Multiple (α-NH-ubiquitin)Protein Endoproteases in Cells*

(Received for publication, February 16, 1989)

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Ubiquitin is encoded as a variable, spacerless repeat of the gene terminating with an additional amino acid or as a gene coding for a single ubiquitin with a carboxyl-terminal extension of 52 to 80 amino acids. We report the identification and partial purification of enzymes that specifically hydrolyze the peptide bond between ubiquitin-ubiquitin conjugate (Ub-Ubase) or ubiquitin fusion proteins (Ub-Xase). The Ub-Ubase was separated from the Ub-Xase by dye-ligand-Sepharose chromatography. The Ub-Xase was purified further by affinity chromatography on ubiquitin-Sepharose. The fidelity of the endoprotease reaction was assessed by measuring the ability of the released ubiquitin to be activated by ubiquitin-activating enzyme (E1) which requires intact ubiquitin and by sequence analysis of the released carboxyl extension protein with 52 amino acids after endoproteolysis of human ubiquitin with 52-amino acid carboxyl extension. The failure of both Ub-Ubase and Ub-Xase to cleave a mutant ubiquitin-Gly-76→Ala-metallothionein showed that the endoproteases distinguish Gly-X from an Ala-X peptide bonds.

In eukaryotes, selective degradation of many short-lived proteins follows the ATP/ubiquitin-dependent proteolytic pathway (for reviews see Refs. 1 and 2). Ubiquitin, a highly conserved 76-amino acid protein, is attached to most cellular proteins destined for proteolysis (3–5). Ubiquitin is also found covalently attached to the ε-NH₂ groups of lysines of proteins which are not degraded (4, 5). The first step in the conjugation of monoubiquitin for the proteolytic pathway is the activation of intact monoubiquitin by ubiquitin-activating enzyme (E₁) (6, 7). Alterations to a single amino acid at the COOH terminus of monoubiquitin prevent activation of the molecule by E₃ (8–10).

Ubiquitin is encoded by a complex multigene family, none of which encodes monoubiquitin (11, 12, and reviewed in Ref. 13). The ubiquitin genes can be separated into two classes. The first codes for contiguous, spacerless, variable repeats (12), and the second for ubiquitin with a carboxyl-terminal extension of 52–80 amino acids (13–16). Monoubiquitin is apparently formed by the post-translational processing of multimeric or conjugated precursor proteins. We have demonstrated the post- or co-translational processing of human ubiquitin with a carboxyl-terminal extension of 52 or 80 amino acids (HUBCEP-52 or -80),¹ to ubiquitin and CEP-52 or -80, respectively (17). Studies with polyubiquitin mutants have shown that the gene is induced under conditions of stress such as heat shock or starvation (1, 18). Monoubiquitin is used to target excess/abnormal proteins produced during the stress for proteolysis (18).

Ubiquitin carboxyl extension proteins provide additional sources of ubiquitin to regulate ubiquitin conjugates in cells (19–21). The gene sequence for the carboxyl extension proteins (CEPs) is well conserved and encodes a basic protein containing ~31% lysine and arginine with features suggesting that CEPs direct natural fusions to the nucleus (22). CEPs contain metal binding motifs or "zinc finger sequences" which are found in factors that regulate transcription, receptors, and regulatory proteins that bind to nucleic acids (23–25).

We report the partial purification and characterization of enzymes that hydrolyze precursor polyubiquitin and ubiquitin-fusion proteins, both of which liberate monoubiquitin. One enzyme cleaves polyubiquitin to monoubiquitin (Ub-Ubase) and others hydrolyze (α-NH-ubiquitin)protein conjugates to monoubiquitin (Ub-Xases).

MATERIALS AND METHODS

Ubiquitin-activating enzyme (E₁) used in these studies was kindly provided by Dr. Avram Hershko, Haifa, Israel. Ubiquitin-aldehyde (Ub-al) and ubiquitin-methionine (Ub-Met) were gifts of Dr. Irwin A. Rose, Philadelphia, PA. Rabbit reticulocyte-rich blood was obtained from Pel-Freeze, and 10–20 or 17–27% polyacrylamide gradient SDS gels were obtained from The Integrated Separation Systems, Hyde Park, MA. The green dye-ligand-Sepharose was obtained from Pierce. [2,3H]ATP was purchased from Du Pont-New England Nuclear.

