenital muscular dystrophies constitute a heterogeneous disease entity that includes defects in the laminin α2 subunit (*9*, *10*). Other myopathies result from defects in the α7 integrin chain (*11*, *12*), which also binds laminin. The two types of laminin receptors (αDG and integrins) are not always functionally distinct in skeletal muscle. As myoblasts differentiate into myotubes, expression of both αDG (*13*, *14*) and α7β1 integrin (*15*) increases. In addition, integrin and DGC components apparently co-localize: both can be identified in costamers (*16*, *17*), immunoprecipitation with β1-integrin antibodies retains DGC components, and α-and γ-sarcoglycans are phosphorylated (on Tyr) when the peptide RGDS binds to integrins (*18*).

Laminins are αβγ heterotrimers. The region of laminin that binds to both αDG and integrins is primarily in the five globular domains (i.e., LG domains) of laminin's α-subunit. Laminin α-chain LG modules 4−5 binds to the acidic polysaccharide chains of αDG (*19*), while the integrin

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[1] Abbreviations: DGC, dystrophin glycoprotein complex; DG, dystroglycan; SG, sarcoglycan; SSPN, sarcospan; PI3K, phosphatidylinositol-3 kinase; LG, laminin globular domain modules; PAK1, $p21^{cdc42,Rac1}$-activated protein kinase; Grb2, growth receptor bound 2; Sos, son of sevenless; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; ECL, enhanced chemiluminescence.

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binding site in this region has not been mapped in detail (*15*). Data exist to suggest that the $\beta\gamma$ chains are needed to keep this region in the correct conformation for integrins to bind (*20*). The binding site for αDG localizes to the LG4−5 modules of laminin α5, while the binding site for α3β1 and α6β1 integrins localizes to LG1−3 (*21*). For laminin-α2, the result is somewhat different with αDG binding to sequences in both LG1−3 and LG4−5, while binding of integrins α7β1 and α6β1 is again via the LG1−3 modules (*22*). In laminin-1 (α1β1γ1), it is LG4 that binds αDG (*23*). The consensus of these various studies is that many integrins bind LG1−3, while, at least in laminin α1 and α5, αDG-binding resides in the LG4−5 region. This region of laminin α1 was expressed, referred to as the E3 protein (*24*), and is used in the studies presented.

The structures of the laminin α1 and α2 LG4−5 domains bound to heparin and to αDG have been described (*19*, *25*, *26*). Furthermore, the LG4−5 region has also been implicated in other forms of laminin-signaling through αDG (*23*). What emerges from these studies is that LG4−5 and αDG form a specific ligand−receptor pair.

Recently, E3 was shown to substitute for laminin in the laminin-induced G$\beta\gamma$-binding to syntrophin and in inhibiting Ca^{2+}-entry via the L-type Ca^{2+}-channels (*7*). Thus, laminin-binding to α-dystroglycan is implicated in these forms of signaling. Blocking antibodies are another way to test whether αDG binds laminin to cause an effect. Two monoclonal antibodies against αDG bind to different regions of αDG: one is VIA4 and does not block laminin−αDG binding, while the other, IIH6, does block this binding (*6*, *27*); the IIH6 αDG antibody also blocked Gβ binding, confirming that αDG is the receptor (*7*). Whether this is also true for the signaling occurring through syntrophin-Grb2 will be addressed here.

Grb2 binds to both syntrophin (*28*) and βDG (*29*, *30*) via PXXP motifs. In syntrophin, there are two different PXXP sequence containing regions, and both bind each of the two Grb2 SH3 domains in vitro with high affinity (*28*). In cell signaling mediated by Grb2, normally Grb2 binds to a phosphorylated tyrosine containing sequence, via its lone SH2 domain, while the N-terminal SH3 (N-SH3) domain of Grb2 binds Sos1 and activates it (*31*). How syntrophin binding to Grb2 can activate Sos1 and Rac1 (*8*) is not known but would be expected to be similar. This would require that laminin binding induces the phosphorylation of syntrophin on a tyrosine. Such phosphorylation of syntrophin has never been reported, although phosphorylation on a serine residue by CaM kinase II has been reported (*32*).

Here, we show that laminin-1 binds to αDG via LG4−5, causing syntrophin to become phosphorylated on a tyrosine and allowing Grb2 to bind via its SH2 domain. Signaling through the Rac1 pathway is initiated. Furthermore, we demonstrate that the laminin α1 LG4−5 domains can substitute for holo-laminin-1 α$\beta\gamma$, and an antibody that blocks laminin-α-dystroglycan binding prevents the downstream binding of Sos1/2 or syntrophin phosphorylation on tyrosine.

MATERIALS AND METHODS

Materials. Antibodies against Grb-2, Sos1/2, and phosphorylated Tyr, were from Santa Cruz Biotechnology. Rac1 antibody was from Upstate Biotechnology. Monoclonal αDG antibodies IIH6 and VIA4−1 were a generous gift from Dr. Kevin Campbell (University of Iowa, Iowa, USA). Affinity purified rabbit polyclonal antibody against β-dystroglycan was a generous gift from Dr. Tamara C. Petrucci (Laboratorio di Biologia Cellulare, Instituto Superiore di Sanita, Via le Regina Elena, Rome, Italy). Monoclonal antibody against laminin β1 chain (2E8) was obtained from Developmental Studies Hybridoma Bank (University of Iowa). Antibody against recombinant mouse α-syntrophin (amino acids 2−503) was produced in rabbit and purified by affinity chromatography on syntrophin A (amino acids 4−274)-Sepharose as previously described (*8*). Goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate and goat anti-rabbit IgG (H+L)-horseradish peroxidase conjugate were from Bio-Rad. Goat anti-mouse IgM (H+L)-horseradish peroxidase conjugate was purchased from Southern Biotechnology Associate Inc. Mouse laminin-1 was obtained from Collaborative Biomedical Products. 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.

Purification of E3. The hLNa1-E3 293 (HEK293) cell line, transfected with the LG4−5 domains of human laminin α1, was generated as described (*24*). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS with G418 (250 μg/mL) and puromycin (10 μg/mL) until confluence. Then cells were washed extensively with PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) and incubate in serum-free DMEM for 24 h, the medium was harvested and replaced by fresh serum-free medium for another 24 h, and again the medium was harvested and the cells were discarded. The pooled collected medium (typically 100 mL) was diluted with two volumes of cold water and 0.5 M EDTA and 0.2 M benzamidine were added to a final concentration of 1 mM each. Diluted medium was loaded onto a 25 mL DEAE-Sepharose column whose outlet was connected to a 5 mL Hi-trap heparin-Sepharose column. The columns were washed with 25 mL of wash buffer (0.1 M NaCl, 20 mM Tris HCl, pH 8.0, 1 mM benzamidine), the columns were separated, and the heparin column washed with an additional 175 mL of wash buffer prior to elution with 0.3 M NaCl, 20 mM Tris HCl pH 8.0. Fraction of 1.5 mL were collected, and the eluted protein was detected by absorption at 280 nm or by gel electrophoresis and pooled. The pooled fractions were dialyzed in 50 mM ammonium bicarbonate, lyophilized, reconstituted in PBS, and filter sterilized. The protein concentration of E3 was determined (*33*) using bovine serum albumin as a standard.

