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Interaction of Human α-Synuclein with VTI1B May Modulate Vesicle Trafficking

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Human α-synuclein is the major component of the protein aggregates known as Lewy bodies or Lewy neurites, which define the intracellular lesions of Parkinson's disease. Despite extensive efforts, the physiological function of α-synuclein has not yet been elucidated in detail. As an approach to defining its function, proteins that interacted with α-synuclein were screened in phage display assays. The SNARE protein vesicle *t*-SNARE-interacting protein homologous 1B (VTI1B) was identified as an interacting partner. A selective interaction between α-synuclein and VTI1B was confirmed by coimmunoprecipitation and GST pull-down assays. VTI1B and α-synuclein were colocalized in N2a neuronal cells, and overexpression of α-synuclein changed the subcellular localization of VTI1B to be more dispersed throughout the cytosol. Considering the role played by VTI1B, α-synuclein is likely to modulate vesicle trafficking by interacting with a SNARE complex.

Key Words : α-Synuclein, Protein interaction, VTI1B, Vesicle trafficking

Introduction

The mutation (A30P, A53T and E46K) or overexpression (duplication and triplication) of α-synuclein causes some forms of familial PD Parkinson's disease (PD).[1-5] Although formation of α-synuclein inclusions may lead to neurodegeneration through an increase in toxicity, sequestration of monomeric α-synuclein into insoluble aggregates may also result in the development of PD due to the loss of an important function. Understanding the physiological role of α-synuclein in normal cells is likely of critical importance to resolution of the role of α-synuclein in the pathology of PD.

Human α-synuclein is a neuronal cytosolic protein that is expressed at high levels, especially in presynaptic terminals.[6] In aqueous solution, purified α-synuclein is unstructured,[7] but upon binding to phospholipid vesicles it can adopt an amphipathic α-helical structure.[8] Studies of α-synuclein function suggest that α-synuclein-associated cytotoxicity might be the consequence of abnormal regulation of vesicle localization and fusion.[9] α-Synuclein-knockout mice release excessive amounts of neurotransmitter at dopaminergic nerve terminals in response to paired stimuli, implicating malfunction in the control of the number of vesicles that fuse.[10] Meanwhile, overexpression of α-synuclein in model organisms consistently revealed vesicle trafficking defects, impairment of the ubiquitin-proteasome system and mitochondrial dysfunction.[11-13] Overexpression of human α-synuclein in transgenic mice induced aggregate formation and abnormal vesicle trafficking, and caused nigrostriatal dopaminergic injury and motor deficits.[14] α-Synuclein-overexpressing PC12 cells interfere with a late step in exocytosis and increase the number of docked vesicles at synapses,[15] while α-synuclein-overexpressing yeasts were defective in endocytosis[16] and endothelial reticulum (ER)-Golgi trafficking.[17] In both yeasts and human PC12 cells, docking and fusion of vesicles with Golgi membranes is hampered.[18,19] The involvement of α-synuclein in endocytosis is also supported by another study, which showed that downregulation of the endocytic pathway in *Caenorhabditis elegans* using siRNAs increased the toxicity of α-synuclein.[20] Consistent with the idea that α-synuclein has a role in vesicle trafficking, it was co-immunoprecipitated with Rab3A, Rab5 and Rab8, which are involved in synaptic vesicle trafficking and exocytosis at the synapse, endocytosis and trans-Golgi transport, respectively.[21] The yeast *v*-SNARE protein ykt6, *Drosophila* Rab1, human Rab1 and *C. elegans* Rab8 mitigated the cytotoxicity of α-synuclein-overexpression,[17,18,22] further supporting the role of α-synuclein in multiple vesicle trafficking steps. However, the detailed mechanism by which α-synuclein regulates multiple vesicle trafficking steps and interactions with other transport proteins remains to be elucidated.

