Interaction between RING1 (R1) and the Ubiquitin-like (UBL) Domains Is Critical for the Regulation of Parkin Activity*

Parkin is an E3 ligase that contains a ubiquitin-like (UBL) domain in the N terminus and an R1-in-between-ring-RING2 motif in the C terminus. We showed that the UBL domain specifically interacts with the R1 domain and negatively regulates Parkin E3 ligase activity. Parkin-dependent mitophagy, and Parkin translocation to the mitochondria. The binding between the UBL domain and the R1 domain was suppressed by carbonyl cyanide m-chlorophenyl hydrazone treatment or by expression of PTEN-induced putative kinase 1 (PINK1), an upstream kinase that phosphorylates Parkin at the Ser-65 residue of the UBL domain. Moreover, we demonstrated that phosphorylation of the UBL domain at Ser-65 prevents its binding to the R1 domain and promotes Parkin activities. We further showed that mitochondrial translocation of Parkin, which depends on phosphorylation at Ser-65, and interaction between the R1 domain and a mitochondrial outer membrane protein, VDAC1, are suppressed by binding of the UBL domain to the R1 domain. Interestingly, Parkin with missense mutations associated with Parkinson disease (PD) in the UBL domain, such as K27N, R33Q, and A46P, did not translocate to the mitochondria and induce E3 ligase activity by m-chlorophenyl hydrazone treatment, which correlated with the interaction between the R1 domain and the UBL domain with those PD mutations. These findings provide a molecular mechanism of how Parkin recruitment to the mitochondria and Parkin activation as an E3 ubiquitin ligase are regulated by PINK1 and explain the previously unknown mechanism of how Parkin mutations in the UBL domain cause PD pathogenesis.


Parkinson disease (PD) is a neurodegenerative disorder that results from degenerated dopaminergic neurons in the substantia nigra. Most cases of PD are sporadic, and ~10% are familial (1). Gene mutations that are known to be responsible for the onset of the disease include PARK2 (Parkin), PARK6 (PINK1), PARK7 (D1L), LRRK2, and SNCA (α-synuclein). LRRK2 and SNCA mutations are believed to cause PD through a gain of function, whereas PARK2, PARK6, and PARK7 mutations cause PD through a loss of function (2–4).

Autosomal recessive early onset parkinsonism is linked to several loci, including PARK2 and PARK6 (5). The PARK2 gene encodes Parkin, an E3 ubiquitin ligase that consists of 465 amino acid residues. Parkin is composed of an ubiquitin-like (UBL) domain at the N terminus and a R1-in-between-ring (IBR)-Rind 2 (R2) motif at the C terminus (6–8). Structurally, Parkin is a RING-type E3 ligase, but functionally it acts as a RING/HECT hybrid E3 ligase (9–12). Parkin functions like a RING-type E3 ligase by interacting with E2 enzymes, Ubch7 and Ubch8, via the IBR domain, whereas the RING1 domain binds to substrates, allowing direct substrate ubiquitination. Parkin can also function like a HECT-type E3 ligase by catalyzing the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to the substrates via the active-site residues, Cys-431 and His-433. E2 enzymes that support Parkin function as a HECT-type E3 ligase are Ubch7, Ubch8, and Ubch13/Ubv1a heterodimer (13, 14).

Substrates that are ubiquitinated by active Parkin include mitofusin (Mfn) 1 and 2, dynamin-related protein 1 (Drp1), voltage-dependent anion-selective channel protein 1 (VDAC1), mitochondrial Rh0 GTPase (Miro), and translocase of outer membrane 20 (TOM20) (15–20). Parkin ligates these substrates with Lys-27, Lys-48, and Lys-63 ubiquitin linkages (19, 21–23). The substrates of Parkin with Lys-48-linked polyubiquitin chains are degraded by the ubiquitin proteasome system (23–26). However, those polyubiquitinated with Lys-63 or Lys-27 ubiquitin linkage recruit ubiquitin-binding adaptors such as histone deacetylase 6 (HDAC6) and p62/SQSTM1 (21, 27–29). The stability of Mfn1 and -2 and Drp1 are reduced by Lys-48-linked polyubiquitination (15–17, 30, 31). TOM20 is both mono- and polyubiquitinated by Parkin by Lys-48 and Lys-63 ubiquitin linkages (20). In the case of VDAC1, Parkin catalyzes polyubiquitination with ubiquitin Lys-27 and Lys-63 linkages, which leads to recruitment of p62/SQSTM1 and subsequent induction of mitophagy (18, 32).

