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Interaction between parkin and mutant glucocerebrosidase variants: a possible link between Parkinson disease and Gaucher disease

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Gaucher disease (GD), a sphingolipidosis characterized by impaired activity of the lysosomal enzyme glucocerebrosidase (GCase), results from mutations in the GCase-encoding gene, *GBA*. We have shown that mutant GCase variants present variable degrees of endoplasmic reticulum (ER) retention and undergo ER-associated degradation (ERAD) in the proteasome. Furthermore, the degree of ERAD of mutant GCase variants correlates with and is one of the factors that determine GD severity. An association between GD and Parkinson disease (PD) has been demonstrated by the concurrence of PD in GD patients and the identification of GCase mutations in probands with sporadic PD. One of the genes involved in PD is *PARK2*, encoding the E3 ubiquitin ligase parkin. Parkin functions in the ERAD of misfolded ER proteins, and it is upregulated by unfolded protein response. Loss of parkin function leads to the accumulation of its substrates, which is deleterious to dopaminergic neurons in PD. We, therefore, tested the possibility that the association between GD and PD reflects the fact that parkin acts as an E3 ligase of mutant GCase variants. Our results showed that mutant GCase variants associate with parkin. Normal parkin, but not its RING finger mutants, affects the stability of mutant GCase variants. Parkin also promotes the accumulation of mutant GCase in aggresome-like structures and is involved in K48-mediated polyubiquitination of GCase mutants, indicating its function as its E3 ligase. We suggest that involvement of parkin in the degradation of mutant GCase explains the concurrence of GD and PD.

INTRODUCTION

Accumulation of glucosylceramides mainly in reticuloendothelial-derived cells is the major cause of Gaucher disease (GD) (1–3), and it results mainly from mutations in the acid-β-glucosidase (GCase)-encoding gene (*GBA*) (4). Owing to its heterogeneity, GD has been subdivided into three different clinical types: the adult chronic non-neuronopathic type 1 disease; the infantile, acute neuronopathic type 2 disease; and the juvenile subacute neuronopathic type 3 GD (reviewed in 1).

We have shown that, in GD, mutant GCase undergoes endoplasmic reticulum-associated degradation (ERAD) (5). In this process, aberrant or mutant proteins are identified as misfolded by the ER quality control machinery and are retained in the ER. After several attempts to refold them by the ER chaperones, the misfolded proteins are retro-translocated from the ER to the cytosol, ubiquitinated and eliminated by the ubiquitin–proteasome system (UPS) (6–8).

We also showed that mutant GCase variants present variable degrees of ER retention and proteasomal degradation and that the degree of ERAD of mutant GCase variants correlates with GD severity (5).

Ubiquitination of proteins is carried out through sequential steps catalyzed by ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiqutin protein ligase (E3) enzymes. Although there are one to two E1 ligases in mammalian cells and several E2 ligases, there are a large number of E3 ligases, which are involved in the recognition of specific substrates for ubiquitination (9–14). Ubiquitin is covalently attached to a substrate protein through the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ϵ-amino group of a lysine residue on the substrate. Polyubiquitination results from the conjugation of ubiquitin molecules to one of the seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) within the preceding ubiquitin molecule (15). K48-linked polyubiquitination acts as a signal for targeting the substrate to the proteasome for

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degradation. In contrast, K63-linked polyubiquitination as well as single or multiple monoubiquitination has been shown to have a proteasome-independent role in the regulation of several cellular processes, including endocytosis, signal transduction and DNA damage (15,16).

The specific E3 ligases that mediate the ubiquitination of mutant GCase in the different tissues are still unknown.

An association between GD and Parkinson disease (PD) has been demonstrated in recent studies of genotypically heterogeneous patients with relatively mild GD, who developed progressive parkinsonian manifestations. In addition, the proportion of GD carriers among PD patients is significantly higher than that in the general population (17–26), indicating that not only GD pathology, even the presence of one mutant GCase allele increases the risk for the development of PD.

PD, a severe neurological disorder of movement, is the second most common neurodegenerative disease (27,28). It is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain. The clinical symptoms include resting tremor, rigidity, bradykinesia and postural instability. There is direct evidence showing that the cause of PD is closely linked to functional abnormality of the UPS (29,30).

Thirteen loci and nine contributory genes have been mapped in familial PD by linkage analysis (31–34). One of them is *PARK2*, which encodes the E3 ligase parkin (35–37) and is mutated in autosomal recessive juvenile parkinsonism (ARJP) (38). Numerous parkin mutations account for ~50% of ARJP cases (39). Parkin is characterized by the presence of a ubiquitin-like domain at its N-terminus and two RING (Really INteresting Gene) finger motifs, flanked by one IBR (In Between RING finger) motif, at its C-terminal region.

The absence of normal parkin leads to improper degradation of some of its substrates and, through their accumulation, to dysfunction and eventually the death of susceptible neurons. Several parkin substrates were already described, including CDCrel-1 (a synaptic vesicle-associated GTPase) (37), Pael-R (parkin-associated endothelin receptor-like receptor) (40), glycosylated α synuclein (SN) (41), synphillin-1 (an αSN interacting protein) (42), cyclin E (43), α/β tubulin (44) and p38, a key structural component of the mammalian aminoacyl-tRNA synthetase complex (45,46).

It is known that the abnormal accumulation of substrates, caused by the loss of parkin function, may be the cause of neurodegeneration in parkin-related parkinsonism. Thus, the misregulation of proteasomal degradation of parkin substrates is deleterious to neurons. Therefore, the accumulation of substrates for the ubiquitination mediated by parkin is probably critical to the pathogenesis of PD (47). Furthermore, parkin is involved in the ubiquitination pathway of misfolded proteins derived from the ER and contributes to protection from neurotoxicity induced by unfolded protein response (UPR). Thus, parkin functions in the ERAD of misfolded ER proteins, and it is upregulated by unfolded protein stress (35).

