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Dominant-negative effects of LRRK2 heterodimers: A possible mechanism of neurodegeneration in Parkinson's disease caused by LRRK2 I2020T mutation

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ABSTRACT

Leucine-rich repeat kinase 2 (LRRK2) is the molecule responsible for autosomal-dominant Parkinson's disease (PD), PARK8, but the etiologic effects of its mutation remain unknown. In the present study, we investigated a novel mechanism for the neurodegeneration induced by I2020T mutant LRRK2. Using native gel electrophoresis and immunoprecipitation, we found that wild-type (WT) LRRK2 formed a heterodimer with I2020T LRRK2 in transfected cells, and that the heterodimer exhibited a markedly lower intracellular protein level than the WT/WT-homodimer. An increased amount of I2020T LRRK2 decreased the protein level of co-transfected WT LRRK2. A pulse-chase experiment revealed that the intracellular protein lifetime of WT LRRK2 was shortened by co-transfection with I2020T LRRK2. These results suggest that I2020T LRRK2 enhances the intracellular degradation of WT LRRK2 through WT/I2020T-heterodimer formation. Overexpression of WT LRRK2 in HEK293 cells increased the phosphorylation level of Akt1 (S473), a possible physiological substrate of LRRK2, and made cells resistant to hydrogen peroxide-induced apoptosis. However, both Akt1 phosphorylation and apoptosis resistance were reduced in WT/I2020T-expressing cells in comparison with WT/WT-expressing cells. Reduction of Akt1 phosphorylation and apoptosis resistance were also evident when a neuroblastoma SH-SY5Y clone overexpressing WT LRRK2 was transfected with the I2020T LRRK2. Altogether, these results suggest that the I2020T mutation enhances the intracellular degradation of LRRK2 through WT/I2020T-heterodimer formation, leading to reduced Akt1 phosphorylation and diminished protectivity against apoptosis. Our findings suggest the possibility of a dominant-negative mechanism of neurodegeneration in PD caused by I2020T LRRK2 mutation.

1. Introduction

Parkinson's disease (PD) is a movement disorder caused by degeneration of dopaminergic neurons. Both environmental and genetic predisposing factors are involved in the development of PD. Leucine-rich repeat kinase 2 (LRRK2) is the causal molecule of the autosomal dominant hereditary form of PD, PARK8, which was originally defined in a study of a Japanese family, the Sagamihara family [1–4], who harbor the I2020T mutation in the kinase domain [4]. The G2019S mutation is that which is detected most frequently, accounting for up to 5% of all cases of familial PD and 1% of cases of sporadic PD in the Caucasian population [5]. Patients with LRRK2 mutations exhibit clinical features indistinguishable from those of patients with sporadic PD, and LRRK2 is postulated to be a key molecule in the etiology of the disease. However, the mechanism of neurodegeneration resulting from the mutation has not been conclusively clarified.

LRRK2 is a 280-kDa multidomain-kinase molecule consisting of LRR (leucine-rich repeat), ROC (Ras of complex), COR (C-terminal ROC), kinase, and WD40 domains [6]. It exists as a dimer under physiological conditions [7,8]. The binding of GTP to the ROC domain, as well as dimer formation, is known to increase the kinase activity of LRRK2 [9–11]. It has been reported that LRRK2 phosphorylates itself (autophosphorylation), and its potential substrates include moesin, 4E-BP1, β-tubulin, and MKK3, MKK6, and MKK7 [12–14]. Although hyper-kinase activity reported for mutant LRRK2 molecules, particularly G2019S LRRK2, may be one possible mechanism for the pathogenesis induced by this molecule [11,15–18], it has not been observed reproducibly in other LRRK2 mutations including I2020T [19]. We recently found that LRRK2 directly phosphorylates Akt1, a central molecule involved in signal transduction for cell survival and prevention of apoptosis [20]. The disease-associated mutations, R1441C, G2019S, and I2020T,

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exhibited reduced interaction with, and phosphorylation of, Akt1. Some studies have reported that LRRK2-containing inclusion bodies and autophagy are responsible for cytotoxicity [19,21].

We have previously demonstrated that wild-type (WT) LRRK2 exerts a protective effect against apoptosis whereas I2020T LRRK2 has impaired protectivity because of high susceptibility to post-translational degradation [22,23]. However, because PARK8 is the dominant hereditary form of PD, it remains to be clarified whether degradation of the I2020T LRRK2 encoded by one of the alleles of affected patients is enough to explain the etiology of PARK8, or whether the WT LRRK2 encoded by the other allele is also affected deleteriously by the mutant LRRK2. In the present study, we found that the lifetime of WT LRRK2 protein was shortened by formation of a heterodimer with I2020T LRRK2. Furthermore, we demonstrated that the kinase activity of WT LRRK2 for phosphorylation of Akt1 was diminished by the presence of I2020T LRRK2. The protective effect of WT LRRK2 against H_2O_2-induced apoptosis was impaired by co-transfection with I2020T LRRK2. These results provide a new insight into the etiology of PD caused by the LRRK2 mutation, i.e., a dominant-negative effect resulting from heterodimer formation.

2. Materials and methods

2.1. LRRK2 cDNA plasmids

The mammalian expression cDNA constructs of wild-type, G2019S, and I2020T mutant *LRRK2* cDNA with a V5 tag at the C-terminus or a FLAG tag at the N-terminus were described previously [22].

