SEVERAL DYSTROPHIN-GLYCOPROTEIN COMPLEX MEMBERS ARE PRESENT IN CRUDE SURFACE MEMBRANES BUT THEY ARE SODIUM DODECYL SULPHATE INVISIBLE IN KCl-WASHED MICROSOMES FROM mdx MOUSE MUSCLE

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Abstract: The dystrophin-glycoprotein complex (DGC) is a large trans-sarcolemmal complex that provides a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. In skeletal muscle, it consists of the dystroglycan, sarcoglycan and cytoplasmic complexes, with dystrophin forming the core protein. The DGC has been described as being absent or greatly reduced in dystrophin-deficient muscles, and this lack is considered to be involved in the dystrophic phenotype. Such a decrease in the DGC content was observed in dystrophin-deficient muscle from humans with muscular dystrophy and in mice with X-linked muscular dystrophy (mdx mice). These deficits were observed in total muscle homogenates and in partially membrane-purified muscle fractions, the so-called KCl-washed microsomes. Here, we report that most of the proteins of the DGC are actually present at normal levels in the mdx mouse muscle plasma membrane. The proteins are detected in dystrophic animal muscles when the immunoblot assay is performed with crude surface membrane fractions instead of the usually employed KCl-washed microsomes. We propose that these proteins form SDS-insoluble membrane complexes when dystrophin is absent.

Key words: Dystrophin, Muscular dystrophy, Dystrophin-glycoprotein complex, mdx mice, SDS insoluble

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Abbreviations used: AU – arbitrary unit; CSM – crude surface membranes; DGC – dystrophin-glycoprotein complex; DMD – Duchenne muscular dystrophy; KCl-Ms – KCl-washed microsomes; mdx – X-linked muscular dystrophy; SDS-PAGE – SDS-polyacrylamide gel electrophoresis
INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive and progressive muscle-wasting disease caused by genetic defects in the dystrophin gene leading to an absence of the protein. Dystrophin is a 427-kDa cytoskeletal protein located on the inner surface of the muscle fibres [1]. In skeletal and cardiac muscle, dystrophin is associated with a large complex of sarcolemmal and cytoskeletal proteins [2-4] which spans the sarcolemma. Together, dystrophin and the dystrophin-glycoprotein complex (DGC) provide a linkage between the extracellular matrix and the actin-based cytoskeleton. The DGC consists of three separate sub-complexes based on their locations within the cell and their physical associations with each other. These are the dystroglycan, sarcoglycan/sarcospan and cytoplasmic sub-complexes [5].

The dystroglycan sub-complex is composed of α- and β-dystroglycan, which are ubiquitously expressed in various tissues. Both are transcribed into a single protein that is subsequently cleaved into two associated proteins [6, 7]. α-dystroglycan is an extracellular protein that binds to extracellular proteins such as laminin and agrin as well as to β-dystroglycan [8]. β-dystroglycan is a transmembrane protein which binds to α-dystroglycan outside the cell, and which interacts inside the cell with several proteins such as dystrophin, dystrophin-related proteins, caveolin-3 and Grb2 [9-12]. In addition, β-dystroglycan binds to transmembrane sarcoglycans [13].

The sarcoglycan/sarcospan sub-complex consists of six transmembrane proteins, namely the α-, β-, γ-, δ-, ε-sarcoglycans and sarcospan. The sarcoglycans are all N-glycosylated transmembrane proteins with a short intracellular domain, a single transmembrane region and a large extracellular domain. Sarcospan is a protein with four transmembrane regions, and it belongs to the tetraspan family [14]. The sarcoglycan/sarcospan sub-complex interacts with the N-terminal region of α-dystrobrevin [15].

The cytoplasmic DGC complex contains syntrophins and dystrobrevins. The syntrophins are modular adaptor proteins. Among the α-, β1-, β2- and γ2- isoforms [16, 17], α-syntrophin is the adult mouse skeletal muscle major isoform. The syntrophins interact with dystrophin, utrophin or dystrobrevins [16], and with several other proteins like ErbB4, Na-channels and nNOS [18]. The dystrobrevins are encoded by two α and β separate genes and are involved in the signalling function of the DGC [19, 20].

The DGC is thought to contribute to the structural stability of the muscle cell membrane during cycles of contraction and relaxation, thereby protecting the muscle from stress-induced membrane damage [21, 22]. In DMD patients and animals displaying muscular dystrophy associated with dystrophin deficiency, such as mdx (X-linked muscular dystrophy) mice, it is currently considered that the absence of dystrophin leads to a drastic reduction in the levels of all the dystrophin-associated proteins in the sarcolemma, thus causing a disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular
matrix [3, 23-25]. This is presumed to cause sarcolemmal instability, which, in turn, may render muscle fibres susceptible to necrosis. That is why the efficacy of dystrophin gene therapy depends not only on replacing dystrophin but also on restoring all of the DGC in the sarcolemma of DMD patients. However, it is not possible to distinguish any difference in the expression levels of dystroglycan and sarcoglycan mRNA between normal and mdx mice [6, 26, 27]. Moreover, in a previous study, we showed that β-dystroglycan is targeted onto the plasma membrane in similar amounts in both normal and mdx mouse muscle: it was detected in crude surface membranes instead of microsomes by routine methods [28].

In this study, we investigate the status of DGC in the skeletal muscle of mdx mice to ascertain whether or not the dystrophin-associated proteins are present in the crude surface membranes (CSM) as shown for β-dystroglycan [28]. Here, we demonstrate that all of the studied dystrophin-associated proteins are actually present and detectable at high levels in the crude surface membranes from mdx skeletal muscle, complementing our previous finding that β-dystroglycan can be detected in mdx mouse muscle. This result suggests that the DGC is synthesized at a normal level and targeted onto the plasma membrane in dystrophin-deficient mouse muscle. However, the organisation of the DGC renders them SDS-invisible in KCl-washed microsomes and SDS-visible in crude surface membranes.

