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Human α-synuclein modulates vesicle trafficking through its interaction with prenylated Rab acceptor protein 1

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ABSTRACT

α-Synuclein has been implicated in the pathogenesis of Parkinson's disease. Although it is highly conserved, its physiological function has not yet been elucidated in detail. In an effort to define the function of α-synuclein, interacting proteins were screened in phage display assays. Prenylated Rab acceptor protein 1 (PRA1) was identified as an interacting partner. A selective interaction between α-synuclein and PRA1 was confirmed by coimmunoprecipitation and GST pull-down assays. PRA1 and α-synuclein were colocalized in N2a neuronal cells. Cotransfection of α-synuclein and PRA1 caused vesicles to accumulate in the periphery of the cytosol in neuronal cells, suggesting that overexpression of α-synuclein hinders proper vesicle trafficking and recycling as a result of the interaction between α-synuclein and PRA1.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, affecting approximately 1–2% of individuals aged over 65 years [1]. PD patients suffer from characteristic movement disorders, including trembling at rest, impaired balance, rigidity, and bradykinesia [2]. The histological hallmark of PD is intracellular protein aggregates, called Lewy bodies and Lewy neurites, in surviving dopaminergic neurons of the substantia nigra [3]. The major fibrillar component of Lewy bodies and Lewy neurites is α-synuclein. Aggregation of α-synuclein is also closely associated with other neurodegenerative disorders, termed synucleinophathies, which include the Lewy body variant of Alzheimer's disease, dementia with Lewy body, and multiple-system atrophy [4]. The connection between α-synuclein aggregation and the pathogenesis of PD is supported by several lines of evidence: three missense mutations in the α-synuclein gene (A30P, A53T, and E46K) alter the kinetics of fibrillation and are associated with PD [5–9]; triplicate and duplicate copies of the wild-type α-synuclein gene increase intracellular α-synuclein protein levels, thereby accelerating α-synuclein fibrillation and causing inherited forms of PD [10–12]; and the overexpression of α-synuclein in transgenic mice results in the formation of amorphous and granular aggregates of α-synuclein, as well as nigrostriatal dopaminergic injury and motor deficits [13].

Although the aggregation of α-synuclein could facilitate toxicity in PD pathology by a gain of toxic function, sequestration of monomeric α-synuclein might result in loss of normal function played by α-synuclein, leading to neurodegeneration. These two hypotheses for explaining PD pathology can be reconciled by a single molecular mechanism, and understanding the role of α-synuclein in normal cells might be of critical importance.

α-Synuclein is highly expressed in the central nervous system, where it is estimated to comprise approximately 0.5–1.0% of cytosolic protein [14], and is especially abundant in the presynaptic terminals of neurons. α-Synuclein is a small, highly conserved protein of 140 amino acid residues, its N-terminal domain is composed of six imperfect repeats of an 11-residue sequence containing the core consensus motif KTKEGV. These repeats can adopt an apolipoprotein-like class A2 helical structure, and are involved in binding phospholipid vesicles. Its sequence conservation suggests that α-synuclein executes an important function. Although the physiological function of α-synuclein has not been fully elucidated, it has been implicated in the regulation of synaptic vesicle localization and fusion [15]. α-Synuclein-knockout mice are viable and fertile and have normal brain architecture, suggesting that α-synuclein is not essential for brain development. However, striatum dopamine content and amphetamine-induced locomotion are reduced, implicating α-synuclein in dopamine transmission in the nigrostriatal system [16]. Cultured hippocampal neurons from α-synuclein-knockout mice have a reduced distal reserve pool of synaptic vesicles, suggesting that the control of the number of

Abbreviations: PD, Parkinson's disease; PRA1, prenylated Rab acceptor protein 1; PBST, phosphate buffered saline with 0.3% Tween 20.

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vesicles released is impaired in the absence of α-synuclein [17]. Meanwhile, overexpression of α-synuclein in PC12 cells increases the number of docked vesicles at synapses, supporting the idea that α-synuclein plays a role in the tethering of vesicles to the presynaptic plasma membrane [18]. In α-synuclein-overexpressing yeasts, defects in endocytosis [19] and endothelial reticulum (ER)–Golgi trafficking [20] have been reported. Vesicles efficiently bud from the ER but fail to dock and fuse with Golgi membranes [21]. Its role in the regulation of membrane trafficking is also supported by the discovery that A30P α-synuclein coprecipitates with Rab3A, Rab5, and Rab8 in Lewy bodies and Lewy neurites in A30P α-synuclein-transgenic mice [22]. Rab3a, Rab5, and Rab8 are involved in synaptic vesicle trafficking and exocytosis at the synapse, vesicle endocytosis, and trans-Golgi transport, respectively [23,24]. However, the detailed mechanism by which α-synuclein regulates vesicle tethering and fusion during multiple transport steps remains unknown.

