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Oligomerization of β-dystroglycan in rabbit diaphragm and brain as revealed by chemical crosslinking

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Abstract

The surface component β-dystroglycan is a member of the dystrophin–glycoprotein complex providing a trans-sarcolemmal linkage between the actin membrane cytoskeleton and the extracellular matrix component laminin-α2. Although abnormalities in this complex are involved in the pathophysiology of various neuromuscular disorders, little is known about the organization of dystrophin-associated glycoproteins in diaphragm and brain. We therefore investigated the oligomerization of β-dystroglycan and its connection with the most abundant dystrophin homologues in these two tissues. Employing detergent solubilization and alkaline extraction procedures of native membranes, it was confirmed that β-dystroglycan behaves like an integral surface molecule as predicted by its cDNA sequence. Immunoblot analysis following chemical crosslinking of native membranes showed that β-dystroglycan has a tendency to form high-molecular-mass complexes. Within these crosslinkable complexes, immuno-reactive overlaps were observed between β-dystroglycan, α-dystroglycan, laminin and 427 kDa dystrophin in diaphragm and skeletal muscle. In synaptosomes, the major brain dystrophin isoform Dp116 also exhibited an immuno-reactive overlap with members of the dystroglycan complex. These findings demonstrate that β-dystroglycan does not exist as a monomer in native membranes and imply that certain dystrophin isoforms and dystrophin-associated components interact with this surface protein in diaphragm and brain as has been previously shown for skeletal and heart muscle. © 1998 Elsevier Science B.V.

Keywords: Dystroglycan; Dystrophin; Dp-116; Dystrophin–glycoprotein complex; Laminin; Crosslinking

1. Introduction

Dystrophin, initially identified as the primary defect in Duchenne/Becker muscular dystrophy [1], is now well established to be associated with a variety of surface proteins [2,3] and was shown to exist in numerous isoforms in many tissues including brain [4,5]. Dystrophin-associated components belonging to the highly glycosylated surface dystroglycan-complex are proposed to provide a trans-sarcolemmal linkage between the basement membrane component laminin-α2 and the actin membrane cytoskeleton in skeletal muscle fibres [6–9]. While peripheral α-dystroglycan of apparent 156 kDa represents a novel non-integrin class of laminin-binding proteins, 43 kDa β-dystroglycan is an integral dystrophin-associated glycoprotein [10]. Both dystroglycan proteins are encoded by a single gene whose expressed precursor protein product is post-translationally processed into two glycoproteins [11,12]. Molecular studies have shown that the extreme COOH-terminus of β-dystroglycan

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constitutes a unique binding site for the second half of the hinge-4 region and the cysteine-rich domain of dystrophin [13] and that α-dystroglycan can interact with the α2-chain of laminin-2, formerly referred to as merosin [11,14,15]. Both proteins co-localize with dystrophin to the muscle surface [16–18], clearly co-purify in subcellular fraction studies with the sarcolemma fraction [16,19] and contain asparagine-linked oligosaccharides [20]. Serine/threonine-linked oligosaccharides on α-sarcoglycan are proposed to confer a stiff conformation to the peptide core and protease resistance to this peripheral cell surface protein [20]. Although the principal sub-plasmalemmal cytoskeleton link to the basement membrane is probably provided by these two tightly associated dystroglycans, other sarcolemmal proteins might also be involved in this process, i.e., components of the sarcoglycan sub-complex ranging in relative molecular mass from apparent 25 kDa to 50 kDa [2,3].

Following the detailed analysis of the spatial configuration of the muscle dystrophin–glycoprotein complex, it was established that primary or secondary defects in the dystrophin–glycoprotein complex lead to a variety of neuromuscular disorders, i.e., Duchenne/Becker muscular dystrophy, congenital muscular dystrophy and certain forms of limb-girdle muscular dystrophy [8,9,21–24]. Recently, β-dystroglycan was described as defective in a novel form of muscular dystrophy [25]. However, although a large number of patients afflicted with Duchenne muscular dystrophy suffer from respiratory complications [26], relatively little work has been done on the biochemical and pathophysiological characterization of dystrophin-associated components in the diaphragm. In addition, the association of brain dystrophins with other neuronal surface components is not well understood and the normal function and potential involvement of these isoforms in brain disorders is mostly unknown. Since one third of Duchenne patients suffer from non-progressive mental retardation, mutations in brain dystrophins may be the primary or contributing factor leading to these clinical symptoms [27–29].

Brain dystrophin of 427 kDa has been localised to the postsynaptic density [27] and is present in a wide range of neurons including cerebellar Purkinje cells and pyramidal neurons in the cerebral cortex [30,31]. Recently, we could show that the major brain dystrophin isoform Dp116 exhibits biochemical properties typical of a membrane cytoskeletal protein [32]. Thus, although Dp116 is lacking the N-terminal actin-binding domain of 427 kDa dystrophin, it appears to be a component of the sub-plasma membrane cytoskeleton possibly involved in anchoring proteins in the periphery of neurons. Another member of the superfamily of spectrin-like components, autosomally-encoded utrophin of 395 kDa, has an even broader distribution in brain [33] and may function in the generation and maintenance of regional substratum-associated membrane specialisations at the blood–brain barrier [34]. Utrophin was originally described as a neuromuscular junction-specific dystrophin isoform [35–37]. Since the utrophin-associated component α-dystroglycan was found to be a functional agrin-receptor, the utrophin–glycoprotein complex is implicated in the anchoring of the nicotinic acetylcholine receptor at the neuromuscular junction [38–40]. Although α- and γ-sarcoglycan appear to be muscle-specific proteins [41], other dystrophin-associated proteins were clearly established to be present in neuronal tissues [42–45]. In neural retina, the dystroglycan complex is expressed in the inner limiting membrane and around blood vessels where it co-localises with laminin and utrophin [43]. Both dystroglycans are also present in the outer plexiform layer of retina, which is devoid of laminin, but contains dystrophin and utrophin [43]. Studies by Mummery et al. [42] showed that β-dystroglycan is enriched in synaptic membranes of adult rat forebrain and that a novel β-dystroglycan-related protein of apparent 164 kDa is enriched in the postsynaptic density. In addition, analysis of dystrophin-associated proteins in rat cerebellum by Tian et al. [44] support the hypothesis that dystrophin interacts with dystroglycans in Purkinje neurons. Binding studies with purified brain α-dystroglycan established that it can interact with α2-chain-containing laminin-2 [44]. In addition, syntrophins were recently shown to co-localize with various dystrophin isoforms and dystroglycans in mouse brain [45].

