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Prolongs the “Active State” of its GTPase Domain

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PARKINSON DISEASE-ASSOCIATED MUTATION R1441H IN LRRK2 PROLONGS THE “ACTIVE STATE” OF ITS GTPASE DOMAIN

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Mutation in leucine-rich-repete kinase 2 (LRRK2) is a common cause of Parkinson disease (PD). A disease-causing point mutation R1441H/G/C in the GTPase domain of LRRK2 leads to overactivation of its kinase domain. However, the mechanism by which this mutation alters the normal function of its GTPase domain [Ras of complex proteins (Roc)] remains unclear. Here, we report the effects of R1441H mutation (RocR1441H) on the structure and activity of Roc. We show that Roc forms a stable monomeric conformation in solution that is catalytically active, thus demonstrating that LRRK2 is a bona fide self-contained GTPase. We further show that the R1441H mutation causes a twofold reduction in GTPase activity without affecting the structure, thermal stability, and GDP-binding affinity of Roc. However, the mutation causes a twofold increase in GTP-binding affinity of Roc, thus suggesting that the PD-causing mutation R1441H traps Roc in a more persistently activated state by increasing its affinity for GTP and, at the same time, compromising its GTP hydrolysis.

Significance

Mutations in the gene encoding for leucine-rich-repete kinase 2 (LRRK2) are a common cause of Parkinson disease (PD). To understand how LRRK2 causes PD, we need to understand its normal functions and how they are altered by disease-causing mutations. This effort has been hampered by the lack of appropriate samples, which led to some confusion in the field. This study shows the construction of a stably folded domain of LRRK2 called Ras of complex proteins (Roc). We use the study to resolve two conflicting models of Roc oligomerization by quantitatively demonstrating its GTPase activity in both monomeric and dimeric states. We further show that a PD-causing mutation affects both GTP binding and GTPase activity of Roc.


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To determine whether this contact patch occurs in human LRRK2, we built a homology model of human Roc domain with extended termini spanning the residues predicted to be in the hydrophobic patch. Our homology model revealed a similar hydrophobic patch that consists of two contiguous segments, residues 1329–1332 and 1518–1524, at the N- and C-terminal extensions, respectively (Fig. 1). Visually, this patch appears to act as the “glue” that bonds the N- and C-terminal extensions together and would prevent a domain-swapped dimer from occurring. To investigate whether the patch affects the structure of Roc, we made a series of human Roc constructs with the N and C termini extending into the hydrophobic patch. A resulting construct consisting of residues 1329–1520 was highly stable and amenable for biochemical and biophysical experiments.

**Roc Domain Forms Stable Monomers.** We expressed our terminal-extended Roc construct (Roc<sub>ext</sub>) in *Escherichia coli* and purified it by sequentially passing it through a column containing Nickel-nitrilotriacetic acid (Ni-NTA), followed by size-exclusion chromatography. During the final step of purification, we noticed that Roc<sub>ext</sub> eluted from the Superdex 200 column in two separate peaks (Fig. 2A). The first peak (at 91.8 mL) corresponded to a molecular mass of about 48 kDa (Roc<sub>48</sub>), and the second peak (at 98.7 mL) corresponded to about 24 kDa (Roc<sub>24</sub>) based on column calibration standard curve using the manufacturer’s molecular mass standard kit. The theoretical mass of Roc<sub>ext</sub> is 23.5 kDa, so Roc<sub>24</sub> and Roc<sub>48</sub> appear to represent the monomeric and dimeric conformations of Roc<sub>ext</sub>, respectively. Consistent with this, Roc<sub>24</sub> and Roc<sub>48</sub> resolved identically on reducing SDS/PAGE with an apparent molecular mass of ~24 kDa, and they both reacted with antibodies against LRRK2 on Western blots (Fig. 2B and C).

To further authenticate our construct and to determine their precise molecular masses, we used matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS). We detected a single mass of 23,484 Da in the Roc<sub>24</sub> sample and 46,839 Da in the Roc<sub>48</sub> sample, which correspond to the molecular masses of monomeric and dimeric forms of Roc<sub>ext</sub>, respectively (Fig. 2 E and F).

