Identification of protein phosphatase 1 as a regulator of the LRRK2 phosphorylation cycle

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Abstract

A cluster of phosphorylation sites in LRRK2 (leucine-rich repeat kinase 2), including Ser[910], Ser[935], Ser[955] and Ser[973], is important for PD (Parkinson's disease) pathogenesis as several PD-linked LRRK2 mutants are dephosphorylated at these sites. LRRK2 is also dephosphorylated in cells after pharmacological inhibition of its kinase activity, which is currently proposed as a strategy for disease-modifying PD therapy. Despite this importance of LRRK2 dephosphorylation in mutant LRRK2 pathological mechanism(s) and in LRRK2's response to inhibition, the mechanism by which this occurs is unknown. Therefore we aimed to identify the phosphatase for LRRK2. Using a panel of recombinant phosphatases, we found that PP1 (protein phosphatase 1) efficiently dephosphorylates LRRK2 *in vitro.* PP1 activity on LRRK2 dephosphorylation was confirmed in cells using PP1 inhibition to reverse LRRK2 dephosphorylation induced by the potent LRRK2 kinase inhibitor LRRK2-IN1 as well as in R1441G mutant LRRK2. We also found that PP1 and LRRK2 can form a complex in cells. Furthermore, we observed that PP1 inhibition modulates LRRK2's cellular phenotype by reducing skein-like LRRK2-positive structures

associated with dephosphorylation. In conclusion, the present study reveals PP1 as the physiological LRRK2 phosphatase, responsible for LRRK2 dephosphorylation observed in PD mutant LRRK2 and after LRRK2 kinase inhibition.

Keywords

leucine-rich repeat kinase 2 (LRRK2); protein phosphatase 1 (PP1); Parkinson's disease; phosphorylation; phosphatase inhibition; skein-like structure

INTRODUCTION

Mutations in the *LRRK2* (leucine-rich repeat kinase 2) gene are a common cause of inherited forms of PD (Parkinson's disease) [1,2]. Moreover, genome-wide association studies have identified LRRK2 as a risk factor for sporadic PD [3–6]. LRRK2 encodes a large multi-domain protein comprising, among others, domains with GTPase and kinase activity. The essential catalytic core encompasses the GTPase domain, termed ROC (Ras of complex proteins), which is linked to the kinase domain via the COR (C-terminal of ROC) domain. The catalytic segment of LRRK2 is flanked at its C-terminus by a WD40 domain and at the N-terminus by armadillo and ankyrin repeats and the namesake leucine-rich repeat domain [7] (Supplementary Figure S1 at http://www.biochemj.org/bj/456/bj4560119add.htm). Of the many LRRK2 substitutions described, the majority of those that are proven to be pathogenic are concentrated in the catalytic core: N1437H, R1441C/G/H and Y1699C in the ROC–COR bidomain and G2019S and I2020T in the kinase domain [8] (Supplementary Figure S1). The most common mutation in patients, G2019S, is located in the kinase domain and has consistently been shown to increase kinase activity 2–3-fold [9], suggesting that targeting kinase activity might be a therapeutic strategy [10]. The other pathogenic substitutions, R1441C/G/H, Y1699C and I2020T, have more modest effects on kinase activity [9,11–13].

The LRRK2 protein is phosphorylated at multiple sites, but the regulation of its phosphorylation is not fully understood [14–18]. LRRK2 displays two distinct clusters of phosphosites, one set of autophosphorylation sites with low or no basal phosphorylation levels in cells and a second set of serine residues with high basal phosphorylation levels located between the ankyrin and leucine-rich repeat domains (Supplementary Figure S1) [19]. Evidence suggests that these sites, including Ser^{910}, Ser^{935}, Ser^{955} and Ser^{973}, are phosphorylated by kinases other than LRRK2 itself ([12,16,17,19,20] and Supplementary Figure S2 at http://www.biochemj.org/bj/456/bj4560119add.htm). Some nominated kinases are PKA (protein kinase A) or the IKK (inhibitor of κB kinase) family which phosphorylate LRRK2 when activated [16,21], although administration of the PKA inhibitor H89 does not significantly block total or Ser^{935} phosphorylation [22,23]. Antibodies against the Ser^{910}/Ser^{935}/Ser^{955}/Ser^{973} sites label LRRK2 extracted from cells or from the brain, showing that these are physiologically relevant modifications of the protein *in vivo* [24].

Changes in the cellular LRRK2 phosphorylation status are linked to the pathogenesis of LRRK2-related PD as disease-associated mutations R1441G/C/H, Y1699C, I2020T and G2385R display reduced phosphorylation at these sites compared with WT (wild-type)

LRRK2 [16,17,20]. Cellular LRRK2 phosphorylation governs a number of LRRK2-mediated processes, including regulation of the cellular LRRK2 complex and subcellular localization. Indeed, decreased cellular phosphorylation correlates with decrements in phospho-dependent 14-3-3 binding and consequent subcellular relocalization of LRRK2 [17]. PD mutations that induce cytoplasmic accumulation of LRRK2 can relocalize to both filamentous and large inclusions in different cell types [17,25–27].

Interestingly, many laboratories have observed that acute inhibition of LRRK2 kinase activity leads to dephosphorylation of LRRK2, loss of 14-3-3 binding [15,16,20,24] as well as subcellular relocalization to skein-like structures [14,15,24]. Typically, these structures are filamentous and co-localize with microtubules [15]. This is a crucial observation, as this potential therapeutic avenue for PD induces cellular phenotypes (decreased phosphorylation, loss of 14-3-3 binding and relocalization) that are similar to pathogenic PD mutations in LRRK2. The nexus of these events is the dephosphorylation of LRRK2.

Taken together, the available data show that phosphorylation of LRRK2 is a highly regulated physiological event that is relevant to the effects of PD-related mutations and represents a potential pharmacodynamic marker for the effects of kinase inhibitors [22]. However, little is known about the regulation of LRRK2 phosphorylation and the data indicate that dephosphorylation is a unifying theme of PD mutations and LRRK2 kinase inhibition. We therefore set out to identify the major phosphatase(s) responsible for regulating LRRK2 phosphorylation. Using a combination of *in vitro*, pharmacological and molecular approaches, we identified PP1 (protein phosphatase 1) as a key LRRK2 phosphatase in physiological and pathological conditions. These results provide a mechanistic explanation for the altered phosphorylation of pathogenic LRRK2 variants and following cellular treatment with the potent LRRK2 kinase inhibitor LRRK2-IN1 [24].

MATERIALS AND METHODS

Cell culture and transfection

HEK (human embryonic kidney)-293T cells, NTH 3T3 cells and SH-SY5Y cells were maintained in DMEM (Dulbecco's modified Eagle's medium) (Gibco-Life Technologies) supplemented with 8% or 15% heat-inactivated FBS (Harlan Sera-Lab) and 50 μg/ml gentamycin at 37 °C in a humidified atmosphere containing 5% CO_2. A549 cells were maintained in RPMI medium with L-glutamine supplemented with 10% FBS, and 0.1 mM MEM (modified essential medium) Non-Essential Amino Acids solution. Overexpression of LRRK2 in HEK-293T and SH-SY5Y cells was obtained by transduction with a lentiviral vector encoding 3×FLAG-LRRK2 or eGFP-LRRK2. Lentiviral vectors encoding human 3×FLAG-LRRK2 and eGFP-LRRK2 under control of the CMV (cytomegalovirus) promoter were prepared as described previously [28]. For transduction in cell culture, 50000 HEK-293T or SH-SY5Y cells were plated in a 24-well plate. The next day, vector was applied to the cells for 2 days. This procedure was repeated twice with the same cells to obtain triple transduced cells. These cells were then expanded for use in experiments. HEK-293 T-REx cell lines were cultured in DMEM supplemented with 10 % FBS and 2 mM glutamine, 1 × antimycotic/antibiotic, 15 μg/ml blasticidin and 100 μg/ml hygromycin. T-REx cultures were induced to express the indicated protein by inclusion of 1 μg/ml

doxycycline in the culture medium for 24 h. Transfections were performed 24 h after seeding for 48 h with linear polyethylenimine (Polysciences Europe) according to the manufacturer's protocol. For co-immunoprecipitation, T-REx HEK-293 cells were transfected using polyethylenimine [29]. All cultures were mycoplasma-free.

