FEBS Letters 572 (2004) 216–220 FEBS 28693

β-Dystroglycan can be revealed in microsomes from *mdx* mouse muscle by detergent treatment

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Received 9 June 2004; revised 6 July 2004; accepted 15 July 2004

Available online 26 July 2004

Edited by Michael R. Bubb

Abstract β-Dystroglycan is the central member of a transmembrane protein complex of the skeletal muscle plasma membrane. Since it was not detected in dystrophin-deficient skeletal muscles, a disruption of the complex was thought to be involved in the dystrophic process. We report here that β-dystroglycan is actually present at normal levels in *mdx* mouse muscle plasma membrane: treatment with cholate detergent is able to reveal its presence by SDS–PAGE and immunoblotting. This result shows that, in dystrophin-deficient muscles, β-dystroglycan is indeed targeted to the plasma membrane but remains inaccessible to classical solubilizing treatments and to antibodies used for immunolocalization.

Keywords: Muscular dystrophy; Dystrophin–glycoprotein complex; *mdx* mouse; β-Dystroglycan; Dystrophin-deficient muscle

1. Introduction

Duchenne muscular dystrophy (DMD) [1] and *mdx* mouse dystrophy [2] result from X-linked genetic defects leading to a lack of dystrophin. Dystrophin [3] is located on the cytoplasmic side of the skeletal muscle plasma membrane [4] and is involved in the dystrophin–glycoprotein complex (DGC). The DGC consists of the integral membrane proteins β-dystroglycan, the sarcoglycans and sarcospan, along with the extracellular protein α-dystroglycan as well as the cytoplasmic proteins dystrophin and the syntrophins (for a review, see [5]). The C-terminal domain of dystrophin binds to the intracellular domain of β-dystroglycan [6,7], which in turn binds to α-dystroglycan, thus establishing a transmembrane link between the extracellular matrix and the cytoskeleton network. F-actin [8,9] and membrane phospholipids [10] are other known partners of dystrophin domains.

Experimental studies have failed to detect DGC in the dystrophin-deficient skeletal muscles of DMD patients [11–13] and *mdx* mouse [14]. Hence, this complex was assumed to be disrupted in the absence of dystrophin, and appeared to be involved in the development of the disease.

However, the level of expression of mRNA encoding for the dystroglycans is similar in dystrophin-deficient and normal mouse and human muscle [12,15]. Therefore, two main hypotheses may be advanced. The DGC proteins could be hydrolysed immediately after translation and are not transported to the sarcolemma, or the DGC proteins could be well targeted onto the sarcolemma but are not detectable due to a secondary modification.

The present work shows for the first time that β-dystroglycan is present as an insoluble protein in dystrophin-deficient muscle plasma membrane.

2. Materials and methods

2.1. Animals

Normal (C57BL/10Sc/Scn) and *mdx* (C57BL/10Sc/Scn/mdx) mice were bred at the Ecole Nationale Vétérinaire in Nantes.

2.2. Antibodies

For immunoblotting, we used mouse monoclonal antibodies: anti-dystrophin (NCL-DYS2), anti-β-dystroglycan (NCL-β DG) from Novocastra Laboratories (Tebu France, Le Peray en Yvelines, France); mouse anti-calsequestrin (VIIID12) was supplied by Affinity Bioreagents (Coger SA, Paris, France); anti-Utrophin (MANCHO-3) was kindly provided by Prof. G. Morris (MRCI Biochemistry Group, Wrexham, UK); anti-Golgi 58K protein (58K-9) was from Sigma. Alkaline phosphatase conjugated donkey anti-mouse secondary antibody was from Chemicon International (Euromedex, Mundolsheim, France).

2.3. Isolation of crude surface membranes

For each preparation, normal and *mdx* mice (8–24 weeks old) were euthanized by i.p. pentobarbital injection, and whole hind-leg muscle samples were taken.

Crude surface membranes were isolated at 4 °C as described in detail elsewhere [4,16] and as shown in Fig. 1A.

2.4. SDS–PAGE and immunoblotting

SDS–PAGE was performed on 8% or 10% polyacrylamide gel following classical procedures of Laemmli [17] and Towbin et al. [18] using secondary antibody coupled to alkaline phosphatase. When quantification of a specific protein was required, the immunoblots were developed using a fluorescent substrate for alkaline phosphatase (ECF, Amersham) and the fluorescence emission was directly measured by fluorescence scanning equipment (Storm instrument, Amersham, and Image-Quant 5.2).

