GTP Binding Is Essential to the Protein Kinase Activity of LRRK2, a Causative Gene Product for Familial Parkinson's Disease[†]

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ABSTRACT: Leucine-rich repeat kinase 2 (LRRK2), a product of a causative gene for the autosomal-dominant form of familial Parkinson's disease (PARK8), harbors a Ras-like small GTP binding protein-like (ROC) domain besides the kinase domain, although the relationship between these two functional domains remains elusive. Here we show by thin-layer chromatographic analysis that LRRK2 stably binds GTP but lacks a GTPase activity in HEK293 and Neuro-2a cells. A ROC domain mutation that converts LRRK2 to a guanine nucleotide-free form (T1348N) abolishes the kinase activity of LRRK2 as well as its phosphate incorporation upon metabolic labeling. The phosphorylation of LRRK2 was inhibited by potential inhibitors for cyclic AMP-dependent protein kinase. These data suggest that binding of GTP to the ROC domain regulates the kinase activity of LRRK2 as well as its phosphorylation by other kinase(s).

Parkinson's disease (PD) is one of the most common neurodegenerative disorders of adulthood affecting the extrapyramidal motor system (*1*). The neuropathological changes of patients with PD are characterized by neuronal loss of the brainstem monoaminergic neurons, e.g., dopaminergic neurons in the substantia nigra or noradrenergic neurons in the locus caeruleus, accompanied by formation of Lewy bodies in remaining neurons (*2*). Lewy bodies are a hallmark lesion of PD that are comprised of α-synuclein and other proteins (*3*, *4*). Most PD patients develop the disease in a sporadic fashion, whereas a subset of patients inherit PD as an autosomal dominant or recessive traits [familial PD (FPD)] (*5*). So far, at least five genes causative for FPD have been identified. Patients with missense mutations (PARK1) or multiplications (PARK4) of α-synuclein genes develop typical PD or dementia with Lewy bodies in an autosomal-dominant fashion, underscoring the significance of α-synuclein deposition (e.g., Lewy bodies) as the cause of PD and related conditions. Parkin, a ubiquitin ligase (PARK2), PINK1, a putative mitochondrial serine/threonine kinase (PARK6), and DJ-1, a multifunctional protein with antioxidative or chaperone activities (PARK7), have also been discovered in early-onset, recessive forms of FPD, implicating abnormalities in proteolysis, mitochondrial function, protein phosphorylation, and oxidative stress, respectively, in nigral degeneration in PD (*5*). In 2004, leucine-rich repeat kinase 2 (LRRK2[1])/dardarin was cloned as the causative gene for PARK8, an adult-onset, autosomal-dominant form of FPD (*6*, *7*). More than 10 missense mutations have been found in the LRRK2 gene in ∼7% of FPD families (*8*), as well as in sporadic PD cases of some ethnic populations (*9*), leading us to consider LRRK2 mutations as a major genetic factor involved in PD. PARK8 patients with mutations of the LRRK2 gene exhibit clinical manifestations of typical PD in adulthood, and pathologically, a somewhat pleomorphic feature in nigral degeneration frequently associated with Lewy bodies and α-synuclein deposition, including cases with pure nigral degeneration without fibrous protein deposits or with tau-positive inclusions, characterizes the disease (*7*). Collectively, analysis of the pathomechanism by which mutations in the LRRK2 gene cause nigral degeneration should provide important clues about the pathogenesis of familial and sporadic PD.

LRRK2 is a large cytoplasmic protein comprised of 2527 amino acids, harboring several putative functional domains: leucine-rich repeat and WD40 repeat motifs involved in protein−protein interaction, ROC (Ras of complex proteins) domain reminiscent of small GTP-binding proteins like Ras, and a kinase domain with a high degree of homology to MAP kinase kinase kinases (MAPKKK) (*6*, *7*). A family of proteins with a similar set of functional domains have been called the ROCO protein family, which are phylogenetically conserved from slime molds to mammals, although their functions are totally unknown (*10*). Recently, LRRK2 has been shown to exhibit an autophosphorylation activity, which

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[1] Abbreviations: HEK, human embryonic kidney; LRRK2, leucine-rich repeat kinase 2; MBP, myelin basic protein; ROC, Ras of complex proteins; TLC, thin-layer chromatography; wt, wild-type

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seems to be upregulated by FPD mutations (e.g., G2019S or I2020T) (*11*, *12*); LRRK2 has been shown to bind to parkin, and expression of FPD mutants induces cell death (*13*). Also, the toxic effect of LRRK2 has been shown to be dependent on its kinase activity (*14*, *15*). However, a couple of important questions remain, e.g., whether LRRK2 binds GTP or GDP to its ROC domain and the functional relationships between the ROC domain and kinase activity.

Here we show that LRRK2 binds GTP but appears to lack a GTPase activity in HEK293 and Neuro-2a cells and that binding of GTP to the ROC domain is essential to the kinase activity of LRRK2 as well as to the phosphorylation of LRRK2 by some upstream kinase(s). These data would also contribute to the understanding of the novel mode of regulation of protein kinase activity of LRRK2, a multidomain protein that harbors a Ras-like domain and a kinase domain within a single molecule.

MATERIALS AND METHODS

cDNA Cloning and Construction of Expression Plasmids. A cDNA encoding full-length human LRRK2 was cloned in four fragments. The most amino-terminal and carboxyl-terminal fragments were amplified from a human brain cDNA library (TaKaRa) by PCR, and the middle two fragments were amplified from the cDNA clones (GenBank accession numbers AK127729 and AK131537) provided by the National Institute of Technology and Evaluation (Tokyo, Japan). The full-length sequence was generated by subsequent ligation of these fragments into the pBluescriptII KS+ vector (Stratagene). Plasmids encoding amino-terminally 3xFLAG-tagged LRRK2 were generated by insertion of full-length LRRK2 into the p3xFLAG-CMV-10 expression vector (Sigma). Single-nucleotide substitution was introduced into a fragment less than 1.5 kb in size by a long PCR protocol, and the following oligonucleotides were used as PCR primers: 5′-gtggctgtgatgatttttaataaac-3′ for K1906M, 5′-tgtgggaaatggtgggagtggta-3′ for T1343G, 5′-gagtggtaaaaacaccttactgcagcaatta-3′ for T1348N, 5′-gattttgcaggtcaagaggaattc-3′ for R1398Q, and 5′-catcagagggcgcaccagggtttc-3′ for T2035A as forward primers and the corresponding complementary sequences as reverse primers. Mutated fragments were subsequently ligated into the full length of sequence by appropriate digestion and ligation. All constructs generated from PCR products were verified by DNA sequencing. cDNAs encoding wild-type human FLAG-*H*-Ras were generated as previously described (*16*).

