Electron microscopic evidence for a mucin-like region in chick muscle α-dystroglycan

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Abstract **α-Dystroglycan has been isolated from chicken cardiac muscle and its molecular weight was estimated to be ≈135 kDa. The avian protein interacts with murine Engelbreth-Holm-Swarm (EHS) tumor laminin via interaction with the C-terminal LG4 and LG5 domains (fragment E3) of the laminin α-chain. This laminin binding is calcium-dependent and can be competed by heparin. Electron microscopy investigation on the shape of α-dystroglycan suggests that the core protein consists of two roughly globular domains connected by a segment which most likely corresponds to a mucin-like central region also predicted by sequence analysis on mammalian isoforms. This segment may act as a spacer in the dystrophin-associated glycoproteins complex exposing the N-terminal domain of α-dystroglycan to laminin in the extracellular space.**

Key words: Dystroglycan; Mucin; Laminin; Electron microscopy; Chick muscle

1. Introduction

α-Dystroglycan is a highly glycosylated protein associated with the external part of the plasma membrane. It is a member of the dystrophin associated glycoproteins (DAGs) complex and its mRNA also contains the β-dystroglycan sequence [1]. An arginine residue at position 640 has been recently proposed as a potential post-translational cleavage site between α- and β-dystroglycans [2]. Previous results have shown that α-dystroglycan binds laminin with high affinity [2–3] and this interaction provides mechanical stability to the muscle cells. In fact it has been proposed that α-dystroglycan links laminin in the extracellular matrix to dystrophin in the cytoskeleton, via interaction with the membrane spanning DAGs complex, [3]. Moreover, a role in the neuromuscular synapsis formation has also been suggested since dystroglycan has agrin binding activity [4].

Glycosylation accounts for approximately 60% of the total molecular mass of α-dystroglycan. It was shown that a part of the core protein expressed as a fusion protein in bacterial cells does not bind laminin [3], suggesting a possible contribution of carbohydrates in the functional behaviour of the protein. In α-dystroglycan primary sequence a potential site for N-linked glycosylation is present at position 141 and its sequence also contains several Ser-Gly (and Ser-Ala) pairs to which glycosaminoglycan-chains may be connected [1]. Enzymatic deglycosylation failed and only treatment with trifluoromethanesulfonic acid (TMFS) [3] led to full deglycosylation, strongly suggesting the presence of a very complex pattern of glycosylation.

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It has been shown that α-dystroglycan binds to the C-terminal heparin and heparan sulfate binding fragment of the laminin α-chain [2], but there is no evidence that α-dystroglycan is a heparan sulfate proteoglycan.

The amount of glycosylation seems to vary depending on the tissue source. Skeletal muscle α-dystroglycan from rabbit has a molecular mass of 156 kDa, and is larger than the 120 kDa protein isolated from central and peripheral nervous system, [1,2,5]. Also experiments on the protein isolated from the ray *T. californica* have indicated the presence of a larger amount of glycosylation (190 kDa), even though the protein primary sequence should be similar to mammalian isoforms [6]. These data indicate that the core protein should have a molar mass of about 67 kDa in all the cases.

Closely related sequences of α-dystroglycan have been reported for rabbit [1], human [7], and mouse [8,9]. No significant homology with other proteins was found [1], and the domain organization remains unclear. Our electron microscopy data on the chicken isoform suggest that the core-protein may have a structure composed of two terminal globular parts connected by an elongated segment. The latter could be formed by a proline- and threonine-rich hydrophylic mucin-like region which is present in the central part of the α-dystroglycan sequence.

2. Materials and methods

The protein has been isolated from chicken cardiac muscles using a procedure similar to that reported by Gee et al. [2]. Namely, 100–150 g of freshly frozen hearts were homogeneized in five volumes of lysis buffer (50 mM Tris, 0.2 M NaCl, 0.02% sodium azide, 2.5 mM *N*-ethylmaleimide (NEM) and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4. After centrifugation (at 39,000 × *g*) the supernatant was filtered using a Whatman filter paper. The solution was loaded on a DEAE-Sephacel (Pharmacia) column (2.5 × 20 cm) equilibrated with lysis buffer, at 4°C. After extensive washing the protein was eluted with two column volumes of the same buffer containing 0.5 M NaCl. The eluted solution was applied directly to a 10 ml Wheat-germ lectin (WGL) affinity column (Sepharose 6MB, Pharmacia), equilibrated with the 0.5 M containing NaCl buffer. After washing (with approximately ten column volumes) elution was carried out with two volumes of the same buffer containing 0.25 M *N*-acetyl-glucosamine. After dialysis against TBS buffer, pH 7.4, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, the DEAE-WGL extract was further purified using a laminin fragment E3 affinity column at room temperature. Protein was eluted with two volumes of 10 mM EDTA solution in TBS buffer, pH 7.4. For preparation of the gel approx 1.5 mg of fragment E3 of mouse tumor laminin was coupled to Sepharose-4B (Pharmacia) according to the manufacturer instruction. EHS laminin and fragment E3 were prepared as previously described [10].

