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Parkinson's disease-associated mutations in *LRRK2* link enhanced GTP-binding and kinase activities to neuronal toxicity

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Mutations in the leucine-rich repeat kinase 2 gene (*LRRK2*) cause late-onset Parkinson's disease indistinguishable from idiopathic disease. The mechanisms whereby missense alterations in the *LRRK2* gene initiate neurodegeneration remain unknown. Here, we demonstrate that seven of 10 suspected familial-linked mutations result in increased kinase activity. Functional and disease-associated mutations in conserved residues reveal the critical link between intrinsic guanosine triphosphatase (GTPase) activity and downstream kinase activity. LRRK2 kinase activity requires GTPase activity, whereas GTPase activity functions independently of kinase activity. Both LRRK2 kinase and GTPase activity are required for neurotoxicity and potentiate peroxide-induced cell death, although LRRK2 does not function as a canonical MAP–kinase–kinase–kinase. These results suggest a link between LRRK2 kinase activity and pathogenic mechanisms relating to neurodegeneration, further supporting a gain-of-function role for *LRRK2* mutations.

INTRODUCTION

Missense mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene redefine the role of genetic variation in Parkinson's disease (PD) susceptibility. *LRRK2* mutations initiate a penetrant phenotype with complete clinical and neurochemical overlap with idiopathic disease (1–3). Genetic studies have broadened the initial role of *LRRK2* mutations beyond familial cases of disease with evidence that mutations occur in high frequency, 1–7% of PD patients of European origin and 20–40% of PD in Ashkenazi Jews and North African Arabs (4–7). Genetic studies suggest the most common *LRRK2* PD-associated haplotypes date several thousand years, independently originating in European and Middle Eastern populations (8). In contrast, *LRRK2*-linked PD appears rare in Asian populations (9). The pathogenic role and associated biochemical pathways responsible for *LRRK2*-linked disease remain unknown, yet represent a unique opportunity to identify therapeutic targets for related neurodegenerative disorders.

The human *LRRK2* gene encodes a unique 280 kDa protein belonging to the newly described ROCO protein family typified by the presence of a characteristic guanosine triphosphatase (GTPase) and C-terminal of Ras (COR) domain most often in conjunction with a kinase domain (10). LRRK2 protein possesses an arrangement of repeat sequences with unknown function beginning at the N-terminus, in addition to a leucine-rich repeat structure near the GTPase domain (11). Subcellular localization efforts from multiple laboratories reveal a cytosolic interaction with membranous structures, including mitochondria, vesicles, ER and golgi, although LRRK2 protein does not possess transmembrane domains (12–14). In the adult human and rodent brain, *LRRK2* is principally expressed in the neurons of the striatum and cortex (15,16). Efforts to biochemically characterize

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LRRK2 protein reveal that the most common disease-associated mutations influence kinase activity *in vitro*, accompanied by increases in apparent neurotoxicity (14,17,18). Intrinsic GTPase activity may modulate kinase activity and neurotoxicity, although the role for the LRRK2 kinase domain in regulating intrinisic GTPase activity has not been explored. The considerable number of described disease-linked missense mutations linked to the development of PD present the opportunity to biochemically explore a unifying hypothesis in understanding the pathogenicity of LRRK2 and whether kinase or other LRRK2-related activities present a viable therapeutic target.

In unraveling the role of *LRRK2* in health and disease, the LRRK2 kinase domain contains sequence homology to MAP–kinase–kinase–kinase (MAPKKK) proteins, specifically a subclass of MAPKKK known as mixed-lineage kinase (MLKs) within the larger tyrosine-kinase-like protein family (Supplementary Material, Fig. S1). MLK proteins are thought to primarily function in programmed cell death pathways by influencing the activation of critical regulators of apoptosis such as c-Jun (19). The importance of MLK proteins in neurodegenerative disease continues to be explored with the MLK inhibitor, CEP-1347, in multiple PD clinical trials (20). The input of LRRK2 into MAPK and programmed cell death pathways has not yet been explored.

In this study, we biochemically characterize an expanded set of 10 PD-linked *LRRK2* mutations by measuring both kinase and GTP-binding activities. We demonstrate that LRRK2 is a serine/threonine kinase capable of activating the MAPK pathway in multiple cell lines in a kinase-independent fashion and does not meet the usual criteria for an MLK protein despite sequence homology. In contrast, LRRK2 potentiates neurotoxicity in a kinase-dependent manner. Our results further support the hypothesis that LRRK2 kinase activity represents the critical component of biochemical activity linking *LRRK2* with neurodegeneration.

RESULTS

LRRK2 GTPase activity regulates kinase activation

Two predicted enzymatic domains exist in the LRRK2 protein: a GTPase domain with sequence similarity to Rab-like proteins and a kinase domain belonging to the tyrosine–kinase-like protein family (Fig. 1A). To probe the potential reciprocity in activity between the two domains, we created a panel of functional mutations predicted to exert specific defects in conserved residues crucial for enzyme activity (Fig. 1B). We established autophosphorylation assays utilizing immunoprecipitated LRRK2 protein to assess kinase activity in combination with phosphorylation of a generic kinase substrate, myelin basic protein (MBP). In comparison with autophosphorylation assays, MBP assays generally demonstrate half the signal-to-noise ratio, although in all assays performed in this study, MBP substrate phosphorylation mimics the activity observed with autophosphorylation, but with more experimental variation (data not shown). Consistent with published results (12,17), the K1906M mutation diminishes kinase activity presumably through disruption of the highly conserved ATP-binding pocket (Fig. 1C). Similarly, the D1994A mutation inhibits kinase activity through mutation of the conserved aspartic acid residue found in all known kinase proteins.