Pharmacological treatment of cells

Cells grown until 50–100% confluency were treated with the indicated compounds, dissolved in pre-warmed culture medium to the final treatment concentration, for the time indicated and with DMSO treatment as a negative control. Except where indicated, cells were rinsed twice in PBS and lysed in lysis buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 10 % (v/v) glycerol, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche)] or as described in [14]. Cell lysates were cleared by centrifugation at 15000 g for 10 min. Membranes were processed as described in the Supplementary Materials and methods section (at http://www.biochemj.org/bj/456/bj4560119add.htm).

Metabolic labelling

Metabolic labelling was performed essentially as described in [30]. Briefly, cells expressing LRRK2 were rinsed twice in DMEM without phosphates and then labelled with 5 μCi/cm^2 [^{32}P]P$_i$ (PerkinElmer) at 37 °C. Following 4–8 h labelling, cells were lysed and LRRK2 was immunoprecipitated using FLAG-M2-agarose beads (Sigma). Immunoprecipitated protein was resolved on Nupage 3–8 % tris-acetate SDS/PAGE gels (Invitrogen) and immunoblotted. Membranes were stained with Ponceau S (Sigma) to visualize proteins. Membranes were processed as described in the Supplementary Materials and methods section.

Statistics

Unless otherwise indicated, the Figures shown are representative of at least three independent experiments. Phosphorylation levels were normalized for expression levels and experimental test conditions were normalized to control conditions. Statistical analysis was performed using one-way ANOVA or one-sample t test comparing test values with the hypothetical value of 1 with Bonferroni correction for multiple comparisons as appropriate. For counting of skein-like structures in cells, analysis was performed with Fisher's exact test on the proportion of cells with skein-like inclusions out of all transfection-positive cells. Statistical significance was set at $P < 0.05$.

RESULTS

PP1a efficiently dephosphorylates LRRK2 *in vitro*

We first identified phosphatases able to dephosphorylate cellular LRRK2 phosphorylation sites *in vitro* from a panel of catalytic subunits of common serine/threonine phosphatases. To visualize total phosphorylation levels, LRRK2 was metabolically labelled with ^{32}P in HEK-293T cells overexpressing 3×FLAG-LRRK2, affinity purified and incubated with phosphatases at a limiting concentration (12.5 units/ml, see the Supplementary Materials and methods section). Alkaline phosphatase (250 units/ml) was included as a positive control

[17]. All phosphatases were active under these experimental conditions against the test substrates phosphorylated MBP (myelin basic protein) or pNPP (*p*-nitrophenyl phosphate) (Supplementary Figure S3 at http://www.biochemj.org/bj/4567bj4560119add.htm). We found that PP1α (HGNC symbol *PPP1CA*) was able to dephosphorylate LRRK2 to the same extent as the positive control. Using antibodies against pSer^{910}, pSer^{935}, pSer^{955} and pSer^{973} LRRK2, we found that all individual sites were efficiently dephosphorylated by PP1α. Partial dephosphorylation was observed after treatment with PP2A1 (HGNC symbol *PPP2CA*) as well as with PPλ (Figure 1), although this did not reach statistical significance. These results identify PP1α as a candidate phosphatase for the constitutive phosphorylation sites of LRRK2.

LRRK2 interacts with PP1α

We next considered whether different PP1 isoforms were LRRK2 phosphatases *in vivo.* First, we examined localization in cells and found that LRRK2 strongly co-localized with PP1α and PP1β in the cytoplasm (Figure 2A). PP1γ was primarily localized to the nucleus, although LRRK2 co-localized with the fraction of PP1γ present in the cytoplasm (Figure 2A). Secondly, we examined whether PP1 isoforms physically bound LRRK2. We transiently expressed FLAG-tagged PP1 isoforms or PP2Aα in HEK-293 T-REx cells expressing GFP or GFP-LRRK2. We found that PP1α formed a non-ionic detergent-stable complex with LRRK2, whereas interaction of LRRK2 with PP1β, PP1γ or PP2Aα was not detected under the same conditions (Figure 2B). We also observed that the LRRK2-PP1α interaction was enhanced after LRRK2-IN1 treatment and abolished after treatment with calyculin A (an inhibitor of PP1 and PP2A). The immunoblot for pSer^{935} and the 14-3-3 overlay assay were used to confirm the induced dephosphorylation of LRRK2 after inhibition [17,20] (Figure 2B).

Thirdly, we verified spatial proximity of these molecules *in situ* using the PLA (proximity ligation assay) [31], We performed *in situ* PLA on cells co-expressing FLAG-tagged PP1α and GFP-LRRK2 and quantified the number of individual ligation amplification signals per cell (Figure 2C). Control amplifications with only single antibody (anti-GFP or anti-FLAG alone) staining, where both antigens are present yielded no PLA signals. Additionally, no PLA signals were observed when both antibody probes were applied to vector-transfected cells, where tagged PP1 is not present but LRRK2 is expressed. We observed specific and significant PLA signals for PP1 and LRRK2 when antibodies against both epitope tags are used and both GFP-LRRK2 and FLAG-PP1 are expressed, verifying *in vivo* interaction of these molecules.

The enhanced interaction of PP1α with LRRK2 after acute inhibition prompted us to test whether there is a similar recruitment of PP1 to mutants of LRRK2 that are dephosphorylated. We compared the interaction of FLAG-PP1α with GFP variants of LRRK2 expressed in inducible HEK-293 T-REx cells and found enhanced binding for mutants with reduced basal phosphorylation, such as N1437H, R1441G and Y1699C, as well as for the combined S910/935/955/973A (Figure 2D). As a positive control, we tested for HSP90 (heat-shock protein 90) co-immunoprecipitation and observed interaction with

LRRK2 that was enhanced with LRRK2 aggregation-prone mutations as seen previously [17,20,32].

We confirmed the LRRK2–PP1 interaction for endogenous LRRK2 and PPlα in A549 cells (Figure 2E). Furthermore, we could show an LRRK2-IN1 -induced increase in the endogenous PPlα–LRRK2 interaction (Figure 2E).

Pharmacological inhibition of PP1 prevents LRRK2 dephosphorylation

We next tested whether inhibition of PP1 with small molecules would prevent dephosphorylation of LRRK2. We first treated HEK-293T cells, stably overexpressing LRRK2, with calyculin A or okadaic acid (a PP2A-selective inhibitor at the used concentration) [33] and found that neither compound affected overall LRRK2 phosphorylation under basal conditions (Figure 3A). Next, we tested phosphatase inhibition in conditions of induced dephosphorylation after inhibition of LRRK2 kinase activity and found that calyculin A prevents the dephosphorylation effect of LRRK2-IN1, whereas okadaic acid did not (Figure 3A). These results suggest that PP1 is an authentic phosphatase for LRRK2, and its effect is strongest under conditions where LRRK2 dephosphorylation is induced.

We confirmed these results using phospho-LRRK2-specific antibodies. First, T-REx HEK-293 cells with WT LRRK2 overexpression were treated with calyculin A or okadaic acid at different concentrations, in the presence or absence of LRRK2-IN1. Consistent with the metabolic labelling data, LRRK2-IN1-induced LRRK2 dephosphorylation could be prevented by calyculin A, but not by okadaic acid (Figure 3B). Interestingly, the low basal phosphorylation level of the LRRK2 R1441G mutant was restored to WT levels by treatment with calyculin A, but not with okadaic acid (Figure 3B). LRRK2-IN1-induced LRRK2 dephosphorylation was counteracted by calyculin A treatment, even at relatively low concentrations of 5 or 20 nM, but not by okadaic acid at concentrations up to 100 nM in a second cell type, stable SH-SY5Y cell lines (Figure 3C). We also employed a recently developed homogeneous and ratiometric assay to detect LRRK2 modification at Ser^{935} [22] in the absence or presence of LRRK2-IN1 (1 μM) across a dilution series of calyculin A. Calyculin A treatment in the low nanomolar range (EC_{50} = 6.98 nM) efficiently suppressed the induced dephosphorylation by 1 μM LRRK2-IN1 (Figure 3D) as well as by 93 nM or 10 μM LRRK2-IN1 (results not shown).