Preparation of Substrates—Pentaubiquitin expressed in Escherichia coli was purified as described previously (10), with the last step (preparative electrophoresis of pentaubiquitin) being replaced by Mono S ion exchange chromatography. 0.1 m KCl-eluted fractions contained pentaubiquitin with varying amounts of contaminating tetra- and triubiquitin but not di- and monoubiquitin. These fractions were used as substrate for Ub-Ubase. The normal and mutant forms of ubiquitin or Ub-Mt used in this study were purified according to published procedures (9, 30). The construction, expression, isolation, and properties of HUBCEP-52 and HUBCEP-80 form E. coli are published (17).

Partial Purification of (α-NH-ubiquitin)Protein Endoproteases—Ub-Ubase and the "Ub-X" endoproteases were partially purified from reticulocyte extracts. "Fraction II" was prepared as described previously (27). Fraction II was applied to an ion exchange column (Fast

¹ The abbreviations used are: HUBCEP-52, human ubiquitin with 52 amino acid carboxyl extension; CE5-52, carboxyl extension protein with 52 amino acids; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Ub, ubiquitin; Ub-al, ubiquitin where the terminal glycine acid group was converted to an aldehyde group; Ub-Met, ubiquitin with an additional methionine; DTT, dithiothreitol; Ub-Mt, ubiquitin-metallothionein; Ub-Ubase, Ub-Ub endoprotease; Ub-Xase, Ub-X endoprotease.
Flow Q Sepharose) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 2 mM DTT and washed with 5 column volumes of this buffer. The adsorbed proteins were eluted with a gradient of 0–1 M KCl. Aliquots were assayed for (α-NH-ubiquitin)protein endoprotease activity with pentaubiquitin or ubiquitin-metallothionein (Ub-Mt), which were expressed and isolated from E. coli (10, 25). The results (Peak-1 and Peak-2 of Fig. 1, A and B) of pentaubiquitin hydrolysis to monoubiquitin were observed. However, when these column fractions were assayed with Ub-Mt as substrate, there was one peak of Ub-Mt hydrolysis, corresponding to Peak-2 of Fig. 1B (data not shown). Ub-Mt is hydrolyzed to ubiquitin and metallothionein. However, in SDS-PAGE monoubiquitin and metallothionein (30) or HUBCEP-52 to monoubiquitin and CEP-52). The Ub-Xases from Peak-2 were purified further by adsorption to acid-washed charcoal. Flow Q Sepharose) equilibrated with 50 mM Tris-HCl, pH 7.4, was applied to a column of Green Dye-Ligand-Sepharose. This column was washed (described above) and eluted with a gradient of 0–0.6 M KCl. The Ub-Ubase (detected by the hydrolysis of pentaubiquitin to monoubiquitin) eluted from this column at ~0.1 M KCl and was not contaminated with Ub-Xases (detected by the hydrolysis of ubiquitin-metallothionein to monoubiquitin and metallothionein (30) or HUBCEP-52 to monoubiquitin and CEP-52). The Ub-Xases from Peak-2 were purified further from the Ub-Ubase by a column (detected by the hydrolysis of pentaubiquitin to monoubiquitin) eluted with 5 volumes of 50 mM Tris-HCl, pH 7.4, and then with the same volume of buffer containing, in addition, 1 M KCl. The affinity matrix was washed with 5 volumes of 50 mM Tris-HCl, pH 7.4. The Ub-Xases were then eluted with the above buffer concentrations and applied to the column, which was washed with 5 volumes of 50 mM Tris-HCl, pH 7.4, and then with the same volume of buffer containing, in addition, 1 M KCl. The affinity matrix was washed with 5 volumes of 50 mM Tris-HCl, pH 7.4. The Ub-Xase(s) was eluted with 5 volumes of Tris-HCl, pH 9, containing 10 mM DTT. Fractions were collected into tubes containing one-tenth the volume of 1 M Tris-HCl, pH 7. The partially purified Ub-Xase had no monoubiquitin or metallothionein activity. The presence of Ub-Ubase and Ub-Xases in Fraction II was shown in Fig. 2A, where pentaubiquitin, Ub-Mt, and HUBCEP-52 were hydrolyzed to monoubiquitin.

