LRRK2 Kinase Activity Is Dependent on LRRK2 GTP Binding Capacity but Independent of LRRK2 GTP Binding

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Abstract

Leucine repeat kinase 2 (LRRK2) is a Parkinson’s disease (PD) gene that encodes a large multidomain protein including both a GTPase and a kinase domain. GTPases often regulate kinases within signal transduction cascades, where GTPases act as molecular switches cycling between a GTP bound “on” state and a GDP bound “off” state. It has been proposed that LRRK2 kinase activity may be increased upon GTP binding at the LRRK2 Ras of complex proteins (ROC) GTPase domain. Here we extensively test this hypothesis by measuring LRRK2 phosphorylation activity under influence of GDP, GTP or non-hydrolyzable GTP analogues GTP\(^c\) or GMPPCP. We show that autophosphorylation and Lrrktide phosphorylation activity of recombinant LRRK2 protein is unaltered by guanine nucleotides, when co-incubated with LRRK2 during phosphorylation reactions. Also phosphorylation activity of LRRK2 is unchanged when the LRRK2 guanine nucleotide binding pocket is previously saturated with various nucleotides, in contrast to the greatly reduced activity measured for the guanine nucleotide binding site mutant T1348N. Interestingly, when nucleotides were incubated with cell lysates prior to purification of LRRK2, kinase activity was slightly enhanced by GTP\(^c\) or GMPPCP compared to GDP, pointing to an upstream guanine nucleotide binding protein that may activate LRRK2 in a GTP-dependent manner. Using metabolic labeling, we also found that cellular phosphorylation of LRRK2 was not significantly modulated by nucleotides, although labeling is significantly reduced by guanine nucleotide binding site mutants. We conclude that while kinase activity of LRRK2 requires an intact ROC-GTPase domain, it is independent of GDP or GTP binding to ROC.

Introduction

Leucine rich repeat kinase 2 (LRRK2) has been identified as a Parkinson’s disease (PD) gene responsible for parkinsonism with a clinical course essentially identical to that in idiopathic PD [1,2]. LRRK2 encodes a 2527 amino-acid multidomain protein including several regions predicted to be involved in protein-protein interactions. Potential protein-protein interaction regions include an ankyrin repeat domain, a leucine rich repeat domain and a WD40 domain as well as two catalytic domains including a GTPase domain of the Ras of complex proteins family (ROC) and a kinase domain of the tyrosine kinase like family [3,4]. Ras family GTPases and tyrosine kinase like kinases are often associated as elements of signal transduction cascades, where Ras GTPases act as molecular switches cycling between a guanosine triphosphate (GTP) bound ‘on’ state and a guanosine diphosphate (GDP) bound ‘off’ state. In the ‘on’ state, Ras GTPases activate an effector protein such as a kinase via direct binding.

For LRRK2, it has been suggested that LRRK2 kinase may be the downstream effector of LRRK2 ROC (reviewed in [5]). Indeed, functional mutant forms of LRRK2 in which guanine nucleotide binding is disrupted have been shown to display very low kinase activity suggesting that the ROC GTPase domain may regulate kinase activity [6,7,8]. Active or inactive states of Ras-GTPases can be mimicked in vitro using GDP for the inactive state and non-hydrolyzable GTP analogues such as guanosine - 5'-O-\([\gamma\text{-thio}]-\)triphosphate (GTP\(^\gamma\)S) or guanosine - 5'-\([\beta,\gamma\text{-methylene}]\)-triphosphate (GMPPCP) for the active state. Enhanced LRRK2 autophosphorylation activity has been reported when the non-hydrolysable GTP analogue GTP\(^\gamma\)S was added to the kinase reaction [9], however the addition of GDP did not have an ‘off’ effect as would be expected. Although enhanced kinase activity has also been reported when GTP\(^\gamma\)S was added to the cellular lysate prior to protein purification [8], this finding could not be reproduced with recombinant protein in solution [10]. Therefore, although widely discussed, the data showing that GTP stimulates LRRK2 kinase activity is difficult to interpret as to whether this is direct binding and therefore a simple intramolecular switch mechanism or whether the mechanism is indirect.

