A Role for the Dystrophin-Glycoprotein Complex as a Transmembrane Linker between Laminin and Actin

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Abstract. The dystrophin-glycoprotein complex was tested for interaction with several components of the extracellular matrix as well as actin. The 156-kD dystrophin-associated glycoprotein (156-kD dystroglycan) specifically bound laminin in a calcium-dependent manner and was inhibited by NaCl (IC_{50} = 250 mM) but was not affected by 1,000-fold (wt/wt) excesses of lactose, IKVAV, or YIGSR peptides. Laminin binding was inhibited by heparin (IC_{50} = 100 μg/ml), suggesting that one of the heparin-binding domains of laminin is involved in binding dystroglycan while negatively charged oligosaccharide moieties on dystroglycan were found to be necessary for its laminin-binding activity. No interaction between any component of the dystrophin-glycoprotein complex and fibronectin, collagen I, collagen IV, entactin, or heparan sulfate proteoglycan was detected by ^{125}I-protein overlay and/or extracellular matrix protein-Sepharose precipitation. In addition, laminin-Sepharose quantitatively precipitated purified dystrophin-glycoprotein complex, demonstrating that the laminin-binding site is accessible when dystroglycan is associated with the complex. Dystroglycan of nonmuscle tissues also bound laminin. However, the other proteins of the striated muscle dystrophin-glycoprotein complex appear to be absent, antigenically dissimilar or less tightly associated with dystroglycan in nonmuscle tissues. Finally, we show that the dystrophin-glycoprotein complex cosediments with F-actin but does not bind calcium or calmodulin. Our results support a role for the striated muscle dystrophin-glycoprotein complex in linking the actin-based cytoskeleton with the extracellular matrix. Furthermore, our results suggest that dystrophin and dystroglycan may play substantially different functional roles in nonmuscle tissues.

SKELETAL muscle dystrophin (Anderson and Kunkel, 1992) has been isolated as part of a large, tightly associated oligomeric complex containing six other sarcolemmal proteins, four of which are glycoproteins (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ohlendieck et al., 1991*b*). Biochemical characterization of the dystrophin-glycoprotein complex suggests that four of the dystrophin-associated proteins are integral membrane proteins (Ervasti and Campbell, 1991) which bind the cysteine-rich and COOH-terminal domains of dystrophin (Suzuki et al., 1992), thus linking dystrophin to a highly glycosylated, extracellular component of 156 kD (Ervasti and Campbell, 1991). In support of its proposed extracellular location (Ervasti and Campbell, 1991), the 156-kD dystrophin-associated gycoprotein (156 kD dystroglycan) was shown to bind laminin (Ibraghimov-Beskrovnaya et al., 1992). Furthermore, dystrophin has been shown to colocalize with laminin in cultured myotubes (Dickson et al., 1992) while the dystrophin-glycoprotein complex and laminin have been shown to codistribute in cardiac muscle (Klietsch et al., 1993). Recently, Hemmings et al. (1992) demonstrated that a chimera comprising the first 233 amino acids of dystrophin and the last 645 amino acids of smooth muscle α-actinin localized to actin-containing structures when expressed in COS cells. In addition, bacterially expressed fusion proteins corresponding to the putative actin-binding domain of dystrophin have been shown to cosediment with F-actin (Hemmings et al., 1992; Way et al., 1992). Nuclear magnetic resonance experiments with synthetic peptides corresponding to defined regions of the NH_2-terminal domain of dystrophin provide evidence for two actin-binding sites on dystrophin located at amino acids 17–26 and 128–156 (Levine et al., 1992). Taken together, these results suggest that the marked reduction of the dystrophin-associated glycoproteins in muscle from *mdx* mice and DMD patients (Ervasti et al., 1990; Ohlendieck and Campbell, 1991*b*; Ohlendieck et al., 1993) disrupts a critical linkage between the actin-based, sarcolemmal cytoskeleton, and the extracellular matrix (Ervasti and Campbell, 1993) thus rendering dystrophic muscle fibers more susceptible to necrosis.

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However, several groups have noted an abnormal accumulation of collagen in Duchenne muscular dystrophy (Duance et al., 1980; Rampoldi et al., 1986; Marshall et al., 1989). These results suggest that the interaction of other extracellular matrix molecules with the sarcolemmal membrane may be affected by the absence of dystrophin. Furthermore, these results raise the question of whether the dystrophin-glycoprotein complex can interact with extracellular matrix molecules other than laminin.