Preparations of Microsomes from Skeletal Muscle and C2C12 Cells. Frozen rabbit skeletal muscle was homogenized in pyrophosphate buffer (20 mM sodium pyrophosphate, 20 mM phosphate, 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 (*8*). The homogenate was centrifuged at 13000*g* for 15 min at 4 °C. The supernatant was then centrifuged for 30 min at 32500*g* to pellet muscle microsomes. For C2C12 cell membrane preparation, 10^7 cells were suspended in 500 μL of pyrophosphate buffer and homogenized using a Dounce homogenizer with type B pestle. The sample was transferred to a 1.5 mL microcen-

trifuge tube and centrifuged for 15 min at 13000*g* at 4 °C. The supernatant was then centrifuged for 30 min at 4 °C at 400000*g* to pellet the membranes. The pellets were suspended in buffer K (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl) or 50 mM Tris, pH 7.5, 100 mM NaCl according to the requirements of the experiment.

Cell Culture. Mouse C2C12 myogenic cells were grown in DMEM containing 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 in air to 80% confluence and then either harvested for experiments or transformed to myotubes. C2C12 myoblast differentiation to myotubes was initiated at 80% confluence by replacing the growth medium with DMEM containing 1% FBS and maintaining the cells in this medium for 6 days, and on the sixth day, the myotubes were harvested for experiments. Myocytes were washed with Hanks' balanced salt solution (HBSS) and were then either harvested by scraping the plate or by adding 1 mL of trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA in HBSS) for 10 min at 37 °C. The cells were then immediately suspended in DMEM/10% FBS and removed from the plate. L6 cells were grown and trypsinized using the same methods as C2C12.

Preparation of Grb2- and PAK1-Agaroses. Chimeric fusion proteins of glutathione *S*-transferase (GST) with Grb2, its individual SH2 and SH3 domains (all the generous gift of Dr. Oreste Segatto, Instituto Regina Elena, Roma, Italy), and the p21-binding domain of PAK1 (the generous gift of Dr. Yi Zheng, Children's Hospital Research Foundation, University of Cincinnati) were expressed in *E. coli* BL21 strain. GST, GST-N-SH3, GST-SH2, GST-C-SH3 and GST-PAK1 were purified by affinity chromatography on glutathione-agarose beads as described (*34*), the only difference being that after washing the beads thoroughly the proteins were not eluted from beads and the beads were used for pull-down experiments.

Preparation of E3- and Laminin-Sepharose. Preactivated CNBr Sepharose (Pharmacia) was suspended in ice-cold 1 mM HCl for 15 min and washed with 1 mM HCl on a sintered glass filter. E3 protein (8.5 μg) in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was mixed with 0.5 mL of this Sepharose in a screwcap plastic tube on a wheel rotator mixer overnight at 4 °C. The E3-Sepharose was washed with blocking buffer (0.1 M Tris-HCl, 0.5 M NaCl pH 8.0), and mixing continued in this buffer overnight. The resin was stored in blocking buffer containing 10 mM NaN_3 as a 1:1 slurry. Laminin-Sepharose was prepared in the same way using 1 mg of laminin/0.5 mL of Sepharose. In both cases coupling, assessed as the difference of the protein added and recovered after coupling, was greater than 95%.

Pull-Down Assay. A total of 100 μL of a 50% slurry of syntrophin-Sepharose (containing 12 μg of syntrophin), the various chimeric GST-fusion protein-glutathione agarose resins, E3- or laminin-Sepharose were equilibrated with buffer K containing 1 mM $CaCl_2$. Microsomes were incubated in buffer K containing 1 mM $CaCl_2$, 1 mM GTP-γS, and 1 mM ATP and with or without laminin (or E3) for 1 h at 4 °C. Then, incubation was continued with the additions of syntrophin-Sepharose, GST-glutathione-agarose, GST-SH2-, GST-N-SH3- and GST-C-SH3-glutathione-agarose, GST-PAK1-glutathione-agarose, E3- or laminin-Sepharose for another 1 h at 4 °C with gentle mixing and then solubilized by addition of 2× Dig (50 mM Tris, pH 7.4, 0.5 M NaCl, 0.5 M sucrose, 2% digitonin). The samples were centrifuged at room temperature for 1 min at 2000 rpm in a microcentrifuge. The resins were washed three times with 1 mL of buffer K containing 1% digitonin. The bound protein was eluted using 60 μL of twice concentrated Laemmli sample buffer (*35*). Samples were heated for 5 min at 95 °C and centrifuged for 5 min to remove the resins. The supernatants were applied to electrophoresis on a 4%-20% Express Gel (ISC BioExpress) SDS−PAGE and electroblotted to nitrocellulose (*8*). The blots were blocked with 5% skim milk in TTBS (0.2% Tween-20, 20 mM Tris, 0.5 M NaCl, pH 7.5). After being washed three times with TTBS, the blot was incubated with affinity-purified anti-syntrophin (1:3000 dilution), anti-phosphorylated Tyr (1:1,000 dilution), or anti-Sos1/2 (1:1000 dilution). Goat anti-rabbit IgG (1:10000) or goat anti-mouse IgG (H+L) − horseradish peroxidase conjugate (1:3000) was used as a secondary antibody as required by the experiment. The blots were then developed using the enhanced chemiluminescence method (*8*).

Laminin Depletion. Laminins were depleted from skeletal muscle membrane using heparin-Sepharose as described previously (*8*). Briefly, microsomes in 50 mM Tris, pH 7.5, 100 mM NaCl, were incubated with either Sepharose as a negative control (retaining endogenous laminins) or heparin-Sepharose (to deplete laminins). After incubation, the resin was removed by slow speed centrifugation (2000 rpm in a microfuge) and the supernatant microsomes were used. For cultured cells, the cells were either scraped from the plate to retain endogenous laminin or were trypsinized briefly in EDTA as described in an earlier section to deplete laminin.

Antibody Blockade. For the antibody blockade experiments, samples were divided into two groups: to one was added VIA4 (1:25 dilution) as control (nonblocking) αDG antibody, the other group was added IIH6 (1:25 dilution), an antibody known to block the laminin-α-dystroglycan interaction (*6*, *27*), and incubated for 1 h on ice. Then, laminin-1 was added and incubation continued for an additional hour.

Immunoprecipitation. Laminins were depleted from rabbit skeletal muscle microsomes using heparin-Sepharose or from cultured myocytes by brief trypsin-EDTA treatment as described above. Microsomes were in buffer K containing 1 mM $CaCl_2$, 1 mM GTP-γS, and 1 mM ATP. To depleted microsomes either nothing (minus) or 3 μg of exogenous laminin-1 or different concentrations of E3 along with 50 μL of protein G-Sepharose ("preclearing") were added, followed by incubation for 1 h at 4 °C with gentle mixing. The supernatants, after centrifugation, were incubated with 2 μL of antibody (against syntrophin, phosphotyrosine, or βDG) for 1 h. Samples were then solubilized by adding 1% Triton X-100, 0.5% IGEPAL, and 0.5% sodium deoxycholate. Incubation was continued for another 1 h at 4 °C with gentle mixing. The immune complexes were then incubated overnight with 50 μL of protein G-Sepharose, centrifuged, and washed extensively with buffer K. The bound proteins were eluted with Laemmli sample buffer and then detected with anti-Grb2 antibody (1:2000 dilution), anti- phosphorylated Tyr antibody (1:1000 dilution), or anti-syntrophin antibody (1:3000 dilution).

