The α-Dystroglycan-β-Dystroglycan Complex

MEMBRANE ORGANIZATION AND RELATIONSHIP TO AN AGRIN RECEPTOR*

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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-plasma membrane, attaching cells to the extracellular matrix, membrane domains, ensuring the structural integrity of the cytoskeleton.

Polymeric and homomeric 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 carboxy-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 plasma membrane 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). 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
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 mrm 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 rm diethylamine, pH 7.4. The membranes were further disrupted in a Branson bath sonicator and centrifuged for 20 min at 7000 x 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 M KCl, and centrifuged for 20 min used in mammalian dystroglycan. These results provide an unambiguous primary structure for α- and β-dystroglycan.

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 M sucrose, 35 m M Tris-HCl, 10 m M EDTA, 10 m M EGTA, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 23 μg/ml apropin, 1.8 mg/ml benzamidine, 0.37 mg/ml iodoacetamide, 0.62 mg/ml N-ethylmaleimide, 0.7 μg/ml N-ethylmaleimide, 0.7 μg/ml aprotinin, 0.02% azide, pH 7.4). The membranes were further digested in a Branson bath sonicator and centrifuged for 20 min at 7000 x 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 M KCl, and centrifuged for 20 min at 7000 x g. The KCl-washed microsomes were collected by centrifugation for 1 h at 40,000 x g, washed once in buffer B (0.3 m M sucrose, 10 m M Tris-HCl, 1 m M EDTA, 1 m M EGTA, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.02% azide, pH 7.4), and stored at -80°C.

Antisera—Polyclonal antisera 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: EGKGRPRKNMTMPRSW. 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 m M Tris, 192 m M glycine, pH 8.3. For immunoblots, the paper was rinsed with phosphate-buffered saline containing 0.02% NaN3 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 modified; Sigma), 1% bovine serum albumin (Sigma), 0.1% Tween 20, and 10% horse serum (Life Technologies, Inc.). Blots were then incubated in a Branson bath sonicator and centrifuged for 20 min at 7000 x 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 M KCl, and centrifuged for 20 min at 7000 x g. The KCl-washed microsomes were collected by centrifugation for 1 h at 40,000 x g, washed once in buffer B (0.3 m M sucrose, 10 m M Tris-HCl, 1 m M EDTA, 1 m M EGTA, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.02% azide, pH 7.4), and stored at -80°C. 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 125I using Iodogen (Pierce). All overlay procedures were at 4°C. 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 125I using Iodogen (Pierce). All overlay procedures were at 4°C. 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 125I using Iodogen (Pierce). All overlay procedures were at 4°C. The identity of this polypeptide was verified by probing adjacent lanes containing identical material with antisera 12031C. 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. 2A). 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 identity rose to 98% 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 antisera 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.
and we will hence refer to them as such.

Cleavage Site of the Dystroglycan Precursor—\(\alpha\) and \(\beta\)-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 \(\alpha\) and \(\beta\)-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 \(\alpha\) and \(\beta\) subunits (28). Sequence \(1\beta\) 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 \(2\alpha\) and \(3\beta\) have been published previously (16).

The apparent molecular mass of Torpedo \(\alpha\)-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, \(\alpha\) and \(\beta\)-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 \(\alpha\)-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.
as compared with the presence, of reducing agents. Because antibody 12031C was generated against a peptide, it is possible that its ability to recognize α-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 (Cys674) is in the intracellular domain, and two (Cys669 and Cys749) are in the extracellular domain. Further, Cys669 is conserved in Torpedo (Fig. 2, sequence 1β). It is thus likely that Cys669 and Cys749 form a disulfide bond in β-dystroglycan (Fig. 4B). These data also indicate that α-dystroglycan, which 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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