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LRRK2 directly phosphorylates Akt1 as a possible physiological substrate: Impairment of the kinase activity by Parkinson's disease-associated mutations

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ABSTRACT

LRRK2 is the causal molecule for autosomal-dominant familial Parkinson's disease, although its true function, including its physiological substrates, remains unknown. Here, using in vitro kinase assay with recombinant proteins, we demonstrated for the first time that LRRK2 directly phosphorylates Akt1, a central molecule involved in signal transduction for cell survival and prevention of apoptosis. Ser473, one of two amino acids essential for Akt1 activation, was the target site for LRRK2. A knockdown experiment using intact cells also demonstrated LRRK2-mediated phosphorylation of Akt1 (Ser473), suggesting that Akt1 is a convincing candidate for the physiological substrate of LRRK2. The disease-associated mutations, R1441C, G2019S, and I2020T, exhibited reduced interaction with, and phosphorylation of, Akt1, suggesting one possible mechanism for the neurodegeneration caused by LRRK2 mutations.

Structured summary of protein interactions:
LRRK2 phosphorylates **Akt1** by protein kinase assay (View Interaction 1, 2, 3).
LRRK2 phosphorylates **MBP** by protein kinase assay (View Interaction 1, 2).
LRRK2 binds to **Akt1** by pull down (View Interaction 1, 2, 3).

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder caused by loss of nigrostriatal dopaminergic neurons. Leucine-rich repeat kinase (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 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 [5]. Most of the disease-associated mutations have been reported in the ROC and kinase domains. 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, neither the true function of LRRK2 nor the mechanism of neurodegeneration resulting from its mutations has been elucidated.

Accumulated data suggest that the kinase activity of LRRK2 may play a key role in neurodegeneration [6,7]. Several candidate molecules have been reported to act as substrates for LRRK2. LRRK2 itself can also serve as a substrate, and autophosphorylation elevates its own kinase activity [8–14]. Other molecules reportedly known to be LRRK2 substrates include the actin-cytoskeleton-related ERM (ezrin/radxin/moesin) proteins, β-tubulin, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and mitogen-activated kinase kinase 3, 4, 6, and 7 [15–20]. However, there is no consensus regarding which of these molecules is the true physiological substrate of LRRK2. For example, it has been reported that LRRK2 may not be the major kinase responsible for phosphorylation of 4E-BP1 in mammalian cells [21].

Akt1 (also known as protein kinase B1) is a serine/threonine kinase implicated in a variety of cellular events. In particular, Akt1 is the central molecule that transduces signals from receptors for growth factors and hormones to insure cell survival and protection of cells from apoptosis [22,23]. In the present study, we found that LRRK2 directly phosphorylated Akt1 at Ser473, an essential site for Akt1 activation. Our results indicate that Akt1 is one of the physiological substrates of LRRK2, and provide new functional insight into LRRK2 and its role in neurodegeneration.

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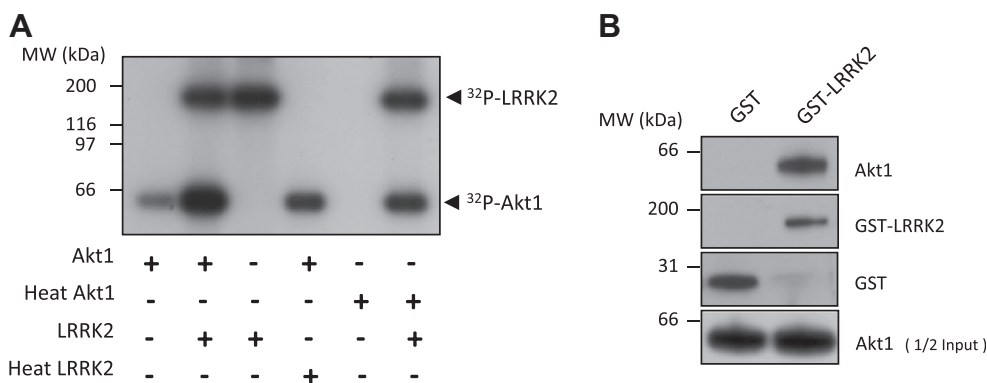
Fig. 1. LRRK2 directly interacts with and phosphorylates Akt1. (A) One hundred nanograms of recombinant human His-Akt1 (native or heat-denatured) was incubated with 50 ng of recombinant wild type GST-LRRK2 (molar ratio: 6.2:1) in the presence of radiolabeled ATP. The ^{32}P-labeled proteins in the reaction mixture were detected by autoradiography following SDS–PAGE. (B) GST-LRRK2 or GST alone was mixed with the recombinant His-Akt1 and subjected to pull-down assay by incubation with glutathione agarose beads. The beads-bound proteins were subjected to Western analysis using antibodies against non-phosphorylated Akt1 and GST.