Because varying results have been reported using different approaches, we sought to further elucidate the issue of how
nucleotides bound to the ROC domain influence kinase activity. For this we compared several modes of application of guanine nucleotides to full length recombinant LRRK2 protein purified from HEK293T cells, coupled to measures of autophosphorylation as well as LRRK2-mediated phosphorylation of lrrktide, a specific in vitro peptide substrate [11]. Our data show that an intact ROC-GTPase domain is required for LRRK2 kinase activity and that kinase activity remains unchanged upon direct application of GDP compared to GTP or non-hydrolyzable GTP analogues, reconciling discrepancies in previous reports.

Results

LRRK2 kinase activity of the affinity purified protein is not altered upon co-incubation or preloading with guanine nucleotides

We first tested whether inclusion of nucleotides in the kinase reaction would alter LRRK2 kinase activity using purified soluble full-length LRRK2 protein (Figure 1A). Via metabolic labeling and thin layer chromatography analysis, we found that our stringent purification procedure yielded protein devoid of guanine nucleotides (supplementary figure S2). Co-incubation of LRRK2 with concentrations of guanine nucleotides varying from 0 to 1 mM did not alter LRRK2 mediated phosphorylation of the lrrktide peptide substrate (Figure 1B-C), while cold ATP was able to compete with radioactive ATP for lrrktide phosphorylation. The apparent $K_m$ for ATP was 4.73+/−1.42 μM, a value comparable to that obtained with truncated LRRK2 [12]. We also found that guanine nucleotides did not alter the time course of phosphorylation either for lrrktide phosphorylation or for autophosphorylation (Figure 2).

In order to better control the nucleotide bound state of LRRK2, we prepared recombinant LRRK2 specifically preloaded with nucleotides via an in vitro loading procedure. In this procedure, purified proteins bound to the affinity resin are equilibrated in buffer containing an excess of nucleotides and incubated at 30°C to allow the loading of nucleotides to the GTP binding site. Unbound nucleotides are then washed away to yield a protein loaded with a specific nucleotide. The efficiency and specificity of the loading was tested using radioactively labeled GTP, which was completely outcompeted by an excess (200 μM) of various cold guanine nucleotides, while 200 μM ATP or CTP did not efficiently compete for GTP binding (Figure 3A). In addition, low binding levels were observed for the T1348N GTP-binding deficient mutant.

Recombinant protein prepared via this procedure and then eluted retained kinase activity both in autophosphorylation and in lrrktide phosphorylation. In these conditions, autophosphorylation was not significantly enhanced by GTP or GTP analogues compared to GDP. On the contrary, GTP or GTP analogues led to unchanged autophosphorylation levels or reduced autophosphorylation levels at the longer time points compared to GDP (Figure 3C). Lrrktide phosphorylation levels were not altered by GTP, GTPγS or GMPPCP compared to GDP (Figure 3D). At the longer time points GDP treated protein had a lowered kinase activity compared to mock treated protein (Figures 3C–D). By comparison, the LRRK2 GTP binding deficient mutant T1348N displayed very weak phosphorylation of lrrktide compared to wild type (Figure 3D) consistent with findings using autophosphorylation as a readout (Figure 3C and ref. [7]).

LRRK2 kinase activity is modestly enhanced by application of GTPγS or GMPPCP to cell lysates prior to protein purification

In a third series of experiments, nucleotides GDP, GTPγS or GMPPCP were added to cell lysates expressing 3xflag-LRRK2 and the lysate-nucleotide mix was incubated at 30°C for 30 minutes. Subsequently purified protein was tested for kinase activity by autophosphorylation assay (Fig. 4A–B) or lrrktide phosphorylation assay (Fig. 4C). 2-way anova analysis revealed a significant enhancement of activity in the GMPPCP and GTPγS groups compared to the GDP group. At 30 minutes incubation, the percent enhancement for lrrktide phosphorylation was 71.8+/−10.2 for GTPγS and 38.6+/−10.4 for GMPPCP while for autophosphorylation these values were +38.0+/−7.6% for GTPγS and +31.4+/−9.6 for GMPPCP (all values are mean +/- SEM).

