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Deficiency of Merosin in Dystrophic *dy* Mice and Genetic Linkage of Laminin M Chain Gene to *dy* Locus*

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Merosin is the predominant laminin isoform in the basal lamina of striated muscle and peripheral nerve, and consists of M, B1 or S, and B2 chains. Here we have demonstrated that merosin is a native ligand for α-dystroglycan, an extracellular component of the dystrophin-glycoprotein complex. We have also mapped the mouse M chain gene, *Lamm*, to the same region of mouse chromosome 10 to which the dystrophia muscularis (*dy*) locus has been mapped. The *dy* mutation represents a severe neuromuscular disease resembling human muscular dystrophy. Analysis of merosin expression of dystrophic *dy* mice revealed a specific deficiency of merosin in skeletal muscle, cardiac muscle, and peripheral nerve. Our results indicate that merosin deficiency may be the primary defect in *dy* mice and suggest that a disruption of the link between α-dystroglycan and merosin may be involved in the pathogenesis of muscle degeneration and peripheral neuropathy in *dy* mice.

Skeletal muscle dystrophin exists in a tightly associated oligomeric complex with dystrophin-associated proteins (DAPs)[1] (1–5): a 59-kDa intracellular dystrophin-associated protein triplet (59 DAP), three transmembrane dystrophin-associated glycoproteins of 35, 43, and 50 kDa (35 DAG, 43 DAG or β-dystroglycan, and 50 DAG or adhalin, respectively), a 25-kDa transmembrane dystrophin-associated protein, and a 156-kDa extracellular dystrophin-associated glycoprotein (156 DAG or α-dystroglycan). The primary structures of α- and β-dystroglycan (6) and adhalin (7) have been determined by cDNA cloning. α-Dystroglycan binds laminin (6, 8), a major component of the basal lamina, and the NH_2-terminal domain of dystrophin binds actin (8–12). These results indicate that the dystrophin-glycoprotein complex (DGC) links the subsarcolemmal actin cytoskeleton to the extracellular matrix.

The prototypical laminin molecule is a cross-shaped heterotrimer consisting of three types of chains, A (~400 kDa), B1, and B2 (~220 kDa) (13). In recent years, various laminin isoforms with unique chain compositions have been identified in various tissues. In striated muscle and peripheral nerve, the tissue-specific laminin variant is merosin, which is defined by having an M chain in place of an A chain (14–17). Merosin has diverse biological functions in mediating cell attachment and spreading and promoting neurite outgrowth (18), as well as forming the basal lamina meshwork with type IV collagen, fibronectin, and heparan sulfate proteoglycan. Moreover, since the expression of merosin is developmentally regulated (14), merosin may play an important role in the maturation or differentiation of the neuromuscular system.

In the present study, we have demonstrated that merosin is a native ligand for α-dystroglycan in skeletal muscle and have mapped the locus of the mouse M chain gene to chromosome (Chr) 10 within the same region as the dystrophia muscularis (*dy*) locus. The *dy* mutation is recessive and represents a severe neuromuscular disease resembling human muscular dystrophy. We analyzed the expression of merosin in skeletal muscle, cardiac muscle, and peripheral nerve from age-matched control and *dy* mice and found a marked reduction of merosin in *dy* mice. Our results indicate that merosin deficiency may be the primary defect causing the *dy* phenotype.

EXPERIMENTAL PROCEDURES

Nitrocellulose Transfer Overlays—Human placenta merosin (Life Technologies, Inc.) was iodinated with [^{125}I]NaI using a lactoperoxidase/glucose oxidase reaction. A nitrocellulose transfer of a SDS-polyacrylamide gel containing purified DGC was overlaid with ^{125}I-merosin as previously described (6).

Immunoaffinity Precipitation—Goat anti-mouse IgG-Sepharose was coupled with anti-adhalin monoclonal antibody $IVD3_1$. 70 μl of concentrated salt-free eluate of succinylated wheat germ agglutinin (sWGA)-agarose column chromatography from 1% digitonin-solubilized rabbit skeletal muscle membranes (1), which contains both DGC and merosin, was incubated with 100 μl of either immunoaffinity or control beads in the absence or presence of 10 mM EDTA overnight at 4 °C. After centrifugation, proteins remaining in the supernatants were analyzed by 3–12% SDS-PAGE and immunoblotting.