Biochemical evidence for a tight association between skeletal muscle surface glycoproteins and dystrophin isoforms exists from chromatographic techniques, analytical gradient centrifugation and domain binding studies [2,3,13,15,20]. Although the dystrophin–glycoprotein complex can be isolated by

laminin affinity columns and labeled laminin binds strongly to α-dystroglycan in overlay assays [11,15], the purified skeletal muscle dystrophin–glycoprotein complex contains no detectable laminin-α2 chain [2,3]. This loss in laminin-α2 may be due to breakage of weak non-covalent bonds during salt washes to remove actomyosin contaminations from vesicular muscle membrane preparations. Thus, to more directly determine the oligomerization of the principal trans-sarcolemma spanning component of the dystrophin–glycoprotein complex, β-dystroglycan, and its association with laminin isoforms and dystrophin, we performed a chemical crosslinking analysis. This is an extremely powerful technique for the determination of tight neighborhood relationships between membrane proteins [46] and this method was exploited in this study to investigate potential protein–protein interactions between key components of the dystrophin–glycoprotein complex in diaphragm and brain. Crosslinking analysis was performed under optimized conditions [47] with the hydrophobic 12 Å crosslinker dithiobis-succinimidyl propionate [48] and changes in the electrophoretic mobility of components of the diaphragm and brain dystrophin–glycoprotein complex were determined using immunoblotting with highly specific antibodies to β-dystroglycan, α-dystroglycan, laminin, dystrophin and Dp116.

2. Experimental procedures

2.1. Materials

Dithiobis-succinimidyl propionate was obtained from Pierce & Warriner (Chester, Chesire, UK). Peroxidase-conjugated and fluorescein-labeled secondary antibodies to rabbit or mouse IgG, as well as chemicals for enhanced chemiluminescence detection and protease inhibitors were purchased from Boehringer Mannheim (Lewis, East Sussex, UK). Immobilon-NC nitrocellulose was from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade and purchased from Sigma Chemical Company (Poole, Dorset, UK).

2.2. Antibodies

Affinity-purified, polyclonal antibodies to the extreme COOH-terminus of dystrophin, which recognize the major brain isoform Dp116, were produced as previously described in detail [32]. Polyclonal antibody to crude laminin preparations from basement membranes of Englebreth–Holm–Swarm (EHS) mouse sarcoma, monoclonal antibody VC1.1 to the HNK-1 antigen, as well as monoclonal antibody SB-SP-1 to human erythrocyte spectrin were purchased from Sigma Chemical Company (Poole, Dorset, UK). The laminin antibody, although produced against laminin-1 (previously named EHS-laminin) [49], recognizes both laminin-1 and laminin-2 (previously named merosin) [49] isoforms. Monoclonal antibody NCL-43 to β-dystroglycan was from Novocastra Laboratories (Newcastle upon Tyne, UK), while monoclonal antibodies $VIA4_1$ to α-dystroglycan, XIXC2 to 427 kDa dystrophin and C464.6 to α_1-Na^+/K^+-ATPase were from Upstate Biotechnology (Lake Placid, NY, USA).

2.3. Membrane preparations

Homogenates from diaphragm, hind leg muscle and heart from New Zealand white rabbits were used to prepare crude microsomal membranes using standard differential centrifugation techniques [16,47]. Rabbit brain synaptosomes were prepared according to the method of Gordon-Weeks [50]. In order to minimize protein degradation, all isolation procedures were performed at 0–4°C and all buffers contained the following protease inhibitors: 0.15 μM aprotinin, 0.3 μM E-64, 1 μM leupeptin, 0.2 mM pefabloc, 1.4 μM pepstatin A, 0.5 μM soybean trypsin inhibitor, and 1 mM EDTA. Protein concentration was determined by the method of Bradford [51] with rat myofibrillar proteins as a standard.

2.4. Extraction procedures

To determine the basic biochemical properties of β-dystroglycan, diaphragm and brain membranes were extracted by detergent solubilization using 1% (v/v) Triton X-100 [19,52] and alkaline extraction was performed at pH 11 as described previously in detail [19,52]. Following solubilization, membranes were centrifuged for 20 min at 150,000 × *g* and then separated fractions were analyzed by immunoblotting.

2.5. Chemical crosslinking

Following isolation, membranes were immediately chemically crosslinked and then electrophoretically analyzed. Membrane vesicles were incubated at room temperature for 30 min with 10 to 200 μg crosslinker per mg protein at pH 8.0 as previously described in detail [47]. To prepare stock solutions of the 12 Å probe dithiobis-succinimidyl propionate [48], hydrophobic crosslinker was dissolved in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in membrane suspensions did not exceed 4% (v/v). Crosslinking was terminated by the addition of 50 μl of 1 M ammonium acetate per ml reaction medium and then an equal volume of sodium dodecyl sulfate-containing sample buffer [53] was added prior to electrophoretic separation.

