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Characterization of the β-Dystroglycan–Growth Factor Receptor 2 (Grb2) Interaction

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The β-dystroglycan/Grb2 interaction was investigated and a proline-rich region within β-dystroglycan that binds Grb2-src homology 3 domains identified. We used surface plasmon resonance (SPR), fluorescence analysis, and solid-phase binding assay to measure the affinity constants between Grb2 and the β-dystroglycan cytoplasmic tail. Analysis of the data obtained from SPR reveals a high-affinity interaction ($K_D \approx 240$ nM) between Grb2 and the last 20 amino acids of the β-dystroglycan carboxyl-terminus, which also contains a dystrophin-binding site. A similar K_D value ($K_D \approx 280$ nM) was obtained by solid-phase binding assay and in solution by fluorescence. Both Grb2-SH3 domains bind β-dystroglycan but the N-terminal SH3 domain binds with an affinity approximately fourfold higher than that of the C-terminal SH3 domain. The Grb2–β-dystroglycan interaction was inhibited by dystrophin in a range of concentration of 160–400 nM. These data suggest a highly regulated and dynamic dystrophin/dystroglycan complex formation and that this complex is involved in cell signaling.

Key Words: **dystrophin; fluorescence spectra; SH3 domains; solid-phase binding assay; surface plasmon resonance; WW domain.**

Dystroglycan is a transmembrane receptor formed by two (α and β) glycoprotein subunits that links extracellular matrix proteins to the actin cytoskeleton. It was originally isolated from rabbit skeletal muscle as component of the dystrophin-associated protein complex and found dramatically reduced in the sarcolemma of patients with Duchenne muscular dystrophy and in dystrophin deficient *mdx* mice (1). Dystroglycan is expressed in a variety of cell types from early development into adulthood indicating that its function is not restricted to muscle (2). It plays a critical role in organizing extracellular matrix proteins on the cell surface and in the basement membranes (3). Recently, it has been found to be the receptor for viral and bacterial agents of severe hemorrhagic fever and leprosy disease (4, 5).

The functional versatility of dystroglycan may be achieved by its interaction with different extracellular and cytoplasmic proteins. α-Dystroglycan, the extracellular highly glycosylated peripheral membrane subunit, binds extracellular matrix proteins containing laminin-type LG domains (i.e., laminin, agrin, and perlecan) with high affinity in a calcium-dependent manner (6–8). β-Dystroglycan spans the membrane, interacting with α-dystroglycan on the outside and with dystrophin and dystrophin-related proteins on the inside of the cell.

The best-characterized interaction of β-dystroglycan is the one with dystrophin. By coprecipitation and gel overlay assays, dystrophin and truncated version of dystrophin, Dp116 and Dp71, were shown to interact with β-dystroglycan carboxyl terminus (9–11) and with a stretch of positively charged amino acids near the β-dystroglycan transmembrane domain (10, 12). Recently, Rentschler *et al.* (12) showed that the region of dystrophin containing the WW domain (13) and two putative Ca^{2+}-binding EF-hand motifs are required for the binding to β-dystroglycan carboxyl terminus and demonstrated that the binding occurs via β-dystroglycan Pro-Pro-x-Tyr (amino acids 890–893) core motif (12).

The cytoplasmic tail of β-dystroglycan binds, *in vitro* and *in vivo,* other cytoplasmic proteins including the growth factor receptor bound protein 2 (Grb2) (14, 15). Mammalian Grb2 is an adapter protein, composed of one SH2 (Src homology 2) domain surrounded by two SH3 domains, involved in signal transduction and cytoskeleton organization (16, 17), which regulates mul-

Abbreviations used: Grb2, growth-factor-receptor-bound protein 2; SPR, surface plasmon resonance; SH3, src-homology domain 3; GST, glutathione *S*-transferase.

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tiple steps in embryonic development and malignant transformation (18). The Grb2/β-dystroglycan association is mediated through β-dystroglycan proline-rich domains and Grb2 src homology 3 domains (14). This interaction may be of biological importance in transducing signals arising from the binding of dystroglycan to extracellular matrix proteins (14) or in transferring information between the dystroglycan complex and other signaling pathways (15).

In the present study we used Surface Plasmon Resonance (SPR) measurements, fluorescence emission spectra analysis and solid-phase binding assay in order to quantify the interaction between β-dystroglycan and Grb2 and identify the sites on β-dystroglycan involved in the binding with Grb2.

