Parkin is recruited to the centrosome in response to inhibition of proteasomes

Jinghui Zhao, Yong Ren, Qian Jiang and Jian Feng*

Department of Physiology and Biophysics, State University of New York at Buffalo, Buffalo, NY 14214, USA

*Author for correspondence (e-mail: jianfeng@buffalo.edu)

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Summary

Parkin is a protein-ubiquitin E3 ligase linked to Parkinson's disease. Although several substrates of parkin have been identified, the subcellular location for parkin to recognize and ubiquitinate its targets is unclear. Here we report that parkin was accumulated in the centrosome when SH-SY5Y or transfected HEK293 cells were treated with the proteasome inhibitor lactacystin. The specific recruitment of parkin was dependent on concentration and duration of the treatment, and was accompanied by the centrosomal accumulation of ubiquitinated proteins and CDCrel-1, a substrate of parkin. The recruitment of parkin was apparently mediated through its binding to γ-tubulin, which has been shown to accumulate in the centrosome in response to misfolded proteins. Furthermore, the effect was abrogated by the microtubule-depolymerizing drug colchicine or the microtubule-stabilizing drug taxol, which indicates that the intact microtubule network is required for the centrosomal recruitment of parkin. Taken together, our data suggest that the lactacystin-induced accumulation of parkin in the centrosome plays a significant role in the ubiquitination of misfolded substrates accumulated there. This process may provide a subcellular locale for parkin to ubiquitinate and degrade protein aggregates critically involved in the pathogenesis of Parkinson's disease.

Key words: Parkin, Centrosome, Aggresome, γ-Tubulin, Parkinson's disease

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder resulting from the selective loss of dopamine (DA) neurons in substantia nigra pars compacta (SNpc). The degeneration of these neurons is thought to be caused by a combination of genetic and environmental factors (Gasser, 2001; Tanner and Langston, 1990). Three genes, encoding α-synuclein (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997), parkin (Kitada et al., 1998) and DJ-1 (Bonifati et al., 2003), have been linked to familial cases of PD. In the α-synuclein gene, two rare mutations (A53T and A30P) have been identified in a very small group of patients with an autosomal dominant form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998). So far, mutations in the DJ-1 gene have been reported in only two families (Bonifati et al., 2003). In contrast, a variety of point mutations, deletions, truncations and duplications of the parkin gene have been found in many patients with an autosomal recessive form (Lucking et al., 2000; Abbas et al., 1999) or sporadic forms (Scott et al., 2001) of PD.

Subsequent studies have found that parkin is a protein-ubiquitin E3 ligase (Shimura et al., 2000) and many of its mutations are clustered in domains critical for its E3 ligase activity (Giasson and Lee, 2001). It suggests that abrogation of parkin's E3 ligase activity and ensuing accumulation of its substrates are critical for the selective degeneration of DA neurons in SNpc. Several substrates of parkin have been identified. They include CDCrel-1 (Zhang et al., 2000), a member of the septin family GTPases that binds to syntaxin-1 (Beites et al., 1999), but appears to be dispensable in neurotransmitter release (Peng et al., 2002). Another substrate of parkin is Pael receptor, a homolog of endothelin receptor type B, whose accumulation in brains with parkin mutations apparently leads to unfolded protein stress in endoplasmic reticulum and may cause selective neuronal death (Imai et al., 2001). More recently, two proteins related to α-synuclein, were found to be substrates of parkin. One of them is an O-glycosylated form of α-synuclein (Shimura et al., 2001), the other is the α-synuclein-binding protein, synphilin-1 (Chung et al., 2001).

Identification of these substrates is a key step towards understanding the biological function of parkin. Another important issue is to determine the subcellular location where ubiquitination and degradation of these substrates occur. Previous studies have shown that accumulation of misfolded proteins, either by inhibition of the 26 S proteasome or by mutation in the protein, leads to the formation of a large aggregate in the centrosomal region of the cell (Wigley et al., 1999; Johnston et al., 1998). In mitotic cells at interphase, the centrosome is a perinuclear structure containing a pair of centrioles, from which a radial array of microtubules is extended. One of the key components of the centrosome is γ-tubulin, which is responsible for nucleating microtubules (Joshi et al., 1992). γ-tubulin also exists in the cytosol in a large complex with several proteins of unknown functions (Wiese and Zheng, 1999). Increasing evidence has shown that the centrosome plays a significant role in the formation of protein aggregates in the cell, in addition to its well-recognized

function as the microtubule-organizing center. Small aggregates of polyubiquitinated proteins are thought to be transported to the centrosome by microtubule-associated motor proteins to form a large inclusion body known as an 'aggresome' (Johnston et al., 1998; Kopito, 2000), which also contains chaperones and components of the 26 S proteasome (Johnston et al., 1998; Wigley et al., 1999; Garcia-Mata et al., 1999). The formation of an aggresome is thought to provide the cell with a mechanism to sequester toxic aggregates of misfolded proteins that cannot be efficiently handled by the ubiquitin-dependent proteolysis system, and a staging ground for subsequent fusion with lysosomes to degrade these protein aggregates by an autophagic pathway (Kopito, 2000).

One of the histological hallmarks of Parkinson's disease is the Lewy body, which is often a large single intracellular inclusion that contains ubiquitinated proteins (Lowe et al., 1988; Galvin et al., 1999), α-synuclein (Spillantini et al., 1997) and parkin (Schlossmacher et al., 2002). However, PD patients with parkin mutations almost invariably lack any Lewy body in their brain tissue (Kitada et al., 1998), suggesting that Lewy bodies cannot be formed effectively in the absence of functional parkin proteins. To understand the role that parkin may play in the formation of protein aggregates, we examined the subcellular location of parkin when cells were treated with specific inhibitors of the 26 S proteasome. Here we report the recruitment of parkin to the centrosome in response to inhibition of proteasomes. The possible molecular mechanism underlying this process and its functional implication are also discussed.

