

Differential Heparin Inhibition of Skeletal Muscle α -Dystroglycan Binding to Laminins*

(Received for publication, August 28, 1995, and in revised form, November 10, 1995)

Elizabeth A. Pall‡, Kevin M. Bolton, and James M. Ervasti§

From the Department of Physiology, University of Wisconsin Medical School, Madison, Wisconsin 53706

The laminin binding properties of α -dystroglycan purified from rabbit skeletal muscle membranes were examined. In a solid phase microtiter assay, ^{125}I -laminin (laminin-1) bound to purified α -dystroglycan in a specific and saturable manner with a half-maximal concentration of 8 nM. The binding of ^{125}I - α -dystroglycan to native laminin and merosin (a mixture of laminin-2 and -4) was also compared using the solid phase assay. The absolute binding of ^{125}I - α -dystroglycan to laminin (6955 ± 250 cpm/well) was similar to that measured for merosin (7440 ± 970 cpm/well). However, inclusion of 1 mg/ml heparin in the incubation medium inhibited ^{125}I - α -dystroglycan binding to laminin by $84 \pm 4.3\%$ but inhibited ^{125}I - α -dystroglycan binding to merosin by only $17 \pm 5.2\%$. Similar results were obtained with heparan sulfate, while de-*N*-sulfated heparin, hyaluronic acid, and chondroitin sulfate had no differential effect. These results were confirmed by iodinated laminin and merosin overlay of electrophoretically separated and blotted dystrophin-glycoprotein complex. In contrast to the results obtained with skeletal muscle α -dystroglycan, both laminin and merosin binding to purified brain α -dystroglycan was significantly inhibited by heparin. Our data support the possibility that one or more heparan sulfate proteoglycans may specifically modulate the interaction of α -dystroglycan with different extracellular matrix proteins in skeletal muscle.

Skeletal muscle dystrophin has been shown to co-purify with a large oligomeric complex of proteins termed the dystrophin-glycoprotein complex (1–3), which is reduced in abundance or abnormal in dystrophic muscle (4). A highly similar complex is associated with utrophin (5), an autosomal homologue of dystrophin (6) that is specifically localized to the neuromuscular junction (7). Both complexes are thought to interact with the extracellular matrix by way of the 156-kDa glycoprotein, now referred to as α -dystroglycan (8), based on binding to the laminins (9–11) and agrins (12–15), two structurally related extracellular protein families with markedly distinct functions (16–18).

The importance of the α -dystroglycan/laminin interaction is inferred from studies that have demonstrated that the absence

or abnormality in various laminin subunits also causes muscular dystrophies (11, 19–22) similar to those involving the dystrophin-glycoprotein complex (4). These findings, coupled with data documenting the importance of the extensive post-translational modification of α -dystroglycan for laminin (10) and agrin binding (15), support further examination of the role that α -dystroglycan post-translational modification plays in binding to proteins of the extracellular matrix. To begin addressing this question, we purified α -dystroglycan from rabbit skeletal muscle membranes. In characterizing the laminin binding properties of purified α -dystroglycan, we were surprised by the significant difference in heparin sensitivity apparent between laminin (laminin-1) and merosin (a mixture of laminin-2 and -4). We propose a mechanism by which this difference may be important in modulating skeletal muscle α -dystroglycan interactions with different extracellular matrix proteins.

EXPERIMENTAL PROCEDURES

Materials—Engelbreth-Holm-Swarm mouse tumor laminin (laminin-1) was the kind gift of Dr. H. Kleinman, and merosin, a mixture of laminin-2 and -4 (23), was obtained from Life Technologies, Inc. or Chemicon International (Temecula, CA). The sodium (H3393) and calcium salts of porcine mucosal heparin were purchased from Sigma. The sodium salts of de-*N*-sulfated heparin (24), heparan sulfate (H7641), hyaluronic acid (H1876), and chondroitin sulfates A (C7571), B (C3788), and C (C4384) were also purchased from Sigma.

Purification of Skeletal Muscle Membranes and Dystrophin-Glycoprotein Complex—KCl-washed rabbit skeletal muscle membranes or total membranes were prepared from 5–6-pound New Zealand White rabbits as described previously (9, 25). Dystrophin-glycoprotein complex was prepared essentially as described previously (26).