2. Materials and methods

2.1. In vitro phosphorylation of Akt1 by LRRK2

One hundred nanograms of recombinant human Akt1 (His-tagged protein) (Calbiochem) was incubated with 50 ng of recombinant human LRRK2 [glutathione-S transferase (GST)-tagged protein consisting of amino acid residues 970–2527; either wild-type, R1441C mutant, G2019 mutant, or I2020T mutant] (Invitrogen) in 25 μl of reaction mixture [40 mM Tris–HCl (pH 7.6), 2 mM dithiothreitol, 10 mM MgCl$_2$, and 5 μM ATP including 3 μCi of [γ-^{32}P]ATP (GE Healthcare)]. After incubation at 30 °C for 30 min, the reaction was stopped by boiling in the sample-loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). The ^{32}P-labeled proteins were detected by autoradiography followed by SDS–PAGE, as described previously [24]. In some experiment, bovine myelin basic protein (MBP) (Sigma) was used as the substrate of LRRK2. For Western analysis, in vitro phosphorylation was performed without radiolabeled γ-ATP.

2.2. In vitro GST pull-down assay

Recombinant GST-LRRK2 or GST alone was mixed with the recombinant His-Akt1 and incubated with glutathione agarose beads overnight at 4 °C in 100 μl of TNE buffer [50 mM Tris–HCl (pH7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA]. After centrifugation, the supernatant was removed, and the beads were washed three times with TNE buffer. The bound proteins were eluted from the beads by boiling in 50 μl of SDS–PAGE sample buffer. The eluted samples were subjected to Western analysis using antibodies against non-phosphorylated Akt1 (Cell Signaling) and GST (Advanced Targeting Systems).

2.3. Western analysis

Proteins after in vitro phosphorylation and those in cell lysates were subjected to SDS–PAGE using a 5–20% gradient of e-PAGEL® (ATTO), blotted onto polyvinylidene fluoride membrane, and analyzed using antibodies with the following specificities: LRRK2 (MJFF2, c41-2) (Epitomics), phospho-Akt1 (Thr308) (Cell Signaling), phospho-Akt1 (Ser473) (Cell Signaling), non-phosphorylated Akt1, phospho-4E-BP1 (Thr37/46) (Cell Signaling), non-phosphorylated 4E-BP1 (Cell Signaling), and horseradish peroxidase (HRP)-labeled secondary antibody against rabbit IgG (BioLegend). HRP-labeled antibody against beta-actin (Abcam) was used as an internal control.