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0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.07.032

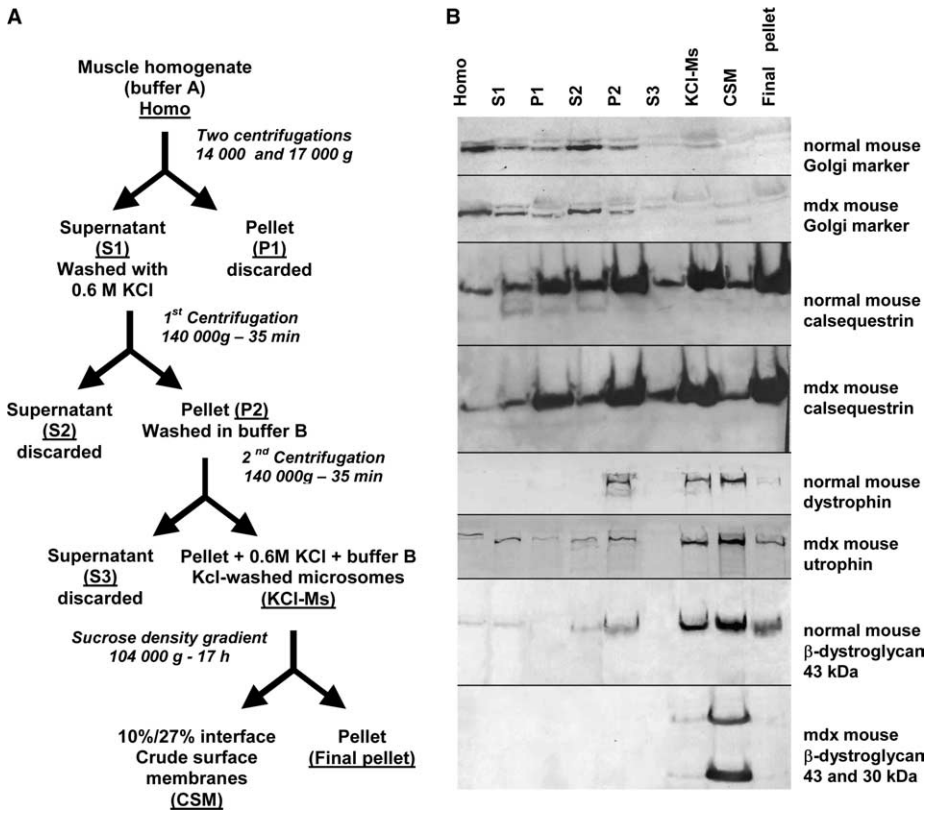

Fig. 1. Skeletal muscle fractionation from normal and *mdx* mouse. (A) Purification protocol: buffer A is 20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM $MgCl_2$, 0.3 M sucrose, 0.5 mM EDTA, at pH 7.0 with protease inhibitors; buffer B is 0.303 M sucrose, 20 mM Tris–maleate, at pH 7.0. (B) SDS–PAGE and immunoblotting of several proteins performed on the successive fractions obtained along with the fractionation procedure of (A).

3. Results

3.1. Muscle fractionation

KCl-washed microsomes (KCl-Ms) and crude surface membranes (CSM) were obtained from skeletal muscle through a well established procedure (Fig. 1A). SDS–PAGE and immunoblotting of the Golgi 58K protein showed that Golgi membranes were highly enriched in supernatant S2 and that KCl-Ms and CSM were largely devoid of these constituents. By contrast, P2, KCl-Ms and CSM were largely enriched in dystrophin from normal mouse muscle and in utrophin when derived from *mdx* mouse muscle (Fig. 1B). In parallel, calsequestrin, a marker of the SR, was highly enriched in P1, P2, KCl-Ms and the final pellet but decreased markedly in S3 and CSM (Fig. 1B). CSM was also enriched in the two plasma membrane markers, acetylcholinesterase and 5′nucleotidase, compared with S1, KCl-Ms and the final pellet (Table 1). Therefore, KCl-Ms appeared to be a mixture of SR and plasma membranes while CSM appeared largely free of SR membranes and enriched in plasma membranes.

