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.

S KELETAL 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., 1991b). 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, 1991b; 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., 1991b).

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 CaCl₂, 1.33 mM MgCl₂, 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/H₂O (3:5 vol/vol) and dialyzed at 4°C overnight after 4 liters of H₂O. The dialyzed sample was extracted with anhydrous ether, lyophilized, and resolubilized in 1 ml of H₂O. 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 NH₂-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 CaCl₂, 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 MgCl₂ to the final concentrations of 100 mM and 3 mM, respectively, in

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1. *Abbreviations used in this paper*: ABB, actin binding buffer (0.1% digitonin, 0.2 mM CaCl₂, 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 CaCl₂, 1 mM MgCl₂, 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 CaCl₂, 1 mM MgCl₂, 10 mM triethanolamine, pH 7.6); TFMS, trifluoromethanesulfonic acid.

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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 VIA4₁, 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), ¹²⁵I-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*). ¹²⁵I-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 ¹²⁵I-laminin to dystroglycan was inhibited by the inclusion of 10 mM EDTA (Fig. 1 *A*). The absence of CaCl₂, but not MgCl₂ from the overlay medium also inhibited ¹²⁵I-laminin to dystroglycan (not shown). ¹²⁵I-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 (IC₅₀) of 250 mM.

The binding of ¹²⁵I-laminin to dystroglycan was inhibited by the inclusion of an excess of unlabeled laminin but
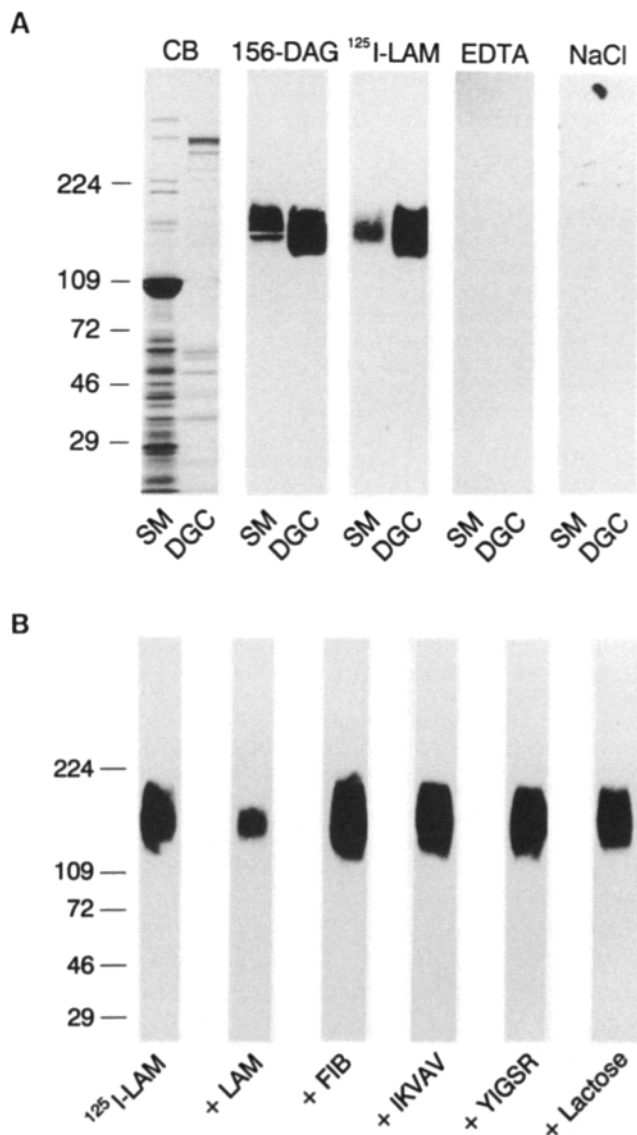
Figure 1. Laminin-binding properties of dystroglycan. Shown in A is 50 μg of rabbit skeletal muscle surface membranes (SM) and 10 μg of dystrophin-glycoprotein complex (DGC) which were electrophoretically separated on a 3–12% SDS polyacrylamide gel and stained with Coomassie blue (CB). Also shown in A is a nitrocellulose transfer stained with monoclonal antibody IIH6 to dystroglycan (156-DAG) or corresponding autoradiograms of transfers overlaid with ^{125}I-laminin in the absence (^{125}I-LAM) or presence of 10 mM EDTA (EDTA) or 0.5 M NaCl (NaCl). Shown in B are the corresponding autoradiograms of nitrocellulose transfers containing 4 μg of electrophoretically separated dystrophin-glycoprotein complex which were overlaid with ^{125}I-laminin in the absence (^{125}I-LAM) or presence of 1,000-fold excesses (wt/wt) of nonradioactive laminin (+LAM), fibronectin (+FIB), IKVAV peptide (+IKVAV), YIGSR peptide (+YIGSR), or in the presence of 0.1 M lactose (+Lactose). The molecular weight standards (×10^{-3}) are indicated on the left.

not fibronectin (Fig. 1 B) or entactin (not shown). Furthermore, no component of the dystrophin-glycoprotein complex bound ^{125}I-fibronectin, ^{125}I-entactin, or ^{125}I-heparan sulfate proteoglycan as assayed by the nitrocellulose transfer overlay method (not shown).

A number of short synthetic peptides corresponding to sequences within laminin can serve as adhesion substrates for a variety of cell types and receptors (Yamada, 1991). Two well characterized peptides, YIGSR (Graf et al., 1987) and IKVAV (Tashiro et al., 1989), had no effect on ^{125}I-laminin binding to dystroglycan when included in 1,000-fold (wt/wt) excess to the overlay medium (Fig. 1 B). Lactose, which inhibits laminin binding to a family of galactoside-binding lectins (Mecham, 1991), also had no effect on ^{125}I-laminin binding to dystroglycan (Fig. 1 B).

Heparin Blocks Laminin Binding to Dystroglycan

The ionic strength sensitivity of laminin binding to dystroglycan and its extensive posttranslational modification (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992) imply that dystroglycan may bind to one of the domains of laminin important in binding heparin (Kouzi-Koliakos et al., 1989; Skubitz et al., 1988). Heparin inhibited ^{125}I-laminin binding to dystroglycan with an IC$_{50}$ of 100 μg/ml while chondroitin sulfate and keratan sulfate were without effect (Fig. 2). While laminin was quantitatively precipitated by heparin-agarose (not shown), only a small fraction of dystroglycan from alkaline surface membrane extracts specifically bound to heparin-agarose (Fig. 2).

The ionic strength dependence (Fig. 1) and heparin inhibition (Fig. 2) of laminin binding to dystroglycan suggest that polyanionic moieties are important to the laminin-binding activity of dystroglycan and further imply that the extensive posttranslational modification of dystroglycan may include glycosaminoglycan addition. However, treatment of dystrophin-glycoprotein complex or alkaline surface membrane extracts with nitrous acid, heparitinase, chondroitinase ABC, or keratanase, all of which specifically remove glycosaminoglycan chains (Soroka and Farquhar, 1991), have no effect on the electrophoretic migration of dystroglycan, its antibody reactivity or ^{125}I-laminin binding while monoclonal antibodies specific for keratan sulfate and chondroitin sulfate failed to stain dystroglycan on nitrocellulose transfers (not shown). Dystroglycan is stained with Stains-All and Alcian Blue (Fig. 3), two dyes which bind polyanionic molecules including sialylated glycoproteins and proteoglycans (King and Morrison, 1976; Kinne and Fisher, 1987). Neuraminidase treatment of dystrophin-glycoprotein complex resulted in the characteristic 10 kD decrease in the size of dystroglycan (Ervasti and Campbell, 1991) which remained strongly reactive with Stains-All and Alcian Blue (Fig. 3), suggesting that the negatively charged sialic acid residues are not responsible for the interaction of these dyes with dystroglycan. Neuraminidase treatment was also without effect on ^{125}I-laminin binding to dystroglycan (Fig. 3) as were N-Glycosidase F and O-Glycosidase treatment (not shown). Finally, 1,000-fold excesses of Jacalin, peanut agglutinin, Maackia amurensis lectin II, Con A or wheat germ agglutinin, lectins specific for sugar moieties common to most O- or N-linked oligosaccharide side chains, had no effect on ^{125}I-laminin binding to dystroglycan as assessed by the blot overlay method (not shown). Taken together, these results suggest that polyanionic moieties that are not a component of the N- and O-linked oligosaccharides of dystroglycan are important in binding laminin.

