Dystrophin-glycoprotein complex and Ras and Rho GTPase signaling are altered in muscle atrophy

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The dystrophin-glycoprotein complex (DGC) was unknown before 1990, and its physiological role is not currently known. We first suggested that the DGC protein complex was a signal transduction complex in 1992 (41). Since then, calmodulin kinase II (40), stress-activated protein kinase (SAPK) 3 (28), nitric oxide synthetase (1, 24), voltage-gated Na+ channels (23), phosphatidylinositol 4,5-bisphosphate (15), growth factor receptor-bound protein 2 (grb2) (48), and other cell signaling components have all been localized to this complex. The interaction of the β-dystroglycan cytoplasmic region with the SH3 domain of grb2, an adaptor protein involved in signal transduction and cytoskeletal organization, has been reported (64). Cavaldesi et al. (13) found that grb2 mediates the interaction of β-dystroglycan from brain synaptosomes with focal adhesion kinase p125FAK, a nonreceptor tyrosine kinase that also participates in the intracellular signaling pathways triggered by integrins. There is now a general agreement that the complex probably serves a role in cell signaling.

Dystroglycan binds to the muscle laminin merosin (29), which resembles fibronectin binding to initiate integrin signaling. There is a great similarity between what is known of the signaling proteins associated with the DGC and that of the integrin signaling, as pointed out by Yoshida et al. (65). Thus signaling cellular attachment to laminin might be the long-sought function of the DGC complex. A vast array of signaling molecules and cascades has been connected to integrin signaling, including p125FAK, protein kinase C, mitogen-activated protein kinase, phosphatidylinositol 3-kinase, Ras, and Rho (16). Ras and Rho proteins are low-molecular-weight GTPases. Ras proteins are central to the control of cellular growth and division. Ras binds to and is regulated by grb2-associated Sos (57). The Rho family of GTPases, which include RhoA, Rac1, and Cdc42, are critical for skeletal muscle differentiation and can regulate the expression of MyoD and myogenin (12, 58). An early effect of integrin-type signaling is the induction of the GTP-bound form of small GTPases, and one of the end results is the event...

INTEGRINS AND DYSTROGLYCAN are coexpressed in skeletal muscle and play critical roles during skeletal muscle differentiation and development (36, 43). Dystroglycan links the extracellular matrix with the actin cytoskeleton, and it exists as a noncovalently linked complex of α- and β-subunits (10, 20, 31, 61). In skeletal muscle cells, dystroglycan forms a tight complex with dystrophin and dystrophin-associated proteins, including α-, β-, γ-, and δ-sarcoglycans, and syntrophins (10, 32, 39, 46, 47, 53, 61). The integrity of the complex is essential for the viability of muscle cells because disruption of the complex due to a defect in dystrophin or any one of the sarcoglycans has been reported to cause various forms of inherited muscular dystrophy (10, 39, 45, 47, 53, 61).
tual NH₂-terminal phosphorylation of c-Jun by c-Jun NH₂-terminal kinase (JNK) (33). Phosphorylation of c-Jun prevents apoptosis and promotes homeostasis and hypertrophy.

We formulated a hypothesis that, just as integrins bind fibronectin to initiate signaling, laminin binding to the DGC complex initiates signaling; i.e., the DGC complex is a laminin receptor. By linking the cytoskeleton to the matrix, the DGC complex could sense the mechanical forces (stretching) of the sarcolemma that accompany contraction. Mechanical stretch, even in the absence of nerve activity, has long been recognized as a powerful modulator of gene expression in skeletal muscle. Muscle that is stretched exhibits a hypertrophic response, whereas an atrophic response is observed in slackened muscle (6, 25, 30, 55, 60). The initiating signal transduction mechanism for stretch has yet to be identified. In our model, the DGC complex is a laminin receptor. By linking the cytoskeleton to the matrix, the DGC complex could sense the mechanical forces (stretching) of the sarcolemma that accompany contraction.

