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Prevention of intracellular degradation of I2020T mutant LRRK2 restores its protectivity against apoptosis

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ABSTRACT

Leucine-rich repeat kinase 2 (*LRRK2*) is the causal gene for autosomal dominant familial Parkinson's disease. We have previously reported a novel molecular feature characteristic to I2020T mutant LRRK2: higher susceptibility to post-translational degradation than the wild-type LRRK2. In the present study, we demonstrated that the protective effect of I2020T LRRK2 against hydrogen peroxide-induced apoptosis was impaired in comparison with the wild-type molecule. When the intracellular level of the protein had been allowed to recover by treatment with proteolysis inhibitors, the protective effect of I2020T LRRK2 against apoptosis was increased. We further confirmed that a decrease in the intracellular protein level of WT LRRK2 by knocking down resulted in a reduction of protectivity against apoptosis. These results suggest that higher susceptibility of I2020T mutant LRRK2 to intracellular degradation than the wild-type molecule may be one of the mechanisms involved in the neurodegeneration associated with this LRRK2 mutation.

Introduction

Parkinson's disease (PD) is a movement disorder caused by degeneration of dopaminergic neurons. *Leucine-rich repeat kinase 2* (*LRRK2*) is the gene responsible for autosomal dominant PD, PARK8, which we originally defined by linkage analysis of a Japanese family (Sagamihara family) [1–4]. LRRK2 belongs to the receptor-interacting protein (RIP) family, which has LRR (leucine-rich repeat), ROC (Ras of complex), COR (C-terminal ROC), kinase, and WD40 domains [5]. The Sagamihara family patients have the I2020T mutation in the kinase domain [4,6]. Up to now, a total of 23 LRRK2 mutations in various domains have been reported worldwide [2–4,7]. 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, its true physiological function or the mechanism of neurodegeneration resulting from the mutation has not been conclusively clarified.

Accumulated data suggest that hyper-kinase activity reported for mutant LRRK2 molecules, particularly G2019S LRRK2, may be one possible mechanism for the pathogenesis induced by this molecule [8–13]. It has also been postulated that autophosphorylation of LRRK2 stabilize the kinase-active dimer and exacerbates the pathogenesis [14]. In the case of I2020T mutation, however, there is a degree of controversy; some studies have reported augmented kinase activity [9,15,16], whereas other studies of this mutation have demonstrated unchanged or impaired phosphorylation activity [11,17,18]. Thus, at least in the case of I2020T mutation, there is no consensus on the mechanism responsible for neurodegeneration.

In the previous study, we demonstrated that I2020T LRRK2 is more susceptible to post-translational degradation than the wild-type LRRK2 and G2019S LRRK2, indicating a novel molecular feature characteristic to I2020T LRRK2 [19]. In the present study, we investigated whether the high degradation rate of I2020T LRRK2 is related to the pathogenesis associated with this mutant molecule. We found that the wild-type LRRK2 exhibited a protective effect against apoptosis whereas I2020T mutant LRRK2 had impaired protectivity. Prevention of the intracellular degradation of I2020T LRRK2 markedly increased its protective effect against apoptosis. Finally, we investigated the relationship between the intracellular protein level of LRRK2 and its protectivity against apoptosis employing a LRRK2-knockdown experiment.

Materials and methods

Transfection of LRRK2. The mammalian expression cDNA construct of wild-type (WT) and I2020T mutant *LRRK2* cDNA with a V5 tag at the C-terminus was described previously [19]. Sequence

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analysis proved that T6059>C (I2020T) at exon 41 was the only difference between the WT and the I2020T *LRRK2* cDNA construct throughout the whole plasmid. HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% FCS and antibiotics. SH-SY5Y cells were cultured in DMEM nutrient mixture F-12 HAM (Sigma) supplemented with 10% FCS, and antibiotics. Transfection of the *LRRK2* cDNA plasmid was performed using Lipofectamine™ 2000 (Invitrogen) for HEK293 cells, and FuGENE® HD Transfection Reagent (Roche) for SH-SY5Y cells in accordance with the manufacturers' protocols. SH-SY5Y clones stably and uniformly expressing WT or I2020T LRRK2 have been described previously [19].

