

approximately 50%. Our data of 21.3% is considerably lower than this. As our data are from a retrospective study, we might have underestimated the frequency of wearing off. In addition, we analyzed dyskinesia separately from wearing off. These factors might in part account for the lower frequency of wearing off. However, the data comparable to ours are also reported in the literature. Koller and associates³ reported 21% frequency of motor fluctuations with mean maintenance dose of L-dopa at 426 mg 5 years after the onset in their controlled prospective study. This frequency is comparable to ours (21.3% and 471.5 mg of L-dopa 5 years from the onset). It has frequently been claimed informally that relatively low frequency of wearing off among Japanese patients is due to lower L-dopa maintenance dose; however, according to the present study, this does not seem to hold true.

Generally, dyskinesia developed after wearing off in our study. If we compare Figures 6 and 7, frequency of dyskinesia is constantly lower than that of wearing off (21.3% vs. 8.4% at the end of the 5th year, 59.4% vs. 35.1% at the end of the 10th year, and 73.2% vs. 62.8% at the end of the 15th year, respectively). In the literature, frequencies of motor fluctuations have been reported variously. Caraceni and coworkers⁴ reported the frequency of motor fluctuations (wearing off and dyskinesia combined) as 29% at 4 years and 60% at 6 years from onset; average maintenance dose of L-dopa was 449 mg at 4 years and 403 mg at 6 years. The Parkinson Study Group⁵ reported the frequency of wearing off as 50% at 3 years with mean L-dopa dose of 329 mg and that of dyskinesia 30% with mean L-dopa dose of 387 mg. Thus, they also reported lower frequency of dyskinesia compared to wearing off with the same duration of the disease from the onset. Schrag and Quinn⁶ also reported higher incidence of wearing off (40%) compared with dyskinesia (28%) in their community based study (total $n = 124$). In their study, the prevalence of motor fluctuations was best predicted by disease duration and dose of L-dopa, whereas dyskinesias could be best predicted by duration of treatment.

Contrary to our and other data shown above, McColl and colleagues⁷ reported that dyskinesia appeared an average of 7 months before the onset of wearing off, which developed in 58% of the patients after a mean treatment period of 35 months. They studied the duration from the start of L-dopa treatment to the onset of motor fluctuations. We evaluated the duration from the onset of the disease to wearing off and dyskinesia, as development of wearing off depends mainly on the severity of nigral neurodegeneration. On the other hand, dyskinesia depends on both disease severity and the dose of L-dopa. Severe

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) victims developed wearing off and dyskinesia shortly after the initiation of L-dopa.⁸

Young-onset patients (50 or before) showed longer duration to reach Stage III, IV, and V suggesting the slower progression compared with late-onset patients. Diamond and associates⁹ also reported a more favorable prognosis for patients with onset under 50 years of age than those whose symptoms began in later years. In addition, in their report expected mortality rate was 1.82 for those with onset under 50, 2.17 for those with onset 50 to 59 years, and 2.20 for those with onset after 60. Our data also suggest a better prognosis for young-onset patients. But young-onset patients developed wearing off and dyskinesia faster than late-onset patients. Therefore, rate of nigrostriatal degeneration may not be slower in young-onset patients compared to late-onset patients. Relatively selective nigrostriatal dopaminergic degeneration without much of nondopaminergic involvement in young-onset patients would explain longer duration to reach Stage III, despite shorter duration to develop motor fluctuations.

Tremor-dominant patients showed significantly longer duration to reach Stage III compared to gait disturbance-onset patients. Also they showed longer duration to develop wearing off. It has been said frequently that tremor-dominant patients tend to show slower progression of symptoms compared to rigid-akinetic patients.¹⁰⁻¹³ Roos and coworkers¹⁴ also reported that tremor-dominant patients reached Stage III significantly later than patients with rigid-hypokinetic patients. Our findings are consistent with these data.

It has been shown in recent prospective studies that starting the treatment of PD with a dopamine agonist delayed the onset of motor fluctuations.^{15,16} Sixty-two percent of our patients received L-dopa as the initial treatment, 11.4% a dopamine agonist, and 26.6% other drugs such as an anticholinergic drug, amantadine, or selegiline. The number of patients who received a dopamine agonist as the initial treatment was too small to make definite conclusion whether or not starting treatment with a dopamine agonist would have delay the onset of motor fluctuations. In 1989, when this study was started, it was not a usual practice to use a dopamine agonist as the initial drug in Japan. Nonetheless, we looked at the effects of initial drug on the long-term outcome.

The initial L-dopa group showed slightly but significantly shorter duration to reach Stage III. But from the small number of the initial dopamine agonist group, we do not believe that this would mean that L-dopa was neurotoxic or other medications were neuroprotective.

The literature also does not support the idea that L-dopa is in some way neurotoxic to nigral neurons.¹⁷⁻²⁰ Types of initial treatment were of no effect on the mortality. Types of initial treatment were also of no effect on the time to develop wearing off or dyskinesia.

What was surprising to us was the significant sex difference between men and women in the time to reach Stage III, time to the onset of wearing off, time to the onset of dyskinesia, and the age at death in comparison with the life expectancy of male and female general Japanese populations. No participating neurologists expected such difference when they were treating their patients. Both time to wearing off and time to Stage III were slightly but significantly shorter for women in our study suggesting that the disease progression was slightly but significantly faster for female patients with PD. The most important contributing factor for the development of wearing off is believed to be loss of nigrostriatal dopaminergic nerve terminals. Therefore, the time to wearing off in some way may be reflecting the speed of the progression as the time to Stage III. Time to Stage IV and V did not show sex difference. Progression into Stage IV and V may be influenced not only by the natural progression of the disease but also by intervening incidents such as severe pneumonia, fractures, malignant syndrome, and other medical complications. Intervention by such events might have obscured the sex difference in advanced stages.

In the literature, Diamond and colleagues²¹ reported disability and mortality as having a sex difference. They analyzed longitudinal disability scores in 47 men and 23 women with PD following them up for 6 years. They found no significant differences between the sexes in mean disability scores in any of the 6 years. But they found a difference in mortality in that the observed to expected ratio for the men was 1.7457 and for the women 2.4740, a significantly greater excess in female mortality. Hely and associates²² reported faster progression as measured by modified Columbia Rating Scale score in female PD patients compared with male patients. Conversely, men did more poorly in other studies.¹¹⁻²³

In our total cohort, during the 14-year study period (1989-2002), 131 patients (71 of 793 men and 60 of 975 women) died. When we compared the average expected age at death in our PD patients from the linear regression curve of the age at death in PD patients (Figs. 9 and 10), there was a striking sex difference. As of year 2003, the life expectancies of the male and female general population were 78.45 years and 85.63, respectively. In contrast, average expected ages at death of the male and female PD patients calculated from the linear regression curves were 76.69 years (97.76% of the male general

population) and 76.23 (89.02% of the female general population), respectively. The age at death for female PD patients was essentially the same as that of male PD patients. Female PD patients lost approximately 8 years of longevity that Japanese women in the general population were entitled to entertain.

By reviewing the literature, the relative risk for mortality of PD patients was variously reported. Among the more than 20 reports on the mortality in PD, two studies reported less than 1.5 mortality (1.17 and 1.2) compared with the general background population,^{24,25} 11 studies reported mortality from 1.5 to 2.0 for PD patients,^{9,26-35} and 10 studies reported higher than 2.0 mortality for PD.^{34,36-44} Five studies reported sex differences: Diamond and coworkers⁹ reported mortality rates of 1.75 for men and 2.47 for women compared with the respective background populations; Wermuth and colleagues³⁴ reported relative risks for mortality of 1.92 for men and 2.47 for women; Minami and associates³⁵ reported relative risks for mortality of 1.74 for men and 1.97 for women; Ben-Shlomo and Marmot⁴⁰ reported a mortality rate of 2.6 without sex difference; Hely and colleagues²⁷ did not find sex differences in the standardized mortality ratios between men and women. On the contrary, Lilienfeld and associates⁴⁵ and Berger and coworkers⁴⁶ reported higher mortality for men compared with women. According to Berger and colleagues,⁴⁶ relative risk was 3.1 for men and 1.8 for women in European populations.

Riggs⁴⁷ reported an annual age-adjusted mortality rate in the United States, which was 19.15/100,000 at age 73.73 for men and 28.64/100,000 at age 78.99 for women each year from 1955 to 1986. Wermuth and associates³⁴ reported median ages of death at 77.29 years for men and 79.11 years for women in contrast with the median ages of death at 80.69 years for men and 84.37 years for women of the respective background populations. Elbaz and coworkers²⁹ reported the median survival of PD patients in Olmsted County, Minnesota, for the period of 1976 to 1995. They found 110 deaths in 196 PD cases and 79 deaths in 185 reference subjects. The median survival was 10.3 years in PD patients and 13.4 years in the reference subjects. The relative risk was 1.81 in women, and 1.49 in men. According to our data, mean disease duration from the onset to death was 13.2 years in men and 12.4 years in women (Table 5).

Imaizumi⁴⁴ studied age-specific mortality rates from PD in Japan from 1950 through 1993. The mortality rate was 2.45 per 100,000 for men and 2.12 per 100,000 for women at age 65.49 years from 1950 to 1951 and 1992 to 1993 compared with the general population. Nakashima and associates⁴⁸ studied mortality in another Japanese cohort of PD cases. They found that the mean

age at death was 75.95 ± 7.25 for men ($n = 57$) and 78.37 ± 6.69 ($n = 57$). According to our data, the mean age at death was 71.9 for men and 74.2 for women (Table 5). In both of these studies, age at death did not differ much between men and women, despite longer life expectancy by 7 to 8 years for women in Japan. Our female PD population will lose approximately 7 years of longevity over men once they get PD.

The reasons why female PD patients reached Stage III slightly but significantly earlier than men and developed wearing off and dyskinesia earlier are not known. Further studies are needed.

In summary, we reported long-term outcome on a large cohort of PD patients. Particularly we were interested in how long it would take to reach Stage III, IV, and V under a usual clinical practice setting. To our knowledge, no report has addressed this question on a large cohort with a long follow-up period.

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14-3-3 η is a novel regulator of parkin ubiquitin ligase

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Mutation of the *parkin* gene, which encodes an E3 ubiquitin-protein ligase, is the major cause of autosomal recessive juvenile parkinsonism (ARJP). Although various substrates for parkin have been identified, the mechanisms that regulate the ubiquitin ligase activity of parkin are poorly understood. Here we report that 14-3-3 η , a chaperone-like protein present abundantly in neurons, could bind to parkin and negatively regulate its ubiquitin ligase activity. Furthermore, 14-3-3 η could bind to the linker region of parkin but not parkin with ARJP-causing R42P, K161N, and T240R mutations. Intriguingly, α -synuclein (α -SN), another familial Parkinson's disease (PD) gene product, abrogated the 14-3-3 η -induced suppression of parkin activity. α -SN could bind tightly to 14-3-3 η and consequently sequester it from the parkin–14-3-3 η complex. PD-causing A30P and A53T mutants of α -SN could not bind 14-3-3 η , and failed to activate parkin. Our findings indicate that 14-3-3 η is a regulator that functionally links parkin and α -SN. The α -SN-positive and 14-3-3 η -negative control of parkin activity sheds new light on the pathophysiological roles of parkin.

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Introduction

In the last decade, people working in the field of Parkinson's disease (PD) witnessed a tremendous progress in uncovering the mechanisms of PD, and several familial PD genes were discovered in succession (Vila and Przedborski, 2004). Of these hereditary PD genes, *parkin* (*PARK2*), the causative gene of autosomal recessive juvenile parkinsonism (ARJP), is of a special interest because it encodes a ubiquitin ligase, a critical component of the pathway that covalently attaches ubiquitin to specific proteins with a polymerization step to

form a degradation signal (Shimura *et al*, 2000). Indeed, parkin catalyzes the addition of ubiquitin to target proteins prior to their destruction via the proteasome, suggesting that the misregulation of proteasomal degradation of parkin substrate(s) is deleterious to dopaminergic neurons (Dawson and Dawson, 2003; Bossy-Wetzel *et al*, 2004; Kahle and Haass, 2004). Consequently, impaired protein clearance can induce dopaminergic cell death, supporting the concept that defects in the ubiquitin–proteasome system may underlie nigral degeneration in ARJP and perhaps sporadic forms of PD (McNaught and Olanow, 2003). On the other hand, it was recently reported that parkin also catalyzes the formation of the K63-linked polyubiquitylation chain, independent of proteasomal destruction, in which the K48-linked polyubiquitylation chain is necessary (Doss-Pepe *et al*, 2005; Lim *et al*, 2005). Thus, it is plausible that parkin shares two roles as an E3 ligase; that is, one linking to and the other independent of the proteasome.

Among the products of major familial PD genes (Vila and Przedborski, 2004), α -synuclein (α -SN) is a product of familial PD gene (*PARK1*) identified as a presynaptic protein of unknown function. α -SN is considered in the molecular mechanisms of PD mainly because it is one of the major components of the cytoplasmic Lewy body (LB) inclusion present in the remaining nigral dopaminergic neurons of PD patients, which is the pathological hallmark of sporadic and some familial PDs (Forno, 1996). Although various studies have been conducted on α -SN (Dawson and Dawson, 2003; Bossy-Wetzel *et al*, 2004; Kahle and Haass, 2004), its pathophysiological role(s) and the interplay between α -SN and parkin are largely unknown.

