Interactions between Dystrophin Glycoprotein Complex Proteins†

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Received February 2, 1995; Revised Manuscript Received July 24, 1995⊗

ABSTRACT: The organization of the dystrophin glycoprotein complex (DGC) was studied by investigating interactions between its components. For this purpose, mouse dystrophin and syntrophin-1 (α-syntrophin) sequences were expressed as chimeric fusion proteins and used in overlay binding experiments to probe gel blots of purified rabbit muscle DGC. In order to identify the DGC proteins that bind to different regions of dystrophin, the amino-terminal 385 amino acids, the unique carboxy-terminal domain (amino acids 3266−3678), and the adjacent cysteine−rich region of dystrophin homologous to α-actinin (amino acids 3074−3265) were expressed as separate fusion proteins. The cysteine-rich sequences of dystrophin predominantly bound adhalin (gp50) and to full length dystrophin suggesting that these sequences may also be important to dystrophin dimerization. The carboxy-terminal domain sequences strongly bound all of the DGC syntrophins and weakly, adhalin, while the amino-terminal sequences of dystrophin bound none of the proteins of this complex. Fusion proteins containing α-syntrophin sequences bound not only to dystrophin but also to all three DGC syntrophins, adhalin, and gp35. The interactions identified here were used to refine the existing model of DGC organization to make it consistent with the current data.

Absence of the protein dystrophin results in Duchenne muscular dystrophy (Hoffman *et al.*, 1987). In the sarcolemma, dystrophin is associated with six proteins and glycoproteins, collectively called the dystrophin glycoprotein complex (DGC)[1] (Ervasti *et al.*, 1991). The DGC apparently links the actin cytoskeleton to the extracellular matrix (Ervasti & Campbell, 1993). The amino-terminal domain of dystrophin binds actin filaments; binding and regulatory sites within this region have been identified (Corrado *et al.*, 1994; Bonet-Kerrache *et al.*, 1994; Jarrett & Foster, 1995, and references therein). The carboxy-terminal domains of dystrophin, on the other hand, were found to bind DGC glycoproteins (Suzuki *et al.*, 1992). Thus, dystrophin has at its two ends sites for binding to cytoskeletal actin and sarcolemmal membrane components which link to laminin in the extracellular compartment. Understanding the organization of DGC has been the focus of several studies and was reviewed recently (Tinsley *et al.*, 1994). A structural model of the DGC (Ervasti & Campbell, 1991) has been refined (Suzuki *et al.*, 1994) and predicts specific interactions of dystrophin's carboxy-terminal region with DGC proteins. In this later model, dystrophin binds to the membrane components of the complex through β-dystroglycan (gp43) and also binds the β-syntrophins. Regions of dystrophin that bind to β-dystroglycan and the β-syntrophins are also identified. Suzuki *et al.* (1994) did not find dystrophin sequences binding to the DGC α-syntrophin. The syntrophin nomenclature described by Tinsley *et al.* (1994) has been used throughout in this report.

Interactions between the DGC components were investigated here using four fusion proteins containing either mouse syntrophin-1 (α-syntrophin) or dystrophin sequences. Gel blots of purified rabbit muscle DGC were probed using these fusion proteins, and several important observations were made. We demonstrate that the syntrophin fusion protein binds to other syntrophins, dystrophin, gp35, and adhalin. For the first time, dystrophin sequences that are potentially involved in dystrophin's dimerization are identified. The binding of distinct domains of dystrophin to different DGC proteins is also shown here. Using these data, a structural model for the organization of the DGC is presented.

MATERIALS AND METHODS

Purification of DGC. Purification of DGC was by the method of Ervasti *et al.* (1991). Starting with 500 g of rabbit skeletal muscle, the preparation was carried out through the DEAE step, to yield 600 μL of purified and concentrated DGC. Affinity chromatography was performed using either 10 or 20 mL succinylated wheat germ agglutinin agarose (Vector Labs) and resulted in DGC preparations with protein concentrations that varied approximately 2-fold (data not shown). Densitometric estimations of dystrophin based on comparisons to protein standards, and other procedures and materials used by us have been described earlier (Madhavan & Jarrett, 1994). Electrophoresis of DGC protein samples was on 5−15% gradient polyacrylamide gels by the method of Laemmli (1970). Silver staining of protein gels was using materials and procedures from Bio-Rad. Other reagents and chemicals used were of the highest purity available.