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells and murine neuroblastoma Neuro-2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. Transient expression in HEK293 and Neuro-2a cells was performed by transfecting the plasmids using FuGENE6 (Roche) and Lipofectamine2000 (Invitrogen), respectively, according to the manufacturer's instructions.

Antibodies and Immunochemical Analysis. A polyclonal antibody against the carboxyl terminus of human LRRK2 (residues 2500−2527) was purchased from NOVUS Biologicals (NB300-268). Anti-FLAG M2 antibody was purchased from Sigma. For immunoprecipitation, 48 h after transfection, transfected cells were lysed in lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 1 mM orthovanadate, 20 mM $MgCl_2$, and protease inhibitor cocktail complete EDTA-free (Roche) (pH 7.4)] for 30 min at 4 °C. Anti-FLAG M2 antibody and protein G-Sepharose 4 Fast Flow (GE Healthcare) were added to the lysates after a preclearing with protein G-Sepharose in the absence of antibodies, and the mixture was incubated for 2 h at 4 °C. Precipitated immunocomplexes were then washed in lysis buffer three times and subjected to immunoblot analysis or in vitro assays. For immunoblot analysis, precipitated proteins were solubilized by boiling in SDS−PAGE sample buffer containing 2% SDS and analyzed by immunoblotting as previously described (*17*).

In Vitro Kinase Assay. Forty-eight hours after transfection, transfected LRRK2 proteins were immunoprecipitated as described above, and the resulting immunocomplexes were resuspended in 20 μL of an assay buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM sodium orthovanadate, 50 μM ATP, and 3 μCi of [γ-^{32}P]ATP (GE Healthcare)]. For the kinase assay, immunoprecipitates of LRRK2 were incubated with 8 μg of dephosphorylated myelin basic protein (Upstate) in the assay buffer for 15 min at 30 °C, and the reaction was stopped by addition of SDS−PAGE sample buffer and boiling. Samples were separated by SDS−PAGE, transferred to a PVDF membrane (Millipore), and subjected to immunoblotting analysis. The same membrane was then subjected to autoradiography and analyzed with a BAS-1800 image analyzer (FujiFilm). To examine in vitro phosphorylation of LRRK2 by cAMP-dependent protein kinase (PKA), the immunoprecipitated K1906M mutant form of LRRK2 was incubated in 20 μL of PKA assay buffer [50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$] containing 2500 units of the purified PKA catalytic subunit (New England Biolabs) and 3 μCi of [γ-^{32}P]-ATP at 30 °C for 15 min. Resultant samples were processed as described above.

Identification of Bound Nucleotides by Thin-Layer Chromatography. Guanine nucleotides bound to the GTP-binding proteins were analyzed as previously described (*16*). Briefly, the cells were cultured for 48 h after transfection and were metabolically labeled with [^{32}P]orthophosphate (1.85 MBq/dish; Perkin-Elmer) in phosphate-free DMEM (Invitrogen) for 4 h. The labeled cells were lysed in an ice-cold lysis buffer, and transfected proteins were immunoprecipitated as described above. After an extensive wash of the immunocomplexes, associated nucleotides were eluted by incubation of the beads in elution buffer [20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 2% SDS, 1 mM GTP, and 1 mM GDP] at 68 °C for 10 min and then separated by thin-layer chromatography (TLC) (Merck) and quantified with a BAS-1800 image analyzer (FujiFilm).

GTPase Activity Assay. Measurement of GTPase activity was conducted essentially as previously described (*18*). Briefly, immunoprecipitated 3xFLAG-LRRK2 was incubated with 5 μCi (33 nM) of [α-^{32}P]GTP (GE Healthcare) in loading buffer [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 0.1 mM EGTA, 10 μM ATP, and 0.1 mM DTT] at 4 °C for 30 min. After incubation, the beads were washed three times in wash buffer [50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 1 mM orthovanadate, and 20 mM $MgCl_2$ (pH 7.4)]. Hydrolysis was carried out at 30 °C for 30 min and stopped by addition of ice-cold wash buffer followed by

repeated washes in the same buffer. Bound nucleotides were eluted and separated by TLC and detected with a BAS-1800 image analyzer.

Guanine Nucleotide Competition Assay. Immunoprecipitated LRRK2 was loaded with [α-^{32}P]GTP in vitro in loading buffer containing various concentrations of guanine nucleotides (i.e., GTP or GDP) as competitors. After incubation, bound nucleotides were eluted and analyzed by TLC as described above.

Estimation of the Stoichiometry of Binding of GTP to LRRK2. Immunoprecipitated LRRK2 was divided into two pools, to analyze bound nucleotides by TLC and to estimate the amount of LRRK2 protein by immunoblotting. One pool was loaded with [α-^{32}P]GTP in vitro in loading buffer (∼444000 dpm/pmol). After incubation, bound nucleotides were eluted and separated by TLC. The spot corresponding to GTP was cut out and analyzed using a liquid scintillation counter, and the amount of GTP was calculated. The other pool was subjected to immunoblotting analysis. Purified 3xFLAG-tagged BAP (amino-terminally 3xFLAG-tagged bacterial alkaline phosphatase; Sigma) was used as a 3xFLAG-tagged protein standard for quantification of the LRRK2 proteins. The blotted membrane was probed with anti-FLAG M2 antibody, and the amount of LRRK2 proteins was estimated within the range where the linear correlations between the band intensity and the amount of proteins were observed.

