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Lrrk2 phosphorylates alpha synuclein at serine 129: Parkinson disease implications

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ABSTRACT

Mutations in the alpha synuclein gene (SNCA) are the most potent cause of autosomal dominant Parkinson disease (PD) while mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are the most common cause. We hypothesized that a direct interaction may exist between their protein products. Here we show that full-length Lrrk2 or fragments containing its kinase domain have a significant capacity to phosphorylate recombinant alpha synuclein (Asyn) at serine 129. Such phosphorylated Asyn is the major component of pathological deposits in PD. We further show that the G2019S mutation in Lrrk2, which is the most common genetic determinant of PD, has a significantly greater capacity than wild-type Lrrk2 to phosphorylate Asyn. This suggests that the G2019S mutant protein may cause PD by generating pathological levels of phosphorylated Asyn. Controlling Lrrk2 Asyn phosphokinase activity may be an approach to disease modifying therapy for PD and other synucleinopathies.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that primarily affects dopaminergic (DA) neurons in the substantia nigra (SN). Most PD cases are sporadic, but about 10% are due to disease causing genetic mutations. Two genes that may have close functional links are alpha synuclein (SNCA) and leucine-rich repeat kinase (LRRK2). Duplication of the SNCA region on one copy of chromosome 4 (three copies of the gene) causes onset of typical PD in late middle age [1–3]. Triplication on one copy of chromosome 4, or duplication on both copies (four copies of the gene) cause a much more severe form of disease, including dementia, with much earlier onset and shorter survival time [3,4].

The pathological deposits in PD and related diseases are composed primarily of alpha synuclein (Asyn) phosphorylated at serine 129 (pS129Asyn) [5–7] indicating that this may be a key step in the pathogenesis. The association between pathological Asyn deposits and causative SNCA mutations has resulted in the synuclein burden hypothesis of PD. It proposes that PD and other synucleinopathies are caused by a failure to clear Asyn and that reducing its expression, or its phosphorylation at serine 129, should be an effective approach to therapy [8].

Although SNCA mutations are the most potent cause of autosomal dominant PD, mutations in *LRRK2* are the most common. More than 20 *LRRK2* mutations involving all domains of the molecule have so far been identified [9] although many have not been associated with disease. The pathological mutations typically cause a late onset form of PD although some disease heterogeneity has been observed.

The most prevalent of all PD causing mutations is the G2019S Lrrk2 mutation. It is particularly common in North African Arabs, accounting for 37–42% of familial cases and 41% of apparently sporadic cases. This compares with 2–3% in Europeans and North Americans [10]. Taken together, these genetic results suggest a general involvement of Lrrk2 in PD pathogenesis [11] with the possibility of a direct functional linkage with Asyn.

To explore this possibility, we cloned vectors to express full-length wild-type Lrrk2 and the G2019S mutant, as well as truncated forms expressing the kinase domain. We found that all proteins expressing the kinase domain phosphorylated Asyn at serine 129. The G2019S full-length protein was more active than wild-type Lrrk2, consistent with the gain of function predicted from clinical studies. These data provide the first evidence that Lrrk2 and Asyn are interacting proteins that may be directly linked to the pathogenesis of PD.

Materials and methods

Plasmid preparation and cloning. A wild-type full-length human *LRRK2* cDNA plasmid was kindly provided by Dr. Matt Farrer (Mayo Clinic, Department of Neuroscience, Jacksonville, FL). For the purpose of generating a myc-tagged vector, this full-length human

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LRRK2 cDNA plasmid was first subcloned by cutting out the *LRRK2* cDNA fragment using Hind III and Xho I restriction enzymes. The product was then inserted into a pCDNA4–myc/his-A vector (Invitrogen). This resulted in a new full-length *LRRK2* plasmid named *pLRRK2*-myc which differed from the original by including a myc/his tag at the C-terminus. The new plasmid was then transformed into DH5a competent cells. The cells were plated and grown overnight on an ampicillin plate. Cell colonies were then further cultured in Luria Broth (LB, Sigma) overnight. The cells were pelleted and the DNA purified using a DNA purification kit (QIAGEN). The product was verified by complete sequence analysis. It was then transfected into HEK293 cells using Lipofectamin 2000 according to the manufacturer's directions. Three fragments incorporating the *LRRK2* kinase domain and a full-length G2019S mutant were then prepared. They included the Roc domain extending to the C-terminus (bp 3945–7581) designated RCKW; the COR domain extending to the C-terminus (bp 4554–7581) designated CKW; and the kinase domain (bp 5535–6429) designated KKK.