Dystrophin Sequences. Dystrophin sequences were expressed as chimeric fusions with the maltose binding protein (MBP) for use in overlay binding experiments. The control fusion protein used in these experiments was an MBP−*lac*Zα fusion (mal−lac) protein. The construction and purification of MBP fusions has been described (Madhavan & Jarrett, 1994). The general cloning techniques were those described by Sambrook *et al.* (1989). Briefly, using the Invitrogen

† This work was supported by The Muscular Dystrophy Association.
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.
[1] Abbreviations: DGC, dystrophin glycoprotein complex; MBP, maltose binding protein; mal−lac, maltose binding protein−*lac*Zα fusion; HRP, horseradish peroxidase; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; glycoproteins are denoted by "gp" followed by a number giving their apparent M_r in thousands, based on SDS gels, e.g., "gp35".

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cDNA Cycle kit, 1 μg of mouse (C57bl) skeletal muscle mRNA, and random hexamers, cDNA was prepared. This cDNA was used with dystrophin sequence-specific primers in the polymerase chain reaction. These primers were, for DysS8, TGTTGGGACCACCCCAAAATG and GGCGAATTCTAATTATTGGCAAATTGGAAGCAGC and, for DysS9, AAACCTGAGATTGAAGCTGC and GGCGAATTCTACATTGTGTCCTCTCTCATTGG. Primer sequences and other procedures used in the preparation of DysS1 are described elsewhere (Jarrett & Foster, 1995). The underlined sequences in the primers are not from dystrophin and helped engineer a unique restriction site and stop codon. The products of the polymerase chain reaction were restriction digested, gel purified, and ligated into pMALc vectors (New England Biolabs) and then used to transform *Escherichia coli* strain TBI. Clones expressing the correct size fusion proteins were restriction mapped, and DNA sequencing confirmed their authenticity. These MBP fusion proteins were purified using maltose−affinity chromatography.

Mouse Syntrophin-1 cDNA. Mouse syntrophin-1 cDNA (Adams *et al.*, 1993) was the generous gift of Drs. Marvin Adams and Stanley Froehner. A filled *Bss*HII/*Xba*I fragment of this cDNA was cloned into a *Stu*I/*Xba*I restricted pMalc vector. A *Bam*HI fragment from this plasmid construct was subcloned into a *Bam*HI restricted pET 28a vector. The resulting pET fusion protein containing syntrophin protein sequences (4−503) was affinity purified (using protocols provided by Novagen) and used to raise rabbit polyclonal antibodies which were affinity purified using the fusion protein coupled to CNBr-activated Sepharose. Specificity of the antibodies was confirmed using syntrophin fusion protein and purified rabbit muscle DGC preparations, with preimmune serum controls (data not shown).

Overlay Binding Experiments. Overlay binding experiments were carried out using SDS−PAGE fractionated DGC proteins electroblotted onto nitrocellulose using conditions described elsewhere (Madhavan & Jarrett, 1994). The volume of the purified DGC (20 μL) loaded onto each gel lane was the same throughout, which in certain cases results in approximately twice as much protein being applied (see above). Experiments using 2-fold larger amounts of DGC were performed for specific reasons that are discussed while describing them (see below and Results and Discussion). Strips of protein blots were either stained for total protein (using Amido Black) or blocked overnight with 1% bovine serum albumin (BSA) in TTBS (0.5 M NaCl, 20 mM Tris, pH 7.5, 0.05% Tween-20) for use in the overlay binding experiments. All probing solutions were 0.1 mg/mL fusion protein in TTBS containing 1 mg/mL BSA. DGC blots were incubated with the fusion proteins for 2 h, washed extensively with TTBS-BSA, and probed with affinity purified anti-MBP antiserum (New England Biolabs, 1:1000 dilution). The general procedures used here for all overlay experiments have been described previously (Jarrett & Madhavan, 1991; Madhavan *et al.*, 1992). In experiments where N-glycosidase F treatment was performed, twice as much DGC protein as used in other experiments (see above) was present in each reaction mixture. The digested samples were concentrated using Microcon 10 concentrators (Amicon) prior to boiling in SDS sample buffer for electrophoresis and blotting. Further, to help intensify fusion protein staining of the digested bands, color development in the final step of the overlay procedure was lengthened 2-fold (to about 10 min).

