The Small Leucine-rich Repeat Proteoglycan Biglycan Binds to α-Dystroglycan and Is Upregulated in Dystrophic Muscle

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Abstract. The dystrophin-associated protein complex (DAPC) is necessary for maintaining the integrity of the muscle cell plasma membrane and may also play a role in coordinating signaling events at the cell surface. The α-/β-dystroglycan subcomplex of the DAPC forms a critical link between the cytoskeleton and the extracellular matrix. A ligand blot overlay assay was used to search for novel dystroglycan binding partners in postsynaptic membranes from *Torpedo* electric organ. An ~125-kD dystroglycan-binding polypeptide was purified and shown by peptide microsequencing to be the *Torpedo* ortholog of the small leucine-rich repeat chondroitin sulfate proteoglycan biglycan. Biglycan binding to α-dystroglycan was confirmed by coimmunoprecipitation with both native and recombinant α-dystroglycan. The biglycan binding site was mapped to the COOH-terminal third of α-dystroglycan. Glycosylation of α-dystroglycan is not necessary for this interaction, but binding is dependent upon the chondroitin sulfate side chains of biglycan. In muscle, biglycan is detected at both synaptic and nonsynaptic regions. Finally, biglycan expression is elevated in muscle from the dystrophic *mdx* mouse. These findings reveal a novel binding partner for α-dystroglycan and demonstrate a novel avenue for interaction of the DAPC and the extracellular matrix. These results also raise the possibility of a role for biglycan in the pathogenesis, and perhaps the treatment, of muscular dystrophy.

Key words: muscular dystrophy • dystrophin-associated protein complex • chondroitin sulfate proteoglycan • agrin • neuromuscular junction

Introduction

The dystrophin-associated protein complex (DAPC)[1] links the cytoskeleton to the extracellular matrix (ECM) and is necessary for maintaining the integrity of the muscle cell plasma membrane. The core DAPC consists of the cytoskeletal scaffolding molecule dystrophin and the dystroglycan and sarcoglycan transmembrane subcomplexes. The DAPC also serves to localize key signaling molecules to the cell surface, at least in part through its associated syntrophins (Brenman et al., 1996; Bredt, 1998). Mutations in either dystrophin or any of the sarcoglycans result in muscular dystrophies characterized by breakdown of the muscle cell membrane, loss of myofibers, and fibrosis (Hoffman et al., 1987; Straub and Campbell, 1997). Moreover, mutations in the ECM protein laminin-α 2, which associates with the DAPC on the cell surface, are the basis of a major congenital muscular dystrophy (Helbling-Leclerc et al., 1995).

The α-/β-dystroglycan subcomplex forms a critical structural link in the DAPC. The transmembrane β-dystroglycan and the wholly extracellular α-dystroglycan arise by proteolytic cleavage of a common precursor. The cytoplasmic tail of β-dystroglycan binds dystrophin, while the highly glycosylated, mucin-like α-dystroglycan binds to several ECM elements, including agrin, laminin, and perlecan (Ervasti and Campbell, 1993; Bowe et al., 1994; Gee et al., 1994; Hemler, 1999). This binding to matrix proteins appears to be essential for assembly of basal lamina, since mice deficient in dystroglycan fail to form these structures and die very early in development (Henry and Campbell, 1998). β-Dystroglycan can bind the signaling adapter molecule Grb2 and associates indirectly with p125FAK (Yang et al., 1995; Cavaldesi et al., 1999). Although the significance of these associations remains unknown, these binding properties suggest that dystroglycan may also serve to localize signaling molecules to the cell surface.

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[1]*Abbreviations used in this paper:* aa, amino acid; AChR, acetylcholine receptor; DAG-125, dystroglycan-associated glycoprotein of 125 kD; DAPC, dystrophin-associated protein complex; ECM, extracellular matrix; GST, glutathione S-transferase; SLRP, small leucine-rich repeat proteoglycan.

Several lines of evidence suggest that dystroglycan may also function in postsynaptic differentiation. α-Dystroglycan binds the synapse organizing molecule agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; O'Toole et al., 1996; reviewed in Fallon and Hall, 1994), and β-dystroglycan binds to the acetylcholine receptor (AChR)-associated protein, rapsyn (Cartaud et al., 1998). Further, agrin-induced AChR clustering is markedly decreased in muscle cells expressing reduced levels of dystroglycan (Montanaro et al., 1998). The precise role of dystroglycan in this process is unknown. Currently available evidence suggests that dystroglycan is not part of the primary agrin receptor, but rather may play a structural role in the organization of postsynaptic specializations (Gesemann et al., 1995; Glass et al., 1996; Jacobson et al., 1998).

The realm of dystroglycan function ranges far beyond muscle. As noted above, mice defective in dystroglycan die long before muscle differentiation. In a surprising development, α-dystroglycan in nonmuscle cells has been shown to function as a receptor for Lassa Fever and choriomeningitis fever viruses (Cao et al., 1998), and on Schwann cells, as a coreceptor for *Mycobacterium leprae* (Rambukkana et al., 1998). Dystroglycan is also abundant in brain, but its function there is not understood (Gorecki et al., 1994; Smalheiser and Kim, 1995).

α-Dystroglycan is comprised of three known domains. An NH_2-terminal domain folds into an autonomous globular configuration (Brancaccio et al., 1995). The middle third of the protein is serine- and threonine-rich and is highly glycosylated (Brancaccio et al., 1997). Indeed, the core molecular weight of α-dystroglycan is ~68 kD, but the native molecule migrates on SDS-PAGE as a polydisperse band whose size ranges from 120–190 kD, depending upon the species and tissue source (Ervasti and Campbell, 1993; Bowe et al., 1994; Gee et al., 1994; Matsumura et al., 1997). Glycosylation of α-dystroglycan, probably in this middle third, is essential for its laminin- and agrin-binding properties. Until the present report, there have been no known structural motifs nor functions associated with the COOH-terminal third of the molecule.

While it is clear that dystroglycan and the DAPC play crucial roles in a variety of processes in muscle, as well as in other tissues, the underlying mechanisms remain obscure. One essential step towards elucidating these functions is to identify and characterize novel dystroglycan-binding molecules. We therefore developed a ligand blot overlay assay to search for such binding partners in synaptic membranes from *Torpedo* electric organ. We purified one dystroglycan-binding molecule and identified it as the small leucine-rich repeat chondroitin sulfate proteoglycan biglycan. We have mapped the binding site to the COOH-terminal third of α-dystroglycan. Although glycosylation of α-dystroglycan is not necessary for this interaction, binding is dependent on the biglycan chondroitin sulfate side chains. In muscle, biglycan is detected at both synaptic and nonsynaptic regions. Finally, we provide evidence that biglycan expression is elevated in the dystrophic *mdx* mouse. These findings thus reveal a novel binding partner for α-dystroglycan, and raise the possibility of a role for biglycan in muscular dystrophy.