Detection of in Vivo Phosphorylation of LRRK2. Immunoprecipitated LRRK2 from transfected and metabolically labeled 293 cells was separated by SDS−PAGE, transferred to a PVDF membrane, and subjected to immunoblotting analysis. The same membrane was then subjected to autoradiography and analyzed with a BAS-1800 image analyzer. For experiments involving kinase inhibitors H-89 (Upstate) and KT5720 (Calbiochem), inhibitors were added at the same time that the [^{32}P]orthophosphate labeling was conducted and were thus present throughout the labeling period. Since both inhibitors were dissolved in dimethyl sulfoxide (DMSO), an equal amount of DMSO was added to the labeling medium of "non-treat" control samples to exclude any effect of DMSO itself.

RESULTS

Kinase Activity of LRRK2. To establish a functional assay for LRRK2, we constructed expression plasmids encoding full-length wild-type (wt) LRRK2 tagged with 3xFLAG at the amino terminus (3xFLAG-LRRK2) as well as some mutant forms (Figure 1A). We transiently transfected human embryonic kidney (HEK) 293 cells with these plasmids, immunoprecipitated LRRK2 using an anti-FLAG antibody, and subjected the immunoprecipitates to an in vitro kinase assay. Full-length wt LRRK2, but not the kinase-dead (K1906M) mutant form of LRRK2 whose invariant lysine residue required for ATP binding was replaced with methionine, phosphorylated myelin basic protein (MBP) as a recombinant substrate in vitro as well as LRRK2 itself, as reported previously (*11*, *12*, *14*) (Figure 1B). We then searched for putative phosphorylation sites of LRRK2 that are required for the kinase activity of LRRK2 and found a well-conserved threonine residue (Thr2035) in the activation loop (subdomain VIII); this threonine is reported to be phosphorylated and essential to the activity in some MAP-KKK family members (*19*−*22*). When we replaced Thr2035 with alanine (i.e., T2035A; Figure 1A), the kinase activity was completely abolished as in the K1906M mutant (Figure 1B). Collectively, these data strongly suggested that LRRK2 functions as a kinase with well-conserved canonical subdomain structures.

GTP Binding Activity of Wild-Type LRRK2. LRRK2 contains a Ras-like putative GTP-binding domain (Figure 1A), although its ability to bind guanine nucleotides has not been elucidated. We examined the binding nucleotides of LRRK2 immunoprecipitated from cells overexpressing human LRRK2 by thin-layer chromatography (TLC). We first transiently transfected HEK293 cells with wt 3xFLAG-LRRK2 as well as amino-terminally FLAG-tagged wt H-Ras (FLAG-Ras) and metabolically labeled them with [^{32}P]-orthophosphate. After immunoprecipitation of transfected proteins with an anti-FLAG antibody, bound nucleotides were eluted and separated by TLC. We found that GTP, but not GDP, was bound to 3xFLAG-LRRK2, whereas both GTP and GDP were bound to FLAG-Ras, suggesting that transfected 3xFLAG-LRRK2 existed predominantly as a GTP-bound form and does not harbor a GTPase activity in vivo in HEK293 cells (Figure 2A). To rule out the possibility that LRRK2 requires some neuronal cell-specific factor (e.g., GTPase activating protein) for its hydrolytic activity for GTP, we examined the binding nucleotides of LRRK2 in Neuro-2a cells and confirmed that LRRK2 binds GTP but not GDP also in Neuro-2a cells (Figure 2A). To test whether immunoprecipitated 3xFLAG-LRRK2 has the potential to bind GTP in vitro, we next incubated immunoprecipitated 3xFLAG-LRRK2 from HEK293 cells with [α-^{32}P]GTP and analyzed the bound nucleotides via TLC. [α-^{32}P]GTP was eluted from 3xFLAG-LRRK2 (Figure 2B, left panel) as well as from FLAG-Ras (data not shown), confirming the GTP binding capacity of LRRK2. Furthermore, the levels of radioactive [α-^{32}P]GTP bound to the immunoprecipitates were reduced by addition of an increasing amount of unlabeled GTP as well as GDP to the incubation mixture (Figure 2B,C), suggesting that LRRK2 harbors an intrinsic affinity also for GDP. Quantitative analyses showed that the percentage of LRRK2 which bound GTP in vitro was 3.5 ± 0.8% (Figure S1 of the Supporting Information).

GTPase Activity of LRRK2. A subset of small GTP binding proteins (e.g., Di-Ras) have been known to lack GTPase activity and persist as GTP-bound forms (*18*, *23*), whereas a number of Ras-like GTPases exist mainly as GDP-bound forms. Thus, the selectivity of the bound nucleotide (i.e., GTP or GDP) for the small G proteins in the resting state is partly attributed to their GTPase activity. Since LRRK2 existed as a GTP-bound form as shown above, we examined whether LRRK2 lacks GTPase activity. We loaded [α-^{32}P]-GTP onto immunoprecipitates from HEK293 cells transfected with wt 3xFLAG-LRRK2 or wt FLAG-Ras in vitro, incubated them at 30 °C, and found that LRRK2 did not produce GDP after incubation for 30 min whereas H-Ras produced a large amount of GDP at this time point (Figure 3A). Several mutations (e.g., G12V and Q61L) are known to convert H-Ras to a GTP-bound form and cause hyperactivation of its oncogenic activity, implicating these specific residues in the GTPase activity of H-Ras. This led us to speculate that the poor conservation of the homologous residues in LRRK2