To date, little is known about the role of parkin as a ubiquitin E3 ligase with respect to the underlying molecular mechanism(s) of ARJP or PD. Here we report for the first time that 14-3-3 η , a member of the 14-3-3 family (β/α , γ , ϵ , η , ζ/δ , σ , and τ/θ) (Berg *et al*, 2003; Bridges and Moorhead, 2004; Mackintosh, 2004) identified in LB (Kawamoto *et al*, 2002; Ubl *et al*, 2002), binds primarily to the linker region of parkin and functions as a novel negative regulator of parkin. We also show that α -SN relieves parkin activity suppressed by 14-3-3 η , indicating that 14-3-3 η is a novel molecule handling both parkin and α -SN, and that functionally links the two familial PD gene products.

Results

Parkin specifically interacts with 14-3-3 η but not with other 14-3-3 isoforms

We first examined the physical association of parkin with 14-3-3 isoforms, which are abundantly expressed in the brain (Martin *et al*, 1994; Baxter *et al*, 2002). Parkin was immunoprecipitated from mouse brain extracts, and the presence of 14-3-3 was analyzed by Western blotting (Figure 1A). 14-3-3 was clearly detected in the parkin immunoprecipitant, but not in those of control IgG or parkin antibody preabsorbed

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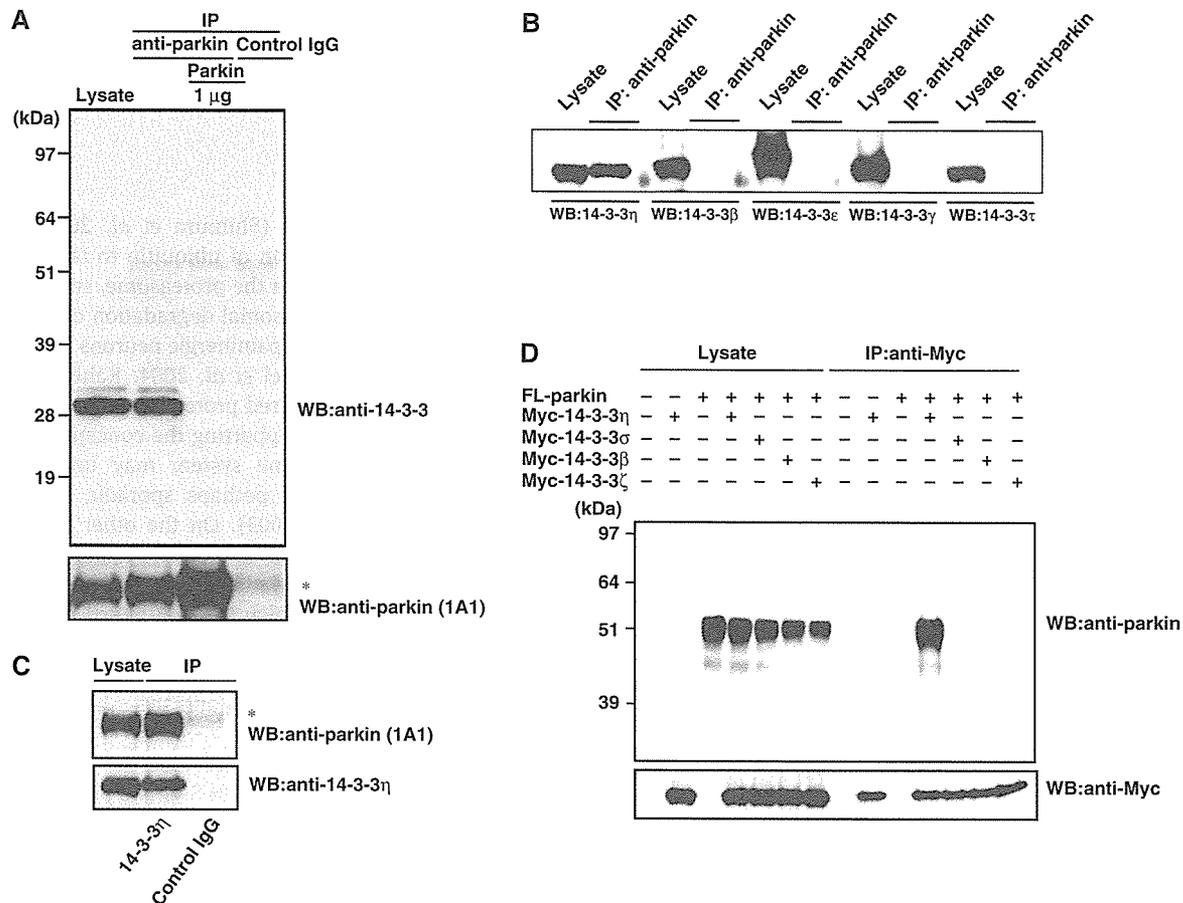


Figure 1 Physical interaction between parkin and 14-3-3 η . (A) Immunoprecipitation by anti-parkin antibody in the mouse brain. Mouse brain lysates were prepared and treated with anti-parkin or control IgG as described in Materials and methods. The resulting immunoprecipitates were subjected to SDS-PAGE, followed by Western blotting with anti-14-3-3 and parkin (1A1) antibodies. In all, 1 μ g of recombinant parkin was pretreated with anti-parkin prior to immunoprecipitation. Left lane: the brain lysate (1.5% input). Asterisk denotes an IgG heavy chain. (B) Specificity analysis of 14-3-3 species. The immunoprecipitation with anti-parkin and subsequent SDS-PAGE were carried out as in (A). Western blotting was conducted with antibodies against various 14-3-3 isoforms as indicated for lysates and anti-parkin immunoprecipitates. (C) Immunoprecipitation by anti-14-3-3 η antibody. After immunoprecipitation with anti-14-3-3 η or control IgG of the brain lysate, the immunoprecipitates were analyzed by Western blotting with anti-parkin (1A1) and 14-3-3 η antibodies, similar to (A). Left lane: the brain lysate (1.5% input). Asterisk denotes an IgG heavy chain. (D) Interaction between parkin and 14-3-3 η in HEK293 cells. FL-parkin (5 μ g), Myc-14-3-3 η , σ , β , or ζ (2 μ g) plasmids were transfected as indicated into HEK293 cells. After 48 h, the cell lysate was prepared and used for immunoprecipitation with anti-Myc antibody. The immunoprecipitates and the lysate (7.5% input) were analyzed by Western blotting with anti-parkin and Myc antibodies, as in (A).

with recombinant parkin protein (1 μ g). Intriguingly, two 14-3-3 signals were evident: a faint band and a strongly stained band, indicating that the 14-3-3 may form homo- and/or hetero-dimers. Subsequently, we determined the type(s) of 14-3-3 species that interacts with parkin in the mouse brain in more detail. In the parkin immunoprecipitant, 14-3-3 η , but not other 14-3-3 isoforms examined, that is, β , γ , ϵ , and τ , was detected (Figure 1B). In the next step, we examined whether parkin is coimmunoprecipitated with anti-14-3-3 η antibody and found parkin in the 14-3-3 η immunoprecipitant (Figure 1C). These reciprocal immunoprecipitation experiments revealed that parkin is associated with 14-3-3 η in the mouse brain.

To confirm the specific interaction of parkin with 14-3-3 η , Myc-tagged 14-3-3 η , σ , β , or ζ was cotransfected with FLAG (FL)-parkin into HEK293 cells, and their interactions were tested. FL-parkin was detected in the immunoprecipitant of Myc-14-3-3 η , but not those of Myc-14-3-3 σ , β , and ζ

(Figure 1D). Taken together with the results of Figure 1B, our data indicate that parkin mainly interacts with 14-3-3 η .

Parkin domain interacts with 14-3-3 η

We next investigated the region of parkin necessary for interaction with 14-3-3 η . Structurally, parkin is characterized by the presence of the N-terminal ubiquitin-like domain (UBL) (which is highly homologous to ubiquitin), the C-terminal RING box, consisting of two RING finger motifs, RING1 and RING2, flanked by one IBR (in between RING finger) motif, and a linker region, which connects these N- and C-terminal regions (Shimura *et al*, 2000). In these experiments, various deletion mutants of FL-tagged parkin were expressed in HEK293 cells and immunoprecipitated by FL-antibody beads (Figure 2A). FL-parkin or its derivatives on the beads were further incubated with cell lysates that expressed Myc-14-3-3 η , and then the amounts of Myc-14-3-3 η bound to the beads were determined (Figure 2B).

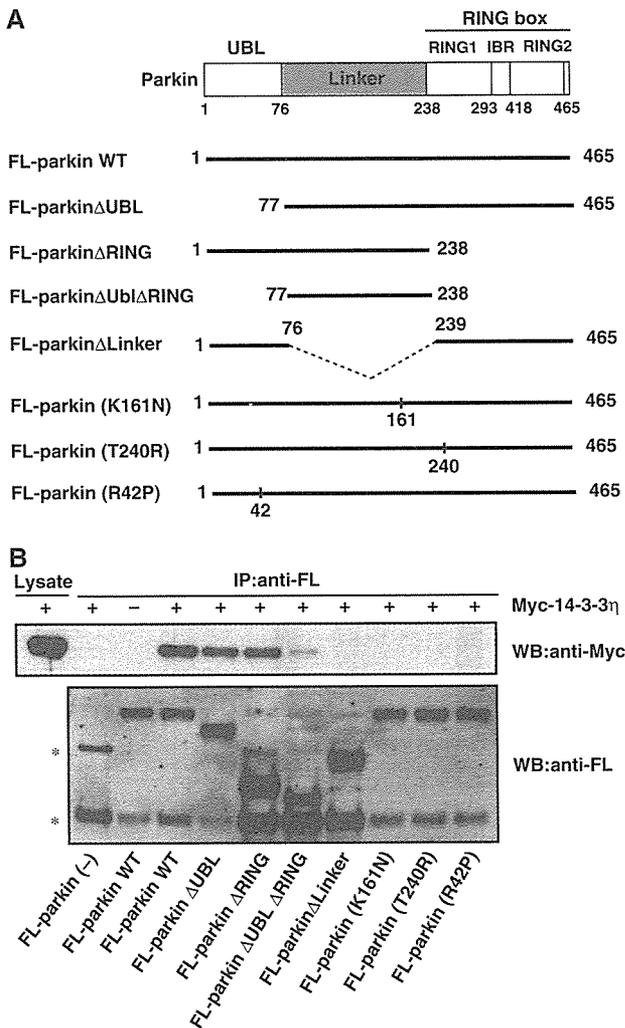


Figure 2 Domain analysis of the parkin region that interacts with 14-3-3 η . (A) Schematic representation of WT parkin and its deletion- and disease-related missense mutants. See text for the domain structures of parkin and mutants. The dotted line denotes the deleted region. (B) Interaction between 14-3-3 η and parkin mutants. FL-parkin (2 μ g) or its mutant (10 μ g) plasmids were transfected into HEK293 cells, as described in Figure 1D. The cell lysates (200–600 μ l) were immunoprecipitated with anti-FL-antibody beads. Note that various amounts of the lysates were used to adjust roughly the levels of expressed parkin mutants. The resulting immunoprecipitates were mixed with other cell lysates (200 μ l) prepared from cells that had been transfected with Myc-14-3-3 η plasmid (2 μ g) and incubated for 6 h at 4°C. Then, the extensively washed immunoprecipitates and cell lysate (7.5% input) were analyzed by Western blotting with anti-Myc and FL antibodies. Asterisks denote nonspecific bands.

The full-length parkin could bind 14-3-3 η . Deletion of either UBL or RING-box domain reduced the binding compared to the full-length parkin, although these deletion mutants retained the ability to bind to 14-3-3 η . Furthermore, mutants with combined deletions of the UBL and RING-box domains, that is, the linker region, could also bind 14-3-3 η to a lesser extent. Conversely, deletion of the linker region resulted in the loss of ability to bind 14-3-3 η . Taken together, it is concluded that the linker region is necessary for the interaction between parkin and 14-3-3 η , although the UBL and RING-box domains may enhance the binding affinity.

Interestingly, the ARJP disease-causing missense mutation within the linker region, that is, parkin(K161N), in which the Lys residue at position 161 was replaced by Asn residue, showed complete loss of binding to 14-3-3 η , confirming the importance of the linker region in the interaction between 14-3-3 η and parkin. Unexpectedly, other disease-causing missense mutations of the UBL region, parkin(R42P), and the RING1 region, parkin(T240R), also showed complete loss of interaction with 14-3-3 η (Figure 2). Thus, although the UBL and RING-box domains are not primarily required for the binding, both R42P and T240R mutations in the UBL and RING-box domains, respectively, deleteriously affect the neighboring linker domain. Alternatively, since 14-3-3 is known to form a homo- or hetero-dimer, and thus has two binding sites (Aitken *et al*, 2002), it is plausible that 14-3-3 η interacts with two distinct regions of parkin, one major site of which is the linker region.

