Identification of Dystrophin-binding Protein(s) in Membranes from Torpedo Electrocyte and Rat Muscle*

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Using solubilized dystrophin isolated from torpedo electric tissue and in vitro blot overlay assay, we have identified dystrophin-binding proteins in membranes from Torpedo electrocyte and rat muscle. In acetylcholine receptor-rich membranes from Torpedo marmorata electric tissue, an extrinsic protein of M, 52,000, known as the 58-kDa protein (Froehner, S. C. (1984) J. Cell Biol. 99, 88-98), represents the major binding site for dystrophin. When membranes were solubilized by non-ionic detergents, the 53-kDa protein as well as a few proteins of M, 200,000, 87,000, and 45,000 coextract and copurify with dystrophin. In rat sarcolemma, a protein doublet of ~58-60 kDa also binds dystrophin in vitro, this protein likely being the DAP 59 characterized by Ervasti and Campbell (Ervasti, J. M., and Campbell, K. P. (1991) Cell 66, 1121-1131). We postulate that the 52- and 59-kDa proteins are functional homologs that play the role of "receptors" for dystrophin in various specialized membrane domains.

Dystrophin, the high molecular weight protein product of the Duchenne muscular dystrophy (DMD)† gene, localizes beneath the sarcolemmal membrane of skeletal muscle fibers. Its subcellular localization as well as its amino acid sequence suggest that dystrophin is a membranous cytoskeletal protein that belongs to the spectrin superfamily (1-5). Dystrophin solubilized from sarcolemmal membrane is part of a large transmembrane complex containing a protein triplet of M, 59,000 (DAP 59) and five glycoproteins of M, 156,000 (DAG 156), 50,000 (DAG 50), 43,000 (DAG 43), 35,000 (DAG 35), and 25,000 (DAG 25) (6). DAG 106 and DAP 59 are extrinsic components readily detached from the core components of the complex by alkali extraction. DAG 156 binds the basement membrane protein laminin and is therefore exposed at the extracellular surface of the membrane (7). DAP 59 is believed to favor anchoring dystrophin at the cytoplasmic surface (6). In skeletal muscles from DMD patients or mdx mice lacking dystrophin, the dystrophin-associated glycoproteins are dramatically reduced, though the overall glycoprotein composition of the sarcolemmal membrane remains unchanged (8, 9). However, the mRNA encoding the 156-43 DAG is expressed at normal amounts in mdx and DMD skeletal muscles compared with controls (7), suggesting that dystrophin is required for the stabilization of the complex. Therefore, the function of dystrophin (mechanical stability, regulation of calcium leak channels, . . .) cannot be envisaged without understanding the activities of the associated proteins. In this context, the knowledge of the precise mode of interaction of dystrophin with these different components is crucial.

Dystrophin is also present at the postsynaptic membrane of the muscle fiber (10-13) as well as in the Torpedo electrocyte in which it represents one of the major extrinsic component of the innervated membrane (14-18). In Torpedo electrocyte, dystrophin separated by immunofluorescence chromatography copurifies with several proteins: a doublet at M, 58,000 and minor proteins at M, 87,000, M, 45,000, and M, 30,000 (19). Among the dystrophin-associated proteins, the M, 58,000 and 87,000 proteins were previously characterized in Torpedo electric tissue as extrinsic components of the acetylcholine receptor (ACHR)-rich membrane (20, 21). Muscle counterparts of these two proteins exist and are associated with the sarcolemma (20, 21). Moreover, there are analogies in the physicochemical properties and polypeptidic composition of the dystrophin complexes isolated from skeletal muscle and Torpedo electrocyte. In particular, the extrinsic M, 58,000/DAP 59 may be involved in the anchoring of dystrophin to the membrane in both tissues.

