Cell, Vol. 96, 635–644, March 5, 1999, Copyright ©1999 by Cell Press

A Novel Ubiquitination Factor, E4, Is Involved in Multiubiquitin Chain Assembly

Manfred Koegl,[†] Thorsten Hoppe,[‡] Stephan Schlenker,[§] Helle D. Ulrich,[‡] Thomas U. Mayer,[‖] and Stefan Jentsch*[‡]
Zentrum für Molekulare Biologie der Universität Heidelberg
Im Neuenheimer Feld 282
69120 Heidelberg
Germany

Summary

Proteins modified by multiubiquitin chains are the preferred substrates of the proteasome. Ubiquitination involves a ubiquitin-activating enzyme, E1, a ubiquitin-conjugating enzyme, E2, and often a substrate-specific ubiquitin–protein ligase, E3. Here we show that efficient multiubiquitination needed for proteasomal targeting of a model substrate requires an additional conjugation factor, named E4. This protein, previously known as UFD2 in yeast, binds to the ubiquitin moieties of preformed conjugates and catalyzes ubiquitin chain assembly in conjunction with E1, E2, and E3. Intriguingly, E4 defines a novel protein family that includes two human members and the regulatory protein NOSA from *Dictyostelium* required for fruiting body development. In yeast, E4 activity is linked to cell survival under stress conditions, indicating that eukaryotes utilize E4-dependent proteolysis pathways for multiple cellular functions.

Introduction

Selective protein degradation plays an important role in cellular regulation. Progression through the eukaryotic cell cycle, for example, is substantially regulated through a precisely scheduled destruction of cyclins, inhibitors of cyclin-dependent protein kinases, and other regulators. Similarly, alterations of transcriptional or developmental programs are often achieved through a coordinated degradation of regulatory proteins. Selective proteolysis is also essential for protecting cells against environmental stress due to its important role in eliminating aberrant proteins generated under normal and, in particular, stress conditions (for recent reviews, see Hochstrasser, 1996; Patton et al., 1998).

In eukaryotes, selective protein breakdown proceeds primarily through the ubiquitin/proteasome system (reviewed by Hochstrasser, 1996). Substrates of this pathway are recognized by components of the ubiquitin conjugation system and earmarked for degradation by conjugation to ubiquitin. Ubiquitin is joined to substrate proteins via an isopeptide linkage between its carboxyl (C) terminus and the ϵ amino group of an internal lysine residue of the target protein. It appears that most substrates are modified by multiubiquitin chains in which single ubiquitin moieties are linked via isopeptide bonds to one another (Chau et al., 1989). Multiubiquitinated substrates are the preferred substrates of the 26S proteasome, whereas proteins modified by single or only a few ubiquitin molecules appear to be long-lived in vivo or are subject to alternative degradation pathways (Jentsch and Schlenker, 1995; Hochstrasser, 1996).

Ubiquitination involves a series of well-defined reactions catalyzed by several classes of enzymes. Ubiquitin-activating enzyme, E1, hydrolyzes ATP and forms a high-energy thioester between a cysteine of its active site and the C terminus of ubiquitin. Activated ubiquitin is then passed on to members of the family of ubiquitin-conjugating enzymes, E2s, which form thioester-linked complexes with ubiquitin in a similar fashion. Finally, ubiquitin is covalently attached to the substrate protein directly by the E2s or, alternatively, by ubiquitin–protein ligases, E3s, which often interact with the substrate directly.

In this article we describe a novel ubiquitination factor that we named E4. In the absence of E4, ubiquitination of a model substrate is initiated, but only a few ubiquitin molecules are ligated to the substrate protein that are insufficient for proteasomal degradation in vivo. E4 binds to the ubiquitin moieties of these conjugates and, in conjunction with the E1, E2, and E3 enzymes, drives multiubiquitin chain assembly, yielding long chains. E4 defines a novel protein family. Members include the yeast protein UFD2, involved in stress tolerance, the developmental regulator NOSA from *Dictyostelium*, and two proteins from human. This suggests that proteolysis of a number of substrates might be regulated by multiubiquitin chain assembly.

Results

Reconstitution of a Ubiquitination System

To examine events downstream of the substrate recognition step in the ubiquitin/proteasome pathway, we reconstituted from recombinant yeast enzymes an in vitro ubiquitination system. We took advantage of specific model substrates, named UFD substrates (ubiquitin fusion degradation), that bear amino (N) terminally positioned ubiquitin moieties that serve as degradation signals for the ubiquitin/proteasome pathway. It has been shown previously that these substrates are modified by multiubiquitin chains, which are attached to specific lysine residues within the ubiquitin moiety of the fusion protein (Johnson et al., 1992, 1995). The enyzmes required for this reaction are the E1 ubiquitin-activating

*To whom correspondence should be addressed (e-mail: jentsch@biochem.mpg.de).
[†] Present address: Lion Bioscience AG, Im Neuenheimer Feld 517, 69120 Heidelberg, Germany.
[‡] Present address: Department of Molecular Cell Biology, Max Planck Institute for Biochemistry, Am Klopferspitz 18a, 82152 Martinsried, Germany.
[§] Present address: Genome Pharmaceuticals Corporation AG, Lochhamer Str. 29, 82152 Martinsried, Germany.
[‖] Present address: Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115.

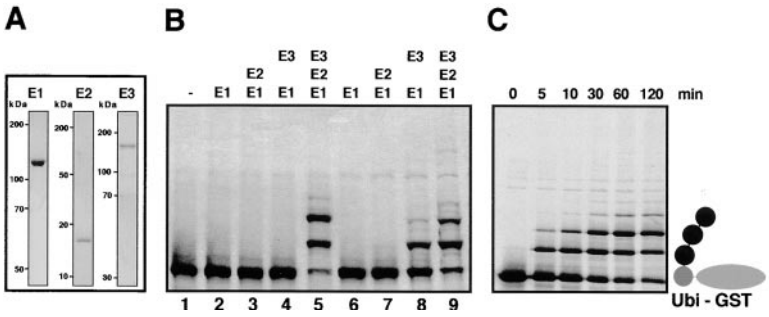

Figure 1. In Vitro Ubiquitination with Recombinant E1, E2, and E3 Enzymes

(A) Expression and purification of enzymes. Recombinant E1 (UBA1), E2 (UBC4), and E3 (UFD4) proteins were produced in baculovirus-infected insect cells and purified by conventional chromatography methods and affinity purification on ubiquitin-Sepharose. Coomassie-stained gels of the purified proteins are shown. The migration position of molecular weight markers is indicated.

(B) Enzyme requirement for ubiquitination of a UFD substrate in vitro. Ubi-GST was ^{35}S-labeled in *E. coli* and purified via glutathione-Sepharose (lane 1). Ubiquitination assays were done with the combination of enzymes indicated using purified enzymes (lanes 2–5) or crude extracts of insect cells expressing the respective clones (lanes 6–9). Crude E3 fractions apparently contain significant levels of insect UBC4. After separation by SDS-PAGE (9%), reaction products were visualized by autoradiography. The migration position of the fusion protein and its ubiquitinated derivatives (indicated by a chain of filled circles) are indicated.

