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Isolated RING2 Domain of Parkin Is Sufficient for E2-Dependent E3 Ligase Activity

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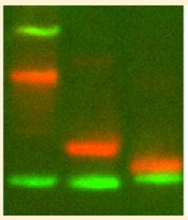
ABSTRACT: The E3 ubiquitin ligase activity of the parkin protein is implicated in playing a protective role against neurodegenerative disorders including Parkinson's, Huntington's, and Alzheimer's diseases. Parkin has four zinc-containing domains: RING0, RING1, IBR (in-between ring), and RING2. Mutational analysis of full-length parkin suggests that the C-terminal RING2 domain contains the catalytic core. Here, a catalytically competent recombinant RING2 containing an N-terminal GB1 solubility peptide is described. In cell-free in vitro ubiqitination reactions, the RING2 construct catalyzes the transfer of ubiquitin from the E2 enzyme UbcH7 to the attached GB1 tag. This intramolecular autoubiquitination reaction indicates that (a) ubiquitination by RING2 can occur in the absence of other parkin domains and (b) UbcH7 can interact directly with RING2 to transfer its bound ubiquitin. Mass spectrometry identified sites of mono- and diubiquitin attachment to two surface-exposed lysine residues (Lys24 and Lys39) on the GB1 peptide. The sites of diubiquitination involved Lys11 and Lys48 linkages, which have been identified as general signals for proteasome degradation. Cleaving the linker between the GB1 tag and RING2 resulted in loss of ubiquitination activity, indicating that the substrate must be tethered to RING2 for proper presentation to the active site. Atomic absorption spectrometry and selective mutation of zinc ligands indicated that only one of the two zinc binding sites on RING2, the N-terminal site, needs to be occupied by zinc for expression of ubiquitination activity. This is consistent with the hypothesis that the second, C-terminal, zinc binding site on RING2 has a regulatory rather than a catalytic function.



Mutations in the parkin gene were initially implicated in hereditary Parkinson's disease, specifically, autosomal recessive juvenile parkinsonism,[1] thereby closely linking the parkin protein to Parkinson's disease. Further cell and animal studies of the parkin gene and protein have shown a greatly increased role for parkin in neuronal health, including clearance of the amyloidogenic Alzheimer's disease $A\beta_{(1-42)}$ peptide[2,3] and, along with the chaperone Hsp70, clearance of Huntington's disease polyglutamine protein.[4,5] As an E3 ubiquitin ligase that ubiquitinates target proteins to earmark them for degradation by the ubiquitin proteasome system (UPS), parkin protein functions either directly or as a member of a multiprotein complex to remove short-lived proteins, altered proteins, proteins that exceed certain concentrations, and improperly folded proteins.[6] Additionally, the UPS is now thought to include interactions and pathways involving chaperone systems and nondegradative ubiquitination, and parkin has been found to form complexes that carry out a variety of critical cellular functions such as DNA repair,[7] aggresome formation,[8] stabilization of microtubules,[9] protection from cytotoxicity, and preservation of the UPS.[5]

Parkin is a 465 amino acid protein (M_w 51 652 Da) that contains five separate domains designated (from N- to C-terminus) as ubiquitin-like (Ubl), RING0, RING1, In Between RING (IBR), and RING2, respectively. The three C-terminal domains, RING1, IBR, and RING2, otherwise known as RBR, place parkin into the RBR family of E3 ubiquitin ligases. Mutations in the RING2 domain resulted in loss of E3 ligase activity, suggesting that RING2 is at least partly responsible for the E3 ligase function.[10−13] Until now it has not been possible to ascertain whether RING2 alone is capable of ubiquitination or if other parts of parkin are required for expression of activity.

A 2.8 Å resolution structure of a large fragment of parkin, containing all but the first 140 amino acids, was recently published.[14] The structure showed that the RING0, RING1, IBR, and RING2 domains form a closely packed core. Sites of interaction between the complex, the Ubl domain, and the E2 enzyme (UbcH7) were identified from the 2.8 Å resolution structure as well as from a low-resolution crystal structure of whole parkin in combination with NMR spectroscopy and molecular modeling of UbcH7. The multidomain core was considered to represent an inhibited form of parkin in which the active site on RING2 is masked by structural elements of the RING0 domain. The E2 binding site was located at an interface with RING1, approximately 50 Å away from the critical active-site residue Cys431 on RING2. An important implication of the structure is that activation of parkin ubiquitination would require a significant change in conformation within the complex to release inhibition caused by

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RING0−RING2 binding to allow the RING2 active site to have access to the E2 bound by RING1 and to allow active-site access to the substrate.[14] The crystal structure of parkin also confirmed an earlier study[6] that indicated that the two zinc binding sites in RING2 form an unusual sequential-topology structure rather than the classical RING cross-brace structure described by Borden and Freemont.[15]

In this study, we have constructed a recombinant RING2 domain from parkin that contains an N-terminal GB1 tag to increase solubility and yield during bacterial overexpression. Using a cell-free in vitro autoubiquitination assay, we show that the recombinant protein possesses an intramolecular E3 ubiquitin ligase activity that results in mono- and diubiquitination of the attached GB1 tag. These observations demonstrate that the RING2 domain, when isolated from other parkin domains, is sufficient for E2 binding and expression of ubiquitination activity.

MATERIALS AND METHODS

Materials. Alkaline phosphatase-conjugated ImmunoPure goat antimouse IgG (H+L) was purchased from Thermo Scientific (Rockford, IL). Monoclonal anti-FLAG M2, clone M2 was from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal [PRK8] antibody to Parkin (ab77924) was obtained from abcam (Cambridge, MA). The epitope recognized by this antibody is located in Parkin RING2. Human recombinant FLAG-tagged ubiquitin, UbcH7, and His_6-ubiquitin activating enzyme (UBE1) were from Boston Biochemicals (Cambridge, MA). Western Lightning CDP-Star chemilumnescence reagent was from PerkinElmer (Boston, MA), and NuPAGE 12% Bis-Tris polyacrylamide gels were from Life Technologies, formerly Invitrogen (Carlsbad, CA). IMMOBILON-P 0.45 μM membranes were purchased from Millipore (Billerica, MA), and an XCell II blot module from Novex (Invitrogen, Carlsbad, CA) was used for western blot transfers.