3.2. β-Dystroglycan in CSM and KCl-Ms

SDS–PAGE and immunoblotting revealed a faint label for β-DG migrating at 43 kDa in normal muscle P2, the label being strongly enhanced in KCl-Ms and CSM (Fig. 1B). In *mdx* mouse muscle, no label appeared in any of the fractions except for CSM where strong labels appeared in two bands migrating at 43 and 30 kDa.

Table 1
Quantification of several markers in the fractions obtained from normal and *mdx* mouse muscle by the skeletal muscle fractionation procedure

	Normal mouse				*mdx* Mouse			
	S1	KCl-Ms	CSM	Final pellet	S1	KCl-Ms	CSM	Final pellet
Total protein μg/g of muscle			20 ± 1				20 ± 1	
ACHase IU/mg protein ($n = 6$)	5 ± 1	8 ± 4	25 ± 1	6 ± 0	4.5 ± 2	105	24 ± 10	6 ± 0
5′N IU/mg protein ($n = 3$)	2	4	15.5	2	3	4	15	2
Calsequestrin (%)	6 ± 1	100	6 ± 0	271 ± 13	5 ± 0	101 ± 2	5 ± 1	263 ± 13
β-DG 43 (%)		100	430 ± 8	4 ± 1			132 ± 90	
β-DG 30 (%)			119				282 ± 100	
Total β-DG (%)		100	440				414	

The fractions are those shown in Fig. 1A.
Calsequestrin, β-DG 43 and 30 kDa levels are obtained from the fluorescence intensity of the bands in the Western blots in Fig. 2 and the 100% reference value is assigned arbitrarily to KCl-Ms. Means ± S.D. are done.

Further on, it appeared that the 30 kDa band represented 70% of the total fluorescence observed for β-DG in CSM from *mdx* muscle, while this band was absent from the normal muscle CSM (Fig. 2 and Table 1). The total fluorescence levels of β-DG of CSM were similar in *mdx* and normal mouse muscle. Calsequestrin was used as a counter marker and showed opposite expressions in these fractions (Fig. 2 and Table 1).

3.3. β-DG revealed by detergent treatment of KCl-Ms

Considering the presence of β-DG in CSM from *mdx* mouse muscle and its non-visibility in corresponding KCl-Ms, we hypothesized that β-DG is indeed present in KCl-Ms but not solubilized by SDS present in the loading buffer for SDS–PAGE. We used several detergents in an attempt to solubilize β-DG from these KCl-Ms. A 2% cholate treatment for 2 h at room temperature yielded a strong label for β-DG in *mdx* and in normal KCl-Ms (Fig. 3). Only one band migrating at 43 kDa was observed in both muscle fractions. The intensity of the band was similar in *mdx* and normal mouse muscle.

We therefore considered that the appearance of the β-DG 30 kDa band in *mdx* CSM could be due to proteolysis occurring during the long duration of the sucrose density gradient centrifugation. To test this hypothesis, KCl-Ms from normal and *mdx* mouse muscle were incubated overnight at 4 °C. This treatment failed to reveal β-DG in KCl-Ms (not shown); however, a 2% cholate treatment was able to reveal one band migrating at 43 kDa (not shown) as observed in Fig. 3. In addition, assuming that β-DG insolubility in *mdx* mouse could be due to intermolecular disulfide bridges, SDS–PAGE was performed under non-reducing conditions. The immunoblots were similar under both reducing and non-reducing conditions for the three fractions S1, KCl-Ms and CSM, with or without cholate treatment (results not shown).

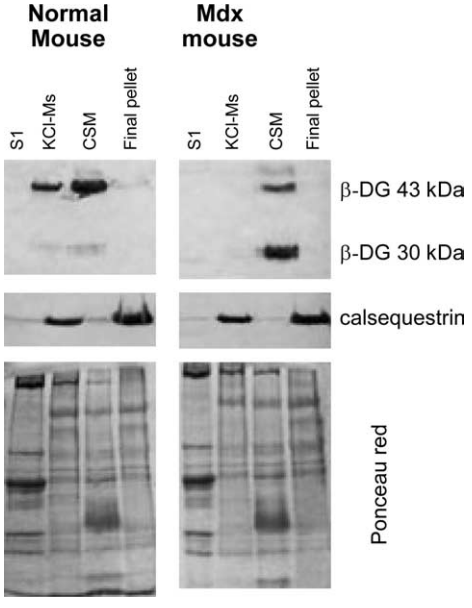

Fig. 2. β-DG is absent in the KCl-Ms from *mdx* mouse muscle but present in CSM of normal as well as *mdx* mouse muscle. Immunoblots were performed for β-DG and calsequestrin on the four fractions: S1, KCl-Ms, CSM and the final pellet obtained from normal and *mdx* mouse muscle. Fluorescence was quantified in three identical blots and reported in Table 1. Ponceau red staining is shown at the bottom.

