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Dysferlin interacts with calsequestrin-1, myomesin-2 and dynein in human skeletal muscle

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ABSTRACT

Dysferlinopathies are a group of progressive muscular dystrophies characterized by mutations in the gene DYSF. These mutations cause scarcity or complete absence of dysferlin, a protein that is expressed in skeletal muscle and plays a role in membrane repair. Our objective was to unravel the proteins that constitute the dysferlin complex and their interaction within the complex using immunoprecipitation assays (IP), blue native gel electrophoresis (BN) in healthy adult skeletal muscle and healthy cultured myotubes, and fluorescence lifetime imaging–fluorescence resonance energy transfer (FLIM–FRET) analysis in healthy myotubes. The combination of immunoprecipitations and blue native electrophoresis allowed us to identify previously reported partners of dysferlin – such as caveolin-3, AHNAK, annexins, or Trim72/MG53 – and new interacting partners. Fluorescence lifetime imaging showed a direct interaction of dysferlin with Trim72/MG53, AHNAK, cytoplasmic dynein, myomesin-2 and calsequestrin-1, but not with caveolin-3 or dystrophin. In conclusion, although IP and BN are useful tools to identify the proteins in a complex, techniques such as fluorescence lifetime imaging analysis are needed to determine the direct and indirect interactions of these proteins within the complex. This knowledge may help us to better understand the roles of dysferlin in muscle tissue and identify new genes involved in muscular dystrophies in which the responsible gene is unknown.

1. Introduction

Dysferlinopathies, also called dysferlin myopathies, are a heterogenous group of progressive muscular dystrophies characterized by mutations in the DYSF gene (Bashir et al., 1998; Liu et al., 1998). These mutations produce reduced or null expression of the dysferlin protein and can cause a variety of phenotypes (Illa et al., 2007, 2001; Nguyen et al., 2007; Paradas et al., 2009; Passos-Bueno et al., 1995), such as limb girdle muscular dystrophy type 2B (LGMD2B) (Bashir et al., 1996) or Miyoshi myopathy (MM) (Miyoshi et al., 1986). Dysferlin myopathies present with muscle weakness and high levels of creatinin kinase in serum, and the muscle biopsy shows dystrophic features and inflammatory infiltrates. There is no correlation, however, between the kind of mutation and a particular phenotype (Krahn et al., 2009). Moreover, all phenotypes show a similar MRI pattern (Paradas et al., 2010).

Dysferlin is a type II protein with a short extracellular tail. It is expressed in several tissues such as monocytes, heart, placenta, liver, lungs, pancreas and kidney (Liu et al., 1998), but most studies focus on skeletal muscle where it plays an essential role in sarcolemma repair (Bansal and Campbell, 2004). In skeletal muscle, dysferlin is expressed in the sarcolemma, in intracellular vesicles and in t-tubules (Glover and Brown, 2007). The analysis of proteins interacting with dysferlin has been addressed in several studies. Most such studies are based on co-immunoprecipitation assays (IP). Co-IP assays have led to the discovery of several dysferlin

Abbreviations: CALQ1, calsequestrin-1; Cav-3, caveolin-3; DHPR, dihydropyridine receptor; DMD, Duchenne muscular dystrophy; DYNC1LI2, cytoplasmic dynein 1 light intermediate chain 2; FLIM–FRET, fluorescence lifetime imaging–fluorescence resonance energy transfer; FSHD, fascio-scapulo-humeral dystrophy; HAC6, histone deacetylase 6; LGMD1C, limb girdle muscular dystrophy 1C; LGMD2B, limb girdle muscular dystrophy 2B; MM, Miyoshi myopathy; MYOM2, myomesin-2; PTRF, polymerase I and transcript release factor; RRC, receptor recycling compartment; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum; trim72/MG53, tripartite containing motif 72/mitsugumin 53.

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partners, such as AHNAK (Huang et al., 2007), Trim72/MG53 (Cai et al., 2009b), caveolin-3 (Cav-3) (Matsuda et al., 2001), and Vinculin (de Morree et al., 2010). These techniques are used to determine which proteins belong to a certain complex but they do not add information on direct or indirect interactions, as two proteins that co-IP do not necessarily interact directly. One of the proteins reported to belong to the dysferlin is AHNAK. It is an enlargeosome marker that colocalizes with dysferlin at the plasma membrane. In the absence of dysferlin, levels of AHNAK are decreased in the sarcolemma (Zacharias et al., 2011). AHNAK participates in membrane repair, vesicle trafficking, and excitation-contraction coupling in skeletal muscle and other tissues (Borgonovo et al., 2002; Haase et al., 1999; Hohaus et al., 2002). The interaction is calcium-independent and mediated by calpain 3 proteolysis (Huang et al., 2008).

Another known partner for dysferlin is Trim72/MG53, a protein that plays a key role in rapid repair of wounded membrane in a Ca^{2+} independent manner (Cai et al., 2009a). It has been reported that Trim72/Mg53 trafficking to the membrane injury site requires polymerase I and transcript release factor (PTRF) (Zhu et al., 2011). PTRF is a protein related to caveolae that is also present in the dysferlin complex (Cacciottolo et al., 2010). Cav-3 is also present in caveolae (Matsuda et al., 2001) and its absence causes limb girdle muscular dystrophy type 1C (LGMD1C) (Minetti et al., 1998). This dominant muscular dystrophy presents reduced levels of Cav-3 in the muscle biopsy, and in some cases, it also shows a secondary reduction of dysferlin (Matsuda et al., 2001), possibly because mutations in CAV3 gene increase dysferlin endocytosis (Hernandez-Deviez et al., 2008). It has been shown that Cav-3 co-immunoprecipitates not only with dysferlin but also with Trim72/MG53 and that this complex has an important role in membrane repair (Cai et al., 2009b). Dysferlin and Cav-3 are also present in the t-tubules, where they colocalize with the dihydropyridine receptor (DHPR) (Ampong et al., 2005). In addition, dysferlin and cav-3 participate in myoblast fusion to form myotubes (Madaro et al., 2011; De Luna et al., 2004; de Luna et al., 2006), a process that requires integrins. Along the same lines, it has been shown that dysferlin interacts with affixin (beta-parvin) and vinculin. These two proteins link dysferlin to integrins and support the role of dysferlin in sarcolemma integrity (Matsuda et al., 2005; de Morree et al., 2010).