To further confirm that the molecular masses determined by MS occur in solution, we used multangle light scattering coupled with size-exclusion chromatography (SEC-MALS) to determine the molecular masses of Roc<sub>24</sub> and Roc<sub>48</sub> in solution. Roc<sub>24</sub> resolved on SEC-MALS as a single peak with a calculated molecular mass of 25 ± 2 kDa, whereas Roc<sub>48</sub> resolved as a single peak with a molecular mass of 46 ± 2 kDa, again consistent with the molecular mass of Roc<sub>ext</sub> monomer and dimer within instrumental errors (Fig. 2D). These results confirm the existence of monomeric and dimeric Roc<sub>ext</sub> in solution in the absence of a Cor domain.

The existence of Roc<sub>ext</sub> as both dimers and monomers in solution indicate that it does not form constitutive dimer as previously suggested (10, 11). This gives rise to the possibility of a dynamic nucleotide-dependent dimerization as a regulation mechanism of Roc activity suggested by Gotthardt et al. (11). To investigate this possibility, we determined whether Roc<sub>48</sub> changes its conformation upon binding to guanine nucleotides by incubating Roc<sub>48</sub> with GDP or GppNHp for 2 h using standard nucleotide-exchange procedures (12). As shown in Fig. 3A, virtually all of Roc<sub>48</sub> was converted to Roc<sub>24</sub> upon binding either GDP or...
GppNHp. We again used SEC-MALS to confirm that this is a bona fide conversion of dimers into monomers (Fig. 3B).

At the onset, we anticipated that dimer–monomer equilibrium might be regulated by GTP/GDP cycling; however, this was not the case because we found that GDP and GTP analog both converted the dimers into monomers with equal potency. Moreover, the physiological concentrations of guanine nucleotides (~500 μM GTP and ~50 μM GDP) are higher than needed for the conversion, which would render Roc<sub>ext</sub> mostly monomeric; therefore, the observed dimer–monomer conversion of Roc<sub>ext</sub> is unlikely to be physiologically relevant. For this reason, our focus turned to understanding the structure and function of the monomeric Roc<sub>24</sub>.

**Monomeric Roc<sub>24</sub> is Catalytically Active.** The literature currently suggests that dimerization of Roc is required for its GTPase activity (10, 11), but this requirement is uncertain because one of the two structures that provided this insight showed an unusual domain-swapped conformation not seen in other GTPases, whereas the second structure required intermolecular exchange of an arginine residue in trans to constitute an active site, but the required arginine does not exist in human LRRK2 (11). Creating a stable monomeric Roc construct enabled us to test these requirements. First, we set out to determine whether Roc<sub>24</sub> has the ability to hydrolyze GTP to generate GDP by using normal-phase hydrophilic interaction liquid chromatography (HILIC) HPLC coupled to electrospray mass-spectrometry (LC/MS). GTP and GDP resolved as two well-separated peaks with determined molecular mass of 524 and 444 Da, which correspond to the theoretical molecular mass of GTP (523 Da) and GDP (443 Da), respectively (Fig. S2). As shown in Fig. 4A, the production of GDP increased in a concentration-dependent manner, thus indicating that GDP was being converted to GDP.

To understand the GTPase activity of Roc<sub>24</sub> in more detail, we determined its steady-state kinetic parameters using the EnzCheck assay kit (Molecular Probes). Initial velocities were measured as a function of GTP concentrations. The resulting saturation curve adheres to the simple Michaelis–Menten equation, which allows us to calculate the steady-state kinetic parameters by nonlinear least-squares fit using the software Prism6 (GraphPad). As summarized in Fig. 4B, k<sub>cat</sub> = 0.020 ± 0.001 min<sup>-1</sup> and K<sub>m</sub> = 553 ± 94 μM. The low turnover rate and high K<sub>m</sub> indicate that isolated Roc<sub>ext</sub> is a slow enzyme and suggest that it might require activating and/or exchanging factors to efficiently hydrolyze GTP.