Western analysis. LRRK2-transfected cells were suspended in cell lysis buffer [Tris–HCl-buffered saline (pH 7.6) containing 1% digitonin, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of Complete mini protease inhibitor cocktail® (Roche)]. Cell lysates were obtained by centrifugation and subjected to Western analysis using horseradish peroxidase (HRP)-labeled antibody against the V5 tag (Invitrogen) for LRRK2 expression and HRP-labeled antibody against β-actin (Abcam) as an internal control.

Prevention of intracellular degradation of LRRK2. After 24 h of transfection with WT and I2020T *LRRK2* cDNA, HEK293 cells were treated with a cocktail of three proteolysis inhibitors, 1 μM MG-132 (Calbiochem) and 1 μM lactacystin (Sigma), both of which are proteasome inhibitors, and with 200 nM chloroquine (Sigma), a lysosome inhibitor. After 24 h of treatment, the cells were harvested and their lysates were analyzed by Western blotting, as described above. The stably LRRK2-expressing SH-SY5Y clones were also treated with the proteolysis inhibitors for 24 h and analyzed in the same manner.

Hydrogen peroxide (H_2O_2)-induced apoptosis. Apoptosis was induced by treatment of LRRK2-transfected cells with various concentrations (1–6 mM) of H_2O_2 for 50 min at 37 °C. In some experiments, the cells were treated with a cocktail of the proteolysis inhibitors MG-132, lactacystin and chloroquine for 24 h before addition of H_2O_2. Percentage of apoptotic cells was measured using an Annexin V-PE apoptosis Kit I™ (BD Biosciences) and an EPICS XL™ Flow Cytometer (Beckman Coulter) in accordance with the manufacturer's protocol. Apoptotic cells were also assessed by Western analysis of the lysates of transfected cells using an antibody against caspase-9 (Cell Signaling). For cell viability analysis, LRRK2-transfected cells were treated with 0.5 mM H_2O_2 for 30 min at 37 °C, and subjected to assay using a Cell Counting Kit-8™ (Dojindo) in accordance with the manufacturer's protocol.

Knockdown of transfected LRRK2. HEK293 cells were transfected with WT LRRK2 cDNA together with 25mer of Stealth™ RNAi for LRRK2 (5′-GAGCUGCUCCUUUGAAGAUACUAAA-3′; Invitrogen) or with an RNAi-control with the scrambled sequence. The effectiveness of knockdown of transfected LRRK2 was confirmed by Western analysis using anti-V5 antibody. After 24 h of co-transfection, the cells were treated with various concentrations (0.05–3 mM) of H_2O_2 for 30 min to induce apoptosis, and cell viability was analyzed.

Results

H_2O_2-induced apoptosis in LRRK2-transfected cells

To elucidate the physiological function of LRRK2 in the maintenance of cell viability, H_2O_2-induced apoptosis in LRRK2-transfected HEK293 cells was analyzed using annexin V staining. Among WT LRRK2-transfected cells treated with H_2O_2, the percentage of apoptotic cells was significantly lower than among untransfected cells, which expressed only endogenous LRRK2 molecules (Fig. 1A). In contrast, the percentage of apoptotic cells among I2020T mutant LRRK2-transfected HEK293 cells was significantly higher than that among WT LRRK2-transfected cells, and not significantly different