Polyacrylamide Gel Assay to Measure Ub-Ubase and Ub-Xase Activities—To estimate the hydrolysis of pentaubiquitin, Ub-Mt, or HUBCEP-52 (25 μg) was incubated with the (α-NH-ubiquitin)protein endoprotease (10 μg), for 30 min at 37°C in the absence or presence of various ubiquitin mutants (30 μg), in a reaction mixture (20 μl) containing 50 mM Tris-HCl, pH 7.4, 1 mM DTT. The reaction was terminated with 10 μl of 5× SDS sample buffer as indicated in Figs. 2 and 3. The ubiquitin bands were separated by SDS-PAGE. The protein bands were visualized with Coomassie Brilliant Blue R. The gel was scanned with a Beckman DU-88 Spectrophotometer equipped with a densitometer to measure the absorbance of the substrate and monoubiquitin. This procedure gave a semiquantitative estimate of the rate of the endoproteolysis.

Assays for Monoubiquitin with Ubiquitin-activating Enzyme (E1)—Ubiquitin formed during the reaction of Ub-Mt or HUBCEP-52 with (α-NH-ubiquitin)protein endoprotease (10 μg), for 30 min at 37°C in the absence or presence of various ubiquitin mutants (30 μg), in a reaction mixture (20 μl) containing 50 mM Tris-HCl, pH 7.4, and 1 mM DTT. The reaction was terminated with 10 μl of 5× SDS sample buffer as indicated in Figs. 2 and 3. The ubiquitin bands were separated by SDS-PAGE. The protein bands were visualized with Coomassie Brilliant Blue R. The gel was scanned with a Beckman DU-88 Spectrophotometer equipped with a densitometer to measure the absorbance of the substrate and monoubiquitin. This procedure gave a semiquantitative estimate of the rate of the endoproteolysis.

RESULTS

Partial Purification and Properties of Ub-Ubase and Ub-Xase—Ion exchange and affinity chromatography separated the Ub-Ubase from the Ub-Xases activities ("Materials and Methods"). Fraction-II after ion exchange chromatography gave a profile of absorbance at 280 nm (Fig. 1A). Two peaks of Ub-Ubase activity were observed; hydrolysis to monoubiquitin was detected by the decrease in the 40-kDa pentaubiquitin band and the simultaneous appearance of a 5-kDa band (data not shown) in addition to the E1, assays (Table I). The column fractions with Ub-Ubase activity are indicated as Peak-1 and Peak-2 in Fig. 1, A and B. The column fractions were also assayed for Ub-Xase activity. With Ub-Mt as substrate, there was only one major peak of Ub-Mt hydrolysis (data not shown). This peak of Ub-Xase activity corresponded to Peak-2 in Fig. 1, A and B. Peak-1 (Fig. 1, A and B) further purified on Green Dye-Ligand-Sepharose hydrolyzed pentaubiquitin to monoubiquitin but did not hydrolyze Ub-Mt or HUBCEP-52 (Ub-Xases) (Fig. 2B). Peak-2 contained Ub-Xase activities and small amounts of Ub-Ubase activity (Fig. 1, A and B, and Fig. 2C). Peak-2 was purified further by adsorption on a ubiquitin affinity column, which separated the HUBCEP-52-hydrolyzing enzyme from the Ub-Mt-hydrolyzing enzyme and Ub-Ubase (Fig. 2D). Most of the Ub-Ubase and the Ub-Mt-hydrolyzing enzymes were found in the unadsorbed column breakthrough and the column wash fractions (data not shown), indicating that ubiquitin affinity chromatography enriched the HUBCEP-52-hydrolyzing enzyme. The Ub-Ubase and the Ub-Xases had molecular mass ranges of ~150 and ~200 kDa, respectively, when chromatographed on G-200 and Superose-12 columns, indicating that they are separate proteins. The Ub-Ubase and the Ub-Xase activities were inhibited by ~5 μM Iodoacetamide.

