Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration


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Parkinson’s disease (PD) is a disorder of movement, cognition, and emotion, and it is characterized pathologically by neuronal degeneration with Lewy bodies, which are cytoplasmic inclusion bodies containing deposits of aggregated proteins. Most PD cases appear to be sporadic, but genetic forms of the disease, caused by mutations in α-synuclein, parkin, and other genes, have helped elucidate pathogenesis. Mutations in leucine-rich repeat kinase 2 (LRRK2) cause autosomal-dominant Parkinsonism with clinical features of PD and with pleomorphic pathology including deposits of aggregated protein. To study expression and interactions of LRRK2, we synthesized cDNAs and generated expression constructs coding for human WT and mutant LRRK2 proteins. Expression of full-length LRRK2 in cells in culture suggests that the protein is predominately cytoplasmic, as is endogenous protein by subcellular fractionation. Using coimmunoprecipitation, we find that LRRK2, expressed in cells in culture, interacts with parkin but not with α-synuclein, DJ-1, or tau. A small proportion of the cells overexpressing LRRK2 contain protein aggregates, and this proportion is greatly increased by coexpression of parkin. In addition, parkin increases ubiquitination of aggregated protein. Also, mutant LRRK2 causes neuronal degeneration in both SH-SY5Y cells and primary neurons. This cell model may be useful for studies of PD cellular pathogenesis and therapeutics. These findings suggest a gain-of-function mechanism in the pathogenesis of LRRK2-linked PD and suggest that LRRK2 may be involved in a pathogenic pathway with other PD-related proteins such as parkin, which may help illuminate both familial and sporadic PD.

Materials and Methods

Materials. Media, N2 and N27 supplements for cell culture, Lipofectamine Plus reagent, and anti-hemagglutinin (HA) polyclonal Ab were obtained from Invitrogen. Hoechst 33342 was obtained from Molecular Probes. Anti-ubiquitin polyclonal Ab was obtained from DAKO. Anti-myc and anti-HA mAbs were obtained from Santa Cruz Biotechnology and Roche Molecular Biochemicals. Cy3-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch. Anti-FLAG Ab was obtained from Sigma. Anti-histone 2B and anti-tubulin Abs were obtained from Upstate Biotechnology (Lake Placid, NY).

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Abbreviations: PD, Parkinson’s disease; LRRK2, leucine-rich repeat kinase 2; IP, immunoprecipitation; Htt, huntingtin; HA, hemagglutinin; Q, polyglutamine.

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mM NaCl membranes were blocked in TBST (10 mM Tris
ferred onto polyvinylidene difluoride membranes (Invitrogen). The
resulting immunoprecipitates and cell
probed with different Abs. Proteins were detected by using en-
defined as having at least one smooth extension (neurite) with twice
an investigator, who was kept unaware of the experimental condi-
cells (neurons) were counted from 40 randomly selected fields by
cotransfected with LRRK2 constructs or full-length htt with 23Q,
DMEM with N2 supplement for 24 h. Primary neurons were
1:15) for 24 h in 10% FBS OPTI-I media and then changed to
affected with pcDNA3.1-GFP, along with vector pcDNA3.1, full-
assays were conducted as described in ref. 29. Cells were cotrans-
taining Hoechst 33342. Lysates were centrifuged at 800
to the manufacturer’s instructions. For counting, images were taken
from 20 randomly selected fields from each experimental group by
apoptotic and necrotic cell death was performed as described in ref.
Hoechst 33342 staining is indicated blue, and green indicates FLAG-
the length of the cell body. The percentage of GFP-positive viable
neurons (neurons) in each experimental group relative to those of cells
transfected with vector and GFP was calculated.
Hoechst 33342/propidium iodide labeling of cells to detect
apoptotic and necrotic cell death was performed as described in ref.
TUNEL staining was performed as described (30) by using the
Texas red in situ cell-death-detection kit (Roche Molecular Bio-
chemicals). SH-SY5Y cells were cotransfected with various con-
structs for 24 h in 10% FBS OPTI-I media, changed to DMEM with
N2 supplement for 24 h, and then subjected anti-FLAG immunohisto-
chemical staining. TUNEL staining was then performed according to
the manufacturer’s instructions. For counting, images were taken from
20 randomly selected fields from each experimental group by
using conventional fluorescence microscopy by an investigator who
was kept unaware of the experimental condition.

**Immunocytochemistry.** Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and processed as described
(31). Cell preparations were incubated with primary Abs: anti-
FLAG, anti-myc, or anti-ubiquitin, then with secondary Abs: Cy3-conju-
gated anti-mouse or FITC-conjugated anti-rabbit; nuclei were
stained with Hoechst 33342, and all signals were analyzed by
fluorescence and confocal microscopy (LSM 510 and Axiowert 100,
The number of cells with cytoplasmic aggregates were counted in 20 randomly selected fields with ~1,000 cells in each experimental condition. Counts were done by an investigator who was kept unaware of the experimental condition.