Effect of suppression of 14-3-3 η on parkin E3 activity

We next investigated the role of parkin-14-3-3 η binding on parkin activity. At first, we tested its effect on the ubiquitin ligase activity of parkin. We incubated recombinant His-parkin with ubiquitin, E1, and E2 (UbcH7) *in vitro*. Under this condition, His-parkin appeared as a smear band, which likely reflects self-ubiquitylation (Figure 3A). Addition of recombinant GST-14-3-3 η (Figure 3A, left panel) or untagged 14-3-3 η (Figure 3A, right panel) to the reaction reduced the smear of His-parkin, and such reduction was proportionate to the added amount of GST-14-3-3 η or 14-3-3 η and resulted in the recovery of His-parkin of intact size. In addition, we found that 14-3-3 η had no effect on the ubiquitylating activity of phosphorylated I κ B α by a fully *in vitro* reconstituted system, containing E1, E2 (Ubc4), and E3 (the SCF $^{\beta$ TrCP complex; Kawakami *et al*, 2001), indicating that 14-3-3 η does not interfere with ubiquitylating reactions in general (data not shown). These results strongly suggest that 14-3-3 η suppresses the intrinsic self-ubiquitylation activity of parkin.

We next tested whether 14-3-3 η also affects the ubiquitylation activity of parkin in HEK293 cells. First, we examined the self-ubiquitylation of parkin, whose activity was observed by cotransfections of HA-ubiquitin and FL-parkin. Myc-14-3-3 η almost completely suppressed the self-ubiquitylation activity of parkin, while Myc-14-3-3 σ , β , and ζ had no inhibitory effect (Figure 3B), indicating the specific role of 14-3-3 η for parkin. Second, we examined the effect of 14-3-3 η on the ubiquitylation of a model substrate for parkin. When V5-tagged synphilin-1, a known parkin substrate (Chung *et al*, 2001), was transfected with FL-parkin and HA-ubiquitin in the cells, V5-synphilin-1 was found in ubiquitylated form, as demonstrated by the poly-ubiquitin chain formation (detected by anti-HA antibody) in anti-V5 immunoprecipitant (Figure 3C, top panel). V5-synphilin-1 was not ubiquitylated when FL-parkin was not cotransfected, suggesting that this ubiquitylation is mediated by coexpressed FL-parkin. Indeed, FL-parkin was found to be associated with V5-synphilin-1, further supporting the above notion (Figure 3C, second panel from the top). Note that the polyubiquitylated bands observed as the smear profile were considered to include not only major synphilin-1 bands over 90-kDa size but also self-ubiquitylated bands of parkin over 52-kDa size.

In the next step, we tested the effects of 14-3-3 η on the ubiquitylation and binding activities of parkin to

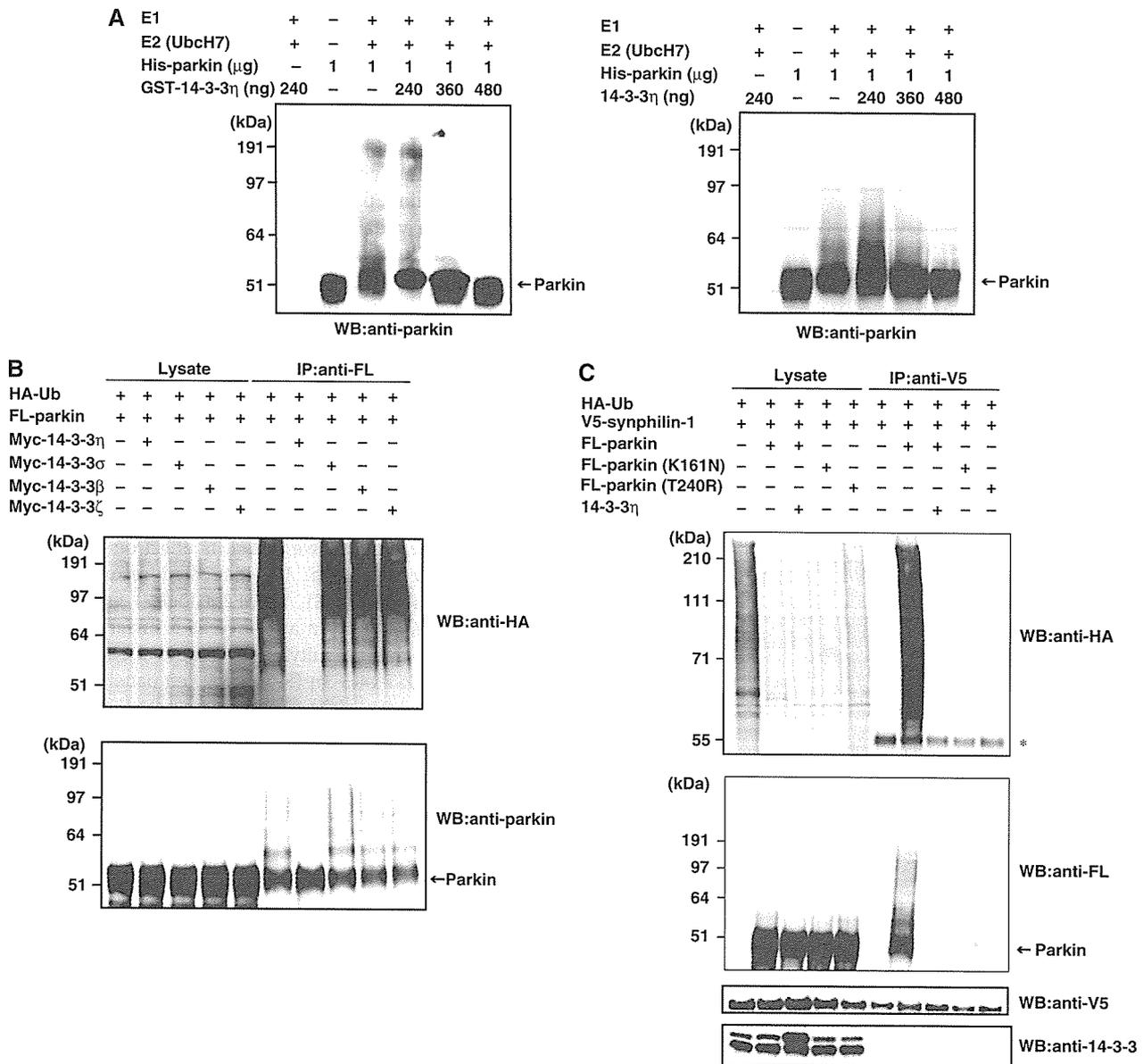


Figure 3 Effects of 14-3-3 η on the E3 activity of parkin. (A) *In vitro* autoubiquitylation. The ubiquitylating assay was conducted as described in Materials and methods with or without various amounts of GST-14-3-3 η (left panel) or 14-3-3 η (right panel). After incubation, the reaction mixtures were subjected to SDS-PAGE, followed by Western blotting with anti-parkin. Arrow on the right indicates the position of His-parkin. (B) *In vivo* autoubiquitylation. HA-Ub (3 μ g), FL-parkin (3 μ g), and Myc-14-3-3 η , σ , β , or ζ (6 μ g) plasmids were transfected for 48 h into HEK293 cells as indicated. After immunoprecipitation with anti-FL, Western blotting was performed using antibodies against HA and parkin. Western blotting of all lysates was performed to test the expression levels (Lysate). (C) Ubiquitylation of synphilin-1 in HEK293 cells. HA-Ub (2 μ g), FL-parkin (3 μ g), FL-parkin(K161N) (3 μ g), FL-parkin(T240R) (3 μ g), Myc-14-3-3 η (6 μ g), and V5-synphilin-1 (4 μ g) plasmids were transfected into HEK293 cells as in (B) at the indicated combinations. After immunoprecipitation with anti-V5 antibody, Western blotting was performed using antibodies against HA, FL, V5, and 14-3-3. Asterisk denotes an IgG heavy chain.

V5-synphilin-1. Cotransfection of 14-3-3 η resulted in almost complete inhibition of the ubiquitylation of synphilin-1 by parkin and/or self-ubiquitylation of parkin (Figure 3C, top and second panels), as well as inhibition of the interaction between synphilin-1 and parkin (Figure 3C, second panel). 14-3-3 η did not interact with synphilin-1 (Figure 3C, bottom panel). Taken together, these results suggest that 14-3-3 η does not only inhibit the intrinsic ubiquitylation activity of parkin, but also its binding activity to the substrate and its ubiquitylation.

The ARJP disease-related parkin(K161N) and parkin(T240R) mutants, which cannot bind with 14-3-3 η ,

could not bind and ubiquitylate synphilin-1 and/or self-ubiquitylation of parkin even in the absence of 14-3-3 η (Figure 3C, top panel). Hence, the linker and RING-box domains of parkin are essential not only for the negative regulation by 14-3-3 η , but also for the substrate recognition and ubiquitin-ligase activity. These results illustrate the importance of these regions of parkin on its positive and negative regulation.

Since parkin is known to associate with E2 (Shimura *et al*, 2000), we also examined the effect of 14-3-3 η on the ability of parkin to recruit E2. For this purpose, we coexpressed HA-parkin with FL-UbcH7 or FL-Ubc7, both of which are known

to bind to parkin (Shimura *et al* 2000; Imai *et al*, 2001). Almost the same amounts of UbcH7 (Figure 4A) and Ubc7 (Figure 4B) were detected in the anti-parkin immunoprecipitants irrespective of cotransfection with Myc-14-3-3 η . These findings indicate that 14-3-3 η does not influence the recruitment of E2, that is, UbcH7 or Ubc7, to parkin.

α -Synuclein abrogates 14-3-3 η -related parkin inactivation

Based on the above findings, we next examined the mechanism that regulates the 14-3-3 η -parkin binding. As α -SN partly has a high homology to 14-3-3 isoforms (Ostrerova *et al*, 1999), we tested the effects of α -SN on the 14-3-3 η -induced suppression of parkin. By cotransfection experiments in HEK293 cells, parkin again ubiquitylated synphilin-1, and 14-3-3 η inhibited the parkin-mediated ubiquitylation (Figure 5A). Coexpression of α -SN resulted in the recovery of ubiquitylation of synphilin-1 and the association of synphilin-1 with parkin, suggesting that α -SN abrogates the 14-3-3 η -induced suppression of parkin (Figure 5A, top panel). Importantly, the familial PD-related mutants of α -SN(A30P) (Kruger *et al*, 1998) and α -SN(A53T) (Polymeropoulos *et al*, 1997) could not abrogate the inhibitory role of 14-3-3 η . Similar results were observed by detection of self-ubiquitylation activity of parkin (Figure 5A, second panel).

We then tested whether α -SN can release the binding of Myc-14-3-3 η from parkin in cotransfection experiment. As shown in Figure 5B (top panel), FL-parkin was self-ubiquitylated in the absence of 14-3-3 η . Coexpression of 14-3-3 η inhibited the self-ubiquitylation of parkin, and this was accompanied by the binding of 14-3-3 η to FL-parkin. Coexpression of α -SN abrogated the binding of 14-3-3 η to

parkin and resulted in the recovery of self-ubiquitylation of parkin. These effects were not seen by coexpression of α -SN(A30P) and α -SN(A53T) (Figure 5B, top panel). In addition, while the 14-3-3 η -parkin interaction was considerably reduced by α -SN, it was not reduced by α -SN(A30P) or α -SN(A53T) (Figure 5B, bottom panel). Taken together, α -SN, but not α -SN(A30P) or α -SN(A53T), binds strongly to 14-3-3 η and thereby releases parkin from the parkin-14-3-3 η complex.

We also tested the interaction of Myc-14-3-3 η with FL- α -SN, FL- α -SN(A30P), and FL- α -SN(A53T). Myc-14-3-3 η interacted only with FL- α -SN, but not α -SN(A30P) nor α -SN(A53T) (Figure 5C, upper-top panel), suggesting that α -SN relieves parkin activity from binding to 14-3-3 η . The 14-3-3 η / α -SN interaction was not affected by parkin (Figure 5C, upper-top panel), and parkin was not associated with α -SN (Figure 5C, upper-second panel). Interestingly, FL- α -SN did not interact with Myc-14-3-3 σ , β , and ζ in the same experiment (Figure 5C, lower panel). These results further strengthen the notion that α -SN specifically activates parkin through binding 14-3-3 η .

We then investigated whether the interaction of 14-3-3 η and parkin is direct or indirect by using purified recombinant His-parkin and GST-14-3-3 η . GST or GST-14-3-3 η was mixed with His-parkin, and pulled down by glutathione beads. His-parkin bound to GST-14-3-3 η , but not GST (Figure 5D, left panel), indicating that parkin directly interacts with 14-3-3 η . On the other hand, a similar *in vitro* binding assay showed that GST-14-3-3 η did not interact with recombinant α -SN (Figure 5D, right panel), suggesting that certain modification(s) of α -SN may be required for the interaction of 14-3-3 η .

Subsequently, we measured the binding affinities of parkin and α -SN for 14-3-3 η by the surface plasmon resonance (SPR) method. As shown in Figure 5E, parkin bound 14-3-3 η with a considerably strong affinity ($K_d = 4.2$ nM, upper), whereas the affinity of α -SN for 14-3-3 η was much lower than that of parkin ($K_d = 1.1$ μ M, lower). These results are consistent with those of the immunoprecipitation/Western analysis using recombinant proteins (Figure 5D).