Time Course for the Hydrolysis of Pentaubiquitin, with (α-NH-ubiquitin)Protein Endoprotease—To estimate the hydrolysis of pentaubiquitin, Ub-Mt, or HUBCEP-52 with the (α-NH-ubiquitin)protein endoproteases a reaction mixture (20 μl) was incubated for 30 min at 37°C (Fig. 3). A 5-kDa band migrated in a gradient SDS-PAGE with the mobility of monoubiquitin (Fig. 3). The absorbance of Coomassie Brilliant Blue stained substrate (pentaubiquitin, 40-kDa band) decreased with time and a 5-kDa band with the mobility of monoubiquitin simultaneously appeared and increased. The estimated rate for pentaubiquitin hydrolysis was ~5–10 ng/min/μg Fraction II.

Estimation of the Ubiquitin Formed During the Endoproteolysis with E1—The 5-kDa protein migrating as ubiquitin (Figs. 1–3), which appeared only when pentaubiquitin, Ub-Mt and HUBCEP-52 were hydrolyzed, was confirmed to be a functional ubiquitin molecule by the E1 assays. These assays estimated only the 76-amino acid ubiquitin released during endoproteolysis. Specificity of the E1 reaction was demonstrated by showing that mutants of ubiquitin were not substrates (Table I). The inability of E1 to activate Ub-al, Ub-Mt, Ub-A-76, Ub-Mt, HUBCEP-52, biotinylated or trypsin, or pentaubiquitin shows the specific requirements of E1 for ubiquitin with the proper COOH terminus. The results of the E1 assays (Table I), demonstrated a stoichiometric recovery of ubiquitin with a proper COOH terminus and thus specific hydrolysis of the peptide bond connecting ubiquitin to the conjugate.

Specificity and Sequence Recognition by the (α-NH-ubiquitin)Protein Endoproteases—The amino-terminal analysis of Ub-Mt and HUBCEP-52 showed that there was only one
Ub-Ub and Ub-X Endoproteases

Table I

Accuracy of the processing of ubiquitin-metallothionein and human ubiquitin carboxyl extension protein with (α-NH-ubiquitin)/protein endoprotease

The procedure and reaction conditions for each assay are given under “Materials and Methods.” ND, not determined.

| Reaction components | Enhancement of ubiquitin-dependent end point | Ubiquitin-dependent exchange with E<sub>i</sub> (10<sup>5</sup> [H]AMP-Ub complex formation) |
|---------------------|--------------------------------------------|----------------------------------|
|                     | pmol/min                                    | pmol/μl                          |
| Fraction II, Peak II, pH 9 eluate, Ub-Mt, or HUBCEP-52 alone | 0 | 0 |
| Peak II + 10 pmol/μl of Ub-Mt | 0.73 ± 0.067 | 9.8 ± 0.5 |
| Fraction II or Peak II + 10 pmol/μl of Ub | 0.77 ± 0.093 | 9.5 ± 0.53 |
| pH 9 eluate + 10 pmol/μl of HUBCEP-52 | ND | 9.4 ± 0.41 |
| Ubiquitin mutants; Ub-al, Ub-Gly-76 to Ala, Ub-Met, Ub treated with trypsin, etc., 10 pmol/μl | 0 | 0 |

FIG. 1. Separation of Peak-1 and Peak-2 by ion exchange chromatography. Panel A, fraction II was applied to an ion exchange column of Mono Q. The fractions eluted with 0–1 M KCl had two peaks of Ub-Ubase activity (hydrolyzed pentaubiquitin to monoubiquitin) indicated as Peak-1 and Peak-2. Panel B, aliquots of the column fractions after incubation with pentauubiquitin were analyzed on 18% SDS-PAGE. Assay conditions are given under “Materials and Methods.” The peaks of pentauubiquitin hydrolysis, where the 40-kDa band of pentauubiquitin was hydrolyzed to a 5-kDa band moving with the mobility of monoubiquitin are indicated as Peak-1 and Peak-2.

Amino terminus in the conjugate and that the first amino acid was methionine. Thus Ub-Mt and HUBCEP-52 isolated from E. coli was a contiguous protein and contained no isopeptide bonds. To confirm that the bond hydrolyzed in HUBCEP-52 was Gly-76-X the CEP-52 released after hydrolysis of HUBCEP-52 by Fraction II was isolated and subjected to amino-terminal analysis. The first two amino acids were isoleucine indicating that the endoproteolytic hydrolysis of HUBCEP-52 was specific.