Materials and Methods

Antibodies and cDNAs

A polyclonal antibody against parkin (P304) was generated by immunizing rabbits with KLH-conjugated peptides derived from mouse parkin sequence (amino acid 304-322). Antisera were purified by affinity chromatography using the same peptide immobilized on SulfoLink gel matrix (Pierce, Rockford, IL) according to the manufacturer's protocol. This antibody happened to be the best one out of the five that we generated, and recognized only the correct band on western blots (Ren et al., 2003). Monoclonal antibodies against γ-tubulin, FLAG (M2), and anti-FLAG-conjugated (M2) agarose were purchased from Sigma (St. Louis, MO). Anti-ubiquitin and rhodamine- or FITC-conjugated secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). Monoclonal anti-HA was purchased from Roche (Indianapolis, IN). Antibodies against MAP kinase, CREB, p38 and S6 kinase were purchased from Cell Signaling (Boston, MA). Polyclonal antibody against myc tag was purchased from Upstate Biotechnology (Lake Placid, NY). Lactacystin was purchased from A G Scientific (San Diego, CA). Mouse parkin cDNA was amplified by RT-PCR from brain total RNA. It was completely sequenced to ensure that no mutation was present. FLAG epitope tag was added to the 5′-end by PCR, and the tagged cDNA was subcloned into pcDNA3.1. CDCrel-1 cDNA was cloned by RT-PCR from rat brain total RNA and subcloned into pCMV-tag3B (Stratagene, La Jolla, CA).

Transfection, immunoprecipitation and western blot

HEK293, BE(2)-C, SH-SY5Y, COS7 and 3T3 cells were purchased from ATCC (Manassas, VA). They were maintained in DMEM with 10% FCS and antibiotics. Transfection of FLAG-tagged parkin in HEK293 cells was performed using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Sixty hours after transfection, cells cultured in 10 cm dishes were lysed on ice in cold lysis buffer (1% Triton X-100, 10 mM Tris pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA and 0.1 mM Na_3VO_4) for 20 minutes. Lysates were centrifuged at 4°C at 16,000 ***g*** and supernatant fractions were incubated with anti-FLAG-conjugated agarose for 1 hour at 4°C. Immunoprecipitates were washed three times with the lysis buffer, boiled in 2× SDS loading buffer for 5 minutes, separated on SDS-polyacrylamide gels, and analyzed by western blotting with anti-γ-tubulin using the ECL method according to the manufacturer's protocol (Amersham, Piscataway, NJ). For experiments using rat brain homogenates, one whole brain was homogenized in 15 ml of ice-cold lysis buffer on ice in a tissue grinder (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 16,000 ***g*** for 20 minutes and ultracentrifuged at 338,000 ***g*** for 30 minutes. The supernatant fractions were incubated with various antibodies for 1 hour at 4°C, followed by incubation with protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) under the same conditions. Gel separation of precipitated proteins and western blot analysis were the same as described above.

Immunocytochemistry

SH-SY5Y, HEK293 and COS7 cells were cultured on collagen-coated coverslips and treated with various drugs at different dosage and for different times as indicated in the figure legends. Cells were fixed with cold methanol at 4°C for 20 minutes, and blocked with 3% of BSA for 1 hour. This was followed by incubation in primary antibodies for 1 day at 4°C and secondary antibodies for another day at 4°C. In some experiments, the cells were also stained with the DNA-binding dye TO-PRO-3 (1:1000 in PBS, Molecular Probes, Eugene, OR) for 5 minutes to visualize the nucleus. Immunofluorescent images were acquired on a Bio-Rad confocal microscope. Monochrome images (512×512 pixels) were pseudocolored and merged with the software Confocal Assistant (freeware by Todd Clark Brelje). The size of the parkin aggregate was measured using the SPOT software (Diagnostic Instruments, Sterling Heights, MI) to calculate the area of a hand-drawn circle around the aggregate. Statistical analysis was performed with the software Origin (Origin Lab, Northampton, MA).

Results

Proteasomal inhibition induces centrosomal accumulation of endogenous parkin in neuroblastoma cell lines

To study the subcellular localization of parkin when protein degradation is inhibited, we chose the widely used human dopaminergic neuroblastoma cell lines SH-SY5Y and BE(2)-C. The expression level of parkin in these cells made it easy to examine the subcellular localization of the endogenous protein. They also express dopaminergic markers such as tyrosine hydroxylase and dopamine transporter (data not shown), which are significant to Parkinson's disease.

When we stained SH-SY5Y with a mono-specific antibody against parkin (Ren et al., 2003), a punctate expression pattern was seen in the cell, with a major spot localized in the perinuclear region (Fig. 1B). After the cells were treated with lactacystin (10 μM for 12 hours), a specific inhibitor of the 26 S proteasome (Fenteany and Schreiber, 1998), we observed significant accumulation of parkin in the perinuclear region (Fig. 1E). Our previous study has shown that parkin puncta are localized along microtubules (Ren et al., 2003). As microtubules are centered on the centrosome in mitotic cells at interphase, we suspected that the major spot of parkin

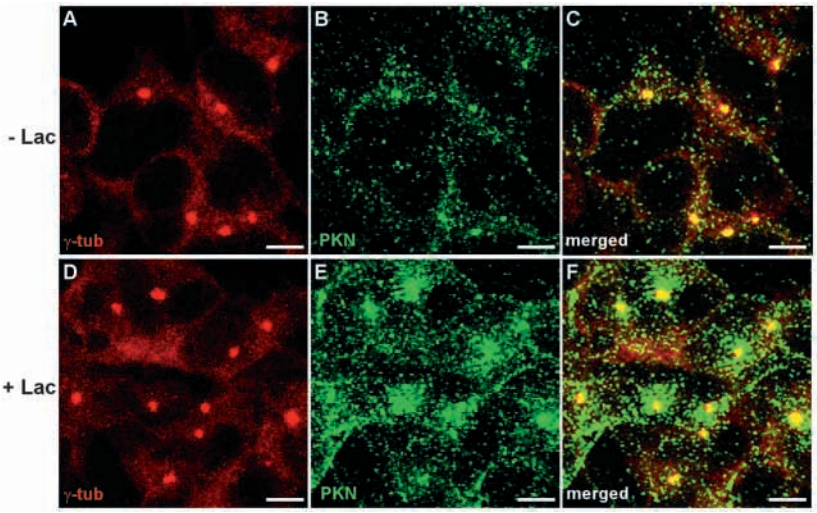

Fig. 1. Lactacystin-induced accumulation of parkin in the centrosome. SH-SY5Y cells were treated without (–Lac; A-C) or with (+Lac, D-F) lactacystin, and co-stained with antibodies against the centrosome marker γ-tubulin (γ-tub; A,D) and parkin (PKN; B,E). Merged images (C,F) showed significant accumulation of parkin in the centrosome after lactacystin treatment (10 μM for 12 hours). The experiment was repeated three times with similar results. Scale bars: 5 μm.