Purification of Skeletal Muscle α -Dystroglycan—KCl-washed skeletal muscle membrane vesicles were osmotically ruptured by diluting 1–2 g of total membrane protein to a protein concentration of 5 mg/ml into 10 mM Tris-HCl, pH 8.5, 1 mM EGTA, and 0.2 mM phenylmethylsulfonyl fluoride. After incubation for 30 min at 4 °C with mixing, the extract was centrifuged for 30 min at 4 °C and $186,000 \times g$. The resulting supernatant was decanted, and the pellet was extracted for 1 h at 4 °C with 8 M urea in 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride. The urea supernatant was filtered through a 0.45 μm Supor-450 membrane filter and circulated overnight at 4 °C on a 14-ml wheat germ agglutinin-Sepharose 6MB (Pharmacia Biotech Inc.) column that was pre-equilibrated with 5 column volumes of TBS.¹ The following day the column was washed with 100 ml of TBS and eluted with 100 ml of 0.3 M *N*-acetylglucosamine in TBS. The *N*-acetylglucosamine eluate was loaded onto a 3-ml DEAE-cellulose (DE52) column pre-equilibrated in 5 column volumes of buffer A (50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride). The DEAE-cellulose column was washed with 25 ml of buffer A and step-eluted with buffer A containing 0.2 M NaCl (25 ml), 0.25 M NaCl (25 ml), and 0.3 M NaCl (25 ml). Five 5-ml fractions were collected from each elution. The pooled 0.2 and 0.25 M NaCl eluates, which contained the majority of α -dystroglycan, were brought to a density of 1.4 g/ml with solid CsCl and the solution heat-sealed in VTi65.1 polypropylene tubes. The tubes were

* This study was funded by grants from the American Heart Association (Wisconsin affiliate) and the Muscular Dystrophy Association and by National Institutes of Health Grant AR42423 (to J. M. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by an American Heart Association of Wisconsin predoctoral fellowship.

§ To whom correspondence should be addressed: Dept. of Physiology, University of Wisconsin, 120 Service Memorial Inst., 1300 University Ave., Madison, WI 53706. Tel.: 608-265-3419; Fax: 608-265-5512; E-mail: ervasti@facstaff.wisc.edu.

¹ The abbreviations used are: TBS, Tris-buffered saline; BSA, bovine serum albumin.

centrifuged at 10 °C for 16–20 h at $152,000 \times g$. Five 2.5-ml fractions were collected from the bottom of the gradients, and those fractions containing purified α -dystroglycan (1 and 2) were dialyzed exhaustively against either TBS or double distilled H_2O , pooled, and concentrated in a Centrifuplus100 (Amicon, Inc.). Purified α -dystroglycan was quantitated by A_{280} using $E_{280} = 0.50 \text{ cm}^2/\text{mg}$, calculated from the predicted amino acid sequence of α/β -dystroglycan precursor (9) with the proteolytic cleavage site located between Gly-653 and Ser-654 (27).

Purification of Rabbit Brain α -Dystroglycan— α -Dystroglycan was prepared from 300 g of frozen rabbit brains (Pel-Freez Biologicals) as described previously (28) and further purified by CsCl gradient centrifugation as described above. Brain α -dystroglycan was recovered in CsCl fractions 2 and 3 and was exhaustively dialyzed against double distilled H_2O prior to use.

Iodination of Purified Proteins—Purified skeletal muscle α -dystroglycan, laminin, and merosin were iodinated with ^{125}I by the School of Veterinary Medicine Radionuclide Laboratory using IODO-GEN (Pierce). Briefly, 2–20 μg of protein was reacted for 3 min at 20 °C in $12 \times 75\text{-mm}$ borosilicate glass tubes coated with 20 μg of IODO-GEN and containing 0.5–1 mCi of $[^{125}\text{I}]\text{NaI}$ in a total volume of 0.1–0.2 ml. The reaction was terminated by removal of the solution from the IODO-GEN-coated tube, and the labeled protein was separated from free ^{125}I on a $0.6 \times 30\text{-cm}$ Sephadex G-100 column.

