plexed antibody already deposited in the airway, and thus amplifying the response. Additionally, the plasma leakage delivers a local source of the complement components, which become fixed by the immune complexes to ultimately generate C5a. The amplification of the formation of immune complexes, as well as the delivery of a local source of complement, occurs through substance P acting at the microvasculature of the airway mucosa.

The present results thus extend the previous model of the inflammatory cascade in immune complex–mediated injury (*4*). In that model, Fc receptor engagement in the skin was shown to be a critical initiating event in the production of edema and neutrophil exudation. However, the identity of the mediators that could link the inciting (Fc) stimulus with the subsequent edema and PMN influx remained conjectural (*16*). Our data provide a plausible linkage between the Fc stimulus and neurohumoral mediators by showing that the complement-dependent inflammatory response is C5aR-dependent and is downstream from the immune complex release of substance P, which amplifies the inflammatory response. The present data heighten interest in investigating the tachykinins as being critical in other inflammatory models such as inflammatory bowel disease and asthma.

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ER Degradation of a Misfolded Luminal Protein by the Cytosolic Ubiquitin-Proteasome Pathway

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Secretion of proteins is initiated by their uptake into the endoplasmic reticulum (ER), which possesses a proteolytic system able to degrade misfolded and nonassembled proteins. The ER degradation system was studied with yeast mutants defective in the breakdown of a mutated soluble vacuolar protein, carboxypeptidase yscY (CPY*). The ubiquitin-conjugating enzyme Ubc7p participated in the degradation process, which was mediated by the cytosolic 26S proteasome. It is likely that CPY* entered the ER, was glycosylated, and was then transported back out of the ER lumen to the cytoplasmic side of the organelle, where it was conjugated with ubiquitin and degraded.

The ER is the site of entry of proteins into the secretory pathway. Such proteins are translocated in an unfolded state through the membrane of this organelle, and, during the subsequent folding process, N- and O-linked glycosylation occurs and disulfide linkages are formed. The large number of unfolded proteins that enter the lumen of the ER must be protected from aggregation and be maintained in a folding-competent state (*1*). Thus, the ER contains a high concentration of molecular chaperones that promote protein translocation, folding, and oligomerization (*2*). Inefficient folding, unbalanced subunit synthesis, or mutations in secretory proteins result in the translocated polypeptides failing to assume their correct conformation (*3*). The ER can eliminate these proteins through selective degradation associated with a pre-Golgi compartment (*3*, *4*). However, little is known about the mechanisms and proteolytic systems that mediate this ER degradation of proteins.

A mutated plasma membrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR), is retained in the ER membrane and degraded by the ubiquitin-proteasome system (*5*). Two mutated soluble vacuolar proteins of the yeast *Saccharomyces cerevisiae*, carboxypeptidase yscY (CPY*) and proteinase yscA (PrA*), are imported into the lumen of the ER but do not reach the vacuole; instead, they remain within the ER and are degraded (*6*). The absence or mutation of the ER membrane protein Der1p abolishes degradation of CPY* and PrA* in yeast (*7*); CPY* is localized in the ER in cells devoid of Der1p (*7*).

We now describe the *DER2* gene which, when mutated, also results in the accumulation of CPY* and PrA* (*7*, *8*). The *DER2* gene was cloned by complementation of the *der2-1* mutation (*9*). Genetic analysis and

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nucleotide sequencing showed that *DER2* is identical to *UBC7* (*QRI8*) (*9*), which encodes the ubiquitin-conjugating enzyme Ubc7p (*10*, *11*). In yeast, Ubc7p catalyzes the conjugation of ubiquitin to the transcriptional repressor protein MATα2 (*12*), which results in destruction of the protein by the proteasome. Metabolic labeling and immunoprecipitation with CPY-specific antibodies revealed that deletion of *UBC7* resulted in the same phenotype as that of the *der2-1* mutant—retardation of degradation of CPY* antigenic material (Fig. 1A).

Introduction of *UBC7* on a single or multicopy plasmid (Fig. 1A) into the *ubc7* deletion strain restored the wild-type phenotype with respect to CPY* degradation. The ubiquitin conjugation activity of Ubc7p depends on the active site cysteine residue at position 89 of the enzyme (*11*). To investigate whether the action of Ubc7p on CPY* degradation required this active site cysteine, we replaced it with serine. The Ubc7p Ser^{89} mutant protein could not fulfill the function of the wild-type protein, indicating that the catalytic activity of Ubc7p was essential for CPY* degradation (*8*). The Ubc6 protein is a ubiquitin-conjugating enzyme that is bound to the ER membrane, its active site facing the cytosol (*13*). Pulse-chase analysis showed that deletion of *UBC6* also reduced the rate of CPY* degradation (Fig. 1B). The half-life of CPY* in the Δ*ubc6* background was more than twice that in the wild-type strain. The Δ*ubc7* and Δ*ubc6* Δ*ubc7* mutant strains were indistinguishable with respect to CPY* degradation, each showing a 3.4-fold increase in the half-life of CPY* (Table 1). Of other ubiquitin-conjugating enzymes tested, deletion of *UBC4* (*14*) also retarded the breakdown of CPY* (*8*).