**Figure 1. Kinase activity of recombinant LRRK2 protein when co-incubated with guanine nucleotides.** A. Silver stain of SDS-PAGE gel of purified LRRK2 used in the kinase assays. B. Lrrktide phosphorylation by LRRK2 (30 minute incubation) was performed with co-incubation of varying concentrations of nucleotides as described in materials and methods. Shown is a representative autoradiogram of P81 filter spotted with $^{32}$P labeled lrrktide from the different assay conditions (n = 4). C. Quantification of Lrrktide phosphorylation levels (y-axis) plotted against concentrations of nucleotides as described in materials and methods. Shown is a representative autoradiogram of P81 filter spotted with $^{32}$P labeled lrrktide from HEK293T cells, coupled to measures of autophosphorylation as well as LRRK2-mediated phosphorylation of lrrktide, a specific in vitro peptide substrate [11]. Our data show that an intact ROC-GTPase domain is required for LRRK2 kinase activity and that kinase activity remains unchanged upon direct application of GDP compared to GTP or non-hydrolyzable GTP analogues, reconciling discrepancies in previous reports.

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The macromolecular properties of purified LRRK2 are unchanged by guanine nucleotides

Because the dimerization state of LRRK2 has been linked to its activity [6,13,14], we decided to test whether LRRK2 macromolecular properties were altered in the presence of GDP compared to GTP. For this, analytical size exclusion chromatography (SEC) was performed on cleared cell lysates of LRRK2 expressing cells or on purified LRRK2 using a column equilibrated with SEC buffer containing 10 μM of either GDP or GTP. The resulting chromatograms are given in Figure 5. These show that LRRK2 displays a peak corresponding to the apparent size of a dimer. Higher molecular weight peaks are observed in the purified protein samples while these higher molecular weight species are absent or very low abundant in the cleared cell lysates. However, GTP addition did not change the SEC profiles compared to GDP.

To confirm this with a second technique, we used Native PAGE analysis of purified LRRK2 protein following lysate treatment with either GDP, GTP, or GMPPCP. All treatments displayed equivalent band profiles and were analogous to SEC results. In silver stained native PAGE gels as well as flag-M2 immunoblots of native gels, a band is observed around the expected size of the dimer with a smear of proteins at higher molecular weights until above 1.2 MDa (Figure 5E–F). Therefore, addition of non-hydrolyzable guanine nucleotides did not change the apparent molecular state of LRRK2.

Nucleotide treatment does not alter 14-3-3 binding nor phosphorylation state of LRRK2

The enhanced LRRK2 kinase activity observed when GTP analogues are applied to the cell lysates points to the presence of LRRK2 cellular cofactors which can modulate LRRK2 activity under influence of guanine nucleotides. One recently reported interactor of LRRK2, 14-3-3 [15,16] can be found in Ras-GTPase pathways [17]. Therefore, we tested whether guanine nucleotide treatment of LRRK2 would modulate the 14-3-3 binding. For this, we treated lysates expressing LRRK2 as described above and further purified LRRK2 under CoIP conditions. However we found that this treatment did not lead to altered 14-3-3 binding as measured by the CoIP assay [Supplementary figure S3]. Next we
assayed autophosphorylation levels of LRRK2 co-incubated with guanine nucleotides after co-immunoprecipitation, but no significant differences were observed (data not shown).

Phosphorylation of LRRK2 in cells depends on an intact ROC GTPase domain

It has been previously established that LRRK2 is phosphorylated in cells, with most sites clustering between residues 855 and 973 [7,8,18]. In order to examine whether nucleotides may influence the phosphorylation status of LRRK2 in cells, we performed metabolic labeling in intact cells and cell lysates. Figure 6A–B shows the incorporation of P\(^{32}\) in LRRK2 in normal cell lysates or after addition of GDP, GTP, GTP\(^{\gamma}\)S or GMPPCP. Although mean values were ~20% higher for GTP and its analogues compared to GDP, these differences were not statistically significant.