Materials and Methods

Membrane Preparation and Solubilization of DAG-125

Postsynaptic and nonsynaptic membrane fractions were prepared by sucrose density centrifugation from *Torpedo* electric organ as previously described (Bowe et al., 1994). All handling of membranes and protein was performed at 4°C. To solubilize DAG-125 (dystroglycan-associated glycoprotein, 125 kD), synaptic membranes were centrifuged at 100,000 *g* for 1 h and resuspended in ddH_2O. The pH was adjusted to 11.0 or 12.0 (as indicated) with NaOH, and the membranes stirred for 1 h. Insoluble material was removed by centrifugation at 100,000 *g* for 1 h. The alkaline extract was neutralized with 10 mM Tris-HCl and adjusted to pH 7.4. DAG-125 remained soluble under these conditions, as determined by resistance to pelleting during a second centrifugation.

In Vitro Transcription/Translation

The in vitro expression plasmids encoding $DG_{1\text{-}891}$ and $DG_{345\text{-}891}$ (human dystroglycan sequence) in the in vitro expression vector, pMGT, developed by A. Ahn (Ahn and Kunkel, 1995), were generously provided by Lou Kunkel (Children's Hospital, Boston, MA). Additional in vitro expression plasmids used in this study were prepared by PCR-based subcloning of these inserts. The PCR primers included restriction sites for religation into the EcoRI site of pMGT. Dystroglycan protein fragments were generated by in vitro transcription/translation using the Promega TNT T7 coupled reticulocyte system as per the manufacturer's instructions. For protein to be used in ligand blot overlay assay, the reaction mixture contained [^{35}S]methionine (with no unlabeled methionine). After incubation for 2 h, the reaction mixture was passed over Bio-Spin desalting columns (BioRad) to remove salts and unincorporated amino acids.

Ligand Blot Overlay Assays

Membrane proteins were separated by SDS-PAGE (5–15% gradient gel) and transferred to nitrocellulose. To detect dystroglycan-binding proteins, the nitrocellulose was rinsed and blocked for 3 h in HBSS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA, 1% nonfat dry milk, 1 mM DTT, 10 mM Hepes, pH 7.4, and was then incubated overnight in the same buffer containing [^{35}S]methionine-labeled dystroglycan fragments produced by in vitro transcription/translation (see above). Blots were rinsed and dried, and bound dystroglycan fragments were visualized by autoradiography. To detect dystroglycan present in the SDS-PAGE sample, an agrin blot overlay assay was performed essentially as described in O'Toole et al. (1996). In brief, the nitrocellulose was rinsed and blocked for 3 h in Hepes-buffered MEM supplemented with 1% BSA and 10% horse serum. It was then incubated for 4 h in this buffer containing recombinant rat agrin (isoform A_0B_0, prepared as described in O'Toole et al., 1996), followed by a second layer containing 1 μg/ml antiagrin antibody ^{125}I-Mab-131 (Stressgen Laboratories). Bound antiagrin antibody was visualized by autoradiography.

Recombinant Dystroglycan from Bacteria

A fusion protein of glutathione S-transferase (GST) and amino acids (aa) 345–653 of dystroglycan was produced by using PCR-based subcloning to introduce dystroglycan coding sequence into the bacterial protein expression vector, pGEX-1λT (Pharmacia Biotech). The resulting bacterial expression plasmid, pGST-$DG_{345\text{-}653}$, was then introduced into the *Escherichia coli* strain BL21 and expressed fusion protein recovered from the cytoplasmic fraction as per manufacturer's instructions. Control protein (GST) was obtained using pGEX-1λT.

Purification and Identification of DAG-125 (Biglycan)

Postsynaptic-rich membrane fractions were first preextracted with 25 mM *N*-octyl-β-D-glucopyranoside to remove detergent-soluble proteins. The bulk of α- and β-dystroglycan is solubilized in this mild detergent (Bowe et al., 1994). DAG-125 was then solubilized by alkaline extraction (pH 12.0). The alkaline extract was diluted in SEN buffer (20 mM Tris-HCl, 100 mM NaCl, 23 μg/ml aprotinin, 0.5 μg/ml leupeptin, 5 mM benzamidine, 0.7 μg/ml pepstatin A, 1 mM phenylmethylsulfonylflouride, 0.02% azide, and 0.1% Tween 20, pH 7.6) and recentrifuged to remove any proteins precipitating upon neutralization. The extract remained in SEN buffer for the remainder of the purification, with only the NaCl concentra-

tion changed as indicated. The extract was passed over a mAb3B3 column (Bowe et al., 1994) to remove the small amount of residual, detergent-insoluble α-dystroglycan. Further analysis of these fractions revealed that this α-dystroglycan was bound to biglycan (see Fig. 2 c); however, since there was relatively little α-dystroglycan in the alkaline extract, the majority of the biglycan flowed through the mAb3B3 column. The flow-through was passed over a combined, non-DAG-125–binding lectin–agarose column (peanut agglutinin and ulex europaeus agglutinin I; Vector Laboratories, Inc.) as a second preclear. The flow-through was next applied to a column of chondroitin sulfate-agarose. The chondroitin sulfate-agarose column was prepared by coupling chondroitin sulfate B (Sigma Chemical Co.; #C-3788) to ω-aminohexyl-agarose (Sigma Chemical Co.) activated with N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (Sigma Chemical Co.). After incubation with the lectin column flow-through, the chondroitin sulfate column was washed extensively and eluted with a 0.1–2.0 M NaCl gradient. DAG-125 eluted in 0.3–0.65 M NaCl. These fractions were pooled, diluted to 0.3 M NaCl, and applied to a heparin-agarose column (Sigma Chemical Co.; #H-0402). The column was washed and eluted with a 0.3–2.0 M NaCl gradient. DAG-125 eluted in 0.6–0.85 M NaCl. These fractions were pooled, concentrated by ethanol precipitation, redissolved in SDS-PAGE sample buffer, separated on a 5–15% gradient gel, and transferred to a PVDF membrane. A portion of the PVDF membrane was analyzed for DAG-125 by blot overlay and the remainder was transiently stained with Ponceau S. Regions of the blot bearing DAG-125 were subjected to tryptic digestion, HPLC analysis, and peptide microsequencing as described previously (Bowe et al., 1994).