The LRRK2 kinase domain possesses highest sequence and predicted structure homology to the MLK kinase proteins, a subfamily within the tyrosine-like kinase family (Supplementary Material, Fig. S1). MLK proteins become activated through phosphorylation of conserved serine and threonine residues within the activation loop of the kinase domain (21). We hypothesized that LRRK2 is similarly regulated through activation loop phosphorylation and created an LRRK2 construct where all three potential sites of phosphorylation, T2031, S2032 and T2035 are mutated to alanines. As predicted, this construct, dubbed 'TripKin', demonstrates kinase activity impairment similar to the K1906M construct, thereby implicating one or more of these residues in kinase activation (Fig. 1C). The TripKin construct likewise cannot phosphorylate MBP (data not shown). Since kinase activation and autophosphorylation may occur simultaneously, we cannot determine whether LRRK2, another kinase, or both stimulate phosphorylation of the activation loop, using the assays described in this study.

Alteration of the conserved lysine residue localized to the nucleotide-binding pocket of the GTPase domain (K1347A) results in ablation of kinase activity (Fig. 1C and D). Kinase activity of wild-type LRRK2 is inhibited by the supplementation of GDP into the cell lysate during immunoprecipitation, whereas activity is increased upon addition of GTPγS (Fig. 1D). Addition of either GDP or GTPγS with pure immunoprecipitated preparations of LRRK2 had no significant effect on kinase activity, suggesting that LRRK2 requires co-factors for the binding and hydrolysis of nucleotides in the GTPase domain, similar to most mammalian GTPase proteins. Likewise, we were unable to establish *in vitro* LRRK2 GTP-hydrolysis assays (data not shown), suggesting that LRRK2, as with many GTPase proteins, requires additional co-factors such as incubation with the corresponding guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) for *in vitro* hydrolysis activity.

In lieu of an assay measuring GTP-hydrolysis, we established a GTP-binding assay which captures the proportion of LRRK2 able to bind GTPγS in cell lysis buffer, reflective of GTPase activity near the time of precipitation (Fig. 1E). As predicted, the K1347A mutant displayed reduced ability to bind GTP, whereas the GTP-binding ability of the kinase-dead LRRK2 proteins was unaffected (Fig. 1F). These results suggest that GTPase activity is required for and modulates downstream kinase activity, but kinase activity does not have a significant effect on GTP binding.

PD-linked mutations increase kinase and GTP-binding activities

Genetic studies have identified a number of missense point-mutations associated with PD spread across the LRRK2 open-reading frame and encoded protein domains. To determine the functional consequences of PD-associated mutations, especially those outside of the kinase domain, we utilized site-directed mutagenesis to create 10 familial-linked mutant LRRK2 constructs (Fig. 1A). The missense mutations were chosen on the basis of

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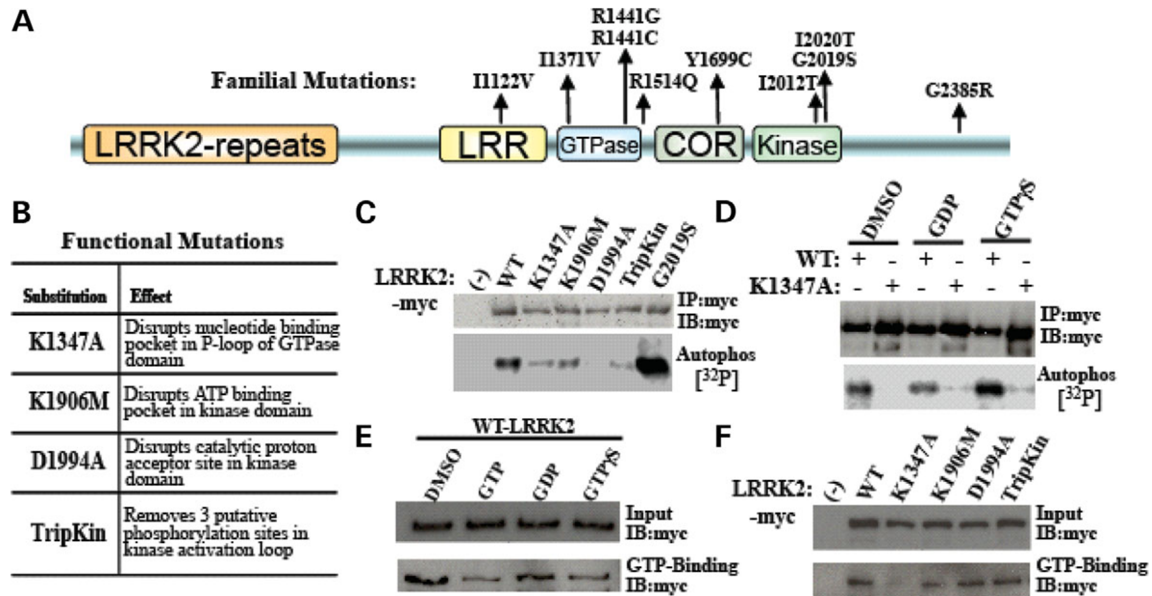

Figure 1. Intrinsic regulation of kinase activity via GTP-binding. (**A**) Domain structure of LRRK2 protein with corresponding alignment of familial-linked PD mutations. Residues 1–660 encode the LRRK2-specific repeat sequences, 984–1278 encode the LRR domain, 1519–1795 encode the COR domain and 1816–2068 encode the kinase domain. (**B**) Table of sequence alterations and predicted functional effect. (**C** and **D**) LRRK2 autophosphorylation assay with signal normalized to input protein. Either 1 mM GDP or 10 μM GTPγS, as indicated, were added to cell lysates 1 h prior to immunoprecipitation. (**E** and **F**) LRRK2 GTP-binding assay, with amount of bound LRRK2 normalized to input levels. Either 1 mM GDP or 10 μM GTPγS was added to cell lysates 1 h prior to immunoprecipitation, as indicated.