One approach to elucidating the function of a protein is to determine the proteins with which it interacts, which may cooperate in regulation of the function of target proteins. However, proteins found in intracellular inclusions, such as Rab3A, Rab5 and Rab8 in Lewy bodies or Lewy neurites,[21] could be innocent bystanders, accidentally incorporated into intracellular inclusions as a secondary consequence of pathogenic conditions. To overcome these limitations, we have screened for interacting partners of α-synuclein under physiological conditions using a phage display human brain cDNA library. In a previous study, prenylated Rab acceptor protein 1 (PRA1) was identified as an interacting partner of α-synuclein.[23] VTI1B was identified during our efforts to identify other α-synuclein-interacting proteins, as reported here. VTI1B is localized on endosomes and vesicles and in the trans-Golgi network.[24,25] It is known to participate in the fusion of vesicles and target membranes during exocytosis and endocytosis.[24,26] In late endosomes, a SNARE complex

composed of VTI1B, syntaxin 8, syntaxin 7, endobrevin/VAMP-8 and VTI1B has been implicated in homotypic late endosome fusion and late endosome-lysosome fusion.[24] VTI1B deficiency in mice results in the loss of a single SNARE partner, syntaxin 8, and slightly delayed lysosomal degradation in hepatocytes.[27] This suggests that α-synuclein regulates vesicle trafficking by interacting with a SNARE complex.

Experimental Section

Strains and Reagents. *E. coli* BLT5615 (Novagen Inc., USA) was used for amplification of phage, and *E. coli* BL21 (DE3) (Novagen Inc., USA) was used for expression of α-synuclein. Q-sepharose™ fast flow and Hybond™ ECL™ nitrocellulose membrane was from GE Healthcare (Piscataway, USA). Bio-Rad detergent-compatible (DC) protein assay kit was purchased from Bio-Rad Laboratories Inc. (Hercules, USA). A rabbit anti-α-synuclein antibody and a rabbit anti-$(His)_n$ antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). A goat anti-mouse and anti-rabbit IgG conjugated to a peroxidase were from Sigma (St. Louis, USA). Curix CP-BU, a medical X-ray film, was purchased from Agfa Co. (Ridgefield Park, USA). All other chemicals were reagent grade.

Phage Library Construction. Normalization of Whole Human Brain Marathon-Ready cDNA (BD Biosciences, USA) was achieved through hybridization with a 20-fold excess of biotinylated cDNA, as described previously.[23] The normalized cDNAs larger than 300 bp in length were digested with *Eco*RI/*Hind*III, and cloned into the T7select 10B vector (Novagen Inc.). Cloned cDNA was incubated with 25 μL T7 packaging extract, and then a host strain (BLT561) was infected according to the manufacturer's protocol.

Isolation of α-Synuclein-Binding Phages. An expression vector for a form of α-synuclein with a biotin acceptor domain (pSyn-B) was constructed, as described before.[23] One milligram of biotinylated α-synuclein was purified and incubated with 20 μL MagPrep Streptavidin beads. A phage library containing 2.1×10^7 phage-forming units (PFU) was incubated with the biotinylated α-synuclein-bead complex for 10 min at room temperature, and the bead-bound phages were then allowed to form plaques in BLT5615 host cell cultures. Isolated plaques were collected, and binding to the biotinylated α-synuclein-bead complex was repeated with increasing stringency to confirm interactions with α-synuclein.

GST Pull-Down Assay. To produce recombinant VTI1B protein, a cDNA encoding human VTI1B was amplified by polymerase chain reaction (PCR) using a pair of primers (5'-CGGCTCGAGGCCATGGCCTCCTCCGCC-3' and 5'-AGAGGATCCATGGCTGCGAAAGAATTTGTA-3'). The PCR products were cloned into pRSET B (Invitrogen Co., Netherlands), a poly histidine-tagging expression vector. $(His)_n$-VTI1B protein was overexpressed in the transformed *E. coli* BL21 (DE3) strain, and cells were disrupted using a Bandelin sonicator. The cell lysates were applied to 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, 60 mM imidazole, pH 7.4). VTI1B protein was eluted with an imidazole gradient.

Human α-synuclein cDNA was cloned into pGEX-4T-1 (Novagen Inc.) for the production of a GST-α-synuclein fusion protein. One μg of recombinant GST or GST-α-synuclein fusion protein was immobilized on 100 μL glutathione agarose beads. The beads were incubated overnight with 1 μg $(His)_n$-tagged VTI1B protein in 1 mL PBST at 4 °C on a rotator. To remove unbound proteins, the beads were washed with PBST. Bound proteins were eluted with PBST containing 1 M NaCl, and detected by immunoblotting using an anti-$(His)_n$ antibody.