Finally, we examined whether 14-3-3 η bound to parkin can be released by α -SN. To test this, we first mixed the lysates coexpressing FL-parkin and Myc-14-3-3 η of HEK293 cells with those expressing α -SN. Then the mixtures were incubated under three different conditions, as indicated in the upper panel of Figure 5F. Next, the lysates were immunoprecipitated with anti-FL antibody, and followed by Western blotting with anti-Myc and anti-parkin antibodies. As shown in Figure 5F (upper panel), the amount of 14-3-3 η bound to parkin was significantly lower in all incubation conditions, when the cell lysates that simultaneously expressed both parkin and 14-3-3 η were incubated with α -SN-expressing lysates. Incubation for 1 h at 37°C reduced the amount of 14-3-3 η bound to parkin in proportion to the added amount of α -SN-expressing cell lysate (Figure 5F, lower panel). Intriguingly, the α -SN(A30P) and α -SN(A53T) mutants had no effect on the release of 14-3-3 η , unlike wild-type (WT) α -SN (Figure 5F, lower panel). These observations strongly indicate that α -SN, but not α -SN(A30P) or α -SN(A53T), can capture and release 14-3-3 η from the parkin-14-3-3 η complex, which supports our notion that the negative regulation of parkin activity by 14-3-3 η is relieved by α -SN (Figure 5A and B).

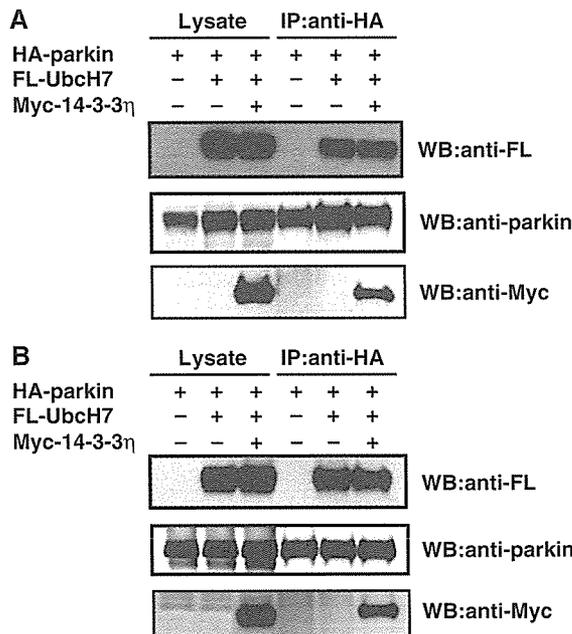
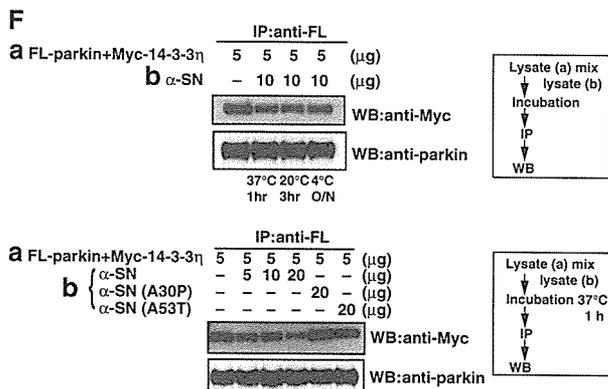
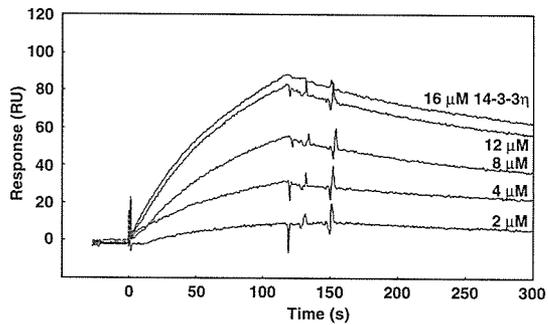
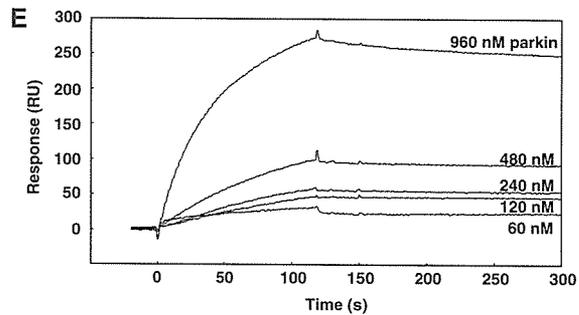
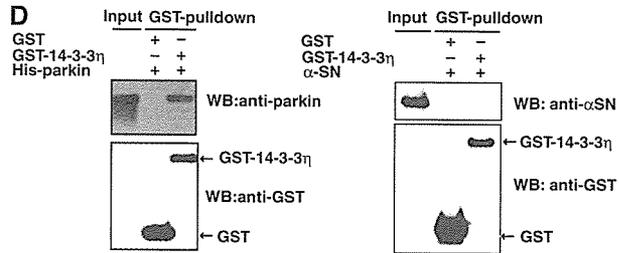
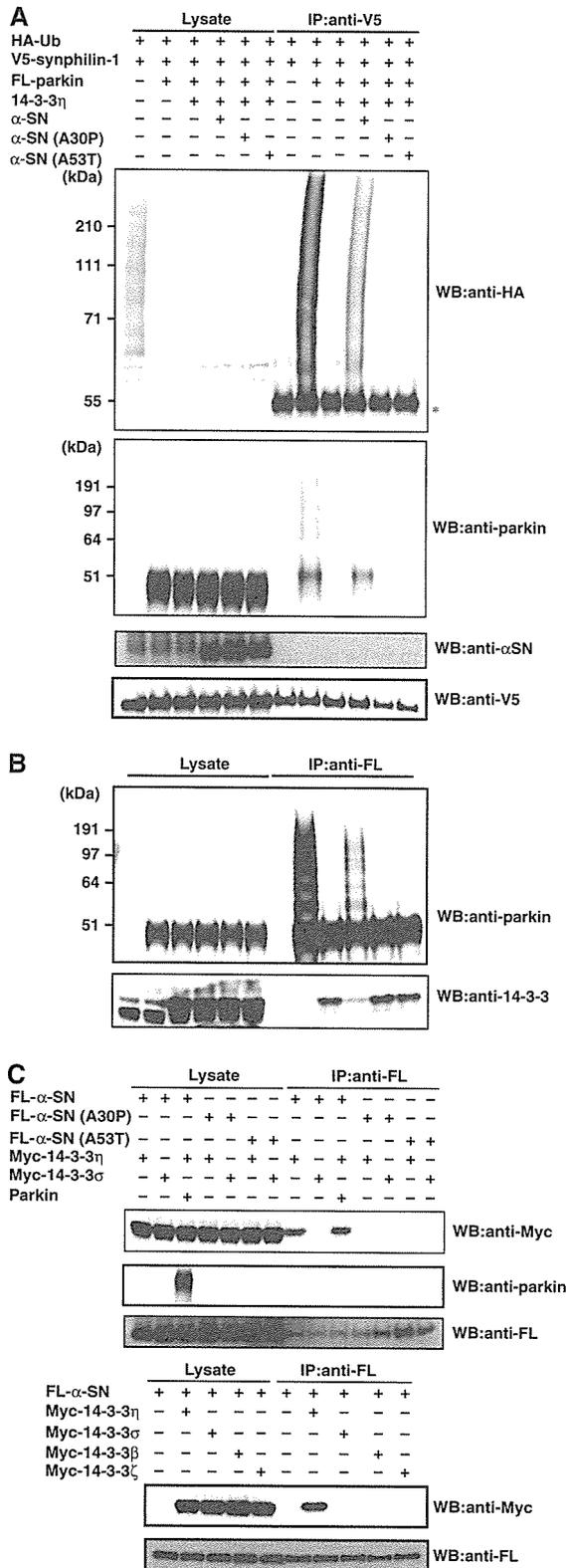


Figure 4 Effect of 14-3-3 η on the recruitment of E2 (UbcH7 or Ubc7) to parkin. (A) HA-parkin (3 μ g), FL-UbcH7 (3 μ g), or Myc-14-3-3 η (6 μ g) plasmids were transfected for 48 h into HEK293 cells at the indicated combinations. After immunoprecipitation with anti-HA antibody, Western blotting was performed using antibodies against FL, Myc, and parkin. (B) The experiment was conducted as in (A), except that FL-Ubc7 was used instead of FL-UbcH7.

Parkin, 14-3-3 η , and α -SN levels in the substantia nigra of PD

Finally, we analyzed the levels of parkin, 14-3-3 η , and α -SN in the substantia nigra of the midbrain from patients with sporadic PD. Western blotting revealed no significant differences in parkin, 14-3-3 η , and actin in the substantia nigra

between control (patients without PD) and PD patients, whereas α -SN was significantly increased in the substantia nigra of PD patients (Figure 6A, upper panel). As parkin did not interact physically with α -SN in our immunoprecipitation analysis (Figure 5C; data not shown), we then examined the interactions of 14-3-3 η with parkin or α -SN by measuring



these proteins in the anti-14-3-3 η immunoprecipitant. Whereas the levels of parkin associated with 14-3-3 η from PD appeared to be decreased relative to the control, the levels of α -SN that interacted with 14-3-3 η were clearly increased in patients with PD (Figure 6A, lower panel). Thus, it is suggested that the elevated levels of α -SN are associated with its interaction with 14-3-3 η and the activity of parkin may be aberrantly regulated in the substantia nigra of sporadic PD.

Discussion

The major finding of the present study was the identification of 14-3-3 η as a novel regulator of parkin. First, parkin was in a complex with 14-3-3 η , but not β , γ , ϵ , or τ isoforms, in the mouse brain (Figure 1). 14-3-3 η could bind primarily to the linker region of parkin, but not with the ARJP-causing missense mutant parkin (K161N), which has a mutation in the

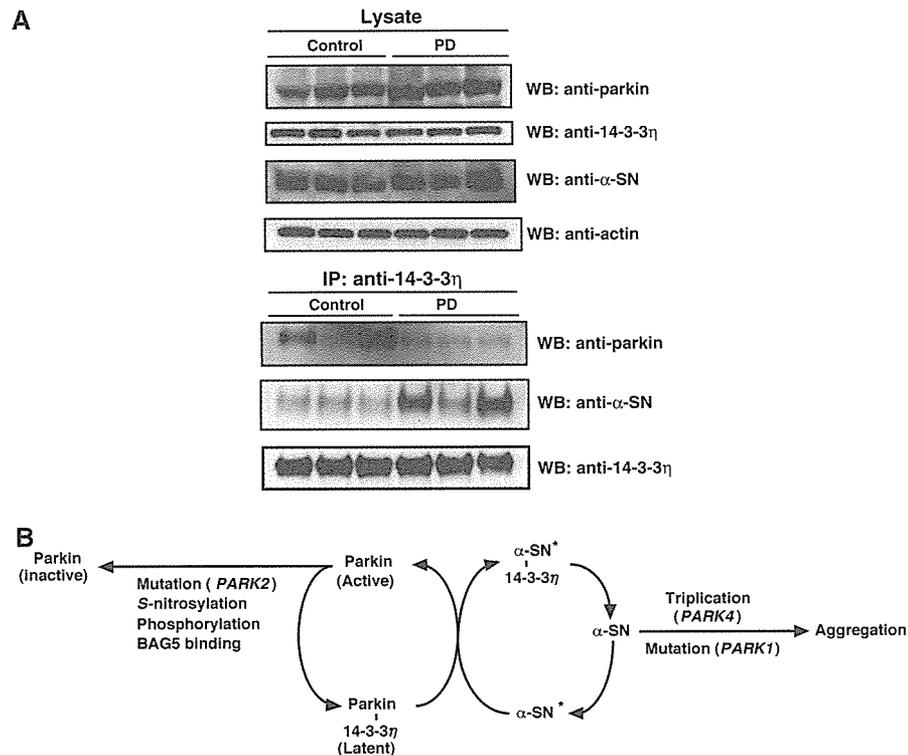


Figure 6 (A) Levels of parkin, 14-3-3 η , and α -SN in the substantia nigra of PD. Brain of a representative patient with PD (upper panel). Samples (30 μ g) of the crude extract of the brains (substantia nigra) of control (patients without PD) and PD patients were subjected to SDS-PAGE, following Western blotting against antibodies against parkin, 14-3-3 η , α -SN, and actin. Physical interaction between 14-3-3 η and parkin or α -SN (lower panel). After the same samples used in the upper panel were immunoprecipitated with anti-14-3-3 η , Western blotting was carried out using antibodies against parkin, α -SN, and 14-3-3 η . (B) A schematic diagram showing the pathways involved in the regulation of parkin activity by 14-3-3 η and α -SN. α -SN, α -synuclein; α -SN*, modified form of α -SN. Note that whether parkin is phosphorylated to bind to 14-3-3 η remains unknown at present. See text for details.

