Effect of leucine-rich repeat kinase 2 (LRRK2) on protein synthesis

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ABSTRACT

Mutations in the leucine-rich repeat kinase 2 (LRRK2) cause Parkinson’s disease (PD) in an autosomal dominant manner. Pathogenic mutations of LRRK2 such as G2019S and R1441C have been observed as common genetic causes of PD. Recently, LRRK2 has been reported to increase the reporter protein synthesis in both cap-dependent and -independent manners via phosphorylation of the ribosomal protein RPS15. In this study, we tested whether LRRK2 recombinant protein would directly increase protein synthesis using a well-defined in vitro coupled transcription/translation system. Addition of commercial full-length LRRK2 or GST-fused N-terminal-deleted LRRK2 recombinant proteins to the system showed no change of protein synthesis, as measured by luciferase reporter activity. In addition, the SUnSET assay to measure newly synthesized cellular proteins showed that G2019S overexpression had a minimal effect on the total protein amount. However, we confirmed the previous result that G2019S overexpression increased the amount of protein synthesized from an exogenous gene, Flag-VAMP2, which was transfected as a reporter, whereas there was no significant change in the amount of the Flag-VAMP2 mRNA. Inhibition of protein degradation showed that protein accumulation in the vector control was higher than that of the G2019S overexpression vector. Our results suggest that LRRK2 protein influences the amount of protein by inhibiting protein degradation rather than by directly stimulating translation.

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Introduction

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene represent the most common pathogenic mutations associated with Parkinson’s disease (PD), which is the second most common neurodegenerative disease after Alzheimer’s disease (Monfrini and Di Fonzo 2017). Studies on LRRK2 are thus important to understand PD pathogenesis since symptoms of patients with LRRK2 mutations are similar to those of patients with idiopathic PD (Monfrini and Di Fonzo 2017). LRRK2 contains active GTPase and kinase domains, and its most prevalent mutant G2019S results in increased kinase activity, suggesting that the kinase activity is related to PD pathology. To elucidate the mechanisms of PD pathogenesis, there has been active research effort focused on identifying the LRRK2 kinase substrates; however, substrates clearly pinpointing the mechanisms remain elusive. Nevertheless, numerous studies have pointed to the critical roles of LRRK2 in regulation of protein degradation and aggregation, vesicle trafficking, neurite outgrowth, and neuroinflammation (Seol 2010; Li et al. 2014; Rideout and Stefanis 2014; Wallings et al. 2015; Ho et al. 2017).

In addition, several studies have reported that LRRK2 increases the translation of a reporter gene. The first study related to the translation of LRRK2 focused on its interaction with and phosphorylation of a negative regulator of cap-dependent translation, eIF4E-BP1 (Imai et al. 2008). The same study further showed that immunoprecipitated LRRK2 increased translation of the luciferase reporter in a rabbit reticulocyte lysate system in a kinase activity-dependent manner. However, subsequent studies found no evidence of the phosphorylation of eIF4E-BP1 by LRRK2 using various systems (Kumar et al. 2010; Trancikova et al. 2012), and thus this interaction remains controversial. More recently, LRRK2 was reported to interact with and phosphorylate several ribosomal proteins, especially RPS15 (Martin et al. 2014). In particular, LRRK2 or RPS15 increased luciferase reporter protein synthesis in both cap-dependent and -independent manners, and the increase of reporter translation was...
dependent on the kinase activity of LRRK2. This result was somewhat surprising because the previous eIF4E-BP1 study suggested that the translation increase by LRRK2 via eIF4E-BP1 occurred in a cap-dependent manner (Imai et al. 2008) since the phosphorylation of eIF4E-BP1 is critical to rescue cap-dependent translation inhibition. However, it is still possible that RPS15 may activate an eIF4E-BP1-independent pathway to stimulate translation. Moreover, these studies showed that the G2019S mutation enhances translation of the reporter, without a corresponding increase of the reporter mRNA transcript (Imai et al. 2008; Martin et al. 2014).

Other studies demonstrated that LRRK2 wild type (WT) and pathogenic mutants impair protein degradation in either an autophagy- or proteasome-dependent manner (Plowey et al. 2008; Lichtenberg et al. 2011; Tong et al. 2012; Manzoni et al. 2013; Manzoni and Lewis 2017) and accumulated specific proteins such as α-synuclein (Lichtenberg et al. 2011). In fact, LRRK2 expression has been reported to result in the accumulation of β-catenin and p53 (Lichtenberg et al. 2011). Since β-catenin and p53 are critical transcription factors that are continuously degraded depending on presence of specific signals (Ashcroft and Vousden 1999; Liu et al. 2002), this result suggested that LRRK2 could accumulate certain degradation-prone proteins by regulating the protein degradation system. Regarding the phosphorylation of a ribosomal protein, phosphorylation of another ribosomal protein, RPS6, by activated S6 kinase has been reported to reduce autophagy (Blommaart et al. 1995), one of the two major regulatory pathways for protein degradation.

