Physical association of ubiquitin ligases and the 26S proteasome

Youming Xie and Alexander Varshavsky*

Division of Biology, California Institute of Technology, Pasadena, CA 91125

Contributed by Alexander Varshavsky, January 19, 2000

The ubiquitin (Ub) system recognizes degradation signals of the target proteins through the E3 components of E3-E2 Ub ligases. A targeted substrate bears a covalently linked multi-Ub chain and is degraded by the ATP-dependent 26S proteasome, which consists of the 20S core protease and two 19S particles. The latter mediate the binding and unfolding of a substrate protein before its transfer to the interior of the 20S core. It is unclear how a targeted substrate is delivered to the 26S proteasome, inasmuch as Rpn10p, the only known proteasomal subunit that binds multi-Ub chains, has been found to be not essential for degradation of many proteins in the yeast *Saccharomyces cerevisiae*. Here we show that Ubr1p and Ufd4p, the E3 components of two distinct Ub ligases, directly interact with the 26S proteasome. Specifically, Ubr1p is shown to bind to the Rpn2p, Rpt1p, and Rpt6p proteins of the 19S particle, and Ufd4p is shown to bind to Rpt6p. These and related results suggest that a substrate-bound Ub ligase participates in the delivery of substrates to the proteasome, because of affinity between the ligase's E3 component and specific proteins of the 19S particle.

E3 | N-end rule | ubiquitin fusion degradation pathway | Ubr1p | Ufd4p

Regulated proteolysis by the ubiquitin (Ub) system plays essential roles in the cell cycle, differentiation, stress responses, and many other processes (1–5). Ub is a 76-residue protein whose covalent conjugation to other proteins, usually in the form of a multi-Ub chain, marks these proteins for processive degradation by the 26S proteasome, an ATP-dependent multisubunit protease (3, 6–8). The conjugation of Ub to other proteins involves the formation of a thioester between the C terminus of Ub and a specific cysteine of the Ub-activating (E1) enzyme. The Ub moiety of E1~Ub thioester thereafter is transesterified to a specific cysteine in one of several Ub-conjugating (E2) enzymes. The Ub moiety of E2~Ub thioester is conjugated, via the isopeptide bond, to the ε-amino group of either a substrate's Lys residue or a Lys residue of another Ub moiety, the latter reaction resulting in a substrate-linked multi-Ub chain (9–11).

Most E2 enzymes function in complexes with proteins called E3. The functions of E3s include the initial recognition of degradation signals (degrons) in the substrate proteins, with different E3s recognizing different classes of degrons. The E2-E3 complexes, referred to as Ub ligases, mediate the formation of substrate-linked multi-Ub chains (10, 12, 13). The ATP-dependent 26S proteasome, which processively degrades a targeted, ubiquitylated[†] substrate, consists of the 20S core protease and two 19S particles (6, 14). A 19S particle mediates the binding and unfolding of a substrate protein before its transfer to the interior of the 20S core (6, 15–19). It is unclear how a targeted substrate is delivered to the 26S proteasome, inasmuch as Rpn10p (Mcb1p/Sun1p), the only known proteasomal subunit that binds multi-Ub chains (20), is not essential for degradation of many proteins in the yeast *Saccharomyces cerevisiae* (21).

Here we show that Ubr1p and Ufd4p, the E3 components of two distinct Ub-dependent proteolytic pathways (13, 22, 23), directly interact with the 26S proteasome. Specifically, Ubr1p is shown to bind to the Rpn2p (Sen3p), Rpt1p (Cim5p), and Rpt6p (Cim3p/Sug1p) proteins of the 19S particle, and Ufd4p is shown to bind to Rpt6p. These results suggest a mechanism for the delivery of protein substrates to the proteasome.

Materials and Methods

Protein Expression in *S. cerevisiae* and *Escherichia coli*. Plasmid construction protocols are available on request. *S. cerevisiae RPN1* was isolated as a multicopy suppressor of the toxicity of co-overproduced Ubr1p and Rad6p (Ubc2p), which were expressed from the bidirectional $P_{GAL1,10}$ promoter as described (24). *RPN2*, *RPN10*, and *RPN12* with their own promoter regions, and the ORFs of *RPN3, RPN11*, *RPT1*, *RPT2, RPT6*, and *PRE6* were amplified by PCR from the DNA of *S. cerevisiae* YPH500. All PCR products were verified by DNA sequencing. For the toxicity suppression assays (Fig. 1), *RPN1*, *RPN2*, *RPN10*, and *RPN12* were subcloned into the high-copy vector pRS425 (25). The *RPT1* and *RPT6* ORFs were subcloned into the low-copy vector pRS314CUP1 derived from pRS314 (25), yielding p314CUP1RPT1 and p314CUP1RPT6, in which *RPT1* and *RPT6* were expressed from the P_{CUP1} promoter. For coimmunoprecipitation assays, the N-termini of Rpt6p, Pre6p, and Ufd4p were extended with the hemagglutinin (HA) epitope (23); alternatively, the FLAG epitope was added to the C termini of Rpt6p and Rpn1p (26). For glutathione *S*-transferase (GST)-pulldown assays, the ORFs of *RPN1*, *RPN2*, *RPN3, RPN10*, *RPN11*, *RPN12*, *RPT1*, *RPT2*, *RPT6*, and *PRE6* were fused in-frame to the 3′ end of GST-coding sequence in pGEX-4T-3 (Amersham Pharmacia). The C-terminally FLAG-tagged alleles of *RPN1* and *RPN10* were subcloned into pET-11c (Novagen). *E. coli* BL21 (DE3) (26) was used to express the GST fusions, as well as Rpn1p-FLAG and Rpn10p-FLAG.

Pulse–Chase and GST-Pulldown Assays. The Ub protein reference (UPR)-based plasmids expressing dihydrofolate reductase (DHFR)-HA-Ub^{R48}-X-βgal (β-galactosidase) (see *Results* for notations) were described (27). The pulse–chase procedures (Fig. 2) were described as well (22, 28). In GST-pulldown assays, $\approx$1 μg of a GST fusion protein or GST alone was diluted to 0.5 ml in the loading buffer (1% Triton X-100/10% glycerol/0.5 M NaCl/1 mM EDTA/50 mM Tris·HCl, pH 8.0), and incubated with 10 μl (bed volume) of the glutathione-agarose beads (Sigma) for 1 h at 4°C. The beads were washed three times with

Abbreviations: Ub, ubiquitin; HA, hemagglutinin; GST, glutathione *S*-transferase; UPR, Ub protein reference; DHFR, dihydrofolate reductase; βgal, β-galactosidase; UFD, Ub fusion degradation.

