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The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI

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Inactivating mutations of the gene encoding parkin are responsible for some forms of autosomal recessive juvenile Parkinson disease. Parkin is a ubiquitin ligase that ubiquitinates misfolded proteins targeted for the proteasome-dependent protein degradation pathway. Using the yeast two-hybrid system and co-immunoprecipitation methods, we identified synaptotagmin XI as a protein that interacts with parkin. Parkin binds to the C2A and C2B domains of synaptotagmin XI resulting in the polyubiquitination of synaptotagmin XI. Truncated and missense mutated parkins reduce parkin-sytXI binding affinity and ubiquitination. Parkin-mediated ubiquitination also enhances the turnover of sytXI. In sporadic PD brain sections, sytXI was found in the core of the Lewy bodies. Since synaptotagmin XI is a member of the synaptotagmin family that is well characterized in their importance for vesicle formation and docking, the interaction with this protein suggests a role for parkin in the regulation of the synaptic vesicle pool and in vesicle release. Loss of parkin could thus affect multiple proteins controlling vesicle pools, docking and release and explain the deficits in dopaminergic function seen in patients with parkin mutations.

INTRODUCTION

Parkinson's disease (PD) is a major neurodegenerative disease characterized by muscle rigidity, tremor and bradykinesia (1). Other symptoms such as postural deficits, gait impairment and dementia are also observed in a subpopulation of PD patients. Although the majority of idiopathic PD cases are sporadic and probably influenced by environmental factors, familial aggregation of cases and rare Mendelian inheritance of PD traits evince the importance of genetic factors. In PD, the level of dopamine is decreased in the striatum, most severely in the putamen. This is largely a result of degeneration of dopamine-producing neurons in the substantia nigra pars compacta (2–4). To date, the three genes that have been associated with autosomal dominant PD are *NR4A2* (5), α-*synuclein* (6), and *ubiquitin C-terminal hydroxylase L1* (UCHL1) (7). The two genes that have been associated with autosomal recessive PD are *parkin* (8) and *DJ-1* (9). Inactivating mutations of the *parkin* gene cause PARK2 autosomal recessive juvenile Parkinsonism (AR-JP). Similar to other PD forms, PARK2 is characterized by loss of dopaminergic neurons in the substantia nigra. However, PARK2 is unique in that Lewy bodies in substantia nigra neurons are absent in most cases of AR-JP (10–12). Mutations in the *parkin* gene cause a form of AR-JP, but are also found in older PD patients demonstrating that *parkin* mutations are not limited to juvenile onset (13).

Parkin is composed of a ubiquitin-like domain in the N-terminal domain and two RING finger motifs toward the C-terminus (8,14). Several inactivating mutations are found in the RING finger domains and suggest that these domains are functionally important (15). To date, only one missense (Arg42Pro) and three frameshift mutations have been found in the ubiquitin-like domain. Shimura *et al.* (14) demonstrated that the ubiquitin-conjugating H7 protein binds to the RING finger domain, and that the RING domains of parkin are required for ubiquitination in human dopaminergic SH-SY5Y neuroblastoma cells. These observations suggest different roles for the ubiquitin-like and RING finger domains. Subsequently,

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the ubiquitin-like domain was found to be important for the stability of parkin (16) and probably for targeting the ubiquitinated substrates to the proteasome. The RING fingers, on the other hand, bind to substrates and other ubiquitin components, such as UbcH7 (E2), required for the ubiquitin-ligase activity. Zhang *et al.* (17) confirmed this observation and further found that parkin bound to CDCrel-1, a member of a synaptic vesicle-associated protein family named septin, and that parkin stimulated the ubiquitination and turnover of this protein. Together, these data led to the suggestion that parkin functions as an E3 ubiquitin ligase.

The E3 ubiquitin ligases together with the activating E1 and often with the conjugating E2 enzymes catalyze the conjugation of ubiquitin chains to cytoplasmic proteins targeted for degradation in the 26S proteasome complex, thus regulating important cellular processes such as cell cycle, cell death and cell differentiation. The ubiquitinated substrate can be degradated either through the proteasome-dependent pathway (18) if the substrate is polyubiquitinated, or through the lysosomal degradation pathway if the protein is monoubiquitinated. Monoubiquitination can cause certain cell surface receptors (e.g. EGF receptor) to internalize or can function as a protein sorting signal in the endosomal pathway (19–21), and direct these monoubiquitinated proteins to the lysosome. Parkin has been found to interact with several proteins which include the Pael-R receptor (22), CDCrel-1 (17), glycosylated α-synuclein (23), synphilin-1 (24), CHIP (25), cyclin E (26), HSP70 (27), α/β-tubulin (28), and the p38 subunit of the aminoacyl t-RNA synthetase complex (29). Parkin-mediated ubiquitination led to the degradation of these proteins by the proteasome system. Absence of parkin-mediated degradation of the Pael-R receptor resulted in the accumulation of the Pael-R receptor causing cell death (22).

Using the yeast two-hybrid screens, we found that parkin interacted with synaptotagmin XI, and this interaction resulted in its ubiquitination and degradation. The interaction with a member of the synaptotagmin family suggests an involvement of parkin in the regulation of proteins involved in controlling neurotransmitter trafficking at the presynaptic terminal.

RESULTS

Identification of parkin interactors by the yeast two-hybrid system

Using the yeast two-hybrid method (30), we screened a human fetal brain pGAD10-cDNA library using the pGBT9-parkin (1–465) construct. Six potential clones were identified in 2×10^6 independent human fetal brain pGAD10-cDNA colonies. These clones were individually isolated, sequenced and subjected to further yeast two-hybrid filter assays to confirm the interactions. Two of these clones had a long open-reading frame and therefore were purified and sequenced. Nucleotide sequences showed that one of the two clones encoded the C2A and C2B domains of the human synaptotagmin XI, and this clone was designated hsytXIAB (for human synaptotagmin XI, domains C2A and C2B). To further determine if the hsytXIAB fragment was a true parkin interactor, the purified pGAD10-hsytXIAB was co-transfected into Y190 yeast cells with either pGBT9-parkin (1–465), or with two unrelated baits, pGBT9-NF2 (encoding the schwannomin tumor suppressor), and pGBT9-Hrs (Hrs = hepatocyte growth factor regulated kinase substrate) constructs (31) or the pGBT9 vector. Filter binding assays demonstrated that only the pGAD10-hsytXIAB clone showed positive interaction with the pGBT9-parkin construct but not with the pGBT9 vector control or unrelated proteins (Fig. 1A). To confirm parkin interaction by yeast two-hybrid liquid assays, transfected yeast colonies were grown in liquid culture and tested for β-galactosidase activity. Yeast cells co-transfected with pGAD10–hsytXIAB and pGBT9–parkin produced about 20-fold higher β-galactosidase activity compared with yeast cells co-expressing pGAD10-hsytX1AB and pGBT9 vector control (Fig. 1B).

