

Parkin-mediated ubiquitination regulates phospholipase C- γ 1

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Abstract

Mutations in parkin cause autosomal recessive forms of Parkinson's disease (PD), with an early age of onset and similar pathological phenotype to the idiopathic disease. Parkin has been identified as an E3 ubiquitin ligase that mediates different types of ubiquitination, which has made the search for substrates an intriguing possibility to identify pathological mechanisms linked to PD. In this study, we present PLC γ 1 as a novel substrate for parkin. This association was found in non-transfected human neuroblastoma SH-SY5Y cells as well as in stable cell lines expressing parkin WT and familial mutants R42P and G328E. Analysis of cortical, striatal and nigral human brain homogenates revealed that the interaction between parkin and PLC γ 1 is consistent throughout these regions, suggesting that the interaction is likely to have a physiological relevance for humans. Unlike many of the previously identified substrates, we could also show that the steady-state levels of PLC γ 1 is significantly higher in parkin KO mice and lower in parkin WT human neuroblastoma cells, suggesting that parkin ubiquitination of PLC γ 1 is required for proteasomal degradation. In line with this idea, we show that the ability to ubiquitinate PLC γ 1 *in vitro* differs significantly between WT and familial mutant parkin. In this study, we demonstrate that parkin interacts with PLC γ 1, affecting PLC γ 1 steady state protein levels in human and murine models with manipulated parkin function and expression levels. This finding could be of relevance for finding novel pathogenic mechanisms leading to PD.

Keywords: parkin • phospholipase C γ 1 • Parkinson's disease

Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by neuronal loss mainly affecting dopaminergic neurons in substantia nigra pars compacta and the appearance of intracellular inclusions termed Lewy bodies [1, 2]. The recent identification and understanding of mutations causing PD has given important clues as to the pathological processes underlying this highly complex neurodegenerative disorder [3]. Various deletion and point mutations in the *parkin* gene have been shown to cause autosomal recessive juvenile Parkinsonism (AR-JP) an inherited form of PD with similar phenotype [4].

Parkin is a 52 kD E3 ubiquitin ligase [5] expressed in multiple tissues. E3 ubiquitin ligases function in the ubiquitin/proteasome

pathway by binding to protein substrates and targeting them to the proteasome for degradation. It has also been reported that parkin can mediate degradation-independent ubiquitination [6]. Parkin has a ubiquitin-like sequence (UBL) at the amino terminal region and two RING (really interesting new gene) finger motifs at the carboxy-terminal region separated by an in-between-RING (IBR) domain [7]. The UBL domain plays a key role in bringing parkin to the proteasome [8]. The RING-IBR-RING motifs are important for interaction with the substrates and the E2 conjugating enzymes UbcH7 and UbcH8 [9].

Several substrates for parkin have been identified, including the septins CDC-rel 1 and 2, cyclin E, aminoacyl-tRNA synthetase cofactor, p38, O-glycosylated α -synuclein (α -Sp22), Pael-R, synaptotagmin XI, synphilin-1, Eps15, far upstream sequence element [FUSE]-binding protein 1 (FBP1) and protein interacting with C-kinase 1 (PICK1) [10–18]. It has been suggested that parkin loss of function (*i.e.* mutation in both alleles) leads to an accumulation of its substrates, which then leads to cell death. To date, only two parkin substrates, aminoacyl-tRNA synthetase cofactor, p38

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and FBP1, have been reported to accumulate in parkin knockout mice as well as in sporadic PD subjects [16, 19], whereas accumulation of α -Syn22 and CDC-rel 2 has been shown in parkin ARJP subjects [12, 20].

Parkin ubiquitination of the substrate Eps15 lead to regulation of the epidermal growth factor receptor (EGFR). Lack of parkin results in enhanced endocytosis and degradation of EGFR and also reduced downstream phosphoinositide 3-kinase (PI(3)K)-Akt signalling. Phospholipase C γ 1 (PLC γ 1) is part of this pathway, being phosphorylated both by EGFR and Akt [21]. Considering these data, we investigated whether parkin is involved in regulation of PLC γ 1, an enzyme known to be degraded in the proteasome following ubiquitination by the E3 ubiquitin ligase, c-Cbl [22].

