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E3 Ubiquitin-Protein Ligase Activity of Parkin Is Dependent on Cooperative Interaction of RING Finger (TRIAD) Elements

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Key Words

Parkin · Parkinson's disease · Proteasome · RING finger · TRIAD/RIR domain

Abstract

The parkin gene codes for a 465-amino acid protein which, when mutated, results in autosomal recessive juvenile parkinsonism (AR-JP). Symptoms of AR-JP are similar to those of idiopathic Parkinson's disease, with the notable exception being the early onset of AR-JP. We have cloned and expressed human Parkin in *Escherichia coli* and have examined Parkin-mediated ubiquitination in an in vitro ubiquitination assay using purified recombinant proteins. We found that Parkin has E3 ubiquitin ligase activity in this system, demonstrating for the first time that the E3 activity is an intrinsic function of the Parkin protein and does not require posttranslational modification or association with cellular proteins other than an E2 (human Ubc4 E2 was utilized in this ubiquitination assay). Mutagenesis of individual elements of the conserved RING TRIAD domain indicated that at least two elements were required for ubiquitin ligase activity and suggested a functional cooperation between the RING finger elements. Since the activity assays were conducted with recombinant proteins purified from *E. coli*, this is the first time TRIAD element interaction has been demonstrated as an intrinsic feature of Parkin E3 activity.

Introduction

Parkinson's disease, a common neurodegenerative disease affecting roughly 1 million people in North America alone, results from the loss of dopaminergic neurons in the pars compacta of the substantia nigra. The classic triad of Parkinson's disease symptoms, including tremor, rigidity and akinesia, is shared by an autosomal recessive form of juvenile parkinsonism (AR-JP) [23] that has been linked to mutations in the parkin gene [10]. Two protein motifs have been identified, an N-terminal ubiquitin-like domain with 32% identity to ubiquitin and a C-terminal RING finger domain [10]. The RING finger domain was later shown to consist of three cysteine-rich elements (fig. 1a), an N-terminal RING, an in-between RING region (IBR) and a C-terminal RING [16], identifying it as a potential member of the TRIAD or RING IBR RING (RIR) family of proteins [24]. These protein motifs suggested that Parkin might play a role in the cellular ubiquitin-proteasome protein degradation pathway.

In this pathway, proteins to be degraded by the proteasome are tagged with a multiubiquitin chain. This process requires a series of reactions. Ubiquitin is first activated in an ATP-requiring step catalyzed by an activating enzyme, E1, then transferred to a ubiquitin carrier protein, E2, and finally conjugated to the substrate protein in a reaction mediated by an E3 ubiquitin-protein ligase (reviewed by Hershko and Ciechanover [6]). The E1 and E2

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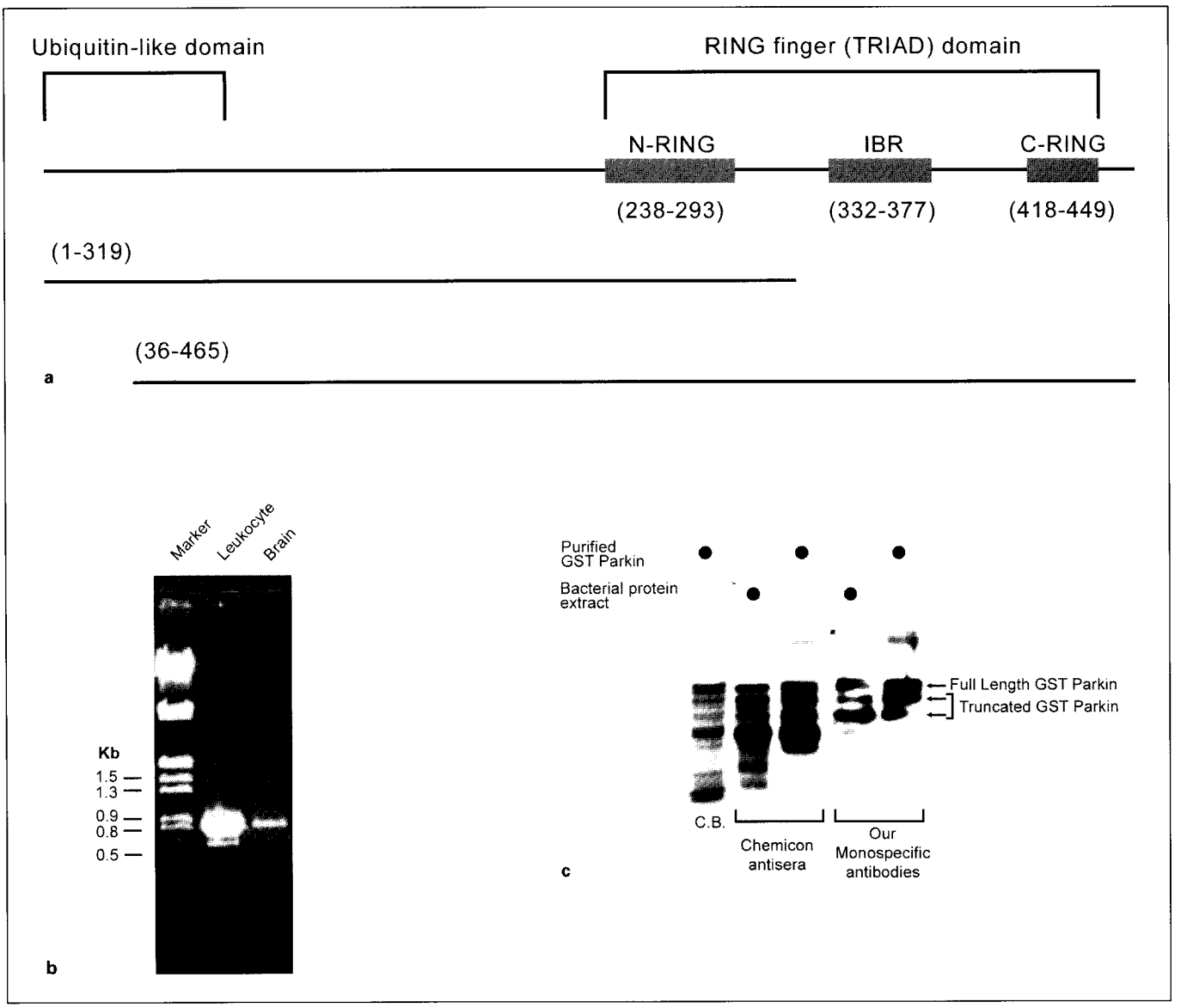