Coimmunoprecipitation of α-Synuclein with VTI1B. For mammalian expression, the α-synuclein gene was subcloned into pcDNA3.1, and VTI1B was subcloned into pEGFP-N3 at *Bam*HI/*Xho*I endonuclease sites. N2a mouse neuronal cells were cultured on 60-mm plates in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS, GIBCO) and antibiotics. The cells were transfected with 1 μg pcDNA3.1-α-synuclein and/or pEGFPN3-VTI1B DNA, using 3 μL TransIT®-LT1 Transfection Reagent (Mirus Bio Co., USA). Cells were grown for 48 h, and treated with lysis 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 polyclonal anti-α-synuclein antibodies. The immune complexes were precipitated through incubation with protein A-Sepharose beads (Amersham) for 2 h, and then washed repeatedly in immunoprecipitation (IP) buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). The final pellet was resolved by 15% SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. The blots were probed with primary antibodies (diluted 1:250) and then with horseradish peroxidase-conjugated secondary antibody (diluted 1:10,000). Bound antibody was visualized by enhanced chemiluminescence (ECL) on an X-ray film using luminol as a substrate.

Colocalization Analysis. N2a cells were transfected with pdsRed-α-synuclein and/or pEGFPN3-VTI1B DNA. Cells were grown on glass cover slips, and fixed through incubation with 3.7% formaldehyde in PBS for 30 min at 37 °C. Cover slips were 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.

Results

VTI1B Specifically Interacts with α-Synuclein *in vitro*. In the present study, the interacting partners of α-synuclein were screened under physiological conditions, using a phage

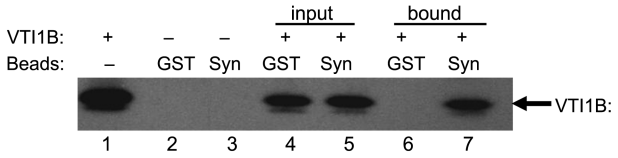

Figure 1. GST pull-down assay for VTI1B. Purified VTI1B was incubated with GST-α-synuclein bound to glutathione-Sepharose. Bound proteins were resolved by 12% SDS-PAGE, and VTI1B was detected by immunoblotting using an anti-$(His)_n$ antibody. Lanes: 1, VTI1B control; 2, GST bound to glutathione-Sepharose; 3, GST-α-synuclein bound to glutathione-Sepharose; 4, input of VTI1B incubated with GST-glutathione-Sepharose; 5, input of VTI1B incubated with GST-α-synuclein-glutathione-Sepharose; 6, bound fraction of VTI1B incubated with GST-glutathione-Sepharose; 7, bound fraction of VTI1B incubated with GST-α-synuclein-glutathione-Sepharose; and Syn, GST-α-synuclein fusion protein.

display of human brain cDNA library. A total of 2.1×10^7 T7 phages displaying a normalized human brain-specific cDNA library were incubated with biotinylated α-synuclein-coated paramagnetic beads. Binding of phages to α-synuclein-immobilized beads and elution were iterated three times with increasing stringency. Nucleotide sequences encoding the proteins on selected phages were determined, and an N-terminal domain encoding VTI1B (up to residue number 81) was isolated.

To investigate the direct interaction between α-synuclein and VTI1B, a GST pull-down assay was performed. Full-length VTI1B was expressed as a $(His)_n$-tagged VTI1B protein, and full-length α-synuclein as a GST-α-synuclein fusion protein. Purified GST-α-synuclein was immobilized on glutathione agarose beads, and VTI1B was added to the mixture. As a control, equivalent amounts of GST and VTI1B were used in a separate pull-down assay. After washing away unbound proteins, bound proteins were eluted by PBST containing 1 M NaCl and resolved by 12% SDS-PAGE. Bound VTI1B was detected by immunoblotting using an antibody against the $(His)_n$-tag (Fig. 1). VTI1B bound to GST-α-synuclein (Fig. 1, lane 7), but not to GST alone (Fig. 1, lane 6). Thus VTI1B specifically interacts with α-synuclein *in vitro*.