We identified PLC γ 1 as a novel substrate for parkin and report that PLC γ 1 interacts with parkin both in dopaminergic neuroblastoma SH-SY5Y cells and in different regions of human brain. Ubiquitination of PLC γ 1 is enhanced in parkin WT cells compared to cells expressing the parkin ARJP R42P and G328E mutants. PLC γ 1 accumulates in the brains of parkin knockout mice, whereas overexpression of parkin WT in SH-SY5Y cells leads to reduced protein levels of PLC γ 1 in comparison to non-transfected (NT) and parkin ARJP mutant cells.

Experimental procedures

Human brain and parkin KO mice brain samples

Post-mortem human brain material was obtained from the Huddinge Brain Bank (Karolinska University Hospital, Sweden) with approval by the Human Ethics Committee of Karolinska University Hospital. Three brains from individuals with non-neurological disorders (two males, 66 and 83 years old, one 85-year-old female) were used. All brains had a post-mortem delay between 24 and 48 hrs. Whole brains from four parkin knockout mice [23] and four wild-type littermates were also used. Homogenization of brain samples was performed on ice in 50 mM Tris-HCl (pH 7.4) plus 150 mM NaCl, 1% (v/v) Triton-X100 and complete protease inhibitors (Hoffmann-LaRoche Inc., Nutley, NJ, USA). Samples were centrifuged at $1000 \times g$ and protein concentration of the supernatants containing soluble proteins were frozen until analysis by immunoblot.

Treatments, DNA-constructs, transfections and cell culture

Human dopaminergic SH-SY5Y neuroblastoma cells were treated with 10 μ M of proteasome inhibitor Lactacystin (Calbiochem, Darmstadt, Germany) for 6 hrs. The constructs used in this study were described previously [24]. Transfections of human dopaminergic SH-SY5Y neuroblastoma cells with various expression vectors were performed using LipofectamineTM2000 (Invitrogen,

Stockholm, Sweden) according to the manufacturer's instructions. Stable transfectants were selected with 200 μ g/ml geneticin (Invitrogen, Stockholm, Sweden). Cells were cultured at 37°C, 5% CO₂, in Eagle's Minimal Essential Medium with Glutamax containing 10% foetal calf serum (FCS). Transfected cells were supplemented with 200 μ g/ml geneticin. All cell culture supplies were purchased from (Invitrogen, Stockholm, Sweden). The expression of WT and mutant parkins were confirmed by sequencing following reverse transcription.

Immunoblot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 2% Nonidet P-40, 2% Triton-X100 and protease inhibitor cocktail (Sigma-Aldrich, Stockholm, Sweden). Brains and cell extract protein amounts were quantified using the BSA protein assay kit (Pierce, Rockford, IL, USA). Equivalent amounts of protein were separated using 10% acrylamide gels. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Western immunoblotting was performed using either anti-parkin (Cell signaling, Danvers, MA, USA), anti-PLC γ 1, anti-PLC β , anti-c-Cbl, anti-ubiquitin, anti-His antibodies or β -actin (BD Biosciences, San Jose, CA) with overnight incubations at a 1:1000 dilution. The secondary antibodies were anti-rabbit or anti-mouse horseradish peroxidase-linked, and were used at 1:2000 dilution for 1 hr in room temperature. Detection was made by the ECL method and exposure to Hyper film MP (Amersham Biosciences, Piscataway, NJ, USA).

Co-immunoprecipitation

The lysates containing 200 μ g protein in 200 μ l lysis buffer were pre-cleared first with Protein A/G-Sepharose beads (Amersham Biosciences, Piscataway, NJ, USA). The lysates were immunoprecipitated overnight with 1 μ g of anti-parkin or anti-PLC γ antibody. Thereafter, new beads were added and lysates incubated for 2 hrs at 4°C with rocking. The immune complexes were washed 3 times with lysis buffer and analysed by Western blot.

Immunocytochemistry

SH-SY5Y cells were grown on glass coverslips and fixed in ice-cold methanol for 10 mins on ice. Methanol was subsequently discarded and cells were washed with PBS. Following 1 h blocking in 5% BSA, 0.2% Triton X-100, 2% goat serum (Sigma Aldrich, Darmstadt, Germany) in PBS, cells were incubated with primary parkin anti rabbit IgG (Cell Signaling, 1:100) and PLC γ 1 anti mouse IgG (Jackson Immuno Research 1:500) together with DAPI (Sigma Aldrich, Darmstadt, Germany) and incubated for 1 h at RT. Finally, cells were rinsed several times in PBS with 0.2% Triton X-100 and mounted with Fluorescent Mounting Medium (Dako, CA, USA).