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3 The abbreviations used are: PD, Parkinson disease; UBL, ubiquitin-like; PINK1, PTEN-induced putative kinase 1; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; RING1, really interesting new gene 1; IBR, in-between-ring; R2, RING2, really interesting new gene 2; Mfn, mitofusin; Drp1, dynamin-related protein 1; VDAC1, voltage-dependent anion channel protein 1; TOM20, translocase of outer membrane 20; USP, ubiquitin-specific protease; FL, full-length; co-IP, co-immunoprecipitation.
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Parkin is activated by a serine/threonine kinase, PINK1, which is encoded by PARK6. As with PARK2, various mutations in PARK6 cause autosomal recessive early onset Parkinsonism (33). PINK1 contains an N-terminal mitochondrial targeting sequence and a kinase domain at the C terminus. With reduction of the mitochondrial membrane potential, for example, by treatment with CCCP, the full-length form of PINK1 accumulates in the mitochondrial outer membrane (34–37). The accumulation of PINK1 in the outer membrane triggers recruitment of Parkin to the mitochondria and subsequent ubiquitination of Parkin substrates (38–42). The ability of PINK1 to recruit Parkin to the mitochondria is absolutely dependent on its kinase activity (41, 43). PINK1 phosphorylates Parkin at Ser-65 of the UBL domain (44). Mutating this residue from serine to alanine results in a delay of Parkin recruitment to the mitochondria compared with WT Parkin upon CCCP treatment (45). Moreover, when phosphorylated at Ser-65, Parkin autoubiquitination activity and polyubiquitination of its substrates, including TOM20 and Miro (mitochondrial Rho GTPase), increase (19, 46), suggesting that the E3 ligase activity of Parkin increases with phosphorylation at Ser-65 by PINK1.

Studies have reported that the UBL domain regulates the activity of the proteins that harbor the domain (47–51). Ubiquitin-specific protease 14 (USP14), a deubiquitinase, associates with the 26S proteasome via its UBL domain and enhances the catalytic function of the proteasome (52). The UBL domain also competes with ubiquitin for binding to the catalytic domain of USP4, suppressing the deubiquitinase mechanism of USP4 (53). Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) is an E3 ligase that contains the UBL domain. The UBL domain of HOIL-1 interacts with the 26S proteasome to promote degradation of its substrates by the ubiquitin-proteasome system (54). The deletion of the UBL domain in Parkin also enhances Parkin autoubiquitination activity (55). Furthermore, an x-ray crystal structure of Parkin revealed that the UBL domain of Parkin binds to its C-terminal catalytic region to block association with E2 (56). These findings raised the possibility that the UBL domain is critical for regulation of Parkin activation.

Here, we investigate the molecular mechanism that underlies the function of the R1 and UBL domains of Parkin. We found that the UBL domain of Parkin suppresses Parkin autoubiquitination, substrate ubiquitination, mitochondria translocation, and mitophagy via interaction with the R1 domain of the E3 ligase. We also showed that the interaction between the R1 domain and the UBL domain is diminished when the UBL domain was phosphorylated at Ser-65 by PINK1. Furthermore, we showed that Parkin activity is regulated by competitive interaction of the R1 domain with the UBL domain and Parkin substrates such as VDAC1. Consistent with these in vitro data, Parkin mutations in the UBL domain of PD patients affected the interaction between the R1 domain and the UBL domain. Together, these results suggest that the interaction between the R1 domain and the UBL domain is critical for proper regulation of Parkin functions both in vitro and in vivo.

Experimental Procedures

Antibodies and Reagents—Rabbit anti-HA (Cell Signaling Technology), mouse anti-GST (Upstate Biotechnology), rabbit anti-GFP (Santa Cruz), mouse or rabbit anti-Myc (Cell Signaling Technology), rabbit anti-ubiquitin (Cell Signaling Technology), mouse anti-FLAG (Medical and Biological Laboratories), mouse anti-MFN2 (Millipore), mouse anti-MFN1 (Abcam), mouse anti-VDAC1 (Santa Cruz), mouse anti-NDUFS3 (Abcam), rabbit anti-COIV4 (Abcam), mouse anti-TIM23 (BD Biosciences), and mouse anti-β-tubulin (Developmental Studies Hybridoma Bank) antibody were used for immunoblot analyses. Rabbit anti-TOM20 (Santa Cruz) mouse anti-GST (Upstate Biotechnology) antibody, rabbit anti-SPQSTM1/p62 (Cell Signaling), and rabbit anti-LC3B antibody (Cell Signaling) were used for immunocytochemistry. CCCP was purchased from Calbiochem. Glutathione-Sepharose 4B beads (GE Healthcare) were used for GST pulldown assays.