2.2. Cell culture and transfection

HEK293 cells, SH-SY5Y cells, and SH-SY5Y clones (WT-2) stably expressing V5-WT LRRK2 were cultured as described previously [22]. Transient transfections with the *LRRK2* cDNA plasmid were performed using Lipofectamine™ 2000 (Invitrogen) for HEK293 cells, and FuGENE® HD Transfection Reagent (Roche) for SH-SY5Y cells in accordance with the manufacturers' protocols. After 48 h of culture, the transfected cells were harvested and used for further analysis.

2.3. Polyacrylamide gel electrophoresis (PAGE) and Western blotting

For blue-native polyacrylamide gel electrophoresis (BN-PAGE), cells were suspended in cell lysis buffer-1 [Tris-HCl-buffered saline (pH 7.6) containing 1% digitonin, 1 mM PMSF, and Complete mini protease inhibitor cocktail® (Roche)], and rotated at 4 °C for 1 h. Cell lysates were obtained by centrifugation at 10,000×*g* for 10 min at 4 °C, mixed with 0.25% Coomassie G-250, and subjected to BN-PAGE using NativePAGE™ Novex® 3–12% Bis-Tris Gels (Invitrogen). For SDS-PAGE using 5–20% gradient e-PAGEL® (ATTO), cell lysates were prepared using cell lysis buffer-2 [20 mM Tris–HCl (pH 8.2) containing 1% Triton X-100, 0.25 mM sucrose, 10 mM EGTA, 2 mM EDTA, and Complete mini protease inhibitor cocktail® (Roche)]. For phosphorylation analysis, PhosSTOP (Roche)] was further included in the lysis buffer-2. Proteins separated by BN-PAGE or SDS-PAGE were blotted onto polyvinylidene fluoride membranes, and analyzed using horseradish peroxidase (HRP)-labeled antibody against the V5 tag (Invitrogen) or the FLAG tag (SIGMA) for LRRK2 expression, and HRP-labeled antibody against beta-actin (Abcam) as an internal control. Phosphorylation of Akt1 was analyzed using an antibody against pospho-Akt1 (Ser473) (Cell Signaling) and one against non-phosphorylated Akt1 (Cell Signaling), in combination with a HRP-labeled secondary antibody against rabbit IgG (BioLegend).

2.4. Immunoprecipitation

HEK293 cells were co-transfected with V5-tagged and FLAG-tagged WT, GS, and IT LRRK2. After 48 h, the cells were lysed and incubated with anti-V5 agarose affinity gel (SIGMA) or anti-FLAG® M2 affinity gel (SIGMA) at 4 °C for 2 h. After four washes, the immunoprecipitates were subjected to SDS-PAGE followed by Western analysis using the HRP-labeled antibody against V5 tag or that against FLAG tag.

2.5. Pulse-chase experiment

HEK293 cells were co-transfected with V5-tagged or FLAG-tagged WT and I2020T LRRK2 cDNAs in various combinations. Experiments were performed using 100 μCi/ml [^{35}S]methionine and cysteine as described previously [22]. The radiolabeled LRRK2 molecules were immunoprecipitated with agarose beads conjugated with the anti-FLAG antibody (SIGMA) and separated on SDS–PAGE. The gel was then subjected to autoradiography and the LRRK2 bands were quantified using a BAS-1800 Image Analyzer (Fuji Film).

2.6. Hydrogen peroxide-induced apoptosis

Cells were treated with various concentrations (0–1 mM) of hydrogen peroxide for 50 min (for LRRK2-transfected HEK293 cells) or 24 h (for LRRK2-expressing SH-SY5Y clones) at 37 °C, and subjected to viability assay using a Cell Counting Kit-8™ (Dojindo) in accordance with the manufacturer's protocol.

3. Results

3.1. The level of WT LRRK2 dimer protein is decreased by co-transfected I2020T LRRK2

We previously reported that I2020T LRRK2 is more susceptible to intracellular degradation than the WT and G2019S LRRK2 [22,23]. Because LRRK2 has been reported to form dimers, in the present study we first examined mutation-dependent degradation at the dimer level using BN-PAGE. We found that the LRRK2 protein level of the I2020T-homodimer was markedly lower than those of the WT- and G2019S-homodimers in both HEK293 and SH-SY5Y cells, confirming our previous results at the dimer level (Fig. 1A).

Next, to investigate how I2020T LRRK2 influences co-transfected WT LRRK2, equimolar amounts of WT and I2020T LRRK2 cDNAs (either V5- or FLAG-tagged) were transfected into HEK293 cells in various combinations. Western analysis for V5-LRRK2 and for FLAG-LRRK2 (Fig. 1B and C, respectively), each detecting one of the subunits, yielded similar results, i.e., the dimer-region signals were much weaker in the WT/I2020T combination than in the WT/WT combination (Fig. 1D and E). These results suggested that I2020T LRRK2 decreased the level of LRRK2 dimer protein when co-transfected with WT LRRK2.

