Identification of A Novel Protein Associated with β-Dystroglycan in Brain

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ABSTRACT

The dystroglycans, α and β-dystroglycan, form the functional core of the dystrophin glycoprotein complex in skeletal muscle. β-dystroglycan is associated with dystrophin inside the cell, and α-dystroglycan binds to laminin outside the cell, thus dystroglycan serves as a transmembrane linkage between the intracellular cytoskeleton and the extracellular matrix. Disruption of dystroglycan gene leads to muscular dystrophy in mice. Dystroglycan has also been implicated in maturation and maintenance of neuromuscular junctions (NMJ). In muscle fibers with reduced levels of dystroglycan, postsynaptic acetylcholine receptor clusters can form, but they are less dense and unstable. In the central nervous system, however, the role of dystroglycan is unknown. It is expressed by astrocytes, epithelial cells of blood-brain barrier, Purkinje cells of cerebellum and in the outer plexiform layer of retina. Many questions remain unanswered, such as: Does dystroglycan have any role in neuronal synapses as in neuromuscular junctions? Are there novel interacting proteins with dystroglycan that might give it new functions? To address these questions, rabbit antisera against last 15 amino acids of β-dystroglycan were raised. The affinity-purified antisera were tested by western blots and they recognize only a 43 kD dystroglycan band in brain extracts. Immunocytochemical methods were used to determine if dystroglycan is expressed in

cultured rat hippocampal neurons. The data have shown that dystroglycan is expressed in cell bodies, dendrites and axons and forms clusters at some dendritic spines and along the shafts. Immunoaffinity purification revealed that β-dystroglycan is associated with α-dystroglycan in rat hippocampus, but association with all isoforms of dystrophin and utrophin are undetectable. In Coomassie-blue stained and silver stained gels, in addition to the 43 kilodalton (kD) of β-dystroglycan band, a band of about 100 kD was also found. The molecular weight of this band does not match any of the proteins known to interact with β-dystroglycan directly, nor any members of dystrophin glycoprotein complex, and we are currently exploring the interaction of this protein with β-dystroglycan. Together, these results suggest that β-dystroglycan is a neuronal protein that complexes with α-dystroglycan and is present at synapses, and that there are interacting proteins in brain which are different from muscle, thus giving us possibility to dissect new functions of dystroglycan in brain.

Résumé

Les dystroglcans, α et β-dystroglycan, forment le noyau fonctionnel du complexe glycoprotéinique de dystrophine dans le muscle squelettique. β-dystroglycan est associé avec la dystrophine à l'intérieur de la cellule et α-dystroglycan se lie à la laminine à l'exteriéur de la cellule, créant ainsi un complexe qui relie la matrice extracellulaire au cytosquelette intracellulaire. Une altération du gène des dystroglycans mène à la dystrophie musculaire dans la souris. Les dystoglycans ont aussi été impliqués dans la maturation et le maintien des jonctions neuromusculaires. Dans les fibres musculaires avec des niveaux réduits de dystroglycans, des aggrégats de récepteurs d'acétylcholine postsynaptique peuvent encore se former, cependant ces aggrégats sont plus petits, plus instables et plus dispersés à travers la membrane. Dans le système nerveux central, les dystroglycans sont exprimés par les astrocytes, les cellules épitheliales de la barrière hémato-meningée, les cellules Purkinje du cervelet et dans la couche extérieure plétiforme de la rétine. Cependant, plusieurs questions concernant leurs fonctions demeurent. Les Dystroglycans ont-ils un rôle dans les synapses neuronales semblable à celui dans les jonctions neuromusculaires? Ya-t-il de nouvelles protéines qui interagissent avec les dystroglycans leur fournissant de nouvelles fonctions? Pour adresser ces questions, de l'anti-sérum de lapin contre les derniers quinze acides aminés de β-dystroglycan a été produit. Le western blot de tissu cérébral utilisant l'anti-sérum purifié par affinité démontre qu'une bande de 43 kDa correspondant à la β-dystroglycan. L'immunocytochimie avec cet anticorps a demontré que le dystroglycan est exprimé dans les cultures de neuron de l'hippocampe de rat. Ces résultats montrent que β-dystroglycan est localisé dans les corps cellulaires et les dendrites. Dans ces derniers, il forme de petits aggrégats dans les épines et le long du tronc. La purification par immunoaffinité a révélé que β-dystroglycan est associé avec α-dystroglycan dans l'hippocamp du rat, mais que

tous les isoformes de dystrophine et utrophine sont indétectables. Les gels teinté au bleu de Coomassie ou à l'argent démontrent la présence d'une protéine de 100 kD en plus de celle de 43 kDa du β-dystroglycan. Le poids moléculaire de cette protéine ne correspond à aucune protéine connue pour intéragir directement avec β-dystroglycan, ni à aucun autre membre du complex glycoproteinique de la dystrophine. Ensemble, ces résultats suggèrent que β-dystroglycan est une protéine localisé dans les neurons qui forme un complexe avec α-dystroglycan et d'autres partenaires différents de ceux qui se trouvent dans le tissu musculaire. Ces résultats permettront a de révéler de nouvelles fonctions des dystroglycans dans le système nerveux central.

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Chapter 1

Literature Review

Part I: Muscular Dystrophy and Dystrophin Associated Glycoproteins

I: Muscular Dystrophy

The muscular dystrophies are a group of genetically determined muscle disorders marked by progressive wasting and weakness of the skeletal muscle, which often affects cardiac and smooth muscles and other tissues. Among all the muscular dystrophies, Duchenne muscular dystrophy (DMD) is the most common, affecting 1 in 3300 boys. DMD leads to severe muscle wasting and eventual death in late teens and early twenties due to cardiac and respiratory failure. Becker Muscular dystrophy (BMD) is a less common and less severe disorder, which affects 1 in 30,000 boys and often has milder clinical symptoms. Both diseases are recessive disorders caused by mutations in a gene located on the short arm of the X chromosome, and this DMD gene, encodes the protein dystrophin whose molecular weight is 427 kDa (2,17). Purification of dystrophin from rabbit skeletal muscle membranes by Kevin Campbell and coworkers (3), led to identification of other proteins that are complexed with dystrophin (3,4,5,6). The dystrophin associated protein complex (DPC) (Figure 1) includes the extracellular matrix protein laminin, the membrane proteins alpha and beta dystroglycans, intracellular proteins syntrophin, dystrobrevin and utrophin, as well as a transmembrane protein complex of sarcoglycans and sarcospan. It is clear now that the primary defects in many muscular dystrophies are mutations in the genes encoding the DPC. As summarized in Table 1, mutations in dystrophin, sarcoglycans, and laminin (alpha2) are responsible for DMD/BMD dystrophy, Limb-Girdle Muscular Dystrophies, and Congenital Muscle

Dystrophy respectively, but no human diseases have been found so far associated with mutations in dystroglycans, syntrophin or dystrobrevin.

In DMD, the protein dystrophin is completely absent whereas in milder BMD the protein is partially lost (7). The limb-girdle muscular dystrophies (LGMD) affect both male and female, and like in DMD and BMD, present clinically with progressive weakness and wasting in the pelvic and shoulder girdle, with elevated serum creatine phosphokinase levels (7). The LGMDs can be classified as LGMD-1 and LGMD-2, which are autosomal dominant and autosomal recessive respectively. The transmembrane sarcoglycans are responsible for the class 2 LGMD (Table 1), and gamma sarcoglycan is responsible for the Severe Childhood Autosomal Recessive Muscular Dystrophy (SCARMD) or LGMD-2C. Typically, the mutations associated with severe LGMD-2C are nonsense mutations that result in a premature stop codon in the gamma sarcoglycan transcript (7). Missense mutations in alpha and beta-sarcoglycans have also been found in severe autosomal recessive muscular dystrophy (LGMD-2D and LGMD-2E) (8,9). Congenital muscular dystrophies encompass several disorders and share common clinical features: neonatal onset of severe weakness, elevated serum creatine phosphokinase levels, and myopathic muscular degeneration. Mutations in the alpha 2-laminin chain, that cause loss expression of laminin alpha2 in muscle basement membranes, are responsible for one form of congenital muscular dystrophy (10).

Table 1: Classification of the major muscular dystrophies(R.H. Brown, 1997)

Disease	Locus	Protein
X-linked recessive		
Duchenne/Becker dystrophy	Xp21	Dystrophin
Emery-Dreifuss dystrophy	Xp28	Emerin
Autosomal dominant		
Myotonic dystrophy	19q	Myotonin
Facioscapulohumeral dystrophy	4q	?
LGMD-1A	5q	?
LGMD-1B	other	?
Autosomal Recessive		
LGMD-2A	15q	Calpain
LGMD-2B	2p	?
LGMD-2C	13q	gamma-sarcoglycan
LGMD-2D	17q	alpha-sarcoglycan
LGMD-2E	4q	beta-sarcoglycan
LGMD-2F	5q	delta-sarcoglycan
Other		
Congenital muscular dystrophy (AR)	6q	alpha2-laminin
Fukuyama disease (AR)	9q13	?
Central core disease (AD)	19q13	Ryanodine receptor
	14q	Myosoin
Nemaline rod disease (AD)	1q22	Tropomyosin
Myotubular myopathies	Xq26	?
Distal muscular dystrophy (AD)	14q	?
Oculopharyngeal dystrophy (AD)	14q	?

LGMD, Limb-girdle muscular dystrophies; AD, autosomal dominant inheritance; AR, autosomal recessive inheritance

II: Dystrophin Associated Protein Complex

In initial studies to understand muscular dystrophy, dystrophin and the DPC were purified (3,4,5) and cloned (162). By using wheat germ agglutinin (WGA)-sepharose and DEAE-cellulose ion exchange chromatography, the 427 kDa dystrophin protein can be purified from the digitonin solubilized rabbit muscle membrane fractions, together with the purification of 156 kDa, 43 kDa, 59 kDa, 50 kDa, 35 kDa, and 25 kDa proteins (3,4,5,6). These proteins were named alpha-dystroglycan (159 kDa), beta-dystroglycan (43 kDa), syntrophin (59 kDa), alpha-sarcoglycan/adhalin (50 kDa), beta-sarcoglycan (43 kDa), gamma and delta-sarcoglycan (35 kDa), and sarcospan (25 kDa). More recent studies had identified a number of other proteins that comprise the DPC, which include

the extracellular basement protein laminin that is associated with alpha-dystroglycan in skeletal muscle (11), the dystrophin and syntrophin binding protein dystrobrevin (144,145), as well as the syntrophin binding protein neuronal nitric oxide synthase (nNOs) (38). The organization of these proteins in muscle membranes is shown as in Figure 1. Based on detergent solubility and their locations, the DPC can be divided into three sub-complexes: the dystroglycan complex, the sarcoglycan complex, and the intracellular cytoplasmic complex. The dystroglycan complex contains α-dystroglycan and the transmembrane β-dystroglycan. The sarcoglycan complex contains the α–,β–,γ–,δ–sarcoglycans, and sarcospan, but not ε-sarcoglycan. The four sarcoglycans are linked to each other and are connected to β-dystroglycan by δ–sarcoglycan within the membrane (147). The cytoplasmic DPC contains the cytoskeletal protein dystrophin, and the dystrophin interacting protein syntrophin as well as dystrobrevin, and the syntrophin interacting protein nNOs. This structure is found around the sarcolemma of skeletal muscle, smooth muscle, and cardiac muscle, and it is critical for muscle integrity.

1: Dystrophin and its Isoforms

Dystrophin gene is a very large, complex gene that spans 2.4 millions base pairs with at least 5 separate promoters and 79 exons in its genomic sequence (13). Alternative splicing results in synthesis of at least seven different dystrophin isoforms. The full-length dystrophin has 3685 amino acids with molecular weight of 427 kDa. Two internal promoters drive production of shorter C-terminal dystrophins that include Dp260, Dp140, Dp116, and Dp71 (Figure 2). Full-length dystrophin contains multiple protein-protein interaction domains.

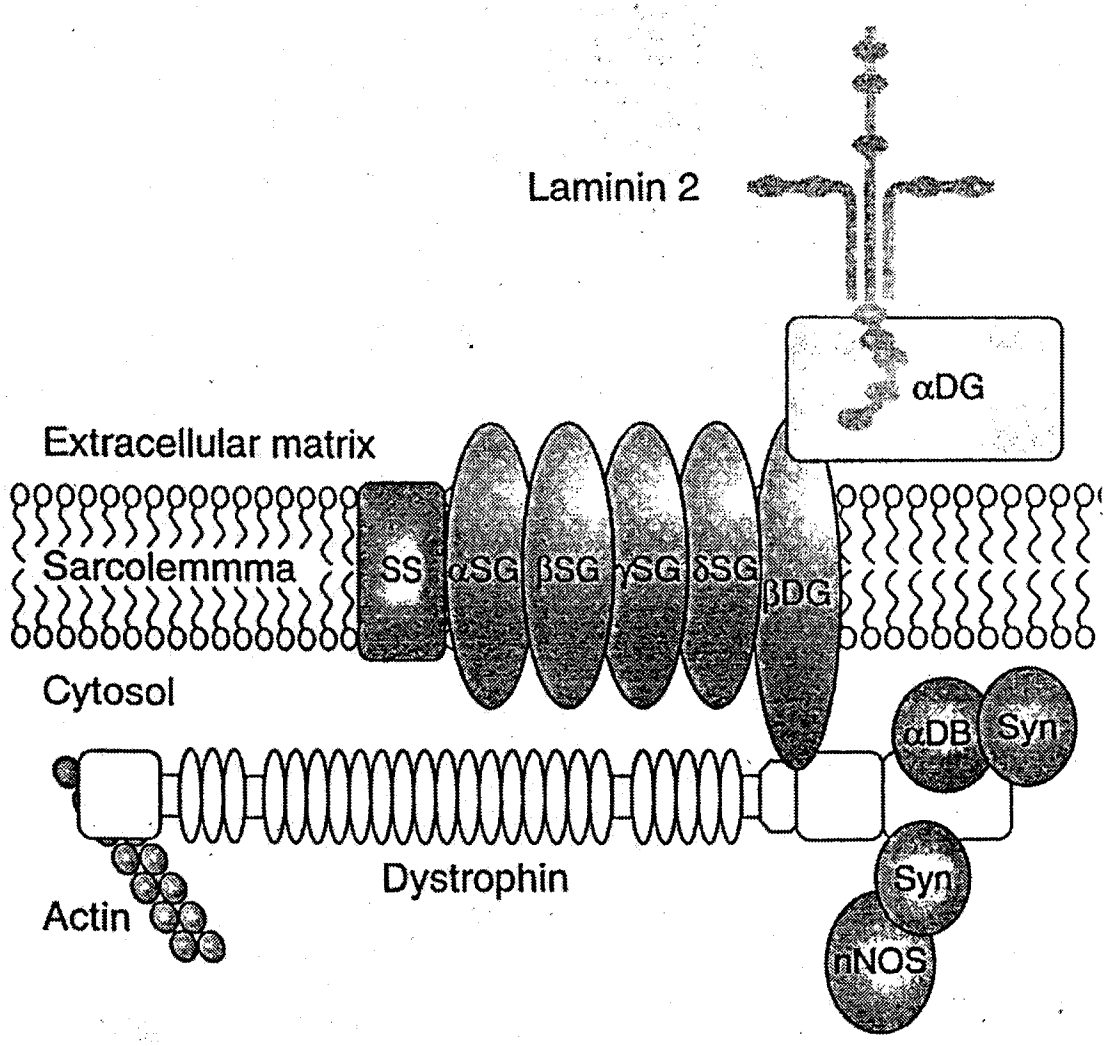

Figure 1: Structure of dystrophin associated glycoprotein complex in muscle

(Blake DJ. 2000)

Figure 1: Structure of dystrophin associated glycoprotein complex in muscle. This complex links the intracellular actin to the extracellular matrix laminin in muscle. The DPC can be divided into three subcomplexes: the dystroglycan complex, containing α- and transmembrane β-dystroglycan and laminin; the sarcoglycan complex, containing the transmembrane sarcoglycans and sarcospan; the intracellular cytoplasmic, complex containing dystrophin/utrophin, syntrophin, dystrobrevin and the syntrophin binding protein nNOs.