**MATERIALS AND METHODS**

**Animals**

Male normal (C57BL/10Sc/Scn) and mdx (C57BL/10Sc/Scn/mdx) mice were bred at the Ecole Nationale Vétérinaire in Nantes. They were given free access to water and standard laboratory chow. All the experiments complied with the French legislation and guidelines for animal experimentation. We have the official authorizations for work on laboratory animals from the French Agriculture Ministry. Thirty animals per series, mdx or normal, were used for these experiments.

**Antibodies**

Mouse anti-Na/K-ATPase was obtained from USABiomax (Euromedex, Mundolsheim, France). Mouse anti-β-dystroglycan (NCL-b-DG), anti-β-sarcoglycan (NCL-b-SARC), anti-γ-sarcoglycan (NCL-g-SARC) and anti-dystrophin (NCL-DYS1 and NCL-DYS2) were supplied by Novocastra Laboratories (Tebu-Bio SA, Le Perray en Yvelines, France). Mouse anti-α-dystrobrevin was purchased from BD Biosciences Laboratories (Le Pont de Claix, France), mouse anti-α-syntrophin (ab11425) from Abcam (Paris, France), and mouse anti-calsequestrin (VIIID12) from Affinity BioReagents (Ozyme, Saint Quentin Yvelines, France). Rabbit anti-α-sarcoglycan (R607) and anti-δ-sarcoglycan (δ-Sarc/12C1) were provided by S.J. Winder (Department of Biomedical
Science, Sheffield, UK). Rabbit anti-ε-sarcoglycan was a gift from J.R. Sanes (Harvard University, Molecular and Cellular Biology, Cambridge, MA, USA). Alkaline phosphatase conjugated anti-mouse and anti-rabbit secondary antibodies were from Sigma-Aldrich (Lyon, France).

**Isolation of crude surface membranes**

Normal and *mdx* mice (16-20 weeks old) were euthanized by intraperitoneal pentobarbital injection, and whole hind-leg muscle samples were taken. KCl-washed microsomes (KCl-Ms) and CSM were isolated by velocity sedimentation at 4°C as described previously [28, 29]. Briefly (see Fig. 1), the muscle was

![Fig. 1. Skeletal muscle fractionation: the purification protocol. Buffer A is composed of 20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM MgCl₂, 0.303 M sucrose and 0.5 mM EDTA, at pH 7.0, with protease inhibitors. Buffer B is composed of 0.303 M sucrose and 20 mM Tris-maleate, at pH 7.0.](image-url)
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homogenized in buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM MgCl₂, 0.303 M sucrose, 0.5 mM Ethylene-diamine-tetra-acetic acid (EDTA), at pH 7.0, with protease inhibitors). The homogenate was centrifuged for 25 min at 14000 g. The pellet was resuspended in buffer A and centrifuged as described in the previous step. The supernatants were combined and subjected to centrifugation for 40 min at 17000 g. The supernatant was incubated with KCl for 30 min at 4°C to obtain a final concentration of 0.6 M KCl for the suspension. After centrifugation for 35 min at 140000 g, the pellet was resuspended in buffer B (0.303 M sucrose, 20 mM Tris-maleate, at pH 7.0, with protease inhibitors) and centrifuged again for 35 min at 140000 g to obtain the KCl-M fraction. The KCl-Ms were resuspended in buffer B containing 0.6 M KCl, loaded onto 0.878 M sucrose, 0.6 M KCl, 20 mM Tris-Maleate, at pH 7, and centrifuged at 104000 g for 17 h. CSM were collected above the 0.303 M / 0.878 M sucrose interface, and three other fractions were obtained above the interface of the two sucrose concentrations (F2), below the interface (F3) and at the bottom as the pellet fraction (F4). The CSM (F1) was further concentrated as a pellet by a final centrifugation for 4 h at 104000 g, and the supernatant was recovered (F5). The proteins were quantified with a colorimetric assay (Bradford Reagent, Bio-Rad, Marne La Coquette, France).

To quantify the total recovery of the gradient, volumes containing 200 μg of protein from each fraction (F1 to F5) were precipitated with 10% trichloroacetic acid. After 30 min incubation at 4°C, the samples were centrifuged at 20000 g for 15 min at 4°C. The protein pellets were washed twice with cold acetone and centrifuged under the same conditions as described above. The final pellets were dried and subjected to SDS-PAGE analysis.

SDS-PAGE and immunoblotting
SDS-PAGE was performed on polyacrylamide gels according to Laemmli [30], with proteins being transferred to nitrocellulose [31]. In brief, 40 μg aliquots of protein were boiled in 2%-SDS buffer with 5% β-mercaptoethanol, separated by SDS-PAGE, and electrophoretically transferred from gel to nitrocellulose filters at 4°C. The efficiency of transfer was evaluated by Ponceau-S-Red staining of membranes, following by destaining in Tris-Buffered Saline (TBS: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) / 0.01% Tween. The filters were blocked with 5% nonfat dry milk / 0.01% Tween 20 in TBS, and then incubated overnight with primary antibody appropriately diluted in blocking buffer. The blots were subsequently washed twice for 15 min in blocking solution and then incubated for 1 h with appropriate alkaline phosphatase-conjugated secondary antibodies. After being washed 3 times for 10 min with TBS, the immunoblots were developed using a chemifluorescent substrate for alkaline phosphatase (ECF, Amersham Biosciences, Orsay, France), and the chemifluorescence emission was directly measured by fluorescence scanning (Storm Instrument, Amersham, and Image-Quant 5.2). The values are expressed as arbitrary units (AU) corresponding to the protein-specific signal corrected for the background (the value used for the
background is the average of all the pixel values in the object outline). The linearity between the chemifluorescence levels and protein contents was first tested. The potential level variations were compared in the same gel with the arbitrary unit reference value of 100 for the KCl-M chemifluorescence.

