Skeletal Muscle Signaling Pathway through the Dystrophin Glycoprotein Complex and Rac1*

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The dystrophin glycoprotein complex has been proposed to be involved in signal transduction. Here we have shown that laminin binding causes syntrophin to recruit Rac1 from the rabbit skeletal muscle. Laminin-Sepharose and syntrophin-Sepharose bind a protein complex containing Rac1 from the muscle membranes. The presence of heparin, which inhibits laminin interactions, prevents recruitment of Rac1. The dystrophin glycoprotein complex recruits Rac1 via syntrophin through a Grb2-Sos1 complex. A syntrophin antibody also prevents recruitment of Rac1, suggesting that the signaling complex requires syntrophin. Pak1 is in turn bound by Rac1. c-Jun NH2-terminal kinase-p56 is phosphorylated and activated only when laminin is present, and the p54 isoform is activated when laminin is depleted or binding is inhibited with heparin. In the presence of laminin, c-Jun is activated in both skeletal muscle microsomes and in C2C12 myoblasts, and proliferation increases in C2C12 myoblasts. We postulate that this pathway signals muscle homeostasis and hypertrophy.

In skeletal muscles, dystrophin, dystroglycans, and syntrophins are found in a complex with other proteins and glycoproteins, the dystrophin glycoprotein complex (DGC),1,2 whose defects cause Duchenne, Becker, various limb girdle, and other inherited muscular dystrophies. The integrity of the complex is essential for muscle cell viability. The DGC provides a link between laminin in the extracellular matrix and the cytoskeleton.

The discovery of calmodulin binding to dystrophin and syntrophin led to the proposal that the DGC was a signal transduction complex. Indeed, the numerous signal transduction proteins now known to have an association with the DGC make it almost certain that this complex is involved somehow in cell signaling. β-Dystroglycan and syntrophin have been shown to bind Grb2 (6, 7), suggesting a role in signal transduction. Grb2 has also been shown to recruit focal adhesion kinase 125 to the DGC (8, 9). Phosphorylated tyrosines on focal adhesion kinase 125 act as docking sites for molecules such as Grb2 which participate in multiple signal transduction pathways (10).

Syntrophin also binds neuronal nitric-oxide synthetase (11, 12), muscle and nerve voltage-gated Na+ channels (13), the mitogen-activated protein kinase SAPK3 (stress-activated protein kinase 3) (14), and calmodulin (15). A domain unique to syntrophins, the SU domain, has been shown to bind Ca2++/calmodulin (15), and the SU domain in addition to other sequences in the COOH-terminal of the protein binds to dystrophin. Ca2++/calmodulin binding inhibits the syntrophin-dystrophin interaction (15). The NH2 terminus of the pleckstrin homology 1 domain and the NH2 terminus of the PDZ domain have been reported to bind calmodulin (16) in a Ca2+-independent manner (15, 17). This domain has also been shown to be involved in the oligomerization of syntrophin in vitro in a Ca2+-dependent manner (17). Calmodulin inhibits oligomerization in a Ca2+-independent manner (17). Recently, syntrophin has been shown to bind Grb2, an SH2/SH3 adapter protein (7). Thus, syntrophins likely act as adapters between the dystrophin complex of proteins and components of the cell signaling apparatus.

Cell signaling often involves small G proteins. Adapter proteins such as Grb2 often recruit other proteins, which activate small G proteins. Rho family GTPases, such as Rac1, Cdc42, and RhoA, regulate a wide spectrum of cellular functions, ranging from cell growth and cytoskeletal organization to secretion (18, 19). They are critical for skeletal muscle differentiation and regulate the expression of MyoD and myogenin.

Within the DGC complex, the dystroglycans bind to muscle laminin-2 (α7β1γ1), merosin, the striated muscle isoform of laminin (20, 21). Signaling cellular attachment to laminin might be the long sought function of this complex (7). Laminin also binds to integrins (11, 22, 23), and cases of congenital myopathy have been linked to mutations in α7 integrin (24), and α7 null mice have the muscular dystrophy phenotype (25). The involvement of integrins and the DGC in muscular dystrophies suggests that both are important for muscle cell viability.

We show here that laminin binds through a complex containing dystroglycans, syntrophin, and several signaling proteins. In the presence of laminin, one particular isoform of c-Jun NH2-terminal kinase (JNK) becomes active, whereas in the absence of laminin, a different JNK isoform is active. c-Jun becomes phosphorylated when laminin is present, and cell proliferation increases in C2C12 myoblasts. We propose that this laminin-induced signaling is a mechanism by which muscle cells are stimulated to grow.

