Biochemical and Biophysical Research Communications 424 (2012) 497–502

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

α-Synuclein modulates neurite outgrowth by interacting with SPTBN1

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ARTICLE INFO

Article history:
Received 18 June 2012
Available online 4 July 2012

Keywords:
α-Synuclein
Protein interaction
SPTBN1
Neurite outgrowth

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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http://dx.doi.org/10.1016/j.bbrc.2012.06.143

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.

2.6. Neurite outgrowth assay

Human SH-SY5Y dopaminergic neuronal cells were transfected with pEGFP-SPTBN1 and pdsRed-α-synuclein. At 24 h after transfection, neurite outgrowth was induced by adding 10 μM retinoic acid to complete culture medium. The differentiated cells were grown on cover slips and observed using an Axhoplan2 confocal laser scanning microscope, as described above.

3. Results

3.1. Normalization of human brain cDNA

Most cDNA libraries contain abundantly expressed genes that can increase false-positive rates and interfere with protein interaction assays. To avoid this, we normalized a human brain cDNA library using a simple PCR-based cDNA subtraction method based on the concept that during a short hybridization time, abundant cDNA species will easily anneal to their complementary strands, whereas less common cDNA species will remain largely unpaired. After a human brain cDNA library was briefly hybridized with biotinylated cDNA, the biotinylated cDNA hybrids, which represented the most abundant cDNAs in the library, were selectively removed from the mixture using streptavidin-linked paramagnetic beads. The reduction in abundant cDNA species was confirmed by PCR amplification of the differentially expressed genes EGFR and RGS5 (expressed at low levels in human brain cells), and G3PDH (an abundantly expressed housekeeping gene) [17,18]. As expected, before normalization, G3PDH PCR products were abundant, while EGFR and RGS5 were present at much lower levels. After normalization, the G3PDH signal intensity decreased significantly, while the EGFR and RGS5 signals increased (Supplementary Fig. S1). The resulting normalized cDNAs were then used to construct a phage display library.

3.2. α-Synuclein specifically interacts with SPTBN1 in vitro

A total of 2.1×10^7 T7 phages displaying a normalized human brain cDNA library were incubated with α-synuclein-coated paramagnetic beads. After repeated binding and elution of phages, the nucleotide sequences carried by bound phages were determined. A phage harboring the N-terminal domain of SPTBN1 (to residue #378) was isolated. The direct interaction between α-synuclein and SPTBN1 was confirmed using a GST pull-down assay. Briefly, α-synuclein was expressed as a GST-α-synuclein fusion protein and immobilized on glutathione-agarose beads. The SPTBN1 N-terminal domain was expressed as a $(His)_n$-tagged SPTBN1 protein and incubated with the beads. After washing away unbound proteins, bound SPTBN1 was detected by immunoblotting using an anti-$(His)_n$ antibody. SPTBN1 bound to the GST-α-synuclein beads (Fig. 1, lane 7), but not to the control GST beads (lane 6). Thus, SPTBN1 specifically interacted with α-synuclein *in vitro*.

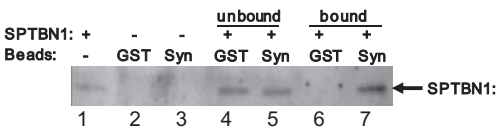

Fig. 1. GST pull-down assay for SPTBN1. Purified $(His)_n$-SPTBN1 was incubated with GST-α-synuclein bound to glutathione-Sepharose. Bound proteins were resolved by 12% SDS–PAGE, and SPTBN1 was detected by immunoblotting using an anti-$(His)_n$ antibody. Lanes: 1, SPTBN1 control; 2, GST bound to glutathione-Sepharose; 3, GST-α-synuclein bound to glutathione-Sepharose; 4, supernatant fraction of SPTBN1 incubated with GST-glutathione-Sepharose; 5, supernatant fraction of SPTBN1 incubated with GST-α-synuclein-glutathione-Sepharose; 6, bound fraction of SPTBN1 incubated with GST-glutathione-Sepharose; and 7, bound fraction of SPTBN1 incubated with GST-α-synuclein-glutathione-Sepharose.