2.4. Knockdown of LRRK2

For knockdown of endogenous LRRK2 expressed by neuroblastoma SH-SY5Y, cells were transfected with 25 mer of Stealth® RNAi
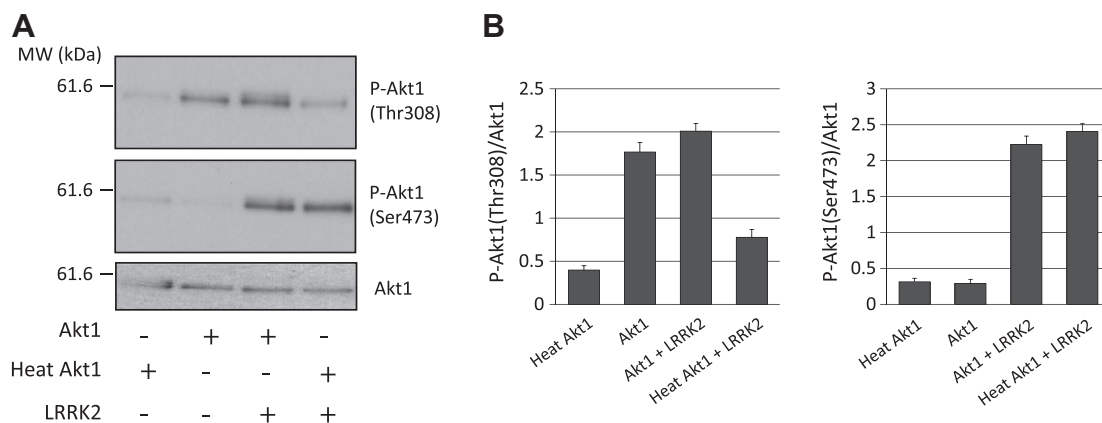
Fig. 2. LRRK2 phosphorylates Akt1 at Ser473. (A) Recombinant human His-Akt1 (native or heat-denatured) was incubated with recombinant GST-LRRK2 in the presence of cold γ-ATP. The phospho-Akt1 (Thr308), phospho-Akt1 (Ser473), and total Akt1 were detected by Western analysis using specific antibodies against each. (B) Graphical representation of the phosphorylation level of Akt1 [phospho-Akt1 (Thr308)/total Akt1 and phospho-Akt1 (Ser473)/total Akt1] (n = 2).
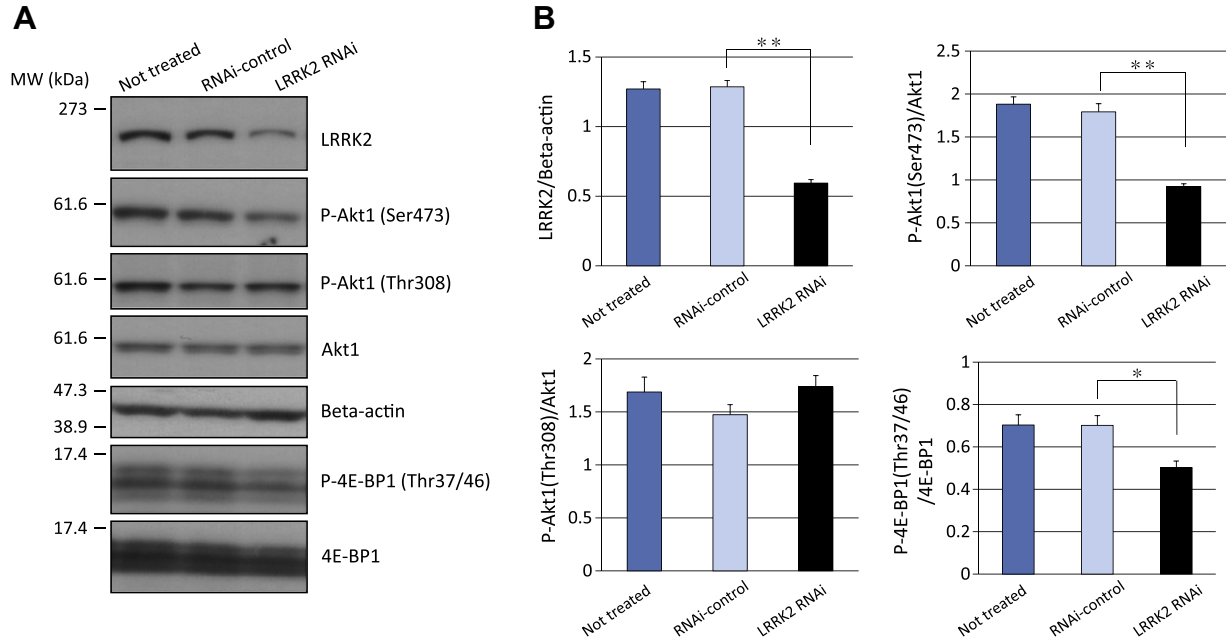
Fig. 3. Knockdown of endogenous LRRK2 expression diminishes Akt1 phosphorylation in intact cells. (A) SH-SY5Y cells were transfected with either the LRRK2-specific RNAi or the RNAi control. After 48 h of transfection, cell lysates were prepared and subjected to Western analysis using antibodies against LRRK2, phospho-Akt1 (Ser473), phospho-Akt1 (Thr308), non-phosphorylated Akt1, phospho-4E-BP1 (Thr37/46), non-phosphorylated 4E-BP1, and beta-actin as a control. (B) Graphical representation of the LRRK2-protein level, Akt1-phosphorylation level, and 4E-BP1-phosphorylation level. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); *: $p < 0.05$, **: $p < 0.005$.