Plasmids—The N-terminal GST-tagged pE8G vector was used to generate truncated Parkin constructs. The N-terminal GFP-tagged pGFPC1 vector was used to generate the Parkin UBL WT domain and its point mutant constructs (S65A and S65D). For site-directed mutagenesis, the QuiChange™ kit (Strategene) was used. Human PARK1 WT 3×Myc, kinase-dead human PINK1 K219A, D362A, and D384A 3×Myc, N-terminal HA-tagged human VDAC1, and N-terminal FLAG-tagged human Parkin were generated using the pcDNA3.1 zeo (+) vector. The pRK5 vector was used to express N-terminal HA-tagged human ubiquitin.

Cell Culture and Transfection—HEK293T and HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO2. HeLa cells stably expressing GFP-Parkin (Parkin-57) were cultured in advanced DMEM (Invitrogen) with 10% fetal bovine serum (Invitrogen), 5 µg/ml puromycin (Invitrogen), 200 mM L-glutamine (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO2. Expression plasmids were transfected using Lipofectamine Plus Reagent (Invitrogen) or polyethyleneimine (Sigma) according to the manufacturer’s instructions.

Cell Lysis and Immunoblotting—Cells were prepared in lysis buffer A (25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM sodium vanadate, 2 mM DTT, 1 mM PMSF, 10 g/ml leupeptin, 1 g/ml pepstatin A, and 0.1% Nonidet P-40) for GST pulldown assays. Mitochondrial protein immunoblot analyses were performed using radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 2 mM DTT, 1 mM PMSF, 10 g/ml leupeptin, and 1 g/ml pepstatin A). Lysis buffers were supplemented with the protease inhibitor mixture (Roche Applied Science). Total protein was quantified using the BCA protein assay kit (Pierce). Lysates were subjected to SDS-PAGE analysis followed by immunoblotting according to standard procedures. The blots were developed and visualized using LAS-4000 (Fujifilm).

Ubiquitination Assay—Cells were lysed with Lysis buffer B (2% SDS, 150 mM NaCl, and 10 mM Tris, pH 8.0) with the protease inhibitor mixture (Roche Applied Science). Transfected cells were harvested and boiled for 10 min at 95 °C. Lysis buffer A and lysis buffer B were mixed at a 10:1 ratio. Samples were incubated at 4 °C for 1 h and centrifuged at 16,000 × g for 20 min. Glutathione-Sepharose 4B beads were added to the supernatant of cell lysates and incubated with rotation at 4 °C for 60
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Results

Parkin UBL Domain Interacts with the R1 Domain—Recently, the UBL domain of Parkin has been linked to the autoinhibitory function of Parkin (55). However, the mechanism of how the UBL domain and other domains regulate Parkin activation is not known. To identify the regions of Parkin that contribute to the autoregulatory activity of Parkin, we generated GST-tagged truncation mutants of Parkin (Fig. 1A), overexpressed the constructs in HEK293T cells expressing HA-tagged human ubiquitin, and tested their function in vitro using an autoubiquitination assay. Compared with full-length (FL) Parkin, autoubiquitination activity was increased by Parkin mutants that lack the UBL domain, namely by the UBL domain deletion mutant (∆UBL) or by the C-terminal RING motif, R1-IBR-R2 (C) (Fig. 1B). To determine whether the UBL domain can suppress the function of Parkin, we tested whether overexpression of the UBL domain with truncation mutants could suppress their autoubiquitination activity. Strikingly, the autoubiquitination activity of ∆UBL or C was noticeably lower with overexpression of the UBL domain (Fig. 1C), indicating that Parkin autoubiquitination is negatively regulated by the UBL domain, in agreement with a previous report (55).

We hypothesized that the autoubiquitination activity of Parkin could be suppressed by direct interaction of the UBL domain with the domains in the C terminus of Parkin. To test this hypothesis, we performed co-immunoprecipitation (co-IP) of the GFP-tagged UBL domain with the GST-fused C-terminal regions of Parkin. We confirmed a direct interaction between the UBL domain and C (Fig. 1D) and, moreover, found that the R1 domain is sufficient for this interaction (Fig. 1E). From these data, we concluded that the UBL domain binds to the R1 domain, through which E2 enzymes interact with Parkin substrates (9, 56) to negatively regulate Parkin activity.

Conserved cysteine residues in the R1 domain, including Cys-238, are important for maintaining the structure of the RING-finger motif of the R1 domain (56, 58). Mutation of cysteine 238 to serine (C238S) prevented binding of the UBL domain to the R1 domain (Fig. 1F). This result indicated that the proper folding of the R1 domain is critical for the interaction between the R1 domain and the UBL domain.

