

13. DYSTROGLYCAN: A NOVEL LAMININ RECEPTOR AND ITS INVOLVEMENT IN THE PATHOGENESIS OF MUSCULAR DYSTROPHY

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Dystroglycan was originally isolated from skeletal muscle as a component of the dystrophin-glycoprotein complex (DGC) and characterized as a novel non-integrin laminin receptor. In skeletal muscle, dystroglycan links the cytoskeletal protein dystrophin and the basal lamina protein laminin. The integrity of this linkage seems critical for maintaining normal muscle function. In various types of muscular dystrophy, the disruption of the linkage leads to muscle cell necrosis. Recent studies have revealed that dystroglycan has amazing functional diversity in various tissues associated with myelination, synaptogenesis, and epithelial morphogenesis. Here we will discuss how dystroglycan is involved in the pathogenesis of muscular dystrophy as well as its diversity in biological functions.

INTRODUCTION: DYSTROPHIN-GLYCOPROTEIN COMPLEX (DGC)

Muscular dystrophy is a group of genetic disorders which primarily affects skeletal muscle and is clinically characterized by progressive muscle wasting and weakness. Histology of dystrophic muscle shows muscle cell necrosis and regeneration, connective tissue proliferation, fiber size variation, and central nucleation. There are various different types of muscular dystrophy caused by various distinct etiologies. Duchenne muscular dystrophy (DMD) is the most severe and common muscular dystrophy whose etiology had been unknown until 1986 when the DMD gene was identified (Monaco *et al.*, 1986). The DMD gene is one of the biggest genes so far identified, spanning 2.4 Mb and containing at least 80 exons and seven promoters regulating several protein products (reviewed in Ahn and Kunkel, 1993 and Tinsley *et al.*, 1993) (Fig. 1). Dystrophin is the protein product of the 14 kb DMD gene transcript in muscle and brain. The primary structure predicts that dystrophin is a rod-shaped cytoskeletal protein of 427 kDa containing four structurally distinct domains (N-terminal actin-binding domain, large spectrin-like rod domain, cysteine-rich domain and unique C-terminal domain) (Koenig *et al.*, 1988). Dystrophin is localized to the inner surface of the sarcolemma in normal skeletal muscle, but is totally missing from skeletal muscle of DMD patients (Arahata *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988; Bonilla *et al.*,

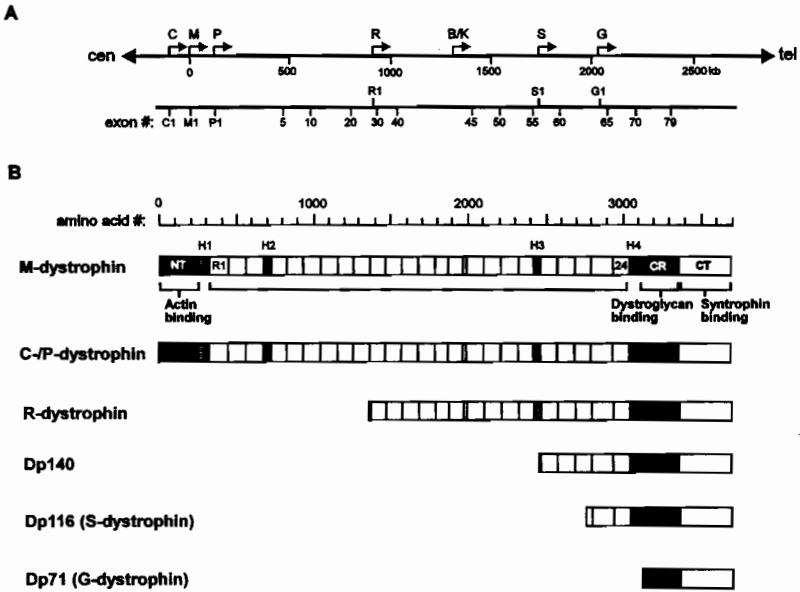


Figure 1 Schematic diagram of the DMD gene and its protein products. (A) Upper line: seven different promoters drive cell-type specific expression of dystrophin. The C (cortical)-, M (muscle)-, and P (Purkinje cell)-dystrophins use their own exon. The R (retinal)-, B/K (brain/kidney)-, S (Schwann cell)-, and G (general or glial)-dystrophin promoters encode retinal dystrophin, C-terminal proteins Dp140, Dp116, and Dp71, respectively. Lower line: a distribution of representative exons including 6 different first exons of dystrophin, spanning 2.4 Mb. (B) Full-length dystrophin consists of 4 functional domains. The N-terminal domain contains actin-binding sites. Rod domain consists of 24 homologous repeats. The cysteine-rich domain contains the dystroglycan-binding site. The C-terminal domain contains syntrophin-binding site(s). There are four different short-forms of dystrophin: a 260-kDa retinal dystrophin, Dp140 (brain and kidney), Dp116 (peripheral nerve), Dp71 (ubiquitous in non-muscle tissues).

1988). However, neither the exact function of dystrophin nor the precise mechanism of muscle cell necrosis caused by the absence of dystrophin was revealed by its primary structure.

Initial biochemical experiments demonstrated that dystrophin was tightly associated with sarcolemmal glycoproteins and suggested that the function of dystrophin could be to link sarcolemmal glycoproteins to the underlying cytoskeleton (Campbell and Khal, 1989). Subsequently, a dystrophin-glycoprotein complex (DGC) was isolated from digitonin-solubilized rabbit skeletal muscle membranes using WGA-Sepharose and DEAE-cellulose and further purified by sucrose density gradient centrifugation in the form of a large (~18S) complex. The complex contained several novel sarcolemmal

protein and glycoprotein components which are called dystrophin-associated proteins (Ervasti *et al.*, 1990; Yoshida and Ozawa, 1990) (Fig. 2). Structural and functional characterization of dystrophin and the dystrophin-associated proteins is now providing clues to the overall membrane organization of the DGC (reviewed in Matsumura and Campbell, 1994, and Tinsley *et al.*, 1994). Table I summarizes the current knowledge of the components of the DGC and Fig. 3 shows a schematic model for molecular organization of the DGC. An extracellular 156-kDa dystrophin-associated glycoprotein (156 DAG), now called α -dystroglycan, mediates the attachment of the sarcolemma to the basement membrane by binding laminin (Ibraghimo-Beskrovnaya *et al.*, 1992, Ervasti and Campbell, 1993). α -dystroglycan is anchored to the sarcolemmal glycoprotein complex, consisting of five different integral membrane proteins: adhalin (50 DAG), 43-kDa glycoprotein doublet (β -dystroglycan and a novel 43 DAG also known as A3b), a 35-kDa glycoprotein (35DAG), and a 25-kDa protein (25 DAP). Among these integral proteins, β -dystroglycan has been shown to bind the cysteine-rich domain of dystrophin (Suzuki *et al.*, 1994). Dystrophin binds the subsarcolemmal cytoskeleton to the sarcolemma by binding F-actin through its N-terminal domain (Hemmings *et al.*, 1992, Wey *et al.*, 1992, Levine *et al.*, 1992) and the glycoprotein complex through its C-terminal domains (Suzuki *et al.*, 1992 and 1994). The syntrophin triplet (59 DAP) (Adams *et al.*, 1993, Yang *et al.*, 1994) also directly associates with the C-terminal domain of dystrophin (Suzuki *et al.*, 1995, Ahn *et al.*, 1995, Yang *et al.*, 1995). Overall the DGC spans the sarcolemma linking the basement membrane and the subsarcolemmal cytoskeleton. Among components of the DGC, dystroglycan is a key molecule as a linker between laminin and dystrophin.