**Data Analysis.** Quantitative data are expressed as arithmetic means ± SE based on at least three separate experiments performed in duplicate. The difference between two groups was statistically analyzed by Student's t test or one-way ANOVA. Significance was defined at P < 0.05.

**Results**

**Cytoplasmic Expression of LRRK2.** To study the expression of LRRK2, we generated a cDNA expression vector containing the full-length LRRK2 ORF with codons optimized for mammalian and bacterial expression (Fig. 1A). Several versions of the construct were generated, including full-length WT LRRK2 with no tag and constructs with N-terminal FLAG tag or C-terminal myc tag. Also, the following three of the reported mutants were generated: R1441C in the ROC domain, Y1699C in the COR domain, and G2019S (the most common one) in the mitogen-activated protein kinase kinase kinase (MAPKKK) domain, with FLAG or myc tags.

We synthesized two peptides to generate Abs against two distinct regions of human LRRK2 (amino acids 1245–1259 and 2396–2408). Expression of LRRK2 in HEK 293T cells yielded a protein of ~280 kDa detected with either Abs to the FLAG tag or to LRRK2 (Fig. 1B). The WT LRRK2 protein without FLAG-tag ran at a similar position (Fig. 1B). Cellular expression of WT or mutant LRRK2 appeared predominately diffuse cytoplasmic (Fig. 1D), although a minority of cells had a punctate label suggestive of aggregates (see below). There was no apparent change in the cytoplasmic distribution for any of the mutants in HEK 293T cells. Subcellular fractionations derived from SH-SY5Y cells confirmed that LRRK2 was excluded from the nucleus and present in cytosol (Fig. 1C).

![Fig. 2. LRRK2 interaction with parkin in HEK 293T cells. (A and B) Lysates prepared from cells transfected with various constructs as indicated were subjected to IP with anti-FLAG (A) or anti-HA (B), followed by anti-HA and anti-FLAG immunoblotting. The experiment was repeated three times with similar results.](image-url)

**Interaction of LRRK2 with Parkin.** Because LRRK2 showed predominately cytoplasmic expression, we investigated whether it interacted with other cytoplasmic PD-related gene products. By coimmunoprecipitation assays, LRRK2 showed a specific interaction with parkin but not with α-synuclein (Fig. 2), DJ-1, or tau (data not shown). Fig. 2A shows IP with FLAG-LRRK2 and detection of either HA-α-synuclein or HA-parkin. Parkin coimmunoprecipitated with LRRK2, but α-synuclein did not. Conversely, either α-synuclein or parkin was immunoprecipitated by using HA, followed by anti-FLAG LRRK2 immunoblotting (Fig. 2B). LRRK2 was detected only with IP of HA-parkin. Even when the blot was considerably overexposed, there was no detection of LRRK2 using IP of HA-α-synuclein (data not shown). The LRRK2 mutations did not alter the interaction between LRRK2 and parkin (data not shown).

To determine which regions of parkin and LRRK2 were responsible for the interaction, a series of constructs containing different domains of parkin tagged with myc were cotransfected with full-length LRRK2. As shown in Fig. 3, only parkin constructs containing the RING2 domain showed an interaction with LRRK2. Conversely, when different domains of FLAG-LRRK2 were cotransfected with full-length HA-parkin, there was a strong interaction between the COR domain of LRRK2 and parkin (data not shown).
Increase of Cytoplasmic Aggregates Containing LRRK2 with Coexpression of Parkin. Although the predominant localization of LRRK2 in transfected cells is diffusely cytoplasmic, a minority of cells show apparent aggregates (Fig. 4A). There was no consistent change in the percentage of cells with aggregates using the mutants (data not shown). However, when LRRK2 was cotransfected with parkin, there was a substantial increase in the percentage of cells with aggregates (Fig. 4). By contrast, there was no change detected when LRRK2 was transfected with H9251-synuclein or full-length htt with 23Q.

Because parkin is an E3 ubiquitin ligase (27, 32–34), we sought to determine whether the aggregates in these cells contain ubiquitinated proteins. As shown in Fig. 5B and C, there was a considerable increase in the percentage of cells containing ubiquitinated aggregates when LRRK2 was cotransfected with parkin. We also found that ~5% of cells with aggregates contained both parkin and LRRK2 labeling (Fig. S4). We did not find evidence that LRRK2 might be directly ubiquitinated by parkin (data not shown). In cotransfection experiments, LRRK2 caused a 25-fold increase in the autoubiquitination activity of parkin in the context of a 3-fold increase in parkin protein level (Fig. 5D).