PINK1 Negatively Regulates the Interaction between the UBL and the R1 Domain—PINK1 is an upstream kinase of Parkin that enhances Parkin activity by phosphorylation of the UBL domain (44–46). We wanted to determine whether PINK1 regulates the binding between the UBL domain and the R1 domain of Parkin. GST pulldown assays were performed in HEK293T cells overexpressing the GST-tagged Parkin R1 domain and C with the GFP-tagged UBL domain. In CCCP-treated cells (20 μM), the interaction between the UBL domain and C and between the UBL domain and the R1 domain were reduced (Fig. 2, A and B, respectively). To test whether the effect of CCCP treatment is PINK1-dependent, we performed co-IP of the R1 domain and the UBL domain in HEK293T cells overexpressing WT PINK1 or a kinase-dead PINK1 mutant (K219A/D362A/D384A). With CCCP treatment, the UBL domain showed a strong interaction with the R1 domain in cells expressing PINK1 kinase-dead mutant at a level comparable with that in cells with no exogenous PINK1 control, whereas the interaction was weak in WT PINK1-expressing cells (Fig. 2C). Together, these results suggested that PINK1 interferes with the interaction between the UBL domain and the R1 domain of Parkin.

Because PINK1 activates Parkin by phosphorylation at Ser-65 of the UBL domain (44), we tested whether the phosphorylation contributes to the binding between the UBL domain and the R1 domain. To do this we performed co-IP of the R1 domain with the WT UBL domain or derivatives of the UBL domain in which Ser-65 was mutated to alanine or glutamate.

The interaction between the UBL phosphomimetic S65D mutant and the R1 domain was very weak, whereas the interaction between the nonphosphorylatable S65A mutant and the R1 domain was at a level comparable with to WT (Fig. 2D). Furthermore, the interaction between the R1 domain and the UBL S65A mutant did not show significant differences upon treatment with CCCP (Fig. 2E). Thus, we concluded that the interaction between the R1 domain and the UBL domain is regulated by phosphorylation of the UBL domain at Ser-65.

The UBL Domain Negatively Regulates Parkin Activity—To determine whether the weakened interaction between the UBL domain and the R1 domain by phosphorylation at Ser-65 has an effect on Parkin activation, we performed an autoubiquitination assay using FL WT Parkin or FL Parkin with S65A or S65D mutation. FL Parkin with S65D mutation showed stronger autoubiquitination activity compared with FL WT Parkin or FL Parkin with S65A mutation (Fig. 3A), indicating that phosphorylation of Parkin at Ser-65 promotes its E3 ligase activity, in agreement with previous studies (44, 45). Furthermore, overexpression of the UBL domain strongly suppressed autoubiquitination of FL WT Parkin or FL Parkin with S65A or S65D muta-
tion (Fig. 3A). These data suggested that exogenous UBL domain blocks Parkin autoubiquitination activity by an intermolecular interaction.

We also co-expressed FL WT Parkin with the WT UBL domain or the UBL domain with S65A or S65D mutation in HeLa cells, which have little or no endogenous Parkin expression, and treated the cells with 20 μM CCCP to induce Parkin autoubiquitination. As expected, the polyubiquitination level in cells expressing FL Parkin alone was comparable with control, and with CCCP treatment the polyubiquitination levels were
significantly increased (Fig. 3B). The CCCP-induced increase in polyubiquitination was noticeably reduced with co-expression of the WT UBL domain or the UBL domain with S65A mutation but less with co-expression of the S65D mutant (Fig. 3B).

We next sought to determine whether the UBL domain affects Parkin E3 ligase activity by checking the ubiquitination levels of Parkin substrates, VDAC1, Mfn1, and Mfn2. We used a HeLa cell line stably expressing GFP-tagged Parkin and transfected with the GFP-tagged UBL domain with WT, S65A, or S65D mutation. We treated the cells with 10 μM CCCP to measure the polyubiquitination levels of endogenous substrate proteins. As in the case of Parkin autoubiquitination, expression of the UBL domain inhibited the CCCP-induced polyubiquitination of Parkin substrates, VDAC1, Mfn1, and Mfn2 (Fig. 3C).

We also observed that the polyubiquitination of substrates was reduced with expression of the UBL domain with WT Parkin or Parkin with S65A mutation; however, expression of Parkin with S65D mutation did not affect the polyubiquitination level of Parkin substrates (Fig. 3C).

