

# Identification and Characterization of the Dystrophin Anchoring Site on $\beta$ -Dystroglycan\*

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**Dystrophin, the product of the Duchenne muscular dystrophy gene, is tightly associated with the sarcolemmal membrane to a large glycoprotein complex. One function of the dystrophin-glycoprotein complex is to link the cytoskeleton to the extracellular matrix in skeletal muscle. However, the molecular interactions of dystrophin with the membrane components of the dystrophin-glycoprotein complex are still elusive. Here, we demonstrate and characterize a specific interaction between  $\beta$ -dystroglycan and dystrophin. We show that skeletal muscle and brain dystrophin as well as brain dystrophin isoforms specifically bind to  $\beta$ -dystroglycan. To localize and characterize the dystrophin and  $\beta$ -dystroglycan interaction domains, we reconstituted the interaction *in vitro* using dystrophin fusion proteins and *in vitro* translated  $\beta$ -dystroglycan. We demonstrated that the 15 C-terminal amino acids of  $\beta$ -dystroglycan constituted a unique binding site for the second half of the hinge 4 and the cysteine-rich domain of dystrophin (amino acids 3054–3271). This dystrophin binding site is located in a proline-rich environment of  $\beta$ -dystroglycan within amino acids 880–895. The identification of the interaction sites in dystrophin and  $\beta$ -dystroglycan provides further insight into the structure and the molecular organization of the dystrophin-glycoprotein complex at the sarcolemma membrane and will be helpful for studying the pathogenesis of Duchenne muscular dystrophy.**

Dystrophin is a large protein with a molecular mass of 427 kDa, which is absent in muscle from patients with Duchenne muscular dystrophy (1). Based on its primary structure, dystrophin can be divided into four domains: the N-terminal actin binding domain, the large triple helical spectrin-like domain, the cysteine-rich domain, and the C-terminal domain (2). Deletion of the cysteine-rich and C-terminal domains is associated with severe muscular dystrophy, which indicates that these domains play important roles in the stability of dystrophin (3, 4).

Immunofluorescence microscopy has established that dystrophin is located at the plasma membrane of skeletal muscle (5).

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Biochemical studies have demonstrated that dystrophin is tightly associated through its cysteine-rich and C-terminal domains with the sarcolemmal membrane to a large glycoprotein complex (6–10). Furthermore, cell membrane fractionation has shown that the dystrophin-glycoprotein complex can only be dissociated by alkaline treatment (6). Dystrophin-glycoprotein complex (DGC)<sup>1</sup> is composed of at least five transmembrane proteins (50-kDa adhalin, 43-kDa  $\beta$ -dystroglycan, 43-kDa dystrophin-associated glycoprotein A3b, 35-kDa dystrophin-associated glycoprotein, and 25-kDa dystrophin-associated protein), one extracellular protein (156 kDa  $\alpha$ -dystroglycan), and four cytoplasmic proteins (syntrophin triplet and dystrophin) (6–9, 11–15). In skeletal muscle, interactions between  $\alpha$ -dystroglycan and laminin  $\alpha$ 2 (11, 12, 16) as well as between dystrophin and cytoskeletal actin filaments (17) have been identified, indicating that one function of the DGC is to provide a link between the extracellular matrix and the cytoskeleton. Furthermore, the loss of dystrophin leads to a great reduction of all the dystrophin-associated proteins in Duchenne muscular dystrophy or *mdx* mouse skeletal muscle (18, 19). Taken together, these results suggest that the marked reduction of the DGC in muscle from Duchenne muscular dystrophy patients and *mdx* mice disrupts the linkage between the extracellular matrix and the cytoskeleton, thereby rendering dystrophic muscle fibers more susceptible to necrosis.

Protein overlay assays have previously indicated that dystrophin cysteine-rich domain interacts with a 43-kDa dystrophin-associated protein ( $\beta$ -dystroglycan or A3b), although the exact identity of this protein has yet to be conclusively determined (20). Recently, interactions between syntrophin and the C-terminal domain (residues 3447–3481) of dystrophin have also been demonstrated (20–23). However, the molecular organization of dystrophin with the membrane components of the DGC is still elusive. Here, we report several experiments aimed at determining whether  $\beta$ -dystroglycan directly binds to dystrophin. We show that dystrophin from solubilized skeletal muscle and brain binds to the cytoplasmic domain of  $\beta$ -dystroglycan expressed as a GST fusion protein and coupled to glutathione-agarose beads. The  $\beta$ -dystroglycan binding site on dystrophin was further localized to the second half of hinge 4 and the cysteine-rich domain, including amino acids 3054–3271, by using *in vitro* translated  $\beta$ -dystroglycan cytoplasmic domain and dystrophin fusion proteins in an *in vitro* binding assay. Finally, we have localized the dystrophin binding site to the extreme C terminus of  $\beta$ -dystroglycan, including amino acids 880–895.

<sup>1</sup> The abbreviations used are: DGC, dystrophin-glycoprotein complex; GST, glutathione *S*-transferase;  $\beta$ -DGct,  $\beta$ -dystroglycan C terminus; PAGE, polyacrylamide gel electrophoresis.

## EXPERIMENTAL PROCEDURES

**Reagents**—TNT-coupled reticulocyte lysate system was purchased from Promega. [<sup>35</sup>S]Methionine was from Amersham Corp., isopropyl-1-thio- $\beta$ -D-galactopyranoside and reduced glutathione were from U. S. Biochemical Corp. Glutathione-agarose, protein G-Sepharose, and cyanogen bromide (CNBr)-activated Sepharose 4B were from Pharmacia Biotech Inc. Horseradish peroxidase-conjugated secondary antibodies were from Boehringer Mannheim. The peptides corresponding to the rabbit skeletal muscle  $\beta$ -dystroglycan sequences were synthesized at the biopolymers facility of the Howard Hughes Medical Institute (University of Texas Southwestern Medical Center). All other chemicals were of reagent grade.

