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Caveolin-1 in Müller Glia Exists as Heat-Resistant, High Molecular Weight Complexes

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Abstract

Caveolin-1 (Cav1), the core structural and scaffolding protein of caveolae membrane domains, is highly expressed in many retinal cells and is associated with ocular diseases. Cav1 regulates innate immune responses and is implicated in neuroinflammatory and neuroprotective signaling in the retina. We have shown that Cav1 expression in Müller glia accounts for over 70% of all retinal Cav1 expression. However, the proteins interacting with Cav1 in Müller glia are not established. Here, we show that immortalized MIO-M1 Müller glia, like endogenous Müller glia, highly express Cav1. Surprisingly, we found that Cav1 in MIO-M1 cells exists as heat-resistant, high molecular weight complexes that are stable after immunoprecipitation (IP). Mass spectrometric analysis of high molecular weight Cav1 complexes after Cav1 IP revealed an interactome network of intermediate filament, desmosomes, and actin-, and microtubule-based cytoskeleton. These results suggest Cav1 domains in Müller glia act as a scaffolding nexus for the cytoskeleton.

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Keywords

Caveolin-1; Caveolae; Müller glia; Immunoprecipitation; Mass spectrometry; Cav1 complexes; Cavin1/PTRF

1 Introduction

Caveolin-1 (Cav1), the signature structural protein of caveolae is known to play a role in cell signaling, lipid metabolism, endocytosis, and mechanotransduction [3, 24, 25]. Cav1 has been associated with ocular neuroinflammation, age-related macular degeneration, diabetic retinopathy, blood-retinal barrier function, and primary open-angle glaucoma [7, 9–11, 30]. It is expressed in a variety of cell types including retinal pigment epithelium (RPE) and choroidal and retinal vascular endothelium [8, 11]. Furthermore, Cav1 is highly expressed in Müller glia [11, 21, 26], and its expression correlates with Müller glia differentiation [8, 23]. We have shown that neuroretinal Cav1 expression overwhelmingly accounts for the majority of Cav1 expression in the retina, with most Cav1 being localized to Müller glia [11]. While Cav1 is highly expressed in Müller glia, its function in these cells is only beginning to be appreciated. Cav1 regulates cytokine secretion and immune cell influx into the retina, as global Cav1 knockout (KO) simultaneously suppresses cytokine secretion and increases immune cell influx into the retina [19]. Neuroretinal deletion of Cav1 suppresses both proinflammatory cytokine secretion and immune cell infiltration into the retina [11], further confirming a role for Müller glial Cav1 in innate immune responses. Cav1 is significantly upregulated in Müller glia in autoimmune uveitis [12]. The role of Cav1 as an immune modulator is likely cell-context dependent as it can either promote or suppress the inflammatory response depending on the cell type examined [19, 31, 32]. Müller glia express toll-like receptors (TLRs), whose activities can be enhanced or suppressed by interaction with Cav1 [22, 31]. Further, we and others have shown Cav1 to be an important regulator of blood-retinal barrier (BRB) function [2, 18, 19, 33].

While Müller glia abundantly express Cav1, it is unclear what proteins interact with Cav1 in these cells. The aim of this study was to identify the Cav1 interactome in MIO-M1 Müller glia by immunoprecipitating Cav1 and analyzing immune complexes by mass spectrometry. We show that Cav1 in MIO-M1 Müller glia exists as high molecular weight aggregates, which are resistant to heating in reducing SDS-PAGE buffer. Mass spectrometric analysis of Cav1 complexes revealed a network of cytoskeletal proteins that interact with Cav1.

2 Materials and Methods

2.1 Cell Lines and Culture Conditions

Immortalized MIO-M1 Müller glia were cultured in DMEM (1X) + GlutaMax™-I (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Prostate cancer cells (PC3s) were cultured in F-12K medium (ATCC Cat#: 30–2004). Retinal microvascular endothelial cells (RMECs) were cultured in Endothelial Cell Basal Medium-2 (Lonza Cat#: CC-3156), supplemented with 2% FBS, human fibroblast growth factor, vascular endothelial growth factor, insulin-like

growth factor-1, ascorbic acid, gentamicin-amphotericin B hydroxycortisone, and human endothelial growth factor. Cells were maintained in a humidified atmosphere of 5% CO_2, at 37 °C.