*To whom reprint requests should be addressed at: Division of Biology, 147–75, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125. E-mail: avarsh@caltech.edu.

[†]Ubiquitin whose C-terminal (Gly-76) carboxyl group is covalently linked to another compound is called the ubiquityl moiety, the derivative terms being ubiquitylation and ubiquitylated. The term Ub refers to both free ubiquitin and the ubiquityl moiety. This nomenclature (5), which also is recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (41), brings ubiquitin-related terms in line with the standard chemical terminology.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.060025497. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.060025497

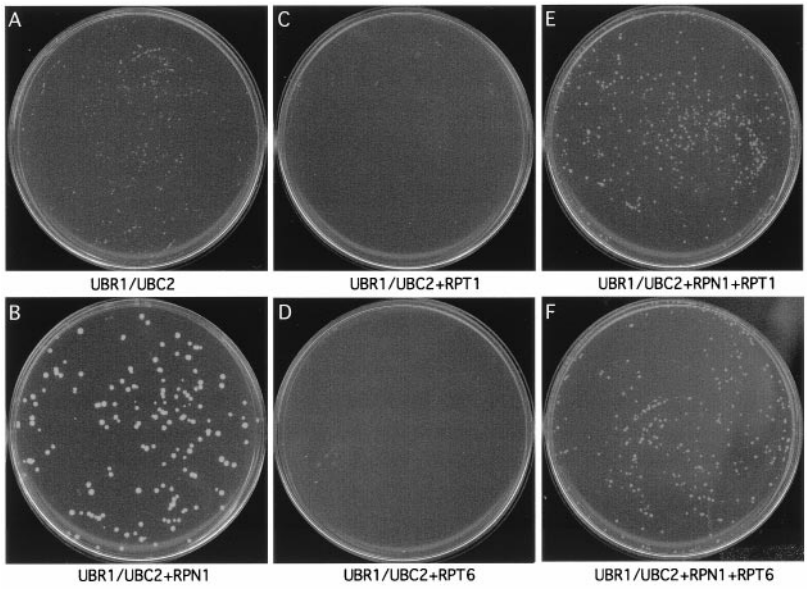

Fig. 1. Toxicity of overexpressed N-end rule pathway is decreased by overexpressed Rpn1p and enhanced by overexpressed Rpt1p or Rpt6p. The *S. cerevisiae* strain JD52 (23) was transformed with pKM1313, a low-copy, *URA3*-based plasmid expressing *UBR1* and *RAD6* from the bidirectional $P_{GAL1,10}$ promoter (24). The pKM1313-containing JD52 cells were transformed either with the pRS425 vector (*A*), *RPN1* (expressed from its own promoter in pRS425) (*B*), *RPT1* (expressed from the P_{CUP1} promoter in p314CUP1RPT1) (*C*), or *RPT6* (expressed from the P_{CUP1} promoter in p314CUP1RPT6) (*D*). (*E* and *F*) JD52 cells carrying both pKM1313 and the *RPN1*-expressing plasmid were further transformed with p314CUP1RPT1 (*E*) and p314CUP1RPT6 (*F*), respectively. Shown here are plates with cells grown in the galactose-containing minimal medium containing 0.2 mM $CuSO_4$ for 4 days at 30°C. On dextrose-containing plates (no overexpression of Ubr1p and Rad6p), all transformants grew at similar rates, irrespective of the presence of overexpressed Rpn1p, Rpt1p, or Rpt6p (data not shown).

1 ml of the binding buffer (0.05% Triton X-100/10% glycerol/50 mM NaCl/50 mM Na-Hepes, pH 7.5). The washed beads were incubated with cell extracts containing FLAG-Ubr1p (Fig. 3*A*), or with purified FLAG-Ubr1p (Fig. 3*B*), or with cell extracts containing either Rpn1p-FLAG or Rpn10p-FLAG (Fig. 4 *A* and *B*), or HA-Ufd4p (Fig. 5*A*) at 4°C for 2 h. The incubation buffer for the assay with purified FLAG-Ubr1p contained ovalbumin (Sigma) at 1 mg/ml in the binding buffer. The beads were washed three times with the binding buffer, followed by SDS/PAGE of the retained proteins and immunoblotting with either anti-FLAG antibody (Sigma) or anti-HA antibody (Babco, Berkeley, CA).

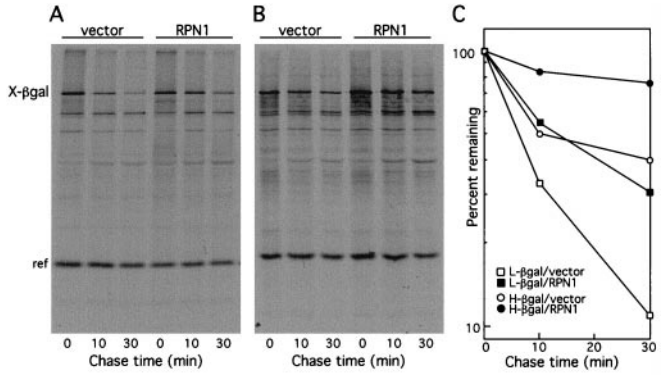

Fig. 2. Overexpression of Rpn1p inhibits degradation of N-end rule substrates. A UPR-based pulse–chase assay was used (see *Results*). *S. cerevisiae* JD52 (*UBR1*) cells coexpressing either DHFR-HA-Ub^{R48}-Leu-βgal (*A*) or DHFR-HA-Ub^{R48}-His-βgal (*B*), and either Rpn1p (expressed from its own promoter in pRS425) or pRS425 alone were labeled with [^{35}S]methionine for 5 min at 30°C, followed by a chase for 0, 10, and 30 min. Cell extracts were immunoprecipitated with both anti-βgal and anti-HA antibodies, followed by SDS/12% PAGE, autoradiography, and quantitation using PhosphorImager (Molecular Dynamics). (*C*) His-βgal and Leu-βgal decay curves calculated from the UPR-based data in *A* and *B* as described (28, 32). The bands of X-βgals and the reference protein DHFR-HA-Ub^{R48} ("ref") are indicated.

