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SH3 Domain-mediated Interaction of Dystroglycan and Grb2*

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Dystroglycan is a novel laminin receptor that links the extracellular matrix and sarcolemma in skeletal muscle. The dystroglycan complex containing α- and β-dystroglycan also serves as an agrin receptor in muscle, where it may regulate agrin-induced acetylcholine receptor clustering at the neuromuscular junction. β-Dystroglycan has now been expressed *in vitro* and shown to directly interact with Grb2, an adapter protein involved in signal transduction and cytoskeletal organization. Protein binding assays with two Grb2 mutants, Grb2/P49L and Grb2/G203R, which correspond to the loss-of-function mutants in the *Caenorhabditis elegans sem-5*, demonstrated that the dystroglycan-Grb2 association is through β-dystroglycan C-terminal proline-rich domains and Grb2 Src homology 3 domains. Affinity chromatography has also shown endogenous skeletal muscle Grb2 interacts with β-dystroglycan. Immunoprecipitation experiments have demonstrated that Grb2 associates with α/β-dystroglycan *in vivo* in both skeletal muscle and brain. The specific dystroglycan-Grb2 interaction may play an important role in extracellular matrix-mediated signal transduction and/or cytoskeleton organization in skeletal muscle that may be essential for muscle cell viability.

Dystroglycan was first identified as a component of the dystrophin-glycoprotein complex (1–5). α-Dystroglycan binds laminin and dystrophin binds actin filaments, indicating that one function of the dystrophin-glycoprotein complex is to provide a link between the extracellular matrix and the cell cytoskeleton (5, 6). Disruption of the dystrophin-glycoprotein complex in several forms of muscular dystrophy suggests that this extracellular matrix-cytoskeleton linkage is important in maintaining muscle cell viability (1, 7–10). Recently, the dystroglycan complex was also shown to serve as an agrin receptor in muscle, where it may regulate agrin-induced acetylcholine receptor clustering at the neuromuscular junction (11–13). The dystroglycan complex has also been suggested to play a role in kidney epithelial morphogenesis.[1] These functional features indicate that dystroglycan may function to transduce extracellular signals into cells or regulate cell cytoskeleton organization in a manner similar to that of growth factor receptors or integrins. The structural analysis of dystroglycan (5, 14) indicates that β-dystroglycan contains a phosphotyrosine consensus sequence and several proline-rich regions that could associate with Src homology 2 and 3 (SH2[2] and 3) domains of cytoskeletal or signaling proteins (15, 16).

Grb2 is a 25–28-kDa protein containing SH2 and 3 domains (17) and has been found to be the mammalian homolog of *sem-5* protein product in *Caenorhabditis elegans*, which is involved in vulval development and in sex myoblast migration (18). Grb2 functions as an adapter protein through its SH2 or SH3 domains to interact with proteins containing phosphotyrosine or proline-rich domains (17–22). Grb2 is also an important protein linking receptor tyrosine kinases to small GTP-binding protein signaling, such as growth factor-induced cytoskeleton organization (17–25). Furthermore, integrin-mediated signal transduction is also coupled to activation of the Grb2/Ras pathway (26).

Here, we demonstrate that β-dystroglycan directly interacts with Grb2. Protein binding assays with two Grb2 mutants, Grb2/P49L and Grb2/G203R, which correspond to the loss-of-function mutants in the *C. elegans sem-5*, demonstrate that the specific association is through β-dystroglycan C-terminal proline-rich domains and Grb2 SH3 domains. Protein affinity purification and immunoprecipitation assays further indicate that Grb2 associates with α/β-dystroglycan *in vivo* in both skeletal muscle and brain. The data suggest that the specific dystroglycan-Grb2 interaction may play an important role in extracellular matrix-mediated signal transduction and cytoskeleton organization in different tissues through the dystroglycan complex.