**Solubilization of Dystrophin from Rabbit Total Membranes by pH 11 Treatment**—Skeletal muscle and brain dystrophin were solubilized from rabbit total membranes by pH 11 treatment as described previously (8) with slight modifications. Briefly, KCl-washed membranes (250 mg) were diluted 20-fold to a volume of 100 ml with buffer A (50 mM Tris-HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, 0.75 mM benzamidine, 2.5  $\mu$ g/ml aprotinin, 2.5  $\mu$ g/ml leupeptin, and 0.5  $\mu$ g/ml pepstatin A) and titrated to pH 11 with 1 M NaOH. After incubation at room temperature for 1 h, the samples were centrifuged for 30 min at 100,000  $\times$  g, and the supernatant was decanted from the membrane pellet. This supernatant was supplemented with NaCl to a final concentration of 50 mM, titrated to pH 7.5, and centrifuged for 30 min at 100,000  $\times$  g. The resulting supernatant was decanted and used for affinity purification and immunoprecipitation.

**Affinity Chromatography with  $\beta$ -Dystroglycan Cytoplasmic Domain GST Fusion Protein**—Rabbit skeletal muscle  $\beta$ -dystroglycan cDNA (11) corresponding to amino acids 775–895 (GenBank accession number X64393) was amplified by polymerase chain reaction and subcloned into *Sma*I-*Eco*RI sites of pGEX-2TK expression vector (Pharmacia). The GST- $\beta$ -dystroglycan cytoplasmic domain fusion protein ( $\beta$ -DGct) construct was transformed into *Escherichia coli* DH5 $\alpha$  cells. Overnight cultures were grown, and the fusion proteins were induced with 1 mM of isopropyl-1-thio- $\beta$ -D-galactopyranoside. The cell cultures were spun down and resuspended in phosphate-buffered saline containing 1% Triton X-100 and sonicated twice for 15 s. The sonicated material was centrifuged at 12,000  $\times$  g for 15 min. The supernatant containing the GST fusion protein was incubated with glutathione-agarose beads. The glutathione-agarose beads were extensively washed with phosphate-buffered saline and stored at 4 °C in the presence of protease inhibitors. All the available  $\beta$ -dystroglycan antibodies were immunoreactive to the expressed fusion protein. GST- $\beta$ -DGct fusion protein immobilized on glutathione-agarose beads and control GST immobilized on glutathione-agarose beads were equilibrated in buffer A + 50 mM NaCl and incubated overnight at 4 °C with the alkaline-solubilized membranes. After incubation, the voids were collected, and the agarose beads were washed with buffer A + 50 mM NaCl. Solubilized membranes (pH 11), voids, and glutathione-agarose beads were resolved on 3–12% gradient SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes.

**Immunoprecipitation of Rabbit Skeletal Muscle Dystrophin**—An immunofluorescence matrix was prepared by coupling anti-dystrophin monoclonal antibody XIXC2 (8) to protein G-Sepharose beads. The beads were washed with buffer A + 150 mM NaCl and incubated overnight at 4 °C with the solubilized muscle membranes. Solubilized dystrophin (pH 11), voids, and beads were resolved on 3–12% gradient SDS-polyacrylamide gels and electrotransferred to nitrocellulose membrane.

**Immunoblotting**—Nitrocellulose transfers were blocked in Blotto (50 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 5% nonfat dry milk) and subsequently incubated overnight with primary antibody. Primary antibodies were either a combination of monoclonal antibody DYS2 (Novocastra Lab, Newcastle) raised against the C terminus of dystrophin and affinity-purified anti-syntrophin antibodies from sheep anti-DGC polyclonal antiserum (13) or affinity-purified anti-dystrophin cysteine-rich and C-terminal (amino acids 3054–3685) antibodies from sheep anti-DGC polyclonal antisera (24). Immunoblots were then washed with Blotto and incubated for 1 h with peroxidase-conjugated secondary antibody (Boehringer Mannheim) at a dilution of 1:1,000. After washing the nitrocellulose blots with Blotto, they were developed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl using 4-chloro-1-naphthol as substrate.

**Generation and Purification of Human Dystrophin GST Fusion Proteins**—Different portions of human dystrophin C-terminal region (GenBank accession number M18533) were amplified by polymerase chain reaction and subcloned into pGEX vectors (Pharmacia). Each fusion protein construct was transformed into *E. coli* DH5 $\alpha$  cells and the

fusion proteins were purified on glutathione-agarose as described above. The fusion protein glutathione-agarose beads were extensively washed with phosphate-buffered saline and either stored at 4 °C in the presence of protease inhibitors or the fusion proteins were eluted with 10 mM glutathione in 50 mM Tris (pH 8.0).

