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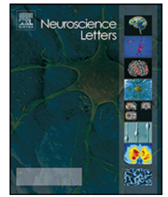

Endosomal sorting related protein CHMP2B is localized in Lewy bodies and glial cytoplasmic inclusions in α-synucleinopathy

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HIGHLIGHTS

- ► CHMP2B is a component of the endosomal sorting complex.
- ► We determined whether CHMP2B is involved in neurodegenerative diseases.
- ► CHMP2B was localized in Lewy bodies and glial cytoplasmic inclusions in α-synucleinopathy.
- ► Endosomal pathway is associated with degradation of α-synuclein.

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ABSTRACT

Charged multivesicular body protein 2B (CHMP2B) is a component of the endosomal sorting complex required for transport-III, which is involved in the degradation of proteins in the endocytic and autophagic pathways. Mutations in the *CHMP2B* gene cause frontotemporal dementia and amyotrophic lateral sclerosis characterized by accumulation of ubiquitinated protein aggregates. Recent studies have shown that autophagosomal proteins are present in α-synuclein aggregates in neurons and glial cells in Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). We therefore immunohistochemically examined the brains of various neurodegenerative diseases using CHMP2B-specific antibody. CHMP2B immunoreactivity was present in intracytoplasmic and axonal Lewy bodies in PD and DLB as well as in neuronal and glial cytoplasmic inclusions in MSA. No CHMP2B immunoreactivity was found in a variety of other neuronal and glial inclusions in TDP-43 proteinopathy and tauopathy. These findings suggest that endosomal and autophagic pathway is associated with degradation or formation of α-synuclein aggregates in α-synucleinopathy.

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1. Introduction

Aggregation of proteins such as tau, α-synuclein and TDP-43, is involved in many neurodegenerative diseases. Charged multivesicular body protein 2B (CHMP2B) is a component of the endosomal sorting complex required for transport-III (ESCRT-III), which is required for the formation of multivesicular body, a late endosomal structure that fuses with the lysosome to degrade endocytosed proteins [29]. CHMP2B is also likely to have a role in macroautophagy (hereafter referred to as autophagy) [29]. Autophagy is a bulk degradation mechanism for long-lived proteins and organelles, whereby cytoplasmic material is sequestered within double-membrane vesicles (autophagosomes) which eventually fuse with lysosomes. Autophagosomes also undergo fusion with late endosomes to form amphisomes [8,23], suggesting an important role for endosomes in autophagic degradation.

TDP-43 is now known to be a major disease protein in ubiquitinated inclusions in sporadic amyotrophic lateral sclerosis (ALS) and sporadic and familial frontotemporal lobar degeneration (FTLD) with or without motor neuron disease, collectively referred to as TDP-43 proteinopathy [3,17]. Mutations in the *CHMP2B* gene cause frontotemporal dementia linked to chromosome 3 (OMIM #600795) characterized by ubiquitin- and p62-positive but tau- or TDP-43-negative cytoplasmic aggregates in affected neurons [13,25]. The mutations also cause ALS-17 (OMIM #614696) characterized by ubiquitin-, p62- and TDP-43-positive but tau-negative cytoplasmic inclusions in surviving motor neurons [5,22]. Recently, Yamazaki et al. [33] demonstrated that CHMP2B

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immunoreactivity is present in granulovacuolar degeneration observed in the hippocampal pyramidal neurons in patients with Alzheimer's disease and aged control subjects, which is morphologically identical to autophagosomes [9,21]. Autophagosomal proteins are present in α-synuclein aggregates in neurons and glial cells in α-synucleinopathy, i.e. Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [1,6,7,12,24,27]. These reports prompted us to investigate whether CHMP2B is involved in a variety of neurodegenerative diseases. Here we report that CHMP2B immunoreactivity is present in neuronal and glial cytoplasmic inclusions in α-synucleinopathy, but not in a variety of other neuronal and glial inclusions in TDP-43 proteinopathy or tauopathy.

2. Materials and methods

2.1. Subjects and immunohistochemical analysis

Fifty post-mortem cases were utilized in the present study; these included cases of PD (PD stage 4; $n = 5$) [4], neocortical-type DLB ($n = 5$) [15], MSA ($n = 5$), Alzheimer's disease ($n = 5$), Pick's disease ($n = 4$), progressive supranuclear palsy ($n = 4$), corticobasal degeneration ($n = 4$), argyrophilic grain disease ($n = 4$), ALS ($n = 5$), FTLD-TDP type B ($n = 4$) [14] and normal controls ($n = 5$). Immunohistochemical analysis was carried out using formalin-fixed, paraffin-embedded sections from the frontal cortex, hippocampus, basal ganglia, midbrain, upper pons, medulla oblongata and cerebellum of patients with PD, DLB, MSA, AD and controls. Sympathetic ganglia were also examined in PD. In other cases, multiple sections taken from the affected regions were immunostained. The primary antibodies used were rabbit polyclonal antibodies against CHMP2B (ab33174; Abcam, Cambridge, UK; 1:50) [33] and TDP-43 (10782-1-AP; ProteinTech Group, Inc., Chicago, IL, USA; 1:4000), and mouse monoclonal antibodies against phosphorylated α-synuclein (#64; Wako, Osaka, Japan; 1:5000) and phosphorylated tau (AT8; Innogenetics, Ghent, Belgium; 1:1000).