VTI1B Coimmunoprecipitates with α-Synuclein in N2a Mouse Neuronal Cells. To determine whether VTI1B interacts with α-synuclein in neuronal cells, pEGFPN3-VTI1B was cotransfected with pcDNA3.1-α-synuclein into N2a cells. Cell extracts were prepared from transfected cells, and incubated with a polyclonal antibody against α-synuclein. Bound complexes were isolated using protein A-Sepharose beads, and coprecipitated VTI1B was detected by immunoblotting using a mouse polyclonal anti-GFP antibody (Zymed Laboratories Inc., USA).

VTI1B coimmunoprecipitated with α-synuclein when both VTI1B and α-synuclein were overexpressed (Fig. 2, lane 6), but not when α-synuclein or VTI1B was not transfected (lanes 4 & 5). This result shows that α-synuclein selectively interacts with VTI1B in cotransfected N2a cells.

VTI1B Colocalizes with α-Synuclein in N2a Cells. N2a cells were transfected with pEGFPN3-VTI1B and pdsRed-

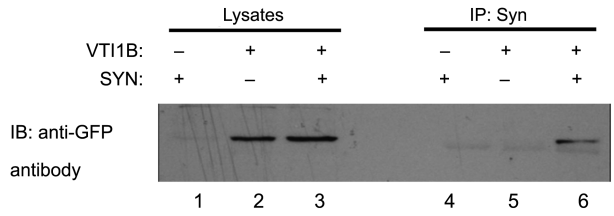

Figure 2. Coimmunoprecipitation of VTI1B with α-synuclein. N2a cells were cotransfected with pEGFPN3-VTI1B and pcDNA3.1-α-synuclein. Cell lysates were immunoprecipitated with a polyclonal anti-α-synuclein antibody, and immunoblotted using a polyclonal anti-GFP antibody.

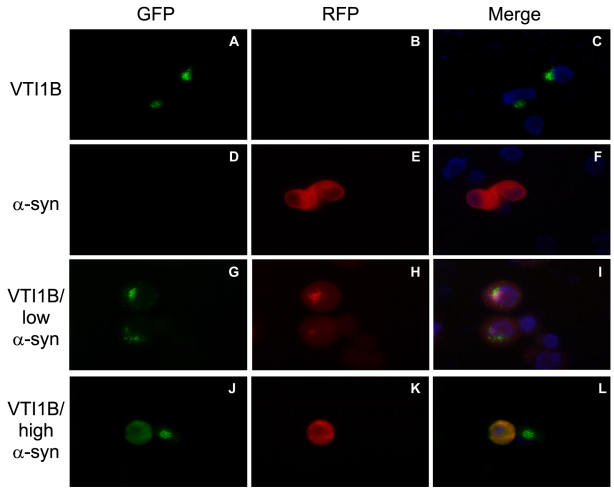

Figure 3. Colocalization of VTI1B and α-synuclein. (A-C) N2a neuronal cells transfected with pEGFPN3-VTI1B. (D-F) N2a cells transfected with pdsRed-α-synuclein. (G-I) N2a cells cotransfected with pEGFPN3-VTI1B and PdsRed-α-synuclein, in which the expression level of α-synuclein was low to medium. (J-L) N2a cells cotransfected with pEGFPN3-VTI1B and PdsRed-α-synuclein, in which the expression level of α-synuclein was high. (A, D, G, J) Green fluorescence emission was monitored. (B, E, H, K) Red fluorescence emission was monitored. (C, F, I, L) Green, red, and blue (DAPI) fluorescence images were merged.

α-synuclein. The subcellular localizations of VTI1B and α-synuclein in transfected N2a cells were determined by green and red fluorescence emission, respectively. Non-transfected cells did not emit any significant signal (data not shown). As reported previously,[24,26] VTI1B was localized to the perinuclear clusters, which is a typical pattern for Golgi apparatus, in the absence of transfected α-synuclein (Fig. 3(c)). α-Synuclein was dispersed throughout the cytosol when transfected alone (Fig. 3(f)). When the expression level of α-synuclein was low-to-medium in VTI1B-cotransfected cells, some α-synuclein colocalized with VTI1B at the Golgi apparatus, while the rest retained a cytosolic distribution (Fig. 3(i)). However, in ~20% of cotransfected cells (80 of the 406 cells counted) in which α-synuclein was expressed at high levels, VTI1B was mistargeted to a more dispersed location, and the fluorescence signals from α-synuclein and VTI1B overlapped (Fig. 3(l)). These results suggest that very high levels of α-synuclein may affect the localization of VTI1B, and thus modulate vesicle trafficking.

