Site-specific Interaction Mapping of Phosphorylated Ubiquitin to Uncover Parkin Activation*

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Background: Phosphorylation of both Parkin and ubiquitin by PINK1 is crucial for Parkin E3 ligase activity; however, the mechanism remains unknown.

Results: Site-specific photo-crosslinking identified the phosphorylation-dependent interaction surface between Parkin and ubiquitin.

Conclusion: IBR along with RING1 domain of Parkin provides an interaction site for ubiquitin.

Significance: A novel binding mechanism with phosphorylated ubiquitin leads to a Parkin conformational change.

Damaged mitochondria are eliminated through autophagy machinery. A cytosolic E3 ubiquitin ligase Parkin, a gene product mutated in familial Parkinsonism, is essential for this pathway. Recent progress has revealed that phosphorylation of both Parkin and ubiquitin at Ser65 by PINK1 are crucial for activation and recruitment of Parkin to the damaged mitochondria. However, the mechanism by which phosphorylated ubiquitin associates with and activates phosphorylated Parkin E3 ligase activity remains largely unknown. Here, we analyze interactions between phosphorylated forms of both Parkin and ubiquitin at a spatial resolution of the amino acid residue by site-specific photo-crosslinking. We reveal that the in-between-RING (IBR) domain along with RING1 domain of Parkin preferentially binds to ubiquitin in a phosphorylation-dependent manner. Furthermore, another approach, the Fluoppi (fluorescence-based technology detecting protein-protein interaction) assay, also showed that pathogenic mutations in these domains blocked interactions with phosphomimetic ubiquitin in mammalian cells. Molecular modeling based on the site-specific photo-crosslinking interaction map combined with mass spectrometry strongly suggests that a novel binding mechanism between Parkin and ubiquitin leads to a Parkin conformational change with subsequent activation of Parkin E3 ligase activity.

Two gene products mutated in autosomal recessive forms of familial Parkinsonism, Parkin and PINK1§ (1, 2), have been identified as essential proteins for eliminating damaged mitochondria through autophagy machinery in a process called mitophagy (3, 4). In healthy mitochondria, a serine/threonine kinase PINK1 is imported to mitochondria in accordance with its N-terminal mitochondrial targeting sequence. After processing by mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like (PARL) proteases (5–8), cleaved PINK1 is retrotranslocated to the cytosol and rapidly degraded by the proteasome through an N-end rule pathway, thereby maintaining low PINK1 levels (9). In contrast, when mitochondrial membrane potential is disrupted, PINK1 is stabilized on the outer membrane with its kinase domain exposing the cytosolic face (10, 11). Another gene product linked to familial Parkinsonism, Parkin, is an E3 ubiquitin ligase that is normally localized throughout the cytosol as an inactivated form (12, 13). The role of E3 ubiquitin ligases is to mediate ubiquitin transfer from an E2 ubiquitin-conjugating enzyme to substrate proteins. E3 ligases are classified into three groups based on the protein structure: HECT, RING, and RBR (RING-between-RING). Parkin is a member of the RBR-type ligases. Although RBR-type ligases are typically composed of three conserved structural domains called RING1, IBR (in-between-RING), and RING2, Parkin has two additional subdomains sit-

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4 The abbreviations used are: PINK1, PTEN-induced putative kinase 1; TcPINK1, Tribolium castaneum PINK1; PTEN, phosphatase and tensin homolog; IBR, in-between-RING; RBR, RING-between-RING; REP, repressor element of Parkin; Ub, ubiquitin; Ubl, ubiquitin-like; HAG homo-tetramer Azami-GFP; Ash, homo-oligomerized protein assembly helper; BPA, p-benzoyl-phenylalanine; Ni-NTA, nickel-nitritrocetic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; aa, amino acid(s); Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; AMBC, ammonium bicarbonate; ACN, acetonitrile; CBB, Coomassie Brilliant Blue; IB, immunoblot.
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uated at the N terminus termed Ubl (ubiquitin-like) and RING0 and a third subdomain termed REP (repressor element of Parkin) between the IBR and RING2 domains (see Fig. 1A). Based on the crystal structure of Parkin, the catalytic Cys431, which forms a thioester intermediate with ubiquitin, and the E2 binding site in the RING1 domain are occluded by the RING0 and REP domains, respectively, indicating that Parkin E3 ligation activity is normally intramolecularly autoinhibited (14–17). Following activation, cytosolic Parkin translocates to depolarized mitochondria (18) where it ubiquitinates various mitochondrial outer membrane proteins (19–21).

Recent accumulating evidence has shown that PINK1 stabilized on the outer membrane phosphorylates both the Ubl domain of Parkin and ubiquitin at Ser65 to convert inactivated Parkin to the fully activated form (22–27). Furthermore, phosphorylated Parkin selectively associates with phosphorylated ubiquitin, and PINK1 promotes phosphorylation of poly-ubiquitin chain as well as mono-ubiquitin. Based on these results, a positive feedforward ubiquitination cycle has been proposed to accelerate Parkin translocation onto mitochondria as well as robust ubiquitination of mitochondrial outer membrane proteins (28–30). Therefore, understanding the molecular mechanisms underlying the critical initial step for mitophagy (i.e. how phosphorylated ubiquitin binds to phosphorylated Parkin and how the interaction promotes Parkin E3 ubiquitin ligation activity) is of significant interest. When the C-terminal glycine of ubiquitin is activated through an E1-E2 cascade reaction, Parkin can load the ubiquitin on its catalytic Cys431 via ubiquitin-thioester intermediate (14, 24, 31, 32). However, both Parkin Cys431 and the ubiquitin C-terminal glycine are dispensable for the interaction between phosphorylated Parkin and ubiquitin (27). Therefore, an uncharacterized, phosphorylation-dependent interaction site between Parkin and ubiquitin must exist.