Muscle membrane preparation for GTPases. For following the GTPases H-Ras, RhoA, Rac1, and Cdc42 that are downstream (of the DGC complex) containing p115-integrin, the final membrane pellet preparation was centrifuged at 13,000 g for 15 min at 4°C. The supernatant was centrifuged for 30 min at 30,600 g at 4°C to pellet total muscle membranes.

To examine dystrophin, syntrophin, β-dystroglycan, and α-sarcoglycan, the final membrane pellet preparation was suspended in 50 mM Tris-HCl (pH 7.5) and 0.3 M NaCl. The protein concentration of normal and atrophied muscle membranes was balanced by diluting 10 μl of each sample with 90 μl of 1% SDS, determining absorption at 280 nm, and diluting samples to give equal absorption. Each sample was mixed with equal volumes of twice concentrated SDS-polyacrylamide gel electrophoresis sample buffer and boiled for 5 min at 95°C. Proteins were fractionated on a Bio-Rad 5–20% Tris-HCl gradient SDS-polyacrylamide gel according to Laemmli (35). Prestained SDS-PAGE molecular weight markers were obtained from Invitrogen (SeeBlue markers).

Muscle membrane preparation for GTPases. For following the GTPases, the powdered muscle sample was divided into three equal portions (by weight). Two portions were homogenized in 7 vols of the same homogenization buffer containing the protease inhibitor cocktail. The third portion was homogenized in homogenization buffer with 10 mM MgCl₂ and protease inhibitors. The total muscle membranes were then isolated as described earlier.

The membrane preparation of the first two portions were suspended in 50 mM Tris, 0.3 M NaCl, and 0.5 mM EDTA (with protease inhibitors) for inducing GTP- and GDP-bound forms. The third portion was suspended in 50 mM Tris, 0.3 M NaCl, 10 mM MgCl₂, and 0.5 mM EDTA (with protease inhibitors) for measuring the endogenous amount of GTP-bound GTPases. The protein concentration of the three different samples was balanced by absorption at 280 nm as described earlier. Triton X-100 (25%) was added to all three samples to a final concentration of 1%. The samples were solubilized by mixing at 4°C for 30 min. Guanosine 5‘-O-(3-thiotriphosphate) (1 mM; for GTP-loaded samples) and GDP (for GDP-loaded samples) were added to the first and second portions, respectively, and mixed for 5 min. This was followed by the addition of 10 mM MgCl₂ to each sample and mixing.

METHODS

Muscle preparation. Sprague-Dawley rats (200–500 g) were used for all experiments. Animals were housed in light- and temperature-controlled quarters where they received food and water ad libitum. Animals were anesthetized with isoflurane for surgery and tissue removal. The Animal Care and Use Committee of the University of Tennessee Health Science Center approved all procedures. Muscle atrophy was induced in four rats (which served as duplicates). Ten days after surgery, rats were checked for weight gain and then anesthetized. The gastrocnemius muscles were removed from both the limbs, weighed, clamped frozen in liquid nitrogen, and stored at −80°C. Muscle samples from two rats (nearly 2 g each of control and test) were used for comparing DGC proteins, and samples from another two rats were used for following the GTPases, and stored at −80°C. Muscle samples from two rats were used for following the GTPases, and stored at −80°C.
for another 15 min. All three samples were centrifuged in microfuge tubes at 14,000 g for 15 min to remove the insoluble material. The final solubilized preparations were used for GTPase trapping.

**Preparation of PAK1, PKN, and Raf-Sepharose.** Glutathione S-transferase (GST) fusions of the p21-binding domains p21-activated kinase 1 (PAK1), protein kinase N (PKN), and Raf were generated in pGEX-2T vector (37). These proteins were purified to homogeneity on glutathione agarose columns (54). The protein concentration was determined by Bradford's method (8) with bovine serum albumin (BSA) as the standard. The purified GST-fusion proteins (5 mg of protein, 2.5 mg/ml) were covalently coupled to 1 g of cyanogen bro-mide-activated Sepharose (Sigma) by following procedures recommended by the manufacturer (Pharmacia). The supports were then washed with the coupling buffer (0.1 M NaHCO₃, pH 8.3, and 0.5 M NaCl) and blocked for 24 h with 0.1 M Tris-HCl, 0.8 mM CoCl₂, and 1% Triton X-100. The purified GST-fusion proteins were fractionated on a 12% SDS-polyacrylamide gel by electrophoresis.