With regard to F-actin binding to dystrophin, the experiments were performed with dystrophin polypeptide fragments (Hemmings et al., 1992; Way et al., 1992; Levine et al., 1992) which may expose binding sites not present in native dystrophin. For example, the targeting of the dystrophin/α-actinin chimera to the actin fibers and adhesion plaques of COS cells (Hemmings et al., 1992) is contrasted by the diffuse cytoplasmic (Ascadi et al., 1991) or plasma membrane location (Lee et al., 1991) of full-length dystrophin constructs expressed in COS cells. Furthermore, proteolytic cleavage of synapsin I results in fragments exhibiting threefold greater affinity for actin than native synapsin (Bahler et al., 1989). Since the NH_2-terminal fusion protein of dystrophin binds F-actin with an estimated K_d of 44 μM (Way et al., 1992), a threefold lower actin-binding affinity for native dystrophin corresponding to the difference between synapsin I fragments and native synapsin would bring into question the physiological relevance of F-actin binding to dystrophin.

To address these issues, we tested the purified dystrophin-glycoprotein complex for interaction with several purified components of the extracellular matrix as well as actin. Our results demonstrate that dystroglycan specifically binds laminin in a calcium- and ionic strength-dependent manner, whether alone or as part of the dystrophin-glycoprotein complex. Nonmuscle dystroglycan also binds laminin. However, the other proteins in the dystrophin-glycoprotein complex in striated muscle tissues appear to be absent, antigenically dissimilar, or less tightly associated in nonmuscle tissues. Finally, we show that the dystrophin-glycoprotein complex cosediments with F-actin but, unlike spectrin or α-actinin, does not bind calcium or calmodulin. Our results support a role for the striated muscle dystrophin-glycoprotein complex in linking the actin cytoskeleton with the extracellular matrix.

Materials and Methods

Isolation of Rabbit Tissue Membranes

KCl-washed rabbit skeletal muscle, cardiac muscle, brain and lung membranes, skeletal muscle triads, and surface membranes were prepared as previously described (Sharp et al., 1987; Ohlendieck et al., 1991*b*).

Preparation of Alkaline Extracts from Surface Membranes

5 mg of skeletal muscle surface membranes were diluted to a volume of 2 ml with 50 mM Tris-HCl, pH 7.4, 0.1 mM PMSF, 0.75 mM benzamidine, 2.5 μg/ml aprotinin, 93 μg/ml iodoacetamide, 2.5 μg ml leupeptin and 0.5 μg/ml pepstatin A, and titrated to pH 12 with 10 M NaOH. After a 1-h incubation at 22°C with mixing, the samples were centrifuged for 30 min at 100,000 *g*. The resulting supernatant (alkaline surface membrane extract) was decanted from the membrane pellets and titrated to pH 7.4 with 1 M HCl.

Purification of Dystrophin-Glycoprotein Complex

The dystrophin-glycoprotein complex was prepared from rabbit skeletal muscle membranes as previously described (Ervasti and Campbell, 1991). Alkaline-dissociated dystrophin-glycoprotein complex was prepared as previously described (Ervasti et al., 1991).

Nitrocellulose Transfer Overlays

EHS laminin (Sigma Chemical Co., St. Louis, MO; Collaborative Research Inc., Lexington, MA, Upstate Biotechnology, or the kind gift of Dr. Hynda K. Kleinman), bovine plasma fibronectin (Sigma Chem. Co.), human placenta merosin (Telios), recombinant mouse entactin (Upstate Biotechnology), heparan sulfate proteoglycan (Collaborative Research), and bovine brain calmodulin (Calbiochem-Novabiochem Corp., La Jolla, CA) were iodinated with [^{125}I]NaI by the Diabetes, Endocrinology Research Center at the University of Iowa using a lactoperoxidase/glucose oxidase reaction.