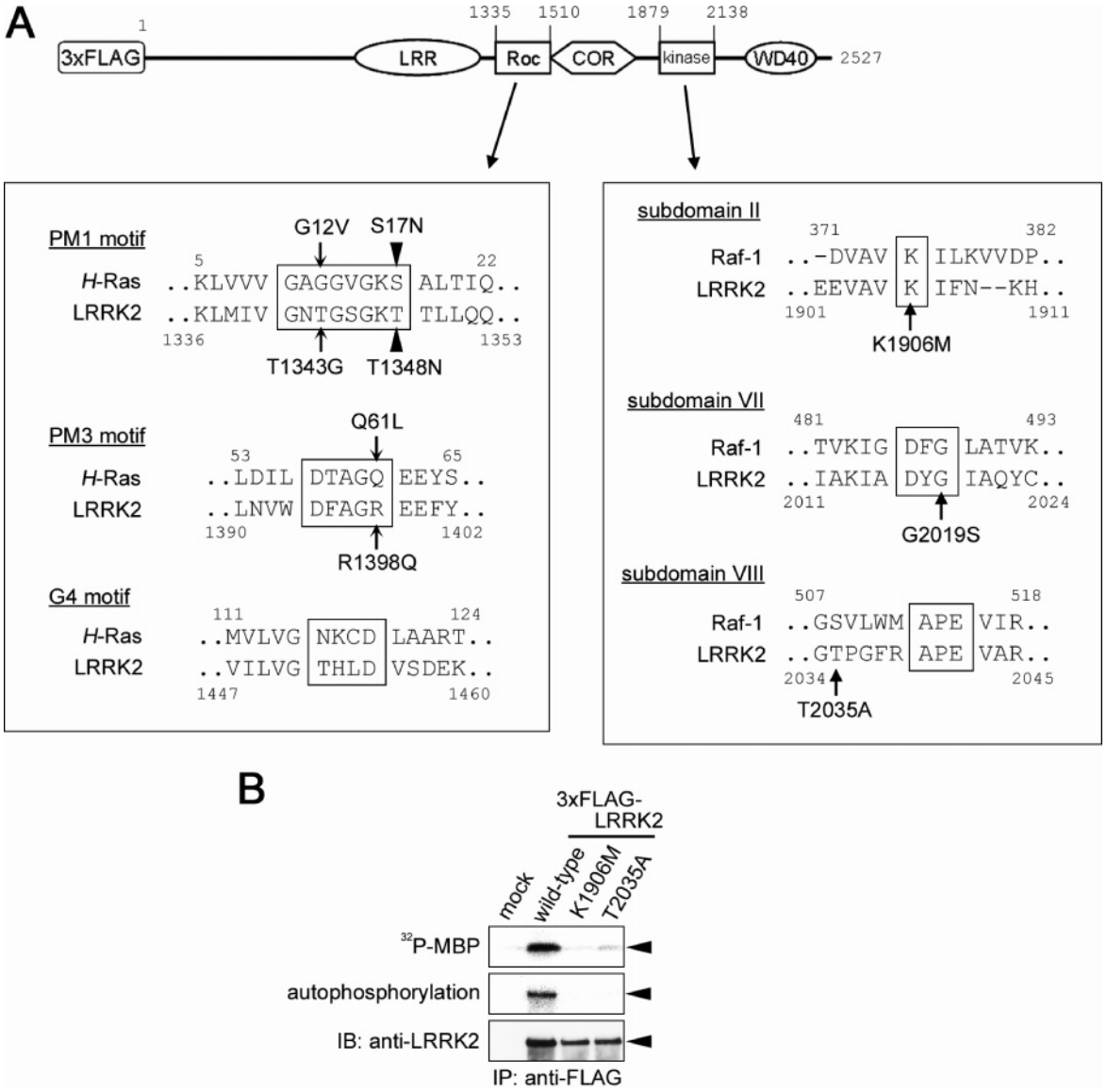

FIGURE 1: (A) Schematic depiction of the domain structure of amino-terminally 3xFLAG-tagged full-length LRRK2 and amino acid substitutions used in this study. The bottom left panel shows the alignment of the conserved motifs in H-Ras (top row) and the ROC domain of LRRK2 (bottom row). Key residues, the mutations of which convert small GTP-binding proteins to the GTPase-inactive form (G12V and Q61L in H-Ras) or nucleotide-free/GDP-bound form (S17N in H-Ras and T1348N in LRRK2), are marked with arrows or arrowheads, respectively. The mutations generating the Ras-like form of LRRK2 (T1343G/R1398Q in LRRK2) are also marked with arrows. The bottom right panel shows the alignment of the canonical subdomains in Raf-1 (top row) and the kinase domain of LRRK2 (bottom row) into which an amino acid substitution was introduced in this study (arrow). (B) In vitro kinase assay of LRRK2. Wild-type, kinase-dead (K1906M), or T2035A mutant LRRK2 was immunoprecipitated from transfected HEK293 cells and subjected to an in vitro kinase assay using MBP as an exogenous substrate (top panel). The bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading. Results are representative of three independent experiments.

might underlie its lack of GTPase activity, keeping its ROC domain in a GTP-bound form (Figure 1A). To test this assumption, we generated the Ras-like form of LRRK2 by substituting Thr1343 and Arg1398 (corresponding to Gly12 and Gln61 in H-Ras, respectively) with Gly and Gln (TGRQ LRRK2) and examined the bound nucleotides after metabolic labeling of cells with [^{32}P]orthophosphate. When we calculated the percentage of the GTP-bound form of LRRK2 considering the number of labeled phosphates in GTP (three) and GDP (two) (see the legend of Figure 3B), we found that ~50% of the guanine nucleotides bound to TGRQ LRRK2 were GDP, presumably due to the recovered GTPase activity, whereas wt LRRK2 predominantly remained a GTP-bound form (Figure 3B). These results further supported the notion that LRRK2 lacks a GTPase activity and remains in a GTP-bound form in the steady state.

Requirement of GTP Binding for the Kinase Activity of LRRK2. It has been repeatedly shown that substitution of the conserved Ser/Thr in the PM1 motif [GxxxxGKS/T (Figure 1A)] of small GTP-binding proteins (e.g., S17N mutation in human H-Ras) dramatically reduces their affinity for guanine nucleotides, especially for GTP, converting them to GDP-restricted or nucleotide-free forms (*24*−*26*). Since this Thr residue is also conserved within the Roc domain of LRRK2 [Thr1348 (Figure 1A)], we replaced Thr1348 of LRRK2 with Asn (T1348N). When T1348N mutant LRRK2 was loaded with [α-^{32}P]GTP in vitro and bound nucleotides were analyzed by TLC, no nucleotide binding was observed