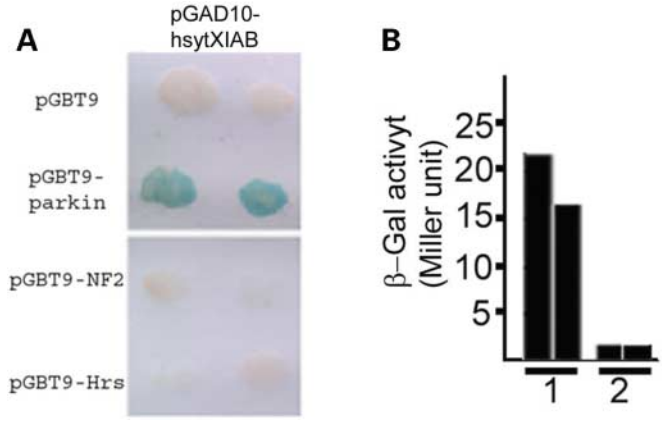

Figure 1. Parkin interacts with the C_2A and C_2B domains of sytXI. (**A**) Yeast two-hybrid filter assay. Yeast Y190 cells transformed with pGAD10–hsytXIAB and pGBT9–parkin produced a positive reaction with β-gal substrate (blue color), while yeast cells transformed with pGAD10–hsytXIAB and the pGBT9–vector control did not. Yeast cells transformed with pGAD10–hsytXIAB and two other controls expressing schwannomin and Hrs (pGBT9–NF2, and pGBT9–Hrs) failed to form positive reactions with the β-gal substrate. These control proteins are functional in the yeast two-hybrid system as previously described in Scoles *et al.* (31,37). (**B**) Yeast two-hybrid liquid assay. Yeast cells transformed with pGAD10–hsytXIAB and pGBT9–parkin plasmids produced 20-fold more β-gal activity than yeast cells transformed with pGAD10–hsytAB (lane 1) and the pGAD10 vector control (lane 2). Each bar graph represents $n = 3$.

Specificity of antibodies to synaptotagmin I and XI

We generated rabbit antibodies to two peptides of synaptotagmin XI (anti-sytXIA and anti-sytXIB) and purchased a mouse antibody to synaptotagmin 1 (anti-syt65). Since sytI and sytXI are related proteins and have close homology to other synaptotagmins, we tested whether the anti-syt65 and anti-sytXI antibodies cross-reacted with each other before using these antibodies to investigate parkin–sytXI interactions. Figure 2A shows western blots of protein extracts isolated from human embryonic kidney (HEK) 293T cells expressing GFP- sytI, GFP-sytIV, GFP-sytXI or GFP. The western blots were separately detected with anti-GFP, anti-syt65 or anti-sytXIA antibodies. As expected, the anti-syt65 and anti-sytXIA antibodies detected only the respective GFP–sytI or GFP–sytXI proteins, while the anti-GFP antibodies detected all GFP-tagged