EXPERIMENTAL PROCEDURES

Generation of Glutathione S-transferase Fusion Proteins—The rabbit β-dystroglycan (GenBank accession number X64393) cytoplasmic domain was amplified from a dystroglycan cDNA clone by polymerase chain reaction (PCR) with the following primers: sense (5′-CCCGGGT-TACCGCAAGAAGCGGAAGG-3′) and antisense (3′-AAGGAATTCGT-GGGCGATGGTCTGC-5′). The resulting product was subcloned into pGEX-2TK vector cDNA encoding. The glutathione *S*-transferase (GST) fusion protein (β-DGct) was introduced into *Escherichia coli* DH5α cells. Overnight cultures were diluted 1:10, incubated for 1 h, and induced for 3 h with 1 mM isopropyl-β-D-thiogalactopyranoside. The GST fusion protein was purified on a glutathione-Sepharose 4B affinity column (Pharmacia Biotech Inc.). The expressed fusion protein was immunoreactive to the antibody against the C-terminal 15 amino acid residues of β-dystroglycan (5). The GST fusion proteins containing different SH3 domains were purified in the same way. The human cDNA-encoding Grb2 protein (GenBank accession number M96995) was amplified using the sense (5′-GGCGGATCCGAAGCCATCGC-CAAATATGACTTC-3′) and antisense (3′-CAGTGGGGGCACTTGGC-CTTGCAGACTTAAGGG-5′) primers and subcloned into pGEX-2TK. The loss-of-binding Grb2 mutant cDNAs were constructed in the pALTER-1 vector using the Altered Sites mutagenesis kit (Promega) with the

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[1] M. Durbeej, E. Larsson, O. Ibraghimov-Beskrovnaya, S. L. Roberds, K. P. Campbell, and P. Ekblom, unpublished data.

[2] The abbreviations used are: SH2 and -3, Src homology 2 and 3; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid.

following primers: Grb2 P49L, antisense (3′-CTATGTAGTTCTTGAG-AATGAAGG-5′); Grb2 G203R, antisense (3′-GCGGGGAAAATCCTG-GTCTGCCCTGG-5′). The underlined codons represent the nucleotide substitutions. These cDNAs were subcloned into pGEX-2TK. These constructs encoding all of the fusion proteins were then transformed into the DH5α competent cells. Fusion proteins were purified as described above.

Protein Overlay Assay—The SH3 domain-containing fusion proteins were electrophoretically separated on 3–12% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The membrane was blocked for 2 h at 4 °C in blocking buffer (0.1% gelatin, 5% bovine serum albumin, and 0.1% Tween 20 in phosphate-buffered saline, pH 7.5) and was then incubated with purified fusion protein β-DGct (10 μg/ml) in overlay buffer (150 mM NaCl, 20 mM Hepes, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 5% bovine serum albumin, pH 7.5) overnight at 4 °C. After washing twice with overlay buffer, the membrane was processed for Western blot with antibody against 15 β-dystroglycan C-terminal amino acid residues.

In Vitro Transcription and Translation of ^{35}S-Labeled β-Dystroglycan Cytoplasmic Domain—The β-dystroglycan cytoplasmic domain was amplified by PCR with the following primers: sense (5′-CCATGGTCT-GCTACCGCAAG-3′) and antisense (3′-TCTAGAGGGTTAAGGGG-GAA-5′). The resulting product was subcloned into pGEM3 containing the 50-nucleotide alfalfa mosaic virus consensus initiation site (27). This construct was used to synthesize a ^{35}S-labeled probe by coupled *in vitro* transcription and translation with the TNT system (Promega) as described. The DNA template was incubated at 30 °C for 2 h with TNT rabbit reticulocyte lysate, T7 RNA polymerase, and an amino acid mixture containing [^{35}S]methionine (Amersham Corp.). The ^{35}S-labeled β-dystroglycan cytoplasmic domain was analyzed by SDS-PAGE and autoradiography.