Chemical deglycosylation with TFMS has been used to remove N- or O-linked oligosaccharides as well as glycosaminoglycan chains from proteoglycans (Soroka and Farquhar, 1991). When alkaline surface membrane extracts were chemically deglycosylated with TFMS before SDS-PAGE, the affinity-purified antibodies to the fusion protein
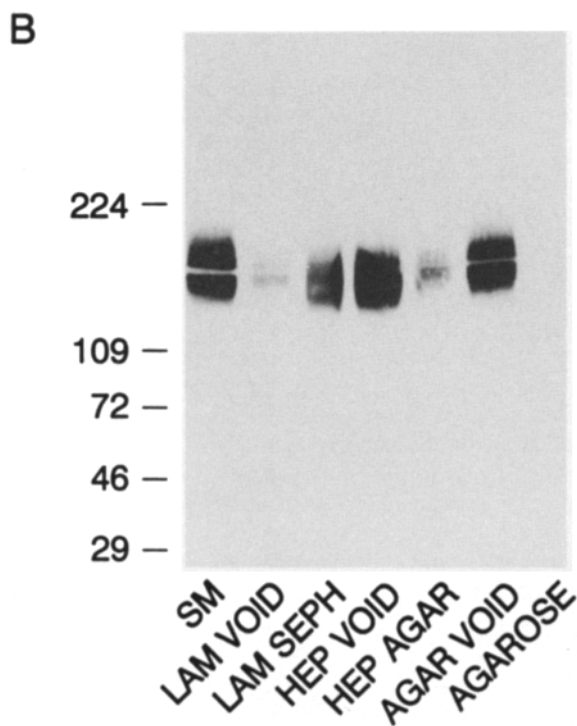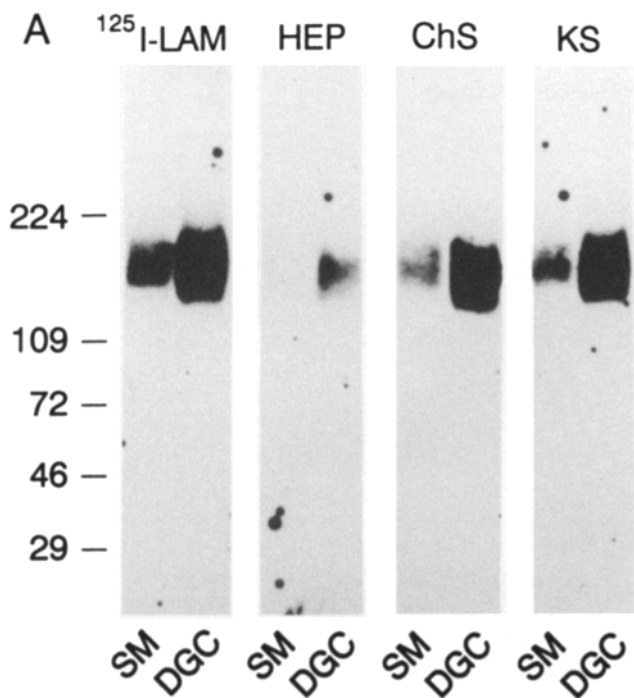
A

¹²⁵I-LAM   HEP   ChS   KS

224 —
109 —
72 —
46 —
29 —

SM DGC   SM DGC   SM DGC   SM DGC

B

224 —
109 —
72 —
46 —
29 —

SM, LAM VOID, LAM SEPH, HEP VOID, HEP AGAR, AGAR VOID, AGAROSE

Figure 2. Heparin inhibition of laminin binding to dystroglycan. Shown in *A* are corresponding autoradiograms of transfers from SDS polyacrylamide gels containing 50 μg of rabbit skeletal muscle surface membranes (*SM*) and 10 μg of dystrophin-glycoprotein complex (*DGC*) which were overlaid with ¹²⁵I-laminin in the absence (¹²⁵I-LAM) or presence of 1,000-fold excesses (wt/wt) of heparin (*HEP*), chondroitin sulfate (*ChS*), or keratan sulfate (*KS*). Shown in *B* is a nitrocellulose transfer stained with monoclonal antibody IIH6 to dystroglycan containing equal volumes of alkaline surface membrane extracts (*SM*), the voids after incubation of alkaline surface membrane extracts with laminin-Sepharose (*LAM VOID*), heparin-agarose (*HEP VOID*) or agarose (*AGAR VOID*), and the washed laminin-Sepharose (*LAM SEPH*), heparin-agarose (*HEP AGAR*), or agarose (*AGAROSE*). The molecular weight standards (×10⁻³) are indicated on the left.

CB   Stains-All   Alcian Blue

224 —
109 —
72 —
46 —
29 —

Con Neur Alk   Con Neur Alk   Con Neur Alk

IIH6   anti-FPD   ¹²⁵I-LAM

224 —
109 —
72 —
46 —
29 —

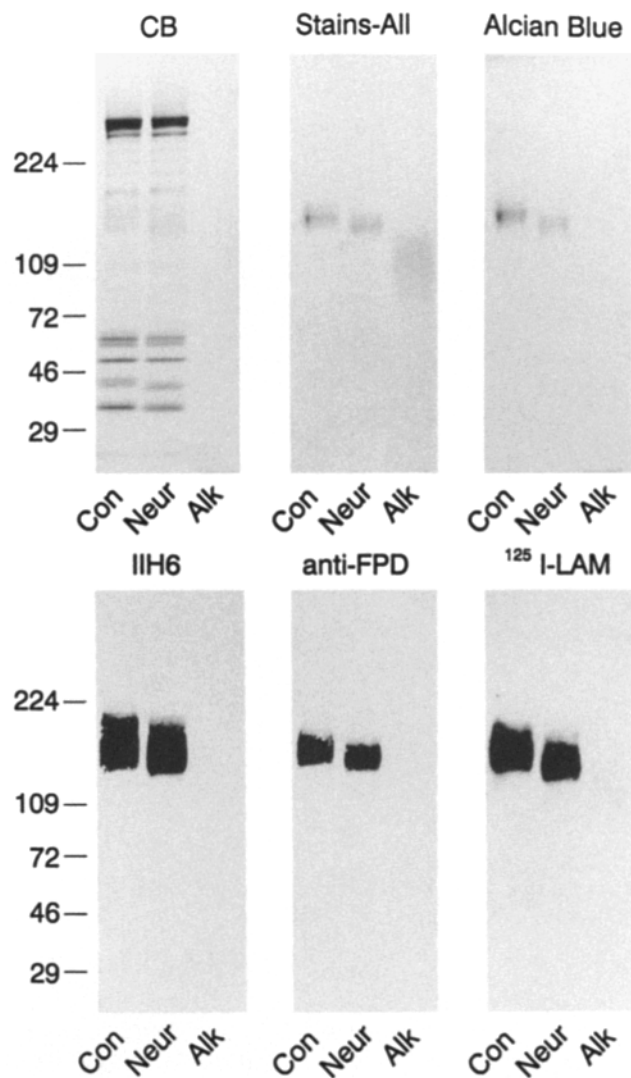Con Neur Alk   Con Neur Alk   Con Neur Alk

Figure 3. Effect of neuraminidase and alkalase treatment on laminin binding to dystroglycan. Shown are SDS polyacrylamide gels stained with Coomassie blue (*CB*), Stains-All (*Stains-All*), or Alcian Blue (*Alcian Blue*) which contain untreated control (*Con*), neuraminidase-treated (*Neur*) and alkalase-treated (*Alk*) dystrophin-glycoprotein complex. Also shown are identical nitrocellulose transfers stained with monoclonal antibody IIH6 to dystroglycan (*IIH6*), polyclonal antibodies specific to fusion protein D representing the core protein sequence of dystroglycan (*Anti-FPD*) or the corresponding autoradiogram of a transfer overlaid with ¹²⁵I-laminin (¹²⁵I-LAM). The molecular weight standards (×10⁻³) are indicated on the left.