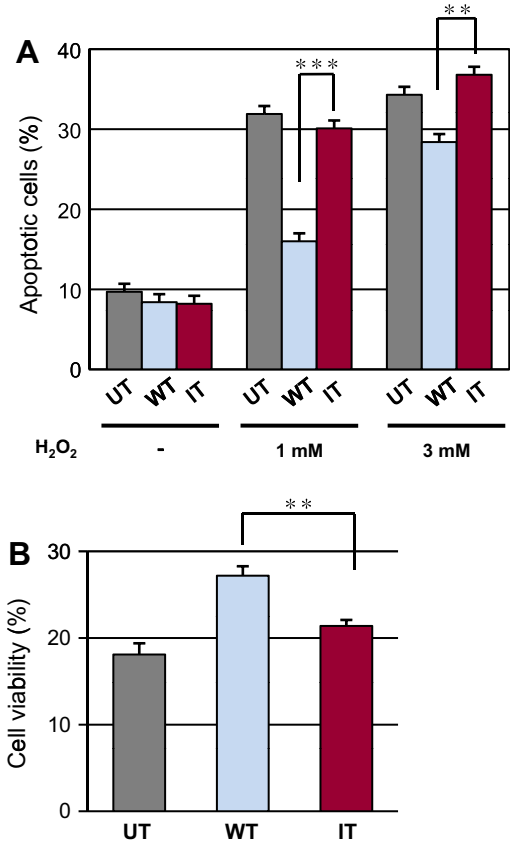

Fig. 1. H_2O_2-induced apoptosis in LRRK2-transfected cells. (A) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated with 1 or 3 mM H_2O_2 for 50 min. The percentage of cells showing apoptosis was measured by annexin V staining. (B) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated with 0.5 mM H_2O_2 for 30 min and the cell viability was measured. UT: Untransfected HEK293 cells. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{**}p < 0.005$. $^{***}p < 0.0005$.

from the situation in untransfected cells. Similar results were obtained for the LRRK2-transfected neuroblastoma cell line SH-SY5Y, although to a less marked extent due to the low transfection efficiency, and for SH-SY5Y clones stably and uniformly expressing WT or I2020T LRRK2 (Supplementary Fig. 1A and B). Consistently, the viability of I2020T LRRK2-transfected HEK293 cells was significantly lower than that of the WT LRRK2-transfected cells (Fig. 1B). These results suggest that WT LRRK2, but not I2020T mutant LRRK2, exerts a protective effect against H_2O_2-induced apoptosis.

Apoptosis of LRRK2-transfected cells after treatment with proteolysis inhibitors

In the previous study, we demonstrated that the I2020T mutant LRRK2 is more susceptible to post-translational degradation than the WT LRRK2 [19]. To investigate whether prevention of degradation of the mutant LRRK2 influences its ability to protect against H_2O_2-induced apoptosis, WT- and I2020T LRRK2-transfected HEK293 cells were treated with a cocktail of proteolysis inhibitors, MG-132 (a proteasome inhibitor), lactacystin (a proteasome inhibitor), and chloroquine (a lysosome inhibitor). As reported, treatment with this inhibitor cocktail increased the I2020T LRRK2 protein to a level similar to that of WT LRRK2 (Fig. 2A). Possibly because of the apoptosis-promoting effect of the protease inhibitors [20,21], the treatment significantly increased the percentage of annexin V-positive apoptotic cells among WT LRRK2-transfected cells, although the percentage was still lower than that among