We, therefore, tested the possibility that the association between GD and PD reflects the fact that parkin is an E3 ligase involved in the recognition and ubiquitination of mutant GCase variants in dopaminergic neurons. In this study, we show that mutant GCase variants are associated with parkin. Normal parkin, but not its RING finger mutants, decreases the stability of mutant GCase. Parkin promotes the accumulation of mutant GCase in aggresome-like structures. Moreover, parkin is involved in K48-mediated polyubiquitination of GCase mutants, indicating its function as an E3 ligase of mutant GCase variants. However, parkin does not affect the stability, nor the ubiquitination of wt GCase. We hypothesize that the involvement of parkin in mutant GCase degradation leads to time-dependent accumulation of its natural substrates, and the death of dopaminergic cells of the substantia nigra, which leads to the development of PD.

RESULTS

Parkin interacts with mutant GCase

We have shown that mutant GCase variants undergo different degrees of ERAD. In this process, mutant proteins are recognized as misfolded in the ER, and following abortive attempts to refold them, they are retro-translocated to the cytosol where they are ubiquitinated and eliminated by the 26S proteasome (5). The components that participate in the recognition and ubiquitination of mutant GCase are still unknown. In order to study whether parkin is an E3 ligase of mutant GCase, we first tested whether parkin recognizes mutant GCase. To do so, HEK293 cells were transfected with plasmids expressing myc-tagged wt or T240R RING finger mutant parkin, which is a 'ligase dead' mutant (48) and His-myc-N370S mutant GCase. The N370S mutation is a mild mutation, most prevalent among Ashkenazi Jews (49,50), which was found in association with PD (20,21,26,51). Following an overnight treatment with carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) to inhibit proteasomal degradation, GCase-containing complexes were isolated and subjected to western blot analysis. The results, presented in Figure 1A, showed that the N370S mutant GCase variant associated with parkin. T240R Parkin, mutated in its first RING finger, which is a 'ligase dead' parkin (48), also interacted with the N370S GCase mutant. We also performed the reciprocal experiment in which parkin-containing immunocomplexes were isolated using anti-parkin antibodies, and the corresponding blot was interacted with an anti-myc antibody. As shown in Figure 1B, mutant GCase was present in the parkin-containing immunocomplexes. Interestingly, wt GCase also interacted with parkin (Fig. 4A and C). It is worth mentioning that under physiological conditions, most normal GCase is in the lysosomes or ER to lysosomal pathway and is not available for interaction with cytoplasmic parkin.

Parkin-mediated degradation of mutant GCase

To determine whether the association between mutant GCase and parkin leads to parkin-mediated degradation of mutant GCase, we tested the effect of parkin expression on GCase steady-state levels. SH-SY5Y cells, which are dopaminergic neuroblastoma cells used as a model for PD (52), stably expressing wt or N370S mutant His-myc-tagged GCase, were transfected with increasing concentrations of myc-tagged parkin. Cell lysates, containing the same amount of protein, were subjected to western blot analysis. The results showed that parkin reduced the amount of mutant GCase in a dose-dependent manner (Fig. 2A). More so, parkin did not affect

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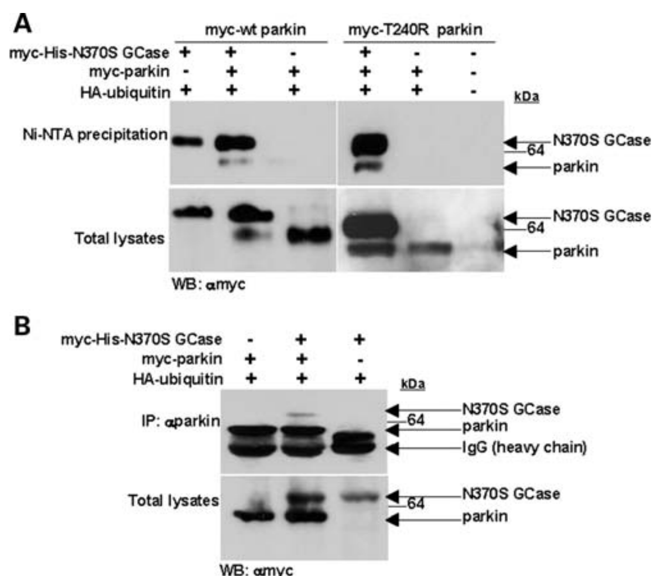

Figure 1. Parkin associates with mutant GCase. HEK293 cells were transfected with myc-tagged-wt or T240R mutant parkin and myc-His-N370S mutant GCase. (**A**) Forty-eight hours later, GCase-containing complexes were isolated by incubating cell lysates with nickel beads (Ni-NTA). Eluted complexes were subjected to western blot analysis and interacted with anti-myc antibody. (**B**) Parkin-containing complexes were immunoprecipitated with anti-parkin antibodies, subjected to western blot analysis and interaction with anti-myc antibody.

the amount of wt GCase (Fig. 2B), indicating that parkin is involved in the degradation of mutant but not wt GCase.