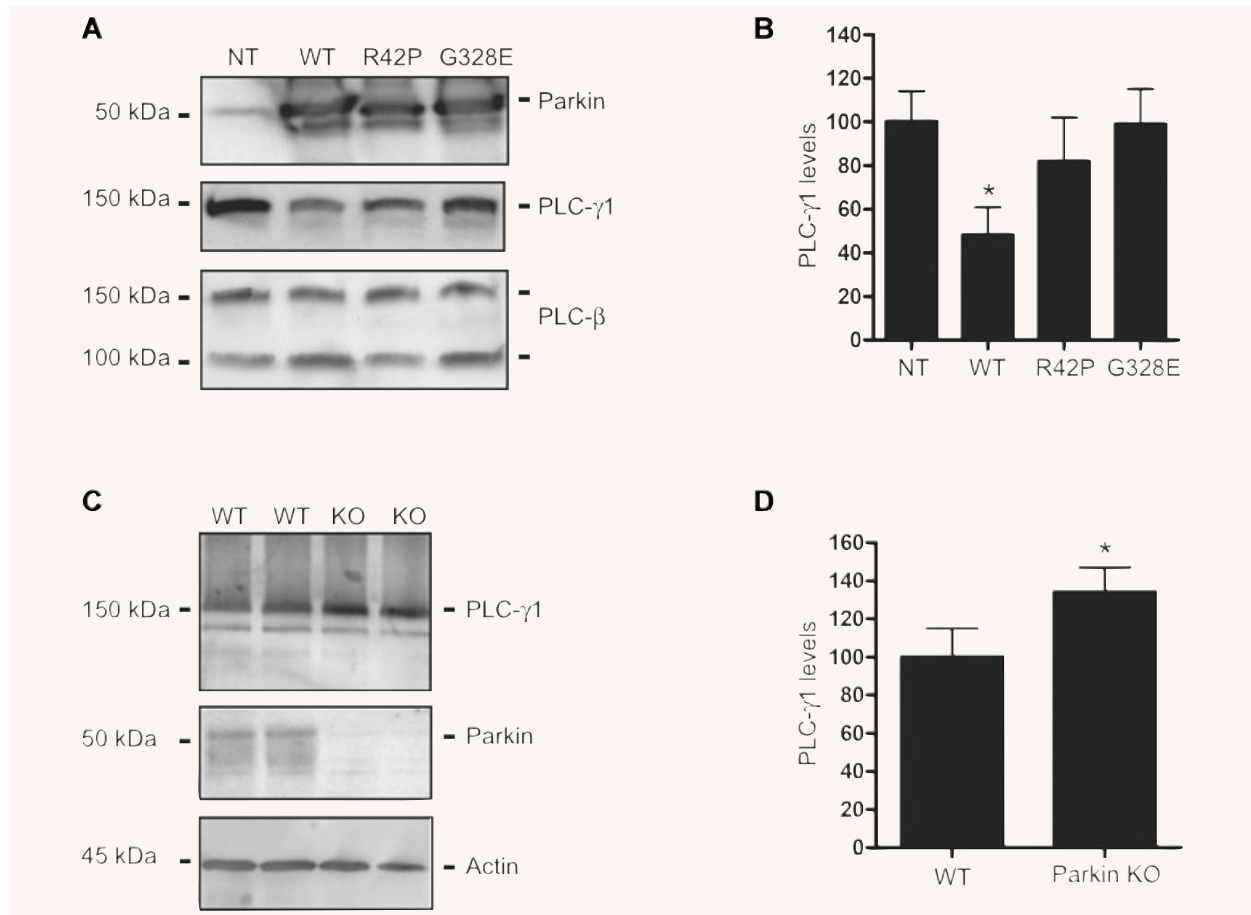


Fig. 1 PLC-γ1 levels are decreased in cells overexpressing WT Parkin, and increased in Parkin knockout mice. **(A)** Immunoblotting of parkin, PLC-γ1 and PLC-β levels in cell lysates from SH-SY5Y cells transfected with parkin (WT) and the ARJP parkin mutations R42P and G328E. **(B)** Quantification of the PLC-γ1 levels from the cells in A. Data are expressed as percentage of values for NT cells and represent the mean ± S.D. of four independent experiments (*, $P < 0.05$; ANOVA followed by Fisher's PLSD *post hoc* test). **(C)** Immunoblots of brain homogenates from wild-type (WT) and parkin knockout mice (KO) using antibodies against PLC-γ1, parkin and β-actin. **(D)** Quantification of PLC-γ1 levels in WT and parkin KO mice. Data were normalized as ratio of PLC-γ1 to actin levels, and expression as percentage of values for WT animals. Bars represent means ± S.D. of four animals per group, (*, $P < 0.05$; ANOVA followed by Fisher's PLSD *post hoc* test).