3.2. Immunoprecipitation of the LRRK2 dimer reveals a reduced level of the WT/I2020T-heterodimer protein

Next, to prove the formation of a heterodimer between the WT and I2020T LRRK2, FLAG-tagged WT LRRK2 cDNA was transfected into HEK293 cells in combination with V5-tagged either WT, G2019S, or I2020T LRRK2 cDNA. The LRRK2 dimers were

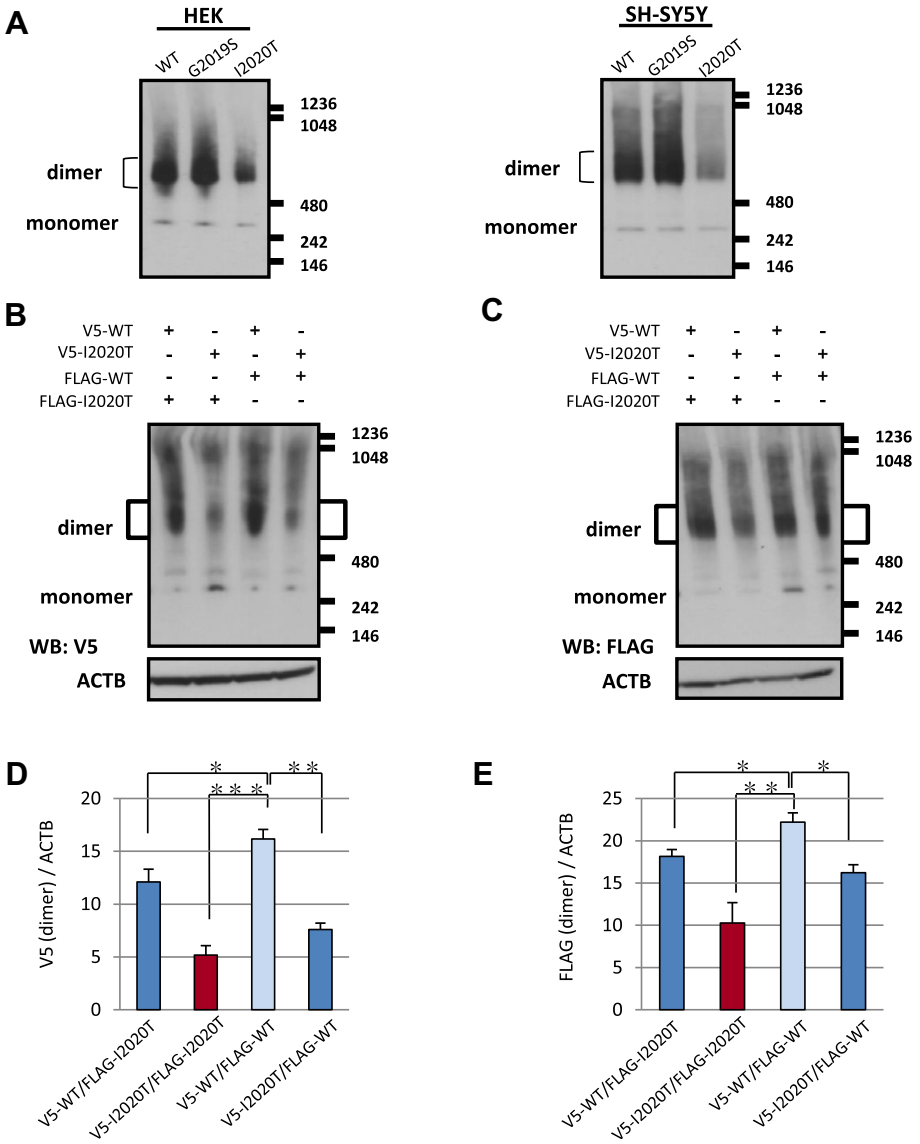

Fig. 1. Blue-native polyacrylamide gel electrophoresis of LRRK2-homodimers and LRRK2-heterodimers. (A) HEK293 and SH-SY5Y cells were transfected with 3.2 μg of each cDNA for V5-tagged wild-type LRRK2 (WT), G2019S LRRK2 (G2019S), or I2020T LRRK2 (I2020T). The cell lysates were subjected to BN-PAGE and Western analysis with the antibody against V5. The left panel shows LRRK2-transfected HEK293 cells, and the right panel shows LRRK2-transfected SH-SY5Y cells. Positions of the LRRK2 dimers and monomers with the expected sizes are indicated. (B, C) HEK293 cells were transfected with V5- or FLAG-tagged WT and I2020T LRRK2, and cell lysates were separated by BN-PAGE (for LRRK2) and SDS–PAGE (for beta-actin), and subjected to Western analysis using antibodies against V5 (B) or FLAG (C). (D, E) Graphical representation of the experiments shown in B and C, in which the amount of dimer level of V5-LRRK2 (D) or FLAG-LRRK2 (E) was normalized against beta-actin. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons with WT-V5/FLAG-WT LRRK2-homodimer by one-way ANOVA: $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunoprecipitated from the cell lysates using an anti-FLAG antibody recognizing WT subunits and subjected to SDS-PAGE. Western analysis using an anti-V5 antibody recognizing the other subunits proved that the WT LRRK2 formed heterodimers with G2019S and I2020T LRRK2 in transfected cells (Fig. 2). Notably, the level of LRRK2 protein in the WT/I2020T-heterodimer, but not that in the WT/G2019S-heterodimer, was markedly lower than that in the WT/WT-homodimer (Fig. 2, lane 1 vs. lane 3).