Statistical analysis
For each protein studied, we performed three independent muscle fractionations for each genotype and three corresponding western blot analyses, with each membrane carrying all the compared fractions. The data is expressed as the mean ± SEM. We used the Mann-Whitney U test to evaluate differences in the labelling levels between the fractions. A p value of less than 0.05 was considered statistically significant.

RESULTS

Muscle fractionation
Before characterizing the DGC, we extensively checked the method of plasma membrane purification, as derived from the work of Ohlendieck and Campbell [2], as regards the yield and composition of marker proteins in the fractions previously described [28]. To confirm successful fractionation, equal amounts of KCl-Ms and CSM, from normal and dystrophin-deficient animals, were analysed by immunoblot assay using antibodies against calsequestrin, a marker of the sarcoplasmic reticulum, and Na/K-ATPase, an established plasma-membrane marker (Fig. 2). In the two animal lines, calsequestrin labelling was strong in the KCl-Ms and markedly reduced in the CSM. By contrast, the CSM was enriched in Na/K-ATPase compared to the KCl-Ms. These results show that the KCl-Ms comprise sarcoplasmic reticulum and plasma membranes, while the CSM fraction appears to be mainly devoid of sarcoplasmic membranes and largely enriched in plasma membranes. On the other hand, comparable levels of calsequestrin expression are found in normal and mdx muscle, both in the KCl-Ms and in CSM fractions (Fig. 2). This indicates that the membrane preparations are similarly affected whether they come from dystrophin-deficient or normal muscle. As illustrated in Fig. 2, the dystrophin isoform Dp427 (detected with two antibodies, NCL-DYS1 and NCL-DYS2, which respectively recognize the dystrophin rod domain and C-terminal region) is not present in KCl-Ms and CSM isolated from mdx muscle homogenates, as expected. This data shows that the fractionation method does not disturb the marker protein composition of dystrophic compared to normal fractions, thus verifying the high degree of purity of the sarcolemmal preparations used in this study. Moreover, equal amounts of sarcolemmal proteins are present in the different membrane preparations, as shown by Ponceau red staining (Fig. 2).
Fig. 2. Detection of subcellular fractionation procedure markers. Nitrocellulose transfers of SDS-PAGE are shown, containing 40 µg of KCl-washed microsomes (1, 3) or crude surface membranes (2, 4) from normal or dystrophic (mdx) mice, stained with the antibodies against calsequestrin, Na/K-ATPase and dystrophin (DYS1 and DYS2 antibodies respectively recognizing the rod domain and C-Terminal region). The Ponceau red staining of membranes, shown at the bottom, reveals a comparable overall protein composition of the normal and dystrophic fractions for KCl-Ms and CSM.

Gradient recovery
Five fractions (F1 to F5) were recovered after loading KCl-Ms onto a sucrose density gradient, and their volumes and protein contents were measured. Two hundred micrograms of proteins from each of the five fractions were precipitated using trichloroacetic acid, and SDS-PAGE was carried out followed by western blotting with β-dystroglycan antibody to quantify the contents of the five fractions (Tabs 1 and 2).

About 30 mg of protein in 1 ml were loaded onto the sucrose density gradient, using preparations from normal and mdx mouse muscle. The whole amount of protein loaded was recovered in the five fractions of the gradient (Tab. 1). The pellet F4 contained 58-75% of the total protein content in the two muscle preparations. Surprisingly, the protein content of the CSM (fraction F1) was twice as high in mdx mouse muscle as in normal muscle.

Firstly, from Fig. 3 and Tab. 2, it is evident from the sum of the contents of the five fractions that there was a total recovery of the total β-dystroglycan content of the normal muscle KCl-Ms loaded onto the sucrose density gradient. By contrast, the total β-dystroglycan content obtained by summing the five fraction
contents from *mdx* mouse muscle was three times higher than the content of the corresponding KCl-M protein loaded on the gradient. This clearly indicates that 2/3 of the β-dystroglycan content of the KCl-Ms from *mdx* mouse muscle is not detected by classic SDS-PAGE and western-blot, in contrast to normal muscle KCl-Ms. This also implies that during application of the sucrose density gradient, a high amount of β-dystroglycan from the *mdx* mouse muscle becomes accessible to SDS in an as-yet unexplained process. This validates and further supports our previous results.

Tab. 1. Gradient recovery data. Total volumes and protein contents of the KCl-Ms and of the five fractions obtained after applying a sucrose density gradient to KCl-Ms from normal and *mdx* mouse muscle.

| Fractions | Normal mouse muscle | mdx mouse muscle |
|-----------|---------------------|------------------|
| KCl-Ms    | Volume (ml) 1       | Protein (mg) 26   | Volume (ml) 1 | Protein (mg) 29 |
| F1, CSM   | 0.21              | 2.00 ± 0.02      | 0.35          | 4.00 ± 0.30     |
| F2        | 0.50              | 1.25 ± 0.25      | 0.50          | 1.10 ± 0.10     |
| F3        | 30.00             | 4.50 ± 0.35      | 29.00         | 7.50 ± 0.35     |
| F4        | 0.90              | 20.00 ± 0.50     | 0.75          | 17.00 ± 0.77    |
| F5        | 20.00             | -                | 20.00         | -               |
| Total F1 to F5 | ~ 27 mg | ~ 29 mg |

Tab. 2. β-dystroglycan recovery over the gradient. The five fractions obtained after the gradient were precipitated with trichloroacetic acid, and then SDS-PAGE and western-blot analysis were performed for β-dystroglycan. The β-dystroglycan level quantified by chemifluorescence is reported for the total volume of the different fractions.