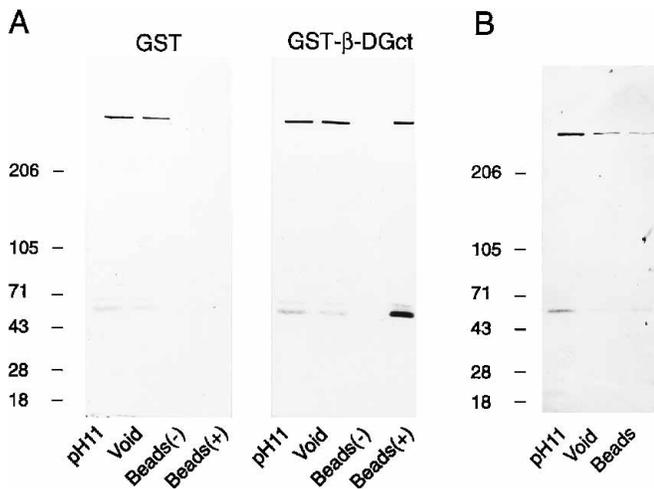
**In Vitro Transcription and Translation of  $\beta$ -Dystroglycan Cytoplasmic Domain**—Rabbit skeletal muscle  $\beta$ -dystroglycan cDNA (GenBank accession number X64393) corresponding to amino acids 775–895 or 775–880 were amplified by polymerase chain reaction using a forward primer that contained an *Nco*I restriction site encoding the translation initiation codon and a reverse primer with an *Xba*I restriction site encoding the termination codon. The amplified fragments were purified and subcloned into *Nco*I and *Xba*I sites of pGEM3 vector (Promega), which was modified to contain a 5'-alfalfa mosaic virus 50-nucleotide consensus initiation site and a 3'-poly(A) tail. All constructs were verified by automated sequencing (Applied Biosystems). Constructs were used to synthesize a [<sup>35</sup>S]methionine-labeled probe, <sup>35</sup>S-labeled  $\beta$ -Dgct, or <sup>35</sup>S-labeled  $\beta$ -Dg775–880 by coupled *in vitro* transcription and translation in the TNT system (Promega) as described previously (25).

**Dystrophin-GST Fusion Protein <sup>35</sup>S-Labeled  $\beta$ -Dystroglycan In Vitro Binding Assay**—Dystrophin-GST fusion proteins (20  $\mu$ g) immobilized on glutathione-agarose beads were equilibrated in binding buffer (150 mM NaCl, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.75 mM benzamidine, 2.5  $\mu$ g/ml aprotinin, 2.5  $\mu$ g/ml leupeptin, 0.5  $\mu$ g/ml pepstatin A, and 10 mM Hepes, pH 7.4) and incubated in a final volume of 1 ml with 5  $\mu$ l of <sup>35</sup>S-labeled  $\beta$ -Dgct or <sup>35</sup>S-labeled  $\beta$ -Dg775–880 for 3 h at 4 °C. For peptide inhibition assays, 1 mM of the different peptides was added to the reaction mixture. The beads were then pelleted by centrifugation and washed three times with binding buffer before resuspension in SDS-PAGE denaturing sample buffer. The samples were resolved on 5–16% gradient SDS-polyacrylamide gels, stained with Coomassie Blue, dried, and exposed to film (X-Omat AR, Kodak). Densitometric scanning of the Coomassie Blue-stained gel and the corresponding autoradiogram was carried out on a Molecular Dynamics 300S computing densitometer. Volume integration of densitometric scans were normalized for the quantity of fusion protein.

**Affinity Chromatography with  $\beta$ -Dystroglycan C-terminal Peptide**—A synthetic peptide representing the 15 C-terminal residues (amino acids 880–895) of rabbit skeletal muscle  $\beta$ -dystroglycan was obtained from the HHMI Peptide Facility (Washington University, St. Louis) as an N-terminal *p*-benzylbenzoyl-peptide photoprobe. The peptide was coupled to bovine serum albumin by UV cross-linking, and the bovine serum albumin-peptide conjugate was then immobilized on CNBr-activated Sepharose 4B (Pharmacia) using standard procedures.  $\beta$ -Dystroglycan C-terminal peptide or control peptide immobilized on Sepharose 4B beads were equilibrated in buffer A + 50 mM NaCl and incubated 3 h at 4 °C with rabbit skeletal pH 11 solubilized membranes or human GST-dystrophin fusion proteins in buffer A + 50 mM NaCl. After incubation, the beads were washed with buffer A + 50 mM NaCl and resolved by 3–12% gradient SDS-polyacrylamide gels and either stained with Coomassie Blue or electrotransferred to nitrocellulose membranes.

## RESULTS

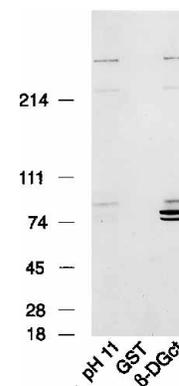
**Dystrophin from Skeletal Muscle Binds to  $\beta$ -Dystroglycan Fusion Protein**—To investigate the existence of a dystrophin- $\beta$ -dystroglycan interaction, the cytoplasmic domain of rabbit skeletal muscle  $\beta$ -dystroglycan was expressed as a GST fusion protein (GST- $\beta$ -DGct). This fusion protein was immobilized on glutathione-agarose beads to form an affinity column for the binding of native dystrophin solubilized from rabbit skeletal muscle membranes by alkaline treatment. The alkaline treatment has been shown to extract dystrophin and syntrophin from the membrane-associated glycoproteins. Syntrophin and dystrophin no longer interact after this treatment (6, 8). The extract was titrated to pH 7.5 and applied to GST- $\beta$ -DGct-agarose or control GST-agarose. After extensive washing, the agarose beads, voids, and extracts were analyzed by SDS-PAGE and immunoblotted using specific antibodies to dystrophin (DYS2) and to the syntrophin triplet. As shown in Fig. 1A, dystrophin and syntrophin cosediment with the GST- $\beta$ -DGct-agarose beads but not with GST-agarose beads. These results indicate that either dystrophin or syntrophin is able to bind the cytoplasmic domain of  $\beta$ -dystroglycan. However, using an over-



**FIG. 1. Skeletal muscle dystrophin binds to  $\beta$ -dystroglycan.** *A*, rabbit skeletal muscle dystrophin solubilized by pH 11 treatment was subjected to affinity chromatography on various columns including GST-agarose (GST) and GST- $\beta$ -dystroglycan cytoplasmic domain (GST- $\beta$ -DGct). The starting material, solubilized membrane (pH 11), flow-through (Void), agarose beads before chromatography (Beads(-)) and after chromatography (Beads(+)) were resolved on SDS-PAGE. The gels were electrotransferred to nitrocellulose membrane and immunoreacted with anti-dystrophin DYS2 monoclonal antibody and affinity-purified anti-syntrophin. Molecular weight standards ( $\times 10^{-3}$ ) are indicated on the left. *B*, rabbit skeletal muscle dystrophin solubilized by pH 11 treatment was immunoprecipitated with anti-dystrophin (Mab XIXC2) protein G beads. The starting material, solubilized membrane (pH 11), flow-through (Void), and protein G beads (Beads) were resolved on SDS-PAGE. The gels were electrotransferred to nitrocellulose membrane and immunoreacted with anti-dystrophin DYS2 monoclonal antibody and affinity-purified anti-syntrophin polyclonal antibodies. Molecular mass standards ( $\times 10^{-3}$ ) are indicated on the left.