We also performed metabolic labeling of LRRK2 proteins, showing that wild type LRRK2 efficiently incorporates phosphates in cells; however, the K1347A and T1348N GTP binding deficient mutations of the ROC domain did not (Fig. 6C–D). Labeling of the N-terminal (1–1245, construct encompassing the constitutive phosphorylation region [18] but lacking ROC and other C-terminal domains) and C-terminal (972–2527, construct encompassing LRR and other C-terminal domains, but lacking most constitutive phosphorylation sites) half sequences of LRRK2 was also undetectable.
Discussion

The LRRK2 protein resembles a group of signaling cascade proteins tethered together in a single polypeptide chain. Indeed, Ras-GTPases homologous to LRRK2 ROC domain and tyrosine kinase like kinases homologous to the LRRK2 kinase domain are often found within the same signaling cascade. Signaling cascades of the Ras-GTPase family include both upstream and downstream kinases. For instance, in the case of Ras, a signaling cascade is initiated by ligand binding to a tyrosine kinase receptor which induces dimerization and autophosphorylation of the receptor [19]. This in turn leads to a series of events including the activation of src tyrosine kinase and the recruitment of accessory proteins that activate Ras protein by promoting GTP binding on Ras leading to a GTP bound form of Ras. Ras-GTP can bind its downstream effector Raf, a mitogen activated protein kinase kinase, which is the first of a three tiered cascade of kinases. Therefore two hypotheses can be formulated to explain the potential interplay between ROC and kinase [5].

A first hypothesis is that the LRRK2 kinase domain is a downstream effector of ROC. In this scenario, kinase activity of LRRK2 is predicted to be turned on when ROC binds GTP and turned off when ROC binds GDP. We first tested this hypothesis by performing kinase assays on purified LRRK2 in the presence of varying concentrations of guanine nucleotides. However, the addition of GTP or non-hydrolyzable GTP analogues GTP\textsubscript{S} or GMPPCP to purified LRRK2 did not significantly enhance LRRK2 activity in the lrrktide assay. Conversely, addition of GDP to the kinase reaction mix had no inhibitory effect on LRRK2 phosphorylation activity, indicating that GDP does not provide an off switch to LRRK2 kinase activity in these conditions. Also, the fact that guanine nucleotides are washed off of the protein in our purification protocol shows that guanine nucleotides are not a requirement for kinase activity. These observations are consistent with results obtained with a similar full length protein purified from a mouse expression system [10].

One potential caveat of testing effects of guanine nucleotides on kinase activity by simple addition of nucleotides to the enzymatic reaction is that two processes are occurring at the same time, namely \textit{in vitro} phosphorylation and guanine nucleotide exchange. In order to dissociate these two processes, we performed an \textit{in vitro} guanine nucleotide exchange such that proteins tested are a homogeneous mix of protein saturated with a specific guanine nucleotide at the moment of kinase activity testing. This loading
Figure 5. Influence of guanine nucleotides on macromolecular properties of LRRK2. A–D. Size exclusion chromatography elution profiles of LRRK2 in cell lysates or purified LRRK2 in the presence of GDP or GTPγS. 3xflag LRRK2 was expressed by transient transfection in HEK293T cells. Cleared cell lysates (A & C) or purified protein (B & D) were prepared as described in Materials and Methods and submitted to SEC in the presence of 10 μM of either GDP (A & B) or GTPγS (C & D). LRRK2 elution profiles were obtained by measuring LRRK2 levels in each elution fraction via immunodot blot and are displayed as relative signal intensity in function of the elution volume, as described in Materials and Methods. The elution peaks of the protein standards are indicated above the dot blot of panel A. E–F. Native PAGE of LRRK2 purified from lysates loaded with different concentrations of nucleotides. 3xflag tagged LRRK2 was expressed in HEK293T cells via transient transfection. Cells were lysed at 48 h post-transfection and incubated with varying concentrations of GDP, GTPγS or GMPCPP (10, 100 and 50 μM) for 30 minutes at 30 °C. Treated lysates were purified as described in materials and methods and separated via native PAGE. Gels were silver stained (top panel) or blotted onto PVDF membranes (bottom panel) to detect flag immunoreactivity E. LRRK2 protein amounts visualized via silver staining on SDS-PAGE are shown under the native PAGE images. F. Signal intensity plotted against migration distance for each lane. The arrow marks the peak corresponding to the band which migrates at the predicted size of a LRRK2 dimer. Data are representative of 3 experiments.