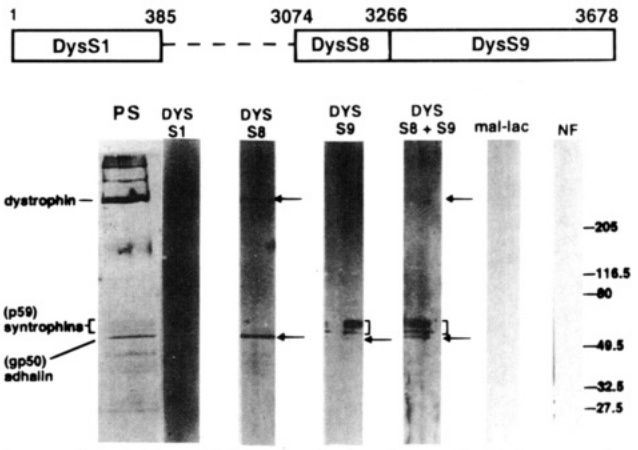

FIGURE 1: Binding of DGC proteins by dystrophin fusion proteins. (A, top) Schematic representation of mouse dystrophin sequences used to produce fusion proteins. The MBP-dystrophin chimeric fusions DysS1, DysS8, and DysS9 contain specific dystrophin sequences shown in the figure. (B, bottom) Binding of dystrophin fusion proteins to components of the DGC. A silver stained SDS−polyacrylamide gel (5−15%) of the purified DGC proteins is shown in the lane marker "PS". Procedures used for blotting and overlay binding experiments are described in Materials and Methods. The blots were probed with the fusion proteins shown in the figure or without fusion protein (NF), followed by anti-MBP antiserum. In panel B, dystrophin, syntrophins (p59 triplet) and adhalin (gp50) and the Bio-Rad prestained molecular mass standards (in kilodaltons) are shown beside the gel and blots of DGC. DGC proteins and molecular mass standards (in kilodaltons) are also indicated beside gel blots in all subsequent figures.

Glycosidase digestion was by the procedure of Ervasti and Campbell (1991). In all overlay experiments, mal−lac and other negative control blots were present and treated side by side (in parallel) with the other DGC blots that were exposed to dystrophin fusion proteins. All of the blots in an experiment were exposed to the color development solution for the same length of time (5 min, unless otherwise specified). Using the procedures described above, DGC blots were also stained with or without 0.1 mg/mL α-syntrophin fusion protein. Detection in these cases was using the affinity purified polyclonal anti-syntrophin antibodies (1:100) or a monoclonal antibody (1:2000) against the T7-antigen generated in the syntrophin fusion protein by the pET 28a expression system. Anti-dystrophin (NCL-DYS2) and anti-adhalin (NCL-50DAG) antibodies were from Novo Castra Labs (U.K.), and used at 1:100 dilution. HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies (both at 1:1000) were used following polyclonal and monoclonal antibodies, respectively.

Concentrations of all the fusion proteins were determined by the method of Bradford (1976) using a BSA standard. Purity of the fusion proteins was assessed using Coomassie stained gels and antibody stained blots and revealed the full length proteins and some minor proteolytic products (data not shown). The novel syntrophin fusion protein used here was purified close to homogeneity (data not shown, but submitted for review) and also showed the full-length protein along with a small amount of proteolysis products.

RESULTS AND DISCUSSION

DGC gel blots were used as the target for binding by dystrophin and α-syntrophin fusion proteins. Figure 1B shows a protein stain (PS) of our DGC which is virtually

identical to that observed by other investigators (Ervasti *et al.*, 1991), and their nomenclature for the DGC components is followed here. Identification of individual proteins in this DGC preparation based on electrophoretic mobilities on SDS gels, differential staining of proteins and glycoproteins by biotinylated wheat germ agglutinin, and antibody staining of dystrophin has been discussed earlier (Madhavan *et al.*, 1992; Madhavan & Jarrett, 1994) and is also further addressed below.

Figure 1A depicts the dystrophin sequences that were used to produce chimeric fusions with MBP. Dystrophin's cysteine-rich region (DysS8) and the carboxy-terminal domain (DysS9) were expressed as separate fusion proteins to distinguish which proteins bind to each of these domains. Figure 1B shows that fusions DysS8 and DysS9 bind DGC proteins, consistent with earlier studies (Suzuki *et al.*, 1992, 1994). The amino-terminal 385 amino acids of dystrophin (DysS1), mal−lac, and the absence of fusion protein (NF) all show no DGC protein staining and provide negative controls. In all experiments described in this report, control blots were treated in parallel and stained for the same length of time as other blots, and negative controls showed no staining in any experiment.

Under the conditions used in Figure 1B, DysS8 binds adhalin (gp50) and full-length dystrophin, while DysS9 binds all three of the DGC syntrophin bands strongly and adhalin weakly. A DGC blot probed with a mixture of DysS8 and DysS9 fusion proteins shows staining of dystrophin, adhalin, and the three DGC syntrophin bands (Figure 1B). Thus DGC binding sites are contained in the carboxy-terminal domains of dystrophin; however, some differences were found when compared to data obtained by others (Suzuki *et al.*, 1994) and are discussed below.