| Fractions | Normal mouse muscle | mdx mouse muscle |
|-----------|---------------------|------------------|
| KCl-Ms    | β-dystroglycan Total | 43-kDa | 30-kDa | β-dystroglycan Total | 43-kDa | 30-kDa |
| F1, CSM   | 65                  | 50      | 15     | 170              | 75     | 95    |
| F2        | 20                  | 16      | 4      | 20               | 10     | 10    |
| F3        | 110                 | 95      | 15     | 200              | 115    | 85    |
| F4        | 270                 | 260     | 10     | 90               | 72     | 18    |
| F5        | -                   | -       | -      | -                | -      | -     |
| Total F1 to F5 | 465 | 421 | 44 | 480 | 272 | 208 |

*The calculations are as follows: (fluorescence of the western blot bands / protein content of the loaded sample) x total protein content of the fraction.
Fig. 3. Immunoblotting of β-dystroglycan in the KCl-Ms and four fractions from the sucrose density gradient of these KCl-Ms from normal and mdx mouse muscle. Forty micrograms of TCA-precipitated proteins from each fraction were loaded on an SDS-PAGE and blotted. β-dystroglycan immunoblots were developed using a chemifluorescent substrate for alkaline phosphatase, and the chemifluorescence emission was directly measured on the blots. The values are shown in Tab. 2.

Secondly, it appears that the two β-dystroglycan species (43 and 30 kDa) show different distributions in the mdx and normal mouse muscle gradient fractions. The 30-kDa band accounted for about 10% of the total content in normal mouse muscle, while this percentage rose to about 43% in the mdx mouse muscle total fractions. The two β-dystroglycan species yielded dissimilar distributions in the different fractions obtained after the gradient: a higher percentage of the 30-kDa β-dystroglycan band was recovered in the CSM (F1) in normal (23%) and mdx (56%) mouse muscle fractions compared to the 43-kDa β-dystroglycan band, while lower percentages were observed in the gradient pellet (F4), which was mainly composed of sarcoplasmic reticulum membranes (3.7 and 20% of β-dystroglycan 30-kDa compared to the β-dystroglycan 43-kDa in F4 from normal and mdx mouse muscle, respectively). This suggests that the β-dystroglycan 30-kDa band is preferentially present in the lightest fractions of the sucrose density gradient. Interestingly, the β-dystroglycan 30-kDa band from normal mouse muscle behaves as if it was obtained from an mdx muscle in the sense that the amount detected in the KCl-M fraction (22 AU) is lower than the total amount detected in F1 to F5 (44 AU).

However, the fact that β-dystroglycan was recovered in great amounts in F3 and F4 does not mean that the protein could be associated with other cellular structures than the sarcolemma. As shown earlier, during the procedure used to obtain sarcolemmal fractions, vesicles can be trapped in each other, i.e. sarcoplasmic reticulum vesicles can trap sarcolemma vesicles and vice versa. Therefore, the isolation procedure essentially removes excess sarcoplasmic reticulum vesicles from the CSM [29], and the F4 fraction contains an excess of sarcoplasmic reticulum vesicles compared to the CSM. We previously showed that the F4 fraction from mdx mouse muscle already contains utrophin. In addition, there is a difference in the distribution of β-dystroglycan when comparing the
fractions F3 and F4 from normal and mdx mouse muscles. However, a sucrose density gradient separates the different membrane vesicles in function of their protein/lipid ratio and their sizes. It is likely that the dystrophin deficiency could modify the sedimentation coefficient of the membranes from mdx mice, and this would account for the modified distribution and for the fact that the CSM were in all cases recovered in higher amounts in the mdx compared to the normal KCl-Ms.

**DGC proteins in KCL-Ms and CSM from normal and mdx mouse muscle**

To quantify the dystrophin-associated protein levels in dystrophin-deficient versus normal skeletal muscle membranes, we performed chemiluminescence analyses of immunoblots from KCl-M and CSM fractions from normal and mdx mice. In the normal mouse muscle KCl-Ms, immunoblot labelling with antibodies against DGC proteins demonstrated the presence of β-dystroglycan, the α-, β-, γ-, δ- and ε-sarcoglycans (Fig. 4A) and α-dystrobrevin and α-syntrophin (Fig. 4B) at their respective expected sizes. For δ-sarcoglycan (Fig. 4A), a close doublet of bands was detected at approximately 35 kDa, corresponding to alternatively spliced products. Dystrobrevin labelling (Fig. 4B) revealed 3 major bands (87, 59 and 35 kDa) corresponding to the three major mouse α-dystrobrevin isoforms (types 1, 2 and 3). For the other proteins, we found a unique band at the expected size.

The labelling of these DGC proteins was strongly and significantly enhanced in the CSM compared to KCl-Ms (Fig. 4A and B), as shown in the histograms reporting the chemiluminescence quantitative values of the western-blots for each protein. For β-dystroglycan, a second band that is absent from the KCl-Ms appeared at 30 kDa in the CSM. These results confirm that the muscle fractionation process induces a concentration of sarcolemmal proteins in the CSM fraction.

