Biochemical and Biophysical Research Communications 415 (2011) 263–269

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Proteomic identification of dysferlin-interacting protein complexes in human vascular endothelium

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ARTICLE INFO

Article history:
Received 3 October 2011
Available online 19 October 2011

Keywords:
Dysferlin
Vascular endothelium
Vesicle trafficking
Protein cargo

ABSTRACT

Dysferlin is a membrane-anchored protein known to facilitate membrane repair in skeletal muscles following mechanical injury. Mutations of dysferlin gene impair sarcolemma integrity, a hallmark of certain forms of muscular dystrophy in patients. Dysferlin contains seven calcium-dependent C2 binding domains, which are required to promote fusion of intracellular membrane vesicles.

Emerging evidence reveal the unexpected expression of dysferlin in non-muscle, non-mechanically active tissues, such as endothelial cells, which cast doubts over the belief that ferlin proteins act exclusively as membrane repair proteins. We and others have shown that deficient trafficking of membrane bound proteins in dysferlin-deficient cells, suggesting that dysferlin might mediate trafficking of client proteins. Herein, we describe the intracellular trafficking and movement of GFP-dysferlin positive vesicles in unfixed reconstituted cells using live microscopy. By performing GST pull-down assays followed by mass spectrometry, we identified dysferlin binding protein complexes in human vascular endothelial cells. Together, our data further support the claims that dysferlin not only mediates membrane repair but also trafficking of client proteins, ultimately, help bridging dysferlinopathies to aberrant membrane signaling.

1. Introduction

Dysferlin is a 230 kDa transmembrane protein found to be associated with rare forms of muscular dystrophies, such as limb-girdle muscular dystrophy type 2B [1], Miyoshi myopathy [2,3] and distal myopathy [3], together referred as dysferlinopathies. Patients with dysferlinopathies present mutations in the dysferlin gene that result in reduction or absence of dysferlin expression and various degrees of muscle weakness and wasting. Studies suggest a direct linkage between genetic mutations of the dysferlin gene and impaired membrane repair in skeletal muscle cells [4,5].

Dysferlin is highly homologous to fer-1, which is essential for the fusion of specialized vesicles to the plasma membrane of sperms [6]. Genetic mutations of fer-1 cause the disruption of fusion events and lead to infertile spermatozoa. Since dysferlin is highly homologous to fer-1, it was proposed that dysferlin also mediates membrane fusion events in skeletal muscles [4]. Evidence from experiments performed in myofibers show dysferlin localizes at the plasma membrane and in cytoplasmic vesicles [7]. In addition to dysferlin, two other ferlin proteins have been identified in human, namely myoferlin and otoferlin. The function of myoferlin in human is not clear, although it is required for the normal fusion of myoblasts to myotubes in mice [8]. Otoferlin is strongly expressed in the auditory inner hair cells that are responsible for transmitting auditory information to central nervous system [9]. Mutations of otoferlin cause autosomal recessive deafness in humans [9] and genetic inactivation of the otoferlin gene impairs calcium-dependent exocytosis in mouse auditory inner hair cells and causes profound deafness [10]. Hence, loss of fer-1, dysferlin or myoferlin function can be summarized as leaded to accumulation of vesicles to the cytoplasmic face of the plasmalemma, suggesting that ferlin proteins are essential for trafficking of intracellular vesicles.

Proteins containing C2 domains such as dysferlin are capable of binding to phospholipids and proteins in both calcium-dependent and independent conditions, processes that are crucial for signal transduction, vesicle trafficking and fusion events. Dysferlin has 7 C2 domains: C2A domain binds to phospholipid in the presence of calcium whereas the other six C2 domains have no phospholipid-binding affinity in cultured mouse myoblasts [11]. In addition to phospholipid, dysferlin also interacts with muscle proteins including AHNAK [12], annexins A1 and A2 [13], caveolin 3 [14,15], calpain 3 [16], and affixin [17] in sarcolemma. These

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doi:10.1016/j.bbrc.2011.10.031

dysferlin-interacting proteins participate in trafficking and fusion of vesicles to the plasma membrane that are necessary for dysferlin-dependent membrane repair. Dysferlin also contains 2 highly conserved DysF domains, namely DysF N in the N-terminus and DysF C in the C-terminus, with unknown function [18].

Although most of the dysferlin research has been focused on understanding how dysferlin mediates membrane repair in ruptured skeletal muscles, increasing evidence demonstrates dysferlin presence in other tissues, such as brain, heart, kidney, liver, lung, placenta and pancreas in humans [1]. We recently found that dysferlin is present in the endothelium and silencing of dysferlin gene results in near complete loss of platelet endothelial cell adhesion molecule 1 (PECAM-1), a membrane-bound adhesion molecule [19]. Although the association of dysferlin and PECAM-1 is poorly understood, we hypothesize that dysferlin regulates the trafficking of vesicles that contain membrane-bound PECAM-1 as protein cargo. This hypothesis is further suggested by our previous findings on myoferlin in which it regulates the expression, stability and function of vascular endothelial growth factor receptor 2 and angiopoietin receptor, tie-2, likely by mediating the traffic of vesicles with these receptors in endothelial cells [20–22]. Using a green fluorescent protein (GFP)-tagged version of dysferlin, we provide further evidence that dysferlin behaves as an intracellular trafficking protein in live cells. Also, by performing glutathione S-transferase (GST) pull-down assay coupled to mass spectrometry, we identified novel dysferlin-interacting protein complexes, although our data suggest that dysferlin is not a highly 'promiscuous' protein. Therefore, our data support the concept that dysferlin indirectly regulates the membrane expression of proteins by trafficking the lipid patches/vesicles that contain membrane proteins, rather than binding directly the cargo proteins.