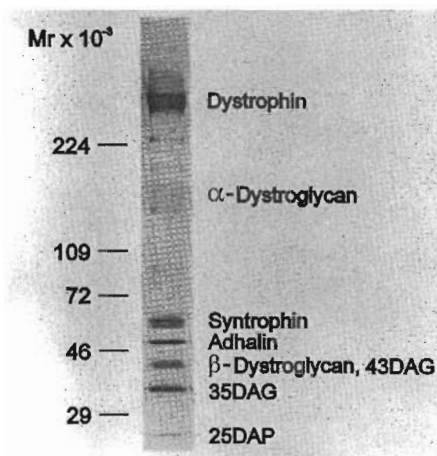


Figure 2 Components of the dystrophin-glycoprotein complex (DGC) separated on a 3–12% SDS-polyacrylamide gel. α -Dystroglycan is poorly stained with Coomassie blue because of its heavy glycosylation.

Table 1 Components of the Dystrophin-Glycoprotein Complex*

Protein	Size (kDa)	Gene Locus	Related Disease	Function
Extracellular matrix	400	6q22-23	CMD <i>dy</i> or <i>dy^β</i> mouse	Basal lamina component that binds α -dystroglycan
Sarcolemma				
Extracellular	156	3p21	—	Binds laminin-2 and is involved in linkage of dystrophin to laminin-2
Transmembrane	43	3p21	—	Binds dystrophin and is involved in linkage to laminin-2
	50	17q21	SCARM1 BIO 14.6 Hamster	?
	43	?	—	?
	35	?	—	?
	25	?	—	?
Intracellular	58	20q11	—	Binds dystrophin/utrophin
	59	16	—	Binds dystrophin/utrophin
	60	8q23-24	—	Binds dystrophin/utrophin
Cytoskeleton	427	Xp21	DMD/BMD <i>mdx</i> mouse <i>grmd</i> dog	Membrane cytoskeletal protein linking transmembrane glycoprotein complex to F-actin

* Reviewed in Matsumura and Campbell, 1994 and Tinsley *et al.*, 1994

† DAG dystrophin-associated glycoprotein; DAP dystrophin-associated protein

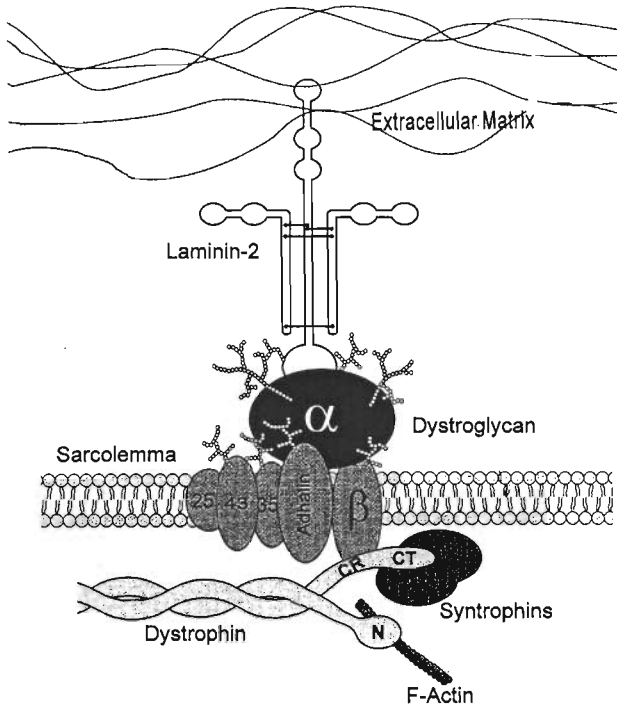


Figure 3 A schematic model for molecular organization of the dystrophin-glycoprotein complex (DGC) as a transmembrane linker between the extracellular matrix and the subsarcolemmal cytoskeleton. CR; cysteine-rich domain, CT; C-terminal domain, N; N-terminal domain of dystrophin

PRIMARY STRUCTURE, GENOMIC STRUCTURE, AND CHROMOSOMAL LOCALIZATION OF DYSTROGLYCAN

Primary structure: The primary structure of dystroglycan was determined by cDNA cloning in 1992 (Ibraghimov-Beskrovnaya *et al.*, 1992). Interestingly, cloning of 43 DAG disclosed that 43 DAG and 156 DAG are encoded by a single 5.8 kb mRNA. Post-translational modification of a 97 kDa precursor protein (dystroglycan) results in two mature proteins (Fig. 4). The 156 DAG and 43 DAG have thus been termed α -dystroglycan and β -dystroglycan, respectively. So far the primary structure of dystroglycan has been determined completely in rabbit (Ibraghimov-Beskrovnaya *et al.*, 1992) and human (Ibraghimov-Beskrovnaya *et al.*, 1993), and partially in mouse (Górecki *et al.*, 1994). Dystroglycan has no significant homology with any known proteins. Both rabbit and human cDNAs encode 895 amino acids with a calculated *Mr* of 97 kDa (Fig. 5). Overall sequence identity is 93%. In addition, 90% of the

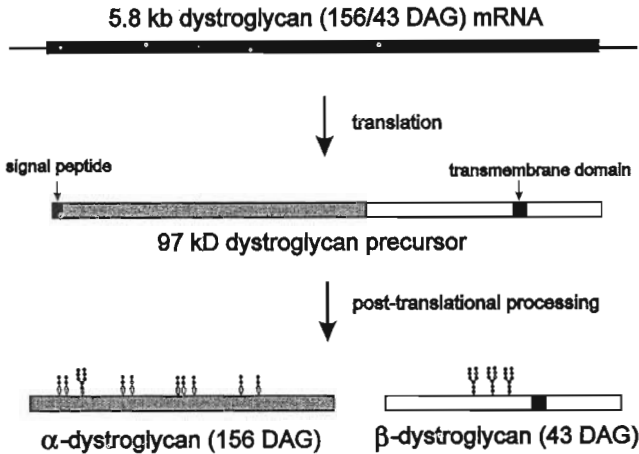


Figure 4 Post-translational processing of the dystroglycan precursor. Cleavage and extensive glycosylation of the 97-kDa precursor generate α -dystroglycan (156 DAG) and β -dystroglycan (43 DAG).