In the KCl-Ms of mdx mouse muscle, the DGC proteins appeared greatly reduced compared to the KCl-Ms in normal mouse muscle (Fig. 4A and B), as previously reported in numerous studies. However, in mdx mouse muscle CSM, most of the DGC proteins exhibited similar or higher contents compared with the values observed in normal mouse muscle CSM (Fig. 4A and B). Fig. 4A and B show a representative assay of this protein label across three similar assays. In particular, the β-dystroglycan 43-kDa band was detected at similar levels in normal and mdx CSM, while the 30-kDa band was highly enriched specifically in CSM extracted from mdx mice (Fig. 4A). Therefore, the total content of β-dystroglycan was higher in mdx than in normal mouse muscle CSM. The α-, β-, γ-, δ- and ε-sarcoglycans were all at a similar level in mdx compared to normal mouse muscle CSM. The 87-kDa α-dystrobrevin-1 isoform was slightly depleted in mdx compared to normal mouse muscle CSM, while the 59- and 35-kDa α-dystrobrevin-2 and -3 isoforms were severely depleted in CSM from mdx mice compared to those from normal mice (Fig. 4B).
Fig. 4. Immunoblotting of DGC proteins in KCl-Ms and CSM from normal and mdx mouse muscle. Immunoblots of the β-dystroglycan and sarcoglycan complex proteins (A) and cytoplasmic complex (B) are presented. Representative nitrocellulose transfers of SDS-PAGE are shown, containing 40 µg of KCl-Ms or CSM from normal or dystrophic (mdx) mice, stained with antibodies against β-dystroglycan, α-, β-, γ-, δ- and ε-sarcoglycan (A), and α-dystrobrevin and α-syntrophin (B). The protein amounts are presented as histograms showing the chemifluorescence measured on the blots. The results are expressed as arbitrary units, with a reference value being assigned to KCl-washed Microsomes (KCl-Ms) from normal mice. Each value is the mean ± SEM from three independent series of subcellular fractionation and immunoblot assays from three identical batches of mice. *significant difference between KCl-Ms and CSM in the same group and **significant difference between the groups for the same fraction, (p < 0.05) according to the Mann-Whitney U test procedure.
In summary, our results show that the KCl-Ms of mdx mouse muscle exhibit a dramatic decrease in DGC proteins compared to normal mouse muscle, as previously reported in numerous studies. However, we show that, in contrast to the KCl-Ms, such a dramatic depletion of the DGC proteins is not observed in the CSM from mdx mouse muscle compared to normal mouse muscle, but rather that these proteins display very similar levels in both types of CSM fraction. The increase in the level of DGC protein in CSM as against KCl-Ms in normal mouse muscle occurs during sarcolemmal purification via the fractionation procedure. However, this purification cannot explain the large variation in protein levels that was observed, ranging from very low contents in KCl-Ms to high contents in CSM from mdx mouse muscle.

DISCUSSION

Our previous study showed that β-dystroglycan is actually present at high levels in the skeletal muscle plasma membrane of mdx mice [28], although this was not detected by the classical immunoblot analysis of the microsomes. The aim of this study was to discover whether this is the general rule for all the DGC proteins. For this purpose, we carried out sub-cellular fractionation of normal and dystrophin-deficient muscles to obtain so-called KCl-washed microsomes (KCl-Ms) and crude surface membranes (CSM) after a final sucrose density gradient centrifugation of the KCl-Ms. Both normal and dystrophin-deficient mdx mouse muscles were subjected to this fractionation procedure.

Many studies report a dramatic decrease in the levels of DGC proteins in dystrophin-deficient muscle homogenates and KCl-Ms [3, 23-25], and the reappearance of the DGC proteins has been used as a significant indicator for quantifying phenotype rescue after therapy strategies performed on dystrophin-deficient animals [32-40]. As in all those studies, we showed that β-dystroglycan, taken as a marker of the DGC proteins, is greatly depleted in KCl-Ms from mdx mouse muscle compared to those from normal mouse muscle. However, we know that the KCl-Ms represent a very heterogeneous mixing of intracellular and sarcolemma membranes, and our current results concur with this point. Therefore, we report here on an attempt to quantify precisely the depletion of the DGC proteins in dystrophin-deficient muscle, based on an estimation of the contents of these proteins in a muscle fraction highly purified in sarcolemma membranes. In this study, we subjected the KCl-Ms to a sucrose density gradient centrifugation to obtain a crude sarcolemma membrane fraction (CSM) highly enriched in specific sarcolemmal proteins such as dystrophin, β-dystroglycan and Na⁺/K⁺-ATPase. In our previous study [28], we showed that the CSM fraction was not dramatically depleted in β-dystroglycan when obtained from mdx mouse muscle. However, we also found that this protein was present at a level similar to that seen in CSM from normal mouse muscle. Here, we showed that: i) the gradient recovery of KCl-Ms in mdx mouse muscle indicates that the sum of the β-dystroglycan levels in the different fractions of
the gradient is three times higher than the level of KCl-M β-dystroglycan loaded onto the gradient; and ii) this increase in the β-dystroglycan level in mdx mouse muscle CSM is the general rule for all members of the DGC.

The gradient recovery is obtained by studying the β-dystroglycan levels in the different fractions obtained after sucrose density gradient centrifugation of the KCl-Ms. In the case of normal muscle, the sum of the β-dystroglycan levels in the fractions after the gradient is equal to the β-dystroglycan content in the KCl-Ms that were loaded onto the gradient, as expected from such an experiment. However, in the case of mdx mouse muscle, the sum of the β-dystroglycan levels in the fractions after the gradient is three times higher than the content of the KCl-Ms loaded onto the gradient, which is a very unusual finding. This result implies that, in accordance with our previous observations, a large proportion of the β-dystroglycan (~2/3) is not visible in the KCl-Ms. After the gradient, it would appear that this large amount becomes visible owing to an as-yet unknown mechanism.

We showed that six out of the seven other DGC proteins studied here are actually present in the CSM of mdx mouse muscle at levels similar to those observed in the CSM from normal mouse muscle. In a similar way to β-dystroglycan, we concluded that these DGC proteins are invisible in the KCl-Ms but become visible in the fractions recovered after the gradient. As these proteins are observed at their expected sizes, the difference in detection between KCl-Ms and CSM cannot be due to altered post-translational modifications as previously shown for α-dystroglycan, for which some authors report a lack of detection by the VIA4-1 antibody [41]. In mdx muscle, most of the DGC proteins display normal mRNA levels [6, 26, 42]. Since these proteins are detected in CSM purified from the KCl-Ms fraction, they appear to be synthesized in normal amounts and not degraded in the absence of dystrophin.