We extended the experiment to HEK293 cells, transiently expressing myc-tagged wt or mutant parkin (P437L, a second parkin 'ligase dead' mutant) (48) and myc-tagged wt or mutant GCase. In this experiment, we included the L444P GCase mutation, which was also found in association with PD (17,24–26,53,54). This is a severe mutation, which leads to type 3 GD in homozygosity (55) and is the most prevalent mutation among non-Jewish patients (49,56). A vector expressing GFP alone was also included, to adjust the total amount of transfected DNA to 6 μg per plate. The results, presented in Figure 2C–F, showed that wt parkin reduced the amount of N370S GCase as well as that of L444P GCase mutant variants in a dose-dependent manner, whereas the RING finger mutant P437L of parkin failed to do so and even stabilized mutant GCase variants. This stabilization reflects, most probably, abortive interaction between mutant parkin and mutant GCase. However, this interaction did not lead to the polyubiquitination and degradation of the latter, but rather to its protection from the activity of other E3 ligases that exist in the transfected cells. Nevertheless, wt or the P437L RING mutant parkin did not affect the stability of wt GCase (Fig. 2G and H), indicating that wt parkin is involved in the recognition of mutant but not wt GCase.

To confirm the results, showing an effect of parkin on the stability of mutant GCase variants, cycloheximide (CHX) chase was performed. CHX blocks *de novo* synthesis of proteins and, thus, allows the detection of their stability (57). SH-SY5Y cells, stably expressing wt or N370S GCase variants, were transfected with myc-tagged parkin and chased with CHX. Cell lysates were prepared after different times of incubation with CHX and analyzed using western blotting. The results (Fig. 3) showed that, although the stability of normal GCase was not affected by the presence of parkin, mutant GCase forms were less stable in the presence of parkin, indicating that parkin mediates degradation of mutant GCase.

Parkin mediates K48 polyubiquitination of mutant GCase variants

The fact that parkin interacted with, and reduced the stability of, mutant GCase variants prompted us to test whether parkin mediates their ubiquitination. Ubiquitination is a dynamic post-translational modification that serves diverse cellular roles (15,58). K48-linked polyubiquitination acts as a signal for targeting the substrate to the proteasome for degradation. In contrast, K63-linked polyubiquitination as well as single or multiple monoubiquitination has been shown to have a proteasome-independent role in the regulation of several cellular processes (15,16). Parkin is known to be a multi-purpose E3 ubiquitin ligase that mediates both K48 and K63 ubiquitination (59–61). Parkin also catalyzes self-multiple monoubiquitination *in vitro* (62,63).

To study whether parkin mediates the ubiquitination of mutant GCase variants, HEK293 cells were transiently transfected with myc-His-tagged wt or mutant GCase variants, in the presence of MG132, and HA-tagged wt or K48R/K63R ubiquitin, with or without myc-tagged wt or RING finger mutant parkin. GCase-containing complexes were isolated and subjected to western blot analysis. The blots were reacted with anti-myc antibody to detect GCase and parkin and with anti-HA antibody to follow the ubiquitination of GCase. As shown in Figure 4, in the absence of MG132 the steady-state level of mutant N370S and L444P GCase variants was reduced, indicating that both underwent ubiquitin-mediated proteasomal degradation, which is inhibited in the presence of proteasome inhibitor. The results also showed that the N370S and L444P mutant GCase variants (Fig. 4A and C) underwent significant ubiquitination in the presence of MG132, wt parkin and wt ubiquitin. In the absence of parkin or in the presence of its RING finger mutants, this ubiquitination was significantly reduced, indicating the importance of functional parkin in mediating it.

Ubiquitin mutated at its lysine 48 (K48R ubiquitin) failed to conjugate and mediate the polyubiquitination of the mutant GCase variants, while lysine 63 mutant ubiquitin (K63R ubiqutin) did not affect the ubiquitination pattern of mutant GCase, strongly indicating that mutant GCase variants underwent K48-mediated polyubiquitination to be degraded by the 26S proteasome. wt GCase interacted with parkin; however, its parkin-mediated ubiquitination was significantly lower compared with that of the mutant variants, indicating that parkin recognizes and mediates K48-linked polyubiquitination of mutant but not wt GCase in order to eliminate it by the proteasome.

Parkin promotes the accumulation of mutant GCase in aggresome-like structures

It is well documented that overexpression of parkin in the presence of proteasome inhibitors leads to its accumulation in aggresomes (64). Aggresomes are proteinaceous inclusion