2.6. Gel electrophoresis and immunoblot analysis

Electrophoretic separation of proteins was performed for 2000 V h in a Hoefer SE-600 system (Hoefer Scientific Instruments, San Francisco, CA, USA) using sodium dodecyl sulfate-containing 4–16% (w/v) gradient polyacrylamide gels [53]. Since very large protein complexes did not properly enter the separating gel after chemical crosslinking, routine gel electrophoretic analysis of crosslinked proteins was carried out in resolving gels which lacked a stacking gel system. Titin, nebulin and myosin from rat myofibrillar preparations served as high molecular mass markers [47]. Electrophoretically separated proteins were transferred to nitrocellulose [54] using a TE-52X transfer unit from Hoefer Scientific Instruments (San Francisco, CA, USA). Blocking and incubation of nitrocellulose sheets was performed by established procedures [16,47] whereby primary and secondary antibodies were used at a dilution of 1:1000 and 1:3000, respectively. Immuno-reactive bands were visualized using the enhanced chemiluminescence kit from Boehringer Mannheim (Lewis, East Sussex, UK).

2.7. Immunofluorescence microscopy

Staining of 10 μm transverse cryosections from rabbit diaphragm and *gastrocnemius* muscle was performed with monoclonal antibody NCL-43 to β-dystroglycan and a polyclonal antibody to a peptide representing the last 15 amino acids of human skeletal muscle dystrophin [32]. Primary and secondary antibodies were used at a dilution of 1:100 and 1:150, respectively. Preparation, blocking, incubation and washing of cryosections, as well as photography was carried out by standard procedures as already described in detail elsewhere [35].

3. Results

3.1. Characterization of the dystrophin-associated glycoprotein β-dystroglycan

Since investigation of the trans-plasmalemmal linkage between dystrophin and laminin-α2 via the dystroglycan complex up to now has been mainly limited to skeletal muscle fibers, we examined the basic biochemical properties of β-dystroglycan in the diaphragm and the brain. Standard extraction procedures using non-ionic detergent or alkaline treatment confirmed that this dystrophin-associated glycoprotein exhibits properties of a typical integral membrane protein also in these two tissues. As illustrated in Fig. 1a, incubation with Triton X-100 solubilized β-dystroglycan from diaphragm microsomes, but alkaline extraction left it with the bilayer fraction. The same results were obtained with solubilization experiments using rabbit brain membrane preparations. Immuno-reactive bands representing the dystrophin-associated glycoprotein of 43 kDa were mostly detected in the detergent-extracted fraction and the synaptosomal pellets following alkaline treatment (Fig. 1b).

Extensive cellular localization studies of the dystrophin- and utrophin–glycoprotein complexes are mostly restricted to skeletal muscles. Thus prior to our crosslinking analysis of diaphragm membranes, we verified the plasmalemmal localization of β-dystroglycan in this tissue. Indirect immunofluorescence microscopy clearly revealed that the antigen recognized by monoclonal antibody NCL-43 is not only localized to the sarcolemma of *gastrocnemius* fibers (Fig. 2d), but is also almost exclusively found in the cell periphery of rabbit diaphragm (Fig. 2b). As already established for rabbit skeletal muscle, β-dystroglycan co-localized with the dystrophin of 427 kDa in the plasma membrane of transversely cut diaphragm cryosections (Fig. 2a,b). Surface staining

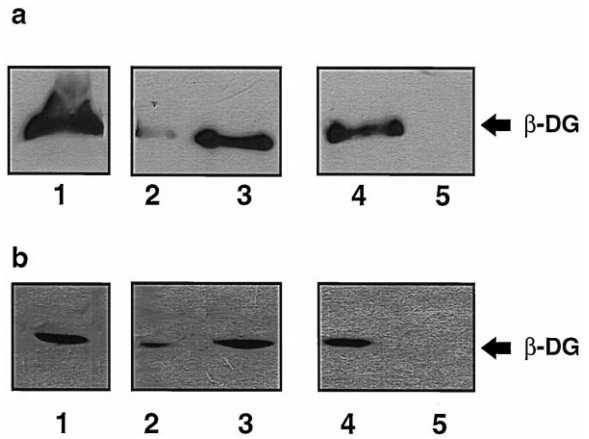

Fig. 1. Immunodetection of β-dystroglycan from rabbit diaphragm and brain following non-ionic detergent treatment or alkaline extraction. Shown are immunoblots of microsomal membranes isolated from rabbit diaphragm (a) and brain (b) homogenates stained with monoclonal antibody NCL-43 to β-dystroglycan. The majority of β-dystroglycan is found in the detergent-extracted supernatant fraction (lane 3) and the non-alkaline sensitive bilayer fraction (lane 4) as compared to the untreated microsomal fraction (lane 1), the detergent-insoluble pellet (lane 2) and the alkaline-extracted supernatant fraction (lane 5). This relative insolubility in alkaline solutions and easy extraction with non-ionic detergents confirms that β-dystroglycan behaves biochemically like a typical integral membrane protein, as predicted by its cDNA sequence [11]. Arrows indicate the position of the immunodecorated 43 kDa band of β-dystroglycan (β-DG).

of the diaphragm was specific, since control sections incubated with fluorescein-labeled secondary antibodies only, did not exhibit significant background staining (not shown). It has previously been shown that β-dystroglycan is associated with blood vessels in brain [34,44]. However, using monoclonal antibody NCL-43, we did not observe enough specific staining patterns in brain for proper interpretation of the subcellular localization of β-dystroglycan. Since immunofluorescence labeling of rabbit brain sections did not result in sufficient staining above background levels, this project was not further pursued.