(C) Time course of in vitro ubiquitination. As in (B), ^{35}S-labeled Ubi-GST was subjected to in vitro ubiquitination using crude extracts of insect cells, expressing the respective enzymes. After incubation for the times indicated, aliquots of the reaction were removed and the reaction was terminated by the addition of SDS-PAGE sample buffer.

enzyme UBA1 (McGrath et al., 1991), the E2 enzyme UBC4 (or its isozyme UBC5; Seufert and Jentsch, 1990; Johnson et al., 1992), and the E3 enzyme UFD4 (Johnson et al., 1995). We reasoned that UFD substrates are particularly suited to study mechanistic aspects of ubiquitination due to the simplicity of their degradation signal and the availability of extensive genetic information about this proteolytic pathway (Johnson et al., 1992, 1995).

The UFD substrate we used (Ubi-GST) is a fusion of ubiquitin with glutathione S-transferase (GST) that allows one-step purification of the protein on glutathione-Sepharose beads. The E1, E2, and E3 enzymes, the gene products of *UBA1*, *UBC4*, and *UFD4*, respectively, were produced in baculovirus-infected insect cells and purified by conventional chromatography methods (Figure 1A). These enzymes were incubated together with the radiolabeled substrate in a buffer containing ATP and ubiquitin. As shown in Figure 1B, the activities of all three enzymes together are required and sufficient for the recognition and ubiquitination of the substrate in vitro. Ubiquitin conjugation proceeded efficiently, but unexpectedly, the majority of conjugates appeared to contain only one to three ubiquitin moieties. Conjugates bearing more than three ubiquitin moieties were generated with greatly reduced efficiency by these enzymes.

The failure to synthesize long multiubiquitin chains could be the result of limited enzyme activity in the assay. Therefore, we tested whether prolonged incubation times can drive the reaction to the formation of longer chains. This was apparently not the case, however, as a time course study (Figure 1C) revealed that after 1 hr, ubiquitin–conjugate formation reached maximum levels, yielding conjugates with rarely more than three added ubiquitin moieties. Even when the reaction was furnished with an additional supply of enzymes or substrates, no significant increase of larger multiubiquitinated species could be detected (not shown). Thus, the limited capacity to generate multiubiquitinated proteins in vitro is inherently characteristic of the combination of E1, E2, and E3 enzymes used in our assay.

UFD2 Is a Novel Ubiquitin Conjugate–Binding Protein

The previously identified multiubiquitin chain–binding protein RPN10 (formerly known as MCB1 or SUN1) prefers chains that are longer than those generated by our in vitro ubiquitination system (van Nocker et al., 1996). Thus, we wondered whether additional ubiquitin-binding proteins with different specificities might exist. To identify such proteins, we set up an affinity-based purification assay in vitro. Ubiquitinated Ubi-GST fusion proteins, and unmodified Ubi-GST as a control, were immobilized on glutathione-Sepharose. A total yeast cell extract was passed over the two resins, and binding proteins were eluted with high salt. SDS–polyacrylamide gel electrophoresis identified an ~110 kDa protein that was significantly enriched in the eluate from the ubiquitin–conjugate column (Figure 2A). This protein was isolated from a preparative SDS gel, digested with trypsin, and the fragments were analyzed by mass spectrometry. Comparison with the predicted tryptic fragments of the yeast protein database identified this protein as UFD2 (data not shown). In fact, UFD2 had been discovered previously in a genetic screen for mutants that stabilize UFD substrates (Johnson et al., 1995). Its function in the proteolytic pathway, however, has remained unclear.

To study the binding properties of UFD2 in more detail, we expressed ^{VSV}epitope-tagged UFD2 (^{VSV}UFD2) in yeast. Extracts of these cells were incubated with immobilized ubiquitinated Ubi-GST or with Ubi-GST in a control reaction, washed, and eluted with SDS. Significantly more ^{VSV}UFD2 could be recovered from the column bearing the ubiquitinated form of Ubi-GST (Figure 2B, lane 2), confirming its specificity for ubiquitinated proteins. When we included an excess of free ubiquitin in our binding assays, no competition was observed (Figure 2B, lane 3), indicating that UFD2 can discriminate between free ubiquitin and ubiquitin–protein conjugates. We also immobilized UFD2 as a GST fusion and allowed ubiquitin–protein conjugates to bind. Since we could not use Ubi-GST as a UFD substrate in this experiment, we used a fusion of ubiquitin with protein A instead of GST (Ubi-ProtA) for these and subsequent assays.

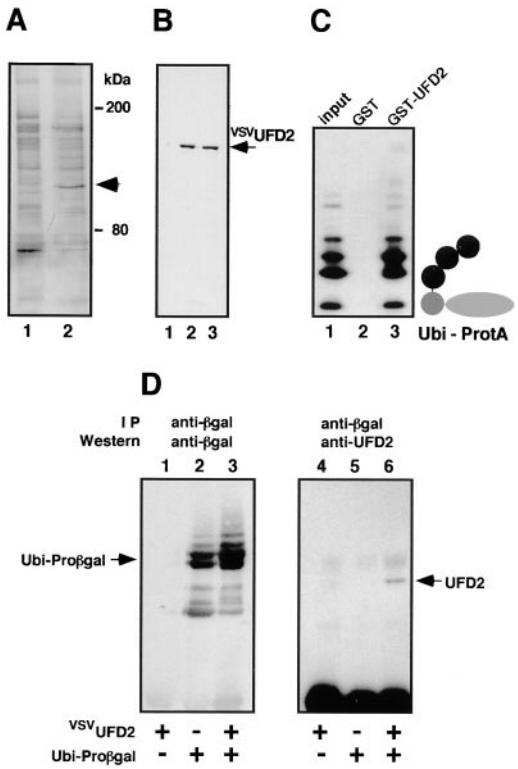

Figure 2. UFD2 Is a Novel Ubiquitin–Protein Conjugate–Binding Protein

(A) Isolation of UFD2 by affinity chromatography. Equal amounts (1 mg) of each Ubi-GST (lane 1) or ubiquitinated Ubi-GST (using E1, E2, and E3 enzymes) (lane 2) were immobilized on glutathione beads. Yeast extract was allowed to bind, unbound material was washed off with a low-salt buffer, and bound proteins were eluted with high salt. After separation by SDS-PAGE, proteins were detected by silver staining. The arrowhead points to a protein in the eluate of the ubiquitin–conjugate column that was identified as UFD2 by mass spectrometry.

(B) Binding of recombinant UFD2 to ubiquitinated Ubi-GST. An epitope-tagged version of UFD2 (^{VSV}UFD2) was expressed in yeast and extracts were prepared. As in (A), extracts (0.6 mg) were allowed to bind to equal amounts (10 μg) of either immobilized Ubi-GST (lane 1) or ubiquitinated Ubi-GST (lanes 2 and 3). After washing and elution by SDS, bound proteins were analyzed by Western blotting using VSV-specific antibodies. In lane 3, free ubiquitin (3 mg/ml) had been added to the binding buffer.

(C) Binding of a ubiquitinated UFD substrate (Ubi-ProtA) to GST-UFD2 immobilized on glutathione-Sepharose. Ubiquitinated Ubi-ProtA was prepared using crude extracts of insect cells expressing E1, E2, and E3 as enzyme sources. The reaction was stopped by addition of EDTA, and aliquots (0.5 μg of substrate) were added to beads displaying bound GST fusion protein (2 μg). After binding, washing, and elution, bound Ubi-ProtA was detected by Western blotting using anti-horseradish peroxidase antibodies. Lane 1, input of ubiquitinated Ubi-ProtA; lanes 2 and 3, material retained on GST (control) or GST-UFD2, respectively.