In vitro ubiquitin conjugation assay

PLC-γ1 and parkin immunoprecipitates were washed 3 times with washing buffer containing 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 25 μM N-Carbobenzoxymethyl-Leu-Leu-Leucinal (MG132), 10 nM dithiothreitol, (Sigma-Aldrich, Stockholm, Sweden), 10 mM N-ethylmaleimide, protease inhibitor cocktail (Sigma-Aldrich, Stockholm, Sweden), 50 mM NaF, 1 mM Na₃VO₄. The immune complexes were analysed by Western blot and normalized to contain similar levels of PLC-γ1 and parkin. Thereafter, they were mixed and incubated together for 10 min. at 30°C with agitation in a reaction buffer containing 10% (vol/vol) reticulocyte lysate (Promega, Nacka, Sweden), 1 μM dithiothreitol, 20 mM HEPES,

10 mM MgCl₂ (Sigma-Aldrich, Stockholm, Sweden), 1 mM ATP, 1 mg/ml hexokinase, 20 mM deoxyglucose, 25 μM MG132 and His-tagged ubiquitin (5 μg/ml) (Sigma-Aldrich, Stockholm, Sweden). The reaction was terminated by washing twice with washing buffer. The immune complexes were analysed by Western blot.

Statistical analyses

Analyses of differences were carried out by ANOVA followed by Fisher's PLSD *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