Experimental Procedures

Antibodies—The following antibodies were used for immunoblotting: mouse anti-Parkin (Sigma, clone PRK8, P6248), mouse anti-ubiquitin (Santa Cruz Biotechnology, clone P4D1, sc-8017), mouse anti-GST (Santa Cruz Biotechnology, clone B-14, sc-138), and mouse anti-HA (MBL, clone TANA2, M180-3). Rabbit anti-TOMM20 (Santa Cruz Biotechnology, sc-11415) antibody was used for immunostaining.

Plasmids—For in vivo site-specific crosslinking, GST-rat Parkin (S65E) and His6-human ubiquitin (S65D) genes were subcloned into the NcoI/NotI and NdeI/Xhol sites of pET-Duet-1 vector (Novagen), respectively. To prepare recombinant GST-rat Parkin, GST-tagged Tribolium castaneum PINK1 (GST-TcPINK1), and His6 human ubiquitin, pGEX6P1-rat Parkin, pGEX6P1-TcPINK1 (gifts from Jean-François Trempe), and pT7-7/His-Ub plasmids were used. For preparation of p-β-phenylalanine (BPA)-incorporated recombinant GST-rat Parkin, first, the stop codon in pGEX6P1-rat Parkin plasmid (originally an amber codon) was changed to TAA (ochre codon), and then the amber codon was introduced at various amino acid positions by primer-based PCR mutagenesis.

pEVOL-pBpF (Addgene, Plasmid 31190) was used for expressing the orthogonal pair of the amber suppressor tRNA and its BPA aminoacyl tRNA synthetase in bacterial cells.

In Vivo Site-specific Photo-crosslinking—Escherichia coli BL21(DE3) cells harboring pEVOL-pBpF and pETDuet/GST-rat Parkin (S65E, Xamber, where X indicates amino acid residues replaced with the amber codon) and His6-human ubiquitin (S65D) were grown in 8 ml of LB medium supplemented with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol at 37 °C until an A600 of ~0.8. The cell culture was then shifted to 16 °C before adding 0.1%(w/v) arabinose (Sigma) and 1 mM BPA (Bachem). BPA-incorporated-GST-rat Parkin (S65E) and His6-human ubiquitin (S65D) were expressed by the addition of 50 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 25 µM ZnCl2 for 16 h at 16 °C. The bacterial culture was split in half with one aliquot subjected to UV irradiation (365 nm) using a B-100AP (UVP) for 5 min. The cell pellet after centrifugation was stored at ~80 °C until needed. The frozen cell pellet was solubilized with 500 µl of B-PER bacterial protein extraction reagent (Thermo Scientific) supplemented with 100 µg/ml lysozyme, 5 units/ml DNase I, and protease inhibitor cocktail (Roche Applied Science) for 10 min at room temperature. The supernatants were clarified by centrifugation and mixed with 500 µl of solubilization buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 6 M urea, 0.5%(v/v) Triton X-100) and 80 µl of 50%(v/v) equilibrated Ni-NTA agarose (Qiagen)). His6-human ubiquitin (S65D) and its crosslinked products were bound to Ni-NTA by mixing for 15 min at room temperature and then washed with 1 ml of wash buffer (solubilization buffer containing 20 mM imidazole) for four times. Proteins were eluted with 40 µl of SDS-PAGE sample buffer.

Preparation of Recombinant Proteins—GST-rat Parkin, GST-TcPINK1, and His6-human ubiquitin were overexpressed in E. coli BL21(DE3) cells from the aforementioned plasmids. The transformants were grown to A600 ~0.8 in LB medium supplemented with 100 µg/ml ampicillin at 37 °C. GST-rat Parkin and GST-TcPINK1 were induced by the addition of 25 µM IPTG for 12 h at 16 °C. For GST-rat Parkin expression, 25 µM ZnCl2 was also added. Bacterial cells were harvested by centrifugation, resuspended in TBS (50 mM Tris-HCl, pH 7.5, 120 mM NaCl) supplemented with protease inhibitor cocktail (Roche Applied Science) and 30 µg/ml lysozyme (Sigma), frozen in liquid nitrogen, and stored at ~20 °C. The thawed cell resuspension was sonicated (Advanced-Digital Sonifier, Branson), and cell debris and insoluble proteins were removed by centrifugation (12,000 × g, 20 min, 4 °C). The soluble fraction was then mixed with glutathione-Sepharose 4B (GE Healthcare) for 30 min at 4 °C. The Sepharose was loaded onto a column and washed with TBS buffer, and GST-tagged proteins were eluted with TBS containing 20 mM L-glutathione reduced (Sigma). 1-β-Glutathione was removed either by dialysis or via a PD MidiTrap G-25 (GE Healthcare). Samples were concentrated using Amicon Ultra centrifugal filters (Millipore). GST-rat Parkin (45 µm) was dissolved in TBS supplemented with 1 mM DTT and 10%(w/v) glycerol, whereas GST-TcPINK1 (12 µm) was dissolved in TBS supplemented with 10%(w/v) glycerol. His6-human ubiquitin was induced with 1 mM IPTG at 37 °C for 3 h. The soluble cell fraction prepared as described above was mixed with Ni-NTA and washed with TBS, and the His6-human ubiquitin was
eluted using 200 mM imidazole. After dialysis and protein concentration, His$_6$-human ubiquitin (750 μM) was dissolved in TBS and stored at −80 °C.