Figure 5 Effects of α -SN on 14-3-3 η -induced suppression of parkin E3 activity and interaction between 14-3-3 η and α -SN in HEK293 cells. (A) Ubiquitylation of synphilin-1. Transfection was conducted at various combinations, as in Figure 3C, except for cotransfection of 4 μ g of α -SN, α -SN(A30P), and α -SN(A53T). After immunoprecipitation with anti-V5 antibody, Western blotting was carried out with antibodies against HA, parkin and α -SN, and V5. Asterisk denotes an IgG heavy chain. (B) Autoubiquitylation of parkin. Transfection was performed as in (A). After immunoprecipitation with anti-FL antibody, Western blotting was carried out with antibodies against parkin and 14-3-3. (C) Interaction of 14-3-3 η and α -SN with or without parkin. Various expression vectors at the indicated combinations were transfected. Immunoprecipitation was conducted by anti-FL antibody and the resulting immunoprecipitates were used for Western blotting with antibodies against Myc, parkin, and FL. (D) Physical interaction between 14-3-3 η and parkin (left panel) or α -SN (right panel) in recombinant proteins. After recombinant His-tagged parkin produced from baculovirus-infected HiFive insect cells (3 μ g) or GST- α -SN expressed in *E. coli* whose GST moiety was removed by PreScission Protease digestion prior to use (3 μ g) was incubated for 1 h at 32°C with 3 μ g of GST or GST-tagged 14-3-3 η expressed in *E. coli*, glutathione-Sepharose was added and the incubation vessels were slowly rotated for 3 h at 4°C. The washed Sepharose resin was eluted with 50 μ l of 50 mM Tris-HCl (pH 8.0) buffer containing 10 mM reduced glutathione, and aliquots (15 μ l) were analyzed by Western blotting with antibodies against parkin (left-top panel), α -SN (right-top panel), and GST (bottom panel). Input: 500 ng of parkin or α -SN. (E) SPR analyses of parkin and α -SN binding to 14-3-3 η . Upper: subtracted sensorgrams of interaction between a subset of parkin concentrations and immobilized 14-3-3 η . Lower: subtracted sensorgrams of interaction between a subset of 14-3-3 η concentrations and immobilized α -SN. (F) Sequestration of 14-3-3 η by α -SN from the parkin-14-3-3 η complex. Various expression vectors were transfected as indicated. Upper panel: 5 μ g of the lysate-(a) from cells co-expressing FL-parkin and Myc-14-3-3 η were mixed with 10 μ g of cellular lysate-(b) expressing α -SN. The mixtures were incubated under various conditions; that is, 37°C for 1 h, 20°C for 3 h, or overnight at 4°C (O/N), then immunoprecipitation by anti-FL antibody was conducted, followed by Western blotting with antibodies against Myc (14-3-3 η) and parkin. Lower panel: the experiments were conducted as for the top panel, except that incubation was carried out at 37°C for 1 h using α -SN-, α -SN(A30P)-, or α -SN (A53T)-expressing lysates as indicated. The experimental protocol is shown in the flow charts on the right.

linker region (Figure 2). Second, the binding of 14-3-3 η to parkin was associated with suppression of the ubiquitin-ligase activity, suggesting that certain parkin bound to 14-3-3 η is present at a latent status in the brain (Figure 3). Third, overexpression of α -SN abrogated the 14-3-3 η -induced suppression of parkin activity, indicating that α -SN relieves the negative regulation of parkin by 14-3-3 η (Figure 5A and B). Intriguingly, PD-causing A30P and A53T mutations of α -SN could not bind 14-3-3 η and failed to activate parkin. These results indicate that 14-3-3 η is a regulator that functionally links parkin and α -SN, as illustrated in Figure 6B.

It is of particular note that we report unusual isoform specificity for 14-3-3 η to interact with parkin among all 14-3-3 species examined. However, the possibility that the other species are also involved in the interaction by forming a heterodimer with 14-3-3 η cannot be excluded *in vivo*, because 14-3-3 bands immunoprecipitated by anti-parkin antibody from the brain extracts showed doublet with one weak signal for Western blotting (Figure 1A). Nevertheless, herein we address that recombinant parkin could directly bind to 14-3-3 η (Figure 5D, left panel), with considerably high affinity (K_d = approximately 4 nM) (Figure 5E) and that the 14-3-3 η homodimer is a negative factor for autoubiquitylating activity of parkin *in vitro* (Figure 3A).

It is known that the 14-3-3 family proteins interact with the majority, but not all, proteins after their phosphorylation (Aitken *et al*, 2002; Bridges and Moorhead, 2004; Mackintosh, 2004). Indeed, parkin contains the RKDSPP sequence in the linker region that resembles the typical binding motifs with a potential phosphorylation residue for 14-3-3 proteins (Yaffe *et al*, 1997; Mackintosh, 2004). It is also known that parkin has several possible phosphorylation sites, and recent studies showed that parkin is phosphorylated *in vitro* (Yamamoto *et al*, 2004), although there is no direct evidence demonstrating phosphorylation of parkin *in vivo* to date. However, it remains elusive whether or not phosphorylation of parkin is responsible for its specific binding to 14-3-3 η , because known potential phosphorylation motifs are capable of associating with many 14-3-3 species in general. The specificities of 14-3-3: client-protein interactions do not result from different specificities for the phosphopeptide-binding motifs, but probably arises from contacts made on the variable surface of 14-3-3 outside the binding cleft, as discussed previously by Yaffe *et al* (1997). In this regard, some reports showed functional specificities of 14-3-3 isoforms (Aitken, 2002; Aitken *et al*, 2002; Roberts and de Bruxelles, 2002), and indeed several enzymes retain several nonphosphorylated binding motifs for 14-3-3s (Hallberg, 2002; Sribar *et al*, 2003), though parkin lacks such 14-3-3-interacting sequences. Thus, parkin, in particular its linker region, may have a new binding motif(s) for 14-3-3 η , but the interacting motif(s) remains to be identified. If 14-3-3 η binds to parkin through two sites as a dimmer, it is plausible that the phosphorylation of parkin is involved in their interactions at least in part.

With regard to the mechanistic action of 14-3-3 η , it may suppress parkin activity by preventing access of the substrate, because the binding of synphilin-1 (used here as a model substrate to parkin) was inhibited by 14-3-3 η (Figure 3C). Accumulating evidence suggests that parkin can bind various targets by the UBL domain or the RING box, in particular the RING 1 domain (Dawson and Dawson, 2003). Accordingly,

14-3-3 η may have function(s) other than suppressing the access of the substrate to parkin. Indeed, 14-3-3 η strongly inhibits substrate-independent self-ubiquitylation of parkin, indicating blockage of the intrinsic E3 activity. It was also anticipated that 14-3-3 η hinders the recruitment of E2 to parkin. However, this was not the case, because 14-3-3 η had no effect on the binding of UbcH7 and Ubc7 to parkin (Figure 4). Thus, while the mechanism of 14-3-3 η -induced suppression of parkin activity remains to be identified, it is possible that it involves preventing the positioning of the ubiquitin-charged E2 toward the target Lys residue by steric hindrance due to the association of 14-3-3 η to parkin.

It is worth noting that parkin does not interact with α -SN directly, because we could not demonstrate the physical binding of parkin to α -SN *in vivo* and *in vitro* (data not shown; see also Dawson and Dawson, 2003). Nevertheless, we found that the negative regulation of parkin by 14-3-3 η was relieved by α -SN, which could bind tightly to 14-3-3 η *in vivo* (Figure 5A–C). In this regard, it is of note that the amounts of 14-3-3 η bound to parkin were decreased when the lysates of cells coexpressing parkin and 14-3-3 η were incubated with those expressing WT α -SN, but not PD-related α -SN(A30P) or α -SN(A53T) mutants *in vitro* (Figure 5F). These results clearly indicate that 14-3-3 η bound to parkin is sequestered by α -SN, but not competition by α -SN toward the binding of 14-3-3 η to parkin. Unlike the association between parkin and 14-3-3 η , there is little or no interaction between α -SN and 14-3-3 η *in vitro*, as recombinant α -SN did not bind to 14-3-3 η (Figure 5D, right panel, and E). Thus, it is plausible that certain modification(s) of α -SN is required for its association to 14-3-3 η in mammalian cells (see our model in Figure 6B). Judging from the characteristic properties of 14-3-3 family proteins capable of binding many phosphorylated proteins (Yaffe *et al*, 1997), certain phosphorylation(s) of α -SN seems quite possible for the interaction with 14-3-3 η . Indeed, there are several reports regarding phosphorylation of α -SN (Fujiwara *et al*, 2002; Hirai *et al*, 2004). Although previous studies clearly demonstrated that α -SN deposited in synucleinopathy brains is extensively phosphorylated at Ser-129 (Fujiwara *et al*, 2002; Hirai *et al*, 2004), this is probably not the case in our study, because the chemically synthesized peptide phosphorylated at Ser-129 of α -SN did not bind to the 14-3-3 η (our unpublished results). However, the possibility that α -SN is phosphorylated at other site(s) cannot be exclusively ruled out. Alternatively, one cannot exclude a possible, though yet unknown, modification(s) of α -SN other than phosphorylation as a mechanism responsible for the increased affinity toward 14-3-3 η . In this regard, α -SN is structurally related to 14-3-3 family proteins (Ostremova *et al*, 1999), but it is unknown whether the homologous region is involved in the physical interaction with α -SN. Further studies are required to clarify the mode of α -SN modification.

In the present study, we found reciprocal regulation of parkin activity by α -SN and 14-3-3 η , whose tripartite control could enhance our understanding of the pathogenesis of PD. As illustrated in Figure 6B, to date there are several reports on the post-translational modification of parkin. Recent findings indicate that the ubiquitin E3 ligase activity of parkin is modified by nitric oxide (NO). Namely, parkin is S-nitrosylated in PD patients and an *in vivo* mouse model of PD, and S-nitrosylation shows inhibition of the E3 activity of

parkin (Chung *et al*, 2004; Kahle and Haass, 2004; Yao *et al*, 2004), which could contribute to the degenerative process in PD by impairing the ubiquitylation of parkin substrates. Moreover, Kalia *et al* (2004) showed that the bcl-2-associated athanogene 5 (BAG5) enhanced the death of dopaminergic neurons in an *in vivo* model of PD by inhibiting the E3 ligase activity of parkin. In addition, recent studies reported that phosphorylation of parkin causes a small but significant reduction of parkin auto-ubiquitylating activity (Yamamoto *et al*, 2004). More recently, it was reported that Nrdp1/FLRF RING-finger E3 ligase binds and ubiquitylates parkin, resulting in reduction of parkin activity, implying its involvement in the pathogenesis of PD (Zhong *et al*, 2005). Considered together, these results indicate that the apparent loss of parkin E3 ubiquitin ligase activity associated with the pathogenesis of PD (see the model displayed in Figure 6B) is in agreement with the ARJP-linked mutations that lead to loss of function of parkin, and that the functional loss of parkin activity is linked to the death of dopaminergic neurons. In addition, we reported herein the imbalance of tripartite interactions among parkin, 14-3-3 η , and α -SN levels in the substantia nigra of sporadic PD, but it is still not clear how these alterations influence parkin activity in neural cells. Thus, parkin is an E3 ubiquitin ligase involved in the ubiquitylation of proteins, irrespective of its involvement of K48- or K63-linked ubiquitylation (Doss-Pepe *et al*, 2005; Lim *et al*, 2005), that are important in the survival of dopaminergic neurons in PD.

In the present study, we found that parkin E3 activity is regulated positively and negatively by α -SN and 14-3-3 η , respectively, suggesting that derangements of this regulation may be responsible for ARJP. For instance, the activated parkin free from 14-3-3 η may be labile, and thus sensitive to other stresses, such as S-nitrosylation, and inactivated secondarily in PD. This situation resembles the effect of S-nitrosylation, in which nitrosative stress leads to S-nitrosylation of WT parkin, which leads initially to a marked increase followed by a decrease in the E3 ligase-ubiquitin-proteasome degradative pathway (Yao *et al*, 2004). The initial increase in the activity of parkin's E3 ubiquitin ligase leads to autoubiquitylation of parkin and subsequent inhibition of its activity, which would impair ubiquitylation and clearance of parkin substrates. In turn, 14-3-3 η may protect against impairment of parkin induced by various environmental stresses, including S-nitrosylation. It is also noteworthy that, although 14-3-3 η acts as a negative regulator of parkin, it may play a positive role in maintaining a large pool of parkin by preventing its self-ubiquitylation in the brain. Finally, we assume that gradual reduction of parkin activity may be associated with the development of ARJP as well as sporadic PD.

Current evidence suggests that α -SN increases in response to various stresses (Sherer *et al*, 2002; Gomez-Santos *et al*, 2003). This finding is compatible with the results of recent studies that dopamine-dependent neurotoxicity (Tabrizi *et al*, 2000; Zhou *et al*, 2000; Junn and Mouradian, 2002) is mediated by the formation of protein complexes that contain α -SN and 14-3-3, which are selectively increased in the substantia nigra in PD (Xu *et al*, 2002). Further studies are needed to determine the levels of parkin, 14-3-3 η , α -SN, and parkin and α -SN-14-3-3 η complexes in the substantia nigra of the midbrain of patients with sporadic PD.