doI:10.1371/journal.pone.0023207.g005
been shown to be regulated by the degree of the cellular phosphorylation of LRRK2 [15,16], this lack of effect of guanine nucleotides on 14-3-3 binding correlates to the findings that guanine nucleotides do not alter the phosphorylation state of LRRK2 (figure 6B). As for the in vitro data, this finding contrasts with data from GTP binding deficient mutants (K1347A, T1348N) for which phospholabeling is quite inefficient (Figure 6).

Interestingly, simple overexpression of the cellular phosphorylation sites but without C-terminal sequences including ROC (amino acids 1–1245), is not sufficient for these sites to be phosphorylated in the cell. Therefore, we can conclude that constitutive phosphorylation of LRRK2 in cells requires the presence of an intact ROC GTPase domain; however it is not significantly modulated by GDP or GTP.

In summary, the present study illustrates that the nature of the guanine nucleotide bound to LRRK2 in cellular lysates. HEK293T cells expressing LRRK2 were lysed and incubated with ATP-32P for 30 minutes at 30 °C without additions (control) or in the presence of 10 μM GDP, GTP or the non hydrolyzable GTP analogues GTPγS and GMPCPP. LRRK2 was subsequently IP purified and submitted to SDS-PAGE and blotting to a PVDF membrane A. Shown here are the representative blot autoradiograms, immunoblot detection and ponceau staining of the phospholabeled samples. B. Quantification of A. C–D. Metabolic labeling of LRRK2 wt, GTP binding deficient LRRK2 mutants (K1347A, T1348N), and LRRK2 C-terminal (972–2527, encompassing ROC domain and lacking most cellular phosphorylation sites) and N-terminal (1–1245, encompassing cellular phosphorylation sites and lacking ROC domain) fragments. C. Representative blot autoradiograms and blot immunodetection of the metabolically labeled samples. D. Quantification of C. Data are representative of 4 experiments. Statistical differences of results in panels B and D were tested by one-way ANOVA as described in the materials and methods section. *** P<0.001.

doi:10.1371/journal.pone.0023207.g006
polyclonal anti 14–3–3 antibody was purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Lrrk2 peptide [11] was synthesized by Enzo life sciences.

Expression and purification of recombinant LRRK2 protein

HEK293T cells (ATCC CRL-11268) were transfected with pCHMWS-3xflag-LRRK2 plasmid using polyethyleneimine and lysed after 48–72 hours in lysis buffer (Tris 20 mM pH 7.5, NaCl 150 mM, EDTA 1 mM, Triton 1%, Glycerol 10%, protease inhibitor cocktail (Roche, Vilvoorde, Belgium)). Lysates were cleared by centrifugation at 20,000 g for 10 minutes and incubated with normal mouse IgGs bound to agarose beads (Sigma, Bornem, Belgium) to remove proteins specifically binding to agarose or mouse IgGs. After removal of the IgG bead slurry, lysates were incubated for 3 to 18 hours with flagM2 bound to agarose beads (Sigma). Beads were washed 4 times with wash buffer (Tris 25 mM pH 7.5, NaCl 400 mM, Triton 1%) and rinsed in kinase buffer (Tris 25 mM pH 7.5, MgCl2 10 mM, dithiothreitol (DTT) 2 mM, Triton 0.02%, beta-glycerophosphate 5 mM, Na2VO4 0.1 mM). For those assays using protein in solution, proteins were eluted in 5 volumes of kinase buffer containing 100 µg/ml 3xflag peptide (Sigma). For assays using purified protein bound to affinity resin, affinity beads were resuspended in an equal volume of kinase buffer unless otherwise indicated. Purity and concentration were assessed by SDS-PAGE containing 100 µg/ml 3xflag peptide (Sigma). For assays using protein in solution, proteins were eluted in 5 volumes of kinase buffer containing 100 µg/ml 3xflag peptide (Sigma). For assays using purified protein bound to affinity resin, purity beads were resuspended in an equal volume of kinase buffer unless otherwise indicated. Purity and concentration were assessed by SDS-PAGE (3–8% tris-acetate SDS gel, Invitrogen, Merelbeke, Belgium) and coomassie brilliant blue staining (Thermo Scientific, Hampton, NH, USA) or silver staining as shown in figure 1A (LRRK2 coomassie brilliant blue staining (Thermo Scientific, Hampton, NH, USA)).

