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The α-Dystroglycan-β-Dystroglycan Complex

MEMBRANE ORGANIZATION AND RELATIONSHIP TO AN AGRIN RECEPTOR*

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Katherine A. Deyst‡, Mark A. Bowe‡, John D. Leszyk§, and Justin R. Fallon‡¶

From the ‡Neurobiology Group and the §W. M. Keck Protein Chemistry Facility, Worcester Foundation for Biomedical Research, Shrewsbury, Massachusetts 01545

Aberrant expression of the dystrophin-associated protein complex is thought to underlie the pathogenesis of Duchenne dystrophy, Becker muscular dystrophy, and severe childhood autosomal recessive muscular dystrophy. Recently, our laboratory identified an agrin receptor from *Torpedo* electric organ postsynaptic membranes. It is a heteromer of 190- and 50-kDa subunits with similarity to two components of the dystrophin-associated protein complex of α- and β-dystroglycan. We now confirm the relationship between the *Torpedo* agrin receptor and mammalian dystroglycans and provide further information about the structure of the α-dystroglycan-β-dystroglycan complex. The sequences of three peptides from each *Torpedo* subunit were 69% identical to mammalian dystroglycans. An antiserum to mammalian β-dystroglycan recognizes the *Torpedo* 50-kDa polypeptide. Additionally, like α-dystroglycan, the 190-kDa agrin receptor subunit binds laminin. Previous studies have indicated that α- and β-dystroglycan arise by cleavage of a precursor protein. Tryptic peptide mapping of both subunits and amino-terminal sequencing of *Torpedo* β-dystroglycan indicate a single cleavage site, corresponding to serine 654 of the mammalian dystroglycan precursor. Gel electrophoresis analysis indicates there is at least one intrachain disulfide bond in β-dystroglycan. These results provide precise primary structures for α- and β-dystroglycan.

Linkages between the extracellular matrix and the cytoskeleton play key roles in forming and maintaining specialized membrane domains, ensuring the structural integrity of the plasma membrane, attaching cells to the extracellular matrix, and cell signaling (1, 2). In skeletal muscle, the dystrophin-associated protein complex (DAPC)[1] is likely to be an essential arbiter of basal lamina cytoskeletal communication in both synaptic and nonsynaptic regions (3–6). Perturbations of this membrane protein complex have been linked to the pathogenesis of muscular dystrophies (MDs). In Duchenne and Becker MD, mutations in dystrophin, a cytoskeletal protein, lead to decreased levels of the DAPC complex in the plasma membrane (4, 7). Some forms of autosomal recessive MD are linked to mutations affecting the expression of adhalin, a transmembrane constituent of the DAPC (8–10). Moreover, the basal lamina protein merosin, a ligand of the DAPC (11), is missing in many cases of congenital MD where the genetic defect is unknown (12, 13). Interestingly, these latter two MDs have a phenotype similar to Duchenne MD, even though the dystrophin gene is unaffected. Together, these results indicate that the DAPC is a functionally interdependent protein ensemble. As such it is essential to characterize the structural and functional features that underlie its organization.

Two components of the DAPC, α- and β-dystroglycan, form a heteromeric membrane-spanning complex that is likely to constitute a principal linkage between the basal lamina and the cytoskeleton. α-Dystroglycan is a highly glycosylated extrinsic peripheral membrane protein that binds the basal lamina elements agrin, laminin, and merosin (11, 14–18). β-Dystroglycan is a transmembrane protein whose cytoplasmic domain has been reported to bind to the carboxyl-terminal tail of dystrophin (19). Utrophin, an autosomally encoded protein that has close structural similarity to dystrophin (20, 21), may also interact with β-dystroglycan. This association is of general interest in view of utrophin's expression at neuromuscular junctions and in a wide variety of nonmuscle tissues (22).

The dystroglycan complex has also been implicated in cell signaling. Agrin is an extracellular matrix molecule that induces the clustering of acetylcholine receptors and other postsynaptic molecules on muscle cell surfaces (reviewed in Ref. 23). Our search for the agrin receptor in postsynaptic membranes of *Torpedo* electroplax led to the discovery of a heteromeric complex of two membrane glycoproteins that based on amino acid sequence of two internal peptides share structural similarity with the dystroglycans (16). Agrin binds to this complex at subnanomolar concentrations in a calcium-dependent manner. Further, the complex spans the plasmalemma and is selectively concentrated in postsynaptic membranes (16, 24). Other investigators have used antibodies to α-dystroglycan to show that it binds agrin (15, 17, 18). Interestingly, agrin induces the redistribution of several other DAPC components, including utrophin and adhalin (reviewed in Ref. 6). Together, these results suggest that the α-dystroglycan-β-dystroglycan complex participates in agrin's signaling pathway. They also suggest that agrin may direct the spatial organization of the DAPC complex on the muscle surface.