Enzymatic Digestions

Enzyme treatments were carried out on alkaline-extracted *Torpedo* electric organ synaptic membrane proteins at 37°C overnight. Enzymes, final concentration, supplier, and catalog numbers are listed in Table I. All reactions were performed in the protease inhibitors present in SEN buffer, with the addition of 1 mM EDTA, 10 mM N-ethylmaleimide, and 0.8% mouse serum albumin. Chondroitinases (all forms) were buffered with 100 mM Tris-acetate (pH 8.0). Hyaluronidase and keratanase were buffered with 50 mM sodium acetate (pH 5.0). Heparinases (I, II, and III), chondro-4-sulfatase, and chondro-6-sulfatase were buffered with 10 mM $NaPO_4$ (pH 7.4). N-Glycanase, O-glycanase, neuraminidase, α-N-acetylgalactosaminidase, and β-N-acetylglucosaminidase, were buffered with 50 mM Tris-HCl (pH 7.3). Control treatments included buffers and protease inhibitors without added enzymes.

Recombinant Biglycan

P16, a cloning plasmid consisting of pBluescript containing cDNA encoding human biglycan, was kindly provided by Larry Fisher (National Institute of Dental Research, National Institutes of Health, Bethesda, MD; Fisher et al., 1989). The sequence coding for the mature secreted protein (aa 38–368) was amplified by PCR and subcloned into the bacterial expression vector pQE9 (Qiagen). The resulting plasmid, pQE-biglycan, adds the sequence MRGSHHHHHHGS to the NH_2 terminus. Recombinant protein was produced in *E. coli* strain M15[pREP4]. Uninduced bacteria provided control protein. Induced or noninduced bacteria were isolated by centrifugation and resuspended in SDS-PAGE sample buffer for analysis by ligand blot overlay.

Histology

Cryostat sections (10 μm) of leg muscle from fresh-frozen wild-type (C57 BL) and *mdx* mice were mounted on the same slide and fixed and treated with chondroitinase, essentially as described in Bianco et al. (1990). Primary antibodies were antibiglycan (LF-106; generously provided by L. Fisher) diluted in PBS containing 5% BSA, 1% normal goat or horse serum, and 0.1% Triton X-100. Incubation in primary antibodies or nonimmune control serum proceeded overnight at 4°C. Except where noted, all subsequent steps were performed at room temperature. Bound antibodies were detected with Cy3-labeled anti-rabbit Ig (Jackson Laboratories). For double-labeling, sections were first fixed for 5 min in 1% formaldehyde, rinsed, and incubated in fluorescein-conjugated α-bungarotoxin (Molecular Probes, Inc.) for 1 h. The sections were then washed, fixed, treated with chondroitinase, and stained for biglycan as described above. Sections were air-dried, mounted in Citifluor (Ted Pella, Redding, CA) and examined on a Nikon Eclipse microscope. Images were acquired on a cooled CCD camera using IPLab Spectrum software, and then imported to Adobe Photoshop. Images from wild-type and *mdx* muscle were acquired and processed for printing under identical conditions.

Results

Characterization of a Dystroglycan-binding Protein, DAG-125

To identify novel dystroglycan-binding partners, we developed a ligand blot overlay assay. *Torpedo* electric organ membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose. The blots were then probed with in vitro translated, [^{35}S]methionine-labeled dystroglycan fragments. α- and β-dystroglycan are encoded by a single polypeptide that is cleaved posttranslationally to yield the mature polypeptides (Fig. 1; Ibraghimov et al., 1992; Bowe et al., 1994). Dystroglycan fragments that included the α-dystroglycan region bound to an ~125-kD, highly glycosylated, polypeptide (Fig. 1) to which we gave the working name DAG-125. Further, DAG-125 is enriched in postsynaptic membrane fractions from *Torpedo* electric organ compared with nonsynaptic membranes.

Coprecipitation of α-Dystroglycan and DAG-125

We confirmed the association between α-dystroglycan and DAG-125 using solution binding. Fig. 2 shows that DAG-125 coprecipitates with both in vitro translated dystroglycan and bacterially produced GST–dystroglycan fusion protein. Finally, we asked if DAG-125 binds to native dystroglycan. Alkaline extracts of *Torpedo* electric organ membranes contain both DAG-125 and α-dystroglycan. We applied this extract to agarose columns conjugated to either control antibody or to an anti-*Torpedo* dystroglycan mAb (mAb3B3; Bowe et al., 1994). DAG-125 was specifically coprecipitated with native α-dystroglycan (Fig. 2 c). Thus, the binding of DAG-125 to α-dystroglycan observed by ligand blot overlay was confirmed using three different coprecipitation methods. Moreover, an α-dystroglycan–biglycan complex can be immunoprecipitated from extracts of synaptic membranes.

Determination of the DAG-125–binding Domain of α-Dystroglycan

We next sought to determine the region of dystroglycan that mediates binding to DAG-125. We generated a panel of dystroglycan fragments by in vitro translation (Fig. 1 b) and tested the ability of each to bind DAG-125 using the ligand blot overlay assay. The COOH-terminal one-third of α-dystroglycan is sufficient to bind DAG-125. Weak binding was observed to a fragment comprised of the middle third of the molecule, suggesting that a contribution from this region is also possible. The ectodomain of β-dystroglycan did not bind to DAG-125. Moreover, these fragments were produced under conditions where the polypeptides are not glycosylated. Therefore, carbohydrate side chains on dystroglycan are not necessary for its binding to DAG-125. We conclude that the major binding domain is contained in ~150-aa region of dystroglycan. The location of this domain and the lack of a carbohydrate requirement indicate that α-dystroglycan's binding site for biglycan is distinct from that mediating association with agrin, laminin, and perlecan (see Discussion).