Fig. 1. Cloning, expression and characterization of Parkin. **a** A map of Parkin protein domains and clones. (1–319) and (36–465) indicate amino acid residue numbers from the sequence of Kitada et al. [10]. **b** Ethidium bromide-stained 0.7% agarose gel containing the DNA fragments obtained from nested PCR with human leukocyte or human brain mRNA as template. Primers and conditions were as described by Sunada et al. [22]. The major product from either leukocyte or brain mRNA was 850 bp. **c** Western blot analysis of bacterial extracts containing recombinant Parkin or of purified recombinant Parkin protein. The dots indicate the presence of the indicated protein or extract. Coomassie blue (C.B.) staining shows the total amount of protein in the GST affinity-purified protein sample. Chemicon antisera and our monospecific antibodies recognized three bands of the same size. These were determined to be full-length GST Parkin and two truncated peptides of GST Parkin by MALDI-TOF MS.

enzymes can be identified through conserved sequences and their function is relatively well understood. In contrast, we are only beginning to understand the nature of the E3 ubiquitin ligases and how proteins are selected for degradation by their specific E3 or E3 complex. There are several recognition factors that have been identified in substrate proteins, including phosphorylation, exposure of a hydrophobic protein surface, the N-end rule, by which certain proteins are ubiquitinated through recognition of specific N-terminal residues, and very short spe-

cific sequences in the carboxy-terminal domain (reviewed by Laney and Hochstrasser [11]). This diversity of substrates predicts the existence of a large number of E3s, although only two main classes of E3 ligases have been described – HECT domain E3s and RING finger E3 ligases which include single chain E3 ligases, such as Ubr1 (an N-end rule E3) and c-Cbl, and multisubunit complexes, such as APC and SCF. The division of labor between the individual subunits in the large multiprotein E3 complexes is instructive in understanding ubiquitin ligase function. For example, the SCF complex is composed of four distinct protein subunits, Skp1, Rbx1 (also known as Hrt1 and Roc1), a cullin and an F-box protein (e.g. Cdc4), which provide multiple functional domains. The variable F-box protein recognizes the substrate protein through specific protein interaction domains. Skp1 binds to the F box itself and links the F-box protein to the remaining subunits, and cullin, together with Rbx1, recruits the E2 and comprises the ubiquitin ligase core [3, 21]. This suggests that single chain RING finger proteins functioning as E3 ligases require multiple functional domains. This is indeed the case, as exemplified by Ubr1 and c-Cb1.

The RING finger protein/domain appears central to the function of E3 ubiquitin ligases since it interacts with and recruits the E2 ubiquitin conjugating enzyme. The essential role of the RING finger protein was first reported with regard to SCF ubiquitin ligase, in ubiquitination assays with natural substrates [19], then with c-Cbl [8]. These reports demonstrated a role for the RING finger in the stimulation of the autoubiquitination of an E2 (in the case of SCF) or an E3 (in the case of c-Cbl), and although the biological significance of E2/E3 autoubiquitination is unknown, the assay has been used to identify putative ubiquitin ligases by virtue of the ubiquitination of the E2 or E3 present in the assay reaction. For proteins such as Parkin, where the natural substrates are not known, this assay is especially useful in identifying E3 ubiquitin ligase activity. In a study utilizing this assay with *Escherichia coli* lysates containing unpurified recombinant proteins in lieu of purified proteins, several more RING finger proteins (BRCA1, Siah-1, TRC8, NF-X1, kf-1 and Praja1) were shown to have E3 ubiquitin-protein ligase activity [13].