ELISA experiments have been performed as described by [11] using a polyclonal anti-serum against mouse (EHS) tumor laminin. For laminin overlay experiments iodinated-laminin was prepared by the chloramine-T procedure, based on the protocol of Hunter and Greenwood

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[12] with minor modifications. Electron microscopy rotary shadowing experiments have been performed as described by Engel [13]. α-Dystroglycan concentration before spraying to mica was about 1–5 μg/ml, as estimated from the amount of signal recovered in overlay experiments. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [14].

3. Results

α-Dystroglycan was isolated and purified from chicken cardiac muscle. The elution profile from the E3 affinity column and the electrophoresis pattern of EDTA eluted α-dystroglycan are reported in Fig. 1. The apparent molar mass estimated by SDS-PAGE electrophoresis is around 135 kDa. This value is in good agreement with the value reported for the rabbit cardiac isoform and this confirms that cardiac muscle isoforms have a molar mass slightly smaller than that of skeletal muscle α-dystroglycan (which is about 156 kDa) [1].

Overlay experiments with iodinated laminin (Fig. 2) and ELISA experiments (Fig. 3) demonstrate binding of the protein to EHS laminin.

For technical reasons overlays experiments were carried out on 7.5% SDS gel and a broader distribution was observed for the iodinated-laminin stained band (Fig. 2) than for α-dystroglycan on 5% SDS gel (Fig. 1). The binding is calcium dependent, as indicated by inhibition with EDTA. Binding was inhibited by heparin (Figs. 2 and 3A) and also by high salt concentration (Fig. 3A). Binding could be demonstrated to the E3 fragment of laminin but not to fragment E8 which lacks the G4 and G5 domains [15], (Fig. 3B). Exact values for the binding constants cannot be estimated from ELISA and from the overlay assays but the data qualitatively suggest that the laminin binding strenght to chick α-dystroglycan is similar to that previously found for mammalian dystroglycans [2,3].

Experiments reported in Figs. 2 and 3 were performed with the α-dystroglycan enriched fraction obtained by DEAE and

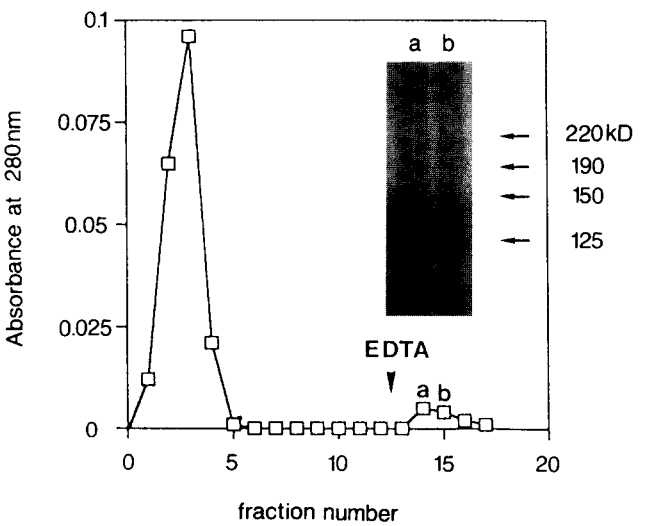

Fig. 1. Elution profile of chick heart α-dystroglycan from a fragment E3 affinity column. 25–30 ml of the α-dystroglycan enriched DEAE-WGL fraction were loaded and, after the first peak which refers to flow through, the column was washed with approx. 10 volumes of TBS buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, at pH 7.4. The arrow indicates addition of 10 mM EDTA for the elution. The insert shows a silver-stained 5% SDS-PAGE with the broad bands corresponding to chicken heart α-dystroglycan. The bands are centered around 135 kDa.

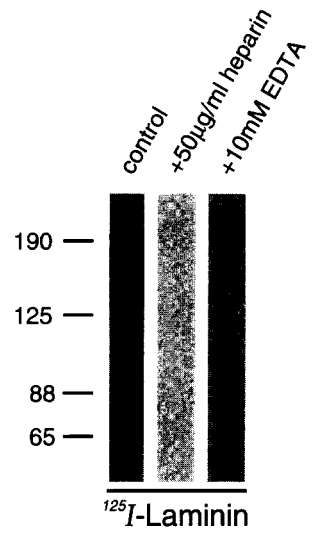

Fig. 2. Autoradiogram of an overlay experiment using iodinated laminin on the chick heart α-dystroglycan enriched DEAE-WGL fraction. In this case a 7.5% SDS-PAGE was carried out. About 0.01–0.05 μg of dystroglycan were loaded in every lane. Incubation condition: TBS buffer, pH 7.4, in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at room temperature.

wheat germ lectin (WGL) affinity chromatography. Similar results were obtained with material purified by the E3 affinity column (data not shown).