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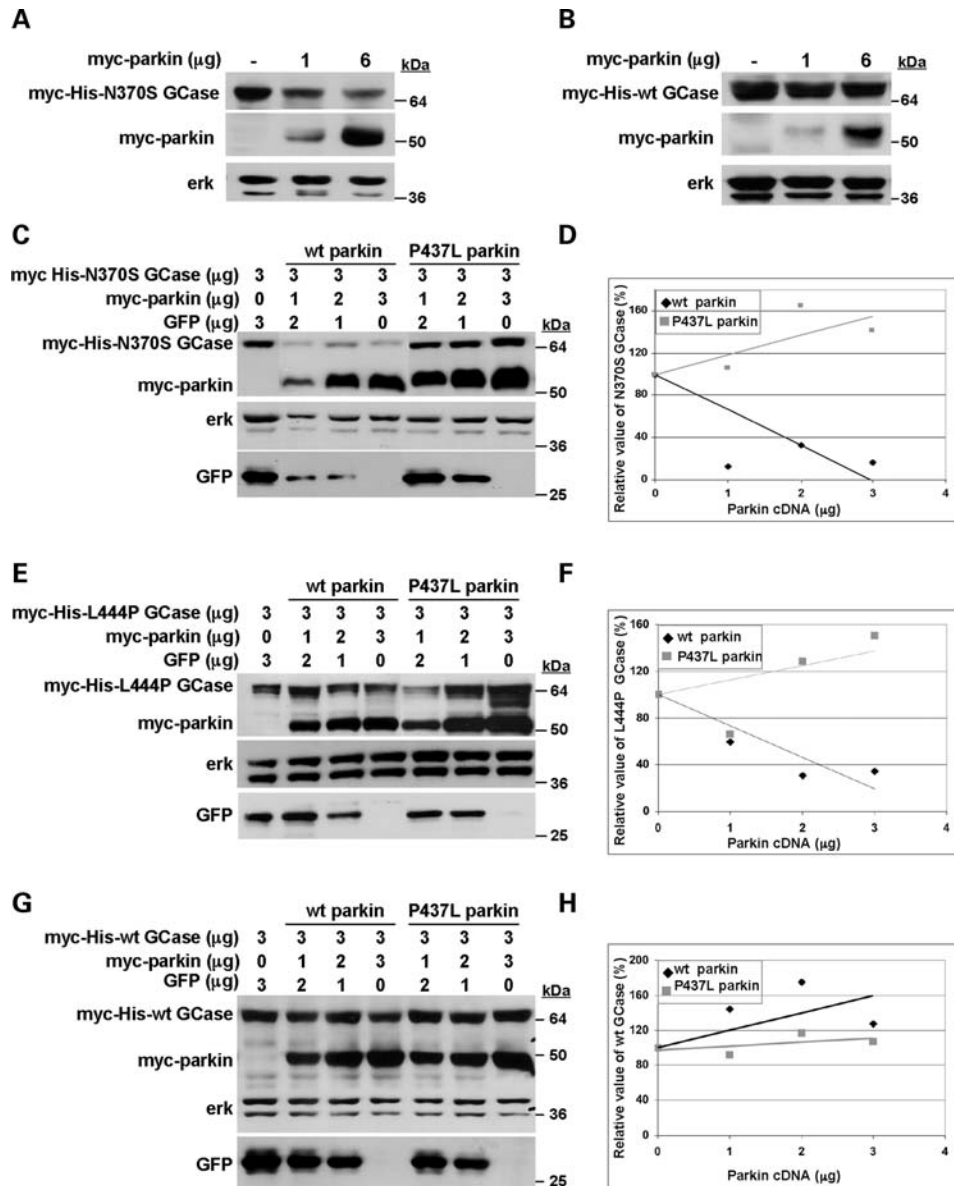

Figure 2. Parkin affects steady-state amounts of mutant GCase. SH-SY5Y cells, stably expressing N370S mutant (**A**) or wt (**B**) myc-His-tagged GCase, were transfected with the indicated amounts of myc-tagged parkin. Twenty-four hours later, cell lysates, containing the same amount of protein, were subjected to western blot analysis, and the blot was reacted with anti-myc antibody and anti-erk antibody, as a protein-loading marker. (**C, E** and **G**) HEK293 cells were co-transfected with 3 μg of plasmids expressing myc-His-tagged N370S (C), L444P (E) or wt (G) GCase and increasing amounts of wt or P437L myc-parkin, as indicated. The total amount of transfected DNA was adjusted to 6 μg with pEGFP C3 plasmid. (**D**, **F** and **H**) To quantify the results, the blots were scanned and GCase intensity was divided by that of erk at each lane and presented as a function of transfected parkin (in micrograms). The no-parkin lane was considered 100%. The results represent the mean of two independent experiments.

bodies that form when cellular degradation machinery is impaired or overwhelmed, leading to the accumulation of misfolded ubiquitinated proteins targeted for degradation (65–67). It has been proposed that aggresome formation is a specific and active cellular response serving to sequester potentially toxic misfolded proteins (67) and that there are similarities between Lewy bodies and aggresomes (68). To determine whether parkin affects the accumulation of mutant GCase, we assessed the effect of GFP-tagged wt or P437L parkin, in the presence of MG132, which inhibits proteasomal degradation, on the accumulation of N370S mutant GCase by immunofluorescence confocal microscopy. The results presented in Figure 5A demonstrated that, in the presence of DMSO vehicle alone, mutant GCase retained in the ER, as we have shown previously (5). Under these conditions, GFP alone was evenly distributed throughout the cell, as expected, and wt parkin was expressed in the cytosol and in the nucleus. However, when co-expressed with mutant parkin, both mutant GCase and mutant parkin, occupied diffuse, perinuclear structures, strongly indicating that mutant parkin binds mutant GCase (Fig. 5A). However, it was unable to mediate its ubiquitination, therefore, they both aggregated together. These

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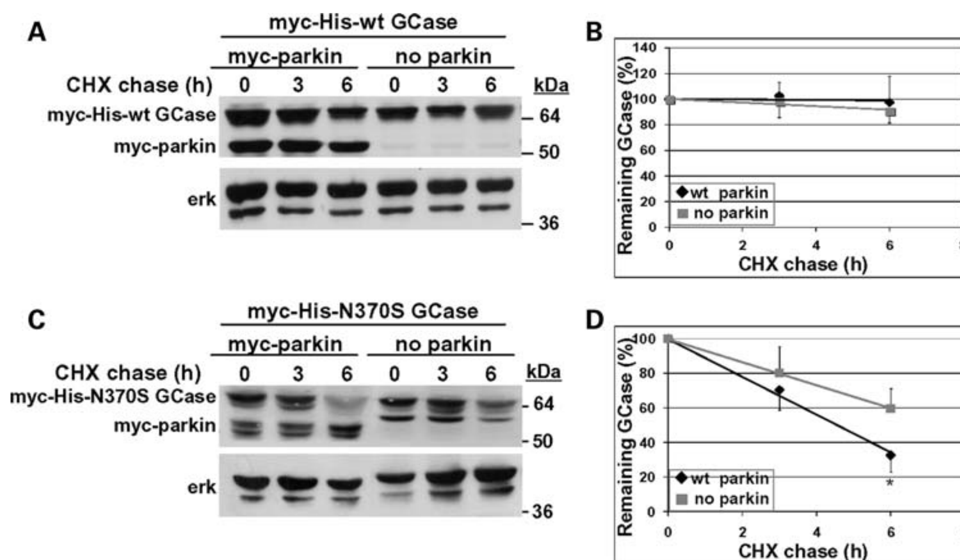

