BRIEF COMMUNICATIONS

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Kinase activity of mutant LRRK2 mediates neuronal toxicity

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Mutations in the the leucine-rich repeat kinase-2 (*LRRK2*) gene cause autosomal-dominant Parkinson disease and some cases of sporadic Parkinson disease. Here we found that LRRK2 kinase activity was regulated by GTP via the intrinsic GTPase Roc domain, and alterations of LRRK2 protein that reduced kinase activity of mutant LRRK2 correspondingly reduced neuronal toxicity. These data elucidate the pathogenesis of *LRRK2*-linked Parkinson disease, potentially illuminate mechanisms of sporadic Parkinson disease and suggest therapeutic targets.

LRRK2, a complex protein of the ROCO family, contains both the predicted kinase effector domain (MAPKKK) and the GTP-binding regulatory domain (ROC-COR)[1,2]. The most common mutation, G2019S in the MAPKKK domain, contributes to 5–6% of all cases of autosomal-dominant Parkinson disease as well as to 1–2% of cases of sporadic Parkinson disease[3–7]. G2019 is part of a highly conserved DYG motif (2,017–1,019 in LRRK2) at the activation segment of the MAPKKK domain of LRRK2 (ref. 8). Parkinson disease–linked LRRK2 mutants augment kinase activity[9–11]. Neuronal loss is a key feature of both familial and sporadic Parkinson disease, and individuals with *LRRK2* mutations exhibit neuronal degeneration in the brain[2]. Mutant LRRK2 causes neuronal toxicity[12]. However, the relation among LRRK2 kinase activity, GTP regulation and toxicity is unknown.

Using the 'Prosite' bioinformatic program and site mutagenesis, we generated constructs predicted to alter the kinase activity of mutant (G2019S and R1441C) LRRK2, including D1994N (DN), in which the predicted proton acceptor is abolished, K1906A (KA), in which a predicted ATP binding site is abolished, and DY2017-2018AL (AL), in which the predicted DYG motif is altered. D1994N and DY2017-2018AL mutations markedly reduced the kinase activity of mutant LRRK2, and the K1906A mutation slightly decreased it (**Fig. 1**), possibly reflecting the involvement of other functional ATP binding sites. Notably, D1994N and DY2017-2018AL mutations correspondingly reduced (and K1906A slightly reduced) mutant LRRK2-induced toxicity in human neuroblastoma SH-SY5Y cells and in primary neurons (**Fig. 2a–f**). These results indicate that kinase activity of mutant LRRK2 is critical for neuronal toxicity.

The Roc domain of LRRK2 comprises conserved motifs for GTPase activity. To determine whether LRRK2 can bind GTP, we conducted a GTP-binding assay using sepharose-coupled GTP. WT-LRRK2 bound to GTP-sepharose and was inhibited by an excess amount of GDP or GTP but not ATP (**Fig. 3a**). G2019S-LRRK2 behaved in a similar fashion (**Fig. 3b**). We mutated the P-loop (refs. 13–15) in the potential nucleotide-binding pocket of the Roc domain, by replacing lysine 1,347 with alanine. LRRK2-K1347A did not bind appreciably to GTP-sepharose (**Fig. 3b**). GTP-γS led to an approximate 2- to 4-fold autophosphorylation of both wild-type and G2019S-LRRK2 (**Fig. 3c**), but GDP binding had no marked effect (**Fig. 3c**). LRRK2-K1347A, which is devoid of GTP binding activity, could not be activated by GTP-γS (**Fig. 3d**), suggesting that a functional Roc