Electron microscopy of DEAE/WGL purified chicken heart α-dystroglycan revealed dumbbell-like molecules with two globular units connected by a 20–30 nm long rod-shaped and frequently curved segment (indicated by arrows in Fig. 4). More elongated particles with two globular structures connected by a flexible chain of up to 100 nm length was also seen (indicated by asterixes in Fig. 4A). In preparations purified by fragment E3 affinity chromatography the dumbbell-like particles were apparently more frequent than the elongated one (data not shown). For a possible demonstration of binding of α-dystroglycan to laminin by electron microscopy, mixtures of laminin and the chicken skeletal muscle form of α-dystroglycan were investigated (Fig. 4B). Laminin molecules as well as the same dumbbell-like particles were clearly seen, but no evidence of binding was detected.

4. Discussion

Our results indicate that the chick cardiac muscle dystroglycan has a similar calcium-dependent binding activity to mouse laminin, as the previously studied embryonic brain α-dystroglycan from the same species [2]. High binding activities were also found for the rabbit skeletal muscle α-dystroglycan [3], and a dissociation equilibrium constant of 90 nM has been reported for the interaction with the bovine brain isoform [2]. We were also able to demonstrate that the LG4 and LG5 domains of laminin represented by heparin and heparan sulfate binding fragment E3 [15] are responsible for recognition of laminin by the chick cardiac α-dystroglycan. The same result was obtained using brain dystroglycan [2]. Heparin inhibition suggests the involvement of heparan sulfate chains in laminin-α-dystroglycan interaction. However, no evidence for presence of gly-

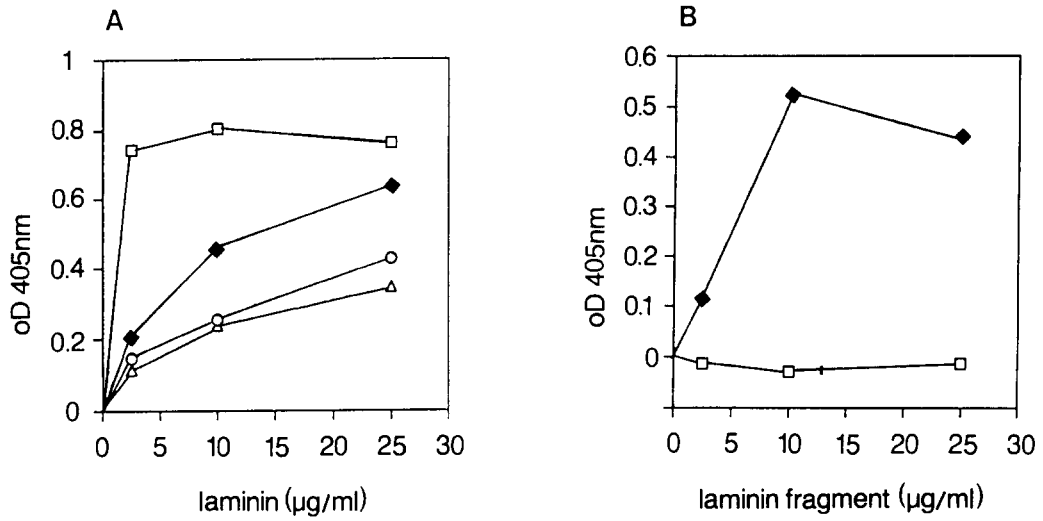

Fig. 3. Chick heart α-dystroglycan binds to laminin as detected by ELISA experiments. The absorbance has been monitored at 405 nm as a function of whole laminin (and E3 or E8 fragment) concentration. The experiments were carried out using the α-dystroglycan enriched DEAE-WGL fraction at room temperature. (A) TBS buffer, pH 7.4, in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (□); binding inhibition by 300 μg/ml heparin (♦), 10 mM EDTA (△) and 0.5 M NaCl (○). (B) Binding specifity for laminin fragments in the same TBS buffer at pH 7.4: E3 (♦) and E8 (□).

cosaminoglycan chains was detected in our electron microscopic analysis of chick muscle α-dystroglycans.