the original estimates that the alteration would be causative for disease. To assess basal kinase activity of immunoprecipitated LRRK2, we performed kinase assays with signal normalized to input levels of proteins. In this assay, the K1906M mutant activity reflects both non-specific incorporation of radionuclide and any residual kinase activity. As previously published (12,14), the mutations G2019S, R1441C and I2020T demonstrate increases in kinase activity relative to wild-type activity. Both the G2019S and I2020T alterations introduce additional potential phosphorylation sites nearby the critical phosphorylation residues as identified with the activation loop mutation construct (construct TripKin, Fig. 1). Additional alterations near the GTPase domain, including R1441G and R1514Q, resulted in increases in kinase activity. A nominal increase in kinase activity is observed with the I1371V mutation, although the increase is not significant when averaged over three experiments (Fig. 2). The I2012T mutation, whose genetic association with PD has not been replicated since the original study, results in a significant reduction in kinase activity presumably through deleterious alteration of the magnesium-binding loop, where the mutation resides. Similarly, the G2385R alteration, originally published as a familial-linked mutation, now suspected as a common polymorphism, does not affect a significant change in kinase activity (Fig. 2). Our results suggest that increased kinase activity represents the common biochemical feature of PD-associated missense mutations, often caused by mutations distant from the kinase domain.

Given the crucial relationship between GTP-binding and kinase activity (Fig. 1), we hypothesized that mutations outside of the kinase domain may perturb GTP-binding activity. As an indirect measure of GTPase activity, we performed the GTP-binding assay on the panel of PD-linked and wild-type LRRK2 proteins. Although the increases relative to wild-type protein are more nominal than those observed with kinase activity, a phenomenon likely due to the inherent variability of the assay, a number of familial-linked mutations near the GTPase domain significantly increased GTP-binding relative to wild-type protein (Fig. 3). Mutations near the kinase domain, including G2019S, I2012T and I2020T, have no significant effect on GTP-binding, consistent with kinase activity acting independently of GTPase activity. Mutations within the GTPase domain, including R1441C, R1441G and I1371V, increase GTP binding and presumably increase kinase activity through alteration of GTPase activity. The Y1699C mutation induces a dramatic increase in kinase activity (Fig. 2B) and simultaneously increases GTP-binding activity, implicating the COR domain as a potential mediator of GTPase and kinase activity. The I1371V alteration induces a consistent increase in GTP-binding yet affects only a non-significant trend towards increased kinase activity. Likewise, the I1122V mutation in the LRR domain nominally increases kinase activity but does not affect a significant change in GTP-binding activity. These alterations may represent more benign polymorphisms that increase susceptibility to disease and therefore not possess the same biochemical properties, as those mutations clearly linked to disease (Table 2). In sum, our results suggest that most mutations outside of the kinase domain likely influence downstream kinase activity through perturbation of intrinsic GTPase activity.

LRRK2 is a serine/threonine kinase independent of the MAPK pathway

In agreement with the placement of LRRK2 within the human kinome, the LRRK2 kinase domain possesses high sequence

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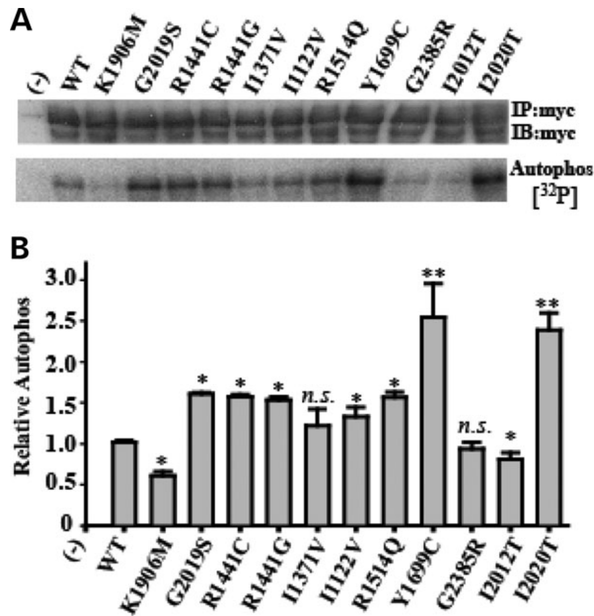

Figure 2. Familial-linked PD mutations increase kinase activity. (**A**) Immunoblot and autoradiogram of autophosphorylated LRRK2, as resolved by SDS–PAGE. LRRK2 protein levels were determined by western blot analysis, using anti-c-myc antibody. (**B**) Normalization of incorporated ^{32}P compared with LRRK2 protein content. Data are representative of at least three independent experiments, in arbitrary units, where WT-LRRK2 kinase activity is defined as 1 and control (−) reactions are defined as 0. Error bars represent mean ± SEM. $^{*}P < 0.01$ and $^{**}P < 0.0001$ compared with WT-LRRK2, assessed by two-tailed unpaired Student's *t*-test. *n.s.* is non-significant versus WT-LRRK2.

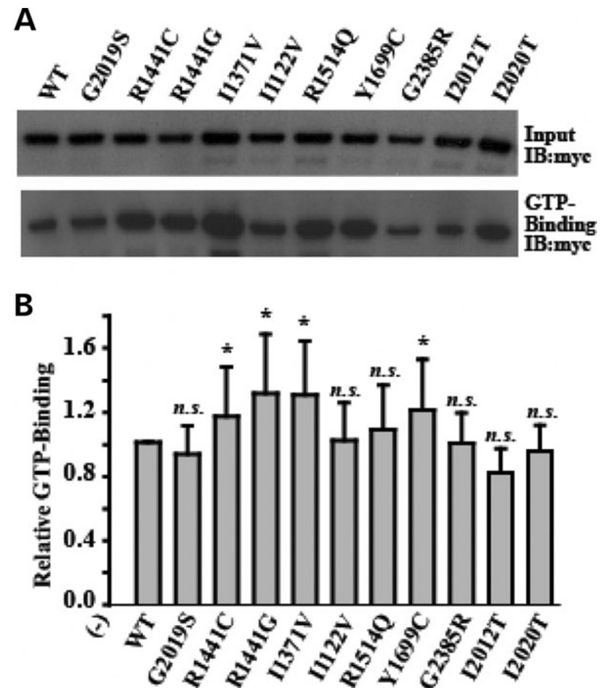

Figure 3. Familial-linked PD mutations increase GTP-binding activity. (**A**) Immunoblots indicating input and GTP-bound LRRK2 levels, as assessed with anti-c-myc antibody. (**B**) Normalization of GTP-bound LRRK2 to LRRK2 protein input. Data are representative of five independent experiments, in arbitrary units, where WT-LRRK2 GTP-binding activity is defined as 1 and control (−) reactions as 0. Error bars represent mean ± SEM. $^{*}P < 0.05$ compared with WT-LRRK2, assessed by one-tailed unpaired Student's *t*-test. *n.s.* is non-significant versus WT-LRRK2.