3.3. The WT/I2020T-heterodimer has a short protein lifetime

To analyze the protein lifetime of LRRK2 dimers, we next performed a pulse-chase experiment. FLAG or V5-tagged WT and I2020T LRRK2 were transfected into HEK293 cells in various combinations. The biosynthetically ^{35}S-labeled LRRK2 molecules were immunoprecipitated using the anti-FLAG antibody, separated by SDS–PAGE, and their degradation rate was chased. We found that the WT/I2020T combination, in both transfections using two differently tagged cDNAs, exhibited a significantly shorter LRRK2 protein lifetime than that of the WT/WT combination (Fig. 3A and B), confirming the above-described findings. As expected, the I2020T/I2020T combination exhibited the shortest protein lifetime.

Next, to examine whether the molecular ratio of I2020T LRRK2 to WT LRRK2 influences the level of LRRK2 protein, a constant amount (1.6 μg) of FLAG-tagged WT LRRK2 cDNA was co-transfected with increasing amounts (0.4–2.0 μg) of V5-tagged WT, I2020T, and G2019S LRRK2 cDNAs into HEK293 cells. As shown in Fig. 3C and D, the protein level of the FLAG-WT subunits was not affected by the increasing amount of V5-WT subunits up to a total of 3.6 μg cDNA. Similar results were obtained for co-transfection with increasing amounts of V5-G2019S LRRK2, indicating that G2019S LRRK2 had no influence on the protein level of WT LRRK2. In marked

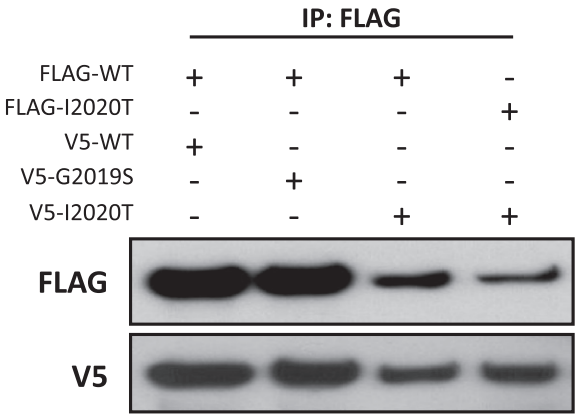

Fig. 2. Detection of WT/I2020T LRRK2 heterodimers. HEK293 cells were transfected with various combinations of 1.6 μg of each cDNA (total 3.2 μg) for WT, G2019S, and I2020T LRRK2 tagged with either V5 or FLAG. The LRRK2-homo and -heterodimers were immunoprecipitated from the cell lysates using the antibody against FLAG and subjected to SDS-PAGE followed by Western analysis with the antibodies against V5 and FLAG.

contrast, the protein level of FLAG-WT LRRK2 was reduced dose dependently as the amount of co-transfected V5-I2020T LRRK2 was increased. These results suggested that increasing the amount of I2020T LRRK2 increased the frequency at which it encountered WT LRRK2 to form the WT/I2020T-heterodimer, thus enhancing degradation.

3.4. Anti-apoptotic protectivity and Akt1 phosphorylation activity of LRRK2 are impaired by WT/I2020T-heterodimer formation

To compare the degree of anti-apoptotic protectivity among LRRK2 dimers, HEK293 cells were transfected with WT and I2020T LRRK2 in various combinations, and then treated with H_2O_2. It turned out that transfection with the WT/WT combination exerted significant protectivity against apoptosis induced by 0.5 mM H_2O_2 in comparison with that resulting from transfection with the empty vector, whereas transfection with the I2020T/I2020T combination exerted little anti-apoptotic protectivity (Fig. 4A). Notably, the anti-apoptotic protectivities afforded by co-transfection of WT LRRK2 and I2020T LRRK2 were significantly lower than that afforded by the WT/WT combination, being rather similar to that resulting from the I2020T/I2020T combination. These results, together with the above findings, suggest that the anti-apoptotic activity of WT LRRK2 is impaired by heterodimer formation with I2020T LRRK2.

Recently, we have reported that LRRK2 directly phosphorylates Ser473 of Akt1, a central molecule involved in cell survival and pre-