As an exception, β-dystroglycan appears as two bands with the light band at 30 kDa becoming prominent in mdx compared to normal muscle preparations. Such a cleavage product was already observed in cancer cells [43] and in ischemic heart tissue [44] being due to the release of the β-dystroglycan extracellular domain by metalloproteinases. It seems likely that such a cleavage is activated during the purification procedure. This hypothesis was reinforced by the observation of a specific increase in this band in the CSM compared to KCl-Ms obtained from normal muscle.

In biochemical studies, the proteins were rendered soluble by the charge buffer [30] before the SDS-PAGE by means of 2% SDS and 5% β-mercaptoethanol and boiling at 95°C. Our results indicate that a large part of the β-dystroglycan and DGC proteins in KCl-Ms are not visible with this charge buffer, which is likely due to non-solubility in SDS. This type of behaviour is already known for membrane proteins where specific strategies are used in proteomic approaches to solubilize these proteins [45, 46]. SDS-insoluble proteins are known to accumulate in several disorders: for example, dysferlin in Alzheimer’s disease.
[47], polyglutamatates in PolyQ neurodegenerative diseases [48], and α-synuclein in Parkinson’s disease [49]. In our study, DGC proteins appear to form oligomers or SDS-insoluble aggregates that accumulate in the sarcolemma. These insoluble aggregates are made up of sarcoglycans, β-dystroglycan, α-syntrophin and α-dystrobrevin-1. Since these aggregates are modified by the density gradient centrifugation step, the proteins become accessible to SDS and antibodies for immunoblotting. At present, we have no explanation for this surprising observation. It appears also clear that if the DGC proteins are not soluble in the SDS-PAGE sample buffer as they are not accessible to the antibodies on the western-blot, they would also be inaccessible to the antibodies in immunocytochemical experiments in which there is no attempt to solubilize the proteins by any treatment.

Our results imply that, in the plasma membrane of mdx mouse muscle, the cytoplasmic proteins α-dystrobrevin-1 and α-syntrophin make up an insoluble sub-complex that is associated with the intrinsic membrane proteins, sarcoglycans and dystroglycans. Because α-dystrobrevin-2 is highly depleted, we may conclude that this cytoplasmic isoform is only linked to dystrophin in normal muscle; α-dystrobrevin-3 is not detected in mdx mouse muscle, even though it links to sarcoglycans, which are still present. However, since sarcoglycans are not accessible to the cytoplasmic side, being embedded in a large membrane complex, this would make them inaccessible to α-dystrobrevin-3, which, in turn, is either not present in the cell or not retained in the plasma membrane fractions. α-dystrobrevin-1 co-purifies with utrophin and is still retained in CSM.

All these findings suggest that while the DGC proteins are produced at normal levels in mdx mice, they may not be properly assembled and/or integrated into the sarcolemma. It appears that they form inaccessible membrane complexes when dystrophin is deficient. This could be reversed by the expression of the dystrophin-related protein Dp71 in mdx skeletal muscle [32, 50], even though the expression of Dp71 results in severe muscle degeneration. Moreover, several of the DGC proteins are partly visible in KCl-Ms in mdx mouse muscle depending on the protocol used. In particular, β-dystroglycan could be detected in mdx mouse muscle at about 50% of the level in normal muscle. This partial visibility is likely due to interactions with other partners apart from dystrophin, such as utrophin or plectin. Urophin is over-expressed in DMD and mdx mouse muscle, and is able to bind to β-dystroglycan [25, 36], while plectin is a newly revealed partner of β-dystroglycan [51]. We propose that the direct interaction between these two proteins and β-dystroglycan could explain the remaining partial visibility of the β-dystroglycan in mdx mouse muscle. In line with the demonstration that the dystroglycan and sarcoglycan sub-complexes may be dystrophin-independent signaling or regulatory modules [52, 53], our results also suggest that the expression of most of the DGC proteins is independent of dystrophin expression, while their functional localization depends partly on the
correct localization of dystrophin and, in particular, on the presence of the β-dystroglycan-binding domain.

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REFERENCES

1. Hoffman, E.P., Brown, R.H. and Kunkel, L.M. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51 (1987) 919-928.
2. Ohlendieck, K., Ervasti, J.M., Snook, J.B. and Campbell, K.P. Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. J. Cell Biol. 112 (1991) 135-148.
3. Ervasti, J., Ohlendieck, K., Kahl, S., Gaver, M. and Campbell, K. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature 345 (1990) 315-319.
4. Campbell, K. and Kahl, S. Association of dystrophin and an integral membrane glycoprotein. Nature 338 (1989) 259-262.
5. Ervasti, J.M. and Sonnemann, K. Biology of the striated muscle dystrophin-glycoprotein complex. Int. Rev. Cytol. 265 (2008) 191-225.
6. Ibraghimov-Beskrovnaya, O., Ervasti, J., Leveille, C., Slaughter, C., Sernett, S. and Campbell, K. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 355 (1992) 696-702.
7. Smalheiser, N.R. and Kim, E. Purification of cranin, a laminin binding membrane protein. Identity with dystroglycan and reassessment of its carbohydrate moieties. J. Biol. Chem. 270 (1995) 15425-15433.
8. Winder, S. The complexities of dystroglycan. Trends Biochem. Sci. 26 (2001) 118-124.
9. Chung, W. and Campanelli, J.T. WW and EF hand domains of dystrophin-family proteins mediate dystroglycan binding. Mol. Cell Biol. Res. Commun. 2 (1999) 162-171.
10. Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M. and Eck, M.J. Structure of a WW domain containing fragment of dystrophin in complex with beta-dystroglycan. Nat. Struct. Biol. 7 (2000) 634-638.
11. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G. and Campbell, K.P. SH3 Domain-mediated Interaction of Dystroglycan and Grb2. J. Biol. Chem. 270 (1995) 11711-11714.
12. Hnia, K., Zouiten, D., Cantel, S., Chazalette, D., Hugon, G., Fehrentz, J.A., Masmoudi, A., Diment, A., Brannham, J., Mornet, D. and Winder, S.J.
ZZ domain of dystrophin and utrophin: topology and mapping of a beta-dystroglycan interaction site. *Biochem. J.* **401** (2007) 667-677.