Coimmunoprecipitation/Immunoblotting. FLAG-Ubr1p, bearing the N-terminal FLAG epitope, was expressed from the P_{ADH1} promoter in the low-copy vector pRS315 (25). The N-terminally HA-tagged HA-Rpt6p, HA-Pre6p, and HA-Ufd4p and the C-terminally FLAG-tagged Rpt6p-FLAG were expressed from the induced P_{CUP1} promoter in the low-copy vector pRS314 (25). The C-terminally FLAG-tagged Rpn1p-FLAG was expressed from its own promoter in pRS425. *S. cerevisiae* was grown in synthetic-dextrose medium (26, 28) containing 0.2 mM $CuSO_4$ to $OD_{600} \approx 1.0$. Cells were disrupted by vortexing with glass beads in 0.05% Triton X-100, 50 mM NaCl, 50 mM Na-Hepes (pH 7.5) containing several protease inhibitors (27).

Results

Ubr1p, the E3 of the N-End Rule Pathway, Binds to the Proteasome. Ubr1p (also called N-recognin), the 225K E3 of the *S. cerevisiae* N-end rule pathway, targets proteins that bear certain (destabilizing) N-terminal residues. Two substrate-binding sites of Ubr1p recognize two classes of destabilizing N-terminal residues: basic and bulky hydrophobic (13). Yet another substrate-binding site of Ubr1p targets proteins that bear internal (non-N-terminal) degrons (29). Similar, but distinct, versions of the N-end rule pathway are present in all organisms examined, from prokaryotes to fungi and mammals (13). Previous work has shown that overexpression of the pathway's Ub ligase, through co-overexpression of its E2 (Rad6p, also called Ubc2p) and E3 (Ubr1p) components, is toxic to *S. cerevisiae* (24).

In the present work, we found that *RPN1* (*NAS1/HRD2*) (30, 31), which encodes a ≈110 K protein of the proteasome's 19S particle, was a multicopy suppressor of the toxicity of co-overexpressed Ubr1p and Rad6p (Fig. 1 *A* and *B*, and data not shown). Because Rpn1p is essential for cell viability (30), it could be that the toxicity of co-overexpressed Ubr1p and Rad6p

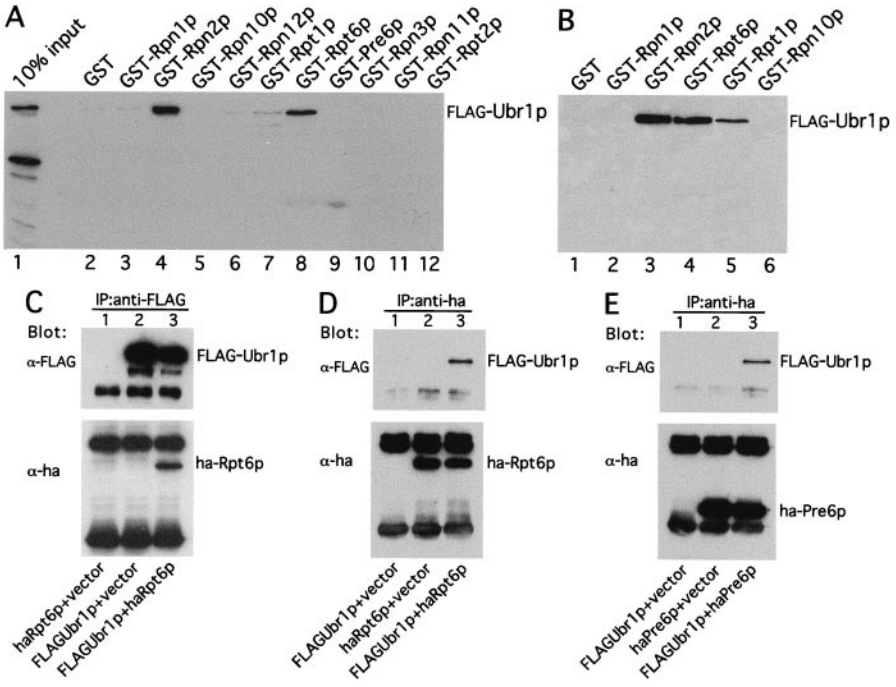

Fig. 3. Ubr1p, the E3 of the N-end rule pathway, is physically associated with the 26S proteasome. (*A* and *B*) Ubr1p interacts with Rpn2p, Rpt1p, and Rpt6p in GST-pulldown assays. Extracts of *S. cerevisiae* containing overexpressed FLAG-Ubr1p (*A*) or the purified FLAG-Ubr1p protein (*B*) were incubated with glutathione-agarose beads preloaded with the indicated GST fusions. The retained proteins were eluted, fractionated by SDS/8% PAGE, and immunoblotted with anti-FLAG antibody. Approximately equal amounts of different GST fusions were immobilized on glutathione-agarose beads in these assays, as verified by Coomassie staining (data not shown). (*C* and *D*) *In vivo* association of Ubr1p and Rpt6p. Extracts of *S. cerevisiae* AVY107 (*ubr1*Δ) expressing either both FLAG-Ubr1p and HA-Rpt6p, or FLAG-Ubr1p alone, or HA-Rpt6p alone were incubated with anti-FLAG antibody (*C*) or anti-HA antibody (*D*). The immunoprecipitated proteins were separated by SDS/12% PAGE and transferred to nitrocellulose membrane. The top halves of *C* and *D* show the results of immunoblotting with anti-FLAG antibody; the bottom halves show the analogous data with anti-HA antibody. (*E*) Coimmunoprecipitation of Pre6p and Ubr1p. Extracts of *S. cerevisiae* AVY107 (*ubr1*Δ) expressing both FLAG-Ubr1p and HA-Pre6p, FLAG-Ubr1p alone, or HA-Pre6p alone were incubated with anti-HA antibody, followed by the immunoprecipitation/immunoblotting described in *C* and *D*.

resulted in part from overdegradation of Rpn1p, which would have accounted for the observed alleviation of toxicity through overexpression of Rpn1p. However, pulse–chase assays with Rpn1p have shown it to be a long-lived protein in either wild-type cells or cells co-overexpressing Ubr1p and Rad6p (data not shown). It also was found that overexpression of Rpn1p had no effect on the expression levels of co-overexpressed Ubr1p and Rad6p (data not shown).