We next asked whether the regulation of LRRK2 phosphorylation is also similar for endogenous protein. Similar to our experiments on overexpressed LRRK2, treatment with calyculin A could also overcome endogenous LRRK2 dephosphorylation induced by LRRK2-IN1 in NIH 3T3 cells (Figure 4A) and in primary neurons (Figure 4B). In addition, calyculin A increased endogenous LRRK2 phosphorylation levels in primary neurons (Figure 4B). Okadaic acid treatment also showed a trend to increased pSer^{935} levels, suggesting that FP2A may also contribute in neurons, although this was to a much lower extent than calyculin A. These results show that, in all cell types tested, including in neurons, PP1 is the major phosphatase for LRRK2.

Molecular inhibition of PP1 counteracts LRRK2 dephosphorylation induced by kinase inhibition

Since no specific pharmacological PP1 inhibitors are available, we wanted to confirm our data using highly specific and potent protein inhibitors of PP1, namely I2 (inhibitor-2, HGNC symbol *PPP1R2*) [34] or the central domain (residues 143–224) of NIPP1 (nuclear inhibitor of PP1, HGNC symbol *PPP1R8*) [35]. We overexpressed these protein inhibitors of PP1 in HEK-293T cells stably overexpressing LRRK2 (Supplementary Figure S4 at http://www.biochemj.org/bj/456/bj4560119add.htm) and investigated the effects on the phosphorylation level of LRRK2. Overexpression of I2 or NIPP1$_{143-224}$ had no effect on basal LRRK2 phosphorylation level compared with an eGFP control. However, overexpression of NIPP1$_{143-224}$ prevented LRRK2-IN1-induced LRRK2 dephosphorylation, consistent with the effects of pharmacological PP1 inhibition using calyculin A (Figure 5).

Pharmacological Inhibition of PP1 prevents formation of LRRK2 skein-like structures

Dephosphorylation of LRRK2 induced by kinase inhibition [14,15,24] or by some pathogenic mutations [17,25–27] causes redistribution into skein-like structures. If PP1 dephosphorylates LRRK2 to induce these structures, then inhibition of PP1 should reverse the effects of mutations or acute kinase inhibition. We transiently expressed WT LRRK2 or a series of mutants (S910A/S935A, R1441C, R1441G, Y1699C, K1906M, G2019S and I2020T) in HEK-293-FT cells (Figures 6A and 6B) and investigated the effect of PP1 inhibition in the presence or absence of LRRK2-IN1. Approximately 45% of cells expressing WT LRRK2 formed skein-like structures after LRRK2-IN1 treatment (1 μM for 30 min), which was significantly reduced in the presence of 5 nM calyculin A (only 22 % of cells had skein-like structures, Figure 6C). A similar decrease in cells with skein-like inclusions was observed for the pathogenic LRRK2 mutants R1441C, Y1699C and G2019S after treatment with both drugs. However, we did not observe a significant reduction in the number of cells with skein-like structures for S910A/S935A, R1441G and I2020T mutants, possibly related to a strong binding of these variants to PP1α (results not shown).

The decreased percentage of cells with skein-like structures after treatment with only 5 nM of calyculin A, both in conditions of LRRK2-IN1 treatment and in mutations, confirms the link between LRRK2 dephosphorylation and the formation of skein-like inclusions and point to a role for PP1 in this process.

DISCUSSION

Similar to many kinases, LRRK2 is also a phosphoprotein and a particular cluster of phosphosites in the interdomain region between the ankyrin repeat and leucine-rich repeat domains, including sites Ser^{910}/Ser^{935}/Ser^{955}/Ser^{973}, is subject to regulation. The identification of regulators of LRRK2 cellular phosphorylation at these sites may provide an insight into the molecular basis of PD as well as into the consequences of kinase inhibition. Although studies researching kinases upstream of these sites have begun to be reported [16,21,23], there are no reports of phosphatases that can affect cellular LRRK2 phosphorylation. In the present study we employed a combination of *in vitro*,

pharmacological and molecular cell biology approaches to identify phosphatases regulating LRRK2 phosphorylation at $Ser^{910}/Ser^{935}/Ser^{955}/Ser^{973}$.

First, using a panel of recombinant serine-threonine phosphatases, we show that only PP1 efficiently dephosphorylated LRRK2 *in vitro* at the four different cellular phosphorylation sites tested. Secondly, pharmacological inhibition of PP1, but not of PP2A, prevents LRRK2-IN1-induced dephosphorylation in five different cell types, including in HEK-293T, SH-SY5Y and U20S cell lines as well as on endogenous LRRK2 in NIH 3T3 cells and mouse primary neuron cultures. It should be noted that a trend towards increased phosphorylation was observed after PP2A inhibition with okadaic acid in primary neurons, suggesting that PP2A may play an auxiliary role in dephosphorylation of LRRK2 under specific conditions or cell types. Inhibition of PP1 also restores the reduced phosphorylation level of the R1441G disease mutant to WT levels. Finally, the functional relevance of PP1-mediated LRRK2 phosphoregulation was confirmed by the observation that PP1 inhibition reduces the formation of skein-like structures, which have previously been shown to be associated with dephosphorylated LRRK2 [14,15,17,24–27], both for mutant forms of LRRK2 as well as after LRRK2 kinase inhibition. From these results, we conclude that PP1 is a regulator of the LRRK2 phosphorylation cycle (Figure 7).

The results of the present study suggest that LRRK2 dephosphorylation involves the enhanced access of PP1 to LRRK2 phosphosites. The PP1α interaction with LRRK2 is weak under basal conditions, consistent with rapid binding kinetics for phosphatases. However, we found that some pathogenic mutant forms of LRRK2, such as R1441G or Y1699C, had increased interaction with PP1α. These data are consistent with the previous observation that most LRRK2 PD mutations have decreased phosphorylation at Ser^{910}/Ser^{935} [14,16,17]. Our data also show that transient inhibition of kinase activity of LRRK2 by small molecules leads to enhanced interaction between PP1α and LRRK2.

Several considerations point to conformational changes in LRRK2 to enable PP1 action as a regulatory mechanism of LRRK2 dephosphorylation. For example, pathogenic mutants with reduced phosphorylation levels such as R1441G and Y1699C display a weakened dimerization which could be explained by an enhanced intramolecular ROC–COR interaction [36,37]. Therefore enhanced PP1 recruitment/activity appears to correlate to altered properties of the LRRK2 catalytic core either via reduced ROC–COR dimerization or through structural or compound-induced changes in the ATP-binding pocket of the kinase domain. Resolving the crystal structure of the entire LRRK2 molecule may be required to further test these hypotheses.

Phosphorylation levels of cellular proteins are determined by the net effect of phosphorylation by kinases and dephosphorylation by phosphatases. To date, PKA [16,23] and IκB (inhibitor of nuclear factor κB) kinases [21] have been suggested to be responsible for the cellular phosphorylation of LRRK2. However, the rapid dephosphorylation induced by LRRK2-IN1 (>80% dephosphorylation after 90 min [21,24]) and the relatively long half-life of LRRK2 in cells [38,39], suggests that enhanced dephosphorylation rather than decreased phosphorylation of LRRK2 by kinases could be the primary mechanism of

LRRK2 dephosphorylation. This conclusion is also in line with our finding that PP1 activity is necessary for LRRK2 to become dephosphorylated.

Cellular PP1 activity is provided by a holoenzyme, which consists of a catalytic subunit bound to one or two regulatory subunits that provide substrate specificity [40,41]. There are three different isoforms of the PP1 catalytic subunit, namely PP1α, PP1β and PP1γ, which have identical catalytic properties and are all sensitive to the inhibitors used in the present study. As our results show that PP1α preferentially interacts with LRRK2, this suggests that PP1α is the primary PP1 isoform active on LRRK2. All three PP1 isoforms are widely expressed with high abundance in the brain where they have been shown to play a role in different neuronal processes such as synaptic plasticity and memory [42,43]. Intriguingly, both LRRK2 [44,45] and PP1α [46] are specifically enriched in the striatum.