and predicted structural homology to MLK1 and MLK2 (Supplementary Material, Figs 1 and 2). Classic kinase subdomain structure analysis usually predicts specificity for serine/threonine or tyrosine phosphorylation capability (22). In the case of MLK proteins, subdomain VI bears homology to serine/threonine kinases, whereas subdomain VIII resembles tyrosine kinases (23). However, all known MLK proteins demonstrate only serine/threonine kinase activity, despite sequence homology to tyrosine kinases (19). Distinct from MLK in this regard, LRRK2 bears sequence homology exclusively to consensus serine/threonine kinase subdomains VI and VIII and does therefore not meet the original criteria for a bone fide member of the MLK proteins (Fig. 4A).

Examination of *in vitro* autophosphorylation using thin-layer chromatography revealed kinase-dependent serine/threonine phosphorylation but not tyrosine phosphorylation (Fig. 4B). Attempts to identify LRRK2 autophosphorylation sites, using high-performance liquid chromatography-tandem mass spectrometry, failed to reveal phosphorylation sites present in wild-type versus kinase-dead LRRK2 (data not shown) despite >90% peptide coverage throughout the protein and complete coverage across the kinase domain. Expected sensitivity for phospho-peptide mapping generally requires between 1 and 5% of a given peptide to contain a phospho-moiety (24). Presumably, a very low proportion of LRRK2 autophosphorylates *in vitro* under the conditions used in this study. Through the course of the analysis, a significant proportion of five independent peptides derived from immunoprecipitated kinase-dead, mutant and wild-type LRRK2 protein contained phosphorylated serine residues, defining LRRK2 as a phospho-protein in HEK-293FT cells (Table 1). The phosphorylated residues, adjacent to the leucine-rich repeat domain, are presumptively not LRRK2 autophosphorylation sites since endogenous LRRK2 cannot be detected in this cell line by immunoblotting (14). Additional phosphorylated residues in the GTPase or kinase domain were not detected.

Although the LRRK2 kinase domain bears some sequence divergence from more canonical MLK domains, the constituency of the tyrosine–kinase-like protein family suggests that LRRK2 may mediate programmed cell death pathways and respond to cellular stressors. MAPKKK proteins respond to cell stress and mitogenic signals to exert a specific biological response via the three-tiered MAPK pathway, from apoptosis to cell division or cessation (25). All known members of the MLK protein family strongly upregulate JNK and c-Jun phosphorylations in transfected cells, ultimately initiating apoptosis (19). The LRRK2 kinase domain possesses highest sequence homology to MAPKKK proteins, such as the MLKs, and therefore may participate in programmed cell death pathways in evoking neurodegeneration in PD.

We evaluated the basal activation of the four MAPK family members in response to overexpressed LRRK2 in two cell lines, SH-SY5Y and HEK-293FT cells, using phosphorylation specific antibodies (Fig. 5A). In both cell lines, >90%

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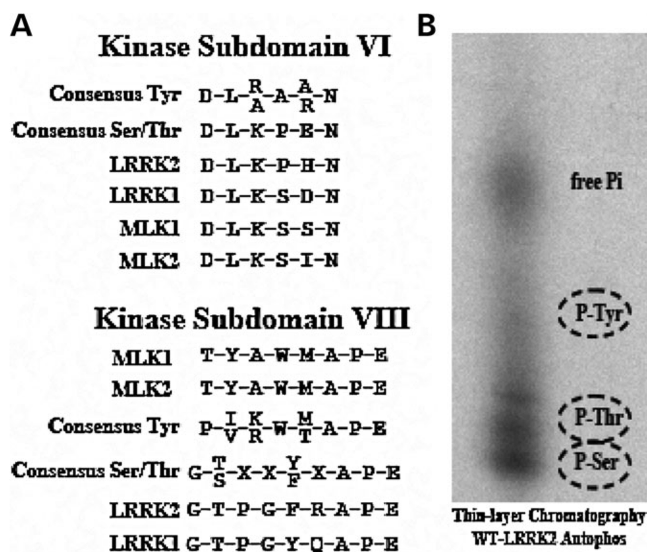

Figure 4. LRRK2 is a serine/threonine kinase. (**A**) Kinase subdomain alignment, where consensus serine/threonine sequences or tyrosine kinase sequences (22) are given. The respective LRRK1 and LRRK2 domains along with MLK1 and MLK2 domain sequences are aligned. (**B**) LRRK2 autophosphorylated residues were resolved with thin-layer chromatography and compared with standards, positions indicated with hashed-circles.

transfection efficiency is achieved as monitored by eGFP (negative control) transfection. We observed an upregulation of total levels of c-Jun tracking with LRRK2 expression in a kinase- and GTPase-independent manner, although the ratio of phosphorylated c-Jun to total c-Jun was not significantly changed. Expression of LRRK2 in SH-SY5Y cells consistently caused an upregulation of phosphorylated JNK1, though in a kinase-independent manner. In HEK-293FT cells, JNK1/2 levels were not affected, although ERK5 dramatically responds to LRRK2 expression along with more nominal increases in ERK1/2 phosphorylation. These pathways presumably result in the increase in c-Jun levels in the HEK-293FT cells in an LRRK2-kinase independent manner. In summary, LRRK2 does not display the characteristic activity of an MAPKKK protein in the cell lines examined and is therefore distinct from MLK proteins.