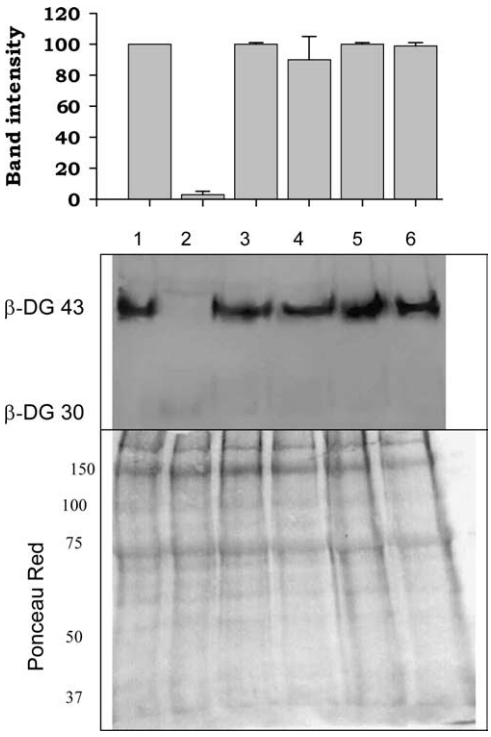

Fig. 3. β-DG can be revealed by cholate treatment of KCl-Ms from *mdx* mouse muscles. A 2% sodium cholate treatment for 2 h, followed by overnight 2% SDS dialysis for half of the fraction. Then, SDS–PAGE and immunoblotting for β-DG were performed on KCl-Ms from normal and *mdx* mouse muscle. Fluorescence was quantified in three identical blots and the levels normalized using a level of 100 for the non-treated KCl-Ms from normal mouse muscle: (1) normal mouse, no treatment; (2) *mdx* mouse, no treatment; (3) normal mouse, 2% cholate; (4) *mdx* mouse, 2% cholate; (5) normal mouse, 2% cholate and SDS dialysis; (6) *mdx* mouse, 2% cholate and SDS dialysis.

4. Discussion

In this paper, we show for the first time the presence of a pool of SDS-insoluble β-DG in microsomes from dystrophin-deficient *mdx* mouse muscle. Furthermore, we describe the presence of a pool of β-DG in crude surface membranes from normal and *mdx* mouse.

The method of plasma membrane purification used in our work was derived from the work of Ohlendieck et al. [7]. We first checked the yield in marker proteins for the fractions and showed that in normal as well as *mdx* mouse muscle: (i) KCl-Ms were composed of SR and plasma membranes devoid of Golgi membranes, (ii) CSM fractions were reasonably free of SR and enriched in plasma membrane markers. Therefore, the dystrophic process does not seem to alter the composition in marker proteins of *mdx* mouse fractions compared to normal mouse. However, we found that dystrophin was absent and utrophin overexpressed in *mdx* mouse preparations, so we could then quantitatively compare further data obtained in normal and *mdx* mouse muscle.

β-DG is a glycoprotein of the plasma membrane that can be detected in KCl-Ms using SDS–PAGE and immunoblotting as a unique band migrating at 43 kDa [5,7]. Accordingly, we were able to reveal β-DG in KCl-Ms of normal mouse. Using the same method, β-DG has been shown to be highly depleted or absent in KCl-Ms from *mdx* mouse [11,14], as

well as in the muscle of DMD patients [13,19,20]. Although our first results were in agreement with the cited studies, we then showed that β-DG can be detected in KCl-Ms from *mdx* mouse after a cholate detergent treatment. The quantitative results indicated that the pools of β-DG were similar in both *mdx* and normal mouse muscle. Although KCl-Ms were largely contaminated by SR but devoid of Golgi membranes, KCl-Ms from both *mdx* and normal muscle have similar protein profiles and are similarly enriched in plasma membrane markers. Therefore, as Golgi membranes are absent from these fractions and β-DG has never been shown to be associated with SR membranes [4,21], we conclude that β-DG is indeed targeted onto the plasma membrane of dystrophin-deficient *mdx* mouse muscle.