MATERIALS AND METHODS

Protein preparation. Dystroglycan–glutathione *S*-Transferase fusion protein, GST–β-C2 (spanning amino acids 821–895) [11], GST–Grb2, GST–Grb2 N-SH3 (amino acids 1–54) and GST–Grb2 C-SH3 (amino acids 163–217) domains (19), (kindly provided by Dr. O. Segatto, Istituto Regina Elena, Rome, Italy), were all expressed in *Escherichia coli* BL21 strain and purified by affinity chromatography on glutathione-agarose beads (Amersham Pharmacia Biotech) as described elsewhere (11, 19). GST was removed from the fusion proteins by digestion with thrombin (20 U/mg) (Sigma) overnight at 4°C. All the recombinant proteins purified after the thrombin digestion also included at their N-terminal a stretch of few foreign amino acids. In the case of Grb2 N-SH3 domain they were GSPGISGGG-GIL. A synthetic peptide spanning the last 20 amino acids of the β-dystroglycan carboxyl-terminus including a cysteine added at the amino terminus (C-KGSRPKNMTPYRSPPPYVPP) was prepared by standard solid phase peptide synthesis and purified by reverse phase HPLC chromatography (11). The N-terminal sequence analysis of the P20 revealed the presence of three additional amino acids: SGC. SG originated from additional reactions during the preparation of the peptide.

Dystrophin-enriched fraction was prepared by alkaline extraction (pH 11) of rabbit skeletal muscle microsomes (20) and concentrated by filtration on Amicon filter (cut off 100 kDa) (Millipore). The amount of native dystrophin present in the sample fraction was evaluated, after SDS–PAGE analysis and staining the gel with Coomassie blue R250, from the intensity of the bands in the region of molecular weight of dystrophin respect to the intensity of the other bands present in the gel as described by others (21).

Recombinant GST–β-C2 was biotinylated in 50 mM sodium phosphate buffer at pH 7.4 with 0.5 mg/ml of sulfosuccinimidobiotin (S-NHS-biotin) (Pierce) as described elsewhere (11) and the optimal dilution for signal detection was determined by dot blot analysis. Protein concentration was determined using a protein BCA assay kit (Pierce) using bovine serum albumin as a standard.

Gel electrophoresis. Small molecular weight peptides were analyzed by tricine–SDS–polyacrylamide gel electrophoresis and gels stained with Coomassie brilliant blue G-250 (22) or transferred to nitro-cellulose membranes (Schleicher & Schuell) for Western blot analysis. The presence of GST was revealed with affinity-purified anti-GST (11) and of Grb2 with a monoclonal antibody (1:4000) (Transduction Laboratory). An antibody against recombinant Grb2 was also produced in rabbit and purified by affinity chromatography.

Surface plasmon resonance experiments. SPR assays were performed using BIAcore instrumentation (BIAcoreX) equipped with two flows cells sensor chip. Immobilization of the proteins was achieved by covalently coupling the proteins to CM-5 sensor chips after activation of the carboxymethylated dextran surface by a mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N′*-3-(dimethylaminopropyl) carbodiimide hydrochloride. The reaction was performed by injecting Grb2 in 10 mM acetate buffer, pH 4.8, Grb2 C-SH3 domain in 10 mM formate buffer, pH 3.2, and Grb2 N-SH3 domain in 10 mM formate buffer, pH 2.8, at a flow rate of 10 μl/min and 25°C for 7 min. The residual activated groups were blocked with 1M ethanolamine-HCl pH 8.5. Experiments were performed in HBS (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.05% surfactant P20, pH 7.4) with a flow rate of 10 μl/min. The soluble ligands (β-C2 and P20) were applied in the concentration range 10 nM–17 μM. At the end of the sample plug, HBS buffer was flowed past sensor surface to allow dissociation. The sensor surface was regenerated for the next sample using a 2- to 15-s pulse of regenerating buffer (50 mM NaOH) at a flow rate of 10 μl/min. The response was monitored as a function of time (sensorgram) at 25°C, as the difference (RU) between signals arising from the cell with the immobilized protein and from the reference cell. The integrity of immobilized proteins was tested with an anti-Grb2 affinity purified polyclonal antibody. The data evaluation was carried out using a BIAevaluation software version 3.0, following the manufacturer's instructions. The kinetics constants were calculated by nonlinear regression of data using the pseudo first order rate equation as described in detail by Herzog *et al.* (23).