We then tested, using pulse–chase assays, whether overexpression of Rpn1p perturbed the degradation of normally short-lived substrates of the N-end rule pathway. In these assays, the *E. coli* βgal-based test proteins were expressed as components of fusions of the form DHFR-HA-Ub^{R48}-X-βgal, where X was either His, a type 1 (basic) destabilizing residue, or Leu, a type 2 (hydrophobic) destabilizing residue (13); DHFR-HA was the C-terminally HA-tagged mouse DHFR; Ub^{R48} was the Ub moiety containing Arg instead of Lys at position 48 (27, 28, 32); and βgal was *E. coli* βgal. Deubiquitylating enzymes (33) cotranslationally cleave these fusions at the Ub-βgal junction, yielding the long-lived DHFR-HA-Ub^{R48} reference protein and a test protein, either His-βgal or Leu-βgal. A reference protein improves the assay's accuracy by serving as a built-in control for variations in the expression levels, immunoprecipitation efficiency, and other sources of data scatter in a pulse–chase assay. The above method for producing a reference protein is called the UPR technique (28, 32). As shown in Fig. 2, the *in vivo* degradation of both His-βgal and Leu-βgal substrates of the N-end rule pathway was significantly impaired in the presence of overexpressed Rpn1p.

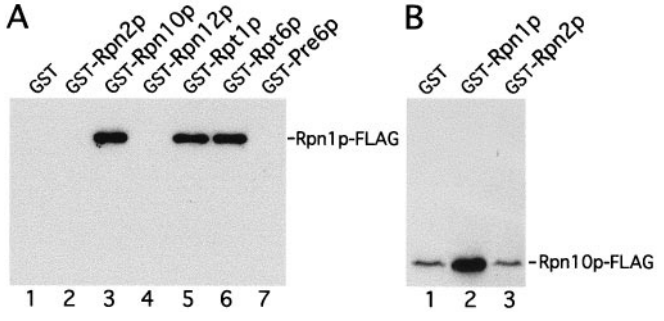

Fig. 4. Rpn1p interacts with Rpt1p, Rpt6p, and Rpn10p. *E. coli*-expressed Rpn1p-FLAG (*A*) and Rpn10p-FLAG (*B*), both carrying the FLAG epitope at the C terminus, were incubated with glutathione-agarose beads preloaded with the indicated GST fusions. The retained proteins were fractionated by SDS/PAGE, followed by immunoblotting with anti-FLAG antibody. Approximately equal amounts of different GST fusions were immobilized on glutathione-agarose beads in these assays, as verified by Coomassie staining (data not shown).

To determine whether other proteins of the 19S particle were similar to Rpn1p in its ability to suppress the toxicity of overexpressed N-end rule pathway, we overexpressed Rpn2p, Rpn10p, and Rpn12p, by transforming cells that co-overexpressed Ubr1p and Rad6p (from the $P_{GAL1,10}$ promoter) with a high-copy vector expressing, separately, Rpn2p, Rpn10p, and Rpn12p from their own promoters. We also tested Rpt1p and Rpt6p, two of the six ATPases of the 19S particle (16), by overexpressing them from the copper-induced P_{CUP1} promoter in a low-copy vector. In contrast to the results with Rpn1p (Fig. 1 *A* and *B*), none of these proteins could alleviate, upon

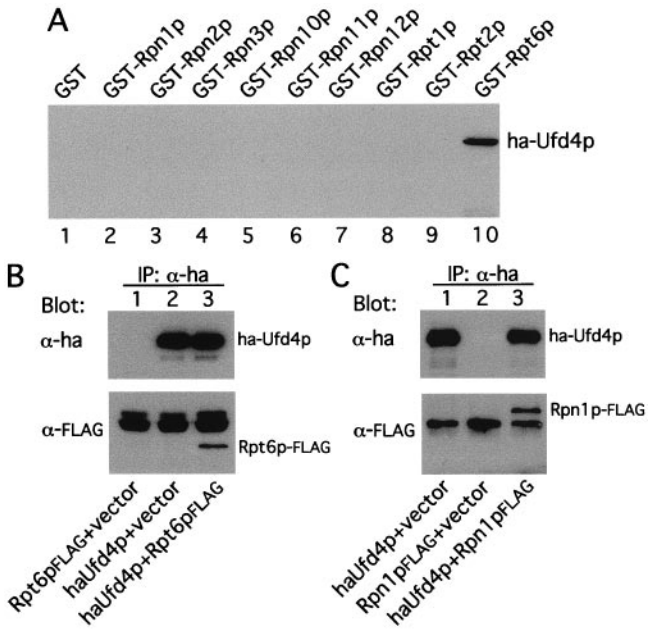

Fig. 5. Ufd4p, the E3 of the UFD pathway, is physically associated with the proteasome. (*A*) Ufd4p interacts with Rpt6p in the GST-pulldown assay. Extracts of *S. cerevisiae* containing overexpressed HA-Ufd4p were incubated with glutathione-agarose beads preloaded with different GST fusions, as indicated. The retained proteins were eluted, fractionated by SDS/8% PAGE, and immunoblotted with anti-HA antibody. Approximately equal amounts of different GST fusions were immobilized on glutathione-agarose beads in these assays, as verified by Coomassie staining (data not shown). (*B*) *In vivo* association of Ufd4p and Rpt6p. Extracts of *S. cerevisiae* JD52 (*UBR1*) expressing both HA-Ufd4p and Rpt6p-FLAG, HA-Ufd4p alone, or Rpt6p-FLAG alone were incubated with anti-HA antibody. The immunoprecipitated proteins were separated by SDS/10% PAGE and transferred to nitrocellulose membrane. (*Upper*) The results of immunoblotting with anti-HA antibody. (*Lower*) The data with anti-FLAG antibody. (*C*) Coimmunoprecipitation of Rpn1p and Ufd4p. Extracts of *S. cerevisiae* JD52 expressing both HA-Ufd4p and Rpn1p-FLAG, HA-Ufd4p alone, or Rpn1p-FLAG alone were incubated with anti-HA antibody, followed by the immunoprecipitation/immunoblotting procedure described in *B*, except that SDS/7% PAGE was used.