In vitro phosphorylation assays

To assay autoprophosphorylation, eluted purified proteins or a suspension of affinity resin bound protein were incubated with 6 µCi of 32P-ATP or 32P-ATP (3000 Ci/mmol; Perkin Elmer) and 10 µM ATP for 40 µl reaction for 5–60 minutes at 30°C. Guanine nucleotides are also added to some kinase reactions to final concentrations as indicated in the results section. Reactions were terminated by adding 6× SDS loading buffer A (for eluted protein, composition: Tris 150 mM pH 6.8, 0.1% SDS, 30% glycerol, 120 µg/ml bromophenol blue, 10% beta-mercaptoethanol) or 2× SDS loading buffer B (for proteins bound to affinity resin, composition: Tris-HCl 160 mM pH 6.8, SDS 2%, DTT 0.2 M, glycerol 40%, bromophenol blue 2 mg/ml). Samples were loaded onto pre-cast Tris-acetate 3–8% gels (Invitrogen) or Tris-glycine 4–20% gels (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene fluoride (PVDF) membranes. Incorporated 33P or 32P was detected by autoradiography using a Storm 840 phosphorescence scanner (GE Healthcare). The same membranes were stained with Ponceau S (Sigma) to correct for protein loading and probed with DRAA/3EDD in house anti-LRRK2 kinase domain antibody [35] to confirm the presence of LRRK2. Densitometric analysis of the bands on the blot autoradiograms and immunoreactivity were performed using Aida analyzer v1.0 (Raytest, Straubenhardt, Germany) or ImagJ software (NIH, USA). Autophosphorylation levels were calculated as the ratio of the autoradiographic signal over the immunoreactivity level.

In enzymatic reactions testing lrrktide phosphorylation, reactions were prepared as described for autophosphorylation above with the addition of 200 µM lrrktide. Reactions were incubated at 30°C and stopped after 5–60 minutes by the addition of 500 mM EDTA containing bromophenol blue. Guanine nucleotides are also added to some kinase reactions to final concentrations as indicated in the results section. All reactions are carried out in the presence of 10 mM ‘cold’ ATP with the exception of the experiment testing varying concentrations of ATP (concentrations as indicated in the results section). Reactions were spotted to P81 phosphocellulose paper (Whatmann) and washed 4 times 10 minutes in 75 mM phosphoric acid. Lrrk2 phosphorylation levels were measured via scintillation counting or via autoradiography [36]. Kinase assays were performed for each condition using at least three independent protein preparations.

Nucleotide loading

For experiments in which LRRK2 was loaded with specific guanine nucleotides, affinity resin bound protein was washed as above, rinsed in loading buffer (Tris 25 mM pH 7.5, NaCl 150 mM, EDTA 5 mM, Triton 0.02%) and incubated with an excess (200 µM) GDP or GTP·S for 30 minutes at 30°C under light shaking [20,37]. Nucleotide exchange was stopped and excess nucleotides removed by rinsing beads 3 times in kinase buffer. Validation of the loading procedure was performed using the same protocol with radioactively labelled GTP (GTP-γ-32P), in the presence or absence of 200 µM ‘cold’ nucleotides.