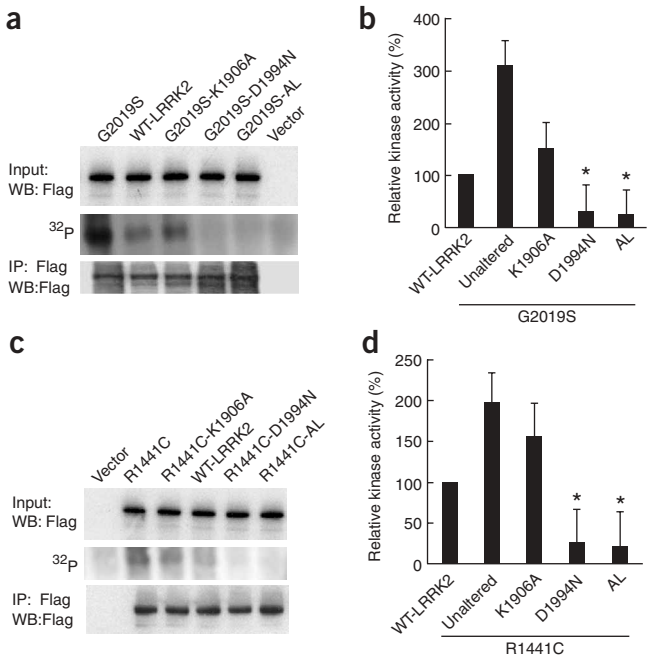

Figure 1 Alteration of the key residues of LRRK2 reduces its kinase activity in an autophosphorylation assay. (**a**–**d**) Immunoprecipitated samples from HEK-293T cells transfected with Flag-*LRRK2* were incubated with [γ-^{32}P]ATP for 90 min, subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes. The samples were then imaged using a phosphoimaging system (**a**,**c**). The incorporation of [γ-^{32}P]ATP into LRRK2 protein was decreased with alteration of key residues. (**b**,**d**) Quantitation of phosphorylation of mutant or altered LRRK2 normalized to phosphorylation of wild-type LRRK2 (five independent experiments). *$P < 0.05$ (one-way analysis of variance, ANOVA) compared to the phosphorylation of G2019S-LRRK2 (**b**) and R1441C-LRRK2 (**d**).

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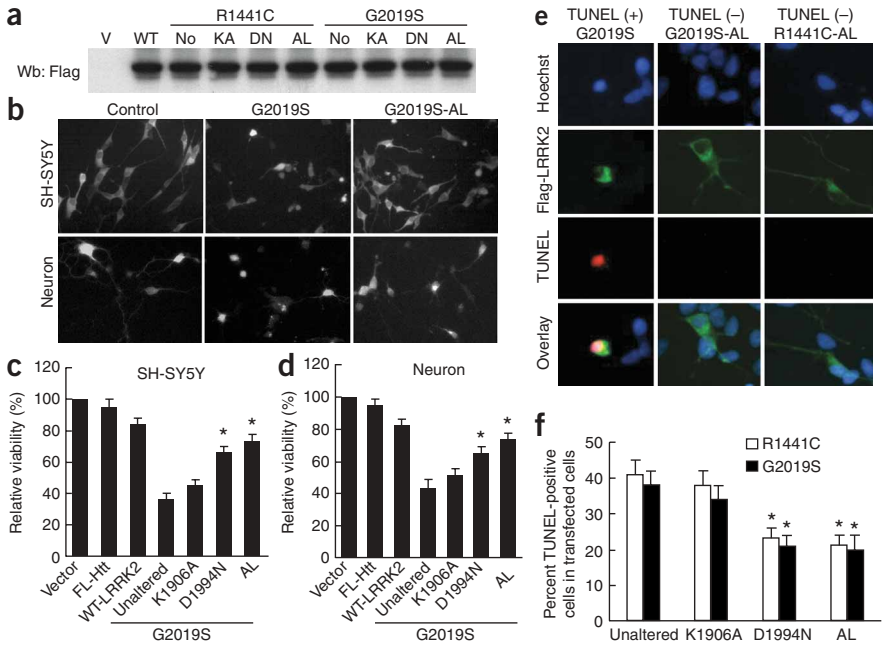

Figure 2 Alteration of mutant LRRK2 activity reduces its neuronal toxicity. (**a**) Western blots showing the comparable expression levels of various altered LRRK2 proteins in SH-SY5Y cells. (**b**) SH-SY5Y cells (top) and mouse primary cortical neurons (bottom) were transfected, respectively, with Lipofectamine Plus or by electroporation, along with pcDNA3.1-GF*P* and various constructs (details in **Supplementary Methods** online). Green fluorescent protein (GFP)-positive cells (neurons) with neurites (defined as continuous extensions twice the diameter of the cell body) were counted using fluorescence microscopy. Shown are representative photomicrographs for each experimental group. (**c**,**d**) Quantitation of cell viability, normalized to number of cells cotransfected with empty vector and GFP in three independent experiments. $^*P < 0.05$ compared to cells expressing G2019S-LRRK2 and GFP (Student's *t*-test). (**e**,**f**) SH-SY5Y cells were cotransfected with constructs expressing various forms of LRRK2, and treated as described in **b**, followed by immunostaining with an antibody to Flag (anti-Flag). Then a TdT-mediated X-dUTP nick end labeling (TUNEL) assay was conducted. The number of TUNEL-positive cells as a percentage of the total LRRK2-transfected cells was calculated. (**e**) Representative confocal images of each experimental group. (**f**) Quantitation of data in **e**. $^*P < 0.05$ compared to cells transfected with unaltered mutant *LRRK2*.

domain is required for GTP-induced LRRK2 autophosphorylation. The G2019S-LRRK2 construct with the K1347A alteration also showed reduced neuronal toxicity in SH-SY5Y cells and primary neurons (**Fig. 3e**,**f**), further suggesting that GTPase activity regulates kinase activity, which mediates the toxicity of mutant LRRK2.