(D) Association of UFD2 with ubiquitin–protein conjugates in vivo. Extracts of yeast cells expressing combinations (indicated by + and − symbols) of ^{VSV}UFD2 and the UFD substrate Ubi-Proβgal were prepared, and Ubi-Proβgal was immunoprecipitated using monoclonal anti-βgal antibodies. Proteins were detected by Western blotting using either the βgal-specific antibody (left panel) or an antibody against the ^{VSV}epitope (right panel).

Interestingly, we found that UFD2 bound all ubiquitinated species with similar affinity (Figure 2C). Thus, the good recovery of UFD2 specifically from the ubiquitin–conjugate column (Figure 2B) probably reflects the larger number of UFD2-binding sites (i.e., ubiquitin moieties) associated with this column. UFD2 thus displays a ubiquitin-binding behavior that is strikingly different from that of RPN10, which has been shown to preferentially bind chains of three or more ubiquitin molecules (van Nocker et al., 1996).

To analyze whether UFD2 also associates with the ubiquitinated form of a UFD substrate in vivo, we used a short-lived fusion of ubiquitin with the bacterial protein β-galactosidase (Ubi-Proβgal) (Johnson et al., 1992, 1995). Indeed, when we precipitated Ubi-Proβgal from extracts of yeast cells expressing ^{VSV}UFD2 with substrate-specific antibodies, a VSV-reactive protein of the predicted size could be detected specifically in cells expressing both proteins, but not in control cells (Figure 2D). Thus, UFD2 possesses ubiquitin conjugate–binding activity also in vivo.

UFD2 Functions as a Ubiquitin Chain Assembly Factor, E4

Next we asked whether this novel ubiquitin-binding protein influences the ubiquitin conjugation reaction. Intriguingly, we found that recombinant UFD2 dramatically stimulated the ubiquitination reaction, yielding protein conjugates with significantly longer chains than those synthesized with E1, E2, and E3 enzymes alone (Figure 3A). The synthesis of long multiubiquitin chains was clearly dependent on the concentration of a GST-UFD2 fusion protein present in the assay, and multiubiquitination was most dramatic if native, baculovirus-expressed UFD2 was used. We performed a time course experiment and started the reaction with E1, E2, and E3 enzymes (Figure 3B). Ubiquitination of the substrate reached a plateau after about 2 hr incubation time, and conjugates with generally no more than two or three added ubiquitin moieties were formed. However, when UFD2 was added at a time when the reaction with E1, E2, and E3 enzymes was already completed (at 140 min), multiubiquitination was reinitiated rapidly and very long multiubiquitin chains were polymerized. Notably, conjugates bearing long multiubiquitin chains accumulated at the expense of the initially synthesized shorter conjugates. Thus, UFD2 is indeed involved in multiubiquitin chain assembly and not needed during the initiation phase of the reaction. In keeping with the common nomenclature of ubiquitination enzymes, we would like to propose the term E4 for ubiquitination factors that, like UFD2, are specifically required for multiubiquitin chain polymerization.

Efficient multiubiquitination of our model substrate requires the complete set of E1, E2, E3, and E4 enzymes (Figure 3C), yet we wondered whether E4 could substitute for E3 during the chain assembly phase of the reaction. To test this possibility, we purified ubiquitin–protein conjugates generated with E1, E2, and E3 enzymes and used these conjugates as substrates in our multiubiquitination assays. As shown in Figure 3D, E4-catalyzed

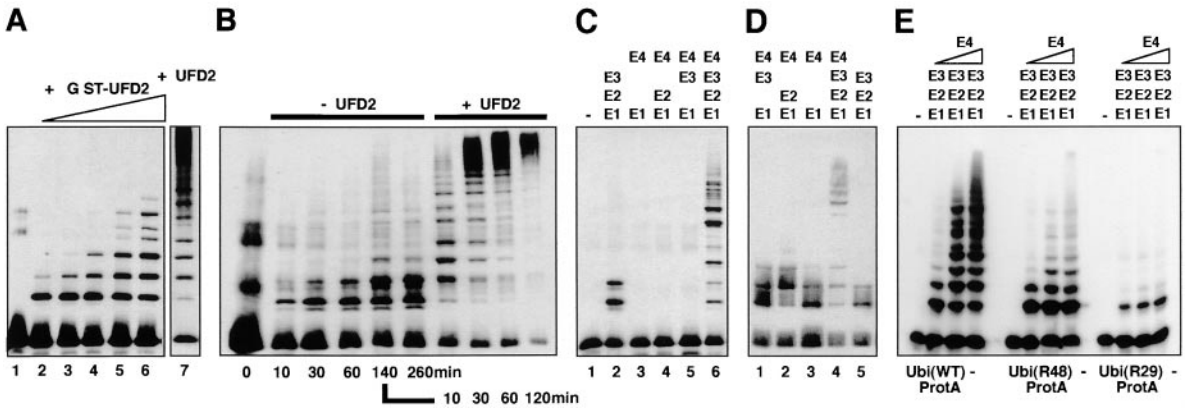

Figure 3. UFD2 Functions as a Ubiquitin-Chain Assembly Factor, E4

(A) Dose-dependent activity of UFD2 in ubiquitin chain elongation. Ubi-ProtA (lane 1) was subjected to a ubiquitination reaction using purified E1, E2, and E3 enzymes (lane 2) in the presence of increasing amounts of purified GST-UFD2 (lanes 3–6; 0.1, 0.3, 1, and 3 μg, respectively). Addition of GST alone to the reaction had no effect (not shown). Even stronger chain assembly activity was observed when nontagged UFD2 from crude fractions of expressing insect cells were used (lane 7).

(B) UFD2 mediates multiubiquitin chain assembly. Time course of a ubiquitination reaction with Ubi-ProtA using crude fractions of E1, E2, and E3 enzymes similar to Figure 1C. After 140 min, UFD2-containing extract was added to one-half of the reaction, and aliquots were taken after an additional 10, 30, 60, and 120 min (labeled +UFD2). The remaining reaction, without UFD2, was incubated for another 120 min (labeled 260 min, -UFD2). Western blots detecting protein A are shown. In lane 1 (0 time point), more Ubi-ProtA was loaded to show that some minor bands correspond to substrate aggregates.

(C) Multiubiquitination of Ubi-ProtA requires the complete set of E1, E2, E3, and E4 enzymes. The experiment was done as above and the enzymes were added to the reaction as indicated.

(D) E4 requires E1, E2, and E3 enzymes for activity. Ubi-ProtA was ubiquitinated as in (B) in the absence of E4. The product of this reaction, ubiquitinated Ubi-ProtA, was purified by an antibody column and again incubated as above with combinations of E1, E2, E3, and E4 enzymes.

(E) Multiubiquitination of Ubi-ProtA requires specific lysine residues of the ubiquitin moiety of the substrate. Ubi-ProtA bearing the wild-type sequence of ubiquitin [Ubi(WT)-ProtA] or variants in which lysine 48 [Ubi(R48)-ProtA] or lysine 29 [Ubi(R29)-ProtA] of the ubiquitin moiety of the substrate had been replaced by arginine residues were incubated with E1, E2, and E3 and either without (−) or with 1 or 5 μl E4 enzyme fractions (0.05 and 0.25 μg E4 protein).

multiubiquitin chain assembly requires the complete set of enzymes, including E3, demonstrating that E4 is a novel, distinct ubiquitination factor.