G proteins that cycle between GTP-bound (active) and GDP-bound (inactive) states as a switching mechanism commonly have low intrinsic GTPase activity. Ras, for example, has equally low intrinsic GTPase activity (k<sub>cat</sub> = 0.028 min<sup>-1</sup>) (13). The conversion between GDP and GTP by Ras family GTPases is usually accelerated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), or oligomerization-induced conformational changes (14, 15). The low k<sub>cat</sub> observed for Roc<sub>ext</sub> is in line with the intrinsic activity of this family of enzymes; however, strong activating or exchange factors of LRRK2 have not yet been identified.

The Michaelis constant K<sub>m</sub> (which is defined as the rate of dissociation of the enzyme–substrate complex [ES] k<sub>d</sub> plus the rate of forming the product and free enzyme [P and E] k<sub>cat</sub> divided by the rate of substrate binding to enzyme forming [ES] k<sub>cat</sub>; K<sub>m</sub> = (k<sub>d</sub> + k<sub>cat</sub>)k<sub>cat</sub>) is commonly used to approximate the affinity of the substrate with the enzyme K<sub>d</sub> (defined as [E][S]/[ES]). When the chemical process is fast, the K<sub>m</sub> approximates K<sub>d</sub>. However, GTPases are usually slow at hydrolyzing its substrate (represented by the rate constant k<sub>cat</sub>) and commonly bind their product GDP with higher affinity than their substrate GTP. This results in slow dissociation of the enzyme–product complex [EP] and renders the K<sub>m</sub> unrepresentative of the K<sub>d</sub> for GTP. To estimate the affinity of Roc<sub>ext</sub> for GTP, we determined the K<sub>d</sub> of Roc<sub>ext</sub> for GTP nonhydrolyzable analog GTP<sub>S</sub> using a fluorescence polarization (FP) assay (16, 17). As shown in Fig. 5A, the dissociation constant (K<sub>d</sub>) of BODIPY-FL-GTP<sub>S</sub> is 7.85 μM, which is two orders of magnitude lower than the K<sub>m</sub>, thus confirming that the K<sub>d</sub> does not represent K<sub>d</sub> in this case. Note that we used BODIPY-FL-GTP<sub>S</sub> instead of GTP to avoid complications associated with GTP hydrolysis during the binding assay as commonly used in the field. Although the FP assay that we used is well established, to confirm that the binding of BODIPY-FL-GTP<sub>S</sub> is an appropriate approximation of GTP binding, we titrated Roc<sub>ext</sub> prebound with BODIPY-FL-GTP<sub>S</sub> with unlabeled natural GTP. We found that GTP effectively displaced BODIPY-FL-GTP<sub>S</sub> (Fig. 5B), thus confirming that BODIPY-FL-GTP<sub>S</sub> binds to the same site as natural GTP on Roc<sub>ext</sub>.

The affinity of Roc<sub>ext</sub> with GTP in the low-micromolar range is relatively weak compared with typical small GTPases (which are in the sub-nanomolar range), but it is similar to that of large GTPases such as dynamin family (which are in micromolar range) (18). The weak affinity of Roc<sub>ext</sub> for nucleotide and the relatively small difference in its affinity for GTP vs. GDP (7.85 vs. 0.47 μM) suggests that it might not require GEFs for nucleotide-exchange cycling (physiological concentrations of GTP and GDP are ~500 and ~50 μM, respectively) (19). However, its low turnover rate of 0.02 molecules per minute suggests that it could require an activation factor for efficient hydrolysis of GTP, as observed in other GTPases.

**Conformations of GDP- and GppNHp-Bound Roc.** GTPases typically function as molecular switches that toggle on/off by undergoing conformational changes upon binding GTP and GDP. Thus, by analogy, the Roc domain of LRRK2 is believed to regulate its kinase domain via a similar mechanism. To investigate this, we characterized the structure of Roc<sub>ext</sub> by using circular dichroism

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**Fig. 3.** Conversion of Roc<sub>ext</sub> to monomers upon guanine nucleotide binding. (A) Size-exclusion chromatography profile of Roc<sub>ext</sub> apo state (green, ~48 kDa), GDP-bound (blue, ~24 kDa), and GppNHp-bound (red, ~24 kDa) shows conversion of the Roc<sub>ext</sub> peak into Roc<sub>ext</sub> peaks upon binding to either GDP or GTP. (B) SEC-MALS profile of GDP- and GppNHp-bound Roc<sub>ext</sub> with calculated molecular masses of 25 kDa for both (black line on the peak).