**In Vitro Kinase Reaction with Recombinant TcPINK1**—
Recombinant His$_6$-human ubiquitin was incubated with recombinant GST-TcPINK1 at a molar ratio of 20:1 in kinase buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 10 mM ATP, 50 mM MgCl$_2$, 2 mM DTT) at 32 °C for 80 min. Samples were solubilized with SDS-PAGE sample buffer. In the case of Parkin phosphorylation, recombinant GST-rat Parkin was incubated with GST-TcPINK1 at a molar ratio of 2:1 in the presence of recombinant His$_6$-human ubiquitin.

**Phos-tag PAGE**—To separate phosphorylated proteins from non-phosphorylated proteins on SDS-PAGE, samples were loaded onto 7.5 or 14% polyacrylamide gels containing 100 μM MnCl$_2$ and 50 μM Phos-tag acrylamide (Wako). After electrophoresis, proteins were stained using an Oriole fluorescent gel staining dye (Bio-Rad) or immunoblotted using anti-ubiquitin antibodies and a 5-bromo-4-chloro-3-indolyl phosphate-nickel conjugated goat anti-mouse or anti-rabbit IgG primary antibodies. Proteins were detected using alkaline phosphatase-conjugated goat anti-mouse IgG antibody and then with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes). The images of the cells were obtained using a LSM710 confocal microscope (Carl Zeiss). For image analysis, ZEN2011 (Carl Zeiss) and Photoshop (Adobe) were used.

**Immunostaining**—Parkin-transfected HEK293T cells grown on 35-mm glass bottom dishes were treated with 10 μM valinomycin (Sigma) for 2.5 h. The cells were fixed with 4% paraformaldehyde in PBS for 25 min at room temperature and permeabilized with 0.15%(v/v) Triton X-100 in PBS for 15 min. After blocking, the fixed cells were incubated with anti-TOMM20 antibody and then with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes). The images of the cells were obtained using a LSM710 confocal microscope.

**Mass Spectrometry and Quantification of Peptides**—GST-rat Parkin (WT) was crosslinked to His-ub (I36BPA) after incubation with GST-TcPINK1. GST-rat Parkin (S65E) was crosslinked to His-ub (S65D, R42BPA) or His-ub (S65D, R72BPA). The appropriate amounts of the crosslinked samples, as well as GST-Parkin (WT and S65E) alone, were subjected to SDS-PAGE followed by CBB staining. CBB-stained bands were gel excised and destained using 50 mM ammonium bicarbonate (AMB), 30%(v/v) acetonitrile (ACN) with agitation for 1 h. The gel pieces were further washed with 50 mM AMB, 50%(v/v) ACN for 1 h and then reduced with 10 mM DTT in 50 mM AMB buffer for 1 h at 56 °C. After washing with 50 mM AMB buffer, cysteine residues were alkylated with 55 mM iodoacetamide for 45 min at room temperature. Samples were washed once with 50 mM AMB buffer, washed twice with 50 mM AMB, 50%(v/v) ACN, and then dehydrated with 100% ACN. Proteins were subjected to in-gel trypsin digestion using 20 ng/μl Trypsin Gold (Promega) in 50 mM AMB, 5%(v/v) ACN buffer at 37 °C overnight. The resulting peptides were solubilized in 0.1% trifluoroacetic acid and analyzed using a LC/MS system.
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nanoflow ultra-HPLC (EASY-nLC, Thermo Scientific) coupled to a Q Exactive mass spectrometer (Thermo Scientific). The mobile phases were 0.1% formic acid in water (solvent A) and 0.1% formic acid in 100% ACN (solvent B). Peptides were directly loaded onto a C18 analytical column (ReproSil-Pur 3-μm, 75-mm inner diameter × 12-cm packed tip column, Nikkyo Technos) with a flow rate of 300 nl/min. For ionization, 1.8 kV of liquid junction voltage and a capillary temperature of 250 °C were used. For peptide identification, MS spectra were analyzed using Protein Discoverer version 1.3 (Thermo Scientific). The fragmentation spectra were searched using amino acid sequences of GST-rat Parkin and His6-human ubiquitin. Methionine oxidation and cysteine carbamidomethylation were chosen as static modifications for database searching. For quantification, raw files were processed using Pinpoint version 1.3 (Thermo Scientific).