We obtained no evidence for a physical interaction of E4 with the relevant E2 and E3 enzymes, UBC4 and UFD4, respectively, either by two-hybrid or biochemical assays (Figure 4A and data not shown). Furthermore, the protein does not seem to stimulate the formation of thioester-linked complexes of E1, E2, or E3 enzymes with ubiquitin (data not shown), indicating that it does not function simply as an activator of one of the other ubiquitination enzymes. In contrast to E3s, the E4 protein has no detectable affinity for substrates other than those that are already modified by ubiquitin, and the protein itself appears to be unable to form a thioester-linked complex with ubiquitin (data not shown). We thus assume that E4 functions primarily through its ubiquitin-binding property and that it may influence the linkage between individual ubiquitin molecules (Figure 3E and Discussion).

E4 Interacts with the AAA ATPase CDC48

To initiate studies aimed to define the steps that follow the multiubiquitination reaction in the ubiquitin/proteasome pathway, we investigated whether UFD2 interacts with other proteins of the UFD pathway. We observed no interaction of UFD2 with UFD1, UFD3, UFD4, and the ubiquitin chain–binding protein RPN10 in two-hybrid assays (Figure 4A and data not shown). Intriguingly, however, we found that UFD2 interacts with CDC48 in two-hybrid and pull-down assays (Figures 4A and 4B). CDC48 belongs to the large family of AAA-type ATPases, which are thought to possess protein folding activity (Fröhlich et al., 1991). It has been shown previously that this protein is required for the degradation of UFD substrates in vivo (Ghislain et al., 1996), but its role in the ubiquitin-dependent proteolytic pathway has remained unclear.

To analyze whether CDC48 plays a role in ubiquitin conjugation, we added extracts of CDC48-overexpressing insect cells to the complete E4-dependent conjugation assay. As shown in Figure 4C, CDC48 does not influence the reaction (Figure 4C). However, when we examined the influence of a purified fusion protein of CDC48 with maltose-binding protein (MBP-CDC48) on binding of UFD2 to immobilized multiubiquitinated Ubi-GST, a dose-dependent, albeit ATP-independent, inhibition of this interaction was apparent (Figure 4D). A similar inhibition of binding was noticed when the interaction was studied using immobilized GST-UFD2 and multiubiquitinated Ubi-ProtA (data not shown). From these data, we assume that CDC48 may function after the multiubiquitin chain has been completely assembled (see Discussion).

E4 Mediates Multiubiquitination In Vivo

To investigate whether initiation and chain assembly phases of the multiubiquitination reaction can also be distinguished in vivo, we performed pulse-chase experiments. Similar to previous studies of *ufd* mutants, we

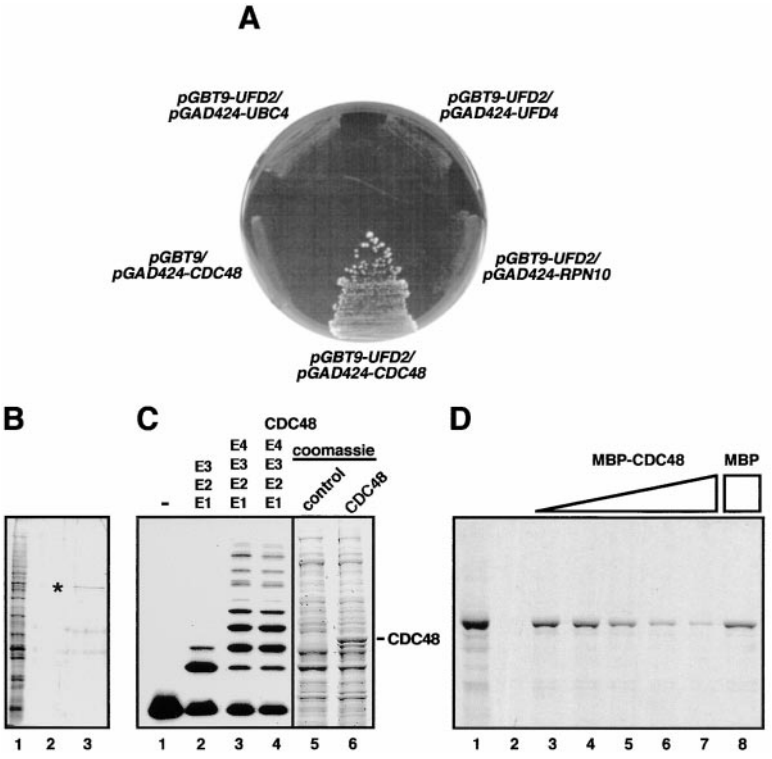

Figure 4. The AAA-type ATPase CDC48 Binds to UFD2

(A) Two-hybrid tests for possible interactions of UFD2 with UBC4, UFD4, RPN10, or CDC48. Yeast cells expressing the indicated plasmids were streaked out on medium plates lacking histidine to assay for interaction-dependent activation of the *HIS3* gene.

(B) Interaction of CDC48 and UFD2 in vitro. An extract from baculovirus-infected insect cells overexpressing CDC48 (lane 1) was incubated with immobilized GST (lane 2) or immobilized GST-UFD2 (lane 3). After extensive washing, bound proteins were eluted with 500 mM NaCl and analyzed by SDS-PAGE and silver staining. The asterisk denotes the position of CDC48.

(C) CDC48 does not influence the ubiquitination reaction. Ubi-ProtA was incubated with E1, E2, E3, and E4 enzymes as indicated. In lane 4, extracts of CDC48-expressing insect cells (lane 6) were added to the reaction. CDC48 (0.3 μg) was in an ~6-fold molar excess to E4 (0.05 μg).

(D) CDC48 interferes with binding of UFD2 to ubiquitinated proteins. ^{35}S-labeled UFD2 (25 μg crude extract from UFD2-expressing insect cells; lane 1 shows a 10 μg aliquot) was allowed to bind either to Ubi-GST protein (5 μg, lane 2) or to ubiquitinated Ubi-GST (5 μg, lanes 3–8). UFD2 was preincubated (lanes 3–7) with MBP-tagged CDC48 in increasing concentrations (0, 1, 3, 10, and 25 μg, respectively). In a control experiment, MBP alone (25 μg, lane 8) was added. After extensive washing, bound proteins were eluted with SDS and analyzed by SDS-PAGE and autoradiography.

used the short-lived Ubi-Proβgal fusion protein as a substrate (Johnson et al., 1992, 1995; Seufert and Jentsch, 1992). As expected, Ubi-Proβgal was rapidly proteolyzed in wild-type (WT) cells but was significantly stabilized in the mutants tested (Figure 5A). As noticed previously, mutants in the genes for the relevant E2 (*ubc4 ubc5* double mutant) and E3 (*ufd4*) enzymes stabilized the substrate completely, yet a fraction became modified by one—rarely two—added ubiquitin moieties, probably by the activities of other E2s and E3s.

When we performed similar experiments with the *ufd2* mutant, we observed that the substrate was stabilized with only a few added ubiquitin moieties. A small fraction of the substrate carried longer multiubiquitin chains, but these were on average notably shorter than those detected with WT cells at early time points of the experiment. These data therefore extend our in vitro finding and demonstrate that E4-catalyzed multiubiquitination is indeed required for the degradation of the substrate in vivo.