The iodinated extracellular matrix protein overlay procedure used was previously described (Ibraghimov-Beskrovnaya et al., 1992). In the case of laminin, for example, nitrocellulose transfers of SDS polyacrylamide gels containing the various samples were blocked overnight at room temperature in 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM triethanolamine, pH 7.6 (1× LBB) containing 5% nonfat dry milk, rinsed briefly in 1× LBB, and incubated for 2–3 h at room temperature in 1× LBB containing 3% BSA and 0.090 μg/ml (0.1 nM) ^{125}I-laminin. The nitrocellulose transfers were washed twice for 30 min at room temperature with 25–50 ml of 1× LBB, dried, and exposed to X-ray film. Lactose, IKVAV and YIGSR peptides, bovine trachea chondroitin sulfate A, bovine cornea keratan sulfate, and porcine intestinal mucosa heparin were all purchased from Sigma Chem. Co. and tested for their effects on laminin binding to dystroglycan by inclusion in the overlay medium at the indicated concentration or wt/wt ratio with respect to the ^{125}I-laminin concentration. The effects of Jacalin, Maackia amurensis lectin II, peanut agglutinin, Con A, and wheat germ agglutinin (all purchased from Vector Labs Inc., Burlingame, CA) on laminin binding to dystroglycan were tested at 1,000-fold (wt/wt) excess of the ^{125}I-laminin concentration.

Overlay of nitrocellulose transfers with $^{45}CaCl_2$ (Dupont New England Nuclear, Boston, MA) was performed by the method previously described to demonstrate calcium binding to erythrocyte and brain spectrin (Wallis et al., 1992).

Overlay of nitrocellulose transfers with ^{125}I-calmodulin in the presence of 1 mM $CaCl_2$ or 1 mM EGTA was performed as previously described (Flanagan and Yost, 1984). Transfers overlaid with ^{125}I-calmodulin also contained 0.3 μg rat brain Calmodulin kinase II (Hashimoto et al., 1987) which was the kind gift of Drs. Roger Colbran and Thomas Soderling.

Affinity Precipitation and Chromatography

EHS tumor laminin (Upstate Biotechnology or the kind gift of Dr. Hynda K. Kleinman), bovine plasma fibronectin (Sigma Chem. Co.), gelatin from porcine skin (Sigma Chem. Co.), rat tail collagen I (Collaborative Research), and EHS tumor collagen IV (Collaborative Research) were coupled to CNBr-activated Sepharose 4B (Sigma Chem. Co.). 0.9 ml of alkaline surface membrane extracts were diluted twofold with 0.28 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM triethanolamine, pH 7.6 (2× LBB) and 0.3 ml applied to 0.1 ml of laminin-, fibronectin-, gelatin-, collagen I-, or collagen IV-Sepharose which had been preequilibrated with 1× LBB containing 3% BSA and washed with three 0.3-ml aliquots of 1× LBB. After incubating overnight at 4°C with mixing, the Sepharose matrices were separated from the supernatants by a brief centrifugation and the supernatants (voids) removed. The Sepharose matrices were washed with three 0.3-ml aliquots of 1× LBB, and then solubilized in 0.3 ml of 1× LBB plus sample buffer for gel analysis. Equal volumes of alkaline surface membrane extracts, Sepharose voids, washes, and Sepharose matrices were analyzed by SDS-PAGE and immunoblotting. The collagen matrices were determined to be functional by their ability to precipitate purified fibronectin (all three matrices) as well as laminin (collagen IV-Sepharose) using the same method under the conditions described above. Dystroglycan binding to heparin was tested under identical conditions except heparin-agarose (Sigma Chem. Co.) was used as the affinity matrix and 8% beaded agarose (Sigma Chem. Co.) was included as a control.

To test for dystrophin-glycoprotein complex binding to the various Sepharose matrices, untreated or alkaline-dissociated dystrophin-glycoprotein complex (44 μg) was diluted fourfold such that the final buffer conditions were 0.1% digitonin, 44 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/

ml *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (Sigma Chem. Co.), 50 mM Tris-HCl, pH 7.4 (DLB)[1]. 0.3 ml of the diluted dystrophin-glycoprotein complex was added to 0.1 ml of laminin-, fibronectin-, gelatin-, collagen I-, or collagen IV-Sepharose which had been first washed with 0.5 ml DLB containing 10 mM Tris-EDTA, pH 8.0, followed by three 0.5-ml washes with DLB. After incubation for 12 h with mixing at 4°C, the Sepharose matrices were washed three times with 0.3-ml aliquots of DLB and subsequently eluted with two 0.3-ml aliquots (1 h each elution) of DLB containing 10 mM Tris-EDTA, pH 8.0 (in the case of laminin-Sepharose), or with one 0.5-ml aliquot of DLB containing 10 mM Tris-EDTA, pH 8.0, and 0.5 M NaCl (all other Sepharose matrices). The Sepharose matrices were then solubilized in 0.3 ml of 1× LBB plus sample buffer for gel analysis. Equal volumes of dystrophin-glycoprotein complex, Sepharose voids, washes, and Sepharose matrices were analyzed by SDS-PAGE and immunoblotting.

