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Summary

Characterization of the sarcolemma in Limb-Girdle Muscular Dystrophy

Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of slowly progressive muscular dystrophies (MD) with common features such as hyperCKemia and skeletal muscle weakness. Mutations in the dysferlin gene cause a spectrum of adult-onset muscular dystrophies including LGMD 2B, Miyoshi myopathy (MM) and distal anterior compartment myopathy (DACM) commonly referred to as dysferlinopathies. Human skeletal muscle from LGMD 2B patients displays known dystrophic features such as variations in fiber size and centralized nuclei, but also a rather unusual accumulation of subsarcolemmal vesicles. Dysferlin is a transmembrane protein and its subcellular localization is predominantly allocated to the plasma membrane, but also to developing T-tubules and intracellular vesicles. At the sarcolemma, dysferlin was shown to be crucial for efficient membrane repair, although it has been proposed to have a role in many other cellular functions such as vesicle fusion and intracellular trafficking, cell adhesion, immune response, and metabolism. LGMD 1C, another type of LGMD, is caused by mutations in the caveolin-3 (cav-3) gene. Cav-3 is the muscle-specific isoform of the caveolin protein family which represent, together with the cavin protein family, the major structural and functional components of caveolae. Caveolae are plasma membrane invaginations of 40-80 nm size and belong to a specialized type of membrane microdomains referred to as lipid rafts. Lipid rafts are highly dynamic membrane domains, distinct in their protein and lipid composition, with multiple roles in cellular signaling, endocytosis, lipid and cholesterol metabolism, and mechanosensing. Cav-3 is known to regulate dysferlin localization and rate of endocytosis at the plasma membrane. However, it remains to be elucidated how both proteins are functionally linked to each other. Recently, recessive mutations in the anoctamin gene could also be related to LGMDs. As seen for dysferlinopathies, *ANO5* mutations can lead to a LGMD or MM clinical phenotype. Anoctamin-5 is a member of the anoctamin protein family, which is characterized by distinct structural features. Anoctamins are thought to contain eight transmembrane domains and to function as calcium-activated chloride channels. Interestingly, the clinical picture of anoctaminopathies display various similarities compared to dysferlinopathies.

In order to reveal the molecular mechanisms underlying LGMD and to investigate the putative interactions of dysferlin, cav-3, and ano5, experiments on primary skeletal muscle

cell lines with disease-related mutations in *DYSF*, *CAV3,* and *ANO5* have been analyzed in this study. Cell lines were characterized by immunofluorescence labelling and western blot analysis. To investigate the impact of mutations in genes causing LGMD on the morphology of the sarcolemma, transmission electron microscopy (TEM) analysis of differentiated myotubes was done. Endocytic structures at the plasma membrane such as caveolae, subsarcolemmal vesicles, and clathrin-coated pits were quantified. To explore the functional association of dysferlin and cav-3, investigations on membrane rafts from normal and LGMD 2B myotubes were done biochemically by purification of detergent-resistant membranes (DRMs). In order to reveal new dysferlin functions an immunopurification assay was established. Intracellular dysferlin-containing vesicles were isolated by subcellular fractionation followed by immunopurification (IP). Characterization of these vesicles was done on ultrastructural level by TEM analysis and on protein level by liquid chromatography–mass spectrometry (LC-MS) analysis.

Immunolabeling studies revealed that dysferlin and cav-3 are partially colocalized in vesicular structures at the plasma membrane of human primary myotubes. Biochemical purification of DRMs from differentiated myotubes showed that dysferlin is associated with lipid rafts linked to the actin-cytoskeleton. TEM analysis of myotubes derived from skeletal muscle of LGMD patients revealed alterations of caveolae abundance at the plasma membrane correlating with the disease-causing mutations. Interestingly, myotubes from patients with *CAV3* mutations displayed morphological intact caveolae at the sarcolemma. Ultrastructural studies on the subcellular localization of dysferlin showed plasma membrane, but also intracellular localization in cytosolic vesicles. IP of intracellular dysferlin-containing vesicles revealed the presence of cav-3 and other known dysferlin interacting partners such as annexin A1 and A2, ahnak, and polymerase I and transcript release factor (PTRF). These vesicles contained a subset of approximately 500 proteins detected by LC-MS, which might represent vesicular structural proteins, vesicle cargo, and putative new dysferlin interaction partners.

Results from this study lead to the conclusion that caveolae play a crucial role in the context of LGMD. Especially for LGMD 2B, caveolae abundance at the plasma membrane in human primary myotubes can be correlated with LGMD-causing mutations. Dysferlin and cav-3 seem to be closely linked on structural as well as on functional level. In this study, we propose that dysferlin function during membrane repair is mediated by actin-linked lipid raft formation, although the underlying molecular mechanisms remain to be elucidated. Our results confirm that dysferlin is localized in cytosolic vesicles, which are involved in multiple cellular processes such as vesicle transport, endo- and exocytosis, cell-adherence, and lipid raft dynamics. Taken together, the close association of dysferlin and cav-3 on cellular functions, especially on caveolae, could be demonstrated in this study.