EXPERIMENTAL PROCEDURES

Materials—GDP, GTP, and GTP-S were purchased from Sigma. [γ-32P]ATP was obtained from PerkinElmer Life Sciences. Antibodies against H-Ras, phosphorylated JNK (specific for phospho-JNK1 or 2), JNK1, phosphorylated (on Ser-63) c-Jun, RhoA, and Sos were from Santa Cruz Biotechnology. Antibody against Rac1 was from Upstate Biotechnology. Antibody against Cdc42 (C70820) was from Transduc-
tion Laboratories. Antibodies against α- and β-dystroglycan and dystrophin were from Novoceastra. Antibody against recombinant mouse α-syntrophin (amino acids 2–503) was produced in rabbit and purified by affinity chromatography on sytophin A (amino acids 4–274)-Sepharose. Antibodies against α- and β-dystroglycan were the generous gifts of Dr. Tamara C. Petrucci (Laboratorio di Biologia Cellulare, Istituto Superiore di Sanita, Via le Regina Elena, Roma, Italy). Goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate and goat anti-rabbit IgG (H+L)-horseradish peroxidase conjugate were from Bio-Rad. Nickel-nitritrocitric acid-agarose was from Qiagen. Cytochrome-bred pre-activated Sepharose and heparin-Sepharose were from Sigma. Mouse laminin was obtained from Collaborative Biomedical Products. All other chemicals were of the highest purity available commercially.

Preparation of myosin kinase His5-Syn—The myosin kinase His5-Syn was prepared and purified as described previously (15). Plasmids for the expression of GST-Rac1 (wild type), GST-Rac1 (V12), GST-Rac1 (N17), and GST-PAK1 were kindly provided by Dr. Yi Zheng (Department of Biochemistry, University of Tennessee, Memphis). GST-Rac1, GST-Rac1 (V12), GST-Rac1 (N17), and GST-PAK1 were expressed in Escherichia coli BL21 strain and purified by affinity chromatography on glutathione-agarose beads (Amersham Biosciences) as described elsewhere (26, 27). The purity of the proteins was determined by 12% SDS-PAGE using the method of Laemmli (28). The major bands of the fusion proteins were of the expected size and relatively high purity (data not shown). The Bradford assay (29) was used to determine the protein concentration of the bovine cerebral cortex homogenate. The purity of the proteins was determined by 12% SDS-PAGE. The homogenate was centrifuged at 13,000 g for 15 min at 4 °C. The supernatant was then centrifuged for 30 min at 32,500 g at 4 °C to pellet total muscle membrane. The precipitated total muscle membranes were suspended using a Dounce homogenizer with 0.25 M sucrose, 20 mM Tris, pH 7.5, 100 mM NaCl, and incubated with 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. Microsomes 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 with protein G-Sepharose for 1 h, washed extensively with buffer K containing 1 mM GTPγS and 2 μg/ml leupeptin, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride) to minimize protein degradation (26). The homogenate was centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was then centrifuged for 30 min at 32,500 × g at 4 °C to pellet total muscle membrane. The precipitated total muscle membranes were suspended using a Dounce homogenizer with 0.25 M sucrose, 20 mM Tris, pH 7.5, 100 mM NaCl, and then for another 1 h with 1% digitonin. The rest of the experiment was performed as described above. Removal of laminin was confirmed by probing the supernatant from heparin-Sepharose with antibodies against merosin (Novoceastra, 1:100 dilution), laminin (1 chain (Santa Cruz Biotechnology, 1:2000 dilution), and 25% anti-laminin (Developmental Studies Hybridoma Bank, 1:500 dilution). The presence of dystrophin, α-syntrophin, and dystroglycans (α and β) in the supernatant was confirmed by their respective antibodies. For the antibody blockade experiments, skeletal muscle membranes were divided into two equal portions. To one portion syntrophin antibody (α-Syn) was added, and to the other portion anti-maltose-binding protein antibody was added as an unrelated antibody (both antibodies were added to a final concentration of 1%) and incubated for 1 h at 4 °C with gentle mixing. Then the muscle membrane preparation was incubated with 100 μl of control-Sepharose or 100 μl of laminin-Sepharose for 1 h at 4 °C with gentle mixing. The immune complexes were then incubated with protein G-Sepharose for 1 h, washed extensively with buffer K containing 1 mM GTPγS and 2 μg/ml leupeptin, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride) to minimize protein degradation (26). The homogenate was centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was then centrifuged for 30 min at 32,500 × g at 4 °C to pellet total muscle membrane. The precipitated total muscle membranes were suspended using a Dounce homogenizer with 0.25 M sucrose, 20 mM Tris, pH 7.5, 100 mM NaCl, and then for another 1 h with 1% digitonin. The rest of the experiment was performed as described above. Immunoprecipitation—Laminin was depleted from rabbit skeletal muscle microsomes using heparin-Sepharose as described above. To the laminin-depleted portion of the microsomes 3 μl of 1 mg/ml exogenous laminin was added to buffer K containing 100 μg/ml anti-laminin (Developmental Studies Hybridoma Bank, 1:500 dilution) and visualized by ECL.