Parkin has been reported to have E3 ubiquitin ligase-associated activity [7, 20, 27]. It associates with the E2s, UbcH7 and UbcH8 via the RIR domain, and has E3 activity in in vitro assays utilizing Parkin recombinant protein expressed and immunoprecipitated from neuroblastoma-derived SH-SY5Y cells [7, 20]. Another study found that Parkin translated in rabbit reticulocytes had E3 ligase activity with UbcH8, UbcH7 and to a lesser extent with UbcH5; however, this system had a high background even without Parkin [27]. Parkin did not stably bind Ubc2, Ubc3, Ubc4, UbcH5 (very similar but not identical to human Ubc4) or UbcH6 in any study. In regard to the protein substrate of Parkin, overexpressed, recombinant Parkin is itself ubiquitinated in transfected cells but did not appear to be ubiquitinated in vitro [7]. Parkin may participate in unfolded protein stress, as shown by increases in Parkin mRNA and protein when cells are stimulated with reagents that result in unfolded protein stress [7]. This same report shows that overexpression of recombinant Parkin renders SH-SY5Y cells resistant to unfolded protein stress [7]. A putative substrate, CDCrel-1, a synaptic vesicle-enriched GTPase, was identified as a Parkin-interacting protein via a yeast two-hybrid system and coimmunoprecipitation [27]. The ubiquitination of CDCrel-1 was increased by wild-type but not mutant Parkin when coexpressed in HEK 293 cells, suggesting that CDCrel-1 might be a Parkin target.

Previous Parkin studies have utilized in vitro E3 assays, with the source of Parkin protein being either immunoprecipitates [7, 20] or in vitro translated Parkin [27]; therefore, in each case, there was the possibility that the observed activity was due to an associated protein (although this association would have to depend on the RIR domain) or required modification of certain Parkin residues. By utilizing purified bacterially expressed recombinant proteins in an in vitro ubiquitination system, we demonstrate that Parkin has intrinsic E3 ubiquitin ligase activity that is not dependent on posttranslational modification or on interaction with cellular proteins other than an E2. We also show that Parkin can utilize the E2, human Ubc4. Further, Parkin ubiquitination activity is dependent on the cooperative interaction of two cysteine-rich elements of the TRIAD/RIR domain.

Methods

Materials

Human frontal cortex brain tissue was obtained from the Brain Tissue Repository of the Institute for Brain Aging and Dementia at the University of California-Irvine, Irvine, Calif., USA. The PolyA-Tract mRNA Isolation System III and sequencing grade modified trypsin was purchased from Promega (Madison, Wisc., USA), and a reverse transcription polymerase chain reaction (RT-PCR) kit and QuikChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, Calif., USA). A TA cloning kit was obtained from Invitrogen (Carlsbad, Calif., USA) and goat polyclonal antiserum raised to Parkin peptide amino acids 83–97 was obtained from Chemicon (Temecula, Calif., USA).

Cloning and Expressing Parkin

A nested PCR strategy was used to clone the Parkin gene. Using human leukocyte mRNA, a forward primer (GAGGATTACCCAGGAGACCGCTGGTG), along with the outer and inner reverse primers of Sunada et al. [22], produced a cDNA fragment of 1,054 bp (encoding amino acid residues 1–319). A reverse primer (CTTCTGCAATTTGGCTGTAGTTGGACTTTG), along with the outer and inner forward primers of Sunada et al. [22], gave a cDNA fragment of 1,449 bp (encoding residues 36–465). The fragments were cloned and their sequences verified. The clones were joined at a common *Kpn*I restriction endonuclease site to generate the entire Parkin cDNA clone.

The entire Parkin cDNA as well as the two fragments (1–319 and 36–465) were subcloned into the *Eco*RI site of pGEX-2T (Pharmacia). The IBR and C-terminal RING components were subcloned as PCR products (encoding residues 314–389 and 408–459, respectively), then inserted into the pET42c vector (Novagen). These two vectors provided a glutathione *S*-transferase (GST) N-terminal tag for recombinant protein purification. Additional Parkin mutations were made by mutating the first cysteine in each element of the RING finger TRIAD to alanine (C238A, C332A and C418A). These mutations were put onto the 36–465 construct, since its ubiquitination activity was comparable to wild-type Parkin and it produced relatively large amounts of protein. All GST-tagged constructs were confirmed by DNA sequence analysis. Proteins were expressed in *E. coli* strain BL21 or BL21(DE3) by induction with 0.2 m*M* isopropyl-β-*D*-thiogalactopyranoside for 4–5 h at 37 °C. Bacteria was harvested, lysed with a French Press in lysis buffer (50 m*M* Tris-HCl, pH 8, 120 m*M* NaCl and 1 m*M* dithiothreitol) and purified on glutathione agarose beads (Sigma). After binding to glutathione agarose, proteins were washed in lysis buffer, eluted with 20 m*M* reduced glutathione in lysis buffer, then dialyzed against lysis buffer. To quantitate the amount of purified protein, proteins were fractionated on SDS-PAGE and blotted to Immobilon-P, and the blots were stained with Coomassie brilliant blue R-250. The intensity of bands representing full-length protein was assessed by SigmaScan/Image (Jandel Scientific) in comparison with BSA standards.