Activated Parkin induces mitophagy (18, 35, 40, 59). We investigated whether expression of the UBL domain may inhibit this process. The WT UBL domain, the UBL domain with S65A mutation, or the UBL domain with S65D mutation was overexpressed in a HeLa cell line stably expressing GFP-Parkin, and the levels of endogenous mitochondrial proteins were analyzed after treatment with or without 10 μM CCCP for 12 h. With CCCP treatment, the protein levels of Mfn1, Mfn2, VDAC1, COXIV, TIM23, and NDUF53 were reduced compared with controls that were not treated with CCCP. Overexpression of the WT UBL domain or the UBL domain with S65A mutation partially prevented the reduction of the protein levels. However, overexpression of the UBL domain with S65D mutation was unable to prevent the CCCP-induced reduction of mitochondrial protein levels (Fig. 3D). Next, we observed mitochondria undergoing CCCP-induced mitophagy by using the mitochondrial marker TOM20 (18, 20). Levels of TOM20 staining were markedly decreased in HeLa cells expressing GST-tagged FL Parkin with CCCP treatment for 12 h (Fig. 3E). In cells co-expressing FL Parkin and GFP-tagged WT or S65A UBL domain, TOM20 staining was not decreased with treatment of CCCP compared with control cells expressing FL Parkin alone; however, in cells co-expressing FL Parkin and S65D UBL domain, TOM20 staining levels were significantly weaker with CCCP treatment compared with control (Fig. 3E). Previous studies have shown that after CCCP treatment Parkin translocates to the mitochondria and ubiquitinates various substrates in the mitochondria and that an adaptor protein containing the LC3B-interaction region, such as p62/SQSTM1, is recruited to the mitochondria followed by recruitment of LC3B (18, 32, 60, 61). This sequence of mitophagy events can be visualized by immunostaining of p62/SQSTM1 and LC3B (18). We further characterized the role of WT, S65A, or S65D UBL domain in the steps leading to Parkin-mediated mitophagy. In HeLa cells overexpressing FL Parkin, we observed co-localiza-
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FIGURE 3. Parkin activity is negatively regulated by its UBL domain. A, autoubiquitination assays for Parkin Ser-65 mutants. GST-tagged FL WT, S65A, or S65D Parkin was co-expressed with GFP-tagged UBL WT, S65A, or S65D domain and HA-tagged human Ub in HEK293T cells. GST pulldown samples were analyzed with anti-HA antibody (first panel) or anti-GST antibody (second panel) as indicated. Whole cell lysates (WCL) were blotted with anti-GFP antibody (third panel) and anti-HA antibody (fourth panel) to detect the same level expression of proteins. B, FLAG-tagged FL Parkin and GFP-tagged WT, S65A, or S65D UBL domain constructs were co-expressed in HeLa cells as indicated. Transfected cells were treated with 20 μM CCCP for 4 h as indicated. Whole cell lysates were prepared and analyzed for immunoblot with anti-Ub antibody (first panel), anti-FLAG antibody (second panel), and anti-GFP antibody (third panel). Immunoblot analyses with anti-β-tubulin antibody were used as loading controls (fourth panel). C, HeLa cells stably expressing GFP-tagged Parkin were transfected with HA-tagged ubiquitin and GFP-tagged UBL WT, S65A, or S65D domain and were treated with 10 μM CCCP for 4 h. Whole cell lysates were quantified and immunoblotted for endogenous mitochondrial proteins using anti-Mfn1, anti-Mfn2, anti-VDAC1, and anti-β-tubulin antibody. Anti-GFP antibody was used to detect the UBL domain and Parkin proteins. D, HeLa cells stably expressing GFP-tagged Parkin were transfected with GFP-tagged UBL WT, S65A, or S65D domain and treated with 10 μM CCCP for 12 h as indicated. Cell lysates were subjected to immunoblot analyses for endogenous mitochondrial proteins using anti-Mfn1, anti-Mfn2, anti-VDAC1, anti-COXIV, or anti-NDUFS3 antibody. Immunoblot analyses with anti-β-tubulin antibody were used as loading controls. Anti-GFP immunoblot was used to detect the UBL domain and FL Parkin proteins. E-G, confocal images of HeLa cells transfected as indicated and treated with 20 μM CCCP for 12 h. Cells expressing both GST-tagged FL Parkin and GFP-tagged UBL were marked by dotted circles. Bars, 20 μm. E, GST-tagged FL Parkin proteins were immunolabeled with anti-GST antibody (red), and the mitochondria were labeled with anti-TOM20 antibody (blue). The WT, S65A, and S65D UBL domain were GFP-tagged (green). F and G, GST-tagged FL Parkin protein was immunolabeled with anti-GST antibody (red). To detect mitophagy, cells were stained with anti-p62/SQSTM1 (p62) antibody (gray, F) or anti-LC3B antibody (gray, G). The WT, S65A, and S65D UBL domain were GFP-tagged (green).

