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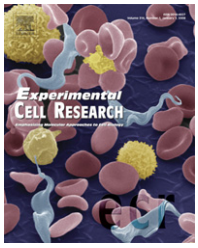

Research Article

LRRK2 regulates synaptic vesicle endocytosis

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ABSTRACT

The leucine-rich repeat kinase 2 (LRRK2) has been identified as the defective gene at the PARK8 locus causing the autosomal dominant form of Parkinson's disease (PD). Although several LRRK2 mutations were found in familial as well as sporadic PD patients, its physiological functions are not clearly defined. In this study, using yeast two-hybrid screening, we report the identification of Rab5b as an LRRK2-interacting protein. Indeed, our GST pull down and co-immunoprecipitation assays showed that it specifically interacts with LRRK2. In addition, subcellular fractionation and immunocytochemical analyses confirmed that a fraction of both proteins co-localize in synaptic vesicles. Interestingly, we found that alteration of LRRK2 expression by either overexpression or knockdown of endogenous LRRK2 in primary neuronal cells significantly impairs synaptic vesicle endocytosis. Furthermore, this endocytosis defect was rescued by co-expression of functional Rab5b protein, but not by its inactive form. Taken together, we propose that LRRK2, in conjunction with its interaction with Rab5b, plays an important role in synaptic function by modulating the endocytosis of synaptic vesicles.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and affects more than 1% of the population over 65 years old [1,2]. Major PD symptoms are bradykinesia, resting tremor, rigidity and postural instability. PD is pathologically characterized by progressive death of midbrain dopaminergic neurons in the substantia nigra and formation of Lewy bodies [1,2]. Recently, leucine-rich repeat kinase 2 (*LRRK2*/*dardarin*) has been identified as a gene responsible for PARK8-linked autosomal dominant inherited familial PD [3,4]. LRRK2 mutations were found in approximately 3 to 5% of familial and 1 to 3% of sporadic PD cases [5–10], with the highest prevalence (up to 40%) in North Africans and Ashkenazi Jews [11]. LRRK2 is predicted to be a 285 kDa multidomain protein coded by 51 exons. LRRK2 belongs to the Roco protein family [12] and contains 12 leucine-rich repeats (LRRs), a GTPase domain of Ras of complex

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proteins (Roc), a C-terminal of Roc (COR) domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) domain and WD40 repeats [3,4]. Among PD-specific LRRK2 mutations, R1441C is located in the GTPase domain, Y1699C in the COR domain, and I2012T, G2019S and I2020T in the kinase domain, implying that these domains' malfunctions lead to PD. Recent studies demonstrated that LRRK2's autophosphorylation activity is enhanced by PD-specific mutations [13,14]. Overexpression of LRRK2 wild type (WT) and pathogenic mutants in various cell lines led to formation of cytoplasmic aggregates whose numbers increased with the expression of various PD-specific mutants and decreased with the expression of kinase-defective mutants [15]. Importantly, expression of PD-specific mutants caused neuronal degeneration in both dopaminergic SH-SY5Y cells and primary neuronal cultures [15,16] and induced a progressive neurite loss in primary cortical neuronal cultures [17]. In addition, it was reported that the GTPase-defective mutant of LRRK2 shows reduced LRRK2's kinase activity and its expression is associated with decreased neuronal death compared to expression of PD-specific mutant(s) [15,18].

Rab proteins are small GTPases which localize to various cellular vesicle compartments and function as regulators of membrane trafficking [19,20]. Rab5 is a key regulator of endocytic vesicular transport from plasma membrane to early endosomes [21]. In hippocampal neurons, Rab5 was detected in endosomes as well as in the membrane of synaptic vesicles at dendrites and axons, suggesting that axonal endosomes may function in biogenesis of synaptic vesicles [22,23]. In *Drosophila*, Rab5 functional defects have been reported to affect endocytosis rates [24].

To investigate the potential biological function of LRRK2, we sought to identify its interacting protein(s). Using yeast two-hybrid screening, we identified Rab5b as an LRRK2-interacting protein. We characterized the LRRK2–Rab5b interaction by biochemical and immunostaining assays. Furthermore, in primary neuronal cell cultures, we found that alterations of LRRK2 expression level by either overexpression or knockdown caused the endocytosis defects which were rescued by co-expression of Rab5b WT or the constitutive active mutant (Q79L), but not by that of the dominant negative mutant (N133I) protein. Our overall data strongly suggest that LRRK2 regulates synaptic function by modulating endocytosis of synaptic vesicles.