immunoreactivity in the perinuclear region may be at the centrosome. To test this, we co-stained the cells with antibodies against parkin and γ-tubulin, a centrosome marker. The major spot of parkin in the perinuclear region before lactacystin treatment (Fig. 1C) and the large accumulation of parkin after the treatment (Fig. 1F) were all co-localized with γ-tubulin signals. When we added the parkin antigenic peptide in the primary antibodies, no signal was observed in FITC channel, while γ-tubulin fluorescence was intact (data not shown). We also obtained the same results with another centrosome marker, pericentrin. In addition, similar effects were found when we treated the cells with MG132, another specific proteasome inhibitor (data not shown). Thus, inhibition of the 26 S proteasome apparently leads to accumulation of endogenous parkin in the centrosome.

Next, we studied the kinetics of this recruitment process by measuring the average area of parkin accumulation in the centrosomal region in SH-SY5Y cells treated with 10 μM lactacystin for different time periods. As shown in Fig. 2, the accumulation of parkin became significant at 4 hours ($P<0.05$, compared to no treatment), and reached a plateau at 8 hours. We also studied the dose response of this effect. After the cells were treated with various concentration of lactacystin for 12 hours, the average area of parkin accumulation was measured and plotted against the dosage. The centrosomal recruitment of parkin was significant at 5 μM lactacystin ($P<0.001$, compared to no treatment), and continued to rise with increasing concentration of the drug (Fig. 2F). At doses above 10 μM, SH-SY5Y cells exhibited significant cell death. Similar results on the lactacystin-induced centrosomal recruitment of endogenous parkin were also obtained when we examined another dopaminergic neuroblastoma cell line BE(2)-C (data not shown).

Lactacystin does not induce non-selective accumulation of proteins in the centrosome

To ascertain that the centrosomal recruitment of parkin is not due to an indiscriminate process of protein accumulation in response to proteasomal inhibition, we examined the subcellular localization of several other proteins before and after lactacystin treatment. Two examples are shown in Fig. 3, in which we co-stained SH-SY5Y cells with antibodies against CREB and γ-tubulin (Fig. 3A,B) or antibodies against MAP

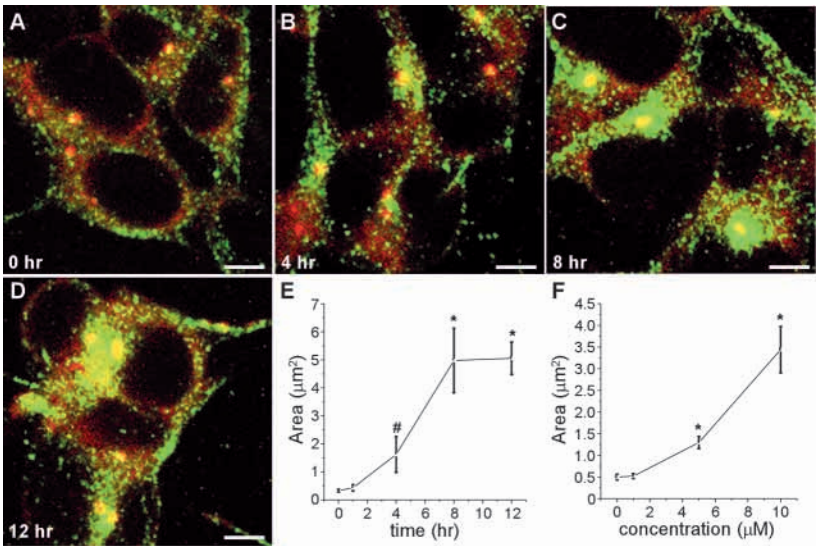

Fig. 2. Recruitment of parkin to the centrosome was dependent on the duration and dosage of lactacystin treatment. Representative confocal images from SH-SY5Y cells treated with 10 μM lactacystin for 0, 4, 8 and 12 hours (A-D). Time-dependent increase of parkin (green) was seen in the centrosome, where γ-tubulin (red) signals overlapped (yellow) with some of parkin signal. The area of the γ-tubulin staining also increased with time of treatment. Scale bars: 5 μm. (E) The average area of parkin accumulation was plotted against the duration of lactacystin (10 μM) treatment. #$P<0.05$ vs. 0 hour; *$P<0.001$ vs. 0 hour (n=19 cells for each point). (F) The average area of parkin accumulation was plotted against the concentration of lactacystin treated for 12 hours. *$P<0.001$ vs. 0 μM (n=16 cells for each point).

