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The Parkinson's disease kinase LRRK2 autophosphorylates its GTPase domain at multiple sites

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Abstract

Mutations in Leucine-rich repeat kinase 2 (LRRK2) are a common cause of inherited Parkinson's disease (PD). The protein is large and complex, but pathogenic mutations cluster in a region containing GTPase and kinase domains. LRRK2 can autophosphorylate *in vitro* within a dimer pair, although the significance of this reaction is unclear. Here, we mapped the sites of autophosphorylation within LRRK2 and found several potential phosphorylation sites within the GTPase domain. Using mass spectrometry, we found that Thr1343 is phosphorylated and, using kinase dead versions of LRRK2, show that this is an autophosphorylation site. However, we also find evidence for additional sites in the GTPase domain and in other regions of the protein suggesting that there may be multiple autophosphorylation sites within LRRK2. These data suggest that the kinase and GTPase activities of LRRK2 may exhibit complex autoregulatory interdependence.

Keywords

Parkinson's disease; kinase; GTPase; autophosphorylation

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Introduction

Protein kinases play key regulatory roles for many processes in cells, influencing signal transduction cascades by the phosphorylation of target proteins. As such, misregulated kinases are associated with many types of human disease. For example, activating mutations are a common and recurrent cause of diverse types of cancer [1]. Mutations are often found in the magnesium binding motif (consensus DY/FG) of the activation loop, a flexible region of many kinases that controls net activity.

Similar types of activating mutations in LRRK2 (leucine-rich repeat kinase 2), cause dominantly inherited form of Parkinson's disease (PD) [2]. A very common mutation, G2019S, is found in the Mg^{2+} binding motif and increases kinase function [3]. However, LRRK2 is a complicated molecule that includes protein-protein interaction domains as well as a second catalytic region, the ROC (ras of complex proteins) domain, which is an active GTPase [4;5;6], and an adjacent COR (C-terminal of ROC) region that may regulate the ROC domain via dimerization [7]. Although there is some controversy, mutations in the ROC domain (R1441C/G/H) do not generally increase kinase activity [3] and are apparently instead associated with decreased GTPase activity [4;5;6;8].

Several models have been proposed to reconcile these two different molecular effects of mutations in LRRK2. GTP binding to the ROC domain can modestly stimulate kinase activity [4;9;10;11], suggesting that decreased GTPase activity might lead to enhanced kinase signaling. However, there is little evidence that mutations in the ROC domain increase kinase activity in the presence of GTP.

Understanding the effects of mutations in LRRK2 on kinase activity has been hampered by a lack of understanding of authentic substrates of the enzyme. Proposed substrates include moesin [12], 4EBP [13], MKK3/6 [14], β -tubulin [15] and α -synuclein [16] but none have been shown to be true *in vivo*, and pathogenic mutations do not consistently have increased activity towards any specific substrate. For example, Jaleel et al examined the effects of LRRK2 mutations on phosphorylation of a peptide derived from moesin (LRRKtide) and found that only G2019S caused a significant increase in kinase activity [12].

These considerations suggest either that kinase activity of LRRK2 is irrelevant for pathogenesis of PD or that current assays do not capture a pathological kinase activity. Regarding the first possibility, we have shown [17], and others have confirmed [18], that kinase activity contributes to toxic effects of mutant LRRK2 *in vitro*, even for pathogenic mutations outside of the kinase domain. This suggests that the kinase activity of LRRK2 is important either as an output of signaling or a modulator of another part of the protein. Potentially relevant to the second point, many assays have been performed using autophosphorylation of LRRK2 as a surrogate for activity in the absence of a known pathological substrate. Autophosphorylation of kinases is often seen *in vitro*, although there are examples where autophosphorylation is a physiologically relevant event. For example, ligand binding to dimeric receptor tyrosine kinases triggers autophosphorylation that leads to recruitment of additional protein partners and initiation of intracellular signaling cascades. In many cases [19], kinases phosphorylate their own kinase domains. LRRK2 forms dimers and full length LRRK2 can autophosphorylate within a dimer [20], although the isolated LRRK2 kinase domain can phosphorylate in *trans* [21].

Such data indicates that understanding autophosphorylation might be interesting in understanding the behavior of LRRK2. Therefore, in the current study we examined the sites of LRRK2 autophosphorylation *in vitro*. Surprisingly, we found that LRRK2 autophosphorylates within the ROC/GTPase domain of the same protein, raising the possibility that complex autoregulation of LRRK2 may occur.