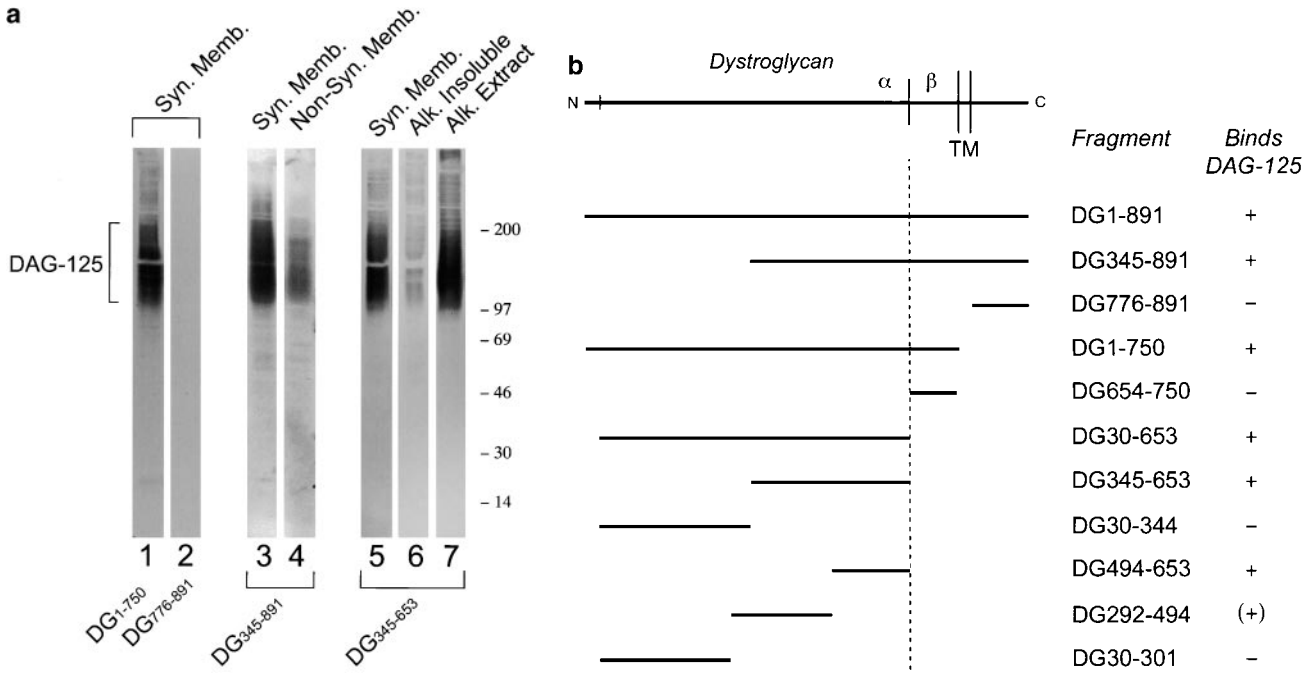

Figure 1. a, Initial characterization of DAG-125. In vitro translated dystroglycan fragments were used to probe the indicated membrane fractions from *Torpedo* electric organ. The subscripts indicate the amino acid numbering for the human sequence (see b for the domain structure of the dystroglycans and the position of these fragments). Lanes 1 and 2, A polydisperse membrane protein (DAG-125) binds to an extracellular portion of dystroglycan. The extracellular domain of dystroglycan (lane 1, $DG_{1\text{-}750}$) bound to DAG-125, whereas the intracellular portion of dystroglycan (lane 2, $DG_{776\text{-}891}$) did not. The region of negativity in the center of the polydisperse DAG-125 band is due to a high abundance, nonbinding protein that does not copurify with DAG-125 (see lanes 5–7). Lanes 3 and 4, DAG-125 is enriched in synaptic as compared with nonsynaptic membranes. Equivalent amounts of protein from each membrane fraction were loaded in both lanes. Lanes 5–7, DAG-125 can be extracted from the membrane by alkaline treatment. Synaptic membranes were extracted at pH 12 and the insoluble (lane 6) and soluble (lane 7) fractions were analyzed. Greater than 90% of DAG-125 is solubilized by pH 12.0 treatment. b, Mapping of the DAG-125–binding region of dystroglycan. A schematic diagram of the in vitro translated recombinant dystroglycan fragments used to probe DAG-125 by blot overlay is shown. The COOH-terminal one-third of α-dystroglycan binds DAG-125. A small contribution from the middle third of α-dystroglycan is also possible. β-Dystroglycan does not appear to contribute to binding of DAG-125.

Identification of DAG-125 as Biglycan

We next identified DAG-125. Although DAG-125 copurified with postsynaptic membranes, it was insoluble in all nonionic detergents tested, including Triton X-100 and *N*-octyl-β-D-glucopyranoside, both of which efficiently extract α-/β-dystroglycan from these membranes (Bowe et al., 1994; Deyst et al., 1995). We determined that even without detergent, ~50% of DAG-125 could be extracted at pH 11, and near-complete solubilization was achieved by a short pH 12 treatment (see Fig 1 a). Importantly, DAG-125 remained soluble when returned to neutral pH. We developed a purification protocol (see Materials and Methods) based upon these properties and the findings that DAG-125 binds to both heparin and chondroitin sulfate columns (data not shown). We estimated the final purity of DAG-125 to be ~30%. This material was separated by SDS-PAGE, blotted to PVDF, and two regions of the DAG-125 band were excised and digested with trypsin. HPLC analysis showed that the two regions (Fig. 3 a, see U and L) had identical peptide maps (data not shown). This finding established the purity of the DAG-125 in these regions and also indicated that the polydisperse DAG-125 band arises from the heterogeneous glycosylation of a common polypeptide core. We then sequenced three tryptic peptides and found that all were highly homologous to mammalian biglycan, with an overall 76% identity (Fig. 3 b). We thus conclude that DAG-125 is a *Torpedo* orthologue of mammalian biglycan.

The Role of Biglycan Chondroitin Sulfate Chains in Binding to α-Dystroglycan

Mammalian biglycan is often substituted with chondroitin sulfate. We therefore asked if *Torpedo* biglycan is also a chondroitin sulfate proteoglycan, and whether glycosylation is important for its binding to α-dystroglycan. We digested DAG-125 with various glycosidases and glycosaminoglycanases and analyzed the products by α-dystroglycan ligand blot overlay (Fig. 4; Table I). Removal of chondroitin sulfate side chains abolished the binding to α-dystroglycan. Chondroitinase B (specific for dermatan sulfate) had a much smaller effect compared with chondroitinases whose activity included chondroitin sulfate A and C. No other glycosidase or glycosaminoglycanase treatment had a detectable effect on α-dystroglycan binding (Table