3.3. SPTBN1 co-immunoprecipitates with α-synuclein in transfected N2a mouse neuronal cells

To determine whether SPTBN1 interacts with α-synuclein in neuronal cells, pEGFP-SPTBN1 and/or pcDNA3.1-α-synuclein was transfected into N2a cells, and co-immunoprecipitation was performed. Cell lysates from the transfected N2a cells were incubated with a polyclonal antibody against α-synuclein, and the immune complexes were precipitated using protein A-Sepharose beads. After resolving the immune complexes on SDS–PAGE, co-precipitated SPTBN1 was detected by immunoblotting using a polyclonal antibody against GFP. When both SPTBN1 and α-synuclein were overexpressed in N2a cells, SPTBN1 co-immunoprecipitated with α-synuclein (Fig. 2A, lane 6). When α-synuclein or SPTBN1 was transfected alone, SPTBN1 did not co-immunoprecipitate with α-synuclein (lanes 4 and 5). Although the polyclonal anti-GFP antibody cross-reacted with some proteins (lanes 1–3; asterisks), these proteins did not co-immunoprecipitate with α-synuclein (lanes 4–6). The results show that SPTBN1 selectively interacted with α-synuclein in cotransfected N2a neuronal cells.

3.4. α-Synuclein colocalizes with SPTBN1 in N2a cells

N2a cells were transfected with pEGFP-SPTBN1 and pdsRed-α-synuclein to analyze the subcellular localization of SPTBN1 and α-synuclein, respectively. Non-transfected cells did not emit any significant signal, as expected. In cells transfected with pEGFP-SPTBN1 or pdsRed-α-synuclein alone, the green fluorescence emission from pEGFP-SPTBN1 was localized mainly to the plasma membrane and Golgi apparatus (Fig. 3C), as previously reported [19], and the red fluorescence signal from pdsRed-α-synuclein was dispersed throughout the cytosol (Fig. 3F). In cotransfected cells, some portion of SPTBN1 colocalized with α-synuclein, which also exhibited a more condensed distribution at specific cytoplasmic loci (Fig. 3I).

3.5. α-Synuclein reverses the disproportionate neurite branching induced by SPTBN1 overexpression

Human SH-SY5Y dopaminergic neuron cells were transfected with pEGFP-SPTBN1 and/or pdsRed-α-synuclein, and neurite outgrowth was induced by addition of 10 μM retinoic acid. In

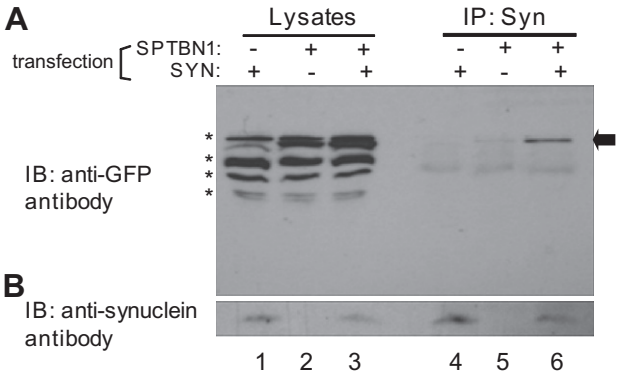

Fig. 2. Co-immunoprecipitation of SPTBN1 with α-synuclein. Lysates of transfected N2a cells were immunoprecipitated with polyclonal anti-α-synuclein antibody and immunoblotted using polyclonal anti-GFP antibody (A) or polyclonal anti-synuclein antibody (B).