Fluorescence experiments. The binding of P20 to Grb2 was studied under the following experimental conditions: 10 mM Tris, 150 mM NaCl, and pH 7.4, at 25°C. Quenching of the intrinsic tryptophan(s) fluorescence of Grb2 was monitored as a function of P20 concentration using a SPEX (Edison, U.S.A.) FluoroMax spectrofluorometer in a 1-cm quartz cell. Fluorescence emission spectra were collected between 300 and 450 nm, selectively exciting tryptophan residues at 295 nm. The titrations were performed adding increasing amounts of P20 and monitoring the fluorescence intensity change at the wavelength corresponding to the peak of the emission spectra of the proteins (342 nm for the full-length protein and 352 nm for its N-terminal fragment). No significant peak shifting was observed upon peptide binding. The P20 fluorescence shows no significant contribute in the examined range, as expected by the absence of tryptophans in the sequence. Fluorescence intensity was corrected for the contribution of the buffer and for the dilution of the protein (24–26). In the experimental conditions used, the fluorescence signal was linear with the concentration of Grb2. To correct for the decrease of fluorescence due to prolonged exposure of the sample to high intensity light beam (25–27) we carried out control experiments in which the Grb2 sample was titrated only with buffer. The data were analyzed according to the model proposed for a single site tight binding system in which free ligand concentration is not much greater than the macromolecule concentration (26–28). Similar experiments were carried out on the N-terminal SH3 domain of Grb2.

Solid-phase binding experiments. Solid-phase binding assays were carried out as described elsewhere (29) with the following modification. Briefly, the microtiter plate (Costar) was coated by incubation of 100 μl/well of 7 μg/ml Gbr2 in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$) at 4°C for 4 h. The remaining binding sites were saturated by overnight incubation 4°C with 300 μl/well of 3% bovine serum albumin in buffer A containing 0.1% Tween 20 (A-T). After washing three times (5 min per wash) with buffer A-T–BSA (AT containing 1 mg/ml bovine serum albumin) wells were incubated with serial dilution of biotinylated GST–β-C2 (11). For competition experiments dystrophin-enriched fraction, prepared from rabbit skeletal muscle, or BSA were added to the wells along with the biotinylated GST–β-C2. The plates were incubated for 2 h. Wells were washed 4 times with buffer A-T–BSA and the biotinylated GST–β-C2 bound fraction was detected with the Vectastain ABC immunodetection kit using 0.4 mg/ml *o*-phenylenediamine dihydrochloride in 80 mM citrate/phosphate buffer containing 0.015% H_2O_2, pH 5.3. The absorption at

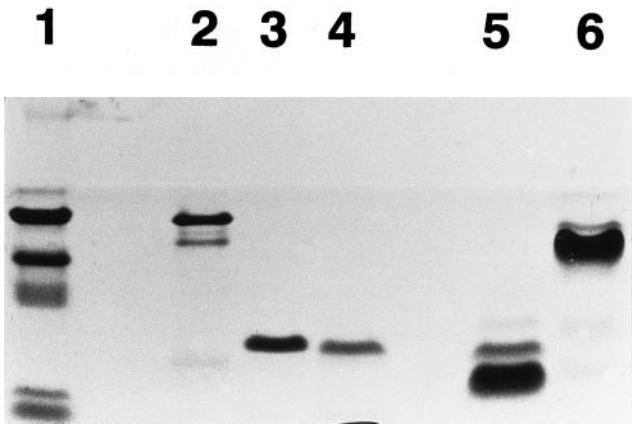

FIG. 1. Tricine–SDS–polyacrylamide gel electrophoresis of recombinant proteins and P20 synthetic peptide. Lane 1, molecular mass markers (26.6, 17, 14, 3.5, and 1 kDa); lane 2, Grb2; lane 3, Grb2 N-SH3; lane 4, Grb2 C-SH3; lane 5, P20 peptide; lane 6, β-C2. Lanes were loaded with 1 μg (lanes 2–5) and 3 μg (lanes 5 and 6).