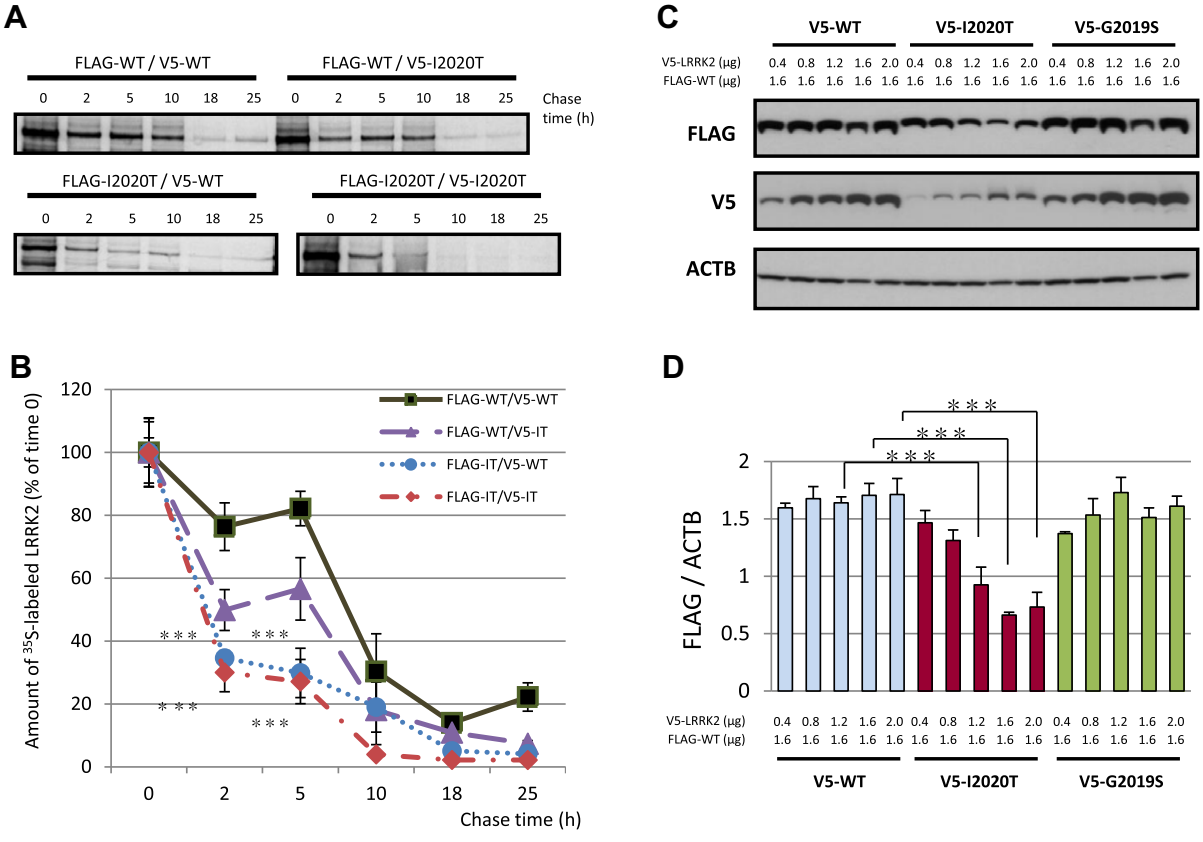

Fig. 3. The WT/I2020T-heterodimer affect protein lifetime. (A) Pulse-chase analysis of LRRK2 dimers. FLAG or V5-tagged WT and I2020T LRRK2 were transfected into HEK293 cells in various combinations. After 24 h of transfection, the cells were pulse-labeled with [^{35}S]methionine and cysteine for 1 h and then harvested at 0, 2, 5, 10, 18, 25 h later, washed, and lysed with digitonin-lysis buffer. The radiolabeled LRRK2 molecules were immunoprecipitated with anti-FLAG antibody, separated on SDS–PAGE, and subjected to autoradiography. (B) The decay curve of the LRRK2 molecules shown in (A). Solid line (———): FLAG-WT/WT-V5 homodimer, dotted line (. . ....): FLAG-I2020T/WT-V5 heterodimer, dashed line (— — —): FLAG-WT/I2020T-V5 heterodimer, dash-dotted line (—·—·—): FLAG-I2020T/I2020T-V5 homodimer. Stars represent statistical comparisons with FLAG-WT/WT-V5 LRRK2-homodimer by one-way ANOVA ($n = 3$); $^{***}p < 0.0005$. (C) Influences of increasing amounts of I2020T or G2019S LRRK2 on the level of WT LRRK2 protein. A constant amount (1.6 μg) of FLAG-tagged WT LRRK2 cDNA was co-transfected with increasing amounts (0.4–2.0 μg) of V5-tagged WT, I2020T, or G2019S LRRK2 cDNA in HEK293 cells. The protein level of LRRK2 in the lysates was analyzed by Western blotting using antibodies against V5 and FLAG. (D) Graphical representation of the experiment shown in (C), in which the amount of FLAG-tagged WT LRRK2 was normalized against beta-actin. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; $^{***}p < 0.0005$.

