Diverse Effects of Pathogenic Mutations of Parkin That Catalyze Multiple Monoubiquitylation in Vitro

Noriyuki Matsuda, Toshiaki Kitami, Toshiaki Suzuki, Yoshikuni Mizuno, Nobutaka Hattori, and Keiji Tanaka

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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). 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 dose not impair E3 activity (8). In the case of other PD mutations, the situation 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 pMAL-p2 (New England Biolabs, Beverly, MA), was prepared to purify maltose-binding protein (MBP)-LVPGRGS-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 by 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 μM 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 μg of MBP-Parkin/ml) was incubated in a reaction buffer (50
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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. 1F), 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. 1A) and then incubated with ATP, ubiquitin, E1, and one of the E2 enzymes indicated in Fig. 1B, 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. 1B, highlighted by the solid circles). Note that the slower migrating bands of more than 160 kDa observed even at reaction time zero (Fig. 1B, 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 autoubiquitylation, the same reaction products were subjected to immunoblotting with anti-ubiquitin antibody. When Ubc7 was used as E2 (Fig. 1D, 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 indeed is autoubiquitylation. When Uev1-Ubc13 was used, a polyubiquitylation signal was observed even in the absence of MBP-Parkin (Fig. 1D, 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. 1D, 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. 1C, lanes 3 and 4), and the exclusion of ubiquitin completely quenched such ladders (Fig. 1C, 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. 1E). Because MBP-Parkin possesses stronger E3 activity.
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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 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. 1F, 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. 2A, 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. 4A), and this construct was cleaved moderately (Fig. 2B, 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. 2B, 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. 2D, 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. 2E). 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. 1D and supplemental Fig. 1A). Motivated by the findings that Parkin catalyzes Lys-63-linked polyubiquitylation (20, 21) and Parkin cooperates with Uev1-Ubc13 in our assay (Fig. 1B), 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 were 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. 3A). The same result was observed when Ubc7 was used as E2 (Fig. 3B), and these results were more evident when IBR-RING2 (Fig. 4A) 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. 3E). It is noteworthy that sole Ubc13 itself assisted autoubiquitylation of Parkin as well as the Uev1-Ubc13 complex (supplemental Fig. 1B), 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”).
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 (ΔUbl) 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 ubiquitination 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 J0 and 12, respectively). These results seemingly suggest that the ubiquitination 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.

**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, auto ubiquitylation; *, oligomerization bands. Anti-MBP antibody was used in all experiments.
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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