Loading Controls. All blots were stained for protein with Ponceau Red (0.2% Ponceau Red, 3% trichloroacetic acid,

3% 5-sulfosalicylic acid), a reversible stain, prior to blocking with milk to observe whether loading was equal. Also, blots were sometimes stripped after the first Western blot by incubation at 55 °C in 2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris, pH 6.8. The blots were then blocked again with milk and Western blotting was repeated with a different primary antibody.

Immunocytochemistry. The gastrocnemius was collected from the rat hind limb, immediately fixed in Tissue-Tek O.C.T. compound, frozen in liquid nitrogen, and stored at −80 °C before using. The muscle cross sections were prepared using a cryostat; sections (10 μM) were collected on the glass slides. To minimize autofluorescence, sections were sliced, stained, and viewed on the same day. Glass slides were coated with 1% gelatin and were fixed in tissue fixative (Histochoice) for 5 min at room temperature. The slides were washed three times with Krebs-Henseleit (KH) bicarbonate buffer. The rabbit polyclonal antibodies against syntrophin (1:500) or βDG (1:100) and the mouse monoclonal antibodies against PO_4-Tyr (1:100) or Rac1 (1:50) were diluted in KH buffer with 3% BSA and were added to the slides of sections. They were incubated for 30 min at room temperature in a humidified container and then washed three times with KH buffer. The labeled secondary antibodies [goat anti-rabbit IgG (H+L)-Alexa Fluor 488 and tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse IgG (Fc)] at 1:100 dilution in KH buffer with 3% BSA were added, and the slides were incubated for another 30 min. Washed slides were mounted with 50% glycerol mounting medium. Red (TRITC) and green (Alexa) immunofluorescence of muscle sections were observed using the Zeiss LSM 5 confocal microscope using the HeNe laser.

RESULTS

Previously, we described a complex putative signaling pathway, which is initiated by the binding of laminin and proceeds through syntrophin-Grb2-Sos1-Rac1-Pak1-JNK, ultimately resulting in the phosphorylation of c-jun on Ser^{65} (*8*). Here, we focus on two important issues that remain: what is the receptor for laminin-1 and how the syntrophin-Grb2-Sos1 interaction initiates signaling. The model pathway will be shown again in a later figure (Figure 8) after we have added further discoveries to it.

Laminin Is Depleted from Trypsinized Myocytes and α*-Dystroglycan Is Expressed in Both Myoblasts and Myotubes.* When cultured cells are briefly trypsinized in EDTA to remove them from the culture dish, this treatment depletes laminins as shown in Figure 1A. This allows us to add laminin-1 to trypsinized cells in later experiments to observe the effect of laminin-1. Densitometry reveals that 79% of the β1-laminin chain is depleted, while αDG (on the outer surface of the cells) and syntrophin (inside the cell) is not affected by trypsin. Laminin is a Ca^{2+}-binding protein, and trypsin digestion in EDTA may make it particularly susceptible.

There is some controversy about whether αDG is expressed (*13*) or not (*14*) in myoblasts, and this is relevant to our studies that use myoblasts for some experiments. Figure 1B shows that while C2C12 myotubes produce much greater amounts of αDG, it is also expressed in myoblasts. Figure 1C shows the medium obtained from the E3 transfected

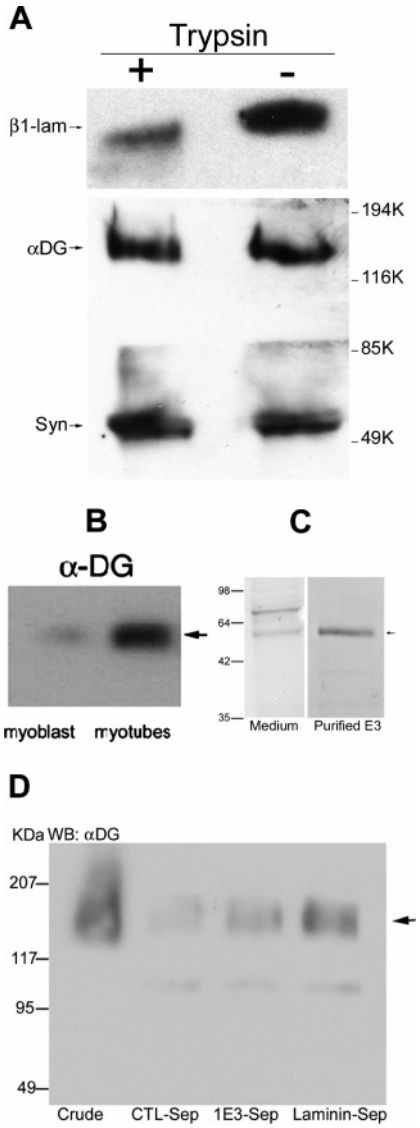

FIGURE 1: Expression of laminin β1 and αDG in C2C12 cells and E3 purification and binding. (A) C2C12 myoblasts were harvested with trypsin digestion or without (by scraping), and microsomes were prepared and solubilized in SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies against laminin β1 chain (2E8 monoclonal), αDG (VIA4) or syntrophin, and detected with enhanced chemiluminescence. (B) Similarly, cells collected by scraping were detected for αDG. (C) Medium from HEK 293 cells stably transfected to produce the E3 protein (hLNa1-E3 293) and purified E3 were applied to SDS−PAGE, and the gels were stained for protein with Coomassie Blue. (D) Rabbit muscle microsomes were solubilized in buffer K containing 1 mM $CaCl_2$ and 1% digitonin and incubated with Sepharose (CTL-Sep.), E3- or laminin-Sepharose. After washing, proteins were eluted in SDS−PAGE sample buffer, electrophoresed, electroblotted, and detected with the IIH6 (1:3000) α-dystroglycan antibody and goat anti-mouse IgM (H+L)-horseradish peroxidase (1:3000). For this antibody, blotting conditions were modified so that 20 mM Tris-HCl, pH 7.5, 100 mM NaCl without Tween replaced TTBS throughout.

HEK293 cells and the E3 protein obtained after purification. Figure 1D demonstrates that both E3- and laminin-1-Sepharose bind muscle microsome αDG.