**Kinase activity** at 280 nm of added protein and that recovered from the beads was determined by the difference between the absorbance of the beads (0.7 × 10⁻⁶) and protein coupled (0.7 × 10⁻⁶) protein. The kinase activity of Raf-Sepharose was determined using the kinase assay described in the manufacturer's manual (54). The protein concentration was determined by Bradford's method (8) with bovine serum albumin (BSA) as the standard. The purified GST-fusion proteins were fractionated on a 12% SDS-polyacrylamide gel by electrophoresis. The membrane preparations were fractionated on a 12% SDS-polyacrylamide gel by electrophoresis.

**Immunoprecipitation** buffer, and bound proteins were examined by electrophoresis.

**Immunoblot analyses.** Transfer of proteins to nitrocellulose was performed according to Towbin et al. (62) followed by immunoblot staining with antibodies. After blocking in TBST (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.2% Tween 20) containing 10 mg/ml BSA overnight, blots were washed three times (for 5 min each) with TBST containing 1 mg/ml BSA and incubated for 4 h at room temperature with 1:100 diluted antibodies for dystrophin, β-dystroglycan, or α-sarcoglycan and 1:200 diluted antibody for syntrophin. For measuring GTPases, the immunoblots were washed and incubated with 1:500 diluted antibody for H-Ras, 1:50 diluted antibody for RhoA, 1:1,000 diluted antibody for Rac1, and 1:200 diluted antibody for Cdc42. After the nitrocellulose membranes were washed three times (for 5 min each) in TBST with 1 mg/ml BSA, immunoblots were incubated for 1 h with 1:2,000 diluted, HRP-conjugated goat anti-mouse IgG for all the proteins except syntrophin. For syntrophin, 1:2,000 diluted HRP-conjugated goat anti-rabbit IgG was used. Finally, immunoblots were again washed three times with TBST with 1 mg/ml BSA and left in 1 M Tris-HCl, pH 8.5, until developed. The blots were developed using the enhanced chemiluminescence method with luminol and coumaric acid. For plasmid injection into muscle, a 1-cm lateral incision in the lower hindlimb was made to expose the soleus muscle (59). Plasmid (25 μg) in 10 μl of saline was injected in small aliquots throughout the muscle.
muscle. The wound was closed, and the animals were allowed to recover for 1 wk. Plasmid constructs were on the pMX-green fluorescent protein (GFP) internal ribosome entry site retroviral backbone, containing either wild-type Cdc42 (67), dominant-negative Cdc42 (T17N) (38), fast-cycling active Cdc42 (F28L) (37), or stuffer DNA (LacZ). This plasmid allows expression of bicistronic mRNA of GFP and small GTPases and permits direct visualization of the small GTPases expressing GTPases and permits direct visualization of the small GTPases (Fig. 1). Densitometric scanning revealed an 88 ± 2% reduction for β-dystroglycan and a 90 ± 3% reduction for α-sarcoglycan in atrophied muscle compared with control muscle membrane. Dystrophin could not be detected in atrophied muscle. The 59-kDa α-syntrophin did not show a significant change in the atrophied muscle compared with the control muscle. Typical of atrophy models and consistent with these data, total protein staining of heavy microsome gels to verify protein loading revealed changes in the expression of other proteins, as well. Therefore, it appears that a specific loss of dystrophin, β-dystroglycan, and α-sarcoglycan occurred during atrophy (per unit protein), whereas α-syntrophin was unaffected. Syntrophin also served as a fortuitous internal control in these experiments.