We hypothesized that one or more familial-linked *LRRK2* mutations may have acquired the ability to upregulate components of the MAPK pathway, given the significant increases they induce in kinase and GTP-binding activities (Figs 2 and 3). In addition, we sought to determine whether LRRK2 may potentiate the phosphorylation of c-Jun in the context of c-Jun activation via anisomycin treatment. In transiently transfected HeLa cells, where >90% transfection efficiency is achieved, none of the familial-linked *LRRK2* mutations caused a significant upregulation of c-Jun activity (Fig. 5B). In the context of anisomycin treatment and c-Jun activation, none of the LRRK2 constructs further potentiated phosphorylated c-Jun compared with control (eGFP) constructs (Fig. 5C). Therefore, familial-linked mutations in *LRRK2* do not obviously perturb the MAPK pathway in these cells, suggesting that LRRK2-linked neurodegeneration may not involve a specific MAPK pathway dysfunction as predicted by LRRK2 kinase sequence homology.

LRRK2 kinase activity is required for neurotoxicity

Numerous reports suggest expression of LRRK2 is toxic to cells in primary cortical cultures. We selected a panel of LRRK2 constructs for toxicity studies, including the D1994A kinase dead, the K1347A GTPase/kinase dead and four PD-linked mutations, including the Y1699C mutation that results in increases in both kinase activity and GTP-binding, the G2019S and I2020T mutations that only increase kinase activity and the G2385R alteration that has no significant effect on kinase activity or GTP-binding activity. Embryonic day 15 mouse cortical preparations were cultured for 10 days *in vitro* prior to transfection. Pilot experiments with G2019S LRRK2 demonstrate significant toxicity 72 h post-transfection, but not 48 h post-transfection. We observed neuronal toxicity associated with WT-LRRK2 expression (Fig. 6). Expression of both the D1994A kinase-dead and the K1347A GTPase/kinase-dead constructs did not result in significant toxicity, suggesting kinase activity mediates cell death caused by overexpressed WT-LRRK2. Expression of the G2385R resulted in toxicity significant from control transfections, but non-significant when compared with WT-LRRK2 toxicity. Thus, WT-LRRK2 and G2385R result in comparable toxicity, in agreement with the G2385R mutation as a benign sequence alteration. In contrast, Y1699C, G2019S and I2020T resulted in dramatic neurotoxicity 72 h post-transfection.

Many protein kinases respond to external stimuli in initiating a cellular response such as apoptosis. Numerous reports suggest that proteins associated with PD respond to oxidative stress, either functioning in a neuroprotective manner in the case of parkin and DJ-1, or through potentiating aggregation and toxicity in the case of α-synuclein (26,27). To determine whether LRRK2 overexpression modulates a peroxide-induced loss of cell viability, we selected a dose and time course of hydrogen peroxide that results in a loss of viability of ~50% in pure neuronal cultures (28). Forty-eight hours post-transfection, mouse cortical cultures were pulsed for 20 min with 25 μM hydrogen peroxide and evaluated for viability 24 h later. WT-LRRK2 expression potentiated peroxide-induced cell death, whereas D1994A or K1347A LRRK2 expression did not result in a decrease in viability versus control (Fig. 6C). Similar to WT-LRRK2, expression of LRRK2 with familial linked-PD-mutations results in near-complete loss of neuron viability. In summary, LRRK2 kinase activity tracks with neuronal toxicity, where GTP-binding relates to toxicity presumably through intrinsic alteration of kinase activity.

DISCUSSION

Mutations in the *LRRK2* gene represent the commonest known cause of PD. As opposed to familial-linked mutations in other PD-linked genes, *LRRK2*-linked disease causes a clinical progression and neurochemical phenotype indistinguishable from typical late-onset disease. In select PD populations, *LRRK2* mutations have been determined at high frequency, analogous to frequencies of *TP53* mutations in solid tumors. Among the *LRRK2*-linked cases evaluated post-mortem, pleomorphic pathology suggests LRRK2 resides far upstream in a

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Table 1. Identification of phosphorylated residues in LRRK2

Peptide	Charge	XCorr	Residue	NetPhosK prediction
K.SAVEEGTA(S*)G(S*)DGNFSEDVLSK.F	2	4.76	S858 or S860	CKII
K.KSNS*ISVGEFYR.D	2	2.68	S910	PKA
R.HSNS*LGPIFDHEDLLK.R	3	4.66	S935	PKA
K.ILSS*DDSLR.S	2	2.66	S955	ATM
R.HSDS*ISSLASER.E	2	3.56	S973	Unknown

The derived sequence of phospho-peptides is given, with phosphorylated residues denoted by asterisks. The position of some phosphorylated residues, denoted in parenthesis, could not be further delineated. Xcorr values represent correlative quality, and predicted kinase phosphorylation sequences were generated by NetPhosK http://www.cbs.dtu.dk/services/NetPhosssK/.