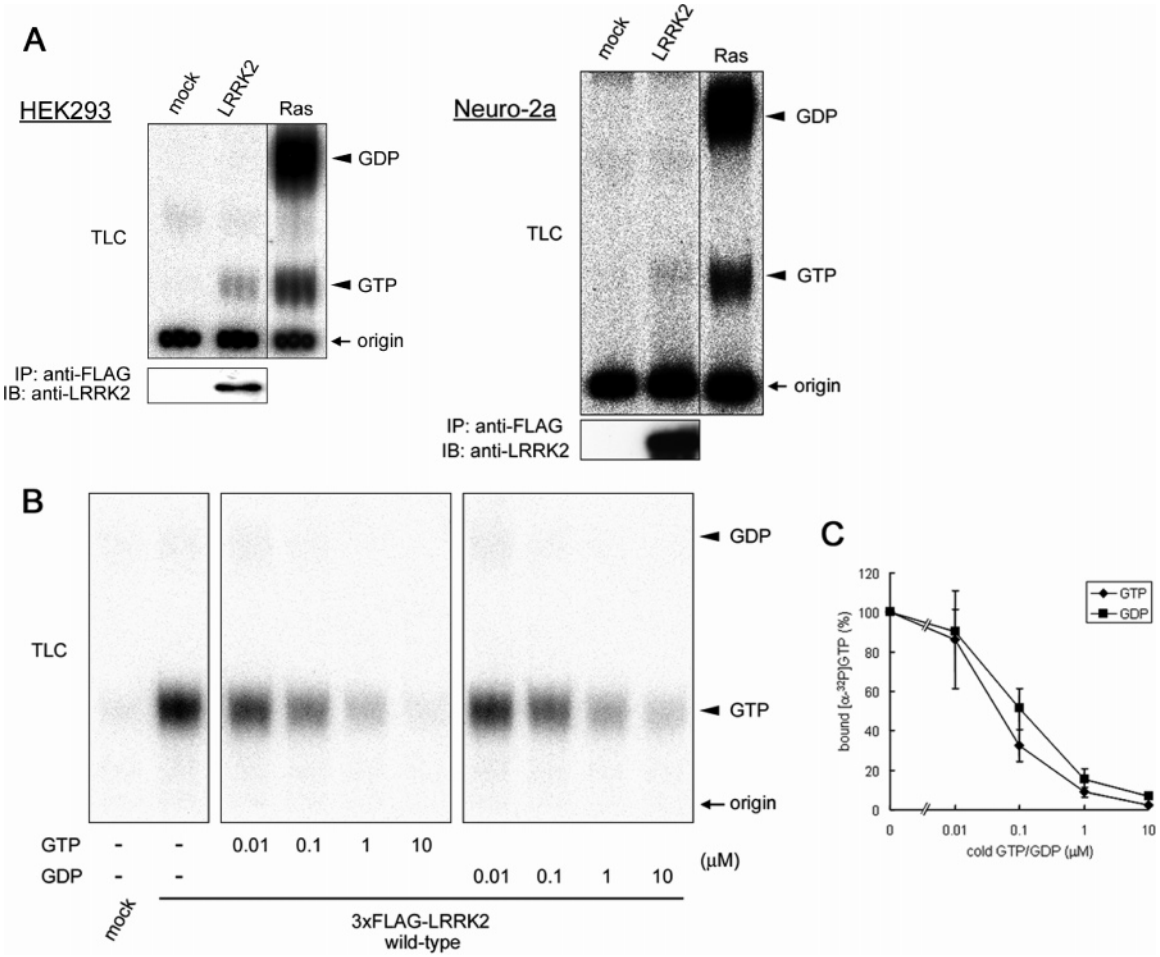

FIGURE 2: Analysis of binding of a guanine nucleotide to LRRK2. (A) Thin-layer chromatographic analysis of bound nucleotides of wild-type LRRK2. LRRK2 immunoprecipitated from transfected HEK293 cells (left) or Neuro-2a cells (right) metabolically labeled with [^{32}P]-orthophosphate was analyzed by TLC. The right-most lane shows TLC separation of bound GTP and GDP to FLAG-tagged H-Ras as a control, and the bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the successful expression and immunoprecipitation. mock means cells were transfected with an empty vector. (B) Displacement of bound [α-^{32}P]GTP with excessive cold GTP (middle) or GDP (right) analyzed by TLC. The concentrations of cold GTP or GDP (micromolar) added to the immunoprecipitates are shown at the bottom of the panels. Results are representative of at least three independent experiments. (C) Quantification of the signal intensity of [α-^{32}P]GTP on TLC analysis shown in panel B. The data are given as the percentage of those observed in the absence of cold GTP (◆) or GDP (■) ($n = 3$, mean ± standard error).

(Figure 4A). TLC analysis also revealed that neither GTP nor GDP was bound to T1348N mutant LRRK2 in vivo in HEK293 cells (Figure 4B), again supporting the notion that the Roc domain in LRRK2 binds GTP and that this affinity for GTP can be compromised by the PM1 motif mutation (i.e., T1348N), in a manner similar to those documented in other GTP-binding proteins (*24*−*26*). Since the coexistence of the GTP-binding domain and the kinase domain in a single molecule is the most characteristic feature of LRRK2, we aimed to clarify the relationship between these two domains. Importantly, the kinase-negative mutant form of LRRK2 (K1906M) that lacks kinase activity retained the capacity to bind GTP (Figure 4B), indicating that kinase activity is not required for the GTP binding activity. Conversely, T1348N mutant LRRK2 that does not bind GTP lacked kinase activities for phosphorylating MBP as examined in an in vitro kinase assay (Figure 4C), strongly suggesting that binding of GTP to the ROC domain is required for the kinase activity of LRRK2.

Phosphorylation of LRRK2 in Vivo in Cultured Cells. Phosphorylation by itself (i.e., autophosphorylation) or by other kinases is one of the typical mechanisms of activation of protein kinases (*20*). When we metabolically labeled the nucleotide pool of HEK293 cells with [^{32}P]orthophosphate, we were able to detect the incorporation of phosphates into wt 3xFLAG-LRRK2 immunoprecipitated from cells (Figure 5A). Since this phosphate incorporation was also observed in the K1906M kinase-negative mutant form of LRRK2, we strongly suggested that LRRK2 undergoes phosphorylation by other kinases in HEK293 cells. Notably, the T1348N nucleotide-free mutant form of LRRK2 did not incorporate any phosphates under the same experimental condition (Figure 5A), suggesting that phosphorylation of LRRK2 by other kinases also is dependent on its GTP binding at the ROC domain. Phosphate incorporation was observed in T2035A mutant LRRK2, which may suggest the presence of multiple phosphorylation sites in LRRK2.