Knowledge of the detailed structure and interactions of the dystroglycan complex will be essential for understanding the DAPC's role in muscular dystrophy pathogenesis. Moreover, these results are important for understanding the role of dystroglycans in postsynaptic differentiation. In this study we extend our examination of the relationship between *Torpedo* agrin receptor subunits and mammalian dystroglycans to three levels: peptide sequence, ligand binding, and antigenicity. In addition

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¶ To whom correspondence should be addressed: Worcester Foundation for Biomedical Research, 222 Maple Ave., Shrewsbury, MA 01545. Tel.: 508-842-8618; Fax: 508-842-9632; E-mail: fallon@sci.wfeb.edu.

[1] The abbreviations used are: DAPC, dystrophin-associated protein complex; MD, muscular dystrophy; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

we have localized the cleavage site of the dystroglycan precursor in *Torpedo*, and present evidence suggesting that a similar site is used in mammalian dystroglycan. These results provide an unambiguous primary structure for α- and β-dystroglycan.

EXPERIMENTAL PROCEDURES

Purification of Agrin Receptor—Agrin receptor was purified over 250-fold from *Torpedo* electric organ as described previously (16). Briefly, proteins were solubilized from a postsynaptic membrane fraction with 25 mM *n*-octyl-β-D-glucopyranoside (Calbiochem) and applied to an immunoaffinity column made with anti-agrin receptor monoclonal antibody 3B3. The column was eluted with 0.1 M diethylamine, pH 11.5. 250 g of *Torpedo* electric organ yielded approximately 20 μg of purified protein.

Preparation of Membranes from Rat Muscle—Preparation of crude rat muscle membranes was based on previous methods (25) with several modifications. Charles River CD rats (postnatal day 10) were euthanized by halothane inhalation. Hindlimb muscle was removed, rapidly frozen, and stored at −80 °C. Muscle was homogenized with a Brinkmann Polytron in two volumes of buffer A (0.3 M sucrose, 35 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 23 μg/ml aprotinin, 1.8 mg/ml benzamidine, 0.37 mg/ml iodoacetamide, 0.62 mg/ml *N*-ethylmaleimide, 0.7 μg/ml pepstatin A, 0.02% azide, pH 7.4). The membranes were further disrupted in a Branson bath sonicator and centrifuged for 20 min at 7000 × *g*. The pellet was resuspended in the same volume of buffer A and centrifuged as above. The supernatants were combined, filtered through cheesecloth, brought to 0.6 M KCl, and centrifuged for 20 min at 7000 × *g*. KCl-washed microsomes were collected by centrifugation for 1 h at 140,000 × *g*, washed once in buffer B (0.3 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.02% azide, pH 7.4), and stored at −80 °C.

Antisera—Polyclonal antiserum 12031C was the generous gift of A. Ahn and L. Kunkel (Children's Hospital, Boston, MA). The antibody was prepared against a synthetic peptide corresponding to a sequence in the carboxyl-terminal region of human β-dystroglycan: EGKGSRPKNMTPYRSP.[2]

Immunoblotting and Ligand Overlay Assays—Proteins were separated on 5–15% gradient gels and electroblotted onto nitrocellulose for 1 h at 2 A in 10% methanol, 25 mM Tris, 192 mM glycine, pH 8.3. For immunoblots, the paper was rinsed with phosphate-buffered saline containing 0.02% NaN_3 and dried overnight. Incubations (30 min each) and washes (in phosphate-buffered saline containing 0.1% Tween 20) were all carried out at room temperature. Blots were first incubated in block solution, consisting of minimal essential medium (HEPES modification; Sigma), 1% bovine serum albumin (Sigma), 0.1% Tween 20, and 10% horse serum (Life Technologies, Inc.). Blots were then incubated sequentially in antiserum 12031C (1:400 in block solution), biotinylated goat anti-rabbit IgG (Vector), and alkaline phosphatase-conjugated ABC reagent (Vector). Color was developed with an alkaline phosphatase substrate kit (Kirkegaard-Perry Laboratories).