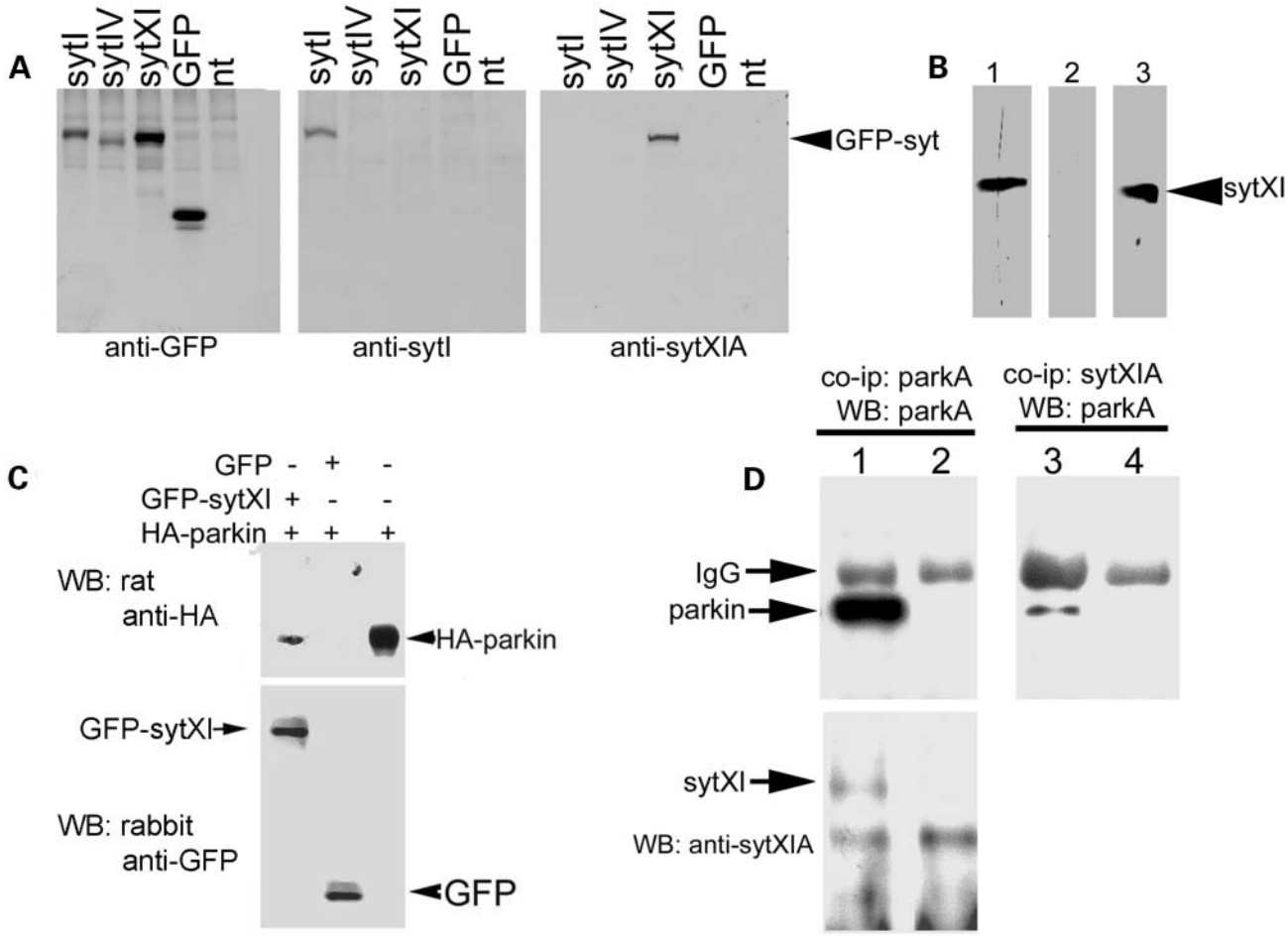

Figure 2. Parkin interacts with synaptotagmin XI. (**A**) Specificity of antibodies to synaptotagmins I and XI. Western blots of protein extracts from HEK293 cells transfected with GFP and GFP-sytI, IV and XI plasmids were detected with antibodies to GFP, sytI and sytXI. Both the anti-sytI and anti-sytXI antibodies specifically detected their respective epitopes but not the GFP-sytIV fusion protein. nt = non-transfected cells. (**B**) Western blots of protein extract from PC12 cells detected with rabbit anti-sytXIA antibody. Lane 1, incubation with 1 μg/ml of anti-sytXIA antibody; lane 2, incubation with anti-sytXIA antibody preincubated with sytXIA peptide; lane 3, incubation with anti-syXIA antibody preincubated with sytXIB peptide. The anti-sytXIA antibody detects a single band at 64 kDa. This band was preabsorbed out with preincubation with the sytXIA peptide but not with sytXIB peptide. (**C**) Parkin-sytXI *in vitro* immunoprecipitation. Protein extracts from HEK293 cells overexpressing HA-parkin and the corresponding GFP fusion proteins were coimmunoprecipitated (co-ip) with a mouse anti-GFP antibody. The IP products were detected with rat anti-HA-peroxidase (top panel) and a rabbit anti-GFP antibody (bottom panel). The sytXI proteins co-precipitated parkin, but the GFP tag did not. (**D**) Co-immunoprecipitation of endogenous parkin and sytXIA. Protein extracts from a human cerebral cortex were co-immunoprecipitated with rabbit anti-parkA antibody (lane 1), or rabbit IgG (lane 2) as a control. The precipitates were detected with anti-parkA (top panel) or anti-sytXIA antibody (bottom panel). The anti-parkA antibody co-immunoprecipitated sytXI but the rabbit IgG control did not. To do the reverse co-ip, protein extracts were co-ip with rabbit anti-sytXI antiserum (lane 3) or the corresponding preserum at identical dilutions (lane 4). The western blot was detected with the chicken anti-parkA (lanes 3 and 4), which detected the endogenous parkin band in the anti-sytXI antiserum (lane 3), but not in the preserum control (lane 4).

proteins. Importantly, the anti-sytXIA antibody did not detect GFP–sytIV, even though sytIV has the closest homology with sytXI.

The specificity of the sytXIA antibody was further confirmed in western blots of protein extracts from PC12 cells (Fig. 2B). PC12 cell protein extracts in strong triple detergent buffer were immunoblotted with anti-sytXIA, anti-sytXIA preabsorbed with sytXIA peptide, or with sytXIB peptide. The anti-sytXIA antibody recognized a single band at 64 kDa (Fig. 2B, lane 1). The p64 band was not detected when the anti-sytXIA antibody was preabsorbed with the sytXIA peptide (Fig. 2B, lane 2), while preabsorption with a different peptide (sytXIB) failed to inhibit the anti-sytXIA immunoreactivity (Fig. 2B, lane 3). Taken together, these observations further confirm the specificity of the anti-sytXIA antibody. Western blots of protein extracts isolated using weak detergent buffer (0.2 or 0.5% NP40 or Triton-X100) gave two sytXI positive bands at 64 and 110 kDa, suggesting that sytXI may form homodimers (data not shown).

Parkin interacts with the full-length synaptotagmin XI

Co-immunoprecipitation of protein extracts from 293T cells coexpressing the GFP–hsytXIAB fusion protein and HA–parkin found that parkin co-precipitated the hsytXIAB fragment (data not shown). To determine whether parkin interacted with the full-length synaptotagmin, we cloned the full-length synaptotagmin XI cDNA into the pEGFP vector.

The pEGFP–sytXI construct was individually cotransfected with the pCMV–HA–parkin plasmid into 293T cells. The pEGFP plasmid was used as a vector control. After 24 h, protein extracts were obtained and co-precipitated with mouse anti-GFP antibody to pull down GFP-fusion proteins. The Co-IP products were immunoblotted with rat anti-HA-conjugated peroxidase (Fig. 2C, top panel) or rabbit anti-GFP (Fig. 2C, bottom panel). The anti-HA peroxidase detected the HA–parkin band only in the sample with GFP-sytXI, but not in the GFP control, suggesting that the HA–parkin specifically co-precipitated with the synaptotagmin fusion proteins but not with the GFP tag.

To determine whether parkin interacted with endogenous sytXI, protein extract from human cerebral cortex was immunoprecipitated with the anti-parkA antibody. The precipitate was then detected with either chick anti-parkA or anti-sytXIA antibody (Fig. 2D, lanes 1 and 2). The anti-parkA antibody detected a single parkin band in the anti-parkA precipitate (Fig. 2D, lane 1, top panel), while the anti-sytXIA antibody detected the sytXI protein band (Fig. 2D, lane 1, bottom panel). When the same protein extract was co-immunoprecipitated with the anti-sytXI antibody, parkin was specifically coprecipitated with sytXI (Fig. 2D, lane 3). The absence of both the sytXI and the parkin bands in the control reactions (Fig. 2D, lanes 2 and 4) demonstrated the specificity of the parkin–sytXI interactions in the cells, confirming that endogenous parkin interacted with endogenous sytXI.

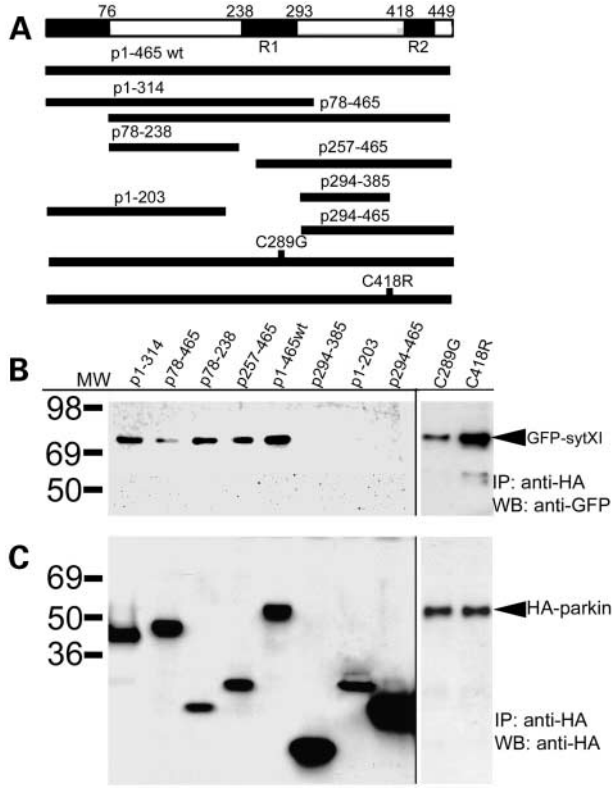

Figure 3. The sytXI binding site maps to a domain containing the RING1 finger motif. (**A**) Map of parkin. Full-length and truncated parkins were constructed by PCR and cloned in-frame with a HA–epitope tag; C289G and C418R denote parkins containing missense mutation at amino acid positions 289 and 418, respectively. (**B**) The sytXI binding site is mapped to the RING1 motif of the parkin. Truncated parkins missing amino acid residues 204–293 which encompass the RING1 finger motif fail to bind to sytXI. The C289G missense mutated parkin interacts weakly with sytXI compared to the C418R mutant. (**C**) Expression of HA-tagged parkins in HEK293 cells. Western blot of HEK293 cells overexpressing the full-length wild-type, missense mutated, or various truncated parkins was detected with anti-HA-peroxidase.