In sum, we provide evidence that LRRK2 is a GTP/GDP-regulated protein kinase, combining both the kinase effector and the GTP-binding regulatory domain in one protein. We identified several key amino residues in LRRK2 as critical for kinase activity. Alterations of key residues of the ROC-COR or the MAPKKK domain reduced the kinase activity of mutant LRRK2 and correspondingly reduced its neuronal toxicity, consistent with dominant gain-of-function inheritance of *LRRK2*-linked Parkinson disease mediated by altered kinase activity. The kinase domain of LRRK2 is homologous to B-RAF kinase,

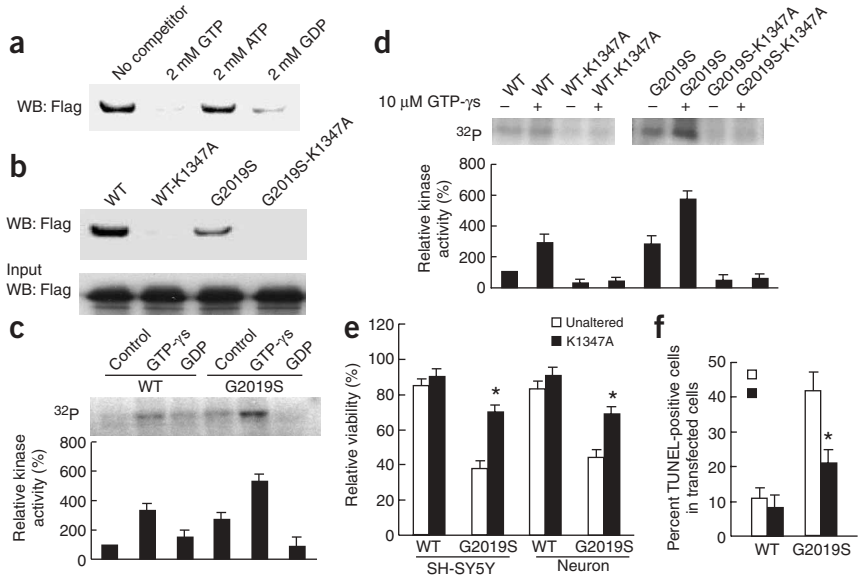

Figure 3 Binding of GTP to LRRK2 requires the GTPase-like Roc domain and leads to stimulation of LRRK2 kinase activity. (**a**) Flag-wt-LRRK2 was affinity-purified from lysates of transfected HEK293T cells, using GTP-sepharose, in the absence or presence of GTP, ATP or GDP. Precipitates were resolved by SDS-PAGE and immunoblotted with anti-Flag. (**b**) Using a GTP binding assay similar to **a**, we found that the wild type-LRRK2-K1347A and G2019S-LRRK2-K1347A did not bind to GTP (top). Equal protein input was controlled by a western blot using anti-Flag (bottom). (**c**,**d**) Top, autoradiographs of immunoprecipitates from HEK-293T cells transfected with various *LRRK2* constructs, subjected to autophosphorylation assays in the presence or absence of 10 μM GTP-γS or GDP (three independent experiments). Bottom, quantitation of kinase activity normalized to that in untreated cell lysates of wt-LRRK2. (**e**) SH-SY5Y cells and primary cortical neurons were cotransfected with pcDNA3.1-*GFP* and various *LRRK2* constructs, and treated as described in **Figure 2b**. Relative cell viabilities were quantified. $^*P < 0.05$ compared to cells cotransfected with G2019S-*LRRK2* and *GFP*. (**f**) SH-SY5Y cells were cotransfected with various *LRRK2* constructs and treated as described in **e**, followed by anti-FLAG immunostaining and TUNEL assay. The number of TUNEL-positive cells as a percentage of the total *LRRK2*-transfected cells was calculated. □ Unaltered construct. ■ Construct with K1347A alteration. $^*P < 0.05$ compared to cells transfected with G2019S-*LRRK2*.

and the mutated G2019 of LRRK2 is equivalent to G595 of B-RAF (ref. 8), suggesting functional analogies between LRRK2 activation in Parkinson disease neurodegeneration and B-RAF kinase activation in cancer. Our findings elucidate the role of LRRK2 kinase activity in neuronal death in *LRRK2*-linked Parkinson disease and suggest that modulation of LRRK2 GTPase and kinase activities could be therapeutic targets.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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