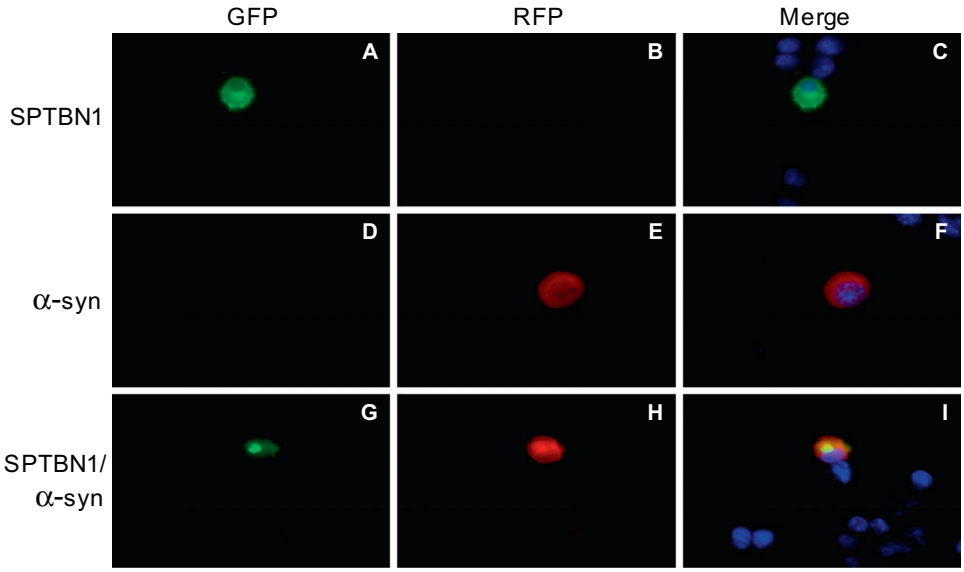

Fig. 3. Colocalization of SPTBN1 and α-synuclein. (A–C) N2a neuronal cells transfected with pEGFP-SPTBN1. (D–F) N2a cells transfected with pdsRed-α-synuclein. (G–I) N2a cells cotransfected with pEGFP-SPTBN1 and PdsRed-α-synuclein. (A, D, G) Green fluorescence emission was monitored. (B, E, H) Red fluorescence emission was monitored. (C, F, I) Green, red, and blue (DAPI) fluorescence images were merged. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

non-transfected SH-SY5Y cells, only one or two neurites extended from the cell body (data not shown). Overexpression of α-synuclein did not promote neurite branching, and most cells retained one or two neurites (Fig. 4E). However, overexpression of SPTBN1 induced excessive branching of neurites from the cell body and inhibited longitudinal growth of neurites. More than 90% of SPTBN1-transfected cells had more than three short neurites (Fig. 4A). Upon cotransfection, α-synuclein reversed the SPTBN1-induced neurite over-branching and resulted in a single neurite per neuron (Fig. 4I). More than 90% of cotransfected cells formed a single long neurite extension. This result suggests that α-synuclein modulates neurite outgrowth by interacting with the cytoskeleton protein SPTBN1 during synaptogenesis.

4. Discussion

SPTBN1 was identified as an α-synuclein-interacting partner in this study. SPTBN1 is a 247-kDa cytoskeletal protein and forms heterodimers with α-spectrins by antiparallel helical association [20,21]. Spectrin heterodimers form a lattice covering the cytoplasmic surface of all cellular membranes, including the plasma membrane, ER, mitochondrial outer membrane, nuclear envelope, and Golgi [19]. SPTBN1 contains an actin-binding domain, synapsin I-binding domain, and 17 homologous repeating units (each about 106 amino acids in length) [20]. SPTBN1 also binds to membrane phospholipids such as phosphatidylserine, phosphatidylglycerol, and cardiolipin [22]. Therefore, spectrin dimers may bind

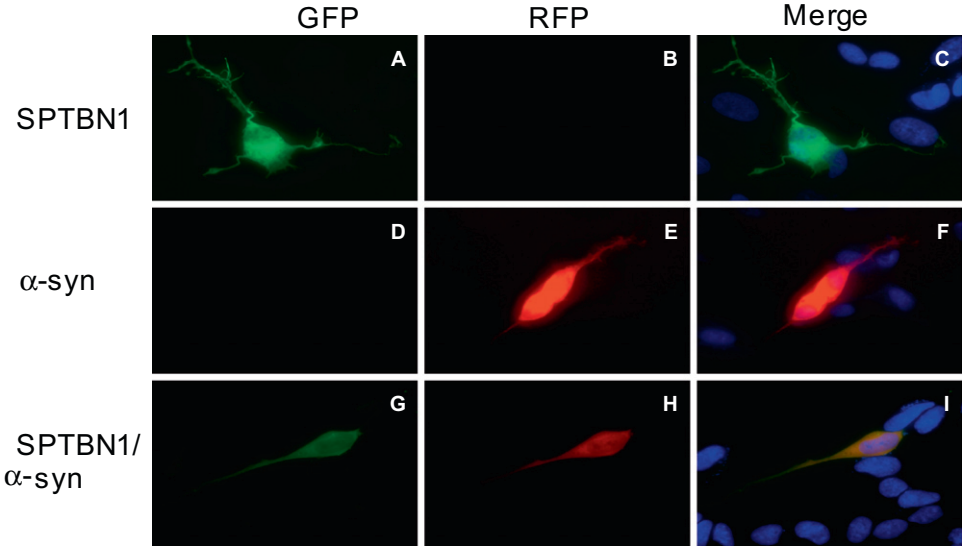

Fig. 4. Effects of SPTBN1 and α-synuclein on neurite outgrowth of human dopaminergic neurons. SH-SY5Y cells were transfected with pEGFP-SPTBN1 and/or PdsRed-α-synuclein. Neurite outgrowth was induced by adding 10 μM retinoic acid to the complete culture medium. (A–C) SH-SY5Y cells transfected with pEGFP-SPTBN1. (D–F) SH-SY5Y cells transfected with pdsRed-α-synuclein. (G–I) SH-SY5Y cells co-transfected with pEGFP-SPTBN1 and pdsRed-α-synuclein. (A, D, G) Green fluorescence emission was monitored. (B, E, H) Red fluorescence emission. (C, F, I) Green, red, and blue (DAPI) fluorescence images were merged. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

membrane surfaces by interacting with coupling proteins such as actin or ankyrin, or through direct interactions with membrane phospholipids.