Protein Binding Assay—GST fusion protein-coupled glutathione-Sepharose beads were equilibrated in binding buffer (150 mM NaCl, 0.1% Tween 20, 10 mM Hepes, pH 7.4) and incubated with *in vitro* translated [^{35}S]β-DGct probe (10 $\mu l \cdot ml^{-1}$ of binding buffer) for 12 h at 4 °C. Due to degradation, more G203R fusion protein was used to achieve an equal amount of intact fusion protein in each lane. The beads were then washed three times with binding buffer and separated on 3–12% SDS-polyacrylamide gel, and the gel was dried and exposed to film (X-Omat AR, Kodak).

Affinity Chromatography—Rabbit skeletal muscle was homogenized in 20 mM Tris-HCl, 1 M NaCl, 1% CHAPS with protease inhibitors: aprotinin (76.8 nM), benzamidine (0.83 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and phenylmethylsulfonyl fluoride (0.23 mM), pH 7.5. The homogenate was incubated at 4 °C for 4 h with mixing and then centrifuged at 100,000 rpm for 30 min at 4 °C. The supernatant was diluted 1:10 with 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ and protease inhibitors as above. The diluted homogenate was further centrifuged at 100,000 rpm for 30 min to remove precipitated protein. After being precleared with glutathione-Sepharose 4B for 3 h, the solubilized skeletal muscle homogenate was incubated with the fusion protein bound to glutathione-Sepharose overnight at 4 °C. After incubation, the Sepharose was washed with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.5% CHAPS. The proteins bound to Sepharose were separated by 3–12% SDS-PAGE and analyzed by immunoblot assay with anti-Grb2 antibody.

Protein Immunoprecipitation—CHAPS-solubilized skeletal muscle or brain homogenate was incubated with anti-α-dystroglycan antibody IIH6-Sepharose overnight at 4 °C after being precleared with Sepharose 6B. After extensive washing with Tris-buffered saline, the proteins attached to beads were resolved by SDS-PAGE with starting material and void in parallel. The gel was transferred to nitrocellulose and subjected to immunoblotting with anti-α/β-dystroglycan antibodies or anti-Grb2 antibody.

Immunoblotting—Proteins were separated on 3–12% gradient SDS-polyacrylamide gels and transferred onto nitrocellulose. Immunoblot staining was performed either with anti-Grb2 antibody (Santa Cruz) or with affinity-purified anti-α/β-dystroglycan antibody from sheep anti-dystrophin-glycoprotein complex polyclonal antisera (1–3).

RESULTS AND DISCUSSION

cDNA containing the rabbit β-dystroglycan cytoplasmic domain was amplified by PCR and subcloned into the pGEX-2TK expression vector to make the GST fusion protein, β-DGct. A protein overlay assay was performed to test the association between β-dystroglycan (β-DGct) and SH3 domain-containing proteins. Fig. 1 shows the interaction between β-dystroglycan

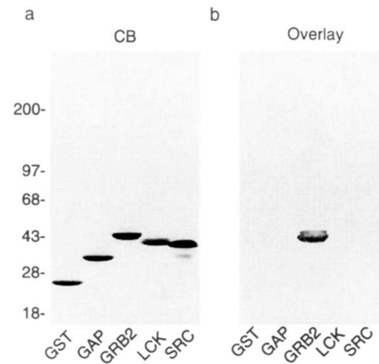

FIG. 1. **β-dystroglycan binds Grb2 *in vitro*.** *a*, Coomassie Blue (*CB*) stained SDS-polyacrylamide gel of different GST fusion proteins of SH3 domain-containing proteins including human GAP (amino acids 275–351, *GAP*), human GRB2 (amino acids 1–217, *GRB2*), murine LCK (amino acids 5–124, *LCK*), and chicken Src SH3 (*SRC*). GST alone serves as negative control. *b*, overlay of the corresponding GST fusion proteins transferred on nitrocellulose with fusion protein β-DGct containing β-dystroglycan cytoplasmic domain and detected with the antibody against the C-terminal 15 amino acid residues of β-dystroglycan (5).