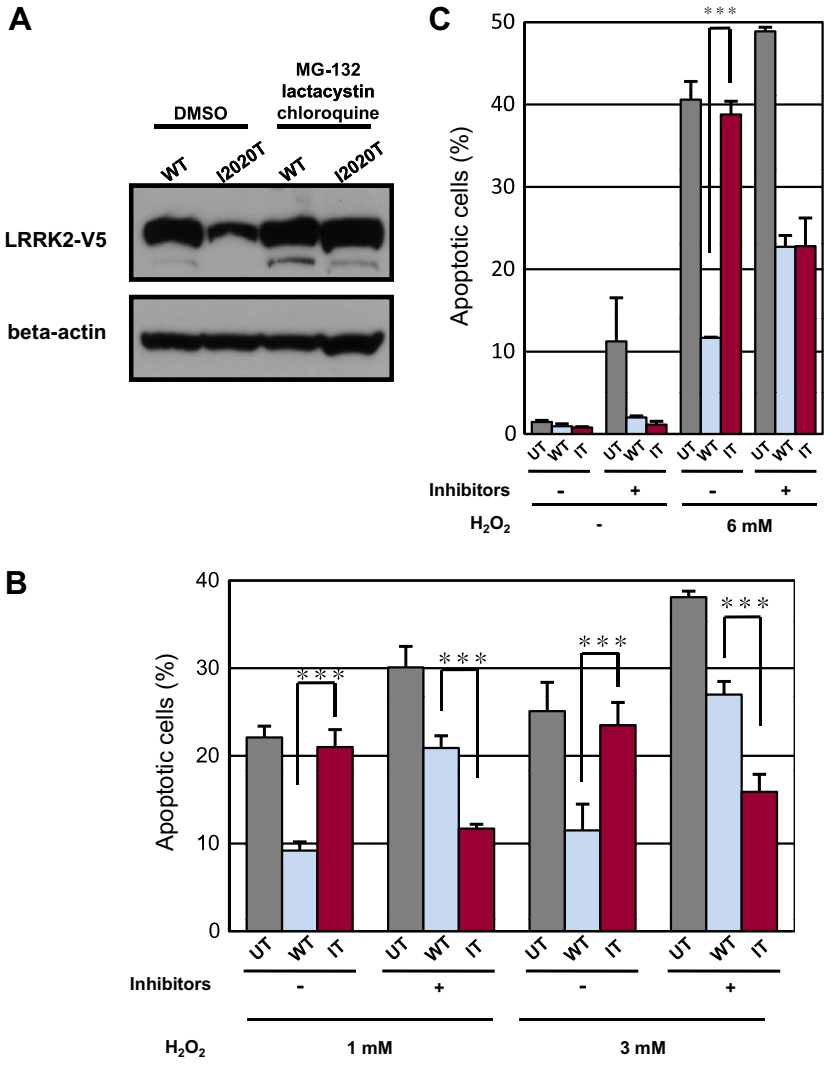

Fig. 2. Effect of proteolysis inhibitors on apoptosis in LRRK2-transfected cells. (A) HEK293 cells were transfected with WT or I2020T LRRK2 cDNA and treated with a cocktail of three proteolysis inhibitors (1 μM MG-132, 1 μM lactacystin, and 200 nM chloroquine) for 24 h. The LRRK2 level in the lysates was then analyzed by Western blotting with an antibody against V5 tag. (B) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated for 24 h with a cocktail of proteolysis inhibitors, and apoptosis was induced with 1 or 3 mM H_2O_2 for 50 min. UT: Untransfected HEK293 cells. (C) SH-SY5Y clones stably and uniformly expressing WT and I2020T LRRK2 (IT) were treated for 24 h with a cocktail of proteolysis inhibitors, and apoptosis was induced with 6 mM H_2O_2 for 4 h. UT: Untransfected SH-SY5Y cells. The percentage of apoptotic cells was measured by annexin V staining. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{***}p < 0.0005$.

untransfected cells subjected to the same treatment (Fig. 2B). Nevertheless, the same treatment of I2020T LRRK2-transfected cells markedly decreased the percentage of apoptotic cells to a level even lower than that among WT LRRK2-transfected cells.

Next, the effect of proteolysis inhibitors on apoptosis was analyzed using SH-SY5Y clones that over-expressed the WT and I2020T LRRK2 molecules stably and uniformly. Treatment with the proteolysis inhibitors increased the percentage of apoptotic cells among the WT LRRK2-expressing clones, although the percentage was still lower than that among the control cells (Fig. 2C). On the other hand, in I2020T LRRK2-expressing clones, the same treatment, which would otherwise have impaired the ability to protect against apoptosis, dramatically reduced the percentage of apoptotic cells to a level similar to that among the WT LRRK2-expressing clones. These results indicated that the ability of I2020T LRRK2 to protect against apoptosis could be restored by preventing its intracellular degradation.

