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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.

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

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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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).

2.8. Statistics

Data are presented as mean ± 95% confidence interval (CI). Differences were evaluated using unpaired Welch *t*-tests, with $p < 0.01$ considered statistically significant.

2.9. Immunocytochemistry

Stable chorein-overexpressing or mock-transfected HEK293 cells were sparsely plated onto poly-D-lysine-coated 18-mm glass cover slips and cultured in MEME containing 10% FBS and 500 mg/mL G418 for approximately 20 h. Cells were fixed for 15 min in 0.1 M phosphate buffer containing 4% (w/v) paraformaldehyde. Cells were washed with PBS–Glycine (0.01 M glycine in PBS, pH 7.4) three times and then permeabilized with PBS containing 0.1% (w/v) Triton X-100 for 5 min. Cells were washed with PBS–Glycine three times and blocked for 1 h at room temperature with 10% (w/v) non-fat dried milk in PBS-T containing 6% (w/v) glycine. Cells were incubated with primary antibodies overnight at 4 °C. After washing with PBS-T, they were incubated with secondary antibodies for 1 h at room temperature. Coverslips were washed, mounted with Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA, USA), and viewed with an LSM 700 confocal microscope system (Carl Zeiss, Jena, Germany).

3. Results

3.1. Protein expression levels in erythrocyte ghosts from a ChAc patient

To identify erythrocyte proteins differentially expressed in ChAc, we performed a comparative proteomic analysis of erythrocyte ghosts from a ChAc patient, *VPS13A* mutant heterozygous carrier, and a healthy control. In total, 35 spots were picked and identified by PMF analysis (data not shown). ACTB protein (β-actin) and adducin 2 isoform a (β-adducin isoform 1) showed lower expression levels in erythrocyte ghosts from the ChAc patient than the control (Table 1).

3.2. β-Adducin and β-actin interact with chorein

We generated HEK293 cells stably overexpressing Myc-DDK tagged chorein. To examine the association between β-adducin and chorein, co-IP and reverse co-IP assays were performed using anti-c-Myc and anti-β-adducin antibodies, respectively. Bands of 97 kDa and approximately 60 kDa were co-immunoprecipitated with chorein (Fig. 1A), corresponding to β-adducin isoform 1 and isoform 2, respectively. A positive chorein signal was also observed in the β-adducin immunoprecipitate (Fig. 1B). IP controls using mock-transfected HEK293 cells failed to precipitate either protein (Fig. 1A and B). These results indicate that overexpressed chorein interacts with β-adducin in HEK293 cells.

To examine the association between β-actin and chorein, co-IP and reverse co-IP assays using anti-c-Myc and anti-β-actin antibodies, respectively, were also performed. A strong positive β-actin signal was observed in the chorein immunoprecipitate (Fig. 2A), and similarly, a chorein positive signal was observed in the β-actin immunoprecipitate (Fig. 2B), suggesting that overexpressed chorein interacts with β-actin.

3.3. Co-localization of chorein with β-adducin and β-actin

Our biochemical findings show that β-adducin and β-actin interact with chorein. Next, we used immunocytochemistry to determine if these proteins co-localize with chorein in chorein-overexpressing HEK293 cells (Fig. 3). Using an anti-chorein antibody, chorein displayed a vesicular staining pattern that strongly co-localized with β-adducin (Fig. 3A), and partially co-localized with β-actin (Fig. 3B).

3.4. Comparison of β-adducin and β-actin immunoreactivity in the brain of ChAc model mice

We analyzed 1% Triton X-100-soluble fractions of brain regions from wild-type (+/+) and ChAc model (−/−) mice by immunoblotting using an anti-β-adducin antibody. In mouse brain, the β-adducin isoform 1 was observed, but isoform 2 was not detected in any brain regions. Immunoreactivity of β-adducin was significantly lower in the striatum of ChAc model mice, but no significant differences were detected in the cerebral cortex or hippocampus (Fig. 4). There was no significant difference in β-actin immunoreactivity in any brain regions (data not shown).

4. Discussion

This study is the first biochemical demonstration of chorein interacting proteins. Chorein is thought to be involved in intracellular trafficking and vesicle-mediated sorting [10–12]. However, its physiological function at the molecular level, and interacting partners, are unclear. In this study, we performed proteomic analysis, co-IP, reverse co-IP, and immunocytochemical analyses, revealing that β-adducin and β-actin are chorein interacting partners.