The ligand overlay assay was performed essentially as described (16). Agrin was purified from *Torpedo* electric organ (24). Laminin (Upstate Biotechnologies Inc.) and anti-agrin antibody MAb-5B1 were labeled with ^{125}I using Iodogen (Pierce). All overlay procedures were at 4 °C. Blots were incubated overnight in block solution (without Tween 20) followed by 100 ng/ml ^{125}I-laminin or 0.2 units/μl *Torpedo* agrin in the presence of 1 mM calcium for 3.5 h. Agrin overlays were incubated in a second layer containing 1 μg/ml ^{125}I-MAb-5B1. Agrin- or laminin-binding polypeptides were revealed by autoradiography.

Microsequencing and Mapping of Agrin Receptor Subunit Peptides—Immunoaffinity-purified agrin receptor subunits (approximately 20 μg of total protein) were electrophoresed on a 1.5-mm-thick discontinuous SDS-PAGE separating gel (upper half, 5–7% gradient; lower half, 11% acrylamide). The resolved polypeptides were electroblotted for 3 h at 2 A onto Immobilon polyvinylidene difluoride, visualized with Ponceau S, and subjected to *in situ* enzymatic digestion with 0.25 μg of trypsin or 0.5 μg endoproteinase Lys-C (26). The released peptides were separated by HPLC and sequenced as described (16).

Amino-terminal Sequencing—Purified agrin receptor was prepared and blotted to polyvinylidene difluoride membrane as described above. The 50-kDa band (approximately 30 pmol) was visualized with Ponceau S. The identity of this polypeptide was verified by probing adjacent lanes containing identical material with antiserum 12031C. The polypeptide was subjected to automated Edman degradation on an Applied Biosystems 492 Procise sequencer equipped with a Model 120A in-line PTH analyzer.

[2] A. Ahn and L. Kunkel, personal communication.

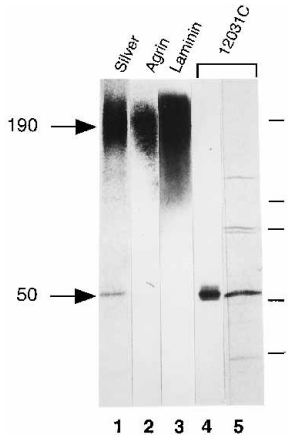

FIG. 1. **Binding of anti-dystroglycan antibodies, agrin, and laminin to purified *Torpedo* agrin receptor.** Agrin receptor subunits from *Torpedo* electric organ (*lanes 1–4*) or membrane proteins from rat muscle (*lane 5*) were separated by SDS-PAGE and either silver-stained (*lane 1*) or blotted to nitrocellulose (*lanes 2–5*). Blots probed with 0.2 units/μl *Torpedo* agrin followed by ^{125}I-labeled anti-agrin antibody 5B1 (*lane 2*); 100 ng/ml ^{125}I-laminin (*lane 3*); or an antiserum, 12031C, raised against a peptide in the predicted cytoplasmic domain of β-dystroglycan (*lanes 4 and 5*). The minor bands in *lane 5* are nonspecific because they are detected with nonimmune serum. The *bars* to the *right* of the *lanes* indicate migration of prestained protein standards (Amersham Corp.): 220, 97.4, 66, 46, and 30 kDa.

RESULTS

The 190-kDa·50-kDa Agrin Receptor from Torpedo Electric Organ Is Closely Related to Mammalian α-Dystroglycan-β-Dystroglycan—We previously purified a candidate agrin receptor from postsynaptic membranes (16). It is a heteromeric complex of two glycoproteins with relative molecular masses of 190 and 50 kDa (Fig. 1, *lane 1*). Sequencing of one internal peptide from each subunit indicated that the 190- and 50-kDa subunits were similar to mammalian α- and β-dystroglycan, respectively. To examine this similarity more closely, we sequenced two additional internal peptides from the 190-kDa subunit and one from the 50-kDa subunit (Fig. 2*A*). All sequences mapped to the predicted amino acid sequence of the mammalian dystroglycan precursor. Identity ranged from 44 to 92% for the individual sequences compared, with an overall value of 69% (82 of 118 amino acids). The similarity rose to 85% when conservative substitutions were taken into account. The amino-terminal sequence of the 50-kDa subunit shows comparable similarity (see below).

We next asked if the *Torpedo* 50-kDa subunit was antigenically related to mammalian β-dystroglycan (Fig. 1). A polyclonal antiserum directed against mammalian β-dystroglycan recognized the 50-kDa *Torpedo* subunit. In addition, the electrophoretic mobility of the *Torpedo* polypeptide was virtually identical to that of rat muscle β-dystroglycan.

