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Cholesterol favors the anchorage of human dystrophin repeats 16 to 21 in membrane at physiological surface pressure

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ABSTRACT

Dystrophin (DYS) is a filamentous protein that connects the cytoskeleton and the extracellular matrix via the sarcolemma, conferring resistance to muscular cells. In this study, interactions between the DYS R16–21 fragment and lipids were examined using Langmuir films made of anionic and zwitterionic lipids. The film fluidity was modified by the addition of 15% cholesterol. Whatever the lipid mixture examined, at low surface pressure (20 mN/m) few differences appeared on the protein insertion and the presence of cholesterol did not affect the protein/lipid interactions. At high surface pressure (30 mN/m), the protein insertion was very low and occurred only in zwitterionic films in the liquid-expanded phase. In anionic films, electrostatic interactions prevented the protein insertion outright, and caused accumulation of the protein on the hydrophilic part of the monolayer. Addition of cholesterol to both lipid mixtures drastically modified the protein–lipid interactions: the DYS R16–21 insertion increased and its organization in the monolayer appeared to be more homogeneous. The presence of accessible cholesterol recognition amino-acid consensus sequences in this fragment may enhance the protein/membrane binding at physiological lateral pressure. These results suggest that the anchorage of dystrophin to the membrane in vivo may be stabilized by cholesterol-rich nano-domains in the inner leaflet of sarcolemma.

1. Introduction

Dystrophin (DYS) is a long filamentous muscular protein of 427 kDa (about 175 nm long) [1,2]. Most of this length is due to a long central rod domain composed of 24 spectrin-like repeats (R1 to R24) folded into a triple-α-helical bundle [3]. The biological function of dystrophin is to protect the membrane against stress during elongation/contraction muscular cycles. Its absence leads to the severe disease Duchenne muscular dystrophy (DMD), whereas the milder Becker muscular dystrophy (BMD) may be observed when truncated proteins are expressed [4]. Due to its high levels of STRs, dystrophin is a member of the spectrin-like superfamily [5]. A relationship has been suggested between the structural organization of the repeat domains in these proteins and the membrane's protection against shearing stresses [3]. This function may result from a homogeneous protein distribution along the membrane caused by protein/lipid interactions. Indeed, spectrin is known to bind to the inner membrane leaflet due to interactions with phosphatidylserine [6–9]. Moreover the presence of 10–20% cholesterol (CHOL) facilitates the insertion of spectrin into phosphatidylethanolamine/phosphatidylcholine monolayers that were preformed at low surface pressure, i.e. from 8 to 11 mN/m [10]. Even during its liquid-expanded phase (LE), spectrin binding capacity depends on the fluidity of the monolayer. Our previous studies showed that fragments of the dystrophin rod domain interact with membrane system models through electrostatic and/or hydrophobic forces [11,12].

This study is focused on the interaction of the specific dystrophin fragment made of repeats R16 to R21 and a membrane model. This construct has several specific features that set it apart from other dystrophin fragments. For instance, it contains a hinge (H3) between the repeats R19 and R20 [13,14]. The presence of H3 in microdystrophin improves the capacity for muscle degeneration prevention, while deletion of this hinge leads to the BMD phenotype [15,16]. In addition, DYS R16–21 is coded by exons 42 to 53, which includes the hot-spot between exons 45 and 53 where about one third of BMD patients have mutations [17]. The fragment also mediates specific protein interactions, such as the anchoring of neuronal NO Synthase (nNOS) to the sarcolemma through R16–R17 [18]. The mislocalisation of nNOS in the membrane leads to a decrease of muscular strength in dystrophin-

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deficient *mdx* mice [19]. R16–21 is located in the last part of the protein, where the cysteine-rich domain is known to bind to β-dystroglycan (β-DG) in a membrane protein complex [20] that is located in detergent-resistant membrane. Sarcolemmal CHOL depletion induces the weakening of force contractions through the redistribution of β-DG, and is accompanied by a reduction of β-DG/dystrophin interactions in favor of β-DG/caveolin-3 interactions [21]. All these studies showed that structural or lipid environmental modifications impact muscular activity. Nevertheless, up to now little was known about the ability of DYS R16–21 to interact with lipid membranes.

Here, we address the question of the DYS R16–21 anchorage in mixed lipid films and more particularly of the effect of CHOL on the interaction. Since phosphatidylserine is specifically abundant in the inner leaflet of the sarcolemma membrane, the effect of the polar head charge was tested using a simplified mixture composed of anionic dioleoylphosphatidylserine (DOPS) and zwitterionic dioleoylphosphatidylcholine (DOPC) lipids. Because we chose unsaturated lipids, the monolayers are in the LE phase regardless of the surface pressure. A mix of dipalmitoylphosphatidylcholine (DPPC) and DOPC was used to mimic a detergent-resistant membrane as described [22,23]. Indeed DPPC has been reported to form liquid-condensed (LC) phase domains and also to create separated phases when mixed with DOPC [24]. Cholesterol, also present in the inner leaflet up to 25% [25], is known to modulate membrane fluidity, and particularly at high surface pressure, it acts as a head group spacer because of its molecular orientation in the monolayer. Moreover, CHOL is located mainly in the condensed phase formed by DPPC [26,27]. Langmuir monolayers were used, which allowed for control of charge densities, of fluidity, and of lipid packing according to specific lipid compositions and surface pressures. Protein–lipid interactions and protein structure were determined by combining tensiometric and ellipsometric measurements with atomic force microscopy imaging. We evaluated the influence of 15% CHOL on the interaction of DYS R16–21 with DOPC/DOPS and DOPC/DPPC lipid mixtures. Using two initial surface pressures, two different lipid packings were tested. The highest of these (30 mN/m) is considered to be comparable to physiological membrane pressure [28,29].