Primers were designed according to the *LRRK2* gene coding sequence (MIM:609007). Forward sequences incorporated the BamH I site and reverse sequences the Xho I site. The forward primer for the Roc initiating site was 5′-cgcggatccgcgccaccatgcaaagacatc ataagg-3′; that for the COR initiating site was 5′-cgcggatccgcgccacc atggctccagagtt tct-3′; and that for the KKK site was 5′-gcaggatccat gcgaatgaaacttatgattgtggga-3′. The reverse sequences were: full-length C-terminus, 5′-ccgctcgagctcaacagatgttcgtctcat-3′; and KKK domain terminus, 5′-ccgctcgagga ctaattcagctgaatt-3′.

The G2019S mutation was introduced into *LRRK2*-WT by site-directed mutagenesis using the QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA). Full-length mutated *LRRK2* plasmids were then generated. The primers were as follows: G2019S-F, 5′-gattg ctgactacagcattgctcagtac; G2019S-R, 5′-gtactgagcaatgctgtagtcagc aatc. They were used according to the manufacturer's instructions to produce a *pLRRK2*(G2019S)-myc plasmid.

In all cases, the PCR-amplified fragments were authenticated by full sequencing. They were cloned into the multicloning site of vector pCDNA4–myc/his-A. The fragments were ligated to the vector using the T4 ligase kit (Invitrogen). The products were transformed into DH5a competent cells and the protein products harvested as described for full-length Lrrk2.

Cell culture and transfection. HEK293 cells were cultured in DMEM medium with 10% FBS at 37 °C in a CO_2 incubator. Plasmid transfections were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Transfected HEK293 cells were incubated for 48 h, pelleted by centrifugation at 6000*g* for 5 min, and washed twice with PBS. The pellet was treated for 30 min on ice with lysis buffer (50 mmol/L Tris–HCl at pH 7.5, 150 mmol/L NaCl, 1% Triton X100, 1 mmol/L EDTA, 1 mmol/L phenylmethanesulphonylfluoride, 10 μg/mL pepstatin and mini complete protease inhibitor cocktail). Cells were then sonicated with a Branson sonifier (10 bursts on ice, 50% pulse, setting 2). The debris was removed by centrifugation at 14,000*g* for 20 min. The supernatants were analyzed for total protein concentration by the Bradford method (Bio-Rad) and then stored at −80 °C until utilized.

To determine the concentration of myc-tagged proteins in the supernatants, a highly sensitive slot blot assay was utilized. Cell lysates were initially diluted 1:10 in PBS and then serially diluted in 2-fold steps. About 10 μl of each dilution was added into apparatus slots (Schleicher & Schuell, Keene NH) containing a nitrocellulose membrane. A vacuum was then applied to pull the samples onto the membrane with drying for at least 2 h. The dried membrane was blocked at room temperature for 1 h with 5% skim milk in tris buffered saline containing 0.05% Tween 20 (TBS-T). After blocking, the membrane was incubated with an anti-c-myc antibody (9E10, Sigma; 1:5000) at room temp for 2 h. It was washed with TBS-T buffer and then incubated with anti-mouse IgG-HRP conjugate (Sigma, 1:4000) for 1 h and detected by ECL. Images were read and quantitated using NIH Image J software. Plots of intensity versus concentration were generated and compared with a c-myc peptide standard (Genway Biotech, San Diego, CA). It was applied to slots at concentrations varying from 5 mg/ml down to 0.312 mg/ml. The calculated c-myc protein concentrations in mg/ml in the original lysates were: KKK, 3.0; Lrrk2 WT, 2.8; Lrrk2 G2019S, 2.66; RCKW, 2.64; CKW, 3.08.