We then examined the ligand binding properties of the agrin receptor using a blot overlay method. Both laminin and agrin bound to the polydisperse 190-kDa subunit (Fig. 1, *lanes 2* and *3*). In agreement with previous results (15), the binding of both ligands was calcium-dependent and inhibited by heparin (data not shown). We also asked if heparin inhibited the binding of either agrin or laminin to intact postsynaptic membranes. Solid phase radioassay showed that heparin (10 μg/ml) reduced agrin and laminin binding by 64 ± 21.2% and 75.5 ± 13.2%, respectively ($p < 0.01$, $n = 3$). Because their ligand binding properties, antigenicity, and sequence are similar to the mammalian dystroglycans, the 190- and 50-kDa agrin receptor subunits are likely to represent *Torpedo* α- and β-dystroglycan,

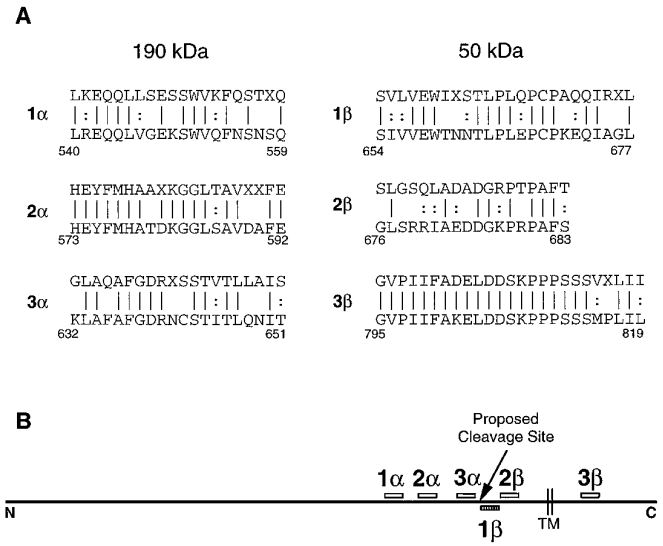

FIG. 2. **Peptide microsequencing of *Torpedo* agrin receptor 190- and 50-kDa subunits.** *A*, amino acid sequences obtained from purified 190- (α-dystroglycan) and 50-kDa (β-dystroglycan) agrin receptor subunits (*top lines*, each pair) aligned with predicted amino acid sequences from human dystroglycan (*bottom lines*) (28). Sequence *1β* was obtained by amino-terminal sequencing of purified 50-kDa subunit; all other sequences were obtained by internal microsequencing. Assignments of amino acid similarity are as defined in the BLASTA algorithm (34). *B*, schematic of the alignment of the *Torpedo* agrin receptor subunit sequences with the predicted full-length human dystroglycan precursor polypeptide. The cleavage site deduced in this study is shown. *TM*, the predicted transmembrane domain (28). Sequences *2α* and *3β* have been published previously (16).

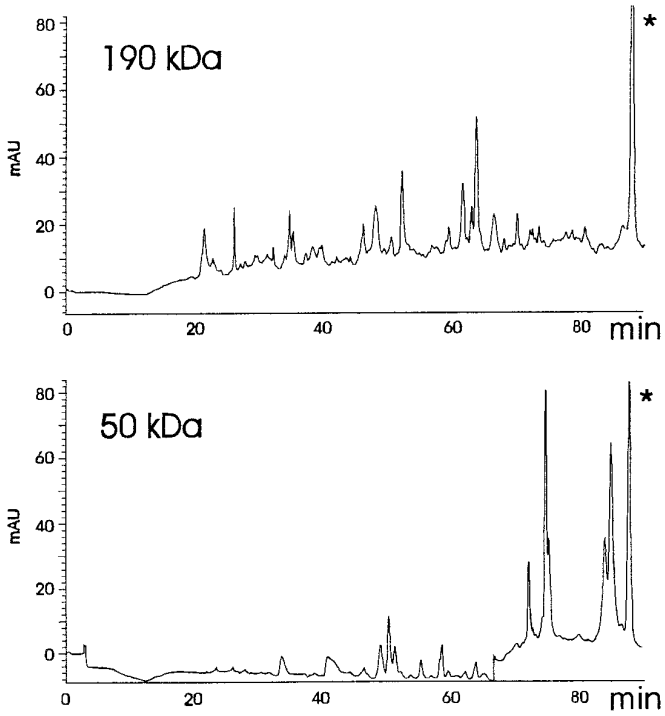

FIG. 3. **Tryptic peptide maps of *Torpedo* agrin receptor 190- and 50-kDa subunits.** Purified *Torpedo* agrin receptor 190- and 50-kDa subunits were separated by SDS-PAGE, electroblotted to polyvinylidene difluoride, and digested *in situ* with trypsin. The released peptides were resolved by HPLC on a C8 column and detected by absorbance at 210 nm. No peptides common to the 190- and 50-kDa subunits were detected. The *asterisk* denotes artefactual peak from digest buffer.