lay assay, we have previously shown that syntrophin only binds to dystrophin and that no interaction of syntrophin with  $\beta$ -dystroglycan was detected (23). Thus, the cosedimentation of dystrophin and syntrophin with the GST- $\beta$ -DGct-agarose beads is likely due to  $\beta$ -dystroglycan-dystrophin interaction rather than the  $\beta$ -dystroglycan-syntrophin interaction. The cosedimentation of syntrophin together with dystrophin may result from a reassociation of the two proteins upon titration of the alkaline extracts to pH 7.5. Indeed, anti-dystrophin monoclonal antibody coimmunoprecipitates dystrophin and syntrophin from alkaline extracts titrated at pH 7.5 (Fig. 1*B*), indicating that dystrophin and syntrophin reassociate. Furthermore, our results suggest that dystrophin is capable of simultaneously binding syntrophin and  $\beta$ -dystroglycan through different binding sites.

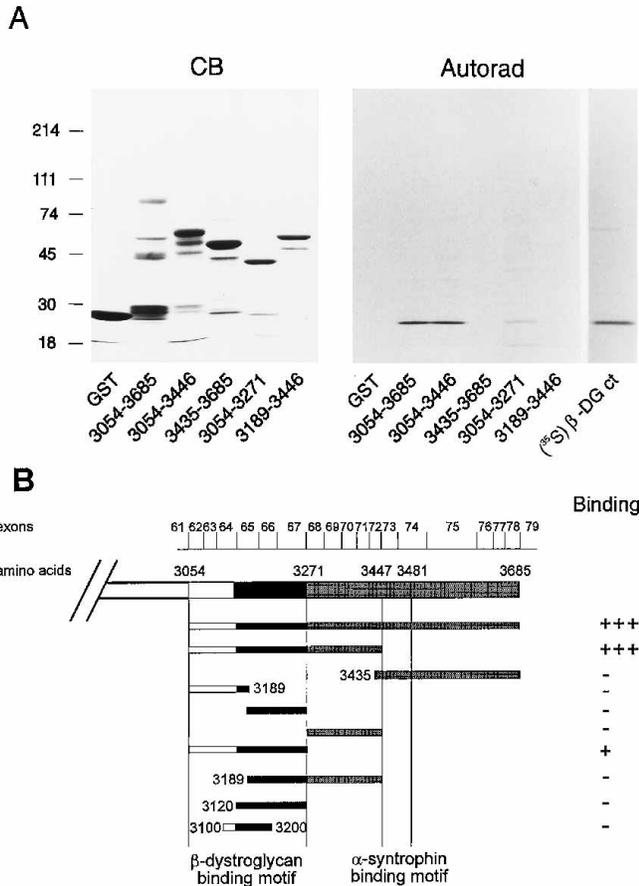
**Dystrophin and Dystrophin Isoforms from Brain Interact with  $\beta$ -Dystroglycan**—Affinity assays with the GST- $\beta$ -DGct-agarose beads were also performed on alkaline extracts of brain microsomes to investigate the ability of  $\beta$ -dystroglycan to bind brain dystrophin and the C-terminal isoforms of dystrophin expressed in this tissue. SDS-PAGE and immunoblot analysis of the proteins bound to the affinity beads were performed using affinity-purified antibodies to the C-terminal portion of dystrophin. As shown in Fig. 2, the GST- $\beta$ -DGct-agarose beads, but not the GST-agarose beads, specifically bound dystrophin from brain extract. In addition to dystrophin, a protein doublet of approximately 80 kDa and two proteins of approximately 260 and 90 kDa also cosedimented specifically with the GST- $\beta$ -DGct-agarose beads. The protein doublet, which is highly enriched in the GST- $\beta$ -DGct-agarose beads compared to the alkaline extract, may correspond to Dp71. Dp71 is an isoform of dystrophin consisting of the cysteine-rich and C-terminal domains of dystrophin. This protein is mainly expressed in non-



**FIG. 2. Brain dystrophin and dystrophin isoform bind to  $\beta$ -dystroglycan.** Rabbit brain dystrophin solubilized by pH 11 treatment was subjected to affinity chromatography on various columns including GST-agarose (GST) and GST- $\beta$ -dystroglycan cytoplasmic domain (GST- $\beta$ -DGct). The starting material, solubilized membrane (pH 11), and agarose beads after chromatography (GST or  $\beta$ -DGct) were resolved by SDS-PAGE. The gels were electrotransferred to nitrocellulose membrane and immunoreacted with affinity-purified anti-dystrophin C-terminal domains. Molecular mass standard ( $\times 10^{-3}$ ) are indicated on the left.

skeletal muscle tissue such as brain and has been described as a protein doublet or triplet with an apparent molecular mass in the range of 77–80 kDa (26–28). The two other proteins may also correspond to dystrophin isoforms, particularly the 260-kDa protein since an isoform of this molecular mass has been previously described in brain (29, 30). Another possibility is that these proteins simply correspond to degradation products of dystrophin, containing the cysteine-rich and C-terminal domains.