Parkin Clones and Constructs. *6×His-GB1-RING2.* The full-length parkin construct in pET42c[11] was the parent plasmid from which the 6×His-GB1-RING2 was cloned. Briefly, the N-terminal glutathione S-transferase (GST) tag in the pET42c-parkin plasmid was replaced with the GB1 tag from the DZG clone (a kind gift from Dr. Roberto DeGuzman, University of Kansas, Molecular Biosciences Department). To obtain parkin RING2, DNA encoding all other parkin domains was deleted from the pET42c construct. This was achieved using a QuikChange Lightning site-directed mutatagenesis kit (Agilent Technologies, Santa Clara, CA) to insert an NdeI site at the starting ATG of parkin in the pET42c plasmid. The pET42c/parkin/NdeI plasmid was cleaved with NdeI and XbaI, and then the larger plasmid fragment was gel purified. This removed the GST tag from the plasmid. Because of the small amounts of DNA obtained by gel purification of the very small GB1 tag, we chose to cleave the pET21a (DZG) plasmid containing the GB1 tag with NdeI, XbaI, and SphI to obtain three DNA fragments. Only the fragment containing the NdeI and XbaI ends ligated successfully into the modified pET42c plasmid, thereby eliminating the need for gel purification. The resulting construct contained the parkin gene in a pET42c plasmid in which the GST tag had been replaced with a GB1 tag. Following insertion of an NdeI site just prior to the parkin R392 amino acid (using the QuikChange kit), the pET42c GB1-parkin was cleaved with NdeI and gel-purified, thereby removing the four parkin domains N-terminal to RING2. The plasmid was ligated, thus joining the two NdeI sites and putting the GB1 tag adjacent to the R392 of parkin.

6×His+14-RING2. Parkin amino acids 392−465 were cloned into the pTBSG plasmid vector[16] by the Cobre Protein Production Group at the University of Kansas. There are 21 amino acids N-terminal to the parkin RING2 domain, including a 6×His tag that is immediately preceded by the start site methionine. The expressed protein (11 kDa) ends at V465, the C-terminal residue of parkin.

RING2 with No Tag. The GB1 cloning tag was removed from purified 6×His-GB1-RING2 protein to produce RING2 with no tag. To remove the imidizole remaining after nickel column purification, the GB1 protein was desalted by use of a G50 Sephadex spin column[17] equilibrated with buffer containing 20 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 1 mM DTT. The concentration of the collected protein filtrate was determined by the Bradford assay.[18] There is a TEV protease cleavage site just preceding R392 in 6×His-GB1-RING2. TEV was added at a 1:100 ratio (TEV/protein), and the mixture was incubated overnight at room temperature to remove the GB1 tag. Following cleavage, the RING2 was separated from the 6×His-GB1 tag using Qiagen Super Flo NTA resin in a batch-binding procedure. After rotating the cleavage mixture plus the NTA beads for 1 h, the NTA beads were separated by centrifugation (15 870*g* for 3 min). The supernatant was saved, and the beads washed once with 20 mM NaCl, 20 mM Tris-HCl (pH 8.0), and the supernatants (containing tagless RING2) were examined by SDS-PAGE. Glycerol was added to the supernatants to a final concentration of 20% (v/v) prior to storage at −80 °C.

Protein Expression and Purification. One liter of LB containing 30 μg/mL of kanamycin was inoculated with overnight cultures of 50 mL of LB, 30 μg/mL of kanamycin. The 1 L cultures were incubated (37 °C, 225 rpm) until the OD_{600} was between 0.4 and 0.5. Isopropylthiogalactoside (IPTG) was added to 0.2 mM and $ZnSO_4$, to 100 μM. Protein production was induced for 3 h, and then the cells were pelleted (15 min at 4012*g*). The brownish-red colored pellets were weighed and stored at −80 °C until lysis.

Pellets were thawed on ice, diluted into lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl) using 10 mL of lysis buffer per gram of cells. Protease inhibitors (leupeptin HCl at a final concentration of 5 μg/mL, bestatin HCl at 1 μM, and 1,10-phenanthroline at 1 mM) were added fresh to the lysis buffer before use. Lysozyme stock (10 mg/mL in 50 mM Tris-HCl, pH 8.0) was added at 100 μL/g of cells after dilution of the cells into lysis buffer. Benzonase at 0.5 μL/g of cells was also added. Cells were incubated at room temperature for 30 min. Following incubation, cells were sonicated for 5 min using 10 s on/10 s off cycles. Cells were kept on ice during sonication and were then centrifuged at 15 950*g* for 15 min to pellet inclusion bodies and debris. After determining the protein concentration of the supernatant, glycerol was added to 20% (v/v).

Proteins were purified with Qiagen Super Flo NTA resin (Qiagen, Valencia, CA). Columns were equilibrated with 25 mM Tris-HCl (pH 8.0). Bacterial supernatants were loaded onto the column, which was then washed with two column volumes of 25 mM Tris-HCl (pH 8.0) followed by a wash containing 25 mM Tris-HCl (pH 8.0) plus 10 mM imidazole. Protein was eluted with 25 mM Tris-HCl (pH 8.0) plus 300 mM imidizole and collected in 2 mL fractions. Glycerol was added to pooled fractions to 20% (v/v) prior to storage at −80 °C. The yield was approximately 40 to 60 mg per liter of cell

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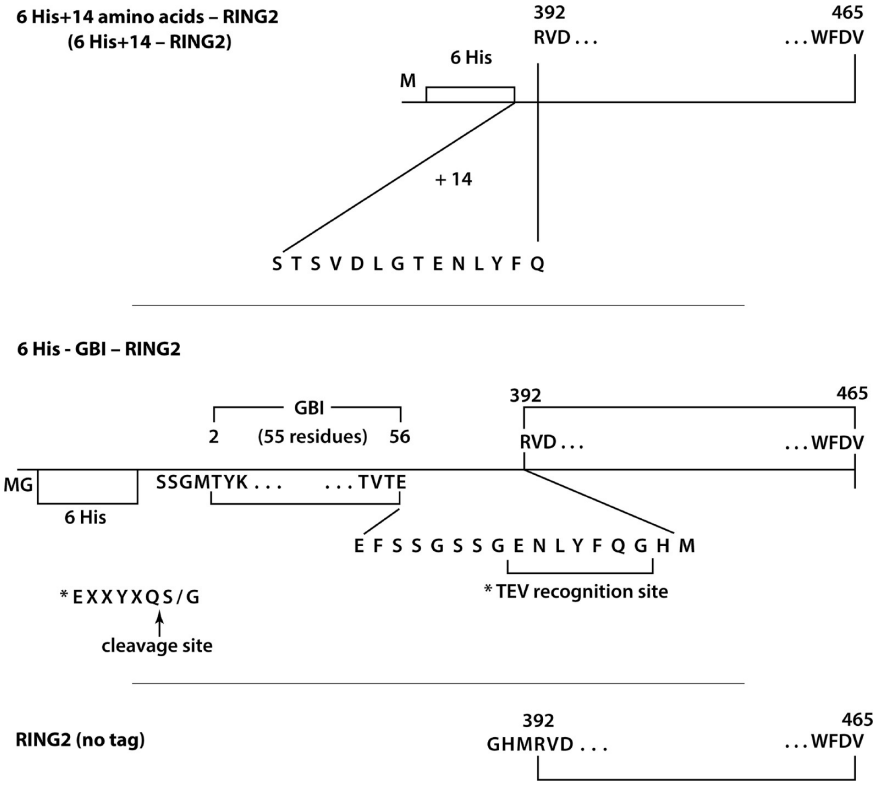