Apoptosis was also analyzed by activation of caspase-9. The molecular ratio of activated relative to inactive caspase-9 in H_2O_2-treated cells was higher in I2020T LRRK2-transfected HEK293 cells than in WT LRRK2-transfected cells (Fig. 3). Although treatment with the proteolysis inhibitors increased the molecular ratio of activated caspase-9 in both WT- and I2020T LRRK2-transfected cells, this treatment reduced the ratio of activated caspase-9 in the I2020T LRRK2-transfected cells to a level lower than that in the WT LRRK2-transfected cells. These results, in terms of both annexin V staining and caspase-9 activation, indicated that the ability of I2020T LRRK2 to protect cells against apoptosis can be increased by preventing its degradation.

Influence of LRRK2-knockdown on protectivity against apoptosis

Finally, the relationship between the intracellular protein level of LRRK2 and protectivity against apoptosis was investigated in a knockdown experiment. Transfection of *LRRK2*-specific RNAi together with WT *LRRK2* cDNA into HEK293 reduced the protein level of transfected WT LRRK2 to 18% in comparison with the use of an RNAi-control (Fig. 4A). As described above, transfection of WT *LRRK2* cDNA into HEK293 markedly improved the viability of H_2O_2-treated cells (Fig. 4B). This protectivity of WT LRRK2 against

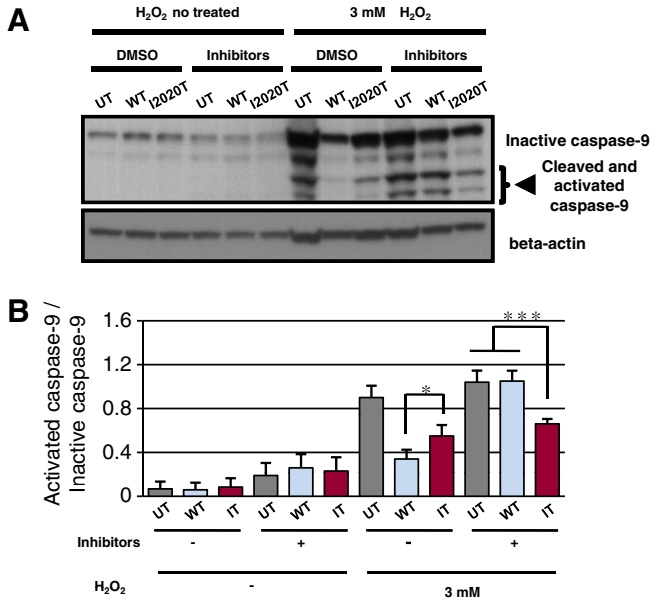

Fig. 3. Caspase-9 activation in LRRK2-transfected cells. (A) WT- and I2020T LRRK2-transfected HEK293 cells were treated with a cocktail of proteolysis inhibitors (MG-132, lactacystin and chloroquine), and apoptosis was induced with 3 mM H_2O_2 for 50 min. The level of cleaved and activated caspase-9 in the lysates was analyzed by Western blotting. UT: Untransfected HEK293 cells. (B) Graphical representation of the molecular ratio of activated caspase-9 relative to inactive caspase-9. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{*}p < 0.05$. $^{***}p < 0.0005$.

apoptosis was significantly abrogated by co-transfection of the LRRK2-specific RNAi but not the RNAi-control. These results indicate that the ability of LRRK2 to protect cells from H_2O_2-induced apoptosis is related with its intracellular protein level.