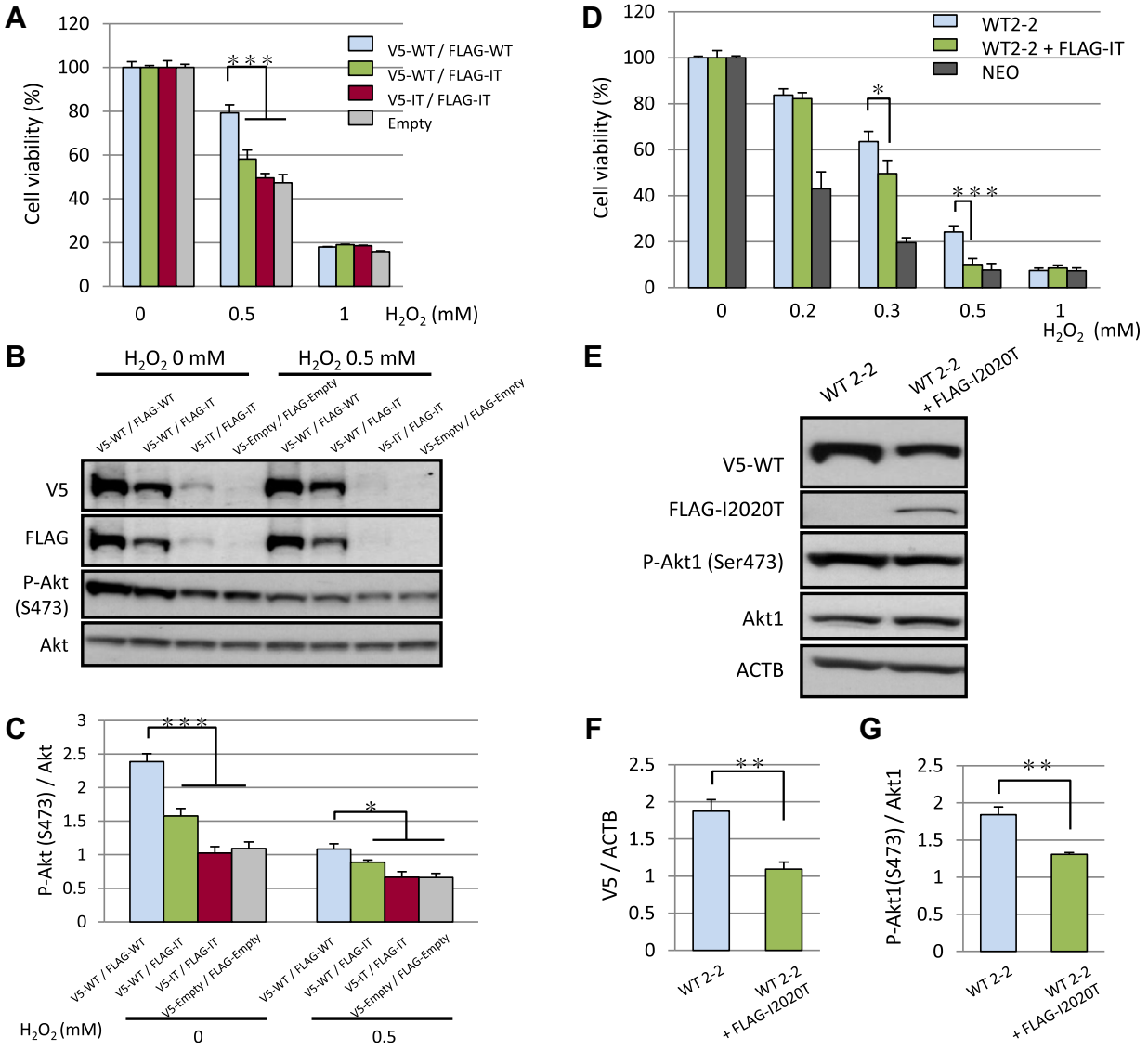

Fig. 4. Comparison of anti-apoptotic protectivity and Akt1 phosphorylation activity among LRRK2 dimers. (A) FLAG- or V5-tagged WT and I2020T LRRK2 were co-transfected into HEK293 cells in various combinations. FLAG- or V5-tagged empty vectors were also transfected as controls. After 24 h, the cells were treated with 0, 0.5, or 1 mM H_2O_2 for 50 min to induce apoptosis, and the cell viability was measured. Without H_2O_2 treatment (0 mM), cell viability was 100% for all transfection combinations. (B) Cell lysates were prepared from LRRK2-transfected HEK293 cells treated with 0 or 0.5 mM H_2O_2 as shown in (A). Western analysis was performed using antibodies against V5, FLAG, phospho-Akt1 (Ser473), and non-phosphorylated Akt1. (C) Graphical representation of phosphorylation levels of Akt1 (Ser473) in the experiment shown in (B) after normalization with the amount of total Akt1. (D) WT2-2, a SH-SY5Y clone stably overexpressing the V5-tagged WT LRRK2 was transfected with I2020T LRRK2, treated with 0, 0.2, 0.3, 0.5, or 1 mM H_2O_2 for 24 h, and cell viability was measured. A SH-SY5Y clone (NEO) expressing only the neomycin gene was used as control. Without H_2O_2 treatment (0 mM), cell viability was 100% in all cases. (E) Cell lysates were prepared from WT2-2 and I2020T-transfected WT-2 and subjected to Western analysis using antibodies against phospho-Akt1 (Ser473), non-phosphorylated Akt1, V5, FLAG, and beta-actin. (F and G) Graphical representation of the experiment shown in (E). V5-LRRK2 protein levels were normalized with beta-actin (F) and phosphorylation levels of Akt1 (Ser473) were normalized with total Akt1 (G). Stars represent statistical comparisons by one-way ANOVA ($n = 6$ in A, D; $n = 4$ in C; $n = 3$ in F, G); $^{*}p < 0.05$. $^{**}p < 0.005$. $^{***}p < 0.0005$.

vention of apoptosis [20]. In keeping with this, transfection of the WT/WT LRRK2, but not the I2020T/I2020T LRRK2 combination, into HEK293 cells markedly increased the degree of Akt1 (Ser473) phosphorylation (Fig. 4B and C). Importantly, the phosphorylation of Akt1 in HEK293 cells transfected with the WT/I2020T combination was significantly lower than in those transfected with the WT/WT combination, both in the absence and presence of 0.5 mM H_2O_2. These results suggested that, through WT/I2020T-heterodimer formation, I2020T LRRK2 impaired the kinase activity targeting Akt1 (Ser473). Such reduced phosphorylation of Akt1, and therefore its reduced activation, would be one of the mechanisms responsible for the above-described decrease in apoptosis resistance observed for WT/I2020T LRRK2-transfected HEK293 cells, i.e., the dominant-negative mechanism of neurodegeneration through heterodimer formation.