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At its N-terminus from amino acid 1-260 is the actin-binding domain, similar to the sequence in the actin-binding domain of actinin. Actin has indeed been shown to bind to this region of dystrophin in vitro and in vivo (14,15,16). Next to the actin-binding domain are the long spectrin-like repeats forming the rod-like domain (17). Adjacent to the rod domain is the cysteine rich (CR) domain, which contains a WW domain, two EF-hand-like regions and a ZZ domain (17). The WW domain is an about 30 amino acid long and contains two conserved tryptophan residues (hence WW). The EF-hand like region is a highly conserved motif involved in the binding of calcium or magnesium ions. The ZZ domain is a putative zinc finger motif found in many proteins. Dystrophin interacts with β-dystroglycan at the WW domain (4,11,18,19). Calcium dependent calmodulin can also bind to this region at the ZZ domain (20). The C-terminus of dystrophin contains a coiled-coil region, which is shown to bind to syntrophin and dystrobrevin (1,34,35,146). All isoforms of dystrophin contain the C-terminal CR domain and the coiled-coil region, and therefore have the ability to form a cytoplasmic complex underneath the membrane, indicating the importance of these C-terminal protein binding domains.

The expression of dystrophin and its isoforms are tissue specific. The full-length dystrophin is expressed in skeletal muscle, epithelial cells, and brain (23, 25, and 26). In brain, full-length dystrophin has been found in retina as well as in the postsynaptic densities of the excitatory synapses (25, 27). Dp260 is found in the outer plexiform layer in the retina in conjunction with Dp427 (31); Dp140 is specific for brain (30), and Dp116

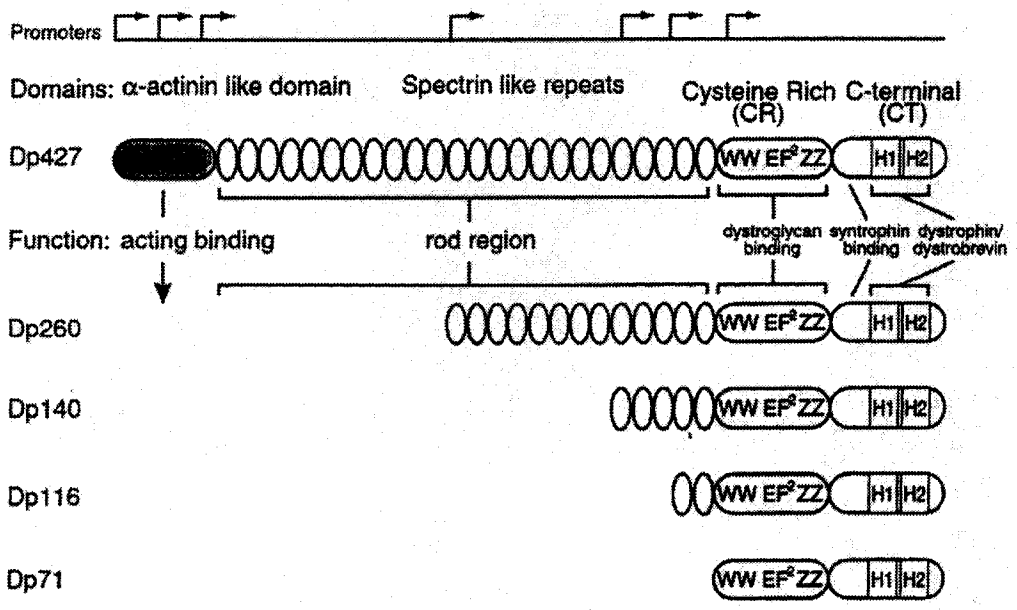

Figure 2: Dystrophin isoforms and its protein binding domains

Figure 2: Dystrophin isoforms and its protein binding domains. Dystrophin isoforms and its related proteins are produced by different promoters. The full-length dystrophin contains multiple domain structure. It contains actin binding domain, rode domain, cysteine rich domain, and C-terminal domain. Its shorter isoforms have only cysteine rich domain, c-terminal domain and a partial rod domain except that Dp71 has complete loss of the rod domain. All these isoforms conserve their abilities to bind to dystroglycan at WW domain and syntrophin, dystrobrevin at C-terminal domain.

is in the Schwann cells of the peripheral nerve (28,29). Dp71 is expressed in a variety of tissues, including skeletal muscle, smooth muscle, cardiac muscle, blood vessels, epithelial cells, glial cells and neurons (23, 25). These specific patterns of expression suggest that DPC with distinct function exist in different tissues (e.g. 151)

There are, in addition to these dystrophin isoforms, several other dystrophin-related proteins coded by separate genes. These genes are autosomal homologues of dystrophin and they encode for a protein called dystrophin related protein 2 (DRP2) (148), and a 395 kDa protein called utrophin or DRP1 as well as several shorter utrophin isoforms such as Up140, Up116 and Up71 (21). The utrophin sequence is quite homologous to dystrophin and only minor differences have been found in its protein binding domains (22). Instead of uniform expression in sarcolemma, like dystrophin, utrophin was found only at the sarcolemma of neuromuscular junction and co-localizes with acetylcholine receptors (23, 24). DRP2 has been found enriched in brain and associated with postsynaptic density (149,150). A recent report showed that it is also in Schwann cells and is associated with a protein called L-periaxin (151), and plays an important role in myelinogenesis (151).

2: Syntrophin and Dystrobrevin

Syntrophin was originally identified in the postsynaptic membrane of *Torpedo* as an intracellular 58kD protein (32). In mammalian tissue, it was co-purified with dystrophin and dystroglycan as one of the DPC components (3, 4), and has been shown to bind to dystrophin, utrophin and dystrobrevin. There are five different syntrophin genes

that encode five highly conserved proteins: α1–, β1–, β2–, γ1–, γ2 -syntrophin (1,33). The γ1–and γ2–syntrophins are exclusively expressed in neurons and found associated with dystrobrevin and dystrophin (Dp71 and Dp140 (1)), whereas the β1- and β2-syntrophins are expressed in a wide variety of tissues including muscle, liver, kidney, brain, lung and pancreas. α1-syntrophin is predominantly expressed in skeletal and cardiac muscle (33,34). In skeletal muscle immunohistochemical studies using specific antibodies revealed that the three syntrophins (α1-,β1-,β2-) are concentrated at neuromuscular junctions, but α1- and β1-syntrophins are also present at the extra-synaptic sarcolemma, whereas β2-syntrophin is exclusively localized at NMJs (35,36). All syntrophin isoforms bind to the C-terminal coiled-coil region of dystrophin or DRPs (1,34,35,37).

Syntrophins appears to function as adapter molecules that recruit signaling proteins to the DPC (35). The muscle specific syntrophins (α1-, β1-,β2-) have two pleckstrin homology (PH) domains, a PDZ domain, so named because of its presence in postsynaptic density protein-95 (PSD-95), the Drosophila discs large tumor suppresser protein, and the zonula occludens-1 protein (ZO-1), and a highly conserved carboxyl-terminal syntrophin unique (SU) domain (33). The γ1- and γ2-syntrophins have somewhat different domain structures with only one PH domain, a PDZ domain and a unique ATP/GTP binding site (1). The PDZ domain is a common protein-protein interaction domain, which could bind to other PDZ domain containing proteins or bind to the motif S/TXV at the C-terminus of a protein. In the case of syntrophin, several PDZ-domain containing proteins have been found to interact with the PDZ domain of syntrophin. These include nNOs (38), and serine/threonine kinases MAST205 and SAST

(39). Syntrophin has also been found to interact with sodium channels in which the C-terminal S/TXV binds to the PDZ domain of syntrophin (40). In brain, the neuronal specific γ1-syntrophin has recently been reported to interact with diacylglycerol kinase-z (DGK-z), an enzyme that has a consensus PDZ domain interacting motif in its C-terminal and can functionally convert diacylglycerol into phosphatidic acid (41). On the other hand, the conserved C-terminal SU domain of syntrophins has been proven to bind to utrophin, dystrophin and its isoforms, which keeps syntrophin in the dystrophin associated protein complex (1,33,35). Unlike the disruption of dystrophin/utrophin, which causes muscular dystrophies, syntrophin has no mutations that have been found to cause human diseases. Ablation of the α-syntrophin gene in mice results in no gross morphological and functional changes or muscular dystrophies although there is a loss of nNOs in sarcolemma (42). Subsequent studies in α-syntrophin deficient mice revealed undetectable level of utrophin, aberrant junctional folds and reduced level of acetylcholine receptors in the postsynaptic membrane (45).

Dystrobrevin was first identified as an intracellular 87 kDa dystrophin associated protein (5), which was also known as A0 (18). It was also found in *Torpedo* electric organ as an 87kD postsynaptic membrane protein (44). Both dystrobrevin and the *Torpedo* dystrobrevin are homologous to the cysteine rich domain and the C-terminal domain of dystrophin (44, 46) and share several features in common with dystrophin, including its coiled-coil structure and the syntrophin binding motif (46). In mice, two dystrobrevin genes are broadly expressed, and these α and β dystrobrevin genes give rise to several distinct splicing variants.

Although at least 5 α-dystrobrevin isoforms are synthesized, there are two dominant ones found in muscle which are the full length α-dystrobrevin-1, and the C-terminal truncated form α-dystrobrevin 2. While α-dystrobrevin-1 is expressed exclusively at NMJs, α-dystrobrevin-2 is expressed throughout the sarcolemma and at deep junctional folds (47). β-dystrobrevin has significant homology to α-dystrobrevin, and it is not expressed in muscle, instead it is found in brain, kidney, and pancreas and is associated with Dp71 and Dp140 (46,48,143).

Targeted deletion of the α-dystrobrevin gene in mice leads to skeletal and cardiac myopathies and impairment in nNOs signaling while the other DPC complex member are unaltered (50). In addition to this, acetylcholine receptors at neuromuscular junctions seem more diffusely distributed in α-dystrobrevin null animals (51). Furthermore, agrin induced acetylcholine receptor clusters in cultured myotubes from null animals seem to be more fragmented and smaller than the wild type animals (51). These experiments indicate the importance of dystrobrevin in cell signaling, structure, maintenance, and NMJ formation. Very recently, a new protein dysbindin was found associated with α-dystrobrevin in muscle, and with β-dystrobrevin in brain (49), and this new member of DPC interacting protein may give new functions to the whole complex.

3: Sarcoglycans and Sarcospan

Sarcoglycans are five transmembrane proteins linked to the DPC through β-dystroglycan (Figure 1). Among them, α-sarcoglycan/adhalin (50 kDa), β-sarcoglycan (43 kDa) and γ-sarcoglycan (35 kDa) were initially co-purified with dystrophin by extracting the dystrophin protein complex with β-D-octyl-glycoside (3,5,18). Later, two

other members were also found and named δ-sarcoglycan and ε-sarcoglycan (43,52). As with other members of the DPC, sarcospan was also co-purified with dystrophin as a 25 kDa protein (3,5,18) that is tightly associated with sarcoglycans in the sarcolemma. Generally, sarcospan and sarcoglycans are called the sarcoglycan complex and this complex is connected to dystroglycan through δ-sarcoglycan (147). The composition of the sarcoglycan complex is different in different tissues. In skeletal muscle, it is α–,β–,γ–,δ-sarcoglycan and sarcospan whereas in smooth muscle β–,δ, ε-sarcoglycan and sarcospan are complexed with dystrophin complex (53), and in Schwann cells the composition of sarcoglycan complex has only the δ–, β–and ε-sarcoglycan with no sarcospan (29). In neurons, both sarcospan and most sarcoglycans are thought not to be expressed (25, 29).

As summarized in Table 1, mutations in α–, β–, γ–, or δ–sarcoglycans lead to muscular dystrophies, and experimental mice deficient for either γ– or δ-sarcoglycan also develop a cardiomyopathy (53). The mechanisms for the disease development are not very clear, but it might involve membrane disruption and increased apoptosis (53) when sarcoglycan is absent. There is also evidence that integrin α5β1 and α-sarcoglycans are associated so that disruption in sarcoglycan also might affect the integrin signaling (54). Only expressed in limited tissues with β-, δ- or γ-sarcoglycan such as in smooth muscle, vascular epithelium and Schwann cells, ε-sarcoglycan is absent from the membrane when β-sarcoglycan is deleted (52). So far, there are no mutations found in ε-sarcoglycan that are linked with any human disease (52).