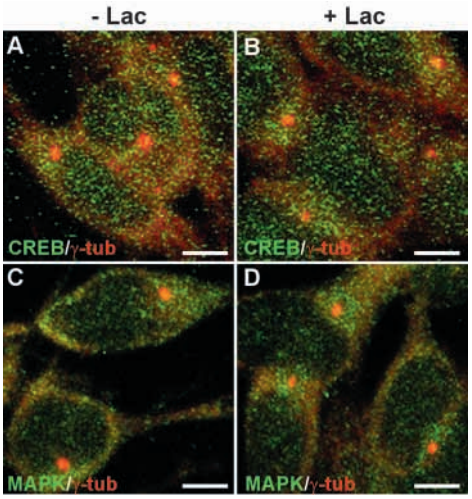

Fig. 3. Lactacystin did not cause a general recruitment of proteins to the centrosome. No significant centrosomal accumulation was seen for CREB (CREB; A,B) or MAP kinase (MAPK; C,D) in SH-SY5Y cells treated without (–Lac; A,C) or with (+Lac; B,D) 10 μM lactacystin for 12 hours. The experiments were repeated three times with similar results. Scale bars: 5 μm.

kinase and γ-tubulin (Fig. 3C,D). Lactacystin treatment (10 μM for 12 hours) did not cause significant accumulation of either protein in the cell. We also examined the subcellular localization of endogenous S6 kinase in HEK293 cells before and after lactacystin treatment. It did not accumulate in the centrosome in response to the treatment (data not shown). Thus, the recruitment of parkin to the centrosome appears to be selective.

Lactacystin induces centrosomal recruitment of transfected parkin in HEK293 cells

To provide more evidence on the generality of this effect, we examined HEK293 cells transfected with FLAG-tagged parkin before and after lactacystin treatment. HEK293 cells express very low level of parkin endogenously (see untransfected cells in Fig. 4C). Transient expression of transfected parkin produced a diffusely cytosolic localization for this protein (Fig. 4C). After lactacystin treatment (10 μM for 12 hours), 9.33±0.67% (mean±s.e.m.) of the transfected cells showed centrosomal accumulation of parkin (Fig. 4B,D,F). Unlike the situation in SH-SY5Y cells where a small amount of endogenous parkin was accumulated in the centrosome even before lactacystin treatment (Fig. 1A-C), transfected parkin in HEK293 cells was not enriched in the centrosome in the absence of lactacystin (Fig. 4E). Thus, we quantified the recruitment process by calculating the percentage of transfected cells that had centrosomal accumulation of parkin. The lactacystin-induced centrosomal recruitment of parkin in HEK293 cells was dependent on the dose (Fig. 4G) and duration (Fig. 4H) of the treatment. Taken together, our results obtained from multiple cell lines on endogenous or transfected parkin indicate that the specific recruitment of this protein in response to inhibition of proteasomes is a general phenomenon.

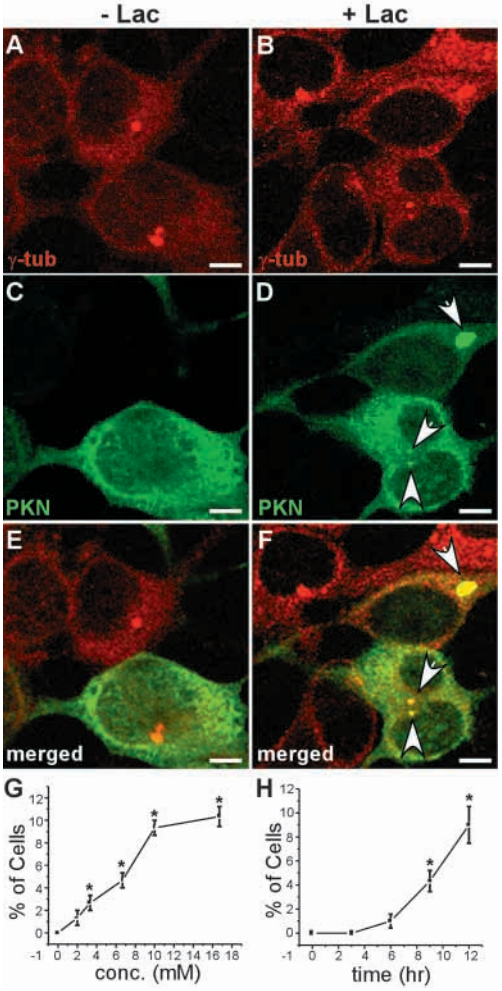

Fig. 4. Lactacystin-induced recruitment of transfected parkin in HEK293 cells. HEK293 cells were transfected with a parkin expression construct, treated without (–Lac; A,C,E) or with (+Lac; B,D,F) 10 μM lactacystin for 12 hours, and co-stained with antibodies against γ-tubulin (γ-tub; A,B) and parkin (PKN; C,D). Merged confocal images (E,F) show that parkin accumulated in the centrosome in some of the transfected cells after lactacystin treatment (indicated by arrows in F). Scale bars: 5 μm. (G) Percentage of transfected cells that have centrosomal accumulation of parkin was plotted against the concentration of the lactacystin applied (12 hours). (H) Percentage of transfected cells that have centrosomal accumulation of parkin was plotted against the duration of lactacystin treatment (10 μM). *****$P<0.05$ vs. 0 μM (G) or 0 hour (H) (n=3 fields of view per data point, with at least 100 transfected cells per field).

Parkin binds to γ-tubulin in the rat brain and HEK293 cells

In untreated SH-SY5Y cells, the biggest parkin spots always seemed to be co-localized with γ-tubulin in the centrosome (Fig. 1A-C). With lactacystin treatment, parkin was recruited to the centrosome, so was γ-tubulin (Fig. 2A,D). These observations suggest that parkin may be physically bound to γ-tubulin. To test this idea, we performed co-immunoprecipitation experiments in rat brain homogenate, which was ultracentrifuged at 4°C to obtain supernatant fractions with no significant amount of microtubules. The

lysates were treated with or without the microtubule depolymerizing drug colchicine (25 μM for 15 minutes at 4°C) and immunoprecipitated with pre-immune serum, anti-parkin, or anti-parkin pre-incubated with its antigenic peptide. As shown in Fig. 5A, γ-tubulin was strongly co-immunoprecipitated with parkin, and this interaction was not affected by colchicine treatment, which ensured the dissociation of any residual microtubules in the ultracentrifuged lysates and the existence of free γ-tubulin.