The sytXI binding domain is located between amino acid residues 204–293 of parkin

To determine which domain of parkin binds to synaptotagmin, several truncated parkins tagged with the HA epitope were constructed (Fig. 3A). The truncated parkin cDNA expression plasmids expressed sufficient truncated parkins for coexpression with GFP-syts (Fig. 3C). Each truncated construct was co-expressed with GFP-sytXI. After 24 h, protein extracts were immunoprecipitated with rat anti-HA-conjugated agarose followed by western blot detection with anti-GFP antibody. Truncated parkins lacking amino acid residues 204–293 (p1–203, p294–385, and p294–465) failed to interact with the full-length synaptotagmin XI. The failure of these truncated parkins to interact with sytXI was not the result of decreased expression levels of the truncated parkins. Expression levels of the p1–203 and p294–465 parkins were much higher than the constructs that interacted with sytXI (Fig. 3C). Truncated parkins containing the whole or part of the p204–293 domain (p1–465, p1–314, p78–465, p78–238, p257–465) interacted with sytXI, all having different binding affinities (Fig. 3B). These observations suggest that amino acid residues 204–293, which contain the RING1 domain, are important for parkin interaction with sytXI.

Data from the binding assays suggest that there are at least two sytXI binding sites on parkin. The first binding site is located between amino acid residues 204 and 238. This was supported by the observation that the p78–238 peptide, which does not contain the RING1 domain, interacted with sytXI while the p1–203 peptide did not bind (Fig. 3). The second binding site is located within the RING1 domain between amino acid residues 257 and 293. This was supported by the observation that peptide p257–465 interacted with sytXI, whereas peptides p294–385 and p294–465 did not bind (Fig. 3). This observation was further supported by the effect of disease-causing amino acid substitutions in parkin. Parkin containing a missense mutation in the RING1 finger motif ($parkin^{C289G}$) interacted weakly with sytXI (~2.5-fold less) compared with $parkin^{C418R}$ (Fig. 3B). In addition, both mutated parkins fails to ubiquitinate sytXI (Fig. 4) although $parkin^{C418R}$ does not lose the ability to bind to sytXI (Fig 3B).

Ubiquitination and degradation of synaptotagmin XI by parkin

Parkin is an E3 ubiquitin ligase, an essential enzyme required for the ubiquitination of specific substrates targeted for degradation by the proteasome complex or the lysosome (14,17). To determine whether sytXI is a substrate of parkin, we performed semi-quantitative *in vitro* ubiquitination assays as described by Zhang *et al.* (17) . In these experiments, HEK293 cells were cotransfected with various combinations of HA-tagged parkin cDNA plasmid or controls, myc-tagged

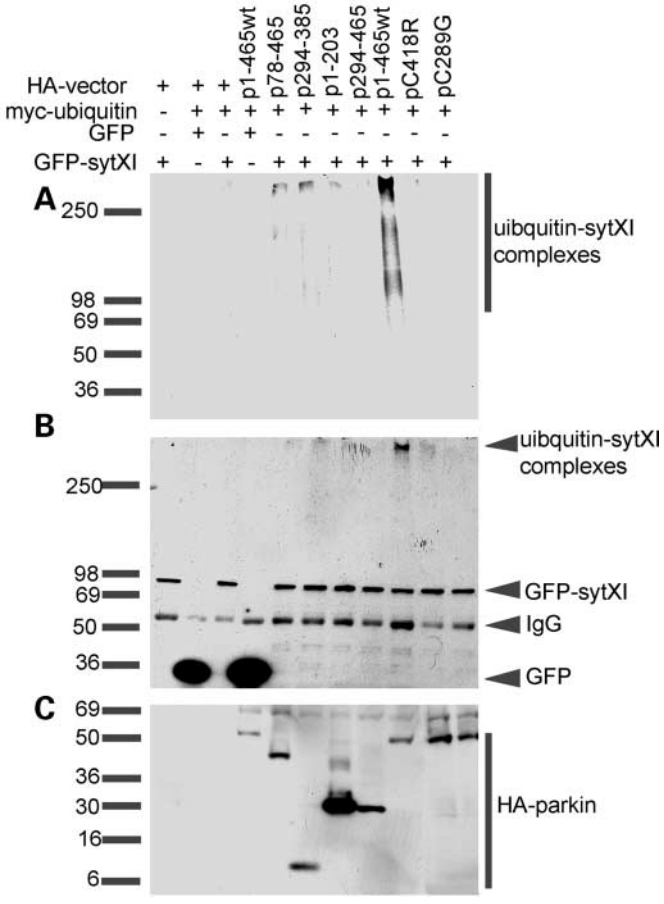

Figure 4. Parkin ubiquitinates sytXI. HEK293 cells overexpressing HA-parkins or controls with the corresponding myc-ubiquitin and GFP-tagged proteins were treated with lactacystin for 4 h, and protein extracts were immunoprecipitated with anti-GFP antibody. IP products of the ubiquitination assays were detected with an antibody to the myc tag (**A**) and anti-GFP antibody (**B**). Note the lack of ubiquitinated products in cells expressing HA-parkin and GFP, and the undetectable level of ubiquitination of GFP–sytXI in other controls. Cells expressing parkin mutants and GFP–sytXI produce a lower amount of ubiquitin-conjugated sytXI compared with the wild-type parkin. The anti-GFP antibody detects near equal amounts of GFP-sytXI monomer in all samples containing GFP-sytXI and a large GFP–sytXI band near the well containing the sample co-expressed with both GFP–sytXI and HA–parkin. (**C**) Western blot of the same lysate with anti-HA antibody indicates that the truncated and mutated parkins are expressed at higher levels than wild-type parkin.

ubiquitin, and GFP-tagged sytXI (Fig. 4). GFP was used as a negative control for substrate specificity, while truncated HA-tagged parkin proteins (p78–465, p1–203, p294–385, p294–465) were used as mutant parkin negative controls. As shown in Figure 4, when cells were incubated with lactacystin, an inhibitor of the proteasome complex, cells expressing the wild-type parkin and GFP–sytXI showed an accumulation of ubiquitin-synaptotagmin conjugates above background controls. Wild-type parkin had no effect on the polyubiquitination of the GFP tag. Truncated parkins showed little effect on the levels of ubiquitinated synaptotagmin conjugates, although one of the peptides (p78–465) could bind to sytXI (Fig. 3), and the levels of expression of the truncated parkins were high compared with the wild-type parkin. The pattern of ubiquitinated syts suggested the presence of a variety of species containing polyubiquitin chains of different lengths.

To determine whether disease-associated point mutations affected the ability to ubiquitinate sytXI, ubiquitination assays were also performed for missense mutated parkin^{C289G} and parkin^{C418R}. Both mutant parkins produced undetectable levels of ubiquitinated sytXI compared with the wild-type parkin (Fig. 4). Under longer exposure, all truncated and missense mutated parkin transfected cells produced weak levels of ubiquitin-conjugated sytXI comparable to cells transfected with only GFP-sytXI, but the levels of the ubiquitinated sytXI were significantly lower than those produced by wild-type parkin (data not shown). This background level of ubiquitinated sytXI was probably produced by endogenous parkin or by an unidentified E3 ubiquitin ligase in HEK293 cells, and are consistent with observations by other investigators using HEK293 cells (28). In both co-ip (Fig. 3) and ubiquitination (Fig. 4) experiments, a majority of mutant parkins could be detected in the pellet fractions that were dissolved in the SDS–PAGE sample buffer.