**Reconstitution of the  $\beta$ -Dystroglycan-Dystrophin Interaction**—To characterize the  $\beta$ -dystroglycan and dystrophin interaction sites, we developed an *in vitro* binding assay. In this binding assay, human dystrophin GST fusion proteins coupled to glutathione-agarose beads were used as ligands for *in vitro* translated cytoplasmic domain of  $\beta$ -dystroglycan labeled with [ $^{35}$ S]methionine ( $^{35}$ S-labeled  $\beta$ -DGct). Since it has been demonstrated that the C-terminal portion of dystrophin is involved in the binding to the glycoprotein complex (10), we used dystrophin C-terminal fusion proteins to investigate the interaction with  $\beta$ -dystroglycan. As shown in Fig. 3*A*,  $^{35}$ S-labeled  $\beta$ -DGct specifically bound with similar intensity to the fusion proteins containing either the entire C-terminal portion (amino acids 3054–3685) or the second half of hinge 4, the cysteine-rich domain, and the first half of the C-terminal domain (amino acids 3054–3446) but did not interact with fusion proteins containing the second half of the C-terminal domain (amino acids 3435–3685). No signals were observed with control GST fusion proteins, indicating the specificity of binding between  $\beta$ -dystroglycan and dystrophin. Thus, these results confirm our previous observation that  $\beta$ -dystroglycan binds to dystrophin. To further define the  $\beta$ -dystroglycan binding site, *in vitro* binding assays were performed with seven GST fusion proteins containing overlapping portions of human dystrophin sequence 3054–3446 (amino acids 3054–3189, 3054–3271, 3120–3271, 3189–3271, 3189–3446, 3271–3446, and 3100–3200). The positions of the fusion proteins in human dystrophin and their association with  $\beta$ -dystroglycan are summarized in Fig. 3*B*. Only GST fusion proteins that contained the second half of hinge 4 and the cysteine-rich domain of dystrophin (amino acids 3054–3271) bind  $^{35}$ S-labeled  $\beta$ -DGct (Fig. 3, *A* and *B*). However, the absence of amino acids 3271–3446 in this fusion protein results in reduced binding of  $\beta$ -dystroglycan (compare lanes 3 and 5 of Fig. 3*A*). Densitometric scanning has



**FIG. 3. Characterization of  $\beta$ -dystroglycan binding motif in dystrophin.** *A*, *in vitro* translated  $\beta$ -dystroglycan cytoplasmic domain (<sup>35</sup>S)  $\beta$ -DGct was incubated with GST or GST-dystrophin fusion proteins (containing amino acids 3054–3685, 3054–3446, 3435–3685, 3054–3271, or 3189–3448) coupled to glutathione-agarose. The beads were subjected to SDS-PAGE, stained with Coomassie Blue (CB), dried, and subjected to autoradiography (Autorad). Autoradiograph of *in vitro* translated  $\beta$ -dystroglycan cytoplasmic domain resolved on SDS-PAGE is shown on the right (<sup>35</sup>S)  $\beta$ -Dgct. Molecular mass standards ( $\times 10^{-3}$ ) are indicated on the left. *B*, alignment of the dystrophin fusion proteins in the C-terminal portion of human dystrophin. The white, black, and gray box represents the hinge 4, the cysteine-rich domain, and the C-terminal domain, respectively. The amino acids of the fusion proteins were numbered based on their locations in the primary structure of human dystrophin. Exons numbers are indicated. The interactions between these fusion proteins and  $\beta$ -dystroglycan cytoplasmic domain are indicated on the left by (+) for interaction or (–) for no interaction.

demonstrated a 5-fold decrease in the binding of <sup>35</sup>S-labeled  $\beta$ -dystroglycan to dystrophin fusion protein containing amino acids 3054–3271 compared to the fusion protein containing amino acids 3054–3446. This suggests that a minimum  $\beta$ -dystroglycan binding motif is confined to amino acids 3054–3271, which include exons 62–67. However, additional amino acids located downstream between residues 3271 and 3446 are required for maximum binding. On the other hand,  $\beta$ -dystroglycan did not bind to fusion proteins that contained the dystrophin amino acid sequence 3054–3189, 3120–3271, 3189–3271, 3189–3446, and 3100–3200. The absence of interaction with these fusion proteins containing a smaller portion of the cysteine-rich domain suggests that the binding motif is composed of several amino acids distributed throughout the second half of hinge 4 and the cysteine-rich domain rather than a consecutive amino acid sequence. Alternatively, disruption of some residues in these two regions could induce a modified tertiary structure of the  $\beta$ -dystroglycan binding domain abolishing the interaction.

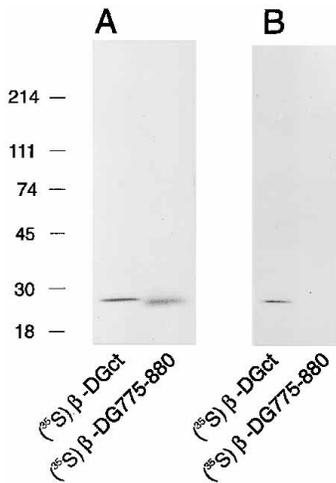


**FIG. 4. Binding of  $\beta$ -dystroglycan to dystrophin fusion protein can be blocked by peptides of the cytoplasmic domain of  $\beta$ -dystroglycan.** *Upper panel*, peptide locations in the cytoplasmic domain of  $\beta$ -dystroglycan; the peptide sequences are underlined. *Lower panel*, *in vitro* translated  $\beta$ -dystroglycan cytoplasmic domain (<sup>35</sup>S)  $\beta$ -DGct was incubated in the presence of 1 mM peptides (+PEP) or the absence of peptides, with GST-dystrophin (amino acids 3054–3446) coupled to glutathione-agarose. Control experiments were performed with GST glutathione-agarose or GST-dystrophin (amino acids 3054–3446) glutathione-agarose in the presence of 1 mM control peptide (+PEPc). The proteins attached to beads were resolved on SDS-PAGE, dried, and subjected to autoradiography. Autoradiograph of *in vitro* translated  $\beta$ -dystroglycan cytoplasmic domain resolved on SDS-PAGE is shown on the right (<sup>35</sup>S)  $\beta$ -Dgct.