Here we suggest that α -SN and parkin function through the same pathway. Indeed, both proteins, if not all, are associated

with presynaptic vesicles (Dawson and Dawson, 2003). So far, however, the physiological role of α -SN is largely unknown, though various roles including its involvement in synaptic plasticity have been suggested (Liu *et al*, 2004). We here provided the first evidence that α -SN acts as a positive regulator of parkin E3 activity. It is worth noting that disease-causing mutations of α -SN(A30P) and α -SN(A53T) could not activate the latent parkin-14-3-3 η complex, and thus, these mutations may accelerate the development of PD by failing to activate parkin. Our results identified a functional link between these two familial PD-gene products, thus highlighting the existence of a novel regulatory mechanism that could help us further understand the pathogenesis of ARJP as well as sporadic PD. However, it must be stressed here that α -SN is the causative gene product of familial PD. It is noteworthy that α -SN is an aggregation-prone protein due to its natively unfolded protein nature. It is of note that the locus of *PARK4* is triplication of the α -SN gene (*PARK1*) (Singleton *et al*, 2003), indicating that overexpression of α -SN itself is toxic and induces dopaminergic neuronal death. Indeed, α -SN tends to self-aggregate, and this tendency, which is augmented in the α -SN(A30P) and α -SN(A53T) mutants (Conway *et al*, 2000) (see our model in Figure 6B), causes autosomal dominant PD (Narhi *et al*, 1999). Both WT and mutant α -SN form amyloid fibrils akin to those seen in LBs, as well as nonfibrillary oligomers termed protofibrils (Dawson and Dawson, 2003; Bossy-Wetzel *et al*, 2004). However, whether aggregation and fibrillary formation of α -SN- and PD-linked mutants play a role in neuronal dysfunction and death of neurons in PD are a matter of fierce debate. At this point of view, we emphasize that the feature of α -SN as an aggregation-prone protein is probably not linked directly to its role as a potent activator of parkin E3 in the pathogenesis of PD. Even if these two unique properties of α -SN account for the development of PD independently or synergistically, however, it is clear that their mechanistic actions differ as illustrated in Figure 6B.

Materials and methods

Immunological analysis

For immunoprecipitation analysis of endogenous proteins in the brains of adult mouse and human, these brains were homogenized in three volumes of ice-cold lysis buffer (20 mM HEPES (pH 7.9) buffer containing 0.2% NP-40, 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (Sigma, Chemical Co., St Louis, MO)). The tissue homogenate was centrifuged at 20 000 g at 4°C for 20 min. The supernatant (2 mg protein) was used for immunoprecipitation with one of the following antibodies: anti-polyclonal parkin (Cell Signaling Technology, Beverly, MA) and anti-14-3-3 η antibodies (Immuno-Biological Lab. Co., Gunma) or control IgG (700 ng). The resulting immunoprecipitates were resolved in 30 μ l of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and one-third of the samples (10 μ l) were subjected to SDS-PAGE, followed by Western blotting with anti-14-3-3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-14-3-3 β , 14-3-3 γ , 14-3-3 ϵ , 14-3-3 η , and 14-3-3 τ (Immuno-Biological Lab. Co., Ltd, Japan) and anti-monoclonal parkin (1A1) antibodies (Shimura *et al*, 1999). In all, 10 μ g of the supernatant (lysate) was used as input (1.5%).

For immunoprecipitation analysis of the cell culture system, HEK293 cells were transfected with the respective plasmids. After 48 h, the cells were washed with ice-cold PBS (in mM, 10 Na₂PO₄, 2 KH₂PO₄, 137 NaCl, and 2.7 KCl), pH 7.4, and harvested in the lysis buffer (600 μ l). The lysate was then rotated at 4°C for 1 h, followed by centrifugation at 20 000 g for 10 min. The supernatant (200 μ l) was then combined with 50 μ l protein G-Sepharose (Amersham Life

Science, Buckinghamshire, UK), pre-incubated with anti-Myc (Santa Cruz Biotechnology, Santa Cruz), V5 (Invitrogen), and HA (Santa Cruz Biotechnology) antibodies or anti-FL antibody beads (Sigma) for 3 h. The protein G-Sepharose or FL-beads were precipitated and the pellets were extensively washed using the lysis buffer containing 500 mM NaCl. The precipitates were used for Western blot analysis using anti-parkin, Myc, FL, HA, V5, 14-3-3, and α -SN (BD Transduction Lab.) antibodies, as mentioned above. A volume of 5 μ l of the supernatant was used as input (7.5%).

In vitro autoubiquitylation assay

Recombinant GST-14-3-3 η was produced in *Escherichia coli*. Untagged 14-3-3 η was produced from GST-14-3-3 η by digestion with PreScission Protease (Amersham Bioscience). Recombinant His-parkin and E1 were produced from baculovirus-infected HiFive insect cells. Reactions were performed for 3 h at 37°C in 50 μ l of assay mixture containing 40 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl₂, 2 mM ATP, 2 mM DTT, 15 μ g ubiquitin (Sigma), 200 ng of E1, and 600 ng of E2 (UbcH7) (Affiniti-Research, Exeter, Devon, UK) in the presence or absence of GST-14-3-3 η or 14-3-3 η . After incubation, the reaction was terminated by the addition of the sample buffer for SDS-PAGE (17 μ l), and aliquots (15 μ l) were subjected to SDS-PAGE followed by Western blotting with anti-parkin antibody.

In vivo ubiquitylation assay

HEK293 cells were transfected for 48 h with pcDNA3.1 expression plasmids, in which FL-tagged parkin or FL-parkin mutants, α -SN or

α -SN mutants, 14-3-3 η , V5-synphilin-1, and HA-ubiquitin cDNAs were ligated. MG132 (50 μ M) was added for 20 min, prior to harvesting of the cells. Then, the cells were washed with cold PBS and lysed by 50 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl, 1% Nonidet-P40, 1% deoxycolate, 0.1% SDS, 5 mM ethylenediaminetetraacetic acid, and protease inhibitor cocktail. Preparation of the cell lysate, immunoprecipitation, and Western blot analyses were essentially the same for the immunological analysis as described above. In all experiments, the cell lysates (10 μ g, 7.5% input) were used for Western blotting as controls to check the expression levels.

For the method sections of 'Cell culture and transfection', 'Plasmids', and 'Surface plasmon resonance (SPR) analysis', see Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Diverse Effects of Pathogenic Mutations of Parkin That Catalyze Multiple Monoubiquitylation *in Vitro**[§]

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Mutational dysfunction of *PARKIN* gene, which encodes a double RING finger protein and has ubiquitin ligase E3 activity, is the major cause of autosomal recessive juvenile Parkinsonism. Although many studies explored the functions of Parkin, its biochemical character is poorly understood. To address this issue, we established an E3 assay system using maltose-binding protein-fused Parkin purified from *Escherichia coli*. Using this recombinant Parkin, we found that not the front but the rear RING finger motif is responsible for the E3 activity of Parkin, and it catalyzes multiple monoubiquitylation. Intriguingly, for autosomal recessive juvenile Parkinsonism-causing mutations of Parkin, whereas there was loss of E3 activity in the rear RING domain, other pathogenic mutants still exhibited E3 activity equivalent to that of the wild-type Parkin. The evidence presented allows us to reconsider the function of Parkin-catalyzed ubiquitylation and to conclude that autosomal recessive juvenile Parkinsonism is not solely attributable to catalytic impairment of the E3 activity of Parkin.

Recessive mutations in the human *PARKIN* gene are the most frequent cause of autosomal recessive juvenile parkinsonism, the common form of familial Parkinson disease (PD).² It has been shown that almost 50% of patients with familial autosomal recessive juvenile parkinsonism carry a series of exon rearrangements or point mutations in *PARKIN*. Moreover, recent findings of the haploinsufficiency of *parkin* and *S*-nitrosylation also imply its association in sporadic PD (1). The causal gene *PARKIN* encodes a double RING finger protein with ubiquitin ligase (E3) activity (2–5) and interestingly, missense mutations in the double RING finger motif resulted in an earlier onset of the disease than mutations in other function-unknown regions (6). To date, numerous biochemical studies have been performed to understand how mutations in Parkin lead to its dysfunction and to pathogenic outcome. However, because the biochemical characterization of E3 activity of Parkin has been difficult, it is still controversial whether the disease-relevant Parkin mutants lose their E3 activity or not. For example, one group of investigators implied that Parkin harboring K161N mutation loses its E3 activity (7), whereas another group suggested the same mutation does not impair E3 activity (8). In the case of other PD mutations, the situa-

tion is even more complex (see supplemental Table 1). Thus, the mode of Parkin-catalyzed ubiquitylation remains poorly understood to date.

Little is known about the reconstituted ubiquitylating experiment using recombinant Parkin. Almost all of the biochemical analyses reported so far have been performed using *in vitro* translated Parkin or immunoprecipitated Parkin. However, it is difficult to avoid trace contaminants of other proteins that could physically interact with Parkin. Indeed it has been reported that Parkin interacts with other E3s such as CHIP (9) and Nrdp1/FLRF (10), and thus the results of experiments using immunoprecipitated or *in vitro* translated Parkin require careful interpretation. To study the E3 activity of intrinsic Parkin, a biochemical approach using bacterially expressed recombinant Parkin that is free from other contaminating E3 enzyme(s) is obviously required. We thus attempted to reconstitute a sensitive E3 assay system using Parkin purified from *Escherichia coli*.

EXPERIMENTAL PROCEDURES

Purification of Recombinant Proteins—To express Parkin in *E. coli*, it was important to use a modified *E. coli* strain BL21(DE3) codon-plus (RIL) strain (Stratagene, La Jolla, CA), because Parkin possesses many rare codons for *E. coli* that might cause low expression and/or amino acid misincorporation. For example, Parkin contains eight AGA codons that lead to mistranslation of lysine for arginine in *E. coli* (11). pMAL-p2T, in which the thrombin recognition site was inserted into pMALp2 (New England BioLabs, Beverly, MA), was prepared to purify maltose-binding protein (MBP)-LVPRGS-Parkin. Parkin cDNAs of wild type and various mutants/deletions were subcloned into BamHI site of pMAL-p2 and pMAL-p2T. All mutants/deletions were generated by PCR-mediated site-directed mutagenesis (details of the plasmid construction processes can be provided upon request). All recombinant fusion proteins were purified from bacterial lysate applying the method advocated by the supplier (New England BioLabs) using a column buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol, and 100 μ M ZnSO₄. The eluted fraction containing 10 mM maltose was not dialyzed because Parkin tends to lose its E3 activity during dialysis. Instead, it was subjected to the ubiquitylation assay directly. We attempted to purify sole IBR-RING2 region of Parkin also by splitting MBP-IBR-RING2 (see “Results”). Specifically, we added E2, various detergents and stabilizers during the cleavage process expecting that they solubilize and/or stabilize free IBR-RING2 in solution. Even in all the above experimental conditions, however, we could not obtain soluble-free IBR-RING2 (data not shown). Six histidine-tagged proteins such as Uev1/Ubc13 were purified by the conventional method and dialyzed by a buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM NaCl and 1 mM dithiothreitol. Glycerol of 6% was added as a stabilizer for preservation of recombinant MBP-Parkin and E2 proteins at -80°C .

In Vitro Ubiquitylation Assay—The *in vitro* ubiquitylation assay was performed as described previously (12–14). Briefly, the purified MBP-Parkin (20 μ g of MBP-Parkin/ml) was incubated in a reaction buffer (50

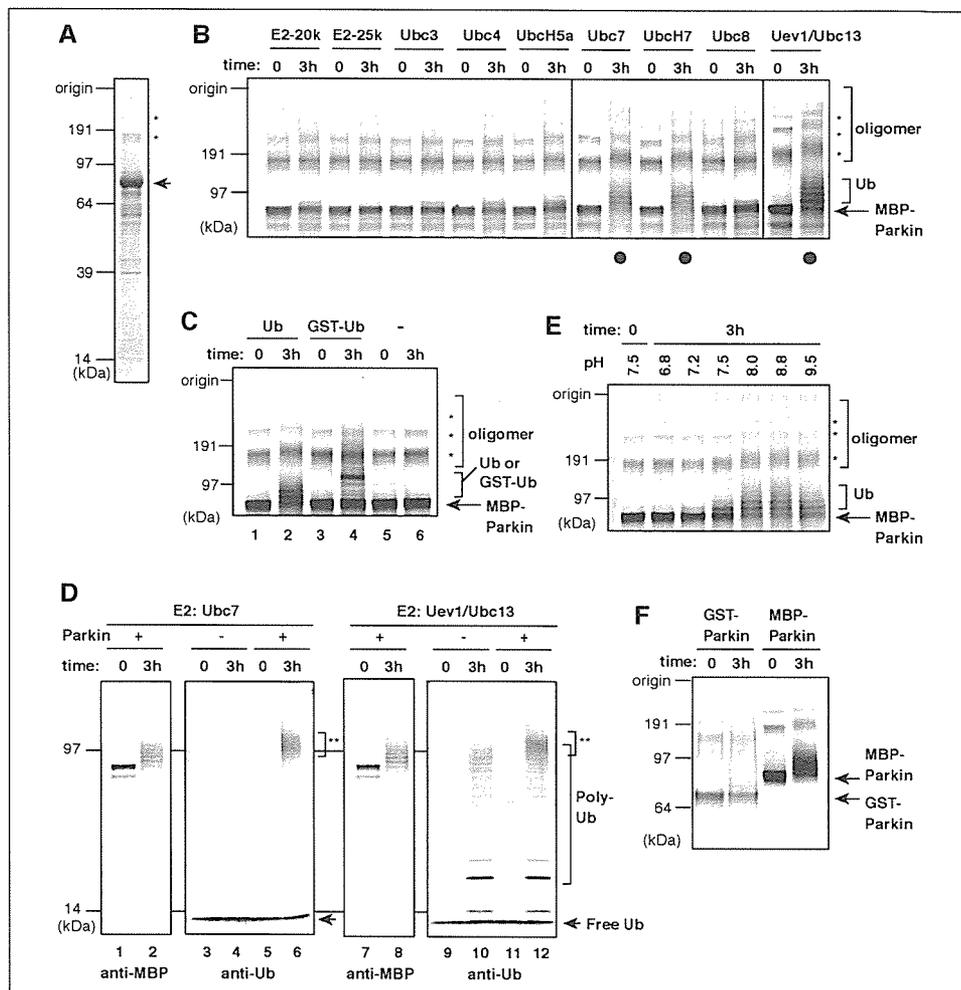
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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1 and Fig. 1.

