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α-Synuclein modulates neurite outgrowth by interacting with SPTBN1

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ABSTRACT

α-Synuclein is the major component of Lewy bodies and Lewy neurites, the pathological hallmarks of surviving neuronal cells in Parkinson's disease patients. However, the physiological role played by α-synuclein remains unclear. In this study, spectrin beta non-erythrocyte 1 (SPTBN1) interacted with α-synuclein in phage display assays using a normalized human brain cDNA library. A direct interaction between α-synuclein and SPTBN1 was confirmed by GST pull-down and co-immunoprecipitation assays. SPTBN1 and α-synuclein proteins colocalized in N2a neuronal cells. Transfection of SPTBN1 caused human SH-SY5Y dopaminergic neuron cells to inappropriately induce neurites, which extended from cell bodies. Cotransfection with α-synuclein reversed SPTBN1-induced excessive neurite branching in SH-SY5Y cells, and only a single neurite extended from each neuron. These results suggest that α-synuclein modulates neurite outgrowth by interacting with cytoskeletal proteins such as SPTBN1.

1. Introduction

In Parkinson's disease (PD), dopaminergic neurons in the substantia nigra are selectively degraded, and Lewy bodies and Lewy neurites are observed in surviving neurons. The major fibrillar component of Lewy bodies and Lewy neurites is α-synuclein, a cytosolic neuronal protein. Lewy bodies and Lewy neurites inhibit proteolysis by proteasomes and increase the sensitivity of cells to a variety of toxic injuries such as mitochondrial damage [1]. The mutation or overexpression of α-synuclein causes some forms of familial PD. Missense mutations in the α-synuclein gene (A30P, E46K, and A53T) cause familial early onset PD [2,3]. The duplication or triplication of the human α-synuclein gene locus increases the intracellular α-synuclein protein levels, thereby increasing the risk for development of some inherited forms of PD [4,5]. Overexpression of α-synuclein affects cellular physiology, including mitochondria and proteasome functions, exocytosis, and protein biosynthesis, and it has been shown to induce the unfolded protein response and oxidative stress [6]. It is not clear which of these effects is the result of α-synuclein-associated cytotoxicity, and which are secondary consequences of cell stress. Determining the native function of α-synuclein may address these questions.

Previous studies have suggested that α-synuclein regulates vesicle trafficking and synaptic plasticity. α-Synuclein knockout mice have reduced pools of synaptic vesicles in paired stimuli, suggesting that α-synuclein plays a role in vesicle fusion [7]. Overexpression of α-synuclein in yeast and human PC12 cells reduced vesicle docking and fusion with Golgi membranes [8,9]. High-copy expression of vesicle transport proteins such as Rabs, COPII coat proteins, and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) mitigated the cytotoxicity of α-synuclein overexpression in yeast [10].

α-Synuclein may also play a role in axonal transport of synaptic materials. Overexpression of PD-associated α-synuclein mutants in cultured neurons inhibited anterograde axonal transport, resulting in the accumulation of proteins proximal to the cell body [11]. When a human α-synuclein mutant A53T was expressed in rat substantia nigra, the levels of proteins involved in synaptic transmission and anterograde transport decreased [12]. Changes in the levels of cytoskeletal proteins (decreases in α-tubulin and increases in actin) accompanied deficits in axonal transport. The accumulation of F-actin induces mitochondrial membrane depolarization, increases ROS, and results in cell death [13]. Furthermore, actin colocalized with α-synuclein in dystrophic and swollen axon terminals. The accumulation of F-actin was also observed in *Drosophila* and mouse models of tauopathy [14].

To understand the physiological role(s) of α-synuclein and the mechanism leading to the development of PD, we screened for protein partners that interact with α-synuclein. Commercially available cDNA libraries originate from differentially expressed mRNAs and thus contain an overrepresentation of abundantly

Abbreviations: PD, Parkinson's disease; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SPTBN1, spectrin beta non-erythrocyte 1; PBST, phosphate buffered saline with 0.3% Tween 20.

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expressed genes. Interference from highly expressed proteins increases false-positive rates during screening and can mask authentic partners present at low abundance. To avoid overrepresentation of proteins, a normalized human brain cDNA library was used in a phage display assay to represent each protein at a similar frequency. In the present study, spectrin beta non-erythrocyte 1 (SPTBN1) interacted with α-synuclein, as confirmed by GST pull-down and co-immunoprecipitation assays. SPTBN1 is also known to interact with actin, and rapid actin remodeling is required for cell adhesion and neurite outgrowth. Therefore, we examined the effect of SPTBN1 and α-synuclein on neurite outgrowth of dopaminergic neuronal cells.

2. Materials and methods

2.1. Phage display of a normalized human brain cDNA library

Whole Human Brain Marathon-Ready cDNA (BD Biosciences, USA) was amplified by PCR (30 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 2 min) using Advantage 2 Polymerase Mix (Clontech Inc., USA) and the two primers, the 5′ adaptor sequence primer (5′-CCTGAATTCACTCACTATAGGGCTCGAGCGGC-3′) and 3′ oligo(dT) primer (5′-GACAAGCTTTTTTTTTTTTTTTTTTTTTTTTT TTTTT-3′). Biotin-14-dCTP (50 nM, 10 μl) (Invitrogen Co., The Netherlands) was included in the PCR reaction to label the PCR fragments.