Adducin in humans and rodents includes three subunits: α, β, and γ. All three adducin subunits contain an N-terminal globular head domain, a neck domain, and a C-terminal tail domain that has myristoylated alanine-rich C kinase substrate (MARCKS)-related domains with clusters of lysine residues [20]. Although α- and γ-adducin are expressed widely in tissues, β-adducin expression is restricted to the brain and hematopoietic tissues [21], corresponding to the main pathological lesions of ChAc. Adducin is an actin capping protein that binds to the barbed end of filamentous (F-) actin to prevent further elongation and depolymerization [20,22]. Adducin promotes the association of spectrin with F-actin in erythrocyte membrane skeletons. Adducin is expressed in the brain at high levels and is a component of synaptic structures, such as dendritic spines and neuron growth cones, and subsequently, is involved in the dynamic assembly–disassembly of the actin cytoskeleton during synaptic plasticity [18]. In addition, β-adducin binds to rabphilin-3A, a protein involved in synaptic vesicle trafficking [23,24]. Moreover, β-adducin gene-deficient mice show long-term synaptic plasticity, behavioral, motor coordination, and learning deficits [18]. We found a decreased protein level and immunoreactivity of β-adducin in erythrocyte membranes from a

Table 1
Mascot search results. Comparison of β-actin and β-adducin protein levels in erythrocyte ghosts.

Spot #	GI number	Mass (Da)	Mascot score	Protein name	Norm Qty		
					Patient[a]	Hetero[b]	Control[c]
SSP 2602	15277503	40,536	162	ACTB protein (beta-actin)	2488.2	3403.9	5731.7
SSP 2805	9257192	81,260	101	Adducin 2 isoform a (beta-adducin isoform 1)	6.8	828.6	1362.3

[a] Patient: ChAc patient, *VPS13A* homozygous c.3889C > T.
[b] Hetero: ChAc mutant carrier, *VPS13A* heterozygous c.3889C > T.
[c] Control: healthy control.

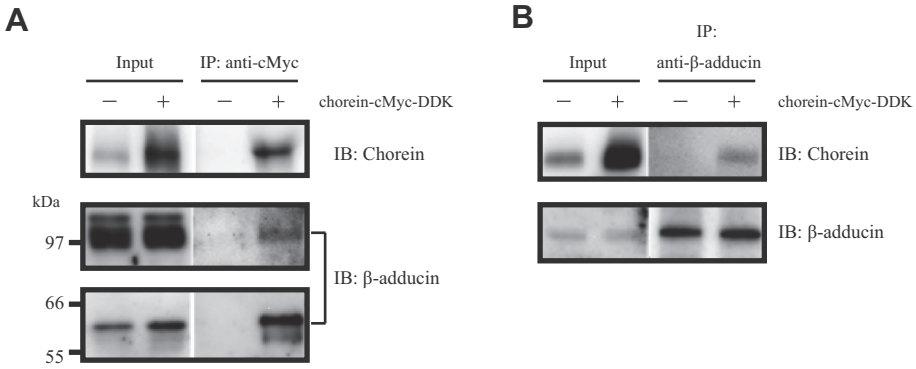

Fig. 1. Chorein co-immunoprecipitates with β-adducin. (A) Co-immunoprecipitation (IP) assay using human embryonic kidney 293 (HEK293) cells stably overexpressing Myc-DDK tagged chorein was performed with anti-cMyc antibody. Immunoblot (IB) analyses used anti-chorein and anti-β-adducin antibodies. The β-adducin isoform 1 (97 kDa) and an alternative product (approximately 60 kDa) were co-immunoprecipitated with chorein. Co-IP using mock-transfected HEK293 cells with an anti-cMyc antibody was performed as a negative control. (B) Reverse co-IP assay confirmed an interaction between β-adducin and chorein. Immunoblot analyses used anti-chorein and anti-β-adducin antibodies.

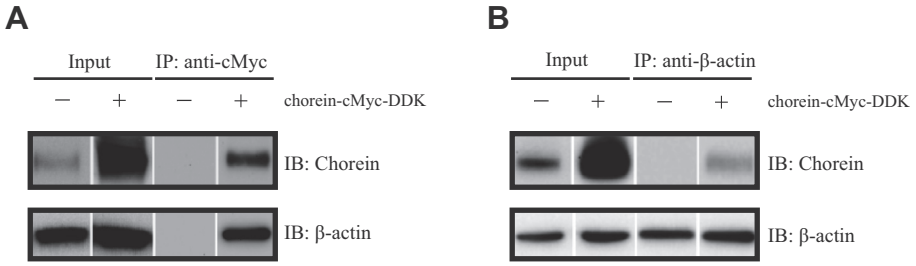

Fig. 2. Chorein co-immunoprecipitates with β-actin. (A) Co-IP assay using human embryonic kidney 293 (HEK293) cells stably overexpressing Myc-DDK tagged chorein. Immunoblot analyses used anti-chorein and anti-β-actin antibodies. (B) Reverse co-IP assay using chorein-overexpressing HEK293 cell lysates. Immunoblot analyses used anti-chorein and anti-β-actin antibodies.