4: Dystroglycan

The dystroglycan gene comprises two exons and encodes a single polypeptide that is post-translationally cleaved to yield two glycoproteins: α-dystroglycan and β-dystroglycan. (Figure 3) (4,55). The dystroglycan protein contains 895 amino acids, and α-dystroglycan is completely extracellular and contains amino acids 1 to 653; β-dystroglycan is a transmembrane protein and contains amino acids 654 to 895. The transmembrane domain within β-dystroglycan contains sequences from 751-774. The primary protein sequence of α-dystroglycan predicts a molecular weight of 72 kDa. However, α-dystroglycan in mammalian skeletal muscle is 156 kDa (4), 140 kDa in cardiac muscle (56), and 120 kDa in brain and peripheral nerve (12,133). There is no evidence for alternative splicing, therefor α-dystroglycan must undergo significant post-translational modification. Several studies revealed that α-dystroglycan resembles mucin proteins and undergoes heavy O-glycosylation within its mucin-like domain (amino acid 315 to 484) (57,58). In addition to the O-linked glycosylation, there are also some potential sites within its sequence for N-linked glycosylation (Figure 3). These oligosaccharide side chains have been suggested to be critical components for interactions between α-dystroglycan and the extracellular matrix proteins such as laminin, agrin and perlecan (59,60,63). β-dystroglycan is also glycosylated. Its protein sequence predicts a molecular weight of 26 kDa, but it actually has a molecular weight of 43 kDa in most tissues. The glycosylation is necessary for correct sorting of the mature α- and β-subunits to the extracellular surface and plasma membrane (55), where they are non-covalently connected. The intracellular region of β- dystroglycan is enriched with

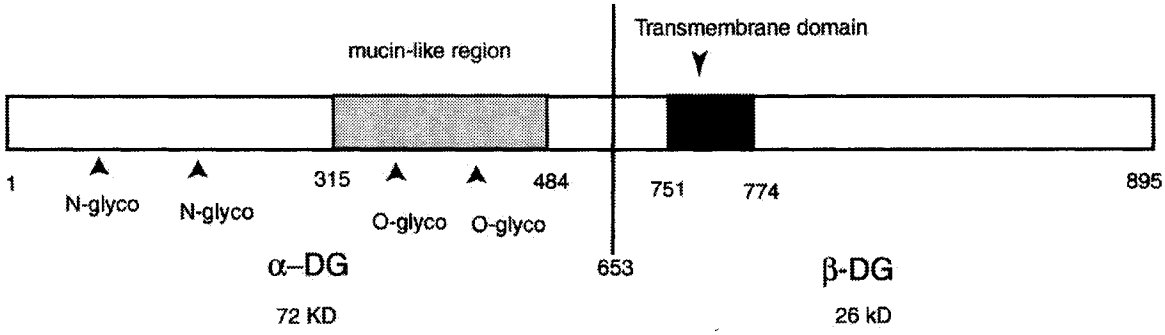

Figure 3: Illustration of α- and β-dystroglycan protein

Figure 3: Illustration of α- and β-dystroglycan. The extracellular N-terminal α-dystroglycan has a mucin-like domain and has, in total, 653 amino acids. Several O-glycosylation sites exist within this domain and several N-linked glycosylation sites are outside the mucin domain. β-dystroglycan contains amino acids from 654 to 895 and the transmembrane domain lies from 751 to 774. Glycosylation on both α- and β-dystroglycan change their apparent molecular weights in tissue from 72 kDa and 26 kDa to 100-200 kDa and 43 kDa respectively.

prolines and contains multiple PxxP and PPxY consensus sequences and has the potential to bind to SH3 and WW domain containing proteins. A number of proteins have been found to interact with dystroglycan (both α- and β-) in muscle (discussed below) and these interactions may give rise to a variety of functions for dystroglycan as well as for the DPC.

Part II: Dystroglycan Interacting Proteins

As noted previously, dystroglycan has been found to associate with multiple proteins inside and outside of the cell and serves as the core component of the DPC. In skeletal muscle, α-dystroglycan interacts with the basement membrane proteins laminin, agrin, perlecan, and biglycan; intracellularly it interacts with dystrophin/utrophin, rapsyn, Grb2 and caveolin-3; on the membrane it associates with δ-sarcoglycan.

Laminin: In skeletal muscle, each muscle fiber is surrounded by an extracellular matrix sleeve, which is composed mainly of laminin, collagen, entactin and heparin sulfate proteoglycan. This matrix is important for muscle differentiation, growth, migration, synapse formation and attachment (61,62). Laminin 1 is a 900 kDa heterotrimer consisting of α, β, and γ chains with many distinctive domains and structures (61). Among many functions of laminin, the most relevant to the DPC is located at the C-terminus of the α-chain. There exist five globular domains (G-domains) (61). These domains were shown to bind to α-dystroglycan with high affinity (Kd=90nM) through the last two G-domains (12). Binding to laminin is calcium-dependent and could be inhibited by heparin and abolished by treating α-dystroglycan with glycosidases including sialidase (5,11,64), indicating that sugar chains of α-

dystroglycan participate in the binding. Other evidence support this idea in that a monoclonal antibody (IIH6) that recognizes the carbohydrate side chains of α-dystroglycan also blocks the binding of α-dystroglycan to the G-domains (69). It is now generally believed that this interaction is a critical linkage between extracellular matrix and the intracellular structure in tissue where dystroglycan and laminin are expressed, including skeletal muscle, neuromuscular junction, smooth muscle, cardiac muscle, epithelia (63), peripheral nerve (29), brain (12) and kidney (65).

Agrin: Agrin is a 400-600 kDa heparan sulfate proteoglycan (66), secreted by neurons and muscle where it is deposited in the basal lamina of neuromuscular junctions. Agrin has long been known as a molecule that can stimulate postsynaptic acetylcholine receptor aggregation at neuromuscular junctions (NMJs), and its receptors in the muscle membrane include the muscle specific tyrosine kinase (MuSK) and α-dystroglycan (67,68,69,70,71). Agrin contains three laminin-like G-domains at its C-terminus, which include G1, the first G-domain; G2, the second G-domain which has either 0- or 4-amino acid insert due to alternative splicing, and G3, the third G-domain, with either a 0-, 8-, 11- or 19- amino acid insert. Agrin's activity to promote AChR clustering resides entirely in the C-terminal three G-domains and four EGF-like domains. Agrin with no inserts has wide tissue origins and displays weak AChR clustering activity, but inserts of 8,11, or 19 amino acids are found only in agrin of neuronal origin and have strong clustering activity. These C-terminal G-domains have been shown to interact with α-dystroglycan dependent upon calcium (69,70,71,72). In addition to α-dystroglycan, agrin also binds to laminin via its N-terminal, helping connect it to the basal lamina (73). The role for the interaction between agrin and α-dystroglycan in promoting postsynaptic AChR clustering was

controversial because dystroglycan was found to bind to both neuron and muscle agrin but only the neuronal agrin has the AChR clustering activity (71). In addition deletion of 15-kDa fragment from the C-terminus of agrin (G3 domain) had no effect on its α-dystroglycan binding ability, but almost abolished its acetylcholine receptor aggregating activity (74).

MuSK has been shown to be downstream of agrin in promoting AChR clustering and is required for neuromuscular junction formation (68,75), but MuSK does not bind agrin in the absence of an unidentified co-receptor (68). Although genetic evidence shows that MuSK and agrin are required for the postsynaptic differentiation process, other evidence indicates that laminin is sufficient to induce AChR clusters in culture, through dystroglycan, but independent of MuSK activity (76,77). Very recent work has demonstrated that the initiation of AChR clustering is independent of any neuronal signal including agrin (78), but intrinsic to muscle itself (78), even though MuSK is still required. This raises the possibility that an unknown muscle signal (instead of neuronal agrin) together with the tyrosine kinase activity of MuSK participate to initiate AChR clustering. However, this does not exclude roles of muscle agrin or dystroglycan in the formation of mature postsynaptic NMJs.

Perlecan and Biglycan: Perlecan is a heparan sulfate proteoglycan with a single chain polypeptide core of 396 kDa that is expressed widely as a component of all basement membranes including in muscle where it is enriched at NMJs (83). Like laminin, perlecan has multiple domains and structures. At its C-terminus it has three G-domains similar to those of the laminin α-chain and agrin (79,80), and these G-domains have shown to bind to α-dystroglycan (81,82). Biglycan is a small, leucine-rich repeat

proteoglycan, and it is found expressed in synaptic and nonsynaptic regions in muscle and enriched at NMJs (83). Biglycan is considered as extracellular matrix protein and it was first found to bind α-dystroglycan in *Torpedo* electric organ by ligand blot overlay assay (83). Unlike the G-domain containing proteins, this protein does not have any globular domains and it binds to the protein core of the last third C-terminal of α-dystroglycan, but not the sugar chains like in laminin, agrin or perlecan (83). Treating the biglycan itself with chondroitinase to remove the chondroitin sulfate side chain abolished the binding to α-dystroglycan, indicating the interaction is dependent on the chondroitin sulfate chains of biglycan (83).

In addition to the above proteins that associate with α-dystroglycan, there are several intracellular proteins and transmembrane proteins that interact with β-dystroglycan.

Rapsyn: Rapsyn, a 43 kDa peripheral membrane protein expressed in skeletal muscle, plays a critical role in organizing postsynaptic structures and acetylcholine receptors (AChRs) during the formation of NMJs. Rapsyn colocalizes with AChRs in the postsynaptic membrane at the earliest stages following innervation (152). Rapsyn-deficient mice die at birth because of failure of neuromuscular transmission (84). These mice have NMJs with little localization of AChRs, and utrophin and dystroglycan are no longer enriched in the postsynaptic membranes (84). Biochemical studies using electric organ of *Torpedo* demonstrated that rapsyn can be cross-linked to the AChR β-subunit (153) and binds directly to the juxtamembrane portion of the cytoplasmic tail of dystroglycan from amino acid 784 to 819, a site distinct from the dystrophin binding site (85,86). When expressed in non-muscle cells, rapsyn can recruit AChRs and dystroglycan

to form membrane associated clusters (87,154). Rapsyn is also required for the MuSK signaling in which rapsyn recruits other synaptic components including AChRs to the MuSK containing scaffold (87,155). These experiments indicate that rapsyn serves as a linkage between AChRs and MuSK, AChRs and dystroglycan/DPC, and is critical for the differentiation of the postsynaptic membrane.

Grb2: Grb2 is a well known signaling molecule that serves as an adapter protein and participates in receptor tyrosine kinase and PI3 kinase and MAP signaling cascade. It contains an SH2 domain that binds to tyrosine-phosphorylated proteins, and two SH3 domains that bind to proline-rich proteins (94). The SH3 domain contains about 60 amino acids and it has been shown to bind to the proline rich tail of SOS (94). The Grb2/SOS complex is thought to be recruited to autophosphorylated receptor tyrosine kinases in the plasma membrane after ligands activate the receptor (91.92,93). This recruitment brings SOS in close proximity to the molecule Ras in the plasma membrane. Genetic and biochemical evidence has demonstrated that SOS can transform inactive GDP-Ras to the active GTP-Ras and that Grb2 couples activation of Ras in response to the receptor tyrosine phosphorylation (94,95,96). Once in an active GTP-bound state, Ras can interact with several effector proteins such as Raf and PI3 kinase to stimulate numerous intracellular processes. Activated Raf stimulates MAP-kinase-kinase (MAPKK) by phosphorylating a key Ser residue. MAPKK then phosphorylates MAPK on a Thr or Tyr residue in the activation-loop to activate it (96). Activated MAPK phosphorylates a variety of cytoplasmic and membrane linked substrates. In addition, MAPK is rapidly translocated into nucleus where it phosphorylates and activates transcription factors (97,98). This signaling cassette is highly conserved in evolution and several MAPK

cascades exist in yeast, in invertebrates and vertebrates (99,100), and they play very important roles in the control of metabolic processes, cell cycle, cell migration, and cell shape as well as in cell proliferation and differentiation (101).

Dystroglycan has been also found to interact with Grb2. The C-terminus of dystroglycan contains several proline rich (PxxP) and hydrophobic consensus sequences, and it binds to both SH3 domains of Grb2 (102), but with higher affinity to the N-terminal SH3 domain than to the C-terminal SH3 domain (102). Mutations in both SH3 domains which abolish the association with Grb2-binding proteins also prevent Grb2 binding to dystroglycan, confirming that the association between Grb2 and dystroglycan is through SH3 and proline rich domains (102). However, there is no evidence so far found that dystroglycan is complexed with other signaling molecules such as receptor tyrosine kinases, SOS or MAPK. The interaction between dystroglycan and Grb2 has been found not only in muscle, but also in brain synaptosomes where the focal adhesion kinase (FAK) P125 was complexed with dystroglycan through Grb2 (103). Further experiments in dissecting β-dystroglycan interacting proteins might be able to find additional proteins complexed to β-dystroglycan through Grb2.

Dystrophin and Utrophin: It has been well documented that the last 15 amino acids of dystroglycan C-terminal bind to the WW domain of dystrophin or utrophin (4,11,18,19). Because dystrophin also interacts with actin in its N-terminal, and α-dystroglycan interacts with basement membrane protein laminin or perlecan, the axis of dystrophin, dystroglycan and laminin serves as the linkage between the intracellular cytoskeleton and extracellular matrix (Figure 1), and this linkage is thought to prevent muscle from the mechanical damage during contraction. It is also interestingly to note

that the interaction between dystroglycan and utrophin at NMJs can be regulated by tyrosine phosphorylation in the PPxY motif of the dystroglycan at Y892 which is a critical point for both utrophin and dystrophin binding (104).

Caveolin 3: Caveolins are principle protein components of caveolae which are 50-100 nm membrane invagination representing an appendage or subcompartment of the plasma membrane (105,106,107). Caveolins consist of caveolin-1, caveolin-2, and caveolin-3, and are encoded by three separate genes (108,109,110). Two isoforms of caveolin-1 (Cav-1α and Cav-1β) are derived from alternate initiation during translation (112). Caveolin-1 and caveolin-2 are most abundantly expressed in adipocytes, endothelial cells and fibroblastic cell types, whereas the expression of caveolin-3 is muscle-specific (110). Caveolin proteins interact with themselves to form homo- and hetero-oligomers (113,114,115), which directly bind cholesterol (116) and require cholesterol for insertion into lipid membranes. Caveolin-1 and caveolin-2 have been found to form a stable hetero-oligomeric complex, in vivo they are strictly co-localized, and the localization of the complex is found within caveolae identified by immunoelectron microscopy (115).