and the four SH3 domain-containing proteins, Gap, Grb2, Lck, and Src. Notably, only Grb2 associates with β-dystroglycan in this assay. Four additional SH3 domain-containing proteins (Abl, Crk, phosphatidylinositol 3-kinase, and spectrin) were also examined and showed no β-dystroglycan binding activity (data not shown). β-Dystroglycan and its cytoplasmic domain were also translated *in vitro* in the presence of [^{35}S]methionine. Using a protein overlay assay, the ^{35}S-labeled β-dystroglycan only bound Grb2 among the eight SH3 domain-containing proteins (data not shown). Additionally, the ^{35}S-labeled β-dystroglycan cytoplasmic domain ([^{35}S]β-DGct) was also retained by Grb2 fusion protein Sepharose beads (Fig. 2, *a–c*). GST or GST-conjugated Sepharose beads, the negative control, did not bind β-dystroglycan (Figs. 1 and 2, *a–c*) .

The primary structure of β-dystroglycan contains several proline-rich regions at its C terminus (5, 14). To test further whether the Grb2-β-dystroglycan interaction occurs via an SH3-proline-rich domain association, two Grb2 mutants, Grb2/P49L and Grb2/G203R, which correspond to the loss-of-function mutants in the *C. elegans* Grb2 homolog, *sem-5* (18, 21, 22), were used in a fusion protein binding assay and an overlay assay (Fig. 2). Each of these Grb2 mutants possesses a point mutation in either the N-terminal or the C-terminal SH3 domain, which abolishes or greatly reduces association with Grb2-binding proteins (18, 21, 22). The ^{35}S-labeled *in vitro* translated β-dystroglycan cytoplasmic domain, [^{35}S]β-DGct, was used in the fusion protein binding assay (Fig. 2*a*). Due to degradation, an increase in G203R fusion protein was used to achieve an equal amount of intact fusion protein in each lane (Fig. 2*b*). As shown in Fig. 2*c*, the N-terminal SH3 domain loss-of-function mutation (P49L) results in loss of β-dystroglycan binding. The C-terminal SH3 domain mutant (G203R) results in reduced binding of β-dystroglycan. In parallel experiments, protein overlay assay using β-dystroglycan as the probe showed that the P49L and the G203R mutants display tremendously decreased binding activity (Fig. 2*d*). There is almost no detectable binding affinity between β-dystroglycan and Grb2/G203R in the protein overlay assay, possibly because of the higher sensitivity of the protein binding assay or overloading of G203R fusion protein in the binding assay. The data above suggest that Grb2 binds to the β-dystroglycan cytoplasmic proline-rich domains with both its N- and C-terminal SH3 domains, but the N-terminal SH3 domain may be the stronger binding site. Like the Grb2-SOS interaction, the association