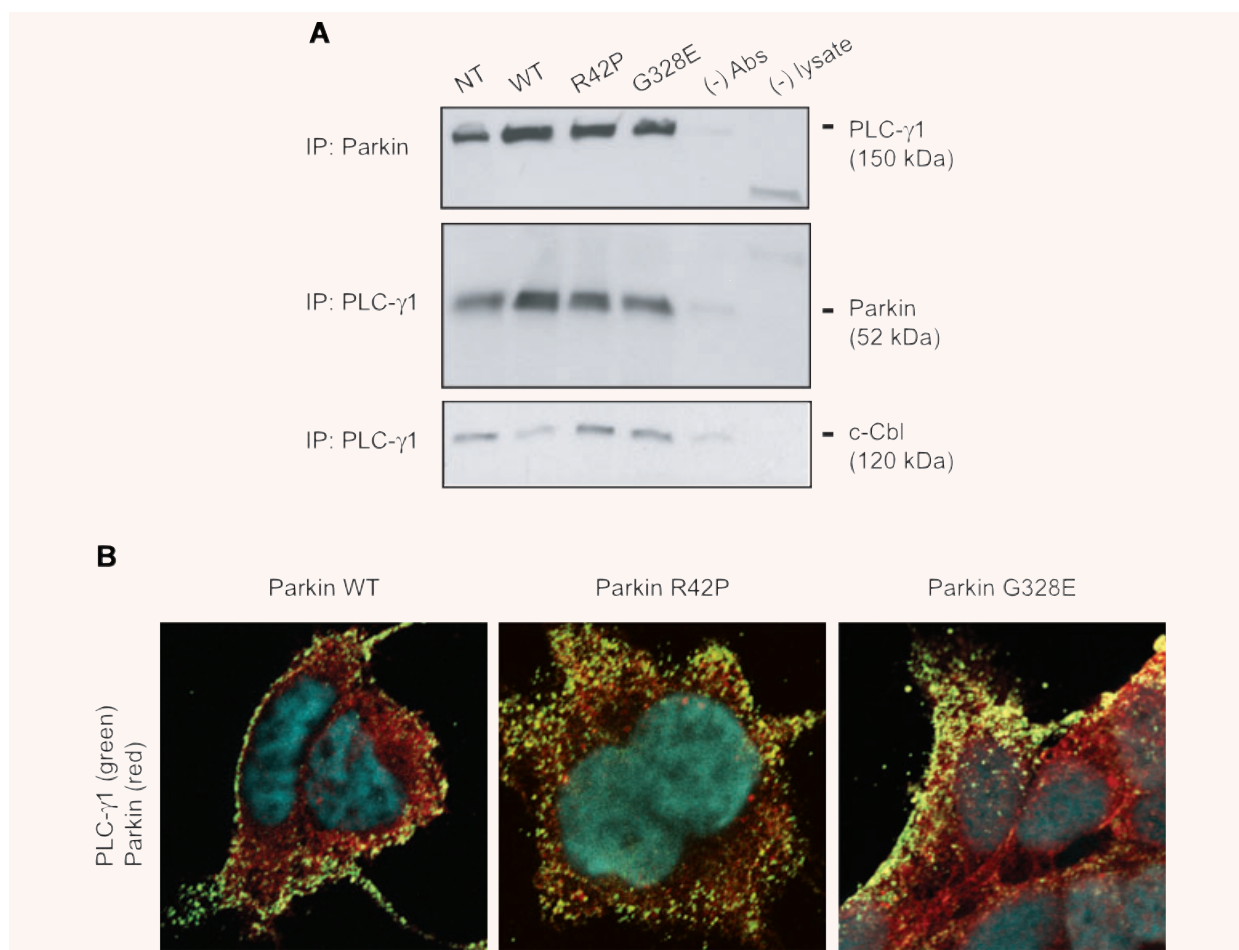


Fig. 2 PLC γ 1 is associated with parkin. **(A)** Lysates from non-transfected (NT) and in stably transfected human SH-SY5Y neuroblastoma cells with wild-type (WT) parkin and the ARJP parkin mutations R42P and G328E were immunoprecipitated with either anti-parkin (upper panel) or anti-PLC γ 1 antibodies (middle and lower panels) and detected by Western blotting with anti-PLC γ 1, anti-parkin or anti-c-Cbl antibodies. **(B)** Confocal micrographs of parkin WT, R42P and G328E cells were double stained with anti-parkin (red) and anti-PLC γ 1 (green) antibodies. Superimposed images showed co-localization of parkin and PLC γ 1 in parkin WT, R42P and G328E cells (yellow).

Results

PLC γ 1 levels are dependent on the expression level of functional parkin

Western blot analysis of human dopaminergic SH-SY5Y stable cell lines expressing WT, R42P or G328E ARJP mutant versions of parkin revealed that PLC γ 1 levels are lower in cells overexpressing WT parkin (Fig. 1A and B). Cells overexpressing ARJP mutant versions of parkin showed similar levels of PLC γ 1 as in NT cells. Protein levels of parkin were approximately 10–12 times higher in transfectants compared to NT cells.

In order to further elucidate whether the impact of functional parkin levels on PLC γ 1 was species specific, we analysed brain material from parkin KO and WT mice. Immunoblotting of whole brain homogenates shows that PLC γ 1 levels are higher when parkin is completely abolished, as compared to WT mice (Fig. 1C). Quantification of the PLC γ 1 band intensity shows that this difference between mouse strains was significant (Fig. 1D).

Parkin and PLC γ 1 are interacting in human dopaminergic neuroblastoma

Since PLC γ 1 has been shown previously to be degraded in the proteasome through ubiquitination by the E3 ubiquitin ligase c-Cbl