Our results show that DYS R16–21 interacts with the lipid monolayer no matter what mixture was used, while the surface pressure has a notable impact on the insertion. In addition, under high surface pressure CHOL was shown to stabilize the fragment at lipid interface, likely through the presence of two cholesterol recognition amino-acid consensus (CRAC) sequences on the protein surface [30]. The presence of CRAC sequences all along dystrophin allows specific protein/lipid interactions that result in homogeneous protein distribution along the membrane. This anchorage could be related to lateral force transmission during muscle elongation/contraction.

2. Experimental sections

2.1. Materials

All lipids were purchased from Avanti Polar Lipids (Alabama, US). The pGEX-4T-1 plasmid vector and GSTrap HP and Sephacryl S-100 columns were bought from GE Healthcare. BL21 bacteria and restriction enzymes were purchased from Ozyme (St.-Quentin-en-Yvelines, France). Purified bovine thrombin came from Stago (Asnières-sur-Seine, France).

2.2. Preparation of recombinant DYS R16–21 protein

The DYS R16–21 fragment begins at residue Ser 1994 and ends with Thr 2686, using the full-length human dystrophin annotation (NCBI NP_003997). In order to improve stability and as previously done for other dystrophin fragments [31,32], we included a three-amino-acid extension in the N-terminus and a seven-amino-acid extension in the C-terminus. The protein was cloned in pGEX-4T-1 then expressed in a protease-deficient *Escherichia coli* BL21 strain as a GST-tagged protein [33]. Cell lysis was obtained by sonication after lysozyme treatment. DYS R16–21 was further purified using GST affinity chromatography. After thrombin proteolysis (50 UI for 2 h at room temperature), the chromatography fractions were separated by salting-out with ammonium sulfate. The target protein was collected at 24% salt saturation and rediluted in TNE buffer (20 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.5). Purification was achieved with size-exclusion chromatography by passing it through a Sephacryl S-100 column (L 2.6 cm × H 60 cm) at 1 mL/min using the same buffer. After purification, the protein was concentrated using a 30 kDa cut-off centrifugal concentrator. Purity was assessed by 10% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), and concentration was determined using a bicinchoninic acid assay with a standard of serum albumin.

Circular dichroism spectra were acquired between 250 nm and 200 nm, with 1.5 μM of protein in TNE buffer at 20 °C (J-815, Jobin-Yvon) using 0.2 cm path-length cells. The α-helical content was calculated from the mean molar residue ellipticity [θ] ($mdeg.cm^2.dmol^{-1}$) at 222 nm [34].

2.3. Tensiometric and ellipsometric measurements

In order to investigate the interfacial properties of the protein with and without lipids, surface pressure and ellipsometric angles were measured in Teflon troughs (4, 8, or 60 mL) at 20 °C. The Wilhelmy method was used to determine the surface pressure using a tensiometer (Nima Technology, Cambridge, UK). Ellipsometric measurements were performed with a conventional polarizer–analyzer null-ellipsometer setup. Briefly, a He–Ne laser (632.8 nm; Melles Griot, Carlsbad, CA) with an angle of incidence of 52.18° (1° away from the Brewster's angle), was used as the light source. Reflecting surface properties were explored by recording the positions of the polarizer and analyzer having the minimal intensity of transmitted light. The analyzer angle was doubled to yield the value of the ellipsometric angle (Δ) [35–37]. Both Δ and the surface pressure (π) were recorded as functions of time. The amphiphilic character was determined by reporting the surface pressure reached at this end of the absorption kinetics as a function of the protein's subphase concentrations (ranging from 0.0001 μM to 1 μM). For lipid/protein experiments, the lipid mixtures (DOPC/DOPS 1:1 molar ratio and DOPC/DPPC 1:1 molar ratio) were prepared in a 2:1 chloroform/methanol solution (v/v) at 0.5 mM, and where used, CHOL was added at 15% (molar ratio). The lipids were gently spread at the air/liquid interface of the TNE subphase at the desired surface pressure. These initial lipid surface pressures (π_i) ranged from 5 to 35 mN/m. At a final concentration of 0.03 μM, the protein was then injected into the subphase just beneath the lipid monolayer. The variations (δΔ and Δπ) induced by protein adsorption at the end of the absorption kinetics were reported as functions of time and π_i. Each experiment was repeated up to four times and means ± standard deviation (SD) were provided for each series. The temperature varied between 19 and 21 °C.

2.4. Atomic force microscopy (AFM) observations

The interfacial film was transferred onto a freshly-cleaved mica plate using the Langmuir–Blodgett technique. The experiment was realized using a 102 cm^2 Langmuir trough equipped with two movable barriers and controlled by a computer (model 601 M, Nima Technology, Cambridge, UK). The transfer was performed at the end of the kinetics and at a constant surface pressure. The dipper speed rate was 1 mm/min. Transferred monolayers were imaged using a PicoPlus atomic force microscope (Agilent Technologies, Phoenix, AZ) equipped with a 10 μm scanner operating in contact mode. Images were acquired under ambient conditions using silicon nitride tips on integral cantilevers (ScienTec) with a spring constant of 0.06 N/m. For each measurement, the set point was adjusted before and during the scanning to minimize