2.2 Western Blotting

Cells were lysed in buffer containing 120 mM octylglucoside, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 0.5 mM EDTA, 0.1% Triton X-100 and protease inhibitor cocktail. Lysates were cleared by centrifugation and protein concentration was determined using a BCA reagent (ThermoFisher Scientific). Equal amounts of proteins were separated by reducing SDS-PAGE and were transferred to nitrocellulose membranes. Membranes were blocked for 1 h in 5% BSA and were probed with primary antibodies of choice: rabbit anti-Cav1 (Cell Signaling Technology, cat. #3267, 1:1000), rabbit anti-PTRF (Abcam, 1:1000) and mouse anti-β-actin (Abcam, 1:5000). Primary antibodies were detected using Horseradish peroxidase (HRP)-conjugated secondary antibodies. To visualize protein bands after SDS-PAGE electrophoresis, gels were stained for 1 h with SimplyBlue™ Safestain (Thermofisher Scientific). Western blot images were captured using the In Vivo F-Pro imaging system.

2.3 Immunoprecipitation

Immunoprecipitation was performed using the Dynabeads™ Protein G Immunoprecipitation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, Cav1 primary antibody (Cell Signaling Technology, cat. #3267) was conjugated to magnetic beads for 20 min at room temperature (RT). Then, the beads-antibody conjugate was incubated with equal amounts of protein lysates for 20 min at RT. After several rounds of washing, beads were resuspended in Laemmli buffer and the immunoprecipitates were separated by reducing SDS-PAGE without heating. The gel was stained for 1 h using SimplyBlue™ SafeStain and washed several times with water. Visible Cav1 complexes were excised and used for mass spectrometry analysis (Fig. 1). A portion of the immune complexes was boiled in Laemmli buffer, separated by reducing SDS-PAGE, and transferred to nitrocellulose membranes for Western blotting.

2.4 Mass Spectrometry

Cav1 immunoprecipitates were separated by SDS-PAGE, gel-stained, and visible high molecular weight Cav1 complexes excised for mass spectrometry (Fig. 2d). Proteins were subjected to the FASP (filter-aided sample preparation) protocol [34] and digested overnight with Sequencing Grade Modified Trypsin (Promega, V5111) at 37 °C in 40 mM NH_4HCO_3. Peptides were desalted, concentrated, and loaded onto C18 sequencing columns (Acclaim™ PepMap™ 100 C18, ThermoFisher). Peptide elution was performed using a 90-min acetonitrile gradient for label-free quantification. Eluted peptides were analyzed by LC-MS/MS analysis using a Thermo Lumos Fusion tribrid Orbitrap mass spectrometer, coupled to an Ultimate 3000 RSLC nano ultra-high-performance liquid chromatography (UHPLC) system. Proteins were identified by Proteome Discoverer 2.4, with SEQUEST as the search engine. Protein identification required the detection of at least two peptides per protein. STRING open-source software was used to identify protein networks.

3 Results

3.1 Cav1 in MIO-M1 Müller Glia Exist as High Molecular Weight Complexes

To determine the protein composition of Cav1 complexes, we first evaluated the expression of Cav1 in authenticated MIO-Müller glia and compared the expression to retinal microvascular endothelial cells (RMEC), which also abundantly express Cav1 [29]. Our results show that MIO-M1 cells, like endogenous Müller glia, abundantly express Cav1 (Fig. 2). To our surprise, we observed that the majority of Cav1 in MIO-M1 cells exist as high molecular weight complexes (Fig. 2a) which are resistant to heating in reducing SDS-PAGE buffer (Fig. 2b). However, upon heating at 98 °C for 10 min, Cav1 complexes dissociate and migrate as monomers on reducing SDS-PAGE (Fig. 2c). High molecular weight Cav1 complexes in MIO-M1 cells remain relatively stable after Cav1 immunoprecipitation (Fig. 2d). On the contrary, Cav1 in RMECs migrates predominantly as monomers on SDS-PAGE gels without heating. While most Cav1 complexes in MIO-M1s remain stable after heating at 70 °C for 10 min, the small fraction of aggregated Cav1 in RMECs dissociates to monomers after heating at 70 °C. These data suggest that different proteins may be interacting with and stabilizing Cav1 complexes in MIO-M1 cells.

3.2 Proteins Associated with Cav1 Complexes Are Involved with the Cell Cytoskeleton

Next, we were interested in identifying the proteins that interact with Cav1 complexes in MIO-M1 cells. To identify the protein composition of Cav1 complexes, we immunoprecipitated Cav1 from MIO-M1 cells and analyzed the high molecular weight complexes by mass spectrometry. We analyzed the same complexes from three replicate samples (Fig. 2d). Thirty-three proteins were found to associate with Cav1 complexes after mass spectrometry. Interestingly, the majority of these proteins including β-actin (ACTB), myosin, vimentin (VIM), plectin (PLEC), and nestin (NES) have been shown to play a role in the cytoskeleton and cell-cell junction structure [4].