2. Materials and methods

2.1. Cell culture

COS-7 cells were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin (Sigma) as published previously [23]. Human umbilical vein endothelial cells (HUVEC) were maintained in EndoGRO™ VEGF complete media kit (Millipore). Both cell lines were cultured in an incubator with 5% CO_2 at 37 °C.

2.2. Plasmid transfection and live cell imaging

COS-7 cells were transfected with human GFP-tagged dysferlin plasmid (a kind gift from Dr. Kate Bushby) as previously described [24]. Live cell images were captured about 8 h using a Leica AOBS SP2 laser scanning inverted confocal microscope (Leica) incorporated with high-resolution Leica Plan-Apocrhromat oil immersion lens adapted to humidified CO_2 incubator. The fluorescence of GFP was excited at 488-nm argon/krypton laser and its emission was detected at 515–540-nm. Time-lapse imaging was acquired and analyzed using Leica Confocal Software TCS SP2. The movement of GFP-tagged dysferlin in the cytosol was analyzed by tracking GFP fluorescence as a function of time.

2.3. Construction of GST-dysferlin plasmids

Four GST-dysferlin fusion proteins were generated, corresponding to Dysf 1 (amino acids 2-515), Dysf 2 (amino acids 1135-1965) and Dysf 3 (amino acids 874-1101). The human GFP-tagged dysferlin plasmid was used as a template for polymerase chain reaction (PCR) to amplify the desired sequences. Restriction sites, EcoRI and NotI (Invitrogen), were respectively added in the N-terminus and C-terminus of the primer sequences in frame with the glutathione S-transferase (GST) gene. The purified PCR fragments were digested with EcoRI and NOT1 and subsequently cloned into EcoRI/NOT1-digested pGEX-4T-3 (GE Healthcare) and transformed in *Escherichia Coli* JM109. Gene sequencing was performed by the University of British Columbia's NAPS facility.

2.4. Expression of GST-dysferlin recombinant proteins

GST and truncated GST-dysferlin fusion proteins were expressed in *E. Coli* BL21 strain. Briefly, GST and GST-dysferlin plasmids were transformed and amplified separately in 5 ml LB media (with 100 μg/ml of ampicillin) overnight at 37 °C at 250 rpm. Overnight cultures were then added into distinctive 250 ml LB media (with 100 μg/ml of ampicillin) and incubated at 37 °C with vigorous shaking (250 rpm) until reaching the optical density at 600 nm of 0.3–0.5. Next, 1 mM isopropyl β-D-1-thiogalactopyranoside was added to induce the expression of GST-dysferlin fusion proteins for 6 h at 34.5 °C, 220 rpm. Bacterial cultures were centrifuged for 30 min at 5100 rpm at 4 °C. Supernatants were removed and the pellets were resuspended in total of 10 ml TrisHCl/NaCl (50 mM TrisHCl pH 8, 150 mM NaCl) buffer. The suspensions were then centrifuged for 10 min at 3000 rpm at 4 °C and supernatants were removed. Pellets were homogenized for 2 min on ice in10 ml of TrisHCl/NaCl buffer with 1 mM phenylmethanesulfenylfluoride, 100 μg/ml of lysozyme, 5 mM of dithiothreitol (DTT) and 1.5% of N-laurylsarcosyl salt. Triton X-100 was added to a final concentration of 2% v/v and centrifuged for 30 min at 13200 rpm at 4 °C. Supernatants were collected, aliquot and stored at −80 °C until use.

2.5. Purification of GST fusion proteins and GST-pulldown assays

GST fusion proteins were purified as previously described [25]. For GST-dysferlin pulldown assays, human umbilical vein endothelial cells (HUVEC) were lysed and homogenized to collect protein extract. After centrifugation, HUVEC lysates were incubated with GST or GST-dysferlin coated glutathione beads overnight at 4 °C. Another set of GST or GST-dysferlin coated beads was incubated with lysis buffer alone (control). Beads were then washed and eluted with 15 mM reduced glutathione. The eluted fractions were combined for each condition and sent to University of Victoria's Genome BC Proteomics Centre for mass spectrometry. Beads were used to run a Coomassie gel to visualize the band patterns.

2.6. Anti-GST Western blotting

Proteins were separated by SDS–PAGE and then transferred to nitrocellulose membrane electrophoretically. Membranes were then incubated with rabbit GST antibody (Santa Cruz biotechnology) at 1:1000 in tween TBS (TTBS) with 10% casein overnight at 4 °C. After subsequent washes, blots were incubated with goat anti-rabbit 700 (Rockland) and scanned with an odyssey scanner (Licor).

2.7. In-solution trypsin digestion, liquid chromatography and mass spectrometry

Protease inhibitors were first removed from the protein samples using Oasis HLB columns (Waters). Proteins were eluted with 50% acetonitrile/0.1% formic acid and vacuum centrifuged. Each sample was incubated with 25 mM ammonium bicarbonate and 200 mM DTT (Sigma) and then alkaylated with 200 mM iodoacetamide (Sigma) at 37 °C. Additional DTT was added to quench residual DTT in the samples. Samples were digested with 20 ng/μl porcine trypsin solution (Promega) for 16 h at 37 °C and then subjected to Oasis HLB columns to desalt and concentrate.