Small GTP-binding protein signals were characterized on the basis of their interaction with a GST-fusion protein derived from their corresponding downstream effector targets. When RhoA becomes active, it exchanges bound GDP for GTP and then binds PKN to initiate a downstream event (50). Likewise, Rac1 and Cdc42 activate PAK1 through binding to its p21-binding domain. This sequence, located in the NH2-terminal regulatory part of the protein, contains a highly conserved 14-amino acid Cdc42/Rac-interactive-binding (CRIB) domain (amino acids 74–88) found in many proteins interacting with Rac- or Cdc42-GTP (4). Ras-GTP binds with the Ras-binding domain of Raf with high affinity (18). It is important to maintain the Mg2+ cofactor in the guanine nucleotide exchange and GTP hydrolysis reactions of Rho family GTP-binding proteins has been discussed by Zhang et al. (66).

The effector fusion proteins used to trap the small GTPases exhibit a selective affinity for the GTP-bound form of GTPases (42). We first verified that this specificity for the active conformation of the GTPases is maintained in the isolated GST-fusion proteins. Purified GST-fusions were used as a probe in an affinity precipitation assay with different nucleotide-bound...
forms of GTPases. There was little or no interaction with the inactive GDP-bound forms obtained by incubation in GDP and EDTA. The results obtained for Cdc42 are shown in Fig. 2 and are representative of findings for the other GTPases (data not shown). We also verified that the GTPases did not bind nonspecifically to GST beads or to glutathione-Sepharose beads alone (data not shown). To solubilize the GTPases in the muscle membrane preparations. GDP- and GDP-loaded forms served as positive (total GTPase, activated and trapped) and negative controls (inactivated and, hence, could not bind to the protein kinase fusion protein), respectively. These controls allowed us to determine the percentage of the protein that was present in the active GTP-bound form in isolated muscle membrane. We found that 94 ± 2% of total H-Ras, 26 ± 5% of total Rac1, and 98 ± 1% of total Cdc42 were present in the active GTP-bound form in normal muscle membrane preparation (Fig. 3). When the total GTPases of normal muscle were compared with those of the atrophied muscle, there was a 67 ± 4% reduction for H-Ras and a 73 ± 5% reduction for Cdc42 in atrophied muscle membrane preparation (Fig. 3). RhoA was either completely lacking or present in low amounts that could not be detected in atrophied muscle membrane. There was no significant change in Rac1. The ratio of the active GTP-bound form to the total GTPases in atrophied muscle was the same as in normal muscle in the case of H-Ras, Rac1, and Cdc42. The ratio for RhoA in atrophied muscle was not determined because RhoA was not detectable in the atrophied muscle.

If the DGC complex is a laminin receptor involved in small GTPase signaling, as we propose, then a signaling complex might form containing laminin, β-dystroglycan, and small GTPases. To examine this possibility, we used an antibody depletion assay. Antibodies against laminin or β-dystroglycan were used to deplete microsomes from normal muscle of these proteins, and the remainder was examined for small GTPase content. The amount of H-Ras, Rac1, and Cdc42 trapped by their respective effector domain fusion proteins was reduced to 5–10% of the normal level following depletion with either of the antibodies (Fig. 4). In contrast, RhoA was considerably less affected by laminin or β-dystroglycan depletion (Fig. 4). As a control for non-specific removal of the small GTPases, we immunodepleted the membrane preparations of the α1-subunit of Na\(^+\)-K\(^+\)-ATPase. Immunodepletion of Na\(^+\)-K\(^+\)-ATPase did not affect the amount of the small GTPases remaining in the membrane preparations (Fig. 4). Immunoblot analysis of the proteins bound to the protein A-Sepharose beads confirmed that the laminin and β-dystroglycan depletion effectively immunoprecipitated the targeted proteins (data not shown). β-Dystroglycan was depleted from the membrane preparations treated with antibodies against laminin or β-dystroglycan, as well as by laminin-Sepharose.