A number of studies have suggested that caveolins provide direct means for signaling molecules to be sequestered within caveolae microdomains, by directly interacting with a conserved 20 amino acid (aa82-101) domain called the caveolin scaffolding domain (111). These signaling proteins include G-proteins, Ras, Src family tyrosine kinases, NOS, epidermal growth factor receptor (EGFR) and phospholipase Cγ (reviewed in 111 and 117). In addition to these signaling proteins, dynamin, a protein critical for endocytosis and synaptic transmission, has also been shown to interact with

caveolin-2 in epithelial cells (118,119), and the presence of dynamin at the neck of caveolae is thought to be important for caveolae-mediated internalization.

In muscle, caveolin-3 is not an integral component of the DPC, but an association between caveolin-3 and β–dystroglycan has been reported (120). The interaction is thought to be through the WW domain in caveolin-3 and caveolin-1 and the PPxY motif in the extreme C-terminus of dystroglycan, which also binds dystrophin/utrophin (120). Thus, there may be competition and regulation between binding of caveolin-3 to dystroglycan and binding of dystrophin/utrophin to dystroglycan. Indirect evidence supports the hypothesis that caveolin-3 interacts with the DPC. First, mutations in caveolin-3 gene cause autosomal dominant limb-girble muscular dystrophy (121,122,123), a phenotype that is similar to the autosomal recessive LGMD-2 caused by mutations in sarcoglycans (Table1). Secondly, in caveolin-3 mutated patients, there is significant loss of α-dystroglycan as well as nNOs in muscle (124), similar to the loss in DMD patients. Other studies in caveolin-3 null mice showed an exclusion of the dystrophin associated protein complex from lipid raft domains (125). Together, these data imply that caveolin-3 interacts biochemically and genetically with the DPC, but further studies are necessary to explain why caveolin mutations lead to myopathies.

Part III: Dystroglycan and the DPC in the Nervous System

1: Dystroglycan and DPC at NMJ

Dystroglycan and the other DPC members are concentrated at NMJs, where utrophin displaces dystrophin to form a complex with other members of the DPC. The function of dystroglycan and the DPC in NMJ formation have been investigated extensively since agrin has been shown to interact with α-dystroglycan. Several observations support a role of dystroglycan in postsynaptic differentiation, if not in initiating AChR clustering. Because dystroglycan knockout mice fail to develop past day 6.5 of embryogenesis due to the disruption of the formation of the extra-embryonic basement membrane (Reichert's membrane) (126), chimeric mice and dystroglycan-deficient embryonic stem cells were used to analyze the formation of NMJs (51,89,127). In these stem-cell induced myotubes, the AChR aggregates form, but they are much larger in size, about half as dense and fail to coalesce into dense clusters (89). AChRs at the NMJs are similarly affected in dystroglycan deficient chimeric mice and also the nerve terminal is larger in size (89). In both in-vivo and in vitro situations, the basement membrane proteins acetylcholinesterase (AChE), laminin and perlecan are all disorganized and do not colocalise with AChRs (89), indicating that dystroglycan functions as an organizer of the basement membrane proteins at NMJs. Another report also pointed out that dystrophin and the intracellular complex are also dislocated from the sarcolemma when dystroglycan is absent (51). A similar phenotype in AChR clustering has been found in the dystrobrevin null NMJs, where AChRs are also diffuse and unstable (51). In utrophin knockout mice, there are decreased postsynaptic foldings,

decreased AChRs and small electrophysiological changes detected (128,130). In dystrophin and utrophin double knock-out mice, in addition to the typical phenotypes for muscular dystrophy, the postsynaptic junction foldings are completely lost and the AChR density is modestly decreased, as in the utrophin knock out mice (129,131). All these data suggested that dystroglycan and dystrophin complex maintain and assemble the postsynaptic specialization.

2: Dystroglycan and DPC in peripheral nerve:

Because of the tissue specific expression of dystrophin isoforms and other complex members, the composition of dystrophin associated complex in other tissues is likely different from that in muscle. In peripheral nerve, where nerve fibers are often wrapped by a myelin sheath, Dp116, in the outermost layer of the myelin sheath, replaces full length Dp427 and is anchored to the membrane by interacting with dystroglycan. Also, instead of four sarcoglycans (α,β,γ, and δ) and sarcospan, peripheral nerve only expresses β–, δ-sarcoglycan and ε-sarcoglycan, an α-sarcoglycan homologue (132,133) (Figure 4). As in the muscle, Dp116 and sarcoglycans are connected to the extracellular matrix through interaction between α-dystroglycan and laminin α2 chain (132,133). Mutations in α2 chain of laminin that results in loss of its expression cause a peripheral dysmyelination (135,136).

An interesting side-light in dystroglycan function comes from studies which show that *Mycobacterium leprae,* a pathogen that invades Schwann cells and causes leprosy in humans, utilizes the laminin-dystroglycan-Dp116 link to invade the Schwann cell (134). *Mycobacterium leprae* binds to α-dystroglycan only in the presence of laminin α2 chain

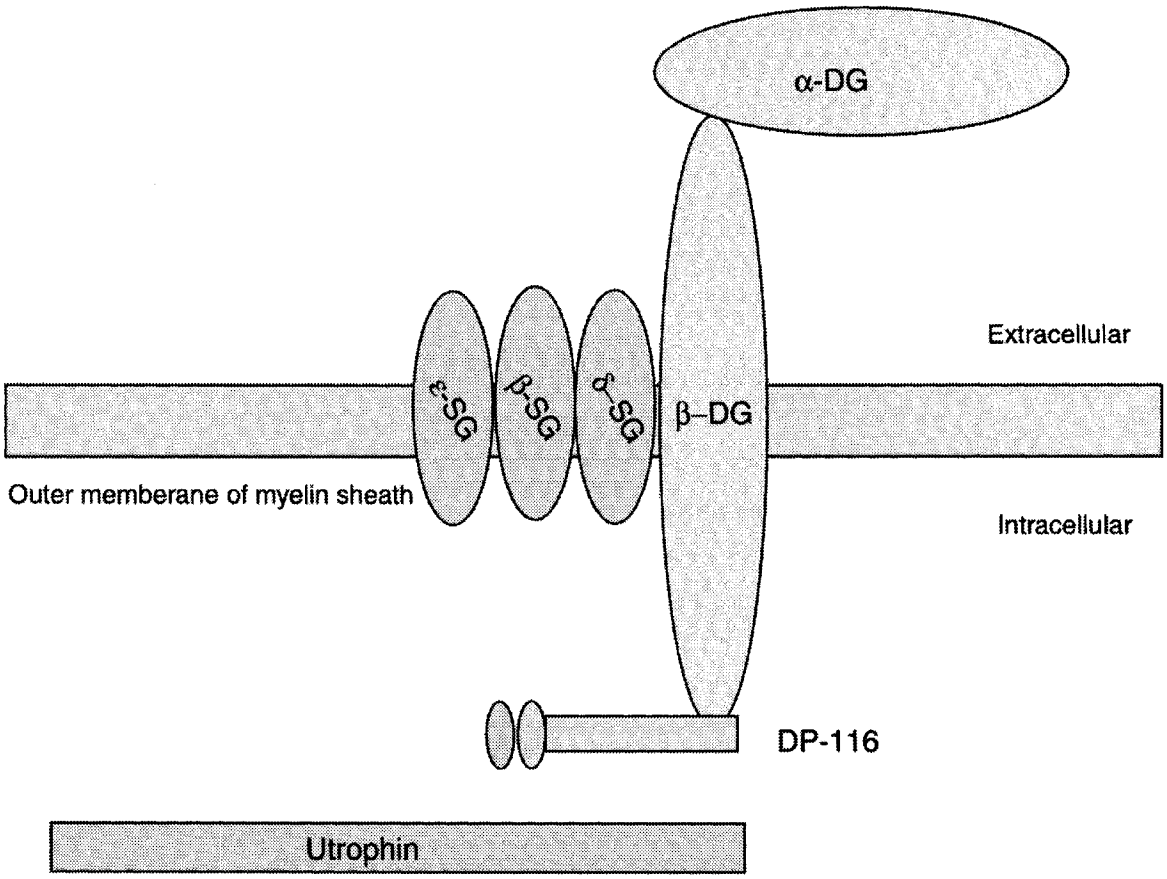

Figure 4: Dystroglycan and the DPC in peripheral nerve

Figure 4: Dystroglycan and the DPC in peripheral nerve. In the outer wrapping of the myelin sheath, Dp116/utrophin connects to β-dystroglycan. δ-sarcoglycan, β-sarcoglycan and ε-sarcoglycan form the transmembrane sarcoglycan complex. α-dystroglycan is also present and connects to transmembrane β-dystroglycan and extracellular laminin 2 (not shown).

(134). Abolishment of the carbohydrate moieties of α-DG or treatment with EDTA will disrupt *Mycobacterium leprae*'s binding to α-dystroglycan, because these treatments eliminate the binding of laminin2 to α-dystroglycan, which is dependent on calcium and carbohydrate moieties (134). These data suggest that existence of α-dystroglycan and laminin 2 as well as their interaction could be a route for *Mycobacterium leprae* to infect the peripheral nerve.

Very recently, a group of scientists also reported that there exists a dystrophin-related protein 2 (DRP2)-dystroglycan complex in peripheral nerve, where DRP2 connects to β-dystroglycan in the membrane and a homodimeric PDZ domain containing protein L-periaxin in the cytoplasm (151). Disruption of the DRP2-dystroglycan complex is followed by hypermyelination and destabilization of the Schwann cell-axon unit in periaxin -/- mice, implying that this complex functions in peripheral nerve myelinogenesis (151).

3: Dystroglycan and DPC in Central Nervous System

Unlike in muscle where the function and composition of DPC is better understood, the component members of DPC in different brain regions and cell types as well as the function of each member in brain are largely unknown. However, the disruption of the dystrophin gene in DMD and BMD patients results in mental retardation in about 1/3 of the patients (156), and in the dystrophin null *mdx* mice behavioral abnormalities such as retention impairment have also been documented (157) even though the spatial learning and hippocampal LTP is little affected (158,159).

Full length dystrophin Dp427, Dp71 and Dp140 are all found expressed in brain

regions including cerebral cortex, hippocampus, cerebellum, striatum and thalamus (25,30). Nevertheless Dp427 is neuronal specific (25), localized to the postsynaptic membrane and is enriched in postsynaptic densities (PSD) (27) together with syntrophin and dystrobrevin (137). Dp427, Dp71 and utrophin are also found expressed in astrocytes, but in differentiated, mature astrocytes, Dp71 replaces both Dp427 and utrophin as the dominant dystrophin isoform in the endfeet of astrocytes (138).

Like Dp427, dystroglycan was found expressed in brain regions including hippocampus, cerebellum, striatum, and thalamus, but not in cerebral cortex (139,140). However, a recent report suggests that dystroglycan immunostaining is found in cerebral cortex (141). α- and β-dystroglycan were also found expressed in blood vessels and astrocytes (140,138), especially on the astrocytic endfeet that surround the basement membrane of blood vessels (140,142), where, not surprisingly, the basement membrane protein laminin 2 is also found (140). It seems possible that laminin 2 forms a linkage with the complex of α-, β-dystroglycan, utrophin or Dp71 between the astrocyte endfeet and the blood vessel basement membrane and it might protect the integrity of the blood brain barrier. In the Purkinje cell layer of cerebellum, α-dystroglycan immunostaining was seen in the Purkinje cell bodies and dendrites (140). Notably the staining appears to be not uniformly distributed (140) which would be expected if it is synaptically localized. A recent report shows that the composition of intracellular cytoplasmic complex of the DPC is different in neurons and glial cells (143). By using co-immunoprecipitation and immunohistochemistry, the authors showed that dystrophin, syntrophin and β-dystrobrevin form a complex underneath the neuronal membrane, whereas Dp71, syntrophin and α1-dystrobrevin form a cytoplasmic complex underneath the glial

membrane (143) (Figure 5). Unfortunately, these authors did not give any evidence about dystroglycan's roles, by showing whether these two complexes in both neurons and glial cells may be connected to plasma membrane by dystroglycan.

To date there have been no clear explanation for the mental impairment in cases of complete or partial loss of dystrophin. However, several studies show possible disrupted synaptic transmission in the retina of dystrophin null mice. This appears as a reduction of the amplitude of the ERG (electroretinogram) b-wave in dystrophin null mice which may be caused by impaired synaptic transmission between rod photoreceptors and their postsynaptic target cells in the outer plexiform layer (25,160,161). Except for that, many questions remain to be answered. For instance, what is the expression profile of β-dystroglycan in neurons? Is the DPC in neurons and glial cells the same as that in the muscle? Are there any novel proteins associated with this dystroglycan and dystrophin complex? Are these novel proteins, if any, involved in synaptic transmission or synaptogenesis early in the development considering the synaptic transmission impairment in the retina of dystrophin null mice? Understanding these questions will help us dissect functions of dystroglycan, dystrophin, and its complex, and will introduce new knowledge to brain structure, function and development.