4 Discussion

In this study, we sought to identify the protein composition of Cav1 complexes in MIO-M1 cells. We show for the first time that Cav1 in MIO-M1 cells exists as heat-resistant, high molecular weight aggregates, which interact with important cytoskeletal proteins. Cav1 is the major protein component of caveolae [5, 6, 27], flask-shaped plasma membrane invaginations whose formation requires another protein called Cavin1 or PTRF (Polymerase I and Transcript Release Factor) [14, 20]. It is currently unclear why Cav1 exist in this aggregated form in MIO-M1 cells, as opposed to the monomeric form in RMEC cells. However, we speculate that the absence of Cavin1/PTRF expression in MIO-M1 cells may provide an explanation for this phenomenon. Cavin1/PTRF stabilizes Cav1 in multiple tissues, as Cavin1/PTRF deficiency downregulates Cav1 protein expression [20]. However, Cav1 is stably expressed in prostate cancer (PC3) cells without Cavin1/PTRF [1, 14, 15] in functional domains described as “Cav1 scaffolds” [16, 17]. Further, multiple studies have shown that approximately 150 Cav1 molecules are required per caveola formed [25]. However, during caveolae biogenesis, Cav1 form oligomers of 12–16 Cav1 molecules, which associate with lipid rafts in the Golgi and adopt detergent-resistant properties [13,

28], similar to Cav1 on the plasma membrane. Thus, it is intriguing to speculate that in the absence of Cavin1/PTRF in MIO-M1 Müller glial cells, these Cav1 high molecular weight oligomers represent non-caveolar Cav1 scaffolds previously described [16, 17]. Therefore, the expression of Cavin1/PTRF in MIO-M1 cells likely provides a mechanism to biochemically resolve Cav1 scaffolds (Fig. 3).

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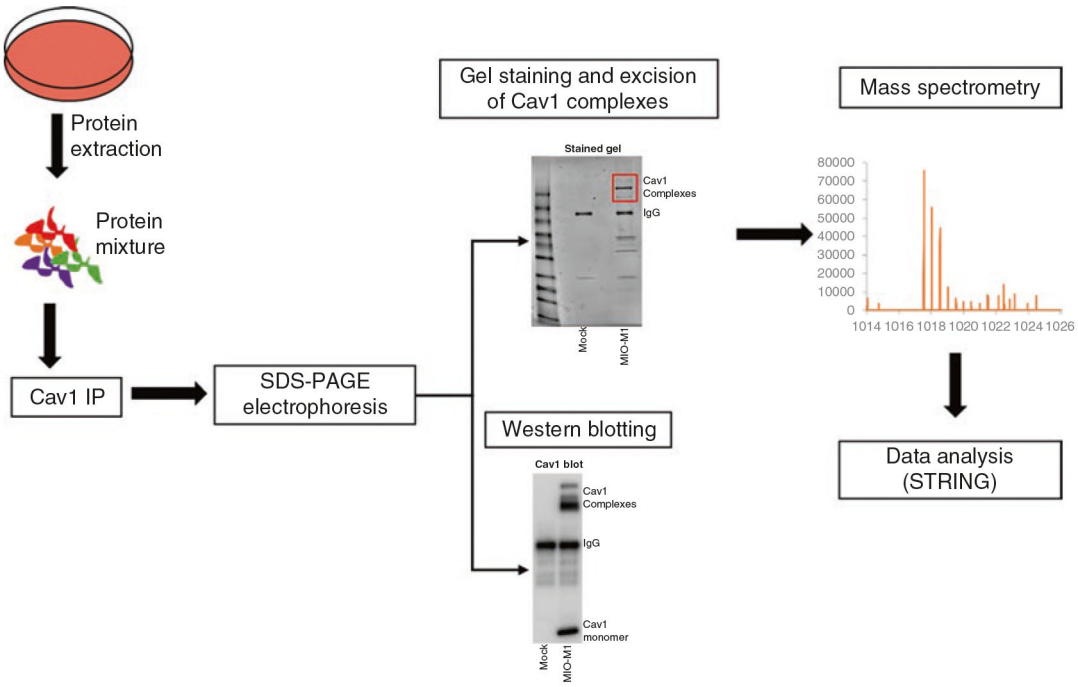

Fig. 1.