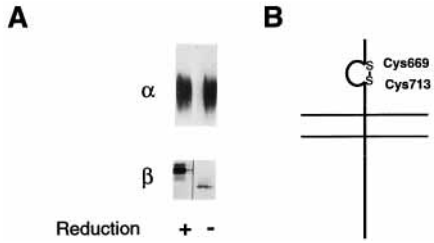

FIG. 4. **Analysis of disulfide linkages in 190- and 50-kDa polypeptides.** *A*, *Torpedo* postsynaptic membranes were incubated for 10 min at 60 °C in sample buffer either in the presence (+) or absence (−) of 20 mM dithiothreitol and then separated by SDS-PAGE. 190 (α) and 50 kDa (β) were visualized by agrin-binding ligand blot overlay and by immunoblot with antiserum 12031C, respectively. The mobility of β-dystroglycan is greater under nonreducing conditions, indicating the presence of intrachain disulfide bonds in this polypeptide. *B*, schematic model of β-dystroglycan topology, drawn approximately to scale. The deduced disulfide bond in the extracellular domain is depicted.

and we will hence refer to them as such.

Cleavage Site of the Dystroglycan Precursor—α- and β-Dystroglycan are translated from a single mRNA (27). This message is thought to encode a common precursor protein that is subsequently cleaved into the two polypeptides. However, the cleavage site(s) has not been determined. Mapping of internal peptide sequences obtained from *Torpedo* α- and β-dystroglycans to the deduced mammalian sequence indicated that this site occurs between Ser^{651} and Ser^{676} (Fig. 2; positions inferred from the mammalian sequence). To localize this site precisely, we obtained amino-terminal sequence from *Torpedo* β-dystroglycan. The amino terminus corresponded to serine 654 of the human dystroglycan precursor protein (Fig. 2). *Torpedo* β-dystroglycan often appears as a closely spaced doublet by both silver staining and immunoblot analysis (Fig. 1, *lane 4*). We therefore sequenced both members of the doublet; each yielded the same sequence. We conclude that the dystroglycan precursor is cleaved between Gly^{653} and Ser^{654}.

The apparent molecular mass of *Torpedo* α-dystroglycan (190 kDa) is substantially greater than that reported for its counterparts in mammalian brain (120 kDa) or muscle (156 kDa) (14, 27). Analysis of all three forms in our gel system confirmed these differences (data not shown), raising the possibility that the *Torpedo* 190-kDa polypeptide might represent the full-length dystroglycan precursor (see below). However, α- and β-dystroglycan share no common peptides, as judged by tryptic peptide mapping (Fig. 3). Additionally, an antiserum directed against the predicted cytoplasmic domain of the dystroglycan precursor fails to bind the *Torpedo* 190-kDa glycoprotein (Fig. 1). Taken together, these data suggest that the 190 kDa glycoprotein represents fully proteolytically processed α-dystroglycan. Moreover, a similar result was obtained with crude *Torpedo* postsynaptic membranes or with electric organ that had been lysed directly with boiling SDS-PAGE sample buffer (data not shown), indicating that cleavage is not an artifact of purification.

Intrachain Disulfide Bonds in β-Dystroglycan—We also asked if inter- or intrachain disulfide bonds play a role in determining dystroglycan structure. We detected no change in the apparent mobility of α-dystroglycan on SDS-PAGE in the presence or absence of disulfide reducing agents (Fig. 4*A*). In contrast, the relative mobility of β-dystroglycan was greater under nonreducing as compared with reducing conditions, indicating the presence of intrachain disulfide bonds in this subunit. However, we did not observe any new immunoreactive bands under these nonreducing conditions. Thus β-dystroglycan is unlikely to form homo-oligomers or to link with any other polypeptide via interchain disulfide bonds. The β-dystroglycan band also showed diminished immunoreactivity in the absence,

as compared with the presence, of reducing agents. Because antibody 12031C was generated against a peptide, it is possible that its ability to recognize β-dystroglycan is partially dependent upon secondary structure.