Western blot analysis of the same protein samples with an anti-GFP antibody detected a high MW GFP–sytXI band near the top of the well loaded with the parkin–sytXI co-expressed sample (Fig. 4). This band was likely the insoluble ubiquitinylated sytXI complex since the anti-myc antibody also strongly detected the same complex. This band was faintly observed in the controls. The smaller MW ubiquitinylated sytXI species were undetectable by the antiGFP antibody. These findings are consistent with ubiquitinylation experiments of α- and β-tubulin (28) and synphilin-1 (24) in HEK293 cells. In these experiments, the ubiquitinylated substrates were undetectable by the antibodies against the respective proteins, but the antibodies against ubiquitin or its tag strongly detected the respective parkin-mediated ubiquitinylated substrates.

Since parkin-mediated ubiquitinylated substrates undergo degradation by the proteasome-dependent pathway (17,22,25,28,29,32), we performed pulse-chase experiments to determine the turnover of GFP–sytXI in HA–vector and HA–parkin$^{1-465}$ transfected HEK293 cells. Parkin increased the degradation of GFP–sytXI in HA–parkin$^{1–465}$ transfected HEK293 cells (Fig. 5). Approximately 40% of newly synthesized GFP-sytXI was degraded after 1.5 h chase in HA–parkin$^{1-465}$ expressing cells, whereas it took 3 h to degrade the equivalent amount of GFP–sytXI in HA–vector transfected cells.

Parkin colocalizes with synaptotagmin XI

The interaction of two proteins is likely to be physiologically relevant if they occupy the same subcellular compartment. To investigate parkin–syt co-localization, we induced non-transfected PC12 cells with NGF for 7 days, and co-labeled the NGF-induced PC12 neuron-like cells with chick anti-parkA and rabbit anti-syt XIA (Fig. 6A–C). Parkin co-localized with sytXI at positions around the nuclear membrane and at boutons (Fig. 6C, white arrows) along the neurites. Parkin or sytXI present in other regions of the cell did not co-localize. The co-localization of parkin and sytXI was further confirmed with immunohistochemical labeling of human substantia nigra neurons (Fig. 6D–L). Both parkin and sytXI proteins had similar distribution patterns in the cytoplasm and neurites of

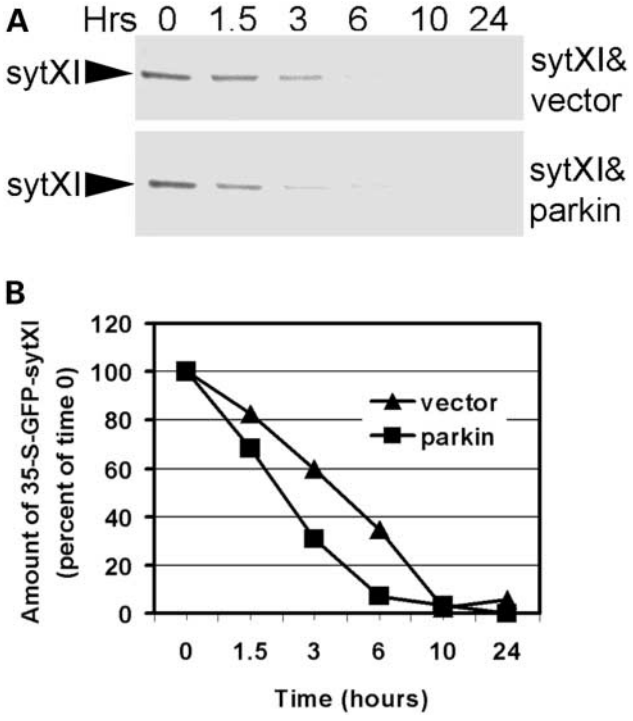

Figure 5. Parkin accelerates the turnover of GFP–sytXI. Pulse-chase analysis of the degradation of GFP–sytXI in HEK293 cells expressing either HA-vector or HA-parkin at 0, 1.5, 3, 6 and 24 h. Data are from one of two independent experiments. The second experiment (not shown) had even stronger parkin effect.

the human nigral neurons. Parkin and sytXI were also co-localized in HEK293 cells co-expressing HA-parkin and GFP-sytXI (data not shown).

SytXI is localized in the cell bodies and neurites of human substantia nigra neurons

The death of substantia nigra neurons and the formation of Lewy bodies are hallmarks of many forms of PD, and some constituent proteins of Lewy bodies are mutated in inherited forms of PD. To establish a potential link between sytXI and parkin in the pathogenesis of neurodegeneration in classic PD, we performed immunohistochemical labeling of substantia nigra sections from two normal and two sporadic PD patients using antibodies to sytXI (anti-sytXIA), parkin and ubiquitin. As shown in Figure 6, the normal human substantia nigra neurons were strongly stained by antibodies to sytXI and parkin. The anti-sytXIA antibody labeled both the cell bodies and neurite extensions of the nigral neurons (Fig. 6D, G and J) similar to the anti-parkA antibody (Fig. 6F, I and L). The sytXI immunoreactivities were specific, since anti-sytXIA preabsorbed with the sytXIA peptide failed to label the nigral neurons (Fig. 6E, H and K).

Figure 6M–O depicts the immunohistochemical labeling of a nigral neuron from an individual with sporadic PD. The anti-sytXIA antibody labeled the core of the intracellular Lewy bodies (LBs; Fig. 6N, black arrow) similar to the anti-ubiquitin antibody (Fig. 6M). The sytXI LBs staining was weak compared with ubiquitin staining; of note is the strong labeling of the neuropil. This labeling was specific since anti-sytXIA antibody preabsorbed with the sytXIA peptide failed to label the Lewy body or the neuropil (Fig. 6O, black arrow). As the authors and others reported previously (33), the Lewy bodies in these two sporadic PD brains did not stain with the anti-parkA antibody (data not shown).

DISCUSSION

Our finding that parkin can directly interact with and ubiquitinate synaptotagmin XI strongly implicates parkin in a role of modifying synaptic vesicle trafficking at the presynaptic terminal. Among the parkin interactors, two proteins, CDCrel-1 and synphilin-1 (34–36), are synaptic vesicle-associated proteins. CDCrel-1 interacts with syntaxin, and overexpression of wild-type CDCrel-1 inhibits secretion in HIT-T15 cells (36). Synphilin-1 interacts with α-synuclein and stimulates the formation of cytosolic inclusion bodies (32). The presence of wild-type parkin appears to be essential for synphilin-1-induced formation of the Lewy bodies. It is currently unknown how CDCrel-1 or synphilin-1 participates in the regulation of presynaptic neurotransmission, and it is also unclear whether CDCrel-1 or synphilin-1 plays any role in the regulation of presynaptic secretion of dopamine.