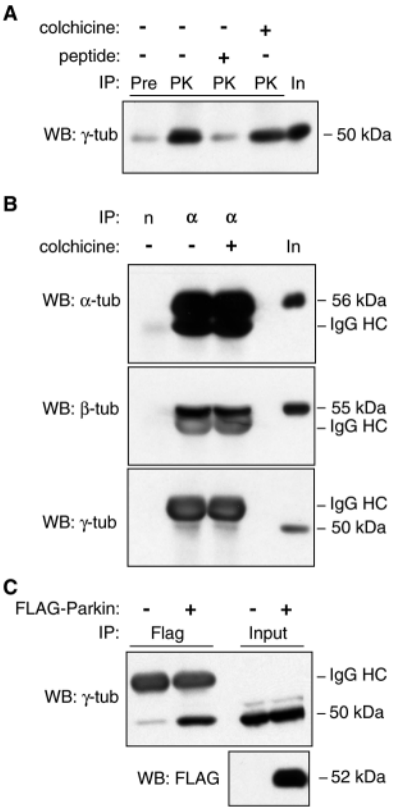

Fig. 5. Binding between parkin and γ-tubulin in the rat brain and HEK293 cells. (A) Ultracentrifuged rat brain homogenates were incubated with or without colchicine (25 μM for 15 minutes at 4°C) and immunoprecipitated with pre-immune serum (Pre) or anti-parkin (PK) that had been pre-incubated with or without its antigenic peptide. Immunoprecipitates and 1% of the input material (In) were analyzed by western blotting (WB) with a monoclonal antibody against γ-tubulin. The co-immunoprecipitation of γ-tubulin with parkin was not affected by colchicine, and was abolished when the antibody was pre-absorbed with its antigen. (B) Ultracentrifuged rat brain homogenates treated with or without colchicine were immunoprecipitated with an irrelevant antibody (n, anti-neurabin) or α-tubulin antibody (α). Precipitated proteins and 1% of the input material (In) were analyzed by western blotting with antibodies against α-, β- or γ-tubulins, respectively. Only a very small fraction of γ-tubulin was co-immunoprecipitated with α/β-tubulin heterodimers. (C) Cleared lysates from HEK293 cells transfected with or without FLAG-tagged parkin were immunoprecipitated with anti-FLAG. Precipitated proteins and 3% of input material were analyzed by western blotting with anti-γ-tubulin. The input was also blotted with anti-FLAG to detect the expression of the parkin construct. γ-tubulin, which was co-immunoprecipitated with parkin, migrated at 50 kDa. IgG HC, heavy chain of IgG. The experiments were repeated at least three times with similar results.

As our previous studies have shown that parkin also binds to α/β tubulin heterodimers very strongly (Ren et al., 2003), we wanted to test whether the binding between parkin and γ-tubulin is a direct interaction or an indirect one mediated by the association of γ-tubulin with α/β heterodimers. Ultracentrifuged rat brain lysates were treated with or without colchicine, and immunoprecipitated with an irrelevant antibody (anti-neurabin) or the α-tubulin antibody. Western blot analysis of the immunoprecipitates showed that only a very small fraction of γ-tubulin was associated with α-tubulin. In contrast, the binding between α- and β-tubulin was much stronger, consistent with existence of α/β-tubulin heteodimers (Fig. 5B). Colchicine treatment had no effect on the co-immunoprecipitation, indicating that the ultracentrifuged brain lysate did not contain any measurable amount of microtubules. These results suggest that the strong binding between parkin and γ-tubulin cannot be entirely attributed to an indirect interaction mediated by the relatively weak association between γ-tubulin and α/β heterodimers. It seems likely that a certain fraction of γ-tubulin may bind to parkin directly and another fraction may do so via association with α/β heterodimers.

To confirm the observation in rat brain homogenate, we performed similar experiment in HEK293 cells. After we transfected HEK293 cells with or without FLAG-tagged parkin, the lysates were immunoprecipitated with anti-FLAG-conjugated agarose to see whether exogenously expressed parkin was bound to γ-tubulin. As shown in Fig. 5C, γ-tubulin was indeed co-immunoprecipitated with parkin.

Centrosomal recruitment of parkin is dependent on intact microtubule networks

Our previous study had shown that parkin binds to microtubules and α/β tubulin heterodimers (Ren et al., 2003). The interaction between parkin and γ-tubulin, and the fact that a fraction of γ-tubulin is associate with microtubules at the centrosome, suggest that the centrosomal recruitment of the parkin/γ-tubulin complex may be dependent on microtubules. To test this, we treated SH-SY5Y cells with the microtubule-depolymerizing drug colchicine or the microtubule-stabilizing drug taxol. As shown in Fig. 6B, colchicine treatment (5 μM for 12 hours) changed the punctate localization of parkin (Fig. 1B) into a more diffusely cytosolic pattern. The existence of parkin in the centrosome was much less apparent than in untreated cells (compare Fig. 6C with Fig. 1C). After the cells were treated with colchicine and lactacystin (both at 5 μM for 12 hours), no significant accumulation of parkin was seen in the centrosome (Fig. 6D-F). In the presence of colchicine, parkin exhibited a similar pattern of subcellular localization with or without lactacystin treatment (compare Fig. 6E with B). In contrast to the situation with a single treatment of lactacystin at 10 μM, a significant degree of cell death was observed when SH-SY5Y cells were treated with 10 μM lactacystin and colchicine at the same time. To test whether 10 μM lactacystin would induce centrosomal recruitment of parkin in the presence of colchicine, we chose COS7 cells, which exhibited very little cell death when treated with both drugs at 10 μM. No significant accumulation of parkin in the centrosome was observed under this condition (data not