Ub-Ubase and Ub-Xases present in Fraction II hydrolyzed pentauubiquitin, Ub-Mt, or HUBCEP-52 as shown by the appearance of 5-kDa monoubiquitin band with increasing time of incubation with all the substrates (Figs. 1–2). However, these endoproteases did not hydrolyze Ub-Gly-76-Ala-Mt (where Gly-76 of Ub-Mt was altered to alanine) as no 5-kDa monoubiquitin band appeared with increasing time of incubation (Fig. 2A).

To understand further the role of ubiquitin in the recognition of the substrates we attempted to inhibit the Ub-Xases with various ubiquitin mutants. Ub-Mt was incubated with Fraction-II and in the absence or presence of various ubiquitin mutants (Fig. 4). Except for Ub-al (where the band of Ub-Mt is not hydrolyzed), attempts to inhibit the Ub-Xase with ubiquitin mutants, methylated ubiquitin, human or yeast ubiquitin, were not successful (recognized by the loss of 16-kDa Ub-Mt band) (Fig. 4). Other results indicate that molar equivalents of Ub-al (compared to Ub-Xase in this case) were
**Ub-Ub and Ub-X Endoproteases**

**Fig. 2. Separation of (α-NH-ubiquitin)protein endoproteases to Ub-Ubase and Ub-Xase.**

Panel A, Fraction II (20 μg) was analyzed for hydrolyzing the substrates (pentaubiquitin, Ub-Mt, HUBCEP-52, and (Ub-Gly-76→Ala-Mt). The reaction was terminated after 0, 15, and 30 min and analyzed by SDS, 10–20% PAGE. The panel shows that Fraction II hydrolyzed pentaubiquitin, Ub-Mt, HUBCEP-52. However (Ub-Gly-76→Ala-Mt) was not hydrolyzed. Panel B, Peak-1 (Fig. 1) (10 μg) after Green-Sepharose chromatography was incubated with substrates according to conditions described above. The panel shows that Peak-1 hydrolyzed pentaubiquitin to monoubiquitin but did not hydrolyze Ub-Mt or HUBCEP-52. Panel C, Peak-2 (Fig. 1) (10 μg) was incubated with the substrates according to the conditions described above. This fraction hydrolyzed all three substrates (pentaubiquitin, Ub-Mt, and HUBCEP-52). Panel D, pH 9 eluate, 1 μg (Peak-2 purified further on ubiquitin affinity column), was incubated with the substrates according to the conditions described above. This fraction hydrolyzed HUBCEP-52 but did not hydrolyze pentaubiquitin or Ub-Mt.

**DISCUSSION**

We have reported that pentaubiquitin expressed in *E. coli* could support the degradation of 125I-bovine serum albumin in the reticulocyte lysate but that isolated pentaubiquitin was not activated by *E.* (10). Thus, pentaubiquitin appeared to have been processed to monoubiquitin in the reticulocyte lysate by endoprotease(s), which presumably cleaved either the peptide bond between ubiquitin-ubiquitin and/or between ubiquitin and asparagine of the terminal repeat of pentaubiquitin (10, 13). A number of other observations suggest the existence of cellular (α-NH-ubiquitin)protein endoproteases. In studies with the ubiquitin-β-galactosidase (Ub-βGal) fusion proteins in yeast, the expression of various Ub-βGal fusions resulted in the release of monoubiquitin (28). In *vitro* translation of diubiquitin and truncated diubiquitin mRNAs yielded monoubiquitin, detected by immunoprecipitation or SDS-PAGE (29). Ubiquitin-metallothionein (Ub-Mt) or some of its mutant fusion proteins can be processed to ubiquitin in yeast or the reticulocyte extracts (26, 30). Finally HUBCEP-52 and -80 are processed to ubiquitin and CEP-52 or -80, with the reticulocyte extracts (17). However, to date multiple enzymes responsible for processing (α-NH-ubiquitin)protein conjugates have not been identified or biochemically characterized.
The present results demonstrate the existence of multiple (α-NH-ubiquitin)protein endoproteases (Ub-Ubase and Ub-Xase) and suggest that there may be different enzymes which hydrolyze various ubiquitinated conjugates (Ub-Mt-hydrolyzing enzyme, ubiquitin carboxyl-terminal hydrolyzing enzyme) (Fig. 2). The result that only one amino terminus was observed in Ub-Mt and HUBCEP-52 showed that the multiplicity observed in these reactions were not due to isopeptidases. The correct amino terminus of CEP-52 released after endoproteolysis of HUBCEP-52 and the quantitative release of monoubiquitin from the substrates during the endoproteolytic reaction (Table I), demonstrate that the hydrolysis catalyzed by the (α-NH-ubiquitin)protein endoproteases is specific. The separation of Ub-Ubase and Ub-Xases activities by ion exchange (Fig. 1) and molecular size columns suggest that they are separate enzymes. The elution of Ub-Ubase both at Peak-1 and Peak-2 may be due to protein-protein interactions because the behavior of the Ub-Ubase from Peak-1 or Peak-2 on Green-Sepharose or the sizing columns was similar. Further purification of the Ub-Xases on ubiquitin Sepharose (pH 9 fraction, Fig. 2) indicated that HUBCEP-52-hydrolyzing enzyme can be separated from the Ub-Mt-hydrolyzing enzyme. This result can be explained by the selective inactivation of the Ub-Mt-hydrolyzing enzyme during its purification on ubiquitin-Sepharose or the separation of the enzymes that hydrolyze Ub-Mt and HUBCEP-52 by the affinity matrix. The results show that the Ub-Mt hydrolyzing enzyme was found primarily in the unadsorbed column breakthrough and wash fractions whereas the HUBCEP-52-hydrolyzing enzyme was found in the pH 9 eluate fractions. Thus they are separate proteins.

