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Chorein, the protein responsible for chorea-acanthocytosis, interacts with β-adducin and β-actin ☆

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ABSTRACT

Chorea-acanthocytosis (ChAc) is an autosomal, recessive hereditary disease characterized by striatal neurodegeneration and acanthocytosis, and caused by loss of function mutations in the vacuolar protein sorting 13 homolog A (*VPS13A*) gene. *VPS13A* encodes chorein whose physiological function at the molecular level is poorly understood. In this study, we show that chorein interacts with β-adducin and β-actin. We first compare protein expression in human erythrocyte membranes using proteomic analysis. Protein levels of β-adducin isoform 1 and β-actin are markedly decreased in erythrocyte membranes from a ChAc patient. Subsequent co-immunoprecipitation (co-IP) and reverse co-IP assays using extracts from chorein-overexpressing human embryonic kidney 293 (HEK293) cells, shows that β-adducin (isoforms 1 and 2) and β-actin interact with chorein. Immunocytochemical analysis using chorein-overexpressing HEK293 cells demonstrates co-localization of chorein with β-adducin and β-actin. In addition, immunoreactivity of β-adducin isoform 1 is significantly decreased in the striatum of gene-targeted ChAc-model mice. Adducin and actin are membrane cytoskeletal proteins, involved in synaptic function. Expression of β-adducin is restricted to the brain and hematopoietic tissues, corresponding to the main pathological lesions of ChAc, and thereby implicating β-adducin and β-actin in ChAc pathogenesis.

1. Introduction

Chorea-acanthocytosis (ChAc; OMIM ID: 200150) is a rare, hereditary, neurodegenerative disorder characterized by adult-onset chorea and acanthocytosis in erythrocytes [1]. ChAc patients also develop psychiatric symptoms (including oral self-mutilation), epilepsy, peripheral neuropathy, and myopathy [2,3]. The main neuropathological feature of ChAc is neurodegeneration of the striatum [4,5]. Using positional cloning, we and others, previously identified ChAc causative mutations in the vacuolar protein sorting 13 homolog A (*VPS13A*) gene [6,7], finding the mutations widely distributed throughout the gene [8,9]. *VPS13A* is located on human chromosome 9q21, spanning an approximately 250-kb region, and encoding chorein, a 360-kDa protein [6]. The *Saccharomyces cerevisiae* homolog, *VPS13p*, is involved in trafficking of membrane proteins from the trans-Golgi network to the prevacuolar compartment [10], and an ortholog of *Vps13p*, the mutant *TipC* gene in *Dictyostelium discoideum*, shows aberrant cell-sorting behavior [11]. In addition, the *Tetrahymena thermophila* VPS13A (TtVPS13A) protein is required for phagocytosis [12], and in PC12 cells, chorein is involved in dopamine release [13]. Altogether, these findings suggest that chorein is involved in intracellular transport and vesicle-mediated sorting.

Chorein is highly expressed in mouse testis, kidney, spleen, and brain [14]. Subcellular distribution studies indicate chorein is localized to the Golgi apparatus in the microsomal fraction, and to dense-core vesicles in synaptosomes [13,14]. Chorein is also present in membrane fractions of erythrocytes [8,9]. Abnormalities of erythrocyte membrane proteins and the cytoskeleton are observed in ChAc patients [15,16]. As human autopsy tissue from ChAc patients is limited, we used gene targeting to develop a mouse ChAc model that encodes the human disease mutation [17]. Chorein functional deficiency led to acanthocytosis and apoptosis of mouse striatal neurons, yet the physiological function of chorein at the molecular level is poorly understood.

Abbreviations: ChAc, chorea-acanthocytosis; *VPS13A*, vacuolar protein sorting 13 homolog A; HEK293, human embryonic kidney 293; TtVPS13A, *Tetrahymena thermophila* VPS13A; MEME, Minimum Essential Medium Eagle; FBS, fetal bovine serum; CBB, Coomassie Brilliant Blue; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PMF, peptide mass fingerprint; PBS-T, PBS containing 0.1% Tween-20.

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In this study, we performed a comparative proteomic analysis of human erythrocyte ghosts, erythrocytes that are devoid of cytoplasmic contents but maintain membrane and cytoskeletal elements. Protein levels of β-adducin isoform 1 and β-actin were markedly decreased in erythrocyte membranes from a ChAc patient. To investigate the association between chorein, β-adducin, and β-actin, we established human embryonic kidney 293 (HEK293) cells stably overexpressing chorein. Co-immunoprecipitation (Co-IP) and reverse co-IP assays showed that β-adducin isoforms 1 and 2, and β-actin, interact with chorein. Adducin and actin are both components of the erythrocyte membrane skeleton and involved in synaptic function [18,19]. Immunocytochemical analysis using HEK293 cells was also performed to confirm the biochemical findings. Immunoblot analysis of β-adducin using ChAc model mice was also performed.

2. Materials and methods

2.1. Human samples and preparation of human erythrocyte ghosts

Erythrocytes from a ChAc patient, a ChAc mutant carrier, and a healthy control were used [2]. Genetic testing of the patient and mutant carrier identified a homozygous and heterozygous nonsense mutation (c.3889C > T), respectively, in *VPS13A*. All participants gave informed consent and the study was approved by the Institutional Review Board of Kagoshima University. Erythrocyte ghost samples were prepared as described previously [9].