3.2. Chemical crosslinking of β-dystroglycan from diaphragm, skeletal muscle and heart

The dystrophin–glycoprotein complex is of extremely large size and therefore our oligomerization studies had to take into account the potentially very limited electrophoretic mobility of chemically crosslinked membrane complexes. Initial studies showed that conventional gel systems employing a stacking gel did not reveal satisfactory results since dystrophin-associated components did not properly enter the resolving gel and/or were not sufficiently

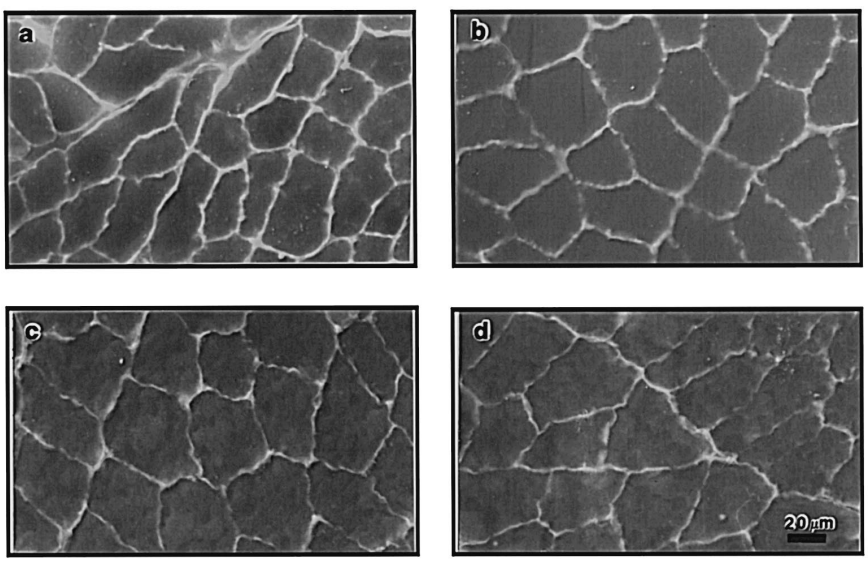

Fig. 2. Distribution of β-dystroglycan in rabbit diaphragm and skeletal muscle. Indirect immunofluorescence labeling of transverse cryosections from rabbit diaphragm (a, b) and *gastrocnemius* muscle (c, d) was performed with primary antibodies to dystrophin (a, c) and β-dystroglycan (b, d) and fluorescein-conjugated secondary antibodies. Both primary antibodies stained in both tissues almost exclusively the cell periphery, while control experiments with secondary antibody only did not result in plasmalemma labeling. Bar equals 20 μm.

transferred from the stacking gel system onto nitrocellulose. However, since the unequivocal identification of β-dystroglycan depended on immunoblotting of electrophoretically separated protein complexes, a gel system lacking a stacking gel was tried out and found to be superior in the detection of very high molecular mass complexes. While increasing amounts of crosslinker resulted, in both gel systems (not shown), in a clearly detectable decrease in the β-dystroglycan monomer of apparent 43 kDa, only the immunoblots from gels which lacked a stacking gel exhibited high molecular mass complexes of this glycoprotein (Fig. 3a–c). Since the lack of linearity between the relative electrophoretic mobility and the molecular mass of very large protein bands prevented a proper determination of the apparent size of the oligomeric complexes, the relative molecular mass of the crosslinked complexes could only be estimated to be approximately 2000 kDa. Diaphragm, skeletal muscle and heart microsomes exhibited a very comparable shift of β-dystroglycan to a much reduced electrophoretic mobility following crosslinking with a hydrophobic 12 Å crosslinker (Fig. 3a–c).

3.3. Chemical crosslinking analysis of β-dystroglycan, α-dystroglycan, dystrophin and laminin in diaphragm and skeletal muscle

Crosslinker to protein ratios were chosen so as to avoid artifacts of general protein clustering. At a concentration of 10 to 200 μg crosslinker per mg membrane protein no general shifts to higher molecular masses were observed. Coomassie-stained protein gels revealed only decreases in the relative electrophoretic mobility of distinct subsets of membrane proteins (not shown). Incubation of diaphragm membranes with 10 μg dithiobis-succinimidyl propionate per mg protein resulted in a dramatic decrease in the 43 kDa monomer of β-dystroglycan and in the concurrent appearance of a very high molecular mass protein band recognized by monoclonal antibody NCL-43 (Fig. 4a). Similar shifts to a greatly reduced electrophoretic mobility were observed for peripheral α-dystroglycan of 156 kDa, the basement component laminin and the membrane cytoskeletal protein dystrophin of 427 kDa (Fig. 4b–d). All four key components of the dystrophin–glycoprotein complex exhib-

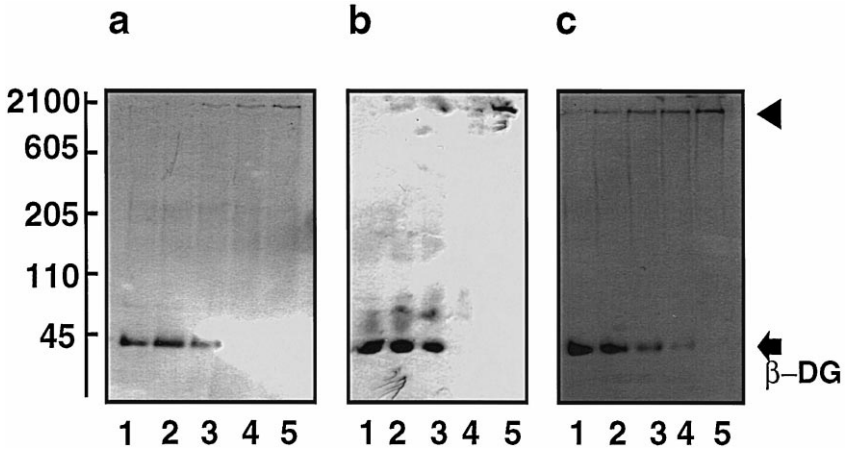

Fig. 3. Chemical crosslinking of β-dystroglycan in microsomes from rabbit diaphragm, skeletal muscle and heart. Shown are immunoblots of crosslinked microsomes from rabbit diaphragm (a), skeletal muscle (b) and heart (c) stained with monoclonal antibody NCL-43 to β-dystroglycan (β-DG). Lanes 1 to 5 represent 0, 10, 50, 100 and 200 μg of crosslinker per mg membrane protein, respectively. Nitrocellulose transfers were obtained from non-reducing 4–16% (w/v) gradient gels run without a stacking gel system. The arrow indicates the position of the 43 kDa β-dystroglycan (β-DG) monomer, while an arrow head marks the position of high molecular mass complexes following crosslinking. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