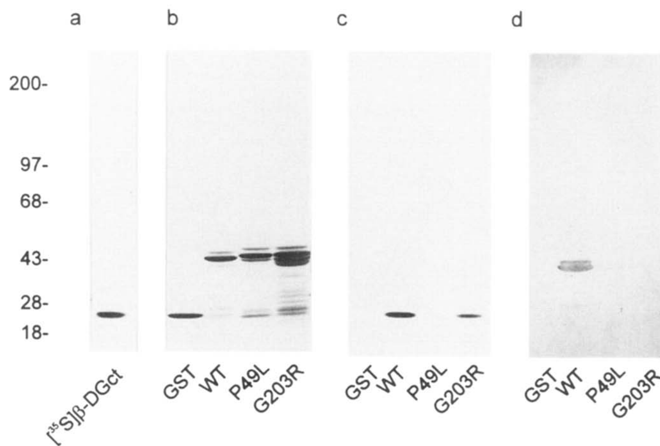

FIG. 2. **Specific association between β-dystroglycan and Grb2 SH3 domains.** *a*, autoradiogram of SDS-polyacrylamide gel of *in vitro* translated ^{35}S-labeled β-dystroglycan cytoplasmic domain, [^{35}S]β-DGct. *b*, Coomassie Blue-stained SDS-polyacrylamide gel of purified GST fusion protein with wild type Grb2 (*WT*) and its mutants Grb2/P49L (*P49L*) and Grb2/G203R (*G203R*). GST was used as the negative control. *c*, autoradiogram of corresponding binding of ^{35}S-labeled β-dystroglycan cytoplasmic domain by these wild type and mutant Grb2 GST fusion protein-conjugated glutathione beads. *d*, overlay of the corresponding GST fusion proteins transferred on nitrocellulose with β-dystroglycan cytoplasmic domain fusion protein and detected with the antibody against the C-terminal 15 amino acid residues of β-dystroglycan (5).

between Grb2 and β-dystroglycan may be mediated primarily through the N-terminal SH3 domain, although high affinity binding may require the coordinate interaction of both Grb2 SH3 domains (21, 22).

Affinity chromatography with β-DGct was used to investigate whether β-dystroglycan can associate with endogenous skeletal muscle Grb2. As shown in Fig. 3, Grb2 in CHAPS-solubilized skeletal muscle homogenate was retained by β-DGct-Sepharose beads but not by the beads conjugated with fusion protein of β-dystroglycan extracellular domain (β-DGnt). In addition, Sepharose beads conjugated with Grb2/R86K, which has the loss-of-function mutation in the SH2 domain, precipitated β-dystroglycan from CHAPS-solubilized muscle homogenate (data not shown). This is consistent with the protein binding and overlay assays, indicating that the association between endogenous Grb2 and β-dystroglycan is mediated through the SH3 domains. To test whether α/β-dystroglycan and Grb2 associate *in vivo* in different tissues, anti-α-dystroglycan antibody IIH6 (6) was used to immunoprecipitate β-dystroglycan and Grb2 from CHAPS-solubilized skeletal muscle and brain homogenates. Both Grb2 and β-dystroglycan were co-immunoprecipitated by IIH6 but not by several other unrelated antibodies (Fig. 4 and data not shown). In Fig. 4*a*, most Grb2 protein in skeletal muscle was immunoprecipitated by IIH6. This suggests that Grb2 mainly associates *in vivo* in skeletal muscle with dystrophin-associated protein dystroglycan, where dystrophin constitutes approximately 5% of the membrane protein (3, 28). In brain, only a small part of Grb2 associates with dystroglycan (Fig. 4*b*). Grb2 and β-dystroglycan were also co-immunoprecipitated by IIH6 from the C2C12 muscle cell line (data not shown). Thus, α- and β-dystroglycan and Grb2 are associated *in vivo* in skeletal muscle and brain.

Through protein overlay and protein binding assays, we have demonstrated that Grb2 directly associates with β-dystroglycan. Experiments performed using mutated GST-Grb2 fusion proteins demonstrated that this association is likely to be mediated through both SH3 domains of Grb2. The fact that both dystroglycan and Grb2 are expressed in many tissues indicates that they may have ubiquitous cellular functions (5, 14, 17).