Another study showed that the LRRK2-mediated increase in translation might be due to regulation of microRNAs, which in turn regulate specific transcripts (Gehrke et al. 2010). However, this mechanism involves negative regulation of the microRNAs let-7 and miR-184*, which would repress the translation of specific e2f1 and dp mRNAs in Drosophila, and is thus distinct from the mechanisms proposed in the other two studies (Imai et al. 2008; Martin et al. 2014).

In the present study, we aimed to determine whether the increased protein levels by LRRK2 (Imai et al. 2008; Martin et al. 2014) may be partially due to impairment of protein degradation rather than to direct stimulation of translation. To this end, we used an in vitro coupled system and the SUNSET assay, a non-radioactive method to measure the amount of newly synthesized protein (Schmidt et al. 2009). Our results showed that LRRK2 has no direct effect on translation in a biochemical assay and that the accumulation of proteins might be, at least partially, due to impairment of protein degradation by LRRK2 G2019S.

**Material and methods**

**Material**

All LRRK2 recombinant proteins were purchased from Invitrogen (Carlsbad, CA, USA), and the construction of the Myc-LRRK2 G2019S mutation was previously reported (Shin et al. 2008). The following antibodies were used: LRRK2 (N241A, NeuroMabs, Davis, CA, USA, #75–253), Flag (Cell signaling Technology, Danvers, MA, USA, #8146), β-actin (Santa Cruz Biotechnology, Dallas, TX, USA, #sc-4778), and puromycin (Merck KGaA, Darmstadt, Germany, #MABE34).

**Cell culture and transfection**

HEK293T cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA) and 1× penicillin–streptomycin (Invitrogen) at 37°C with 5% CO₂. Transfection of the indicated plasmids was carried out with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

**Reverse transcription-polymerase chain reaction (RT–PCR)**

Total RNA was isolated from the cells, used for cDNA synthesis, and the target mRNAs were amplified by PCR using the cDNAs as templates and specific primer sets (h-β-actin-F: CGCCGCCACCTCACCAG, h-β-actin-R: CAC-GATGGAGGGGAAGCG; Flag-VAMP2-F: ATGGGGAACATCTTAGGCAAC, Flag-VAMP2-R: GGTATGCATTTTATCATCATC; Myc-LRRK2-F: GTCATGATGACAGCACAGC, Myc-LRRK2-R: CTCTCTGATGAGTTTTATCCGAG). The PCR products were analyzed by agarose gel analysis.

**Western blot analysis**

HEK293T cells were seeded at 1 × 10⁵ cells/well in a 24-well plate. To overexpress LRRK2 G2019S, plasmids containing the Myc-tagged LRRK2 G2019S were transiently transfected using Lipofectamine 2000 (Invitrogen). One day after transfection, the culture medium was replaced with a medium containing dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), 100 nM bafilomycin A1 (Sigma-Aldrich), or 25 µM MG132 (Sigma-Aldrich) and incubated further for 24 h. The cells were washed with cold d-phosphate buffered saline twice and lysed.
with 50 µl of 1× sample buffer. After sonication, the total cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis with the indicated antibodies. Their densities were analyzed with the Multi-Gauge V 3.0 program (Fuji Photo Film, Tokyo, Japan).

In vitro coupled transcription/translation assay

The in vitro coupled transcription/translation assay was conducted using the TNT® T7 coupled reticulocyte lysate system (Promega, Madison, WI, USA). Each of two sets of recombinant LRRK2 proteins (full length-WT and -G2019S; GST-ΔN-WT, -G2019S, -R1441C, and -D1994A) was incubated with the TNT lysate reaction components as recommended by the manufacturer. The mixtures were incubated at 30°C and the same portion of each reaction was sampled after incubation for 0, 15, 30, 60, and 120 min. The luciferase assay of each sample was then performed using the dual luciferase assay kit (Promega).