In overlay binding experiments, the gel blots can be stained for short times to reveal preferentially the strongest interactions between overlayed fusion proteins and target proteins. These were the conditions used in Figure 1B (also see Materials and Methods), and the results show that DysS8 interacts most strongly with adhalin (gp50) and dystrophin, while DysS9 binds most strongly to the syntrophin triplet and weakly to adhalin. In other experiments discussed below, higher amounts of DGC and prolonged staining times were used to reveal other, weaker interactions which may also be important to the organization of the DGC. In all cases, however, appropriate negative controls such as the mal−lac protein showed no staining, and the staining observed is due to dystrophin-derived sequences.

The identity of DGC protein bands stained by the dystrophin fusion proteins was confirmed next, using different antibodies. The high molecular weight DysS8 binding band is dystrophin, and it is also stained by NCL-DYS2, a monoclonal antibody specific for dystrophin's carboxy-terminus (Figure 2). In this and subsequent figures, certain regions of blots are shown to provide greater detail. Any staining observed in regions of the blots not shown is either described here or did not occur.

The 50 kDa band bound by DysS8 is adhalin, and it also binds the anti-adhalin monoclonal antibody (Figure 3A). This band is not stained by the mal−lac negative control, and thus dystrophin's cysteine-rich sequences are responsible for this binding. In addition to this strong interaction with adhalin, weaker interactions with gp43 (β-dystroglycan) and gp35 are also revealed here by using larger amounts of the

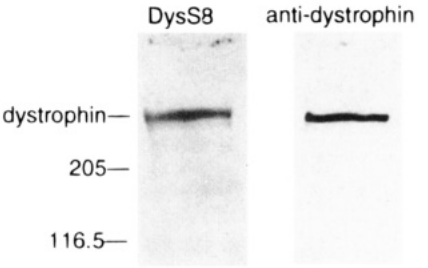

FIGURE 2: DGC dystrophin binds the dystrophin cysteine-rich domain (DysS8). The high molecular mass protein band bound by the DysS8 fusion protein is also bound by a monoclonal antibody (NCL-DYS2) against dystrophin. NCL-DYS2 was used to identify dystrophin, and its binding was detected with HRP-conjugated goat anti-mouse antibodies.

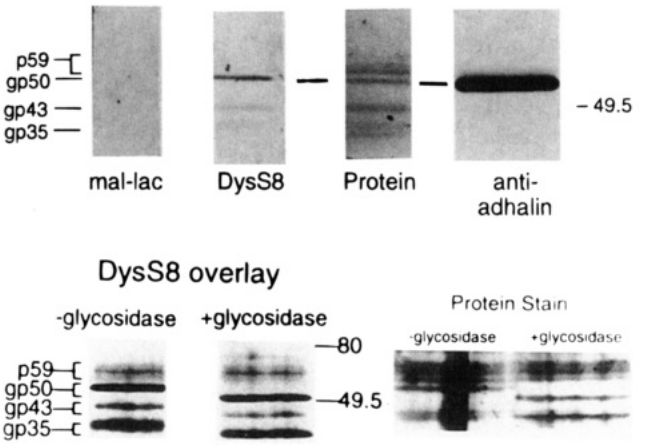

FIGURE 3: Identity of the DGC proteins bound by the dystrophin DysS8 fusion protein. This experiment uses a 2-fold larger amounts of DGC than in Figure 1. (A, top) Overlay binding experiments with DysS8 and mal−lac control fusion proteins (first two lanes), Amido Black total protein staining of a DGC gel blot ("Protein" lane), and a western blot of DGC stained with NCL-50DAG monoclonal antibody ("anti-adhalin" lane) are all shown. (B, bottom) Effect of glycosidase treatment on DGC proteins. A silver stained SDS gel (right-hand side) shows the syntrophin (p59) triplet, adhalin (gp50) and β-dystroglycan (gp43). The position of gp35 is not shown on the protein stain because the glycosidase has a similar molecular weight. The samples were either untreated or treated with N-glycosidase F prior to electrophoresis. On the left-hand side, the untreated and treated samples were blotted onto nitrocellulose and probed with DysS8. Differences in sample preparation and blot staining procedures used here are described in Materials and Methods.

DGC target proteins in the gel blots (see Materials and Methods). Although not very apparent in Figure 3A, the original blot also clearly shows some weak staining of the syntrophin bands by DysS8; this additional staining will be made clearer below.