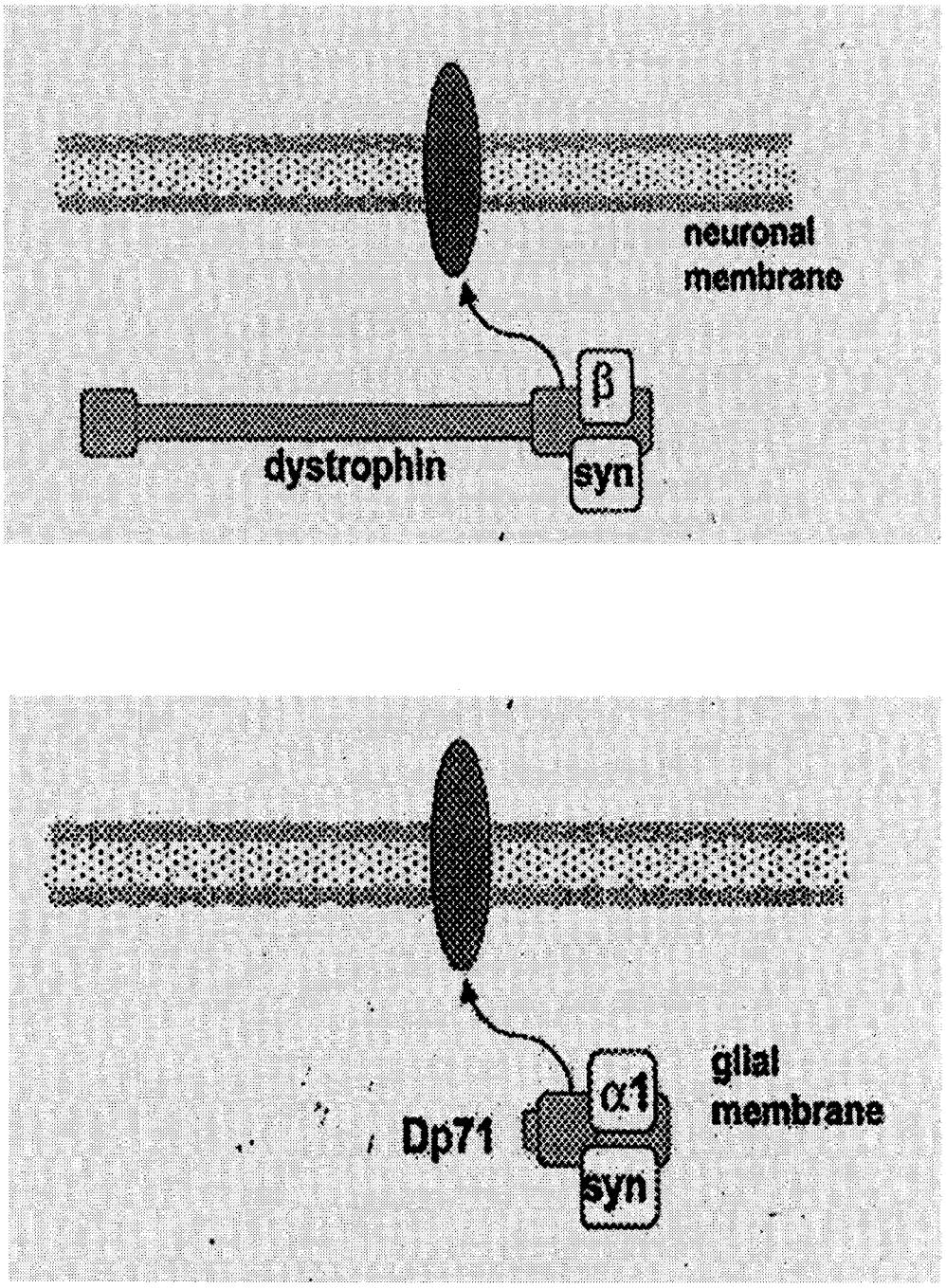

Figure 5: Comparison of DPC cytoplasmic complex in brain neurons and glia

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Figure 5: Comparison of DPC cytoplasmic complex in brain neurons and glia. In neurons, full-length dystrophin is complexed with syntrophin and β-dystrobrevin to form the cytoplasmic DPC under the neuronal membrane; whereas Dp71, syntrophin and α1 form the cytoplasmic complex under the glial membrane.

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Chapter 2

The Expression Profile of Dystroglycan in Neurons

Introduction

In mature muscle membrane α- and β-dystroglycan are uniformly expressed along the sarcolemma and also concentrated at neuromuscular junctions. Indeed dystroglycan colocalizes with postsynaptic AChRs and clustered agrin as early as AChR clusters begin to form during NMJ formation (13). As indicated by experiments done by P. Cote , C. Jacobson and coworkers (25,71), dystroglycan functions in postsynaptic maturation and maintenance of neuromuscular junctions. The more general role for dystroglycan is to serve as a linkage between the intracellular dystrophin cytoskeleton, actin and extracellular matrix. α- and β-DG are also expressed in peripheral myelin (26), where β-DG is associated with one of the alternatively spliced dystrophin isoforms, Dp-116, and α-DG is connected to laminin-2 in the basal lamina around the Schwa cell (28). In astrocytes, β-DG is thought to associate with Dp71 or utrophin during cell differentiation (44). In the cerebellum α-dystroglycan is non-uniformly expressed in Purkinje cell bodies and dendrites. It is colocalized with the laminin α2 chain at the glial-vascular interface, suggesting a role for dystroglycan in maintaining the blood-brain barrier (31). In other regions of brain such as retina, hippocampus and striatum, dystroglycan is also expressed (49). Despite this wealth of data regarding dystroglycan in glial cells and brain, there are still many fundamental questions concerning the expression, and especially the distribution and function of α- and β-dystroglycan in neurons. To address these questions, I have designed experiments to visualize dystroglycan in individual neurons by using primary neuronal cultures dissected from rat hippocampi.

Materials and Methods

Antibodies

The polyclonal rabbit dystrophin antibody is a gift from Dr. Paul Holland's lab and was used at 1:500 dilution (71). The monoclonal mouse β-dystroglycan antibody was purchased from *Novocastra Laboratories, Ltd.* and was used at 1:250 dilution. Mouse monoclonal anti-MAP2 antibody was purchased from Sigma and diluted 1:2000 for immunocytochemistry. Mouse monoclonal anti-synaptophysin antibody was from *Boehringer Mannheim Biochemicals* and was used at 1:50 dilution for immunocytochemistry. Anti-utrophin mouse monoclonal antibody was purchased from *Novocastra Laboratories, Ltd* and used at 1:50 for immunoblotting. A mouse monoclonal anti α-dystroglycan antibody was generated and characterized by this lab and was used at 1:50 dilution for immunoblotting (69). The secondary horseradish peroxidase–conjugated goat anti-mouse and goat anti-rabbit antisera (*Jackson ImmunoResearch Laboratories*) were used at 1:2000 and 1:4000 dilution respectively for immunoblotting. Oregon Green labeled goat anti rabbit (*Molecular Probes*) and Rhodamine(TRITC)-conjugated goat anti-mouse antisera (*Jackson ImmunoResearch Laboratories)* were both used at 1:100 dilution for immunocytochemistry.

Immunolocalization in rat brain hippocampal cultures

Hippocampi of postnatal day 1-3 rats were dissected and dissociated by papain (*Worthington Inc.*) treatment for 1 hour following trituration in neurobasal media (*Gibco*) containing 0.1 mg/ml of DNAase. The dissociated cells were plated into chamber slides

(*Nalge Nunc International)* that had been first coated (2-4 hrs) with 0.5 mg/ml of poly-D-lysine and 30 μg/ml of laminin, and the neurons in the chamber slides were allowed to grow in B27-supplemented neurobasal medium for 3-4 weeks with addition of 5 μM of cytosine arabinoside for the first 4 days and 2 μM for additional one week to kill dividing cells.

Coverslips containing the cultured neurons are removed from wells for immunostaining, and the cells were washed once with phosphate-buffered saline (PBS) and fixed in methanol at 20°C for 20 minutes. The fixed cells were then "blocked" with 5% horse serum for 1 hour, followed by 1 hour of primary antibody incubation, and three washes with blocking solution. The neurons were then incubated with secondary antibody for 1 hour followed by 3 washes with blocking solution. Anti β-dystroglycan rabbit polyclonal antibody was used at concentration of about 1μg/ml, mouse monoclonal MAP2 antibody (*SIGMA*) was diluted 1:2000, and mouse monoclonal anti-synaptophysin antibody (*Boehringer Mannheim Biochemica*) was diluted 1:100. Coverslips were mounted on the chamber slides with Immuno Floure Mounting Medium (*ICN Biomedicals*). Photomicrographs were taken with ZEISS Axioskop immunofluorescence microscope. All the micrographs were scanned and processed under identical conditions by computer with Adobe Photoshop and Adobe Illustrator software.

Brain Extracts and SDS-PAGE

Rat brains were dissected on ice, and homogenized in a glass/teflon homogenizer in phosphate-buffered saline (pH7.2) containing 1x protease inhibitor cocktail (*Boehringer Mannheim*). After homogenization, the protein concentration was

determined (Bio-Rad protein assay kit), and 30 μg of homogenate were mixed with 1/3 volume of sample buffer (*New England Biolabs*) containing 2% SDS, 10% glycerol, 0.01% bromophenol blue and DTT. The samples were then boiled 5 minutes before loading for SDS-PAGE. The samples (20μg) were loaded into the wells of a SDS-PAGE electrophoresis system and electrophoresed on 5 or 7.5 % polyacrylamide gel according to method of Laemmli (72) in a buffer containing 25mM of Tris-HCL (pH8.2), 200 mM of glycine and 0.05% SDS. Pre-stained and unstained broad range molecular weight markers (*New England Biolabs*) are loaded at the same time. The samples are electroblotted (100mV, 10mA) onto a supported nitrocellulose membrane in a buffer containing 20% methanol, 10 mM Tris-HCL (pH8.0) and 2.5 mM glycine. The nitrocellulose filters are blocked for 1hr in a solution containing 5% skim milk, 150 mM NaCl, 10 mM Tris-HCL (pH7.5) and 0.1%Tween-20. Subsequently the appropriate primary antibody is incubated with the membrane overnight in the milk solution, and then the membrane is rinsed 3 times (10 minutes each) in washing buffer (150 mM NaCL, 10 mM Tris, and 0.1%Tween-20). After the rinsing, the membrane was labeled by secondary antibody conjugated to HRP for 1 hour, followed by 4 times of washing 15 minutes each. The signal was revealed by chemiluminescence (ECL from *NEN*) for 60 seconds and exposure to Kodak autoradiographic films.

Preparation of anti β-dystroglycan antibodies

The 0.2 mg of synthetic polypeptide (PKNMPYRSPPPYVP) which represents the last 15 amino acids of the dystroglycan C-terminus were conjugated to keyhole limpet hemacyanin (KLH), mixed with 0.5ml of complete Freund's Adjuvant (Gilbco) and 0.3ml

sterilized distilled H_2O, and injected into rabbits subcutaneously. Two weeks later, another 0.2mg of polypeptides were injected with incomplete Freund's Adjuvant (Gilbco). After another two weeks, 15ml of rabbit blood was taken and incubated at 37°C for 1 hour to inactivate the complement system, after the incubation, the blood was left at 4°C degree overnight, and then centrifuged at 8000 x g for 15 minutes. The serum was collected and saved at –80°C.

The agarose beads that have been coupled with the peptide listed above were prepared by Research Genetics. 2ml of that 50% agarose slurry was poured into a 10 ml column and the final volume of the agarose bed in the column was 1 ml. 1 ml of serum was mixed with 9 ml of 10 mM Tris-HCL (pH7.5) and loaded onto the column pre-equilibrated with 10 ml of 10mM Tris-HCL (pH7.5). The follow-through was collected and re-loaded into the column two more times. After that, the column was washed with 10 ml of 10 mM Tris-HCL (pH7.5) and 10 ml of Tris-HCl (pH7.5) containing 500 mM of NaCL. Then the bound proteins were eluted with 3 ml of 100 mM glycine (pH2.5) and the eluate was directly collected in 300 µl of 1M Tris-HCL (pH8.0) to neutralize the pH. The column was then washed with 10 ml of Tris-HCl (pH8.8). Finally 3 ml of triethylamine (pH11.5) was used, and the eluate was collected in 300µl of 1M Tris-HCl (ph8.0). The column was finally washed with 10 ml of Tris-HCL (pH7.5) and stored in 10 mM Tris-HCl (pH7.5) containing 0.02% sodium azide. The two fractions of eluent were pooled and subject to dialysis against 4L of phosphate-buffered saline (PBS) overnight, and 0.01%BSA was added to the antibody to prevent nonspecific binding in western blots. All the procedures were done at 4°C degree. The concentration of the

antibody was determined by spectrophotometry at OD280 before BSA was added. The specificity of the purified antibody was tested by western blots.

Results

1: Specificity of the polyclonal anti β-dystroglycan antibodies

Equal amounts of rat brain homogenate (about 20 μg each) were subject to SDS-PAGE, transferred and probed with monoclonal anti β-dystroglycan antibody (Figure 1, lane B), or polyclonal anti β-dystroglycan antibody (lane A) or a secondary antibody alone (lane C), and visualized with HRP labeled goat-anti-rabbit antibody (lane A and C), or HRP labeled goat-anti-mouse antibody (lane B). The polyclonal antibody recognizes a major band at 43 kDa in the homogenate, which corresponds well to the band recognized by the commercial monoclonal antibody (Figure 1, lane B) and the known apparent molecular weight of β-dystroglycan (1). The lower bands at the about 25 kDa recognized by both monoclonal and polyclonal antibodies are likely degradation product of the 43 kDa β-dystroglycan. The control lane (lane C) shows no bands. Taken together, these results indicate that the purified antibody is specific for β-dystroglycan. This antibody was also used in previous studies (25), and its specificity was confirmed by pre-absorption experiment in which the addition of peptide used for immunization abolished the immunoreactivity of β-DG in muscle sections (25).

2: β-Dystroglycan is distributed in cell bodies, dendrites and axons, and present in a punctate pattern in some of these regions of hippocampal neurons (10 day) in culture.

Cultured hippocampal neurons were labeled first with antibodies to the dendritic marker protein, microtubule-associated protein 2 (MAP-2), (Figure 2.A and B). At 10 days in culture, most of cells in dish are of neuronal origin as indicated by MAP-2 staining. When the polyclonal anti β-dystroglycan antibodies were used (Figure 2C), immunostaining was seen in neuronal bodies, and dendrites (Figure 2B and C). Along with the expressions in cell bodies and dendrites, dystroglycan staining is also present as bright puncta, mainly on the distal dendritic shafts and on the dendritic spines (Fig.2.C). It is well known that distal dendrites are often synaptic sites, thus these clusters might be synaptic (53, 54, 55). The soma and some proximal dendrites also have dystroglycan-stained puncta, but not as many as on the distal dendrites (Figure 2.C). We also observed sites of enrichment along dendritic shafts, and it is possible that some or all of these sites correspond to spines oriented perpendicular to the culture substrate. In addition to dendrite and cell body staining, there is also observed dystroglycan staining in the axons of neurons where MAP-2 staining is negative but β-dystroglycan staining is positive and present as puncta (arrows in Figure 2. B and Figure 2.C). In each experiment cultures were treated with secondary antibody alone as a control, and in these control experiments, the fluorescent signal was always very weak and neurons cannot be visualized (not shown). Over five culture experiments have been done, and the Figure 2 is the representative of all experiments.