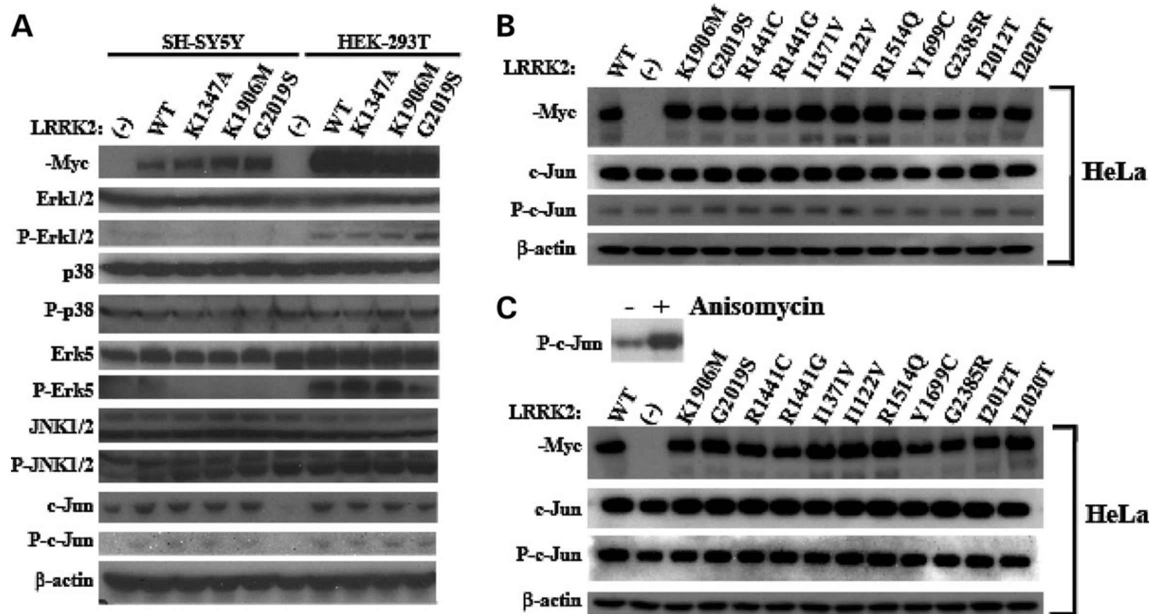

Figure 5. LRRK2 activates the MAPK pathway in a kinase-independent manner. (**A** and **B**) The indicated LRRK2 construct or (−) control (eGFP) were transfected in the indicated cell line. Forty-eight hours post-transfection, cells were harvested and lysates analyzed with SDS–PAGE and immunoblotted with the indicated antibody. (**C**) HeLa cells were treated with 1 μM anisomycin for 20 min prior to harvesting. A representative immunoblot estimating phospho-c-Jun activation, compared with non-treated cells, is given.

pathway related to neurodegeneration. As such, the present study seeks to further elucidate the pathological dysfunction occurring in *LRRK*-linked disease in addition to identifying viable therapeutic targets for use in rationale drug designs.

We confirm that intrinsic GTP-binding activity modulates downstream kinase activity (18) and further demonstrate the lack of reciprocity by showing kinase activity does not significantly modulate GTP-binding activity. The conserved serine and threonine residues in the activation loop of LRRK2 likely track with enzyme activity, given their apparent critical input in kinase activation. The above-average size of the LRRK2 protein in addition to poor activity in autophosphorylation assays proves standard methods to map autophosphorylation sites as ineffective. Antibodies specific for phosphorylated residues within the activation loop may provide the best option for understanding LRRK2 activation. We identify overexpressed LRRK2 as a phospho-protein in HEK-293T cells, containing five phosphorylated serine residues in a small cluster immediately downstream of the leucine-rich repeat domain (Table 1). Of note, a possible *LRRK2* missense mutation, Q930R, reported in a European PD case, localizes within the phosphorylation cluster nearby the S935 phosphorylated residue (29). We are currently generating constructs and antibodies to determine the potential biological significance of phosphorylated LRRK2.

We hypothesized that evaluating an expanded set of missense mutations associated with PD would provide molecular clues to guide the generation of transgenic models and drug design strategies. Precedent for the importance of such studies in understanding pathogenic mechanisms relating to neurodegeneration is given by the *presenilin 1* gene, where dominant missense mutations result in increased production of Aβ-42 peptide and therefore Alzheimer's disease (30). In this study, we show that a potential unifying hypothesis for the pathogenic gain-of-function of LRRK2 relates to increased kinase activity, either through alteration of conserved residues

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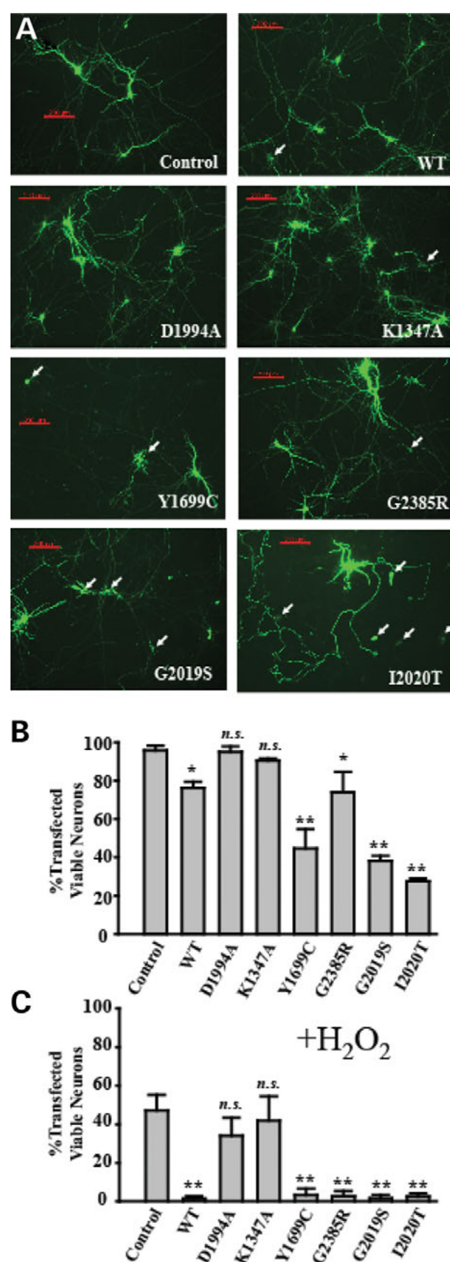

Figure 6. LRRK2 toxicity requires kinase activity and potentiates peroxide-induced neurotoxicity. (**A**) Representative photomicrographs with red scale bars indicating 200 μM. Mouse primary cortical neurons were analyzed 72 h post-transfection (13 total days *in vitro*) with the indicated LRRK2 construct in a 10:1 molar ratio with eGFP. White arrows indicate neurons counted as non-viable (positive propidium iodide staining and/or obvious nuclear and process fragmentation). (**B**) Data are representative of at least three independent experiments, where percent transfected and viable neurons are given for each condition. Error bars represent mean ± SEM. $^{*}P < 0.01$ and $^{**}P < 0.0001$ compared with control (eGFP only) transfections, assessed by one-tailed unpaired Student's *t*-test. *n.s.* is non-significant versus control (eGFP only) transfection.

in the kinase or GTPase domain or through perturbation of predicted protein-binding domains (Table 2 and Fig. 7). Biochemical characterizations of mutations that clearly segregate with disease in multiple families and are therefore unambiguously causative for PD support the role for increased LRRK2 kinase activity as responsible for disease. A number of mutations studied here will likely prove as relatively benign polymorphisms in future genetic analyses. Nevertheless, polymorphisms that alter kinase activity may represent risk factors for PD susceptibility.