SUnSET assay

HEK293T cells were seeded at 1 × 10^5 cells/well in a 24-well plate and transiently transfected with LRRK2 G2019S and the pcDNA3.1 control vector (Invitrogen) using Lipofectamine 2000. Twenty minutes before 48-h incubation, puromycin (5 µg/ml, Sigma-Aldrich) was added to the cells, and they were incubated further for 20 min to label the newly synthesized proteins with puromycin (Schmidt et al. 2009). The total cell lysates were prepared and analyzed with the puromycin antibodies as described above. To confirm that similar amounts of proteins were loaded, the protein gel was stained using GelCode™ blue stain reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

All statistical analyses were carried out with the Prism5 program (GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean ± standard error of the mean.

Results and discussion

Recombinant LRRK2 WT or mutant proteins have no direct effect on protein synthesis in the in vitro coupled transcription/translation system

To determine whether LRRK2 directly increases translation, we utilized commercial recombinant LRRK2 proteins and a well-known in vitro coupled transcription/translation system. Although this system has been mainly used to synthesize proteins in vitro with the addition of an exogenous gene, it has also been used to investigate the effect of specific recombinant proteins on protein synthesis (Velasquez et al. 2016). Moreover, according to the manufacturer, the system can carry out post-translation modifications such as phosphorylation.

A luciferase plasmid is provided as a control in the commercial coupled transcription/translation kit. In the present study, we used the luciferase gene as a reporter and measured its synthesis by a highly sensitive luciferase assay. First, we measured the basal level of protein synthesis in the system. Figure 1(A) shows that protein synthesis became saturated between 1 and 2 h of incubation. Therefore, we incubated LRRK2 in the system for 2 h. We initially tested the effects of full-length WT and G2019S proteins. The result showed no significant difference between LRRK2 G2019S and the control protein bovine serum albumin (BSA) (Figure 1(B)). Silver staining of a gel loaded with the tested proteins confirmed that the same amount of proteins was added to each reaction system (Figure 1(C)). As shown in Figure 1(B), LRRK2 WT showed a slight, but not significant, increase of protein synthesis compared to that of the mutant G2019S. To test whether the LRRK2 kinase activity affects protein synthesis, we utilized a commercially available GST-ΔN-LRRK2 fusion of kinase-dead D1994A along with the R1441C and G2019S mutant and WT proteins. This assay confirmed that there was no significant difference among the LRRK2 WT, mutant, and control BSA proteins (Figure 1(D)), indicating that neither LRRK2 nor its kinase activity itself has a direct effect on protein synthesis. This result is in contrast to the previous result showing that LRRK2 immunoprecipitates increased the protein, but not mRNA, amount of the luciferase reporter (Imai et al. 2008). Because a similar in vitro coupled system was used in the previous study, this discrepancy suggests that the observed kinase-dependent stimulation of the translation was due to the proteins co-immunoprecipitated with LRRK2, rather than to LRRK2 itself. Furthermore, the candidate proteins may be present specifically in HEK293T cells, which they used to overexpress LRRK2, but not in the rabbit reticulocyte used in the present study.

Overexpression of LRRK2 G2019S increases the exogenous reporter protein level but not the total cellular protein level

Next, we tested whether LRRK2 might affect the protein synthesis of cellular rather than exogenous reporter proteins. For this purpose, we employed the SUnSET assay,
which is a non-radioactive in vivo assay, to measure the total cellular protein synthesis utilizing an antibody against puromycin, a translation inhibitor (Schmidt et al. 2009). After puromycin treatment for a relatively short time, the SUnSET assay can detect newly synthesized cellular puromycin-labeled proteins by western blot analysis using a puromycin antibody. Because previous studies suggested that LRRK2 kinase activity is a critical regulator of protein synthesis (Imai et al. 2008; Martin et al. 2014), we focused on the G2019S pathogenic mutation, which results in increased kinase activity. Treatment of puromycin to the cells

Figure 1. Direct addition of LRRK2 recombinant WT or mutant proteins shows no effect on protein synthesis. (A) Basal activity of the in vitro coupled system. (B) LRRK2 WT or G2019S full-length recombinant proteins have no effect on protein synthesis. The indicated proteins were added to the in vitro coupled transcription/translation system with the luciferase reporter plasmid as a template. The same portion from each reaction was sampled after incubation for 0, 15, 30, 60, and 120 min and assayed for its luciferase activity. (C) A silver-stained gel showing that the same amount of proteins was added to the system. (D) LRRK2 ΔN-WT, pathogenic (G2019S and R1441C), and kinase-dead (D1994A) mutants also had no effect on protein synthesis. (n = 3). A.U.: arbitrary unit.