Cloning and Chromosomal Mapping of Mouse M Chain Gene—Genomic DNA was prepared from embryonic fibroblasts derived from 15-day gestation 129/SvJ mouse embryos. Based on a cDNA sequence for the carboxyl portion of the mouse M chain,[2] two oligonucleotide primers were prepared in a region having low homology to laminin A chain. A 2.1-kb segment of M chain DNA was amplified from the mouse genomic DNA by the polymerase chain reaction and used to screen a 129SVJ normal mouse genomic library (Stratagene, La Jolla, CA). A 4-kb subclone from one of the clones isolated was generated for chromosomal mapping. This subclone contains one exon of 183 base pairs encoding a part of the COOH-terminal globular domain of the M chain with 88% amino acid sequence identity to the corresponding sequence of the human M chain (15).

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[1] The abbreviations used are: DAP, dystrophin-associated protein; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; 59 DAP, 59-kDa dystrophin-associated protein; 35 DAG, 35-kDa dystrophin-associated glycoprotein; 43 DAG, 43-kDa dystrophin-associated glycoprotein; 50 DAG, 50-kDa dystrophin-associated glycoprotein; 156 DAG, 156-kDa dystrophin-associated glycoprotein; sWGA, succinylated wheat germ agglutinin; Chr, (mouse) chromosome; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.

[2] S. M. Bernier, manuscript in preparation.

The genetic locus encoding the M chain was mapped by analysis of the progeny of two genetic crosses: (NFS/N or C58/J × *Mus musculus*) × *M. m. musculus* (19) and (NFS/N × *Musculus spretus*) × *M. spretus* or C58/J (20). DNAs extracted from parental and progeny mouse livers and spleens were analyzed by Southern blotting as described (19) for restriction enzyme polymorphisms of M chain DNA using the probe pgmm4–3. The progeny of these crosses had previously been typed for over 580 markers distributed over all 19 autosomes and the X chromosome. These markers include the Chr 10 markers *Ifgr* (interferon γ receptor), *Myb* (myeloblastosis oncogene), *Zfa* (zinc finger protein, autosomal), *Pfp* (pore-forming protein), and *Tpi-rs6* and *Tpi-rs5* (triosephosphate isomerase-related sequences 6 and 5). *Myb*, *Igfr*, and *Pfp* were typed in the *M. spretus* cross following digestion with *Bam*HI, *Eco*RI, and *Bgl*II using probes described previously (19). *Zfa* was typed following digestion with *Eco*RI using as probe a 6.5-kb insert of the clone pDR1115 obtained from ATCC (Rockville, MD) (21). *Tpi-rs6* and *Tpi-rs5* were identified, respectively, as 6.3-kb *Pst*I and 3.5-kb *Sca*I fragments reactive with the probe pHTPI-5A kindly provided by Dr. L. Marquat (Roswell Park, Buffalo, NY) (21).

Recombinant percentages and standard errors were calculated according to Green (22). Where no recombinants were identified, a recombinational distance is given, which is the upper limit of the 95% confidence interval (22). Data were stored and analyzed using the program LOCUS designed by C. E. Buckler (NIH).

Immunofluorescence—Quadriceps muscle and sciatic nerve cryosections (7 μm) were immunostained with antibodies against laminin M chain, B1/B2 chain, or type IV collagen as previously described (23).