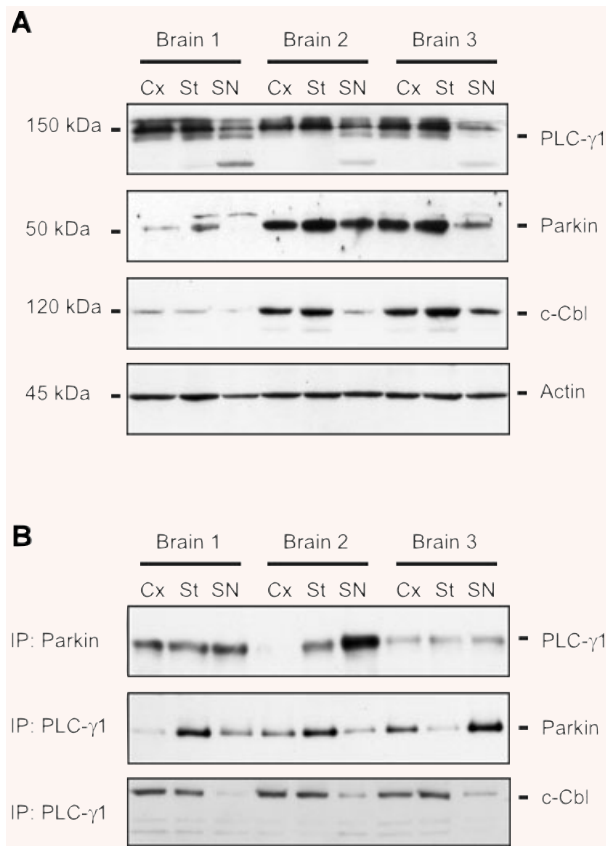


Fig. 3 The association of PLC γ 1 in different brain regions. **(A)** Immunoblotting of PLC γ 1 and parkin in homogenates Cortex (Cx), Striatum (St) and Substantia Nigra (SN) for three human brains. **(B)** Homogenates for the same samples seen in figure A were immunoprecipitated with either anti-parkin (upper panel) or anti-PLC γ 1 (lower panels) antibodies and detected by Western blotting with anti-PLC γ 1, anti-parkin or anti-c-Cbl antibodies.

which is a member of the RING domain family, and parkin is also an E3 ubiquitin ligase in the same family, we analysed if there was a ligase-substrate interaction between parkin and PLC γ 1 using two different approaches. Protein extracts from NT and parkin transfected cells immunoprecipitated with parkin and PLC γ 1 antibodies revealed that there was an interaction between parkin and PLC γ 1 in all cell lines (Fig. 2A). The input for this immunoblot is presented as Figure 1A and the interaction with c-Cbl is shown as a positive control for the immunoprecipitation. In order to visualize the interaction between parkin and PLC γ 1, we performed confocal microscopy of parkin transfectants. We noted that in parkin WT overexpressing cells PLC γ 1 and parkin co-localize mostly in plasma membrane, whereas in the parkin ARJP mutants the interaction was more scattered. Consistent with the immunoprecipitation results, the detected fluorescence representing parkin (red) and PLC γ 1 (green) overlapped in all the parkin overexpressing cell lines. Since endogenous parkin was impossible to be detected

with this method, we were not able to confirm the interaction in NT cells. As negative controls, immunoprecipitation and co-staining experiments were also performed with the IgG antibody PKC ϵ together with parkin, where neither overlapping signal in confocal microscopy nor IgG interaction was found (Supporting Fig. S1).

Parkin and PLC γ 1 are associated in human cortical, striatal and nigral areas

The observed interaction between parkin and PLC γ 1 in human neuroblastoma cells led us to further investigate if the same observations could be seen in human brain material. Since one of the pathological hallmarks of PD is nigral degeneration affecting dopaminergic input to the striatum, we decided to analyse substantia nigra, striatum and cortex. Immunoprecipitation of human brain homogenates showed that the interaction between parkin and PLC γ 1 is consistent in all the three examined regions from three different individuals (Fig. 3A). Western blot analysis of the input for the precipitation uncovered a regional difference in protein levels of PLC γ 1 and c-Cbl, which were lower in the nigral fractions but present in striatum and cortex (Fig. 3B). Though c-Cbl could be recognized as a co-precipitate to PLC γ 1 in the substantia nigra, but persistently at very low levels. This finding suggests that in the substantia nigra parkin may play a stronger regulatory role in regulating PLC γ 1 levels than does c-Cbl. A lower molecular weight band was also detected with the PLC γ 1 antibody in all the fractions, but more abundant in the substantia nigra.

Parkin WT successfully ubiquitylates PLC γ 1 and this function is impaired in parkin ARJP mutants

As previously reported by others, we found that proteasome inhibition by lactacystin results in an accumulation of PLC γ 1 (Fig. 4A). One suggested mechanistic impairment caused by parkin ARJP mutations is a deficient E3 ubiquitin ligase activity. To test this function on PLC γ 1, we performed an *in vitro* ubiquitin conjugation assay. Two major bands were detected, corresponding to full length (150 kD) and a highly ubiquitylated PLC γ 1 (approximately 200 kD), respectively. Levels of PLC γ 1 associated ubiquitin were identified with immunoblotting with a His-tag antibody (Fig. 4B). Quantification of ubiquitin revealed that parkin ARJP mutants had significantly lower levels of PLC γ 1 associated ubiquitin (Fig. 4C).