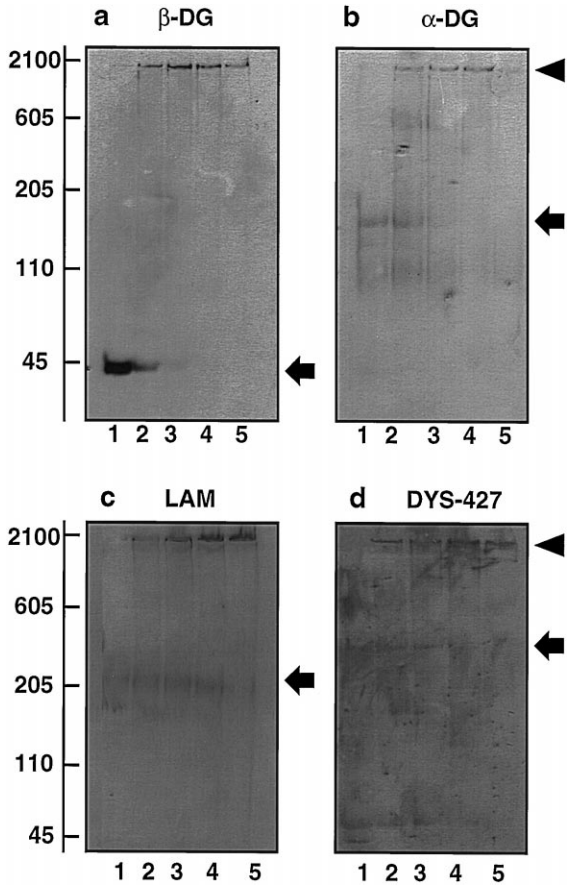

Fig. 4. Chemical crosslinking of the dystroglycan complex in microsomes from rabbit diaphragm. Shown are immunoblots of crosslinked microsomes from rabbit diaphragm stained with monoclonal antibodies NCL-43 to β-dystroglycan (β-DG) (a), VIA4$_1$ to α-dystroglycan (α-DG) (b) and XIXC2 to 427 kDa dystrophin (DYS-427) (d), as well as a polyclonal antibody to laminin (LAM) (c). Lanes 1 to 5 represent 0, 10, 50, 100 and 200 μg of crosslinker per mg membrane protein, respectively. Nitrocellulose transfers were obtained from non-reducing 4–16% (w/v) gradient gels run without a stacking gel system. Arrows indicate the position of monomers, while arrow heads mark the position of high molecular mass complexes following crosslinking. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

ited a distinct overlap in immunoreactivity. Immunoblotting with antibodies to adhalin (α-sarcoglycan), a member of the sarcoglycan subcomplex, did not result in strong enough labeling for proper interpretation of oligomerization following crosslinking (not shown). Increasing amounts of crosslinker caused no further decrease in electrophoretic mobility of the members of the dystrophin–glycoprotein complex studied (Fig. 4a–d). Monomers of α-dystroglycan, laminin and dystrophin were relatively weakly labeled by their respective antibodies, possibly due to their low abundance in the diaphragm membrane preparations. However, for an accurate crosslinking analysis it was necessary to perform our experiments with crude, non-salt washed microsomes so as to avoid potential artifacts introduced by excessive density gradient

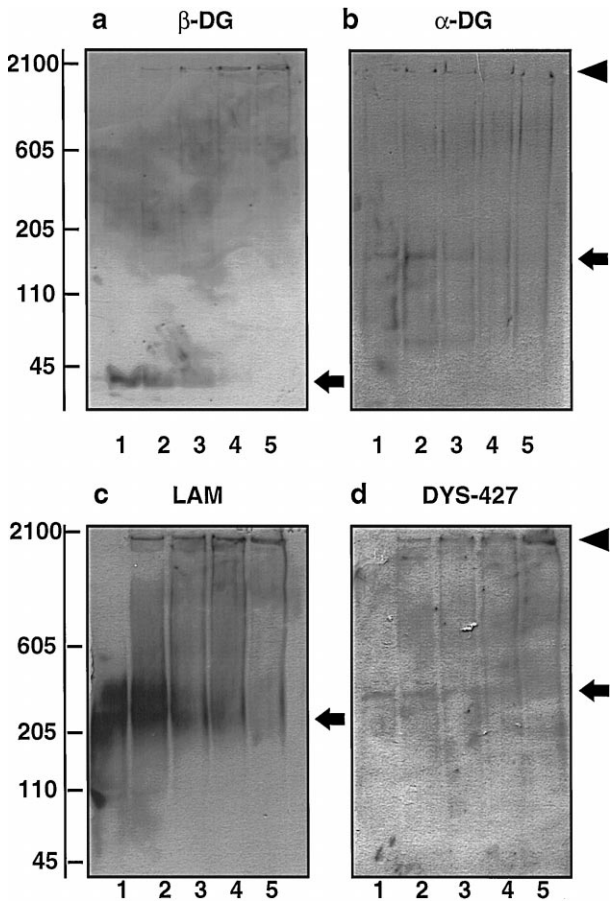

Fig. 5. Chemical crosslinking of the dystroglycan complex in microsomes from rabbit skeletal muscle. Shown are immunoblots of crosslinked microsomes from rabbit skeletal muscle stained with monoclonal antibodies NCL-43 to β-dystroglycan (β-DG) (a), VIA41 to α-dystroglycan (α-DG) (b) and XIXC2 to 427 kDa dystrophin (DYS-427) (d), as well as a polyclonal antibody to laminin (LAM) (c). Lanes 1 to 5 represent 0, 10, 50, 100 and 200 μg of crosslinker per mg membrane protein, respectively. Nitrocellulose transfers were obtained from non-reducing 4–16% (w/v) gradient gels run without a stacking gel system. Arrows indicate the position of monomers, while arrow heads mark the position of high molecular mass complexes following crosslinking. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