for LRRK2 (5′-GAGCUGCUCCUUUGAAGAUACUAAA-3′; Invitrogen) or with an RNAi-control with the scrambled sequence using FuGENE®HD Transfection Reagent (Roche). After 48 h of transfection, the cells were suspended in cell lysis buffer [20 mM Tris–HCl (pH 8.2) containing 1% Triton X-100, 0.25 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 tablet of Complete mini protease inhibitor cocktail® (Roche), and 1 tablet of PhosSTOP® (Roche)]. Cell lysates were obtained by centrifugation at $10\,000 \times g$ for 10 min at 4 °C and subjected to Western analysis using anti-LRRK2 antibody. A SH-SH5Y clone stably and uniformly overexpressing the V5-tagged wild-type full-length LRRK2 was described

previously [25,26]. A SH-SY5Y clone expressing only the neomycin gene was used as a vector control. LRRK2 was knocked down using the same procedure as that described above.

3. Results

3.1. LRRK2 directly interacts with and phosphorylates Akt1

We have previously reported that LRRK2 exerts a protective effect against H_2O_2-induced apoptosis [26]. Akt1 is a signal-transducing molecule playing a central role in cell survival and inhibition of
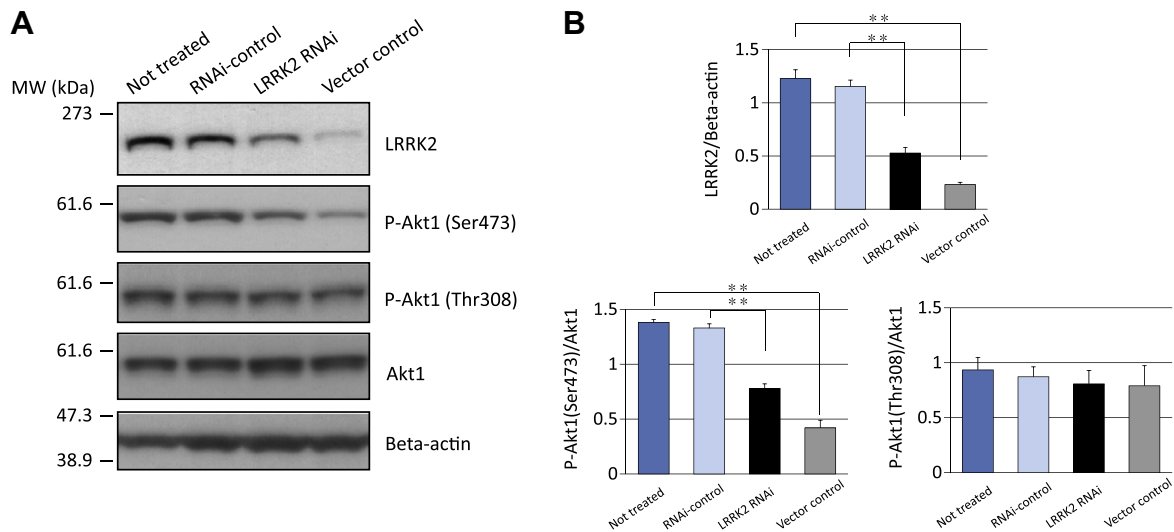
Fig. 4. Knockdown of overexpressed LRRK2 reduces Akt1 phosphorylation. (A) A SH-SY5Y clone stably overexpressing wild-type LRRK2 was transfected with either the LRRK2-specific RNAi or the RNAi control. After 48 h of transfection, cell lysates were prepared and subjected to Western analysis using antibodies against LRRK2, phospho-Akt1 (Ser473), phospho-Akt1 (Thr308), non-phosphorylated Akt1, and beta-actin as a control. A SH-SY5Y clone expressing only the neomycin gene was used as a vector control. (B) Graphical representation of the LRRK2-protein level and Akt1-phosphorylation level. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); **: $p < 0.005$.
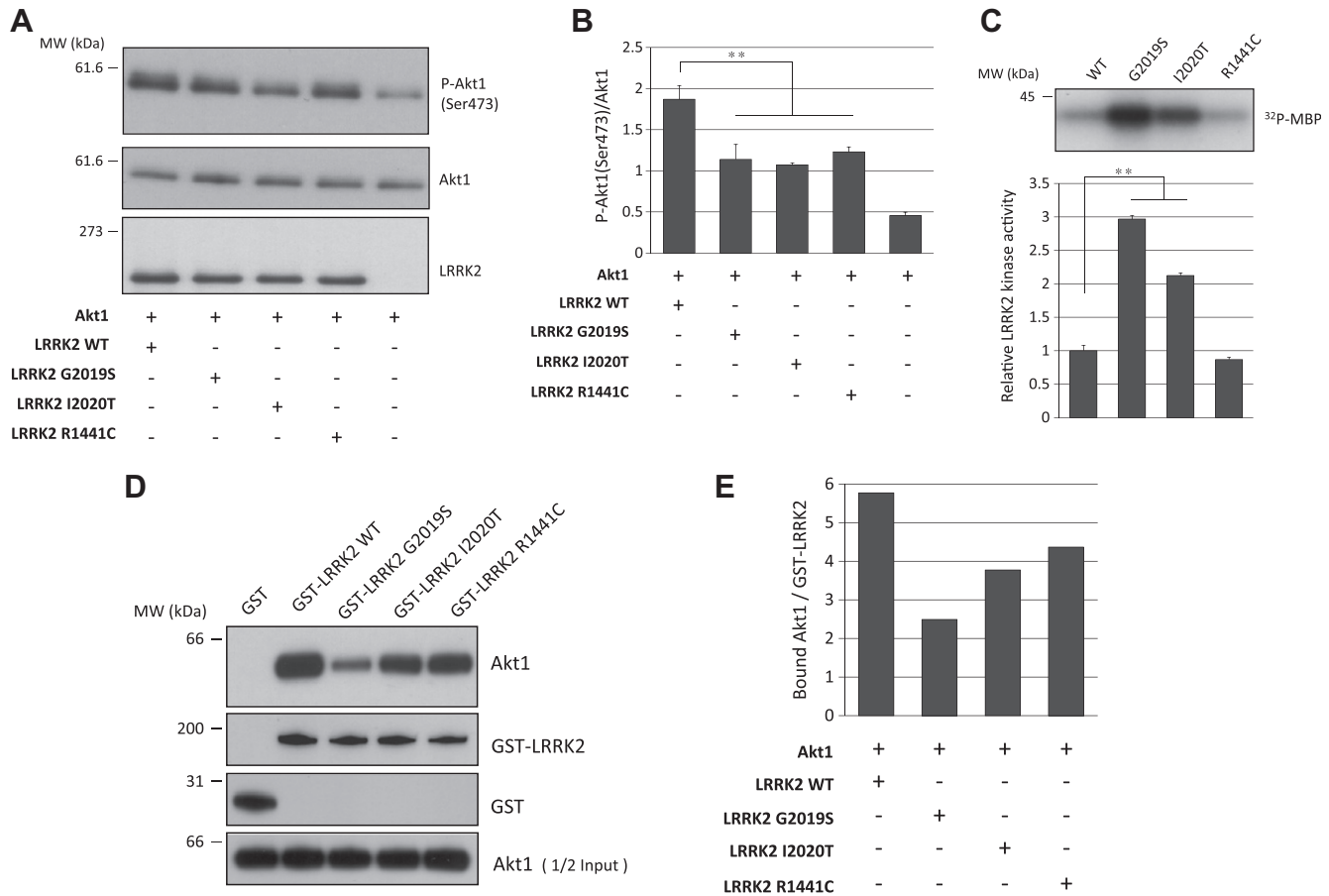
Fig. 5. Mutant LRRK2 exhibits lower Akt1-phosphorylation activity than wild-type LRRK2. (A) Recombinant His-Akt1 (native) was incubated with recombinant GST-LRRK2 of the wild-type (WT), G2019S mutant, I2020T mutant, or R1441C mutant in the presence of cold γ-ATP. The phosphorylated Akt1 was subjected to Western analysis using antibodies specific to phospho-Akt1 (S473). (B) Graphical representation of the Akt1 (S473) phosphorylation level. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); **: $p < 0.005$. (C) Bovine MBP was incubated with recombinant LRRK2 in the presence of radiolabeled ATP. ^{32}P-Labeled proteins in the reaction mixture were detected by autoradiography following SDS–PAGE. Relative LRRK2 kinase activities to that of WT LRRK2 are shown. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); **: $p < 0.005$. (D) Recombinant GST-tagged wild-type (WT), G2019S, I2020T, or R1441C LRRK2 was mixed with the recombinant His-Akt1 and subjected to GST pull-down assay by incubation with glutathione agarose beads. The proteins bound to the beads were subjected to Western analysis using antibodies against non-phosphorylated Akt1 and GST. (E) Graphical representation of the bound Akt1 normalized to GST-LRRK2.