Schematic overview of Cav1 immunoprecipitation and mass spectrometry workflow. Proteins were extracted from untreated MIO-M1 Müller glia and were immunoprecipitated using Cav1 primary antibodies. Cav1 immunoprecipitates were separated by reducing SDS-PAGE without heating. Gels were stained for 1 h and visible Cav1 complexes were excised and analyzed by mass spectrometry. A portion of the immunoprecipitate was transferred to nitrocellulose membranes to evaluate interactions by Western blotting

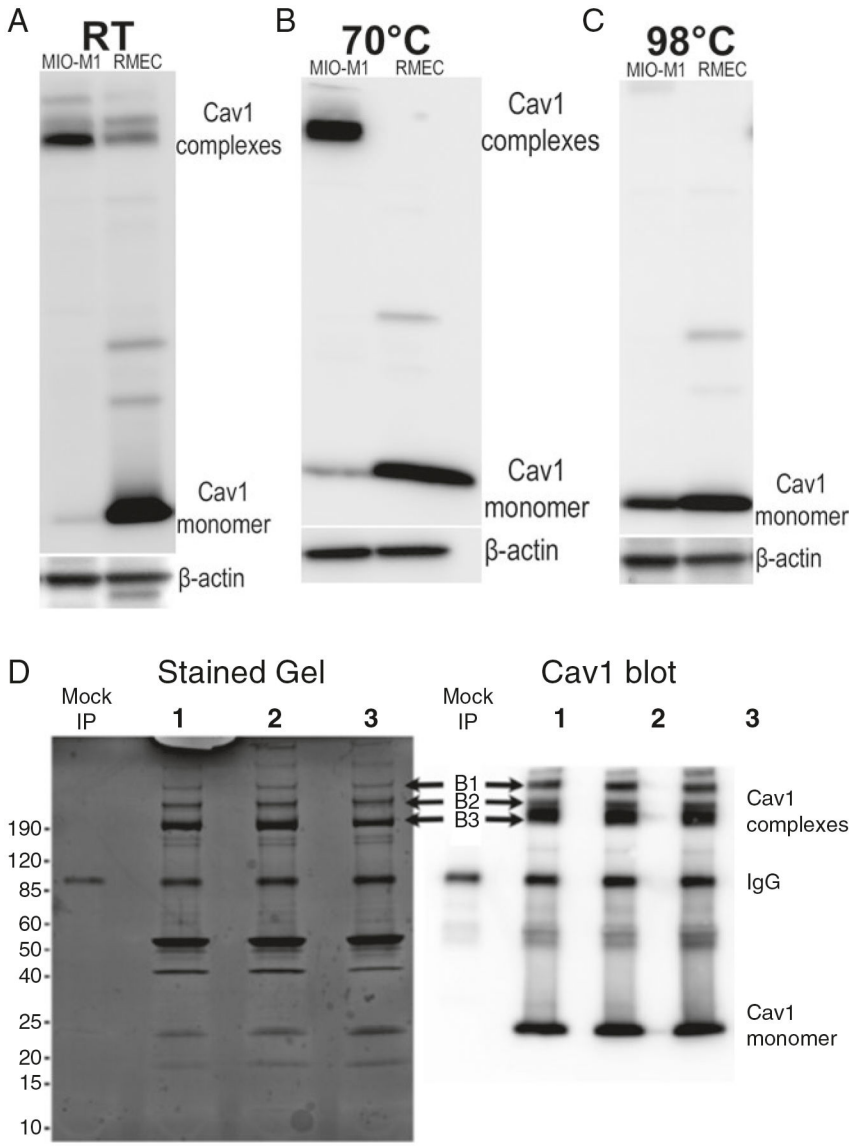

Fig. 2.

Cav1 in MIO-M1 cells exists in heat-resistant, high molecular weight complexes. (**a**) Representative Western blots showing expression of Cav1 in MIO-M1 and RMEC cells. MIO-M1 cells, like endogenous Müller glia, abundantly expresses Cav1. Interestingly, Cav1 in these cells exists in high molecular weight complexes. (**b**) Cav1 complexes are resistant to heating at 70 °C for 10 min in reducing SDS-PAGE. On the contrary, Cav1 in RMEC cells was mostly monomeric both at RT and after heating at 70 °C for 10 min. (**c**) Representative Western blots showing that Cav1 migrates as a monomer in reducing SDS-PAGE buffer only after rigorous heating at 98 °C for 10 min. (**d**) Stained gel and representative Western blot showing stable Cav1 complexes after Cav1 immunoprecipitation. Bands from the stained gel (B1, B2, and B3 indicated by arrows) were excised and analyzed by mass spectrometry