Laminin-1 LG4−5 Domains (E3) Can Substitute for Intact Laminin-1. Figure 2 presents data from experiments to test whether the E3 protein, containing the LG4−5 modules of laminin α1, has the same effect as laminin-1. Microsomes from trypsinized rat L6 myoblast were immunoprecipitated

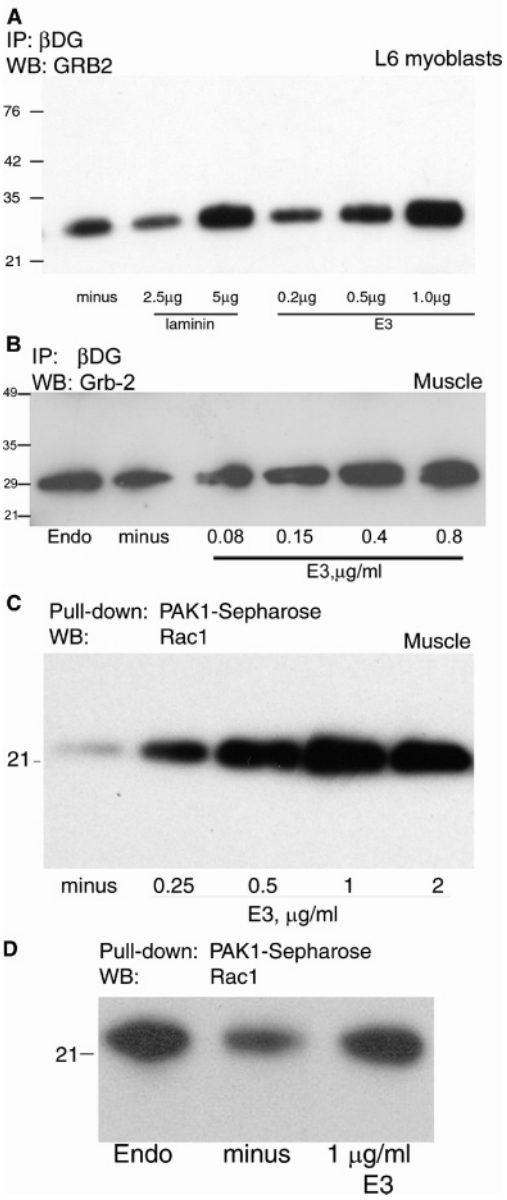

FIGURE 2: E3 can substitute for laminin-1 in inducing signaling. (A) L6 myoblasts were harvested by trypsin digestion and microsomes were isolated. The microsomes were made 1 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM GTP-γS and the concentrations of laminin-1 or purified E3 shown were added. After preclearing with protein G-Sepharose, 5 μg of a rabbit polyclonal antibody against β-dystroglycan was added. After an hour incubation at 4 °C, the membranes were solubilized in 0.5% deoxycholate, 1% Triton, and 0.5% IGEPAL, and incubation was continued for another an hour. A fresh portion of protein G-Sepharose was then added and incubated overnight at 4 °C with gentle mixing. After washing with buffer K, bound proteins were eluted with SDS−PAGE sample buffer. After electrophoresis and electroblotting, samples were probed with antibody against Grb2 and visualized by ECL. (B) Rabbit muscle microsomes were treated with Sepharose (Endo) or with heparin-Sepharose to deplete laminin (minus). To portions of depleted microsomes the concentrations of E3 shown were also added. After incubation for 1 h at 4 °C, the membranes were solubilized in 1% digitonin, and the samples were then treated as described in panel A. (C) Rabbit skeletal muscle microsomes were treated as in panel B except that after solubilization in digitonin, the samples were incubated with PAK1-GST-glutathione-Sepharose (PAK1-Sepharose) as previously described (*8*). After washing and elution in SDS−PAGE sample buffer, samples were Western blotted (WB) for Rac1. (D) As in panel C except that active Rac1 was compared for endogenous laminin and with E3.

with a β-dystroglycan polyclonal antibody and Western blotted for Grb2 in Figure 2A. This β-dystroglycan antibody has previously been used to isolate the dystrophin-associated complex because it does not block the laminin response, unlike some other antibodies (e.g., the syntrophin antibody (*8*) and see below). Clearly, laminin induces greater Grb2 binding to this complex, in agreement with a previous report (*8*), and E3 can substitute for laminin. The data in Figure 2A also shows that 1 μg of E3 has about the same effect as 5 μg of laminin. In several experiments comparing the effects of laminin on various signaling components, about 5−20-fold less E3 is required to have the same effect as laminin-1 on a mass basis (data not shown). Because laminin has about 20 times the mass of E3, this suggests that the E3 sequences account for a substantial fraction of laminin's effect. The data in Figure 2A also show that the lowest dose of laminin or E3 actually diminished the amount of Grb2 bound. This was only observed with L6 myoblasts and not with other cells or tissue (data not shown), and its cause is not known. Whatever its cause, it is interesting that both laminin and E3 cause this decreased Grb2 binding at low concentration as well as the increased Grb2 binding at higher concentrations, further showing their similar effects.

Similar results to those shown have also been obtained in mouse C2C12 myoblasts and myotubes; thus, human laminin α1 LG4−5 sequences bind to mouse (not shown), rat (Figure 2A), or rabbit (Figures 1D and 2B) microsomes showing these events and protein sequences must be common among mammals.

Previously, we showed that heparin-Sepharose can be used to deplete skeletal muscle microsomes of laminin (*8*). As shown in Figure 2B, before depletion the complex isolated with the βDG antibody contains Grb2, presumably because of the presence of endogenous (Endo) laminins; after depletion (minus), less Grb2 is found, and as the laminin is replaced with E3, again more Grb2 is found. Heparin depletion is somewhat variable. In Figure 2B, it is modest although still sufficient to observe the effect of E3. The effect of laminin after depletion with heparin was shown previously (Figure 8A in ref *8*) and is similar that shown in Figure 2B for E3.

We previously showed that laminin activates Rac1 (*8*). Figure 2C shows that E3 also has this activity. Only when Rac1 is activated (e.g., GTP-bound) does it bind to the $p21^{cdc42,\ Rac1}$-activated protein kinase (PAK1) (*36*). In microsomes depleted of laminin (minus), less Rac1 is active and bound, and the addition of E3 increases the amount of active Rac1. This effect appears to be dose-dependent and saturable at around 1 μg/mL. The amount of active Rac1 at saturating levels of E3, 1 μg/mL, is similar to that observed at endogenous levels of laminin (Figure 2D). Comparison with Figure 2B shows that the endogenous Grb2-binding or Rac1-activation is similar to that seen with E3 concentrations near 1 μg/mL. Thus, E3 causes similar Rac1 activation (Figure 2D) or grb2 recruitment (Figure 2B) as exogenous laminin-1or the endogenous muscle laminin (merosin, primarily laminin-2).