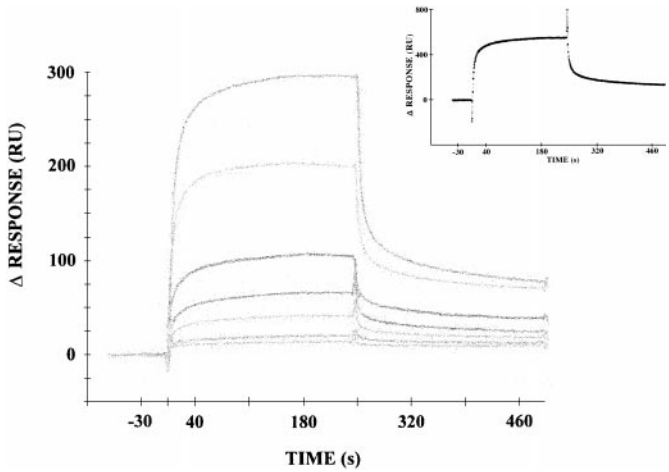

FIG. 2. Surface plasmon resonance profiles of the binding of P20 peptide to Grb2 immobilized to the sensor chip. Individual curves from the bottom to the top obtained with P20 concentrations of 0.068, 0.68, 3.4, 6.8, 13.6, 34, and 68 μM are reported. Association and dissociation phases were started at 0 and at 240 s.

405 nm was determined. Data were fitted using a single class of equivalent binding site equation (30).

RESULTS

Purification of the Proteins under Analysis

Purified polypeptides, Grb2, Grb2 C-, and N-SH3 domains, and β-dystroglycan cytoplasmic domain β-C2, after digestion with thrombin, and P20 peptide used are shown in Fig. 1. The apparent molecular weights of β-C2 and P20 on the gel do not correspond to the expected mass. This anomalous electrophoretic behavior has also been observed for the native (3) as well as for recombinant cytoplasmic (11) and extracellular regions of β-dystroglycan (30).

Kinetic Measurement of Grb2-β–Dystroglycan Interaction

Sensorgrams for the binding of Grb2 and P20 are shown in Fig. 2. Similar SPR sensorgrams were obtained for Grb2/β-C2 or for Grb2-SH3 domains/β-C2 or/P20 interactions (data not shown). The initial part of the curve corresponds to the signal of the buffer flowing on the sensor surface. The rising part of each curve corresponds to the difference of the signals (RU) observed upon injection through the cells of the sensor chip, the cell with immobilized Grb2 and the reference cell. The decreasing phase of each curve corresponds to the dissociation of bound P20 from immobilized Grb2 after the sample volume has finished and the buffer flows on the sensor surface again. Increasing concentrations of P20 ranging between 0.01–17 μM were allowed to flow over the sensor surface. The dissociation rate constant k_{off} was evaluated from traces obtained at saturation of ligand (β-C2 or P20) and used to calculate the observed association rate constants (k_{on}) (Table 1).

Immobilized Grb2 and Grb2 N-SH3 bind to either P20 or β-C2 in a similar fashion, with K_D values in the range of 220–400 nM (Table 1). Whereas, Grb2 C-SH3 binds P20 and β-C2 with an affinity approximately fourfold lower (K_D values were in the range of 800–1400 nM) (Table 1).

Fluorescence Study of Grb2–P20 Interaction

Figure 3 shows the fractional saturation of Grb2 as a function of free P20 concentration. The steady-state fluorescence intensity was measured at constant Grb2 concentration as a function of increasing P20 concentrations. The peptide binding to Grb2 affects the intrinsic fluorescence of the protein and is compatible with the quenching of tryptophan residue(s) originating from a conformational change reflecting the interaction between the two molecules. The K_D value measured in solution (≈280 nM) is in

TABLE 1

Comparison of P20 and β-C2 Binding to Grb2 and Grb2 N-/C-SH3 Domains

Immobilized ligand	β dystroglycan region (μM)	k_{off} (s^{-1})	k_{on} ($M^{-1}\ s^{-1}$)	K_D (nM)
Grb2	β-C2 (0.1–17)	1.19×10^{-3}	3.95×10^{3}	301 ± 54
	P20 (0.01–17)	1.78×10^{-3}	7.49×10^{3}	237 ± 45
Grb2 N-SH3	β-C2 (0.1–17)	1.21×10^{-3}	3.03×10^{3}	399 ± 42
	P20 (0.01–17)	1.56×10^{-3}	7.08×10^{3}	220 ± 45
Grb2 C-SH3	β-C2 (0.1–17)	2.13×10^{-3}	1.52×10^{3}	1404 ± 170
	P20 (0.01–17)	1.95×10^{-3}	2.52×10^{3}	774 ± 72

Note. SPR assays were carried out at 25°C in HBS buffer. The concentration range of soluble ligands is indicated in parentheses (μM).