Proteomic analyses of cortical Lewy bodies from dementia with Lewy bodies (DLB) patients have previously identified SPTBN1 in Lewy bodies with α-synuclein [23]. However, it was possible that the identified proteins might have been contaminants or other proteins accidently incorporated into Lewy bodies under pathological conditions. Another proteomics analysis using α-synuclein C-terminal peptide in pull-down assays identified several cytoskeletal elements, including non-erythrocyte αII and βII spectrins, along with the known spectrin-binding proteins ankyrin, band 4.1B, and actin [24]. Although proteomics analysis is a robust and powerful screening method for identifying proteins, the identified proteins must be confirmed or validated using a second method. No further validation of the SPTBN1 interaction with α-synuclein has been performed in previous studies, and false-positive results or indirect interactions through an association in a complex mediated by actual binding partner(s) have remained possible. Here, SPTBN1 was identified as an α-synuclein-interacting partner using a phage display assay under physiological conditions. Furthermore, direct interaction between α-synuclein and SPTBN1 was confirmed by GST pull-down (Fig. 1) and co-immunoprecipitation assays using neuronal cell extracts (Fig. 2). In addition, SPTBN1 proteins colocalized with α-synuclein in cotransfected neuronal N2a cells (Fig. 3). Thus, SPTBN1 is likely an authentic interacting partner of α-synuclein.

In neuronal cells, SPTBN1 and α-synuclein modulated neurite outgrowth (Fig. 4). In the growing cones of neural dendrites, structural rearrangement of F-actin filaments at the leading edge is critical [25]. Considering the actin-binding activity of SPTBN1, overexpression of exogenous SPTBN1 may interfere with the balanced remodeling of cytoskeletal actin and may affect neurite outgrowth in dopaminergic neuronal cells. Cotransfection of α-synuclein may recover the balance and reverse the SPTBN1-induced neurite over-branching. It has been also suggested that α-synuclein can reduce neurite outgrowth, possibly by reducing cell adhesion [26]. Given that SPTBN1 is essential for stabilization of synaptic cell adhesion [27], overexpression of α-synuclein may reduce synaptic adhesion by occupying SPTBN1 binding sites. Our results are also consistent with a previous observation by Chung et al. [12] that actin colocalized with α-synuclein in dystrophic axon terminals. As SPTBN1 has an actin-binding site, α-synuclein may colocalize with actin filaments through its interaction with SPTBN1.

α-Synuclein may regulate vesicle trafficking by interacting with SPTBN1. SPTBN1 is involved in the regulation of exocytosis, in particular, the regulation of neurotransmitter release, in which its interaction with small synaptic vesicles via synapsin I plays a crucial role [28,29]. Actin filaments, which are a binding partner of SPTBN1, also participate in the transport of clear and dense-core vesicles along the axon and in their clustering at the plasma membrane site where exocytosis occurs. Upon cell stimulation, a dense network of actin filaments beneath the plasma membrane is reorganized to allow access of vesicles to fusion sites. Because the binding sites for synapsin I and actin are spatially close on SPTBN1, vesicle tethering to the plasma membrane would release the N-terminal domain of SPTBN1 from its contact with F-actin, thus destabilizing the membrane skeleton [30]. Fusogenic proteins such as v-SNAREs, t-SNAREs, and SNAPs would then engage to form the fusion pore. Indeed, an antibody against the synapsin-binding domain of SPTBN1 blocks synaptic transmission and inhibits excitatory postsynaptic currents in hippocampal neurons [29]. Thus, our finding that SPTBN1 is an α-synuclein-interacting protein suggests that α-synuclein inhibits synaptic vesicle fusion by competing for synapsin I-binding sites on SPTBN1. Further studies are required to address this hypothesis. The information obtained from this study contributes to the identification of novel targets for the development of therapeutic compounds for PD, for which no effective therapy is currently available.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2012-0002875).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.143.

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