EDTA Extraction of Merosin and Immunoblotting—Freshly prepared cardiac muscle and sciatic nerve tissues or cryosections of skeletal muscle from age-matched control +/+ and dystrophic *dy/dy* mice were incubated with 20 volumes of EDTA extraction buffer (10 mM EDTA, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.75 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) on ice for 2 h. After centrifugation, samples containing 100 μg of protein were separated on 3–12% SDS-PAGE (24) in the presence of 1% 2-mercaptoethanol and stained with Coomassie Blue or transferred to nitrocellulose (25). Nitrocellulose transfers were stained with a polyclonal antibody against mouse M chain as previously described (3). For quantitative analysis, the immunoblots were labeled with ^{125}I-labeled protein A (~250,000 cpm/ml, DuPont NEN) and exposed to x-ray films. Densitometric scanning of autoradiograms was carried out on a computing densitometer (model 300S; Molecular Dynamics, Inc., Sunnyvale, CA) and analyzed using Image Quant (Molecular Dynamics, Inc.) software.

Animals and Antibodies—129/ReJ and C57BL/6J strain dystrophic *dy/dy* mice (6–8 weeks) and age-matched normal control +/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Polyclonal antibodies specific for each dystrophin-associated protein were affinity-purified from sheep antiserum against the DGC as previously described (5, 6). The preparation and characterization of monoclonal antibodies IIH6 and IVD3$_1$, specific for α-dystroglycan and adhalin, respectively, have been previously described (2, 4). A rabbit polyclonal antibody was raised against a recombinant human M chain fragment, and its IgG fraction was used for immunoblotting. Since sheep anti-DGC serum also recognized merosin, a polyclonal antibody specific for a 300-kDa fragment of M chain was affinity-purified using purified human placenta merosin. Rabbit polyclonal antibodies specific for mouse laminin B1/B2 chain or mouse type IV collagen were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) or Biodesign International (Kennebunkport, ME), respectively.

RESULTS AND DISCUSSION

During the process of DGC purification described previously (26), the majority of merosin is removed by washing membranes with 0.6 M KCl. However, residual merosin was coeluted with DGC from an sWGA-agarose column under salt-free conditions and cosedimented with DGC on a 5–30% sucrose density gradient.[3] In addition, sheep anti-DGC serum recognized merosin, but not laminin A chain.[4] ^{125}I-Merosin overlay demonstrated that merosin binds α-dystroglycan following transfer to a nitrocellulose membrane (Fig. 1*A*). To confirm the binding of merosin to α-dystroglycan, immunoadsorption experiments of salt-free sWGA eluate from solubilized rabbit muscle membranes were performed using anti-adhalin immunoaffinity beads (Fig. 1*B*). Immunoblot analysis of proteins in the supernatants revealed that anti-adhalin beads precipitated merosin as well as dystrophin and all DAPs in the absence of EDTA. In the presence of 10 mM EDTA, dystrophin and all DAPs were also precipitated, although less effectively. However, precipitation of merosin was completely abolished, indicating that the binding of merosin to α-dystroglycan was inhibited by addition of 10 mM EDTA. Although we have already shown that α-dystroglycan is a non-integrin laminin receptor (6, 8), these results demonstrated that merosin is a native ligand of α-dystroglycan in skeletal muscle.

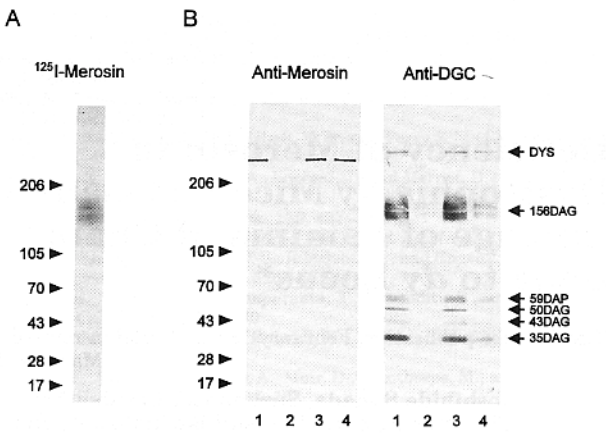

FIG. 1. **Specific binding of merosin and α-dystroglycan.** *A*, autoradiogram of a nitrocellulose transfer containing purified DGC overlaid with ^{125}I-merosin. *B*, immunoadsorption of merosin and components of DGC by anti-adhalin immunoaffinity beads. Anti-adhalin immunoaffinity column (goat anti-mouse IgG-Sepharose coupled with anti-adhalin antibody) void (*lanes 2* and *4*) or control beads (goat anti-mouse IgG-Sepharose) void (*lanes 1* and *3*) after incubation in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of 10 mM EDTA, immunostained with an antibody against mouse M chain (*Anti-Merosin*) or a mixture of antibodies against components of DGC (*Anti-DGC*). Molecular weight standards (×10^{-3}) are indicated on the *left*.