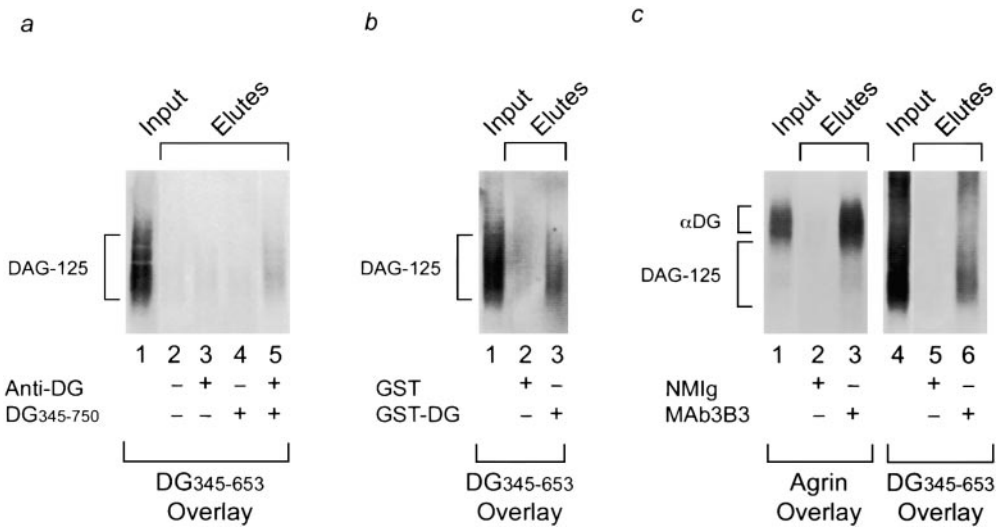

Figure 2. Coprecipitation of DAG-125 and dystroglycan (αDG). DAG-125 was solubilized by alkaline treatment, neutralized, and incubated with column matrices and recombinant or native dystroglycan as indicated. The input material and eluates from the beads were analyzed by ligand blot overlay assay for the presence of DAG-125 (^{35}S-DG345-653 as probe) or native α-dystroglycan (agrin overlay, see Materials and Methods). a, Coimmunoprecipitation of DAG-125 and in vitro synthesized dystroglycan. DAG-125 was incubated with goat anti–mouse Ig-conjugated agarose beads in the presence or absence of in vitro translated dystroglycan polypeptide ($DG_{345-750}$) and/or antidystroglycan mAb (NCL-β-DG; Novocastra). DAG-125 coprecipitated with dystroglycan plus antidystroglycan antibody (lane 5), but was not precipitated in the absence of either or both (lanes 2–4). b, Coaffinity precipitation of DAG-125 and bacterially produced dystroglycan. DAG-125 was incubated with glutathione Sepharose beads that had been preincubated with either bacterially produced GST or a bacterially produced GST–dystroglycan fusion protein ($GST–DG_{345-653}$). DAG-125 was coprecipitated with the dystroglycan fusion protein (lane 3), but not with GST alone (lane 2). c, Coimmunoprecipitation of DAG-125 and native α-dystroglycan. DAG-125 and α-dystroglycan were solubilized by alkaline treatment, and incubated with agarose beads conjugated to either normal mouse Ig (NMIg) or anti-*Torpedo* dystroglycan mAb, mAb3B3. Native α-dystroglycan and DAG-125 were coprecipitated by the antidystroglycan antibody (lanes 3 and 6), but not by control antibody (lanes 2 and 5). Western blots indicate that mAb3B3 does not recognize DAG-125 (data not shown; see Bowe et al., 1994).

I). Several lines of evidence indicate that the effects of chondroitinase digestion are due to chondroitinase activity and not to contaminating proteases: the digestions were performed in a cocktail of protease inhibitors (see Materials and Methods); the same result was seen with four different preparations of chondroitinase, including two which had been affinity-purified to remove proteases; and the effect was prevented by addition of 5 mM Zn^{2+}, an inhibitor of chondroitinase, but not of proteases. We conclude that biglycan from *Torpedo* synaptic membranes is substituted with chondroitin sulfate chains, which are predominantly chondroitin sulfate A and/or C. Finally, chondroitin sulfate substitution of biglycan is necessary for binding to dystroglycan.

Table I. The Role of Biglycan Glycosylation in Binding to α-Dystroglycan

Enzyme	Inhibits binding?	Enzyme concentration	Source	Catalog #
		U/ml		
Chondroitinase ABC	+	0.5	Sigma Chemical Co.	C-2905
Chondroitinase ABC +5 mM $ZnCl_2$	−	0.5	Sigma Chemical Co.	C-2905
Chondroitinase ABC, protease-free	+	0.5	Sigma Chemical Co.	C-3667
Chondroitinase ABC, protease-free	+	0.5	Roche	1080717
Chondroitinase AC	+	0.5	Sigma Chemical Co.	C-2780
Chondroitinase B	±	25	Sigma Chemical Co.	C-8058
Heparinase I	−	25	Sigma Chemical Co	H-2519
Heparinase II	−	5	Sigma Chemical Co	H-3812
Heparinase III (heparitinase)	−	5	Sigma Chemical Co.	H-8891
Chondro-4-sulfatase	±	0.5	Sigma Chemical Co.	C-2655
Chondro-6-sulfatase	−	0.5	Sigma Chemical Co	C-2655
Keratanase	−	0.02	Roche	982954
α-N-acetylgalactosaminidase	−	2	Sigma Chemical Co	A-9763
β-N-acetylglucosaminidase	−	8	Sigma Chemical Co	A-2264
N-Glycanase	−	15	Genzyme Corp.	N-Gly-1
O-Glycanase	−	0.03	Genzyme Corp.	B2950
Neuraminidase	−	1	Genzyme Corp.	NSS-1