Computational Modeling of Fully Activated Parkin with Phosphorylated Ubiquitin—The proposed model for fully activated Parkin with phosphorylated ubiquitin was constructed using the protein-protein docking and molecular dynamics procedure as follows. We first simulated protein-protein docking of phosphorylated ubiquitin (Protein Data Bank ID code: 4wzp) with an inactivated Parkin structure (Protein Data Bank ID code: 4k95). ClusPro version 2.0 (33) was used to generate docked conformations with the lowest docking energies and clustering properties. Phosphorylated Ser65 in phosphorylated ubiquitin was computationally mutated to glutamate because the ClusPro program only supports native amino acids. During the protein-protein docking procedure, photo-crosslinking data were utilized as constraints for residues (Ile36, Arg42, and Arg72 of phosphorylated ubiquitin and Tyr315–Arg344 and Val350–Arg366 of Parkin) involved in complex formation of the protein-protein interaction interface. The best model in the first step was selected using criteria consisting of: docking energy, number of cluster members, and compatibility with the BPA photo-crosslinking experimental results. In the second step, the best model in the first step was subjected to 100 ns molecular dynamics-based energy minimization using Desmond version 3.8 (34); this generated the final model. To prepare the protein structure for molecular dynamics, the missing loop regions of Parkin were constructed using Prime (Schrödinger, LLC). Because the missing loop region from Pro73 to Lys140 is long and predicted to be disordered, we truncated the loop by 10 residues leading out of the Ubl domain and 10 residues leading into the RING0 domain. Ser65 in the Parkin Ubl domain was also computationally mutated to phosphorylated serine to generate activated Parkin. The OPLS2005 force field was used for simulations. Initial model structures were placed into TIP3P water molecules solvated with 0.15 M NaCl. After minimization and relaxation of the model, the production molecular dynamics phase was performed for 100 ns in an isothermal-isobaric (NpT) ensemble at 300 K and 1 bar using Langevin dynamics. Long-range electrostatic interactions were computed using the Smooth Particle Mesh Ewald method. All system setups were performed using Maestro (Schrödinger, LLC). All molecular figures were generated using PyMOL (Schrödinger, LLC).

Results

RING0-RING1-IBR of Parkin Is Important for the Interaction with Ubiquitin—To identify the minimal Parkin domains required for the interaction with phosphorylated ubiquitin, we applied the Fluoppi (fluorescent-based technology detecting protein-protein interaction) technique in which protein interactions are detected as foci in mammalian cells. We previously reported that the Ser65 phosphomimetic pair of Parkin and ubiquitin, but not the WT pair, formed Fluoppi-mediated foci (27). We first confirmed that Ash-tagged phosphomimetic Parkin (Ash-Parkin (S65E)) and hAG-tagged phosphomimetic ubiquitin (hAG-Ub (S65D)) formed foci in HeLa cells (Fig. 1B). When Ash-Parkin (S65E) with Ub, REP, or RING2 domain deletions was expressed with hAG-Ub (S65D), foci formed with similar efficiency to full-length Ash-Parkin (Fig. 1, B and C), suggesting that these domains are not required for the interaction with ubiquitin. In contrast, deletion of the RING0, IBR, or RING1 domains impeded foci formation (Fig. 1B). Next, we examined whether pathogenic mutations in the RING0-RING1-IBR region affected the physical interaction between Parkin and ubiquitin. Foci were apparent in cells co-expressing hAG-Ub and Ash-Parkin with R234Q, R256C, N273S, D280N, G328E, T351P, or R334C mutations (Fig. 1, B and C), whereas R275W mutation reduced the numbers of foci. In sharp contrast, L283P, G284R, and C352G mutations inhibited foci formation (Fig. 1, B and C). Because Cys284 participates in zinc coordination, the C352G mutation likely disrupts the conformational integrity of the IBR domain. Orientation of the Leu283 and Gly284 side chains toward the exterior of the structure suggests that these residues may be directly involved in ubiquitin binding. Taken together, these results indicate that the Parkin RING0-RING1-IBR region is important for interacting with phosphorylated ubiquitin and potentially functions to provide a binding surface or to induce conformational flexibility to facilitate stable interactions with phosphorylated ubiquitin.