The M chain genomic DNA probe, pgmm4–3, identified *Bam*HI fragments of 18.8 kb in NFS/N and C58/J and 15.0 kb in *M. spretus*. Analysis of the parental mice of the *M. m. musculus* cross failed to identify polymorphic fragments using this probe following digestion with 13 different enzymes. Analysis of the progeny of the *M. spretus* cross for inheritance of *Lamm*, the gene for the M chain, demonstrated that this locus was linked to markers on proximal mouse Chr 10 (Fig. 2) and positioned *Lamm* just distal to *Myb*. These data indicate that the *Lamm* gene maps to a region of mouse Chr 10 that shows conserved linkage to human chromosome 6q, which is consistent with a map location for the human homolog of this gene (27). The map location of *Lamm* in the mouse places this gene in the same region of the genome known to contain the gene *dy*. Although composite genetic maps position *dy* proximal to *Myb* (28, 29), *dy* and *Myb* have never been mapped relative to one another, and, furthermore, *dy* had not been mapped relative to any other molecular markers. Thus, our mapping data of *Lamm* is consistent with the suggestion that a mutation in this sequence may be responsible for the *dy* phenotype, prompting us to examine the status of merosin expression in dystrophic *dy* mice.

Immunofluorescence analysis of merosin and type IV collagen, another component of the basal lamina, in control +/+ and *dy/dy* skeletal muscle and sciatic nerve is shown in Fig. 3. In control skeletal muscle, M chain, B1/B2 chain, and type IV collagen were localized throughout the basal lamina surrounding the sarcolemma. In *dy/dy* skeletal muscle, the immunostaining intensity of M chain was significantly decreased,

[3] Y. Sunada and K. P. Campbell, unpublished data.
[4] Y. Sunada, J. M. Ervasti, and K. P. Campbell, unpublished data.

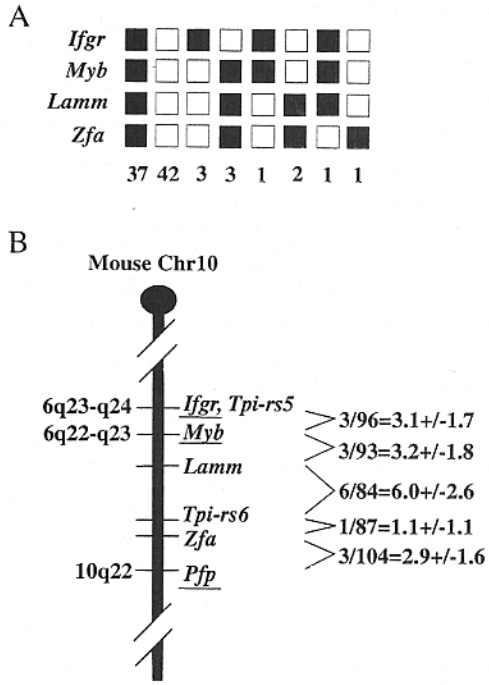

FIG. 2. **Map location of the M chain gene, *Lamm*, on Chr 10.** *A*, segregation of *Lamm* and flanking genes in the progeny of a *M. spretus* cross. *Black squares* represent heterozygous mice; *open squares* represent homozygous mice. *Numbers* under each *column* represent the number of mice with each genotype. *B*, an abbreviated map of Chr 10 illustrating the position of *Lamm* relative to other markers on Chr 10. The map locations of the additional markers *Tpi-rs5*, *Tpi-rs6*, and *Pfp* are also indicated. Percent recombination between adjacent locus pairs and standard errors were calculated according to Green (22), and these numbers are based on the total number of mice typed for the locus pair. Map locations of the human homolog of the *underlined* genes are indicated to the *left*.