3: In 3 week old hippocampal cultures, dystroglycan expression is reduced in dendrites, and present as puncta, some of which colocalize with synaptophysin staining

Hippocampal neurons of 3 week old culture were double-labeled with antibodies to dystroglycan and the presynaptic marker, synaptophysin. Over 3 weeks in culture, dystroglycan staining was seen at cell bodies and in some regions of dendrites, and there is reduction in expression along dendritic shafts (Fig.3) although clusters of β-dystroglycan staining still can be seen long the neuronal processes (Fig.3). Synaptophysin staining appeared as puncta along processes. Some of the puncta of dystroglycan staining colocalize with synaptophysin staining (indicated by arrows and arrow-heads in Figure 3) indicating that in the 3 weeks cultures, dystroglycan is synaptically localized.

Discussion

Dystroglycan is a widely expressed protein, which has been found in membranes of muscle, epithelial cells, blood vessels, Schwann cells, astrocytes and Purkinje cells (31,46,47). However, there are few reports talking about the detailed expression pattern of β-dystroglycan in neurons in culture except for that of Belkin and Smalheiser (74) who pointed out that dystroglycan is enriched at focal adhesion sites and at growth cones of E17 rat hippocampal axons prior to synapse formation (74). Our results show that dystroglycan, in cultured hippocampal neurons, is highly expressed in the cell body, dendrite and that it forms clusters along the dendrites in 10 day old cultures. As in the previous report (74), our results indicate that β-dystroglycan is expressed in axons and present as puncta as well. In addition we show that in 10 day cultures, β-dystroglycan is expressed on dendrites. As the neurons grow older (3 weeks), preliminary studies indicate

that the dystroglycan expression pattern changes. Staining in cell body is still strong, but the staining in dendritic membrane is weaker although clusters are still seen on dendrites. This may suggest that dystroglycan is especially required during the processes of neuron maturation. At both stages, the dystroglycan staining is seen as puncta, and these puncta appear at times to be synaptic by their colocalization with synaptophysin, suggesting that dystroglycan may be a synaptic protein. However, only portion of these dystroglycan puncta correlate with synaptophysin staining, and only small portion of synaptophysin-labeled nerve terminals colocalize with dystroglycan puncta. This may indicate that only portion of dystroglycan is present at presynaptic sites and portion of it is at postsynaptic sites. Preliminary studies from others suggest that dystroglycan (both α-and β-) colocalize with $GABA_A$ receptor, a major inhibitory receptor in the hippocampus (72). Only two subunits (α2 and γ2) of $GABA_A$ receptor colocalize with dystroglycan in both hippocampal and spinal neurons in culture. These data further confirm our results that dystroglycan is expressed in neurons and present in some synapses. Future studies should address at what kind of post-synaptic sites dystroglycan is located, when β-dystroglycan clusters first appear (10 day or sooner), and whether dystroglycan is presynaptically , postsynaptically localized or both .

Chapter 3

Identification of A Novel Dystroglycan Interacting Protein in Brain

Introduction

Dystroglycan is the core component protein of the DPC and consists of α and β subunits with multiple interacting proteins and various functions. The targeted complete disruption of the dystroglycan gene in mice is lethal and results in failure of development of Reichert's membrane(60). Reduced expression of this gene leads to muscular dystrophy and impairment of NMJs (71). The α and β subunits are produced by proteolytic cleavage of a precursor proteins of 97 kDa molecular mass (1,58,59) and are both glycosylated. In muscle sarcolemma, α-dystroglycan has been shown to bind to proteins of the basal lamina such as agrin, laminin, perlecan and biglycan (14,5,15). Intracellularly, the β-dystroglycan subunit with molecular weight of 43 kDa binds to dystrophin underneath the sarcolemma membrane (1), utrophin at neuromuscular junction, Grb2 an adapter protein (33), caveolin 3 in muscle and the AChR effector protein rapsyn (32) at NMJs. The dystrophin binding region of β-dystroglycan has been localized to the C-terminal 15 amino acids (880-895) (63,64), the binding region for the SH3 domain has been localized to the proline-riched region (7), and the possible rapsyn binding sites are between amino acid 787-819 (32). As a member of dystrophin associated protein complex, dystroglycan has also been found associated with syntrophin and dystrobrevin indirectly through dystrophin (1,4,12), it also associates with a group of transmembrane proteins called sarcoglycans, through a possible interaction between dystroglycan and δ-sarcoglycan (11,12).

In CNS, the DPC is different because of the distinctive tissue specific expressions of dystrophin isoforms (46), dystroglycan and others members. As demonstrated by Blake et al (34), Dp-71, syntrophin and alpha-1 dystrobrevin comprise the dystrophin

complex in glial cell membranes, and Dp-427, syntrophin, and beta-dystrobrevin comprise the dystrophin complex in neuronal membranes. However, the authors did not provide evidence showing dystroglycan is associated with dystrophin. In a separate experiment Hakima Moukhles had shown that β-DG in brain is associated with individual dystrophin isoforms with or without syntrophin in different parts of brain (76). Considering the cognitive impairment in DMD and BMD patients (46), it would be essential to be sure if, and how, dystroglycan is linked to dystrophin complex in CNS neurons or glial cells.

Dystroglycan appears concentrated in CNS synapses in culture (discussed above) and may function in CNS synapses as it does in neuromuscular junctions. Current evidence shows that dystroglycan is associated with the tyrosine kinase adapter protein Grb2 in synaptosomes (65) as in muscle, and also interacts with prion protein (66). It is plausible dystroglycan interacts with synaptic proteins because of its expression there and these interactions may be mediated by its proline-rich regions.

To begin to address these questions, I designed experiments to identify proteins that interact with β-dystroglycan biochemically. To accomplish this, rat hippocampi were dissected and extracted and antibody affinity chromatography was used to purify dystroglycan and any associated proteins. My data show that both α-dystroglycan and β-dystroglycan can be purified by this method and they are present in hippocampus in about the same ratio. Unlike in the muscle, I did not detect dystrophin and utrophin associated with dystroglycan. By staining the purified protein complex with Coomassie blue, we found several bands associated with dystroglycan that seem to be novel and specific with a prominent band located around 97 kDa.

Materials and Methods

Preparation of the Immunoaffinity Column

Polyclonal anti-dystroglycan antibody was purified as described in Material and Methods (Chapter 2). 2-3 ml of the antibody was dialyzed in 4 L of phosphate-buffered saline (PBS) at 4°C overnight. The antibody concentration was determined by spectrophotometer at OD-280, and 5mg of antibody in 2-3 ml of PBS were coupled to Affi-gel 10 matrix (*Bio-Rad*) according to recommendations of the company. Briefly, the Affi-gel 10 was thawed at room temperature for 30-60 minutes, and a 2 ml slurry of gel was poured into a funnel covered with filter paper and then rinsed with distilled water 3 times quickly. The beads were added to the antibody solution in a total volume less than 5ml at a concentration of 1-2 mg/ml for the antibody. This mixture was shaken for 4 hours at room temperature and transferred to a 10 ml column. The follow-through was collected and the column was washed 3 times with PBS. The protein concentration of the follow-through was determined and the coupling efficiency was calculated at about 78%. After that, the column was treated with 200mM ethanolamine at pH7.4 overnight to neutralize the capacity of the Affi-gel to bind additional proteins non-specifically. The column was washed with PBS 3X and stored 4°C at 0.02 % sodium azide in PBS for future use. Control columns had 5 mg of bovine serum albumin (BSA) or 5 mg of normal rabbit IgG (NRIgG) (*SIGMA*) coupled to 1 ml of Affi-gel beads by same procedure.

Hippocampal Extracts

Four rats were sacrificed and hippocampi of brains were dissected on ice. The dissected hippocampal tissue was homogenized 10 seconds for four times with a Polytron

tissue homogenizer in PBS containing 1% of sodium deoxylcholate (pH9.0), 0.5mM of $CaCl_2$, and 1x protease inhibitor cocktail (*Boehringer Mannheim*). The homogenized tissue was incubated at 37°C for 30 minutes followed by addition of TritonX-100 to a final concentration of 0.5% in about 6 ml. After 2 hours of incubation at 4°C with occasional vortexing, the homogenates were centrifuged at 37,000x g for 30 minutes. The supernatant was collected and the protein concentration was detected with a Bio-Rad detergent compatible DC Protein Assay kit.

Immunoaffinity Chromatography

5 mg of above supernatant in about 2-3 mls were incubated with Affi-gel 10 coupled with β-dystroglycan antibody or BSA or rabbit immunoglobulin overnight with constant shaking. The conditions for washing and eluting are as follows: The unbound proteins were collected and the columns were each washed first with 3 ml of PBS, and then washed with 3 ml of PBS containing 1% of sodium deoxycholate, 0.5% Triton X-100 and 0.15 m sodium chloride. This was followed by washing with 3 ml of PBS with 1% sodium chloride, 0.5% Triton X-100, and 0.5 M of sodium chloride, and finally the columns were washed with 3 ml of PBS containing 5 mM of EDTA. The proteins still bound to the column were eluted with 0.1 M of glycine (pH2.5) into 0.3ml of 1M Tris-HCl (pH8.0). The columns then were equilibrated each with 10 ml of PBS and remaining proteins in the columns then eluted with 3 ml of 0.1M of triethylamine (pH11.5). Both glycine and triethylamine eluates were pooled, and all eluentes from columns were concentrated to equal volumes by centrifugal filter device from Centricon (*Millipore*), and subjected to further analysis.

Procedures for Coomassie Blue staining

Western blots were done as described above. After completion of SDS-PAGE, gels were incubated in 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% distilled H_2O for a minimum of 1 hour with constant shaking. After the incubation, the gels were de-stained in 10% acetic acid, 50 % methanol, 40% of distilled H_2O with at least two changes of solution for at least two hours until the gel background is clear. Then the gels were scanned into a computer with Adobe Photoshop software, documented and processed with Adobe Illustrator software. After the documentation of the gel, the individual bands were cut out and sent out for mass spectrometry.

Results

1: Characterization of Immunoaffinity columns

To first confirm that the columns are working and the conditions are right for purifying β-dystroglycan, I analyzed the eluate of all the columns for β-dystroglycan immunoreactivity. As shown in the Figure 4 in β-dystroglycan immunoblots, β-dystroglycan is greatly enriched in the antibody column eluate (Lane β-DG Ab of Figure 4) when compared with the starting material (Lane H.H.). Moreover there is no β-dystroglycan detected in eluentes of the normal rabbit IgG (NRIgG) and BSA control lanes (Lane NRIgG and BSA of Figure 4). This result indicates that hippocampal β-dystroglycan specifically binds to the antibody column and is enriched following immunoaffinity chromatography.

2: Detection of other DPC members

I next asked if α-dystroglycan is present in the eluate with β-dystroglycan. As shown in the immunoblots with the antibody specific to α-dystroglycan (69), α-dystroglycan is also greatly enriched by the β-dystroglycan immunoaffinity chromatography when compared with the pre-loading material (Figure 5; lane H.H.), and there is no α-dystroglycan detected in the control column (Figure 5; lane BSA). These data suggest that dystroglycan α- and β- are associated with each other in hippocampus as in muscle. I also asked if dystrophin and utrophin are associated with β-dystroglycan in hippocampus, As shown in Figure 6, with the polyclonal anti-dystrophin antibody which recognizes all dystrophin isoforms, we found that dystrophin is not detectable in the column eluate (lane B, figure 6.1), although several isoforms of dystrophin were detected in the starting material, including Dp71, Dp116, Dp140 and full length Dp427 (lane C, figure 6.1). Like dystrophin, utrophin is also undetectable in the eluate (lane B, figure 6.2), but the 395 kDa utrophin band is present in the starting material (lane c, figure 6.2). These data suggest that dystrophin/utrophin is not complexed with dystroglycan in this particular tissue or it is disrupted during extracting/purification.

3: Identification of a novel DPC in brain

To find out if there are any novel proteins that might be associated with β- and α-dystroglycan, I stained with Coomassie blue gels loaded with equal amounts of eluents from each column. As shown in the Figure 7, there are several bands appearing in the lane of the antibody affinity column, but not in the normal rabbit IgG column or BSA column. One band is located around 97 kDa (lane 1,arrowhead). Since there is no band in

the same position of the IgG control and BSA control, I consider that the protein located in this band is binding specifically to the anti dystroglycan antiserum. As there are no proteins of this size which have been reported to interact with β-dystroglycan, this protein located in 97 kDa is likely a novel protein. In addition there are two less prominent bands around 70 kDa and 32 kDa, that specifically associate with β-dystroglycan. In the β-DG antibody lane of figure 7, a 43 kDa band which is β-dystroglycan as indicated by β-dystroglycan antibody (Figure 4) is also apparent. In addition to the Coomassie blue staining, silver stains have also been done in different eluents in two experiments. The 100 kDa band was constantly visible and there were no similar bands appearing in NRIgG and BSA lanes (Data not shown).

Discussion

In this study, I used immunoaffinity chromatography to purify β-dystroglycan from the hippocampus. β-dystroglycan and α-dystroglycan are both purified and enriched, and the degree of enrichment is similar for both. These data clearly indicate that α- and β-dystroglycan are biochemically linked, and with results from Chapter 2 it can be concluded that β-dystroglycan complexes with α-dystroglycan and this complex may constitute synaptic structure or function at CNS synapses.