Polyclonal Antiserum to Parkin Peptides

Rabbit polyclonal antiserum was raised to a Parkin peptide, GAHPTSDKETPVA (amino acids 213–225). Monospecific antibodies were obtained by antigen affinity purification using recombinant Parkin bound to PVDF membrane [4].

Mass Spectrometry

Purified recombinant protein was fractionated on SDS-PAGE (10%), and three bands, identified both by our monospecific antibodies as well as the commercially available Chemicon goat polyclonal antisera (raised to Parkin peptide amino acids 83–97), were subjected to in-gel trypsin digestion [5, 18]. The trypsin fragments were analyzed by the KU Biochemical Resource Service Laboratory on a Voygeo DE-STR matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) instrument.

In Vitro Ubiquitination Assays

The in vitro ubiquitination assays were conducted as described previously [8]. In brief, His-tagged mouse E1 and human Ubc4 were produced in *E. coli* BL21(DE3) by induction with 0.4 m*M* isopropyl-β-*D*-thiogalactopyranoside for 3 h at 30 °C prior to lysis in 50 m*M* Tris-HCl (pH 8) and 20 m*M* NaCl. TALON beads (Clontech) and 100 m*M* imidazole were used for E1 and Ubc4 protein isolation. Ubiquitination reactions (10–15 μl) contained E1 (50–500 n*M*), Ubc4 (0.5–5 μ*M*), Parkin or Parkin mutants (10 μg), ubiquitin (5 μ*M*) and ATP (2 m*M*) in ubiquitination buffer (50 m*M* Tris-HCl, pH 7.5, 2.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol). Reactions were incubated for 90 min at 23 °C and stopped with 2 × SDS sample buffer.

Results

The Cloning and Expression of Parkin

A cDNA clone of the human parkin gene was obtained by utilizing RT-PCR. In the search for a suitable mRNA template, we used the same primers and conditions for leukocyte and brain poly A+ mRNA as described previously by Sunada et al. [22]. An 850-bp product was obtained from both human leukocyte and brain poly A+ mRNA templates (fig. 1b), indicating that the Parkin transcripts from leukocyte and brain were similarly processed. By contrast, the results of Sunada et al. [22] indicated that transcripts from these sources were processed differently. The mRNA we isolated from human brain showed degradation on Northern blot analysis (data not shown) and, when used as an RT-PCR template, gave less PCR product than the intact mRNA from leukocytes (fig. 1b). Therefore, we used leukocyte mRNA for the RT-PCR step in cloning Parkin cDNA (detailed in Methods). PCR products included cDNA fragments of 1,054 bp (encoding amino acid residues 1–319) and 1,449 bp (encoding residues 36–465) (fig. 1a). These overlapping fragments were cloned, then joined to generate wild-type Parkin cDNA. Sequence analysis of our entire Parkin cDNA clone confirmed identity with human Parkin [10] except for one base substitution 768C→T. As this 768C→T was found in cDNAs from separate PCR reactions and has also been reported by other laboratories, it may be an error in the initial cDNA sequence [1]. The entire Parkin cDNA as well as the two fragments encoding residues 1–319 and 36–465 were subcloned into an *E. coli* expression vector containing an N-terminal GST tag, and proteins were expressed and purified on glutathione agarose beads (detailed in Methods).

Identification of Purified Parkin by Antibody and Mass Spectrometry

Purified full-length recombinant protein was analyzed by immunoblot and mass spectrometry. Two separate sources of peptide antibodies were used for Western blot analysis, one raised to Parkin peptide residues 83–97 (Chemicon) and our monospecific antiserum raised to Parkin peptide residues 213–225. Both identified three