Syntrophin Becomes Phosphorylated on a Tyrosine when E3 or Laminin Is Bound. Figure 2 shows that endogenous laminins, laminin-1, and E3 all cause increased binding of Grb2 and activation of Rac1. How this is initiated was next investigated. For these experiments, the DGC was again

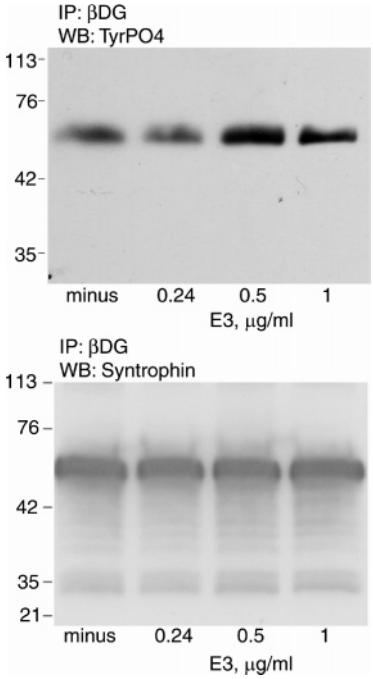

FIGURE 3: E3 induces the tyrosine phosphorylation of a protein the size of syntrophin. C2C12 myoblast were harvested with trypsin-EDTA to deplete laminin (minus), and microsomes were prepared. Microsomes in buffer K containing 1 mM $CaCl_2$, 1 mM ATP, and 1 mM GTP-γS were then incubated with the concentrations of E3 shown, and immunopreciptiated (IP) with 5 μg of the β-dystroglycan antibody. After washing, the immune complexes were eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with the phosphorylated tyrosine antibody ($TyrPO_4$, upper panel). The blot (top) was then stripped and reprobed with antibody against syntrophin (Syn, lower panel).

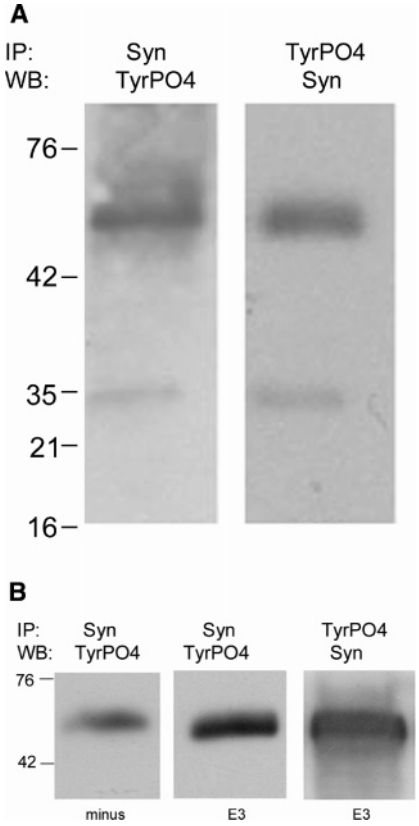

FIGURE 4: Syntrophin is tyrosine phosphorylated in the presence of E3. (A) The laminin-deleted rabbit skeletal muscle microsomes in buffer K with 1 mM $CaCl_2$, 1 mM GTP-γS, and 1 mM ATP were incubated with 1 μg/mL of E3 as described in Figure 3. Immunoprecipitation (IP) with the syntrophin antibody was probed with antibody against phosphorylated tyrosine (left). To the right is shown the same sample immunoprecipitated with the phosphotyrosine antibody and detected with syntrophin antibody. (B) Trypsinized C2C12 myoblasts microsomes with or without 1 μg/mL E3 were immunoprecipitated and Western blotted as in panel A.

immunoprecipitated with the polyclonal βDG antibody. Figure 2A,B shows that these immunoprecipitates again bind greater amounts of Grb2 as increasing amounts of E3 are added. In Figure 3, when these immunoprecipitates are probed with the phosphotyrosine antibody, a single band the size of syntrophin is detected (upper panel) and a band of the same size is detected with the syntrophin antibody (lower panel), indicating that syntrophin is tyrosine phosphorylated in the presence of E3. The same result is also obtained when laminin-1 is used instead of E3 (data not shown).

To confirm that the tyrosine phosphorylated protein is syntrophin, reverse IP/Western blots were used as shown in Figure 4A. Microsomes were depleted of laminins and incubated with 1 μg/mL E3. Immunoprecipitation with the syntrophin antibody reveals a band detected with the phosphotyrosine antibody, and when the reverse experiment is performed (immunoprecipitate with the tyrosine phosphate antibody and detect with the syntrophin antibody), a band of the same size is detected. These results clearly show that syntrophin is phosphorylated on tyrosine. This phosphorylation is increased by E3 (Figure 4B), in agreement with Figure 3, or by laminin-1 binding (data not shown).

An α-Dystroglycan Antibody Blocks Laminin-Induced Recruitment of Sos and the Phosphorylation of Syntrophin. Grb2-binding and Rac1 activation is highest when endogenous laminins, exogenous laminin-1, or E3 is present (Figure 2). Sos1 is known to activate Rac1 and bind to Grb2, and it is present in these syntrophin-containing complexes (*8*). Syntrophin forms oligomers (*37*) and binds to complexes containing syntrophin (*8*). Figure 5A shows that when syntrophin-Sepharose is used to pull-down these complexes, they also contain Sos1/2, in agreement with previous results (*8*). The αDG antibody VIA4 does not block laminin-binding to αDG, while the IIH6 antibody does block laminin binding (*6*, *27*). When the nonblocking antibody (VIA4) is used, adding exogenous (exo) laminin-1 restores Sos1 binding to laminin-depleted microsomes (minus). However, the blocking antibody (IIH6) prevents this, showing that laminin binding to αDG is responsible for Sos1 recruitment. Sos1 is an activator of Rac1, and it is likely that this recruitment of Sos1 (Figure 5A) causes the activation of Rac1 (Figure 2). Interestingly, the blocking antibody (IIH6) did not apparently displace endogenous (endo) laminin but can prevent exogenous (exo) laminin binding (Figure 5A). The blot (Figure 5A) also shows some smaller molecular weight bands, which are probably due to partial proteolysis of Sos1.

Since Grb2 binds this complex of proteins (Figure 2), GST-Grb2-glutathione-agarose (abbreviated Grb2-Sep. in Figure 5B) can be used to pull down these complexes. We used this as an independent means to investigate the effect of these antibodies on the laminin-1-induced syntrophin phosphorylation. When the antibodies were added along with laminin-1 to laminin-depleted microsomes, only the blocking antibody (IIH6) prevented the tyrosine phosphorylation of a