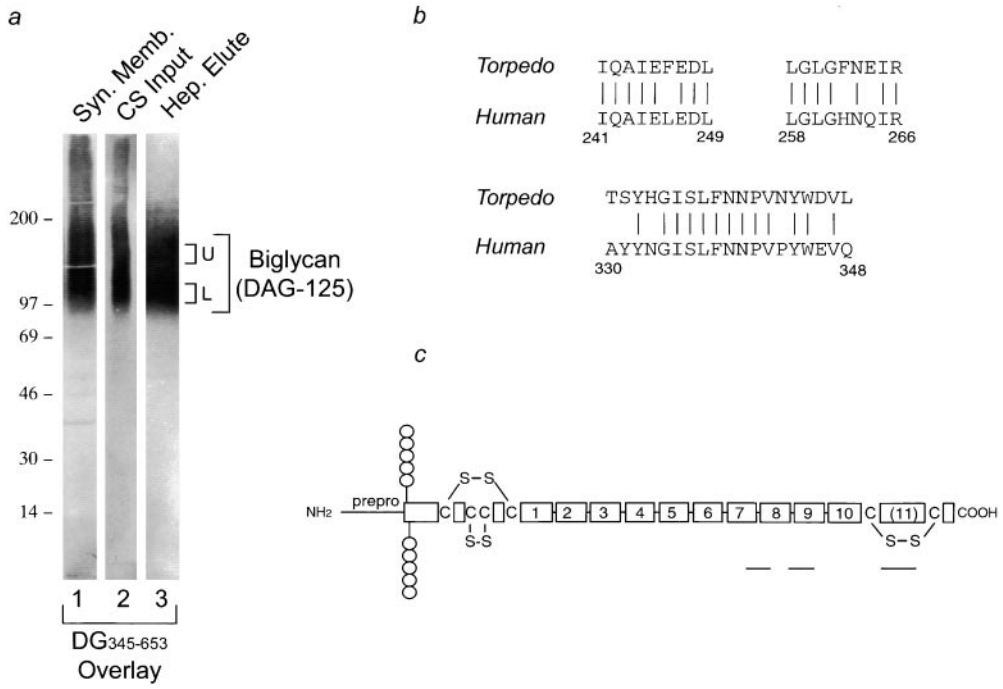

Figure 3. Purification of DAG-125 and its identification as biglycan. a, Blot overlay analysis of DAG-125 in selected purification steps. DAG-125 was purified using biochemical and affinity methods before performing peptide microsequencing. Represented are the initial (synaptic membrane protein, lane 1), middle (input to chondroitin sulfate column, lane 2), and final (concentrated eluate from heparin sulfate column, lane 3) steps of purification. See Materials and Methods for details of the purification scheme. The final purified product was subjected to SDS-PAGE and blotted to PVDF. An upper (U) and a lower (L) region of the Ponceau-stained membrane was excised and digested with trypsin. The released peptides were analyzed by HPLC using a C8 column and UV detection. The column profiles were virtually identical, indicating that the polydisperse band is due to the presence of a single, heterogeneously glycosylated protein. b, Peptide microsequencing of DAG-125. Three peptides from the trypsin digest were collected as fractions from the HPLC analysis and subjected to automated Edman degradation. The sequences obtained were compared with public databases. The alignment of the *Torpedo* DAG-125 peptides to the deduced sequence of human biglycan is shown (aa 241–249, 258–266, and 330–348). c, Domain structure of human biglycan (Fisher et al., 1989; Hocking et al., 1998). Biglycan is one of a family of small leucine-rich repeat proteins. It consists of a prepropeptide that is not present in the mature polypeptide. This domain is followed by a short unique sequence with two chondroitin sulfate attachment sites (shown as stacked beads). There are two pairs and one pair of disulfide-linked cysteines at the NH_2- and COOH-terminal domains, respectively. Finally, the bulk of the protein is comprised of ten (or 11, depending upon the classification of the region within the COOH-terminal cysteine pair) leucine-rich repeats. The position of the three *Torpedo* peptides relative to the human sequence is indicated by horizontal lines.

We next tested the binding of α-dystroglycan to biglycan derived from a variety of sources, as well as to decorin, a small leucine-rich proteoglycan that is ~50% identical to biglycan. Bacterially expressed biglycan, which contains no chondroitin sulfate side chains, did not bind α-dystroglycan (Fig. 5), consistent with a requirement for chondroitin sulfate chains. Biglycan purified from articular cartilage bound α-dystroglycan poorly, even at >100-fold higher loading than that used for *Torpedo* biglycan analysis. These findings indicate that specific chondroitin sulfate chains are required to mediate α-dystroglycan binding to biglycan. Note that these experiments show that chondroitin sulfate side chains are necessary for biglycan–α-dystroglycan binding, but they do not establish whether or not they are sufficient. Studies to determine the possible contribution of the biglycan core to this binding are underway.

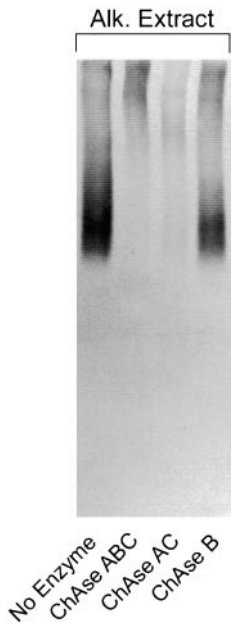

Figure 4. Analysis of *Torpedo* biglycan glycosylation. Alkaline-solubilized synaptic membrane proteins were digested as indicated, in the presence of protease inhibitors, and then analyzed for dystroglycan binding by blot overlay assay with ^{35}S-DG345-653. Both chondroitinase ABC and chondroitinase AC greatly reduced the binding of dystroglycan to DAG-125/*Torpedo* biglycan. Chondroitinase B, which degrades dermatan sulfate, had a much smaller effect. See also Table I.

Biglycan Is Expressed at Synaptic and Nonsynaptic Regions and Is Upregulated in Dystrophic Muscle

Previous reports have shown that biglycan mRNA and protein are expressed in muscle (Bianco et al., 1990; Bosse et al., 1993). Since we purified biglycan from synaptic membranes, we asked whether it is also expressed at the neuromuscular junction. Biglycan is localized around the periphery of the muscle fiber and at all synapses. Further, biglycan is enriched at a subset of neuromuscular junctions (Fig. 6). Finally, since biglycan binds to a component of the DAPC, we asked whether or not its expression was altered in a mouse model of muscular dystrophy where dys-

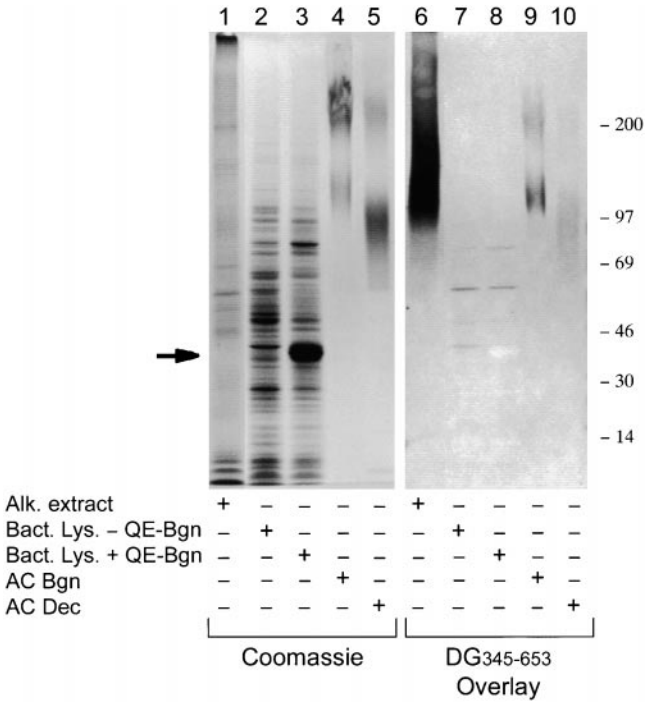

Figure 5. The binding of dystroglycan to biglycan is dependent upon specific chondroitin sulfate side chains. Biglycan (or decorin) was analyzed by SDS-PAGE and Coomassie Brilliant blue staining for protein (lanes 1–5), or blot overlay assay for dystroglycan binding (lanes 6–10). Lanes 1 and 6, alkaline extract of *Torpedo* synaptic membranes (1 μg total protein); lanes 2 and 7, lysate of noninduced bacteria; lanes 3 and 8, lysate of induced bacteria expressing recombinant human biglycan (QE-Bgn; prominent band at ~37 kD, arrow); lanes 4 and 9, biglycan purified from bovine articular cartilage (4 μg; Sigma Chemical Co.); lanes 5 and 10, decorin purified from bovine articular cartilage (4 μg; Sigma Chemical Co.). Biglycan present in electric organ binds dystroglycan much more strongly then biglycan or decorin purified from articular cartilage (compare Coomassie staining to dystroglycan overlay). Note that 4 μg of purified biglycan are present in lanes 4 and 9, compared with only 1 μg of total protein in lanes 1 and 6, of which biglycan is estimated to be <2%.

trophin is absent, *mdx*. We examined adult mice, which contain almost exclusively regenerated muscle fibers that survive due to utrophin compensation (Grady et al., 1997). Immunostaining revealed that the level of biglycan expressed in *mdx* muscle is elevated compared with control animals (Fig. 7). These observations raise the possibility that biglycan could be part of the compensatory mechanism that allows survival of dystrophin-negative muscle fibers.