Besides large *in vitro* assays that biochemically characterize LRRK2 protein, a number of previous studies have applied exogenous *LRRK2* expression to various cell systems (17,18). In general, the expression of mutant *LRRK2* results in a significant loss of viability, whereas kinase-dead LRRK2 reduces such toxicity. Here, we present a cell system paradigm whereby the entirety of LRRK2 toxicity appears related to kinase activity. Peroxide-induced oxidative stress and related loss of viability clearly contrast the effects of WT-LRRK2 versus kinase-dead LRRK2 overexpression. In the context of cellular insult, in this case, hydrogen-peroxide exposure, LRRK2 kinase activity appears to mediate neuron death, potentially consistent with a role in a cell death program.

We were unable to assign LRRK2 as an MAPKKK protein, using standard techniques usually capable of placing kinases within the three-tiered system. Perhaps coherent with this notion, the MLK drug CEP-1347, which strongly inhibits MLK activity and prevents JNK and c-Jun activations in neurons, has proved ineffective as a treatment for PD in initial trials. We therefore hypothesize that LRRK2 functions in a kinase-dependent pathway, potentially independent of the MAPK pathway, which links cell stress to programmed cell death. Given the successful development of kinase inhibitors and translation to the clinic, it seems reasonable to expect small molecules exist that can inhibit LRRK2 kinase activity. Also, we demonstrate that the GTPase domain, in addition to other protein interaction motifs, may strongly modulate kinase activity and therefore act as potential small molecule targets for indirect kinase activity inhibition. In summary, LRRK2 kinase activity is critically linked to toxicity and presents a viable target for therapeutic modulation.

MATERIALS AND METHODS

Plasmid generation and antibodies

The LRRK2 open-reading frame fused to an myc-his c-terminal tag has been described previously (14). Functional and familial-linked mutations were introduced into LRRK2 cDNA by PCR-mediated, site-directed mutagenesis, using the QuickChange kit (Stratagene), followed by the sequencing of the entire insert to confirm their correct incorporation. Plasmid DNA was prepared using Maxi-prep columns (Qiagen) and concentration of plasmid DNA normalized by transient transfection assays with subsequent western blot analysis.

Antibodies to Erk1/2, phospho-Erk1/2, p38, phospho-p38, Erk5, JNK1/2, phospho-JNK1/2, c-Jun and phospho-c-Jun were obtained from Cell Signaling. Antibodies to β-actin and phospho-ERK5 were purchased from Sigma. Anti-myc conjugated to horse-radish peroxidase or anti-myc antibody (clone 9E10) were purchased from Roche Biochemicals.

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Table 2. Summary of biochemical characterization of *LRRK2* missense mutations

Amino acid substitution	Disease segregation	Number of probands	Domain	GTP-binding activity	Kinase activity
I1122V	Possible	1	LRR	No change	Increase
I1371V	Possible	2	GTPase	Increase	No change
R1441C	Yes	Many	GTPase	Increase	Increase
R1441G	Yes	Many	GTPase	Increase	Increase
R1514Q	Possible	2	COR	No change	Increase
Y1699C	Yes	2	COR	Increase	Increase
I2012T	Possible	2	Kinase	No change	Decrease
G2019S	Yes	Many	Kinase	No change	Increase
I2020T	Yes	2	Kinase	No change	Increase
G2385R	Unlikely	Many	None	No change	No change

Disease segregation and number of probands correspond to published literature as of November 2006. Where disease segregation is possible but unconfirmed by genotype analysis, the designation of possible or unlikely is given. GTP-binding activity and kinase activity are summarized with respect to WT-LRRK2 activity.

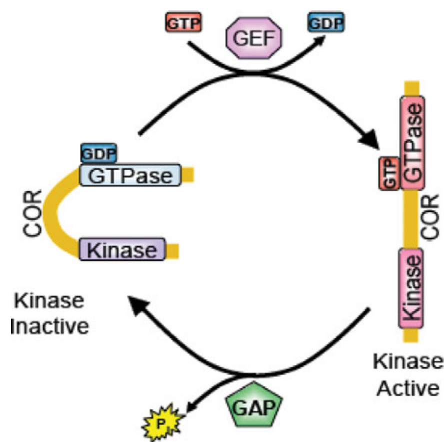

Figure 7. GTPase control over LRRK2 kinase activity. Hypothetical model, in which GTP-binding mediates a potential conformational shift allowing kinase activity.

Autophosphorylation assay

HEK-293FT (Invitrogen) cells were maintained in Opti-MEM media (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). For each experiment, cells were plated in 10 cm dishes and transfection was accomplished using FugeneHD reagent (Roche) with 12 µg of plasmid DNA and 36 µl FugeneHD reagent in serum-free Opti-MEM media. Cell media were replaced with fresh serum-free Opti-MEM media 12 h post-transfection, and cells were harvested 48 h post-transfection into lysis buffer containing 0.5% NP-40, 150 mM NaCl, 50 mM HEPES, pH 7.4, 5 mM EGTA, 1× complete protease inhibitors (Roche) and 1× phosphatase inhibitor cocktails 1 and 2 (Sigma). In this cell line, we did not observe significant toxicity as a result of transfection of any construct, with cell viability estimated by trypan-blue counting and glucose 6-phosphate dehydrogenase activity (Molecular Probes). Cells in lysis buffer were incubated for 1 h at 4°C with rotation, and insoluble material was removed with a 20 000*g* centrifugation for 10 min. Magnetic dynabeads (50 µl; Invitrogen) were pre-complexed with anti-myc antibody (clone 9E10, Roche) and added to the pre-cleared lysate. The bead–lysate mixture was allowed to rotate overnight at 4°C, and the dynabeads were subsequently washed five times with lysis buffer supplemented to 500 mM NaCl.