The present study points to new possibilities in targeting LRRK2 function. PP1 can be targeted in a highly specific manner to its substrate through its interaction with regulatory subunits [41]. This unique complex, including the PP1 substrate, PP1 and PP1 regulatory subunits could be disrupted with specific protein–protein interaction inhibitors of the interaction between the PP1 catalytic subunit (PP1α) and its regulatory subunits or its substrate [47]. Further research is needed to identify the specific regulatory components of the LRRK2-specific PP1 holoenzyme in order to test this approach.

In summary, we provide the first report confirming a phosphatase, PP1, as a physiological regulator of LRRK2 cellular phosphorylation in cell lines and in neurons. Mechanistically, we find that dephosphorylation is perhaps mediated by enhanced accessibility of PP1 to the LRRK2 complex, thereby explaining why LRRK2 is dephosphorylated following kinase inhibition or in disease mutants. By revealing a major regulator of LRRK2 phosphorylation in cells, the present study provides new insight into the regulation of LRRK2 physiological and pathological functions and points to the molecular consequences of a much discussed potential therapy for PD, namely LRRK2 kinase inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

COR	C-terminal of ROC
DMEM	Dulbecco's modified Eagle's medium
HEK	human embryonic kidney
I2	inhibitor-2
LRRK2	leucine-rich repeat kinase 2
NIPP-1	nuclear inhibitor of protein phosphatase 1
PD	Parkinson's disease
PKA	protein kinase A
PLA	proximity ligation assay
PP	protein phosphatase
ROC	Ras of complex proteins
WT	wild-type

References

1. Paisan-Ruiz C. LRRK2 gene variation and its contribution to Parkinson disease. Hum Mutat. 2009; 30:1153–1160. [PubMed: 19472409]

2. Gasser T. Molecular pathogenesis of Parkinson disease: insights from genetic studies. Expert Rev Mol Med. 2009; 11:e22. [PubMed: 19631006]

3. Satake W, Nakabayashi Y, Mizuta I, Hirota Y, Ito C, Kubo M, Kawaguchi T, Tsunoda T, Watanabe M, Takeda A, et al. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. Nat Genet. 2009; 41:1303–1307. [PubMed: 19915576]

4. Simon-Sanchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, Paisan-Ruiz C, Lichtner P, Scholz SW, Hernandez DG, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet. 2009; 41:1308–1312. [PubMed: 19915575]

5. Lill CM, Roehr JT, McQueen MB, Kavvoura FK, Bagade S, Schjeide BM, Schjeide LM, Meissner E, Zauft U, Allen NC, et al. Comprehensive research synopsis and systematic meta-analyses in Parkinson's disease genetics: the PDGene database. PLoS Genet. 2012; 8:e1002548. [PubMed: 22438815]

6. Ross OA, Soto-Ortolaza AI, Heckman MG, Aasly JO, Abahuni N, Annesi G, Bacon JA, Bardien S, Bozi M, Brice A, et al. Association of LRRK2 exonic variants with susceptibility to Parkinson's disease: a case-control study. Lancet Neurol. 2011; 10:898–908. [PubMed: 21885347]

7. Mata IF, Wedemeyer WJ, Farrer MJ, Taylor JP, Gallo KA. LRRK2 in Parkinson's disease: protein domains and functional insights. Trends Neurosci. 2006; 29:286–293. [PubMed: 16616379]

8. Corti O, Lesage S, Brice A. What genetics tells us about the causes and mechanisms of Parkinson's disease. Physiol Rev. 2011; 91:1161–1218. [PubMed: 22013209]

9. West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, Dawson VL, Dawson TM. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proc Natl Acad Sei USA. 2005; 102:16842–16847.

10. Vancraenenbroeck R, Lobbestael E, De Maeyer M, Baekelandt V, Taymans JM. Kinases as targets for Parkinson's disease; from genetics to therapy. CNS Neurol Disord: Drug Targets. 2011; 10:724–740. [PubMed: 21838679]

11. Greggio E, Cookson MR. Leucine-rich repeat kinase 2 mutations and Parkinson's disease: three questions. ASN NEURO. 2009; 1 art:e00002. doi: 10.1042/AN20090007

12. West AB, Moore DJ, Choi C, Andrabi SA, Li X, Dikeman D, Biskup S, Zhang Z, Lim KL, Dawson VL, Dawson TM. Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. Hum Mol Genet. 2007; 16:223–232. [PubMed: 17200152]

13. Webber PJ, Smith AD, Sen S, Renfrew MB, Mobley JA, West AB. Autophosphorylation in the leucine-rich repeat kinase 2 (LRRK2) GTPase domain modifies kinase and GTP-binding activities. J Mol Biol. 2011; 412:94–110. [PubMed: 21806997]

14. Doggett EA, Zhao J, Mork CN, Hu D, Nichols RJ. Phosphorylation of LRRK2 serines 955 and 973 is disrupted by Parkinson's disease mutations and LRRK2 pharmacological inhibition. J Neurochem. 2011; 120:37–45. [PubMed: 22004453]

15. Dzamko N, Deak M, Hentati F, Reith AD, Prescott AR, Alessi DR, Nichols RJ. Inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser^{910}/Ser^{935}, disruption of 14-13-3 binding and altered cytoplasmic localization. Biochem J. 2010; 430:405–413. [PubMed: 20659021]

16. Li X, Wang QJ, Pan N, Lee S, Zhao Y, Chait BT, Yue Z. Phosphorylation-dependent 14-13-3 binding to LRRK2 is impaired by common mutations of familial Parkinson's disease. PLoS ONE. 2011; 6:e17153. [PubMed: 21390248]

17. Nichols RJ, Dzamko N, Morrice NA, Campbell DG, Deak M, Ordureau A, Macartney T, Tong Y, Shen J, Prescott AR, Alessi DR. 14-13-3 binding to LRRK2 is disrupted by multiple Parkinson's disease-associated mutations and regulates cytoplasmic localization. Biochem J. 2010; 430:393–404. [PubMed: 20642453]

18. Lobbestael E, Baekelandt V, Taymans JM. Phosphorylation of LRRK2: from kinase to substrate. Biochem Soc Trans. 2012; 40:1102–1110. [PubMed: 22988873]

19. Gloeckner CJ, Boldt K, von Zweydorf F, Helm S, Wiesent L, Sarioglu H, Ueffing M. Phosphopeptide analysis reveals two discrete clusters of phosphorylation in the N-terminus and the Roc domain of the Parkinson-disease associated protein kinase LRRK2. J Proteome Res. 2010; 9:1738–1745. [PubMed: 20108944]

20. Doggert EA, Zhao J, Mork CN, Hu D, Nichols RJ. Phosphorylation of LRRK2 serines 955 and 973 is disrupted by Parkinson's disease mutations and LRRK2 pharmacological inhibition. J Neurochem. 2012; 120:37–45. [PubMed: 22004453]

21. Dzamko N, Inesta-Vaquera F, Zhang J, Xie C, Cai H, Arthur S, Tan L, Choi H, Gray N, Cohen P, et al. The IκB kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during toll-like receptor signaling. PLoS ONE. 2012; 7:e39132. [PubMed: 22723946]

22. Hermanson SB, Carlson CB, Riddle SM, Zhao J, Vogel KW, Nichols RJ, Bi K. Screening for novel LRRK2 inhibitors using a high-throughput TR-FRET cellular assay for LRRK2 Ser935 phosphorylation. PLoS ONE. 2012; 7:e43580. [PubMed: 22952710]

23. Ito G, Okai T, Fujino G, Takeda K, Ichijo H, Katada T, Iwatsubo T. GTP binding is essential to the protein kinase activity of LRRK2, a causative gene product for familial Parkinson's disease. Biochemistry. 2007; 46:1380–1388. [PubMed: 17260967]

24. Deng X, Dzamko N, Prescott A, Davies P, Liu Q, Yang Q, Lee JD, Patricelli MP, Nomanbhoy TK, Alessi DR, Gray NS. Characterization of a selective inhibitor of the Parkinson's disease kinase LRRK2. Nat Chem Biol. 2011; 7:203–205. [PubMed: 21378983]