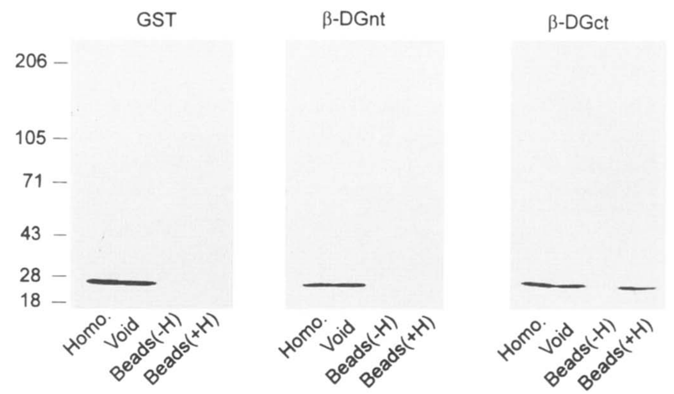

FIG. 3. **Skeletal muscle Grb2 binds to β-dystroglycan.** CHAPS-solubilized skeletal muscle homogenate was subjected to affinity chromatography on various columns including GST-Sepharose (*GST*), β-dystroglycan N-terminal domain Sepharose (*β-DGnt*), and β-dystroglycan C-terminal domain Sepharose (*β-DGct*). The starting material, skeletal muscle homogenate (*Homo.*), flow-through (*Void*), Sepharose beads before incubating (*Beads(−H)*) and after incubating (*Beads(+H)*) with muscle homogenate, and washing were analyzed on SDS-PAGE. The gels were transferred to nitrocellulose and subjected to immunoblotting with anti-Grb2 antibody.

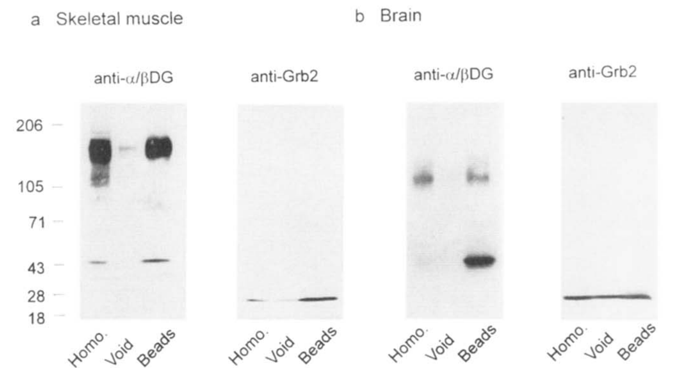

FIG. 4. **In vivo association between dystroglycan and Grb2 in skeletal muscle and brain.** CHAPS-solubilized skeletal muscle (*a*) or brain (*b*) homogenate was subjected to immunoprecipitation with anti-α-dystroglycan monoclonal antibody IIH6. The starting material, skeletal muscle homogenate (*Homo.*), flow-through (*Void*), and IIH6 Sepharose beads after incubation with muscle homogenate (*Beads*) were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose blots were stained with affinity-purified anti-α/β-dystroglycan antibody (*anti-α/β-DG*) or anti-Grb2 antibody (*anti-Grb2*).

Protein immunoprecipitation assays suggest that α- and β-dystroglycan and Grb2 are associated in both skeletal muscle and brain. This gives rise to the hypothesis that the dystroglycan complex may regulate cellular functions, such as certain cytoskeletal processes, through Grb2 in different tissues. Consistent with this hypothesis is the finding that growth factor-induced stress fiber formation at focal adhesions and ruffle induction involves small GTP-binding protein (Ras, Rac, and Rho) activation (23–25). Grb2 has been shown to modulate the activities of these small GTP-binding proteins that stimulate cytoskeleton organization *in vivo* (23–25). Furthermore, integrin-mediated signal transduction in NIH3T3 fibroblasts is coupled to activation of the Grb2/Ras pathway (26). As a laminin or agrin receptor, the dystroglycan complex may function in an analogous manner and regulate cytoskeleton organization through Grb2-involved signal transduction triggered by the extracellular matrix or neurally released agrin.

It has been demonstrated that the extracellular matrix affects many aspects of cell behavior such as cell adhesion, migration, proliferation, and differentiation (29, 30). Attachment to the extracellular matrix is also required for maintaining cell

viability (31–33). It is possible that the extracellular matrix-dystroglycan-Grb2 interaction is essential for muscle cell viability. The disruption of this interaction may affect normal muscle cell function and lead to muscle cell apoptosis or necrosis in several types of muscular dystrophy where muscle cell attachment to the extracellular matrix is perturbed (1, 7–10).

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