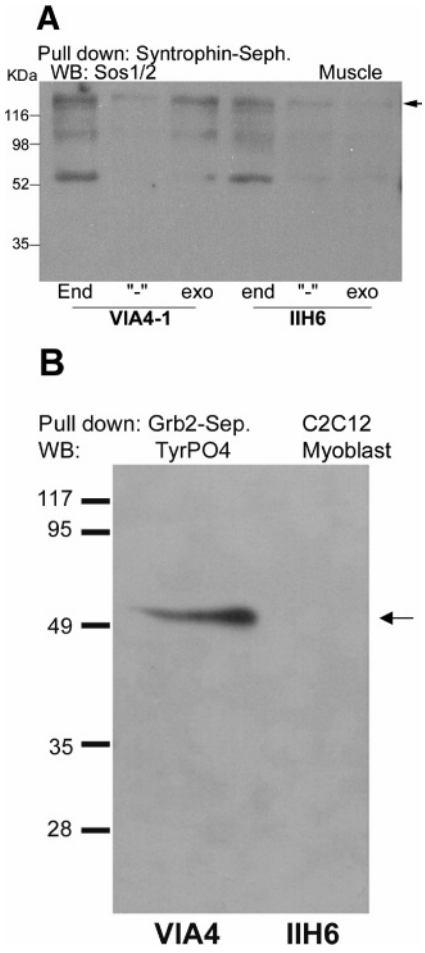

FIGURE 5: An antibody that blocks laminin binding to α-dystroglycan blocks Sos 1/2 recruitment and syntrophin phosphorylation. (A) Rabbit muscle membranes were either not depleted (Endo) or depleted of laminin (minus). The membranes, in buffer K containing 1 mM $CaCl_2$, 1 mM ATP, and 1 mM GTP-γS, were then incubated for 1 h at 4 °C with either the nonblocking VIA4 α-dystroglycan antibody as a negative control or with the blocking IIH6 antibody. Then, 3 μg of exogenous (exo) laminin-1 was added to a portion of the depleted microsomes. Syntrophin-Sepharose (100 μL) was then added, and the samples were made 1% digitonin (by adding 5% digitonin in water), and incubation was continued for a further 1 h. After washing, bound proteins were eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies against Sos1/2. (B) Microsomes from trypsinized C2C12 myoblasts were prepared in buffer K with 1 mM $CaCl_2$, 1 mM GTP-γS, and 1 mM ATP. Either the VIA4 or IIH6 antibody was then added along with 3 μg of laminin, and the samples incubated for 1 h at 4 °C. The samples were then mixed with GST-Grb2-glutathione-agarose (Grb2-Sep), made 1% digitonin, and incubated for a further 1 h. After washing, the bound proteins were eluted with SDS−PAGE and Western blotted using the phosphotyrosine antibody ($TyrPO_4$) for detection.

single protein the size of syntrophin (Figure 5B). Other experiments identify this protein as syntrophin (data not shown but see Figure 4 and Figure 6 below). Thus, laminin-1 binding to αDG is necessary for both the recruitment of Sos1/2 (Figure 5A) and for the phosphorylation of syntrophin (Figure 5B).

Tyrosine Phosphorylation of Syntrophin Alters the Way in Which Grb2 Binds. How this tyrosine phosphorylation affects Grb2 binding is shown in Figure 6. The two SH3 domains (N-terminal and C-terminal SH3) and the single SH2 domain of Grb2 were expressed as GST fusion proteins, bound to glutathione-agarose, and used in a pull-down experiment. The GST protein negative control binds little protein in the presence or in the absence of 3 μg of laminin-1. The SH2 domain binds a tyrosine phosphorylated band of the same size as syntrophin, which increases in the presence of laminin-1 (upper panel). When detected with the syntrophin antibody, the band is identified as syntrophin (lower panel) and its binding to the SH2 domain increases in the presence of laminin-1 (Figure 6), presumably because of the increased tyrosine phosphorylation of syntrophin under these conditions (Figures 3 and 4, and data not shown). Similar results were also obtained using laminin-depleted microsomes prepared from rabbit skeletal muscle or when E3 was used with either C2C12 cells (data not shown) or muscle microsomes (see Supporting Information Figure S1). The upper panel also shows that additional tyrosine phosphorylated proteins bind to the Grb2 SH2 and N-SH3 domains in these long exposures (Figure 6). In Figure 5B using shorter exposures, only the more intense syntrophin staining was observed.

Previously, we showed that syntrophin binds both the N-terminal and the C-terminal SH3 domains of Grb2 in vitro (*28*). However, the results in Figure 6 indicate that, in these C2C12 myoblasts or in skeletal muscle (data not shown), it is the C-terminal SH3 domain that binds. This binding is also increased somewhat in the presence of laminin-1 (lower panel, right-hand side), suggesting that perhaps the PXXP motif on syntrophin is more exposed in laminin's presence. Furthermore, the C-terminal SH3 domain is binding only to the syntrophin that is not tyrosine phosphorylated (compare to upper panel).

It is also clear that the C-SH3 is predominantly binding higher molecular weight syntrophin oligomers (Figure 6) as well as the monomer (arrows). The oligomers are not phosphorylated, while the SH2 domain binds only monomers, which are phosphorylated. In other experiments, the SH2 domain also bound syntrophin oligomers (see Figure S1, Supporting Information). This will be discussed further below.

Syntrophin, Tyrosine Phosphorylation, βDG, and Rac1 Co-Localize in the Sarcolemma. The experiments presented so far were performed in isolated microsomes and cells but are supported by immunohistochemistry experiments performed on sectioned rat gastrocnemius muscle (Figure 7). As shown in the upper row, syntrophin co-localizes with tyrosine phosphorylation over large areas of the sarcolemma. There are exceptions to this indicated by the arrowheads, which show regions where co-localization is not observed. The lower row shows that βDG has a similar distribution to that observed for syntrophin as expected and furthermore co-localizes with Rac1. Control experiments leaving out the primary antibody show no significant staining with either of the two secondary antibodies (see insets on lower panels). In other experiments, sections stained for Rac1 were overlayed with GST-PAK1 and stained with the GST antibody. These experiments show that PAK1 binds and co-localizes with Rac1, indicating that the Rac1 is active. However, since PAK1 also binds active CDC42, we cannot be sure if it is active Rac1 or CDC42 that is detected in those experiments (data not shown).

DISCUSSION

Here, we have shown that the effect of laminin on the Rac1 signaling pathway we previously reported (*8*) can be

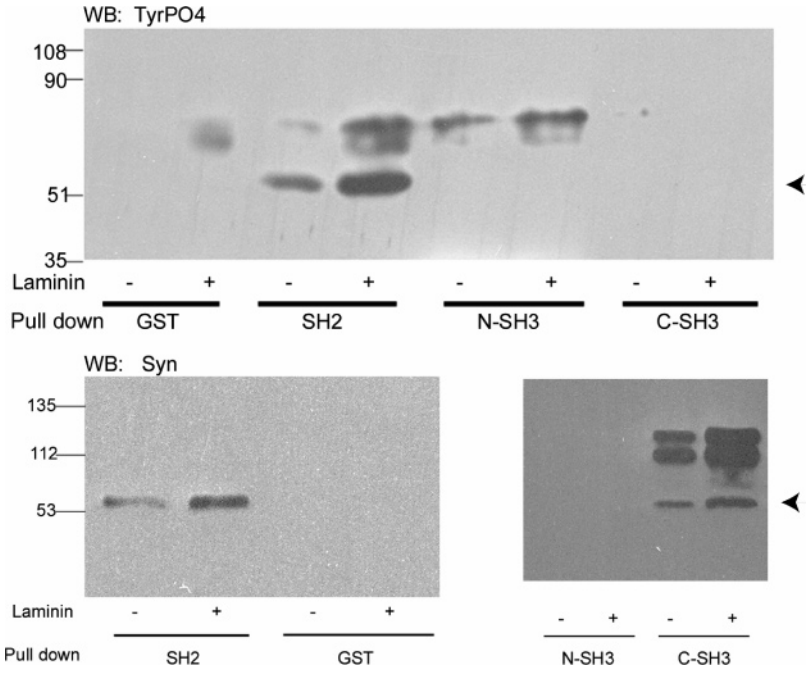

FIGURE 6: The SH2 domain of Grb2 binds tyrosine phosphorylated syntrophin, while the SH3 domains do not. The microsomes prepared from trypsin-EDTA treated C2C12 myoblasts were incubated in buffer K containing 1 mM ATP, 1 mM GTPγS, 1 mM $CaCl_2$ and with or without 3 μg of laminin at 4 °C for 1 h. Then incubation was continued with the additions of GST-glutathione-agarose (GST), the GST-SH2- (SH2) and the C- and N-terminal GST-SH3-glutathione-agaroses (C-SH3 and N-SH3, respectively) for another 1 h at 4 °C with gentle mixing and then solubilized by addition of 2× Dig. After washing, bound proteins were eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies against phosphorylated tyrosine ($TyrPO_4$, upper), and syntrophin (Syn, lower). For data from muscle microsomes and E3 stimulation, see Supplementary Figure S1, Supporting Information.