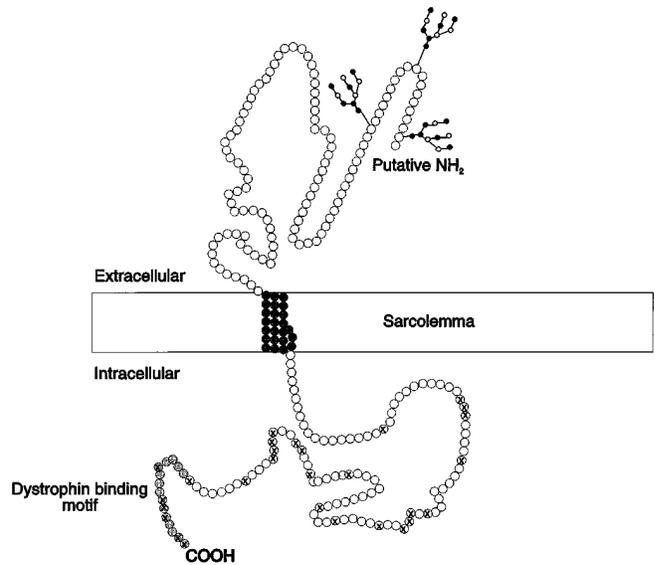
**Identification of the Dystrophin Binding Site on  $\beta$ -Dystroglycan**—To characterize the portion of the  $\beta$ -dystroglycan cytoplasmic domain involved in the binding of dystrophin, six peptides covering the entire  $\beta$ -dystroglycan cytoplasmic domain were synthesized (peptides 1–6) (Fig. 4). These synthetic peptides were used in competitive binding experiments to test whether they form part of a binding site for dystrophin. As shown in Fig. 4, an unrelated peptide corresponding to the  $\beta_{1b}$  Ca<sup>2+</sup> channel subunit sequence (25) did not inhibit <sup>35</sup>S-labeled  $\beta$ -DGct binding to GST fusion proteins containing amino acids 3054–3446 of dystrophin. However, peptide 6 competed with <sup>35</sup>S-labeled  $\beta$ -DGct for the binding to dystrophin fusion protein, whereas peptides 1, 2, 3, 4, and 5 did not interfere with this interaction. The 15 C-terminal amino acid sequence of  $\beta$ -dystroglycan, which corresponds to peptide 6, is therefore sufficient by itself to entirely prevent the association between  $\beta$ -dystroglycan and dystrophin. Thus, this result suggests that the extreme C-terminal domain (amino acids 880–895) of  $\beta$ -dystroglycan is a unique binding motif for dystrophin.

To confirm the localization of a unique dystrophin binding site at the C-terminal domain of  $\beta$ -dystroglycan, we determined whether or not  $\beta$ -dystroglycan lacking the 15 C-terminal amino acids is still able to bind dystrophin. For this purpose, the cytoplasmic domain of  $\beta$ -dystroglycan lacking the 15 C-terminal amino acids was translated *in vitro* (<sup>35</sup>S-labeled  $\beta$ -DG775–880). *In vitro* binding assays were performed with this <sup>35</sup>S-labeled probe and dystrophin fusion protein. As shown in Fig. 5, the binding of  $\beta$ -dystroglycan to the cysteine-rich domain of dystrophin was completely abolished by the truncation of the C-terminal 15 amino acids of  $\beta$ -dystroglycan. This result demonstrated that the C-terminal 15 amino acids of  $\beta$ -dystroglycan are part of the dystrophin binding motif and that no other binding motif is present on  $\beta$ -dystroglycan.

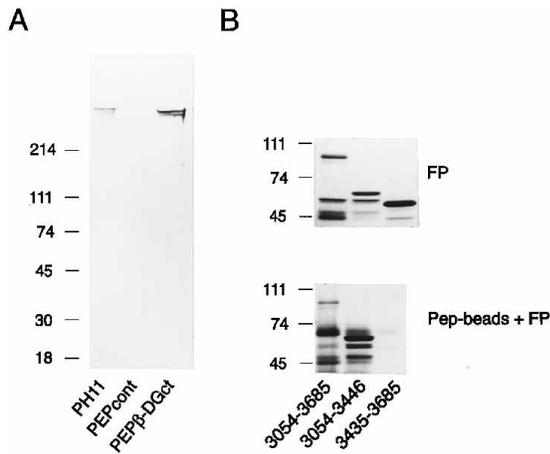
However, despite the fact that the truncation of the C-terminal 15 amino acids of  $\beta$ -dystroglycan abolished the binding to dystrophin, it does not rule out the possibility that flanking N-terminal sequences are contributing to the interaction. To test this possibility, we performed a dystrophin affinity purification assay with a  $\beta$ -dystroglycan C-terminal synthetic peptide (amino acids 880–895) immobilized on Sepharose beads.



**FIG. 5. Truncation of the 15 C-terminal amino acids of  $\beta$ -dystroglycan abolish the binding to dystrophin.** *A*, autoradiograph of *in vitro* translated  $\beta$ -dystroglycan containing the full-length cytoplasmic domain ( $^{35}\text{S}$ )  $\beta$ -DGct and the cytoplasmic domain lacking the 15 C-terminal amino acids ( $^{35}\text{S}$ )  $\beta$ -DG775-880 resolved on SDS-PAGE. *B*, *in vitro* translated  $\beta$ -dystroglycan ( $^{35}\text{S}$ )  $\beta$ -DGct or ( $^{35}\text{S}$ )  $\beta$ -DG775-880 were incubated with GST-dystrophin fusion protein, containing amino acids 3054-3446, coupled to glutathione-agarose. The beads were washed and subjected to SDS-PAGE, dried, and subjected to autoradiography. Molecular mass standards ( $\times 10^{-3}$ ) are indicated on the left.