Synaptotagmin XI is a member of a large family of ∼50 calcium-binding proteins with high homology at the C_2A and C_2B domains. These proteins include synaptotagmins I–XIII, raphilin-2a, protein kinase C, GTPase-activating protein (GAP), rat/yeast ubiquitin ligase Nedd4, and phospholipaseA. Together, these proteins serve a common function as regulators of cell signal transduction ranging from calcium sensors (syts and protein kinase C) to phosphorylation (GAP) and phospholipid degradation (phospholipase C). Among the synaptotagmins, sytI has the highest homology with sytII, and it is expressed abundantly in synaptic vesicles and secretory granules (37). SytI and sytII function as calcium sensors in fast presynaptic neurotransmission (38,39) similar to sytIII, V–VII, and X. Synaptotagmin XI, in contrast, is similar to sytIV owing to a conserved substitution of an aspartate by a serine residue in the C_2A domain resulting in lack of Ca^{+2} binding to this domain (40). SytXI is predominantly expressed in the brain (40). The functions of most synaptotagmins are currently speculative, although sytXI is thought to function as a down-regulator of fast presynaptic neurotransmission (41). Overall, members of the syt family have high homology at the C_2 domains with amino acid identity ranging from 30 to 50%. Since parkin binds to the C_2A and C_2B domains of sytXI, it is likely that parkin interacts with other syts as well. The observation that parkin also interacts with and regulates sytI (Huynh, D.P. *et al.*, manuscript in preparation), a protein that contains the lowest C_2 domain homology with sytXI (30% identity), suggests that parkin may interact with a wide range of proteins containing domains related to the C_2A and C_2B sequences.

SytXI is found in the central core of LBs in substantia nigra neurons from patients with idiopathic PD (Fig. 6). This distribution is also observed for other parkin substrates, p38 subunit of the aminoacyl tRNA synthetase complex and

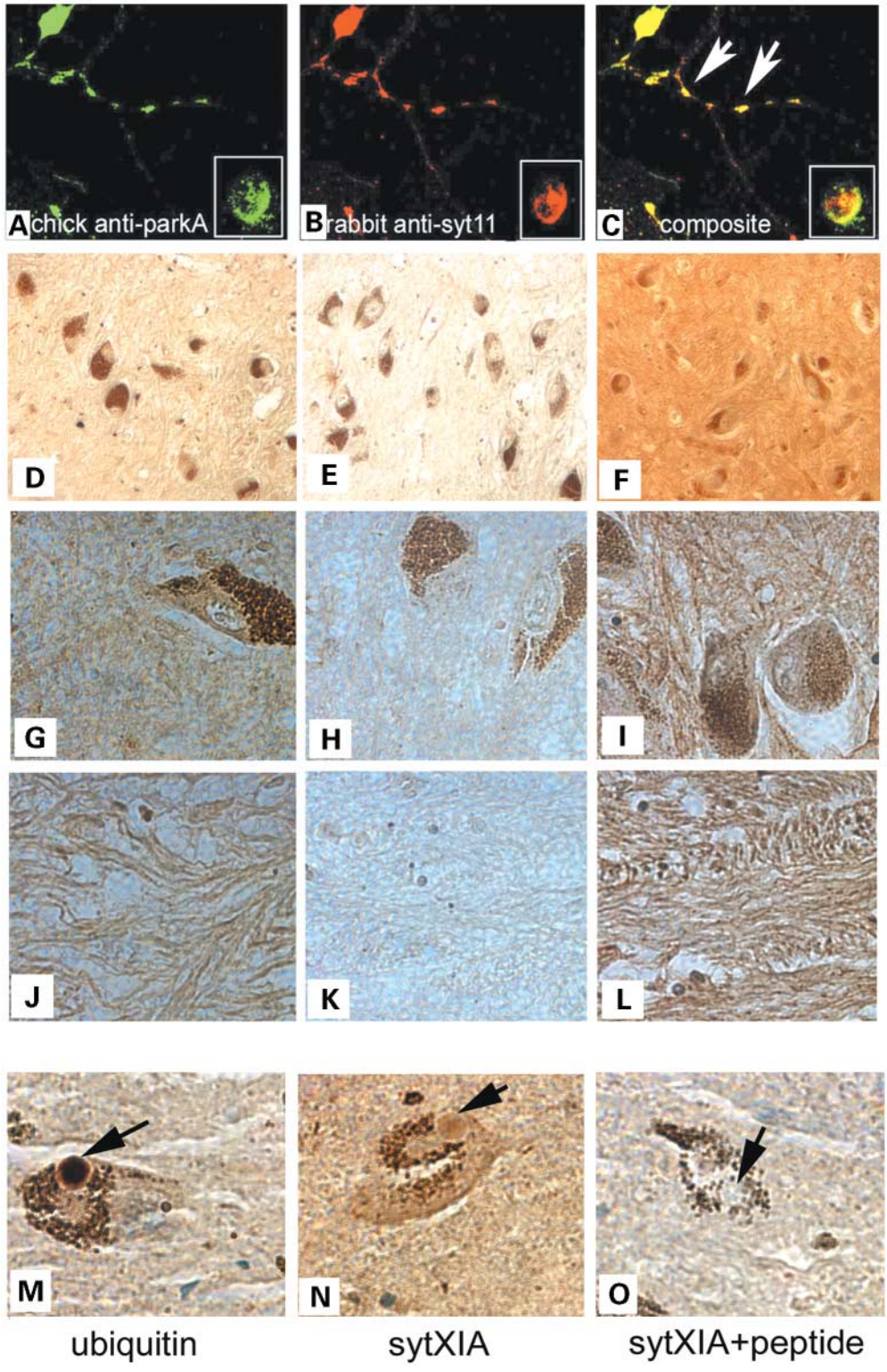

Figure 6. Subcellular distribution of endogenous synaptotagmin XI in nontransfected PC12 cells and human substantia nigra neurons. (**A**– **C**) Endogenous parkin co-localizes with endogenous sytXI in NGF-induced PC12 cells. PC12 cells were induced with 50 ng/ml NGF for 7 days, and immunofluorescently co-labeled with antibodies to parkin (green, A) and sytXI (red, B). Images were acquired by Leica TCSSP microscopy using a 100× oil immersion lens. Stacked images were merged (C). Yellow indicates co-localization of two proteins. Inserts show the cell body of the same cell from which the long neurite arises. Parkin and sytXI co-localize in the perinuclear area and boutons (arrows) along the neurite. (**D**–**L**) Distribution of synaptotagmin XI and parkin in a normal human substantia nigra section. Human substantia nigra sections were labeled with the rabbit anti-sytXIA antibody (D, G, J), anti-sytXIA antibody preabsorbed with 100×sytXIA peptide (E, H, K), or rabbit anti-parkA antibody (F, I, L). Images show the cell bodies and neurites of dopaminergic neurons in the substantia nigra. (**M**–**O**) Adjacent PD brain sections labeled with rabbit anti-ubiquitin (M), anti-sytXIA (N), and anti-sytXIA + sytXIA peptide (O); black arrows point to Lewy bodies. Note the absence of Lewy body labeling by preabsorbed sytXIA antibody. Images were acquired using a 20× lens (D–F), and 63× oil immersion lens (G–O).

synphilin-1 (29,42,43). The finding of sytXI in LBs suggests a potential link of abnormal processing of synaptotagmins in PD. Whether LBs play a role in dopaminergic neuronal death in Parkinsonism is speculative. The absence of LBs in parkin-associated parkinsonism (44–46) implies that these inclusions are not the primary cause of dopaminergic neuronal degeneration in parkin-associated parkinsonism.