Immunocytochemistry of the control medial gastrocnemius muscle showed uniformly arranged intact fibers, whereas the tenotomized muscle fibers were smaller in size compared with the normal fibers and were pulled apart from each other in some areas. These data are consistent with numerous reports from other laboratories. Because H-Ras and Cdc42 were reduced in muscle atrophy (Fig. 3) and found to be associated with laminin and β-dystroglycan in the depletion assay (Fig. 4), it was of interest to confirm these observations by immunostaining. β-Dystroglycan, H-Ras, Cdc42, and α-syntrophin were all primarily localized to the sarcolemma (Fig. 5). The β-dystroglycan fluorescence at the sarcolemma (Fig. 5A) was reduced in the tenotomized medial gastrocnemius muscle fibers (Fig. 5B). Unlike β-dystroglycan, intracellular nonsarcolemmal

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![Fig. 2](image-url) Cdc42 expression and activities in normal and atrophied muscle heavy microsomes. Microsomes for the endogenous GTP-bound form were prepared in the presence of 10 mM Mg\(^{2+}\). Microsomes for the GDP- and GDP-loaded forms were prepared in the absence of Mg\(^{2+}\), followed by addition of 1 mM guanosine 5’-O-(3-thiotriphosphate) (GTP\(^{\gamma}\)S) or GDP and then 10 mM Mg\(^{2+}\). The GTP-bound form of the GTPase was trapped with p21-activated kinase 1-glutathione-S-transferase (PAK1-GST) fusion protein coupled to Sepharose beads. The amount of protein trapped was measured from immunoblots of the SDS-PAGE fractionated protein. Ras, RhoA, and Rac1 were similarly analyzed by trapping with Raf1, protein kinase N (PKN)-, and PAK1-GST fusion proteins, respectively.

![Fig. 3](image-url) Comparison of the differences in small GTPase signaling between normal and atrophied gastrocnemius muscle. The amount of GTP-loaded control represents “total” GTPase, and the endogenous GTP-bound form represents “active” GTPase. Data are normalized to the total of each small GTPase in normal muscle.
bodies. Active small GTPases remaining in the membrane preparations was used to test for nonspecific depletion of the small GTPases. Fluorescence was detectable in addition to the fluorescent immunodepletion of the \-dystroglycan was depleted by treating the muscle membrane preparation with antibodies against the protein and protein A-Sepharose. Immunodepletion of the \( \alpha \)-subunit of Na\(^+\)-K\(^+\)-ATPase was used to test for nonspecific depletion of the small GTPases. Active small GTPases remaining in the membrane preparations were trapped with their respective effector GST-fusion targets as described in Figs. 2 and 3. The trapped proteins were separated on a gel and detected by immunoblot analysis with the appropriate antibodies.

Immunofluorescence was detectable in addition to the sarcolemmal fluorescence for H-Ras (Fig. 5C) and Cdc42 (Fig. 5E). In tenotomized muscle, approximately one-half of the fibers lacked H-Ras and Cdc42 staining at the sarcolemma, and the immunoreactivity was found in aggregates (as punctate fluorescence) in the remaining fibers. The overall fluorescence was reduced significantly in tenomized muscle fibers for both H-Ras and Cdc42 (Fig. 5, D and F, respectively). Labeling with \( \alpha \)-syntrophin antibodies indicated no difference between the control and tenotomized muscle fibers in syntrophin staining (Fig. 5, G and H). These data are consistent with the abundance of these proteins in the heavy microsomes isolated from control and tenotomized muscle (Figs. 1 and 3).