Phosphorylation of JNK—Laminin was depleted from rabbit skeletal muscle microsomes using heparin-Sepharose as described above. To the laminin-depleted portion of the microsomes 3 μl of 1 mg/ml exogenous laminin, 1 μM RGDS, or 1 μM RGES peptide was added in buffer K containing 1 mM GTPγS, 1 mM CaCl2, and 1 mM ATP for 1 h at 4 °C with gentle mixing. Microsomes were then solubilized by adding 1% Triton X-100, 0.5% IGEPIAL, and 0.5% sodium deoxycholate. Incubation was continued for another 1 h at 4 °C with gentle mixing. The immune complexes were then incubated with protein G-Sepharose for 1 h, washed extensively with buffer K containing 1 mM GTPγS, 1 mM CaCl2, and 1 mM ATP for 1 h at 4 °C with gentle mixing. The immune complexes were then incubated with protein G-Sepharose for 1 h, precipitated, and washed extensively with buffer K. The bound proteins were detected by anti-Grb2 antibody (1:2000 dilution) and visualized by ECL.

RESULTS

Two components of the DGC (β-dystroglycan and syntrophin) bind Grb2 (6, 7), and recruitment and activation of small G proteins are usually a consequence of the action of Grb2. Because the DGC binds laminin we investigated whether laminin binding to the DGC initiates signaling by activating small G proteins. Laminin-Sepharose or control-Sepharose was incubated with rabbit skeletal muscle microsomes, the membranes...
were detergent solubilized, and the presence of bound small GTPase proteins was investigated using specific antibodies. As shown in Fig. 1A, laminin-Sepharose binds Rac1 but not other small G proteins tested. Furthermore, most of the Rac1 found in crude microsomes binds laminin-Sepharose.

Laminin is known to bind two receptors on the outside of the sarcolemma, integrins (23) and α-dystroglycan in the DGC (4). Although integrins have no known association with syntrophin, syntrophin is a component of the DGC. Syntrophin oligomerizes (17), and thus syntrophin-Sepharose would bind complexes containing syntrophin, such as the DGC, as well as bind those proteins that associate more directly with syntrophin. Fig. 1B shows that syntrophin-Sepharose, like laminin-Sepharose, binds Rac1 but not the other small G proteins. Thus, laminin, which presumably binds on the outside of the sarcolemma, and an intracellular DGC protein, syntrophin, show similar binding.

Presumably, there is some complex of proteins containing laminin, syntrophin, and Rac1. To determine whether syntrophin was directly involved in Rac1 binding, laminin-Sepharose was again used to bind this complex, and a specific, affinity-purified syntrophin polyclonal antibody was added to determine its effect on Rac1 binding. As shown in Fig. 2, the syntrophin antibody blocks the Rac1 association with laminin, whereas another, irrelevant antibody had no effect. Thus, the order of the components must be laminin-syntrophin-Rac1.

Rac1 is known to oligomerize (31). Fig. 2 not only shows p21 Rac1 but also shows some higher molecular mass forms that also bind the specific Rac1 antibody. These have molecular masses consistent with Rac1 dimers and tetramers and are apparent in the crude microsome sample, whereas the dimer is apparent in the third lane in Fig. 2. These are also seen in some of the other experiments. The significance of Rac1 oligomerization is not known.

To determine whether syntrophin binds to Rac1 directly, an enzyme-linked immunosorbent assay type experiment was performed in which a microtiter plate was coated with syntrophin and overlaid with GST-Rac1 (WT), GST-Rac1 (V12) or GST-Rac1 (N17). None of the forms of Rac1 such as wild type, GDP-bound, or GTP-bound forms, respectively, showed any direct association with syntrophin (data not shown), whereas a positive control, GST-Grb2, does bind in this assay (7). Thus, binding of Rac1 to syntrophin-Sepharose is caused by other components in the microsome complex and not by a direct interaction with syntrophin.