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² The abbreviations used are: PD, Parkinson disease; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; GST, glutathione *S*-transferase; MBP, maltose-binding protein.

FIGURE 1. *In vitro* ubiquitylation assay using Parkin derived from *E. coli*. *A*, purified MBP-Parkin was visualized by CBB staining. The *arrow* indicates MBP-Parkin, and the *asterisks* indicate oligomerization bands. *B*, MBP-Parkin catalyzes autoubiquitylation in cooperation with Ubc7, UbcH7, and Uev1-Ubc13 (*solid circles*). MBP-Parkin was subjected to *in vitro* ubiquitylation assay and to immunoblotting with anti-MBP antibody. *Ub*, ladders derived from autoubiquitylation, *, oligomerization bands. *C*, confirmation of autoubiquitylation of MBP-Parkin. To demonstrate that the slower migrating ladders are because of autoubiquitylation, a reconstitution assay was repeated in the absence (-) or presence of ubiquitin (*Ub*) or GST-ubiquitin (*GST-Ub*). *D*, the high molecular weight forms of MBP-Parkin are recognized by anti-ubiquitin antibody. *Double asterisks* indicate the signal derived from MBP-Parkin autoubiquitylation. The *arrow* indicates free ubiquitin. *E*, Parkin prefers weak alkaline conditions to exert its E3 activity. *F*, GST-Parkin rarely exhibits E3 activity. Bacterial recombinant GST- and MBP-Parkin were concurrently subjected to ubiquitylation assay. Ubc7 was used as E2 in *C*, *E*, and *F*. Anti-MBP antibody was used in *B*, *C*, and *E*, and anti-Parkin antibody was used in *F*.



mm Tris-HCl, pH 8.8, 2 mM dithiothreitol, 5 mM MgCl₂, and 4 mM ATP) with 50 μ g of ubiquitin/ml (Sigma), 1.6 μ g of recombinant mouse E1/ml and 20 μ g of purified E2 or 100 μ g of various E2-expressing *E. coli* lysate/ml at 32 °C for 2 h and subjected to immunoblotting with anti-MBP antibody (New England BioLabs), anti-parkin (1A1) antibody (15), or anti-ubiquitin antibody (DakoCytomation, Carpinteria, CA). In some cases, GST-ubiquitin or methylated ubiquitin (BostonBiochem, Cambridge, MA) was used instead of native ubiquitin. The subsequent thrombin cleavage was performed by incubation on ice for 3 h in the presence of thrombin and 2 mM CaCl₂.

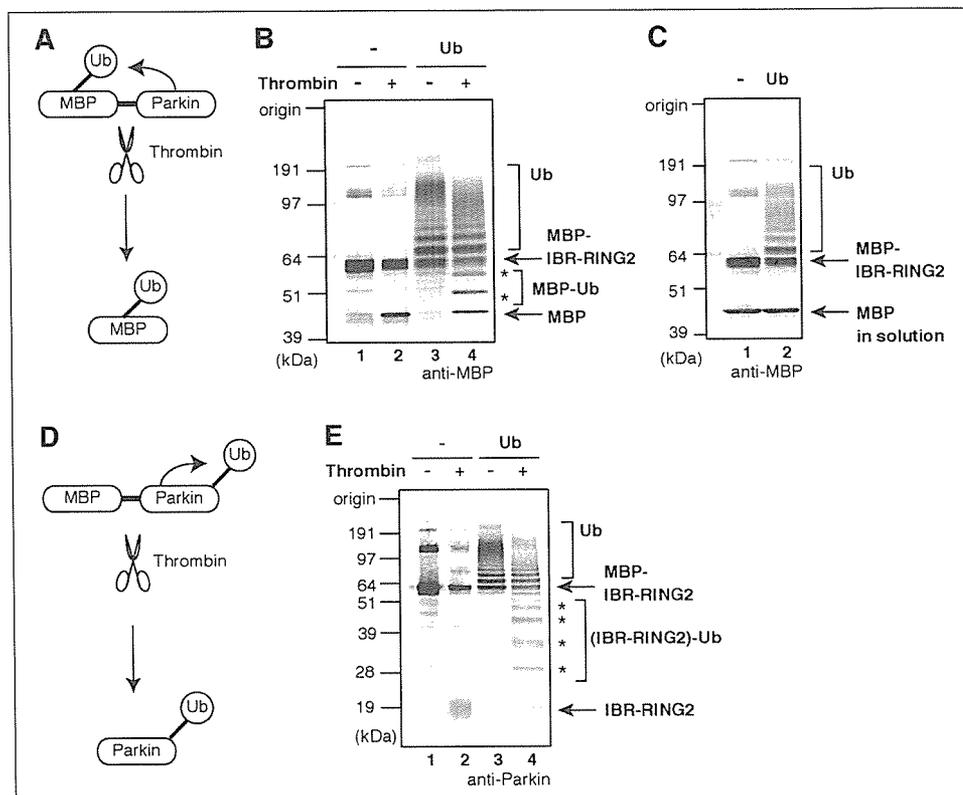
RESULTS

Autoubiquitylation by MBP-Parkin Fusion Protein—In 2001, Rankin *et al.* (16) reported that glutathione *S*-transferase (GST)-tagged Parkin purified from *E. coli* possesses E3 activity. However, we found that the E3 activity of this GST-Parkin is very weak (Fig. 1*F*), and thus there is a need for a more sensitive E3 assay system for bacterially expressed recombinant Parkin. During studies of other RING finger proteins, we recognized the superiority of the MBP-tag relative to GST-tag in purifying RING finger proteins that retain their E3 activities (12, 13, 17, 18) and hence decided to use MBP-Parkin. MBP-Parkin was purified using a modified *E. coli* strain BL21(DE3) codon-plus-RIL (Fig. 1*A*) and then incubated with ATP, ubiquitin, E1, and one of the E2 enzymes indicated in Fig. 1*B*, and we subjected it to immunoblotting with anti-MBP antibody. High molecular mass ladders derived from autoubiquitylation (see below) were observed when MBP-Parkin was incubated with Ubc7,

UbcH7, and Uev1-Ubc13 (Fig. 1*B*, highlighted by the *solid circles*). Note that the slower migrating bands of more than 160 kDa observed even at reaction time zero (Fig. 1*B*, *asterisks*) or without ubiquitin (Fig. 1, *A* and *C*, *asterisks*) are derived from MBP-Parkin oligomerization. To test whether the modification acquired by MBP-Parkin is due to ubiquitylation, the same reaction products were subjected to immunoblotting with anti-ubiquitin antibody. When Ubc7 was used as E2 (Fig. 1*D*, lanes 1–6), only modified MBP-Parkin was detected by anti-ubiquitin antibody (lane 6, *double asterisks*), indicating that the modification acquired by MBP-Parkin was indeed autoubiquitylation. When Uev1-Ubc13 was used, a polyubiquitylation signal was observed even in the absence of MBP-Parkin (Fig. 1*D*, lanes 9 and 10), because Uev1-Ubc13 complex itself can catalyze polyubiquitin chain formation (19). Also in this case, modified MBP-Parkin reacted with anti-ubiquitin antibody, confirming the above conclusion (see Fig. 1*D*, *double asterisks* in lane 12; note that the difference between lanes 10 and 12 corresponds to the autoubiquitylation signal of MBP-Parkin). Moreover, the replacement of ubiquitin with GST-ubiquitin retarded the mobility of these ladders (Fig. 1*C*, lanes 3 and 4), and the exclusion of ubiquitin completely quenched such ladders (Fig. 1*C*, lanes 5 and 6). Based on these results, we concluded that MBP-Parkin catalyzes autoubiquitylation in cooperation with Ubc7, UbcH7, and Uev1-Ubc13. Interestingly, autoubiquitylation became evident when the pH of the reaction buffer was increased to 8.0 and 8.8, indicating that Parkin prefers weak alkaline conditions to exhibit its E3 activity *in vitro* (Fig. 1*E*). Because MBP-Parkin possesses stronger E3

Parkin Catalyzes Multiple Monoubiquitylation *In Vitro*

FIGURE 2. In-frame-fused MBP can be a good pseudosubstrate of Parkin. *A*, a schematic diagram of Parkin catalyzed ubiquitylation of MBP. If the MBP portion is ubiquitylated, a change in its mobility would be recognized by immunoblotting after cleavage. *B*, the MBP-(IBR-RING2) fused protein (see Fig. 4) was subjected to *in vitro* ubiquitylation, subsequent cleavage into MBP moiety, and immunoblotting with anti-MBP antibody. The MBP portion of IBR-RING2 was ubiquitylated (asterisks). *C*, MBP can be ubiquitylated only when it is in the physical vicinity of Parkin. Note that free MBP in solution was not ubiquitylated. *D*, a schematic diagram of Parkin-catalyzed autoubiquitylation. *E*, IBR-RING2 portion is also ubiquitylated. Asterisks in lane 4 show the ubiquitylated IBR-RING2 moiety (compare lane 4 with 2). Ubc7 was used as E2 in these experiments.



activity than GST-Parkin, as shown in Fig. 1*F*, we used MBP-Parkin in the following experiments.

Fused MBP Is a Good Pseudo-substrate to Monitor E3 Activity of Parkin—We next determined whether the MBP portion or Parkin portion (or both) is ubiquitylated. To check this, we purified MBP-LVPRGS-Parkin, in which a thrombin-digestion sequence is inserted between MBP and Parkin. As depicted in Fig. 2*A*, if the MBP moiety is ubiquitylated, its molecular weight would increase by ubiquitylation and subsequent digestion, but if not ubiquitylated, its molecular weight would remain unchanged. First we tried to split MBP-LVPRGS-Parkin by thrombin; however, this recombinant protein was hardly digested for some unknown reason (data not shown). We next fused the C-terminal IBR-RING2 region of Parkin to MBP-LVPRGS (hereafter dubbed IBR-RING2, see Fig. 4*A*), and this construct was cleaved moderately (Fig. 2*B*, lanes 1 and 2). When IBR-RING2 was subjected to an ubiquitylation assay and subsequently separated into MBP and IBR-RING2 portions by thrombin digestion, the molecular weight of MBP moiety was clearly increased (see the asterisks in Fig. 2*B*, lanes 3 and 4), meaning that the MBP portion is ubiquitylated. Does this result mean that bacterial MBP protein is the substrate for Parkin? The answer is no. When sole MBP protein was incubated with IBR-RING2, this free MBP was not ubiquitylated at all, even though IBR-RING2 was autoubiquitylated as described (Fig. 2, compare *C* with *B*). This result indicates that the IBR-RING2 region of Parkin ubiquitylates fused-MBP, but not unbound MBP, and strongly suggests that Parkin recognizes MBP as a substrate not because of its amino acid sequence but because of its physical vicinity to Parkin. As depicted in Fig. 2*D*, when the same experiment was repeated using an anti-Parkin antibody, the molecular weight of the IBR-RING2 moiety was also increased meaning that both MBP and IBR-RING2 portions were ubiquitylated (Fig. 2*E*). Although many putative substrates of Parkin have been reported, the lack of a good *in vitro* substrate makes any biochemical study difficult. Our study revealed that

fused MBP could be a good pseudo-substrate to monitor the E3 activity of Parkin.

Parkin by Itself Catalyzes Multiple Monoubiquitylation—The Uev1-Ubc13 heterodimer is an E2 involved in the formation of Lys-63-linked polyubiquitylation (19). We confirmed that our Uev1-Ubc13 complex is functional (Fig. 1*D* and supplemental Fig. 1*A*). Motivated by the findings that Parkin catalyzes Lys-63-linked polyubiquitylation (20, 21) and Parkin cooperates with Uev1-Ubc13 in our assay (Fig. 1*B*), we investigated the mode of Parkin-catalyzed ubiquitylation. Parkin could either catalyze multiple monoubiquitylation, Lys-48-linked polyubiquitylation, or Lys-63-linked polyubiquitylation. Lys-48-linked polyubiquitylation has been studied most and it essentially directs the substrate to degradation by the proteasome. In contrast, the Lys-63-linked polyubiquitylation and monoubiquitylation serve as a signal other than proteasomal-proteolysis (22–24). We first used methylated ubiquitin (hereafter referred to as Met-Ub) in which all lysine residues are blocked by methylation and is incapable of polyubiquitylation. If Parkin catalyzes polyubiquitylation, the use of Met-Ub would shorten the ladder of ubiquitylation but if not, the ubiquitylation pattern would remain unchanged. Unexpectedly, the use of Met-Ub and Uev1-Ubc13 did not change the ubiquitylation pattern, indicating that Parkin catalyzes multiple monoubiquitylation *in vitro* (Fig. 3*A*). The same result was observed when Ubc7 was used as E2 (Fig. 3*B*), and these results were more evident when IBR-RING2 (Fig. 4*A*) was utilized (Fig. 3, *C* and *D*). Repeated experiments using lysine-less ubiquitin, in which all lysine residues were changed to arginine, showed it cannot form a polyubiquitin chain, again confirmed the consequence (Fig. 3*E*). It is noteworthy that sole Ubc13 itself assisted autoubiquitylation of Parkin as well as the Uev1-Ubc13 complex (supplemental Fig. 1*B*), again supporting this conclusion. These results allowed us to conclude that the mode of ubiquitylation catalyzed by intrinsic Parkin *in vitro* is multiple monoubiquitylation rather than polyubiquitylation (see “Discussion”).