13. Yoshida, T., Pan, Y., Hanada, H., Iwata, Y. and Shigekawa, M. Bidirectional signaling between sarcoglycans and the integrin adhesion system in cultured L6 myocytes. *J. Biol. Chem.* **273** (1998) 1583-1590.

14. Crosbie, R.H., Lebakken, C.S., Holt, K.H., Venzke, D.P., Straub, V., Lee, J.C., Grady, R.M., Chamberlain, J.S., Sanes, J.R. and Campbell, K.P. Membrane targeting and stabilization of sarcospan is mediated by the sarcoglycan subcomplex. *J. Cell Biol.* **145** (1999) 153-165.

15. Yoshida, M., Hama, H., Ishikawa-Sakurai, M., Imamura, M., Mizuno, Y., Araihi, K., Wakabayashi-Takai, E., Noguchi, S., Sasaoka, T. and Ozawa, E. Biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex as a basis for understanding sarcoglycanopathy. *Hum. Mol. Genet.* **9** (2000) 1033-1040.

16. Ahn, A.H., Freener, C.A., Gussoni, E., Yoshida, M., Ozawa, E. and Kunkel, L.M. The three human syntrophin genes are expressed in diverse tissues, have distinct chromosomal locations, and each bind to dystrophin and its relatives. *J. Biol. Chem.* **271** (1996) 2724-2730.

17. Peters, M.F., Adams, M.E. and Froehner, S.C. Differential association of syntrophin pairs with the dystrophin complex. *J. Cell Biol.* **138** (1997) 81-93.

18. Ervasti, J.M. Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. *Biochim. Biophys. Acta* **1772** (2007) 108-117.

19. Peters, M., O’Brien, K., Sadoilet-puccio, H., Kunkel, L., Adam, M. and Froehner, S. β-dystrobrevin, a new member of the dystrophin family. *J. Biol. Chem.* **272** (1997) 31561-31569.

20. Blake, D.J. Dystrobrevin dynamics in muscle-cell signalling: a possible target for therapeutic intervention in Duchenne muscular dystrophy? *Neuromuscul. Disord.* **12 Suppl 1** (2002) S110-117.

21. Campbell, K.P. Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell* **80** (1995) 675-679.

22. Petrof, B.J., Shragger, J.B., Stedmann, H.H., Kelly, A.M. and Sweeney, H.L. Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. USA* **90** (1993) 3710-3714.

23. Ohlendieck, K. and Campbell, K.P. Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J. Cell Biol.* **115** (1991) 1685-1694.

24. Ohlendieck, K., Matsumura, P., Ionasescu, V., Towbin, J., Bosch, E., Weinstein, S., Sernetz, L. and Campbell, K.P. Duchenne muscular dystrophy: deficiency of dystrophin-associated proteins in the sarcolemma. *Neurology* **43** (1993) 795-800.

25. Ozawa, E., Yoshida, M., Suzuki, A., Mizuno, Y., Hagiwara, Y. and Noguchi, S. Dystrophin-associated proteins in muscular dystrophy. *Hum. Mol. Genet.* **4 Spec No** (1995) 1711-1716.
26. Hack, A., Lam, M., Cordier, L., Shoturma, D.I., Ly, C.T., Hadhazy, M.A., Hadhazy, M.R., Sweeney, H.L. and McNally, E.M. Differential requirement for individual sarcoglycans and dystrophin in the assembly and function of the dystrophin-glycoprotein complex. J. Cell Sci. 113 (2000) 2535-2544.

27. Rouger, K., Cunff, M.L., Steenman, M., Potier, M., Gibelin, N., Dechene, C. and Leger, J. Global/temporal gene expression in diaphragm and hindlimb muscles of dystrophin-deficient (mdx) mice. Am. J. Physiol. Cell Physiol. 253 (2002) C773-C784.

28. Cluchague, N., Moreau, C., Rocher, C., Pottier, S., Leray, G., Chérel, Y. and Le Rumeur, E. β-dystroglycan can be revealed in microsomes from mdx mouse muscle by detergent treatment. FEBS Letters 572 (2004) 216-220.

29. Ohlendieck, K. Characterisation of the dystrophin-related protein utrophin in highly purified skeletal muscle sarcolemma vesicles. Biochim. Biophys. Acta 1283 (1996) 215-222.

30. Laemmli, U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680-685.

31. Towbin, H., Staehelin, T. and Gordon, J. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76 (1979) 4350-4354.

32. Cox, G.A., Sunada, Y., Campbell, K.P. and Chamberlain, J.S. Dp71 can restore the dystrophin-associated glycoprotein complex in muscle but fails to prevent dystrophy, Nature Genet. 8 (1994) 333-339.

33. Wells, D.J., Wells, K.E., Asante, E.A., Turner, G., Sunada, Y., Campbell, K.P., Walsh, F.S. and Dickson, G. Expression of human full-length and minidystrophin in transgenic mdx mice: implications for gene therapy of Duchenne muscular dystrophy. Hum. Mol. Genet. 4 (1995) 1245-1250.