The possibility remains that binding of DysS8 may be to minor contaminating proteins underlying the gp50, gp43, and gp35 bands. To test this, we carried out glycosidase treatment of our DGC samples by the method of Ervasti and Campbell (1991). Consistent with the observations of these investigators, adhalin (gp50) undergoes a characteristic increase in electrophoretic mobility following glycosidase digestion (Figure 3B). An identical change in migration is shown by the 50 kDa band stained by DysS8. Thus it is to adhalin, and not an underlying contaminant protein, that DysS8 binds. The conditions for increased DGC protein staining by fusion proteins used here (see Materials and Methods) also made the staining of gp35, β-dystroglycan

(gp43), and the syntrophin triplet by DysS8 quite clear (Figure 3B). These results are consistent with those in Figure 3A, but the staining of adhalin by DysS8 in Figure 3B appears only slightly stronger than that of gp35 and β-dystroglycan. This is caused by the last step of blot staining being twice as long (10 min), resulting in saturating levels of color development. At shorter color development times, the predominant staining of adhalin by these dystrophin sequences is clearer (see Figures 1B and 3A) and identifies adhalin as the DGC protein that binds dystrophin sequences in DysS8 best. Following glycosidase digestion, β-dystroglycan (gp43) and gp35 also migrate more rapidly and are seen in front of adhalin, while the migration of the three syntrophin bands (behind adhalin) is unaffected by this treatment (Figure 3B). These results are also consistent with those of Ervasti and Campbell (1991) and demonstrate typical features of the DGC glycoproteins. Since these mobility shifts are due to removal of carbohydrate from these glycoproteins and do not affect DysS8 binding, DysS8 is interacting with the protein component as expected for an interaction envisioned as occurring on the inner face of the sarcolemma.

In summary, we find that the cysteine-rich sequences of dystrophin (DysS8) bind most strongly to adhalin; weaker interactions also occur with β-dystroglycan (gp43), gp35, and the syntrophins. This disagrees somewhat with the conclusions of Suzuki *et al.* (1994), who, using different fusion protein constructs, concluded that this region of dystrophin interacted most strongly with β-dystroglycan. The different experiments carried out in this study show conclusively that adhalin is the protein bound best by DysS8. The binding of β-dystroglycan (and also gp35 and the syntrophins) by DysS8 in our blots occurs, albeit to a lesser extent than adhalin (Figures 1 and 3). A lack of staining by mal−lac controls in all of our experiments confirms that the observed binding is due to the overlayed dystrophin sequences (Figures 1 and 3A and data not shown) and is not an artifact. While the reason for the discrepancy between these results (ours and those of Suzuki *et al.*) is unclear, differences in the DGC purification procedures, fusion protein construction, and the detection methods used could be responsible. The DCT264 fusion used by Suzuki *et al.* (1994) also contains regions other than the cysteine-rich sequence domain of dystrophin present in DysS8, and these additional sequences may alter binding. Differences in the overlay binding assay procedures used may also be important, since the extent to which proteins renature on gel blots may differ. However, perhaps the major difference is that we have used anti-MBP antibodies to detect DysS8 binding while Suzuki *et al.* (1994) used antibodies directed against dystrophin sequences to detect binding. It is possible that these dystrophin antibodies, by binding to dystrophin sequences, alter their binding properties. We have observed such differences in Figure 5 below where a syntrophin antibody detects different staining by syntrophin than when an antibody against non-syntrophin sequences is used (see below).

Two other differences between this report and the results of Suzuki *et al.* (1994) must also be addressed. Dystrophin is probably a dimer (Ervasti & Campbell, 1991), and the association that occurs between dystrophin and DysS8 (Figures 1B and 2) suggests that sequences in DysS8 may serve a role in dimerization. This is an important result. Most current models take the dimerization of dystrophin into account when describing DGC organization. However, specific regions of dystrophin that may be involved in this self-association had not been identified before. Suzuki *et al.* (1994) could not have observed these putative dimerization sequences since they used anti-dystrophin antibodies for detection in their experiments. Secondly, we did not observe staining of the 87 kDa protein described by Suzuki *et al.* (1994) in our experiments. This protein is not a major component of DGC prepared by the method of Ervasti *et al.* (1991) and is not enriched in our DGC preparations (Figure 1B). This could result from the DGC being purified using different procedures by our two groups.