FIGURE 4. The UBL domain delays Parkin translocation to the mitochondria. A, confocal images of Parkin translocation to the mitochondria in HeLa cells. HeLa cells transfected with GST-tagged FL Parkin or GFP-tagged UBL domain constructs (green) were treated with DMSO (top panel) or 20 μM CCCP (bottom panel) for 4 h. GST-tagged Parkin protein was immunolabeled with anti-GST antibody (green), and the mitochondria were labeled with anti-TOM20 antibody (red). Bars, 20 μm. B, HeLa cells were transfected with plasmids expressing GST-tagged FL Parkin and GFP-tagged UBL domain with WT or S65D mutation. The cells were treated with 10 μM CCCP for 4 h and measured for the subcellular localization of GFP-tagged UBL WT or S65D (green). The same cells were also immunostained for GST-tagged FL Parkin with anti-GST antibody (red) and for mitochondria with anti-TOM20 antibody (blue). Bars, 20 μm. C, quantification of the percentage of Parkin localized to the mitochondria. n = 200. Error bars: S.D. *, p < 0.05 by analysis of variance Tukey’s test.
Parkin, Parkin showed less localization to the mitochondria. However, when the UBL S65D mutant was expressed with FL Parkin, Parkin highly localized to the mitochondria (Fig. 4B). Quantitative analyses revealed that of 200 cells that were counted, ~20% of cells showed reduced translocation of FL Parkin to the mitochondria when the WT UBL domain was co-expressed; however, co-expression of the UBL domain S65D mutant showed no difference in the mitochondrial translocation of Parkin compared with control cells that did not express exogenous UBL domain (Fig. 4C). Therefore, we concluded that the UBL domain of Parkin negatively regulates CCCP-induced mitochondrial translocation of Parkin.

The UBL Domain Regulates Interaction between Parkin and Its Substrate VDAC1—The Parkin R1 domain is necessary for Parkin binding to the E2 enzyme or its substrates (62). We found above that polyubiquitination or mitophagy of Parkin substrates, Mfn1, Mfn2, and VDAC are regulated by the direct interaction of the Parkin UBL domain and R1 domain (Fig. 3). This suggested that the interaction may affect Parkin binding to its substrates. Before testing this possibility, we first sought to identify Parkin substrates that directly bind to Parkin among previously reported substrates that showed direct binding, namely Mfn1, Mfn2, VDAC1, and Drp1, by co-IP with FL Parkin (16, 17, 63, 64). We observed that only VDAC1 directly interacts with Parkin (Fig. 5A). Next, to identify which region of Parkin interacts with VDAC1, we examined the ability of Parkin deletion mutants to bind to VDAC1. We found that C (Fig. 5B) and, more specifically, the R1 domain of Parkin, are sufficient for Parkin interaction with VDAC1 (Fig. 5C).

To determine whether this inhibition occurs by interfering with the interaction between the R1 domain and VDAC1, we performed co-IP to measure the interaction between the R1 domain and VDAC1 when the WT UBL domain or the UBL S65A or S65D mutant was co-expressed. With co-expression of the WT UBL domain or the UBL domain with S65A mutation, the interaction between the R1 domain and VDAC1 was noticeably reduced, but co-expression of the UBL domain with S65D mutation showed little or no difference in the ability to interact (Fig. 5D). Thus, we concluded that the UBL domain and VDAC1 competitively binds to the R1 domain of Parkin. Furthermore, these results suggested that the UBL domain of Parkin simultaneously regulates the activation of Parkin E3 ligase activity and the interaction between Parkin and its substrates.

Mutations of the UBL Domain Identified in PD Patients Affect Parkin Activity—To determine whether the UBL domain plays a role in the pathogenesis of Parkinson disease, we utilized five mutants of the UBL domain (K27N, R33Q, R42P, A46P, and K48A) in Parkin that were previously reported in PD patients (62, 65–69).

We observed the intracellular localization of Parkin in HeLa cells expressing FL WT Parkin or FL Parkin with the pathogenic mutations in the UBL domain. As a negative control, we utilized Parkin with T240R mutation in the R1 domain or C431S mutation in the R2 domain, which are known to hinder interaction with the E2 enzyme or prevent activation of Parkin, respectively (9, 62). When treated with CCCP, WT, R42P, and K48A FL Parkin localized to the mitochondria, but K27N, R33Q, and A46P FL Parkin mutants distributed throughout the cytosol (Fig. 6A). Also, in agreement with previous reports (13, 18, 70, 71), the T240R or C431S FL Parkin mutant did not translocate to the mitochondria (Fig. 6A). Quantitative analysis revealed that ~84% of cells showed translocation of WT FL Parkin to the...
mitochondria with CCCP treatment; however, in cells expressing K27N, R33Q, or A46P mutant, Parkin localization to the mitochondria was rare or not detected (Fig. 6B). In cells expressing FL Parkin with R42P or K48A mutation, Parkin was localized to the mitochondria in 67% or 78% of the cells, respectively (Fig. 6B).