It is not known why cholate is able to reveal β-DG from this muscle while SDS does not. At present, we have no data to explain this result. It could be related to the fact that SDS has a poorer phospholipid solubilization action in contrast to cholate [22]. To modify the protein structure, SDS needs to interact with accessible charged residues of a protein via its hydrophilic charged domain, which thus produces solubilization and unfolding. This is not the case for certain membrane proteins such as bacteriorhodopsin [22], which are embedded in the phospholipid bilayer and which need long detergent interaction to achieve solubilization. Therefore, we may hypothesize that β-DG of *mdx* mouse muscle plasma membrane is not accessible to SDS. Moreover, the non-solubility of β-DG in SDS could not be due to an aggregation of the molecules by intermolecular disulfide bridges because SDS–PAGE under non-reducing conditions does not modify the migration profile of the β-DG.

The only additional data about the non-solubility of *mdx* KCL-Ms β-DG in SDS are from the CSM obtained by sucrose density gradient centrifugation of KCl-Ms. SDS–PAGE and immunoblotting reveal the presence of β-DG in the CSM fraction from both *mdx* and normal mouse muscle. However, in *mdx* mouse CSM, β-DG appears as two bands migrating at 43 and 30 kDa, while in normal CSM, only one band at 43 kDa could be observed. The antibody used here recognizes the β-DG intracellular domain. A cleavage product migrating at 30 kDa has already been observed in ischemic heart [23] and in cancer cells [24], being due to the release of the β-DG extracellular domain that interacts with the extracellular α-DG by activation of metalloproteinases. In dystrophic muscle, this cleavage could arise from the proteases known to be highly activated in these muscles.

Our results on β-DG obtained with *mdx* mouse muscle preparations appear quite surprising considering that β-DG could not be revealed in these preparations by the usual SDS–PAGE method in agreement with numerous previous studies. Instead, we obtained the following results:

(i) β-DG can be revealed in KCl-Ms using detergent treatment, indicating that it is SDS-insoluble in KCl-Ms;
(ii) β-DG is detected in CSM by routine methods, indicating that it is SDS-soluble in CSM;
(iii) β-DG found in CSM consists of 70% of the cleaved product and 30% of the native protein;
(iv) there are similar levels of β-DG 43 kDa in *mdx* and normal mouse muscle KCl-Ms after cholate treatment;
(v) β-DG 30 and 43 kDa of *mdx* mouse muscle CSM show similar levels to β-DG 43 kDa in CSM from normal mouse muscle.

Therefore, it appears that proteolysis of β-DG occurs specifically in the KCl-Ms of *mdx* mouse muscle during the sucrose density gradient centrifugation, thus allowing SDS-solubilization of β-DG in the corresponding CSM. This proteolysis does not occur without the centrifugation since the overnight incubation is not followed by β-DG labelling of *mdx* KCl-Ms and a cholate treatment reveals only the β-DG 43 kDa. The striking feature is that such a cleavage does not occur in normal muscle.

Our results are in line with the fact that immunofluorescence analysis fails to detect β-DG in *mdx* mouse muscle. While it appears that β-DG is present, its intracellular domain is not accessible to the antibody, and this prevents its labelling. Therefore, in *mdx* mouse muscle plasma membrane, the intra- and extra-cellular domains of β-DG become inaccessible to the SDS of the loading buffer for SDS–PAGE analysis as well as to antibodies used for immunofluorescence analysis.

We have no explanation for the real difference in solubility of β-DG between normal and *mdx* mouse muscles. Nevertheless, we are able to reconcile the occurrence of normal β-DG mRNA levels in *mdx* compared to normal mouse muscle [12,15] with the observation that β-DG could not be revealed by either SDS–PAGE or the usual immunofluorescence methods due to an inaccessibility of its intra- and extra-cellular domains. Therefore, the major conclusion from our data is that β-DG is targeted onto the plasma membrane in similar amounts in both normal and *mdx* mouse muscle, but that, in *mdx* mouse muscle, β-DG is insoluble in SDS and soluble in cholate. Research is in progress in order to ascertain whether the same results can be obtained with the other members of the DGC and with human DMD skeletal muscle.

Acknowledgements: The authors thank M. Philippe and A. Bondon for encouraging discussions and suggestions. This work was supported by the Association Française contre les Myopathies, and the COREC of the Université de Rennes I. N.C. benefited from a fellowship of the Conseil Régional de Bretagne. M.S.N. Carpenter post-edited the English style.

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