25. Alegre-Abarrategui J, Christian H, Lufino MM, Mutihac R, Venda LL, Ansorge O, Wade-Martins R. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. Hum Mol Genet. 2009; 18:4022–4034. [PubMed: 19640926]

26. Greggio E, Jain S, Kingsbury A, Bandopadhyay R, Lewis P, Kaganovich A, van der Brug MP, Beilina A, Blackinton J, Thomas KJ, et al. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. Neurobiol Dis. 2006; 23:329–341. [PubMed: 16750377]

27. Kett LR, Boassa D, Ho CC, Rideout HJ, Hu J, Terada M, Ellisman M, Dauer WT. LRRK2 Parkinson disease mutations enhance its microtubule association. Hum Mol Genet. 2011; 21:890–899. [PubMed: 22080837]

28. Geraerts M, Michiels M, Baekelandt V, Debyser Z, Gijsbers R. Upscaling of lentiviral vector production by tangential flow filtration. J Gene Med. 2005; 7:1299–1310. [PubMed: 15906396]

29. Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE. Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. J Virol Methods. 2006; 138:85–98. [PubMed: 16950522]

30. Taymans JM, Vancraenenbroeck R, Ollikainen P, Beilina A, Lobbestael E, De Maeyer M, Baekelandt V, Cookson MR. LRRK2 kinase activity is dependent on LRRK2 GTP binding capacity but independent of LRRK2 GTP binding. PLoS ONE. 2011; 6:e23207. [PubMed: 21858031]

31. Weibrecht I, Leuchowius KJ, Clausson CM, Conze T, Jarvius M, Howell WM, Kamali-Moghaddam M, Soderberg O. Proximity ligation assays: a recent addition to the proteomics toolbox. Expert Rev Proteomics. 2010; 7:401–409. [PubMed: 20536310]

32. Rudenko IN, Kaganovich A, Hauser DN, Beylina A, Chia R, Ding J, Maric D, Jaffe H, Cookson MR. The G2385R variant of leucine-rich repeat kinase 2 associated with Parkinson's disease is a partial loss of function mutation. Biochem J. 2012; 446:99–111. [PubMed: 22612223]

33. Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, et al. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun. 1989; 159:871–877. [PubMed: 2539153]

34. Hurley TD, Yang J, Zhang L, Goodwin KD, Zou Q, Cortese M, Dunker AK, DePaoli-Roach AA. Structural basis for regulation of protein phosphatase 1 by inhibitor-2. J Biol Chem. 2007; 282:28874–28883. [PubMed: 17636256]

35. Beullens M, Van Eynde A, Vulsteke V, Connor J, Shenolikar S, Stalmans W, Bollen M. Molecular determinants of nuclear protein phosphatase-1 regulation by NIPP-1. J Biol Chem. 1999; 274:14053–14061. [PubMed: 10318819]

36. Daniëls V, Vancraenenbroeck R, Law BM, Greggio E, Lobbestael E, Gao F, De Maeyer M, Cookson MR, Harvey K, Baekelandt V, Taymans JM. Insight into the mode of action of the LRRK2 Y1699C pathogenic mutant. J Neurochem. 2011; 116:304–315. [PubMed: 21073465]

37. Klein CL, Rovelli G, Springer W, Schall C, Gasser T, Kahle PJ. Homo-and heterodimerization of ROCO kinases: LRRK2 kinase inhibition by the LRRK2 ROCO fragment. J Neurochem. 2009; 111:703–715. [PubMed: 19712061]

38. Liu Z, Lee J, Krummey S, Lu W, Cai H, Lenardo MJ. The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. Nat Immunol. 2011; 12:1063–1070. [PubMed: 21983832]

39. Wang L, Xie C, Greggio E, Parisiadou L, Shim H, Sun L, Chandran J, Lin X, Lai C, Yang WJ, et al. The chaperone activity of heat shock protein 90 is critical for maintaining the stability of leucine-rich repeat kinase 2. J Neurosci. 2008; 28:3384–3391. [PubMed: 18367605]

40. Shi Y. Serine/threonine phosphatases: mechanism through structure. Cell. 2009; 139:468–484. [PubMed: 19879837]

41. Bollen M, Peti W, Ragusa MJ, Beullens M. The extended PP1 toolkit: designed to create specificity. Trends Biochem Sci. 2010; 35:450–458. [PubMed: 20399103]

42. Ceulemans H, Bollen M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. Physiol Rev. 2004; 84:1–39. [PubMed: 14715909]

43. Munton RP, Vizi S, Mansuy IM. The role of protein phosphatase-1 in the modulation of synaptic and structural plasticity. FEBS Lett. 2004; 567:121–128. [PubMed: 15165904]

44. Galter D, Westerlund M, Carmine A, Lindqvist E, Sydow O, Olson L. LRRK2 expression linked to dopamine-innervated areas. Ann Neurol. 2006; 59:714–719. [PubMed: 16532471]

45. Taymans JM, Van den Haute C, Baekelandt V. Distribution of PINK1 and LRRK2 in rat and mouse brain. J Neurochem. 2006; 98:951–961. [PubMed: 16771836]

46. da Cruz e Silva EF, Fox CA, Ouimet CC, Gustafson E, Watson SJ, Greengard P. Differential expression of protein phosphatase 1 isoforms in mammalian brain. J Neurosci. 1995; 15:3375–3389. [PubMed: 7751917]

47. McConnell JL, Wadzinski BE. Targeting protein serine/threonine phosphatases for drug development. Mol Pharmacol. 2009; 75:1249–1261. [PubMed: 19299564]
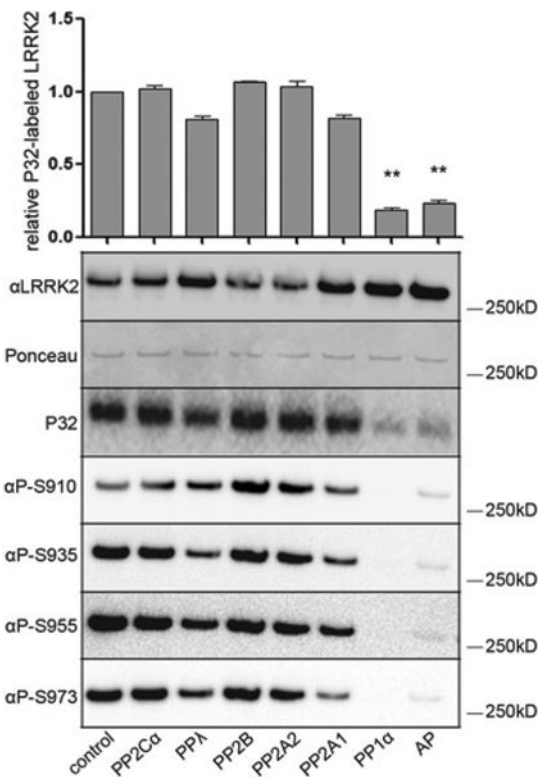
Figure 1. Protein phosphatase 1 can dephosphorylate LRRK2 *In vitro*