The four G proteins fall into three distinct groups. One of these, represented by Rac1, is little affected by atrophy (Fig. 3). In contrast, RhoA is dramatically affected by atrophy (Fig. 3) but does not appear to be physically associated with either laminin or \( \beta \)-dystroglycan (Fig. 4). The third group contains H-Ras and Cdc42, which are both diminished by atrophy and associated with laminin and \( \beta \)-dystroglycan. Like H-Ras, Cdc42 is also found predominantly in the active form in both control and tenotomized muscle (Fig. 3). This last group is clearly relevant to muscle atrophy. To further probe whether Cdc42 activity was a potential signal transduction component affecting muscle structure, we injected adult rat soleus muscle with retroviral plasmids coding for forms of Cdc42 with different intrinsic activities. Skeletal muscle is well known to be able to incorporate and express from DNA injected directly into the muscle. The soleus muscle was chosen because of its small mass, its exquisite sensitivity to mechanical activity for its gene expression, and its surgical accessibility. We were able to identify muscle fibers expressing the Cdc42 forms by their coexpression of the GFP that was encoded behind an internal ribosome entry site in the retroviral Cdc42 construct. Plasmid vector (Fig. 6E), wild-type Cdc42 (Fig. 6, A and B), and a fast-cycling active mutant Cdc42 (data not shown) produce normal fiber appearance. The expression of GFP was in a subsarcolemmal or variegated pattern that was identical to the expression observed previously in muscle injected with retrovirus coding for \( \beta \)-galactosidase (59). In contrast, the dominant-negative form of Cdc42 (Fig. 6, C and D) caused the muscle to exhibit fibers that were misshapen and had lost fiber-to-fiber contacts. The dominant-negative Cdc42-injected muscle also had areas that apparently had lost muscle fibers (see e.g., Fig. 6C). The pattern of GFP expression within the fibers did not appear different in the dominant-negative Cdc42-injected muscles, except that the fluorescence was now localized to subsarcolemmal regions that were not in cell-cell contacts in many cases. A comparison of the frequency and distribution of muscle fiber area in GFP-expressing regions of wild-type and dominant-negative Cdc42-injected muscle is shown in Fig. 7. It demonstrates the significantly diminished size of the fibers that received the dominant-negative mutant (\( P < 0.001 \), power > 0.999).

**DISCUSSION**

Our experiments support the hypothesis that the DGC complex in skeletal muscle may participate in the transduction of mechanical forces to intracellular signals. Upon tenotomy, the muscle is no longer subjected to normal stretching and the DGC receptor downregulates (Fig. 1). Dystrophin can no longer be detected 10 days posttenotomy, and members of the dystroglycan and sarcoglycan complex are also greatly diminished. \( \alpha \)-Syntrophin is not changed over this same time period. Immunocytochemistry for \( \beta \)-dystroglycan and syntrophin further support these data. \( \beta \)-Dystroglycan staining was reduced significantly, and syntrophin remained the same (Fig. 5). \( \alpha \)-Syntrophin is also known to form complexes with utrophin at neuromuscular junctions that are devoid of dystrophin (2); the \( \alpha \)-syntrophin in these other complexes may be more abundant or exhibit a compensatory increase with tenotomy.
such that an effect on DGC syntrophin is masked. Furthermore, the role of syntrophin in binding Na\(^+/\)H\(^-\) channels suggests that there may be other complexes rich in syntrophin (23), as do the observations that the syntrophins are often abundant in tissues other than muscle or brain that express very little dystrophin or utrophin (e.g., testes) (34). All the activities involving DGC complex proteins appear to be related in some way to cell signaling, making it likely that they form a signal transduction complex. That it is altered in atrophy suggests that it may be important for regulating normal muscle growth and homeostasis.

Fig. 5. Immunofluorescence of dystrophin-glycoprotein complex (DGC) components in control (A, C, E, and G) and contralateral tenotomized (B, D, F, and H) medial gastrocnemius muscle cross sections (10 μm). Reduced expression of β-dystroglycan (A and B), H-Ras (C and D), and Cdc42 (E and F) was observed in the atrophied muscle. α-Syntrophin expression was not noticeably different between normal and atrophied muscle (G and H, respectively). Calibration bar, 50 μm.
Dystrophin is located in the sarcolemma, the myotendinous junctions, and costameres (52). Myotendinous junctions are extensive and highly specialized subsarcolemmal adherens junctions, and costameres provide myofibril-to-sarcolemma attachment sites. We extracted and detected dystrophin from whole gastrocnemius muscle sarcolemmal membrane preparations (heavy microsomes), the source for all purifications of the DGC. This may explain, in part, why we report decreased dystrophin in the tenotomized gastrocnemius muscle, whereas other investigators have reported increased dystrophin in denervated muscle (5, 7, 51). In addition to contrasting atrophy models with respect to the integrity of the neuromuscular junction, the reports mentioned above also used cruder protein extracts from muscle (only one of which was gastrocnemius). Whereas this earlier report used a crude membrane fraction from the superficial portion of the gastrocnemius muscle dissected free of adhering connective tissue (7), we used isolated sarcolemma-derived microsomes from the whole gastrocnemius muscle for our experiments. In our membranes enriched in the DGC, we observed that dystrophin, β-dystroglycan, and α-sarcoglycan are all decreased during the atrophy. We conclude that the DGC is downregulated under these conditions. Nevertheless, the proposed role of the DGC as a mechanical receptor is not excluded by either an upregulation (5, 7, 51) or downregulation (this report) that may occur in different animal models.