Involvement of cAMP-Dependent Protein Kinase-like Activity in the Phosphorylation of LRRK2. To identify the kinase responsible for the phosphorylation of LRRK2, we treated HEK293 cells with a set of well-characterized kinase inhibitors during the labeling period and found that H-89, a potent inhibitor of cAMP-dependent protein kinase (PKA) (*27*), significantly inhibited the incorporation of phosphate

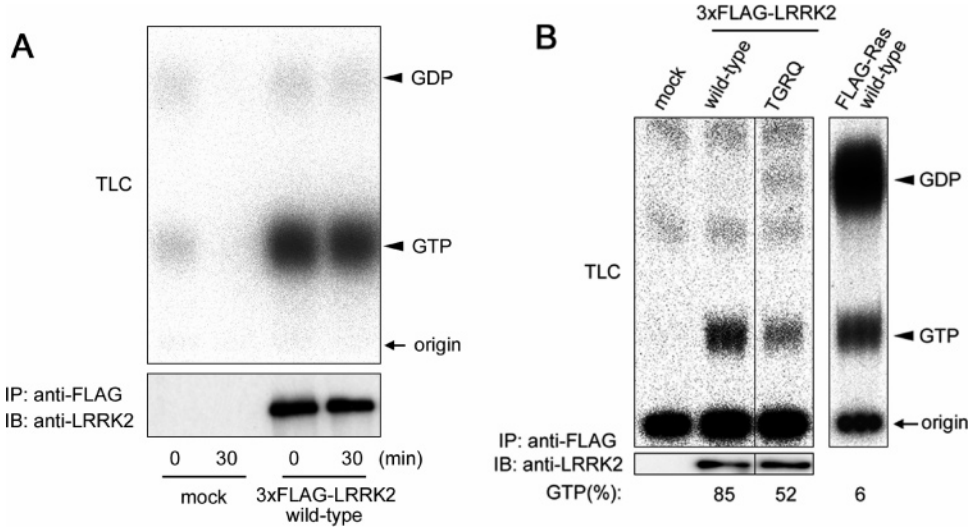

FIGURE 3: GTPase activity of LRRK2. (A) LRRK2 immunoprecipitated from HEK293 cells and loaded with [α-^{32}P]GTP in vitro was incubated for 30 min at 30 °C to elicit hydrolysis of bound GTP and then analyzed by TLC. The bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading. mock means cells were transfected with an empty vector. (B) Comparison of bound nucleotides between wild-type and TGRQ mutant LRRK2, and H-Ras. Relative densities of bands representing bound GTP [GTP(%)] to total (GTP + GDP × 1.5) are shown below the panels. Results are representative of three independent experiments.

into LRRK2 in a dose-dependent manner (Figure 5B). KT5720, another potent inhibitor of PKA (*28*), also inhibited the phosphorylation of LRRK2 (Figure 5B). Furthermore, purified PKA efficiently phosphorylated the K1906M kinase-inactive mutant of LRRK2 immunoprecipitated from HEK293 cells in vitro (Figure 5C). Collectively, these data suggested that PKA is one of the candidate protein kinases involved in the phosphorylation of LRRK2.

DISCUSSION

LRRK2 is mutated in a large number of patients with FPD linked to PARK8 as well as those with sporadic PD, although the biochemical properties of LRRK2 in relation to its small GTP binding protein-like (ROC) domain have not been extensively studied. In this study, we have shown that (i) LRRK2 is constitutively bound to GTP but lacks a GTPase activity in non-neuronal (HEK293) and neuronal (Neuro-2a) cells, (ii) GTP binding is required for the kinase activity as well as phosphorylation of LRRK2, and (iii) PKA might be involved in the phosphorylation of LRRK2.

Both unlabeled GTP or GDP competed for binding of [α-^{32}P]GTP to LRRK2 immunoprecipitated from cells in vitro (Figure 2B,C), leading us to conclude that LRRK2 has an intrinsic affinity for both GTP and GDP. It has recently been shown that human LRRK1 binds GTP (*29*), with which GDP can compete, suggesting that the intrinsic affinity for both GTP and GDP is a common feature of ROCO proteins, including LRRK1 and LRRK2. However, whether the GDP-bound form of LRRK2 exists under a physiological condition in cells remains unknown, because only the GTP-bound form of LRRK2 was detected upon TLC analysis of cells metabolically labeled with [^{32}P]orthophosphate. The percentage of LRRK2 which binds GTP was estimated to be ~3.5% under our experimental conditions (Figure S1 of the Supporting Information). This relatively low binding stoichiometry compared to those with authentic small GTP-binding proteins overexpressed in cells might be attributed to the small proportion of properly folded LRRK2 that comprises the total amount of immunoprecipitated LRRK2 because of its unusually large size as a GTP-binding protein, as well as its strong propensity to aggregate upon overexpression (*13*, *14*). Moreover, specific binding of GTP to wt LRRK2 was assured by the fact that T1348N mutant LRRK2 failed to bind GTP under the same experimental condition (Figure 4A).

We further showed that wt LRRK2 existed predominantly as a GTP-bound form in cultured cells, suggesting that LRRK2 is unable to hydrolyze GTP into GDP in cultured cells, whereas conversion of LRRK2 to the Ras-like form by substitution of two amino acid residues (TGRQ) restored its GTPase activity, as judged by the emergence of a GDP-bound form of LRRK2 (Figure 3B). The lack of in vitro GTP hydrolysis by wt LRRK2 after incubation for 30 min also supports this speculation (Figure 3A). Since the endogenous level of GTP is much higher than that of GDP (*30*), it is not likely that the GDP-bound form of LRRK2 (TGRQ) was generated by direct binding of GDP to LRRK2, but by a GTPase activity. Taken together, LRRK2 appears to lack a GTPase activity like a subset of small GTP-binding proteins (e.g., Di-Ras). However, it is possible that some GTPase-activating protein (GAP) associates with LRRK2 upon specific stimulation or in a specific type of cells in vivo and elicits its GTPase activity, as observed with a number of small GTP-binding proteins.