Identification of the Parkin-Ubiquitin Interaction Surface via Site-specific Photo-crosslinking—To clarify the interaction site between Parkin and ubiquitin in more detail, we applied a site-specific photo-crosslinking technique (35). This technique enables us to introduce a photo-reactive crosslinker, BPA, at desired amino acid positions within a target protein in bacterial cells by using an orthogonal pair of amber suppressor tRNA and its cognate aminoaicyl-tRNA synthetase specific for BPA (BpaRS) (36). Subsequent UV irradiation results in covalent bonding of BPA with nearby proteins. Therefore, BPA photo-crosslinking can capture protein-protein interactions at a spatial resolution of amino acid level even if the interaction is weak or transient. A phosphomimetic GST-rat Parkin (GST-rat Parkin (S65E)) gene with an amber codon in a desired position was inserted into pETDuet vector together with a phosphomimetic His6-tagged ubiquitin (His6-Ub (S65D)) gene. The plasmid was then introduced into bacterial cells that have the amber suppressor tRNA and BpaRS under an arabinose-inducible promoter. The GST-rat Parkin (S65E, K220amb) fragment, which has an amber codon at Lys220 position, was converted to the full-length form only when both arabinose and BPA were added to culture medium, indicating that BPA was precisely incorpo-
rated into the amber codon position (Fig. 2A). Because Fluoppi analysis indicated that the RING0-RING1-IBR domains are important for ubiquitin binding, we replaced various amino acids in this region with BPA. After UV irradiation, bacterial cells were subjected to immunoblotting, which revealed that when BPA was introduced at residues 266, 282, 327, 329, 344, 355, 356, and 357 in GST-rat Parkin (S65E), bands migrating at ~80 kDa were detected in a UV-dependent manner with anti-Parkin antibody (Fig. 2B). The absence of the crosslinked bands in cells lacking exogenous ubiquitin expression confirmed that the crosslinked products were ubiquitin adducts (Fig. 2C). Despite relatively weak intensities, quantification of the crosslinked products indicated that BPA at residues 220, 222, 224, 269, 270, 279, 305, 320, 342, and 347 was also preferentially crosslinked to His-Ub (Fig. 2D). Mapping the ubiquitin-interacting residues onto the inactivated Parkin structure revealed that they segregated across several regions (Fig. 2E). The amino acid positions Met227, Gly329, Glu344, Gly355, Asn356, and Gly357, whose replacement with BPA generated robust crosslinked products, are clustered within a region on the edge of the IBR domain that is located on the opposite side of the Ubl domain. Other crosslinked residues (score >0.5), Leu266 and Gln282, are on the α-helix of RING1 domain that directly interacts with Ile44 of the Ubl domain and on the small β-hairpin loop in the RING1 domain, respectively. Weak crosslinked residues (score between 0.25 and 0.5) such as Lys220, Thr222, and Val224 are in the RING0 domain.

Next, we tested whether these crosslinked products are PINK1 phosphorylation-dependent. For this purpose, we performed in vitro photo-crosslinking using recombinant GST-rat Parkin harboring BPA at the desired positions in conjunction with recombinant GST-TcPINK1 (37) and recombinant His-Ub (Fig. 3A). GST-rat Parkin and His-Ub were subjected to in vitro kinase reaction with or without GST-TcPINK1 prior to gel filtration analysis. We confirmed that PINK1 efficiently phosphorylates both Parkin and ubiquitin in vitro (Fig. 3B, IB: Parkin and IB: Ub) as reported previously (22, 26). Furthermore, results from the gel filtration analysis indicated that a stable complex only formed when both Parkin and ubiquitin were phosphorylated (Fig. 3B and C). This result is consistent with previous isothermal calorimetry methods, which found that the phosphorylated Parkin and ubiquitin pair bound each other tightly with a calculated $K_d \sim 17$ nM (29). We then performed in vitro photo-crosslinking using GST-rat Parkin with BPA incorporated at 218, 222, 224, 266, 269, 327, 329, 355, or 357 (Fig. 3D). BPA at residues 327, 329, 355, and 357 in the IBR domain effectively crosslinked with His-Ub in a PINK1 phosphorylation-dependent manner. BPA at residues 222 and 224 also crosslinked with His-Ub, although less efficiently (Fig. 3D and E). On the other hand, Leu266, which exhibited significant crosslinking capability in bacterial cells (Fig. 2B and D), did not generate a strong crosslinked signal (Fig. 3D and E). In summary, crosslinking occurs in a PINK1-dependent manner, and the crosslinking efficiencies in bacterial cells and in vitro are compatible except for Leu266.
Next, to determine which region of ubiquitin interacts with Parkin, we prepared recombinant BPA-incorporated ubiquitin (Fig. 4A) and performed in vitro photo-crosslinking. We unexpectedly found that His-Ub containing BPA at residues 42, 45, 44, 49, 70, and 72 was not efficiently (≤H1102120%) phosphorylated by GST-TcPINK1 (Fig. 4B and C). These residues are located adjacent to the β-sheet that is centered at Ile44 (Fig. 4D). Because the BPA side chain is bulky, we assume that steric hindrance blocks the phosphorylation by PINK1. Although further analysis is required, the ubiquitin Ile44 patch (38) may be important for association with PINK1 prior to phosphorylation. We next sought to further define the ubiquitin interaction surface with Parkin. When the position of BPA was varied, crosslinking to Parkin was the most evident at residue 36 with 70% crosslinking efficiency (Fig. 5A and B). In addition to this, BPA introduction into other ubiquitin residues also resulted in crosslinking to Parkin (Fig. 5A and B). In the case of the amino acid residues where the introduction of BPA inhibited Ser65 phosphorylation (Fig. 4C), we substituted the phosphomimetic pair of GST-Parkin and His-Ub (Fig. 5C and D). As the crosslinked efficiency of His-Ub I36BPA (and also R54BPA) to Parkin was comparable between phosphorylated and phosphomimetic Parkin-Ubiquitin Interaction upon Phosphorylation