amino acid substitutions are conservative. The first 29 (rabbit) or 27 (human) amino acids are predominantly hydrophobic and represent a signal sequence. Near the C-terminus, there is a single transmembrane domain of 24 amino acids which is completely conserved between these two species. The intracellular C-terminal tail is also highly conserved and has several features consistent with signal transduction and/or cytoskeletal organization; several phosphotyrosine consensus sequences and proline-rich regions that could serve as binding sites for SH2 and SH3-like domains, respectively. There are 4 potential N-linked glycosylation sites and numerous potential O-linked glycosylation and phosphorylation sites. Biochemical characterization demonstrated that β -dystroglycan has hydrophobic properties characteristic of a transmembrane protein and contains N-linked oligosaccharides, whereas, α -dystroglycan is not an integral membrane protein and contains both N-linked and O-linked glycosylation (Ervasti and Campbell, 1991). Furthermore, the elegant experiment using site-specific fusion proteins demonstrated that β -dystroglycan is derived from the C-terminal half and α -dystroglycan is derived from the N-terminal half of the 97-kDa precursor polypeptide (Ibraghimov-Beskrovnaya *et al.*, 1992). The precise cleavage site in the 97-kDa precursor protein has not been determined, however, there are two candidate cleavage sites; one is Arg 640 based on the microsequencing data of brain α -dystroglycan (Gee *et al.*, 1993), and the other site is Arg 652 based on the microsequencing data of a *Torpedo* β -dystroglycan homologue which was isolated as part of the agrin receptor (Fallon *et al.*, unpublished data).

RABBIT	50	KKPRTFVPRVTTKAPITRLESTASPPTRIRITTSVPRGGEPRORPELAK	500
HUMAN	50	KKPRTFVPRVTTKVSITRLESTASPPTRIRITTSVPRGGEPRORPELAK	500
RABBIT	100	NHIDVDAMVGTFFVKVIPSDDTFYDKEDTTDKLKLTLKLAQQQLVGEKS	550
HUMAN	100	NHIDVDAMVGTFFVKVIPSDDTFYDHDHTTDDKLLKTLKLAQQQLVGEKS	550
RABBIT	150	VYQFNNSQLMYGLDPSVSVGHEVTHMATHDKGLSANDAVFIHVHRPQ	600
HUMAN	150	VYQFNNSQLMYGLDPSVSVGHEVTHMATHDKGLSANDAVFIHVHRPQ	600
RABBIT	200	GDKAPAFKAFVGDPAFVVDNDHKKALKVKLAFADGRNCSTVTLQNI	650
HUMAN	200	GDRAPAFKAFVGDPAFVVDNDHKKALKVKLAFADGRNCSTVTLQNI	650
RABBIT	250	TRGSIVVFWNTNTPLEPCFKSQITGLSRIEAEDNGQPAPAFYNALPDF	700
HUMAN	250	TRGSIVVFWNTNTPLEPCFKSQIAGLSRIEAEDNGQPAPAFYNALPDF	700
RABBIT	300	KATSLAITGSGSCRHLQFIQVAPGIPISVTPTEVPDRDPEKSEDDVY	750
HUMAN	300	KATSLITGSGSCRHLQFIQVPPRPRVPEAPTEVPDRDPEKSEDDVY	750
RABBIT	350	LHTVPAVVAAILLAGLMIYCKRKRKGLTLEDQATFIKGVPIIF	800
HUMAN	350	LHTVPAVVAAILLAGLMIYCKRKRKGLTLEDQATFIKGVPIIF	800
RABBIT	400	ADELDDSPFSSMPLILIQEKAPLPPPEYPSQVPEYETPLNQDTVGY	850
HUMAN	400	ADELDDSPFSSMPLILIQEKAPLPPPEYPSQVPEYETPLNQDTVGY	850
RABBIT	450	TPLRDEDPNAPFYQPPPPFFAPMKGKGRPKMFTYRSPPFPVPP	895
HUMAN	450	TPLRDEDPNAPFYQPPPPFTVMKKGSRPKMFTYRSPPFPVPP	895

Figure 5 Primary structure of human and rabbit dystroglycan. Both human and rabbit dystroglycan consists of 895 amino acids and share 93% identity. Identical amino acids are indicated by asterisks and conservative amino acids by dots. The putative signal peptide is underlined and the single transmembrane domain is **boldly underlined**.

Genomic structure: As shown in Fig. 6, human dystroglycan coding sequence is organized into two exons (Ibraghimov-Beskrovnaya *et al.*, 1993). The first exon encodes the 5'-untranslated region and 285 nucleotides of coding sequence, including the initiating methionine codon. The second exon encodes the rest of the coding sequence (starting at nucleotide 286) together with part of the 3'-untranslated region. The rest of the 3'-untranslated region is organized in other exon(s) to be characterized. There is a large intron sequence which is more than 10 kb in size between exon 1 and 2.

Chromosomal localization: The human dystroglycan gene was first localized to chromosome 3 by Southern blot analysis of human/hamster cell hybrids using a radiolabelled dystroglycan cDNA probe. Further refined localization on chromosome 3p21 was determined by fluorescence in situ hybridization (FISH) (Ibraghimov-Beskrovnaya *et al.*, 1993). The somatic cell hybrid analysis and FISH demonstrated a single site of specific hybridization, suggesting that the dystroglycan gene is a single copy gene. A conserved region of synteny exists between human chromosome 3p21-q23 and a distal portion of mouse Chromosome 9. In fact, recently mouse dystroglycan cDNA has been partially cloned and mouse dystroglycan gene has been mapped to Chromosome 9. Interestingly, two neurological mouse mutation loci, ducky (*du*) and tippy (*tip*), are located on distal mouse Chromosome 9 close to *GNAI2/gnai-2* and *GNAT1/gnat-1* whose homologs map to human chromosome 3p21. Homozygous ducky mice (*du/du*) show waddling or reeling gait and have dysgenesis of hindbrain and spinal cord, and dysmyelination and axonal dystrophy in the spinocerebellar and vestibospinal tracts. Homozygous tippy mice (*tip/tip*) are small, hyperactive and unstable in walking, and die by 20 to 25 days of age. The mapping of the mouse dystroglycan gene to the region

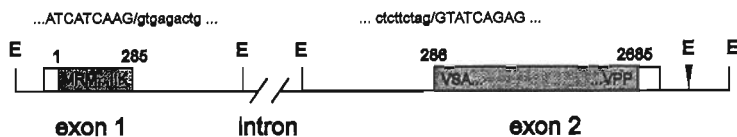


Figure 6 Diagram of the genomic structure of human dystroglycan. The gene is represented by the line with an interruption at the location of the intron. Exons are shown by boxes and coding sequences are shaded. Three amino acids at 5' and 3' ends of each exon are indicated. The nucleotide sequences at exon/intron junctions are presented above. The nucleotides in uppercase are exon sequence while those in lowercase are intron sequence. A polymorphic *EcoRI* site is indicated by the arrowhead.

containing *du* and *tip* loci suggests possible involvement of dystroglycan for these mutant phenotypes.