Materials and Methods

Mutagenesis

All point mutations were inserted into LRRK2 full length cDNA [17] or fragments using the Quick Change site-directed mutagenesis kit (Stratagene). Primers sequences are available upon request. Constructs were fully sequenced to confirm fidelity.

Recombinant proteins

ROC (amino acids 1333–1516), ROC-COR (1333–1845) and kinase (1887–2091) domains were cloned into pSKB3 vectors with hexa-histidine tags and expressed in *E. coli*. The kinase domain was co-expressed with two different molecular chaperones (trigger factor, C2; GroEL/ES, C3). All proteins were purified as described [8]. For N-terminal GST tags, ROC (1333–1516) was cloned into pGEX-5X1 (GE Life Sciences), expressed in *E. coli*, purified with glutathione sepharose beads (GE Life Sciences) and eluted with 10 mM glutathione in PBS (pH 8.0). ROC (1335–1548) was also cloned into pRSET-A with an N-terminal histidine tag. Proteins were expressed in *E. coli*, collected as inclusion bodies, resolubilized using 6M guanidinium hydrochloride, purified on Ni-TED affinity resin and refolded on the column using a GuHCl gradient. Proteins were eluted in 50 mM NaH₂PO₄, 300 mM NaCl, 0.1 mM DTT and 1% Empigen pH 8.0 with imidazole. Proteins displayed >95% purity by SDS-PAGE and Coomassie staining.

Recombinant LRRK2^{970–2527} with an N-terminal GST tag purified from insect cells was purchased from Invitrogen. LRRK2^{1326–2527} was cloned into pFastBac1 (Invitrogen) with N-terminal Flag tag and C-terminal His tags and used to generate baculovirus with the Bac-to-Bac Expression system (Invitrogen). Sf9 cells were infected with viruses and harvested after 48 hours. Protein was purified by anti-Flag M2 affinity chromatography according to the manufacturer's instructions (Sigma), confirmed by MALDI analysis and stored in 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 0.01% Brij-35, 0.1 mg/ml Flag peptide at –80°C.

For mammalian expression, full length human LRRK2, the N-terminal region of LRRK2 (amino acids 1–1248), LRR (982–1280), ROC (1335–1548), COR (1550–1880), kinase (1880–2138) and WD40 (2150–2500) were cloned into the pCHMWS expression plasmid [22], incorporating an N-terminal 3×flag tag. HEK293T cells were transfected using Fugene and lysed after 48–72 hours in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton, 10% Glycerol and protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 20,000 g for 10 minutes, pre-cleared with normal mouse IgG bound to agarose beads (Sigma), incubated for 3 h to 18 h with flagM2 bound to agarose beads (Sigma), washed with 25 mM Tris (pH 7.5), 400 mM NaCl, 1% Triton and rinsed in kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na₃VO₄, 10 mM MgCl₂), then eluted from beads with 3×flag peptide (Sigma).

Kinase assays

Recombinant domain proteins were incubated with recombinant GST tagged LRRK2^{970–2527} or recombinant full length 3×flag tagged LRRK2 in 40 µl kinase buffer containing 6 µCi of ³³P-ATP (3000 Ci/mmol; Perkin Elmer) for 1 hour at 30°C. Reactions were terminated by adding 6× SDS loading buffer, loaded onto 4–20% SDS-PAGE gels (Biorad) and transferred onto Immobilon-P membranes (Millipore). Incorporated ³³P-ATP was detected by autoradiography using a Storm 840 scanner (GE Healthcare). The same membranes were stained with Coomassie blue or Ponceau S (Sigma) or probed with anti-GST (GE Life Sciences), Flag M2 (Sigma) or DR4A/3EDD anti-LRRK2 [23] to confirm the presence of proteins. Densitometric analysis of the bands on the blot autoradiograms and immunoreactivity

were performed using Aida Image Analyzer Software v4.0 (Raytest GmbH, Straubenhardt, Germany).

Autophosphorylation assays were performed using 30 μ M LRRK2^{1326–2527}, 1 μ Ci [γ -³²P]ATP and 25 μ M of ATP and incubated at RT for 0h or 17h. Reactions were stopped by adding Laemmli buffer and boiling the samples for 5 minutes. Samples were separated on a gradient 4–20% SDS-PAGE (BioRad). Gels were stained with coomassie brilliant blue, destained overnight, dried on 3 mm Whatman paper using a gel dryer (Biorad) and exposed to film to generate an autoradiogram.