Figure 1. Recombinant RING2 constructs. All constructs contain the entire parkin RING2 domain extending from R392 through C-terminal V465. The first construct contains a short 6× His tag linked to the RING2 by 14 amino acids that contain a TEV recognition sequence for tag removal. The initiating methionine is located just prior to the 6×His purification tag. In the second construct, a 55 amino acid GB1 solubility tag is inserted between the TEV recognition site and the 6×His purification tag. The initiating methionine, located before the 6×His tag, is separated from the tag by glycine. In the third construct, the 6×His-GB1 tag was removed by TEV cleavage three amino acids before R392 of RING2.

culture for the 6×His-GB1-RING2 construct and 10 mg per liter of culture for the 6×His+14 amino acid-RING2 construct. Protein concentrations were measured by the Bradford[18] method.

Ubiquitination Reactions and Immunoblots. In vitro reactions contained 50 mM Tris-HCl (pH 7.0), 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1.39 μM UbcH7 (E2), 22 nM UBE1 (E1), 4 mM ATP, 5 or 10 μM FLAG-tagged ubiquitin, and 5 or 10 μM RING2 protein (added last). Reaction mixtures were incubated for 1 h at 37 °C. Samples were dissolved in SDS sample buffer (25 mM Tris-HCl (pH 6.8), 0.8% (w/v) SDS, 3.5% (v/v) glycerol, 2 M urea, 2% (v/v) β-mercaptoethanol, and 0.08% (w/v) bromophenol blue) and then applied to 12% Bis-Tris gels for fractionation.

Immunoblots were blocked, and proteins were identified with monoclonal FLAG antibody (to identify the FLAG-tagged ubiquitin) or parkin RING2 antibody at a 1:10 000 dilution. The secondary antibody (alkaline phosphatase-conjugated goat antimouse IgG) was used at a dilution of 1:15 000 or 1:30 000 followed by reaction with Western Lightning CDP-Star and detection of chemiluminescence with a Kodak imager station 4000R.

Capillary Liquid Chromatography–Mass Spectrometry Analysis (Cap LC–MS/MS). Ubiquitinated products of the 6×His-GB1-RING2 purified by electrophoresis on a polyacrylamide gel, along with unreacted 6×His-GB1-RING2 and ubiquitin, were excised from the gel. Gel slices were destained by incubation with 200 mM NH_4HCO_3/50% (v/v) acetonitrile at 37 °C, reduced by incubation with dithiothreitol (20 μL of 100 mM) at 60 °C for 30 min, and alkylated by incubation with iodoacetamide (40 μL of 100 mM at room temperature for 30 min). Trypsin (0.5 μg) was added to the gel slices in 200 mM NH_4HCO_3/10% (v/v) acetonitrile, and the gel slices were incubated overnight at 37 °C.

Digested protein samples were analyzed by LC–MS/MS using a NanoAcquity chromatographic system (Waters Corp., Milford, MA) coupled to an LTQ-FT mass spectrometer (ThermoFinnigan, Bremen, Germany). Peptides were separated on a reverse-phase C18 column, 15 cm, 300 μm i.d. (Thermo Acclaim PepMap300, 300 Å, 5 μm). A gradient was developed from 1 to 40% B in 50 min, ramped to 95% B in 4 min, and held at 95% B for 5 min at a flow rate of 10 μL/min with solvents A (99.9% H_2O, 0.1% formic acid) and B (99.9% acetonitrile, 0.1% formic acid). The ESI (electrospray ionization) source was operated with spray voltage of 2.8 kV, a tube lens offset of 200 V, and a capillary temperature of 200 °C. All other source parameters were optimized for maximum sensitivity of the MRFA peptide (MetArgPheAla tetrapeptide) MH^+ ion at m/z 524.26. The instrument was calibrated using an automatic routine based on a standard calibration solution containing caffeine, peptide MRFA, and Ultramark 1621, a commercially available mixture of fluorinated phosphazines.

The data-dependent acquisition method for the mass spectrometer (configured version LTQ-FT 2.5.5) was set up using Xcalibur software (Thermo Scientific, version 2.1). Full MS survey scans were acquired at a resolution of 50 000 with an automatic gain control (AGC) target of 5×10^5. The five most abundant ions were fragmented in the linear ion trap by collision-induced dissociation with an AGC target of 2×10^3 or maximum ion time of 300 ms. The ion-selection threshold was

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500 counts. The LTQ-FT scan sequence was adapted from Olsen and Mann.[19]

Analysis of MS/MS spectra for peptide and protein identification was performed by protein database searching using Mascot (Matrix Science, London, UK, version 2.3) and X! Tandem (The Global Proteome Machine Organization, version 2010.12.01.1). The programs were set up to search the UniProt-Sprot database as well as the GB1-RING2 sequence assuming trypsin as the digestion enzyme. The fragment ion mass tolerance was 0.20 Da, and the parent ion tolerance was 20 ppm. A delta mass of 114 (Gly−Gly) was specified as variable modification for lysine residues. The iodoacetamide derivative of cysteine (delta mass 57) was specified as a fixed modification. Scaffold software (Proteome Software Inc., Portland, OR; version 3.6) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm. Intensities of MS1 signals were measured as chromatographic areas of monoisotopic peaks using Qual Browser of Xcalibur software (Thermo Scientific, version 2.1).

Atomic Absorption. Zinc-to-protein ratios were determined (1) in protein following purification by Qiagen Super Flo NTA resin or (2) in purified protein that was then desalted on Sephadex G50 columns.[17] A series of Zn standards was prepared by spiking portions of the buffer with aliquots of a Zn reference standard solution (Fisher Chemical, Fair Lawn, NJ) to a total volume of 1 mL. The initial protein sample was undiluted prior to injection, whereas 0.5 mL aliquots of the second and third protein samples were diluted with an equal volume of buffer to a final volume of 1 mL before injection. Background measurement was made with no solution injected into the graphite furnace (GTA 110, Varian) atomic absorption spectrometer (GFAAS) (SpectrAA 220 FS, Varian, Walnut Creek, CA), and the resulting value was subtracted from each subsequent measurement. A 10 μL aliquot of solution was injected into the GFAAS with a micropipet (Finnpipette, Thermo Scientific, Vantaa, Finland) for all other absorbance measurements. A total of six replicate injections each were performed for the buffer, for each standard, and for each protein sample. A calibration curve for the determination of the concentration of Zn was constructed from a least-squares fit of the plotted Zn standard data. The concentration of Zn in each protein sample was calculated on the basis of the standard calibration.