So far no known human neuromuscular disorders are linked to this region, however, in chromosomal band 3p21 deletions or chromosomal rearrangements are associated with particular malignancies such as breast cancer, renal cell carcinoma, lung cancer and testicular cancer. (Yokota *et al.*, 1987; Lothe *et al.*, 1989; Yamakawa *et al.*, 1991; van der Hout *et al.*, 1991). These findings suggest a possibility that the dystroglycan gene could be a tumor suppressor gene.

TISSUE DISTRIBUTION AND TISSUE-SPECIFIC ISOFORMS

Tissue distribution of dystroglycan expression was examined by both northern blot and western blot analyses in rabbit and human (Ibrahimov-Beskrovnaya *et al.*, 1992 and 1993). A 5.8 kb transcript was detected in various muscle (skeletal and cardiac) and non-muscle (brain, liver, kidney, pancreas, etc.) tissues (Fig. 7A). The mRNA is most abundant in skeletal muscle and heart and less abundant in non-muscle tissues. By immunoblot analysis dystroglycan was detected in rabbit skeletal muscle, heart, brain, and lung with an affinity-purified antibody recognizing the core protein of dystroglycan (Fig. 7B). α -dystroglycan detected in cardiac muscle has a slightly lower molecular weight compared with that in skeletal muscle. In brain and lung, the molecular

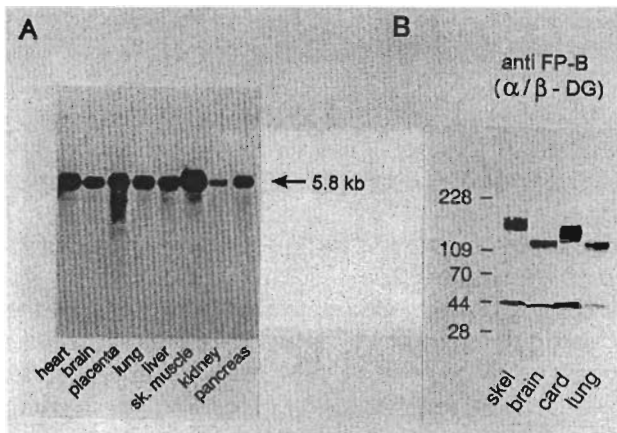


Figure 7 Tissue distribution of dystroglycan. (A) Northern blot analysis of human adult tissues. Each lane contains 2 μ g of human poly(A)⁺RNA from various tissues indicated. RNA was probed with ³²P-labeled dystroglycan cDNA. (B) Immunoblot analysis of total microsome fractions isolated from rabbit tissues. Nitrocellulose transfer was immunostained with anti FP-B antibody recognizing the core polypeptide of α -/ β -dystroglycan.

weight of α -dystroglycan is not 156 kDa but 120 kDa. Interestingly, LBP-120, laminin-binding protein of 120 kDa, isolated from the brain is closely related to α -dystroglycan; 5 peptides from bovine brain LBP-120 are almost identical to the amino acid sequence of rabbit muscle α -dystroglycan (Gee *et al.*, 1993). These results strongly suggest that the LBP-120 is a brain form of α -dystroglycan. Recently, brain localization of dystroglycan was examined by *in situ* hybridization (Górecki *et al.*, 1994). Dystroglycan mRNA is co-localized with dystrophin mRNA in the hippocampal formation (Ammon's horn and the dentate gyrus) and cerebellum (mostly Purkinji neurons). Dystroglycan mRNA was also detected in the olfactory bulb, thalamus, and the choroid plexus. These distribution patterns of dystroglycan could provide a clue to understand the cognitive impairment in DMD patients as discussed later.

How are these tissue-specific dystroglycan isoforms produced? Since the 5.8 kb single is detected using probes specific for exon 1 or exon 2 in muscle and non-muscle tissues (Ibraghimov-Beskrovnaya *et al.*, 1993), dystroglycan transcripts in these tissues seem identical. Additionally, the relatively simple two-exon genomic structure implies unlikely opportunity for alternative splicing. RT-PCR using two different reverse primers from exon 1 or exon 2 amplified identical products of predicted size based on the skeletal muscle cDNA from brain and heart RNA (Ibraghimov-Beskrovnaya *et al.*, 1993). These data demonstrate that tissue specific isoforms do not differ by primary structure. Thus, the variability in the molecular weight for α -dystroglycan may be due to differential glycosylation of the core protein in muscle versus non-muscle tissues.

LAMININ BINDING PROPERTIES OF α -DYSTROGLYCAN

α -Dystroglycan has been identified as a peripheral membrane protein based on several experimental findings: (1) a hydrophobic probe does not label α -dystroglycan (Ervasti and Campbell, 1991), (2) extraction from surface membrane at high pH (Ervasti and Campbell, 1991), and (3) extracellular localization observed by immunoelectronmicroscopy (Cullen *et al.*, 1995). An extracellular localization of α -dystroglycan suggests that it may interact with the extracellular matrix. Overlay studies and affinity precipitation experiments using major extracellular matrix components such as laminin, fibronectin, entactin, and heparan sulfate proteoglycan revealed that α -dystroglycan binds laminin specifically (Ervasti and Campbell, 1993) (Fig. 8). Affinity precipitation experiments demonstrated that non-muscle dystroglycan (brain and lung) also binds laminin (Ervasti and Campbell, 1993).

α -Dystroglycan binds laminin with high affinity. The K_d of the laminin binding to LBP-120/brain α -dystroglycan was determined to be 90 nM by Scatchard analysis (Gee *et al.*, 1993), whereas affinity co-electrophoresis of muscle α -dystroglycan with laminin yielded an apparent K_d of 4.7 nM (Lander *et al.*, unpublished data). This difference may be caused by the different

assays used to determine the K_d or by the different binding affinity between muscle and non-muscle dystroglycan. However, both K_d values demonstrated the high affinity laminin-binding property of α -dystroglycan, suggesting its structural role in muscle and brain. The binding of laminin to dystroglycan is inhibited by the inclusion of 10 mM EDTA (Fig. 8). The presence of CaCl_2 , but not MgCl_2 restores this binding. Thus, the interaction between α -dystroglycan and laminin is calcium ion dependent. The binding is also inhibited by inclusion of NaCl with an average half-maximal concentration of inhibition (IC_{50}) of 250 mM (Fig. 8). Heparin also inhibits laminin binding to dystroglycan with an IC_{50} of 100 $\mu\text{g}/\text{ml}$, while chondroitin sulfate and keratan sulfate are without effect (Fig. 8). The ionic strength dependence and heparin inhibition of laminin binding to dystroglycan suggest that polyanionic moieties are important to the laminin-binding activity of dystroglycan. Since neuraminidase and N-glycosidase F treatment of α -dystroglycan does not affect laminin binding, negatively charged oligosaccharides other than sialic acid are necessary for the laminin binding activity of dystroglycan (Ervasti and Campbell, 1993).