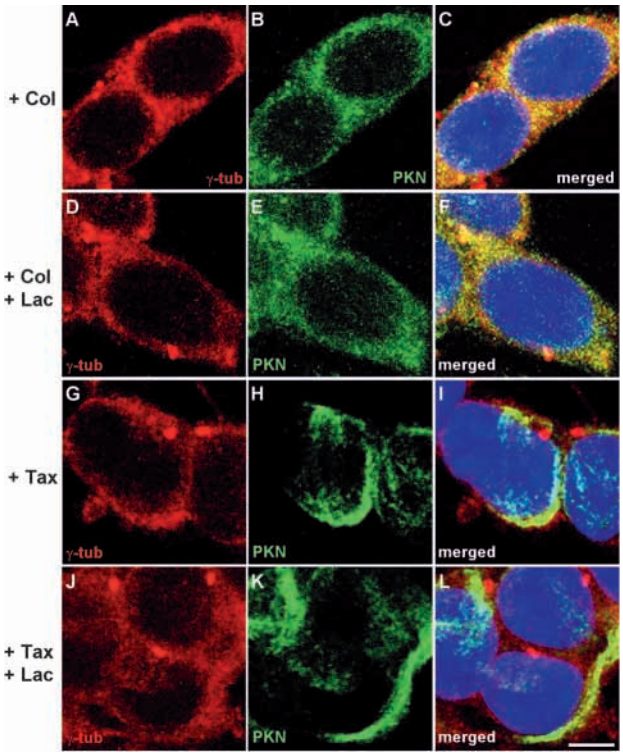

Fig. 6. Colchicine or taxol treatment blocked the centrosomal recruitment of parkin induced by lactacystin. SH-SY5Y cells were treated with colchicine (+Col; A-C), colchicine plus lactacystin (+Col +Lac; D-F), taxol (+Tax, G-I), or taxol plus lactacystin (+Tax +Lac, J-L) for 12 hours, all at 5 μM. They were fixed and co-stained with anti-γ-tubulin (red), anti-parkin (green) and the DNA-binding dye TO-PRO-3 (blue). The experiments were repeated at least three times with similar results. Scale bars: 5 μm.

shown), which is consistent with the results from SH-SY5Y cells.

We also treated SH-SY5Y cells with taxol (5 μM for 12 hours). The treatment increased the intensity of parkin staining along microtubules, however, parkin was not observed in the centrosome (Fig. 6G-I). After the cells were incubated with taxol and lactacystin (both at 5 μM for 12 hours), parkin was still absent in the centrosome (Fig. 6J-L). In addition, there was no accumulation of parkin elsewhere in the cell, compared to cells treated with taxol alone (Fig. 6K and H). Similar results were obtained in COS7 cells treated with taxol alone (10 μM for 12 hours) or together with lactacystin (both at 10 μM for 12 hours) (data not shown). Thus, the centrosomal recruitment of parkin appears to be dependent on intact microtubules. Both the disruption of microtubules (by colchicine) and their overt stabilization (by taxol) blocked the movement of parkin towards the centrosome.

The expression level of parkin is not significantly affected by lactacystin, colchicine or taxol

As the fluorescence intensity of parkin appears to differ in cells treated with vehicle, lactacystin, colchicine or taxol (Figs 1 and 6), we performed western blotting on lysates from SH-SY5Y cells after various treatment (5 μM of each drug for 12 hours) to examine the expression level of parkin under these conditions. As shown in Fig. 7, almost all parkin was in the supernatant fraction in untreated cells, and the amount was not significantly changed by treatment with lactacystin, colchicine or a combination of the two. However, when the cells were treated with taxol or taxol plus lactacystin, the amount of parkin was significantly reduced in the supernatant fraction and increased commensurately in the pellet fraction. As a control for equal loading, we examined the level of p38 MAP kinase, a cytosolic protein that has no known interaction with microtubules. The amount of p38 in the supernatant fraction was not changed by these treatments, and it did not go into the pellet fraction after taxol treatment. Similar results were obtained from COS7 and 3T3 cells with regard to the expression level of parkin in response to these drugs (10 μM of each drug for 12 hours, data not shown).

Thus, these data showed that the total amount of parkin was not significantly affected by lactacystin, colchicine or taxol treatment, either alone or in combination. However, taxol-induced microtubule polymerization brought down a portion of parkin from the supernatant to the pellet fraction. This is consistent with the strong interaction between parkin and microtubules (Ren et al., 2003). Taken together, our results showed that the centrosomal recruitment of parkin in response to lactacystin was caused by change of localization, rather than alteration of expression.

Lactacystin induces centrosomal accumulation of the parkin substrate CDCrel-1 and ubiquitinated proteins

As parkin is a protein-ubiquitin E3 ligase, we hypothesize that its recruitment to the centrosome may be linked to ubiquitination of misfolded proteins accumulated there. We transfected HEK293 cells with myc-tagged CDCrel-1, a known substrate of parkin (Zhang et al., 2000), and treated the cells with or without lactacystin (10 μM for 12 hours). When we co-stained the cells with anti-γ-tubulin and anti-myc tag, it was

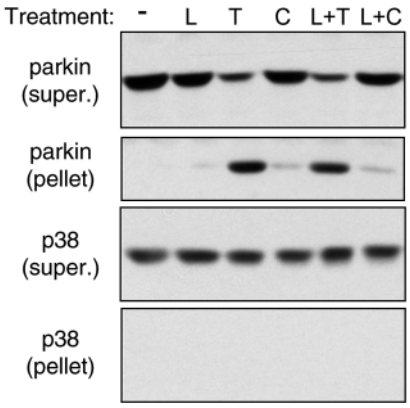

Fig. 7. Lactacystin, colchicine or taxol treatment did not alter the total amount of parkin expression in the cell. SH-SY5Y cells were treated with vehicle (–), lactacystin (L), taxol (T), colchicine (C), lactacystin plus taxol (L + T), or lactacystin plus colchicine (L + C), all at 5 μM, for 12 hours. Cell lysates were separated by centrifugation at 4°C into supernatant and pellet fractions, and analyzed by western blotting with anti-parkin or anti-p38. Experiments were repeated three times, each with similar results.

clear that lactacystin induced the centrosomal accumulation of transfected CDCrel-1 (compare Fig. 8E with B, and F with C). The antibody against myc tag specifically recognized transfected myc-CDCrel-1 in the cell (Fig. 8M). We also co-stained these cells with antibodies against γ-tubulin and ubiquitin. Prior to lactacystin treatment, ubiquitin staining was diffuse in the cell, with a large portion in the cytosol (Fig. 8H). Most of the staining represented ubiquitinated proteins, rather than free ubiquitin, as evidenced by western blots of total cell lysates with anti-ubiquitin (Fig. 8N). After lactacystin treatment, ubiquitinated proteins were accumulated in the centrosome (Fig. 8K,L), which is consistent with previous reports on the recruitment of proteasome components and ubiquitinated proteins to the centrosome (Wigley et al., 1999; Garcia-Mata et al., 1999). The presence of CDCrel-1, parkin and ubiquitinated proteins simultaneously in the centrosome after lactacystin treatment suggests that parkin is recruited to the centrosome to ubiquitinate its substrates, such as CDCrel-1, which is accumulated there.