Discussion

In this paper, we report the identification and characterization of a novel α-dystroglycan binding protein from postsynaptic membranes of *Torpedo* electric organ and provide evidence that it is the small leucine-rich repeat proteoglycan (SLRP) biglycan. Mapping studies indicate that the biglycan binding site on α-dystroglycan is distinct from that of its previously described ligands, laminin, agrin, and perlecan (Figs. 1 and 8). Moreover, the involvement of biglycan's chondroitin sulfate side chains in binding to α-dystroglycan is of particular interest in view of a large body of evidence implicating chondroitin sulfate proteoglycans in synaptogenesis. Finally, the association of biglycan with α-dystroglycan, coupled with its upregulation in dystrophic muscle, suggest that biglycan could play a role in normal DAPC function and perhaps in the pathophysiological responses of dystrophic muscle.

Several lines of evidence indicate that biglycan is an α-dystroglycan–binding protein. We demonstrated this interaction using two assays: blot overlay and solution binding. Further, we showed that several forms of α-dystroglycan, in vitro translated, bacterially produced, and native, bind to biglycan. Biglycan and α-dystroglycan are both enriched in postsynaptic membranes of *Torpedo* electric organ (Figs. 1 and 2; see also Bowe et al., 1994). Importantly, biglycan–α-dystroglycan complexes can be coimmunoprecipitated from extracts of these membranes. Biglycan is localized on the muscle cell surface in a pattern similar to that previously reported for α-dystroglycan (Fig. 7; Durbeej et al., 1998). Taken together, these results strongly support the proposal that biglycan and dystroglycan interact at the muscle cell surface.

Work in other systems has shown that biglycan can be substituted with either chondroitin or dermatan sulfate (Hocking et al., 1998). The enzymatic analysis in the current study indicates that *Torpedo* synaptic membrane biglycan is predominantly substituted with chondroitin sulfate A and/or C, with less or no chondroitin sulfate B (dermatan sulfate). Further, these GAG chains are required for binding to α-dystroglycan. The specific structure of the chondroitin sulfate side chains may also be important since α-dystroglycan binds less well to articular cartilage biglycan, which contains both chondroitin and

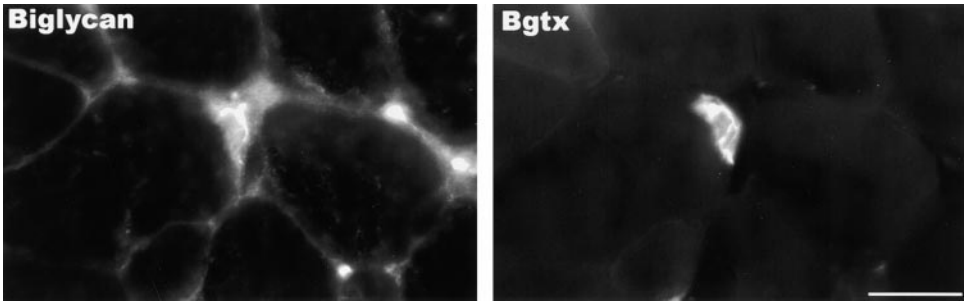

Figure 6. Biglycan expression at the neuromuscular junction. Frozen sections of normal adult mouse muscle were double-labeled with α-bungarotoxin (Bgtx; to localize AChRs) and antibodies to biglycan. Biglycan immunoreactivity is distributed over the entire periphery of the myofibers (see also Fig. 7). Biglycan immunoreactivity is also concentrated at some neuromuscular junctions. Bar, 20 μm.

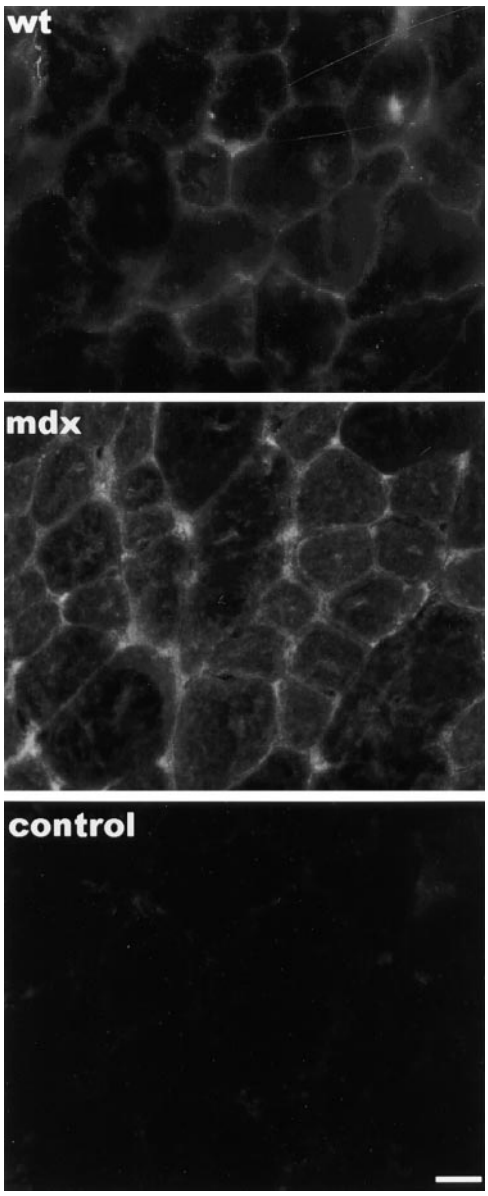

Figure 7. Upregulation of biglycan expression in dystrophic muscle. Frozen sections of normal (wt) and *mdx* muscle from 6-wk-old mice were mounted on the same slides and incubated with rabbit antibiglycan (wt and *mdx*) as described in Materials and Methods. The overall pattern of immunoreactivity was similar in both cases. However, the expression of biglycan was elevated in *mdx* as compared with normal muscle. Very low levels of staining were observed when either normal (control) or *mdx* muscle sections (not shown) were incubated with nonimmune rabbit IgG in the first layer. Similar results were obtained in sections prepared from two other animals. Bar, 20 μm.

dermatan sulfate side chains (Cheng et al., 1994). It is possible that synapse-rich tissues contain specific enzymes that modify the chondroitin sulfate side chains. Interestingly, at least one such enzyme, the chondroitin-6-sulfotransferase NSIST, is selectively enriched in *Torpedo* electric organ and brain (Nastuk et al., 1998). Together, these results suggest that the interaction between dystroglycan and biglycan may be highly regulated through post-translational modification.