Figure 3. Parkin mediates the degradation of mutant GCase. (**A** and **C**) SH-SY5Y cells, stably expressing wt or N370S mutant myc-His-tagged GCase, were transfected with wt myc-parkin. Non-transfected cells served as a control. Twenty-four hours later, cells were treated with CHX (100 μg/ml) for the indicated times. Cell lysates, containing the same amount of protein, were subjected to western blot analysis and the blots were reacted with anti-myc and anti-erk antibodies. (**B** and **D**) To quantify the results, the blots were scanned and GCase intensity was divided by that of erk at each lane and presented as a function of chase time (h). The results represent the mean ± standard deviation (error bars) of three to four independent experiments, as percentage of the protein level at time 0, which was considered 100%. $^*P < 0.01$ as analyzed by Student's *t*-test.

results corroborate the stabilization effect exerted by mutant parkin on mutant GCase (Fig. 2C–F). In the presence of proteasome inhibitor (MG132), wt parkin and mutant GCase aggregated together in a perinuclear aggresome-like structure (Fig. 5A). The morphology and localization of the N370S mutant GCase inclusions appeared similar to that of aggresomes (65–67). To confirm that mutant GCase co-localizes with parkin in aggresome-like structures, an additional immunofluorescence confocal microscopy was performed using γ-tubulin, as a marker of centrosomes where aggresomes are assembled (66). As evident from the results presented in Figure 5B, mutant GCase co-localized with wt parkin in aggresome-like, γ-tubulin-positive structures. These results strongly indicate that functional parkin promotes the redistribution of mutant GCase into aggresome-like structures and emphasize its role in targeting mutant GCase for degradation.

DISCUSSION

An association between GD and PD has been established lately. Thus, the proportion of GD patients and probands who developed PD or parkinsonism was higher than that in the general population (69), and the percent of GD carriers among PD patients was higher compared with that in the general population (20,21,26,51,54). Since PD individuals who are carriers of GCase mutations do not exhibit glucosylceramide storage, the accumulation of glucosylceramide cannot be the pathological cause in the development of PD. We assume that the ERAD of mutant GCase, mediated by parkin in dopaminergic neurons of the substantia nigra, is a major cause for the development PD among GD patients or carriers. In this study, we show that mutant GCase interacts with parkin, an E3 ubiquitin ligase, whose loss of function mutations result in ARJP. Parkin mediates K48 polyubiquitination of mutant, but not, wt GCase variants and their degradation in the 26S proteasome. In the presence of proteasome inhibitors parkin leads to their accumulation in aggresome-like structures. Moreover, mutant parkin interacts with mutant GCase; however, this is an abortive interaction which does not lead to the polyubiquitination and proteasomal degradation of mutant GCase, but rather to its stabilization (Fig. 2C–F). It is worth mentioning that overexpressed normal GCase interacted with parkin. However, it did not undergo parkin-mediated ubiquitination (Fig. 4), indicating that only mutant GCase is recognized as misfolded and undergoes parkin-dependent ubiquitination and degradation. Yet, under physiological conditions, most normal GCase is not a substrate for ERAD (5) and is unavailable for interaction with cytoplasmic parkin.

Accumulation of unfolded and/or misfolded proteins in the ER lumen induces ER stress. To withstand this stress, UPR processes are activated. The UPR includes translational attenuation, induction of ER-resident chaperones and degradation of misfolded proteins through ERAD. In case of prolonged ER stress, cellular signals leading to cell death are activated. ER stress, induced by aberrant protein degradation, has been implicated in PD (70). Thus, parkin interacts with Hsp70 and CHIP and plays a role in the degradation of proteins during ER stress (35,71,72). Moreover, overexpression of parkin was shown to reduce ER stress-mediated toxicity, caused by its substrate, Pael-R (35,40). Furthermore, the ubiquitin E2-conjugating enzymes Ubc6 and Ubc7, which are parkin partners, are ER-associated and are involved in ERAD (40). Therefore, ER retention of mutant GCase variants and their ERAD seem to underlie the association between GD and PD.

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Figure 4. Parkin mediates K48-linked polyubiquitination of mutant GCase. HEK293 cells were co-transfected with plasmids expressing myc-His-tagged wt, N370S (**A**), or L444P (**C**) mutant GCase variants, in the presence or absence of myc-wt or RING finger (T240R and P437L) parkin mutants and HA-tagged wt or K48R/ K63R mutant ubiquitin. Thirty-six hours later, following treatment with MG132, or vehicle alone, for 16 h, cells were lysed under denaturing conditions and interacted with nickel beads. Eluted fractions were subjected to western blot analysis. The blots were reacted with anti-myc and anti-HA antibodies. (**B** and **D**) 2.5% of total cell lysates were subjected to western blot analysis and the blots were reacted with anti-myc and anti-HA antibodies to demonstrate GCase + parkin and ubiquitin expression, respectively. The blots were reacted with anti-actin, as a protein-loading marker.

We suggest that, since GCase is not a natural substrate of parkin, the enduringly ER retention and proteasomal degradation of mutant GCase, mediated by parkin, affect its activity toward its natural substrates. Time-dependent accumulation of these substrates leads to the death of cells in the substantia nigra and, eventually, to the development of PD.