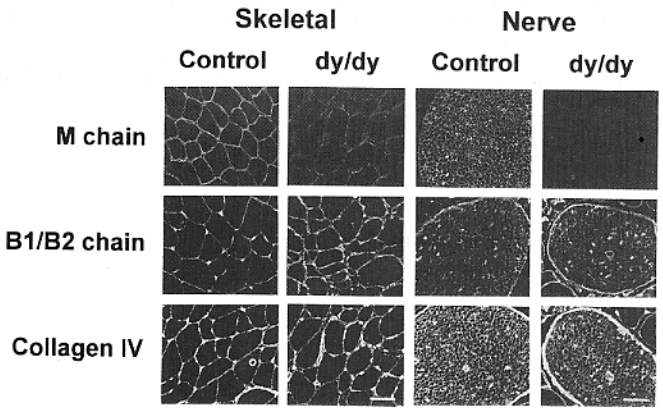

FIG. 3. **The immunohistochemical localization of merosin M chain, B1/B2 chain, and type IV collagen in skeletal muscle and peripheral nerve from control +/+ and *dy/dy* mice.** Seven-μm-thick transverse cryosections of the quadriceps skeletal muscle (skeletal) and sciatic nerve (nerve) from 7-week-old 129/ReJ strain +/+ (control) and 7-week-old 129/ReJ strain dystrophic *dy/dy* (*dy/dy*) mice were labeled by indirect immunofluorescence with affinity-purified antibodies against a 300-kDa fragment of M chain (M chain) or polyclonal antibody against B1/B2 chain (B1/B2 chain) or type IV collagen (collagen IV). Indistinguishable results were observed using skeletal muscle and femoral nerve obtained from 7-week-old C57BL/6J strain +/+ and *dy/dy* mice. *Bar*, 50 μm.

whereas the immunostaining of B1/B2 chain and type IV collagen was indistinguishable between control and *dy/dy* skeletal muscle. Laminin M chain was localized to the endoneurial basal lamina surrounding Schwann cells in control nerve. In *dy/dy* nerve, M chain immunostaining was very faint, although the immunostaining of B1/B2 chain and type IV collagen in the endoneurial basal lamina and the perineurium was similar to that in control nerve.

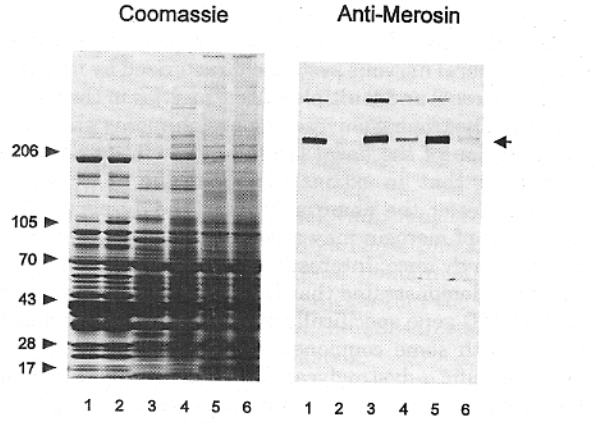

FIG. 4. **Immunoblot analysis of merosin M chain in EDTA extracts from control +/+ and dystrophic *dy/dy* mouse skeletal muscle, cardiac muscle, and peripheral nerve.** EDTA extracts (100 μg/*lane*) of skeletal muscle (*lanes 1* and *2*), cardiac muscle (*lanes 3* and *4*), and sciatic nerve (*lanes 5* and *6*) from C57BL/6J strain control +/+ mice (*lanes 1*, *3*, and *5*) and dystrophic *dy/dy* mice (*lanes 2*, *4*, and *6*) were separated on a 3–12% gradient SDS gel and stained with Coomassie Blue (*Coomassie*), or a nitrocellulose transfer from an identical gel was immunostained with a polyclonal antibody against a recombinant mouse M chain fragment (*Anti-Merosin*). Molecular weight standards ($\times 10^{-3}$) are indicated on the *left*. An *arrow* indicates the 300-kDa fragment of M chain.