Neuronal Degeneration Caused by Expression of Mutant LRRK2. To determine whether mutant LRRK2 could alter cell viability, SH-SY5Y cells were cotransfected with GFP and various LRRK2 constructs by Lipofectamine. Viable cells were defined as having at least one smooth extension with twice the length of the cell body, and they were counted by an investigator who was kept unaware of the experimental condition. Transfection efficiencies were similar in all groups. There was mild, nonsignificant, decreased viability with WT LRRK2. All three mutations of LRRK2 caused significant cell toxicity compared with WT LRRK2, control protein (full-length htt with 23Q), and vector (Fig. 6A and B). We confirmed these results using mouse primary cortical neurons and electroporation. Mutant LRRK2 caused significant neuronal degeneration in primary cultures compared with WT LRRK2, control protein (full-length htt with 23Q), and vector (Fig. 6C and D). Expression of mutant LRRK2 in SH-SY5Y cells caused highly condensed and fragmented nuclei as measured by Hoechst 33342/propium iodide staining and significantly increased the TUNEL-positive cells (Fig. 6E and F), indicating that mutant LRRK2 induced primarily apoptotic cell death. Coexpression of parkin did not significantly protect against mutant LRRK2-induced neuronal degeneration (data not shown).
Discussion

In this study, we found that LRRK2 protein was predominantly cytoplasmic. Using coimmunoprecipitation, we found that LRRK2 interacted with parkin, but not with α-synuclein, DJ-1, or tau. LRRK2 interacted preferentially with the C-terminal R2 RING-finger domain of parkin, and parkin interacted with the COR domain of LRRK2. Coexpression of LRRK2 and parkin increased cytoplasmic protein aggregates that contain LRRK2 and enhanced the ubiquitination of these aggregates. Last, expression of mutant LRRK2 caused neuronal degeneration in both SH-SY5Y cells and mouse primary neurons. These findings suggest a gain-of-function mechanism in the pathogenesis of LRRK2-linked disease.

The cytoplasmic localization of LRRK2 protein is similar to that of several other PD-related gene products, including α-synuclein and parkin (35, 36). LRRK2 appears to be expressed in most tissues although at low levels (14). The cytoplasmic localization is consistent with the LRRK2 amino acid sequence, which does not contain any predicted hydrophobic membrane spanning domains or targeting sequences for other cellular organelles (15). However, we cannot exclude the possibility that some fraction of LRRK2 protein might be associated with cytoskeleton or with the cytoplasmic faces of mitochondrial, endoplasmic reticulum, or other membranes.

We found that LRRK2 specifically associated with parkin but not α-synuclein, DJ-1, or tau protein. Parkin is an E3 ubiquitin ligase, and several of its substrates and interaction partners are relevant for PD pathogenesis (11, 27, 37–39). Parkin associates with and ubiquitinates synphilin-1 (27), a protein that interacts with α-synuclein (28) and is highly enriched in Lewy bodies (40). Also, parkin can interact with other proteins, such as p38/JIV-1 (38).

The site of interaction between LRRK2 and parkin appears to be in the RING2 domain of parkin. This site is also the site of parkin interaction with synphilin-1 (27). When expressed in cells, LRRK2 had a tendency to aggregate, and aggregates were strikingly increased when LRRK2 was coexpressed with parkin but not other proteins. We also found that cytoplasmic aggregates were ubiquitinated in a parkin-dependent fashion. Previously, we found that parkin promotes the formation of ubiquitinated aggregates with cotransfected α-synuclein and synphilin-1 (31). Other studies also found that parkin can accumulate in aggresomes under conditions of proteasome impairment (41, 42). These observations are consistent with the idea of a role for the ubiquitin proteasome pathway in PD (43, 44). In this study, the effect of parkin on the ubiquitin-
The study of α-synuclein has provided insight into both genetic and sporadic PD (49). Similarly, the study of LRRK2 cell biology has the potential to identify the contribution of LRRK2 to neuronal cell death and aggregate formation, and to the molecular pathogenesis of both familial and sporadic PD, which may suggest approaches to therapeutic development. For PD, like Alzheimer’s disease, the identification of rare genetic forms has led to the identification of critical gene products that are likely to be involved in the pathogenesis of sporadic disease. Protein interactions are believed to be central to Alzheimer’s disease pathogenesis. For example, the presenilin gene product has a key role in the proteolysis of amyloid precursor protein (APP), yielding the toxic amyloid β peptide (50–52).

Our results indicate that LRRK2 is a cytoplasmic protein and that mutant LRRK2 can be directly toxic to cells, although these observations would need to be extended to endogenous protein in vivo and human tissue studies. The cell model reported here may be useful for studies of PD pathogenesis and, possibly, therapeutics. LRRK2 interacted with parkin, and parkin can potentiate the formation of aggregates containing LRRK2 and ubiquitin. These findings may be relevant to the formation of Lewy bodies or other protein aggregates in PD and related disorders. Identification of LRRK2 interaction partners and toxicity is likely to provide insights into the molecular pathway of PD pathogenesis and the targets for therapeutic intervention.

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