representing the core protein of dystroglycan stained a 57-kD band (Fig. 4 *A*) which corresponds well with the size of the core protein predicted from cDNA sequence (Ibraghimov-Beskrovnaya et al., 1992). Neither did the monoclonal antibody IIH6 stain the 57-kD band recognized by the fusion protein antibodies nor did the 57-kD band bind ¹²⁵I-laminin (Fig. 4 *A*). Periodic acid treatment (Wu et al., 1991) of alkaline surface membrane extracts also resulted in loss of monoclonal antibody IIH6 staining and ¹²⁵I-laminin binding to dystroglycan (not shown). Fusion proteins corresponding to sequences in the 43/156-kD dystrophin-associated glycoprotein precursor protein (Ibraghimov-Beskrovnaya et al., 1992) failed to bind ¹²⁵I-laminin as assayed by the nitrocel-
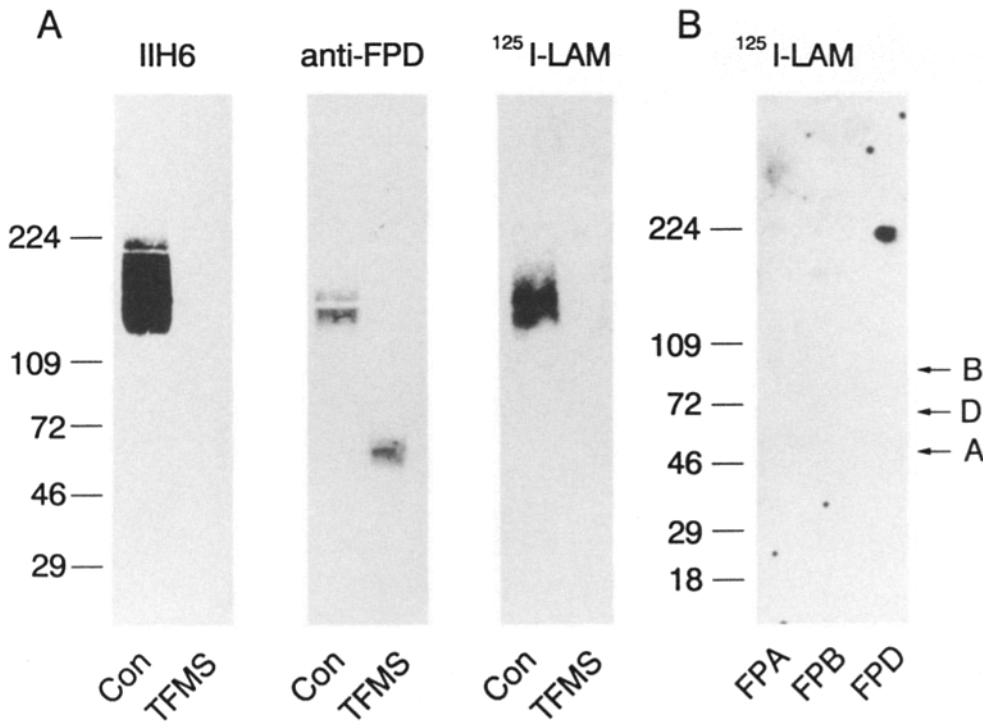
Figure 4. Chemically deglycosylated dystroglycan or its corresponding fusion proteins do not bind laminin. Shown in *A* are nitrocellulose transfers containing untreated control (*Con*) and TFMS-treated (*TFMS*) alkaline surface membrane extracts stained with monoclonal antibody IIH6 to dystroglycan (*IIH6*) or affinity-purified polyclonal antibodies specific to fusion protein D corresponding to the core protein sequence of dystroglycan (*Anti-FPD*) or the corresponding autoradiogram of a transfer overlaid with ^{125}I-laminin (*^{125}I-LAM*). Shown in *B* is the corresponding autoradiogram of a transfer containing fusion proteins A (*FPA*), B (*FPB*), and D (*FPD*) representing sequences present in the core protein of dystroglycan overlaid with ^{125}I-laminin (*^{125}I-LAM*). The arrows on the right mark the migration of the fusion proteins which were detected with Ponceau S before the ^{125}I-laminin overlay procedure. The molecular weight standards ($\times 10^{-3}$) are indicated on the left.

lulose overlay method (Fig. 4 *B*). These results suggest that negatively charged oligosaccharides other than sialic acid are necessary for the laminin-binding activity of dystroglycan. On the other hand, digestion of the dystrophin-glycoprotein complex with the nonspecific protease alkalase (Linhardt et al., 1992) yielded a 100-kD smear detected with Stains-All which did not bind ^{125}I-laminin, monoclonal antibody IIH6, or fusion protein D-specific antibodies (Fig. 3). This result suggests that the core protein of dystroglycan is also necessary for laminin binding to dystroglycan.

Laminin-Sepharose Binding of Dystroglycan and the Dystrophin-Glycoprotein Complex

Various Sepharose-conjugated extracellular matrix proteins were also tested for their ability to bind dystroglycan from alkaline surface membrane extracts of skeletal muscle. SDS polyacrylamide gel analysis demonstrated that dystroglycan was completely removed from the voids of alkaline surface membrane extracts incubated with laminin-Sepharose and remained bound to the laminin-Sepharose after several washes (Fig. 5 *A*). Dystrophin and the 59-kD dystrophin-associated protein, which are also present in alkaline surface membrane extracts (Ervasti and Campbell, 1991), remained in the laminin-Sepharose voids (not shown), indicating that neither protein bound to laminin-Sepharose. Dystroglycan was not bound by fibronectin-, gelatin-, collagen I-, or collagen IV-Sepharose (Fig. 5 *B*), further demonstrating that the

interaction between laminin and dystroglycan is selective and specific.

One of the RGD recognition sequences present in laminin has been reported to be cryptic in the intact molecule (Aumailley et al., 1990). Likewise, the SDS-denaturation and alkaline treatment necessary to isolate dystroglycan for use in the above assays may also have exposed laminin binding-sites on dystroglycan which are not accessible when it is part of the dystrophin-glycoprotein complex. Thus, laminin-Sepharose binding of purified dystrophin-glycoprotein complex was attempted. <25% of the dystrophin-glycoprotein complex was bound by laminin-Sepharose in the presence of 0.1% digitonin and 140 mM NaCl (not shown). An inhibitory effect of detergents used to solubilize membrane-bound laminin receptors which bind ^{125}I-laminin with high affinity in a blot overlay assay but fail to bind laminin-Sepharose has been noted (Lesot et al., 1983; Smalheiser and Schwartz, 1987; Douville et al., 1988). That the inhibitory effect of detergents on laminin binding is reportedly ionic strength dependent (Lesot et al., 1983) led us to attempt laminin-Sepharose binding of the dystrophin-glycoprotein complex in the presence of lower NaCl concentrations. Gel analysis of the laminin-Sepharose voids after incubation with dystrophin-glycoprotein complex in the presence of 0.1% digitonin and 44 mM NaCl demonstrated specific and quantitative removal of the dystrophin-glycoprotein complex from the void while a 109-kD contaminant of dystrophin-glycoprotein complex preparations was not bound by the laminin-Sepharose (Fig. 6). Recovery of the dystrophin-glycoprotein
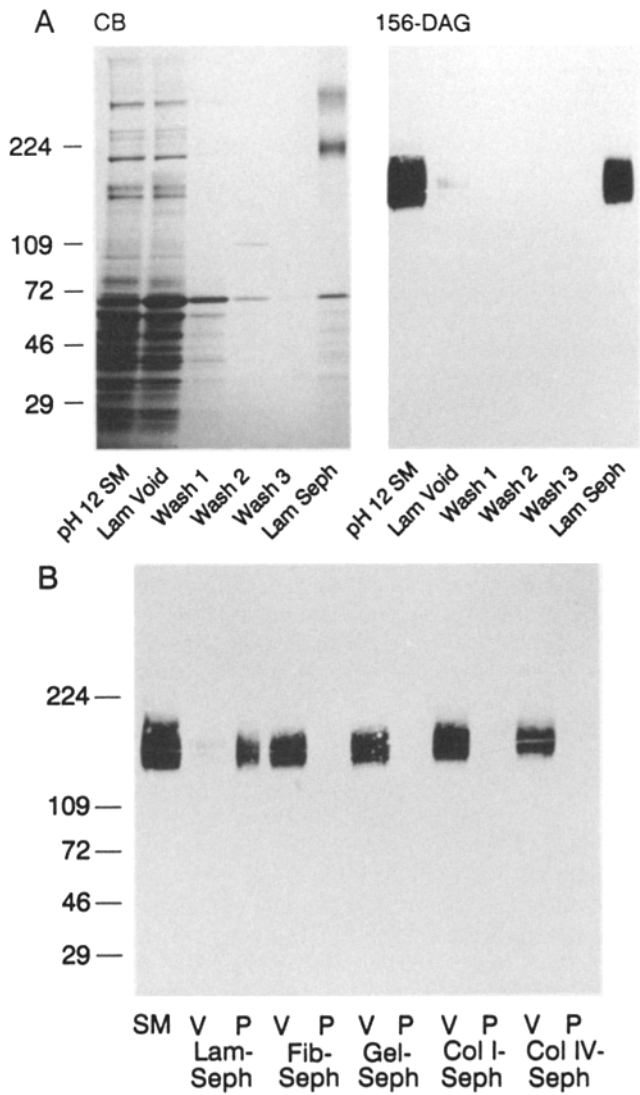
Figure 5. Laminin-Sepharose binding of dystroglycan from alkaline surface membrane extracts. Shown in A is a Coomassie blue–stained gel (CB) or identical nitrocellulose transfer stained with monoclonal antibody IIH6 to dystroglycan (156-DAG) containing equal volumes of alkaline surface membrane extracts (pH 12 SM), laminin-Sepharose void (Lam Void), three washes of the laminin-Sepharose (Wash 1, 2, and 3), or the washed laminin-Sepharose (Lam Seph). Shown in B is a nitrocellulose transfer containing equal volumes of alkaline surface membrane extracts (SM) and the voids (V) and Sepharose pellets (P) of alkaline surface membrane extracts after incubation with laminin-, fibronectin-, gelatin-, collagen I-, or collagen IV-Sepharose which was stained with monoclonal antibody IIH6 to dystroglycan. The molecular weight standards (×10⁻³) are indicated on the left.