bands in the recombinant bacterial extracts as well as in glutathione agarose-purified protein (fig. 1c). An additional band of purified Parkin recognized by the Chemicon antiserum likely represented an N-terminal peptide too short to contain the epitope(s) recognized by our antisera. No prominent bands were detected by the Chemicon antiserum when the control, GST alone, was expressed (fig. 2). Purified GST Parkin was fractionated on SDS-PAGE and the three protein bands that had been recognized by both peptide antibodies were cleaved in the gel fragment with trypsin. The resulting peptide fragments were subjected to MALDI-TOF MS analysis. MALDI-TOF MS results were compared to the predicted tryptic digestion from the known amino acid sequence of GST-tagged Parkin (82.128 kD). Tryptic peptides representing 27% of the entire protein were identified from analysis of the highest molecular weight band. These tryptic fragments were scattered throughout the protein from the N- to the C-terminal. MALDI-TOF MS analysis of the approximately 66-kD protein band showed 32% coverage, and 41% coverage with the 46-kD band. Tryptic fragments located only within the N-terminal portion of the protein were identified from the 66- and 46-kD protein bands. These mass spectrometry results indicate that the bacterial expression system produces full-length GST-tagged Parkin as well as C-terminally truncated proteins. Further evidence for the production of C-terminally truncated Parkin is shown by glutathione agarose affinity isolation via the N-terminal GST tag during purification. These immunoblot and MALDI-TOF data established (1) expression of the Parkin protein and (2) recognition of Parkin by both Chemicon antisera and our monospecific antibodies.

The identity of wild-type and mutant forms of Parkin used in this study was confirmed by Western blot (fig. 2). Truncated Parkin proteins 36–465 and 1–319, as well as point mutants C238A, C332A and C418A were identified by Chemicon antiserum in protein preparations purified by glutathione agarose chromatography (fig. 2). As seen above (fig. 1c), C-terminally truncated as well as full-length protein products were present. The Chemicon antiserum did not recognize either GST alone or the molecular weight protein standards, suggesting that the Parkin immunoreactivity is specific. The IBR (314–389) and C-RING (408–459) Parkin protein fragments could not be identified by the Chemicon antibody or our polyclonal antiserum because the peptide antigens to which the antisera were raised are located outside of these regions.

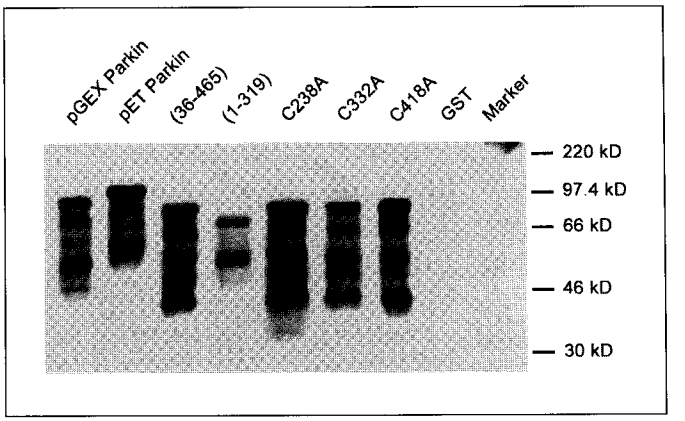

Fig. 2. Identification of wild-type and mutant Parkin used in this study. The Chemicon antisera were used to identify the purified proteins. pGEX Parkin = Wild-type Parkin subcloned into pGEX-2T (Pharmacia); pET Parkin = wild-type Parkin subcloned into pET42c (Novagen); (36–465) = truncated Parkin lacking the first 35 amino acids from the ubiquitin-like domain but containing an intact TRIAD/RIR domain; (1–319) = amino-terminal portion of Parkin through the N-RING of the TRIAD domain; C238A, C332A, C418A = point mutations of the first cysteine in each element of the TRIAD/RIR. These mutants are on the background of the 36–465 construct.

E3 Activity of Parkin

To determine whether Parkin has E3 ubiquitin ligase activity, a cell-free in vitro ubiquitination assay containing *E. coli*-purified His-tagged E1 and E2 (human Ubc4), ubiquitin and ATP was used. Although the eukaryotic 20S proteasome core particle has both prokaryotic and archaebacterial ancestors, the ubiquitin-dependent proteasomal pathway of protein degradation is restricted to eukaryotic cells [2, 25]. Therefore, by expressing ubiquitin-conjugating components in *E. coli,* contamination by other eukaryotic components is prevented, and we are confident that any ubiquitination activity results from known reagents in this defined system. Ubiquitination activity was detected by the ability to promote the synthesis of isopeptide bonds between ubiquitin and proteins in the reaction mixture, leading to the formation of stable, high-molecular-weight ubiquitin conjugates [8]. Thioester bonds linking E2 with ubiquitin and possibly Parkin with ubiquitin are unstable in the reducing SDS-PAGE sample buffer, whereas isopeptide bonds formed by the conjugation of ubiquitin to proteins remain stable. We found that both wild-type and 36–465 RING TRIAD proteins supported the formation of high-molecular-weight ubiquitin conjugates in the in vitro assay (fig. 3). Since the first 35 amino acids are missing from Parkin 36–465, it appears that an