Discussion

Parkin mutant ARJP patients have a similar neuropathological profile as the idiopathic disease, making studies on the functions of parkin important for distinguishing PD pathology. How parkin dysfunction participates in ARJP pathogenesis is still under debate, but it is likely that deficient protein ubiquitination by parkin

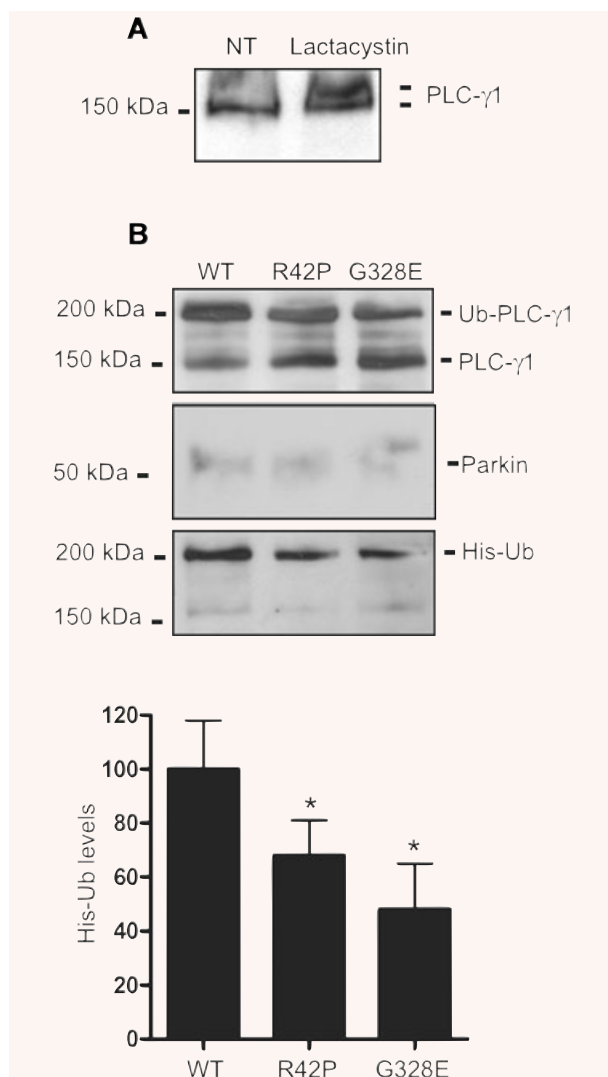


Fig. 4 ARJP parkin mutations cause a defective PLC γ 1 ubiquitination. **(A)** Immunoblotting of PLC γ 1 in human SH-SY5Y neuroblastoma cells untreated or treated with proteasome inhibitor Lactacystin (10 μ M, 6 hrs). **(B)** *In vitro* ubiquitin conjugation assay of PLC γ 1 in cells transfected with parkin WT or the ARJP parkin mutations R42P and G328E. Parkin and PLC γ 1 immunoprecipitates were normalized to contain similar levels of these proteins and incubated in the presence of reticulocyte extracts and His-tagged-ubiquitin. Samples were analysed by Western blotting with antibodies against PLC γ 1 (upper panel), parkin (middle panel), anti-his (lower panel).

would lead to impaired degradation, aberrant localization or altered activity of substrates and that this consequence takes part in the pathological mechanisms behind PD. This idea has stimulated the search for parkin substrates of which defective ubiquitination could be linked to neurodegeneration. Previous studies have identified a range of important substrates. However, few are

accumulated and others are not targets for degradation depending on the type of ubiquitination by parkin.

Our major finding in this study is the identification of PLC γ 1 as a novel substrate for the E3 ubiquitin ligase parkin. This interaction is consistent in human SH-SY5Y cells as well as in cortical, striatal and nigral regions of the human brain. We also show increased ubiquitination of PLC γ 1 by parkin WT compared to R42P and G328E mutants, resulting in a lower steady state PLC γ 1 protein level in parkin WT overexpressing SH-SY5Y neuroblastoma cells. In line with previous studies, the R42P and G328E mutants appear to be loss-of-function mutations, thus cells with ectopic expression of these mutants do not differ in PLC γ 1 levels compared to NT cells due to the preserved endogenous expression of parkin between all cell lines. The R42P mutation that is situated in the UBL domain and the G328E mutation that is found in the IBR-domain can both interact successfully with PLC γ 1. Thus, the apparent difference in ubiquitination and PLC γ 1 degradation could be resulting from deficient catalytic activity. Though we did not detect a typical poly-ubiquitination pattern, but possibly multiple mono-ubiquitinations, directing us to think that the difference in PLC γ 1 degradation could result from the activity of other ubiquitin ligases, where parkin mono-ubiquitination is a prerequisite. There is also a possibility that parkin is initiating PLC γ 1 translocation, which would lead to impaired degradation as a downstream consequence. We detected that PLC γ 1 co-localize differently in parkin ARJP mutants, supporting this hypothesis but further experiments have to be done to confirm this observation.