RESULTS

Recombinant RING2 Constructs. Two recombinant parkin RING2 constructs were prepared for these studies: one with an N-terminal 6×His tag (6×His+14-RING2) and the other with a 6×His tag attached to the N-terminus of a 55 amino acid residue GB1 solubilization peptide (6×His-GB1-RING2). These are illustrated in Figure 1. A third construct was obtained by cleaving the 6×His-GB1-RING2 construct at a TEV protease cleavage site located between the GB1 and RING2 proteins (RING2-no tag). Recombinant constructs were subcloned into pET expression vectors that were transformed into and overexpressed in *Escherichia coli* BL21-(DE3) cells, as described in the Materials and Methods. After cell lysis and centrifugation, the supernatants containing the His-tagged RING2 proteins were applied to Ni-NTA affinity columns, and eluted proteins were obtained at >90% purity as determined by SDS gel electrophoresis (Figure 2). This

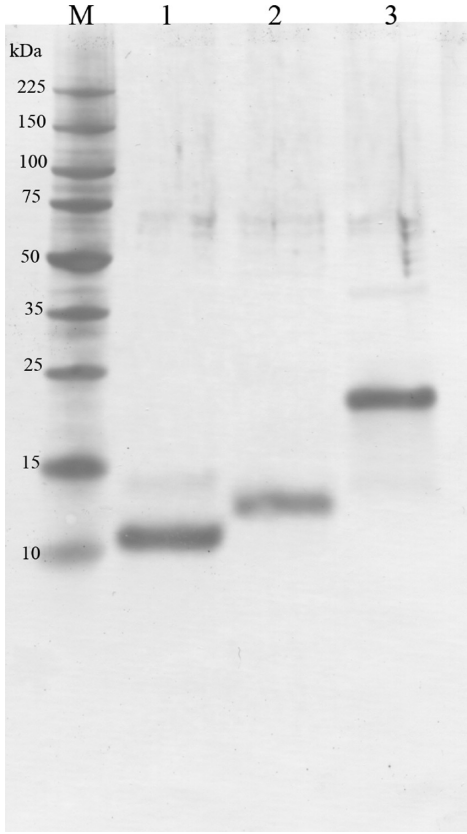

Figure 2. Analysis of purified recombinant proteins. The proteins were fractionated by SDS PAGE, transferred to Immobilon-P membrane, and stained. The sample buffer was 2.5% SDS, 250 mM DTT, 250 mM $NaCO_3$, and 2.5% glycerol. Lane 1 = RING2 (no tag), 9.103 kDa; lane 2 = 6×His+14-RING2, 11.3 kDa; and lane 3 = 6×His-GB1-RING2, 18.4 kDa. M = size marker.

procedure yielded approximately 10 mg of purified 6×His +14-RING2 protein per liter of bacterial cell culture. In contrast, the construct containing the N-terminal 6×His-GB1 tag (6×His-GB1-RING2) routinely yielded 40−60 mg of purified protein per liter of bacterial culture.

E3 Ubiquitin Ligase Activity of RING2 Constructs. Cell-free in vitro ubiquitination reactions contained an E1, an E2, FLAG-tagged ubiquitin, ATP, and buffer in addition to the recombinant RING2 protein. In these reactions, there was no added substrate; therefore, the recombinant RING2 acted as both the E3 ligase and the substrate via autoubiquitination. Reaction components and products were separated on SDS-PAGE gels and transferred to PVDF membranes, and specific bands were identified by a FLAG antibody to recognize ubiquitin or by the monoclonal PRK8 antibody to identify parkin RING2. Protein bands were decorated with secondary chemilumiscent antibodies and analyzed with the computer program ImageJ. Blots were assigned separate colored channels: red for RING2 and green for ubiquitin; these blots were then merged to identify overlap.

In Figure 3, lane 1, the merged result from the autoubiquitination reaction of 6×His-GB1-RING2 contains three strong bands and three faint bands. Utilizing the coomassie blue-stained marker (M), the lower green band (~10 kDa) is the unattached free ubiquitin. The red band (~21 kDa) is the 6×His-GB1-RING2 protein with no attached ubiquitin. The next highest band (~30 kDa) shows a

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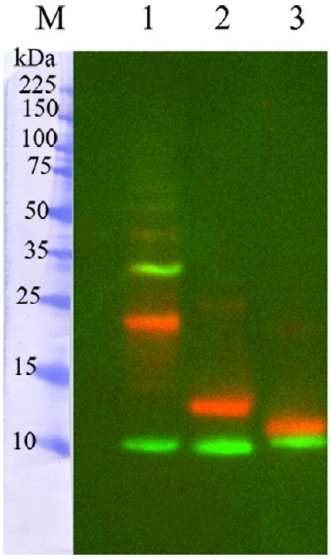

Figure 3. Ubiquitination activity of recombinant RING2 proteins. The in vitro reactions are as follows: Lane 1 = 6×His-GB1-RING2, lane 2 = 6×His+14-RING2, and lane 3 = RING2 alone following removal of the GB1 tag. The marker is the same as in Figure 2. The red band identifies RING2 that is not ubiquitinated. The green band at the bottom, migrating as 10.11 kDa, is the unattached free ubiquitin. All bands appearing above the red RING2 band in each lane are identified as autoubiquitinated RING2 protein. Only the 6×His-GB1-RING2 protein construct consistently produced at least one robust ubiquitinated band. The concentration of FLAG-tagged ubiquitin and RING2 was 5 μM for these reactions.

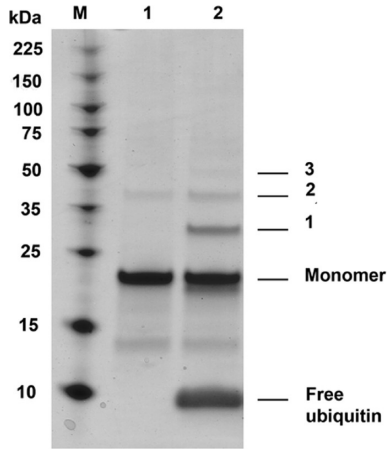

Figure 4. SDS-PAGE gel showing protein bands excised for mass spectrometry. Lane 1 contains unreacted 6×His-GB1-RING2 protein in reaction buffer. Lane 2 is an in vitro ubiquitination reaction. The molecular weights of bands labeled 3, 2, and 1 indicated proteins with three, two, or one attached ubiquitin(s) per RING2, respectively.