FIGURE 2. Site-specific photo-crosslinking using BPA-incorporated Parkin in bacterial cells. A, bacterial cells harboring the plasmid encoding His-Ub (S65D) and GST-rat Parkin (S65E) with no amber mutations (none) or an amber mutation at position Lys220 (K220) were cultured under the indicated conditions. The total cell lysates were analyzed by immunoblotting with anti-GST antibody. Asterisks, double asterisks, and triple asterisks represent N-terminal truncated GST-rat Parkin with BPA incorporated at Lys220, a GST-rat Parkin fragment truncated at Lys220, and an N-terminal truncated GST-rat Parkin fragment truncated at Lys220, respectively. B, GST-rat Parkin (S65E) with BPA at the indicated amino acid positions and His-Ub (S65D) were expressed in bacterial cells. The cell culture was split into two with one aliquot subjected to UV irradiation. GST-rat Parkin (S65E) and its crosslinked products were immunoblotted using anti-Parkin antibody. His-Ub (S65D) was detected by CBB staining. Arrowheads indicate crosslinked products of GST-rat Parkin (S65E) and His-Ub (S65D). Asterisks denote GST-rat Parkin (S65E) translated from an ATG codon other than the first one. w/o denotes GST-rat Parkin (S65E) without BPA incorporation. Molecular mass markers are shown to the right as kDa. C, GST-rat Parkin (S65E)-incorporated BPA at the indicated amino acid positions was expressed with or without His-Ub (S65D) in bacterial cells. The cell cultures were subjected to UV irradiation. GST-rat Parkin (S65E) and its crosslinked products were analyzed by immunoblotting with anti-Parkin antibody. His-Ub (S65D) was detected by CBB staining. Arrowheads indicate crosslinked products of GST-rat Parkin (S65E) and His-Ub (S65D). Asterisks denote GST-rat Parkin (S65E) translated from an ATG codon other than the first one. D, the crosslinked products in B were quantified. The amounts of crosslinked products (arrowheads in B) relative to those of GST-rat Parkin without UV irradiation are indicated. E, the relative amounts of crosslinked products with His-Ub (S65D) in D were mapped onto the corresponding amino acid residues in the rat Parkin structure (Protein Data Bank (PDB) ID: 4k95) via white-blue gradation. The RING0-RING1-IBR domains are shown as molecular surface projections. Ser65 residue is shown in green.
metric forms, we directly compared the crosslinking efficiency between the results generated in Fig. 5, B and D. Mapping the Parkin-interacting residues onto a phosphorylated ubiquitin structure revealed that Lys6, Thr7, Leu8, Gly10, Lys11, Ile36, Arg42, and Arg72 cluster to form the surface that preferentially interacts with Parkin (Fig. 5E). However, BPA at residues 16 and 63, which are located on the other side of ubiquitin, was also efficiently crosslinked (Fig. 5E).

Site-specific Crosslinking Combined with Mass Spectrometry Identifies Contact Points between Parkin and Ubiquitin—In general, the carbonyl oxygen of the benzophenone group of BPA reacts with any carbon-hydrogen bond of a nearby protein, which means that it is difficult to differentiate the crosslinked region of the partner protein at the amino acid level. However, we assumed that, when compared with non-crosslinked Parkin, a trypsin-treated peptide of Parkin that directly crosslinked to BPA would be shifted. To test this hypothesis, we utilized quantitative mass spectrometry (Fig. 6A). The His-Ub containing BPA at residues 36, 42, and 72 was crosslinked to GST-Parkin, and following SDS-PAGE separation, the resulting crosslinked bands were subjected to in-gel trypsin digestion followed by mass spectrometry analysis. When compared with peptides derived from GST (198–218 aa) and Parkin (7–27 aa), the amount of the peptide corresponding to 350–366 aa of Parkin in the His-Ub (I36BPA) crosslinking band was significantly reduced when compared with that of non-crosslinked GST-Parkin. Moreover, the amount of the Parkin 315–334 aa peptide in the His-Ub (R42BPA) or His-Ub (R72BPA) crosslinked products was also reduced when compared with the control non-crosslinked GST-Parkin (S65E). A ubiquitin-derived peptide (12–27 aa) was present only in the crosslinked samples, indicating that these bands contained ubiquitin. These results indicate that the spatial coordinates of ubiquitin Ile36 are in close proximity to the IBR 350–366-aa region of Parkin, and that ubiquitin Arg42 and Arg72 are in close proximity to 315–334 aa, which corresponds to the RING1-IBR junction of Parkin. Of note, BPA introduced into these peptides, at 355, 356, 357, 327, and 329, was crosslinked to ubiquitin with high efficiency (Figs. 2D and 3E).