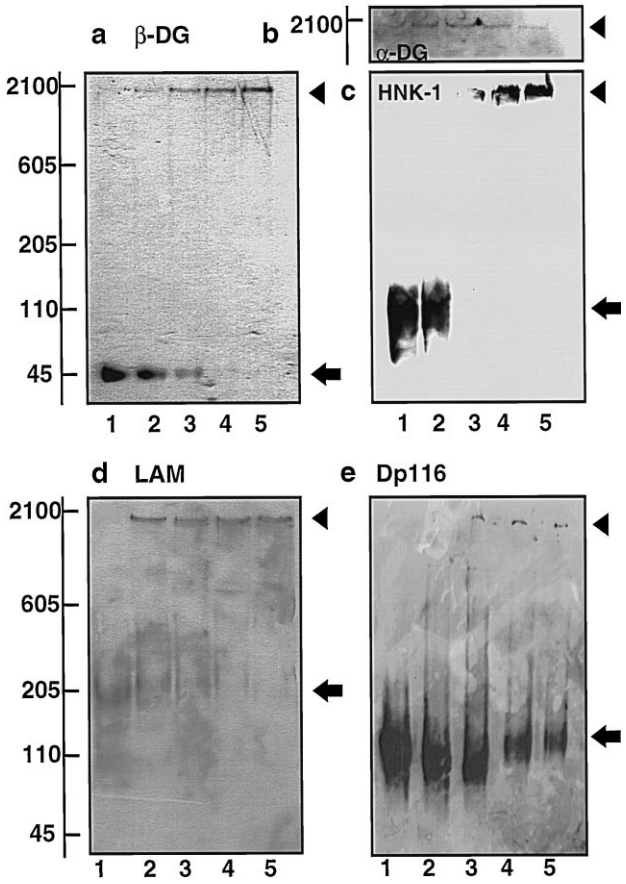

Fig. 6. Chemical crosslinking of the dystroglycan complex in microsomes from rabbit brain. Shown are immunoblots of crosslinked synaptosomes from rabbit brain stained with monoclonal antibodies NCL-43 to β-dystroglycan (β-DG) (a), VIA4$_1$ to α-dystroglycan (α-DG) (b) and VC1.1 to the HNK-1 antigen (HNK-1) (c), as well as polyclonal antibodies to laminin (LAM) (d) and 116 kDa brain dystrophin (Dp116) (e). Lanes 1 to 5 represent 0, 10, 50, 100 and 200 μg of crosslinker per mg membrane protein, respectively. Nitrocellulose transfers were obtained from non-reducing 4–16% (w/v) gradient gels run without a stacking gel system. Arrows indicate the position of monomers, while arrow heads mark the position of high molecular mass complexes following crosslinking. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

centrifugation or subcellular affinity purification. In skeletal muscle, very comparable results to those of the diaphragm were obtained. Chemical crosslinking caused a shift to higher relative molecular masses for all members of the dystrophin–glycoprotein complex analyzed (Fig. 5a–d). A definite overlap between immunoreactive bands representing β-dystroglycan, α-dystroglycan, laminin and dystrophin was established.

3.4. Chemical crosslinking analysis of β-dystroglycan, α-dystroglycan, Dp116 and laminin in brain

In synaptosomes, β-dystroglycan of 43 kDa exhibited a distinct shift to very high molecular mass complexes after chemical crosslinking. A decrease in monomer staining and an increase in labeling of oligomeric complexes was synchronous in appearance (Fig. 6a). Labeling of the 156 kDa monomer of α-dystroglycan was extremely weak (not shown), but a clear overlap of immuno-reactivity was observed for both dystroglycans following crosslinking (Fig. 6b). A shift of α-dystroglycan to higher molecular mass complexes following crosslinking was indicated by immunoblotting with a monoclonal antibody which recognizes the HNK-1 epitope (Fig. 6c). The fact that α-dystroglycan contains the HNK-1 epitope was recently established by Yamada et al. [55]. However, other proteins such as the neuronal cell adhesion molecule might also be recognized by this monoclonal antibody. Antibodies to laminin showed sufficient staining of monomers and oligomers of this basement membrane component and the oligomeric band exhibited an overlap with α- and β-dystroglycan (Fig. 6d). With respect to brain dystrophin isoforms, Dp116 appears to be of relatively high abundance in synaptosomal vesicles. Our affinity-purified antibody to the extreme COOH-terminus of dystrophin recognized this protein of apparent 116 kDa extremely well (Fig. 6e). Increasing amounts of chemical crosslinking of synaptosomes resulted in a marked decrease in the immunolabeling of the monomer band and concurrently produced the appearance of a high molecular mass oligomeric complex. The Dp116 band with decreased electrophoretic mobility showed a potential overlap with the immuno-reactive bands representing laminin and the dystroglycans (Fig. 6a–d). Immunoblotting of crosslinked brain membranes with antibodies to 427 kDa dystrophin and 395 kDa utrophin did not reveal satisfactory labeling (not shown) and the potential complex formation of this minor brain dystrophin isoform and its autosomal homologue with the dystroglycan proteins could thus not be investigated.