Protein Overlay Assay—A modification of the procedure previously used for ^{125}I -laminin overlay (10) was used. Briefly, samples were electrophoretically separated on 3–12% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes that were blocked in 0.15 M NaCl, 8 mM sodium phosphate monobasic, 42 mM sodium phosphate dibasic, pH 7.5, and 5% nonfat dry milk for 1 h at room temperature. Blocked nitrocellulose transfers were rinsed briefly with TBS and incubated for 2 h at room temperature in TBS containing 3% BSA, 1 mM CaCl_2 , 1 mM MgCl_2 , and 1×10^6 cpm/ml ^{125}I -labeled protein. Finally, transfers were washed 2×10 min in TBS containing 1 mM CaCl_2 and 1 mM MgCl_2 and exposed to x-ray film. Alternatively, nitrocellulose transfers were incubated for 2 h at room temperature in TBS containing 3% BSA, 1 mM CaCl_2 , 1 mM MgCl_2 and 1 $\mu\text{g}/\text{ml}$ native laminin or merosin, and laminin/merosin binding proteins were detected with affinity-purified polyclonal laminin antibodies as described previously (29) using SuperSignal CL-HRP (Pierce) as substrate.

Solid Phase Microtiter Binding Assay—Immulon 1 removable microtiter wells (Dynatech Laboratories, Chantilly, VA) were incubated overnight at 4 °C with 1 μg of laminin or merosin in 0.1 ml of TBS, aspirated, and blocked for 2 h at room temperature with 0.35 ml of TBS containing 3% BSA, 1 mM CaCl_2 , and 1 mM MgCl_2 . Wells were then incubated for 2 h at room temperature with 30,000 cpm/well ^{125}I - α -dystroglycan in 0.1 ml of TBS containing 3% BSA, 1 mM CaCl_2 , and 1 mM MgCl_2 in the absence or presence of the indicated concentration of heparin. Finally, the wells were rapidly rinsed twice with 0.35 ml of TBS containing 3% BSA, 1 mM CaCl_2 , and 1 mM MgCl_2 and counted in a Packard 5650 γ counter. Each experiment was performed in triplicate wells, and the n reported in the figure legends refers to the number of independent experiments performed. Averaged data are expressed \pm S.E. Binding data depicted in Fig. 1 were analyzed by nonlinear regression analysis with the computer program Kinetics (30).

SDS-Polyacrylamide Gel Electrophoresis and Densitometry—SDS-polyacrylamide gel electrophoresis (31) was carried out on 3–12% gradient mini-gels in the presence of 1% 2-mercaptoethanol, and gels were stained with Coomassie Blue. Molecular weight standards shown in the figures were purchased from Sigma. Coomassie Blue-stained gels and films were analyzed densitometrically using a Bio-Rad model GS-670 imaging densitometer. The intensities of the scanned bands were quantitated by volume integration after background subtraction.

RESULTS

^{125}I -Laminin binding to purified skeletal muscle α -dystroglycan was examined with a solid phase assay previously used to characterize the laminin binding properties of brain α -dystroglycan (28). The concentration curve for ^{125}I -laminin binding to purified skeletal muscle α -dystroglycan (Fig. 1) was virtually identical to that previously published for brain α -dystroglycan (see Fig. 7 of Ref. 28). ^{125}I -Laminin bound to purified skeletal muscle α -dystroglycan in a saturable manner with a half-maximal concentration of 8 nM (Fig. 1).

The binding of ^{125}I - α -dystroglycan to immobilized laminin and merosin was also compared using the solid phase microtiter assay. In side-by-side experiments, the absolute binding of