overexpression, the growth defect caused by co-overexpressed Ubr1p and Rad6p (data not shown). Moreover, we found that overexpression of either Rpt1p or Rpt6p had an effect opposite to that of overexpressed Rpn1p: the toxicity of co-overexpressed Ubr1p and Rad6p was higher in the presence of overexpressed Rpt1p or Rpt6p (Fig. 1 *C* and *D*; compare with Fig. 1 *A* and *B*). In a control experiment, the overexpression of Rpt1p or Rpt6p in cells that did not co-overexpress Ubr1p and Rad6p was not at all toxic (data not shown). These results suggested that Rpt1p and Rpt6p may interact with a component(s) of the N-end rule pathway, perhaps with Ubr1p, the pathway's E3. To verify this conjecture, we carried out GST-pulldown assays.

In these experiments, Ubr1p was N-terminally tagged with the FLAG epitope. The N-terminal FLAG tag did not impair the function of Ubr1p (A. Webster and A.V., unpublished data). Rpt1p and Rpt6p were expressed in *E. coli* as fusions to the C terminus of GST. Extracts of *S. cerevisiae* overexpressing FLAG-Ubr1p were incubated with glutathione-agarose beads preloaded with GST-Rpt1p, GST-Rpt6p, or GST alone. The bound proteins were eluted, fractionated by SDS/PAGE, and immunoblotted with a monoclonal anti-FLAG antibody. Remarkably, GST-Rpt6p was found to bind FLAG-Ubr1p; a smaller, but significant, amount of FLAG-Ubr1p also was bound by GST-Rpt1p, whereas no FLAG-Ubr1p was bound by GST alone (Fig. 3*A*, lanes 2, 7, and 8).

Could Ubr1p interact with other proteins of the 26S proteasome as well? Using the same assay, we discovered that GST-Rpn2p was also able to bind FLAG-Ubr1p, whereas all of the other tested proteasomal components (GST-Rpn1p, GST-Rpn3p, GST-Rpn10p, GST-Rpn11p, GST-Rpn12p, GST-Rpt2p, and GST-Pre6p, the latter a component of the 20S core proteasome) did not bind to FLAG-Ubr1p (Fig. 3*A*). Coomassie staining of the eluted, SDS/PAGE-fractionated proteins confirmed that the amounts of different GST fusions prebound to glutathione-agarose beads in the pulldown assays were approximately equal (data not shown).

To determine whether Ubr1p binds to Rpt1p, Rpt6p, and Rpn2p directly, we carried out pulldown assays with purified FLAG-Ubr1p (a gift from F. Du and A. Webster, California Institute of Technology) (Fig. 3*B*). FLAG-Ubr1p was overexpressed in *S. cerevisiae* and purified in three consecutive steps: anti-FLAG antibody affinity chromatography; Rad6p (E2) affinity chromatography; and gel filtration (F. Du, A. Webster, and A.V., unpublished data). In agreement with the results obtained by using extracts from cells overexpressing FLAG-Ubr1p (Fig. 3*A*), the purified FLAG-Ubr1p bound to the purified GST-Rpt1p, GST-Rpt6p, and GST-Rpn2p, but not to GST-Rpn1p, GST-Rpn10p, or GST alone (Fig. 3*B*). FLAG-Ubr1p used in this assay was purified from *S. cerevisiae* overexpressing this protein. Although FLAG-Ubr1p was at least 95% pure by Coomassie staining, one would like to purify *S. cerevisiae* Ubr1p from a noneukaryotic host as well, to preclude the unlikely possibility that a trace amount of a Ubr1p-interacting protein in the preparation of purified Ubr1p could mediate the binding of Ubr1p to Rpt1p, Rpt6p, and Rpn2p. Attempts to overexpress the 225K Ubr1p in *E. coli* have not been successful thus far.

Further tests used a coimmunoprecipitation/immunoblotting assay to examine the *in vivo* interaction between Ubr1p and Rpt6p. FLAG-Ubr1p was co-overexpressed in *ubr1*△ *S. cerevisiae* with HA-Rpt6p, which bore the N-terminal HA-derived epitope. The controls included congenic cells expressing either FLAG-Ubr1p alone or HA-Rpt6p alone. Proteins were immunoprecipitated from cell extracts with anti-FLAG antibody, followed by SDS/PAGE and immunoblotting with either anti-HA antibody (Fig. 3*C Lower*) or anti-FLAG antibody (Fig. 3*C Upper*). The results indicated that HA-Rpt6p was specifically coprecipitated with FLAG-Ubr1p by anti-FLAG antibody. We also carried out the reciprocal coimmunoprecipitation assay and found that FLAG-Ubr1p was specifically coprecipitated with HA-Rpt6p by anti-HA antibody (Fig. 3*D*).

In the *in vitro* pulldown assays, FLAG-Ubr1p did not interact with GST-Pre6p, a GST fusion to a protein of the 20S core protease (Fig. 3*A*, lane 9). To determine whether Ubr1p is associated with the mature 26S proteasome *in vivo*, FLAG-Ubr1p and HA-Pre6p (N-terminally tagged with the HA epitope) were co-overexpressed in *ubr1*Δ cells. Proteins were precipitated from cell extracts with anti-HA antibody and separated by SDS/PAGE, followed by immunoblotting with either anti-FLAG or anti-HA antibody. FLAG-Ubr1p was found to be specifically coprecipitated with HA-Pre6p by anti-HA antibody (Fig. 3*E*). Thus, GST-pulldown assays with either crude or purified FLAG-Ubr1p (Fig. 3 *A* and *B*) and the coimmunoprecipitation assays (Fig. 3 *C–E*) demonstrated that Ubr1p, the E3 of the N-end rule pathway, is physically associated with the 26S proteasome. Moreover, Ubr1p was found to interact with more than one protein of the 19S particle (Fig. 3 *A* and *B*).