Although the precise binding site(s) on the laminin molecule have not been determined, LBP-120/brain α -dystroglycan has been shown to bind to a proteolytic laminin E3 fragment containing the major heparin binding domain (Gee *et al.*, 1993). Interestingly, monoclonal antibody IIH6 specific for α -dystroglycan blocks the laminin binding to α -dystroglycan, suggesting that the binding sites for IIH6 and laminin overlap (Ervasti and

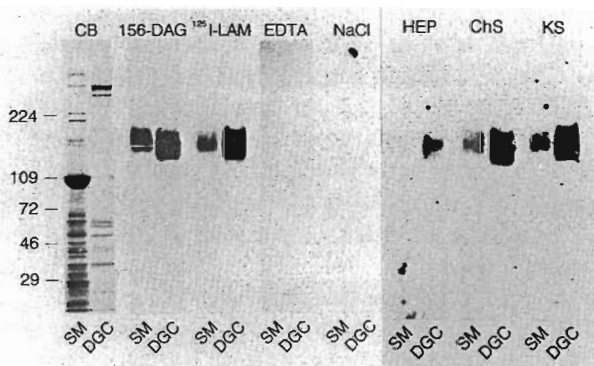


Figure 8 Laminin-binding properties of α -dystroglycan. Rabbit skeletal muscle surface membrane (SM) and the dystrophin-glycoprotein complex (DGC) were separated on a 3–12% SDS polyacrylamide gel and transferred to nitrocellulose. The gel was stained with Coomassie blue (CB). A nitrocellulose transfer was stained with monoclonal antibody IIH6 against α -dystroglycan (156-DAG) or overlaid with ^{125}I -laminin in the absence (^{125}I -LAM) or in the presence of 10 mM EDTA (EDTA) or 0.5 M NaCl (NaCl). Laminin-binding to dystroglycan is inhibited by 1,000-fold excesses of heparin (HEP) in an overlay medium, but not by chondroitin sulfate (ChS) or keratan sulfate (KS).

Campbell, 1993).

DYSTROGLYCAN IN DMD

Mechanism for muscle cell necrosis: Although early immunochemical studies revealed that dystrophin is totally missing from skeletal muscle of DMD patients, the precise mechanism by which the absence of dystrophin leads to muscle cell necrosis has not been fully elucidated. Identification of the DGC linking the actin cytoskeleton to the extracellular matrix provided a possibility that the DGC has a mechanical function to stabilize the sarcolemma and protect it from stresses developing during muscle contraction. Thus, the absence of dystrophin could disrupt the laminin-DGC-cytoskeletal linkage and render the sarcolemma more susceptible to damage from muscle contraction. Several functional experiments supported the proposed mechanical function of the DGC. Dystrophin-deficient muscle fibers of the *mdx* mouse, a murine model for DMD, exhibit an increased susceptibility to contraction-induced sarcolemmal rupture (Petrof *et al.*, 1993). Measurement of surface membrane stiffness evaluated as the increment force required for 1mm indentation demonstrated that normal myotubes are four-fold stiffer than myotubes cultured from *mdx* mice (Pasternak *et al.*, 1995). Further supportive evidence came from extensive analyses of the correlation between deleted regions in truncated dystrophin and the phenotype of the disease. In general, patients lacking the C-terminal domains which contain binding sites for dystrophin-associated proteins or the N-terminal actin binding domain show severe phenotype (DMD), whereas patients having deletions in the rod domain show milder phenotype (BMD). These findings suggest that the disruption of the linkage mediated by the DGC may be critical for pathogenesis of DMD.

Additionally, immunohistochemical studies revealed a secondary drastic reduction in all of the dystrophin-associated proteins in the sarcolemma of DMD muscle (Ervasti *et al.*, 1991, Ohlendieck *et al.*, 1993). A similar reduction in dystrophin-associated proteins was observed in the *mdx* mouse (Ervasti *et al.*, 1991, Ohlendieck and Campbell, 1991). The specificity of the loss of the dystrophin-associated proteins is demonstrated in DMD carriers where the only fibers positive for dystrophin are also positive for the dystrophin-associated proteins. Interestingly, the severity of the reduction in dystrophin-associated proteins correlates well with the severity of the phenotype. In BMD patients there is a mild reduction of the dystrophin-associated proteins. In DMD patients lacking the C-terminal domains of dystrophin, all of the dystrophin-associated proteins are drastically reduced despite proper localization of the truncated dystrophin to the sarcolemma (Matsumura and Campbell, 1994). Thus, secondary reduction of the dystrophin-associated proteins may contribute to the pathogenesis by further disruption of the muscle cell attachment to the extracellular matrix. In this context, dystroglycan could

be involved in the pathogenesis of muscle cell necrosis. Actually, secondary deficiency of α -dystroglycan correlates with elevated serum creatine kinase and decreased muscle contraction tension in dystrophin-deficient dogs with golden retriever muscular dystrophy (Ervasti *et al.*, 1994).

Cognitive impairment in DMD: DMD is associated with a variable degree of non-progressive mental retardation, where the verbal skills are the most affected, probably as a consequence of memory deficits (Billard *et al.*, 1992). The brain is the major site of dystrophin expression. Dystrophin proteins from several different transcripts are localized to the postsynaptic regions of several types of neurons in areas associated with cognitive function and learning (Górecki *et al.*, 1992). Although no specific histopathological abnormalities have been demonstrated in the brains of DMD patients, subtle functional abnormalities associated with dystrophin absence in special neurons could be involved in the cognitive impairment of DMD. Dystroglycan colocalizes with dystrophin in the hippocampal formation which is associated with cognitive function (Górecki *et al.*, 1994). Hippocampal damages are known to cause loss of memory storage and verbal and learning deficits which are consistent with the features of mental retardation in DMD patients. Dystrophin and dystroglycan also colocalize in the cerebellum which is involved in movement coordination and learning and memory of motor tasks. Thus, impaired function of the cerebellum could affect language processing. The lack of dystrophin in DMD skeletal muscle drastically reduces the amount of dystroglycan in the sarcolemma, which may contribute to instability of the linkage. Thus, colocalization of dystrophin and dystroglycan in the hippocampal formation and cerebellum raises the intriguing possibility that dystroglycan could be involved in cognitive impairment of DMD patients.