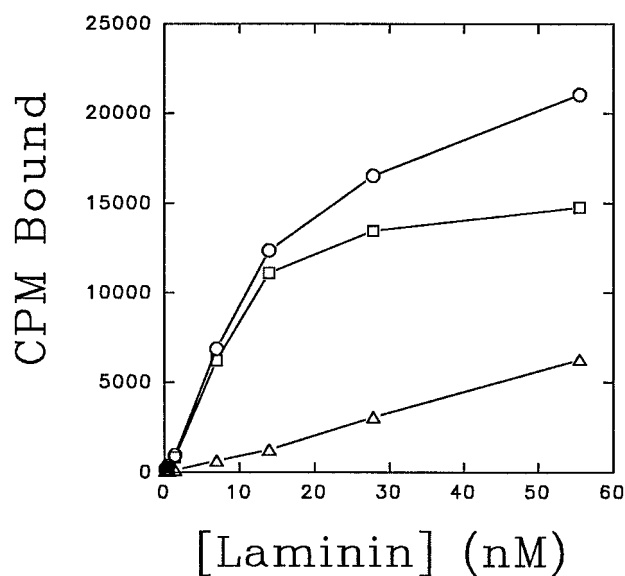


FIG. 1. Binding of ^{125}I -laminin to purified skeletal muscle α -dystroglycan. Shown is the binding of eight different concentrations (0.14–56 nM) ^{125}I -laminin to purified skeletal muscle α -dystroglycan in the absence (\circ) or presence (Δ) of 1 mg/ml heparin. Specific binding (\square) is taken as the difference of values obtained with and without heparin. Each point represents the average of triplicate determinations. Nonlinear regression analysis yielded a half-maximal concentration of 8 nM.

^{125}I - α -dystroglycan to laminin (6955 ± 250 cpm/well) was similar to that measured for merosin (7440 ± 970 cpm/well). ^{125}I - α -Dystroglycan binding to both laminin and merosin was similarly competed by increasing concentrations of unlabeled laminin (IC_{50} , 2 nM). As further evidence of the specificity of the interaction, it was observed that ^{125}I - α -dystroglycan binding to equivalent amounts of BSA (246 ± 38 cpm/well), collagen IV (407 ± 15 cpm/well), or heparan sulfate proteoglycan (671 ± 51 cpm/well) was substantially less than that observed for laminin (6955 ± 250 cpm/well) and merosin (7440 ± 970 cpm/well). In agreement with our previous results using the ^{125}I -laminin overlay (10), ^{125}I - α -dystroglycan binding to laminin and merosin was similarly inhibited by increasing ionic strength with an IC_{50} for NaCl of 250 mM (not shown).

^{125}I - α -Dystroglycan binding to laminin and merosin was further compared by examining the concentration dependence of heparin inhibition (0–2 mg/ml) using the microtiter assay. Surprisingly, ^{125}I - α -dystroglycan binding to laminin was significantly more sensitive to heparin over the range of 0.5–2 mg/ml in comparison with merosin (Fig. 2). ^{125}I - α -Dystroglycan binding to merosin was notably insensitive to heparin at concentrations greater than or equal to 0.2 mg/ml (Fig. 2). Performance of the microtiter assay using wells coated with purified α -dystroglycan and probing with iodinated laminin and merosin yielded heparin inhibition curves virtually identical to those illustrated in Fig. 2 (not shown).

The specificity of heparin in differentially inhibiting ^{125}I - α -dystroglycan binding to laminin and merosin was examined by comparing the relative effect of various glycosaminoglycans (1 mg/ml) in the solid phase assay. The sodium salt of heparin inhibited ^{125}I - α -dystroglycan binding to laminin by an average of $84 \pm 4.3\%$ (Fig. 3). Ca^{2+} heparin and heparan sulfate also dramatically inhibited ^{125}I - α -dystroglycan binding to laminin, although heparan sulfate appeared less effective than either heparin salt (Fig. 3). In contrast to the results obtained with laminin, Na^+ heparin inhibited ^{125}I - α -dystroglycan binding to merosin by only $17 \pm 5.2\%$, which was similar to the inhibition effected by the other glycosaminoglycans tested (Fig. 3). These data document the specificity of heparan sulfate-like glycos-