Normalization of the cDNA library was performed as described previously [15] with slight modifications. Briefly, 2 μg of cDNA were hybridized with a 20-fold excess of biotinylated cDNA (40 μg) in 50 μl of hybridization buffer (10 mM Tris–Cl, pH 7.8, 5 mM EDTA, and 0.5% SDS) by heating the mixture at 95 °C for 3 min to denature the cDNA to single-stranded DNA and then incubating this at 65 °C for 3 h to anneal the unlabeled and labeled cDNAs. The mixture was diluted with 1 ml of pre-chilled 10× SSC buffer (1.5 M NaCl, 150 mM sodium citrate, pH 7), and the biotinylated cDNA hybrids were selectively removed using MagPrep streptavidin beads (Novagen Inc., USA). The remaining cDNA was precipitated with ethanol and dissolved in 20 μl of TE buffer. Normalization was confirmed by amplifying representative differentially expressed genes: epidermal growth factor receptor (EGFR) and regulator of G-protein signaling 5 (RGS), which are normally expressed at low levels, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), which is normally expressed at a high level. For PCR amplification, the following primers were used (5′ → 3′): for EGFR, ATGGCACCCTCCGGGACGGCCGGG (forward) and CCAAAGCT GTATTTGCCCTCGGGG (backward); for RGS5, CACCATGTGCAAAGG ACTTGCAGCTTTGC (forward) and GCCTGGCTAAATTACTACTTGA TTAACTCCTGA (backward); and for G3PDH, GGTCTTACTCCTTGGAG GCCATGT (forward) and GACCCCTTCATTGACCTCAACTACA (backward).

The remaining non-biotinylated cDNAs larger than 300 bp in length were cloned into T7select 10B vector (Novagen Inc.). Assembled T7 phages were used to infect *Escherichia coli* BLT5615 (Novagen Inc.), according to the manufacturer's protocol.

2.2. Isolation of α-synuclein-binding phages

Biotinylated α-synuclein was expressed in *E. coli* strain BL21 (DE3) (Novagen Inc.), and purified as described previously [16]. In brief, 1 mg of biotinylated α-synuclein was applied to 20 μl MagPrep Streptavidin beads, and unbound proteins were washed with phosphate buffered saline containing 0.3% Tween 20 (PBST). A phage library containing 2.1×10^7 phage-forming units (PFU) was incubated with the biotinylated α-synuclein-bead complex, and the bead-bound phages were allowed to form plaques in *E. coli* BLT5615 cultures. Isolated plaques were collected, and binding to the biotinylated α-synuclein-bead complex was repeated with increasing stringency.

2.3. GST pull-down assay

To produce recombinant SPTBN1 protein, a cDNA encoding human SPTBN1 N-terminal domain was amplified by polymerase chain reaction (PCR), using primers of sequence (5′ → 3′): CAGGGATCCATGACGACCACAGTAGCCAC (forward) and CTTGAATTCCTATGTGATGCGCTTGATGTCGTG (backward). The PCR products were digested with *Xho*I and *Eco*RI, and then cloned into pRSET B (Invitrogen Co., The Netherlands), a poly histidine-tagging expression vector. $(His)_n$-SPTBN1 protein was overexpressed in the transformed *E. coli* BL21 (DE3) strain, and purified using a Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Peptron Co., Korea) column that had been pre-equilibrated with loading buffer (20 mM Tris–HCl, 0.5 M NaCl, and 5 mM imidazole, pH 7.4). SPTBN1 protein was eluted with an imidazole gradient.

The production of a GST-α-synuclein fusion protein and GST pull-down assay is done as described previously [16], with slight modification. GST or GST-α-synuclein fusion protein attached to glutathione-agarose beads were incubated overnight with 1 μg $(His)_n$-tagged SPTBN1 protein in 1 ml PBST at 4 °C on a rotator. Bound proteins were eluted with PBST containing 2 M NaCl, and detected by immunoblotting using a rabbit anti-$(His)_n$ antibody (Santa Cruz Biotechnology Inc., USA).

2.4. Co-immunoprecipitation of α-synuclein with SPTBN1

For mammalian expression, the α-synuclein gene was subcloned into pcDNA3.1, and SPTBN1 was subcloned into pEGFP-C1 at *Bam*HI/*Eco*RI endonuclease sites. N2a mouse neuronal cells were transfected with 1 μg pcDNA3.1-α-synuclein and/or pEGFP-SPTBN1 DNA, using 3 μl TransIT®-LT1 Transfection Reagent (Mirus Bio Co., USA). After 48 h, cells were lyzed in a buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin). Crude cell lysates were incubated overnight with a rabbit polyclonal anti-α-synuclein antibody (Santa Cruz Biotechnology Inc., USA) in immunoprecipitation buffer (50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), and the immune complexes were precipitated using protein A-Sepharose beads. Thirty μg of the immune complexes was resolved on 15% SDS–PAGE and analyzed by immunoblotting using primary antibodies (diluted 1:250) and then with horseradish peroxidase-conjugated secondary antibodies (diluted 1:10,000). A mouse anti-GFP antibody was purchased from Zymed Laboratories Inc., and a goat anti-mouse and anti-rabbit IgG conjugated to a peroxidase were from Sigma. Bound antibody was visualized by enhanced chemiluminescence (ECL) on an X-ray film (Curix CP-BU, Agfa Co., USA) using luminol as a substrate.

2.5. Colocalization analysis

N2a cells were transfected with pdsRed-α-synuclein and/or pEGFP-SPTBN1 DNA. Cells grown on glass cover slips were fixed with 3.7% formaldehyde in PBS, and mounted using 90% glycerol containing 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Images were captured using an Axhoplan2 confocal laser scanning microscope (ZEISS). The excitation and emission wavelengths were 488 and 509 nm, respectively, for eGFP, and 563 and 581 nm, respectively, for dsRed.