**Fig. 4.** GTPase activity of Roc<sub>ext</sub> (A) Background-subtracted LC/MS spectra of GDP generated by Roc<sub>ext</sub> GTP hydrolysis activity. Reaction was conducted with a range of GTP concentrations (0.1–1 mM). This graph shows proportional production of GDP with respect to the concentration of GTP. (B) Steady-state kinetics of Roc<sub>ext</sub> GTP hydrolysis. Data (open circles) is an average of three independent measurements with error bars (± SEM) indicated.
After establishing that the Roc domain of LRRK2 is as a bona fide self-contained GTPase, we turned our focus to investigate how the PD-causing mutation in Roc R1441H (Rocext) affects its properties. We introduced the mutation by using PCR-based site-directed mutagenesis using our Rocext DNA template and confirmed by DNA sequencing. Protein expression and purification procedures are as described for Rocext above. The mutated amino acid sequence was authenticated by mass spectrometry (Fig. S3).

We determined the GTPase activity of RocR1441H using the same EnzCheck assay described above and compared it to wild-type Rocext. We found that RocR1441H showed significantly lower activity than Rocext (Fig. 7A), suggesting that the point mutation does indeed change the GTPase activity of LRRK2 and might constitute a basis for PD pathogenesis. This is consistent with previously reported observations of two other amino acid substitutions at R1441 (R1441C and R1441G) using proteins pulled down from rat brains (24).

To investigate the GTPase activity of RocR1441H in more detail, we determined its enzyme kinetics. We found that it is more than twofold less active than Rocext with $K_{cat} = 0.009$ vs. 0.020 min$^{-1}$, respectively, with $K_{m}$ = 112 ± 22 μM (Fig. 7B), suggesting that the point-mutation R1441H affects both GTP-binding affinity, as well as its biochemical hydrolysis, which would undoubtedly trap Roc in a more persistent active state. GTP-bound state of Roc has been shown to activate kinase activity of LRRK2, which is associated with PD pathogenesis (8, 25).

**RocR1441H Has Higher Affinity for GTP than Wild Type.** To understand how the R1441H mutation led to a reduction in GTPase activity of Roc, we performed a battery of biochemical assays to examine its effects on structural features, thermal stability, and guanine nucleotide-binding affinity. We found that the point mutation does not affect the structure or thermal stability of Roc (Figs. 6B and 8A), thus suggesting that the reduction in GTPase activity of the mutant might be caused by biochemical perturbations as opposed to structural.

When we compared guanine nucleotide-binding affinity of RocR1441H with Rocext using a fluorescence polarization assays as described above, we found no significant difference in their affinity for GDP (Fig. 5D). However, the GTPγS-binding affinity of RocR1441H is twofold higher than that of Rocext, $K_a = 3.39$ vs. 7.85 μM, respectively (Fig. 5C). This seems counterintuitive because higher substrate affinity usually results in higher activity instead of lower as we observed.

To confirm the observed difference in GTP-binding affinity, we determined the thermal denaturation of Rocext and RocR1441H bound with GDP and GppNHp using differential scanning fluorimetry (26). We found no significant difference in the denaturation curve between GDP-bound RocR1441H and Rocext, which is consistent with their similar affinities for GDP (Fig. 8B). However, GppNHp-bound RocR1441H has a melting temperature (Tm) ~3.1 °C higher than Rocext, thus confirming the above observation that the R1441H mutation does indeed lead to increased GTP-binding affinity. More importantly, the observed increase in GTP-binding affinity and decreased in GTPase activity would independently result in a prolonged active state of Roc, having both features in a single mutation is a “double-whammy” that together would trap Roc in a more persistent active state.