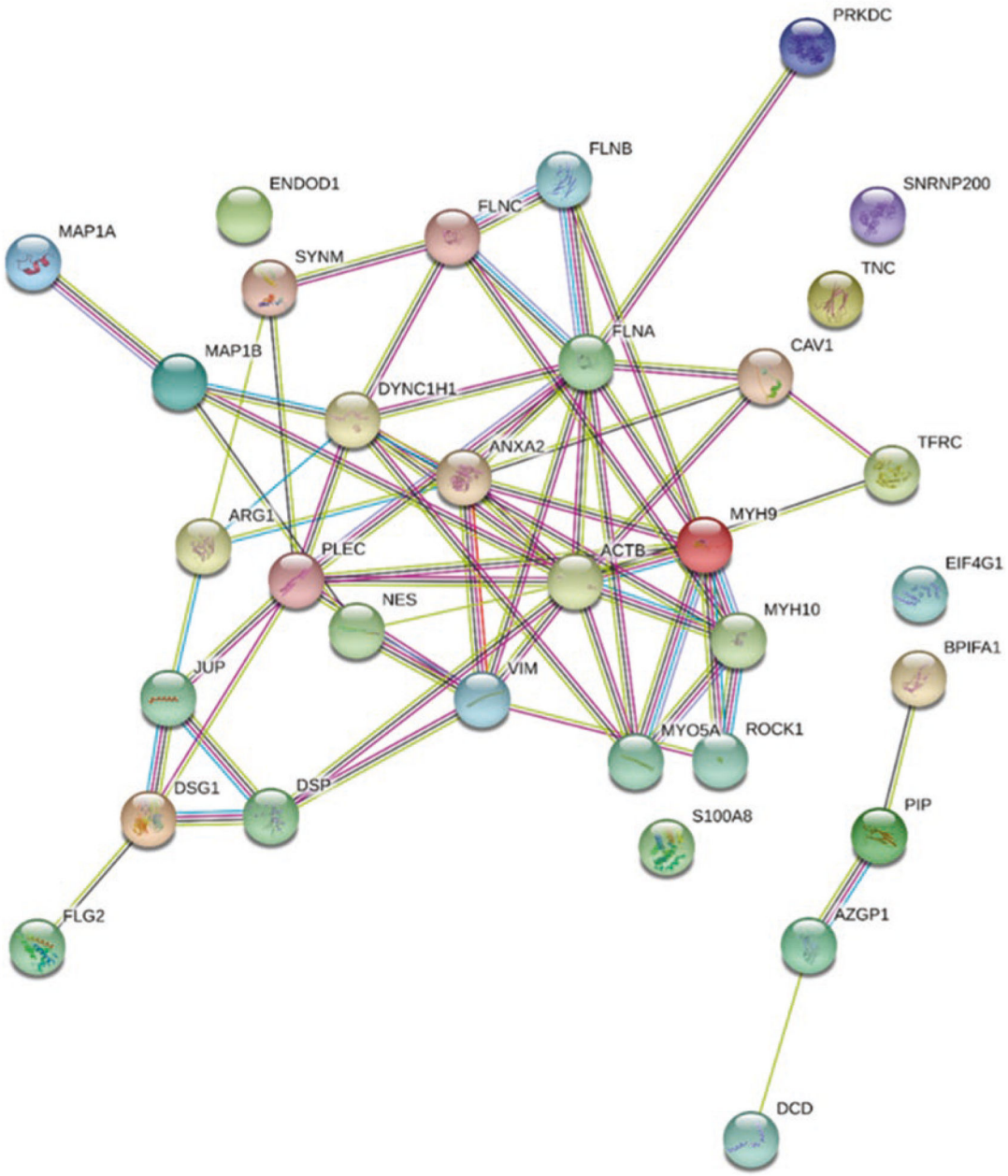

Fig. 3.

Proteins that interact with Cav1 complexes are associated with the cell cytoskeleton. Cav1 protein-protein interaction by STRING analysis. Cav1 complexes were analyzed by mass spectrometry and STRING open-source database was used to identify protein-protein interactions. A total of 33 proteins were found to interact with Cav1 complexes, most of which play a role in the cell cytoskeletal architecture and include β-actin (ACTB), myosin (MYO5A), vimentin (VIM) plectin (PLEC), and nectin (NEC)