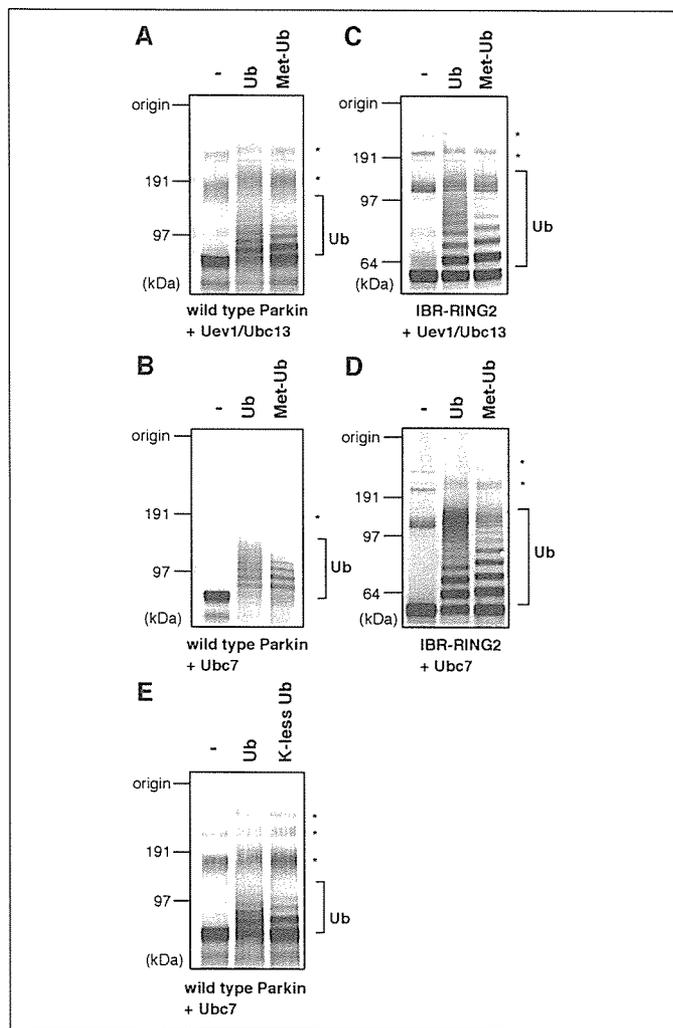


FIGURE 3. Parkin catalyzes multiple monoubiquitylation. *A* and *B*, *in vitro* ubiquitylation assay was performed in the absence (–) or presence of ubiquitin (*Ub*) or methylated-ubiquitin (*Met-Ub*; cannot form polyubiquitylation chain). Uev1-Ubc13 was used as E2 in *A* and Ubc7 in *B*. Almost identical ubiquitylation patterns were observed in *Ub* and *Met-Ub*, indicating that Parkin catalyzes multiple monoubiquitylation. *C* and *D*, the result was more evident when IBR-RING2 (see Fig. 4) was utilized. Uev1-Ubc13 was used as E2 in *C* and Ubc7 in *D*. *E*, the same experiment was performed using lysine-less ubiquitin. *Ub*, autoubiquitylation; *, oligomerization bands. Anti-MBP antibody was used in all experiments.

Mode of E3 Activity of Parkin with Pathogenic Missense Mutations—At present, dozens of disease-relevant mutations of Parkin have been reported, and the primary cause of autosomal recessive juvenile parkinsonism is assumed to be impairment of the E3 activity of Parkin by such mutations. However, it is still contentious whether Parkin with PD-causing mutation loses its E3 activity or not (see supplemental Table 1), primarily because of the absence of a sensitive E3-activity assay system using recombinant Parkin. To settle this problem, we examined the E3 activity of MBP-Parkin harboring various mutations and deletions. Three in-frame exonic deletions and 19 PD-linked mutations distributed throughout Parkin were selected (Fig. 4A). In addition, two Parkin species, one lacks its Ubl domain (Δ Ubl) and the other possesses only C-terminally IBR-RING2 domain (IBR-RING2), were also generated. When these MBP-Parkin mutants were incubated with Ubc7 as E2, only mutations neighboring the second RING finger motif (Fig. 4A, *solid circles*) abolished the E3 activity completely (Fig. 4B). A nonsense mutation lacking the rear RING finger motif had no E3 activity and sole IBR-RING2 retained the E3 activity (Fig. 4, *light gray circles*), indicating

that the second RING finger motif is the catalytic core for the E3 activity of Parkin. Contrary to what was assumed, all disease-relevant mutations other than those in RING2 still possessed E3 activities equivalent to that of the wild-type Parkin (Fig. 4B). The same results were observed when UbcH7 or Uev1-Ubc13 was used as E2 (data not shown). In these assays, we used a bacterially expressed recombinant Parkin, and to our knowledge, this is the first direct evidence that E3 activity of the strictly pure Parkin harboring various pathogenic mutations is not compromised.

DISCUSSION

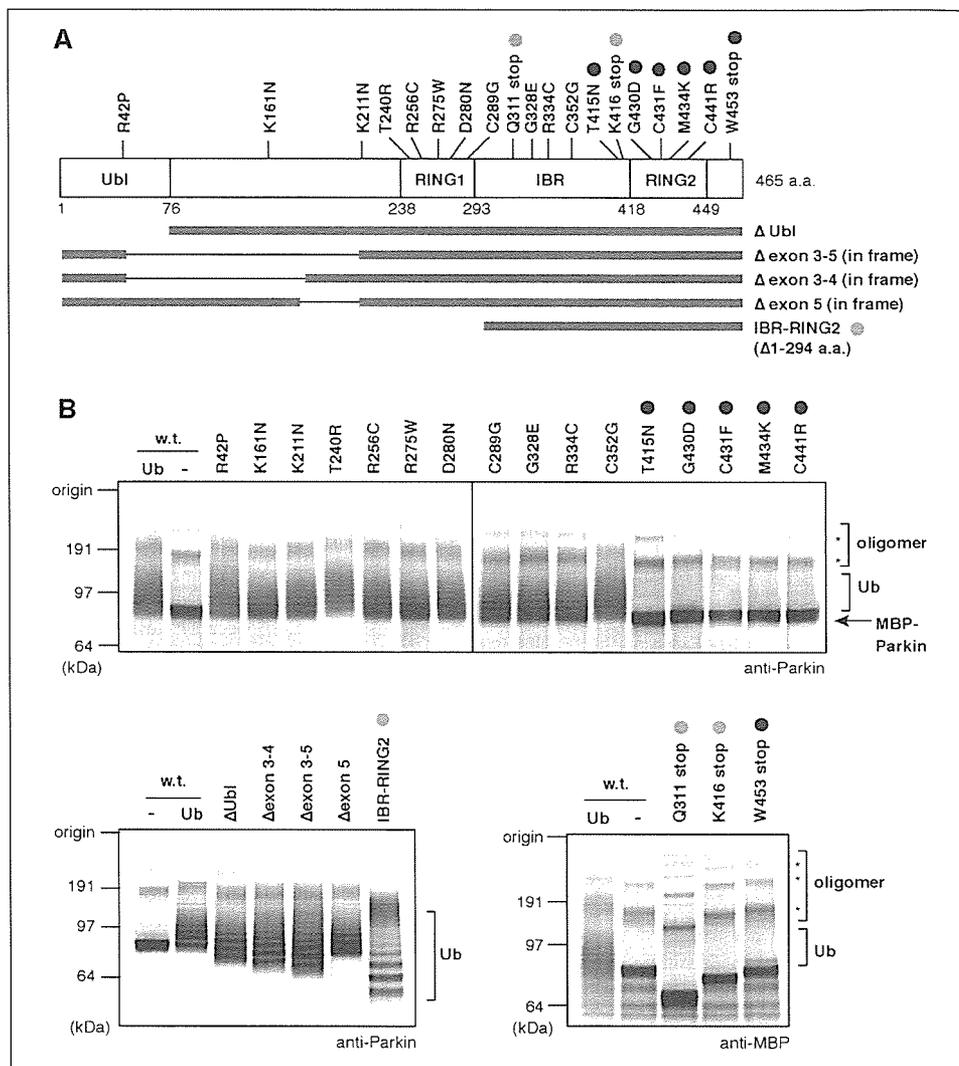
To date, numerous biochemical studies have been performed to understand the E3 activity of Parkin. However, it is difficult to rule out the possible involvement of other E3s (see Introduction). Furthermore, the lack of a good model substrate spurs the difficulty to check the intrinsic E3 activity of Parkin. We thus set up a sensitive E3 assay system using bacterially expressed recombinant Parkin. Our assay system has some advantages; namely, we can obtain a large quantity of MBP-Parkin with higher E3 activity than GST-Parkin (Fig. 1). In addition, this fusion protein was already primed for ubiquitylation even in the absence of model substrate, because fused MBP can work as a good pseudo-substrate (Fig. 2). More importantly, because MBP-Parkin is purified from *E. coli*, it is free from possible contamination of other E3(s). The establishment of this assay allowed us to perform a thorough biochemical characterization of Parkin protein.

Interestingly, sole Parkin catalyzes multiple monoubiquitylation *in vitro* (Fig. 3). Moreover, although Doss-Pepe *et al.* (20) reported that Parkin accelerates polyubiquitin chain formation, the MBP-Parkin in our assay did not stimulate the assembly of polyubiquitin chain (Fig. 1D, compare lanes 4 and 6, and 10 and 12, respectively). These results seemingly suggest that the ubiquitylation catalyzed by Parkin functions not for proteasomal degradation but for non-proteasomal-proteolytic function(s), such as transcriptional regulation and/or membrane trafficking *in vivo*. However, it is still premature to make such conclusion. Although we showed that pure Parkin catalyzes multiple monoubiquitylation *in vitro* (Fig. 3), some additional factor(s) like E4 can work together *in vivo*, and this needs to be considered. E4 can extend the ubiquitin chain by recognizing the ubiquitin moiety of a ubiquitylated-protein as a substrate (25). If such an E4-like factor(s) cooperates with Parkin *in vivo*, it is still possible that monoubiquitylation catalyzed by Parkin is used as the scaffold for further polyubiquitylation and finally functions for proteasomal degradation. All things considered, further studies are obviously required; in particular, the authentic substrate and the function of Parkin-catalyzed ubiquitylation need to be addressed.

Another unexpected result was that most of the PD-relevant missense mutations do not abrogate E3 activity of Parkin (Fig. 4). Only missense mutations in the rear RING finger motif abolished the E3 activity, revealing that not the first but the second RING finger motif is the catalytic core of Parkin. Recently, several studies that focused on the pathophysiological mechanisms of Parkin have been published (26–30). Although our results on enzymatic activities of mutant Parkin are not fully consistent with previous reports (see supplemental Table 1), methodological differences in the E3 assay may account for the conflicting observation. For example, in one study immunoprecipitated Parkin was used as the source of E3 *in vitro* (31) and in other studies, E3 activity of Parkin was checked by whether or not coexpression of Parkin in cells enhances the ubiquitylation of the putative substrate (7, 30). Although there is little discrepancy, recent studies and our present work drew the same conclusion that the dysfunction of Parkin is not simply attributable to catalytic impairment of its E3 activity. Indeed, several missense mutations cause Parkin to be sequestered into an aggresome-like structure, and this phenomenon may be involved in disease pathogenesis

Parkin Catalyzes Multiple Monoubiquitylation in Vitro

FIGURE 4. A, schematic diagram of disease-relevant mutations and exonic deletions of Parkin. B, E3 activities of various Parkin proteins bearing PD-linked mutations and deletions. Note that mutations neighboring the second RING finger motif (solid circles) abolished E3 activity of Parkin. Light gray circles indicate that the RING2 is the catalytic core of Parkin (see text for details). Conversely, pathogenic mutants other than RING2 mutants retain E3 activities equivalent to that of the wild-type (w.t.) control. Ub, autoubiquitylation; *, oligomerization bands.



(27–30). We think that disease-relevant mutations cause not only attenuation of E3 activity but also a variety of primary defects such as sequestration into aggresome and dissociation from its partner protein, and possibly a complex of such defects may eventually lead to Parkin dysfunction and autosomal recessive juvenile parkinsonism.

PD is the second most prevalent neurodegenerative disorder, and thus, analysis of Parkin is important in terms of public welfare. Indeed, a large number of articles on Parkin have been published; however, because of fierce scientific competition, not all Parkin-related phenomena were critically scrutinized although there remains room for close examination. For example, Pawlyk *et al.* (32) recently inspected the anti-Parkin antibodies and uncovered a high non-specificity of the available Parkin antibodies. This also holds true for the E3 activity of Parkin, because precedent works could not exclude the possible involvement of another E3(s). Herein we investigated thoroughly the enzymatic activity of bacterially expressed recombinant Parkin. Although our work is not conspicuous, we hope that our biochemical characterization using pure Parkin would be a solid cornerstone for further studies, as the preceding works were.

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