**Conclusions**

Over the past 10 y, the field has been pursuing disparately to understand the functions of LRRK2 and how it is affected by...
E. coli (filled triangle with solid line) is less active than γA polyclonal antibody against the Roc domain of human were collected from Roc peak and Roc and Roc A (Novagen) by inducing with 0.5 mM isopropyl-
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showing similar thermal stability. (C) is more ther-
Molecular masses of Roc and Roc |
|C is more ther-

PD-associated mutations. A major hurdle in the field has been the difficulty in obtaining sufficient quantity and quality of samples (both full-length and individual domains) suitable for quantitative investigations. We have produced a suitable Roc domain of LRRK2 that enabled us to quantitatively demonstrate that the Roc domain of LRRK2 is a bona fide GTPase, which clarifies a current confusion in the field regarding the necessity of dimerization for Roc activity. The kinase activity of LRRK2 has been shown to be overactivated in PD-associated R1441H mutant; here, we show that it traps Roc in a more persistently activated state by increasing its affinity for GTP and, at the same time, decreasing its GTPase activity, thus providing insights into the mutant’s mechanism of PD pathogenesis.

Methods

Homology Modeling. Homology models of human LRRK2 GTPase domain were built based on the structure of C. tepidum Roco (PDB ID code 3DPV) by using the program Modeler 9.12 (Andrej Sali, University of California, San Francisco, CA). Molecular graphics display and presentation were made using PyMol (www.pymol.org).

Protein Expression and Purification. An extended GTPase domain of LRRK2 consisting of residues 1329–1520 (Rocext) was subcloned into a pETDuet-1 vector (Novagen, Merck) using PCR cloning techniques. The resulting protein consisting of an N-terminal hexahistidine tag was expressed from Rosetta2 vector (Novagen, Merck) using PCR cloning techniques. The resulting protein samples were desalted by using ZipTip-C18 columns (EMD MILLIPORE) and eluted with acetone and acetonitrile. 1.5 μL of eluent was spotted on a stainless steel MALDI plate and allowed to dry. A “sandwich” of sample-matrix-sample was formed by layering 1.5 μL of matrix (sinapinic acid (stock, 10 mg/mL) in 75% acetonitrile and 0.1% TFA) on top of the dried protein sample spot, followed by another layer of protein sample on top of the dried matrix. Sample was analyzed using standard MALDI-TOF methods. To determine GTPase activity of Rocext by MS, we used normal-phase ZIC-HILIC (EMD Millipore) HPLC coupled to electrospray MS; 20 μM Rocext was incubated with various concentration of GTP (0.1, 0.2, 0.4, and 1 mM) at 25 °C for 1 h. After incubation, protein samples were be denatured at 100 °C for 10 min, followed by centrifuging to remove denatured protein. Samples were loaded into ZIC-HILIC HPLC equilibrated in 90% acetonitrile and 30 mM ammonium acetate and eluted in 70% acetonitrile and 30 mM ammonium acetate isocratically. The eluted nucleotides molecular mass was determined by inline electrospray MS.

CD Spectroscopy. CD spectra were collected on a Biologic Science Instruments MOS450 A/F/C/D spectrometer with slit width of 1.0 mm and data acquisition of 1.0 s. The protein samples with concentrations ranging from 0.46 to 0.86 mg/mL (based on absorbance at 280 nm) were dissolved in the buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 5% glycerol. The data used for graphical presentation and analyses were each an average of five different scans.

Fluorescence Polarization Nucleotide-Binding Assay. To estimate the binding affinity of guanine nucleotides, BODIPY-FL-GTP/γS (100 nM) or 150 nM BODIPY-FL-GDP (150 nM) (Life Technologies) were titrated with Rocext (starting at 0.1 μM) until saturation was reached (15 and 10 μM, respectively). Fluorescence polarization was read using an EnVision 2102 Multilabel Plate Reader (Perkin-Elmer) with excitation at 485 nm and emission at 535 nm. Experiments were performed at 25 °C in buffer 10 mM Heps (pH 7.4), 150 mM NaCl, 10 mM MgAc, 10 mM glycine, 4 mM EDTA, 1 mM DTT, 5% glycerol. Data were analyzed using Prism 6 (GraphPad Software).