Upon binding of GTP, small GTP-binding proteins dramatically convert their conformation and activate downstream effectors by means of physical interaction. For example, Raf, a downstream kinase of Ras, is known to require an interaction with the GTP-bound form of Ras to elicit its kinase activity and activate ERK (*31*). Our finding that LRRK2 possesses a GTP binding activity together with a kinase activity prompted us to hypothesize that the kinase activity of LRRK2 is regulated by GTP binding. Analysis of a mutant form of LRRK2 homologous to the dominant-negative form of Ras (T1348N) clearly showed that binding of GTP to the Roc domain of LRRK2 is required for the

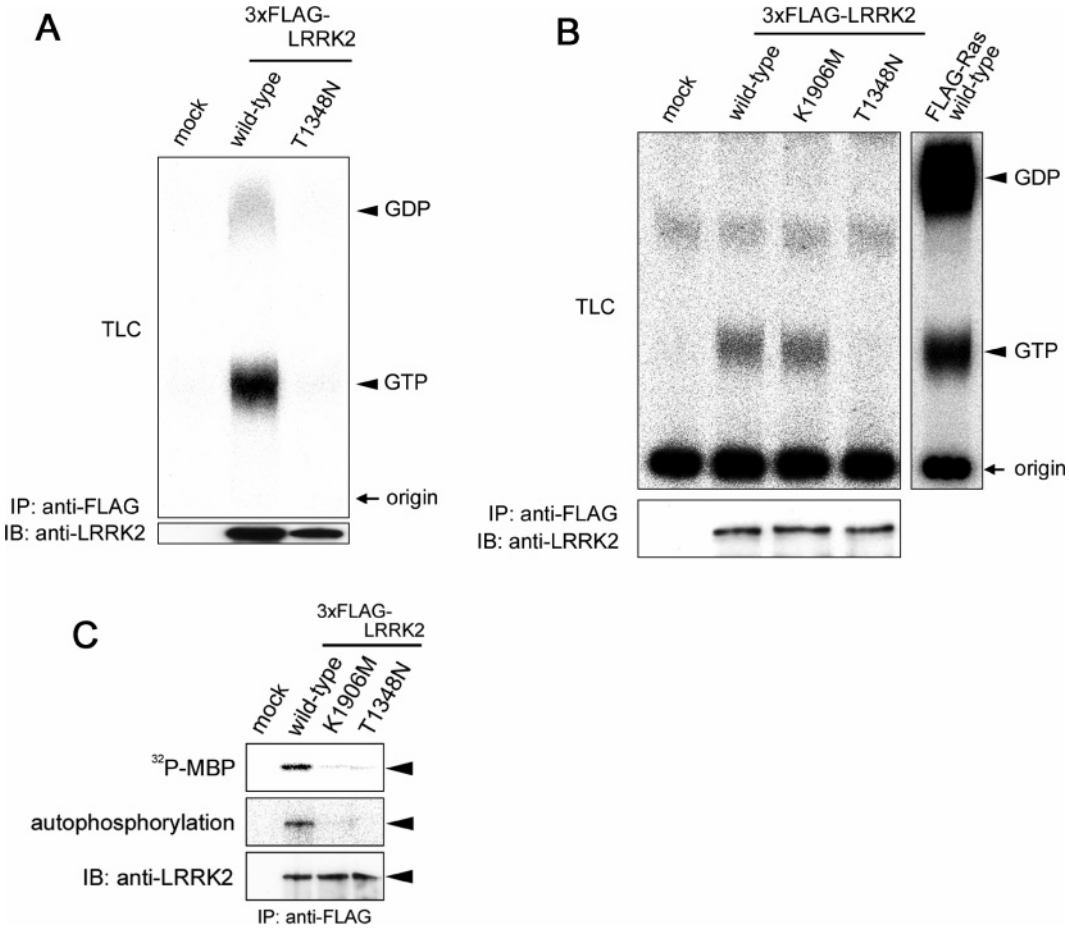

FIGURE 4: Effect of the T1348N mutation on GTP binding and kinase activity. (A) Wild-type and T1348N mutant LRRK2 were immunoprecipitated from HEK293 cells and loaded with [α-^{32}P]GTP in vitro, and bound nucleotides were analyzed by TLC. The bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading. mock means cells were transfected with an empty vector. (B) Thin-layer chromatographic analysis of bound nucleotides of wild-type, kinase-dead (K1906M), and T1348N mutants of LRRK2. LRRK2 immunoprecipitated from transfected HEK293 cells metabolically labeled with [^{32}P]orthophosphate was analyzed by TLC. The right panel shows the TLC separation of GTP and GDP bound to FLAG-tagged H-Ras as a control, and the bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading. mock means cells were transfected with an empty vector. (B) In vitro kinase activity of LRRK2. Wild-type, kinase-dead (K1906M), or T1348N nucleotide-free mutant LRRK2 was immunoprecipitated from transfected HEK293 cells and subjected to an in vitro kinase assay for analysis of phosphorylation of MBP (top panel) or autophosphorylation (middle panel). The bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading. Results are representative of three independent experiments.