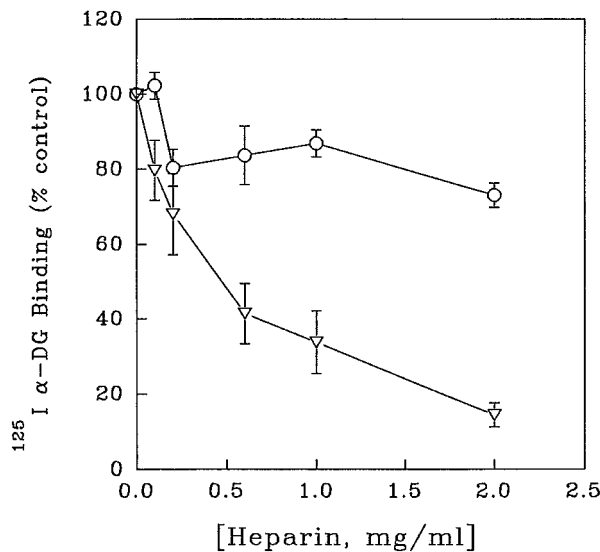


FIG. 2. The effect of heparin on ^{125}I - α -dystroglycan binding to laminin and merosin. ^{125}I - α -Dystroglycan (^{125}I - α -DG) binding to laminin (∇) and merosin (\circ) in the presence of the indicated concentration of heparin was measured using the solid phase microtiter assay described under "Experimental Procedures." Binding data were normalized as a percent of control for individual experiments performed in triplicate, and the graph represents the mean \pm S.E. of three independent experiments.

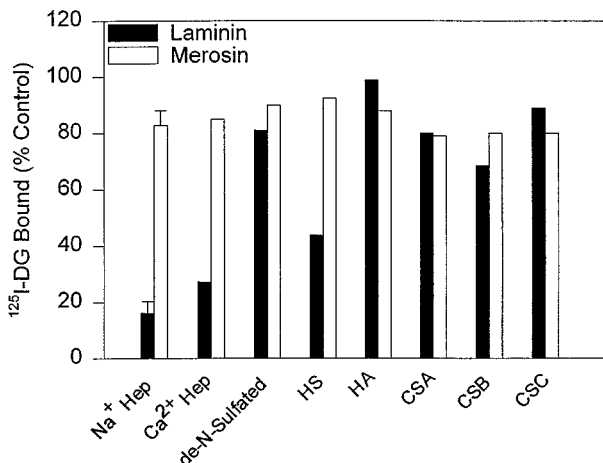


FIG. 3. Relative effect of various glycosaminoglycans on ^{125}I - α -dystroglycan binding to laminin and merosin. ^{125}I - α -Dystroglycan (^{125}I - α -DG) binding to laminin and merosin in the presence of 1 mg/ml Na⁺ heparin (Hep), Ca²⁺ heparin, de-N-sulfated heparin, heparan sulfate (HS), hyaluronic acid (HA), or chondroitin sulfates A (CSA), B (CSB), and C (CSC) was measured using the solid phase microtiter assay described under "Experimental Procedures." Binding data were normalized as a percent of control performed in the absence of added glycosaminoglycan. The data for Na⁺ heparin represent the mean \pm S.E. of nine independent experiments, each performed in triplicate. The data for heparan sulfate and chondroitin sulfate B represent the mean of two independent experiments, each performed in triplicate. All other data represent the mean of triplicate determinations.

aminoglycans in differentially inhibiting α -dystroglycan binding to laminin *versus* merosin.

The binding of ^{125}I -laminin and ^{125}I -merisin to α -dystroglycan was also compared by the protein overlay assay using nitrocellulose transfers containing electrophoretically separated dystrophin-glycoprotein complex (10). ^{125}I -Laminin and ^{125}I -merisin binding to α -dystroglycan in the dystrophin-glycoprotein complex were similarly inhibited by the inclusion of 10 mM EDTA or 0.5 M NaCl to the overlay medium (Fig. 4A). However, addition of 1 mg/ml heparin completely inhibited ^{125}I -laminin binding to α -dystroglycan but had little effect on