Results from control experiments as illustrated in Fig. 7 demonstrate the specificity of the immunological overlap between members of the dystrophin–glycoprotein complexes in skeletal muscle, di-

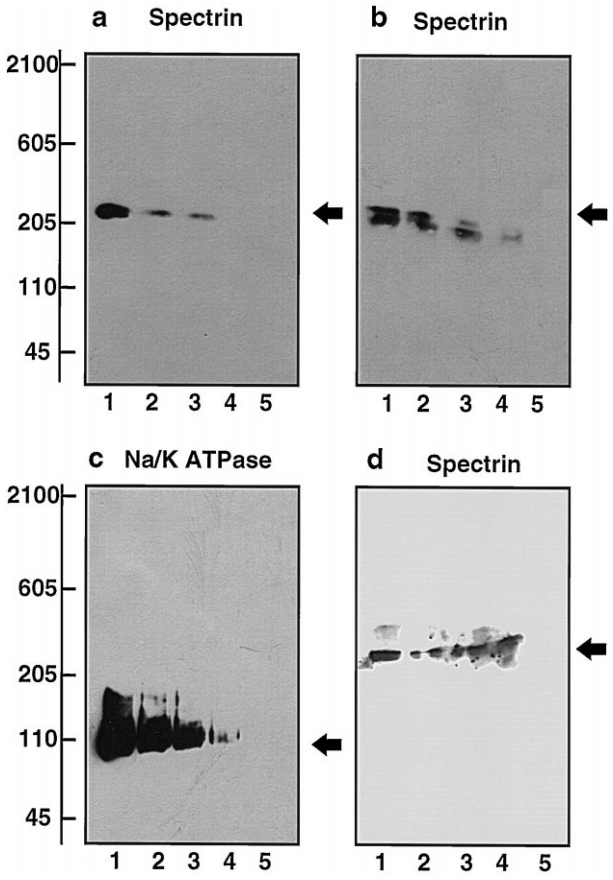

Fig. 7. Chemical crosslinking of microsomes from rabbit skeletal muscle, diaphragm and brain. Shown are control immunoblots of crosslinked membranes from rabbit skeletal muscle (a), diaphragm (b) and brain (c, d) stained with monoclonal antibodies SB-SP-1 to spectrin (a, b, d) and C464.6 to α_1-Na^+/K^+-ATPase (c). Lanes 1 to 5 represent 0, 10, 50, 100 and 200 μg of crosslinker per mg membrane protein, respectively. Nitrocellulose transfers were obtained from non-reducing 4–16% (w/v) gradient gels run without a stacking gel system. Arrows indicate the position of monomers. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

aphragm and brain following chemical crosslinking (Figs. 4–6). Immunodecoration of the 220 kDa monomer of the membrane cytoskeletal component spectrin decreases with increasing amounts of crosslinker used, but no high molecular mass band is detectable in the region of the crosslinked β-dystroglycan protein band. While the crosslinking-induced decrease in the relative abundance of spectrin monomers is very pronounced for skeletal muscle (Fig. 7a) and diaphragm (Fig. 7b) membranes, brain synaptosomes exhibit a much less prominent reduction in immunodecoration of the apparent 220 kDa band (Fig. 7d). Thus, brain spectrin does not appear to be extensively crosslinkable using 10 to 100 μg DSP per mg protein which clearly induces complex formation of dystrophin-associated glycoproteins (Fig. 6). The lack of high molecular mass bands containing spectrin could be due to the failure of very large spectrin complexes to be able to enter the gel system employed or possibly the epitope(s) recognized by monoclonal antibody SB-SP-1 were destroyed during the crosslinking process. The fact that the immunological overlap between dystrophin and dystrophin-associated glycoproteins is specific is furthermore demonstrated by previously published findings [47]. Crosslinking of rabbit skeletal muscle membranes, under the same conditions as employed here, does not result in an immunoreactive overlap between dystrophin-associated proteins and various muscle membrane proteins present in crude microsomal fractions including the surface dihydropyridine receptor. Complexes between ryanodine receptor tetramers and α_1-subunits of the transverse tubular dihydropyridine receptor are larger in relative molecular mass [47] than the dystrophin–glycoprotein complexes described here. With respect to brain membranes, staining of the α_1-subunit of the Na^+/K^+-ATPase showed a marked decrease of the 110 kDa monomer following chemical crosslinking using dithiobis-succinimidyl propionate (Fig. 7c). However, as already observed for spectrin, no appearance of a high molecular mass band containing this ubiquitous surface membrane marker was observed in the region of crosslinked β-dystroglycan (Fig. 7c). Therefore, the results presented in Figs. 4–6 strongly suggest that β-dystroglycan does not exist as a monomer in native membranes and that the immunological overlap with other members of the dystrophin–glycoprotein complex is a specific result of chemical crosslinking of surface membrane proteins which have a very close neighbourhood relationship.

4. Discussion

Although early symptoms of Duchenne muscular dystrophy include a decrease in strength of limb and torso muscles, the great majority of patients die of

respiratory and cardiac failure [26]. In addition, cognitive impairments may be the primary manifestation of the disease process preceding muscular weakness [26]. This lends central importance to studies into dystrophin-associated glycoproteins in diaphragm and brain with respect to understanding the overall pathogenesis of muscular dystrophies [8,9]. Considering the world-wide efforts to develop suitable therapeutic strategies for the treatment of inherited diseases such as muscular dystrophies [56], it is important to take into account the complex nature of the pathophysiological mechanisms occurring down-stream from the initial genetic defect(s). Efficient up-regulation of dystrophin and its isoforms by myoblast or gene transfer therapies in muscular dystrophy patients is still elusive [57,58]. For gene therapy to be successful the pathophysiological problems in tissues such as the diaphragm and nervous system have also to be addressed. In this respect, elucidation of the normal cellular functions of diaphragm and brain dystrophins and its associated glycoproteins might shed light on respiratory and neuronal abnormalities associated with certain neuromuscular disorders.