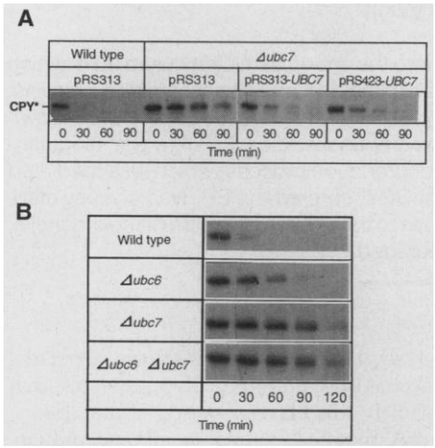

Fig. 1. Effect of *UBC7* deletion on the degradation of CPY*. Cells were labeled for 20 min with [^{35}S]methionine and then incubated in the absence of label for the times indicated (*30*). Cell lysates were prepared and subjected to immunoprecipitation with antibodies to CPY, and the immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography. (**A**) Pulse-chase analysis of CPY* degradation in the isogenic strains W303-1C (wild type) and W303-CQ (Δ*ubc7*) containing either a CEN plasmid without insert (pRS313) or the *UBC7* gene integrated into a CEN plasmid (pRS313-*UBC7*) or into a multicopy plasmid (pRS423-*UBC7*) as indicated (*31*). The less than maximal restoration of degradation of CPY*, especially in the strain harboring the multicopy plasmid, is attributable to plasmid loss during cell growth (*8*). (**B**) Pulse-chase analysis of CPY* degradation in the isogenic strains W303-1C (wild type), W303-CP (Δ*ubc6*), W303-CQ (Δ*ubc7*), and W303-CPQ (Δ*ubc6* Δ*ubc7*) (*31*).

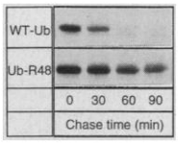

Fig. 2. Effect of blocking polyubiquitination with Ub-R48 on CPY* degradation. CPY* degradation in the wild-type strain W303-1C expressing plasmids encoding either wild-type ubiquitin (WT-Ub) or Ub-R48 (*31*) was examined by pulse-chase analysis as described in the legend to Fig. 1.

Degradation of proteins by the ubiquitin pathway requires the synthesis of a polyubiquitin chain on a lysine residue within ubiquitin (*15*, *16*). Lysine residues 29, 48, and 63 of ubiquitin are the main targets in this process, with Lys^{48} being the most commonly used site (*16*). Overexpression of plasmid-encoded ubiquitin species with lysine residues 29, 48, or 63 replaced with arginine (Ub-R29, Ub-R48, or Ub-R63) (*16*) in a strain expressing CPY* revealed that only Ub-R48 markedly inhibited CPY* degradation (Fig. 2) (*8*). Thus, polyubiquitination at Lys^{48} of ubiquitin was required for CPY* degradation. Degradation of two substrates of the proteasome, the MATα2 repressor (*17*) and fructose-1,6-bisphosphatase (*18*), also depends on polyubiquitination at Lys^{48} of ubiquitin.

Degradation of polyubiquitinated proteins is mediated by the 26*S* proteasome complex (*19*). We investigated the possible role of this enzyme complex in the degradation of CPY* with the use of mutants that are defective either in the 20*S* proteolytic core (*20*, *21*) or in the 19*S* cap subunits of the complex (*22*, *23*). Pulse-chase analysis revealed that the rate of CPY* degradation was markedly reduced in proteasome mutants defective in one (*pre1-1*) or two (*pre1-1 pre2-2* or *pre1-1 pre4-1*) subunits of the proteolytic core, otherwise being of identical genotype (Fig. 3A). The half-life of CPY* was increased about twofold in the *pre1-1* mutant strain and 2.5-fold in the *pre1-1 pre2-2* and the *pre1-1 pre4-1* double mutants, relative to that in the wild type (Table 1). The previously undetected participation of the proteasome in the degradation of CPY* (6) can be explained by the use of nonisogenic strains in these experiments. The involvement of the entire 26*S* proteasome in the degradation of CPY* was demonstrated with the use of mutants defec-

Table 1. Summary of the half-lives of CPY* in wild-type and mutant strains. Protein bands corresponding to the pulse-chase experiments shown in Fig. 1B and Fig. 3A were quantified with either a Fuji BAS 2000 or a Molecular Dynamics imaging system. The members of each set of strains were isogenic and varied only in the *ubc* or *pre* background.