Table 1. Molecular Weights of Ubiquitinated Proteins

band in Figure 3	molecular weight of protein band on gel[a]	calculated molecular weight
3	48.75	46.3
2	40.36	37
1	30.20	27.7
monomer	20.90	18.4

[a]The molecular weights are expressed in kilodaltons (kDa). The 6×His-GB1-RING2 monomer migrated as a 20.90 kDa protein in the preparatory gel for mass spectrometry (Figure 4). Bands 1, 2, and 3, representing ubiquitinated reaction products, migrated as shown in the middle section of Table 1 and are comparable to the calculated molecular weights in the third column.

combination of red and green, indicating that this band contains ubiquitin that is bound to 6×His-GB1-RING2. The upper faint bands appear to be 6×His-GB1-RING2 with two and three attached ubiquitins. No significant ubiquitination activity was observed when 6×His-GB1-RING2 was replaced with either 6×His+14-RING2 (Figure 3, lane 2) or the tagless RING (Figure 3, lane 3) construct or if any of the reaction components (ATP, E1, E2, ubiquitin, or 6×His-GB1-RING2) was omitted from the reaction mixture (data not shown).

Identification of Ubiquitination Sites by Mass Spectrometry. 6×His-GB1-RING2 ubiquitination reactions were fractionated on SDS-PAGE gels, and protein bands were stained with coomassie blue (Figure 4). The recombinant 6×His-GB1-RING2 monomer migrates as an ~21 kDa protein (Figure 4, lane 1). Three suspected ubiquitinated bands were present, as indicated in lane 2 (bands 1, 2, and 3). Band number 1 migrates as an ~30 kDa protein, consistent with the attachment of one ubiquitin/protein molecule (Table 1). The second band migrates as an ~40 kDa protein, consistent with the attachment of two ubiquitins/RING2 molecule, and the third band migrates as an ~49 kDa protein, consistent with the attachment of three ubiquitins/RING2 molecule. The three bands were excised from gels containing multiple identical lanes equivalent to lane 2 in Figure 4 along with control bands of 6×His-GB1-RING2 monomer or free ubiquitin. Pooled samples were digested with trypsin.

Ubiquitin is attached to targeted lysines via an isopeptide bond formed between the carboxyl terminal glycine of the ubiquitin and the ε amino group of a lysine residue. Trypsin cleaves between the R and G of the ubiquitin C-terminal RGG amino acids, leaving a GG tag from the ubiquitin attached to the target lysine. Hence, ubiquitinated protein fragments are identified by mass spectrometry as Lys−Gly−Gly.[20] This isopeptide bond is stable in the reducing SDS urea sample buffer, whereas the thioester bond between the ubiquitin and the E2 or, as hypothesized by Wenzel and Klevit,[21,22] between the ubiquitin and the C431 in the RING2 of parkin, would not be stable.

LC−MS analysis applied to the ubiquitinated GB1-RING2 construct identified three sites of ubiquitination: K24, K39, and K101 (Figure 5A and Table 2). The extent of these modifications was estimated on the basis of the assumption that the MS response factor of closely related peptides was the same. Thus, measuring chromatographic areas of ion intensities for modified versus nonmodified peptides (Table 2) pointed to two residues, K24 and K39 on the GB1 tag, as predominant sites of ubiquitination. The peptide mapping procedure was also applied to the ubiquitin itself, and fragments that contained evidence of diubiquitination (Lys48- and Lys11-linked chains) were found (Figure 5B and Table 3).

The rate of attachment of a single ubiquitin to the 6×His-GB1-RING2 protein was obtained by estimating the density of monoubiquitinated 6×His-GB1-RING2 on SDS-PAGE gels (Figure 6). The rate of product formation, determined from the slope of the linear portion of the curve shown in Figure 6, was 52 nmol/min (0.2 s^{-1}). This value underestimates the actual rate because it does not account for the smaller amounts of di- and triubiquitinated species present. From Figure 6, it is also apparent that there is a lag in the initiation of ubiquitination

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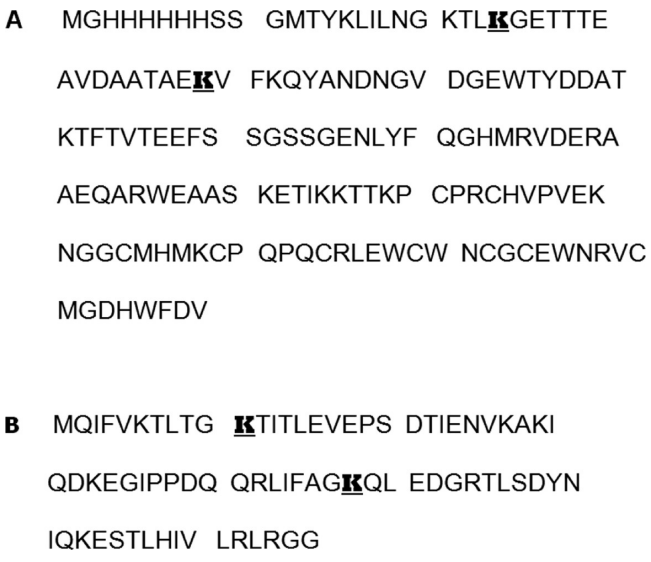

Figure 5. Identity of ubiquitinated lysine residues. (A) 6×His-GB1-RING2 sequence showing ubiquitinated lysines identified by mass spectrometry analysis. Ubiquitin was attached to either lysine 24 or 39 in peptides from all of the three excised bands. Ubiquitinated lysines are bold and underlined. (B) Ubiquitinated ubiquitin from bands 2 and 3 in Figure 4. Analysis of trypsinized ubiquitin peptides by mass spectrometry showed that Lys11 and Lys48 were ubiquitinated, indicating formation of Lys11- and Lys48-linked diubiquitin chains. The concentration of FLAG-tagged ubiquitin and RING2 in reactions for mass spectrometry was increased to 10 μM.

activity of approximately 10 min, which is possibly due to an initial coordination between the E1, E2, and E3 enzymes.