LAMININ α 2 CHAIN: A NATIVE LIGAND FOR DYSTROGLYCAN IN SKELETAL MUSCLE

Laminin α 2 chain (merosin, new nomenclature based on Burgeson *et al.*, 1994) was first isolated from human placenta as a laminin α 1 (A) chain homologue (Leivo and Engvall, 1988). The α 2 chain is a constituent of the laminin-2 heterotrimer, the major laminin isoform in muscle and peripheral nerve, together with β 1 (B1) and γ 1 (B2) chains (Ehrig *et al.*, 1990, Engvall *et al.*, 1990, Paulsson *et al.*, 1991). Recently the complete amino acid sequence and the gene locus of the human (Vuolteenaho *et al.*, 1994) and mouse α 2 chain (Bernier *et al.*, 1995) have been determined. The α 2 chain has a domain structure similar to that of the α 1 chain; domains VI, IVa and IVb of the α 2 chain are predicted to form globular structures. Domains V, IIIb, and IIIa contain cysteine-rich EGF-like repeats and are predicted to form rigid rod-like structures. Domains I+II are involved in the formation of a triple

stranded coiled-coil structure that forms the long arm of the laminin heterotrimer together with laminin $\beta 1$ and $\gamma 1$ chains. The C-terminal region contains five internal homologous repeats to form a large globular domain (G domain). The most N-terminal globular domain is highly conserved between the $\alpha 2$ and $\alpha 1$ chains, whereas the α -helical domains I+II have low homology.

As a member of the laminin family, laminin-2 has various biological functions in cell attachment, neurite outgrowth promotion, and Schwann cell migration and participates in the formation of supramolecular structures by self aggregation or by interacting with other components of the basal lamina. Laminin $\alpha 2$ chain is specifically expressed in skeletal muscle, cardiac muscle, Schwann cells, and placental trophoblasts. Thus, the $\alpha 2$ chain is a reasonable candidate for a native ligand for α -dystroglycan in skeletal muscle. The association of the $\alpha 2$ chain with the DGC in skeletal muscle was demonstrated by sucrose density gradient centrifugation and immunoprecipitation using anti-adhalin immunoaffinity beads (Sunada *et al.*, 1994). As will be discussed later, agrin is a ligand for α -dystroglycan at the neuromuscular junctions. Thus, laminin $\alpha 2$ chain is a native ligand for α -dystroglycan at extrajunctional regions in skeletal muscle, suggesting that the laminin-dystroglycan interaction is a major mechanism of attachment between muscle cells and the extracellular matrix.

LAMININ $\alpha 2$ CHAIN-DEFICIENT MUSCULAR DYSTROPHIES

Recently, deficiency of laminin $\alpha 2$ chain has been identified in one form of congenital muscular dystrophy and its murine models. The deficiency of the most extracellular component of the linkage between the extracellular matrix and the cytoskeleton leading to muscle degeneration provides a novel etiology of muscular dystrophy.

Laminin $\alpha 2$ chain-deficient congenital muscular dystrophy: Demonstration that laminin $\alpha 2$ chain is a native ligand for α -dystroglycan prompted investigation of whether the $\alpha 2$ chain deficiency could be involved in autosomal recessive muscular dystrophy. A specific absence of the $\alpha 2$ chain was observed in 13 patients affected by a classical, non-Fukuyama type congenital muscular dystrophy (Tomé *et al.*, 1994). Congenital muscular dystrophy is characterized by its early clinical onset. Manifestations of CMD are evident at birth or in the first few months of life and consist of muscle weakness and hypotonia, delayed motor milestones, and early joint contractures. Several forms of CMD have been described. CMD associated with brain anomalies, prevalent in Japan, is known as Fukuyama-type CMD (Fukuyama *et al.*, 1981) and was recently mapped to chromosome 9q31–33 (Toda *et al.*, 1993). The most frequent form observed in occidental countries is clinically characterized by exclusive involvement of skeletal muscle and thus called classical type or pure type

CMD. Immunofluorescence analysis showed that laminin $\alpha 2$ chain is totally missing from the basal lamina of skeletal muscle fibers from classical CMD patients. A 300-kDa fragment of the $\alpha 2$ chain was undetectable by immunoblotting. The absence of the $\alpha 2$ chain in CMD patients also leads to a disruption of the laminin-DGC-cytoskeletal linkage. Recently, laminin $\alpha 2$ chain-negative CMD has been linked to chromosome 6q2 in the region containing the $\alpha 2$ chain gene (Hillaire *et al.*, 1994). Although these data strongly suggest that a mutation in the $\alpha 2$ chain gene may be responsible for this form of CMD, such a mutation has not been identified yet. In addition, there is a subgroup of classical CMD patients who show normal $\alpha 2$ chain expression and are excluded from chromosome 6q2 linkage.

dy (*dystrophia muscularis*) and *dy*^{2J} mice: The *dy* mouse and its allelic strain the *dy*^{2J} mouse presenting muscle degeneration have been studied as murine models for autosomal recessive muscular dystrophy (Meier and Southard 1970, Bray and Banker 1970, McPike and Meier 1976). Recently, the primary structure of mouse laminin $\alpha 2$ chain cDNA was determined by cDNA cloning (Bernier *et al.*, 1995) and its gene (*Lama2*) was mapped to a proximal portion of Chromosome 10 close to the *dy* locus (Sunada *et al.*, 1994). The immunohistochemical analysis using an affinity-purified antibody against the $\alpha 2$ chain showed a drastic reduction (Sunada *et al.*, 1994) or absence (Arahata *et al.*, 1993, Xu *et al.*, 1994a) of the $\alpha 2$ chain in *dy* mouse skeletal muscle and peripheral nerve compared with control mice. Western blot analysis data were consistent with more than 95% reduction of the $\alpha 2$ chain with normal molecular weight (Sunada *et al.*, 1994). Consistent reduction of the $\alpha 2$ chain mRNA in amount was confirmed by northern blot analysis (Xu *et al.*, 1994a). Together with the *Lama2* locus close to the *dy* locus, these data suggest that $\alpha 2$ chain deficiency may be a primary cause of the *dy* phenotype.

The allelic *dy*^{2J} mouse shows a less severe phenotype. Immunohistochemistry showed less severe reduction of laminin $\alpha 2$ chain in amount. Interestingly, *dy*^{2J} mice expressed a mutant $\alpha 2$ chain with smaller molecular weight suggestive of a deletion in the $\alpha 2$ chain transcript (Xu *et al.*, 1994b Sunada *et al.*, 1995). RT-PCR amplified several different variant transcripts from *dy*^{2J} mice with deletions and/or insertions. Sequencing of genomic PCR product identified an G to A transition at the first nucleotide of an intron sequence resulting in a disruption of a splice donor site. Subsequently, various transcripts arise from various aberrant splicing (Xu *et al.*, 1994b). We characterized a major variant transcript which may be translated to a truncated $\alpha 2$ chain polypeptide in the *dy*^{2J} mouse. This transcript has a single in-frame deletion of 171 nucleotides eliminating 57 amino acids (residues 34 to 90) with a substitution of glutamine for glutamic acid at residue 91 (Sunada *et al.*, 1995). The deletion also eliminates two potential N-linked glycosylation sites. Interestingly, the deletion is located in the N-terminal domain VI which is presumed to be involved in self-aggregation of laminin-2 heterotrimers as

in the case of laminin $\alpha 1$ chain (Schittny and Yurchenco 1990). Thus, a deletion in domain VI could disrupt the formation of the laminin-2 network in the basal lamina of *dy^{2J}* mice and loosen the integrity of the laminin-DGC-cytoskeletal linkage and, in turn, result in muscle cell degeneration.