In addition, SH-SY5Y clone overexpressing WT LRRK2 (WT2-2) [22] exhibits much higher resistance to apoptosis over a wide range of H_2O_2 concentration in comparison with a vector-control clone (Fig. 4D). Transfection of FLAG-I2020T LRRK2 into WT2-2 significantly reduced its resistance to apoptosis induced by 0.3 mM and 0.5 mM H_2O_2. Both the protein level of WT LRRK2 and the phosphorylation level of Akt1 (Ser473) in WT2-2 were significantly reduced by transfection of I2020T LRRK2 (Fig. 4E, F and G). The degree of reduction in the Akt1 phosphorylation level in the presence of I2020T LRRK2 was almost the same as that observed when the WT2-2 clone was treated with a specific inhibitor, LRRK2-IN1

(Supplementary Fig. 1). These results are consistent with those of co-transfection into HEK293 cells, and lend further support to the notion of a dominant-negative mechanism of neurodegeneration through heterodimer formation.

4. Discussion

We have previously reported that I2020T LRRK2 has a unique molecular feature, i.e., it is more susceptible to post-translational degradation than WT and G2019S LRRK2 [22]. In the present study, we confirmed this at the dimer level using BN-PAGE. Although G2019S and I2020T are mutations that occur in amino acid residues neighboring each other, they may induce characteristic molecular changes in LRRK2 that are distinct from each other, as has been observed for kinase activity [11,13–18]. More importantly, using immunoprecipitation, BN-PAGE, and a pulse-chase experiment, we have proven that I2020T LRRK2 forms a heterodimer with WT LRRK2 and decreases the intracellular level of LRRK2 protein. It is possible that I2020T mutation might affect the stability of the heterodimer molecule or binding between the dimer and molecules such as chaperone HSP90, p50cdc37, heat shock cognate 71 kDa, and the carboxyl terminus of HSP70-interacting protein, which have all been reported to associate with, and may stabilize, LRRK2 dimers [16,24].

Several studies have reported that LRRK2 forms oligomers larger than dimers [7,8]. Although using BN-PAGE we observed some oligomers at around 1–2 MkDa, the level of LRRK2 protein was not affected by the appearance of oligomers (Fig. 1). In addition, transfection with the WT/I2020T combination did not increase the amount of monomers in comparison with the WT/WT combination, suggesting that the decrease of LRRK2 dimers was not due to an increase of WT-monomers. Our BN-PAGE results contrast with those of Sen et al. [7], who demonstrated that both G2019S and I2020T LRRK2 showed higher proportions of dimers in comparison with WT LRRK2. On the other hand, Ito et al. reported that LRRK2 showed as dimers on BN-PAGE and operated as monomeric form predominant within cells [25]. Although the reason for the discrepancy is currently unclear, instead of PBS-freeze-thawing, we prepared our cell lysate using digitonin, a mild detergent that enables solubilization of membrane proteins without breaking weak protein–protein interactions, as the LRRK2 dimer is reported to exist predominantly in the membrane fraction [26]. It has also been postulated that autophosphorylation of LRRK2 promotes the formation of stable dimers [7,8]. Although it would be difficult to measure the actual autophosphorylation status of LRRK2 in intact cells [10], it is possible that the kinase activity of I2020T LRRK2 might affect the dimer protein level.

It has been reported that LRRK2 kinase activity is dependent on dimer formation [7,26], suggesting that a decrease in the level of dimer protein would largely affect the kinase activity of LRRK2. In the present study, a decrease in the protein level of WT/I2020T-heterodimers was accompanied by a reduction of phosphorylation activity directed toward Ser473 of Akt1, which we have recently identified as a possible physiological substrate of LRRK2 [20]. Using recombinant proteins, we also found that I2020T LRRK2 has intrinsically impaired Akt1 (Ser473) phosphorylation activity [20]. Therefore, both the decreased dimer protein level and the impaired kinase activity would account for the lowered Akt1 phosphorylation activity of the WT/I2020T-heterodimer LRRK2.

In the present study, the LRRK2 dimers WT/WT, WT/I2020T, and I2020T/I2020T exhibited protective effects against H_2O_2-induced apoptosis in accordance with their intracellular protein level. However, the notion of a cell-protective function of LRRK2 does not appear compatible with a report indicating that LRRK2-knockout (KO) mice exhibit no neurological abnormality in a steady state or any susceptibility to neurotoxins [27,28]. On the other hand, the KO mice reportedly show pathology in the kidney and lung, where the LRRK2 protein is expressed at a much higher level than in the brain of WT mice [28]. It could be possible that the function of LRRK2 in KO mice is compensated by a homologous molecule, LRRK1, in the brain but not in other organs requiring a higher level of LRRK2 protein, such as the kidney and lung. In the human brain, it is speculated that the long-term (>50 year) persistence of a low protein level of LRRK2 could cause vulnerability in neurons, particularly dopaminergic neurons that are thought to be exposed to continuous oxidative stress resulting from dopamine metabolism.

In conclusion, the present data indicate that I2020T mutant LRRK2 forms a heterodimer with WT LRRK2, resulting in reductions of the total LRRK2 protein level, Akt1 phosphorylation, and apoptosis resistance. Our results provide a new insight into the etiology of autosomal-dominant PD, PARK8, which is caused by I2020T mutation of one of the LRRK2 alleles.

Acknowledgments

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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.2012.11.113.