As noticed earlier (Ghislain et al., 1996), *cdc48* mutants stabilized the UFD substrate with relatively long chains. These chains were on average significantly longer than those from *ufd2* mutant cells (Figure 5). Remarkably, however, in *ufd2 cdc48* double mutants, short chains similar to those found in *ufd2* single mutants were detected. Together with our previous in vitro data, this epistasis analysis suggests that CDC48 functions downstream of the multiubiquitination reaction in the proteolysis pathway.

E4 Function Is Linked to Stress Tolerance

Protein degradation by the UFD pathway is not essential for viability under normal growth conditions (Johnson et al., 1992, 1995). Mutants in *ufd2* exhibit no growth defect under normal conditions. However, compared to WT cells, they show a significant sensitivity to 6% ethanol (not shown). This mutant phenotype was not exacerbated when other mutations in the UFD pathway were

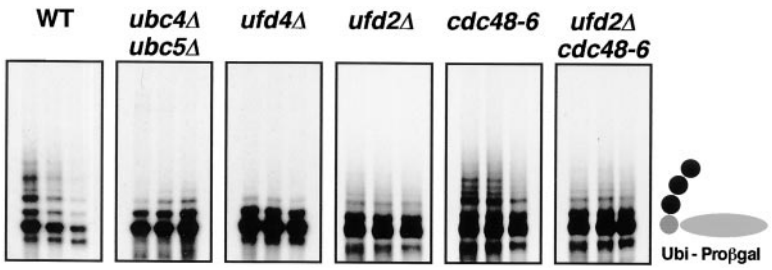

Figure 5. UFD2 Is Required for Efficient Multiubiquitination of Ubi-Proβgal In Vivo

Pulse-chase analysis of Ubi-Proβgal in WT cells and stabilizing *ufd* mutants. Epistatic relationship between the respective genes is indicated by the extent of multiubiquitination. Time points were 0, 10, and 30 min.

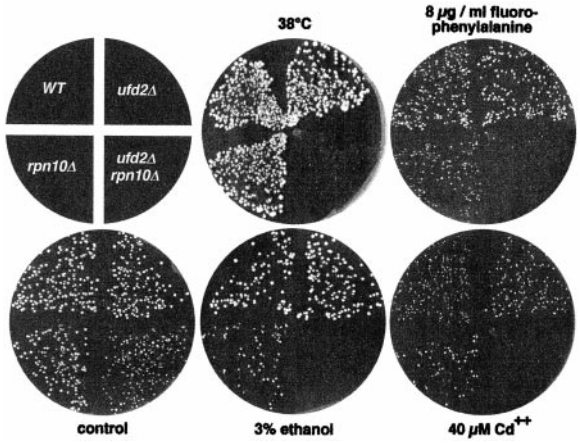

Figure 6. E4 Activity Is Linked to Stress Tolerance in Yeast

Approximately equal numbers of cells from WT, *ufd2* or *rpn10* single mutants, or *ufd2 rpn10* double mutants were streaked out on SD agar plates containing the indicated additions. Hypersensitivity to stress is indicated by the lack of colony formation.

crossed into the *ufd2* mutant strain, confirming an epistatic relationship between these genes. Notably, however, we observed that double mutants lacking UFD2 and the only other known ubiquitin-binding protein, RPN10, exhibited a profound sensitivity to a variety of stress conditions that was not observable in either of the two single mutants. They were hypersensitive to elevated temperatures and exposure to the heavy metal cadmium, ethanol (3%), and the amino acid analog fluoro-phenylalanine (Figure 6). Thus, the yeast E4 is functionally linked to stress tolerance and most likely mediates the degradation of stress-induced aberrant proteins.

Discussion

Initiation and Polymerization Phases of Multiubiquitination

Multiubiquitination is a polymerization reaction by which single ubiquitin moieties become connected to one another through isopeptide linkages. The reaction starts with the conjugation of the first ubiquitin moiety to the side chain of a lysine residue of a target protein. In the following reaction, the conjugation enzymes switch the target molecule and link the next ubiquitin moiety to a lysine of the previously conjugated ubiquitin molecule. This reaction is repeated several times, yielding chains of sometimes more than ten polymerized ubiquitin molecules.

Reconstituting ubiquitination of a model substrate in vitro, we unexpectedly discovered that the reaction can be conceptionally divided into an initiation and a polymerization phase. In the initiation phase of the reaction, which requires E1, E2, and E3 enzymes, the substrate used in our studies is recognized, but only a few ubiquitin molecules are ligated to the substrate. The synthesis of long multiubiquitin chains, however, requires the same set of E1, E2, and E3 enzymes, plus an additional ubiquitination factor, which we named E4. Unlike E1, E2s, and some E3s, E4 does not participate in the ubiquitin–enzyme thioester cascade. Moreover, in striking contrast to E3s, the E4 protein does not interact with the substrate directly, but apparently with the ubiquitin moieties of ubiquitin–substrate conjugates.

Why the ubiquitination machinery stops after a few cycles in the absence of E4 is not clear at the present time. It could be the result of steric constraints. Depending on the number of ubiquitin moieties in a chain, multimers of ubiquitin are thought to adopt distinct quaternary structures (Cook et al., 1994), and some of these may prevent further elongation by the E3 enzyme. Binding of E4 may then rearrange this structure in a chaperone-like way that allows optimal recognition by E3. In a variation of this model, the multiubiquitination reaction might be sensitive to the type of ubiquitin–ubiquitin linkage, that is, the identity of the lysine residue of ubiquitin used for the formation of the isopeptide bond. In line with published data (Johnson et al., 1995), we noticed that the ubiquitination of the UFD substrate requires the presence of lysine 29 of the ubiquitin moiety of the substrate (Figure 3E), suggesting that ubiquitination commences at this site. Replacing lysine 48 of the ubiquitin moiety of the substrate by arginine, on the other hand, leads to a significant inhibition in E4-dependent chain assembly. Consistent with these data is a model by which the binding of UFD2 to the conjugate triggers the formation of a different ubiquitin–ubiquitin linkage (e.g., via lysine 48) that may facilitate the addition of further ubiquitin molecules to the substrate.

Can Proteolysis Be Regulated by Ubiquitin Chain Assembly?

The distinctive feature of the ubiquitin/proteasome pathway is the apparent division between substrate-selecting and substrate-degrading components. Substrate selection is mediated by the ubiquitin system, and it is thus generally assumed that multiubiquitination of a substrate is a direct consequence of its interaction with the ubiquitination machinery. Although degradation of a large share of substrates might in fact be regulated at this early stage, our data suggest that alternative control points might be operative and could be of crucial physiological importance.

Analogous to other polymerization reactions such as transcription, translation, or oligoglycosylation, multiubiquitination might be regulated at the initiation or the polymerization phase. Proteins whose turnover might be regulated via E4 activities have presumably passed the recognition step and are primed for rapid degradation by modification with a few ubiquitin moieties. It is thus conceivable that some of the "monoubiquitinated" or weakly ubiquitinated proteins previously observed in vivo, for example histones or actin, are among those substrates. Regulating chain assembly may thus provide the cells with an alternative mechanism to selectively degrade individual molecules among a pool of otherwise identical proteins.