Proteins that function at the same steps in a pathway tend to be associated with each other physically. Therefore, an experimental approach to elucidating the function of a protein is the identification of its interacting partners. Early studies aimed at identifying proteins that interact with α-synuclein were carried out under pathogenic conditions, for example, in the presence of intracellular inclusions, Lewy bodies, or Lewy neurites. Therefore, the coprecipitated proteins in those studies could have been ''innocent bystanders,'' accidentally incorporated into intracellular inclusions under abnormal conditions. To overcome these limitations, in the present study, we screened for the interacting partners of α-synuclein under physiological conditions, using a phage display of human brain cDNA library. After biochemical association of interacting partners was confirmed *in vitro* and in neuronal cultures, the effect on vesicle trafficking was evaluated.

2. Materials and methods

2.1. Strains and reagents

Escherichia 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 mouse anti-GFP antibody was purchased from Zymed Laboratories Inc. (South San Francisco, USA), and 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.

2.2. Phage library construction

Whole Human Brain Marathon-Ready cDNA was purchased from BD Biosciences (Palo Alto, USA). Normalization of the cDNA library was achieved through hybridization with a 20-fold excess of biotinylated cDNA, as described previously [25]. The hybrids with biotinylated cDNA were removed using MagPrep Streptavidin Beads (Novagen Inc.). The remaining nonbiotinylated cDNAs larger than 300 bp in length were selected and digested with *Eco*RI/*Hin*dIII, and cloned into the T7select 10B vector (Novagen Inc.). 1 μg of cloned cDNA was incubated with 25 μl T7 packaging extract at room temperature for 2 h, and then a host strain (BLT561) was infected according to the manufacturer's protocol.

2.3. Preparation of biotinylated α-synuclein

An expression vector for a form of α-synuclein with a biotin acceptor domain (pSyn-B) was constructed. A pair of oligonucleotides encoding the biotin acceptor domain (5′-AGCTTTGAACGACATCTTCGAAGCTCAGAAGATCTTCTGGCA-3′ and 5'-CTAGTGCAAG AAGATCTTCTGAGCTTCGAAGATGTCGTTCAA-3′) was synthesized. The two oligonucleotides were phosphorylated at their 5′-termini and annealed to generate double-stranded DNA. This was then ligated with *Hind*III-*Xba*I-digested pSyn [26]. pSyn-B was transformed into *E. coli* strain BL21 (DE3), and biotinylated α-synuclein was purified as described previously [26]. Protein concentrations were measured using a Bio-Rad DC protein assay kit with bovine serum albumin as the standard. Biotinylation of α-synuclein was confirmed by modified immunoblotting using a streptavidin-peroxidase-conjugated protein (Sigma).

2.4. Isolation of α-synuclein-binding phages

One milligram of biotinylated α-synuclein was incubated with 20 μl MagPrep Streptavidin beads for 10 min, and unbound biotinylated α-synuclein was eliminated by washing three times with 1 ml 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 for 10 min at room temperature. The reaction mixture was placed on a Magnetight™ Separation Stand (Novagen Inc.) for 10 min, and then washed five times with 1 ml PBST. 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.

2.5. GST pull-down assay

To produce recombinant PRA1 protein, a cDNA encoding human PRA1 was amplified by polymerase chain reaction (PCR), and then cloned into pRSET B (Invitrogen Co., Netherlands), a poly histidine-tagging expression vector. $(His)_n$–PRA1 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 sodium acetate, 0.5 M NaCl, 5 mM imidazole, pH 5.5). PRA1 protein was eluted with an imidazole gradient.

The cDNA encoding human α-synuclein was amplified by PCR and cloned into pGEX-4T-1 (Novagen Inc.) for the production of a GST–α-synuclein fusion protein. 1 μg of recombinant GST or GST–α-synuclein fusion protein was immobilized on 100 μl gluta thione agarose beads. After washing with 1 ml PBST, the beads were incubated overnight with 1 μg $(His)_n$-tagged PRA1 protein in 1 ml PBST at 4 °C on a rotator. To remove unbound proteins, the beads were washed with 1 ml PBST. Bound proteins were eluted with PBST containing 1 M NaCl, and detected by immuno blotting using an anti-$(His)_n$ antibody.

2.6. Coimmunoprecipitation of α-synuclein with PRA1

For mammalian expression, the α-synuclein gene was subcloned into pcDNA3.1, and *PRA1* 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. When the cells reached about 60% confluence, they were transfected with 1 μg pcDNA3.1-α-synuclein and/or pEGFPN3–PRA1 DNA, using 3 μl TransIT®-LT1 Transfection Reagent