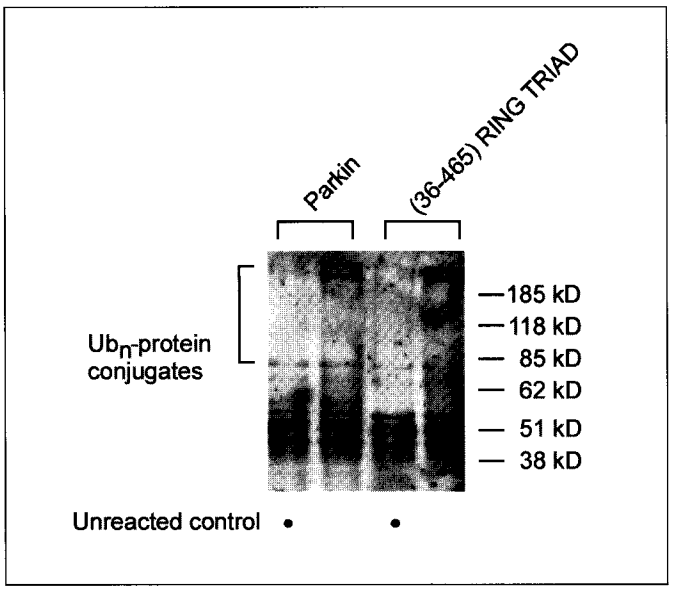

Fig. 3. Ubiquitination reactions. Purified wild-type Parkin protein and the N-terminally truncated Parkin protein from construct 36–465 were incubated with mouse E1, the human E2 Ubc4, free ubiquitin and ATP. Reactions were fractionated on SDS-PAGE, blotted and probed with anti-ubiquitin antibody. SDS-PAGE sample buffer was added prior to the addition of Parkin proteins in the unreacted control. High-molecular-weight ubiquitin-protein conjugates show the attachment of ubiquitin to protein/s in the reaction mixture and demonstrate ubiquitination activity.

intact ubiquitin-like domain is not required for E3 ligase activity.

TRIAD RING Finger Elements Are Important to Ubiquitination Activity

The data in figure 3 suggest that the TRIAD RING finger domain rather than the ubiquitin-like domain mediates Parkin activity in this in vitro cell-free system. As the TRIAD/RIR domain is composed of three separate cysteine-rich elements, we performed a mutational analysis of this region to determine which elements were required for ubiquitination. Since some E2s do not require an E3 for ubiquitination activity [6], we assayed for that possibility by omitting Parkin from the reaction. No high-molecular-weight conjugates were formed by Ubc4 in the absence of Parkin (lane labeled 'No E3', fig. 4a). A repeat reaction with the protein product from Parkin 36–465 containing the entire TRIAD domain again produced high-molecular-weight ubiquitin conjugates, whereas the protein product from Parkin 1–319, which contained only the N-RING of the TRIAD, did not (fig. 4a). The isolated C-RING as well as the isolated IBR were also inactive. This suggested that the overall architecture of the TRIAD domain is important and that there are critical interactions between the individual motifs that are required for E3 activity.

To further investigate cooperative interaction of the RIR elements, we constructed a series of inactivating mutations on the background of the Parkin 36–465 construct, by which the first cysteine of each element was mutated to alanine (C238A, C332A and C418A mutations; fig. 4b). Mutation of the first cysteine in a RING finger has had a detrimental effect on E3 activity for most RING fingers examined [unpubl. observations] [13]. Mutation of the first cysteine in the N-RING (C238A) did not affect ubiquitination (fig. 4a), nor did mutation of the first cysteine in the IBR (C332A). However, mutation of the first cysteine in the C-RING (C418A) completely inactivated the Parkin TRIAD/RIR. This, along with the inactivity of the isolated C-RING, suggests that the C-RING is necessary, but not sufficient for E3 activity. These results indicate that the E3 activity of Parkin requires a functional C-RING, together with either the N-RING or the IBR.

Discussion

A novel class of proteins identified by the TRIAD/RIR domain has only recently been described [16, 24]. Here, we report that Parkin, a protein containing a TRIAD/RIR domain, has intrinsic E3 ubiquitin ligase activity. Another member of this family, the human homologue of *Drosophila ariadne* (HHARI), has been shown to bind a human E2 [17], suggesting that it may also function as an E3 ubiquitin-protein ligase. Since a number of proteins containing a single RING finger domain function quite well as E3 ubiquitin ligases [8, 13], the purpose of the TRIAD/RIR architecture remains obscure. In Parkin, we found that no single element of the TRIAD/RIR was active, perhaps because the Parkin RINGs deviate from the consensus RING motif (C-X_2-C-$X_{(9-39)}$-C-$X_{(1-3)}$-H-$X_{(2-3)}$-C-X_2-C-$X_{(4-48)}$-C-X_2-C). In Parkin, the last two cysteines of the N-RING are separated by three rather than two amino acids, and the fourth and fifth cysteines of the Parkin C-RING are separated by four rather than two amino acids [16]. It is of interest that the distinctive spacing of cysteines in the Parkin C-RING is common to the C-RING of other TRIAD domain proteins. And, although other TRIAD proteins also differ from the consensus sequence in the spacing between the last two cysteines