The total number of inclusions immunostained with anti-CHMP2B and anti-phosphorylated α-synuclein was quantified in contiguous sections [20]. Cortical Lewy bodies (LBs) were counted in deeper layers of the temporal cortex of each DLB patient. Brainstem-type LBs were quantified in the substantia nigra in PD. Glial cytoplasmic inclusions were quantified in the pontine base in MSA.

Double-immunofluorescence staining was performed to detect overlapping expression of CHMP2B and phosphorylated α-synuclein. Deparaffinized sections were blocked with goat and horse serum and then incubated overnight at 4 °C with a mixture of polyclonal anti-CHMP2B (1:25) and monoclonal anti-phosphorylated α-synuclein (1:500). Following a wash for 5 min at 3 times with phosphate-buffered saline (PBS), the sections were incubated for 1 h with Alexa Fluor 488- and 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA).

2.2. Cell culture and siRNA-mediated knockdown

Human neuroblastoma cell line (SH-SY5Y, the European Collection of Cell Culture) was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. The siRNA was purchased from Dharmacon (Lafayette, CO, USA). The siRNA (final concentration 10 or 20 nM) for CHMP2B (L-004700-01-0005) or non-targeting control (D-001810-10-20) was transfected into cultured cells using Lipofectamine RNAi MAX (Invitrogen). After 24 h of incubation, the cells were lysed with lysis buffer containing 4% sodium dodecyl sulfate (SDS) for immunoblotting. A lysosomal inhibitor, bafilomycin A, was purchased from Wako, and used for 24 h at a final concentration of 100 nM.

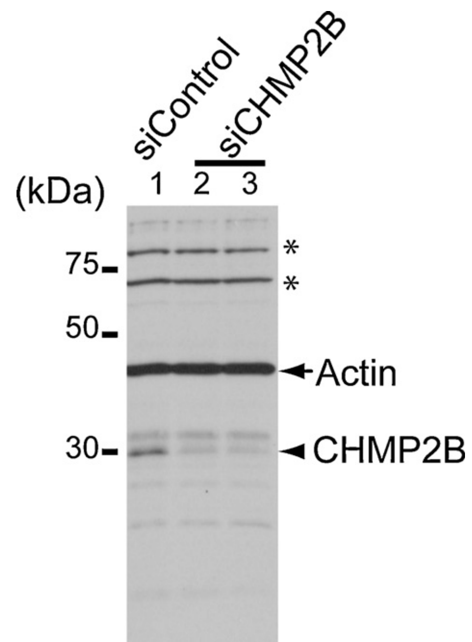

Fig. 1. Specificity of CHMP2B antibody. SH-SY5Y cells were subjected to siRNA-mediated knockdown of CHMP2B at final concentration of 10 nM (lane 2) or 20 nM (lane 3), and controls at final concentration of 20 nM (lane 1). Anti-CHMP2B antibody reacts with a several bands (asterisks and arrowhead), whereas the 30 kDa-band disappears in cells with CHMP2B knockdown (lanes 2 and 3). Molecular mass is indicated on the left side of the panel. Actin is used as a loading control.

Cultured cells were double-immunolabeled with rabbit anti-CHMP2B (1:200) and mouse anti-α-synuclein (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:400). Alexa Fluor 594- and 680-conjugated secondary antibodies (Invitrogen) were used. After a rinse in PBS, the cells were mounted with ProLong gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and examined using a confocal microscope (EZ-Ci, Nikon, Tokyo, Japan). Adobe Photoshop CS5 software (Adobe systems, San Jose, CA, USA) was used for image processing.

2.3. Immunoblot analysis

Western blot analysis was performed as described previously [28]. Transfer and detection were carried out according to the protocol provided with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) was used as a secondary antibody.

3. Results

3.1. Specificity of the primary antibody

We first confirmed the specificity of the anti-CHMP2B antibody (Fig. 1). The antibody detected several bands (asterisks and arrowhead) in SH-SY5Y cells, whereas the band with 30 kDa was almost abolished in cells subjected to siRNA-mediated knockdown of CHMP2B (lanes 2 and 3 in Fig. 1). This antibody was therefore employed for subsequent immunohistochemistry.

3.2. Immunohistochemical analysis

In normal controls, the neuronal cytoplasm and neuropil were weakly immunolabeled with anti-CHMP2B antibody (Fig. 2A). Consistent with previous study [33], granulovacuolar bodies observed in the hippocampus in controls and neurodegenerative diseases

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