Because the parkin RING2 domain can strongly ubiquitinate the attached GB1 cloning tag, it was of interest to determine if the parkin RING2 domain can support the intermolecular ubiquitination of unattached GB1. In these experiments, RING2 was separated from GB1 following cleavage of the linker by the TEV protease. Figure 7A shows a coomassie-stained gel to identify the individual protein bands used in the reaction mixtures. To determine whether free GB1 is ubiquitinated by the RING2 E3 ligase, duplicate reactions were performed as shown in Figure 7B. In this experiment, reaction mixture components were transferred to an Immobilon-P membrane. In Figure 7B, lanes 1−4 of the membrane were stained with commassie blue, whereas lanes 5−8 were decorated with an antibody to the FLAG tag on the ubiquitin protein. Lanes 1 and 5 show the GB1-RING2 protein alone (no reaction), lanes 2 and 6 are GB1-RING2 ubiquitination reactions showing the full complement of ubiquitinated bands, lanes 3 and 7 show equimolar amounts of parkin RING2 domain along with unattached GB1 (no reaction), and lanes 4 and 8 show the products from the ubiquitination reaction containing RING2 ubiquitin and unattached GB1. The ubiquitinated bands (1, 2, and 3) in lane 6 of the GB1-RING2 reaction are striking in their clarity and strength. The antibody shows slight cross-reactivity with the GB1-RING2 monomer (lanes 5 and 6). The only strong band in lane 8 is unattached ubiquitin.

Zinc Content and Requirement for E3 Ligase Activity. Freshly isolated GB1-RING2 contained 1.4 mol of zinc ions per mol of protein, as measured by atomic absorbance. The same protein sample was used for the ubiquitination reaction shown in Figure 3, lane 1, and also for the mass spectrometry data (Figure 4) (Materials and Methods). The zinc content of the protein was reduced to 0.83 ± 0.12 mol of zinc per mole of protein following desalting on Sephadex G50,[17] suggesting that a single tightly bound zinc ion remained. The E3 ubiquitination activity of the desalted GB1-RING2 (Figure 8A) was essentially identical to that of the RING2 preparation before desalting (compare Figure 8A to Figures 4 and to 8B, lane 1, where wild-type GB1-RING2 was not desalted), suggesting that only one bound zinc ion is required for ubiquitination activity.

Previous studies[11,23] indicated that mutation of C418 and/or C441 that form ligands to the zinc bound in the first (N-terminal) site resulted in loss of ubiquitination activity. Therefore, the first site must be occupied by zinc for ligase activity. Here, we mutated the last two zinc binding ligands in the second C-terminal site. The activity of the double mutant (C457A and H461A) (Figure 8B, lane 3) was essentially the same as that of the wild-type enzyme (Figure 8B, lane 1). Therefore, these data indicate that only the first zinc binding site on RING2 must be occupied to elicit ubiquitination activity when utilizing the isolated RING2. Attempts to fill the second site by adding an excess of exogenous zinc resulted in protein aggregation and precipitation.

DISCUSSION

Three recombinant proteins containing the isolated RING2 domain of parkin were used in this study. They differed in the cloning tag attached N-terminal to the parkin RING2 domain. Cell-free in vitro ubiquitination reactions showed that the tagless RING2 construct was inactive. The construct containing the 6×His tag plus a 14 amino acid linker exhibited a very low amount of activity that varied significantly from one reaction to another. In contrast, the 6×His-GB1-RING2 variant consistently exhibited a significant amount of ubiquitination activity. Mass spectrometry confirmed ubiquitination and identified the targeted lysines, both mono- and diubiquitinated, within the GB1 tag.

The significance of these observations is 3-fold. First, they demonstrate that the RING2 domain of parkin is catalytically active in the absence of any other parkin domains. It has been proposed that full-length RBR proteins function according to a hybrid RING/HECT mechanism.[21,22] In RING E3 ligases, the RING structure brings the E2 and substrate together so that the ubiquitin is transferred directly from the E2 to the substrate, whereas HECT ligases ubiquitinate by transferring the ubiquitin to a cysteine within the E3 ubiquitin ligase, from which the ubiquitin is then transferred to the substrate. The

Table 2. Extent of Ubiquitination of 6×His-GB1-RING2-Specific Residues Calculated as the Fraction of Modified vs Nonmodified Peptide

ubiquitinated residue	sequence of identified peptide	GB1-control	GB1-1ub	GB1-2ub	GB1-3ub
K24	(K)TLkGETTTEAVDAATAEK(V)	0.0	8.2	3.7	3.3
K39	(K)GETTTEAVDAATAEkVFK(Q)	0.0	9.7	4.5	7.7
K101	(R)WEAASkETIK(K)	0.0	1.2	0.9	0.7

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Table 3. Extent of Ubiquitination of Ubiquitin-Specific Residues Calculated as the Fraction of Modified vs Nonmodified Peptide

ubiquitinated residue	sequence of identified peptide	GB1-control	GB1-1ub	GB1-2ub	GB1-3ub
K11	(K)TLTGkTITLEVEPSDTIENVK(A)	0.0	0.0	3.6	3.8
K48	(R)LIFAGkQLEDGR(T)	0.0	0.0	5.3	7.3

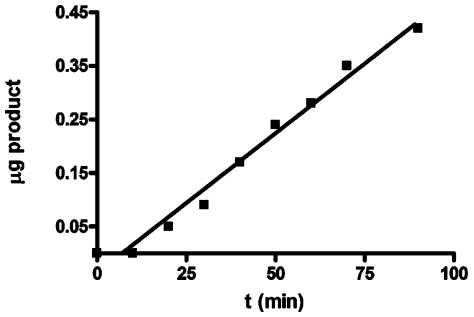

Figure 6. Time dependence of ubiquitinated product formation. The rate was determined by measuring the density of band 1 (Figure 4) that contained one ubiquitin attached to the 6×His-GB1-RING2 construct identified by mass spectrometry. Samples from a ubiquitination reaction were withdrawn over a period of 90 min; SDS-PAGE sample buffer was immediately added to the sample to stop the reaction. Following fractionation on SDS-PAGE and staining with coomassie blue, the density of band 1 was determined by Image Quant software (Amersham, GE Healthcare Life Sciences), and the data were analyzed using GraphPad Prism software.