Co-immunoprecipitation (Co-IP)

HEK293T cells were transfected with pCHMWS-3xflag-LRRK2 with polyethyleneimine and lysed after 48–72 hours in Co-IP buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM Na3EDTA, 1 mM PMSF, 0.1% Triton X-100, 10% glycerol, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche)). Lysates were centrifuged at 4°C for 10 minutes at 20,000 g and supernatant further cleared by incubation with normal mouse IgGs bound to agarose beads at 4°C with end over end mixing. After removal of the IgG beads by centrifugation, cleared lysates were incubated for 3 to 18 hours with flag M2 beads at 4°C. Beads were washed 4 times with Co-IP buffer. After four washes, immunoprecipitates were eluted by addition of 2× SDS loading buffer B. Samples were resolved on 3–8% tris-acetate gels. For detection of the 14-3-3 interaction, gels were blotted onto PVDF membranes and probed with goat anti 14-3-3 antibody (Santa Cruz). For those Co-IP preparations further submitted to in vitro autophosphorylation assay, beads were rinsed in kinase buffer, resuspended in equal volumes of kinase buffer and submitted to autophosphorylation in the presence or absence of nucleotides as described under ‘kinase assays’.

Metabolic labeling

For labeling in intact cells, LRRK2 or LRRK2 fragments were expressed via transient transfection in HEK293T cells. At 48–60 hours post-transfection, cells were rinsed two times in DMEM without phosphates then metabolically labeled with 5 µCi/cm2 orthophosphate-P32 (Perkin-Elmer) in DMEM without phosphates at 37°C. Following labeling, cells were lysed and LRRK2 was immunoprecipitated using flag-M2 agarose beads. Immunoprecipitated protein was resolved on 3–8% SDS-PAGE gels and blotted to PVDF membranes. Membranes were processed as described above for the autophosphorylation assay. To identify nucleotides to LRRK2, nucleotides were eluted from purified LRRK2 protein in kinase buffer with an excess of GTP (1 mM), then separated by thin-layer chromatography (TLC) (Merck, Darmstadt, Germany) and visualized by autoradiography using a Storm 840 phosphorescence scanner (GE Healthcare).

For metabolic labeling of LRRK2 in cell lysates, LRRK2 was expressed in HEK293T cells as described above and lysed in kinase buffer (see above) with EDTA-free protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Sigma) and 0.1%...
triton. Lysates were cleared by centrifugation at 20.000 g for 10 minutes and by incubation with normal mouse IgGs bound to agarose beads (Sigma). The cleared lysate was pooled and distributed into 5 tubes (500 µl lysate per tube). 20 µCi ATP-P32 was added to each tube, either without further additions (control reaction) or with addition of 10 µM of GDP, GTP, GTPyS or GMPPCP. Reactions were incubated at 30°C for 30 minutes under light shaking to keep protein in suspension. After incubation, the pan-kinase inhibitor staurosporine was added to a final concentration of 100 nM to halt kinase phosphorylation processes and lysates were incubated with flag-M2 affinity resin for 1–2 hours at 4°C. After washing affinity resin bound protein 4 times in IP wash buffer, 2× SDS loading buffer B was added and analyzed using SDS-PAGE as described above for the autophosphorylation assay. The labeling was repeated using at least three independent protein preparations per condition tested.

Analytical size-exclusion chromatography (SEC)

SEC was performed on cleared cell lysates as well as on purified protein. 3xflag-LRRK2 was expressed in HEK293T cells as described above. Cleared cell lysates were made by lysis of cells in SEC lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.1% Triton, 1 mM DTT, protease inhibitor cocktail (Roche)) then clearing via centrifugation at 20.000 g for 10 minutes followed by clearing with normal mouse Igs bound to agarose beads. Lysates were supplemented with either GDP or GTPyS 10 µM before loading onto the column. Purified protein for SEC analysis was obtained as described above using flag-M2 agarose beads, with the exception that proteins were eluted in SEC running buffer (25 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.02% Triton, 1 mM DTT) containing 100 µg/ml 3xflag peptide supplemented with 10 µM of either GDP or GTPyS.