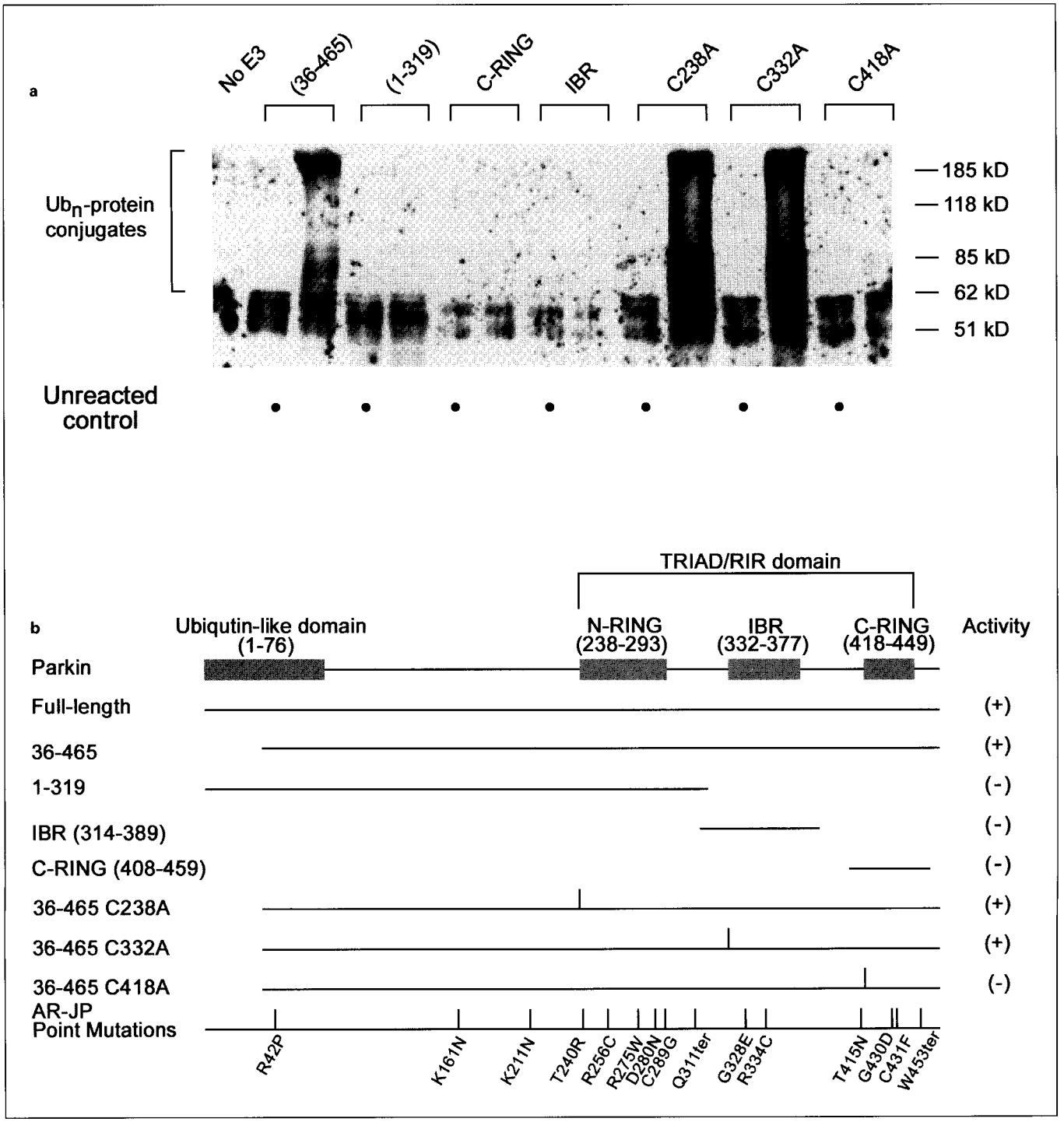

Fig. 4. Elements in the TRIAD domain cooperate in ubiquitination. **a** PAGE gel of ubiquitination reaction products. The reactions were performed with free ubiquitin and developed with an antiubiquitin antibody. Controls included a reaction containing all reagents except E3 (No E3) and unreacted reactions as in figure 3. **b** Map showing Parkin domains and GST fusion proteins used in the ubiquitination assays. The level of ubiquitination activity was noted by (–) for no activity and (+) for readily visible activity.

of the N-RING (X_4 instead of X_2), the X_3 amino acid spacing in Parkin is unique.