Model of Interaction between Phosphorylated Parkin and Phosphorylated Ubiquitin—Ubiquitin-vinyl sulfone is a ubiquitin-derived probe with an electrophilic C-terminal end that targets the active-site cysteine (Cys341) of Parkin. Ubiquitin-vinyl sulfone can thus be used as a tool to monitor Cys341 accessibil-
ity. Following binding to phosphorylated ubiquitin, the Parkin catalytic Cys^{431}, which is normally occluded by the RING0 domain, becomes exposed as demonstrated by Ub-vinyl sulfone accessibility (Fig. 6B) (14, 24, 39, 40). This result strongly suggests that Parkin structural remodeling occurs after binding to phosphorylated ubiquitin. We finally constructed superposition model of Parkin and ubiquitin binding by computational modeling. Based on the crosslinking experiments that were coupled to mass spectrometry, we constructed the first model in which ubiquitin Ile^{36}, Arg^{42}, and Arg^{72} residues are in close proximity to the IBR and RING 1 domains (Fig. 6C). Interestingly, computational simulations suggest that the ubiquitin Ser^{65} phosphorylation site oriented toward the RING1 domain is captured by the positively charged residues, Lys^{151} and Arg^{305} (Fig. 6D and supplemental movie). Further computational simulations also showed that a separate positively charged region

**FIGURE 4.** Phosphorylation of BPA-incorporated ubiquitin by recombinant TcPINK1. A, CBB staining of recombinant His-Ub with BPA incorporation at the indicated amino acid positions. w/o denotes His-Ub without BPA incorporation. B, the indicated BPA-incorporated His-Ub was phosphorylated in vitro with or without GST-TcPINK1. The samples were subjected to Phos-tag PAGE followed by Oriole staining. C, the amount of phosphorylated His-Ub relative to that of His-Ub in a reaction without GST-TcPINK1 in B was quantified. D, amino acid residues where BPA replacement inhibited ubiquitin Ser^{65} phosphorylation by GST-TcPINK1 (~20% as in C) are shown in blue on the surface model of the ubiquitin structure (PDB ID: 1ubq). Ser^{65} residue is shown in green.
composed of Arg^{170} (and Lys^{220}) comes into close proximity to the negatively charged surface (Glu^{16} and Glu^{18}) of ubiquitin (Fig. 6D and supplemental movie). The resulting electrostatic interactions drive pushing of the IBR domain with phosphorylated ubiquitin to the RING0 domain, which in turn triggers a conformational change in REP that removes the occlusion blocking as well as bringing the E2 binding site and catalytic Cys^{431} residue into close proximity (Fig. 6D).

Computational modeling predicted that the ubiquitin Ile^{44} patch interacts with the Parkin RING1 domain α-helix (309 – 326 aa) via hydrophobic-hydrophobic interactions. To test this prediction, we used Fluoppi to monitor Parkin-ubiquitin complex formation following disruption of the hydrophobic-hydrophobic interactions by arginine replacements of Ala^{320} or Val^{324} in Parkin or alanine substitution of Ile^{44} in ubiquitin. All three mutations completely blocked foci formation (Fig. 7, A and B). We also mutated Lys^{151} and Lys^{305}, which are predicted to be critical for coordination with the phosphorylated Ser^{65}. Although the single alanine mutations (K151A and R305A) did not impair the foci formation, the K151A/R305A double mutation did reduce foci formation (Fig. 7, A and B). Similar reduction in foci formation in cells expressing a R170E/K220E double mutant suggests that Arg^{170} and Lys^{220} in Parkin also contribute to interactions with ubiquitin (Fig. 7, A and B). We also performed a Parkin translocation assay using these Parkin mutants. Although YFP-Parkin WT and the R170E/K220E mutant were efficiently recruited to TOMM20-labeled mitochondria within 2.5 h of valinomycin treatment, mitochondrial translocation was completely impeded by the Parkin K151A/R305A, A320R, and V324R mutations (Fig. 7, C and D).