2.2. Cell culture and generation of stably transfected cell lines

HEK293 cell lines, obtained from the Health Science Research Resources Bank (Osaka, Japan) were grown in Minimum Essential Medium Eagle (MEME) (Sigma, St. Louis, MO, USA) supplemented with 10% (w/v) fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 50 U/mL penicillin, and 50 μg/mL streptomycin (both Nacalai Tesque, Inc., Kyoto, Japan). Cells were grown in an incubator at 37 °C with a humidified atmosphere of 5% CO_2.

To generate stable cell lines overexpressing chorein, HEK293 cells were transfected with pCMV6 vector, containing an ORF clone of *Homo sapiens VPS13A* (transcript variant A) with a Myc-DDK tag at the C-terminus and a neomycin selectable cassette (Origene, Rockville, MD, USA), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After selection with 500 mg/mL G418 (Nacalai Tesque, Inc.) for >2 weeks, mono clones were selected by single-cell dilution and expansion. Chorein overexpression was confirmed by immunoblot analysis and immunofluorescence (data not shown).

2.3. ChAc model mice and mouse brain preparation

ChAc model mice encoding a human disease mutation with deletion of exons 60–61 in *VPS13A*, were produced by gene targeting as previously described [17]. ChAc model mice were backcrossed for at least 10 generations on a C57BL/6J background (CLEA JAPAN, Tokyo, Japan).

Brain tissue was obtained from C57BL/6J wild-type (+/+) and ChAc model mice with the homozygous deletion genotype (−/−), and prepared as described previously [14]. Triton X-100 (1%) soluble fractions were subjected to NuPAGE followed by immunoblot analysis. This study was approved by the Committee on Animal Experimentation of Kagoshima University (Japan) and carried out in accordance with its guidelines.

2.4. Mass spectrometry and database analysis

Using erythrocyte ghosts from the ChAc patient, *VPS13A* mutant heterozygous carrier and healthy control, two-dimensional polyacrylamide gel electrophoresis was performed. First, isoelectric focusing using Immobiline DryStrips pH 3–10 non-linear, 13 cm (GE Healthcare, Little Chalfont, UK) was performed, followed by SDS (10–18%) polyacrylamide gradient gel (Bio Craft, Tokyo, Japan) electrophoresis. Gels were stained with 0.1% Coomassie Brilliant Blue (CBB) G-250, and samples for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis were excised. In-gel digestion of individual protein spots was performed using trypsin. Peptide solutions were desalted using ZipTip C18 columns (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. The AXIMA-CFR Shimadzu model MALDI-TOF mass spectrometer (Shimadzu, Kyoto, Japan) was used for mass analysis, with α-cyano-4-hydroxycinnamic acid as the matrix. Database searches of peptide mass fingerprints (PMF) were performed using Mascot software (Matrix Science, http://www.matrixscience.com/).

2.5. Antibodies

Rabbit polyclonal anti-chorein [14], mouse monoclonal anti-β-actin (Sigma), and goat polyclonal anti-N-terminal-β-adducin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used as primary and immunoprecipitation antibodies. Anti-rabbit IgG, horseradish peroxidase (HRP)-linked species-specific (GE Healthcare), anti-mouse IgG, HRP-linked species-specific (GE Healthcare), anti-goat IgG-HRP (Santa Cruz Biotechnology), Alexa-488-labeled anti-rabbit IgG, Alexa-555-labeled anti-mouse IgG, and Alexa-555-labeled anti-goat IgG (all Invitrogen) antibodies were used as secondary antibodies.

2.6. Immunoprecipitation (IP)

Co-IP and reverse co-IP assays were performed using the Pierce Co-immunoprecipitation or c-Myc-Tag IP/Co-IP Kits (Thermo Scientific, Rockford, IL, USA). HEK293 cell lysates were solubilized using Nonidet P-40 at a final concentration of 0.5%. Soluble fractions (200–600 μg) from cell lysates (input) were incubated overnight at 4 °C with antibody-immobilized beads. Beads were then centrifuged at 1000*g* for 1 min, and washed three times with PBS before elution. Protein samples eluted by NuPAGE LDS Sample buffer (Invitrogen) were heated to 99 °C for 5 min. Samples of input proteins and eluates were analyzed by immunoblot analysis.

2.7. Immunoblot analysis

Protein samples were denatured by NuPAGE LDS Sample buffer (Invitrogen), separated on NuPAGE® 4–12% Bis–Tris gels (Invitrogen), and electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare). Equal loading of protein was confirmed using the MemCode Reversible Protein Stain Kit (Thermo Scientific). Membranes were blocked for 1 h at room temperature with 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), and incubated overnight at 4 °C with primary antibodies for each target protein in PBS-T milk. After rinsing in PBS-T, membranes were incubated with appropriate second antibodies for 1 h at room temperature. Proteins were visualized using ECL Plus Western Blotting Detection System or ECL Prime Western Blotting Detection Reagent (GE Healthcare) and images recorded by digital analyzer (Fujifilm LAS-1000; Fujifilm, Tokyo, Japan).

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