**FIG. 7. Schematic structure of  $\beta$ -dystroglycan.** Each amino acid is represented by a circle. The putative N-terminal amino acid, corresponding to the cleavage site of the 97-kDa dystroglycan precursor, is based on the results of Gee and colleagues (35). Potential N-linked glycosylation sites (branched chains), transmembrane domain (gray circles), and proline residues from the cytoplasmic domain (X) are indicated. The dystrophin anchoring site is indicated by the light gray circles.



**FIG. 6. Dystrophin binds to the 15 last amino acids of  $\beta$ -dystroglycan.** *A*, rabbit skeletal muscle dystrophin solubilized by pH 11 treatment was subjected to affinity chromatography on Sepharose beads coupled to a synthetic peptide covering the sequence of the 15 last amino acids of  $\beta$ -dystroglycan (PEP- $\beta$ -DGct) or a control peptide (PEP-cont). The starting material, solubilized membrane (pH 11), and the Sepharose beads after chromatography were resolved on SDS-PAGE. The gels were electrotransferred to nitrocellulose membrane and immunoreacted with anti-dystrophin DYS2 monoclonal antibody. *B*, *Upper panel* shows a Coomassie Blue-stained polyacrylamide gel of 15  $\mu\text{g}$  of purified human dystrophin fusion protein (FP) containing amino acids 3054-3685, amino acids 3054-3446, and amino acids 3435-3685. *Lower panel*, purified human dystrophin fusion protein described above were incubated with Sepharose beads coupled to a synthetic peptide covering the sequence of the 15 last amino acids of  $\beta$ -dystroglycan (PEP- $\beta$ -DGct). After an extensive wash, the beads were resolved on SDS-PAGE, and the gel was stained with Coomassie Blue. Molecular mass standards ( $\times 10^{-3}$ ) are indicated on the left.

As shown in Fig. 6A, dystrophin from pH 11 solubilized skeletal muscle membranes was retained by  $\beta$ -dystroglycan C terminus peptide-Sepharose beads but not by beads conjugated to an unrelated peptide. In addition,  $\beta$ -dystroglycan C-terminal peptide-Sepharose beads successfully bound human dystrophin fusion proteins containing amino acids 3054-3685 or amino acids 3054-3446 but did not bind to fusion proteins containing

amino acids 3435-3685 (Fig. 6B). Taken together, these experiments demonstrate 1) the existence of a unique dystrophin binding domain on  $\beta$ -dystroglycan C terminus, which contains amino acids 880-895 and 2) that this 15-amino acid sequence is sufficient by itself for the association to dystrophin.

DISCUSSION

In conclusion, our results demonstrate that  $\beta$ -dystroglycan directly binds to dystrophin. Furthermore, we identified the interacting motif on both proteins. One function of dystrophin is to link, by way of the associated glycoprotein complex, the actin cytoskeleton to the extracellular matrix (11-17). Two domains of dystrophin responsible for its interaction with the associated glycoprotein complex have now been characterized. The C-terminal domain binds syntrophin (20-23), and, as we demonstrated here, the second half of hinge 4 and cysteine-rich domain binds the C terminus of  $\beta$ -dystroglycan. Recently, Rafael and colleagues (31) have generated a transgenic *mdx* mouse expressing a dystrophin gene missing exons 71-74. Dystrophin expression in this *mdx* transgenic mouse restored all the dystrophin-associated proteins in muscle sarcolemma and prevented dystrophic pathology, even in the absence of the syntrophin binding motif in dystrophin. Consequently, the integrity of the  $\beta$ -dystroglycan motif in dystrophin, which appears to lie outside exons 71-74, is sufficient to maintain the link between the subsarcolemmal cytoskeleton and the extracellular matrix and maintain the structural integrity of the muscle cells. Furthermore, syntrophin is a dystrophin binding protein but does not exhibit any dystrophin-associated glycoprotein binding activity (23). Therefore, it is unlikely that syntrophin is a direct molecular linker that connects dystrophin to the associated glycoprotein complex. This function may be fulfilled by  $\beta$ -dystroglycan.

$\alpha$ - and  $\beta$ -dystroglycan are tightly associated and therefore likely exist as a unique complex in various tissues (32-34). It is reasonable to assume that this dystroglycan complex could link the underlying plasma membrane cytoskeleton to the extracellular matrix by itself especially in non-muscle tissues. So far,

no pathologies resulting from the absence of dystroglycan have been reported. However, this may be because dystroglycan, which is ubiquitously expressed, plays an essential role in cell function and/or development, and cells harboring such mutations may be inviable.

On the basis of these results and previous data (11, 35), a schematic representation of  $\beta$ -dystroglycan structure can be drawn (Fig. 7). This model of  $\beta$ -dystroglycan includes the putative N-terminal amino acid, the potential N-linked glycosylation sites, the transmembrane domain, and the dystrophin anchoring site. It is interesting to notice that the dystrophin binding is located in a proline-rich environment within the cytoplasmic domain of  $\beta$ -dystroglycan. We recently demonstrated that the cytoplasmic domain of  $\beta$ -dystroglycan binds to the SH3 domains of Grb2 (36). Therefore, it is reasonable to assume that the interaction between dystrophin and  $\beta$ -dystroglycan could be structurally related to a SH3-proline-rich sequence interaction.

The identification of the interaction sites in dystrophin and  $\beta$ -dystroglycan provides further insight into the structure and the molecular organization of the dystrophin-glycoprotein complex at the sarcolemma membrane and will be helpful for studying the pathogenesis of Duchenne muscular dystrophy.