However, the identification of the components responsible for the anchoring of dystrophin to the membrane cannot simply be deduced from the detergent extraction procedures used so far. As currently reported, cytoskeleton-plasma membrane interactions that are founded essentially on polar interactions should resist extraction by non-ionic detergents, since non-ionic detergents disrupt hydrophobic but not polar protein-protein or protein-lipid interactions (see Ref. 22 for a discussion). Indeed, the yield of dystrophin-protein complex extracted from the purified postsynaptic membrane by detergents is always low (14-19) and is not necessarily representative of the mode of interaction of dystrophin with the membrane. An alternative approach would be the in vitro study of interaction of solubilized dystrophin with isolated membrane components. In this work, using a blot overlay binding assay, we show that dystrophin binds principally to the extrinsic 52/58-kDa protein of Torpedo postsynaptic membrane and to a 58-60 kDa protein doublet of mammalian sarcolemma.

Part of this work was previously released in abstract form (23).

EXPERIMENTAL PROCEDURES

Antibodies and Other Materials—Polyclonal antisera against dystrophin were raised in rabbit and characterized previously (18). Dystrophin antibodies were affinity-purified on dystrophin separated on 6% SDS-polyacrylamide gel electrophoresis and electrotransferred on nitrocellulose according to Ref. 24. Human spectrin and polyclonal antibodies against spectrin were a gift from Drs. R. Cassoly and L. A. Pradel.

Preparation of Acetylcholine Receptor-rich Membranes—Nicotinic receptor-rich membranes were prepared from fresh Torpedo marmorata.
electric tissue (Institut de Biologie Marine, Arcachon, France) as previously described (25). Typically 10–20 mg of protein of membranes were obtained from 100 g of electric tissue. The protein concentration was determined by the method of Bradford (26).

Alkali Extraction of Dystrophin—Alkaline extraction (pH 11) was carried out using the protocol of Neubig et al. (27). The alkali extract was recovered in 0.01% bacitracin-treated glassware to prevent adsorption and neutralized to pH 7.8 with 2 x Tris-HCl, pH 7.0, β-mercaptoethanol (1 mM), and anti-proteolytic agents leupeptin (5 μg/ml) and aprotinin (0.5 μg/ml). The alkali extract (S11) was recovered in 0.01% bacitracin-treated glassware to prevent adsorption and neutralized to pH 7.8 with 2 x Tris-HCl, pH 7.0, β-mercaptoethanol (1 mM), and anti-proteolytic agents leupeptin (5 μg/ml) and aprotinin (0.5 μg/ml). The alkali-extracted dystrophin sample was added to 10 μg of total protein and a doublet protein of 130 kDa that was not detected in the control experiment. Lanes 2 and 7, dystrophin binding assay on alkali-stripped membranes. Lanes 5 and 7, dystrophin binding assay on alkali-stripped membranes. Lanes 5 and 7, dystrophin binding assay on alkali-stripped membranes.

Preparation of Sarcolemmal Membranes—Sarcolemmal membranes from rat skeletal muscles were prepared according to Mitchell et al. (25) in the presence of benzamidine (0.8 mM) leupeptin (0.5 μg/ml), aprotinin (0.5 μg/ml), pepstatin (5 μg/ml) were added. Dystrophin concentrations (typically 1–10 μg/ml) were estimated by comparison with bovine serum albumin standards after gel electrophoresis in the presence of SDS.

Immunofluorescence of the Dystrophin Complexes—AChR-rich membranes were resuspended to 2 mg/ml in 25 mM Tris, pH 7.4, buffer containing 150 mM NaCl, 2.5 mM EGTA, 2.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin (buffer A), and the non-ionic detergent Triton X-100, for 30 min at 150 V, 4 h at 200,000 Xg for 30 min to remove detergent. The supernatant was diluted with the same buffer (v/v) and incubated on a rocking platform overnight at 4 °C with hydrazide gel (M-Gel, Bio-Rad and Avidgel, Bioprobe International Inc.) on which dystrophin IgG were previously coupled. The IgG were purified from the serum on a protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) column, and the coupling was achieved according to the supplier’s instructions.