Figure 6. Laminin-Sepharose binding of the dystrophin-glycoprotein complex. Shown are a Coomassie blue–stained gel (CB) or identical nitrocellulose transfer stained with monoclonal antibody IIH6 to dystroglycan (IIH6) containing equal volumes of untreated or alkaline-dissociated dystrophin-glycoprotein complex (DGC), the voids after incubation with laminin-Sepharose (LAM VOID), and the two subsequent EDTA washes of the laminin-Sepharose (EDTA 1 and 2). The asterisk marks the position of the ~109-kD contaminant typically found in dystrophin-glycoprotein complex preparations. The molecular weight standards (×10⁻³) are indicated on the left.

complex in EDTA-elutions of the laminin-Sepharose (Fig. 6) demonstrated that binding of the complex to laminin-Sepharose is reversible and dependent on divalent cations. As observed for uncomplexed dystroglycan (Fig. 5 B), the dystrophin-glycoprotein complex was not bound by fibronectin-, gelatin-, collagen I-, or collagen IV-Sepharose (not shown). Thus, these data demonstrate that the laminin-binding site is not a cryptic site, but is accessible when dystroglycan is associated with the dystrophin-glycoprotein complex.
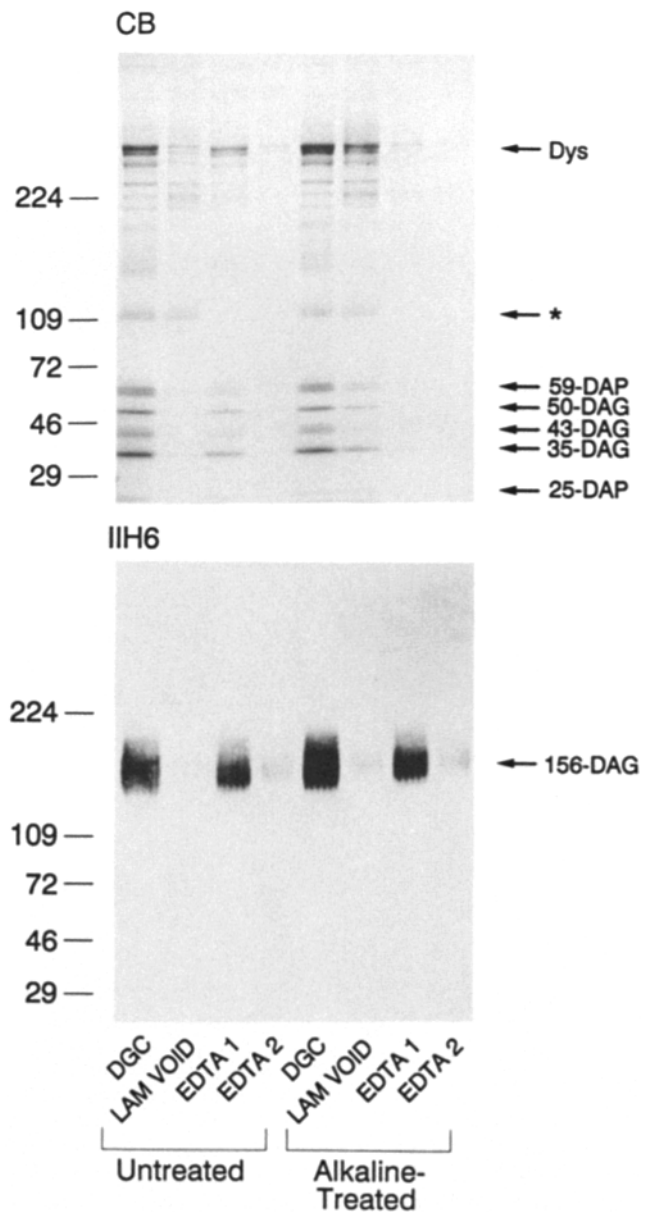
We have previously demonstrated that all of the components in the dystrophin-glycoprotein complex no longer cosediment on sucrose density gradients (Ervasti et al., 1991) or coimmunoprecipitate (Ervasti and Campbell, 1991) after alkaline dissociation. To determine whether the interaction between laminin and dystroglycan is solely responsible for the binding of the dystrophin-glycoprotein complex by laminin-Sepharose, the void and the proteins bound to laminin-Sepharose after incubation with alkaline-dissoci-
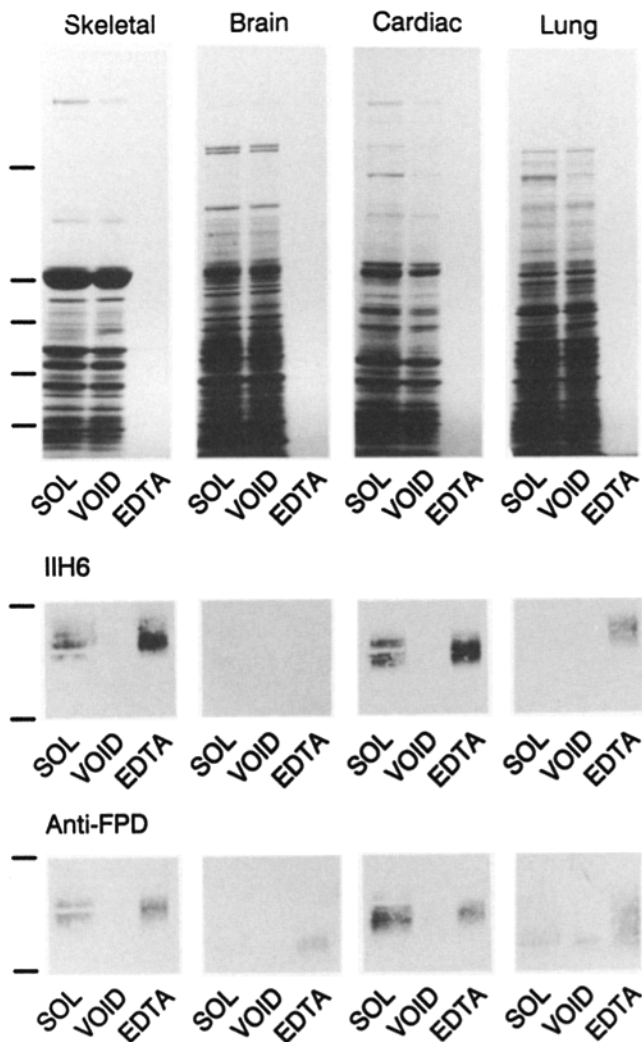
Figure 7. Laminin-Sepharose binds dystroglycan from various tissues. Shown are Coomassie blue–stained gels and portions of identical nitrocellulose transfers stained with monoclonal antibody IIH6 to dystroglycan (*IIH6*) or affinity-purified polyclonal antibodies specific to fusion protein D corresponding to the core protein sequence of dystroglycan (*Anti-FPD*) containing equal volumes of digitonin-solubilized skeletal muscle, brain, cardiac muscle, or lung membranes (*SOL*), the voids after incubation of the solubilized membranes with laminin-Sepharose (*VOID*), or the EDTA eluates from each laminin-Sepharose after extensive washing (*EDTA*). Because the laminin-Sepharose was EDTA-eluted in a volume that was only one-half that of the original solubilate, the amount of EDTA eluate loaded on the gels represents twice the protein equivalents loaded for the solubilates and voids. The migration of the molecular weight standards (224, 109, 72, 46, and 29 $\times$ 10³ from *top to bottom*) are indicated on the left of the Coomassie blue–stained gel. Only the portion of the nitrocellulose transfers that lies between the 224,000 and 109,000 molecular weight standards is illustrated.

ated dystrophin-glycoprotein complex was analyzed by SDS-PAGE and immunoblotting (Fig. 6). The laminin-Sepharose quantitatively bound dystroglycan from the alkaline-treated dystrophin-glycoprotein complex while dystrophin, the 59, 50, 43, 35, and 25 kD dystrophin-associated proteins remained largely in the void (Fig. 6). Because dystroglycan appears to be the only link between dystrophin and laminin, the quantitative binding of the dystrophin-glycoprotein complex

by laminin-Sepharose further demonstrates that dystroglycan is a stoichiometric component of the complex.