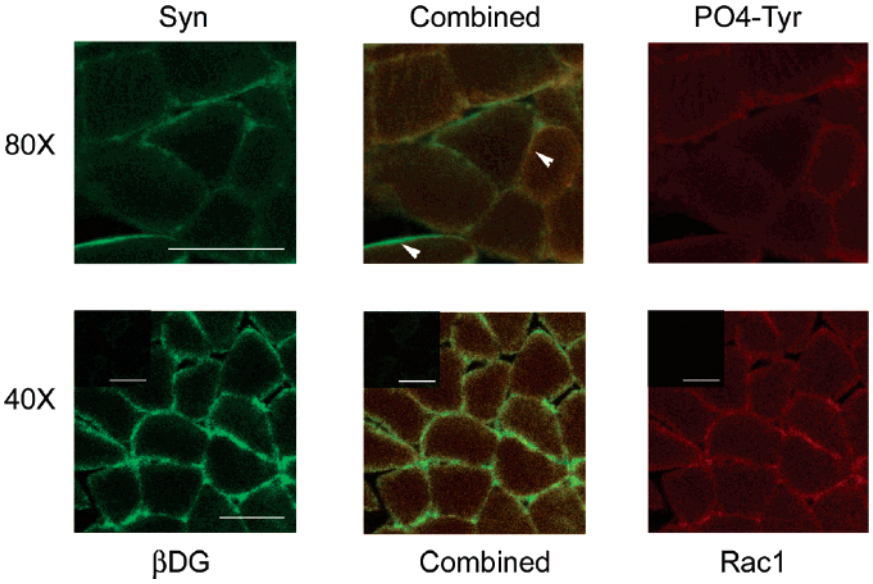

FIGURE 7: Syntrophin, tyrosine phosphorylation, β-dystroglycan, and Rac1 co-localize over much of the sarcolemma. The upper row shows staining of the same section with the syntrophin polyclonal (stained green) and tyrosine phosphate (red) monoclonal antibodies. Combined is the merged image showing yellow wherever the two co-localize at this resolution. The arrowheads show places where the two do not co-localize (green or red regions). The lower row is stained for β-dystroglycan and the Rac1. The bar in each is 50 μm. The inset in the lower panels shows staining at the same microscope settings without primary antibody but when both secondary antibodies are used.

attributed to the LG4−5 modules of laminin α1. Other laminin sequences may also be involved in vivo and certainly would be necessary for the normal polymerization of laminin and interaction with other constituents of the basement membrane, but clearly this small region of laminin is sufficient to induce the Rac1 signaling. It is the LG4−5 region of laminin-1 or -2 which binds αDG, heparin, and other ligands (*19*, *25*). In skeletal muscle, the predominant laminin in the mature muscle fiber basement membrane is laminin-2 (α2β1 γ1), not laminin-1 (α1β1γ1); however, laminin α1 has over 40% sequence identity with laminin α2. It is also known that laminin-1 and laminin-2 have many of the same effects on muscle cells, and laminin-1 was used previously in our studies of this pathway. Interestingly, the similarity of laminins is even greater in the E3 region with laminin-1 and -2 having 57% identity and 87% conserved sequences over the E3 region (data not shown). Furthermore, the endogenous laminin is causing similar effects to what is

observed with exogenous laminin-1 or the E3 region derived from it (Figures 2B,D and 5A). Laminin-1 was used here for an additional reason: the binding site for αDG has been localized to the LG4 module (*23*), while integrins bind to LG1−3 (*21*−*23*) of laminin-1. Thus, the similar effects of laminin-1 and E3 argues that laminin is binding to αDG to cause this signaling.

This is also supported by the ability of the antibody IIH6 to block Sos1/2 recruitment and syntrophin phosphorylation, both necessary steps of this pathway. This antibody is known to bind αDG and specifically block laminin binding while the antibody VIA4 which also binds αDG but does not block laminin binding serves as a negative control (*6*, *27*). Furthermore, a syntrophin antibody has been shown to also block this signaling (*8*). Upon the basis of all these data, there can be little doubt that laminin α1 LG4−5 (E3) binds to αDG through a complex containing syntrophin to cause signaling through the Rac1 pathway.

How this signaling is initiated probably involves the tyrosine phosphorylation of syntrophin. When laminin-1 or E3 bind, the C-SH3 domain of Grb2 binds somewhat better to syntrophin than in the absence of laminin, but the syntrophin being bound is not tyrosine phosphorylated (Figure 6). This suggests two features of this binding: The PXXP motif bound by C-SH3 must be more accessible to binding when laminin binds to αDG, suggesting that laminin binding on the outside of the cell triggers a conformational change in syntrophin on the inner surface of the membrane. This greater accessibility to C-SH3 may also make this region more accessible to the protein kinase responsible for phosphorylation. Second, when syntrophin is tyrosine phosphorylated, it no longer binds to C-SH3 but does bind to the Grb2 SH2 domain (Figure 6).

These two features of binding suggest a possible site for phosphorylation. There are six tyrosines in α-syntrophin, and two are located adjacent to one of the PXXP motifs of syntrophin previously identified in a Grb2 binding site (*28*). This sequence is

YVSRRCTPTDPEPR**Y** (α-syntrophin 215−229)

PXXP motifs are apparently 12−16 residues long (*38*) with the PXXP sequence in the approximate middle of this stretch. Including the two tyrosines, the sequence above is 15 residues long, with 7 residues N-terminal to the PTDP and 4 residues C-terminal of it. Phosphorylation of either or both of these tyrosines (Y^{215} or Y^{229}) could prevent C-SH3 binding to the phosphorylated syntrophin (Figure 6). Furthermore, searches of mouse α-syntrophin's sequence with the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) produces a score of 0.971 (one a scale of 0 to 1.0 with 1.0 being highly likely) for Y^{229}, while no other syntrophin tyrosine has a score above 0.306. While this is a rather speculative model, it accounts for the data and can be tested. However, since the C-SH3 binding and phosphorylation are indicative of a conformational change (see above), any tyrosine in syntrophin's sequence is possible.