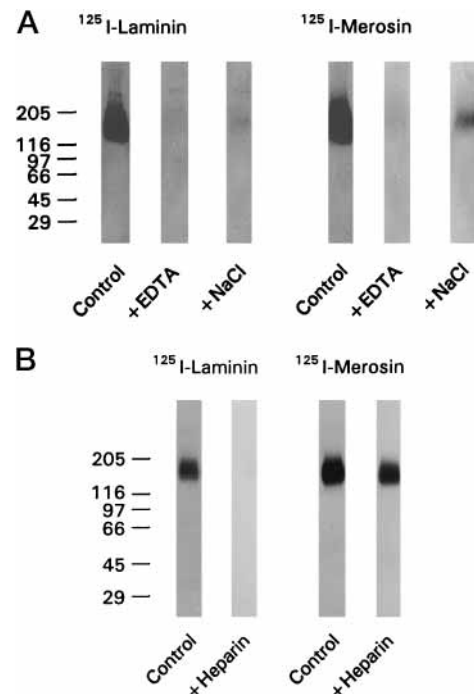


FIG. 4. Effect of heparin on ^{125}I -Laminin and ^{125}I -merisin overlay of dystrophin-glycoprotein complex. Shown in A are identical nitrocellulose transfers containing electrophoretically separated dystrophin-glycoprotein complex overlaid with ^{125}I -laminin or ^{125}I -merisin in the absence (Control) or presence of 10 mM EDTA or 0.5 M NaCl. Shown in B are identical nitrocellulose transfers containing electrophoretically separated dystrophin-glycoprotein complex overlaid with ^{125}I -laminin or ^{125}I -merisin in the absence (Control) or presence of 1 mg/ml heparin (+Heparin). The molecular weight standards ($\times 10^{-3}$) are indicated on the left.

^{125}I -merisin binding to α -dystroglycan (Fig. 4B). Comparison of heparin's effect on nonradioactive laminin and merosin overlay of dystrophin-glycoprotein complex, as detected with a polyclonal laminin antibody (29), yielded results similar to those presented in Fig. 4 (Fig. 5). In contrast to our results with 156-kDa skeletal muscle α -dystroglycan, merosin binding to 120-kDa brain α -dystroglycan was markedly inhibited by heparin (Fig. 5), which agrees with previous findings on 120-kDa peripheral nerve α -dystroglycan (29). In addition to confirming the results obtained with the solid phase assay (Figs. 2 and 3), our overlay results (Figs. 4 and 5) suggest that the more extensive post-translational modification of skeletal muscle α -dystroglycan is necessary for the observed differences in heparin sensitivity of its binding to laminin *versus* merosin.

DISCUSSION

The differential heparin sensitivity of skeletal muscle α -dystroglycan binding to laminin *versus* merosin (Figs. 2–5) is surprising in light of the fact that both laminin and merosin bind heparin (32, 33). Variations in the purity or integrity of commercial merosin preparations are likely not the reason for this difference because experiments with four different merosin lots from two vendors yielded similar results. Other than differential heparin inhibition, laminin and merosin exhibited very similar α -dystroglycan binding properties. Furthermore, unlabeled laminin was equally effective at inhibiting ^{125}I - α -dystroglycan binding to laminin and merosin. Our data imply that laminin and merosin have similar α -dystroglycan binding sites that overlap with distinct heparin binding sites. Consistent with our data, it was shown that laminin and merosin binding to heparan sulfate proteoglycan (perlecan) were differentially inhibited by heparin with IC₅₀ values of 0.8 and >500 $\mu\text{g}/\text{ml}$, respectively (34). The overall sequence similarity between the

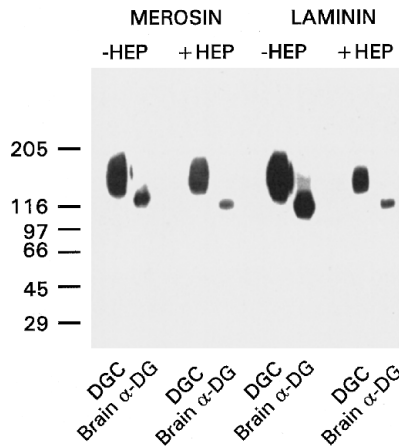


FIG. 5. Effect of heparin on laminin and merosin overlay of brain α -dystroglycan. Identical nitrocellulose transfers containing purified rabbit skeletal muscle dystrophin-glycoprotein complex (DGC) and brain α -dystroglycan (α -DG) were incubated with 1 μ g/ml native laminin or merosin in the absence (-HEP) or presence (+HEP) of 1 mg/ml heparin. Transfers were washed and then sequentially incubated with affinity-purified polyclonal antibodies to laminin followed by peroxidase-coupled secondary antibody. Laminin and merosin binding was detected by chemiluminescence using SuperSignal CL-HRP (Pierce) as substrate. Densitometric analysis indicated that heparin inhibited merosin binding to skeletal and brain α -dystroglycan 26 and 63%, respectively, while heparin inhibition of laminin binding to skeletal and brain α -dystroglycan was 68 and 87%, respectively. The molecular weight standards ($\times 10^{-3}$) are indicated on the left.