Concurrent with the downregulation of the DGC complex, we also observed changes in the small GTPases associated with the DGC. Small GTPases have been

Fig. 6. Injection of soleus muscle with retroviral plasmids expressing green fluorescent protein (GFP) and either wild-type Cdc42 (A and B), dominant-negative Cdc42 (C and D), or an empty construct (E) indicates differences in muscle structure. GFP expression (asterisks) was both cytosolic and subsarcolemmal, with the subsarcolemmal expression tending to be opposed in adjacent fibers (arrowheads) of the wild-type Cdc42-injected fibers (A and B). This opposition was disrupted in the dominant-negative Cdc42-injected muscles (C and D). Daggers (†) indicate areas with missing fibers. Calibration bars, 50 μm.
found to be important for integrin signaling and, by analogy, may be important in DGC signaling. The effects on Cdc42 and Ras appear quite similar (Fig. 3) in that almost all of the GTPase present is active and the amount present is greatly diminished by atrophy. The immunocytochemistry results for H-Ras and Cdc42 (Fig. 5) support these results from the in vitro assays using muscle membrane preparations. RhoA is the most dramatically affected of all the small GTPases, becoming undetectable in atrophied muscle (Fig. 3). Although RhoA was not present in detectable levels in our protein extractions from atrophied muscle, this does not mean that it was completely absent. Rac1 is seemingly unaffected by atrophy. Paradoxically, this may suggest that it is particularly important to atrophy and its recovery. Muscles recover from atrophy when stretching and contractile activities are resumed, so the atrophied muscle must contain within it the components necessary for recovery. Because Rac1 is available and only modestly activated in normal and atrophied muscle, it has a potentially large range of response to changes in the muscle.

The results of the laminin and β-dystroglycan depletion assay show that there is a physical link between the DGC and the small GTPases. The association of H-Ras, Rac1, and Cdc42 with laminin and β-dystroglycan (Fig. 4) and the localization of β-dystroglycan, H-Ras, and Cdc42 at the sarcolemma (Fig. 5) are notable in this regard. In support of this suggestion, we also found that the DGC proteins are directly associated with the small GTPase Rac1 through grb2 and Sos (48). Laminins are major components of the basement membrane. Cells bind directly to laminins via a subset of integrins and other non-integrin-type receptors, such as dystroglycans. Reduction of the small GTPases H-Ras, Rac1, and Cdc42 due to laminin depletion implies that these GTPases may be signal transduction elements reflecting mechanical attachment between the cell cytoskeleton and the extracellular matrix. The similar magnitude of reduction in these GTPases due to β-dystroglycan depletion shows the involvement of DGC in GTPases signaling.

The retroviral transformation data (Fig. 6) complement the GTPase trapping data (Fig. 3). The fast-cycling active mutant of Cdc42 does not alter fiber morphology, similar to the vector and wild-type controls. Because of its rapid cycling property and the >10-fold excess of GTP over GDP in cells, this protein functions as though it were constitutively active. Because Fig. 3 shows that virtually all of the Cdc42 present in muscle is active, the fast-cycling active mutant form is in the same high-activation state as the native protein, and this may be why it had no effect. However, when constitutively inactive (dominant negative) Cdc42 is introduced, it clearly alters the muscle fiber morphology (Figs. 6 and 7). Muscle fiber diameter decreases in atrophy, so in that sense, the dominant-negative phenotype is similar to atrophy. We also performed analogous experiments using retroviral constructs containing variants of RhoA. All were without any discernable effect (data not shown). Thus we conclude that Cdc42 has the special property of affecting muscle fiber morphology, a property not shared with RhoA.