Whatever the actual site of phosphorylation, this has the potential to provide a switch that activates Rac1 signaling as depicted in Figure 8. Panel B depicts a modified model similar to that proposed previously (*8*) but refined to add new observations made in the current report. Figure 8A shows a more detailed portion of the model. The C-SH3 domain of Grb2 binds syntrophin (Figure 6), presumably at one of the PXXP motifs identified previously (*28*). This complex is envisioned as unable to bind Sos but would retain Grb2 in the complex in the absence of laminin binding. Once laminin-1 binds, syntrophin becomes phosphorylated and now no longer interacts with the C-SH3 domain (Figure 6) and instead interacts with the SH2 domain of Grb2 (Figure 6). This new interaction then induces the N-SH3 domain to bind and activate Sos, which in turn activates Rac1 and the remainder of the pathway (Figure 8B).

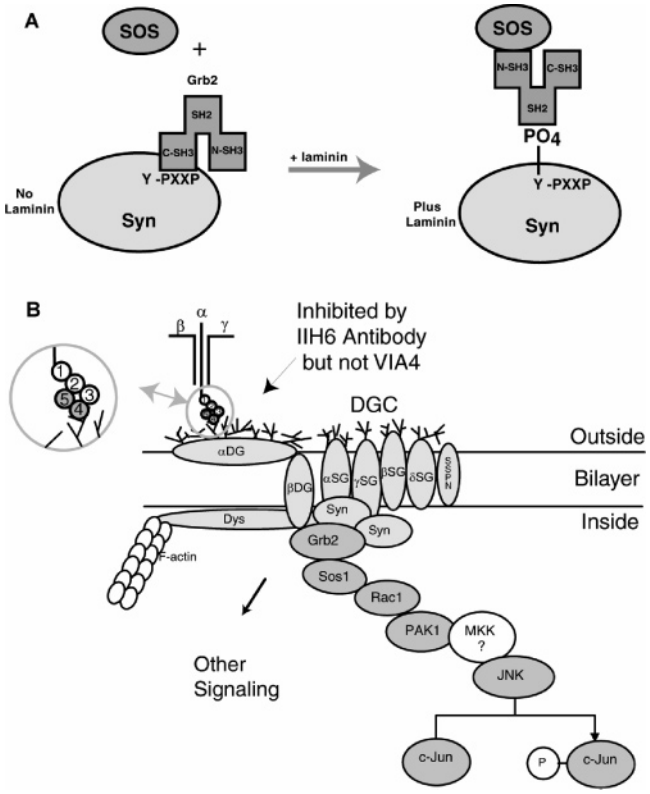

FIGURE 8: The Grb2 switch model. In panel A is shown a hypothetical scheme to account for how the laminin-induced syntrophin phosphorylation alters Grb2-binding to initiate downstream signaling. In panel B, the Rac1 signaling model (*8*) has been modified to show that the laminin LG4−5 region binding to α-dystroglycan is required.

The B panel modifies the original model of the pathway to incorporate new observations: that it is LG4−5 of the laminin-α chain that interacts with αDG to initiate signaling.

Figure 6 also shows that the C-SH3 domain of Grb2 binds syntrophin oligomers, while the SH2 domain did not in this experiment. We have included Supplementary Figure S1 (Supporting Information) which shows E3 has similar effects but in that experiment, and in other experiments, the SH2 domain bound syntrophin oligomers regardless of whether laminin or E3 was used. The possibility that syntrophin phosphorylation affects its oligomerization was considered but discarded. If only syntrophin monomers are phosphorylated, and then no longer oligomerize, why would the SH2 domain of Grb2, which binds phosphotyrosine sequences, bind syntrophin oligomers at all? It is more likely that the relatively small SH2 domain can bind phosphorylated syntrophin monomers or oligomers while the larger antibody against phosphotyrosines can only bind the phosphorylated syntrophin monomers and not the oligomers. This suggest

the phosphorylation site(s) may be near the protein−protein interaction sites in the oligomers.

It has been previously reported that βDG is also tyrosine phosphorylated (*29*, *39*−*41*); however, we found little evidence for it in the experiments presented here. The previous studies were done in HeLa (*39*), 3T3 (*29*), Cos7 (*29*), 293T (*41*), or C2C4 (*40*) cells of which only the latter is a muscle cell line such as were used in our study. In only one case, skeletal muscle was characterized (*41*). Many (*39*, *40*) were done in the presence of 2 mM H_2O_2 and 1 mM vanadate to inhibit phosphatases; however, it is also likely these toxic conditions stress cells (HeLa cells rounded and detached from the plate upon prolonged exposure (*39*)), and the phosphorylation may be in response to this stress. Other phosphatase inhibitors were used in other experiments (*29*, *39*, *40*) and may not be easily compared to our experiments in the absence of phosphatase inhibitors. Other experiments were done with cells transfected with v- or c-src (*29*, *40*, *41*). In the absence of transfection and c-src overexpression or H_2O_2/vanadate treatment, βDG phosphorylation was either not detected or shown to be at very low levels (*29*, *39*−*41*). In untreated muscle cells, no tyrosine phosphorylation of βDG was shown using commercially available antibodies (*40*) and was only observable in skeletal muscle using an antibody raised specifically against a phosphopeptide sequence derived from βDG (*41*). While the data showing that βDG is phosphorylated to some extent under some unusual conditions and in some cells are persuasive, we conclude that βDG is phosphorylated either transiently or at very low levels in muscle. Our studies differ in that we did not use phosphatase inhibitors or transfection, used a different phosphotyrosine antibody and reflect the phosphorylation under conditions we had previously used to characterize Rac1 signaling (*8*). Under these conditions, we do not observe βDG phosphorylation (Figures 3−6), although in Figure 4 we do detect a band at roughly 35 000 molecular mass, which is the size of γSG, previously reported to be tyrosine phosphorylated (*18*). We did not however confirm its identity. However, our data do not prove that β-dystroglycan is not phosphorylated, only that it is not phosphorylated to a detectable extent under the conditions we used.

Thus, the present work shows that the LG4−5 domain region of the laminin 1α chain binds α-dystroglycan, and this results in syntrophin phosphorylation on a tyrosine, which alters the association with Grb2. Under these conditions, Sos is bound, and Rac1 becomes activated. Many questions remain. The protein tyrosine kinase responsible for syntrophin's phosphorylation remains to be identified, and the site(s) of phosphorylation on syntrophin remains to be characterized. This phosphorylation however is likely to function as an elegant biochemical switch regulating Rac1 signaling in muscle.

The physiological function of this Rac1 signaling is not currently known. It seems likely that basement membrane laminin does not bind and unbind frequently in mature muscle. However, the binding of laminin is important in cell migration and attachment, the development of muscle (*42*), and the survival of myotubes (*43*). This signaling may serve roles in these processes. Alternatively, muscle contraction may stress the laminin−DGC interaction, perhaps signaling trophic effects, i.e., this signaling could serve as a mechanoreceptor similar to what has been proposed in smooth muscle (*44*). We are currently testing these hypotheses. However, since muscular dystrophies arise from defects in both laminin and laminin receptors, it is likely this signaling has important health consequences. Pharmacological modification of this signaling may provide a means of treatment.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Supplementary Figure S1 provides the results of an experiment on rabbit skeletal muscle microsomes analogous to Figure 6 (which was performed on C2C12 microsomes). It shows that syntrophin oligomers are bound by Grb2 SH2 and SH3 domains. This material is available free of charge via the Internet at http://pubs.acs.org.

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