In a very recent work, it was shown that dysferlin interacts with tubulin (Azakir et al., 2010) and histone deacetylase 6 (HAC6) (Di Fulvio et al., 2011). The authors related this interaction to muscle differentiation as they observed that dysferlin promoted alpha-tubulin acetylation, preventing HDAC6 activity by binding both proteins. It has previously been reported that a high acetylation state of alpha-tubulin is needed for the microtubule elongation and stabilization required for myogenesis and myotube elongation (Di Fulvio et al., 2011).

Fluorescence lifetime imaging–fluorescence resonance energy transfer (FLIM–FRET) analysis is the most robust way to demonstrate direct interactions between two proteins (Sun et al., 2011; Badiola et al., 2011; Guardia-Laguarta et al., 2009; Lennon et al., 2003). Using this technique, Lennon et al. (2003) showed that dysferlin interacts directly with annexin A1 and with annexin A2, the only direct interactions for dysferlin described to date. The authors showed that these interactions require the presence of calcium, and also that the interaction of annexin A1 and dysferlin is disrupted after sarcolemmal injury. To further understand the different functions of dysferlin it is necessary to determine if there are other proteins present in the dysferlin complex. Moreover, dysferlin partners are putative genes causing non-filiated dystrophies and could help to understand the phenotypic variability in dysferlinopathies.

The aims of our study were, first, to find new members of the dysferlin interactome by combining three techniques: IP, blue native electrophoresis (BN), and FRET–FLIM analysis; and second, to distinguish whether proteins in the dysferlin complex interact directly or indirectly.

2. Materials and methods

2.1. Primary muscle cultures

Human muscle biopsies were minced and cultured in monolayer following the method described by Dr. Askanas (Askanas et al., 1971) with some modifications (De Luna et al., 2004). The culture media for myoblast proliferation contains 75% DMEM and 25% M199, complemented with 10% Fetal bovine serum (FBS), 10 μg/ml insulin, 2 mM glutamine and penicillin–streptomycin–fungizone, 10 ng/ml epidermal growth factor and 25 ng/ml fibroblast growth factor. Cells were grown to 80% confluence. The culture media was then substituted for one without growth factors and with only 2% of FBS.

To injure cells, myotubes were treated with 0.25 mM SDS for 2 min at 37 °C and fixed with cold methanol for 10 min.

2.2. Biopsies

Blue native experiments were performed using a muscle biopsy from the semitendinous muscle of a healthy patient. The immunoprecipitation assays were performed using muscle biopsies from a vastus externus and a facia lata from healthy patients. All patients signed an informed consent and the project was approved by the Ethics Committee at Hospital de la Santa Creu i Sant Pau (code 12/2009).

2.3. Immunoprecipitation assays

Protein extracts from myotubes and adult skeletal muscle were obtained by sonication in a lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100 and 1× protease inhibitor cocktail (GE Healthcare Lifesciences, Freiburg, Germany) on ice. The lysates were spun down at maximum speed, at 4 °C for 30 min. Protein A sheparose CL-4B (GE Healthcare Lifesciences) was prepared following the manufacturer's instructions and used to pre-clear the homogenates for at least 1 h, at 4 °C while tumbling. After removing the Protein A sepharose, samples were incubated with 20 μg of antibody at 4 °C overnight in a head-over-head shaker. Dysferlin IP was performed using F4 antibody, a monoclonal llama-derived antibody (kindly provided by Dr. Van der Mareel) or commercial antibodies against different dysferlin partners (mouse anti-cytoplasmic dynein (Sigma, Saint Louis, MO) or mouse anti-Trim72/MG53 (AbCam, Cambridge, UK). As a control for non-specific unions, a commercial control isotype antibody was used for dynein and trim72/MG53 IP, and a llama-derived antibody anti-β-amiloid was used as a control of isotype for the F4 antibody (kindly provided by Dr. Van der Mareel) (Huang et al., 2005). The immunocomplexes were precipitated by adding protein A sepharose incubated for at least 2 h at 4 °C while tumbling. The immunoprecipitated complexes were eluted by boiling in sample buffer 2×, and analyzed in a SDS-PAGE at 10% acrylamide treated with silver staining. The bands obtained were sequenced by MS/MS. Each experiment was performed at least twice.

Some of these results were confirmed by Western blot, transferring the proteins to a nitrocellulose membrane (Whatman, Dassel, Germany) that was blocked using the Odyssey blocking reagent (Li-COR, Lincoln, Nebraska, USA). For the immunodetection of dysferlin, the Hamlet I monoclonal antibody (Novocastra, Newcastle Upon Tyne, UK) was used and revealed using a goat-anti-mouse IR_Dye 800 (Li-COR). The membrane was visualized using the Odyssey infrared scanner (Li-COR).

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