Loss of parkin was shown to result in the accumulation of some of its substrates, leading to the death of nigra neurons. Thus, non-ubiquitinated forms of Pael-R, cyclin E, CDCrel-1, p38/AIMP2 and FBP1, known substrates of parkin, have been shown to accumulate in the brain tissue from parkin-deficient ARJP patients, suggesting that parkin may normally contribute to their turnover *in vivo* (37,40,43,46,73,74). Yet, the accumulation of another substrate, CDCrel-1, caused cell death restricted to the substantia nigra *in vivo* in rat brains expressing CDCrel-1 (75). Overexpression of the parkin substrate Pael-R (76) led to dopaminergic cell death, which could be rescued by parkin but not by its E3-inactive mutants. Of all its known substrates, p38/AIMP2 and FBP1 accumulated in the brain tissue from parkin null mice, MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine—a neurotoxin that causes permanent symptoms of PD)-treated mice and sporadic PD subjects by killing certain neurons in the substantia nigra, suggesting that both proteins are authentic substrates of parkin and are targeted for proteasomal degradation through parkin-mediated polyubiquitination (46,74).

It is of note that microarray analysis of brain cerebellum tissues, derived from mice homozygous for the V394L GCase mutation, which was found in association with PD (20,24,26), showed an increase in parkin mRNA, as well as an increase in the mRNA levels of Hsp70 and ubiquitin C-terminal hydrolase L1 (UCH-L1), in comparison with normal mice (77).

Hsp70 is an ER chaperone which is induced upon ER stress and the UPR (78,79). It undergoes parkin-mediated monoubiquitination (80). Moreover, Hsp70 enhances parkin binding and ubiquitination of expanded poly-glutamine proteins (81), suggesting that Hsp70 helps to recruit misfolded proteins as substrates for the E3 ligase activity of parkin. Parkin also forms a complex with Hsp70 and CHIP (C-terminus of Hsp70-interacting protein), which is an E3 ubiquitin ligase crucial for the ubiquitination of several heat shock proteins that are involved in neurodegenerative disease (82). This complex enhances the E3 ligase activity of parkin and, thus, enhances its ability to inhibit cell death induced by unfolded protein stress (71).

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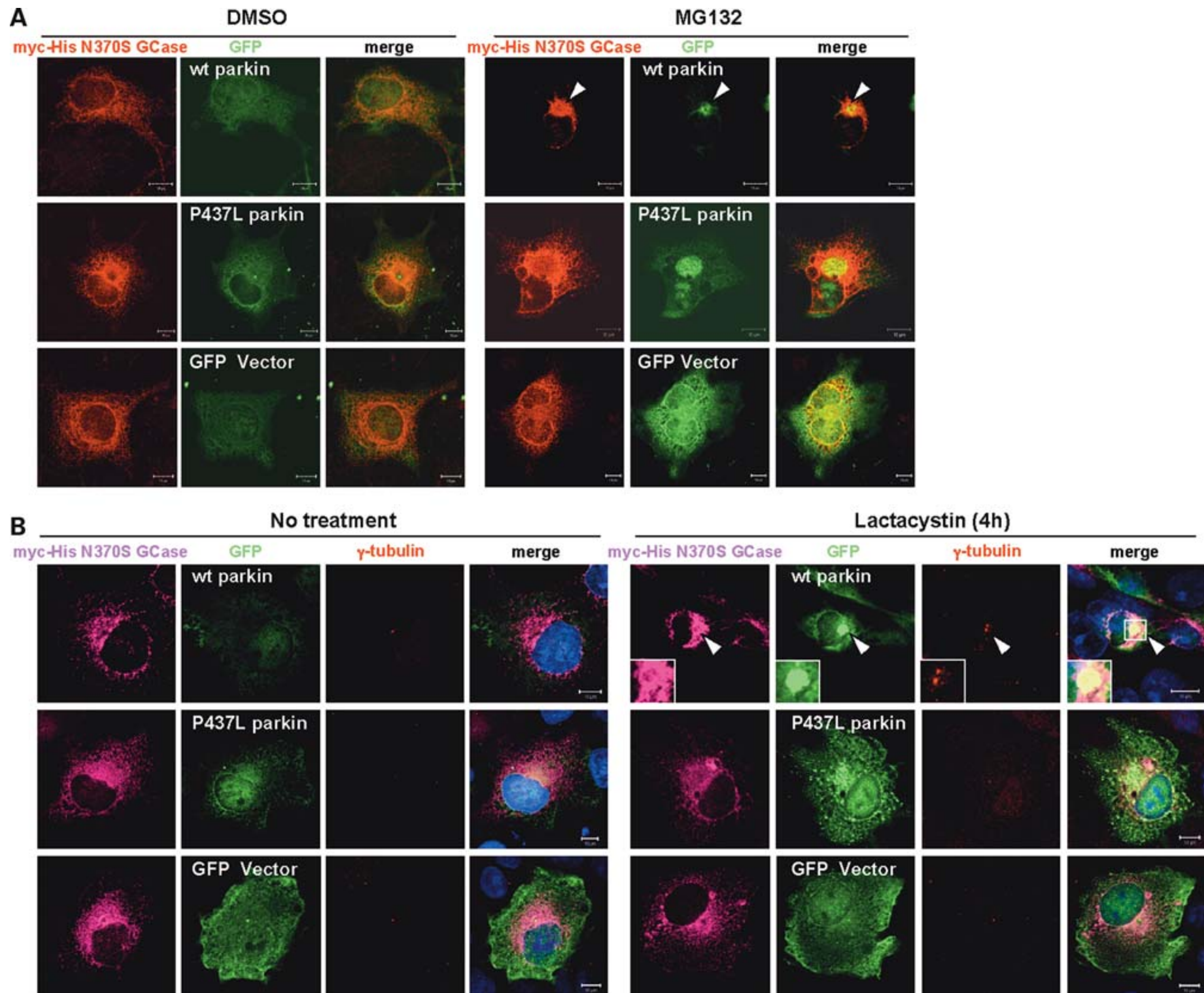

