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Subcellular localization and putative role of VPS13A/chorein in dopaminergic neuronal cells

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ABSTRACT

Chorea-acanthocytosis (ChAc) is a rare hereditary neurodegenerative disorder caused by loss of function mutations in the vacuolar protein sorting 13 homolog A (*VPS13A*) gene encoding chorein. Although a deficiency in chorein function leads to apoptosis of striatal neurons in ChAc model mouse, its detailed subcellular localization and physiological role remain unclear. In this study, we produced two anti-chorein polyclonal antibodies and examined the intracellular localization of endogenous chorein in neuronal cells. Immunocytochemically, chorein was observed in the termini of extended neurites and partially colocalized with synaptotagmin I in differentiated PC12 cells. Subcellular localization analysis by sucrose density gradient fractionation showed that chorein and synaptotagmin I were located in dense-core vesicles (DCVs), which contain dopamine. In addition, PC12 cells stably expressing carboxyterminal fragment of chorein increased K^+-induced dopamine release. Taken together, these results suggest that chorein is involved in exocytosis of DCV.

1. Introduction

Chorea-acanthocytosis (ChAc; OMIM ID: 200150) is a rare autosomal recessive neurodegenerative disorder, which is characterized by adult-onset chorea and erythrocyte acanthocytosis. Clinically, ChAc patients show various symptoms such as psychiatric features, epilepsy, peripheral neuropathy, myopathy, and oral self-mutilation [1]. Their symptoms have been thought to resemble those of Huntington's disease [2]. The main neuropathological feature of ChAc is degeneration of the striatum [3]. The inherited pattern of ChAc is considered to be autosomal recessive, and ChAc is caused by loss of function mutations in the *VPS13A* gene, encoding a protein named chorein [4,5]. *VPS13A* is located on human chromosome 9q21, spanning approximately 250 kb. Concerning the structure of *VPS13A*, two main splicing variants have been reported: transcript A (exons 1–68, 70–73, GenBank Accession No. NM_033305) and transcript variant B (exons 1–69, GenBank Accession No. NM_015186) [4,6]. Chorein is a 360 kDa protein that is absent or markedly reduced in ChAc patients with *VPS13A* mutations [7]. The null mutant of *Saccharomyces cerevisiae* homolog, *VPS13*, exhibits deficiency in vacuolar protein sorting [8]. A mutant *TipC* gene (an ortholog of *Vps13p*) in *Dictyostelium discoideum* has abnormality in cell-sorting behavior [9]. These findings suggest that chorein is involved in the cytoskeleton and intracellular transport system.

Chorein is highly expressed in testis, kidney, spleen, and brain. In wild-type mouse brain, chorein is predominantly localized to microsomal and synaptosomal fractions, the neuronal perinuclear region, cytoplasm, and fibers [10]. In ChAc model mouse, a deficiency in chorein function leads to apoptosis of striatal neurons [11]. Although there have been some findings about the biological function of chorein, its detailed subcellular localization and physiological role remain unclear.

In this study, we investigated the detailed subcellular localization and physiological role of chorein in neuronal cells to clarify the molecular pathogenesis of ChAc.

2. Materials and methods

2.1. Plasmid constructions

We used partial cDNAs encoding human VPS13A (transcript variant B) (hVPS13A). pCold-TF/hVPS13A-(1-604) and pCold-TF/hVPS13A-(2610-3095) were constructed for bacterial His6-tagged

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protein expression using standard techniques. pcDNA-His-Flag/hVPS13A-(1-609) and pEF-BOS-HA/hVPS13A-(2610-3095) were constructed for mammalian expression using standard techniques.

2.2. Materials and chemicals

pcDNA-His-Flag vector and mouse N1E-115 neuroblastoma cells were provided by Dr. M. Fukata (National Institute for Physiological Sciences, Okazaki, Japan). Rat pheochromocytoma PC12 cells were provided by Dr. A. Kikuchi (Osaka University, Suita, Japan). The pCold™ TF DNA vector was purchased from Takara Bio Inc. (Ohtsu, Japan). PC12 cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% horse serum (HS). PC12 cells stably expressing hVPS13A-(1-609) and hVPS13A-(2610-3095) were generated by selection with G418 as described previously [12]. His6-tagged hVPS13A-(1-604) and His6-tagged hVPS13A-(2610-3095) expressed in BL21/DE3 proteins were purified using HiTrap™ Chelating HP columns (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. After the immunizations of guinea pigs against these recombinant proteins, polyclonal antisera were affinity-purified using HiTrap™ NHS-activated HP columns (GE Healthcare) coupled with hVPS13A(1-604) or hVPS13A(2610-3095) according to the manufacturer's instructions. Anti-synaptotagmin (Syt) I antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-synaptotagmin and anti-GM130 antibodies were purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Anti-Syt V antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-synaptophysin antibody (clone SVP38) was purchased from Sigma (St. Louis, MO, USA). An anti-HA antibody was purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA). Alexa-546-labeled anti-mouse IgG and Cy3-labeled anti-guinea pig IgG were purchased from Invitrogen Inc. (Carlsbad, CA, USA) and Jackson ImmunoResearch (West Grove, PA, USA), respectively. Nerve growth factor (NGF) 7.5S was purchased from Invitrogen Inc. Perchloric acid (PCA) was purchased from Wako (Osaka, Japan).