There are only three cysteines in the predicted amino acid sequence of mammalian β-dystroglycan (Fig. 2 and Refs. 27 and 28). One (Cys^{774}) is in the intracellular domain, and two (Cys^{669} and Cys^{713}) are in the extracellular domain. Further, Cys^{669} is conserved in *Torpedo* (Fig. 2, *sequence 1β*). It is thus likely that Cys^{669} and Cys^{713} form a disulfide bond in β-dystroglycan (Fig. 4*B*). These data also indicate that α-dystroglycan, which is tightly associated with the plasma membrane (16, 24, 29), does not do so via interchain disulfide bonding to β-dystroglycan.

DISCUSSION

Mammalian dystroglycan isolated from muscle plasma membranes is a detergent-soluble heteromer comprised of α- and β-subunits (30). We assessed the relationship between the 190-kDa · 50-kDa agrin receptor complex from *Torpedo* electric organ and mammalian dystroglycan at three levels: ligand binding, sequence similarity, and antigenicity. First, like mammalian α-dystroglycan, the 190-kDa subunit requires calcium to bind laminin and agrin, and this binding is inhibited by heparin. Second, the peptides that we sequenced from the *Torpedo* subunits (118 total amino acids) are 69% identical and 85% similar to mammalian dystroglycan. Third, antibodies raised against mammalian β-dystroglycan recognize the *Torpedo* 50-kDa polypeptide. Finally, our previous findings that the 190-kDa · 50-kDa subunits form a stable complex in *n*-octyl-β-D-glucopyranoside (16) are in agreement with those obtained for mammalian dystroglycan (30). Taken together, these results indicate that the *Torpedo* agrin receptor 190- and 50-kDa subunits are homologues of α- and β-dystroglycan in mammals.

In this study, we have deduced the location of a cleavage site in the dystroglycan precursor that yields α- and β-dystroglycan. Alignment of the predicted mammalian sequence with the amino-terminal sequence of *Torpedo* β-dystroglycan and the internal sequences from α-dystroglycan indicates that cleavage occurs between Gly^{653} and Ser^{654} (Fig. 2). Moreover, this locus is likely to be the major if not the only cleavage site: 1) a single amino-terminal sequence was obtained from β-dystroglycan; 2) all internal peptide sequences for the α- and β-dystroglycan subunits were located on the amino- and carboxyl-terminal sides, respectively, of this site; and 3) the tryptic peptide maps of α- and β-dystroglycan were distinct and nonoverlapping. Cleavage is also efficient; we find no evidence for intact dystroglycan precursor in *Torpedo* postsynaptic membranes (Fig. 1).

Based upon a Gly^{653} cleavage site, the predicted molecular masses of the mammalian α- and β-dystroglycan polypeptides are 72- and 27-kDa, respectively (27, 28). Interestingly, as judged by SDS-PAGE, the apparent molecular mass of α-dystroglycan ranges from 120 to 190 kDa, whereas that of β-dystroglycan is approximately 43 kDa (50 kDa in the gel system used here). Both subunits must therefore undergo significant post-translational modification. Some of these modifications include asparagine-linked glycosylation and sialylation (14, 16, 29). However, because only a fraction of the carbohydrates are sensitive to any of the glycosidases or glycosaminoglycanases surveyed to date, additional, perhaps novel, carbohydrate structures are probably present. Such structures are likely to be key to the function of α-dystroglycan. For example, variant glycosaminoglycan-deficient muscle cells show greatly attenuated agrin-induced acetylcholine receptor clustering (31–33), and the α-dystroglycan they produce shows reduced agrin binding (15). Elucidation of the carbohydrate structure of dystroglycan is thus an important area for future investigation.

The ability of the dystroglycan complex to function as a receptor is likely to depend upon interaction between its subunits. α-Dystroglycan is tightly associated with the plasma membrane, probably via a strong, albeit noncovalent, interaction with β-dystroglycan. β-Dystroglycan spans the plasma membrane and is thus well positioned to mediate signal transduction events initiated by ligand binding to α-dystroglycan. The localization of the cleavage site provides a starting point for delimiting the domains of α-dystroglycan that are required for association with β-dystroglycan. These domains may also be important for the interaction of α- and/or β-dystroglycan with other members of the DAPC, such as adhalin. It is noteworthy that α-dystroglycan is also found in soluble pools (14), raising the possibility that the association between α- and β-dystroglycan may be regulated. Such regulation would have important implications for whether or not binding of extracellular matrix components to α-dystroglycan leads to signaling events.

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