LRRK2, metabolically labelled with ^{32}P, was immunopurified from HEK-293T cells overexpressing 3×FLAG–LRRK2, and incubated with the indicated phosphatases *in vitro.* Shown are the incorporated ^{32}P levels as well as the levels of phosphorylation at Ser^{910}, Ser^{935}, Ser^{955} and Ser^{973} measured using phosphosite-specific anti-LRRK2 antibodies. Total LRRK2 levels were detected using both Ponceau S and immunoblotting. The graphs show the quantification of incorporated ^{32}P in LRRK2 after *in vitro* dephosphorylation normalized to total LRRK2 levels using immunoblotting of three independent experiments, ±S.E.M. Blots show representative results. Statistical significance was assessed by single sample *t* tests combined with Bonferroni correction for multiple testing. ***P* < 0.01. Ap, alkaline phosphatase.
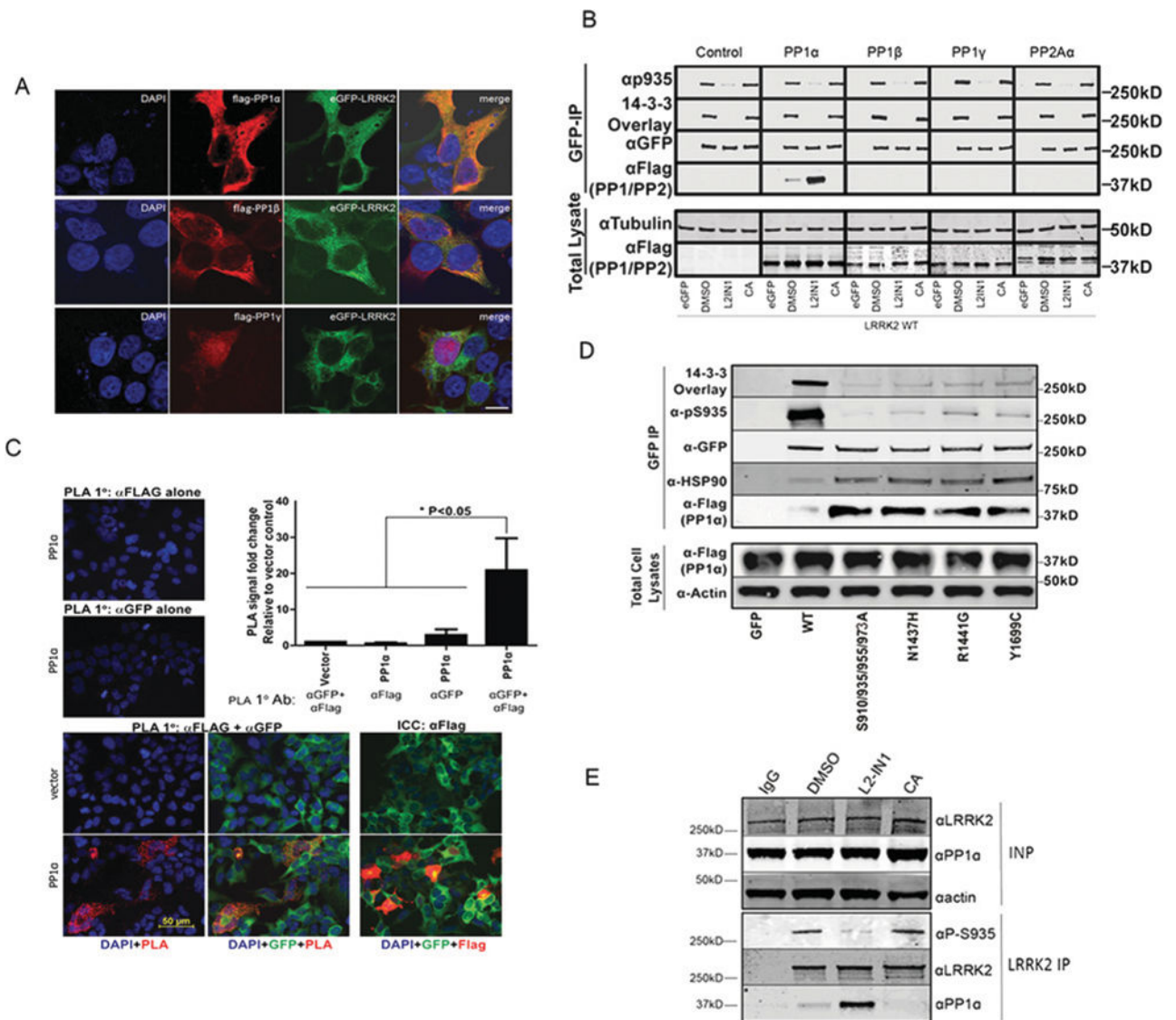
Figure 2. PP1α Interacts with LRRK2

(**A**) HEK-293T cells with stable eGFP–LRRK2 expression were transfected with 3 × FLAG-tagged PP1α, PP1β or PP1γ. Cells were stained with DAPI (blue, left-hand panels), and proteins were detected by immunofluorescence using FLAG for PP1 isoforms (red, second panels) and eGFP for LRRK2 (green, third panels). Merged images are shown in the right-hand panels. The scale bar represents 10 μm and applies to all photomicrographs. (**B**) Co-immunopurification (IP) of PP1α with LRRK2, T-REx lines, stably expressing eGFP alone or WT eGFP–LRRK2 and transiently expressing FLAG-tagged PP1 isoforms or PP2Aα, were treated with 1 μM LRRK2-1N1 for 90 min or 20 nM calyculin A (CA) for 30 min. GFP complexes were isolated with GFP nano trap beads and analysed by immunoblotting with the indicated antibodies. Representative blots are shown for three independent experiments. p935, pSer[935] (**C**) PLA. T-RExcells, stably expressing WT GFP–LRRK2, were transfected with FLAG vector or FLAG–PP1α as indicated. At 48 h after transfection, cells were subjected to PLA following the manufacturer's protocol using the indicated primary

antibodies. The far left-hand panels show the merged image of cells stained with DAPI (blue) and ligation amplifications (red). A merged image of GFP-LRRK2 with DAP/PLA is presented in the middle column. PLA signals per cell were quantitated by Duolink ImageTool and are presented as fold change from the number of vector signals per cell from n=3 experiments. Results are means+ S.D., analysis was one-way ANOVA against each control ($*P < 0.05$). The right-hand panels show immunocytochemistry with anti-FLAG antibody staining of transfected PP1α. Merge image shows DNA (blue), GFP–LRRK2 (green) and FLAG–PP1 (red). 1°, primary; ICC, immunocytochemistry, (**D**) Transfection of FLAG-tagged PP1α in T-REx HEK-293 cell lines overexpressing eGFP–LRRK2 WT or the mutants indicated. GFP-overexpressing cells were included as a negative control. Cells were lysed using Nonidet P40 lysis buffer and cell lysates were immunoprecipitated by GFP-Trap agarose. Immunopurified samples and total cell lysates were analysed by immunoblotting with the indicated antibodies. Representative blots are shown for three independent experiments. (**E**) Co-immunopurification of endogenous PP1α with endogenous LRRK2 in A549 cells after treatment with DMS0, 2 μM LRRK2-IN1 or 20 nM calyculin A. Input (INP) and co-immunoprecipitation (IP) samples were analysed with immunoblotting with indicated antibodies. Representative blots are shown for four independent experiments. Molecular masses in kDa are indicated to the side of Western blots.
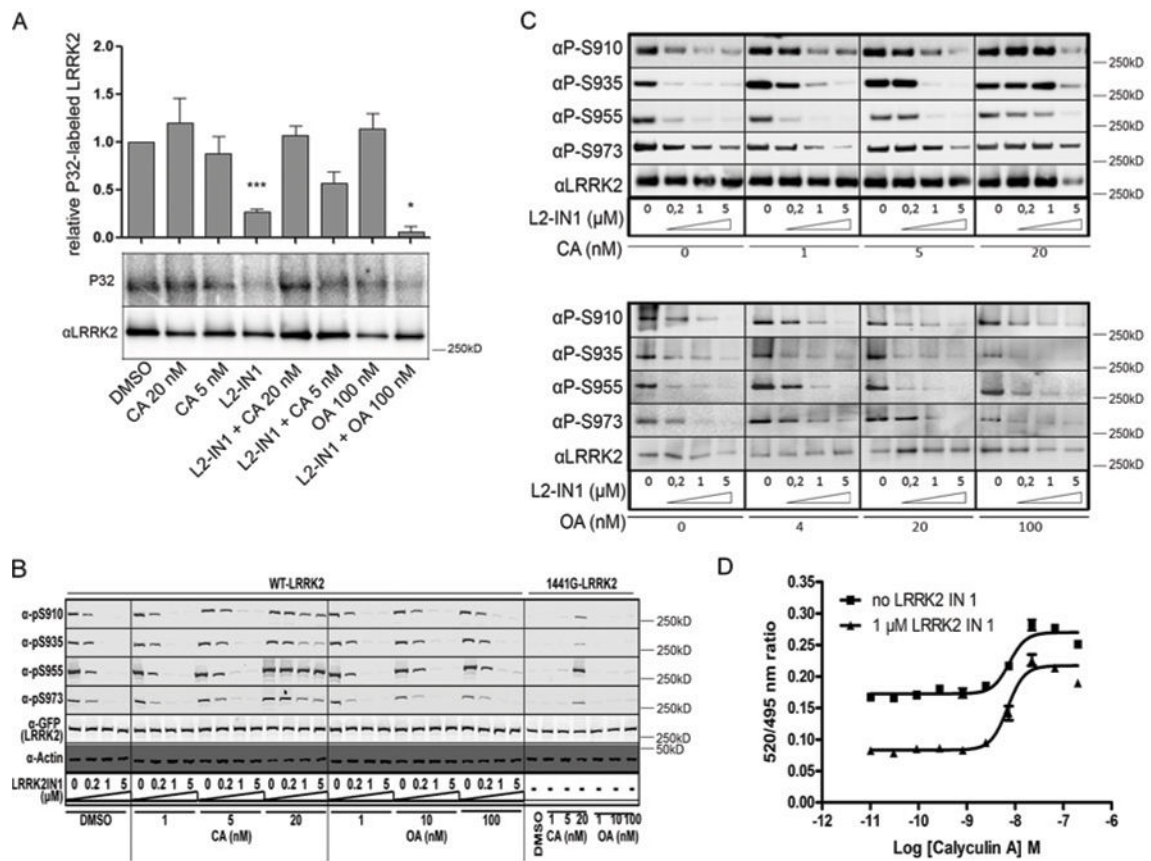
Figure 3. Calyculin A treatment prevents LRRK2 dephosphorylation induced by LRRK2-IN1 and restores reduced LRRK2 R1441G phosphorylation levels to WT LRRK2 levels