apoptosis [22,23]. Activation of Akt1 requires phosphorylation by various kinases such as 3′-phosphoinositide-dependent kinase-1 (PDK-1), mammalian target of rapamycin complex 2 (mTORC2), and integrin-linked kinase (ILK) [23,27]. To examine the possibility that the LRRK2-mediated protectivity against apoptosis involves Akt1 activation, recombinant human Akt1 (His-tagged protein) was subjected to in vitro [γ-^{32}P]ATP phosphorylation assay with recombinant human LRRK2 (GST-tagged wild-type protein consisting of all the functional domains in the stretch incorporating amino acid residues 970–2527). As reported previously, both Akt1 and LRRK2 exhibited a significant level of autophosphorylation (Fig. 1A, lanes 1 and 3). We found that LRRK2 directly and effectively phosphorylated the heat-denatured (i.e., autophosphorylation-disrupted) Akt1 molecule (Fig. 1A, lane 6). On the other hand, Akt1 did not phosphorylate heat-denatured LRRK2 (Fig. 1A, lane 4), indicating that Akt1 is the downstream substrate of LRRK2. Curiously, heat-inactivated LRRK2 appeared to promote Akt1 phosphorylation, although the extent of which was significantly lower than that mediated by active LRRK2. The GST-pull down assay confirmed the direct interaction between LRRK2 and Akt1 (Fig. 1B).

3.2. LRRK2 phosphorylates Akt1 at Ser473

The phosphorylation of Thr308 by PDK-1 and phosphorylation of Ser473 by mTORC2 and ILK are well known to be involved in

the activation of Akt1 [23,27]. To clarify whether LRRK2 phosphorylates these sites, the recombinant Akt1 that had been phosphorylated by recombinant LRRK2 was subjected to Western analysis using antibodies specific to each Akt1-phosphoamino acid. As shown in Fig. 2, LRRK2 scarcely phosphorylated the heat-denatured (i.e., autophosphorylation-disrupted) Akt1 at Thr308, i.e., at a degree of phosphorylation much weaker than that of autophosphorylation. On the other hand, LRRK2 effectively phosphorylated the heat-denatured Akt1 at Ser473 [about 7.3-fold of autophosphorylation in the phosphorylation level (phospho-Akt1/total Akt1)].

3.3. Knockdown of LRRK2 diminishes Akt1 phosphorylation in intact cells

To confirm the kinase activity of LRRK2 on Akt1 in intact cells, we next performed a LRRK2-knockdown experiment using neuroblastoma SH-SY5Y cells. As shown in Fig. 3, transfection of LRRK2-specific RNAi into the cells reduced the level of LRRK2 protein to 46% in comparison with the use of an RNAi control. The knockdown of LRRK2 resulted in a marked reduction of phospho-Akt1 (Ser473), whereas it had no influence on the total level of Akt1 protein. Consequently, the phosphorylation level of Akt1 [phospho-Akt1 (Ser473)/total Akt1] in the cells with LRRK2-knockdown was reduced to 51% in comparison with that in the RNAi control cells. The LRRK2-knockdown also diminished the phosphorylation of

4E-BP1, a reported LRRK2 substrate [16], but to a lesser degree than that observed for Akt1 (71% in comparison with the control RNAi).

We also analyzed a SH-SY5Y clone stably and uniformly over-expressing the full-length LRRK2 [25,26]. This clone exhibited 3.4-fold higher Akt1 (Ser473) phosphorylation than the vector-control clone (Fig. 4). The knockdown of the overexpressed LRRK2 in this clone (46% in comparison with the control RNAi) resulted in a marked reduction of the phosphorylation level of Akt1 [phospho-Akt1 (Ser473)/total Akt1] (55% in comparison with the control RNAi). Altogether, these results indicated that LRRK2 indeed phosphorylated Akt1 at Ser473 in intact cells.