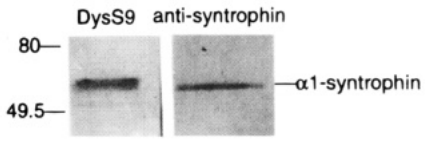

FIGURE 4: DysS9 binds primarily to the syntrophin (p59) triplet. DGC gel blots were either overlayed with the DysS9 fusion protein or with affinity purified polyclonal antibodies against a mouse syntrophin-1 fusion protein (see Materials and Methods). Mouse syntrophin-1 is an α-syntrophin, and the antibody identifies the smallest band of the triplet as rabbit α-syntrophin. DysS9 binds to all three bands in the triplet including the one detected by the antibody. DysS9 contains the last 17 amino acids of dystrophin which is bound by the NCL-DYS2 antibody. Here instead of anti-MBP, NCL-DYS2 antibodies were used to detect DysS9 binding.

Thus, we conclude that the cysteine-rich region of dystrophin serves at least two roles: it binds to dystrophin sequences and may be important to dystrophin dimer formation and it binds most strongly to adhalin. This direct interaction of dystrophin with adhalin could explain the importance of adhalin in maintaining an intact DGC *in vivo*. Roberds *et al.* (1993) showed that the absence of this DGC glycoprotein results in disruption of the complex.

The interaction of the unique carboxy-terminal domain of dystrophin (DysS9) with all three of the syntrophin bands was further characterized. As observed in Figure 4, the DysS9 fusion protein binds to all three of the syntrophin bands, of which the fastest migrating one is also stained by our affinity purified antibodies against the mouse α-syntrophin fusion protein. These results show that both α- and β-syntrophins bind to the carboxy-terminal domain of dystrophin.

To confirm that dystrophin and α-syntrophin bind to each other directly and to understand other DGC interactions, we also carried out overlay experiments using fusion proteins that contained mouse syntrophin-1 (α-syntrophin) sequences and DGC gel blots. Figure 5A shows binding of dystrophin by the α-syntrophin fusion protein and demonstrates a direct interaction between these two proteins. This is consistent with the results in Figures 1B and 4, which showed dystrophin's carboxy-terminal domain sequences (DysS9) binding to all DGC syntrophins. We also find that α-syntrophin binds to gp35 and to a lesser extent adhalin (gp50). Syntrophin fusion binding was detected in this experiment using anti-syntrophin antibodies. The antibodies bind none of the DGC proteins other than α-syntrophin when the syntrophin fusion protein is absent from the probing solution ("−syntrophin" lane, Figure 5A). In this figure both blots (in the presence and absence of syntrophin fusion protein) were part of the same experiment, treated in parallel, and stained for the same amount of time. The staining of α-syntrophin by its antibody is diminished when the blot is

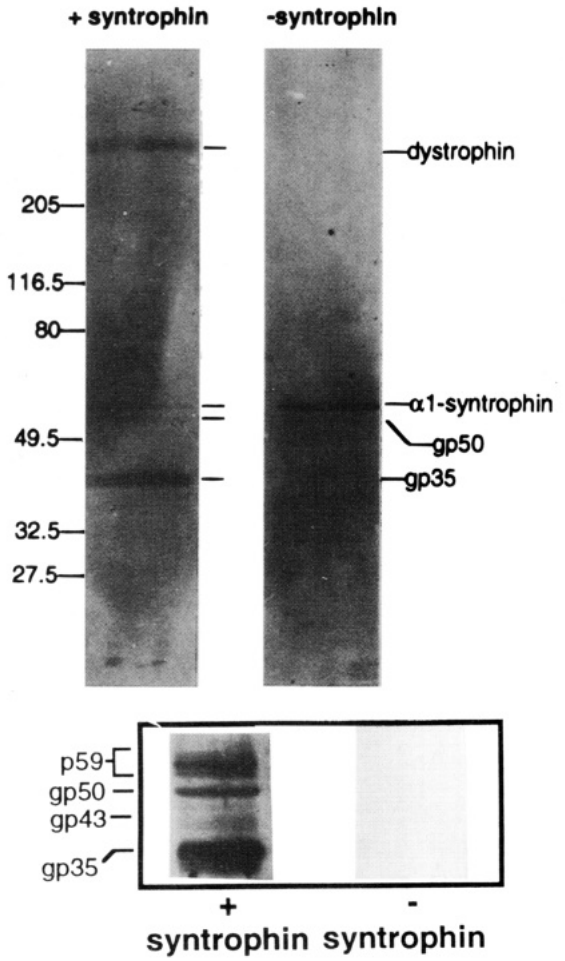

FIGURE 5: DGC syntrophin triplet is bound by α-syntrophin. (A, top) Fusion proteins containing cloned mouse syntrophin-1 (α-syntrophin) sequences bind dystrophin, gp35, and to adhalin (gp50). DGC blots were incubated with or without the syntrophin fusion protein and probed with affinity purified polyclonal anti-syntrophin antibodies (see Materials and Methods). (B, botom) The syntrophin triplet in the DGC is bound by the α-syntrophin fusion protein. The blots were incubated with or without the syntrophin fusion protein, and detection was using the anti-T7 monoclonal antibody (see Materials and Methods) and HRP-conjugated goat anti-mouse secondary antibodies. The syntrophin triplet is labeled as "p59".