Nonmuscle Dystroglycan Binds Laminin

While skeletal muscle dystroglycan exhibits binding properties consistent with its hypothesized function as a laminin receptor, the broad tissue distribution of dystroglycan (Ibraghimov-Beskrovnaya et al., 1992) raises the question of whether this protein also functions as a laminin receptor in nonmuscle tissues. To address this question, digitonin-solubilized rabbit skeletal muscle, brain, cardiac muscle, and lung membranes were incubated with laminin-Sepharose. After removal of the voids and extensive washing, the laminin-Sepharose matrices were eluted with incubation buffer containing 10 mM EDTA. The volume of the EDTA elution was equal to one-half that of the original solubilate applied to the laminin-Sepharose. Equal volumes of the resulting voids and EDTA eluates were then compared to the original solubilate by SDS-PAGE and immunoblotting (Fig. 7). The Coomassie blue–stained gel revealed that none of the major protein constituents of the original solubilates were depleted from the laminin-Sepharose voids while very few proteins were detected in the laminin-Sepharose eluates (compare SOL, VOID, and EDTA Fig. 7, *Top*). Identical immunoblots were stained with monoclonal antibody IIH6 to dystroglycan or affinity-purified polyclonal antibodies to a fusion protein corresponding to the core protein of dystroglycan (Fig. 7, *Bottom*). Immunoblot analysis demonstrated that an immunoreactive band corresponding to dystroglycan was significantly reduced in the laminin-Sepharose voids of solubilized skeletal muscle, cardiac muscle, and lung membranes which was recovered in the EDTA eluates. Monoclonal antibody IIH6 reacts very weakly with brain dystroglycan (Ibraghimov-Beskrovnaya et al., 1992) and was not useful in evaluating laminin-Sepharose binding to the brain. In addition, the immunoreactive bands detected with the fusion protein antibodies were barely detectable in the original solubilates from brain making it impossible to evaluate their depletion in the void. However, the fusion protein antibody clearly detected a band of ∼120 kD, which is characteristic of brain dystroglycan (Ibraghimov-Beskrovnaya et al., 1992), in the laminin-Sepharose EDTA eluate of solubilized brain (Fig. 7). These results demonstrate that dystroglycan of muscle and nonmuscle tissues binds laminin.

The presence of dystroglycan in nonmuscle tissues presents the possibility of the existence of a dystrophin-glycoprotein complex in non-muscle tissues which is similar to that found in skeletal muscle (Ervasti et al., 1990; Ohlendieck et al., 1991b, Ervasti and Campbell, 1991). The laminin-Sepharose EDTA eluates from solubilized skeletal muscle, brain, cardiac muscle, and lung membranes were concentrated tenfold and analyzed by SDS-PAGE, immunoblotting, and ¹²⁵I-laminin overlay (Fig. 8). Although the Coomassie blue–stained gel revealed numerous proteins in the concentrated EDTA eluates from each tissue, only dystroglycan was detectable by ¹²⁵I-laminin overlay (Fig. 8). Most notably apparent from the Coomassie blue–stained gel, however, was a 400-kD protein present in the eluates from skeletal and cardiac membranes but absent from brain and lung eluates. The 400-kD protein in the skeletal and cardiac eluates was stained very intensely with sheep antiserum raised against the dystrophin-glycoprotein complex
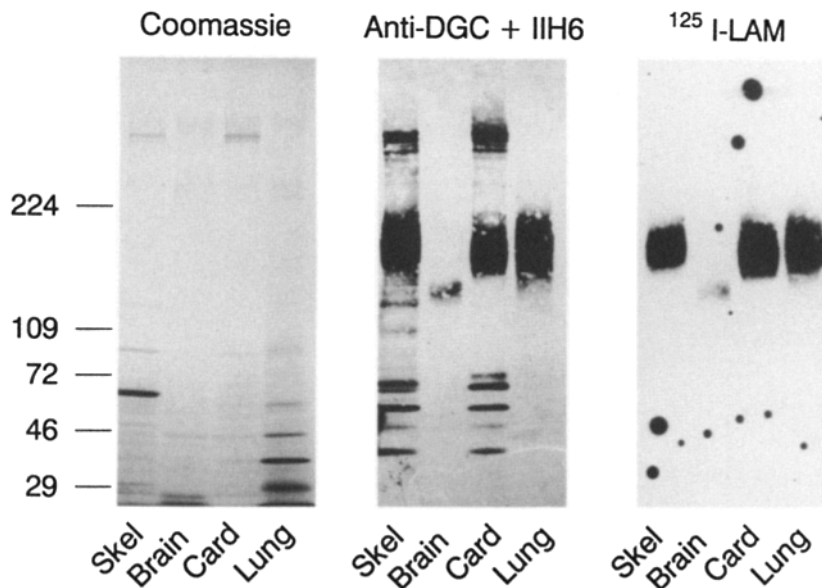
Coomassie   Anti-DGC + IIH6 ^{125} I-LAM

224 —

109 —
72 —

46 —

29 —

Skel Brain Card Lung   Skel Brain Card Lung   Skel Brain Card Lung

Figure 8. Analysis of laminin-Sepharose eluates from various tissues. Shown are the laminin-Sepharose EDTA eluates described in Fig. 7 concentrated 10-fold, electrophoretically separated on an SDS polyacrylamide gel and stained with Coomassie blue (*Coomassie*), an identical nitrocellulose transfer stained with sheep antiserum against the dystrophin-glycoprotein complex and monoclonal antibody IIH6 to dystroglycan (*Anti-DGC+IIH6*), or the corresponding autoradiogram of a transfer overlaid with ^{125}I-laminin (*^{125}I-Lam*). The molecular weight standards ($\times 10^{-3}$) are indicated on the left.

(Fig. 8) as well as by monoclonal antibody XIXC2 (not shown) which is specific for dystrophin (Ervasti et al., 1990; Ohlendieck et al., 1991b; Ohlendieck and Campbell, 1991a; Ervasti and Campbell, 1991). In addition to dystrophin and dystroglycan, immunoreactive bands corresponding to the 59, 50, 43, and 35 kD dystrophin-associated proteins were detected in both the skeletal and cardiac laminin-Sepharose EDTA eluates (Fig. 8). With respect to the brain and lung laminin-Sepharose EDTA eluates, however, immunoreactive bands corresponding only to dystroglycan and the 43-kD dystrophin-associated glycoprotein were detected with a cocktail of monoclonal antibody IIH6 and sheep antiserum to the dystrophin glycoprotein complex (Fig. 8). These results demonstrate that the dystrophin-glycoprotein complex is similar in skeletal and cardiac muscle. Furthermore, the data suggest that if a dystrophin-glycoprotein complex exists in nonmuscle tissues, it is of grossly different structure and/or composition than the complex expressed in muscle.

The Binding Sites on Dystroglycan for Monoclonal Antibody IIH6 and Laminin Overlap

It is apparent from the results presented in Figs. 3 and 4 that treatments that result in the loss of staining of dystroglycan by monoclonal antibody IIH6 are accompanied by the loss of ^{125}I-laminin binding by dystroglycan, suggesting that the binding sites for monoclonal antibody IIH6 and laminin overlap. On the other hand, brain dystroglycan is not detectable with monoclonal antibody IIH6 but is retained by laminin-Sepharose, which implies that while the binding sites for monoclonal antibody IIH6 and laminin overlap, they are not identical. To test whether the binding of monoclonal antibody IIH6 affects laminin binding to dystroglycan, nitrocellulose transfers containing dystrophin-glycoprotein complex were preincubated with tissue culture media containing either monoclonal antibody IIH6, VIA4₁, which is also against dystroglycan (Ervasti et al., 1990), or monoclonal antibody XIXC2 to dystrophin (Ervasti et al., 1990; Ohlendieck et al., 1991b) and subsequently overlaid with ^{125}I-laminin (Fig. 9 *A*). Preincubation with monoclonal IIH6 almost completely inhibited ^{125}I-laminin binding to dystro-

glycan. ^{125}I-laminin binding to dystroglycan was not affected by preincubation of the blots with monoclonal antibodies VIA4₁ and XIXC2 (Fig. 9 *A*). Inclusion of increasing concentrations of purified IIH6 also inhibited ^{125}I-laminin binding to dystroglycan with complete inhibition observed at a IIH6/laminin ratio (wt/wt) of 100 (Fig. 9 *B*). Conversely, inclusion of increasing concentrations of laminin to a limiting concentration of purified IIH6, blocked IIH6 staining of dystroglycan with complete inhibition observed at a laminin/IIH6 (wt/wt) ratio of 10 (Fig. 9 *C*). These results demonstrate that the binding sites for IIH6 and laminin on dystroglycan overlap.