As also reported by others [2,3], we have detected laminin (or E3 fragment) binding to α-dystroglycan using solid phase assays. We were, however, not able to demonstrate binding by electron microscopy experiments. This could be due to the very low proteins concentration (below 50 nM) used in electron microscopy experiments. A further explanation is that the binding is more easily detected in solid phase experiments for the increase of overall affinity constants due to multivalent interactions.

The primary sequence of chick α-dystroglycan is not yet known. The high similarity of the sequences of rabbit, human and mouse α-dystroglycans suggests that the proteins are similar in different species, as indicated by results on *T. californica* dystroglycan [6]. It has been also reported that the dystrophin C-terminus in chicken and man is almost identical [16], and furthermore this domain of dystrophin is thought to be responsible for the interaction with β-dystroglycan in the DAGs-complex [17,18]. This is striking evidence for the presence of high cross-species identity and conservation of the proteins belonging to the DAGs complex. The data indicate that α-dystroglycan serves the general function, also in different species, of a mediator between laminin in the extracellular matrix and dystrophin, or dystrophin-related protein as utrophin, in the cytoskeleton of myocites.

Our electron microscopic data suggest that α-dystroglycan core protein can have a dumbbell-like architecture. This provides a first information about the structure of the laminin binding part of the DAGs complex. The presence of an elongated segment between N- and C-terminal globular regions of the α-dystroglycan molecule suggests a bridge between the membrane bound part of the DAGs complex and the basement membrane. It is worth noting that the G-domains of laminin are located at the interface between the basement membrane and cells in the epithelial layer of cornea [19,20]. A similar geometry may be present in basement membranes surrounding myotubes making an interaction between the LG4/5 domains of laminin and α-dystroglycan possible.

In the central region of the α-dystroglycan sequences [1,7–9] a mucin-like motif is detectable. Region from residue 300 to 490 containing 40% of proline and threonine residues with $(Pro)_n(Thr)_m$ repeats (n = 1,2; m = 1–4). Homology (30% of identity) was found with intestinal human mucin-2 and with other mucin-like proteins. Mucins are proteins which cover and lubricate epithelia, containing high levels of serine, threonine, glycine, alanine and proline and also characteristically containing tandemly repeated amino acid sequences [21]. Mucin-like regions have also been found in cell surface molecules respon-

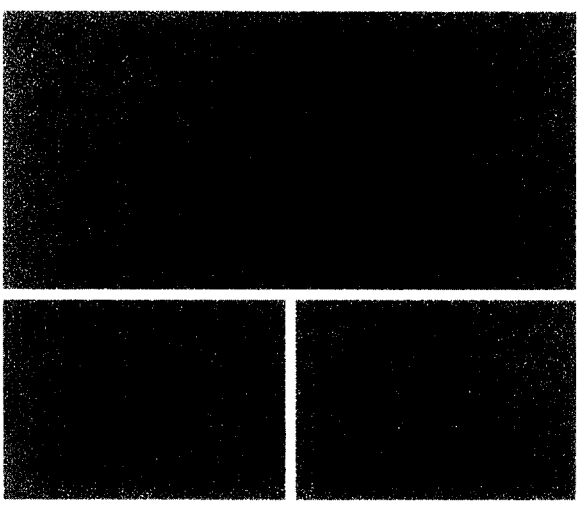

Fig. 4. Visualization of chick α-dystroglycan by electron microscopy after rotary shadowing. (A) Cardiac muscle α-dystroglycan enriched DEAE-WGL fraction was sprayed in 10 mM Tris, 5 mM $CaCl_2$ at pH 7.4 . Arrows refer to the dumbbell-like particles and asterisks to the more elongated ones. (B) Skeletal muscle α-dystroglycan (DEAE-WGL fraction) and laminin were sprayed in TBS buffer, pH 7.4, in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$, after 1 h incubation at room temperature. Arrows refer to the dumbell-like particles.

sible for cell–cell adhesion like MAdCam-1 and Sgp50 [22,23]. Electron microscopy analysis of mucins reveals that the presence of extensive O-linked glycosilation hinders the formation of elaborated three-dimensional structure (secondary and tertiary structure) and causes the organization of the polypeptidic chain in rod-shaped elongated chains [24].

As previously discussed, our observations suggest that a similar situation could also be present in the α-dystroglycan: a mucin-like repeat, by similarity with the situation present in other cell surface molecules could constitute a bridge to trespass the glycocalix surrounding the membrane and to point towards the external space and interact with extracellular matrix molecules [24].

Knowing that α-dystroglycan is linked to β-dystroglycan at its C-terminal domain, the N-terminal domain is a good candidate for interactions with laminin. Recombinant expression of various fragments of the mouse skeletal muscle isoform, we have recently cloned [9], will be used in further work to test this hypothesis.

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