FIGURE 5. In vitro site-specific photo-crosslinking using BPA-incorporated ubiquitin. A, recombinant His-Ub-incorporated BPA at the indicated amino acid positions and GST-rat Parkin were incubated with or without GST-TcPINK1. The samples were split into two with one aliquot subjected to UV irradiation. Proteins were detected by CBB staining. Arrowheads indicate crosslinked products of GST-rat Parkin and His-Ub. w/o denotes His-Ub without BPA incorporation. B, the crosslinked products in A were quantified as a ratio of the amount of crosslinked GST-rat Parkin to that of GST-rat Parkin without UV irradiation. C, recombinant His-Ub- (WT or S65D) incorporated BPA at the indicated amino acid positions was incubated with GST-rat Parkin (WT or S65E) for 10 min at room temperature. The samples were split into two with one aliquot subjected to UV irradiation. Proteins were detected by CBB staining. Arrowheads indicate crosslinked products of GST-rat Parkin and His-Ub. D, the crosslinked products in C were quantified as a ratio of the amount of crosslinked GST-rat Parkin to that of GST-rat Parkin without UV irradiation. E, the amounts of GST-rat Parkin crosslinked products with His-Ub in the presence of GST-TcPINK1 in B and those using a phosphomimetic pair of GST-rat Parkin and His-Ub in D were mapped onto the corresponding amino acid residues of the phosphorylated ubiquitin structure (PDB ID: 4wzp) via white-blue gradation. Phosphorylated Ser^{65} is shown in green.
Numerous studies have shown that PINK1-mediated phosphorylation of both Parkin and ubiquitin at Ser65 is required for efficient Parkin recruitment to damaged mitochondria. More recently, Parkin interactions with phosphorylated ubiquitin, which activate the latent E3 ligase activity of Parkin, have also been shown to be essential to the process. In the current study, we used two independent and unique experimental approaches, Fluoppi and site-specific photo-crosslinking, to elucidate the mechanism by which phosphorylated ubiquitin interacts with Parkin. The Fluoppi-based assay showed that the RING0-RING1-IBR domain of Parkin is essential for binding with phosphomimetic ubiquitin (Fig. 1), and pathogenic mutations of this domain, such as L283P, G284R, and C352G, impede those interactions (Fig. 1). Although no ubiquitin cross-linked products were observed following BPA incorporation at Parkin Leu283 and Gly284, incorporation at Gln282, which is close to Leu283 and Gly284, efficiently crosslinked to phosphomimetic ubiquitin (Fig. 2, B and D). This suggests that L283P and G284R mutations affect ubiquitin binding directly.
ever, we cannot rule out the possibility that the L283P and/or G283R mutations restrict molecular motion, thereby affecting the flexibility of the small β-hairpin loop comprising Val278–Ser286 aa. Another pathogenic mutation, R275W, also reduced foci formation (Fig. 1). When this mutant was expressed in SH-SY5Y neuroblastoma cells, it was found in the Triton X-100-insoluble fraction (41), suggesting that the mutation affects the overall structural folding and stability of Parkin. The Fluoppi foci in Parkin R275W-expressing cells, which were observed as clusters near the perinuclear region rather than spherical dots throughout the cytosol (Fig. 1B), are consistent with a structurally impaired Parkin as suggested above. Further site-specific photo-crosslinking analyses allowed us to generate a map of the interactions linking the phosphorylated forms of Parkin and ubiquitin and to construct a computational model of the Parkin-ubiquitin complex. Based on the inactivated human Parkin crystal structure (16), Lys161, Arg163, and Lys211 coordinate a sulfate ion structure, suggesting that they are viable sites for binding the phosphorylated serine of ubiquitin. However, our computational modeling predicts that Parkin Lys151 and His302 residues are responsible for capturing the phosphorylated serine of ubiquitin (Fig. 6D). Using site-directed mutagenesis, we confirmed that Lys151/His302 are directly involved in binding phosphorylated ubiquitin as well as efficient translocation of Parkin to damaged mitochondria (Fig. 7). On the other hand, Parkin was efficiently crosslinked following incorporation of BPA at Arg42 in non-phosphorylated ubiquitin (Fig. 4C). Consequently, interactions between the Parkin RING1 domain α-helix (309–326 aa) and the Ile34 hydrophobic patch of ubiquitin are phosphorylation-independent. Given

![Image](49x308 to 563x733)

FIGURE 7. Parkin mutants that have a defect of binding with phosphorylated ubiquitin. A, S65E phosphomimetic Ash-Parkin (referred to as WT) and its mutants were expressed with S65D phosphomimetic hAG-Ub (referred to as WT) and its I44A mutant in HeLa cells as the indicated pair in B. Foci of the hAG-Ub (S65D) were observed using confocal microscopy. Scale bars, 20 μm. B, quantification of foci formation in A. The percentages of cells forming foci are shown. The error bars represent ± S.D. from three independent replicates. Over 100 cells were counted in each of three replicate wells. C, the indicated YFP-human Parkin mutants were transiently expressed in HeLa cells. Cells were treated with valinomycin for 2.5 h and subjected to immunostaining with anti-TOMM20 antibody. Scale bars, 20 μm. D, quantification of Parkin translocation in C. The percentages of cells having YFP-Parkin on mitochondria are shown. The error bars represent ± S.D. from three independent replicates. Over 100 cells were counted in each of three replicate wells.
that BPA crosslinking can capture transient protein-protein interactions, our data show that Parkin and ubiquitin utilize at least two different interaction sites. Although interactions between Lys151/Arg305 of Parkin and phosphorylated Ser65 of ubiquitin stabilize the Parkin-ubiquitin complex, it is the interactions between the RING1 domain α-helix (309–326 aa) and the ubiquitin Ile44 hydrophobic patch that likely serve as the initial and most essential contact site. Our mutational analyses in which the Parkin A320R and V324R mutations and the ubiquitin I44A mutation completely impair complex formation support this conclusion.

During the manuscript revision process, two independent studies examining the interactions between Parkin and phosphorylated ubiquitin were published. The Muqit group (42) identified Parkin Lys151/His302 residues as critical residues for binding phosphorylated ubiquitin. Of note, two of the four Parkin residues (Lys151, His302, Arg305, and Gln316) that form a phosphate binding pocket were identified in our computational modeling approach. In addition, the Komander (43) group revealed the crystal structure of Pediculus humanus corporis Parkin complexed with phosphorylated ubiquitin. To our surprise, both the overall structure and the interaction sites at the amino acid level are very similar to those predicted by our modeling.

In this study, we elucidated a novel binding mechanism that uses the RING1-IBR domain to promote Parkin interactions with phosphorylated ubiquitin, and demonstrate that site-specific crosslinking coupled with mass spectrometry and computational molecular modeling can serve as a powerful tool to provide insight into a dynamic protein conformational change.

**Author Contributions**—K. Y. conceived and designed the main body of experiments. K. Y., B. B. Q., and F. K. performed experiments. Y. S. contributed to the mass spectrometry experiments and analysis. T. H. contributed to the computational molecular modeling. K. Y., K. T., and N. M. interpreted data and wrote the manuscript.

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