The washing and the elution of the dystrophin-associated protein complexes were performed either through a column or a series of batch-extraction steps. The gels were successively washed with buffer A containing 0.02% detergent, buffer A containing 0.02% detergent plus 0.5 % NaCl, and buffer A containing 0.02% detergent. The bound dystrophin complexes were eluted from Avidgel with 0.1 M NaC1, and anti-proteases or with 0.1 M NaC1, and anti-proteases or with 0.1 M sodium acetate buffer, pH 3.5, 0.5 M NaCl, and anti-proteases or with 0.1 M glycine buffer, pH 2.5, and anti-proteases from Avid-Gel. The eluted fractions (1 ml) were collected in 1.5 ml microtubes. The samples were analyzed by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with 10 μg/ml of dystrophin antibody, washed with wash buffer (Tris buffer at pH 7.8) and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Promega Biotec, Madison, WI) followed by enhanced chemiluminescent (ECL) detection (Amersham, UK).

Results and Discussion

Dystrophin binding experiments were carried out using a blot overlay assay (35). Experiments were performed using soluble dystrophin preparations obtained by alkaline extraction of highly purified AChR-rich membranes. Alkaline extraction was chosen for its high yield and for its low denaturing effect on dystrophin shape as shown by electron microscopy (37) and sedimentation experiments (38). Dystrophin extracted at alkaline pH occurs as defined molecular entities (dimers) and is not associated with other membrane components (38). After alkaline extraction solubilized dystrophin was stable at pH 7.8 for a few hours while longer storage resulted in loss of its binding properties.

Fig. 1 shows the binding of dystrophin to a single polypeptide of M, ~52,000 of AChR-rich membrane fractions (lane 2). After extraction of the membrane with alkaline, the dystrophin-binding 52-kDa protein was present in alkali extracts (lane 4) but absent from residual stripped membranes (lane 6). These data indicate that the 52-kDa protein is an extrinsic component of the AChR-rich membrane. A protein of similar mass (previously described as the 58-kDa protein) is one of the major extrinsic components of the AChR-rich membrane (39). This protein was further shown to display an apparent mass of 52,000 (19–21) and is composed of several isoelectric variants (isoelectric point (pI) = 6.4–6.7) (39). The specificity of the binding of dystrophin to the 52-kDa component was confirmed by control experiments that show that spectrin, a protein of the superfamily of dystrophin which in electrophoresis is specifically associated with the non-innervated membrane (40), does not bind to the 52-kDa protein in the same experimental conditions (not shown).

In a second series of experiments, we checked that the 52-kDa dystrophin-binding component of the membrane was indeed part of the dystrophin-containing complexes that can be isolated from membranes with non-ionic detergents. Fig. 2A shows that the isolated complexes contain, in addition to dystrophin, proteins of M, 200,000, 87,000, 52,000, and 45,000. As observed by Butler (19), the 52-kDa protein is one of the major components of the complexes. Two-dimensional isoelectric focusing analysis of the 52-kDa protein (Fig. 2B) shows that it occurs in a range of isoelectric points (pI) of 6.4–6.7.

**Fig. 1. Dystrophin binding on proteins in AChR-enriched membranes, alkali extracts, and alkali-stripped membranes by blot overlay assay. Lane 1, Coomassie Blue staining of purified AChR-rich membrane proteins separated by 8% SDS–gel electrophoresis. D, corresponds to the position of dystrophin (430 kDa). Lanes 2 and 3, dystrophin binding assay on purified AChR membrane fraction. Dystrophin binds to a 52-kDa protein (lane 2, arrow). The band at ~80 kDa revealed in both lanes 2 and 3 (control) was an artifact of the detection method in this assay. Lane 2, binding assay; lane 3, control experiment in which dystrophin incubation was omitted. Lanes 4 and 5, dystrophin binding assay on alkali extract (S11). Dystrophin binds to a 52-kDa protein and a doublet protein of 130 kDa that was not detected in purified AChR-rich membrane proteins. Lane 4, binding assay; lane 5, control experiment. Lanes 6 and 7, dystrophin binding assay on alkali-stripped membranes. Lane 6, binding assay; lane 7, control experiment. Note that a high percentage of dystrophin was solubilized by alkali treatment (compare lanes 5 and 7). All fractions (AChR-rich membranes, alkali extracts, and alkali-stripped membranes) were recovered in the same volume of sample buffer, and an equal volume was deposited in each well to allow direct comparison.**
The binding of dystrophin to the proteins of the isolated complexes (Fig. 2A) shows that the 52-kDa component binds dystrophin (lane 2, binding assay; lane 3, control experiment). B, lane 1, isoelectrofocusing experiment of dystrophin-associated proteins reveals that the 52-kDa protein occurs as four isoelectric variants with isoelectric points ranging from 6.4 to 6.9 ± 0.1; lane 2, blot overlay experiment demonstrating that dystrophin binds to the charge variants of the 52-kDa protein. The spots on the left of the blot probably correspond to aggregated proteins that have not migrated in the isoelectrofocusing gel.