Based on biochemical studies and its cDNA sequence, skeletal muscle β-dystroglycan is predicted to be a glycosylated transmembrane protein [2,10–12,20]. Here, we could clearly show that diaphragm and brain β-dystroglycans also exhibit properties typical of integral membrane proteins. With respect to solubility in non-ionic detergent and resistance to alkaline extraction procedures, diaphragm and brain β-dystroglycan behave in an exactly opposite way to dystrophin [19], utrophin [52] and the dystrophin brain isoform Dp116 [32]. These findings agree with the current model of the dystrophin–glycoprotein complex [7–9] which assumes a membrane cytoskeletal function for dystrophin isoforms and a transplasmalemma spanning role for β-dystroglycan [10]. Binding of laminin-α2 by peripheral α-dystroglycan has been demonstrated by overlay assays, but highly purified preparations of the dystrophin–glycoprotein complex from skeletal muscle fibers are essentially devoid of the α2-chain-containing laminin-2 [2,20]. However, here we could show by chemical crosslinking of skeletal muscle microsomes that laminin-α2 and the dystroglycans exist in a very close neighborhood relationship. This confirms the prediction that 427 kDa dystrophin is an integral part of a surface membrane system that links the sub-sarcolemmal membrane cytoskeleton and the extracellular basement membrane [7–9]. Approximately the same size dystrophin-associated complexes were found in skeletal muscle and diaphragm preparations following crosslinking, indicating that very similar spatial configurations might be present in the two different tissues. Therefore dystrophin may have the same function in diaphragm as in skeletal and heart muscle and disruption of its proper organization in muscular dystrophy could be the initial step in the pathophysiological processes rendering all muscular tissues more susceptible to necrosis [8,9].

In contrast to skeletal muscle fibers, very little is known about the function of brain dystrophins and dystrophin-associated proteins and their fate during muscular dystrophies [27–34,42–45]. The crosslinking analysis presented here indicates that in synaptosomes both α-dystroglycan of 156 kDa and β-dystroglycan of 43 kDa form a complex. In analogy to the muscle dystrophin–glycoprotein complex, laminin was also found to exist in a close neighbourhood relationship to the dystroglycans. It is unclear how and with which brain laminin isoform α-dystroglycan and thus indirectly β-dystroglycan may interact in synaptosomes. Since Tian et al. [44] could show that brain α-dystroglycan and α2-chain-containing laminin-2 can interact, we presume that both dystroglycans and the laminin-2 isoform are integral parts of this proposed brain complex. However, studies by Mummery et al. [42] indicate that β-dystroglycan, although present in synaptic membranes, is not detectable in the postsynaptic density fraction of rat forebrain. This would exclude a major link between 427 kDa brain dystrophin and β-dystroglycan in the postsynaptic density. On the other hand, little is known about the subcellular localisation of the other brain dystrophin isoform, Dp116, which was investigated in this report. Dp116 might have a broader synaptic distribution than the less abundant 427 kDa dystrophin.

Since immuno-decoration for Dp116 overlapped with that of the dystroglycans, these components might form a complex within a synaptic region other than the postsynaptic density. However, currently, it cannot be concluded that β-dystroglycan, α-dystroglycan, Dp116 and utrophin are all closely linked at only one specific subcellular region in brain tis-

sues. It is however clear that Dp116, since it is lacking the N-terminal actin-binding domain of 427 kDa skeletal muscle dystrophin [6], cannot be involved in anchoring a cortical actin membrane cytoskeleton to the brain cell surface. A potential indirect connection of Dp116 with the extracellular matrix of brain cells could have a similar function as proposed for the muscular dystrophin–glycoprotein complex and the neuromuscular utrophin–glycoprotein complex, namely stabilization of the surface membrane and/or anchoring of peripheral receptor molecules [8,9]. Our crosslinking studies of synaptosomes isolated from brain tissue is in agreement with reports by Yamada et al. [55,59] who described a complex formation of Dp116 in peripheral nerve using laminin affinity chromatography. Thus, Dp116 appears to form a linkage with some of the glycoproteins originally described in skeletal muscle both in the central and peripheral nervous system. Recently, it was suggested that this interaction between α-dystroglycan and laminin is at least partially mediated by complex carbohydrate moities on dystroglycan [60].

The overall spatial configuration of dystrophin–glycoprotein complexes, based on extensive studies into the structure of dystrophin and its many associated surface components [6–10], is confirmed by the chemical crosslinking data as presented in this report. In the periphery of fibers from skeletal muscle, heart and diaphragm, the plasmalemma-spanning dystroglycan complex is in close contact with sarcoglycans, dystrophin and the cortical actin membrane cytoskeleton, as well as laminin-α2. Besides dystrophin, a major cytoskeletal anchor in the neuromuscular junction appears to be utrophin which exists also in close association with the same set of surface glycoproteins [61]. In contrast, sarcoglycans do not appear to be present in brain tissues [41] and possibly the major brain dystrophin isoform Dp116 is an integral part of a surface membrane complex including laminin-α2 and the two dystroglycans. Since diaphragm dystrophin of 427 kDa is missing in muscular dystrophy patients, a disruption of the dystroglycan-complex might destabilize the diaphragm fiber periphery and cause an impaired respiratory function. Whether abnormalities in various brain dystrophin isoforms play a direct or indirect role in cognitive impairment of Duchenne muscular dystrophy patients and which dystrophins and dystrophin-associated proteins are complexed at which synaptic regions remains to be determined.

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