As described above, overexpression of Rpn1p, a non-ATPase component of the 19S particle, inhibited the activity of the N-end rule pathway (Fig. 2). What might be the mechanism of this inhibition? Overexpression of Rpn1p apparently did not have a significant effect on the function of the 26S proteasome, inasmuch as cells overexpressing Rpn1p were not hypersensitive to either an arginine analog canavanine or high temperature (data

not shown). Hypersensitivity to these stressors is characteristic of yeast strains that have impaired proteasome components (6, 14). Overexpression of Rpn1p in wild-type cells (in the absence of co-overexpression of Ubr1p and Rad6p) did not result in a growth defect (data not shown). Furthermore, GST-pulldown assays indicated that GST-Rpn1p did not directly interact with Ubr1p (Fig. 3), ruling out the possibility that overproduced Rpn1p may decrease the toxicity of overexpressed N-end rule pathway by sequestering the overproduced Ubr1p. It was also unlikely that the overproduced, free Rpn1p competed with the 26S proteasome by interacting with substrate-linked multi-Ub chains, because Rpn1p was unable to bind isolated multi-Ub chains (data not shown).

As described above, overexpression of either Rpt1p or Rpt6p, the ATPase subunits of the 19S particle, was found to increase the toxicity of co-overexpressed Ubr1p and Rad6p (Fig. 1 *C* and *D*; compare with Fig. 1*A*). Therefore, we examined the possibility that the overexpressed Rpn1p may alleviate the toxicity of co-overexpressed Ubr1p and Rad6p by sequestering Rpt1p and Rpt6p, both of which were shown to interact with Ubr1p (Fig. 3). We began by determining, using the GST-pulldown assay, whether Rpn1p could directly bind to Rpt1p and/or Rpt6p. *S. cerevisiae* Rpn1p-FLAG (bearing the C-terminal FLAG epitope) was expressed in *E. coli* and incubated with glutathione agarose beads containing prebound GST-Rpt1p, GST-Rpt6p, or GST alone. The bound proteins were eluted, separated by SDS/PAGE, and immunoblotted with anti-FLAG antibody. Rpn1p was found to interact directly with both GST-Rpt1p and GST-Rpt6p, but not with GST alone (Fig. 4*A*, lanes 1, 5, and 6).

We also found that Rpn1p-FLAG directly interacted with GST-Rpn10p, the protein of the 19S particle that binds to multi-Ub chains (Fig. 4*A*). Rpn1p-FLAG did not interact, in this assay, with other tested proteins of the 19S particle (Rpn2p and Rpn12p) and also did not interact with the Pre6p protein of the 20S core (Fig. 4*A*). A direct Rpn1p-Rpn10p interaction also was observed in a reciprocal GST-pulldown assay, in which the *E. coli*-expressed Rpn10p-FLAG was shown to be retained by the prebound GST-Rpn1p, but not by GST-Rpn2p (Fig. 4*B*). Rpn1p, Rpn2p, Rpn10p, and the six Rpt ATPases Rpt1p-Rpt6p form the base of the 19S particle that is proximal to the 20S core of the 26S proteasome (16, 31).

Further tests indicated that overexpression of either Rpt1p or Rpt6p antagonized the ability of overexpressed Rpn1p to suppress the toxicity of overexpressed N-end rule pathway (Fig. 1 *E* and *F*; compare with Fig. 1*B*). Taken together, these results (Figs. 1, 3, and 4) strongly suggested that Rpn1p, which does not bind to Ubr1p, suppresses the toxicity of co-overexpressed Ubr1p and Rad6p at least in part through the demonstrated binding of Rpn1p to both Rpt1p and Rpt6p, either of which binds to Ubr1p. Schauber *et al.* (34) have shown that Rad23p, which bears an N-terminal Ub-like moiety, is another high-copy suppressor of the toxicity of overexpressed N-end rule pathway. Rad23p was found to interact with Rpt1p, Rpt6p, and Rpn10p (35, 36). In the present work, the same three proteins of the 19S particle were shown to interact with Rpn1p, another protein of this particle, suggesting that overexpression of Rpn1p and Rad23p suppresses the toxicity of overexpressed N-end rule pathway through similar mechanisms.

Ufd4p, the E3 of the Ub Fusion Degradation (UFD) Pathway, Binds to the Proteasome. The substrates of another Ub-dependent proteolytic system, termed the UFD pathway, include proteins bearing at their N termini a "nonremovable" Ub moiety (23, 37, 38). A partial or complete resistance of these Ub-containing proteins to deubiquitylating enzyme-mediated cleavage stems from either alterations of the last residue of Ub moiety or the presence of proline at the C-terminal side of the Ub-protein junction (23). Ufd4p, a member of the HECT family of E3 proteins (39), is the E3 of the *S. cerevisiae* UFD pathway (23). Sequence comparisons did not detect statistically significant similarities between the 225K Ubr1p and the 167K Ufd4p. We used the GST-pulldown and coimmunoprecipitation/immunoblotting assays to determine whether Ufd4p also could bind to the proteasome.

Ufd4p was N-terminally tagged with the HA epitope, which did not impair the function of Ufd4p (data not shown). In the GST-pulldown assay, extracts from *S. cerevisiae* overexpressing HA-Ufd4p were incubated with glutathione-agarose beads preloaded with GST-Rpn1p, GST-Rpn2p, GST-Rpn3p, GST-Rpn10p, GST-Rpn11p, GST-Rpn12p, GST-Rpt1p, GST-Rpt2p, GST-Rpt6p, or GST alone. The retained proteins were fractionated by SDS/PAGE and immunoblotted with anti-HA antibody. As shown in Fig. 5*A*, HA-Ufd4p was found to be specifically bound to GST-Rpt6p, one of three proteins of the 19S particle that interacted with Ubr1p, the E3 of the N-end rule pathway. None of the other tested proteasomal proteins were able to interact with HA-Ufd4p in the GST-pulldown assay (Fig. 5*A*).