(**A**) Effect of phosphatase and kinase inhibitors on the overall LRRK2 phosphorylation level. HEK-293T cells stably overexpressing eGFP–LRRK2 were metabolically labelled and treated with phosphatase inhibitors and/or LRRK2-IN1 (1 μM) as indicated. Incorporated ^{32}P was measured and normalized to total LRRK2 levels detected by immunoblotting, as described in the Materials and methods section. The graph shows quantification of three independent experiments as means ± S.E.M. Statistical significance was assessed by column statistics combined with Bonferroni correction for multiple testing. *$P < 0.05$, ***$P < 0.001$. (**B**) T-REx HEK-293 cells overexpressing eGFP–LRRK2 WT or the R1441G variant were treated with the indicated concentrations of okadaic acid or calyculin A alone, or combined with the indicated concentration of LRRK2-1N1. LRRK2 phosphorylation at Ser^{910}, Ser^{935}, Ser^{955} and Ser^{973} was determined by phosphorylation-site-specific anti-LRRK2 antibodies. Representative blots are shown for three independent experiments. (**C**) SH-SY5Y cells stably overexpressing eGFP–LRRK2 were treated with a concentration range of phosphatase inhibitors combined with LRRK2-IN1. Detection of phosphorylation level at the four cellular phosphorylation sites was performed as in (**B**). Blots show the representative results of three independent experiments (**D**) U2OS cells expressing LRRK2–GFP were treated with 1 μM LRRK2-IN1 in the presence of the indicated dilution series of calyculin A. Cells were lysed in the presence of Terbium-labeIled anti-pSer^{935} antibody. TR-FRET (time-resolved FRET) was measured and the emission

ratios of 520 nm/490 nm are plotted against calyculin A concentration. CA, calyculin A; OA, okadaic acid.
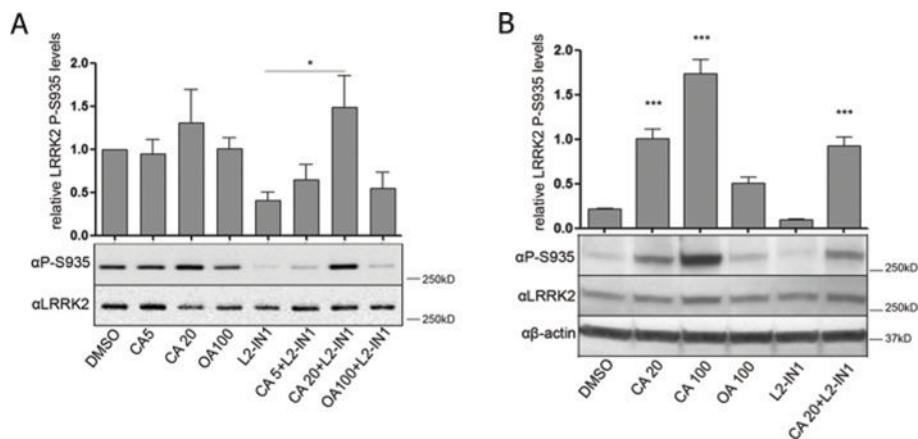
Figure 4. Calyculin A treatment prevents endogenous LRRK2 dephosphorylation induced by LRRK2-IN1

NIH 3T3 cells (**A**) or primary neurons from C57BL/6J mice (**B**) were treated with phosphatase inhibitor (5,20 or 100 nM), with LRRK2-IN1 (1 μM for 30 min) or with the combination of both. Monoclonal anti-pSer^{935} antibody was used to detect the phosphorylation level at Ser^{935}. Histograms show the means ± S.E.M. of three independent experiments, *P < 0.05, ***P < 0.001. CA, calyculin A; OA, okadaic acid.
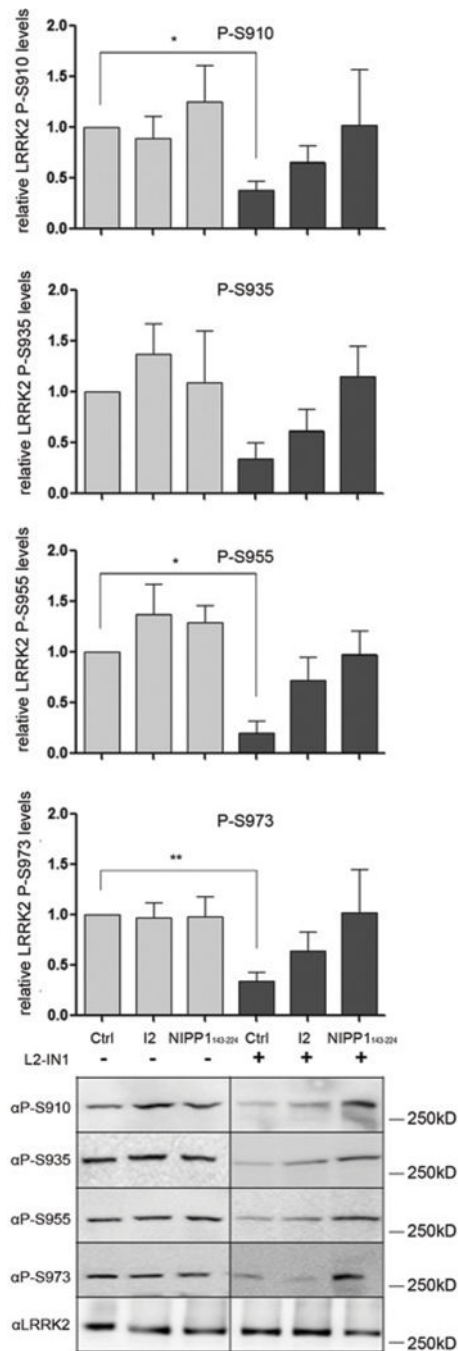
Figure 5. Molecular Inhibition of PP1 prevents LRRK2 dephosphorylation induced by LRRK2-IN1