Discussion

We have previously reported a novel molecular feature characteristic to I2020T LRRK2: that it is more susceptible to post-translational degradation than the wild-type LRRK2 and G2019S mutant LRRK2 [19]. In the present study, we found that the increased intracellular protein level achieved by preventing degradation of I2020T LRRK2 restore its protectivity against apoptosis. Indeed the protease inhibitors used in this study have been reported to show various additional cellular effects, e.g., promotion of apoptosis (MG-132, lactacystin, and chloroquine) and activation of the CMV promoter (lactacystin) [20–25]. However, such effects, if any, would have appeared in both WT- and I2020T LRRK2-transfected cells in a similar manner. Therefore, an increased amount of I2020T LRRK2 after treatment with proteolysis inhibitors would be the most plausible explanation for the increased protective effect against apoptosis. The notion that the intracellular protein level of LRRK2 determines its protective effect against apoptosis is further supported by the fact that knockdown of transfected WT LRRK2 impaired its cell-protective effect. Similarly, the apparently opposite effects in WT- and I2020T LRRK2-transfected cells after proteolysis treatment would have been due to the fact that, in the latter case, the extent of the increased protective effect might have overcome the toxic effects of the inhibitors. When its degra-

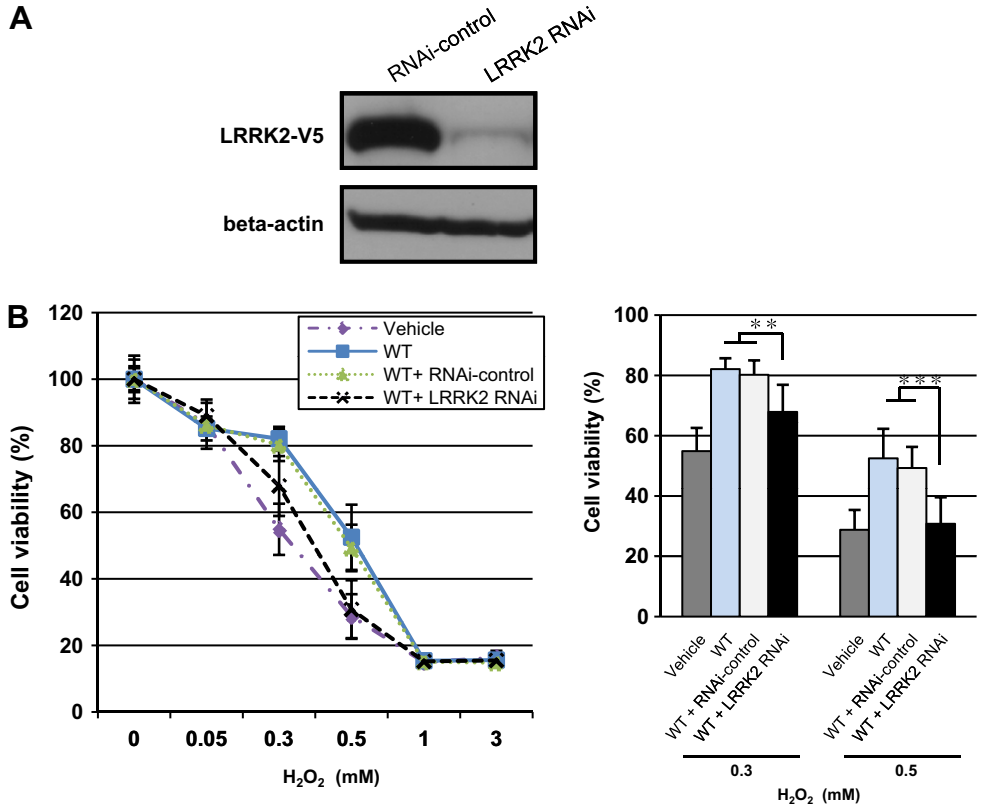

Fig. 4. Effect of a decrease in LRRK2 protein level on protectivity against apoptosis. HEK293 cells were transfected with WT *LRRK2* cDNA together with the LRRK2-specific RNAi or with the RNAi-control having the scrambled sequence. (A) After 24 h of co-transfection, the protein level of transfected WT LRRK2 was analyzed by Western blotting using anti-V5 antibody. (B) The cells after 24 h co-transfection were treated with 0.05, 03, 0.5, 1, or 3 mM H_2O_2 for 30 min to induce apoptosis, and cell viability was measured. Dash-dotted line (–·–·): vehicle, solid line (—): WT *LRRK2* cDNA, dashed line (– –): WT *LRRK2* + LRRK2 RNAi, dotted line (·····): WT *LRRK2* + RNAi-control. The results of treatment with 0.3 and 0.5 mM H_2O_2 are also represented by bar graph. Stars represent statistical comparisons by one-way ANOVA ($n = 6$); $^{**}p < 0.005$. $^{***}p < 0.0005$.

dation was prevented, the I2020T LRRK2 expressed by HEK293 exhibited an even stronger protective effect against apoptosis, in terms of both annexin V and caspase-9 analysis, than WT LRRK2, suggesting that I2020T LRRK2 might have a higher intrinsic potential than WT LRRK2 to activate a yet unknown apoptosis–protection pathway.