The Dystrophin-Glycoprotein Complex Interacts with Actin but Not Calcium or Calmodulin

Sequence comparisons have shown that the NH₂-terminal domain of dystrophin is homologous to the actin-binding domain of α-actinin, β-spectrin, and actin-binding protein 120 (Koenig et al., 1988; Karinch et al., 1990; Bresnick et al., 1990) and recently, fusion proteins corresponding to the sequence of the NH₂-terminal domain of dystrophin have been shown to cosediment with F-actin (Hemmings et al., 1992; Way et al., 1992). To test whether the dystrophin-glycoprotein complex is capable of interacting with actin, a cosedimentation assay based on that used to demonstrate dystrophin fusion protein/actin cosedimentation (Hemmings et al., 1992) was performed. In the absence of actin or in the presence of actin concentrations below the apparent critical concentration for polymerization, the dystrophin-glycoprotein complex was found completely in the supernatant after a 30-min centrifugation at 100,000 *g* while incubation of dystrophin-glycoprotein complex with actin concentrations above the apparent critical concentration for polymerization resulted in sedimentation of up to 50% of the dystrophin-glycoprotein complex with the actin pellet (Fig. 10). These results indicate that the native dystrophin-glycoprotein complex is capable of binding F-actin.

As reviewed by Dubreuil et al. (1991), calcium has been shown to inhibit the binding of nonmuscle α-actinin and some isoforms of spectrin with actin and some spectrins have
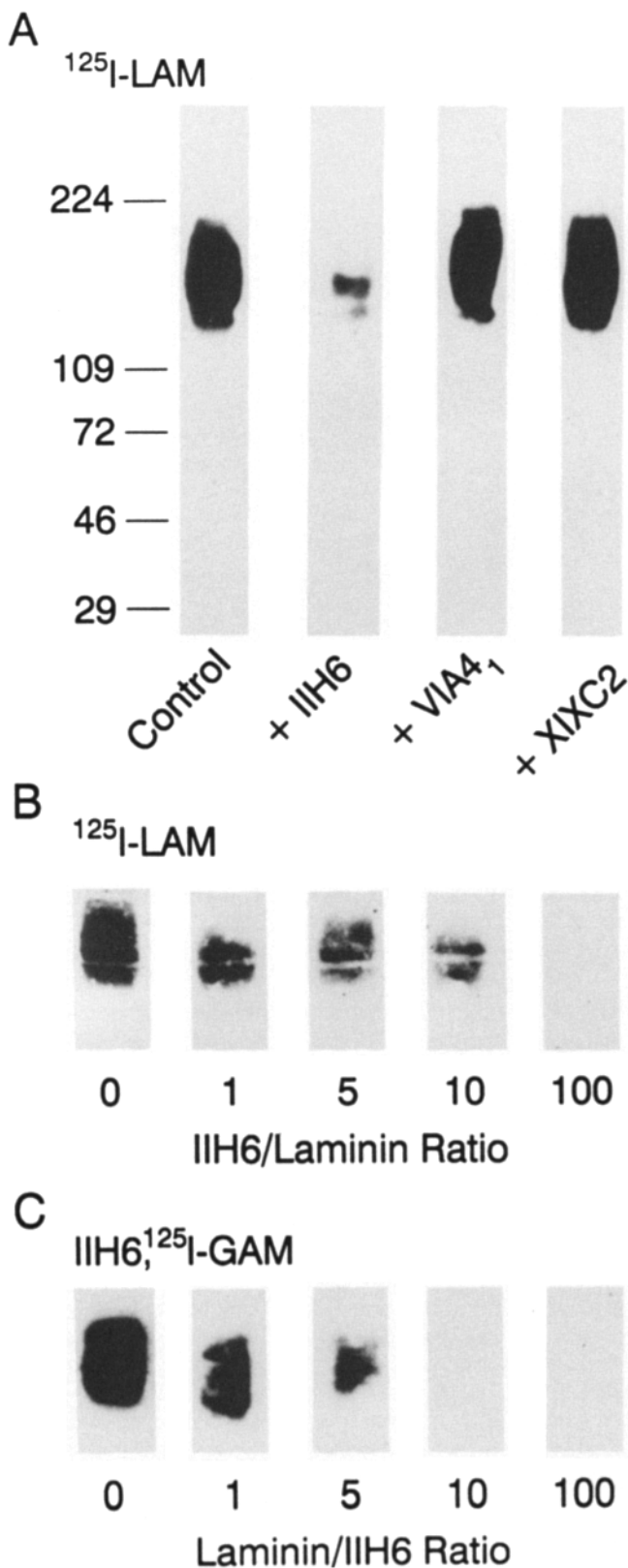
A

^{125}I-LAM

224 —

109 —

72 —

46 —

29 —

Control + IIH6 + VIA4$_1$ + XIXC2

B

^{125}I-LAM

0 1 5 10 100

IIH6/Laminin Ratio

C

IIH6,^{125}I-GAM

0 1 5 10 100

Laminin/IIH6 Ratio

Figure 9. The binding sites on dystroglycan for IIH6 and laminin overlap. Shown in *A* are the corresponding autoradiograms of nitrocellulose transfers containing 4 μg of electrophoretically separated dystrophin-glycoprotein complex which were untreated (*Control*) or first incubated with tissue culture media containing monoclonal antibodies IIH6 (*IIH6*), VIA4$_1$ (*VIA4$_1$*), or XIXC2 (*XIXC2*) and subsequently overlaid with ^{125}I-laminin. The molecular weight

Coomassie

224 —

109 —
72 —

46 —

29 —

← DYS

← Actin

Anti-DGC + IIH6

224 —

109 —

72 —

46 —

29 —

S P S P S P S P

[Actin] 0 50 250 500

← DYS
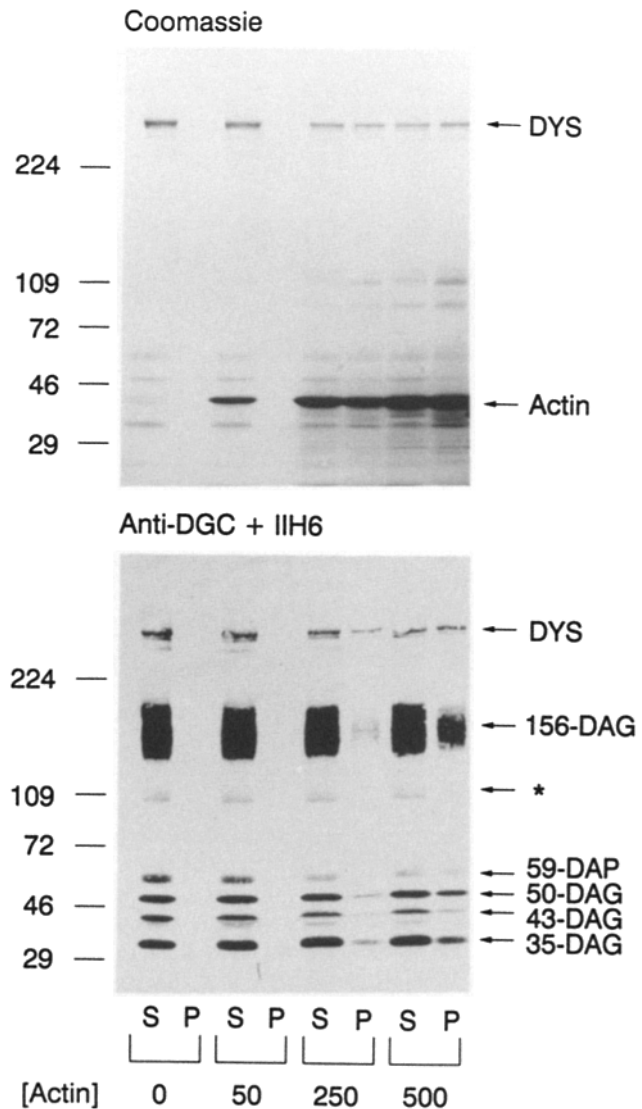← 156-DAG
← *
← 59-DAP
← 50-DAG
← 43-DAG
← 35-DAG