Laminin affinity chromatography of detergent-solubilized membranes was performed under conditions identical to those used for affinity precipitation of the dystrophin-glycoprotein complex. Twenty-five mg of rabbit KCl-washed skeletal muscle, brain, cardiac muscle, and lung membranes were solubilized in 5 ml of 1% digitonin, 0.44 M NaCl, 0.1 mM PMSF, 0.75 mM benzamidine, 2.5 μg/ml aprotinin, 2.5 μg ml leupeptin, and 0.5 μg/ml pepstatin A and 50 mM Tris-HCl, pH 7.4. 3.0 ml of the solubilized membranes were diluted tenfold with 1.33 mM $CaCl_2$, 1.33 mM $MgCl_2$, 13.3 μg/ml *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 50 mM Tris-HCl, pH 7.4, to reduce the digitonin and NaCl concentrations to 0.1% and 44 mM, respectively. After incubation overnight with mixing at 4°C with 0.75 ml of laminin-Sepharose which had been preequilibrated with DLB, the laminin-Sepharose was separated from the supernatant (void) by a brief centrifugation, and then washed with five 0.75-ml aliquots of DLB. The laminin-Sepharose was subsequently eluted with two 0.75-ml aliquots (1 h each solution) of DLB containing 10 mM Tris-EDTA, pH 8.0. The two EDTA eluates were pooled and 15 ml of each laminin-Sepharose void was concentrated tenfold in a Centriprep 30 for gel analysis. Equal volumes of solubilized membranes, Sepharose voids, and EDTA eluates were analyzed by SDS-PAGE and immunoblotting.

Chemical and Enzymatic Treatments

Chemical deglycosylation of alkaline surface membrane extracts using trifluoromethanesulfonic acid (TFMS) was performed as previously described (Burgess and Norman, 1988). Briefly, 1 ml of alkaline surface membrane extracts were lyophilized in a 5-ml Reactivial (Pierce, Rockford, IL) and incubated under nitrogen for 4 h on ice with 0.392 ml anisole and 0.588 ml TFMS (Sigma Chem. Co.). The reaction was terminated with 1.568 ml ice-cold pyridine/H_2O (3:5 vol/vol) and dialyzed at 4°C overnight after 4 liters of H_2O. The dialyzed sample was extracted with anhydrous ether, lyophilized, and resolubilized in 1 ml of H_2O. Dystrophin-glycoprotein complex was treated with neuraminidase as previously described (Ervasti and Campbell, 1991). The dystrophin-glycoprotein complex was digested with alkalase (Novo, New York, NY) by incubating 72 μg of dystrophin-glycoprotein complex which had been titrated to pH 9 using 1 M NaOH, for 2 h at 60°C in the presence of 0.1% alkalase (Linhardt et al., 1992).

Actin Cosedimentation Assay

The buffer conditions in the actin cosedimentation assay were based on those recently used to demonstrate actin cosedimentation with a fusion protein corresponding to the NH_2-terminal domain of dystrophin (Hemmings et al., 1992). 0.5 ml of dystrophin-glycoprotein complex (0.116 mg/ml) was applied to a Pharmacia PD-10 column (Sephadex G-25 M), which had been preequilibrated with 25 ml of 0.1% digitonin, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT, 10 mM Tris-HCl, pH 7.4 (ABB), and eluted according to the manufacturer's instructions. The PD-10 eluate was concentrated to 0.25 ml in a Centricon 100 (Amicon, Beverly, MA) for use in the actin cosedimentation assay. Various amounts of rabbit muscle actin (Sigma Chem. Co.) dissolved in ABB were added to 40 ml of the concentrated PD-10 eluate and actin polymerization was initiated by the addition of NaCl and $MgCl_2$ to the final concentrations of 100 mM and 3 mM, respectively, in a total volume of 0.1 ml. After incubation at room temperature for 1 h, the samples were centrifuged at 100,000 *g* for 30 min, the supernatants removed, and the actin pellets resuspended in ABB plus SDS sample buffer. Equal volumes of the supernatants and resuspended actin pellets were analyzed by SDS-PAGE and immunoblotting.