EDTA extracts from skeletal muscle, cardiac muscle, and sciatic nerve were separated by SDS-PAGE. Coomassie Blue staining of the gel revealed that the overall protein composition in each sample was similar between control and *dy/dy* mice (Fig. 4). M chain polypeptide migrates as two fragments of 300 and 80 kDa under reducing conditions (15). A rabbit polyclonal anti-M chain antibody detected a COOH-terminal 300-kDa fragment of M chain and a 600-kDa component. The latter consists of a 300-kDa M chain fragment (15) and B1 and/or B2 chains[3] as determined by immunoblot analysis. However, the relative abundance of a 300-kDa fragment and a 600-kDa component was significantly reduced in *dy/dy* skeletal muscle, cardiac muscle, and peripheral nerve (Fig. 4). The reduction of merosin in *dy/dy* mice was quantitated using ^{125}I-protein A-labeled immunoblots. Densitometric scanning of autoradiographs revealed a 94% reduction in skeletal muscle, a 96% reduction in cardiac muscle, and a 97% reduction in peripheral nerve when compared with age-matched control mice. Taken together with the gene mapping data, our results are highly suggestive that an M chain gene mutation is the cause of the *dy* phenotype. The size of a 300-kDa fragment of M chain detectable in *dy/dy* mice was indistinguishable from that in control +/+ mice, and the merosin whole molecule of 700 kDa was detected under non-reducing conditions in *dy* mice,[3] suggesting that a large deletion in the M chain gene is unlikely.

dy mice present severe dystrophic muscle pathology, which mimics human muscular dystrophy like DMD. In DMD patients (2, 6, 23) and *mdx* mice (5), the absence of dystrophin leads to a drastic reduction of all DAPs. In severe childhood autosomal recessive muscular dystrophy with DMD-like phenotype, a specific deficiency of adhalin was identified (30–32). Thus, disruption of a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix caused by the deficiency of DGC component(s) may play a crucial role in the pathogenesis of these muscular dystrophies. However, our previous study demonstrated that all components of the DGC appear normal in *dy* mice (5). Since merosin is a native ligand for α-dystroglycan in skeletal muscle, the deficiency of merosin could contribute to a similar disruption of this critical link and

lead to muscle cell necrosis in *dy* mice.

A striking feature of the *dy* phenotype is the involvement of the peripheral nervous system characterized by naked axons in the nerve roots and multiple discontinuities in the basal lamina (33, 34). Dystrophic/shiverer mouse chimeras show a striking amelioration of the basal lamina defect in *dy* Schwann cells, suggesting that an extrinsic factor to the *dy* Schwann cell is responsible for the abnormalities in the *dy* nerve (35). Thus, deficiency of merosin may account for peripheral nerve abnormalities in *dy* mice. Interestingly, a recent immunohistochemical study demonstrated that Dp116, a 116-kDa protein product of the DMD gene specifically expressed in Schwann cells, complexed with some components of DAPs including peripheral nerve-specific α-dystroglycan (36). Moreover, ^{125}I-merosin overlay demonstrated that peripheral nerve α-dystroglycan binds merosin.[3] Thus, disruption of the linkage between merosin and peripheral nerve α-dystroglycan may play a role in the pathogenesis of peripheral neuropathy in *dy* mice.

In conclusion, our results suggest that merosin deficiency may be the primary defect of dystrophic *dy* mice and suggest a novel hypothesis to explain the etiology of both muscular dystrophy and peripheral neuropathy based on a common molecular pathogenesis. A disrupted linkage between the subsarcolemmal cytoskeleton and the basal lamina mediated by DGC may cause muscle cell necrosis or peripheral neuropathy. It remains to be determined whether merosin deficiency is involved in the pathogenesis of human muscular dystrophy.

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