ROLE OF THE LAMININ-DGC-CYTOSKELETAL LINKAGE IN THE PATHOGENESIS OF MUSCULAR DYSTROPHIES

SCARMD: Extensive analysis of dystrophin-associated proteins in various types of muscular dystrophy revealed a specific deficiency of adhalin (50 DAG) in severe childhood autosomal recessive muscular dystrophy (SCARMD) patients (Matsumura *et al.*, 1992a). SCARMD resembles DMD phenotype but affects both male and female at equal rates (Ben Hamida *et al.*, 1983). SCARMD was first identified in Tunisia and has since been found in other populations. In several North African families, SCARMD has been linked to a pericentromeric region of chromosome 13q (Ben Othmane *et al.*, 1992). Recently, the adhalin gene was mapped to chromosome 17q12–21.33, excluding the gene from involvement in 13q-linked SCARMD (Roberds *et al.*, 1994). However, the adhalin gene was linked to SCARMD in one large family and missense mutations of the adhalin gene were identified in this family (Roberds *et al.*, 1994). Since then, several additional adhalin gene mutations including nonsense, missense, splice site and insertional mutations have been identified in SCARMD patients from different ethnic origin (Piccolo *et al.*, 1995).

Adhalin deficiency was observed in the BIO 14.6 cardiomyopathic hamster which experiences both autosomal recessive cardiomyopathy and myopathy, suggesting that this could be an animal model for SCARMD (Roberds *et al.*, 1993). Biochemical experiments on isolated membranes from BIO 14.6 cardiomyopathic hamster skeletal muscle have demonstrated that dystrophin and α -dystroglycan are not tightly associated with the sarcolemma (Roberds *et al.*, 1993). Thus, loss of adhalin can lead to the functional disruption of the dystrophin-glycoprotein complex.

Diseases of the laminin-DGC-cytoskeletal linkage: The observation in three different types of muscular dystrophy where defects of particular components of the laminin-DGC-cytoskeletal linkage leads to muscle cell degeneration (Fig. 9) suggests that these muscular dystrophies are diseases of muscle cell attachment to the extracellular matrix. Since the laminin-DGC-cytoskeletal linkage is the major attachment site in skeletal muscle, the integrity of this linkage probably stabilizes the muscle cell attachment to the extracellular matrix and protects the sarcolemma from stresses which develop during muscle contraction. In this critical laminin-DGC-cytoskeletal linkage, dystroglycan plays a major role in linking laminin-2 and dystrophin.

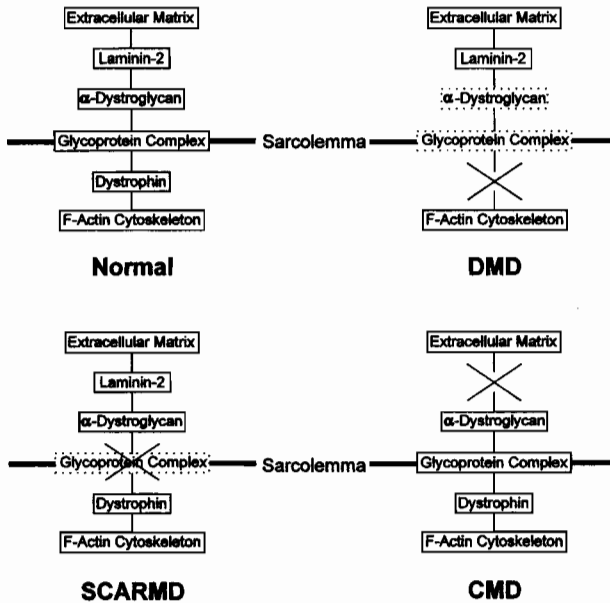


Figure 9 Involvement of the laminin-DGC-cytoskeletal linkage in the pathogenesis of muscular dystrophies. X indicates a primary deficient component. Dotted boxes indicate secondary deficient components. In normal skeletal muscle, α -dystroglycan links laminin-2 with the sarcolemma. Integral membrane glycoprotein complex including β -dystroglycan and adhalin, links α -dystroglycan and dystrophin. Finally, dystrophin binds F-actin through the N-terminal domain. Disruptions of this critical linkage caused by the deficiency of particular components (dystrophin absence in *DMD*, adhalin deficiency in *SCARMD*, laminin $\alpha 2$ chain deficiency in *CMD*) lead to muscle cell necrosis in muscular dystrophy.

FUNCTIONAL DIVERSITY OF DYSTROGLYCAN IN VARIOUS TISSUES

As described above, dystroglycan was originally isolated from skeletal muscle as a component of the DGC. Its ubiquitous expression in various tissues as well as the presence of tissue-specific isoforms suggest its diverse biological functions. Recent biochemical studies revealed that α -dystroglycan interacts with different ligands in the extracellular matrix and β -dystroglycan binds different DMD gene products or its homologues. Thus, there is a family of dystroglycan complexes in several different tissues as shown in Fig. 10. Utrophin (DRP), the dystrophin homologue encoded by a gene on chromosome 6q24 which is ubiquitously expressed in most tissues and localized to the neuromuscular junction in normal muscle, is associated with dystrophin-associated proteins (Matsumura *et al.*, 1992b). Moreover, α -dystroglycan is a major agrin binding protein. Thus, the agrin-dystroglycan-utrophin complex may occur at

Dystroglycan Complexes

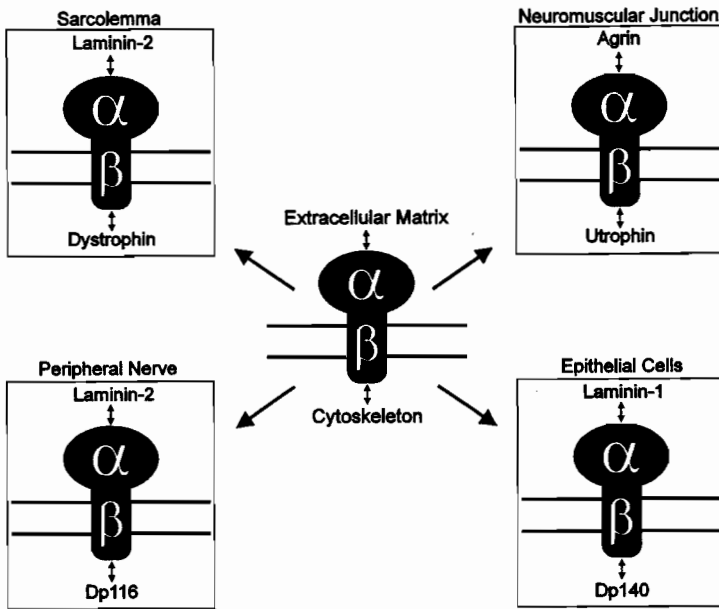


Figure 10 Heterogeneity of the dystroglycan complex linking the extracellular matrix and cytoskeleton in different tissues.