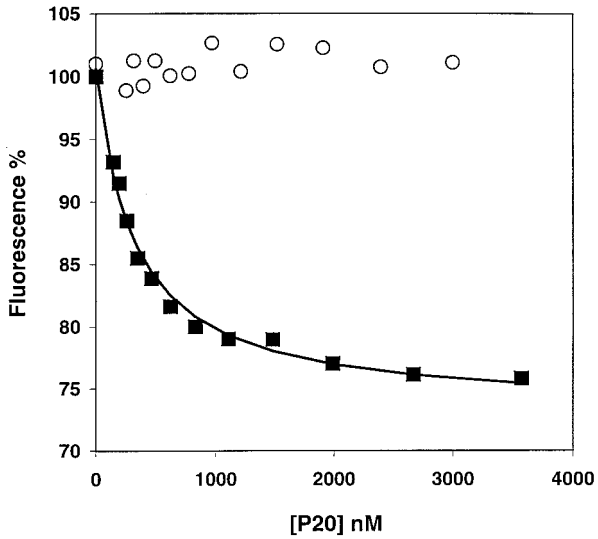

FIG. 3. Titration of Grb2 with P20 recorded by fluorescence change. The saturation of Grb2 as a function of free P20 concentration (■) was calculated as described under Materials and Methods. Data refer to experiments carried out at three different Grb2 concentrations, 80, 120, and 200 nM. The continuous line was obtained by nonlinear least-squares fitting of the experimental data, resulting in an equilibrium dissociation constant (K_D) equal to 280 nM. In the case of Grb2 N-SH3 domain titration with P20 no fluorescence change was detected (○) (see Discussion).

full agreement with the value obtained by SPR analysis (see Table 1).

It is noteworthy that the Grb2 full-length protein harbors five tryptophan residues while its N-terminal fragment, whose intrinsic fluorescence is apparently not sensitive to the binding of P20, only one (in position 36) (16, 30). In fact, the Grb2 N-terminal fragment shows an emission peak at 352 nm and no change in the fluorescence of the protein was observed when titrated with the peptide (Fig. 3).

Dystrophin Inhibits Grb2-β–Dystroglycan Interaction

β-Dystroglycan interacts with Grb2 via its carboxy-terminus that was previously shown to bind dystrophin (9–11). A dystrophin-enriched fraction was obtained from skeletal muscle (20, 21) and checked on Western blot using dystrophin-specific antibodies (data not shown) (see Materials and Methods). Dystrophin from several preparations was estimated to be 2–5% of total protein fraction in agreement to Ohlendieck and Campbell (21). To investigate whether β-dystroglycan–Grb2 interaction is affected by dystrophin we performed competition experiments using solid-phase binding assay. Consistent with the data obtained by SPR (Table 1) we have measured high-affinity binding (K_D value of about 0.3 μM) of GST–β-C2 to Grb2 (Fig. 4), and found that the presence of skeletal muscle dystrophin-enriched fraction (0.15–0.4 μM) but not BSA (4 μM) inhibited Grb2/GST–β-C2 interaction (Fig. 4).

DISCUSSION

Dystroglycan is a receptor molecule which binds several extracellular matrix and cytoplasmic proteins through its α and β subunits, and plays important biological roles both in development and disease (31). Only the binding of α-dystroglycan to laminin, agrin and perlecan has been extensively studied so far and the affinity constants quantitatively determined (8). The present study contains the first quantitative analysis of β-dystroglycan/Grb2 interaction using different experimental approaches, SPR, fluorescence and solid phase assay. All assays demonstrate that β-dystroglycan carboxy-terminus binds Grb2 with a K_D value in the high nanomolar range (220–300 nM).

We found interaction with both Grb2s SH3 domains. This was also observed using two Grb2 mutants (14), which correspond to the loss-of-function mutants in the *C. elegans sem-5*. Grb2 N-SH3 domain contributes more to β-dystroglycan binding than its C-terminal counterpart, similarly to what found for Grb2/Sos interaction (32, 33), whereas, only the Grb2 N-SH3 domain was found to be essential for Grb2/dynamin high-affinity binding (34).