The reduction of H-Ras, Rac1, and Cdc42 signals in response to laminin or β-dystroglycan depletion (Fig. 4) strongly suggests that they are physically linked to one another in a signaling complex. However, this alone does not establish a cause-and-effect relationship. Thus much remains to be done to establish their functional links. Nonetheless, the studies presented arose from a hypothesis that predicts such a link and would be hard to explain by any other existing model for the function of the DGC complex. Because muscle is a complex tissue, there are many possible transduction mechanisms that may influence muscle structure and function. Members of the Rho family of GTPases have previously been implicated in the sarcromere assembly: Rac1 activity inhibits normal assembly during myotube differentiation (22), and RhoA activity initiates reorganization during cardiac hypertrophy (14). Our studies show the possible involvement of H-Ras and Cdc42 GTPases signaling in muscle atrophy. Of these two, we have shown that the dominant-negative Cdc42 results in abnormal muscle cells. It is important to determine the role of H-Ras by studying the effect of dominant-negative H-Ras on muscle fibers, which we plan for future work. Furthermore, we have recently shown that grb2 binds α-syntrophin (48), and it is already known that grb2 also binds to β-dystroglycan (64), suggesting that signaling through the Ras family small GTPase proteins is likely to be relevant to the DGC complex.

The signal transduction mechanisms for stretch in striated muscle are not well understood. Several studies have demonstrated heterotrimeric G protein acti-
vation (17, 27, 63) or focal adhesion kinase (FAK) activation (26). However, there is little information linking skeletal muscle stretch to modulation of small G protein activity. Nonetheless, results from smooth and cardiac muscle support the model presented here. For example, the elastin-laminin receptor functions as the stretch receptor in vascular smooth muscle, and a peptide derived from elastin can inhibit it. This stretch signaling involves grb2 recruitment, and cRas-dominant-negative transfection prevents signaling whereas an inhibitor of stretch-activated cation channels does not (56), showing that grb2/small GTPase signaling is important to stretch signaling in smooth muscle. In bladder smooth muscle, stretch activates SAPK2 and JNK (44), a frequent consequence of grb2/small GTPase signaling. In cardiomyocytes, stretch activation of another mitogen-activated protein kinase, extracellular signal-regulated kinase 2 (ERK2), requires an intact Rac1/RhoA pathway. Dominant-negative constructs of Rac1 and RhoA prevent activation of ERK2 by stretch, as does inhibition of RhoA (3). The data presented here are consistent with these reports of modulation of small G proteins by stretch. Because the DGC complex is also present in cardiac and smooth muscle, it may be a common upstream transducer of mechanical stretch.

The model proposed here of DGC being involved in mechanotransduction mechanisms regulating muscle size has thus survived our initial examination. This hypothesis predicts that the DGC complex, involved in many devastating muscular dystrophies, is also involved in muscle atrophy (a normal process of muscle remodeling). We now have experimental data to support this prediction. Our prediction is also supported by Brown et al. (9), who showed that the treatment of cultured myotubes with a monoclonal antibody that blocks α-dystroglycan binding to laminin leads to the induction of a dystrophic phenotype in vitro. The phenotype is characterized by reduced myotube size, myofibril disorganization, loss of contractile activity, and reduced spontaneous clustering of acetylcholine receptors; this phenotype is reversed by addition of exogenous laminin 2. They concluded that α-dystroglycan may be part of a signaling pathway for the maturation and maintenance of skeletal myofibers.

The hypothesis presented here, if it survives subsequent testing, could represent an important advance. Detailed knowledge of this signaling may provide insights into the molecular pathology of the various inherited muscular dystrophies, and identify valuable pharmacological targets and new therapeutic strategies. A consequence of the model is that these therapies may also be effective against muscle atrophy in the convalescing and elderly.

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