exposed to the fusion protein ("+syntrophin" lane). This result was obtained consistently and probably results from some combination of the following: (1) the anti-syntrophin antibodies being raised against the nondenatured mouse α-syntrophin fusion protein, which causes them to bind preferentially the overlayed fusion protein rather than the SDS-denatured rabbit DGC α-syntrophin. Mouse syntrophin-1 is 92% identical to rabbit α-syntrophin (p59A) (Yang et al., 1994); however, these slight differences could also account for some of the differences in the antibody staining discussed. (2) The antibodies may also preferentially bind to epitopes on α-syntrophin which are more exposed when syntrophin is bound by some of its target proteins as in the overlay lane, and, finally, (3) the limited amount of anti-syntrophin antibodies (used to avoid nonspecific staining) binding to multiple targets in the syntrophin fusion overlay case. Thus, α-syntrophin binds dystrophin [in agreement with the work of Kramarcy *et al.* (1994) but in contrast to the results of Suzuki *et al.* (1994)] and also to gp35, adhalin, and the syntrophin triplet which were previously unknown (Tinsley *et al.*, 1994) and potentially important interactions.

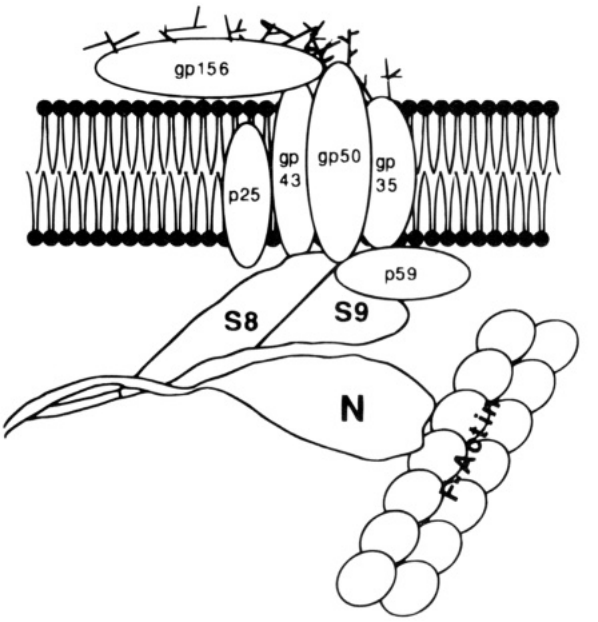

FIGURE 6: Model for DGC organization. Existing models (Ervasti & Campbell, 1991; Suzuki *et al.*, 1994) were refined to be consistent with the data presented in this report. DysS1, DysS8, and DysS9 regions of dystrophin are labeled as "N", "S8", and "S9", respectively.

The original blot for Figure 5A shows staining of all three DGC syntrophin bands, but this staining was partially masked by the staining of the DGC α-syntrophin by the anti-syntrophin antibodies. To test this further and learn if the anti-syntrophin antibody was influencing the result, we used another feature of the pET 28a-derived syntrophin fusion protein. Cloning in this vector results in a fusion containing the T7 antigen sequence at the amine terminus of the fusion protein. Again, DGC gel blots were overlayed (or not) with the α-syntrophin fusion protein (Figure 5B) but now stained using the anti-T7 monoclonal antibody (see Materials and Methods). The results confirm that the α-syntrophin fusion protein binds to all three DGC syntrophin bands. The staining of gp35 and adhalin by the α-syntrophin fusion is also apparent in this figure (Figure 5B). Consistent with the results in Figure 5A, α-syntrophin binding to full length dystrophin was also observed on the blots used for Figure 5B (data not shown). While the same proteins bind α-syntrophin in Figure 5, panels A and B, the intensity of stained bands differs somewhat, showing that using an antibody against syntrophin sequences affected the intensity of staining of the target bands. For this reason, the best approach in such fusion overlay experiments may be to use fused sequences rather than putative binding sequences for detection.