Figure 10. Cosedimentation of the dystrophin-glycoprotein complex with actin. Shown are a Coomassie blue–stained gel (*Coomassie*) or a nitrocellulose transfer stained with sheep polyclonal antisera to the dystrophin-glycoprotein complex and monoclonal antibody IIH6 to dystroglycan (*Anti-DGC + IIH6*) containing equal volumes of supernatants (*S*) and pellets (*P*) of dystrophin-glycoprotein complex incubated with the indicated concentrations of rabbit muscle actin (μg/ml) and subsequently centrifuged at 100,000 *g* for 30 min. The asterisk marks the position of the ∼109-kD contaminant typically found in dystrophin-glycoprotein complex preparations. The molecular weight standards (× 10^{-3}) are indicated on the left.

standards (× 10^{-3}) are indicated on the left. Shown in *B* are nitrocellulose transfers containing 100 μg surface membranes which were overlaid with ^{125}I-laminin in the absence or presence of added pure monoclonal antibody IIH6 to attain the indicated IIH6/^{125}I-laminin ratio (wt/wt). Shown in *C* are nitrocellulose transfers containing 100 μg surface membranes which were incubated with pure monoclonal antibody IIH6 in the absence or presence of added laminin to attain the indicated laminin/IIH6 ratio (wt/wt). After extensive washing, IIH6 staining of the transfers illustrated in *C* was detected with ^{125}I-labeled goat anti–mouse IgM. Only the portion of the nitrocellulose transfer that lies between the 224,000 and 109,000 molecular weight standards is illustrated in *B* and *C*.
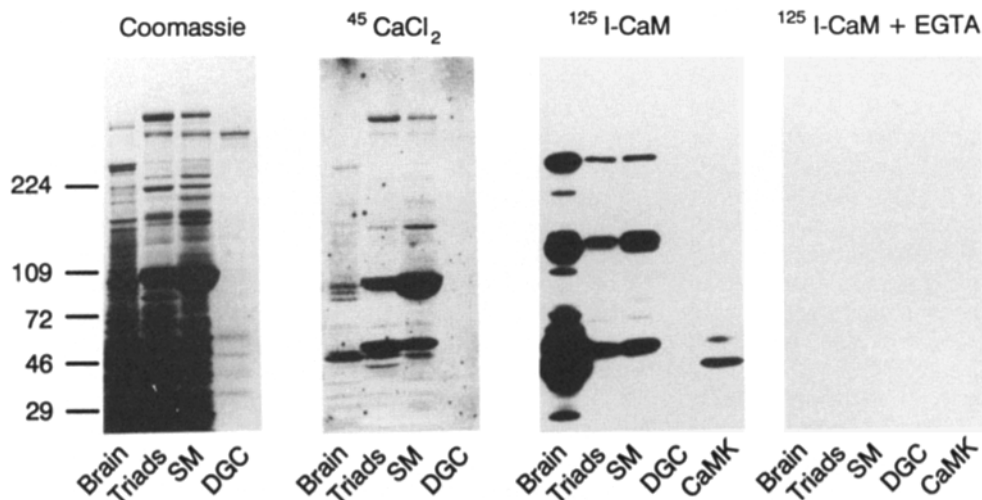
Figure 11. The dystrophin-glycoprotein complex does not bind Ca^{2+} or Calmodulin. Shown is a Coomassie blue-stained gel (*Coomassie*) containing rabbit brain membranes, triads, surface membranes (*SM*), and dystrophin-glycoprotein complex (*DGC*). Also shown are identical nitrocellulose transfers overlaid with $^{45}CaCl_2$ (*$^{45}CaCl_2$*) or ^{125}I-Calmodulin in the presence of 1 mM $CaCl_2$ (*^{125}I-CaM*) or 1 mM EGTA (*^{125}I-CaM + EGTA*). The transfers used for ^{125}I-Calmodulin overlay also contained 0.3 µg rat brain Calmodulin kinase II (*CaMK*). The molecular weight standards ($\times 10^{-3}$) are indicated on the left.

been shown to bind calcium and calmodulin directly. The sequence similarity between dystrophin, spectrin, and α-actinin suggests the possibility that calcium may regulate dystrophin-actin interactions in a similar manner. As an initial test of this hypothesis, $^{45}Ca^{2+}$ overlays of nitrocellulose transfers containing rabbit brain membranes, triads, and dystrophin-glycoprotein complex were performed under conditions which have been used to demonstrate calcium binding to erythrocyte and brain spectrins (Wallis et al., 1992). $^{45}Ca^{2+}$ bound to the upper band of a doublet of ~ 260 kD in rabbit brain membranes which is presumably brain α-spectrin (Fig. 11, Brain). $^{45}Ca^{2+}$ bound to three known calcium-binding proteins of skeletal muscle; the ryanodine receptor, the sarcoplasmic reticulum Ca^{2+}-ATPase and calsequestrin present in triads and as contaminants of crude surface membrane preparations (Fig. 11, Triads, SM). However, $^{45}Ca^{2+}$ did not bind to any component of the dystrophin-glycoprotein complex (Fig. 11, DGC). Neither did ^{125}I-calmodulin overlay reveal any component of the dystrophin-glycoprotein complex, in the presence of 1 mM $CaCl_2$ or 1 mM EGTA (Fig. 11), nor was dystrophin-glycoprotein complex affinity-precipitated by calmodulin-Sepharose (not shown). These data indicate that skeletal muscle dystrophin does not bind calcium or calmodulin which suggests that the dystrophin-actin interaction in skeletal muscle may not be regulated by calcium. The $^{45}CaCl_2$ overlay data further suggest that the calcium effect on laminin binding to dystroglycan (Fig. 1) is exerted through laminin and not dystroglycan.

Discussion

The results of the present study demonstrate that the native skeletal muscle dystrophin-glycoprotein complex is capable of binding both actin and laminin which supports a role for the complex in linking the actin-based cytoskeleton with the extracellular matrix. The drastic reduction of the dystrophin-associated glycoproteins in muscle from *mdx* mice and DMD patients (Ervasti et al., 1990; Ohlendieck and Campbell, 1991b; Ohlendieck et al., 1993) is evidence that alteration in dystrophin expression profoundly affects integral

components of the sarcolemmal membrane. Absence of dystrophin thus may compromise the integrity and flexibility of the sarcolemma, leading to either mechanical damage (Weller et al., 1990; Menke and Jockusch, 1991; Stedman et al., 1991) or alteration in specific calcium-regulatory mechanisms (Franco and Lansman, 1990; Turner et al., 1991) of the sarcolemmal membrane. That dystrophin comprises two percent of sarcolemmal protein (Ohlendieck et al., 1991b) and five percent of the sarcolemmal cytoskeleton (Ohlendieck and Campbell, 1991a) also supports a role for dystrophin in maintaining skeletal muscle architecture. Finally, dystrophin-associated glycoproteins are specifically reduced (Matsumura et al., 1993) or absent (Matsumura et al., 1992b) from the muscle of patients with severe, Duchenne-like muscular dystrophies in which the abundance and structure of dystrophin is normal. These results suggest that the cytoskeletal-sarcolemma-extracellular matrix linkage formed by the dystrophin-glycoprotein complex can be disrupted in a manner independent of a defect in dystrophin.

Implication of the four spectrin-like repeats of α-actinin in binding the β_1-integrin subunit led to the suggestion that the spectrin-like repeat domain of dystrophin may also interact with the sarcolemma through an integrin (Otey et al., 1990). However, there is currently no evidence for a direct interaction between dystrophin and integrin. While the fibronectin-specific $\alpha_5\beta_1$ integrin has been observed to codistribute with dystrophin at specific stages during development of chick embryonic myotubes (Lakonishok et al., 1992), Dickson et al. (1992) have observed distinct and nonoverlapping distribution patterns for dystrophin and the β_1 integrin in mouse myotubes. The results of the present work indicate that dystroglycan and the purified dystrophin-glycoprotein complex interact with laminin but do not bind entactin, heparan sulfate proteoglycan, fibronectin, or collagens. While many other extracellular matrix constituents remain to be tested for interaction with the dystrophin-glycoprotein complex, these data argue in favor of a specific interaction between laminin and the dystrophin-glycoprotein complex.