Phosphopeptide identification

LRRK2^{21326–2527} protein (50 μ g) was digested using trypsin [24], and peptides were desalted using a C18 cartridge. Phosphopeptides were subjected to beta-elimination using d0 or d6 labeled DTT as the base for Michael addition, combined and enriched using Thiopropyl Sepharose 6B beads (GE Life Sciences). After extensive washing, peptides were eluted using 100 mM DTT and analyzed using a Thermo Finnigan FT-LTQ mass spectrometer. The peptides were identified through database search, and potential phosphorylated peptides were further verified by manual inspection of the raw MS and MS/MS spectra. Only those peptides that were first identified to be DTT labeled and also displayed as ion pairs, 2–3 Da apart, in the MS spectra were concluded to be authentic phosphopeptides.

Selected reaction monitoring (SRM) analysis

To determine phosphorylation sites in LMIVGNTGSGK and DYHFNATEESDALAK, all possible phosphorylated peptides were synthesized and analyzed using TSQ-Quantum to determine the peptide retention time and their fragmentation pattern. Phosphopeptides released from digestion of LRRK2 were then compared with these peptide standards.

Results

LRRK2 phosphorylates its isolated ROC/GTPase domain

To identify the site(s) of LRRK2 autophosphorylation, we screened different LRRK2 fragments as potential substrates for LRRK2 kinase activity. We used recombinant LRRK2^{970–2527} from an insect cell expression system that is highly active for autophosphorylation [25]. Approximately 25 nM of LRRK2^{970–2527} was used against ROC (1 μ M), ROC-COR (0.2 μ M) or kinase (1 μ M; two different chaperones were used to maintain solubility) domains expressed in *E. coli*. LRRK2^{970–2527} was able to phosphorylate the ROC fragment and the ROC-COR bidomain but not the kinase domain (Fig 1A). We also expressed and purified LRRK2 domains (LRRs, ROC, COR, Kinase and WD40) in HEK293T cells and found that LRRK2^{970–2527} can efficiently transfer a phosphate group to the ROC domain (Fig. 1B). The COR domain alone did not incorporate any detectable radiolabel, and we found only minor phosphorylation of the kinase, LRR and WD40 domains. These results suggest that the ROC domain is a major target of LRRK2 autophosphorylation activity.

To confirm this result with full-length protein, we used ~50 nM 3 $\times$ flag-tagged wild-type, K1906M or G2019S LRRK2 from mammalian cells and ~2 μ M of his-tagged ROC domain from *E. coli*. Wild-type LRRK2 efficiently phosphorylated ROC while the K1906M variant showed decreased activity (Fig. 1C). The G2019S mutant not only displayed increased autophosphorylation, but also higher ROC phosphorylation compared to wild-type (Fig. 1C). Collectively, these results suggest that LRRK2 can phosphorylate its own isolated ROC domain.

Mass spectrometry reveals multiple potential phosphosites

We next identified candidate phosphoacceptor sites within the ROC domain using 3 μ M of recombinant protein and 25 nM LRRK2^{970–2527}. To allow for the possibility that there may be additional sites outside of the ROC domain, we also performed similar assays with 200 nM LRRK2^{970–2527} alone. In both experiments, we analyzed resultant digested and enriched phosphopeptides by liquid chromatography and tandem mass spectrometry (LC-MS/MS; Table 1).

Seven potential phosphosites were found in either LRRK2^{970–2527} or isolated ROC. Only threonine or serine but not tyrosine residues were identified. Among the peptides located within ROC, three were recovered multiple times; a peptide phosphorylated at T1343/S1345 (recovered 20 times from ROC and 7 times from LRRK2^{970–2527}); the tandem site T1348/T1349 (10 times from ROC, twice from LRRK2^{970–2527}) and T1491 (10 times from ROC). Two peptides were identified outside of the ROC domain using LRRK2^{970–2527}, one peptide located in the LRR domain containing T1024/S1025 and another peptide in the kinase domain at position T2031/S2032, each recovered once.

Confirmation of T1343 as an autophosphorylation site

In several of the above peptides, there were multiple Ser/Thr residues. We used two independent techniques to attempt to confirm the authentic sites within each peptide. We first repeated the above experiments with a slightly different LRRK2 recombinant construct expressed in Sf2 cells, LRRK2^{1326–2527}, that is also highly active [12]. We isolated four peptides that overlapped with those found in the above experiments in the ROC domain and, using SRM analysis (Figure 2), were able to unambiguously identify T1343 and T1491 as phosphosites (Table 2). We also found evidence of phosphorylation at T2031 in the kinase domain as well as minor sites in the COR and WD40 domains.