We then examined the *in vivo* association of Ufd4p with Rpt6p. HA-Ufd4p was co-overexpressed in *S. cerevisiae* with Rpt6p-FLAG, which bore the C-terminal FLAG epitope. The controls included congenic cells expressing either HA-Ufd4p alone or Rpt6p-FLAG alone. Proteins were immunoprecipitated from cell extracts with anti-HA antibody, followed by SDS/PAGE and immunoblotting with either anti-HA antibody (Fig. 5*B Upper*) or anti-FLAG antibody (Fig. 5*B Lower*). The results indicated that Rpt6p-FLAG was specifically coprecipitated with HA-Ufd4p by anti-HA antibody.

If Ufd4p interacts with the mature proteasome, one would expect Rpn1p, which did not directly interact with Ufd4p (Fig. 5*A*), to be coimmunoprecipitated with HA-Ufd4p. To test this conjecture, HA-Ufd4p was co-overexpressed in *S. cerevisiae* with Rpn1p-FLAG, which bore the C-terminal FLAG epitope. The controls included congenic cells expressing either HA-Ufd4p alone or Rpn1p-FLAG alone. Extracts were immunoprecipitated with anti-HA antibody, followed by SDS/PAGE and immunoblotting with either anti-HA antibody (Fig. 5*C Upper*) or anti-FLAG antibody (Fig. 5*C Lower*). Rpn1p-FLAG was indeed specifically coprecipitated with HA-Ufd4p by anti-HA antibody. Taken together, the GST-pulldown and coimmunoprecipitation results (Fig. 5) demonstrated that Ufd4p, the E3 of the UFD pathway, was physically associated with the proteasome.

Discussion

It has been unclear how the Ub system delivers its substrates to the 26S proteasome for processive degradation. Two structural elements of a targeted, Ub-conjugated substrate could serve as ligands for the substrate's docking at the 26S proteasome: a substrate-linked multi-Ub chain and/or a cognate E3-E2 complex reversibly bound to the substrate's degron. Thus far, Rpn10p is the only proteasomal component known to bind multi-Ub chains; it also binds to chain-bearing model substrates (refs. 10, 20, and 21, and our unpublished data). However, *S. cerevisiae* stains lacking Rpn10p were found to be impaired in the degradation of only some of the model substrates and did not exhibit aberrant phenotypes characteristic of proteolysis-impaired proteasome mutants (10, 21).

Our discovery that Ubr1p and Ufd4p, the E3 components of, respectively, the N-end rule pathway and the UFD pathway, directly interact with specific proteins of the 26S proteasome suggests a general mechanism for the delivery of targeted substrates to the proteasome. In this model, the E3 component of a Ub-dependent pathway binds (as an E3-E2 complex) to a cognate degron of a target protein and initiates, either sequentially or concurrently, two sets of processes: (*i*) the formation of a substrate-linked multi-Ub chain, through the activity of substrate-bound Ub ligase (E3-E2) complex, and (*ii*) the delivery of an E3-bound substrate to the chaperone-like proteins of the 26S

proteasome, through interactions between the E3 and specific proteins of the 19S particle. A key assumption of this model is that the demonstrated physical affinity between an E3 and the proteasome is essential for the activity (at least for the normal level of activity) of an E3-mediated proteolytic pathway. In the case of the N-end rule pathway, this prediction can be tested, for example, through the mapping of a site(s) in the 225K Ubr1p that mediates its binding to Rpn2p, Rpt1p, and Rpt6p of the 19S particle. The site(s) thus identified then could be mutated in ways that leave intact the other functions of Ubr1p, such as its binding to substrates, to the Rad6p E2 enzyme, and the previously described, RING finger-dependent activity of Ubr1p in the Rad6p/Ubr1p-mediated formation of a substrate-linked multi-Ub chain (27). A similar approach could be used to test the model's assumption in regard to the demonstrated Ufd4p-Rpt6p interaction.

Specific functions of either the Ufd4p-Rpt6p interaction or the interactions of Ubr1p with Rpn2p, Rpt1p, and Rpt6p of the 19S particle are unknown, in part because it is largely unknown how the proteins of this particle contribute to the proteasome-mediated proteolysis. It has been shown that Rpt1p-Rpt6p, the six ATPases of the 19S particle, are not functionally redundant, and that they cooperate in preparing individual substrates for degradation by the 20S core of the proteasome (40). It is likely that the functional role of the Ubr1p-Rpn2p interaction is different from those of the Ubr1p-Rpt1p and Ubr1p-Rpt6p interactions, because overexpression of Rpn2p was found to have virtually no effect on the toxicity of co-overexpressed Ubr1p and Rad6p, whereas overexpression of Rpt1p and Rpt6p increased this toxicity (see above). One possibility is that the non-ATPase Rpn2p protein of the 19S particle functions as the main Ubr1p affinity anchor, whereas the Ubr1p-binding ATPases Rpt1p and Rpt6p mediate the unfolding of a Ubr1p-bound substrate before its translocation into the 20S core. If so, the observed increase in the toxicity of overexpressed N-end rule pathway in the presence of free (overproduced) Rpt1p or Rpt6p may result from their binding to the Ubr1p moiety of the Ubr1p-Rad6p complex and their facilitation of the unfolding of Ubr1p-bound substrates before docking at the proteasome. Given the differences between Ubr1p and Ufd4p E3s in regard to their ligands in the 19S particle (unlike Ubr1p, the Ufd4p E3 did not bind to either Rpt1p or Rpn2p), it should be of interest to determine whether Rpt6p is, in fact, the only significant ligand of Ufd4p in the proteasome.

Several aspects of the proposed delivery mechanism remain unconstrained by the available evidence. For example, if the demonstrated Ubr1p-proteasome and Ufd4p-proteasome interactions (Figs. 3–5) prove relevant to the functional activity of the N-end rule and UFD pathways, one would like to determine how many of the distinct E3s in a cell interact with the proteasome and what proteins of the proteasome these E3s bind to. Another important question is whether the Ubr1p-dependent formation of a substrate-linked multi-Ub chain is required, *in vivo*, for the docking of a Ubr1p-bound substrate at the Ubr1-binding site of the proteasome. An alternative model is that these two Ubr1p-mediated, substrate-centered processes (the chain formation and the docking at the proteasome) take place concurrently and independently.