Attempts to inhibit the endoproteolytic reaction with various mutants of ubiquitin were unsuccessful (Fig. 4). However, results indicate that (Ub-al) at concentrations equal to those of Ub-Ubase and Ub-Xases irreversibly inhibits both endoproteases (Fig. 4). The likely mechanism for the inhibition of the endoprotease activity is the binding of the substrate analogue (Ub-al) to the substrate binding site on the enzymes. The endoproteases may also be specific for a particular ubiquitin conjugate and the inability of the enzymes to hydrolyze Ub-Gly-76→Ala-Mt (Fig. 2A), indicate their specificity for a Gly-X peptide bond. This hypothesis is supported by the result that yeast with plasmids expressing Ub-Gly-76→Ala-Mt grow at a reduced rate compared to yeast containing plasmids expressing Ub-Mt (30).

Our results suggest that several (α-NH-ubiquitin)protein endoproteases are involved with the maintenance of the cellular content of ubiquitin. Ub-Ubase may be induced when cells are subjected to stress conditions, such as heat shock or starvation. This hypothesis is supported by the previous observation in yeast that deletion of the polyubiquitin gene results in marked susceptibility to heat, starvation, sporulation, or conditions for spore germination (1, 18). The enhanced susceptibility of these cells may have been due to the increased accumulation of structurally and/or functionally abnormal proteins. The polyubiquitin gene in normal yeast induced under similar stress conditions could provide the cells with quantities of ubiquitin needed to complex with and target the abnormal proteins for energy-dependent proteolysis. Factors regulating the induction of the polyubiquitin gene may, in addition, regulate the amounts of Ub-Ubase in cells. Normal cell growth is not affected by deleting the yeast polyubiquitin gene (1, 18). Thus cells may have other enzymes, for example, Ub-Xase(s), ubiquitin carboxyl-terminal hydrolases, ubiquitin-depent proteases, or isopeptidases to maintain homeostasis between ubiquitin and ubiquitin conjugates under normal growth conditions (19).

Ub-Xase(s) not only help in maintaining appropriate levels of ubiquitin in cells but may play a major role in cell regulation. For example, the (α-NH-ubiquitin)CEP conjugates appear to be regulated by post- or co-translational processing to give ubiquitin and CEPs in cells (19, 20). Genetic analyses with the yeast natural fusion protein genes (UBI-1, UBI-2, UBI-3 coding for the yeast ubiquitin carboxy extension proteins) show that mutations in any of the three UBI loci lead to a prolonged G1 phase in the cell cycle and that a double mutation of UBI-1/UBI-2 is lethal (1). Purification and characterization of the individual (α-NH-ubiquitin)protein endoproteases will help in understanding further the cellular roles of these enzymes.

Acknowledgments—We thank Dr. Michael M. Mattern for editing the manuscript. We thank Dr. James E. Strickler for conducting the amino-terminal analysis for Ub-Mt and HUBCEP-52.

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