Although no isolated TRIAD element of Parkin showed E3 ubiquitin activity, we did find evidence for cooperative interactions between the individual motifs. Additional evidence for cooperative interaction between elements in the TRIAD comes from the study of the HHARI gene mentioned above [17]. In that study, the N-RING element and part of the adjacent IBR were required for E2-E3 (UbcH7-HHARI, respectively) binding. In our studies, ubiquitination activity required a functional C-RING together with either the N-RING or the IBR. If the IBR is not a single functional unit, an alternative interpretation for our results might be that proteins containing the C238A or C332A mutation have the required intact C-RING plus the adjacent portion of the IBR needed for activity.

The importance of the Parkin RIR domain was confirmed by three other Parkin research groups [7, 20, 27]. An intact RIR was found to be essential for UbcH7 binding [7, 20], whereas the C-RING element of the RIR was shown to be the minimal region required for binding of UbcH8 [27]. Both IBR and C-RING elements were needed to obtain the same high-affinity UbcH8 binding that was seen with the intact RIR [27]. There was some discrepancy in regard to the minimal region required for Parkin E3 ligase activity. The two studies which utilized the E2 UbcH7 needed full-length wild-type Parkin to obtain E3 ligase activity in either in vivo or in vitro ubiquitination assays [7, 20]. However, since it is not known whether the role of the TRIAD RING finger elements in the E3 autoubiquitination assays is the same as in assays with natural substrates, the demonstration of intrinsic E3 activity in this in vitro assay with purified proteins does not eliminate the possibility that the E3 requires other protein domains for specific recognition and ubiquitination of its natural substrates. Similar to our findings with human Ubc4, the C-RING appeared to be required but not sufficient for optimal E3 ligase activity in the study utilizing the E2, UbcH8 [27], although the high background in their assay made interpretation difficult.

Interestingly, stable UbcH5-Parkin binding was not observed in the previous studies [7, 20, 27], yet we found that the very similar human Ubc4 supported Parkin E3 ubiquitin ligase activity. Stable Parkin-E2 binding may not be a requirement for ubiquitination, since UbcH5 partially supported Parkin-dependent ubiquitination in the study of Zhang et al. [27], and Parkin was ubiquitinated under conditions in which UbcH7 did not bind Parkin in the study of Imai et al. [7] (perhaps a different E2 functions in the ubiquitination of Parkin). The fact that Ubc4 worked in our in vitro assay could be because of the unphysiologically high concentration of recombinant Ubc4 protein used. However, a study of functional interactions between the E2, Ubc2p, and Ubr1p, the E3 component of the N-end rule pathway in *Saccharomyces cerevisiae,* showed that transient E2-E3 interactions are sufficient for ubiquitination and that stable binding between E2 and E3 is not required [26].

Our results indicated that the C-RING of the Parkin TRIAD domain is required for ubiquitination activity. Thus, we would predict that disease-causing point mutations would be found in that region. Thus far, two have been located in adjacent amino acids within the C-RING, G430D [14] and C431F [15]. Another, a potential phosphorylation site (T415N), immediately precedes the C-RING, while a terminating mutation (W453ter) follows the C-RING [1]. The group of Zhang et al. [27] found that the disease mutation T415N eliminated UbcH8 binding and E3 ligase activity, and W453ter reduced both binding and E3 activity. We expect that disease-causing point mutations would also be found within other elements of the TRIAD since our evidence indicated cooperative interactions between the C-RING and other elements of the TRIAD domain. Consistent with our expectations, this appears to be the case (fig. 4b) (reviewed in the report of Kahle et al. [9]). The T240R mutation, mapping between the first and second cysteines of the N-RING, places a positively charged residue in a position in which only hydrophobic residues are normally found [16]. This mutation reduces UbcH8 binding and E3 ligase activity and eliminates UbcH7 binding and supported E3 activity [7, 20, 27].

One point mutation in particular is informative in regard to Parkin E3 ligase function. Q311ter is very similar to our 1–319 construct, containing the ubiquitin-like region plus the N-RING of the TRIAD domain. Our studies showed that 1–319 was unable to support ubiquitination. This was confirmed by three other research groups; the Q311ter mutation neither bound E2 nor supported E3 ligase activity [7, 20, 27]. The inability of Q311ter to support ubiquitination as well as the localization to the RING TRIAD domain of most naturally occurring Parkin mutations observed in AR-JP suggests that inactivation of ubiquitin ligase activity is a direct cause of AR-JP.

An accumulating body of evidence (including our results) suggests that defects in the proteasome degradation pathway may be important in Parkinson's disease. Another inherited form of Parkinson's disease is caused

by a mutation in the ubiquitin carboxy-terminal hydrolase (UCH-1) gene [12]. The UCH-1 hydrolase is thought to release ubiquitin from degraded protein residues, thus replenishing cellular ubiquitin pools. The E3 ubiquitin ligase activity of Parkin and the carboxy-terminal hydrolase activity of UCH-1 function at different points in the ubiquitin-proteasome pathway, and since inactivation of either protein causes parkinsonism, impaired protein degradation and the resulting accumulation of proteins may be a basic molecular mechanism of the pathology of Parkinson's disease.

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