Expression and purification of recombinant Asyn. A full-length human A-syn cDNA (140 bp residues) was kindly provided by Dr. Matt Farrer (Mayo Clinic, Department of Neuroscience, Jacksonville, FL). This recombinant *ASYN* was transformed into *E. coli* BL21 (DE3) cells and grown overnight in Luria Broth. When the OD reached a level of 0.6–0.8, isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma) was added at a concentration of 0.8 M, with further culturing for 4–6 h to induce protein expression. The collected pellet was resuspended in extraction buffer (0.75 M NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM DTT). Cells were sonicated five times on ice. The extraction solution was boiled for 20 min. The supernatant was collected by centrifugation at 20,000*g* for 20 min. Finally, the extracted supernatant was concentrated and dialyzed at 4 °C against 10 mM Tris–HCl pH 7.4 using the Amicon Ultra 15 Centrifugual Filter. The dialyzed recombinant Asyn was purified on a Mono Q column.

In vitro phosphorylation of Asyn. Recombinant human Asyn (0.4 mg) was incubated in 50 ml of 30 mM Tris–HCl at pH 7.5 containing 4 mM magnesium chloride, 2 mM ATP, for up to 6 h with various protein preparations being tested for their phosphokinase activity. The positive control against which Lrrk2 preparations were compared was casein kinase 2 (CK2.1, MBL International Corporation, MA). It was typically added to the standard incubation mixture at 2 ml (0.2 μg). Amounts of the Lrrk2 preparations that were added varied from 5 to 14 ml depending on the relative total protein concentration of the lysate supernatant. These were equalized to 100 μg of total protein. Negative control incubations were carried out with an empty vector transfected cell lysate and by omitting Asyn or ATP.

Western blotting. Cell lysates and products of phosphokinase reactions were resolved by SDS–PAGE and further analyzed by Western Blotting using standard techniques. Proteins were transferred to PROTRAN membranes and then incubated with appropriate primary and secondary antibodies. To detect phosphorylated Asyn, the primary antibody was EP1536Y (pS129, 1:1000, Epitomics) and the secondary antibody goat-anti-rabbit-HRP (1:2500, Jackson Immuno Research). To detect non-phosphorylated Asyn, the primary antibody was a rabbit anti-Syn antibody EP1646Y (1:1000, Epitomics). To detect myc-tagged proteins, the primary antibody was 9E10 (1:5000, Sigma) and the secondary antibody goat anti-mouse-HRP (1:2500, Invitrogen). All antibodies were diluted in 5% (w/v) skim milk and incubated with the PDVF blot overnight at 4 °C. Incubation with the secondary peroxidase-conjugated anti-mouse or anti-rabbit antibodies was performed at room temperature for 1 h. Blots were developed by using the ECL system (Amersham Pharmacia Biotech) and quantitated by Image J software Analysis.

Results

Fig. 1A shows a schematic diagram of Lrrk2 indicating the boundaries of the five major domains and those incorporated in the RCKW, CKW and KKK constructs. It also shows representative Western blot results of the domains detected with the anti-myc antibody 9E10. There was a correspondence of the molecular