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#### REFERENCES

- Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C., and Kunkel, L. M. (1987) *Cell* **50**, 509–517
- Koenig, M., Monaco, A. P., and Kunkel, L. M. (1988) *Cell* **53**, 219–228
- Koenig, M., Beggs, A. H., Moyer, M., Scherpf, S., Heindrich, K., Bettecken, T., Meng, G., Müller, C. R., Lindlöf, M., Kaariainen, H., de la Chapelle, A., Kiuru, A., Savontaus, M.-L., Gilgenkrantz, H., Récan, D., Chelly, L., Kaplan, J.-C., Covone, A. E., Archidiacono, N., Romeo, G., Liechti-Gallati, S., Schneider, V., Braga, S., Moser, H., Darras, B. T., Murphy, P., Francke, U., Chen, J. D., Morgan, G., Denton, M., Greenberg, C. R., Wrogemann, K., Blonden, L. A. J., van Paassen, H. M. B., van Ommen, G. J. B., and Kunkel, L. M. (1989) *Am. J. Hum. Genet.* **45**, 498–506
- Bies, R. D., Caskey, C. T., and Fenwick, R. (1992) *J. Clin. Invest.* **90**, 666–672
- Zubrzycka-Gaarn, E. E., Bulman, D. E., Karpati, G., Burgles, A., Belfall, B., Klamut, H. J., Talbot, J., Hodges, R. S., Ray, P. M., and Worton, R. G. (1988) *Nature* **333**, 466–469
- Ervasti, J. M., Kahl, S. D., and Campbell, K. P. (1991) *J. Biol. Chem.* **266**, 9161–9165
- Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990) *Nature* **345**, 315–319
- Ervasti, J. M., and Campbell, K. P. (1991) *Cell* **66**, 1121–1131
- Yoshida, M., and Ozawa, E. (1990) *J. Biochem. (Tokyo)* **108**, 748–752
- Suzuki, A., Yoshida, M., Yamamoto, H., and Ozawa, E. (1992) *FEBS Lett.* **308**, 154–160
- Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) *Nature* **355**, 696–702
- Ervasti, J. M., and Campbell, K. P. (1993) *J. Cell Biol.* **122**, 809–823
- Yang, B., Ibraghimov-Beskrovnaya, O., Moomaw, C. R., Slaughter, C. A., and Campbell, K. P. (1994) *J. Biol. Chem.* **269**, 6040–6044
- Roberds, S. L., Anderson, R. D., Ibraghimov-Beskrovnaya, O., and Campbell, K. P. (1993) *J. Biol. Chem.* **268**, 23739–23742
- Campbell, K. P. (1995) *Cell* **80**, 675–679
- Sunada, Y., Bernier, S. M., Kozak, C. A., Yamada, Y., and Campbell, K. P. (1994) *J. Biol. Chem.* **269**, 13729–13732
- Corrado, K., Mills, P., and Chamberlain, J. (1994) *FEBS Lett.* **334**, 255–260
- Ohlendieck, K., and Campbell, K. P. (1991) *J. Cell Biol.* **115**, 1685–1694
- Ohlendieck, K., Matsumura, K., Ionasescu, V. V., Towbin, J. A., Bosch, E. P., Weinstein, S. L., Sernett, S. W., and Campbell, K. P. (1993) *Neurology* **43**, 795–800
- Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y., and Ozawa, E. (1994) *Eur. J. Biochem.* **220**, 283–292
- Suzuki, A., Yoshida, M., and Ozawa, E. (1995) *J. Cell Biol.* **128**, 373–381
- Ahn, A. H., and Kunkel, L. M. (1995) *J. Cell Biol.* **128**, 363–371
- Yang, B., Jung, D., Rafael, J. A., Chamberlain, J. S., and Campbell, K. P. (1995) *J. Biol. Chem.* **270**, 4975–4978
- Greenberg, D. S., Sunada, Y., Campbell, K. P., Yaffe, D., and Nudel, U. (1994) *Nat. Genet.* **8**, 340–344
- De Waard, M., Pragnell, M., and Campbell, K. P. (1994) *Neuron* **13**, 495–503
- Jung, D., Filliol, D., Metz-Boutigue, M., and Rendon, A. (1993) *Neuromusc. Disorders* **3**, 515–518
- Fabrizio, E., Nudel, U., Hugon, G., Robert, A., Pons, F., and Mornet, D. (1994) *Biochem. J.* **299**, 359–365
- Cox, G. A., Sunada, Y., Campbell, K. P., and Chamberlain, J. (1994) *Nat. Genet.* **8**, 333–338
- Yoshioka, K., Zhao, J., Uchino, M., and Miike, T. (1992) *J. Neurol. Sci.* **108**, 214–220
- D'Souza, V. N., Thi Man, N., Morris, G. E., Karges, W., Pillers, D. M., and Ray, P. N. (1995) *Hum. Mol. Genet.* **4**, 837–842
- Rafael, J. A., Sunada, Y., Cole, N. M., Campbell, K. P., Faulkner, J. A., and Chamberlain, J. S. (1994) *Hum. Mol. Genet.* **3**, 1725–1733
- Campanelli, J. T., Roberds, S. L., Campbell, K., and Scheller, R. H. (1994) *Cell* **77**, 663–674
- Yoshida, M., Suzuki, A., Yamamoto, H., Noguchi, S., Mizuno, Y., and Ozawa, E. (1994) *Eur. J. Biochem.* **222**, 1055–1061
- Ibraghimov-Beskrovnaya, O., Milatovich, A., Ozcelik, T., Yang, B., Koepnick, K., Francke, U., and Campbell, K. P. (1993) *Hum. Mol. Genet.* **2**, 1641–1657
- Gee, S. H., Blacher, R. W., Douville, P. J., Provost, P. R., Yurchenco, P. D., and Carbonetto, S. (1993) *J. Biol. Chem.* **268**, 14972–14980
- Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) *J. Biol. Chem.* **270**, 11711–11714