As noted above, dystrophin/utrophin connects to β-dystroglycan to form part of a large DPC in muscle, and other reports indicate that there is a distinct cytoplasmic DPC in neurons and glial (34). Surprisingly, in our purified dystroglycan fraction, neither dystrophin nor utrophin was detected. This result implies that, in neurons and in glial

cells, dystroglycan might not form complex with dystrophin/utrophin, instead dystrophin may form an independent cytoplasmic complex with syntrophin and β-dystrobrevin, and might connect to plasma membrane through other protein(s). Indeed a report (44) showing dystroglycan is linked to Dp71 in brain was based on immunohistochemistry, and there is no direct biochemical evidence to support they are connected in astrocytes or neurons. This notion is consistent with the genetic evidence in muscle, where deficiency of dystroglycan in some muscle fibers has no effect on distributions of dystrophin, syntrophin and nNOs, and nor to their anchorage to sarcolemma (75). A previous report also showed that deletion of dystrophin binding site in the last 15 amino acid of β-dystroglycan has no effect on localization of dystrophin to the plasma membrane (Society of Cell Biology Meeting 1999, Abstract from K. Campbell's lab). On the other hand the polyclonal antiserum used is made against the last 15 amino acid of dystroglycan, a region same for dystrophin to bind to dystroglycan, and this might disrupt dystrophin β-dystroglycan interaction. In future studies, other antisera which do not recognize any of the binding sites in dystrophin and dystroglycan should be used to do immunoprecipitation to confirm the relationship in neurons or astrocytes, such as antibodies recognizing α-dystroglycan or antibody recognizing the N-terminal dystrophin/utrophin.

A number of proteins have been shown to bind to dystroglycan in skeletal muscle. In brain, however, few proteins have been found associated with dystroglycan. These include Grb2 (33,65) and the prion protein (40 kDa) (66). Very recently another protein neurexin-α has also been reported to physiologically complex with α-dystroglycan possibly at synapses (70). In my experiments, a new protein around 100 kDa appeared in

the purified dystroglycan fraction, which is likely a novel protein. I am presently obtaining protein sequence data to identify this potentially new protein and try to characterize the interaction.

In summary, this study shows that dystroglycan, both β and α, are neuronal proteins. They form a complex in hippocampal neurons and cluster in cell bodies, in axons, and along dendrites. Some of these clusters are localized at synaptic sites. This study does not support that dystrophin/utrophin is associated with dystroglycan in hippocampus, instead a new 100 kDa band has been found, which appears to be part of dystroglycan complex in brain.

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Figure 1: Specificity of the polyclonal antiserum for β-dystroglycan. Total brain homogenates were subjected to SDS-PAGE, transferred to nitrocellulose and probed with A: polyclonal anti β-dystroglycan, B: monoclonal anti β-dystroglycan antibody (Novocastra). Both antibodies recognize a major band at about 43 kDa (molecular weight markers are on the left). A lower band is also recognised by antibodies in both A and B, that may be breakdown products of β-DG. The lane C was probed with only the secondary HRP conjugated goat-anti rabbit antiserum.

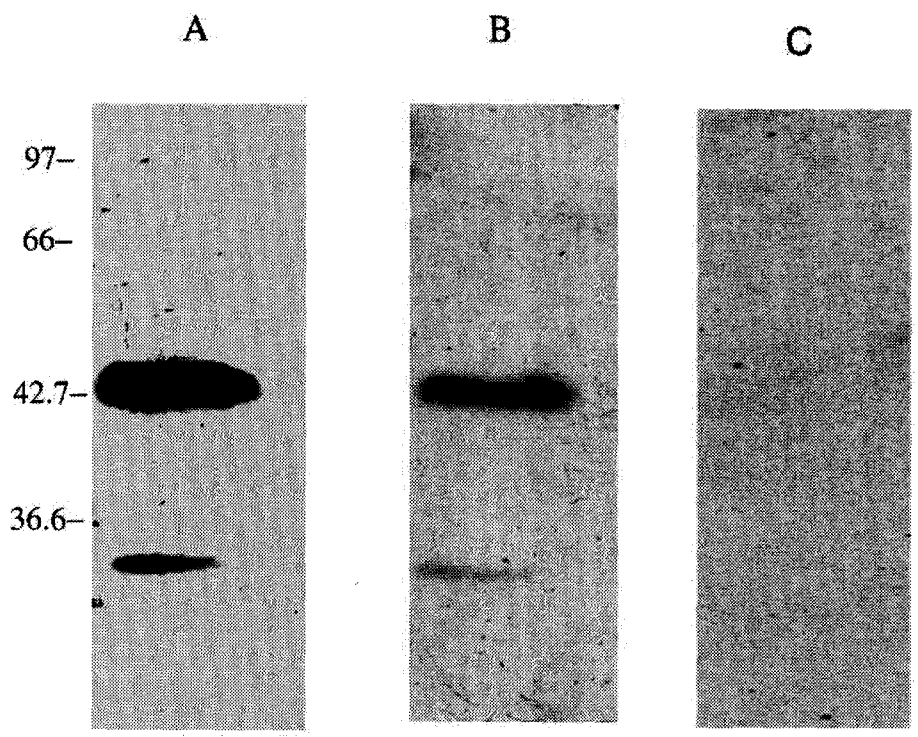
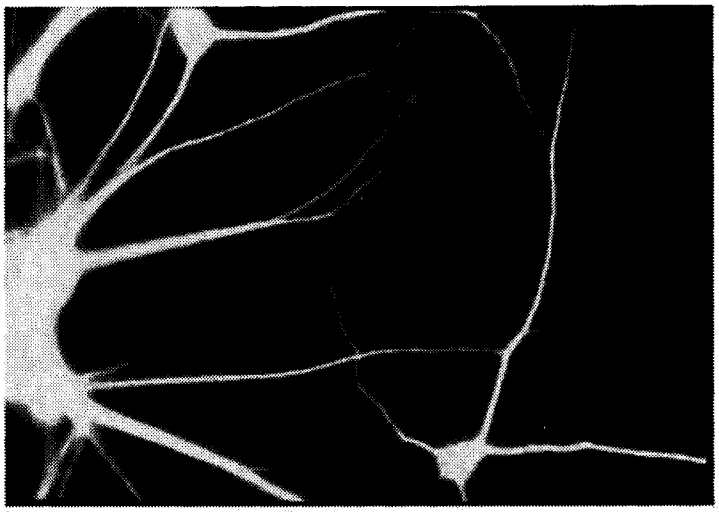


Figure 1

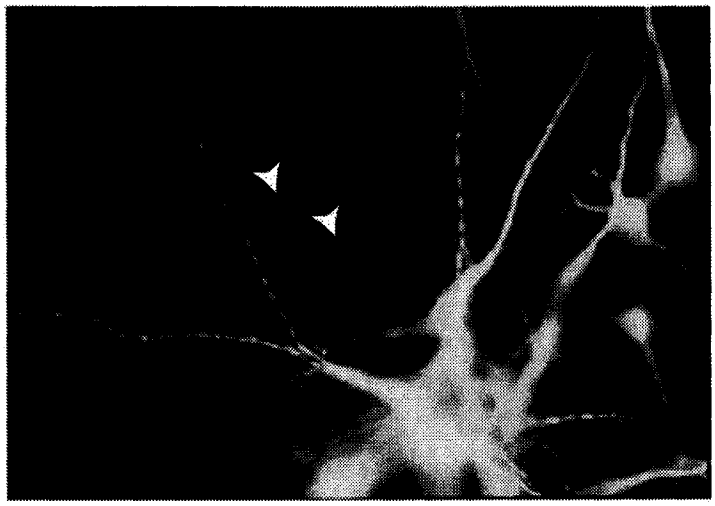
Figure 2:Immunocytochemistry of hippocampal neurons (10 days) in culture. Neurons were stained by antibodies to A: anti-MAP2 antibody, B: anti-MAP2 antibody, and C: polyclonal anti β-dystroglycan antibody. Note that in B and C, the neurons are double labeled with MAP2 and β-dystroglycan. The arrowheads point to the puncta present in β-dystroglycan positively staining processes (C), which are MAP2 negatively stained (B).

A



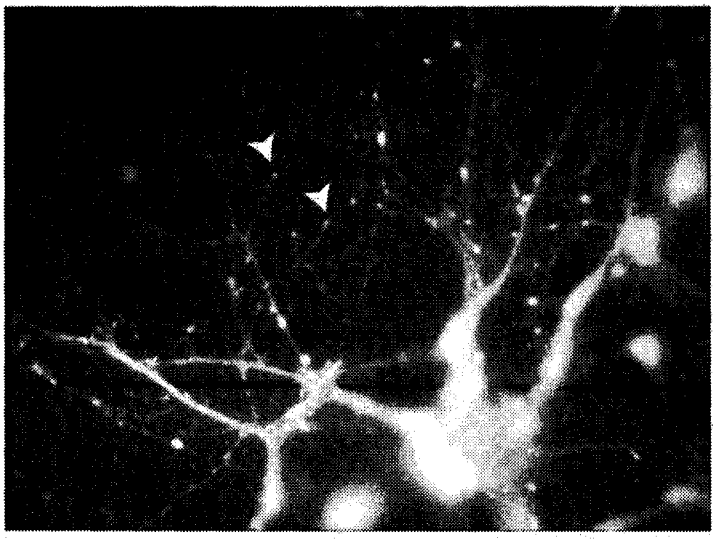
MAP-2

B



MAP-2

C

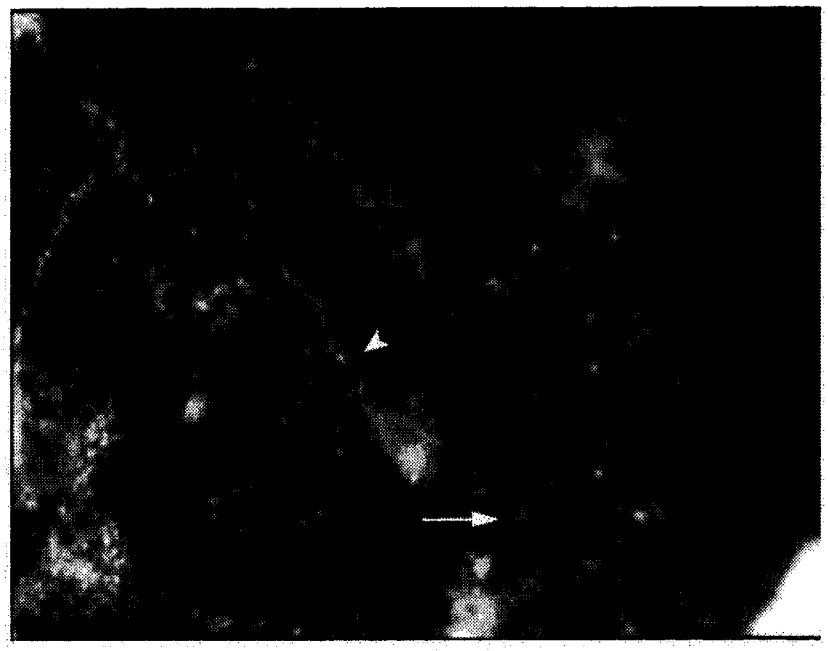


β-dystroglycan

Figure 2

Figure 3: Immunocytochemistry of hippocampal neurons (3 weeks) in culture. Neurons were stained with synaptophysin and β-dystroglycan antiserum. The arrowhead and arrow (Top) show clustered synaptophysin staining. The arrow and arrowhead (Bottow) indicate clusters of β-DG.

Synaptophysin



β-DG

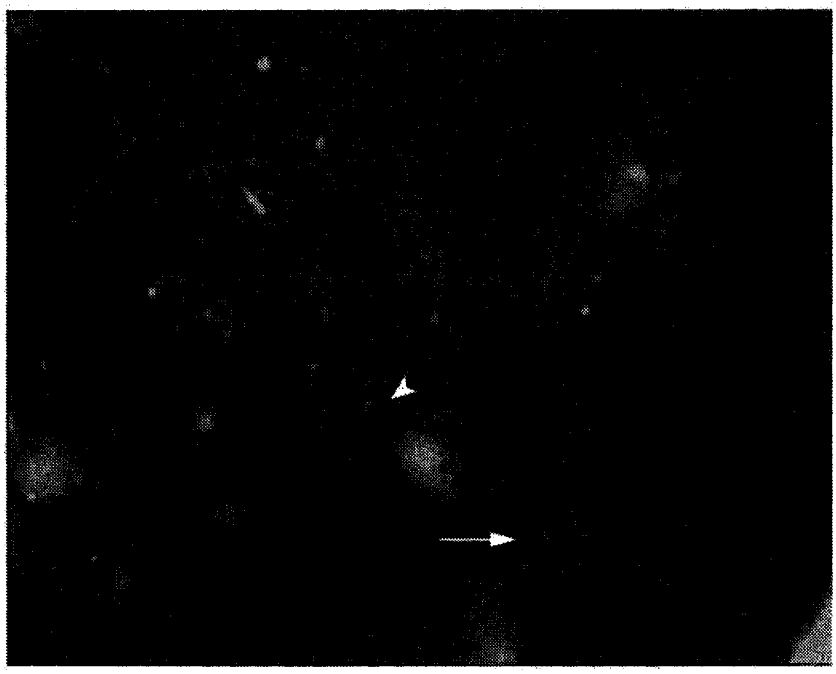


Figure 3

Figure 4: Immunoaffinity purification of β-dystroglycan. Equivalent amounts of eluate from antibody affinity column (β-DG Ab) that is $1/10^{th}$ of the total eluates, normal rabbit IgG column (NRIgG), BSA column (BSA), and 1/25 of hippocampal homogenates (H.H.) were loaded onto the gel, subject to SDS-PAGE, transferred to nitrocellulose and probed with monoclonal anti β-DG antibody (Novocastra). The signal for β-DG in the antibody affinity column eluate is greatly enriched when compared with those in other lanes, and there is no signal (β-dystroglycan) present in NRIgG and BSA column eluates.