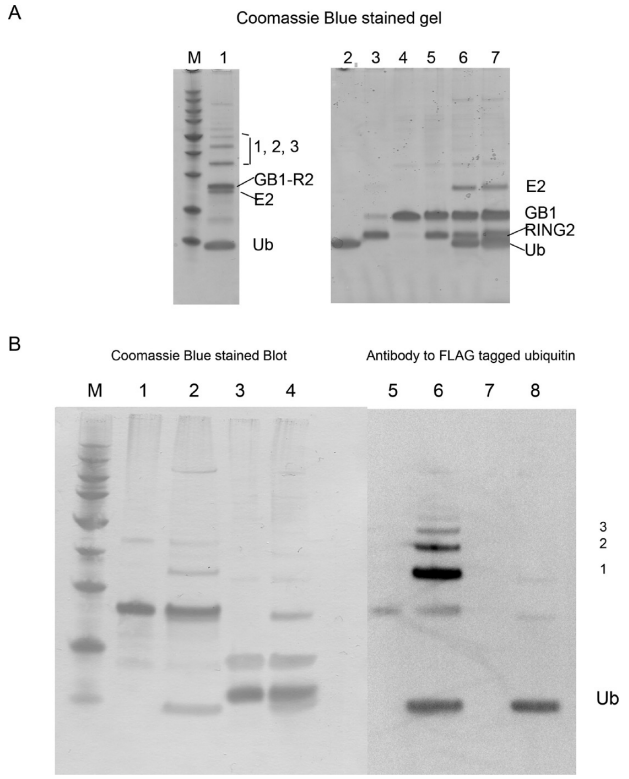

Figure 7. Lack of ubiquitination of unattached GB1. (A) Protein bands in the ubiquitination reactions identified following staining of the SDS-PAGE gel was with coomassie blue. The calculated molecular weights are RING2, 9.1 kDa; 6×His-GB1, 9.3 kDa; FLAG-tagged ubiquitin, 9.3 kDa; 6×His-GB1-RING2, 18.4 kDa; and UbcH7, 18 kDa. Although the calculated molecular weight of the 6×His-GB1 tag is 9.3 kDa, it migrated as a 12 kDa protein. The protein markers are the same as in Figures 2, 3, and 4. (B) Ubquitination reactions and controls were fractionated on an SDS-PAGE gel; the proteins were transferred to Immobilon-P and either coomassie-stained (lanes 1 to 4) or immunodecorated with FLAG-tagged ubiquitin antibody (lanes 5 to 8). Lanes 1 and 5 = GB1-RING2 protein alone; lanes 2 and 6 (positive control) = GB1-RING2 ubiquitination reactions; lanes 3 and 7 = parkin RING2 domain plus unattached GB1; and lanes 4 and 8 (experimental) = RING2, ubiquitin, and unattached GB1 products from a ubiquitination reaction.

hybrid RING/HECT hypothesis[21,22] proposes that the ubiquitin-charged E2 binds to RING1, which is brought to the catalytic center in RING2 by release of the RING0−RING2 binding inhibition and a supposed conformational change in parkin.[14] The charged ubiquitin is then transferred to a cysteine in RING2, from which it is attached to the substrate via a stable isopeptide bond.

Considering that our cell-free in vitro ubiquitination results were obtained with only the isolated parkin RING2 domain (no RING1 or IBR in the reaction), parkin RING2 must interact directly with the E2, in this case UbcH7, to effect ubiquitination. Indeed, direct E2−E3 interactions are well-known.[24] For example, the crystal structure of the complex formed between the E2 (UbcH7) and the E3 of the c-Cbl RING domain[25] showed that there are two specific c-Cbl residues, Trp 408 and Ile383, both bulky hydrophobic amino acids, that are solvent-accessible in the E2 binding cleft. Bulky hydrophobic residues are found in equivalent positions in other E3 ubiquitin ligases.[24] The parkin RING2 Trp453 (W453) is comparable to the c-Cbl Trp408, and parkin RING2 Asn428 is in the same position as c-Cbl Ile383. Asparagine has also been found in the equivalent position in other E2−E3 pairs.[24] Therefore, these residues, which are conserved in parkin, are potential sites for E2 interaction and will be tested for this function in future experiments.

The second significant result of this study is the identification of the ubiquitinated lysine residues on the GB1 tag. The Chaugule et al.[26] hypothesis of parkin activation suggests that ubiquitination of actual substrates may be similar to a modified autoubiquitination mechanism. This hypothesis is based on the identification of binding motifs near the parkin N-terminal ubiquitin-like binding domain (Ubl) as well as near the RING2 domain. On the N-terminal side of the RING2 domain, there is a putative ubiquitin interacting motif (UIM). This motif is thought to interact with the Ubl domain to inhibit the ubiquitination activity of RING2 unless a specific substrate is presented.[26] The parkin UIM region (W403−E409) is contained within our RING2 constructs. This parkin UIM region is shorter than the UIM consensus sequence (ΦxxAlaxxxSerxxAc), where Φ is a bulky hydrophobic residue, Ac is an acidic residue, and x can be any residue.[27] The Chagule et al.[26] hypothesis requires that substrates are presented at the UIM site, which, in its inhibited form, is bound by the N-terminal Ubl. The presenting molecule was named an effector wherein presentation of the substrate by the effector breaks the Ubl-UIM inhibition and activates parkin. We would therefore expect that the substrate would be located in the vicinity of the UIM.

There are three lysines in the region between the first RING2 zinc ligand, C418, and the upstream UIM (Figure 9),

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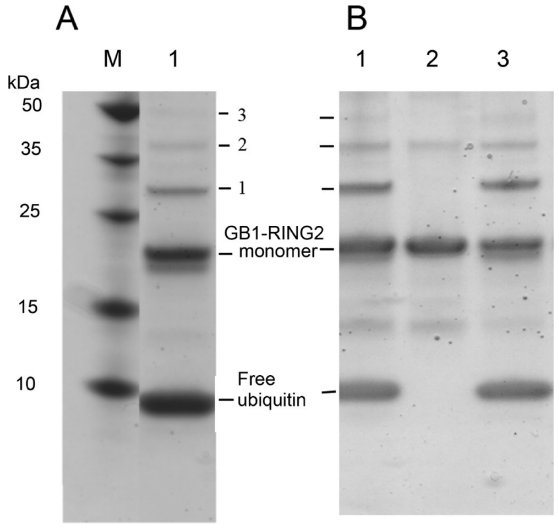

Figure 8. Requirement of one filled zinc site for ubiquitination activity. (A) Ubiquitination reaction products resulting from use of desalted 6×His-GB1-RING2 fractionated on SDS-PAGE and stained with coomassie blue. The desalted RING2 construct contains 0.832 ± 0.12 mol zinc per mol of GB1-RING2 protein. (B) Ubiquitination activity of mutated 6×His-GB1-RING2 in which the last two ligands in zinc ion site II (C457 and H631) were mutated to alanines to prevent zinc ions from binding in site II. Lane 1 = ubiquitination of wild-type GB1-RING2, lane 2 = unreacted mutated GB1-RING2, lane 3= ubiquitination reaction products from the 6×His-GB1-RING2 mutant (C457A plus H461A). Lanes 1 and 3 appear the same, showing that the zinc site II mutant has the same activity as wild-type RING2.