References

[1] M. Funayama, K. Hasegawa, H. Kowa, et al., New locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1, Ann. Neurol. 51 (2002) 296–301.
[2] A. Zimprich, S. Biskup, P. Leitner, et al., Mutations in LRRK2 cause autosomal-dominant Parkinsonism with pleomorphic pathology, Neuron 44 (2004) 601–607.
[3] C. Paisan-Ruiz, S. Jain, E.W. Evans, et al., Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease, Neuron 44 (2004) 595–600.
[4] M. Funayama, K. Hasegawa, E. Ohta, et al., An LRRK2 mutation as a cause for the Parkinsonism in the original PARK8 family, Ann. Neurol. 57 (2005) 918–921.
[5] T. Gasser, Mendelian forms of Parkinson's disease, Biochim. Biophys. Acta 1792 (2009) 587–596.
[6] E. Meylan, J. Tschopp, The RIP kinases: crucial integrators of cellular stress, Trends Biochem. Sci. 30 (2005) 151–159.
[7] S. Sen, P.J. Webber, A.B. West, Dependence of leucine-rich repeat kinase 2 (LRRK2) kinase activity on dimerization, J. Biol. Chem. 284 (2009) 36346–36356.
[8] E. Greggio, I. Zambrano, A. Kaganovich, et al., The Parkinson disease-associated leucine-rich repeat kinase 2 (LRRK2) is a dimer that undergoes intramolecular autophosphorylation, J. Biol. Chem. 283 (2008) 16906–16914.
[9] J. Deng, P.A. Lewis, E. Greggio, et al., Structure of the ROC domain from the Parkinson's disease-associated leucine-rich repeat kinase 2 reveals a dimeric GTPase, Proc. Natl. Acad. Sci. USA 105 (2008) 1499–1504.
[10] G. Ito, T. Okai, G. Fujino, et al., GTP binding is essential to the protein kinase activity of LRRK2, a causative gene product for familial Parkinson's disease, Biochemistry 46 (2007) 1380–1388.
[11] W.W. Smith, Z. Pei, H. Jiang, et al., Kinase activity of mutant LRRK2 mediates neuronal toxicity, Nat. Neurosci. 9 (2006) 1231–1233.
[12] E. Lobbestael, V. Baekelandt, J.M. Taymans, Phosphorylation of LRRK2: from kinase to substrate, Biochem. Soc. Trans. 40 (2012) 1102–1110.
[13] Y. Imai, S. Gehrke, H.Q. Wang, et al., Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in drosophila, EMBO J. 27 (2008) 2432–2443.
[14] M. Jaleel, R.J. Nichols, M. Deak, et al., LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity, Biochem. J. 405 (2007) 307–317.
[15] A.B. West, D.J. Moore, C. Choi, et al., Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity, Hum. Mol. Genet. 16 (2007) 223–232.

[16] C.J. Gloeckner, N. Kinkl, A. Schumacher, et al., The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity, Hum. Mol. Genet. 15 (2006) 223–232.

[17] B. Luzon-Toro, E.R. de la Torre, A. Delgado, et al., Mechanistic insight into the dominant mode of the Parkinson's disease-associated G2019S LRRK2 mutation, Hum. Mol. Genet. 16 (2007) 2031–2039.

[18] V.S. Anand, L.J. Reichling, K. Lipinski, et al., Investigation of leucine-rich repeat kinase 2: enzymological properties and novel assays, FEBS J. 276 (2009) 466–478.

[19] E. Greggio, M.R. Cookson, Leucine-rich repeat kinase 2 mutations and Parkinson's disease: three questions, ASN NEURO 1 (2009) e00002.

[20] E. Ohta, F. Kawakami, M. Kubo, F. Obata, LRRK2 directly phosphorylates Akt1 as a possible physiological substrate: impairment of the kinase activity by Parkinson's disease-associated mutations, FEBS Lett. 585 (2011) 2165–2170.

[21] E.D. Plowey, S.J. Cherra 3rd, Y.J. Liu, C.T. Chu, Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells, J. Neurochem. 105 (2008) 1048–1056.

[22] E. Ohta, Y. Katayama, F. Kawakami, et al., I2020T leucine-rich repeat kinase 2, the causative mutant molecule of familial Parkinson's disease, has a higher intracellular degradation rate than the wild-type molecule, Biochem. Biophys. Res. Commun. 390 (2009) 710–715.

[23] E. Ohta, M. Kubo, F. Obata, Prevention of intracellular degradation of I2020T mutant LRRK2 restores its protectivity against apoptosis, Biochem. Biophys. Res. Commun. 391 (2010) 242–247.

[24] T. Li, D. Yang, S. Sushchky, et al., Models for LRRK2-linked Parkinsonism, Parkinsons Dis. (2011) 942412.

[25] G. Ito, T. Iwatsubo, Re-examination of the dimerization state of leucine-rich repeat kinase 2: predominance of the monomeric form, Biochem. J. 441 (2012) 987–994.

[26] Z. Berger, K.A. Smith, M.J. Lavoie, Membrane localization of LRRK2 is associated with increased formation of the highly active LRRK2 dimer and changes in its phosphorylation, Biochem. 49 (2010) 5511–5523.

[27] E. Andres-Mateos, R. Mejias, M. Sasaki, et al., Unexpected lack of hypersensitivity in LRRK2 knock-out mice to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), J. Neurosci. 29 (2009) 15846–15850.

[28] Y. Tong, H. Yamaguchi, E. Giaime, et al., Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice, Proc. Natl. Acad. Sci. USA 107 (2010) 9879–9884.