Function of E4

UFD2, encoded by a single copy gene in yeast, is not essential for viability (Johnson et al., 1995). Thus, substrates of a UFD2-dependent degradation pathway are

supposedly proteins whose stabilization does not interfere with vital functions of the cell under normal growth conditions. Interestingly, however, we found that UFD2-dependent degradation becomes crucial when cells are exposed to stress. Mutants in *ufd2* are hypersensitive to ethanol, and *ufd2 rpn10* double mutants are severely sensitive to heat and exposure to ethanol, heavy metals, or amino acid analogs. Elevated temperatures and exposure of cells to these reagents are known to affect protein folding, thereby generating elevated levels of aberrant proteins (Jungmann et al., 1993; Hochstrasser, 1996). UFD2's ability to mediate ubiquitin chain elongation is therefore linked to the role of the ubiquitin/proteasome–dependent proteolytic system in abnormal protein degradation. Our finding that *ufd2 rpn10* double mutants display synthetic lethality under stress conditions is intriguing, as it suggests an additive or synergistic function of both gene products in the proteolytic stress tolerance pathway. RPN10 exists in a free and a proteasome-bound form and is required for proteasomal targeting of ubiquitinated UFD substrates in vivo (van Nocker, 1996). Thus, it seems attractive to speculate that the ubiquitin chain elongation activity provided by UFD2 assists and amplifies RPN10's role in the reception of multiubiquitinated proteins by the proteasome.

In addition to abnormally folded proteins, substrates of a UFD2-dependent pathway may include proteins that structurally resemble UFD substrates. In fact, eukaryotes express several proteins that harbor ubiquitin-related domains within their N-terminal regions in analogy to UFD substrates. Examples from yeast are the related proteins RAD23 and DSK2, which are important for DNA repair and spindle pole body duplication, respectively (Watkins et al., 1993; Biggins et al., 1996). RAD23 has a known mammalian homolog (Masutani et al., 1994), and the human protein Parkin, which is linked to Parkinson's disease, is another example (Kitada et al., 1998). Whether all these proteins are subject to ubiquitin-dependent proteolysis has not been investigated systematically. RAD23, however, was recently shown to be degraded by the proteasome under certain conditions, and indeed, this reaction depended on the presence of the ubiquitin-like domain of the protein (Schauber et al., 1998).

Intriguingly, UFD2 is a highly conserved protein and defines a novel protein family (Figure 7A). Database searches identified two homologs in human (accession numbers Q14139 and AB014584) and one each in *C. elegans* (Q09349), *Dictyostelium* (AF044255), and the fission yeast *Schizosaccharomyces pombe* (AF059906), respectively. The similarity between these proteins extends over their entire lengths but is particularly striking within their C-terminal domains. In fact, binding of UFD2 to ubiquitin–protein conjugates is mediated by this conserved domain (data not shown). This strongly suggests that these proteins are indeed functional orthologs. In addition to full-length homologs, we identified several proteins in the database that possess a domain similar to the highly conserved C-terminal ~100 residues of UFD2. In these proteins, this domain, which we designate the U box (UFD2-homology domain), is flanked by sequences that often represent putative protein–protein interaction domains. An intriguing example is a human

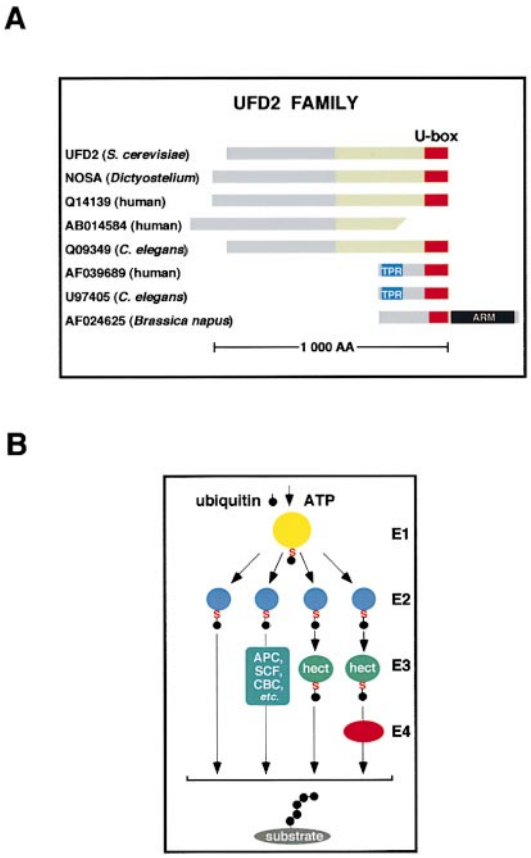

Figure 7. UFD2 Protein Family and Ubiquitination Pathways

(A) Schematic diagram of UFD2 relatives. The first bars represent full-length homologs (AB014584 is presumably a partial sequence). The highly conserved C-terminal domain is shaded in light green and red. The most highly conserved C-terminal 100 amino acids of UFD2 (the U box, shown in red) is present in several additional proteins that are often linked to other protein–protein interaction domains (TPR, tetratricopeptide repeat; ARM, armadillo repeat).

(B) Multiubiquitination of proteins proceeds via variations of a ubiquitin–enzyme thioester cascade. E1 (yellow) is always required, forms a thioester-linked complex (S) with ubiquitin (filled circles), and transfers activated ubiquitin onto E2s (blue). In some reactions, E2s can directly multiubiquitinate substrates, whereas others involve E3s (different shades of green). In contrast to some E3s (APC, SCF, CBC, etc.), a subfamily of E3s (hect) can form thioester-linked complexes with ubiquitin, similar to E1 and E2. At least one E1, E2, E3-thioester cascade involves an additional E4 activity for efficient substrate multiubiquitination.

U box protein (AF039689) that possesses TPR repeats which are strikingly similar to those of the Hsp70 co-chaperone HOP. It will be interesting to see whether this protein recruits ubiquitin–protein conjugates to the Hsp70 chaperone system.

Intriguingly, NOSA, the full-length UFD2 homolog from *Dictyostelium*, was recently shown to be required for normal differentiation (Pukatzki et al., 1998). During development, *nosA* mutants fail to form fruiting bodies and arrest as tight aggregates. At this stage, *nosA* mutants lose collective cell synergy and display a strong cell-autonomous phenotype. The proteins that are degraded in *Dictyostelium* by a NOSA/E4–catalyzed pathway are presently not known, but the phenotype suggests that

important developmental regulators are among those substrates. The identification of at least two UFD2 homologs in humans also suggests that alternative E4-dependent degradation pathways may exist.

Alternative Ubiquitination Pathways

Ubiquitination can proceed by variations of the typical ubiquitin–enzyme thioester cascade (Figure 7B). Some E2 enzymes can directly transfer ubiquitin to the substrate in vitro. One example is $UBC2^{RAD6}$, a yeast E2 required for a variety of functions, including DNA repair (Jentsch et al., 1987). This protein possesses a highly negatively charged C terminus by which the protein interacts with and ubiquitinates positively charged substrates such as histones (Jentsch et al., 1987; Sung et al., 1988). In most cases, however, E2s seem to collaborate with substrate-interacting E3s (Figure 7A). E3s can be distinguished by their mode of action. Some E3s (hect E3s) extend the ubiquitin–thioester cascade by an additional thioester-linked E3-enzyme-ubiquitin intermediate (Scheffner et al., 1995), whereas others, such as APC or SCF, do not (Patton et al., 1998). A common property of E3s, however, is that they may be furnished with alternative specificity factors (Patton et al., 1998).