kinase activity of LRRK2. This observation is in good agreement with the recent finding by Smith and colleagues, showing that another ROC domain mutant LRRK2, i.e., K1347A that abolished GTP-sepharose binding, also lacked autophosphorylation (*15*). Furthermore, we showed by metabolic labeling by [^{32}P]orthophosphate that wt LRRK2 undergoes phosphorylation, which is completely absent in T1348N mutant LRRK2, suggesting that phosphorylation of LRRK2 also requires GTP binding. The finding that the kinase-negative mutant form of LRRK2 (K1906M) bound GTP (Figure 4B) and was phosphorylated to an extent similar to that of wt LRRK2 (Figure 5A) suggests that the activation of LRRK2 occurs downstream of GTP binding and phosphorylation, and that upstream kinase(s) other than LRRK2 may be involved in the phosphorylation of LRRK2. Our mutagenesis analysis suggested that the well-conserved Thr2035 in the activation loop may be one of the phosphorylation sites required for the LRRK2 activity. Also, an inhibitor study revealed that a kinase activity that phosphorylates LRRK2 in cultured cells is partially inhibited by two potent PKA inhibitors, H-89 and KT5720. Considering the relatively wide spectrum of kinases suppressed by these inhibitors (*32*, *33*), however, the in vivo kinase(s) that phosphorylates LRRK2 should further be determined by additional rigorous analyses, e.g., systematic inhibition of kinases by RNAi. Autophosphorylation, which may initially be triggered by phosphorylation by other kinases, is one of the typical mechanisms of activation of protein kinases, as demonstrated in apoptosis signal regulating kinase 1 (*20*). An exact identification of the phosphorylation sites of LRRK2 by other kinases (including PKA) or by itself (autophosphorylation) will be crucial to the elucidation of the relationship between phosphorylation of LRRK2 elicited by binding of GTP to the ROC domain and the kinase activity of LRRK2 (Figure 6).

In summary, we have shown that the GTP binding is essential to the protein kinase activity of LRRK2, possibly through phosphorylation by upstream kinase(s), although the precise regulatory mechanism of LRRK2 awaits further characterization, especially by rigorous in vitro studies using recombinant LRRK2. Our results suggest that LRRK2 is a constitutively active kinase, because self-inactivation would

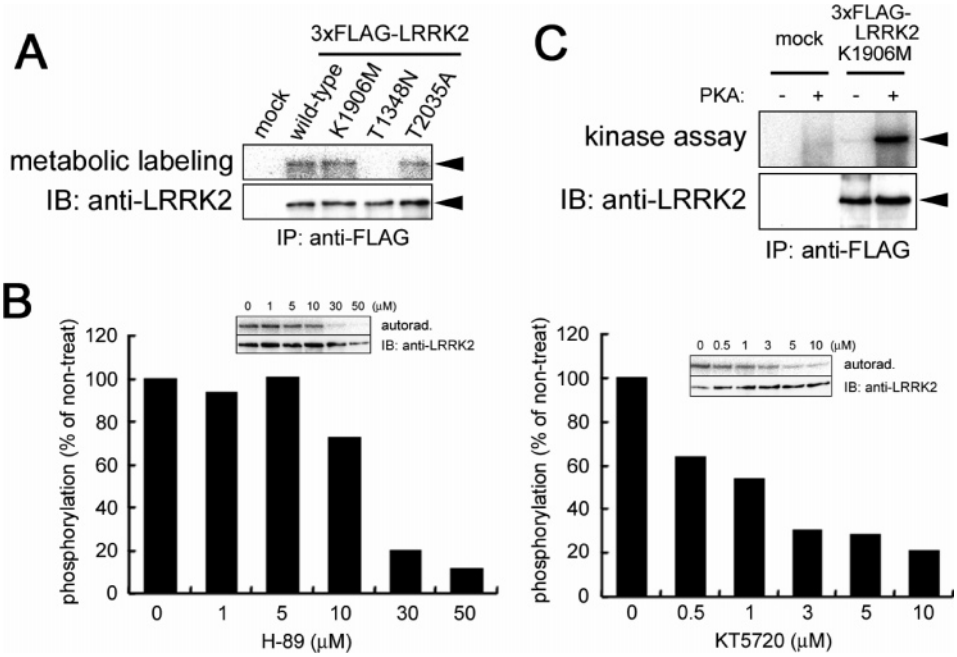

FIGURE 5: (A) Phosphorylation of LRRK2 as determined by autoradiographic analysis of immunoprecipitated LRRK2 after metabolic labeling of cells with [^{32}P]orthophosphate (top panel). The bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading. Results are representative of three independent experiments. (B) Dose-dependent inhibition of phosphorylation of LRRK2 by H-89 (left) and KT5720 (right). LRRK2 immunoprecipitated from transfected HEK293 cells metabolically labeled with [^{32}P]orthophosphate was analyzed by SDS−PAGE and immunoblotting followed by autoradiography (inset, top panel). H-89 and KT5720 were added into the labeling medium throughout the labeling period at the indicated concentration. The phosphorylation level of LRRK2 was quantified and normalized by the expression level determined by immunoblotting (inset, bottom panel). The experiment was carried out in duplicate, and the mean values are indicated as the percentage of those observed in solvent-only control. autorad means autoradiography and IB immunoblot (anti-LRRK2). (C) In vitro phosphorylation of LRRK2 by purified PKA. The immunoprecipitated K1906M mutant form of LRRK2 was subjected to an in vitro PKA assay according to the manufacturer's instructions. The bottom panel shows immunoblots of immunoprecipitated LRRK2 using anti-LRRK2 to show the equal level of loading.

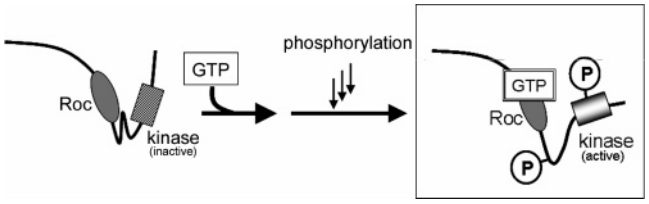

FIGURE 6: Hypothetical scheme of the regulation of kinase activity of LRRK2 through binding of GTP to the ROC domain and phosphorylation of LRRK2. P denotes possible phosphorylation.

be impossible due to a lack of GTPase activity. Accordingly, search for specific stimuli that activate or inactivate the kinase activity of LRRK2, as well as identification of physiologically relevant substrates of LRRK2, would be crucial to understanding the physiological and pathological functions of LRRK2. Elucidation of the downstream pathway of LRRK2 and the effects of FPD mutations therein would provide us with the clues about the role of LRRK2 in PD.

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SUPPORTING INFORMATION AVAILABLE

Estimation of the amount of LRRK2 in the immunoprecipitates and the amount of GTP bound to LRRK2 and a summary of the results of three independent experiments (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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