The previously proposed function of a substrate-linked multi-Ub chain is to serve as a dissociation-slowing device (9). Specifically, if the rate-limiting step that precedes the first proteolytic cleavages of a proteasome-bound substrate is the substrate's unfolding by chaperones of the 19S particle, then a decrease in the rate of dissociation of the proteasome-substrate complex, brought about by the multi-Ub chain, should facilitate substrate's degradation: the longer the allowed "waiting" time, the greater the probability of a required unfolding event (13). Similar considerations may apply to the function of E3-mediated binding of a substrate to the proteasome.

The main result of this work is that the E3 components of two distinct Ub ligases are physically associated with specific proteins of the 26S proteasome. We discussed the mechanistic and functional implications of this advance.

We thank F. Du and A. Webster for the gifts of purified FLAG-Ubr1p and the UPR-based plasmids, C. Pickart for isolated multi-Ub chains, H. Rao for the strain AVY107, and E. Johnson for the pRS313UFD4 plasmid. This work was supported by grants to A.V. from the National Institutes of Health (GM31530 and DK39520). Y.X. was supported in part by a postdoctoral fellowship from the National Institutes of Health.

1. Maniatis, T. (1999) *Genes Dev.* **13,** 505–510.
2. Nasmyth, K. (1999) *Trends Biochem. Sci.* **24,** 98–104.
3. Hershko, A. & Ciechanover, A. (1998) *Annu. Rev. Biochem.* **76,** 425–479.
4. Peters, J.-M., King, R. W. & Deshaies, R. J. (1998) in *Ubiquitin and the Biology of the Cell*, eds. Peters, J.-M., Harris, J. R. & Finley, D. (Plenum, New York), pp. 345–387.
5. Varshavsky, A. (1997) *Trends Biochem. Sci.* **22,** 383–387.
6. Baumeister, W., Walz, J., Zühl, F. & Seemüller, E. (1998) *Cell* **92,** 367–380.
7. Scheffner, M., Smith, S. & Jentsch, S. (1998) in *Ubiquitin and the Biology of the Cell*, eds. Peters, J.-M., Harris, J. R. & Finley, D. (Plenum, New York), pp. 65–98.
8. Hochstrasser, M. (1996) *Annu. Rev. Genet.* **30,** 405–439.
9. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. & Varshavsky, A. (1989) *Science* **243,** 1576–1583.
10. Pickart, C. M. (1997) *FASEB J.* **11,** 1055–1066.
11. Haas, A. J. & Siepman, T. J. (1997) *FASEB J.* **11,** 1257–1268.
12. Laney, J. D. & Hochstrasser, M. (1999) *Cell* **97,** 427–430.
13. Varshavsky, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12142–12149.
14. Rechsteiner, M. (1998) in *Ubiquitin and the Biology of the Cell*, eds. Peters, J. M., Harris, J. R. & Finley, D. (Plenum, New York), pp. 147–189.
15. DeMartino, G. N. & Slaughter, C. A. (1999) *J. Biol. Chem.* **274,** 22123–22126.
16. Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A. & Finley, D. (1998) *Cell* **94,** 615–623.
17. Rivett, A. J., Mason, G. G., Murray, R. Z. & Reidlinger, J. (1997) *Mol. Biol. Rep.* **24,** 99–102.
18. Coux, O., Tanaka, K. & Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–817.
19. Hilt, W. & Wolf, D. H. (1996) *Trends. Biochem. Sci.* **21,** 96–102.
20. Deveraux, Q., Ustrell, V., Pickart, C. & Rechsteiner, M. (1994) *J. Biol. Chem.* **269,** 7059–7061.
21. van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D. & Vierstra, R. D. (1996) *Mol. Cell. Biol.* **16,** 6020–6028.
22. Bartel, B., Wünning, I. & Varshavsky, A. (1990) *EMBO J.* **9,** 3179–3189.
23. Johnson, E. S., Ma, P. C., Ota, I. M. & Varshavsky, A. (1995) *J. Biol. Chem.* **270,** 17442–17456.
24. Madura, K. & Varshavsky, A. (1994) *Science* **265,** 1454–1458.
25. Sikorski, R. S. & Hieter, P. (1989) *Genetics* **122,** 19–27.
26. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (1998) *Current Protocols in Molecular Biology* (Wiley, New York).
27. Xie, Y. & Varshavsky, A. (1999) *EMBO J.* **18,** 6832–6844.
28. Suzuki, T. & Varshavsky, A. (1999) *EMBO J.* **18,** 6017–6026.
29. Byrd, C., Turner, G. C. & Varshavsky, A. (1998) *EMBO J.* **17,** 269–277.
30. Glickman, M. H., Rubin, D. M., Fried, V. A. & Finley, D. (1998) *Mol. Cell. Biol.* **18,** 3149–3162.
31. Finley, D., Tanaka, K., Mann, C., Feldmann, H., Hochstrasser, M., Vierstra, R., Johnston, S., Hampton, R., Haber, J., McCusker, J., *et al*. (1998) *Trends Biochem. Sci.* **23,** 244–245.
32. Lévy, F., Johnsson, N., Rumenapf, T. & Varshavsky, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4907–4912.
33. Wilkinson, K. & Hochstrasser, M. (1998) in *Ubiquitin and the Biology of the Cell,* eds. Peters, J.-M., Harris, J. R. & Finley, D. (Plenum, New York), pp. 99–125.
34. Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W. & Madura, K. (1998) *Nature (London)* **391,** 715–718.
35. Lambertson, D., Chen, L. & Madura, K. (1999) *Genetics* **153,** 69–79.
36. Hiyama, H., Yokoi, M., Masutani, C., Sugasawa, K., Maekawa, T., Tanaka, K., Hoeijmakers, J. H. J. & Hanaoka, F. (1999) *J. Biol. Chem.* **274,** 28019–28025.
37. Johnson, E. S., Bartel, B., W. & Varshavsky, A. (1992) *EMBO J.* **11,** 497–505.
38. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U. & Jentsch, S. (1999) *Cell* **96,** 635–644.
39. Scheffner, M., Huibregtse, J. M., Vierstra, R. D. & Howley, P. M. (1993) *Cell* **75,** 495–505.
40. Rubin, D. M., Glickman, M., Larsen, C. N., Dhruvakumar, S. & Finley, D. (1998) *EMBO J.* **17,** 4909–4919.
41. Webb, E. C. (1992) *Enzyme Nomenclature* (Academic, New York), p. 527.