Syntrophin has one of the highest affinities known for Grb2 (7). Furthermore, Grb2 binds Sos1, and Sos1 is known to bind to Rac1 and activate it (32). To determine whether Sos1 is present in microsomes and whether it associates with syntrophin, the experiment in Fig. 3 was performed. Crude muscle microsomes do indeed contain Sos1, and Sos1 binds syntrophin-Sepharose. Although Sepharose alone does bind a small amount of Sos1, syntrophin clearly binds much more.

Sos1 is a GTP/GDP exchange factor that activates Rac1 (32). Activated Rac1 (i.e. GTP-bound) in turn binds to the p21

-activated protein kinase, PAK1. The p21-binding domain of PAK1 was produced as a GST fusion protein and immobilized on glutathione-Sepharose. The fused GST sequence was used as a negative control. As shown in Fig. 4, PAK1 binds Rac1 as expected but also reveals the association of Rac1 with syntrophin and α-dystroglycan, two components of the DGC. Thus, the complex under investigation contains laminin-dystroglycan-syntrophin-Sos1-Rac1-PAK1. α- and β-Dystroglycan are tightly associated in the sarcolemma, and α-dystroglycan binds laminin (33); thus, presumably, laminin and β-dystroglycan are associated with α-dystroglycan.

As a further control, the experiment was repeated using an
Molecular mass markers are shown on the right. Antibody against Sos. The buffer. Samples, after electrophoresis and electroblotting, were probed with antibody against Sos. The arrow shows the expected size of Sos. Molecular mass markers are shown on the right.

FIG. 3. Sos is recruited by syntrophin. Rabbit skeletal muscle microsomes (C) were incubated syntrophin-Sepharose (Syn) or control-Sepharose (S) in buffer K containing 1 mM CaCl₂ and 1 mM GTPγS. After washing, bound proteins were eluted with SDS-PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibody against Sos. The arrow shows the expected size of Sos.

FIG. 4. Syntrophin recruits p21-activated kinase. Rabbit skeletal muscle membranes were incubated with GST (GST) or GST-PAK1 (PAK1) bound to glutathione-Sepharose in buffer K containing 1 mM CaCl₂ and 1 mM GTPγS for 1 h at 4 °C. After extensive washing, bound proteins were eluted with SDS-PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies against syntrophin (Syn), β-dystroglycan (βDG), and Rac1 (Rac1). C represents crude microsomes. The top arrow shows the expected size of α-syntrophin detected by its antibody, the middle arrow shows the band detected by β-dystroglycan antibody, and the bottom arrow shows the band detected by the Rac1 antibody. Molecular mass markers are shown on the right.

antibody against the Na⁺,K⁺,Cl⁻-cotransporter, an unrelated sarcolemma protein; PAK1-GST-glutathione-Sepharose did not bind this protein (data not shown), showing that binding is specific for DGC components rather than binding all sarcolemma proteins indiscriminately.

Furthermore, much of the Rac1 present in the microsomes (C) must be in the activated, GTP-bound form under these conditions because it is only the GTP-bound, activated form of Rac1 which binds to PAK1 (Fig. 4). These experiments are performed in 10 mM MgCl₂, conditions under which Rac1 is known to exchange GDP only very slowly (34) unless stimulated to do so by a GDP/GTP exchange factor such as Sos1. This experiment was performed in the presence of nonhydrolyzable GTPγS to trap the activated Rac1 formed by this exchange. Thus, binding to PAK1 (Fig. 4) strongly suggests that the Rac1 present in these microsomes is being activated, probably by the interaction with Sos1.

To investigate further whether this complex of proteins serves some signaling function, it was desirable to deplete laminin from these microsomes. It has been reported that laminin is not bound by the DGC in EDTA and that millimolar Ca²⁺ is required for binding (11, 35). Thus, to remove laminin, microsomes were washed thoroughly with EDTA in the absence of Ca²⁺. Surprisingly, Western blots of these microsomes with three different anti-laminin antibodies revealed that laminin was still present (data not shown and Fig. 5A). Heparin is known to inhibit the interaction of laminin with α-dystroglycan (11), and heparin binds the α-subunit of laminin (36). When microsomes are incubated with heparin-Sepharose, most of the laminin is removed from the microsomes, as shown in Fig. 5A. The heparin-Sepharose binds several other proteins in addition to laminin as shown in B. However, as shown in C, α- and β-dystroglycan are not appreciably removed by treatment with heparin-Sepharose. Densitometry of the results in Fig. 5 reveals that although 94% of the laminin was removed (A), only 0.3% of α- and 8% of β-dystroglycan are removed by heparin-Sepharose treatment (C). Other experiments (data not shown) demonstrated that dystrophin and syntrophin, two other components of the DGC, were not appreciably depleted by heparin-Sepharose treatment. Thus, although heparin-Sepharose depletes microsomes of laminin, it does not significantly affect the amount of DGC components (C), although other, unidentified proteins are bound by heparin-Sepharose (B).