Figure 5. Parkin promotes the accumulation of mutant GCase in aggresome-like structures. (**A**) COS7 cells, grown on cover slips, were transfected with myc-His-tagged N370S GCase and GFP-tagged wt or P437L parkin or GFP vector alone. Twenty-four hours later, cells were treated with 25 μM MG132 or vehicle alone for an overnight and subjected to indirect immunofluorescence using anti-myc antibody. Detection was performed with cy3-conjugated goat anti-mouse antibodies to demonstrate myc-GCase localization (red), and with GFP (green) to follow parkin localization. The slides were visualized with a confocal microscope. Co-localization was illustrated by merging GFP and cy3 images (merge). (**B**) COS7 cells were transfected as described in (A). Twenty-four hours later, they were treated with 10 μM lactacystin or vehicle alone for 4 h and subjected to indirect immunofluorescence using anti-myc and anti-γ-tubulin antibodies. Detection was performed with cy5-conjugated goat anti-mouse antibody to demonstrate myc-GCase localization (pink), with rhodamine goat anti-rabbit antibodies to follow centrosome marked by γ-tubulin (red) and with GFP (green) to follow parkin localization. DAPI represents nuclei (blue). The slides were visualized with a confocal microscope. Co-localization was presented in merge. Accumulation of N370S GCase in the aggresome region is marked by arrows. Scale bar, 10 μm.

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UCH-L1 is a neuronal-specific ubiquitin-recycling enzyme, also abundantly found in Lewy bodies (83). It belongs to the family of deubiquitinating enzymes, which cleave polymeric ubiquitin chain to monomers (84,85). UCH-L1 is suspected to be associated with PD, since in one family, mutation in the UCH-L1-encoding gene led to autosomal dominant PD (86,87). Moreover, a common polymorphism in UCH-L1 has been associated with risk for sporadic PD (88).

In conclusion, we have provided evidence that parkin mediates K48-linked polyubiquitination of misfolded, ER retained, mutant GCase, which directs it for proteasomal degradation.

We suggest that the involvement of parkin in mediating the degradation of mutant GCase explains the recently established association between GD and PD. Thus, according to our hypothesis, the enduringly occupation of parkin with mutant GCase affects its activity toward its natural substrates. This leads to the accumulation of proteins detrimental to neuronal survival, thereby contributing to their demise and development of PD.

MATERIALS AND METHODS

Materials

The following primary antibodies were used in this study: mouse monoclonal anti-myc (9B11 Cell Signaling

Technology, Beverly, MA, USA), rabbit polyclonal anti-GFP (FL sc-8334), mouse monoclonal anti-HA probe (F-7 sc-7392), rabbit polyclonal anti-erk (C16 sc-93) and rabbit polyclonal anti-γ-tubulin (sc-10732), which were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-actin (C4, 0869100) was from MP Biomedicals, Solon (OH, USA).

Secondary antibodies used were cy3 and rhodamine-conjugated goat anti-mouse, horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit, which were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

MG132 was purchased from Calbiochem (San Diego, CA, USA). Restriction enzymes were purchased from several companies and employed according to the manufacturers' recommendations. CHX, leupeptin, phenylmethylsulfonyl fluoride (PMSF) and aprotinin were from Sigma-Aldrich (Rehovot, Israel). Lactacystin was from Calbiochem (La Jolla, CA, USA).

Cell lines

COS7 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SH-SY5Y cells were grown in DMEM supplemented with 20% FBS. All cells were grown at 37°C in the presence of 5% CO_2.

Construction of plasmids

GCase cDNA, encoding its 38 amino acid leader, was cloned into the *Eco*RI and *Xho*I sites of pcDNA4 myc-His-plasmid (Invitrogen Life Technologies Co., Carlsbad, CA, USA) to produce myc-His-wt GCase plasmid. *In vitro* site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene Life Technologies Co., Austin, TA, USA) to create variant forms with the mutations: N370S and L444P, as described elsewhere (5). Prk5-myc-parkin was a kind gift of Professor Simone Engelender (Technion-Israel Institute of Technology, Haifa, Israel). A *Bam*HI–*Bgl*II fragment isolated from the prk5 myc-parkin vector was cloned between the *Bgl*II and *Bam*HI sites of pEGFPC3 plasmid (Clontech, Laboratories, Inc., CA, USA) to produce GFP-parkin. *In vitro* site-directed mutagenesis was performed on prk5-myc-parkin and pEGFPC3 parkin, using the QuikChange site-directed mutagenesis kit, to create T240R and P437L myc-parkin and P437L GFP-parkin RING finger mutants. HA-ubiquitin as well as its K48R and K63R mutants was kindly provided by Professor Yossef Yarden (The Weizmann Institute of Science, Rehovot, Israel).

SDS–PAGE and western blotting

Cell monolayers were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in lysis buffer (10 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$ and 1% Triton X-100) containing 10 μg/ml aprotinin, 0.1 mM PMSF and 10 μg/ml leupeptin. Lysates were incubated on ice for 30 min and centrifuged at 10 000*g* for 15 min at 4°C. Samples, containing the same amount of protein, were electrophoresed through 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Schleicher and Schuell BioScience, Keene, NH, USA). Membranes were blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline (TBS) for 1 h at room temperature (RT) and incubated with the primary antibody for 1–2 h at RT or an overnight at 4°C. The membranes were then washed three times in 0.1% Tween-20 in TBS and incubated with the appropriate secondary antibody for 1 h at RT. After washing, membranes were reacted with ECL detection reagents (Santa Cruz Biotechnology, Inc.) and analyzed by luminescent image analyzer (Kodak X-OMAT 2000 Processor Kodak, Rochester, NY, USA).