none of which were ubiquitinated. Instead, the two lysines identified by mass spectrometry as the main sites of ubiquitination (Lys39 and Lys24) are located upstream of the UIM on the α helix (Lys39) and on the turn between β-sheet 1 and 2 (Lys24) of the GB1 tag, where they are surface-exposed and easily accessible to ubiquitination components. Furthermore, they are on opposite sides of the GB1 molecule, suggesting that there is flexibility in either the presentation of this particular substrate or in the positioning of the RING2 ubiquitination components. Our data showed that the substrate must be attached to RING2 for ubiquitination to occur. This was also reported by Matsuda et al.[28] in which a maltose binding protein (MBP) tag attached to the combined IBR–RING2 domains was ubiquitinated, whereas the unattached MBP tag was not. Although further in vitro studies utilizing the recombinant MBP-tagged IBR–RING2 showed E2–independent autoubiquitination, ubiquitination of the parkin substrate synphilin-1 was E2 dependent.[29] Because synphilin-1 binds to parkin RING2,[30] synphilin-1 can be considered attached to RING2 in the in vitro ubiquitination reactions.[29] Under physiological conditions, perhaps substrate–RING2 attachment is promoted by the effector molecule, as introduced in the Chaugule et al.[26] hypothesis.

Mass spectrometry data also showed diubiquitin formation. Formation of ubiquitin chains is an important feature of ubiquitin signaling. Lys48-linked chains target proteins to the proteasome. The Lys11-linked chain may also function as a proteosomal degradation signal but may have other functions as well.[31] Interestingly, the specific atypical Lys-11 linkage identified here by mass spectrometry has been identified in deposits of Huntington's disease patients (HD)[32] and in deposits of tau neurofibrillary tangles (NFT) taken from Alzheimer's patients.[33]

The third significant observation made in this study is that only one of the two zinc binding sites is required for ubiquitination activity of the RING2 construct. The crystal structure reported by Trempe et al.[14] identified RING2 as a zinc finger domain with two zinc ions bound, supporting earlier data suggesting that parkin RING2 contains two zinc binding sites.[6] Atomic absorbance measurements indicated that the active, desalted RING2 retained ~1 zinc atom per molecule. The apparent lack of effect of simultaneous mutation of two ligands in the second zinc binding site indicated that zinc binding to the first site was sufficient for catalysis. This result is consistent with observations of the analogous HHARI E3 ligase, which requires just one bound zinc for activity.[34] The four ligands binding the first zinc ion in parkin are comparable to those binding the single zinc ion in HHARI.[34]

The observation that just one of the two zinc binding sites in RING2 is required for catalysis is also consistent with the observation that mutation of Cys457, one of the four ligands in the second zinc binding site in full-length parkin, led to loss of autoinhibition[14] (i.e., activation of E3 ligase). This latter observation further suggests that binding zinc to the second site on RING2 may facilitate formation of the inhibited complex. That is, this site has a regulatory rather than a catalytic role. This idea is supported by the fact that two of the zinc ligands in

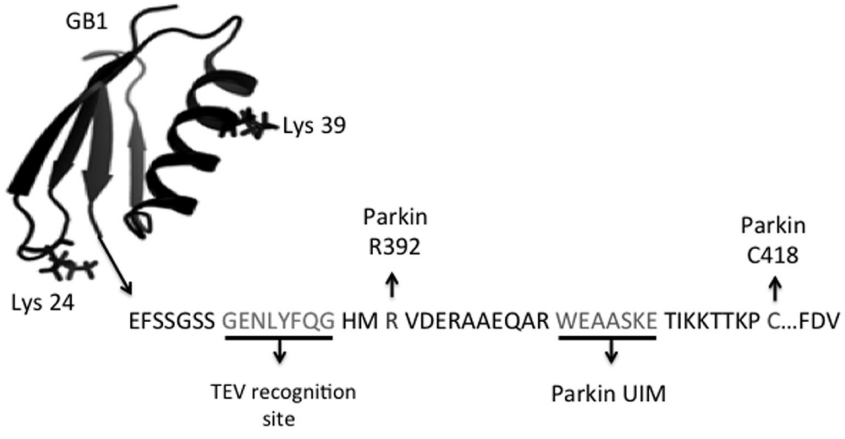

Figure 9. Model showing the 6×His-GB1-RING2 construct. The location of the parkin ubiquitin interacting motif (UIM) as well as the linker sequence between the tag and RING2 may determine which lysines are ubiquitinated.

the second site, Cys457 and His461, form part of a helix that makes multiple contacts with RING0, leading to the inhibited state identified in the crystal structure.[14]

SUMMARY AND CONCLUSIONS

The in vitro 6×His-GB1-RING2 system developed here has focused on the isolated RING2 and has provided new insight into the protein structural requirements for substrate ubiquitination by parkin. With regard to E3 ligase activity, the other domains of parkin appear to be involved in substrate specificity by inhibiting ligase activity of RING2.[14,26] However, removal of these other domains does not in itself activate the catalytic center in RING2. In at least two instances, ubiquitination of MBP by the IBR–RING2[28] and, as reported here, ubiquitination of GB1 by RING2 require that the MBP or GB1 substrate is attached to RING2; neither free MBP nor free GB1 is ubiquitinated by the RING2 catalytic core. This suggests that effective ubiquitination occurs only if the substrate is immobilized and colocalized with the RING2 catalytic center. Although in this study the GB1 substrate was covalently attached, it is assumed that under normal circumstances the substrate would bind specifically to another part of parkin to be presented to the active site for ubiquitination. An effector molecule might provide immobilization of substrate and colocalization with RING2 by binding to both substrate and the UIM that is located just upstream of RING2.[26]

Although the small isolated RING2 of parkin used in this study is unlikely to recognize any relevant substrates, the information obtained suggests that attachment and location of the substrate (GB1 tag in this study) is important for successful ubiquitination. Several studies are now feasible using this in vitro model system including mutational analysis to identify the E2 binding site and the catalytic mechanism as well as to determine the structural requirements for effective ubiquitination of the GB1 tag (e.g., length of the GB1-RING2 linker) and whether other tags may be ubiquinated in place of the GB1 tag.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

E1, (HbE1) ubiquitin activating enzyme; FLAG, hydrophilic epitope tag (DYKDDDDK); GB1, B1 domain of Streptococcal protein G; HECT, homologous to the EbAP carboxyl terminus; IBR, in between RING domain separates two linked RING domains in the same protein; RING, really interesting new gene, describing a cysteine-rich motif; TRIS, tris-(hydroxymethyl)aminomethane; UbcH7, human recombinant E2 (UBE2L3) ubiquitin-conjugating enzyme; UPS, ubiquitin proteasome system

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