2.3. ChAc-model mice

ChAc-model mice, with a deletion of exons 60–61 in *VPS13A* corresponding to a human disease mutation, were produced by gene targeting as previously described [11]. The ChAc-model mice were backcrossed for at least ten generations in a c57BL6J background (CLEA JAPAN, Tokyo, Japan). Brain tissues were obtained from c57BL/6J wild-type and ChAc-model mice. This animal study was approved by the Committee on Animal Experimentation of Kagoshima University (Japan) and carried out in accordance with its guidelines.

2.4. Human samples

Human erythrocyte ghost samples were obtained from normal healthy controls with no mutation. They provided written informed consent. The research protocol and consent form were approved by the Institutional Review Board of Kagoshima University, Japan.

2.5. Preparation of protein extracts

Cerebral tissues from wild-type and ChAc-model mice with the homozygous deletion genotype were homogenized in five volumes of ice-cold lysis buffer (20 mM Tris–HCl (pH 8.0), 1% (w/v) Nonidet® P-40 (Nacalai Tesque, Inc., Kyoto, Japan) (NP-40), 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin) with a Teflon glass Potter homogenizer, and centrifuged at 20,000*g* for 30 min at 4 °C. PC12 and N1E-115 cells (35-mm-diameter dishes) were lysed with 100 μL of NP-40 buffer (20 mM Tris–HCl (pH 8.0), 1% (w/v) NP-40, 137 mM NaCl, 10% (w/v) glycerol, 10 μg/mL leupeptin, 1 mM PMSF) with 20 μg/mL aprotinin and NP-40 buffer with 20 mM sodium β-glycerophosphate, respectively, and then centrifuged at 20,000*g* for 15 min at 4 °C. Erythrocyte ghost samples were prepared as described previously [13]. These clarified samples were then used for the Western blot analysis.

2.6. Western blot analysis

Samples were subjected to SDS–PAGE using Tris–glycine buffer system, as well as NuPAGE® Gel system (Invitrogen) for detection of endogenous chorein as previously described [10], and transferred onto nitrocellulose or polyvinylidene difluoride membranes. They were visualized with an ECL Western blotting system (GE Healthcare) using primary antibodies for each target protein and appropriate horseradish peroxidase-conjugated secondary antibodies.

2.7. Immunocytochemistry

To observe neuron-like cells, PC12 cells sparsely plated onto poly-D-lysine-coated 18-mm glass cover slips were cultured with DMEM containing 1% FBS, 0.5% HS, and 100 ng/mL NGF for 48 h. The cells were fixed for 10 min in PBS containing 4% (w/v) paraformaldehyde (PFA) with 10% (w/v) trichloroacetic acid (TCA), or 4% (w/v) PFA and 10% (w/v) TCA sequentially. The cells were washed with PBS three times and then permeabilized with PBS containing 0.2% (w/v) Triton® X-100 (Nacalai Tesque, Inc., Kyoto, Japan) and 2 mg/mL BSA for 2 h. The cells were washed with PBS four times and incubated with primary antibodies for 1 h. After being washed with PBS, they were further incubated with Alexa-546-labeled anti-mouse IgG and Cy3-labeled anti-guinea pig IgG for 1 h. The coverslips were washed, mounted on glass slides, and viewed with an LSM 700 confocal microscope system (Carl Zeiss, Jena, Germany). All procedures were performed at room temperature.

2.8. Subcellular fractionation

One wild-type mouse brain (cerebrum) was homogenized by twenty strokes of a Teflon glass Potter homogenizer in ten volumes of homogenization buffer (25 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 1 mM dithiothreitol (DTT), and 10 μg/mL leupeptin). The homogenate was then centrifuged at 270*g* for 10 min at 4 °C. PC12 cells (four 100-mm-diameter dishes) were harvested and washed with PBS once. The cells were homogenized in 2 mL of homogenization buffer (10 mM HEPES-NaOH (pH 7.4), 0.32 M sucrose, 1 mM PMSF, 20 μg/mL aprotinin, and 20 μg/mL leupeptin) with fifty strokes in a Teflon glass Potter homogenizer and homogenates were centrifuged at 50*g* for 5 min at 4 °C. The supernatants from mouse brain or rat PC12 cells (1 mL, approximately 1.25 mg of protein) were loaded on a 4-mL discontinuous sucrose density gradient (0.6 M, 0.9 M, 1.2 M, 1.6 M, 1 mL each) and centrifuged at 100,000*g* for 2 h at 4 °C in a P55ST2 rotor (Hitachi Koki, Co., Ltd., Tokyo, Japan). After centrifugation, twenty fractions of 0.5 mL each were collected from the top of the gradient and the aliquots were analyzed by Western blotting.

2.9. Dopamine release assay

PC12 cells were plated onto 35-mm-diameter dishes at a density of 1.5×10^6 cells per dish and cultured overnight. The cells