HEK-293T cells with stable eGFP–LRRK2 overexpression were transfected with expression constructs for the PP1 molecular inhibitors I2 or NIPP1$_{143–224}$, or with a control construct encoding eGFP. Cells were treated with 1 μM LRRK2-IN1 or DMSO as a control for 30 min followed by cell lysis and immunoblotting with the different phosphorylation-site-specific anti-LRRK2 antibodies. Representative blots of three independent experiments are shown. Quantification of the blots are represented as a histogram. For quantification, the ratio of phosphorylation over total LRRK2 signal was normalized to control condition and are

means ± S.E.M. The blots show representative results. Statistical significance was assessed by column statistics combined with Bonferroni correction for multiple testing. *P<0.05, **P<0.01.
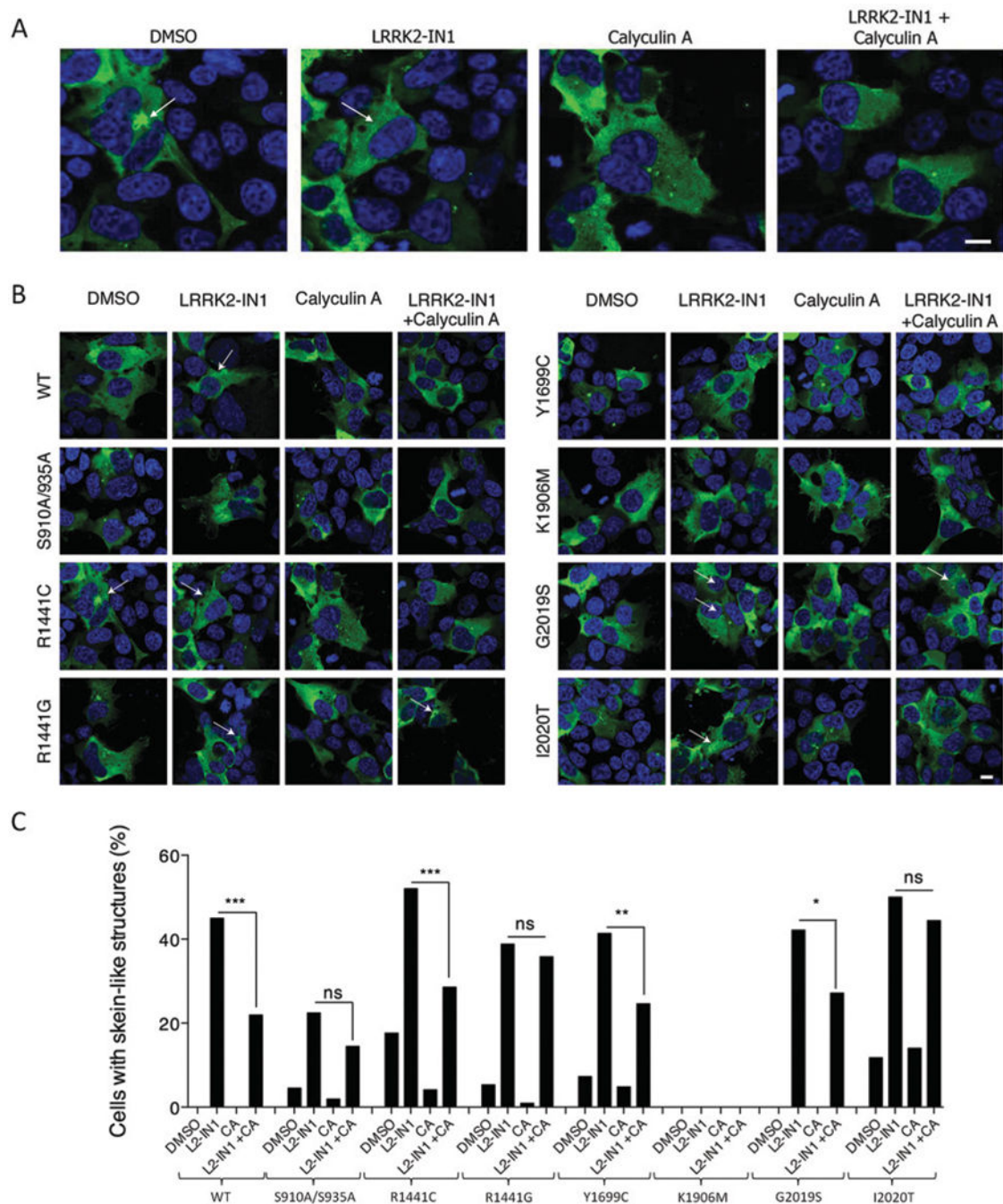
Figure 6. PP1 Inhibition prevents formation of skein-like structures
(**A** and **B**) Immunocytochemistry of HEK-293-FT cells transiently expressing 3×FLAG–
LRRK2 WT and series of mutants (S910A/S935A, R1441C, R1441G, Y1699C, K1906M,
G2019S and I2020T) were treated for 30 min with DMSO, 1 μM LRRK2-IN1, 5 nM
calyculin A, or both 1 μM LRRK2-IN1 and 5 nM calyculin A. Arrows indicate skein-like
inclusions. Enlarged images for R1441C are given in (**A**), whereas images for all tested
LRRK2 isoforms are given in (**B**), Scale bar represents 10 μm and applies to all
photomicrographs, (**C**) Quantification of cells with skein-like structures for samples

described in (**A**) and (**B**), CA, calyculin A. Bars represent percentage of cells with inclusions out of all LRRK2-transfected cells counted per sample (n=100–150 cells). Statistical significances were tested using a Fisher's exact test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns, not significant.
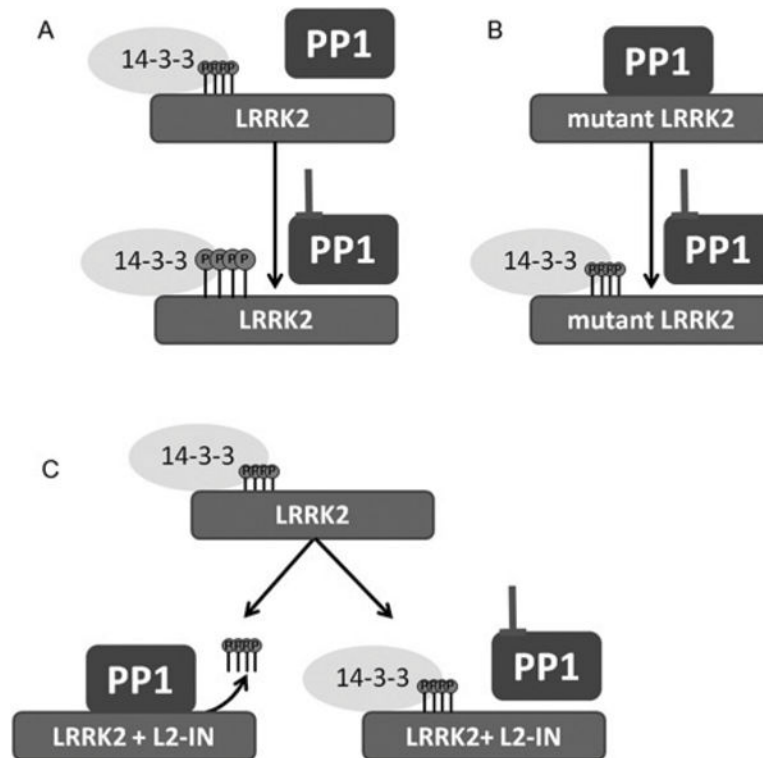
Figure 7. Model of PP1-mediated LRRK2 dephosphorylation
(**A**) WT LRRK2 is basally phosphorylated in cells at multiple sites clustering in the N-terminal half of the protein (see Supplementary Figure S1 at http://www.biochemj.org/bj/456/bj4560119add.htm for a schematic representation of the cellular phosphorylation sites relative to the functional domains). In its phosphorylated state, LRRK2 binds 14-3-3 proteins tightly and PP1 is loosely associated with the cellular LRRK2 complex Under conditions of PP1 inhibition (pharmacological inhibition with calyculin A or molecular PP1 inhibition with NIPP1$_{143-224}$, see text), LRRK2 phosphorylation level is only modestly increased. (**B**) Several disease mutants are basally dephosphorylated and this coincides with the observation reported in the present study that PP1 binds tightly to dephosphorylated disease mutants. Mutant LRRK2 can be rephosphorylated by PP1 inhibition. (**C**) LRRK2 kinase inhibition (L2-IN) leads to dephosphorylation of LRRK2. The dephosphorylation induced by LRRK2-IN is blocked by PP1 inhibition.