SEC-MALS. To determine the absolute molecular mass of Rocext in solution, we used multiple angle light scattering. Our experimental setup includes an AKTA FPLC (GE Healthcare Biosciences) with a silica-based size-exclusion chromatography column (WTC-0305S; Wyatt Technology) as an LC unit. Down from the LC is a refractive index detector (Optilab T-Rex; Wyatt Technology), followed by a multiple light scattering detector (Dawn HeleosI; Wyatt Technology) for determining protein concentration and particle size, respectively. Each sample injection consisted of ~1 mg of purified Rocext in buffer containing 10 mM Heps (pH 7.4), 0.15 M NaCl, 10 mM MgAc, 10 mM glycine, 1 mM DTT, and 5% glycerol. Flow rate was set at 0.4 mL/min, and data were collected every 2-s interval. Data processing and analysis were performed using the ASTRA software (Wyatt Technology).

Mass Spectrometry. Molecular masses of Roc and Rocext were determined using MALDI-TOF (AB Sciex). Protein samples were desalted by using ZipTip-C18 columns (EMD MILLIPORE) and eluted with acetone and acetonitrile. 1.5 μL of eluent was spotted on a stainless steel MALDI plate and allowed to dry.
GTPase Activity Assay. GTPase activity of Roc<sub>ext</sub> was assessed by using the EnzCheck assay kit (Life Technologies) according to manufacturer's instructions. Briefly, Roc<sub>ext</sub> (15 μM) was incubated with different concentration of GTP (0.15, 0.3, 0.45, 0.75, 1.5, and 3 mM) in in buffer containing 10 mM Hepes, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 5% glycerol at 25 °C. Absorbances at 360 nm were recorded every 4 min for a duration of 60 min using a microplate reader. The amount of inorganic phosphate released from GTP hydrolysis at each time point was determined by extrapolation using a phosphate standard curve. Kinetic parameters were determined by fitting the data to Michaelis-Menten equation using GraphPad Prism 6.

Fluorescence Polarization-Based Nucleotide Competition Assay. To ensure that the binding that we observed between GTP/GDP and Roc<sub>ext</sub> was mediated by the nucleotides and not the BODIPY molecules attached to them, we performed nucleotide-competition assays for each nucleotide. Roc<sub>ext</sub> (2 μM) was preincubated with 100 nM BODIPY-FL-GTP or 150 nM BODIPY-FL-GDP (Life Technologies) for 10 min at 25 °C in 384-wells black plate (NUNC). Then, the Roc<sub>ext</sub>-BODIPY–nucleotide complex was titrated with unlabeled natural GTP or GDP (started at 25 nM) until the binding between Roc<sub>ext</sub> and labeled nucleotide was inhibited completely (20%). The fluorescence polarization was read in an EnVison 2102 Multiplicable Plate Reader (Perkin-Elmer) using excitation at 485 nm and emission at 535 nm. Experiments were performed at 25 °C in buffer containing 10 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM MgAc, 10 mM glycerin, 4 mM EDTA, 1 mM DTT, 5% glycerol. The titration plots were fitted in a nonlinear regression curve using GraphPad Prism 6.

ThermoFluor Assay. Solutions of 7.5 μL of 16x Sypro Orange (prepared from 5,000x stock concentrate; Life Technologies) in buffer containing 10 mM Hepes (pH 7.4), 0.15 M NaCl, 10 mM MgAc, 10 mM glycerin, 1 mM DTT, and 5% glycerol, 12.5 μL of 4 mM GDP or GppNHp (Sigma), and 5 μL of 25 μM Roc<sub>ext</sub> or Roc<sub>ext</sub> R1441H were added to a 96-well thin-walled PCR plate. The plate was heated in the Real Time PCR Detection System (Masterycer Realplex; Eppendorf) from 20 °C to 85 °C, and fluorescence was recorded in increments of 0.4 °C. The emission wavelength was set at 550 nm.

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