The majority of experiments in this study used laminin purified from mouse EHS tumors which is composed of three subunits: a 400-kD A chain and two distinct 200-kD

chains designated B1 and B2 (Timpl et al., 1987). Recent studies have shown that laminin is a member of a family of proteins which vary in their subunit structure and composition. Skeletal muscle predominately expresses a protein named merosin, which differs from laminin in that the A chain is replaced by a structurally homologous M chain (Sanes et al., 1990; Engvall et al., 1990). Laminin, and merosin to a lesser extent, can be further substituted by replacement of the B1 chain with a homologous subunit named S-laminin (Sanes et al., 1990; Engvall et al., 1990). More recently, it was shown that cardiac muscle laminin contains a 300-kD heavy chain which is immunochemically similar to the M chain of merosin (Paulsson et al., 1991). We previously noted that both S-laminin (Sanes et al., 1990) and dystrophin-related protein are specifically localized to the neuromuscular junction (Ohlendieck et al., 1991a) and it is apparent that merosin (Sanes et al., 1990) and dystrophin (Ohlendieck et al., 1991a) exhibit similar distributions throughout the sarcolemmal membrane, including the neuromuscular junction. Recently, we demonstrated (Matsumura et al., 1992a) that dystrophin-related protein is associated with a complex of glycoproteins that is identical or antigenically similar to the complex associated with dystrophin (Ervasti and Campbell, 1991). In the present work, we have noted that merosin purified from human placenta also bound dystroglycan as detected by the blot overlay method. Curiously, the autoradiographic intensity of ^{125}I-merosin binding to dystroglycan did not appear as strong as that observed for ^{125}I-laminin when performed in parallel. While this is not the anticipated result, there are several possible explanations for our observation. SDS-polyacrylamide gel electrophoretic analysis has demonstrated that purified merosin preparations contain polypeptides of 600, 300, and 180-200 kD as well as minor components of 60-90 kD (Ehrig et al., 1990; Ervasti, J. M., and K. D. Campbell, unpublished observations). Thus, it is possible that the commercial merosin preparation is impure or proteolyzed, either of which could account for its reduced signal intensity (in comparison to laminin) in the blot overlay assay. Furthermore, there is precedent for an integrin binding to a nonnative laminin with greater affinity than it exhibits for its native ligand (Sonnenberg et al., 1991). Additional experiments will be necessary to understand the nature of this apparent difference between laminin and merosin in binding dystroglycan.

The emerging importance of laminin in skeletal muscle development (Goodman et al., 1989; von der Mark et al., 1991) raises the question of whether dystroglycan plays a role in skeletal muscle differentiation. In cultured muscle cells, dystrophin expression is not evident in myoblasts before fusion (Lev et al., 1987). Thus, laminin binding by dystroglycan would not be expected to mediate any early events in skeletal muscle differentiation, assuming that expression of the components of the dystrophin-glycoprotein complex is coordinately regulated. However, our finding that monoclonal antibody IIH6 blocks laminin binding to dystroglycan (Fig. 9) suggests that this antibody may be useful in further delineating the function of the dystrophin-glycoprotein complex through the possible perturbation of laminin-dystroglycan interactions in vivo.

Until recently, the actin-binding properties of dystrophin have largely been speculated from its sequence homologies with well characterized actin-binding proteins (Koenig et al.,

1988; Karinch et al., 1990; Bresnick et al., 1990). Hemmings et al. (1992) demonstrated that a chimera comprised of the first 233 amino acids of dystrophin and the last 645 amino acids of smooth muscle alpha-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). However, the apparent dissociation constant of actin binding for one of these fusion proteins was estimated at 44 μM (Way et al., 1992) which the authors noted was ten times greater than the K_d value obtained for filamin and two orders of magnitude greater than the apparent K_d of α-actinin dimer binding to actin. Although the concentration dependence of dystrophin-glycoprotein complex binding to F-actin was not rigorously determined, we observed significant (50%) dystrophin-glycoprotein complex cosedimentation with F-actin using an effective dystrophin concentration of 0.1 μM (Fig. 10), suggesting that the native dystrophin-glycoprotein complex binds F-actin with an affinity similar to native α-actinin. Whether the difference between dystrophin fusion proteins and the dystrophin-glycoprotein complex with respect to F-actin binding affinity is due to an intact dystrophin molecule, dystrophin dimerization or a modulatory effect by one of the dystrophin-associated glycoproteins will require further investigation. Furthermore, dystrophin does not appear to be directly associated with the myofibrillar actin filaments (Watkins et al., 1988; Bonilla et al., 1988) which raises the issue of what actin-based structures skeletal muscle dystrophin may interact with in vivo. Peripheral actin filaments emanating from the Z lines and M lines of skeletal muscle myofibers have recently been identified (Bard and Franzini-Armstrong, 1991) while γ-actin (Craig and Pardo, 1983) and dystrophin (Porter et al., 1992) are two of several cytoskeletal proteins which exhibit discrete, lattice-like organizations comprised of a longitudinal element and transverse elements coincident with the I bands and M lines. The low abundance of γ-actin in adult skeletal muscle would also favor its interaction with dystrophin from the standpoint of stoichiometry. In addition, the recent identification of novel actin-related proteins (Lees-Miller et al., 1992; Clark and Meyer, 1992) raises the possibility for discovery of a unique actin-like protein in skeletal muscle which specifically binds dystrophin. In the meantime, our present results demonstrate that the dystrophin-glycoprotein complex has the capacity to bind F-actin.

The cysteine-rich region of dystrophin shows significant homology to a domain of *Dictyostelium* α-actinin that contains two Ca^{2+}-binding sites (Koenig et al., 1988). Thus, like nonmuscle α-actinin and some spectrins (Dubreuil et al., 1991), dystrophin-actin interactions are conceivably affected by calcium. However, calcium was not found to bind any component of the skeletal muscle dystrophin-glycoprotein complex (Fig. 11) under $^{45}CaCl_2$ overlay conditions identical to those used in demonstrating direct calcium binding to spectrin (Wallis et al., 1992). At variance with Madhavan et al. (1992), we have detected no interaction between any component of the dystrophin-glycoprotein complex and calmodulin (Fig. 11). Since calmodulin is a cytosolic protein, the observation that biotinylated calmodulin interacts with 156 kD dystroglycan (Madhavan et al., 1992) conflicts strikingly with the proposition that it is wholly extracellular

based on its extensive glycosylation (Fig. 7 and Ervasti et al., 1990; Ervasti and Campbell, 1991), laminin-binding properties (Figs. 1–9), membrane extraction properties (Ohlendieck and Campbell, 1991a; Ervasti and Campbell, 1991), and lack of a predicted transmembrane domain (Ibraghimov-Beskrovnaya et al., 1992). While the sum of these results provide support for calcium independent dystrophin-actin interactions in skeletal muscle, they leave open the possibility that nonmuscle isoforms of dystrophin or its autosomal homologue dystrophin-related protein (Love et al., 1989) may function in a calcium-dependent manner as is the case for nonmuscle α-actinin.

While the distribution of dystrophin in skeletal muscle (Porter et al., 1992) supports a role for dystrophin in stabilizing the sarcolemmal membrane, its distribution in cardiac and smooth muscle (Byers et al., 1991), cortical neurons (Lidov et al., 1990), and Torpedo electrocytes (Yeadon et al., 1991; Sealock et al., 1991) suggest that dystrophin may play more varied roles in noncontractile tissues. The 43-kD dystrophin-associated glycoprotein and 156-kD dystroglycan are also expressed in nonmuscle tissues (Ibraghimov-Beskrovnaya et al., 1992). However, while nonmuscle dystroglycan binds laminin (Fig. 7), it does not appear to form a complex with full-length dystrophin as in cardiac and skeletal muscle (Fig. 8). Nonmuscle dystroglycan may bind to the novel dystrophin isoforms which are expressed in some nonmuscle tissues (Rapaport et al., 1992; Blake et al., 1992; Lederfein et al., 1992) at levels comparable to that of full-length dystrophin in striated muscle (Hoffman et al., 1987). On the other hand, actin binding as a universal function of dystrophin must also be reconsidered in light of these novel dystrophins, which completely lack the putative actin-binding domain (Rapaport et al., 1992; Blake et al., 1992; Lederfein et al., 1992).

In conclusion, our results demonstrate that the skeletal muscle dystrophin-glycoprotein complex can bind both actin and laminin and suggest that dystrophin serves as a specialized link between the actin cytoskeleton and the extracellular matrix. It is clear, however, that the same function cannot be immediately extrapolated to all tissues. Clarification of the role of dystrophin in nonmuscle tissues awaits further investigation.

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