By depleting microsomes of laminin and then adding laminin back, the effect of laminin binding can be investigated. Such an
experiment is shown in Fig. 6. Again, syntrophin-Sepharose was used to bind the complex containing Rac1. Microsomes treated with Sepharose alone (−, heparin-Sepharose) still contain abundant endogenous laminin (Fig. 5), and syntrophin-Sepharose binds a complex containing Rac1 (Fig. 6). When laminin is depleted, syntrophin-Sepharose no longer binds Rac1, but adding exogenous laminin to the depleted microsomes reconstitutes Rac1 binding. In this experiment as in a previous experiment (Fig. 2), a band (indicated by an asterisk) is also observed, which is probably the Rac1 dimer.

When PAK1-Sepharose was used to bind complexes containing activated Rac1, Fig. 7 shows that the DGC proteins syntrophin and β-dystroglycan are present. After depleting laminin with heparin-Sepharose, these components are no longer detected (Fig. 7). Because we showed previously that heparin-Sepharose does not significantly deplete DGC components (Fig. 5), this experiment suggests that the complex containing β-dystroglycan-syntrophin-Rac1-PAK1 does not form in the absence of laminin. This result would be found if laminin binding causes the activation (GTP binding) of Rac1 and subsequent binding to PAK1. Although other possibilities exist, this hypothesis is supported by the other experiments presented here.

If there indeed is a complex containing laminin-dystroglycan-syntrophin-Grb2-Sos1-Rac1 as suggested by the above experiments, then a β-dystroglycan antibody would precipitate a complex containing Grb2 when laminin is present. This is shown in Fig. 5A. When laminin is depleted with heparin-Sepharose, much less Grb2 is present in the complex, and adding back laminin reconstitutes the binding of Grb2 to the β-dystroglycan-containing complex, presumably the DGC. This suggests that laminin binding to the DGC reversibly alters the binding of Grb2 to the DGC.

Activated PAK1 frequently causes activation of one or more JNKs. To determine whether this was also the case here, the experiment in Fig. 8B was performed. Each sample shown in the figure was immunoprecipitated with the β-dystroglycan antibody, and after electrophoresis and electroblotting, the result was detected with an antibody specific for the activated, threonine/tyrosine-phosphorylated forms of JNK. Microsomes containing endogenous laminin show activation of JNK-p46. When laminin is depleted with heparin-Sepharose, JNK-p54 is activated. When 1 μg of laminin is added back to the depleted microsomes, JNK-p46 is again phosphorylated. When heparin is added to microsomes containing endogenous laminin, it results in JNK-p54 activation similar to that observed by laminin depletion. Because heparin inhibits laminin binding to α-dystroglycan (11), it would be expected to give an effect similar to laminin depletion.

RGD sequences bind to and activate some, but not all, integrins (37), and some of these RGD-binding integrins are found in skeletal muscle sarcolemma and myoblasts (23). C2C12 myoblasts bind to RGD-containing sequences cross-linked to alginate in vitro, and this binding affects both proliferation and differentiation (38), and laminin α5 chains, which are present in skeletal muscle (39), are known to interact with integrins present in muscle by way of RGD sequences (40). However, RGDS peptide at 1 μM (Fig. 8B) or at concentrations of 10 μM, 100 μM, 1 mM, or 2 mM (data not shown) was unable to mimic the effect of laminin, and the RGES-negative control always has the same effect. Thus, the effects of laminin are not mediated by RGD-binding integrins. However, the experiment does not exclude the possibility that other integrins, binding laminin by other sequence motifs, are involved. However, because the JNK isoforms being probed were isolated using β-dystroglycan antibody immunoprecipitation (Fig. 8B), such integrins would have to have a close association with β-dystroglycan, an association that is not known to occur.

In other experiments, the JNK-p46 and JNK-p54 isoforms bound a JNK1-specific antibody (data not shown). Thus, JNK1
To characterize further the laminin effects on c-Jun and cell proliferation, cultured C2C12 myoblasts were used in the experiments in Fig. 9, B and C. These myoblasts contain laminin detectable with the laminin β1 chain monoclonal antibody (data not shown). As shown in Fig. 9B, c-Jun is phosphorylated to some extent in the presence of this endogenous laminin as detected with the phosphorylated c-Jun-specific antibody; adding additional laminin causes greater c-Jun activation.