34. Matsumura, K., Tome, F.M., Collin, H., Leturcq, F., Jeamari, M., Kaplan, J.C., Fardeau, M. and Campbell, K.P. Expression of dystrophin-associated proteins in dystrophin-positive muscle fibers (revertants) in Duchenne muscular dystrophy. Neuromuscul. Disord. 4 (1994) 115-120.

35. Rafael, J.A., Sunada, Y., Cole, N.M., Campbell, K.P., Faulkner, J.A. and Chamberlain, J.S. Prevention of dystrophic pathology in mdx mice by a truncated dystrophin isoform. Hum. Mol. Genet. 3 (1994) 1725-1733.

36. Mizuno, Y., Yoshida, M., Nonaka, I., Hirai, S. and Ozawa, E. Expression of utrophin (dystrophin-related protein) and dystrophin-associated glycoproteins in muscles from patients with Duchenne muscular dystrophy. Muscle Nerve 17 (1994) 206-216.

37. Crawford, G.E., Faulkner, J.A., Croesbie, R.H., Campbell, K.P., Froehner, S.C. and Chaberlain, J.S. Assembly of the Dystrophin-associated Protein Complex Does Not Require the Dystrophin COOH-terminal Domain. J. Cell Biol. 150 (2000) 1399-1409.
correction of adult mdx mouse muscle using gutted adenoviral vectors expressing full-length dystrophin. **Proc. Natl. Acad. Sci. USA** **99** (2002) 12979-12984.

39. Harper, S.Q., Hauser, M.A., DelloRusso, C., Duan, D., Crawford, R.W., Phelps, S.F., Harper, H.A., Robinson, A.S., Engelhardt, J.F., Brooks, S.V. and Chamberlain, J.S. Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. **Nature Med.** **8** (2002) 253-261.

40. Gardner, K., Kearney, J., Edwards, J. and Rafael-Fortney, J. Restoration of all dystrophin protein interactions by functional domains in trans does not rescue dystrophy. **Gene therapy** **13** (2005) 744-751.

41. Ervasti, J. and Campbell, K. A Role for the Dystrophin-Glycoprotein Complex as a Transmembrane Linker between Laminin and Actin. **J. Cell Biol.** **122** (1993) 809-823.

42. Rouger, K., Brault, M., Daval, N., Leroux, I., Guigand, L., Lesoeur, J. and Chérel, Y. Muscle satellite cell heterogeneity: in vitro and in vivo evidences for populations that fuse differently. **Cell Tissue Res.** **317** (2004) 319-326.

43. Yamada, H., Saito, F., Fukuta-Ohi, H., Zhong, D., Hase, A., Arai, K., Okuyama, A., Maekawa, R., Shimizu, T. and Matsumura K. Processing of β-dystroglycan by matrix metalloproteinase disrupts the link between the extracellular matrix and cell membrane via the dystroglycan complex. **Hum. Mol. Genet.** **10** (2001) 1563-1569.

44. Armstrong, S., Latham, C. and Ganote, C. An ischemic beta-dystroglycan degradation product: correlation with irreversible injury in adult rabbit cardiomyocytes. **Mol. Cell. Biochem.** 242 (2003) 71-79.

45. Bierczynska-Krzysik, A., Kang, S.U., Silberring, J. and Lubec, G. Mass spectrometrical identification of brain proteins including highly insoluble and transmembrane proteins. **Neurochem. Int.** **49** (2006) 245-255.

46. Zhou, J., Zhou, T., Cao, R., Liu, Z., Shen, J., Chen, P., Wang, X. and Liang, S. Evaluation of the application of sodium deoxycholate to proteomic analysis of rat hippocampal plasma membrane. **J. Proteome Res.** **5** (2006) 2547-2553.

47. Galvin, J.E., Palamand, D., Strider, J., Milone, M. and Pestronk, A. The muscle protein dysferlin accumulates in the Alzheimer brain. **Acta Neuropathol.** **112** (2006) 665-671.

48. Wong, S.L., Chan, W.M. and Chan, H. Y. Sodium dodecyl sulfate-insoluble oligomers are involved in polyglutamine degeneration. **FASEB J.** (2008)

49. Cappai, R., Leck, S.L., Tew, D.J., Williamson, N.A., Smith, D.P., Galatis, D., Sharplies, R.A., Curtain, C.C., Ali, F.E., Cherry, R.A., Culvenor, J.G., Bottomley, S.P., Masters, C.L., Barnham, K.J. and Hill, A.F. Dopamine promotes alpha-synuclein aggregation into SDS-resistant soluble oligomers via a distinct folding pathway. **FASEB J.** **19** (2005) 1377-1379.
50. Greenberg, D.S., Sunada, Y., Campbell, K.P., Yaffe, D. and Nudel, U. Exogenous Dp71 restores the levels of dystrophin-associated proteins but does not alleviate muscle damage in mdx mice. *Nature Genet.* 8 (1994) 340-344.

51. Rezniczek, G.A., Konieczny, P., Nikolic, B., Reipert, S., Schneller, D., Abrahamsberg, C., Davies, K.E., Winder, S.J. and Wiche, G. Plectin 1f scaffolding at the sarcolemma of dystrophic (mdx) muscle fibers through multiple interactions with beta-dystroglycan. *J. Cell Biol.* 176 (2007) 965-977.

52. Batchelor, C.L. and Winder, S.J. Sparks, signals and shock absorbers: how dystrophin loss causes muscular dystrophy. *Trends Cell Biol.* 16 (2006) 198-205.

53. Hack, A., Groth, M. and McNally, E.M. Sarcoglycans in muscular dystrophy. *Microsc. Res. Tech.* 48 (2000) 167-180.