Several new interactions of dystrophin and syntrophin with DGC proteins have been identified here. While some of these are in contrast with previously accepted DGC interactions (discussed above), others have been recently confirmed by the investigations carried out in other laboratories (see below). Using the findings described in this report and building upon earlier models (Ervasti & Campbell, 1991; Suzuki *et al.*, 1994), we present a model for DGC organization in Figure 6. In the model (showing one end of the dystrophin antiparallel dimer, lower left) a direct interaction between dystrophin sequences (DysS8 and DysS9) and adhalin (see Figures 1 and 3) is depicted. Other DGC transmembrane glycoproteins (Ervasti & Campbell, 1991),

namely, gp43 (β-dystroglycan) and gp35, are shown binding dystrophin (DysS8) sequences (Figure 3) and are probably required for maintaining an intact complex. The syntrophins are shown collectively as a single "p59" component to signify their self-association (Figure 5A,B) and also since the differences in binding amongst the three syntrophins is presently poorly characterized. Whether the β-syntrophins bind to any DGC proteins other than dystrophin (and now, α-syntrophin) is not known, and hence in our DGC model (Figure 6) the syntrophins are shown as a complex (p59). The binding of p59 to dystrophin, adhalin, and gp35 (Figure 5) as well as dystrophin's self-association (Figures 1B and 2) are all included in this model. These data add significantly to our previous understanding of interactions within the DGC [see Tinsley *et al.* (1994)].

Some of the results presented here were independently demonstrated by other investigators while this paper was under editorial review and are relevant to the DGC model (Figure 6) presented here. The binding of β1-syntrophin to an alternatively spliced exon of dystrophin (located in our DysS9 fusion) was shown by Ahn and Kunkel (1995). Suzuki *et al.* (1995) have obtained new data demonstrating that both α- and β-syntrophins bind to the carboxy-terminal domain of dystrophin as shown here, but in contrast with their earlier report (Suzuki *et al.*, 1994) where association of dystrophin with only the β-syntrophins was shown. Interestingly, also observable in the newer report of Suzuki *et al.* (1995) is the faint staining by dystrophin fusion protein DCT264 of an approximately 50 kDa band (probably adhalin).

A report by Yang *et al.* (1995), which also appeared while this paper was under review, examines rabbit α-syntrophin binding to DGC proteins. They found dystrophin and all the syntrophin bands bind α-syntrophin, much as demonstrated here. These investigators (Yang *et al.*, 1995), however, did not detect the binding of gp35 using their *in vitro* translated ^{35}S-labeled α-syntrophin. Their results also showed the staining of a band which appears to have same M_r as adhalin, but this was not discussed. Rabbit and mouse α-syntrophins are 92% identical, but the small difference may be capable of altering the results with gp35 binding. More likely, however, are differences due to the DGC probing procedure. Yang *et al.* (1995) discuss how the low concentration of *in vitro* translated proteins used may have caused the failure to detect other protein binding (between dystrophin and β-dystroglycan). Similar reasons may explain why Yang *et al.* (1995) did not observe some of the interactions reported here. As described in Materials and Methods, we use 0.1 mg/mL syntrophin fusion protein in our probing solutions, and it is possible that gp35 staining may be strongly influenced by syntrophin concentrations.

The DGC model that we have presented is consistent with our results and also with results obtained recently by other investigators. This model describes the currently known interactions within the DGC. The trans membrane components of the DGC except p25 all interact directly (albeit with differences in their binding) with peripheral dystrophin. α-Syntrophin binds not only to dystrophin and the syntrophins but also to two of the DGC glycoproteins, adhalin, and gp35. Dystrophin binds F-actin, and the transmembrane DGC proteins interact with α-dystroglycan, which binds to the extracellular matrix protein laminin. The DGC thus links the actin cytoskeleton to the extracellular matrix (Ervasti & Campbell, 1993), which is important in maintaining the integrity of nondystrophic muscle (Campbell, 1995). Since signal transducing mechanisms, namely, calmodulin binding [Madhavan *et al.*, 1992; Bonet-Kerrache *et al.*, 1994; Jarrett & Foster, 1995; this has been disputed (Ervasti & Campbell, 1993)] and protein phosphorylation (Milner *et al.*, 1993; Madhavan & Jarrett, 1994, and references therein), operate on DGC proteins, some of these DGC interactions could potentially be regulated.

ACKNOWLEDGMENT

We are grateful to Drs. Marvin Adams and Stanley Froehner for the mouse syntrophin-1 cDNA. We also thank Elizabeth Brown Helms, R. Preston Rogers, and J. Todd Anderson for excellent technical assistance.

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BI9502405