This work revealed an unexpected, novel mechanistic aspect of ubiquitin conjugation pathways. The E4-dependent pathway described here is a modification of an E1, E2, E3-thioester cascade pathway, with the important accessory option to regulate proteolysis by ubiquitin chain elongation. If E4 activities are linked to, for example, heterooligomeric E3 complexes, the E3 complex could be activated at a specific time (e.g., of the cell cycle), yet the presence of a regulated ubiquitin chain assembly factor could allow different degradation times for different substrates of the same E3 complex.

Lastly, we discovered an intriguing relationship between the ubiquitination factor E4 and the ATPase CDC48. We found that these proteins physically interact and that CDC48 functions in the proteolysis pathway subsequent to the E4-catalyzed ubiquitination step. Because of the assumed function of this enzyme in protein folding reactions, we postulate that CDC48 may catalyze the assembly or disassembly of protein complexes involved in ubiquitination or proteasomal targeting. Remarkably, p97 (VCP), the apparent mammalian ortholog of the yeast CDC48 enzyme, was recently found to be specifically complexed with the ubiquitinated form of IκBα (Dai et al., 1998). It is thus conceivable that IκBα is among those substrates that are degraded by a mechanism similar to the one described here.

Experimental Procedures

Cloning and Yeast Techniques

All strains are derivatives of DF5 (Finley et al., 1985; *ura3-52 leu2-3, -112 lys2-801 trp1-101 his3Δ200*). The yeast strain HF7c was used for two-hybrid studies and the assay was done as described (Matchmaker, CLONTECH). The *ufd2* knockout strain was a kind gift of Erica Johnson and Alex Varshavsky. *RPN10* was amplified by PCR and subcloned into the *E. coli* vector pBS (pBS-RPN10). An *rpn10* deletion construct was made by replacing the AvrII/PstI fragment of *RPN10* with the *HIS3* gene. The resulting plasmid, pBS-RPN10::HIS3, was used to create the *RPN10* knockout strain.

UBA1 and *UBC4* were amplified by PCR and cloned into pVL1392 (pVL1392-UBA1, pVL1392-UBC4) and pGAD424 (pGAD-UBC4). The *UFD4* open reading frame (ORF) was cloned into pVL1393 (pVL1393-UFD4) and pGAD424 (pGAD-UFD4). The *UFD2* ORF was cloned by PCR into pBluescript (pBS-UFD2), pGBT9 (pGBT-UFD2), and pVL1393 (pVL1393-UFD2). The VSV tag was inserted as a double-stranded oligonucleotide into pBS-UFD2 and was cloned into YCP22-G (in YCP22-G, the *GAL1-10* promoter is inserted into the multiple cloning site as an EcoRI–BamHI fragment). *RPN10* was cloned into pGEX 4T-1 (pGEX-RPN10) and pGAD424 (pGAD-RPN10). The *CDC48* ORF was amplified via PCR and subcloned into pVL1392 (pVL1392-CDC48) and pGAD424 (pGAD-CDC48). The ORF for *MBP* was amplified from pMal c-2 and inserted in frame into pVL1392-CDC48, yielding pVL1392 MBP-CDC48.

To create Ubi-GST, the ORFs of GST and ubiquitin were cloned by PCR downstream of ubiquitin into pET3a. The ubiquitin fragment was C terminally joined to lacI sequences as derived from Ubi-Val-DHFR (Johnson et al., 1995) (pET3a Ubi-GST). The lacI insertion was required for efficient ubiquitination of the substrate. To clone Ubi-ProtA, the protein A (ProtA) ORF was cloned by PCR with 5′ sequences encoding a PKA site and a stop codon at the 3′ end. The fragment was inserted into pET3a Ubi-GST, replacing the GST part of the construct and yielding Ubi-ProtA. The mutant variants of Ubi-ProtA [Ubi(K48)-ProtA and Ubi(K29)-ProtA] were generated by PCR. The GST and ProtA fusion proteins were expressed and purified by standard methods. Details on each construct are available on request. Pulse-chase studies were done essentially as described (Seufert and Jentsch, 1992; Johnson et al., 1995).

Expression and Purification of Proteins

Recombinant baculovirus was generated using the Baculo Gold system as described by the manufacturer (Pharmingen). For protein production, 150 ml of infected cells (3×10^6 cells/ml) were grown for 2 days. Cells were lysed in twice the volume of the cell pellet in buffer A (10 mM KPO_4 [pH 7.0], 10 mM β-mercaptoethanol [β-ME], Complete protease inhibitors [Boehringer]) using a dounce homogenizer. For volumes below 1 ml, cells were lysed in the same buffer containing 0.1% Triton X-100. Recombinant proteins were flash frozen in liquid nitrogen and stored at −80°C. E1 was purified as described (Jentsch et al., 1987) with the following modification: after elution from ubiquitin-Sepharose, the protein was purified using a MonoQ anion exchange column in 20 mM Tris (pH 7.2), 10 mM β-ME using a KCl gradient. E1 eluted around 350 mM KCl.

UBC4-expressing cells were lysed in buffer A. The lysate was cleared by centrifugation at 20,000 rpm, and glycerol was added to 10%. Throughout the purification, E2 was detected using the assay described in this article supplemented with E1 and E3 using Ubi-ProtA as a substrate or by Western blotting using an antibody against yeast UBC4 (S. J., unpublished). The lysate (20 ml, 12.5 mg/ml) was passed over a 25 ml hydroxyapatite column using a 250 ml gradient of KPO_4 (pH 7.0) (10 to 215 mM). The buffer in the eluate was changed to 10 mM Tris (pH 8.0), 10% glycerol, 10 mM β-ME using an online dialysis device (VariPerm M, Bitop). E2 was recovered in fractions corresponding to 70 to 110 mM KPO_4. These fractions were pooled and applied to a 5 ml DEAE column in the same buffer containing 0.03% Triton X-100. Proteins were eluted by a 120 ml NaCl gradient (40 to 120 mM NaCl). E2 activity peaked in fractions corresponding to 56 to 72 mM NaCl. These fractions were pooled and the buffer was changed to 10 mM Tris (pH 7.5), 10% glycerol, 10 mM β-ME by dialysis. The proteins were applied to a 1 ml MonoQ column and eluted by a 22 ml NaCl gradient (0 to 130 mM NaCl) in the same buffer containing 0.01% Triton X-100. One milliliter fractions were collected. E2 peaked around 50 mM NaCl. Five hundred micrograms of the peak was purified over a Superose 12 gel filtration column (Pharmacia, HR10 30). E2 (UBC4) eluted around 12.5 ml, corresponding to 40 kDa. About 100 μg of UBC4 was purified from 250 mg insect cell protein.