Analysis was performed using a Superose 6 10/300 GL column (GE Healthcare) coupled to an AKTA purifier 10 UPC-900 system (GE Healthcare). The column was calibrated using protein standards (Gel Filtration Calibration Kit HMW, GE Healthcare: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and ovalbumin (44 kDa)) in 50 mM Tris pH 7.5, 100 mM KCl, 5% glycerol (supplemental figure S1). Before analysis, the column was equilibrated in SEC running buffer containing 10 µM of either GDP or GTPyS. SEC runs were performed at 4°C with 100 µl cell lysate or purified protein sample. Fractions (300 µl) were analyzed via dot blotting (Bio-Dot Microfiltration Apparatus, BioRad) onto nitrocellulose membrane (Bio-Rad) and detecting fractions immunoreactive to flag-M2 antibody. Signals were quantified by densitometry using Aida analyzer v1.0 (Raytest, Straubenhardt, Germany) and the elution profile was plotted as a percentage of the maximum signal. Molecular weights and Stokes radii were calculated from the standard curve obtained from the elution volumes of the standard proteins (supplementary figure S1), showing a resolution sufficient to discern alterations of 50–100 kDa in size.

Immunoblotting procedures

Protein samples were resolved by electrophoresis on 3–8% tris-acetate (Invitrogen) or 4–20% tris-glycine (Bio-Rad) gradient gels, and electroblotted to PVDF membranes. Membranes were blocked with 5% skimmed milk (w/v) in 30 mM Tris/HCl, pH 7.5, 0.15 M NaCl and 0.1% (v/v) Tween 20 (TBST Buffer). Antibodies were used at 1:10.000 in 5% (w/v) milk in TBST. Detection of immune-complexes was performed using horseradish-peroxidase-conjugated secondary antibodies (Dako, Heverlee, Belgium) and an enhanced-chemiluminescence reagent (Bio-Rad).

Statistics

For the statistical comparisons, test values were normalized to control (for instance the GDP group). In the dose range experiment (Figure 1C), changes in kinase activity in the guanine nucleotide groups was tested for by 2-way ANOVA with concentration and treatment as factors followed by a Bonferroni post test for each concentration using GDP as the control group. In the time course experiments (Figures 2, 3, 4), changes in kinase activity in the test groups compared to the GDP control group was tested for by 2-way ANOVA with time and treatment as factors followed by a Bonferroni post test for each time point. In other experiments, values from test groups were tested for significant differences from the control group using a one-way ANOVA followed by a Dunnett post-hoc test. Statistical significance was set at p<0.05.

Supporting Information

Figure S1 Calibration of size exclusion column. A. Chromatographic calibration curve for the standard proteins on Superose 6 10/300 GL column. The retention volume (Vt) of thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and ovalbumin (44 kDa) was determined from the A280 nm elution profile. Blue dextran was used to determine the void volume (V0) of the column (not shown). B. The experimental and calculated parameters for the equilibration of the Superose 6 10/300 GL column, with the apparent molecular weight (Mw), the elution volume (Vt), the void volume (V0), the gel phase distribution coefficient (Kav = (Vt-V0)/(Vt-V0)) and the Stokes’s radius. C. Calibration curve displaying the relationship between Ln(Mw) and Vt/V0, obtained with the standard proteins as run on Superose 6 10/300 GL column. (D) Calibration curve displaying the relationship between the Stokes radius and the Blue/Empty((log(Kav))).

Figure S2 Analysis of guanine nucleotide bound to LRRK2 as purified in this study. LRRK2 constructs (as in figure 6) were metabolically labeled with [32P]-orthophosphate and submitted to the affinity purification procedure described in the materials and methods. Thin-layer chromatographic analysis of bound nucleotides for LRRK2 WT, LRRK2 K1347A, LRRK2 K1340N and N-terminal and G-terminal fragments shows that the purification procedure washes out all nucleotides.

Figure S3 Evaluation of the effect of guanine nucleotides on the binding of 14-3-3 to LRRK2. Displayed is the western blot detection of 14-3-3 protein co-immunoprecipitated with 3xflag-LRRK2 following treatment of cell lysates with different guanine nucleotides. Representative of 2 experiments.

Acknowledgments

The authors are grateful to Fangye Gao for technical assistance.

Author Contributions

Conceived and designed the experiments: J-MT MRC. Performed the experiments: J-MT RV PO AB EL. Analyzed the data: J-MT RV AB MRC. Contributed reagents/materials/analysis tools: J-MT MDM VB MRC. Wrote the paper: J-MT MRC.
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