To attempt to confirm this data, we mutagenized all the candidate residues to alanine in a substrate construct. Recombinant GST-ROC proteins (~ 0.5 μ M) were then used as substrates for phosphorylation by full-length 3 $\times$ flag-LRRK2 (Figure 3). Quantitation of the amount of radiolabeled phosphate transferred to each GST-ROC mutant shows that T1343A had ~ 50% phosphorylation of wild-type.

We next quantified the proportion of peptides that were phosphorylated for the two most promising candidate sites, T1343 and T1491. We used recombinant LRRK2^{1326–2527} and compared kinase dead (D2017A) with the activating G2019S mutant under basal conditions and under conditions of extended incubation of kinase reactions (Table 3). Neither T1343 nor T1491 were phosphorylated in the kinase dead construct. Consistent with the higher number of recovered peptides for T1343 in Table 1, the proportion of peptide phosphorylated at T1343 was higher (~23%) than for T1491 (~6%). However, neither site was completely phosphorylated nor was the proportion of phosphopeptides increased with longer incubation times (Table 3). We did find increased ³²P incorporation into WT or G2019S, but not kinase dead, LRRK2 after similar 17h incubations (Supplementary figure S1). Collectively, these results support the identification of T1343 as a site for autophosphorylation of LRRK2 but suggest that additional sites exist.

Discussion

Several mutations in the complex multi-domain kinase LRRK2 are linked to PD but the mechanism(s) by which they affect protein function are poorly understood. Data on kinase activity has been controversial for all mutations apart from G2019S, which is activating. However, our incomplete understanding of the normal physiological substrates of LRRK2

complicates interpretation of this data. As several laboratories have used autophosphorylation as a proxy for kinase activity, the aim of the current study was to identify the autophosphorylation sites of LRRK2.

We expected that we might see evidence of autophosphorylation within the kinase domain of LRRK2, as this would be a likely regulatory site. However, we found only minor evidence for phosphorylation of one residue, T2031, in this domain. In contrast, several lines of evidence suggest that residues in the GTPase/ROC domain of LRRK2 are autophosphorylated.

Of the sites identified with the ROC domain, the most consistent candidate is T1343. Previous data has shown that a ras-like T1343G mutation in full length LRRK2 has decreased kinase activity when combined with R1398Q [8]. T1348N, which converts LRRK2 to a GTP deficient version, also decreases kinase activity [9]. One interpretation of these results is that they reflect a requirement of guanine nucleotides for kinase activity but they may also represent loss of autophosphorylation of the protein when T1343 is mutated. This may explain why such mutations have a dramatic effect, decreasing kinase activity by about 50% when addition of GDP to immunoprecipitated LRRK2 has no effect on kinase activity [18].

Compared to T1343, T1491 appears to be a relatively minor site based on quantitation by mass-spectrometry and from experiments showing that T1491A in GST-ROC domain has a minimal effect on overall phosphorylation. Another minor site is at T2031, which we have previously shown can impact kinase activity as expected for an activation loop residue [20].

Phosphorylation of the ROC domain suggests that autoregulation within LRRK2, which has previously been proposed to be of the kinase domain by the ROC domain, might occur from kinase activity to the GTP binding region. One possibility is that phosphorylation of one or more threonine residues may alter GTPase activity. A hyperactive kinase mutation such as G2019S would be predicted to decrease GTPase activity and would be functionally similar to R1441 mutations in the ROC domain that have lower inherent GTPase activity [4;5;6;8]. If correct, then kinase inhibition, which has been suggested as a possible therapeutic approach for LRRK2 PD [26] would likely be effective in maintaining GTPase activity by inhibiting such repression.

Overall, our results identify two novel sites for autophosphorylation of LRRK2. It is of interest that these sites are within the other enzymatic domain of LRRK2, the ROC/GTPase region as this suggests a complex autoregulation of LRRK2. These sites now need to be confirmed *in vivo*, perhaps using phosphospecific antibodies that are yet to be developed. However, several considerations suggest that T1343 and T1491 are not the only two autophosphorylation sites within LRRK2, including that we did not find quantitative changes at either T1343 or T1491 under conditions where autophosphorylation proceeds further. Further work is required to identify these additional sites in future experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

COR, C-terminal of ROC; LRRK2, leucine-rich repeat kinase 2; PD, Parkinson's disease; ROC, ras of complex proteins.