Discussion

In this report, we describe the lactacystin-induced centrosomal recruitment of parkin. Several lines of evidence indicate that it is a specific response of the cell in reaction to inhibition of protein degradation by the 26 S proteasome. The dose- and time-dependent accumulation of parkin in the centrosome was observed for endogenous parkin in SH-SY5Y cells (Figs 1, 2) and BE(2)-C cells (data not shown), and for ectopically expressed parkin in HEK293 cells (Fig. 4). In addition, the recruitment of parkin was accompanied by the centrosomal accumulation of a parkin substrate CDCrel-1, ubiquitinated proteins (Fig. 8), and components of the 26 S proteasome (Wigley et al., 1999; Garcia-Mata et al., 1999). As parkin is an E3 ligase, it is reasonable to assume that the recruitment of parkin may serve to ubiquitinate its substrates accumulated in the centrosome. Further evidence on the specificity of this process was provided by the lack of accumulation for proteins such as MAP kinase and CREB (Fig. 4), as well as S6 kinase (data not shown). It clearly shows that not all proteins go to the centrosome when proteasomes are inhibited.

The potential mechanism for the centrosomal recruitment of parkin may be the binding between parkin and γ-tubulin, a protein that shuttles between a cytosolic pool and a centrosomal pool (Schiebel, 2000; Wiese and Zheng, 1999). Accumulation of misfolded proteins leads to significant increase of γ-tubulin in the centrosome (Johnston et al., 1998; Wigley et al., 1999; Garcia-Mata et al., 1999). Although the detailed mechanism for the centrosomal recruitment of γ-tubulin is unclear, it appears to be dependent on transport proteins on microtubules (Johnston et al., 1998; Garcia-Mata et al., 1999), whose minus ends are anchored on γ-tubulin ring complexes in the centrosome (Schiebel, 2000; Wiese and Zheng, 1999). The binding between parkin and γ-tubulin appears to be independent of microtubules, as colchicine added to rat brain lysate did not disrupt the co-immunoprecipitation

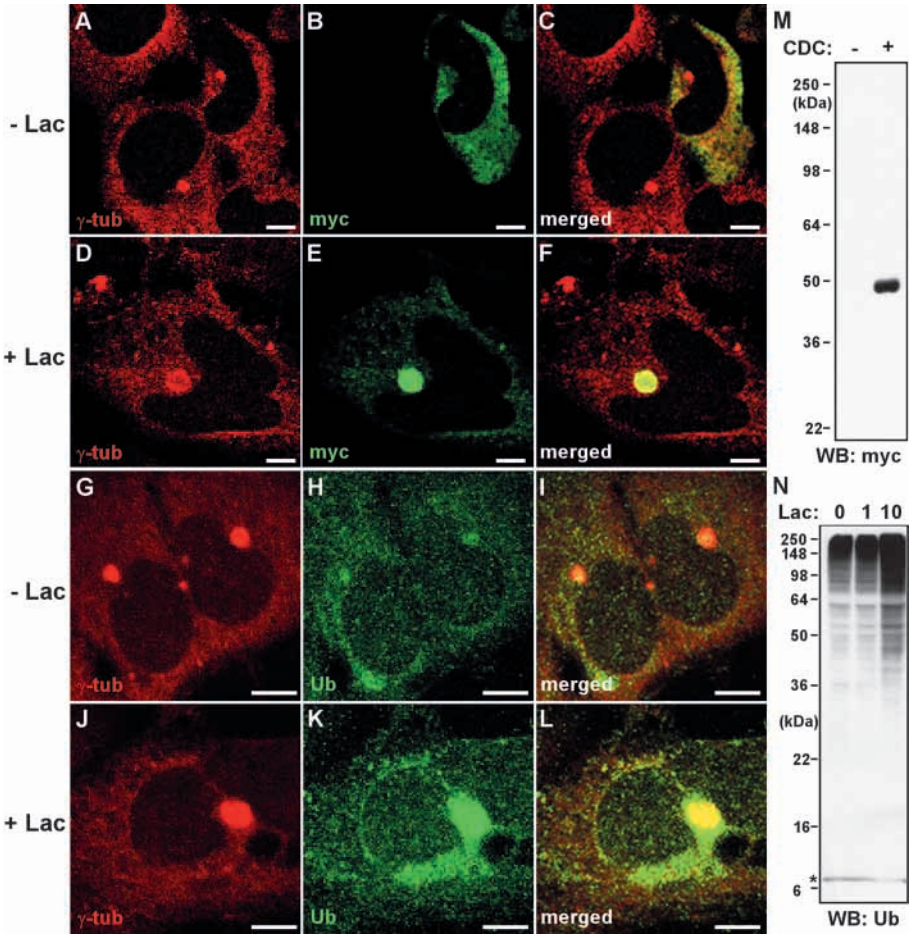

Fig. 8. Lactacystin-induced accumulation of CDCrel-1 and ubiquitinated proteins in the centrosome. HEK293 cells transfected with myc-tagged CDCrel-1 were treated without (– Lac; A-C,G-I) or with (+Lac; D-F,J-L) lactacystin (10 μM for 12 hours) and co-stained with antibodies against γ-tubulin (γ-tub; A,D) and myc-tag (myc; B,E), or anti-γ-tubulin (γ-tub; G,J) and anti-ubiquitin (Ub; H,K), respectively. Merged confocal images showed that CDCrel-1 (F and C) and ubiquitinated proteins (L and I) accumulated in the centrosome after lactacystin treatment. Scale bars: 5 μm. Similar results were obtained from at least five sets of confocal images taken from two to three coverslips at each condition. (M) Anti-myc western blot of lysates from cells transfected without (–) or with (+) myc-CDCrel-1 to show expression of the protein and specificity of the antibody (only one band at the correct size was recognized). (N) Anti-ubiquitin western blot of lysates from cells treated with 0 μM, 1 μM, or 10 μM lactacystin (Lac) for 12 hours. Most of the ubiquitin signals represented polyubiquitinated proteins with very little free ubiquitin running at 8.5 kDa (*).