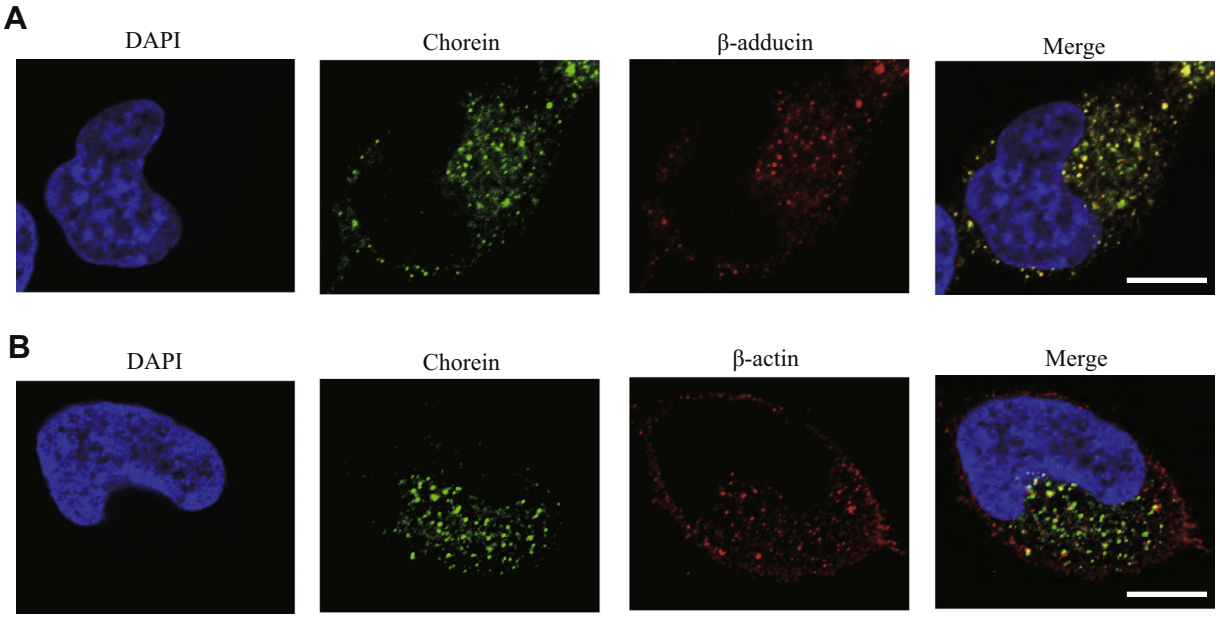

Fig. 3. Chorein partially co-localizes with β-adducin and β-actin in human embryonic kidney 293 (HEK293) cells stably overexpressing chorein. HEK293 cells stably overexpressing chorein were stained with anti-chorein (green) and anti-β-adducin (red) (A) or anti-β-actin (red) (B) antibodies. Cell nuclei were visualized using DAPI staining. Scale bars, 10 μm.

ChAc patient, and in the striatum of ChAc model mice, suggesting β-adducin may be involved in ChAc molecular pathology.

Actin is a ubiquitous, essential cytoskeletal protein. The actin cytoskeleton is required for modulation of the synaptic vesicle release cycle via multiple mechanisms [19,25,26]. F-actin indirectly binds to gephyrin, a $GABA_A$ receptor-anchoring protein that shows enhanced expression in the striatum and hippocampus of ChAc model mice [27,28]. Recent investigations found actin