Antibodies

Preparation of sheep polyclonal antisera against the dystrophin-glycoprotein complex was previously described (Ohlendieck and Campbell, 1991*b*). Polyclonal antibodies specific for fusion protein D, which corresponds to the core protein of dystroglycan, were affinity-purified from the sheep polyclonal antisera against the dystrophin-glycoprotein complex as previously described (Ibraghimov-Beskrovnaya et al., 1992). The preparation and characterization of monoclonal antibodies IIH6 and $VIA4_1$, specific for dystroglycan and the dystrophin-specific monoclonal antibody XIXC2 have previously been described (Ervasti and Campbell, 1991; Ervasti et al., 1990; Jorgensen et al., 1990; Ohlendieck et al., 1991*b*). Monoclonal antibody IIH6 was purified from tissue culture media by a previously described method (Imagawa et al., 1987) except Sephacryl S-400 was used instead of Sepharose CL-4B. Monoclonal antibodies specific for chondroitin sulfate and keratin sulfate were obtained from Sigma Chem. Co. and ICN Biomedicals, Inc., Costa Mesa, CA, respectively.

SDS-PAGE and Immunoblotting

SDS-PAGE (Laemmli, 1970) was carried out on 3–12% gradient gels in the presence of 1% 2-mercaptoethanol and stained with Coomassie blue, Stains-All (Campbell et al., 1983), Alcian Blue (Al-Hakim and Linhardt, 1991), or transferred to nitrocellulose (Towbin et al., 1979). Molecular weight standards shown in the figures were purchased from GIBCO BRL (Gaithersburg, MD). Nitrocellulose transfers were stained with polyclonal antisera, affinity-purified polyclonal antibodies, or monoclonal antibodies as previously described (Campbell et al., 1987). Coomassie blue–stained gels and autoradiograms were analyzed densitometrically using a model 300S scanning densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Laminin-binding Properties of Dystroglycan

A number of commercially available purified extracellular matrix components were radiolabeled and tested for binding to dystrophin-glycoprotein complex which had been electrophoretically separated on SDS polyacrylamide gels and transferred to nitrocellulose. As previously reported (Ibraghimov-Beskrovnaya et al., 1992), ^{125}I-laminin bound to a protein band in crude skeletal muscle surface membranes and purified dystrophin-glycoprotein complex corresponding to 156 kD dystroglycan (Fig. 1 *A*). ^{125}I-Merosin also labeled dystroglycan, albeit more weakly than EHS laminin (not shown). Overexposed autoradiograms revealed additional laminin-binding proteins of 100 and 60 kD in the crude surface membrane preparation (not shown). However, these additional laminin-binding proteins were less abundant in pure sarcolemma than in crude muscle membranes, suggesting that they are either peripheral proteins which were removed by the KCl wash step or were a component of a distinct vesicle population.

The binding of ^{125}I-laminin to dystroglycan was inhibited by the inclusion of 10 mM EDTA (Fig. 1 *A*). The absence of $CaCl_2$, but not $MgCl_2$ from the overlay medium also inhibited ^{125}I-laminin to dystroglycan (not shown). ^{125}I-laminin binding to dystroglycan was also completely inhibited by inclusion of NaCl to the overlay medium (Fig. 1 *A*) with an average half-maximal concentration for inhibition (IC_{50}) of 250 mM.

The binding of ^{125}I-laminin to dystroglycan was inhibited by the inclusion of an excess of unlabeled laminin but

1. *Abbreviations used in this paper*: ABB, actin binding buffer (0.1% digitonin, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT, 10 mM Tris-HCl, pH 7.4); DLB, digitonin laminin binding buffer (0.1% digitonin, 44 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane, 50 mM Tris-HCl, pH 7.4); LBB, laminin binding buffer (140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM triethanolamine, pH 7.6); TFMS, trifluoromethanesulfonic acid.