Transfections

Transfection of COS7 cells was performed using Fugene-6 transfection reagent (Roche Diagnostic, Mannheim, Germany) or TransIT®-LT1 Reagent (Mirus Bio LLC, WI, USA) according to the manufacturer's instructions.

SH-SY5Y cells were transfected using Lipofectamine 2000™ (Invitrogen). HEK293 cells were transfected using calcium phosphate solutions. A mixture of DNA in 250 μl of 250 mM $CaCl_2$ was dropped into a tube containing HBSX2 solution (50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.09) and incubated for 20 min at RT. The mixture was then added dropwise to subconfluent cells for 36–48 h.

Immunoprecipitation

Forty-eight hours after transfection, HEK293 cells were washed three times with ice-cold PBS and lysed at 4°C in 1 ml of lysis buffer (10 mM HEPES pH 8, 100 mM NaCl, 1 mM $MgCl_2$ and 0.5% NP-40) containing 10 μg/ml aprotinin, 0.1 mM PMSF and 10 μg/ml leupeptin. Following incubation on ice for 30 min and centrifugation at 10 000*g* for 15 min at 4°C, the supernatants were pre-cleared for 1–2 h at 4°C with protein A-agarose (Roche Diagnostic). Samples were centrifuged at 15 000*g* for 1 min at 4°C, and the supernatants were incubated overnight at 4°C with antibodies, immobilized on protein A–Sepharose (Sigma-Aldrich). Following four washes with 1 ml of lysis buffer containing protease inhibitors, proteins were eluted for 5 min at 100°C in 5x Laemmli loading buffer, electrophoresed through 10% SDS–PAGE and blotted. The corresponding blot was interacted with the appropriate antibodies.

Nickel beads (Ni-NTA) precipitation

Cell were lysed forty-eight hours after transfection. Following incubation on ice for 30 min and centrifugation at 10 000*g* for 15 min at 4°C, the supernatants were pre-cleared for 1 h at 4°C with protein A-agarose. Samples were centrifuged at 15 000*g* for 1 min at 4°C, and the supernatants were incubated with Ni-NTA agarose (Qiagene GmbH, Hilden, Germany) for 3 h at 4°C. Following four washes with wash buffer (5% sucrose, Tris, pH 7.5, 50 mM NaCl, 0.5% NP40, 20 μM imidizole), the Ni-NTA precipitates were eluted for 5 min at 100°C

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with 5x Laemmli loading buffer and subjected to SDS–PAGE and western blot analysis.

Ubiquitination in tissue culture

Twenty-four hours after the transfection of HEK293 cells, MG132 (25 μM) in DMSO or DMSO (vehicle only, final concentration 0.05% DMSO) was added to the cells. Following an overnight incubation, they were harvested and lysed in 200 μl of denaturing buffer (1% SDS, 50 mM Tris, pH 7.4, 140 mM NaCl) by boiling for 10 min after vigorous vortexing. An amount of 800 μl of renaturation buffer (2% Triton X-100, 50 mM Tris, pH 7.4, 140 mM NaCl) was added to the lysates. After centrifugation for 15 min at 10 000*g* at 4°C, the supernatants were collected for Ni-NTA precipitation, as described earlier, and subjected to western blot analysis.

Generation of SH-SY5Y cell lines stably expressing myc-His-GCase

SH-SY5Y cells stably expressing myc-His-GCase variants were generated by co-transfecting SH-SY5Y cells with pBABE-puro plasmid (Aldgene, Inc., Cambridge, MA, USA) and wt or N370S myc-His-GCase, at 1:10 (w:w) ratio, respectively, using Lipofectamine 2000™ (Invitrogen), according to the manufacturer's protocols. Forty-eight hours later, cells were split and grown in a medium containing 0.8 μg/ml of puromycin (Sigma-Aldrich). Puromycin-resistant cultures were tested for myc-His-GCase expression by western blot analysis, using anti-myc antibody.

Immunofluorescence and confocal laser scanning microscopy

Subconfluent COS7 cells, grown on cover slips, were washed twice with PBS, fixed for 5 min at −20°C in methanol, followed by 5 min at −20°C in methanol–acetone (1:1 v/v). Following washes, cells were blocked by incubating with PBS containing 1% BSA and 20% normal goat serum for 30 min at RT after which they were incubated for 1 h with the corresponding primary antibody (1:5000 for mouse anti-myc; 1:50 for rabbit anti-γ-tubulin) in 1% BSA/PBS at RT. Cells were washed three times with PBS and then immunostained with mouse-cy3 or rhodamine-conjugated secondary antibodies (1:200 dilution) in 1% BSA/PBS for 45 min at RT. Following three washes with PBS, the cover slips were mounted with galvanol or DAPI (Golden Bridge International, Inc., WA, USA) and imaged using an LSM 510 or LSM 510 meta confocal laser scanning microscope (Carl Zeiss, Germany).

CHX chase experiments

SH-SY5Y cells, stably expressing wt or N370S myc-His-GCase, were transiently transfected with myc-wt or T240R parkin or GFP expressing plasmid as a control. Twenty-four hours later, the cells were treated with CHX (100 μg/ml) to inhibit *de novo* protein synthesis. At the indicated times, cell lysates were prepared, and the same amount of lysates were subjected to western blot analysis using anti-myc, anti-erk and anti-GFP antibodies.

Quantitation

The blots were scanned using Image Scan scanner (Amersham Pharmacia Biotech) and the intensity of each band was measured by the Image Master 1DPrime densitometer (Amersham Pharmacia Biotech).

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Conflict of Interest statement. None declared.

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