Finally, c-Jun phosphorylation is often associated with cell proliferation. As shown in Fig. 9C, the addition of exogenous laminin to the growth medium causes a significant increase in the proliferation of C2C12 myoblasts for the two higher laminin doses (2.4 and 4 µg/0.1 ml). The effect is clearly dose-dependent. This effect of laminin on myoblast proliferation has also been reported previously (for review, see Ref. 23).

**DISCUSSION**

There is a complex of proteins which links laminin binding on the outside of the sarcolemma to a stress-activated protein kinase signaling pathway, and c-Jun activation shown by the experiments presented here. A model consistent with these experiments is shown in Fig. 10. To investigate this pathway, both ends of the pathway were probed to confirm the linkage and order of the pathway steps involved. Thus, the experiments are highly interrelated, and the approach was to jump from one end to the pathway and back to confirm the results obtained. In this model, the DGC is shown in light gray, and signaling components that were probed in experiments are shaded dark gray. The model proposes that laminin binding initiates assembly of parts and perhaps the entire signaling complex. The model will be discussed further below. In muscular dystrophies, muscle cells death increases, suggesting defects in these signaling pathways.

Laminin binds α-dystroglycan and integrins (11, 22), and laminin-Sepharose binds a protein complex containing Rac1 but not other small G proteins (Fig. 1A), and a specific syntrophin antibody blocks this interaction (Fig. 2). These data suggest that Rac1 is bound through a complex like the DGC, which contains syntrophin, and not through integrins, which have no known association with syntrophin. Syntrophin-Sepharose also binds Rac1 but not other small G proteins (Fig. 1B), and enzyme-linked immunosorbent assay type binding assays show that Rac1 does not bind syntrophin directly. This interaction must be indirect, involving other microsomal proteins. Because syntrophin oligomerizes (17), syntrophin-Sepharose may well be binding to microsomal syntrophin-containing complexes that in turn are interacting with Rac1. Syntrophin-Sepharose binds microsomal Sos1 (Fig. 3), and previously we had shown that syntrophin binds Grb2 (7), which is known to bind Sos1. Thus, the indirect interaction between syntrophin and Rac1 is indicative of a syntrophin-Grb2-Sos1-Rac1 complex. This is supported by other experiments. This would explain why the syntrophin antibody prevents recruitment of Rac1 (Fig. 2). When the DGC is immunoprecipitated with anti-β-dystroglycan, Grb2 is present (Fig. 8A). Also, when activated Rac1 is bound by PAK1-Sepharose, the isolated complex also contains syntrophin.
Cell counts were performed in triplicate. The number of cells is shown on the hemocytometer in trypan blue dye. The plate using trypsin and counted using a coordinate ordinate. The cells were then removed from the presence or absence of added laminin. The arrowhead indicates the expected position of c-Jun. B, myoblasts (1 x 10⁶ cells) were cultured in 10% fetal calf serum and Dulbecco’s modified Eagle’s medium for 24 h. The medium was then replaced with either fresh medium or medium containing 1 μg/ml laminin, and the culture was continued for another 24 h. The cells were then scraped from the plate and dissolved in sample buffer, and electrophoresis was performed on 4–12% polyacrylamide gels. After electrophoresis and electroblotting, the filters were probed with the phospho-c-Jun-specific antibody, followed by second antibody-horseradish peroxidase conjugate and developed using ECL detection. The position of c-Jun is indicated by the arrow on the left, and molecular mass markers are shown on the right. C, C2C12 cells (1 x 10⁶) were cultured as described in A for 24 h in the presence or absence of added laminin. The cells were then removed from the plate using trypsin and counted using a hemocytometer in trypan blue dye. The number of cells is shown on the ordinate. Cell counts were performed in triplicate (n = 3), and the bars show the mean and the error bars the S.D. Student’s t test (two tailed, unpaired values) was performed and showed that the control grown in the absence of added laminin was significantly different (indicated by the asterisk, *) from the cells grown in the presence of 2.4 (p = 0.02) and 4 μg (p = 0.035) of laminin.