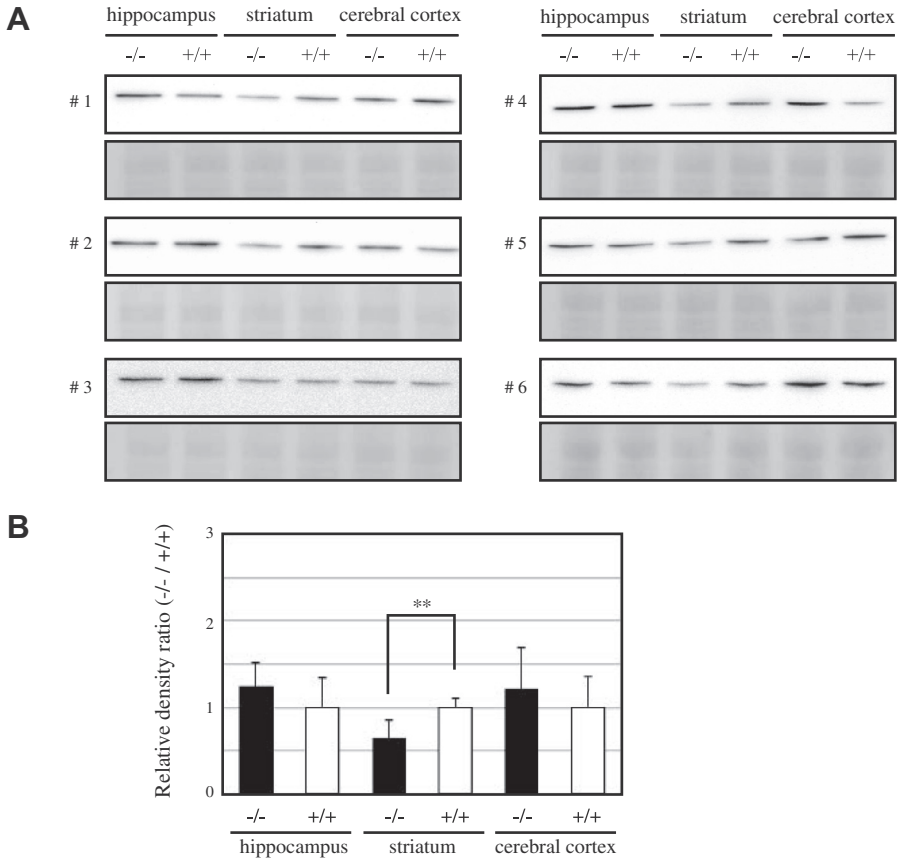

Fig. 4. Protein levels of β-adducin in mouse brain. (A) Six independent immunoblot results were shown. Immunoblot analysis of β-adducin in 1% Triton X-100-soluble fractions of the hippocampus, striatum, and cerebral cortex from wild-type (+/+) and ChAc model (−/−) mice. Bands of 97 kDa corresponding to β-adducin isoform 1 were shown. Decreased β-adducin isoform 1 immunoreactivity is observed in the striatum of −/− mice. No differences in band densities between +/+ and −/− mice were detected in the hippocampus or cerebral cortex. Identical gels were run and stained with Coomassie dye to confirm uniform protein loading. (B) Histograms showing β-adducin isoform 1 relative density ratio in −/− to +/+ mice. Error bars represent 95% confidence intervals ($n = 6$). $^{**}p < 0.01$.

depolymerization induced in erythrocytes and platelets from ChAc patients [29,30]. As β-actin is an abundant housekeeping protein, we were unable to detect any differences in β-actin immunoreactivity, although a subtle difference may exist.

We found chorein exhibited a vesicular pattern of staining in HEK293 cells, similar to other vacuolar protein sorting (VPS) proteins [31,32]. Human VPS52, 53, and 54 co-localize with the Golgi marker, GM130, and late endosome marker, mannose-6-phosphate-receptor [31], and human VPS11, 16, and 18 co-localize with the late endosome/lysosome marker, Lamp-1 [32]. Recently, chorein and COH1 (VPS13B) were also reported to co-localize with GM130 [13,33]. Thus, chorein is likely to be associated with the Golgi apparatus and endosomes/lysosomes. Chorein-positive enlarged vesicular structures showed co-localization with β-adducin and partially co-localized with β-actin in HEK293 cells. Therefore, chorein may co-localize with its interacting proteins, β-adducin and β-actin, in neuronal cells to regulate the synaptic vesicle release cycle.

The actin-capping activity of β-adducin requires both the neck (amino acids 335–436) and C-terminal MARCKS-related (amino acids 700–726) domains, and can be inhibited by protein kinase A (PKA) and C (PKC) phosphorylation, and calmodulin [18,34]. However, in this study, a β-adducin alternative product, predicted to be β-adducin isoform 2 (559 amino acids) that does not contain the highly basic carboxy terminus of isoform 1, was also co-immunoprecipitated with chorein. The β-adducin isoform 2 may have novel functions in ChAc pathology.

Human chorein contains a chorein_N motif, a proposed leucine zipper, a DUF1162 motif (conserved within vacuolar protein sorting-related proteins), and an ATG_C motif that may function by targeting it to vacuoles. Chorein also contains 10 Tetratrico Peptide Repeat motifs (Swiss-Prot entry Q96RL7) that potentially interact with other binding partners, and is predicted to have a coiled-coil structure by the online program PSORT II [13,35]. However, chorein does not contain an actin-binding domain. The actin and adducin binding sites in chorein remain unknown, and may not bind directly to chorein.

Although further analyses are required, this study demonstrates that β-adducin and β-actin are chorein-interacting proteins and may be involved in brain pathology and acanthocytosis in ChAc. Our findings provide a basis for the development of investigation into chorein functions and the molecular pathogenesis of ChAc.

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