Figure 2. SUnSET assay of cells expressing G2019S or the vector control, showing no change in protein synthesis. HEK293T cells were transfected with G2019S or the vector control, and total cell lysates were analyzed by western blot analysis using puromycin antibodies. Loading of the same amount of proteins was confirmed by staining of the gel using GelCode™ blue stain reagent. The western blot (A) and quantitative analysis (B) are shown. (n = 3). RLU = relative light units, N.S.: not significant.
expressing LRRK2 G2019S exhibited no significant difference in protein accumulation from the cells transfected with the vector control, although G2019S expression was clearly detected (Figure 2). However, it might be still possible that the degradation-prone proteins such as p62, p53, or β-catenin are accumulated by G2019S expression as previously reported (Lichtenberg et al. 2011; Manzoni et al. 2013) since the SUnSET assay detects changes of major cellular component proteins.

Inhibition of protein degradation increases the reporter protein level in cells transfected with the vector control but not with G2019S

Our results demonstrated that neither the LRRK2 protein itself nor cellular expression of LRRK2 G2019S affects protein synthesis. The previous studies measured only reporter proteins synthesized from a luciferase or α-synuclein gene that was exogenously added to the system (Imai et al. 2008; Lichtenberg et al. 2011; Martin et al. 2014). Therefore, we decided to test whether LRRK2 expression might increase different reporter proteins. For this purpose, we chose Flag-tagged VAMP2, a synaptic vesicle protein. LRRK2 G2019S expression significantly increased the amount of protein synthesized from co-transfected Flag-VAMP2 by more than 2-fold, but did not significantly increase its mRNA level (Figure 3).

To test whether this increase of protein synthesis might be due to impairment of protein quality control, we treated HEK293T cells overexpressing LRRK2 G2019S with the autophagy inhibitor bafilomycin A1 or the proteasome inhibitor MG132. As shown in Figure 4, MG132

Figure 3. LRRK2 G2019S overexpression in HEK293T cells increases the protein level of Flag-VAMP2, which was transiently transfected to the cells. (A) Total cell lysates were analyzed by western blot with the indicated antibodies. (B) Total RNA was isolated from the cell lysates and RT-PCR was carried out with specific primers for the indicated genes. mRNA levels of the indicated genes are normalized to that of β-actin. *: p < 0.05 by Student’s t-test (n = 3). N.S.: not significant.

Figure 4. MG132 treatment results in a greater increase of the amount of exogenously expressed protein in cells transfected with the vector control than in cells transfected with G2019S. HEK293T cells were transfected with the same amount of G2019S (GS) or the vector (V) control, incubated for 24 h, and then treated with MG132 (25 μM), bafilomycin A1 (100 nM), or vehicle (DMSO, -) for another 24 h. Total cell lysates were analyzed by western blot with the indicated antibodies (A) and protein levels were quantified (B). Activity of bafilomycin A1 (Baf) treatment was confirmed by LC3B western blot analysis (C). n = 8.
increased the VAMP2 protein level by 4.4-fold in the vector-expressing cells, but only increased the protein level by less than 2-fold in cells expressing G2019S (Figure 4(B)). This result suggests that the increase of the reporter protein level by LRRK2 expression might be due to impairment of protein degradation, especially proteasome activity, as previously reported (Lichtenberg et al. 2011). In addition, we repeatedly observed that MG132 treatment increased LRRK2 protein level, suggesting that LRRK2 itself is regulated by proteasome activity (Figure 4(A)). In contrast, bafilomycin A1 treatment resulted in marginal increase of Flag-Vamp2 in cells expressing the vector, but its decrease in cells expressing G2019S. This is rather surprising because relation of LRRK2 to autophagy has been well established (Manzoni and Lewis 2017). To validate activity of bafilomycin A1, we checked LC3B-II level after bafilomycin A1 treatment by western blot analysis. The result showed increase of LC3B-II in the bafilomycin A1 treated samples, confirming activity of bafilomycin A1 (Figure 4(C)). It is unclear at present why the bafilomycin A1 treatment reduced VAMP2 level in cells expressing G2019S.

Based on our study of in vitro coupled transcription/translation system and SUnSET assays, we suggest that LRRK2 does not regulate translation, at least directly. LRRK2 has been reported to impair protein degradation (Plowey et al. 2008; Lichtenberg et al. 2011; Tong et al. 2012; Manzoni et al. 2013; Manzoni and Lewis 2017) and to accumulate specific proteins such as α-synuclein, β-catenin and p53 (Lichtenberg et al. 2011). Our additional data suggests that increase of protein amount by LRRK2 expression might be, at least partially, due to impairment of protein degradation through proteasome pathway that results in protein accumulation, rather than stimulation of translation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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