neuromuscular junctions. In peripheral nerve, the 116 kDa protein product (Dp116) of the DMD gene could complex with dystroglycan and laminin-2. In epithelial cells, dystroglycan may be associated with Dp140 (the 140-kDa protein product of the DMD gene) and laminin-1 (Ekblom *et al.* unpublished data).

Dystroglycan and synaptogenesis: Mechanism of synapse formation (synaptogenesis) is a major problem in neuroscience to understand the complexity of the nervous system. The most well-characterized synapse is the peripheral synapse between nerve and muscle, the neuromuscular junction. As the accumulation of acetylcholine receptors (AChRs) at the postsynaptic membrane is the initial event of synaptogenesis (Hall and Sanes, 1993) and is thought to be mediated by the basal lamina protein agrin (McMahan, 1990; Bowe and Fallon, 1994), agrin is thus positioned to play a key role in the process of synaptogenesis. Since agrin does not bind to the AChR itself (Nastuk *et al.*, 1991; Ma *et al.*, 1993), recent efforts have been directed to discover the receptor that mediates the clustering of AChRs and have led to a surprising conclusion that α -dystroglycan is the only agrin-binding protein so far identified

(Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994).

The biochemical evidence was obtained by two independent approaches. In one approach, an agrin-binding protein was purified from *Torpedo* electric organ postsynaptic membranes by immunoaffinity or agrin affinity chromatography. The purified complex contains two glycoproteins of 190 kDa and 50 kDa. Microsequencing of these components revealed sequence nearly identical to α - or β -dystroglycan, respectively (Bowe *et al.*, 1994). The separate approach was started from the hypothesis that agrin is a possible ligand for α -dystroglycan based on the following facts; (1) α -dystroglycan binds the G-domain of laminin α 1 chain (Gee *et al.*, 1993), and (2) the C-terminal portion of agrin contains homologous sequence to the G domain of the laminin α 1 chain (Rupp *et al.*, 1992). Agrin binds an α -dystroglycan-like protein in cultured C2 muscle cells and purified rabbit α -dystroglycan. This binding was inhibited by laminin, heparin, and removal of calcium ion. The binding of agrin to α -dystroglycan was blocked by I1H6 monoclonal antibody specific for α -dystroglycan (Gee *et al.*, 1994). These results demonstrated that α -dystroglycan is an agrin-binding protein and makes it a strong candidate for the agrin receptor which is involved in signaling for AChR clustering. This means that dystroglycan could play a role in signal transduction other than its structural role linking the extracellular matrix and the cytoskeleton.

Dystroglycan and myelination: The *dy* or *dy^{2J}* phenotype also presents developmental dysmyelination of peripheral nerve in addition to severe muscle degeneration (Bradley and Jenkinson 1973, Madrid *et al.*, 1975). This drastic change is predominantly observed at the spinal nerve roots where the paucity of Schwann cells and a defective myelination process result in the presence of large bundles of naked axons, thin myelin sheath, short internode and long nodal gap. At birth, Schwann cells are deficient in the *dy* mouse spinal roots, but many undifferentiated cells capable of cell division are present, which are probably uncommitted Schwann cells that can differentiate when transplanted to a normal environment (Perkins *et al.*, 1981). Thus, Schwann cells, responsible for wrapping and myelinating neuronal cells, appear to lack the ability to differentiate and migrate on the axonal surface of neuronal cells in the *dy* mouse. Laminin-2 has been shown to promote neurite outgrowth and Schwann cell migration (Engvall *et al.*, 1992; Anton *et al.*, 1994), which are the two most critical events during the normal development of the nervous system. These biological effects are mediated by specific interactions between structural domains of laminin-2 and different cell surface laminin receptor. Since α -dystroglycan is a major α 2 chain receptor in Schwann cells (Yamada *et al.*, 1994), the α 2 chain-dystroglycan interaction could be involved in the normal myelination process of peripheral nerve.

Dystroglycan and epithelial morphogenesis: The formation of organized

epithelial sheets with polarized cells is an important event during organogenesis. Cell-cell adhesion mediated by cadherins (Gumbiner, 1992; Larue *et al.*, 1994) and adhesion of cells to the basal lamina (Klein *et al.*, 1988) are critical for initiation of epithelial cell polarity. In the developing kidney, the interaction of laminin $\alpha 1$ chain with its receptors is critical for the onset of epithelial cell polarization (Klein *et al.*, 1988). Both E8 and E3 fragments of laminin-1 are involved in the interaction. The former binds to $\alpha 6\beta 1$ integrin (Sonnenberg *et al.*, 1990) and antibodies against the $\alpha 6$ subunit of the integrin interfere with renal tubule development (Sorokin *et al.*, 1990). Since renal tubule development can be partially inhibited by antibodies to the E3 fragment (Klein *et al.*, 1988; Sorokin *et al.*, 1992), additional laminin receptors which bind to the E3 fragment could be involved in renal tubule development. Recently, a remarkable co-expression of dystroglycan with laminin $\alpha 1$ chain was demonstrated in embryonic Kidney epithelium. Furthermore, monoclonal antibody IIH6 against α -dystroglycan perturbed development of kidney epithelium in organ culture of mouse embryonic kidney (Durbeej *et al.*, 1995). These results strongly suggest that α -dystroglycan is the receptor for the E3 laminin fragment and is involved in the regulation of epithelial morphogenesis.

CONCLUSION

Dystroglycan is a novel non-integrin laminin receptor. As a major component of the DGC linking the extracellular matrix and the cytoskeleton, dystroglycan is involved in the pathogenesis of muscular dystrophies including DMD/BMD, laminin $\alpha 2$ chain-deficient CMD, and SCARMD. On the other hand, characterization of the dystrophin-glycoprotein complex in various tissues revealed multiple variants in components, suggesting that there is a family of dystroglycan complexes that serve different functions specific for particular cells or subcellular regions. Besides having a structural role in muscle cell attachment to the extracellular matrix, dystroglycan may have diverse biological functions including a possible role in signal transduction. Thus, dystroglycan is a key molecule attracting the general interest of numerous scientists studying neuroscience, developmental biology, and cell biology as well as neuromuscular disorders.

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