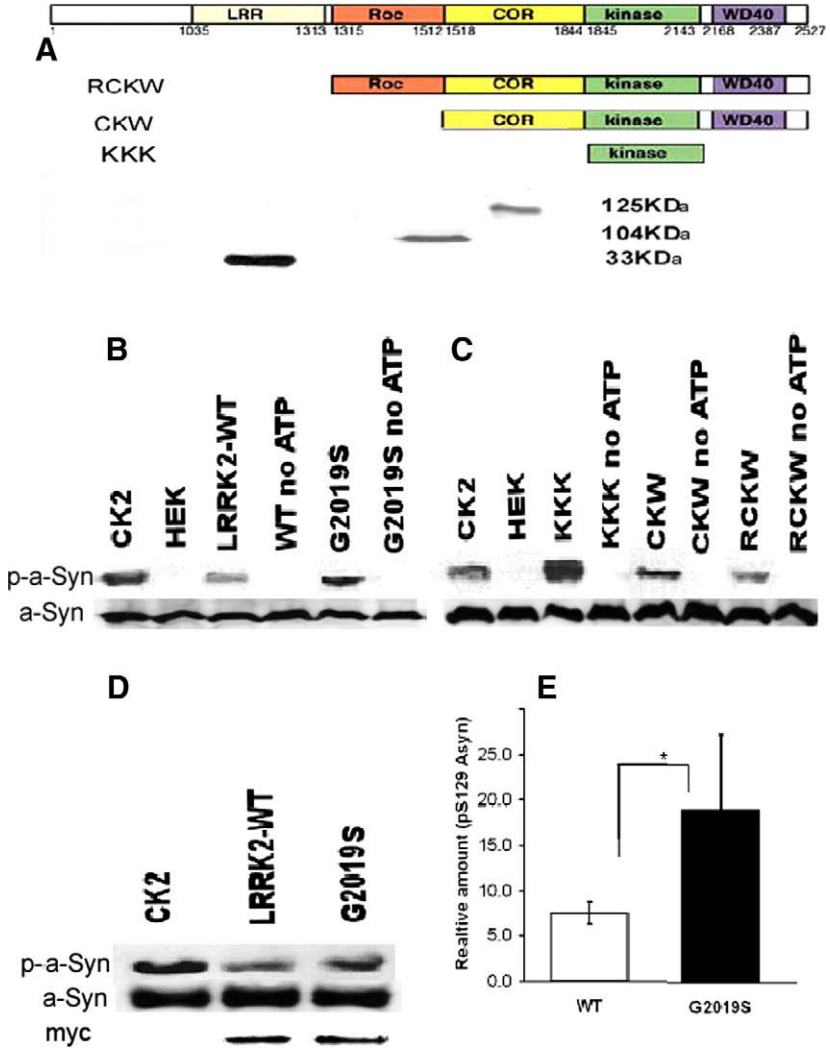

Fig. 1. (A) Schematic representation of the *LRRK2* domain structure, with the domain boundaries being shown by the amino acid residue numbers. The domains included in the three constructs are shown along with their calculated molecular weights, including the myc-tag on the C-terminus. Shown below are bands from Western blotting of lysates derived from HEK cells expressing myc-tagged constructs detected with the 9E10 anti-myc antibody. Bands are consistent with the calculated molecular weights of RCKW 124 kDa, CKW 104 kDa, and KKK 33 kDa. See Methods for details. (B) Upper lanes showing Western blot bands (ca 16–17 kDa) for alpha synuclein phosphorylated at serine 129, as detected with the specific antibody EP1536Y. Lower lanes show equivalent loading of total alpha synuclein as detected with the non-phosphorylation sensitive antibody EP1646Y. HEK cell lysates were loaded with 100 μg of total protein and CK2 was added at 0.2 μg. See Methods for details. (C) Upper lanes showing Western blot bands of alpha synuclein phosphorylated by CK2 and the three truncated Lrrk2 products RCKW, CKW, and KKK. Lower lanes show equivalent loading of total synuclein. Strong pS129A bands were obtained with CK2 and the three constructs but no bands were obtained when ATP was omitted or when an empty vector HEK lysate was incubated. (D) A representative Western blot where the phosphorylating capacity of LRRK2-WT was compared with the G2019S mutant and CK2. Upper lanes show phosphorylated alpha synuclein detected with the EP1536Y antibody. Middle lanes demonstrate equivalent loading of total synuclein as detected with the EP1646Y. Lower lanes demonstrate equivalent loading of myc-tagged proteins in the LRRK2-WT and G2019S lanes as detected with the anti-myc antibody 9E10. (E) Quantitative results of the comparison showing a 2.36-fold greater capacity for the G2019S mutant ($n = 3$, $p < .001$).

weight observed with the calculated domain sizes for RCKW (125 kDa), for CKW (104 kDa) and for KKK (33 kDa).

Fig. 1B–D show representative Western blot bands of various incubates in which recombinant alpha synuclein that has been phosphorylated at serine 129 (p129SA) is detected with the specific antibody EP1536Y. The bands are located at about 16–17 kDa by SDS–PAGE as detected by molecular weight markers, which is slightly above the calculated molecular weight of 14 kDa.