Although in the present study we found that the WT LRRK2 had a protective effect against H_2O_2-induced apoptosis and that knockdown of the WT LRRK2 abrogated this effect, there has been some controversy as to whether LRRK2 is protective or toxic for cells [12,15,26–28]. Under our experimental conditions, we did not observe any increase of apoptosis in WT LRRK2-transfected cells without H_2O_2 treatment. However, we could not exclude the possibility that over-expressed WT LRRK2 exerts a cytotoxic effect on cells in a steady state, whereas it functions as a maintenance or protective molecule when cells are exposed to oxidative stress. Interestingly, loss of the LRRK2-orthologue in *Drosophila* has been reported to induce an increase in susceptibility to oxidative stress and a lower survival rate, being consistent with the results of our LRRK2-knockdown experiments [29].

Although hyper-kinase activity of mutant LRRK2 molecules, particularly G2019S LRRK2, has been reported to be one possible mechanism for the pathogenesis induced by this molecule [8–13,15], there is controversy in the case of I2020T mutation. Some studies have reported augmented kinase activity [9,15,16], whereas other studies of this mutation have demonstrated unchanged or impaired kinase activity [11,17,18]. The results presented here suggest a new neurodegenerative mechanism induced by I2020T LRRK2, i.e., higher susceptibility to degradation gives rise to insufficiency of functional molecules to protect neurons from apoptosis. Several reports have revealed that insufficiency of gene products can cause dominant hereditary neurodegeneration, e.g., progranulin in frontotemporal lobar degeneration linked to chromosome 17 [30], transforming growth factor beta 2 and neurotrophin receptor trkB/C in the mouse PD model [31,32], and p73 in the mouse Alzheimer's disease model [33]. In addition, because LRRK2 has been reported to form dimers [9,34], any postulated molecular instability leading to degradation of I2020T LRRK2 may influence the stability and/or function of not only the I2020T/I2020T-homodimer but also the WT/I2020T-heterodimer, as is the case for GTP cyclohydrolase I in DYT5 dystonia [35,36] and KIT (mast/stem cell growth factor receptor) in piebaldism [37,38]. Finally, as in the case of I2020T LRRK2, the G2019S mutant LRRK2 exhibited impaired protectivity against H_2O_2-induced apoptosis (data not shown). As we reported previously, the G2019S LRRK2 does not differ from WT LRRK2 in susceptibility to degradation [19]. It cannot be excluded that each type of LRRK2 mutation affects a different molecular aspect of LRRK2, i.e., kinase activity, dimer formation, or susceptibility to degradation, all of which finally lead to neurodegeneration through a common and/or an independent pathway.

Conclusion

The intracellular protein level of LRRK2 determines protectivity against H_2O_2-induced apoptosis. The protective effect of I2020T mutant LRRK2 against apoptosis can be restored by preventing its intracellular degradation. Our results suggest a new etiology of neurodegeneration in PD caused by the LRRK2 mutation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.043.

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