To test whether the defect in mitochondrial localization of the pathogenic Parkin mutants is a result of altered interaction between the UBL domain and the R1 domain, we performed an autoubiquitination assay to test the inhibitory function of the UBL domain. The autoubiquitination activity of FL Parkin with R42P or K48A mutation was higher than that of FL WT Parkin without CCCP treatment, indicating that the UBL domain with R42P or K48A mutation loses the ability to tightly regulate Parkin activation (Fig. 7A). We also observed that the autoubiquitination activity of FL WT Parkin and FL Parkin with R42P or K48A mutation was induced by CCCP treatment compared with that in untreated controls (Fig. 7A). However, the autoubiquitination activity of FL Parkin with K27N, R33Q, or A46P mutation was low or barely detectable even with CCCP treatment (Fig. 7A). These results indicated that K27N, R33Q, or A46P mutation in the UBL domain blocks Parkin activation upon CCCP treatment.
We tested whether autoubiquitination levels differ between cells expressing Parkin mutants that do not lead to an increased autoubiquitination, K27N, R33Q, or A46P, and Parkin T240R or C431S mutants with CCCP treatment. We found that autoubiquitination levels were comparable in cells expressing K27N, R33Q, or A46P Parkin mutant and the T240R or C431S mutant controls, even with CCCP treatment (Fig. 7B).

To investigate whether the changes in the autoubiquitination level of the mutant Parkin proteins are due to changed interactions between the R1 domain and the UBL domain, we examined whether the ability of the UBL domain with K27N, R33Q, R42P, A46P, or K48A mutation to bind to the R1 domain is altered compared with the WT UBL domain. Interaction between the R1 domain and the UBL domain with R42P or K48A mutation was decreased compared with the WT UBL domain, as determined by co-IP (Fig. 7C, left). Strikingly, although the interaction between the R1 domain and the WT UBL domain or between the R1 domain and the UBL domain with R42P or K48A mutation was dramatically decreased upon CCCP treatment, the interaction between the R1 domain and the UBL domain with K27N, R33Q, or A46P mutation was not affected by CCCP treatment (Fig. 7C, right). Interestingly, this unaffected interaction between the R1 domain and the UBL domain by CCCP treatment correlated well with the decreased autoubiquitination and mitochondria localization levels of FL Parkin with K27N, R33Q, or A46P mutation (Figs. 7A and 6).

Together, these results strongly suggested that the mechanism of regulating Parkin activation by the interaction between the UBL domain and the R1 domain is linked to PD pathogenesis and that the interaction can be a key target in developing effective treatments against Parkin-mediated pathogenesis.

Discussion

In this study we presented evidence that the R1 domain and the UBL domain of Parkin interact and that this interaction leads to reduced Parkin E3 ligase activity. We also showed that the R1 domain directly binds to VDAC1 and that competitive binding of the UBL domain to the R1 domain prevents Parkin translocation to the mitochondria. Moreover, we demonstrated that PINK1-dependent phosphorylation of Parkin at Ser-65 negatively regulates the interaction between the R1 domain and the UBL domain, suggesting a novel mechanism of how PINK1 regulates Parkin activation (Fig. 8).
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Various proteins and functional domains, including ubiquitin-associated proteins (UBAs), ubiquitin-binding domains (UBPs), ubiquitin binding domains (UBDs), and UBLs share structural similarities with ubiquitin and consist of a Leu-8–Ile-44–Val-70 hydrophobic patch that interacts with ubiquitin or other target proteins (54, 55, 72–74). The UBL domain of Parkin harbors a hydrophobic patch with a similar structure with conserved Ile-44 and Val-70 residues. Recently, the determination of the structure of rat FL Parkin by low resolution x-ray crystallography raised the possibility that the R1 domain may interact with the UBL domain via the Ile-44 residue of the hydrophobic patch and the surrounding hydrophobic surface (56). We tested whether the hydrophobic patch in the UBL domain is required for binding between the R1 domain and the UBL domain by generating UBL domain mutants where the Ile-44 or Val-70 residue was mutated to an alanine. Using co-IP and autoubiquitination assays, we showed that residues 1–35 of the UBL domain are necessary for binding of the UBL domain to the R1 domain. Three-dimensional structural analysis of the Parkin UBL domain obtained from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank revealed that four amino acid residues of the UBL domain, Phe-4, Arg-6, Pro-14, and Glu-16, are externally exposed and lie in the same plane (75, 76). Furthermore, through sequence analysis, we found that the four amino acid residues are conserved in humans and Drosophila. To investigate whether these residues are required for binding of the UBL domain to the R1 domain, we mutated Phe-4, Arg-6, Pro-14, and Glu-16 into alanines and observed that the interaction between the R1 domain and the UBL domain was abolished. From this we concluded that the N terminus of the UBL domain mediates binding to the R1 domain.