Dynabeads were suspended into 50 µl of kinase buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EGTA, 20 mM β-glycerol phosphate and 10 µM mouse MBP), and kinase reactions were initiated with the addition of 10 µM ATP [0.5 µCi γ-^{32}P-ATP (Perkin Elmer)] and 20 mM $MgCl_2$. Reactions were incubated at 30°C for 20 min with shaking, placed on ice, and supernatant was removed into 1× Laemmli buffer. Beads were washed once and then suspended into 2× Laemmli buffer and heated for 10 min at 75°C. Then beads were removed and discarded. Kinase reactions were resolved onto SDS–PAGE gels and transferred onto PVDF membrane. Autophosphorylation and MBP signal was estimated using a Storm Phosphoimager and ImageQuant 6.0. Input levels of protein present on the PVDF membrane were determined by western blot.

Phosphorylated residues contained in LRRK2 protein were resolved using thin-layer chromatography. LRRK2 protein was excised from PVDF membrane, rinsed with water, and protein digested with 6 N HCl at 110°C for 1 h. Supernatant was transferred and evaporated and resulting residue dissolved in water. Samples were loaded onto aluminum sheets precoated with silica gel [0.2 mm layer thickness, 20 cm height (EMD Biosciences)] in addition to 10 µg of each phosphoamino acid standard (O-phospho-DL-serine, threonine and tyrosine; Sigma) and a combination of the three standards adjacent to samples. Samples were resolved in one-dimension with an ethanol:25% NH_4OH (3.5:1.6) solvent, cycling three times with air drying between cycles. Plates were sprayed with 0.25% ninhydrin in acetone and developed at 65°C for 5 min. Standards were aligned to isotope signal using Biomax Film (Kodak).

Select autophosphorylation reactions incubated without radionuclides or protein directly immunoprecipitated from cells were analyzed by high-performance liquid chromatography-tandem mass spectrometry. LRRK2 protein stained with BioSafe G-250 dye (Biorad) was excised from SDS–PAGE gels and rinsed two times with water. Samples were submitted to the Taplin Biological Mass Spectrometry Facility (Harvard Medical School) for in-gel digestion and micro-capillary LC/MS/MS analysis.

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GTP/GDP-binding studies

HEK-293FT cells were seeded into 10 cm dishes and transiently transfected with 8 μg plasmid DNA for each myc-tagged LRRK2 variant, using Lipofectamine Plus reagent (Invitrogen) as per the manufacturer's instructions. Cells were lysed at 48 h post-transfection in lysis buffer G [1 × PBS, pH 7.4, 1% Triton X-100, 1 × phosphatase inhibitor cocktail 1 and 2 (Sigma)] containing Complete Mini Protease Inhibitor Cocktail (Roche) by rotating at 4°C for 1 h, and lysates were clarified by centrifugation at 17 500*g* for 15 min at 4°C. Supernatants were incubated with 50 μl γ-aminohexyl-GTP-sepharose suspension (Jena Bioscience) by rotating at 4°C for 2 h and then sequentially pelleted and washed three times with 1 ml lysis buffer G and once with PBS alone. For guanine nucleotide competition experiments, incubation was allowed to proceed for 60 min at 4°C and nucleotides, i.e. GTP, GDP or GTPγS (all Sigma), were added to a final concentration of 2 mM, and incubation was continued for a further 60 min at 4°C followed by washing. GTP-bound proteins were eluted in 50 μl Laemmli sample buffer by heating for 10 min at 70°C. GTP-bound proteins or inputs (1% total lysate) were resolved by SDS–PAGE and subjected to western blot analysis with anti-myc-HRP (Roche) antibody, as previously described.

Primary cortical cultures and cell viability

Primary cortical neuron cultures were prepared from gestational day 15–16 fetal mice. Cortices were dissected and the cells dissociated by trituration in modified Eagle's medium (MEM), 20% horse serum, 25 mM glucose and 2 mM L-glutamine, following a 15 min digestion in TrypLE (Invitrogen). The cells were plated on 48 well plates coated with poly-L-ornithine and were maintained in MEM, 10% horse serum, 25 mM glucose and 2 mM L-glutamine in a 7% CO_2 humidified 37°C incubator. The glial cell growth was inhibited by addition of 5-fluoro-2′-deoxyuridine (5F2DU, Sigma, 30 μM) to the culture medium on DIV 4. The growth medium was refreshed once every third day. At DIV 10, neurons represent >90% of the total number of cells (31). For assessment of LRRK2-toxicity, LRRK2 and eGFP constructs were combined in a molar ratio of 10:1, respectively, and added to NeuroFect reagent (Genlantis) in serum-free media. DNA:NeuroFect complexes were added to DIV 10 cultures. On DIV 13, cultures were incubated with 2.5 μM propidium iodide (Sigma) for 10 min at room temperature (Invitrogen). Images were collected on a Zeiss Automatic stage with Axiovision 6.0. Neurons with propidium iodide-positive nuclei and/or obvious process and nuclear fragmentation were counted as non-viable cells by investigators blinded to the identity of the experiment. Percent viability was calculated by normalizing that data with total eGFP-positive cells in each culture. Cultures were subsequently fixed with 4% paraformaldehyde and incubated with anti-myc antibody (Roche) and anti-mouse IgG-Cy5 antibody (Jackson ImmunoResearch). LRRK2 expression was confirmed in >95% of eGFP-positive neurons (data not shown).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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