3.4. Mutant LRRK2 exhibits reduced Akt1-phosphorylation activity than wild-type LRRK2

Finally, we examined the influence of disease-associated LRRK2 mutations on its kinase activity toward Akt1. The recombinant Akt1 was phosphorylated by the recombinant LRRK2 with the three known mutations, i.e., R1441C in the ROC domain, G2019S in the kinase domain, and I2020T in the kinase domain. We found that the three LRRK2 mutants exhibited significantly weaker kinase activity of Akt1 (Ser473)-phosphorylation than the wild-type LRRK2 (Fig. 5A and B). When MBP was used as substrate, on the other hand, G2019S as well as I2020T mutant LRRK2 exhibited much stronger kinase activity than the wild-type LRRK2, in accordance with several other reports [6] (Fig. 5C). The pull-down assay using the three LRRK2 mutants showed that the reduced kinase activity toward Akt1 (Ser473) was ascribable to impaired ability to bind Akt1, particularly in the case of G2019S LRRK2 (Fig. 5D and E).

4. Discussion

LRRK2 is postulated to be a key molecule in the etiology of both familial and sporadic PD. However, the molecular mechanism whereby mutant LRRK2 causes loss of dopaminergic neurons has not yet been confirmed. This is due to the lack of consensus about the true physiological substrate or true function of LRRK2. Here, for the first time, we demonstrated that LRRK2 interacts with and phosphorylates Akt1, a central molecule that transduces signals necessary for cell survival. In vitro pull-down and kinase assays using recombinant proteins proved that Akt1 is the direct downstream substrate of LRRK2. LRRK2-mediated phosphorylation of Akt1 was also demonstrated in intact cells, suggesting that Akt1 is a convincing candidate for the physiological substrate of LRRK2.

Like mTORC2 and ILK [23,27], LRRK2 phosphorylated Akt1 at Ser473, one of the two amino acids essential for Akt1activation. Therefore, it is possible that LRRK2 may activate Akt1 in concert with PDK-1, which phosphorylates another essential amino acid, Thr308, in a manner that is dependent on phosphatidylinositol-3,4,5-triphosphate [23]. The serine is rather unique as a target site for LRRK2, which has previously been thought to preferentially phosphorylate Thr rather than Ser, based on the phosphorylation sites of known substrates [12,15,28]. The amino acid sequence surrounding Ser473 is also distinct from those reported as the LRRK2 consensus phosphorylation motif [12,28].

Once activated, Akt1 phosphorylates and negatively regulates a wide variety of apoptosis-associated molecules such as Bad, Bax, Bim, caspase-9, and forkhead box factor FoxO1. In addition, Akt1 activates several signal-transduction cascades for cell survival, which are mediated by ASK-1, GSK-3β, or NFκB [23,27,29–32]. Notably, LRRK2 reportedly activates NFκB, although any involvement of Akt1 has not been investigated [18,20,33,34]. We have reported previously that LRRK2 has an ability to protect cells against

H_2O_2-induced apoptosis, and that knockdown of LRRK2 abrogates this ability [26]. It is possible that the cell-protective ability of LRRK2 is exerted through phosphorylation and activation of Akt1, which in turn activates one or several of the downstream signal cascades described above. On the other hand, there has been some controversy as to whether LRRK2 is protective or toxic for cells [14,26,33,35–37]. LRRK2 has been reported to activate FoxO1 [38] in Drosophila, but in this case resulting in elevated expression of Bim and neural cell death. The LRRK2-mediated phosphorylation of Akt1 might transduce different downstream signals depending on the source of the cells, growth conditions, and presence of oxidative stress or other factors.

It is noteworthy that all the disease-associated mutations analyzed here, i.e., R1441C, G2019S, and I2020T, reduced both interaction with, and phosphorylation of, Akt1. In particular, G2019S LRRK2, which exerts hyperkinase activity toward most known substrates, showed diminished kinase activity toward Akt1 (Ser473), being ascribable to impaired Akt1-binding ability. Our results suggest that neurons expressing the mutant LRRK2 may exhibit lower resistance to apoptosis, due to lower phosphorylation and activation of Akt1, than those expressing the wild-type LRRK2. Furthermore, a transfection experiment using a combination of wild-type and mutant LRRK2 revealed that a mutation at one of the two LRRK2 alleles was enough to explain the significant reduction in both Akt1 phosphorylation and resistance to apoptosis (unpublished data), suggesting one possible mechanism for neurodegeneration in the dominant-heredity form of PD, PARK8.

Acknowledgments

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