Fig. 1B shows that significant bands were obtained following incubation with 100 μg of HEK cell lysates transfected with LRRK2WT (wild-type) or the G2019S mutant. The G2019S lysate gave a stronger band than the wild-type lysate. A strong band was also obtained with 0.2 μg of purified CK2 added to the standard incubate in place of a cell lysate. No bands were obtained when ATP was omitted from the reaction mixtures, and no band was obtained from a HEK cell lysate that had been transfected with empty vector only.

Fig. 1C shows comparable results with HEK cell lysates that had been transfected with the three kinase-containing constructs. The RCKW, CKW, and KKK lysates all demonstrated significant bands with the KKK lysate being the strongest. Again, a significant band was also obtained with 0.2 μg of CK2. Bands were not obtained from incubates lacking ATP or from an incubate from empty vector transfected HEK cells. The lower panels in Fig. 1B,C demonstrate equivalent loading in comparative lanes of non-phosphorylated Asyn as detected with antibody EP1646Y.

These data establish that cells transfected with full-length LRRK2, the G2019S mutant, or constructs containing the KKK domain can phosphorylate alpha synuclein at serine 129 at rates comparable with 0.2 μg of CK2.

Fig. 1D is representative of a series of 3 incubates where the phosphorylating capacity of wild-type LRRK2 and the G2019S mutants were directly compared in 6 h incubations, again using CK2 as the positive control in each incubate. The middle panels again demonstrate equivalent loading of Asyn in each lane while the lower panels demonstrate equivalent loading of myc-tagged proteins in the Lrrk2-WT and G2019S mutant lanes. Fig. 1E is an analysis of the band strength showing that the G2019S mutant has a significant 2.36-fold stronger capacity to phosphorylate alpha synuclein ($p < .001$) than wild-type Lrrk2.

Discussion

We have previously reported that Lrrk2 mRNA is found in human brain, heart, and liver, as well as in cultured human astrocytes, microglia and oligodendroglia [12]. We also reported that a specific C-terminal antibody to Lrrk2 (NB300-268) intensely labeled Lewy bodies, oligodendroglial occlusions, thorn shaped astrocytes and a subset of tangles in AD and the PDC syndrome of Guam [12]. Others, using different antibodies to Lrrk2 have reported labeling of Lewy bodies [13–15] and oligodendroglia in multiple system atrophy [14,16]. Taken together, these results establish a close association of Lrrk2 with the lesions of PD and other synucleinopathies. As far as a direct association of Lrrk2 with Asyn is concerned, we have demonstrated coprecipitation of Asyn and Lrrk2 from soluble brain extracts in diffuse Lewy body disease [17]. It has also been reported that in PD lesions, Asyn is extensively phosphorylated at serine 129 [5,6,15,18–21] and that ubiquitin binds primarily to this phosphorylated form [22].

In this study we have shown that Lrrk2, and constructs which include its KKK domain, are able to carry out this specific Asyn phosphorylation. Previously it had been reported that Lrrk2 does not phosphorylate Asyn [23] but the incubation times were short and the temperature low. Therefore attention has been focused on CK1 and CK2 [18,20,21] and pololike kinase 2 [24] where such phosphorylation has been reported. However our results, showing that Lrrk2 can also carry out this phosphorylation, provide a direct link between disease causing mutations and the pathology observed in post mortem brains. Our finding that the G2019S mutation is a more active pS129Asyn phosphokinase than wild-type Lrrk2 may explain how the predicted gain of function [25] of this mutation may be linked to PD.

In the present study, we have demonstrated *in vitro* the capacity of Lrrk2, and truncated constructs which included the KKK domain, to carry out the phosphorylation of Asyn at serine 129. This is the reaction that may underlie the pathology of PD. Furthermore, we have shown that the disease causing G2019S mutation enhances that kinase activity. This suggests a contributing mechanism to PD pathogenesis and indicates that Lrrk2 may be a target for future therapeutic intervention [9,10].

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