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Figure 1. LRRK2 phosphorylates the isolated LRRK2 ROC domain

(A) Recombinant GST-LRRK2⁹⁷⁰⁻²⁵²⁷ was incubated alone (lane 1), with recombinant kinase (lanes 2–3), ROC (lane 4) or a ROC-COR domains (lane 5) and radiolabeled ATP. The ROC domain is phosphorylated by LRRK2⁹⁷⁰⁻²⁵²⁷, visualized by autoradiography (upper panel). Lower panel shows total protein loadings visualized by coomassie blue staining (* and ** are chaperones coexpressed with kinase domain). (B) A similar experiment using FLAG-tagged LRR (lane 1), ROC (lane 2), COR (lane 3), kinase (lane 4) or WD40 (lane 5) domains expressed in mammalian cells. Lower panel shows loading of the individual domains by immunoblot after FLAG immunoprecipitation. (C) Full length, FLAG-tagged wild type (lane 1), K1906M (lane 2) or G2019S (lane 3) LRRK2 were incubated with His-tagged ROC domain. ROC phosphorylation was lower with K1906M and higher with G2019S compared to WT. For all panels, molecular weight markers on the right are in kilodaltons. Each result is representative of at least triplicate independent experiments.

Figure 2. Identification of the phosphorylation site in LRRK2¹³³⁷⁻¹³⁴⁷ using selected reaction monitoring

Mass spectrometry analyses of synthesized peptide standards: unphosphorylated (A), pT1343 (B) and pS1345 (C). SRM spectra for the unphosphorylated (D) and phosphorylated (E) peptides from a G2019S digest compared to these standards support the identification of T1343 as a phosphorylated residue.

Figure 3. Confirmation of possible phosphorylation sites by mutagenesis

(A) Full length FLAG tagged LRRK2 was incubated with wild type or the indicated Alanine mutants of the ROC domain and phosphorylation was assessed by autoradiography (upper panel). Anti-GST immunostaining (lower panel) shows loading of the ROC domain. (B) Autophosphorylation was quantified, corrected for loading and expressed relative to WT ROC domain on each experiment. Error bars indicate range from duplicate independent experiments.

Table 1

Identification of possible phosphosites in recombinant LRRK2

Domain	Sequence	# peptides recovered		Possible Sites
		LRRK2 ⁹⁷⁰⁻²⁵²⁷	ROC	
LRR	ELHQNALT ^{2S?} FPQQLCET	1	-	T1024/S1025
ROC	LMIVGNT ^{2GS?} GKTTLLQQLM	7	20	T1343/S1345
ROC	LMIVGNTGSGKT ^{2T?} LLQQLM	2	10	T1348/T1349
ROC	EFYS ^{2T?} HPHFMTQRA	2	1	S1403/S1404
ROC	EFYSTHPHFM [*] TQRA	1	-	T1410
ROC	RASSSPVILVGT [*] HLDVSDEKQ	3	-	T1452
ROC	YHVNAT [*] EESDALAKLR	-	10	T1491
ROC	KLRKT [*] IINESLNFK(I)	3	5	T1503
Kinase	RMGIKT ^{2S?} EGTPGFRA	1	-	T2031/S2032

Using tandem MS/MS on phospho-purified proteins, we identified several possible threonine residues within the ROC domain.

* For many peptides, a single threonine residue was present and seen to be modified on the spectra.

? However, in some peptides, the exact site could not be unambiguously identified.

Table 2Selective reaction monitoring (SRM) identification of phosphorylation sites in LRRK2^{1337–1347}

Domain	Sequence	Position	Phosphorylation site
ROC	LMIVGNTGSGK	1337–1347	T1343
ROC	KSDLGMQSATVGIDVK	1359–1374	undetermined ^I
ROC	ASSSPVILVGTHLDVSDEK	1442–1460	undetermined
ROC	DYHFNATEESDALAK	1484–1499	T1491
COR	LTIPISQIAPDLILADLPR	1848–1866	undetermined
Kinase	MGIKTSEGTPGFR	2027–2039	T2031
WD40	GQLSFLDLNTEGYTSEEVADSR	2177–2198	undetermined
WD40	ILNIGNVSTPLMCLSESTNSTER	2290–2312	undetermined

^I The exact phosphorylation site in the peptide was not determined.

Table 3

T1343 and T1491 represent autophosphorylation sites

	Thr1343		Thr1491	
	0 hrs	17 hrs	0 hrs	17 hrs
D2017A	0%	0%	0%	0%
G2019S	22.9 ± 0.7%	16.7 ± 4.2%	5.9 ± 0.8%	4.2 ± 0.8%

Percentage of peptide phosphorylation at residue Thr1343 and Thr1491 in LRRK2 before (0 h) and after (17 h) *in vitro* kinase reactions. Each data point is the average of three independent reactions ± SEM.