As phosphorylation of the Parkin UBL domain at Ser-65 leads to a decreased interaction between the R1 domain and the UBL domain, we investigated whether Ser-65 phosphorylation affects the amino acid residues that participate in binding of the two domains. To do so we measured the distance between the residues in a three-dimensional configuration. Interestingly, Ser-65 was located not far from the Phe-4 and Arg-6 residues of Parkin, suggesting the possibility that phosphorylation at Ser-65 may affect the interaction between the R1 domain and the UBL domain by interfering these two amino acid residues.

Recently, it has been reported that phosphorylation of Parkin Ser-65 forms a cleft in the three-dimensional structure formed between the UBL domain and C and that the cleft is filled with an increased number of water molecules (77). With the conformational change, the distance between the UBL domain and the R1 domain increased from 20 Å to >50 Å, and the binding site of ubiquitin-conjugated E2 increased within the R1 domain is revealed, leading to docking of the UbcH5a/UBE2D1 E2 enzyme to the R1 domain. This promotes Parkin ubiquitination at Cys-431 and its E3 ligase activity (77).

In agreement with these structural analyses, we found that phosphorylation of Parkin UBL domain at Ser-65 by PINK1 leads to a decreased interaction between the R1 domain and the UBL domain (Fig. 2D). Consistent with this finding, the UBL domain with S65D phosphomimetic mutation failed to suppress polyubiquitination of Parkin endogenous substrates, in contrast to wild-type or the UBL domain with S65A mutation (Fig. 3C). Moreover, we further demonstrated that interaction between Parkin R1 domain and Parkin substrate VDAC1 is regulated by Ser-65 phosphorylation of the UBL domain. Collectively, we provided evidence that PINK1 phosphorylation of Parkin at Ser-65, by preventing binding between the UBL domain and the R1 catalytic region, exposes the R1 domain and, consequently, promotes Parkin E3 ligase activity and translocation to the mitochondria (Fig. 8).
Regulation of Parkin by R1 and UBL Domains

The PINK1 phosphorylation site in the UBL domain is conserved in ubiquitin; ubiquitin is also phosphorylated by PINK1 at Ser-65 (78, 79). Phosphorylation of ubiquitin at Ser-65 leads to increased activation of FL Parkin (78, 79). Furthermore, Parkin activation is increased in an in vitro ubiquitin assay when phospho-ubiquitin is coexpressed with WT or S65A mutant of FL Parkin (78, 79). For Parkin to be fully activated, phosphorylation at the Ser-65 residue of both ubiquitin and Parkin UBL domain is required (80 – 84). In a recent study the crystal structure of phospho-ubiquitin and a UBL domain-deleted mutant Parkin (amino acids 140 – 461) of Pediculus humanus corporis was reported (85). This report suggested the possibility that Parkin activation may be regulated not only by interaction between the R1 domain and the UBL domain but also by interaction between the R1 domain and phospho-ubiquitin (85). In this study we showed that binding to the R1 domain is decreased when the UBL domain is phosphorylated at Ser-65 (Fig. 2) and that activation of FL Parkin is suppressed when co-expressed with the WT UBL domain or the UBL domain with S65A mutation, but not efficiently (Fig. 3). This may be explained by the fact that phospho-ubiquitin also regulates Parkin activation. Further study is needed to address whether phosphorylation of Ser-65 in ubiquitin, in addition to phosphorylation of Ser-65 in the UBL domain, regulates the interaction between the R1 domain and the UBL domain of Parkin.

We found that the interaction between the R1 domain and the UBL domain with K27N, R33Q, or A46P mutation was not dissociated by CCCP treatment (Fig. 7C). The tight interaction between the R1 domain and the UBL domain with K27N, R33Q, or A46P mutation in the presence of CCCP prevents Parkin translocation to the mitochondria and inhibits Parkin autoubiquitination (Figs. 6 and 7A). These data suggested that, of the mutations found in PD patients, K27N, R33Q, or A46P mutation in the UBL domain leads to a continuous interaction of the UBL domain with the R1 domain and that the UBL domain interferes with interaction of the R1 domain with its substrates or E2 enzymes to prevent Parkin activation. From this we suggest a new mechanism of PD pathogenesis in which failure to regulate the interaction between the R1 domain and the UBL domain to regulate Parkin activation leads to the development of PD.

In summary, our findings indicated that the interaction between the R1 domain and the UBL domain is critical for proper regulation of Parkin function and that interruption of this regulation may lead to PD pathogenesis. Given the importance of modulating the binding between two domains in Parkin, development of a new drug that can regulate this interaction may offer exciting possibilities of hindering PD pathogenesis by regulation of Parkin activity and of controlling various cellular processes, such as mitochondrial homeostasis, mitochondrial dynamics, and mitophagy.

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