Fig. 9. Laminin increases phosphorylation of c-Jun and proliferation in C2C12 myoblasts. A, rabbit skeletal muscle microsomes were depleted of laminin with heparin-Sepharose (–), treated, and then either 3 or 6 μg of laminin was added, or mock treated with Sepharose (end) and immunoprecipitated with the β-dystroglycan antibody as described for Fig. 8. After electrophoresis and electroblotting, the blot was probed with an antibody specific for phosphorylated c-Jun. The arrowhead indicates the expected position of c-Jun. B, myoblasts (1 x 10⁶ cells) were cultured in 10% fetal calf serum and Dulbecco’s modified Eagle’s medium for 24 h. The medium was then replaced with either fresh medium or medium containing 1 μg/ml laminin, and the culture was continued for another 24 h. The cells were then scraped from the plate and dissolved in sample buffer, and electrophoresis was performed on 4–12% polyacrylamide gels. After electrophoresis and electroblotting, the filters were probed with the phospho-c-Jun-specific antibody, followed by second antibody-horseradish peroxidase conjugate and developed using ECL detection. The position of c-Jun is indicated by the arrow on the left, and molecular mass markers are shown on the right. C, C2C12 cells (1 x 10⁶) were cultured as described in A for 24 h in the presence or absence of added laminin. The cells were then removed from the plate using trypsin and counted using a hemocytometer in trypan blue dye. The number of cells is shown on the ordinate. Cell counts were performed in triplicate (n = 3), and the bars show the mean and the error bars the S.D. Student’s t test (two tailed, unpaired values) was performed and showed that the control grown in the absence of added laminin was significantly different (indicated by the asterisk, *) from the cells grown in the presence of 2.4 (p = 0.02) and 4 μg (p = 0.035) of laminin.

These interactions clearly depend upon laminin binding. When syntrophin-Sepharose is used, it binds Rac1 from laminin-containing microsomes, does not bind it when laminin is depleted, and Rac1 binding is reconstituted by adding laminin to the depleted microsomes (Fig. 6). Immunoprecipitation with anti-β-dystroglycan shows that laminin is also required for Grb2 recruitment (Fig. 8A). Probing further downstream in the putative complex (Fig. 10) using PAK1-Sepharose to bind active Rac1, depletion of laminin also prevents the association of upstream syntrophin and β-dystroglycan (Fig. 7). Thus, the complex apparently assembles only in the presence of laminin.

The consequences of this complex assembly were also probed. PAK1 is implicated in mediating signaling from Rac1 to the JNKs (41). When laminin is present, JNK-p46 is activated (Fig. 8B), and c-Jun becomes phosphorylated (Fig. 9, A and B), whereas when it is depleted, or when laminin binding is inhibited with heparin, JNK-p54 is activated (Fig. 8B), and c-Jun is phosphorylated to a lesser extent (Fig. 9A). Because these results were obtained with proteins immunoprecipitated with a specific β-dystroglycan antibody (Figs. 8 and 9A), laminin presumably is binding to the DGC. In support of this idea, RGDS peptide, which activates many but not all integrin complexes, was ineffective (Fig. 8B).

However, although these experiments are most consistent with laminin acting through the DGC signaling complex, we cannot at this time exclude the possibility that there may be some cross-talk between integrins and the DGC, and this is also suggested in the model (Fig. 10). The data show that the Grb2-Sos1-Rac1-PAK1 components of the putative signaling pathway are associated with the DGC proteins syntrophin and dystroglycan. Although the simplest model would be that laminin binds directly to the DGC to cause all of the interactions shown, it is also possible that laminin binds to integrins to influence some of these associations. For example, laminin binding to integrins could cause cytoskeletal rearrangements or post-translational modifications, which facilitate or induce the association of these signaling components with the DGC we have shown here. Regardless of whether such cross-talk occurs, the direct involvement of DGC proteins is clear for several reasons. That the syntrophin antibody can block Rac1 recruitment, that the β-dystroglycan antibody can recover the activated JNK isoforms and c-Jun, and the association of Rac1, Sos1, or Grb2 with syntrophin or β-dystroglycan would all be difficult to account for in another way.

An interesting question to consider is how this putative pathway would function in myoblasts such as those used in Fig. 9, B and C. Myoblasts have diminished amounts of dystroglycan, and dystrophin is expressed at only a low level (42, 43). However, sufficient dystroglycan to initiate signaling may be present, and shortened dystrophin forms such as dp71 are expressed in myoblasts (43), and this or utrophin may replace dystrophin. We have also shown that syntrophin and Rac1 are...
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expressed in these C2C12 myoblasts. Thus, although the constituents of the laminin-binding complex may be somewhat different in myoblasts, most of the key components are present and could function in a similar way.

Kolodziejczyk et al. (44) have shown that murine models of DMD display a muscle-specific activation of JNK1, primarily the p54 isoform. They also reported that independent activation of JNK1 resulted in defects in myotube viability and integrity in vitro, similar to a dystrophic phenotype. In addition, direct muscle injection of an adenoviral construct containing the JNK1 inhibitory protein, JIP1, dramatically attenuated the progression of dystrophic myofiber destruction.