E3-expressing cells were lysed, and the E3 protein was prepared as above and stored in buffer A containing 10% glycerol and 0.03% Triton X-100. E3 was detected by the assay described with Ubi-ProtA as a substrate. The lysate was filtered (45 μm) and passed over a hydroxyapatite column (22 ml). Proteins were eluted with a 250 ml gradient of KPO_4 (pH 7.0) (0 to 215 mM KPO_4) and 5 ml

fractions were collected. The buffer was changed to 20 mM Tris (pH 7.5), 10% glycerol, 10 mM β-ME during the elution by online dialysis, and Triton X-100 was added to 0.03%. E3 peaked around 130 mM KPO_4. Fractions were pooled and passed over a 1 ml DEAE column. The column was washed with 180 mM NaCl and proteins were eluted with a 40 ml gradient (200 to 440 mM NaCl). NaCl was removed by online dialysis. One milliliter fractions were collected and tested for E3 activity. E3 peaked around 290 to 330 mM NaCl. These fractions were pooled and passed over a MiniQ column on a SMART system (Pharmacia). Proteins were eluted with a 2 ml gradient (0 to 350 mM: 0.3 ml; 350 to 500 mM: 1.7 ml). Fifty microliter fractions were collected. E3 eluted in fractions corresponding to 380–420 mM NaCl. About 15 μg of E3 was recovered from 100 mg insect cell protein.

Ubiquitination Reactions

Reactions were done in 25 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, 10 mM ATP with 0.5 μg/μl ubiquitin (SIGMA). Purified enzymes were used at a concentration of 6 ng/μl, crude extracts at a concentration of 0.6 μg/μl. After incubation at 30°C for 60 min, reactions were stopped by addition of SDS-PAGE sample buffer. Labeling of proteins expressed in *E. coli* with ^{35}S methionine was done as described (Ausubel et al., 1994).

Purification of UFD2 from Yeast

The yeast cake from 4 l of culture grown in YPD to $OD_{600} = 2.0$ was lysed in a French press in 20 mM Tris (pH 7.5), 60 mM NaCl, 10 mM DTT, Complete protease inhibitors (Boehringer). After centrifugation, the cleared lysate was allowed to bind to 1 mg of ubiquitinated Ubi-GST immobilized on glutathione-Sepharose beads. After extensive washing in the same buffer with 0.02% Triton X-100 without protease inhibitors, bound proteins were eluted in the same buffer with 500 mM NaCl. A 110 kDa band specifically eluting from the ubiquitinated substrate column was analyzed by mass spectrometry (MALDI; Mortz et al., 1994).

Binding Assays

Assays studying the interaction of UFD2 or RPN10 with multiubiquitin chains were done in 20 mM Tris (pH 7.5), 10 mM β-ME, 0.1% Triton X-100. Binding was done in volumes of 50 to 100 μl at 4°C for 30 min, the beads were washed four times in the same buffer, and bound proteins were eluted with SDS-PAGE sample buffer. UFD2-CDC48 interactions were studied in 50 mM HEPES (pH 7.5), 10 mM β-ME, 100 mM NaCl, 5 mM EDTA. Binding was done in a volume of 300 μl in a shaker at 4°C for 30 min, the beads were washed five times in the same buffer, and bound CDC48 was eluted with 500 mM NaCl.

Acknowledgments

We are grateful to Kathrin Jepsen for excellent technical assistance; K. Ashman (EMBL, Heidelberg) for the identification of UFD2 by mass spectrometry; E. Johnson, A. Varshavsky, D. Finley, and M. Scheffner for plasmids and strains; and Eva Löser for technical help. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Fonds de Chemischen Industrie, and European TMR ubiquitin network to S. J.

Received October 2, 1998; revised January 22, 1999.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmna, J.G., Smith, J.A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Green and Wiley).

Biggins, S., Ivanovska, I., and Rose, M.D. (1996). Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. J. Cell Biol. *133*, 1331–1346.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576–1583.

Cook, W.J., Jeffrey, L.C., Kasperek, E., and Pickart, C.M. (1994). Structure of tetraubiquitin shows how multiubiquitin chains can be formed. J. Mol. Biol. *236*, 601–609.

Dai, R.-M., Chen, E., Longo, D.L., Gorbea, C.M., and Li, C.-C.H. (1998). Involvement of valosin-containing protein, an ATPase co-purified with IκBα and the 26S proteasome, in ubiquitin-proteasome-mediated degradation of IκBα. J. Biol. Chem. *273*, 3562–3573.

Finley, D., Özkaynak, E., and Varshavsky, A. (1985). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell *48*, 1035–1046.

Fröhlich, K.-U., Fries, H.W., Rüdiger, M., Erdmann, R., Botstein, D., and Mecke, D. (1991). Yeast cell cycle protein Cdc48p shows full length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. J. Cell Biol. *114*, 443–453.

Ghislain, M., Dohmen, R.J., Levy, F., and Varshavsky, A. (1996). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. EMBO J. *15*, 4884–4899.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. Annu. Rev. Genet. *30*, 405–439.

Jentsch, S., and Schlenker, S. (1995). Selective protein degradation: a journey's end within the proteasome. Cell *82*, 881–884.

Jentsch, S., McGrath, J.P., and Varshavsky, A. (1987). The yeast DNA repair gene *RAD6* encodes a ubiquitin-conjugating enzyme. Nature *329*, 131–134.

Johnson, E.S., Bartel, B., Seufert, W., and Varshavsky, A. (1992). Ubiquitin as a degradation signal. EMBO J. *11*, 497–505.

Johnson, E.S., Ma, P.C.M., Ota, I., and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. *270*, 17442–17456.

Jungmann, J., Reins, H.-A., Schobert, C., and Jentsch, S. (1993). Resistance to cadmium mediated by ubiquitin-dependent proteolysis. Nature *361*, 369–371.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature *392*, 605–608.

Masutani, C., Sugasawa, K., Yanagisawa, J., Sonoyama, T., Ui, M., Enomoto, T., Takio, K., Tanaka, K., van der Spek, P.J., and Bootsma, D., et al. (1994). Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23. EMBO J. *13*, 1831–1843.

McGrath, J.P., Jentsch, S., and Varshavsky, A. (1991). *UBA1*: an essential yeast gene encoding ubiquitin-activating enzyme. EMBO J. *10*, 227–236.

Mortz, E., Vorm, O., Mann, M., and Roepstorff, P. (1994). Identification of proteins in polyacrylamide gels by mass spectrometric peptide mapping combined with database search. Biol. Mass. Spectrom. *23*, 249–261.

Patton, E.E., Willems, A.R., and Tyers, M. (1998). Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. Trends Genet. *14*, 236–242.

Pukatzki, S., Tordilla, N., Franke, J., and Kessin, R.H. (1998). A novel component involved in ubiquitination is required for development of *Dictyostelium discoideum*. J. Biol. Chem. *273*, 24131–24138.

Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. (1998). Rad23 links DNA repair to the ubiquitin/proteasome pathway. Nature *391*, 715–718.

Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81–83.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. EMBO J. *9*, 543–550.

Seufert, W., and Jentsch, S. (1992). *In vivo* function of the proteasome in the ubiquitin pathway. EMBO J. *11*, 3077–3080.

Sung, P., Prakash, S., and Prakash, L. (1988). The RAD6 protein of *Saccharomyces cerevisiae* polyubiquitinates histones, and its acidic domain mediates this activity. Genes Dev. *2*, 1476–1485.

van Nocker, S., Sadis, S., Rubin, D.M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Viestra, R.D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. *16*, 6020–6028.

Watkins, J.F., Sung, P., Prakash, L., and Prakash, S. (1993). The *Saccharomyces cerevisiae* DNA repair gene *RAD23* encodes a nuclear protein containing a ubiquitin-like domain required for biological function. Mol. Cell. Biol. *13*, 7757–7765.