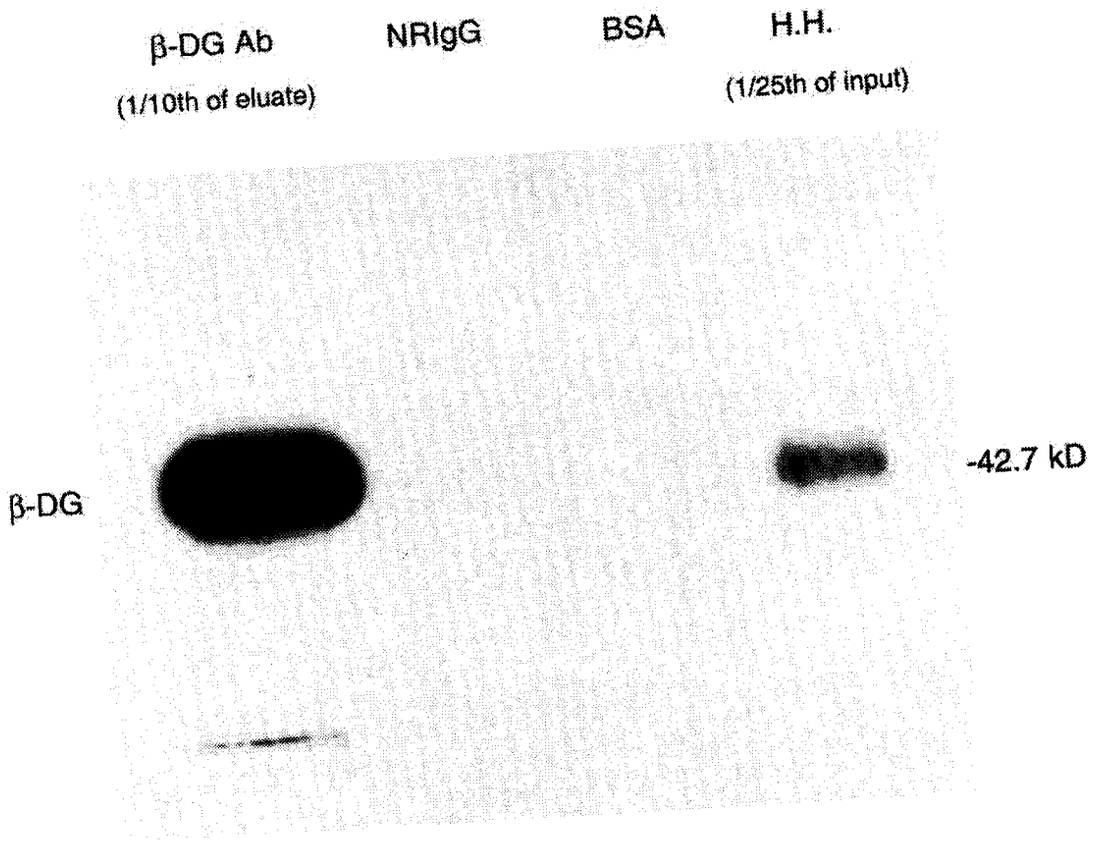


Figure 4

Figure 5: α-dystroglycan interacts with β-dystroglycan in hippocampus. Equivalent amounts of protein eluted from anti β-DG antibody affinity column (β-DG Ab), BSA column (BSA) and 1/20th of hippocampal homogenates (H.H.) were loaded onto the gel, subjected to SDS-PAGE, transferred to nitrocellulose and probed with monoclonal anti α-dystroglycan antibody. Note that α-dystrgolycan signal is greatly enriched in the β-DG antibody column eluate when compared with other eluate, and there are no signals present in BSA column eluate. The molecular weight marker is on the right.

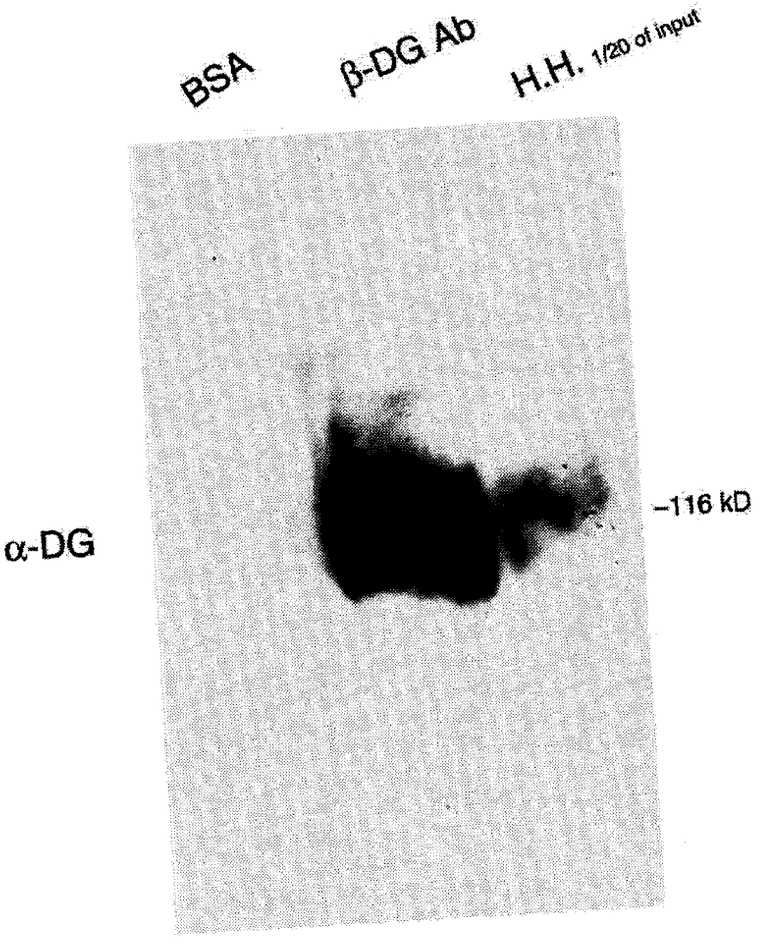


Figure 5

Figure 6.1: Dystrophin is not associated with β-dystroglycan in hippocampus. Lane A: eluate from BSA column. Lane B: eluate from anti β-dystroglycan affinity column. Lane C: starting material of hippocampal homogenate. The loaded amount in Lane A and B are equivalent. All lanes were subject to SDS-PAGE and probed with polyclonal anti-dystrophin antibody. The molecular weight markers are on the left, and the name of the proteins identified by this antibody in the hippocampal homogenate (pre-loading material) are on the right. Note that no dystrophins are detected in the β-DG antibody affinity column (B) and BSA column (A), but there are 4 dystrophin isoforms present in the hippocampal homogenates (C).

Figure 6.2: Utrophin is not associated with β-dystroglycan in hippocampus. All lanes were probed with anti utrophin antibody. Lane A: eluate from BSA column. Lane B: eluate from anti-dystoglycan antibody affinity column. lane C: hippocampal homogenate (pre-loading material). Molecular weight marker is on the left. Note that there is no utrophin signal in both anti β-DG antibody affinity column and BSA column, but a utrophin signal is present in the starting materials.

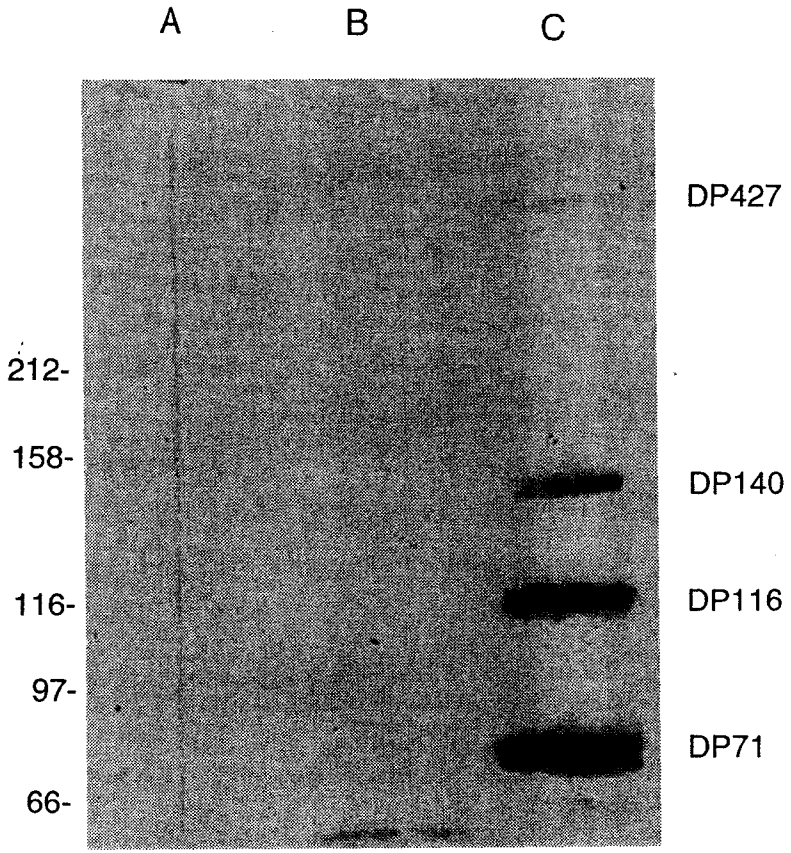


Figure 6.1

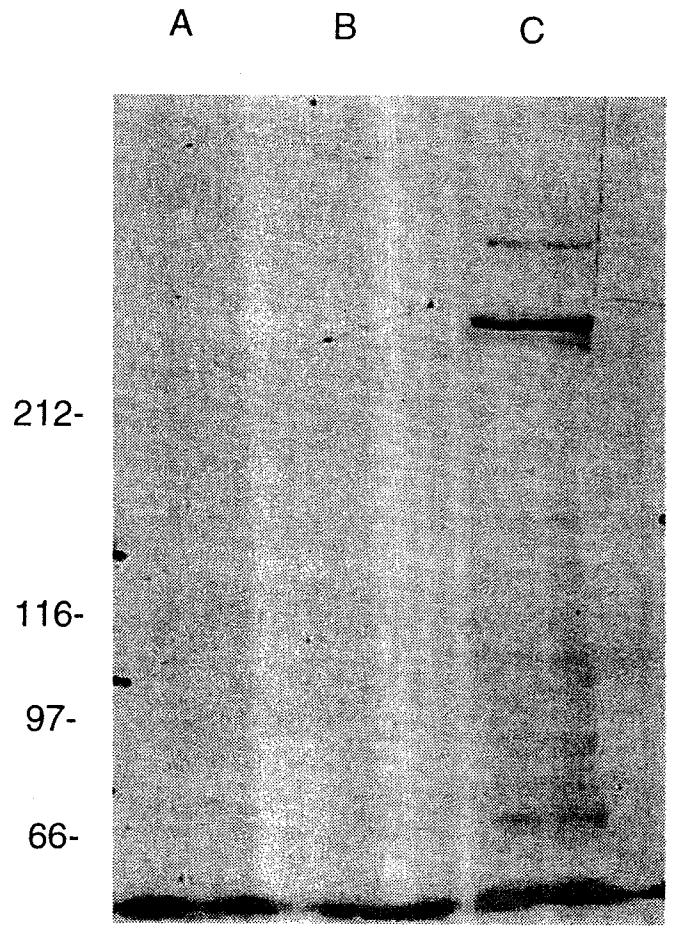


Figure 6.2

Figure 7: Identification of a new DPC in Brain. Equivalent amounts of eluate from anti β-DG antibody affinity column (β-DG antibody), normal rabbit IgG column (NRIgG), BSA column (BSA), and 2 μg of hippocampal homogenates (H.H.) were loaded, and subjected to SDS-PAGE and stained with Coomassie blue. The molecular weight markers are shown. Arrowheads indicate bands seen only in β-DG antibody affinity column, but not in NRIgG and BSA, and those bands were cut out and sent for sequencing. Note that the band arround 100 kDa is the most prominent one. The antibody heavy chains (55kD) were seen in both β-DG antibody affinity column eluate and NRIgG eluate. Also the 43 kD β-dystroglycan band is apparent in β-DG antibody affinity column eluate.

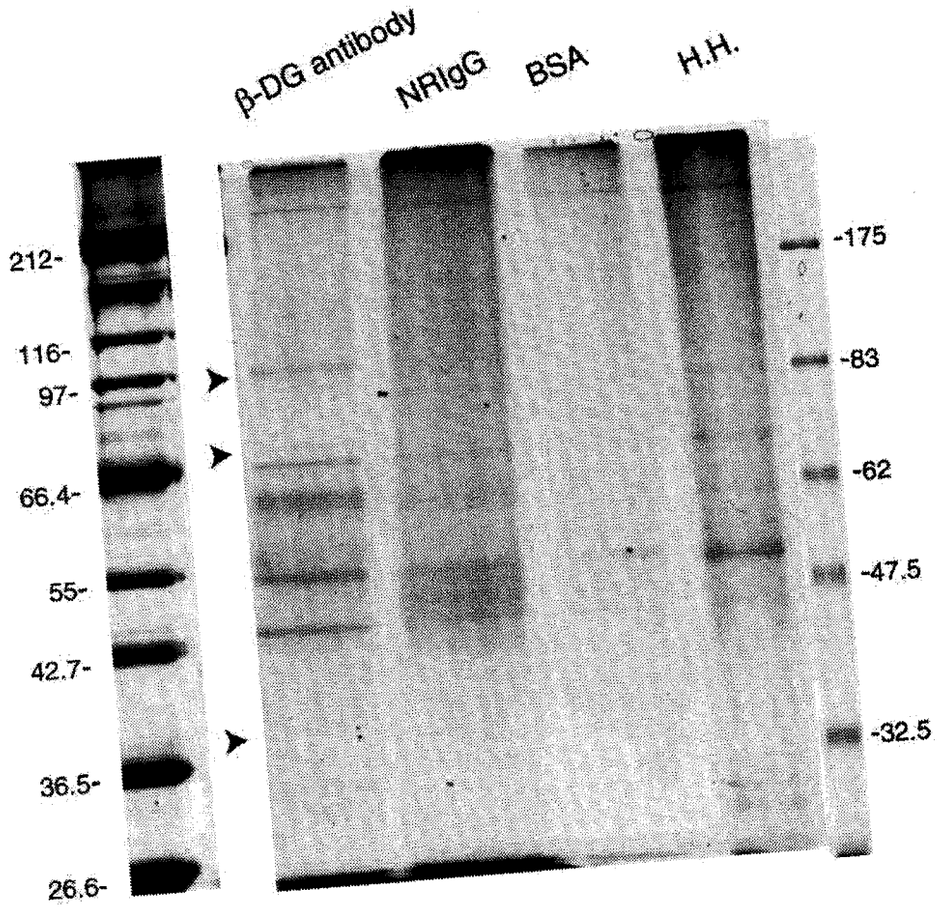

β-DG antibody
NRIgG
BSA
H.H.
212-
116-
97-
66.4-
55-
42.7-
36.5-
26.6-
-175
-83
-62
-47.5
-32.5

Figure 7