Our studies indicate that the α-dystroglycan–binding site for biglycan is distinct from that which binds agrin, laminin, and perlecan. Dystroglycan binding has been mapped to G-domains in each of these basal lamina proteins (Campanelli et al., 1996; Gesemann et al., 1996; Hopf and Hoch, 1996; Talts et al., 1999). Further, this binding requires O-linked glycosylation of α-dystroglycan, which occurs in the middle one-third of the molecule (Brancaccio et al., 1995; Fig. 8). In contrast, biglycan does not contain a G-domain and binds to a COOH-terminal, nonglycosylated α-dystroglycan fragment. Together, these observations suggest that α-dystroglycan could interact simultaneously with a G-domain–containing protein and biglycan. These findings also raise the possibility for an alternative mode of association of α-dystroglycan with the ECM. It is interesting to speculate that such a complex could confer unique signaling and/or structural properties to specific cell surface domains.

The identification of DAG-125 as *Torpedo* biglycan rests on its size, chondroitin sulfate substitution, and sequence homology to mammalian biglycan (76%, 28/37 identical residues over three peptides). It should be noted that although we purified biglycan from highly enriched postsynaptic fractions, its biochemical properties indicate that it is an ECM protein. Biglycan is a member of the SLRP family that includes decorin, fibromodulin, lumican, keratocan, PRELP, osteoadherin, epiphycan, and osteoglycin (reviewed in Iozzo, 1998). The SLRPs have been further subdivided into three classes based upon sequence comparison, phylogenetic relationships, and genomic structure. Biglycan and the predominantly dermatan sulfate proteoglycan, decorin, comprise one class (Hocking et al., 1998). Biglycans from rodent, rabbit, dog, sheep, cow, horse, and human are >95% identical. In contrast,

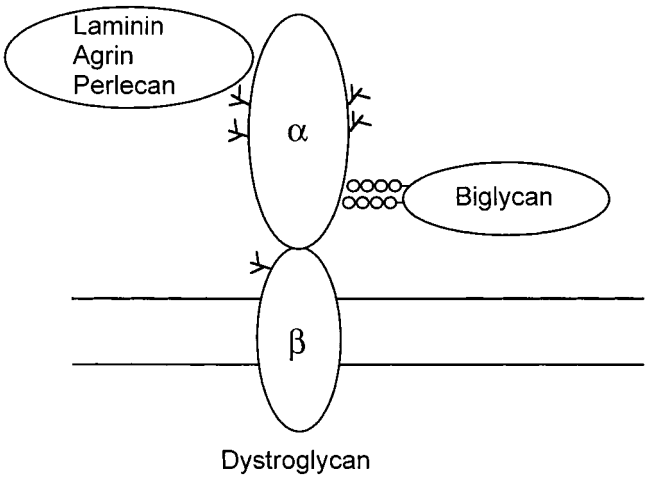

Figure 8. A model of α-dystroglycan interactions in the muscle plasma membrane. α-Dystroglycan binds the G-domain containing proteins agrin, laminin, and perlecan in a manner that requires glycosylation of the mucin-like middle third of the molecules. Biglycan, in a fashion dependent on its chondroitin sulfate side chains, binds to the protein core of the COOH-terminal third of α-dystroglycan. See text for details.

human decorin and biglycan are only 55% identical. The three peptides from *Torpedo* DAG-125 are 51% identical to human decorin. Thus, it is possible that DAG-125 is a *Torpedo* decorin or a primitive SLRP that combines functions of this molecule and biglycan (Blaschke et al., 1996). At present, this issue cannot be resolved, since neither biglycan nor decorin have been cloned from *Torpedo*. However, based upon the stronger sequence homology and our preliminary observations that biglycan more closely matches the distribution of α-dystroglycan in rodent muscle (Mendis, D.B., and J.R. Fallon, unpublished observations), we favor the identification of DAG-125 as biglycan.

Our results show that biglycan binding to α-dystroglycan is mediated by its GAG chains. However, it seems likely that the biglycan protein core will also play an important role in muscle. For example, collagen I binding to decorin is mediated wholly by the protein core, mainly by leucine-rich repeats #4–6 (Svensson et al., 1995; Kresse et al., 1997). Decorin null mice have fragile skin and defects in collagen fibril structure (Danielson et al., 1997). Biglycan also binds to collagen I, however, the affinity is >100-fold less, and collagen I fibril defects are not observed in biglycan null mice (Schonherr et al., 1995; Xu et al., 1998). Further, both biglycan and decorin bind to TGFβ via their cores, raising the possibility that these SLRPs could be involved in the presentation and/or sequestration of growth factors. A signaling role for this class of molecules has been suggested by the finding that nanomolar concentrations of decorin directly bind to and activate EGF receptors (Patel et al., 1998). It also remains possible that an interaction between the biglycan core and α-dystroglycan could have been missed in the assays used here. We are currently using properly folded recombinant biglycan core protein to test its binding to α-dystroglycan and other DAPC components.

Although we discovered biglycan in a highly enriched synaptic fraction and it is expressed at the neuromuscular junction, at present we do not know its function at these sites. Several studies have shown that chondroitin sulfate proteoglycans are important for postsynaptic differentiation (Gordon et al., 1993; Mook-jung and Gordon, 1995; Bowen et al., 1996), although the proteoglycans involved have not been identified. Further, α-dystroglycan may function in the formation of AChR clusters. It is possible that biglycan could play a role here as well. Finally, it is noteworthy that MuSK (muscle-specific kinase) is the agrin signaling receptor, but agrin does not bind it directly. The signaling properties of SLRPs in other systems invite speculation that biglycan could be an element in the hypothetical MuSK/coreceptor complex, (Glass et al., 1996) either by binding MuSK directly or presenting a growth factor-like ligand to it.

Our findings could also have important implications for understanding muscular dystrophy. The integrity of the DAPC and its association with the ECM are essential for muscle cell viability. The binding of biglycan to α-dystroglycan represents an entirely new mode for DAPC–ECM association. This mode could act in concert with, or as an alternative to, binding via the G-protein–containing basal lamina proteins agrin, perlecan, and laminin. Such an alternative pathway for DAPC association with the ECM could provide a new avenue for developing therapeutic interventions for muscular dystrophies and perhaps other neuromuscular disorders.

We thank Dr. Larry Fisher and Lou Kunkel for generously providing reagents used in this study and for valuable discussions. We thank John Leszyk of the Worcester Foundation Keck Peptide Chemistry Facility for tryptic peptide analysis and sequencing, and Beth McKechnie for expert technical assistance. Finally, we thank K. Deyst, M. Rafii, and other members of the laboratory for useful and timely insights.

This work was supported by postdoctoral fellowships from the National Institutes of Health and the American Cancer Society to M.A. Bowe, and grants from the Muscular Dystrophy Association and the National Institutes of Health (HD23924) to J.R. Fallon.

Submitted: 29 September 1999
Revised: 15 December 1999
Accepted: 20 January 2000

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