Parkin consists of three functional domains, the ubiquitin-like, RING1 and RING2 domains (14). The RING2 domain was found to be required for binding to ubiquitin-conjugating enzymes (14,17,47) and the ubiquitin-like domain is important for the stability of parkin (16). However, the RING finger motifs were found later to be essential for parkin binding to its two substrates, CDCrel-1 (17) and Pael-R (47). Consistent with these findings, we found that sytXI bound to the region between amino acid residues 204 and 293 (Fig. 3). This region contains the RING1 motif. Furthermore, a parkin peptide lacking only the ubiquitin-like domain (p78–465) bound more weakly to synaptotagmins than parkins containing the ubiquitin-like domain (Fig. 3B). These observations suggest that the ubiquitin-like domain is important for the correct folding of the full-length parkin to expose the RING finger motif for synaptotagmin binding. We suggest that the three parkin domains serve distinct functions: the ubiquitin-like domain is required for the correct folding and stability of parkin, the p204–293 domain, which contains the RING1 finger motif, is essential for the interaction with the C2 domain containing proteins such as sytXI, whereas the RING2 finger motif is important for complex formation with the E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugate proteins.

Parkin is an E3 ubiquitin ligase (14,17) that catalyzes the ubiquitination of targeted proteins. Polyubiquitination will lead to the degradation of the ubiquitin-conjugated substrate by the proteasome. Wild-type parkin strongly catalyzes the polyubiquitination of sytXI compared with truncated parkins, missense mutated parkins, or negative controls (Fig. 4). Parkin-dependent ubiquitination also led to rapid turnover of sytXI (Fig. 5) further supporting the hypothesis that parkin regulates the level of sytXI. Cells expressing truncated parkins or missense mutated parkins (C289G and C418R) produced the same amounts of ubiquitinylated sytXI as cells expressing only GFP-sytXI (Fig. 4). These observations suggest that truncating or missense mutations of parkin reduce or eliminate the ubiquitination of sytXI. In PARK2 AR-JP, mutations of parkin probably cause a decrease in the ubiquitination of specific proteins, resulting in an increase in their intracellular levels of sytXI and other proteins regulated by parkin. The net effect of the abnormal increase in the intracellular levels of parkin-regulated proteins probably contributes to the pathological conditions of AR-JP.

SytXI mRNA is expressed abundantly in the brain, but at lower levels in nonneural tissues (40). However, information on the specific subcellular distribution of endogenous sytXI protein is unknown. Exogenous sytXI in PC12 cells was mainly localized in the Golgi network (48). In non-transfected NGF-induced PC12 cells, endogenous parkin and sytXI were found co-localized in a perinuclear distribution and in dense-core vesicles in the NGF-induced processes (Fig. 6). The distribution pattern of endogenous parkin was similar to previous observations (33). The distribution of sytXI was also similar to the subcellular distribution of sytIV, a protein with 48% identity to sytXI. In PC12 cells, sytIV is localized mainly in the Golgi and immature vesicles (49–52). When PC12 cells are treated with NGF, sytIV protein redistributes to the mature dense-core vesicles (52). Dense-core vesicles are secretory granules that carry neuropeptides or biogenic amines, and release their contents under the stimulation of calcium ions. Therefore, the observation that both sytXI and parkin co-localize in the dense-core vesicles suggests that both proteins probably play a role in the calcium-dependent exocytosis. This hypothesis is further supported by the observation that both parkin and sytXI have similar distribution patterns in the neurites and cell bodies of neurons in the human substantia nigra (Fig. 6).

One can speculate that the loss of parkin function in patients with AR-JP alters synaptotagmin XI function resulting in altered dopamine release, which in turn causes the symptoms of dystonia and parkinsonism. Altered vesicle functioning, be it at the stages of release or recycling, may cause an increase of cytoplasmic dopamine resulting in increased oxidative damage, and subsequently in cell death explaining the neurodegeneration seen in patients with parkin mutations.

MATERIALS AND METHODS

Plasmid constructs

To prepare the yeast two-hybrid bait plasmid pGBT9-parkin (1–465), we excised the full-length parkin cDNA encoding amino acids 1–465 from pEGFPC1-parkin [previously described in Huynh *et al.* (33)] and ligated it into the pGBT9 plasmid (Clontech). The full-length cDNAs of human sytI and sytXI were obtained by PCR from a human adult brain cDNA library cloned in the pGAD10 expression plasmid (Clontech) using primer pairs spanning the entire reading frame. All others expression plasmids were similarly constructed using specific PCR primer pairs. The mutant parkin cDNAs, $parkin^{G289G}$ and $parkin^{C418R}$, were gifts from Professor Alexis Brice of INSERM U289, Hôpital de la Salpetrière, Paris, France.

Identification of parkin interacting proteins

A yeast two-hybrid screen of a human adult brain cDNA library cloned in the GAL4 activation domain vector pGAD10 was performed using as the bait pGBT9-parkin (1–465), encoding parkin amino acids 1–465 fused to the GAL4 binding domain (vectors and library from Clontech). As previously described (53,54), the bait plasmid was co-transformed in yeast strain Y190 and grown on synthetic media without leucine, tryptophan and histidine, and with 25 mM 3-amino-1,2,4-triazole and 2% glucose. The Y190 strain allows for nutritional selection of the *HIS3* gene that allows growth in media lacking histidine, and color selection using the *LacZ* gene encoding β-galactosidase. The β-galactosidase reporter was assayed on stamped nitrocellulose filters by incubating freeze-fractured colonies in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 0.03 mM β-mercaptoethanol, and 2.5 μM X-gal) at 37°C for 15 min to

8 h. Positive clones were restreaked on synthetic media without leucine or tryptophan, and retested for β-galactosidase activity, and then pGAD10 'prey' plasmids were isolated. A pGAD10 plasmid containing a partial sequence encoding the human synaptotagmin XI gene was purified and then retransformed with pGBT9-parkin (1–465) or negative control plasmids (pGBT9 vector, pGBT9-NF2, pGBT9-HRS) and retested for β-galactosidase activity. To obtain semi-quantitative estimates of interaction strengths between various parkin and synaptotagmin XI protein fragments, liquid assays for β-galactosidase were conducted by incubating Y190 yeast cells extracted in Z-buffer and 5% chloroform with 0.6 mg/ml *o*-nitrophenylgalactoside for 2 min to 1 h. Standard deviations were calculated from triplicate measures of replicate cultures. β-Galactosidase activity was expressed as Miller units (Miller unit = $1000 \times [OD_{420}/(OD_{600} \times \text{time} \times \text{volume})]$ (55).

In vitro co-immunoprecipitation

To perform the co-ip, we first co-transfected HA-tagged parkin expression cDNAs (pCMV–HA–parkin, pCMV–HA–truncated parkins, pCMV–HA–parkin^{C418R}, pCMV–HA–parkin^{G289R}) with the respective GFP tagged fusion protein expression vectors (pEGFP–hsytXIAB, pEGFP–sytI, pEGFP-sytXI, or pEGFP) into HEK293 cells growing at 60–80% confluency in 100 mm^2 dishes. Controls were cells transfected only with the pCMV–HA–parkin or non-transfected cells. After 48 h, proteins were extracted with detergent buffer containing 0.5% NP40 and protease inhibitor mixture (Roche). Protein extracts were immunoprecipitated (ip) with anti-GFP (Chemicon), or rat anti-HA agarose matrix (Roche). IP products were immunoblotted with anti-GFP antibody and anti-HA conjugated peroxidase.