Discussion

To develop a therapeutic strategy for PD, it is first necessary to determine the biological function of α-synuclein in the central nervous system. Many researchers have attempted to determine the role of α-synuclein both in the central nervous system and in PD. α-Synuclein has been shown to interact with tau, tubulin, synphilin, Rab3A, syntaxin and lipid vesicles.[8,19,28-30] However, most previous studies have examined α-synuclein function under abnormal pathogenic conditions, such as in Lewy bodies, Lewy neurites and cytoplasmic inclusions. To determine its functions under normal conditions, we isolated the proteins that interacted with α-synuclein using a phage display method in which human brain peptides or proteins are displayed at the phage surface. Since no thioflavin T fluorescence was emitted from the biotinylated α-synuclein preparation (data not shown), the bait for interacting partners was not in amyloid form. Some phages that selectively bound to α-synuclein contained VTI1B, a SNARE component.[26] This interaction was confirmed by GST pull-down assays (Fig. 1), co-immunoprecipitation (Fig. 2) and colocalization (Fig. 3) experiments. Localization of VTI1B was typical for the Golgi apparatus in VTI1B-transfected mouse neuronal cells, as reported previously (Fig. 3(a)), and α-synuclein localized to the cytosol when transfected alone (Fig. 3(d)). In cotransfected cells, some α-synuclein colocalized with VTI1B in the Golgi apparatus, even when the expression levels of α-synuclein was low (Fig. 3(i)). These results suggest that α-synuclein interacts with VTI1B in neuronal cells.

α-Synuclein has been suggested to play a role in the regulation of vesicle recycling and synaptic plasticity. However, the detailed mechanism has not been elucidated fully. The involvement of α-synuclein in vesicle trafficking *via* SNARE complexes is supported by studies using cysteine-string protein α (CSPα)-deficient mice.[31] CSPα is a presynaptic molecular chaperone that is crucial for neurotransmitter release, vesicle recycling and the integrity of synaptic nerve terminals. SNARE complex levels are significantly reduced in CSPα-deficient mice. Overexpression of α-synuclein compensates for the loss of CSPα, restores SNARE complex levels and suppresses presynaptic degeneration. The ability of α-synuclein to compensate the reduced SNARE level in CSPα-deficient mice suggested a positive role for α-synuclein in vesicle trafficking. Meanwhile, overexpression of α-synuclein delayed vesicle docking and fusion,[15-17] and thus had a negative effect on vesicle transport. Therefore, α-synuclein is likely in a delicate balance between the supporting for synaptic function and the slowing of biosynthetic secretory pathways through disruption of SNAREs, depending on its concentration. Overexpression or mutation of α-synuclein in PD may simply amplify the negative features of this normal cellular trade-off.

We previously identified PRA1 as an α-synuclein-interacting partner[28] that regulates the cycling of small Rab GTPases during exocytosis and endocytosis in eukaryotic cells.[32,33] PRA1 plays regulatory roles in SNARE assembly, vesicle budding, vesicle tethering and fusion,[34] which further supports the putative role of α-synuclein in modulation of vesicle trafficking *via* SNARE complexes. Although human Rab1 and yeast Ykt6, the proteins involved in vesicle trafficking, mitigated the cytotoxicity of α-synuclein-overexpression,[17] direct interaction between Rab1 (or ykt6) and α-synuclein has not been addressed. It will be of interesting to examine whether they rescues α-synuclein-mediated toxicity *via* direct interaction, and their binding sites on α-synuclein, if they indeed binds, overlaps with that of VTI1B. Further studies would lead to a better understanding of the biological function of α-synuclein and the cellular dysfunction associated with α-synuclein toxicity, and enable the development of novel therapeutic strategies. The knowledge obtained through this study might contribute to the identification of new therapeutic targets for the treatment of PD.

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