A chain of laminin and the M chain of merosin is 46.6%, with a 41.8% sequence identity in the carboxyl-terminal G domain (18), which is responsible for laminin binding to α -dystroglycan (28). While laminin and merosin exert similar effects on cell attachment and neurite outgrowth (33), it has been noted that merosin promoted a significantly greater level of neuronal cell migration than did laminin (35). Finally, that increased expression of laminin fails to correct for genetic (22) merosin deficiency in the dystrophic *dy/dy* mouse (20) is additional proof that laminin and merosin are not functionally redundant. Thus, there is sufficient sequence variability as well as precedent for a functional difference between laminin and merosin like that implied by our observation of differential heparin sensitivity in α -dystroglycan binding.

One question raised (36) by the observations that skeletal muscle α -dystroglycan binds to both laminins (9–11) and agrins (12–15) is whether and how α -dystroglycan might discriminate between different extracellular matrix proteins, even when all are present in the same tissue (37, 38). This issue is particularly relevant because laminin has been shown to inhibit agrin binding to α -dystroglycan (14), while nerve and muscle agrins bind α -dystroglycan with similar affinity (15) yet exhibit dramatically different potencies in clustering acetylcholine receptors (39). Our results support the possibility that unique heparan sulfate-containing proteoglycans may differentially modulate α -dystroglycan interactions with various extracellular matrix proteins. In support of this hypothesis, we have demonstrated that heparin and heparan sulfate, but not de-N-sulfated heparin, hyaluronic acid, or chondroitin sulfates, are effective in inhibiting α -dystroglycan binding to laminin (Fig. 3). However, the high concentration of heparin necessary to inhibit α -dystroglycan binding to laminin (Fig. 2) further suggests that a minor subpopulation of heparin is responsible for our observed effects. As reviewed by Rapraeger (40), specifically sulfated microdomains of heparan sulfate are important in the mechanism of action of antithrombin and fibroblast growth factor. Furthermore, unique heparan sulfate proteoglycans can exhibit restricted localization at the neuromuscular

junction (41) and have been implicated in the development and/or repair of muscle (42–44) and nerve (45–48). Finally, laminin and other extracellular matrix proteins bind to structurally distinct subpopulations of heparin with variable affinities (49). Taken together, these findings support the possibility that one or more heparan sulfate proteoglycans may specifically modulate the interaction of α -dystroglycan with different extracellular matrix proteins in skeletal muscle.

Because dystroglycan is encoded by a single gene (8), the apparent size difference between neuronal and skeletal muscle α -dystroglycan is likely due to differential post-translational modification (3, 9). While recent progress has been made in the characterization of brain α -dystroglycan post-translational modification (27), little is presently understood concerning the differences in post-translational modification between different tissue forms of α -dystroglycan and how they translate into variations in α -dystroglycan function. However, since post-translational modification is important to the laminin binding activity of α -dystroglycan (10), it seemed reasonable that merosin binding to neuronal and skeletal muscle α -dystroglycan may also be differentially inhibited by heparin. In support of this possibility, Gee *et al.* (28) demonstrated that heparin inhibited 125 I-laminin binding to brain α -dystroglycan with an IC_{50} of less than 0.1 μ g/ml, while our previous (10) and present (Fig. 2) results indicate that heparin inhibited skeletal muscle α -dystroglycan with an IC_{50} of 250 μ g/ml. We have further demonstrated that heparin does indeed inhibit both laminin and merosin binding to purified brain α -dystroglycan (Fig. 5). Yamada *et al.* (29) also observed heparin inhibition of merosin binding to 120-kDa peripheral nerve α -dystroglycan, which is similar in size to brain α -dystroglycan (9, 10, 28). Thus, it would appear that both differential post-translational modification of α -dystroglycan and structural variations between the identified extracellular ligands for α -dystroglycan may be involved in our hypothesized role for heparan sulfate containing proteoglycans in modulating α -dystroglycan/extracellular matrix interactions.

Acknowledgments—We are grateful to Dr. Hynda Kleinman for the generous gift of laminin. We thank Dr. Inna Rybakova for providing purified dystrophin-glycoprotein complex and Aimee Geissler for expert preparation of the skeletal muscle membranes used in this study.

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