Co-immunoprecipitation of human brain extracts

A 1.4 g sample of human cerebral cortex was chopped into small pieces and resuspended in 7 ml of cold lysis buffer (100 mM Tris–HCl, pH 7.5, 150–mM NaCl, 1% Triton X-100, 0.05% SDS, 0.05% deoxycholic acid, and 1 protease inhibitor pellet/10 ml buffer). The cell suspension was homogenized by a glass homogenizer. Protein lysate was aliquoted into 1 ml aliquots and microfuged at top speed in the cold room. Protein extracts were precleared with rabbit IgG conjugated agarose and protein A conjugated agarose for 3 h at 4°C, and the precleared lysate was incubated with rabbit anti-parkA or mouse anti-sytXI antibody overnight in the cold room. The primary antibodies were pulled down with protein A conjugated agarose for 4 h at 4°C. The final pellets were washed five times with co-ip buffer, and the coprecipitates were eluted from the secondary antibody-conjugated agarose with SDS–PAGE buffer. Co-ip products were immunoblotted with either chick anti-parkA or anti-sytXIA antibody.

Ubiquitination and degradation assays

HEK293 cells were transfected with 5 μg of pCMV–Myc–Ubiquitin, pEGFP–sytI or –SytXI, and different pCMV-HA-parkins (wild-type, truncated, and missense). After 24 h, cells were incubated in normal media containing 20 μM lactacystin or 20 μM proteasome inhibitor I for 4 h. Cells were washed with cold DPBS, and proteins were extracted with RIPA buffer containing protease inhibitor pellet (Roche, 1 pellet per 10 ml buffer), and 2 μM *N*-ethylamimide to inhibit deubiquitination enzymes. Protein extracts were immunoprecipitated with mouse antiGFP antibody, and the IP products were detected with anti-HA, anti-myc, and rabbit anti-GFP.

To determine whether parkin accelerates GFP-sytXI degradation, HEK293 cells were co-transfected with GFP-sytXI and HA-vector or GFP-sytXI and HA-parkin plasmids. After 24 h, cells were washed once with DMEM containing 5% dialyzed FBS and no Met and Cys amino acids. Cells were incubated in this media for 30 min, and grown in the same media containing 100 μCi/ml of ^{35}S-Met/Cys (EXPRE^{35}S^{35}S [^{35}S]Protein Labeling Mix, Amersham) for 30 min. Cells were then chased at the indicated time points. Protein extracts were isolated using RIPA buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton-X100, 1 mM EDTA, protease mixture pellet). GFP-sytXI was immunoprecipitated with rabbit anti-GFP antibody (CHEMICON) as described above.

Antibodies

Mouse monoclonal antibody to syt65 (Stressgen, β-actin (Sigma), β-COP (Sigma), and rabbit ubiquitin antibody (DAKO) were purchased. The following reagents were purchased from Roche Diagnostic: mouse anti-HA, anti-HA-peroxidase, anti-myc-peroxidase, anti-HA-agarose. The rabbit anti-sytXIA antibody was made against the sytXIA peptide (HQQAEKKQKNPPYKF) by QCP. Another antibody against the sytXIB peptide (KVRRDKDGPRRESGRG) was made, but this antibody recognized multiple bands in western blots of human and PC12 cells protein extracts (data not shown). The anti-sytXIA antibody was affinity purified by a sepharose-sytXIA column. The rabbit and chicken antiparkA was made against peptide ParkA as described in detail in Huynh *et al.* (33).

Cell cultures

COS1 cells were grown in DMEM medium supplemented with 10% FBS and penicillin/streptomycin, in 37°C incubator with 5% CO_2. Media were changed every 3 days. One day prior to transfection, 50 000 cells were seeded in a 1 cm coverslip previously coated with 20 μg/ml collagen IV. For PC12 cells, cells were grown in DMEM containing 10% heat-inactivated serum, 5% fetal bovine serum, and penicillin/streptomycin in a 37°C incubator with 10% CO_2. Media were changed every 3 days.

Immunohistochemical labeling of human substantia nigra sections

Human brain 7 μm sections were stained with rabbit anti-sytXIA (10 μg/ml), parkA (5 μg/ml), ubiquitin (1/500) antibodies using the immunohistochemical labeling protocols described in Huynh *et al.* (33). Briefly, brains sections were deparafinized and demasked by Biomedia's Autozyme solution (Fisher). Sections were then blocked with 3% normal goat serum, and incubated with the primary antibody overnight in

the cold room. Next day, sections were developed using the *Elite* Vector ABC kit (Vector, San Diego, CA, USA), and the Biomedia's diaminobenzidene substrate kit (Fisher). For peptide preabsorption, 10 µg of anti-sytXIA antibody were preincubated with 1000-fold sytXIA peptide overnight in the cold room. The next day, the preincubated antibody was microfuged for 10 min, and diluted in 1 ml of the staining buffer. Images were acquired using the 20× and 63× oil immersion lenses, and captured by a SPOT digital camera.

Transfection methods

Cells were plated 24 h prior to transfection. On the following day, cDNA plasmids were treated with polyfect transfectant reagent (Qiagen) and transfected into HEK293 cells according to the manufacturer's protocol. At desired time point (24, 48, and 72 h) after transfection, cells were fixed for immunofluorescence labeling, or extracted for immunoprecipitation and western blots. For cells that were examined longer than 24 h after transfection, the media were changed once.

Immunofluorescent labeling and confocal laser microscopy

Cells were fixed with 4% paraformaldehyde in DPBS for 20 min on ice, and incubated in solution A (DPBS, 3% goat serum, 0.05% Triton X-100) for 30 min. Cells were then incubated with selected mouse or rabbit primary antibody diluted in solution A for 1 h at room temperature. Cells were then washed five times with cold DPBS, and incubated with the corresponding secondary antibody conjugated to either FITC or TRITC diluted in solution A for 1 h at room temperature. Cells were then again washed five times with cold DPBS and covered with a slide in 80% glycerol and 10 mM sodium gallate for fading protection.

Cells were viewed with a Leica TCSSP (true confocal scanner spectrophotometry) microscope through the oil immersion 100× lens. Images were acquired sequentially to prevent bleaching between FITC and GFP with TRITC fluorescence.

Protein extraction and western blots

At predetermined time point after transfection, cells were extracted with weak detergent CO-IP buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 0.5% Triton X100). For ubiquitination assays and antibody analysis, cells or tissues were extracted with strong triple detergent buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate). The protein extracts were clarified by 30 min centrifugation using a tabletop Beckmann Microfuge. For western blot, 10 µl of the protein extract was loaded per well of a 15-well 4–20% gradient, mini SDS–polyacrylamide gel. Proteins were resolved at 100 V for 2 h and transferred to Amersham's nitrocellulose filter overnight at 30 V in the cold room. The filter was then removed from the western blot apparatus, and blocked with 5% non-fat milk for 1 h at room temperature. The blocking solution was then replaced with blocking solution containing the desired concentration of primary antibody. The western blot was visualized with the Amersham Chemiluminescent western blot detection kit.

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