of parkin and γ-tubulin (Fig. 5A). Thus, it seems that parkin/γ-tubulin complexes are normally present in the cytosol in the absence of lactacystin treatment. Inhibition of proteasomes triggers a response that recruits the complex to the centrosome.

This recruitment process is apparently dependent on intact microtubule networks in the cell. When microtubules were depolymerized by colchicine, recruitment of parkin did not occur (Fig. 6A-F). However, when microtubules were rendered overtly stable by taxol, parkin became more concentrated on bundled microtubules (Fig. 6H,K), and never moved to the centrosome (Fig. 6I,L). A large portion of parkin became so strongly associated with microtubules that it entered the pellet fraction with them (Fig. 7). Even the portion of parkin that was normally in the centrosome (Fig. 1B,C) was no longer present when SH-SY5Y cells were treated with taxol (Fig. 6H,I). As parkin bound to γ-tubulin (Fig. 5), as well as α/β tubulin (Ren et al., 2003), the data suggest that taxol could perhaps change the dynamic partition of parkin between γ-tubulin and α/β tubulin. With taxol treatment, parkin-α/β tubulin complexes are polymerized into microtubules, leaving very little parkin to interact with γ-tubulin, which may prevent the movement of parkin toward the centrosome. The other possibility is that taxol treatment may cause centrosome-independent nucleation of microtubules, which would prevent the centrosomal recruitment of parkin even if its trafficking is not affected by taxol. Further studies are necessary to elucidate the mechanism(s) by which taxol blocks the accumulation of parkin at the centrosome.

Although the lactacystin-induced centrosomal recruitment of parkin was observed in several cell lines, the effect was not seen in cultured neurons. The subcellular localization of parkin was punctate, decorating along microtubules (Ren et al., 2003), but exhibited no significant accumulation anywhere in the neuron with or without lactacystin treatment (data not shown). The expression level of γ-tubulin was very low in neurons, compared to mitotic cells. Almost all γ-tubulin proteins are localized in the centrosome in neurons (Baas and Joshi, 1992), however, microtubules are not anchored on the centrosome in these postmitotic cells (Yu et al., 1993). It is believed that microtubules are still nucleated from γ-tubulin in the centrosome, but soon after their formation, they are released into the cytosol, probably with the help of katanin (Baas, 1999). The lack of microtubules anchored on the centrosome perhaps makes it impossible for protein aggregates to be transported along the microtubules to the centrosome, which in turn may cause the formation of inclusion bodies at a different site. Regardless of this, it is still thermodynamically favorable for small aggregates to coalesce into a single large inclusion body, primarily because of gains in entropy, as misfolded proteins expose large amounts of their hydrophobic surface to water. The presence of parkin (Schlossmacher et al., 2002), its substrate α-synuclein (Spillantini et al., 1997; Shimura et al., 2001) and many ubiquitinated proteins (Lowe et al., 1988; Galvin et al., 1999) in the Lewy body suggests that parkin may be involved in the formation of this cytoplasmic inclusion. This is corroborated by the lack of Lewy bodies in PD patients with parkin mutations (Kitada et al., 1998). It appears that functional parkin may be required for the formation of Lewy bodies. Further studies using human postmortem tissues are necessary to find out whether γ-tubulin or the centrosome is involved in the formation of Lewy bodies in neurons, and if so, whether the interaction between γ-tubulin and parkin plays a role in the process.

The lactacystin-induced centrosomal accumulation of parkin (Figs 1, 4), its substrate such as CDCrel-1 (Fig. 8), ubiquitinated proteins (Fig. 8) (Johnston et al., 1998), chaperons (Garcia-Mata et al., 1999), and proteasomes (Wigley et al., 1999) appears to be a concerted response to an overwhelming increase of misfolded proteins caused by inhibition of their degradation. However, our data on MAP kinase, CREB (Fig. 3) and S6 kinase (data not shown) clearly showed that these proteins did not go to the centrosome when their degradation was inhibited. It is possible that they represent another category of proteins that do not easily misfold and aggregate. Proteins that are known to form aggresomes tend to be transmembrane proteins (e.g. CFTR, PS1) or membrane-associated proteins (e.g. CDCrel-1) that are prone to misfold in the cytosol (Johnston et al., 1998). These misfolded proteins may expose large patches of hydrophobic surface that would bind to and inactivate many cellular proteins non-specifically (Taylor et al., 2002). Their aggregation at the centrosome would minimize the toxic effect and expedite their degradation en masse by fusion with lysosomes in an autophagic manner (Klionsky and Emr, 2000).

In summary, our results demonstrate that parkin, an E3 ligases linked to PD, was recruited to the centrosome in response to inhibition of the 26 S proteasome. The recruitment is accompanied by the centrosomal accumulation of γ-tubulin, to which it binds, as well as its substrates CDCrel-1, which it ubiquitinates (Zhang et al., 2000). Thus, the centrosomal accumulation of parkin may enhance the ubiquitination of its substrates and facilitate their aggregation in the centrosome so that they can be efficiently degraded later. This novel function of parkin in mitotic cells may also play a role in the formation of inclusion bodies in neurons, which is an important process in neurodegenerative disorders including Parkinson's disease.

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