Taken together, these results suggest that a JNK-mediated signal cascade, such as the one proposed in Fig. 10, could contribute to the progression of the disease pathogenesis. Interestingly, we find the p46 isoform active when laminin binds to a dystrophic state, to become active in the absence of laminin. The dystrophic phenotype often results in vivo from disruptions of the DGC complex and would result in attenuated laminin signaling through this complex. Thus, our study and that of Kolodziejczyk et al. are consistent with one another. Here, we have shown that JNK-p46 is active in the presence of laminin (Fig. 8B) and that also in the presence of laminin c-Jun becomes phosphorylated (Fig. 9, A and B) and myoblasts proliferate (Fig. 9C). Our model (Fig. 10) proposes that laminin, acting through the DGC pathway, results in c-Jun phosphorylation, although there may be alternative explanations for the experiments performed in whole cells (Fig. 9, B and C). In our model, we have shown a question mark next to the activated JNK-p54 because we have no direct evidence for its function.

However, the results of Kolodziejczyk et al. (44) would suggest that activation of this JNK isoform is associated with the dystrophic phenotype.

Along depicted in the model in Fig. 10 is a partial list of other signaling components that have been linked to the DGC. Many of these, including calmodulin, focal adhesion kinase 125, and Akt, have already been discussed elsewhere in this paper.

Recently, it has been shown that the COOH terminus of β-dystroglycan becomes tyrosine-phosphorylated by c-Src, and this interferes with binding to dystrophin; recruits other SH2 domain proteins including c-Src, Fyn, and Shc; and causes relocalization of β-dystroglycan to intracellular vesicles (45, 46). Recently, we have found that the heterotrimeric G protein, Gs, binds to syntrophin. Clearly, the DGC is involved in many different kinds of cell signaling.

We show that the dystrophin glycoprotein complex, or some related complex containing β-dystroglycan and syntrophin, is a laminin receptor that signals, and we propose that the effect of signaling is to cause the cell to grow normally whenever it is attached to merosin (muscle laminin). This is consistent with the effect of laminin on C2C12 myoblast proliferation (Fig. 9C) and c-Jun activation (Fig. 9, A and B). Activation (phosphorylation) of c-Jun induces cell proliferation and prevents apoptosis and premature cell senescence (47). Thus, we propose that the DGC, by acting as a laminin receptor, signals the normal muscle cell to grow rather than die. Recently, blocking laminin binding to α-dystroglycan was shown to increase apoptosis and interfere with Akt (protein kinase B) activation (48), which further supports this hypothesis and suggests that other signaling arises at the DGC. Laminin-induced cell proliferation (Fig. 9C) was shown and is consistent with previous reports (22, 49).

It has also been reported that agrin, a major component of the basal lamina of the neuromuscular junction which also binds to α-dystroglycan, leads to muscle-specific activation of Rac and Cdc42 in differentiated myotubes, and this activation is essential for acetylcholine receptor clustering at synaptic sites (50). In our laboratory, we have found that after tenotomy the muscle is no longer subjected to normal stretching, and the DGC receptor down-regulates. In addition to the down-regulation of the DGC, there is a large effect on G protein signaling through Ras, RhoA, and Cdc42 without having an effect on Rac1 signaling (53). Because atrophied muscle has the potential to recover rapidly from atrophy, it must maintain all of the components necessary for the recovery including the ability to signal growth through Rac1 as we have proposed here. Thus, Rac1 may not be diminished in atrophy precisely because it is required for recovery from atrophy.

We have proposed earlier that the DGC may function as a stretch receptor (53). The signal transduction mechanism for this stretch receptor is not known. However, stretch signaling of an elastin-laminin receptor involves Grb2 and small GTPase in smooth muscles (52). In bladder smooth muscles, JNK (stress-activated protein kinase 2) is activated as a result of stretch (51), a frequent consequence of Grb2 and small GTPase signaling. Thus, our model that DGC-laminin signaling promotes muscle growth and its putative function as a stretch receptor regulating atrophy/hypertrophy are similar concepts. In such a stretch receptor paradigm, laminin would remain attached to its DGC receptor but would be stressed by stretching.

Acknowledgments—We thank Inga Warr for excellent technical assistance and Drs. Himanshu Gadgil and Yi Zheng for many helpful discussions. We acknowledge gratefully the generous gifts of plasmids

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2 Y. W. Zhou and H. W. Jarrett, unpublished data.

3 S. A. Oak, Y. W. Zhou, and H. W. Jarrett, unpublished data.