When we expanded this finding to a murine model, we found that the level of PLC γ 1 was moderate but significantly increased in parkin KO animals, suggesting that parkin E3 ubiquitin ligase activity is important for maintaining balanced levels of PLC γ 1. Among the many previously reported parkin substrates, only aminoacyl-tRNA synthetase cofactor p38 and FBP1 have been shown to accumulate in tissue from parkin null mice. These proteins are believed to be authentic parkin substrates targeted for proteasomal degradation by poly-ubiquitination, since they have also been shown to accumulate in MPTP-treated mice and in the brain of sporadic PD subjects.

Supporting the finding of Wang *et al.* (2006) that parkin is a regulator of EGFR endocytosis, by ubiquitination of Eps15 and PI3K-Akt signalling, we show that parkin also regulates PLC γ 1 that is part of the same signalling pathway. It is therefore likely that parkin regulates tyrosine kinase receptor downstream signalling in several critical steps of the cascade. Additionally, PLC γ 1 plays an important role in cellular proliferation, differentiation and motility. It is part of the PLC superfamily that is divided into six isoforms, namely, β , γ , δ , ϵ , ζ , η [25–29]. Tyrosine kinases activate PLC γ 1, which catalyses the hydrolysis of phosphoinositides (PI) to inositol triphosphate (IP $_3$) and diacylglycerol, two second messengers that regulate the mobilization of the intracellular calcium and protein kinase C (PKC) activation, respectively [30]. PKC signalling results in various cellular responses including differentiation, gene expression and apoptosis [31]. Also calcium is central for different cellular processes from regulation of enzyme activity and gene expression to apoptosis [32, 33]. Dopaminergic

neurons in SN are more exposed to oxidative stress and higher intracellular calcium concentrations due to their pace-making feature, making them more vulnerable compared to other neurons in the human brain [34]. It has been suggested that cell death in Parkinson's disease may be associated with increased calcium concentrations [35].

Interestingly, PLC γ 1 levels are significantly lower in substantia nigra compared to striatum and cortex. One reason for this regional discrepancy could be a higher sensitivity to PLC γ 1 downstream signalling cascades within the nigral regions, which then would require high proteasomal regulation of PLC γ 1 to maintain accurate protein level. Changes in PLC γ 1 protein levels would probably have a substantial effect. Since c-Cbl is the only other known E3 ubiquitin ligase responsible to maintain this balance, the relative lower levels of c-Cbl in the substantia nigra fractions may suggest that the E3 ubiquitin ligase of parkin is particularly important in this region.

Taken together, we have demonstrated that parkin is associated and participates in the ubiquitination of PLC γ 1. PLC γ 1 is one of the few parkin substrates that accumulate in parkin knockout mouse. Moreover, ARJP parkin mutants have a defective PLC γ 1 ubiquitination. Parkin has most likely a physiological important role in the regulation of PLC γ 1 in substantia nigra, since we found a low c-Cbl/PLC I interaction in this area. Our studies provide new insights for understanding the function of parkin by the identification of the new substrate PLC γ 1.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Parkin does not interact with protein kinase C- ϵ (PKC- ϵ). To ensure the specificity of the parkin/PLC- γ 1 interaction, (A) lysates from stably transfected human SH-SY5Y neuroblastoma cells with wild-type parkin (WT) were immunoprecipitated with either anti-PKC- ϵ or anti-PLC- γ 1 antibodies and detected by Western blotting with anti-PKC- ϵ , anti-PLC- γ 1 or anti-parkin antibodies. (B) Confocal micrograph of parkin WT cells double stained with anti-parkin (red) and anti-PKC ϵ (green) antibodies. Neither association nor colocalization between parkin and PKC ϵ was found.

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