Materials and methods

Plasmid and oligomers

The full-length human LRRK2 cDNA was generated by PCR amplification of short cDNA fragments using a human cerebral cortex cDNA pool as the template and gene-specific primers based on the human LRRK2 sequences from the NCBI database. The resulting cDNA was sequenced and the sequence was compared to that of the LRRK2 full-length clone (NCBI AY792511). Our clone contained three nonsynonymous substitutions (635C>T, 4939T>A, 7190T>C) and six synonymous substitutions (457C>T, 2196A>G, 2857C>T, 4872C>A, 4911A>G and 6324G>A; the numbers indicated are from the LRRK2 clone AY792511). The three nonsynonymous substitutions are due to LRRK2 polymorphism since two of them (4939T>A, 7190T>C) were already reported as polymorphisms [25] and the 635C>T was also found in other independent human EST clone in the NCBI databases (BC117180). All synonymous substitutions except 2196 A>G were also reported [25]. To generate myc-tagged vector, full-length human LRRK2 cDNA was subcloned into pCDNA3.1–myc/his-A (Invitrogen), resulting in pCDNA3.1–myc/his-hLRRK2 which was named as Myc–LRRK2 and contains the myc/his tag at the C-terminus. Two more copies of the myc tag were inserted into Myc–LRRK2 to make Myc3–LRRK2. The Rab5b clone was synthesized by PCR from human brain cDNA library as templates and cloned into the pcDNA3.1 plasmid as a Flag-tagged form, resulting in the pcDNA3-Flag–Rab5b plasmid. Both full-length LRRK2 and Rab5b genes were cloned into the pGEX4T-1 plasmid to express GST fusion proteins.

To make LRRK2 G2019S, R1441C and K1906M and Rab5b Q79L and N133I mutations, site-directed mutagenesis was carried out with a pair of oligomers containing the corresponding mutated bases (G2019S-F: 5′ GATTGCTGACTACAGCATTGCTCAGTAC; G2019S-R: 5′ GTACTGAGCAATGCTGTAGTCAGCAATC; R1441C-F: 5′ CTTCAATATAAAGGCTTGCGCTTCTTCTTC; R1441C-R 5′ GAAGAAGAAGCGCAAGCCTTTATATTGAAG; K1906M-F: 5′ GAAGAAGTGGCTGTGATGATTTTTAATAAACATAC; K1906M-R 5′ GTATGTTTATTAAAAATCATCACAGCCACTTCTTC; Q79L-F: 5′ CTGGGACACAGCTGGGCTGGAGCGATATCACAGC; Q79L-R: 5′ GCTGTGATATCGCTCCAGCCCAGCTGTGTCCCAG; N133I-F: 5′ ATTGCCCTGGCAGGGATCAAAGCTGACCTGGCCAAC; N133I-R: 5′ GTTGGCCAGGTCAGCTTTGATCCCTGCCAGGGCAAT) and pcDNA3–Myc–LRRK2 and pcDNA3-Flag–Rab5b as template, respectively, using *in vitro* site-directed mutagenesis kit (Stratagene, CA, USA). The specific mutations were confirmed by sequencing of the resulting clones. LRRK2-specific siRNAs (siRNA-1: 5′ GGAUCUUCCUUUGGAUGAACUG; SiRNA-2 5′ AGCCUUGGCUCUUCAAUAUAAA) were synthesized.

Cell culture and transfection

HEK293T cells were cultured in DMEM with 10% FBS at 37 °C in CO_2 incubator. Transfections were done using Lipofectamine2000 (Invitrogen) with indicated plasmids according to the manufacturer's direction. E-18 primary rat hippocampal neurons were prepared as described [26]. Neurons were transfected at DIV 10 for endocytosis assay using the calcium-phosphate method with synaptopHluorin (spH) alone or with indicated LRRK2 plasmids in the ratio of 1:2 [26] and then used 5 days after transfection. The amount of spH construct transfected was fixed for constant fluorescent signal.

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using Matchmaker Two-hybrid system 3 (Clontech, CA, USA). LRRK2 was divided into LRR (amino acids 967–1360), GTPase-MAPKKK (amino acids 1334–2128), and WD40 (amino acids 2168–2510) domains, fused to GAL4 DNA binding domain (DBD) of pGBKT7 and each fusion protein expression was determined by Western analysis using GAL4 antibody. Each of three plasmids was used as bait for the screening against human brain cDNA library (Clontech). Several positive clones were