As four isoelectric variants (pI = 6.4–6.9), the 52-kDa protein is likely the 58-kDa protein described by Froehner that has similar molecular properties (39). The binding of dystrophin to the proteins of the isolated complexes (Fig. 2B) is additional experimental evidence of the capacity of the 52-kDa protein to bind to dystrophin in vitro. Moreover, all isoelectric variants were shown to be able to associate with dystrophin. Taken together, these in vitro binding studies suggest that the 52-kDa protein represents the "membrane receptor" for dystrophin in the postsynaptic membrane of the electrocyte. Its colocalization in situ with dystrophin and presence in the extracted dystrophin complexes is in full agreement with this conclusion. However, the nature of the interactions of the other components of the complexes is still unsolved. According to Butler et al. (19), the 52-kDa protein is the most constant element of the various complexes of dystrophin immunopurified either from anti-dystrophin or anti-58-kDa antibodies (19). Our data that stress the possibility for direct association of dystrophin with the 52-kDa protein suggest that the other components of the complexes, i.e., the 87-kDa protein and the other minor components, may be either loosely attached to dystrophin or indirectly associated with dystrophin via the 52-kDa protein.

Mammalian sarcolemmal and Torpedo synaptic dystrophin complexes disclose structural analogies. In particular, the extrinsic DAP 59 identified in rabbit sarcolemma by Campbell and co-workers (6, 38) is likely to represent the muscle counterpart of the Torpedo 52/58-kDa protein. Using monoclonal antibodies directed against the Torpedo 58-kDa protein, Froehner et al. (20) have shown that both the sarcolemmal and the neuromuscular junctions in rat muscle are labeled. The labeling, which corresponds to a 58-kDa protein, vanished from the sarcolemma in mdx mice skeletal muscle but persisted at the neuromuscular junction (19). The fact that the 59-kDa protein can be chemically cross-linked to dystrophin (41) indicates that these two molecules are close neighbors. Consequently, it was important to check the binding of dystrophin to the sarcolemmal 59-kDa protein. Using the same blot overlay assay, we show (Fig. 3) that dystrophin binds to a doublet of proteins of 58,000–60,000 in the rat sarcolemmal membrane. The present in vitro binding studies thus support the hypothesis that dystrophin complexes in postsynaptic membrane from Torpedo electrocyte and mammalian sarcolemma share common features and in particular point to a common functional anchoring site for dystrophin at the membrane.

The functional significance of the dystrophin accumulation in the excitatory membrane in Torpedo electrocyte is still unclear. At variance with sarcolemmal membranes, the subsynaptic membrane of the electrocyte is not particularly subjected to mechanical stress and therefore is not merely involved in membrane resilience. A peculiar structural feature of this membrane is its highly ordered supramolecular organization in which the AChR molecules are assembled into well ordered arrays in the plane of the membrane (42, 43). It has been shown that alkaline extraction of peripheral proteins (mainly the 43 kDa (ν1), 58 kDa, 87 kDa, and dystrophin) induces a striking structural disorganization of the membrane (reviewed in Ref. 44) but has little, if any, functional consequences (ligand binding, ionic fluxes) (27). During ontogenesis of the AChR-rich membrane, dystrophin (15) as well as 58-kDa protein (45) are among the first components of the membrane to accumulate in the early stages of the differentiation of the membrane (reviewed in Ref. 44), concomitantly with basal lamina components (i.e., laminin (46–47)). Despite the fact that dystrophin is apparently not tightly associated with the AChR molecules (Ref. 19 and this work), the involvement of dystrophin and associated proteins in the assembly and long range structural organization of the postsynaptic membrane is to be expected.

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