Interestingly, SPR data were also confirmed by tryptophan fluorescence analysis in solution suggesting that P20 binding to Grb2 produces some conformational rearrangement of the protein, which is

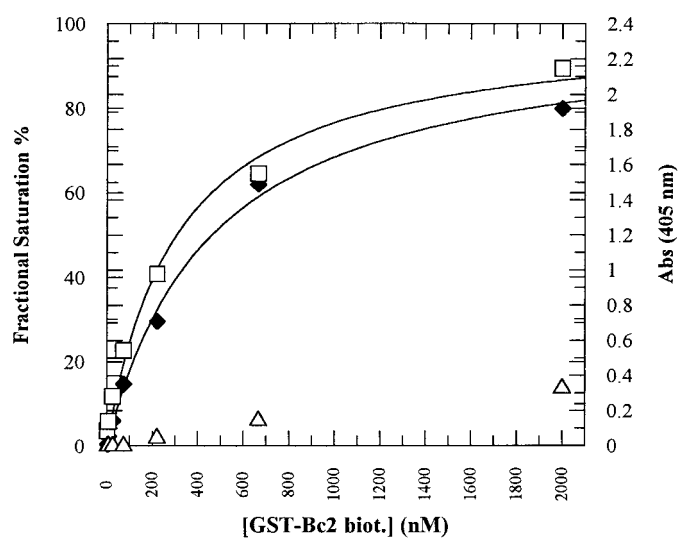

FIG. 4. Solid-phase biotinylated-ligand binding assay. Biotinylated GST–β-C2 binding to Grb2 in absence (□) and in presence of rabbit skeletal muscle dystrophin-enriched fraction (△). The presence of BSA slightly affects biotinylated GST–β-C2/Grb2 binding (◆). Data were normalized using the OD_{tot} parameter and experimental values were fitted according to (30).

likely to involve tryptophan residues belonging to the SH2 and/or the C- terminal SH3 domain of Grb2 (30).

The β-dystroglycan cytoplasmic region contains several proline-rich sequences. Recognition motifs for the SH3 domains are present in the β-C2 protein (two) and in the P20 peptide (one). The values obtained by SPR indicate that Grb2 N-SH3 domain interacts with the same affinity with both polypeptides indicating that the C-terminus proline-rich motif of β-dystroglycan is the primary Grb2 binding site. The minimal sequence requirement for the SH3 domain ligands (35), Pro-x-x-Pro (where x is any amino acid), present in the P20, amino acids 891–894, overlaps with the Pro-Pro-x-Tyr sequence (amino acids 890–893), identified as the β-dystroglycan core motif for the dystrophin binding through the WW domain (12).

Since Grb2 binds β-dystroglycan on a site, which overlaps the dystrophin-binding site, it is possible that the Grb2/β-dystroglycan complex can be established only in some physiological conditions. In fact, dystrophin indeed competes *in vitro* with Grb2 for the binding to β-dystroglycan (see Fig. 4) suggesting that the β-dystroglycan–Grb2–β-dystrophin complex formation is highly dynamic. Although we could not measure the dystrophin-β-C2 affinity constant, we observed a full inhibition of Grb2-β-C2 interaction by dystrophin in a range of concentration of 160–400 nM. This would imply that dystrophin binds-β-C2 with higher affinity compared to Grb2.

Recently, it has been found that utrophin, a ubiquitous protein homologous to dystrophin, binds to the carboxy-terminus β-dystroglycan PPxY motif through its WW-domain and that the binding is inhibited by calcium/calmodulin (36) and by β-dystroglycan tyrosine phosphorylation (37).

In nonmuscle cells the binding of β-dystroglycan to dystrophin isoforms, Dp116 expressed in Schwann cells (10), and Dp71, which is the major product of the dystrophin gene in non-muscle tissues, is weaker compared to that to dystrophin in skeletal muscle (10, 38). It is noteworthy that Dp116 contains the WW domain while Dp71 does not, although it still binds β-dystroglycan *in vitro* (11) and *in vivo* (39). This suggests that for the stabilization of the dystroglycan/dystrophin-isoform interaction other components may be required (10).

In conclusion, the high affinity of Grb2/β-dystroglycan interaction and the finding that the binding site overlaps with that of dystrophin further support that notion of a highly regulated and dynamic dystrophin/dystroglycan complex formation. Further studies are needed to investigate the role of extracellular matrix proteins in the formation of Grb2/β-dystroglycan complex and which pathways are activated in the cells following Grb2–β-dystroglycan interaction.

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