Strain	Genotype	CPY* half-life (min)
W303-1C	wild type	38
W303-CP	Δ*ubc6*	86
W303-CQ	Δ*ubc7*	130
W303-CPQ	Δ*ubc6* Δ*ubc7*	130
WCGY4a	wild type	24
WCGY4-11a	*pre1-1*	46
WCGY4-11/22a	*pre1-1 pre2-2*	64
YHIY29/14	*pre1-1 pre4-1*	59

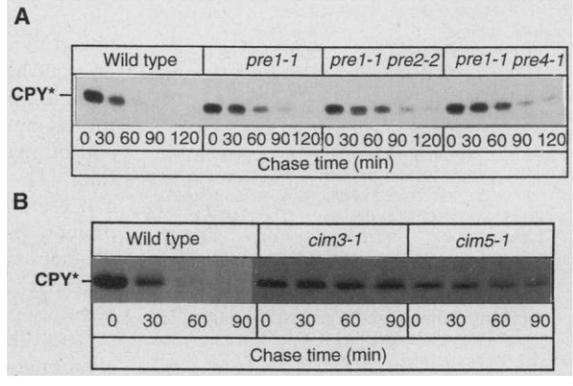

Fig. 3. Effects of mutations in the proteasome on the degradation of CPY*. The experiments were performed as described in the legend to Fig. 1. (**A**) Pulse-chase analysis of CPY* degradation in the isogenic strains WCGY4a (wild type), WCGY4-11a, WCGY4-11/22a, and YHIY29/14, the latter three of which contain *pre1-1*, *pre1-1 pre2-2*, and *pre1-1 pre4-1* mutations, respectively, affecting the catalytic core of the proteasome (*31*). (**B**) Pulse-chase analysis of CPY* degradation in the wild-type strain YPH499Y and the congenic mutants CMY762Y and CMY806Y harboring the *cim3-1* and *cim5-1* mutations, respectively, affecting components of the 19*S* cap of the 26*S* proteasome (*31*). Incorporation of ^{35}S into the wild-type strain was about four times that in the mutant strains, which was probably attributable to an effect of the *cim* mutations [as was independently shown for the *cim3-1* mutant by complementation (*8*)].

tive in the Cim3p (Sug1p) or Cim5p subunits of the 19S cap of the complex: Proteolysis of CPY* was markedly reduced in *cim3-1* and *cim5-1* mutants (Fig. 3B).

After entering the ER, CPY* undergoes core glycosylation (6). The misfolded enzyme accumulates in the ER of mutants defective in Der1p (7). On the other hand, the proteasome is located in the cytoplasm and nucleus of cells of higher eukaryotes (24), and ubiquitin is not present in the ER of these cells (25). Ubc7p is a soluble yeast enzyme with no ER targeting sequence (11), and Ubc6p is located on the cytoplasmic face of the ER membrane in yeast (13). Together, these observations suggest that the degradation of CPY* by the cytosolic ubiquitin-proteasome system may occur via retrograde transport of CPY*, or fragments thereof, from the ER lumen to the cytoplasmic side of the ER. Studies on the transport of small peptides (26) indicate that such a retrograde transport system might exist. To search for and localize ubiquitinated species of CPY*, we overexpressed ubiquitin tagged with an epitope of influenza virus hemagglutinin (HA) (HA-Ub) (17) or, as a control, untagged ubiquitin (WT-Ub) (17) in various yeast strains. Crude extracts were prepared, and CPY* was immunoprecipitated with CPY-specific antibodies (7), separated by SDS–polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose. Immunoblot analysis with HA-specific antibodies revealed no immunoreactive material in cells synthesizing HA-Ub that either expressed wild-type CPY or harbored a deletion in the *PRC1* gene (coding for CPY); cells expressing WT-Ub and CPY* similarly lacked immunoreactivity (Fig. 4A). In contrast, substantial amounts of immunoreactive material were apparent in cells expressing HA-Ub and CPY*. Reduced amounts of HA-Ub–conjugated CPY* were detected in cells in which *UBC7* was deleted (Fig. 4A); the residual HA-Ub–conjugated CPY* in these cells is attributable to the action of other ubiquitin-conjugating enzymes such as Ubc6p (Fig. 1B). HA-Ub–conjugated CPY* was also virtually absent from cells in which the *DER1* gene was deleted (Fig. 4A). We separated crude extracts of cells expressing CPY* and HA-Ub into soluble and particulate fractions. Only the particulate fraction contained HA-Ub–conjugated CPY* (Fig. 4B). However, treatment of the membrane fraction with trypsin resulted in complete disappearance of the HA-tagged protein material, whereas Kar2p, which is localized in the lumen of the ER (27), remained resistant to trypsin digestion (Fig. 4B). The additional treatment of the particulate fraction with Triton X-100 rendered Kar2p also susceptible to trypsin digestion. Thus, the HA-Ub–conjugated CPY* did not reside inside the ER but was most probably located on the cytosolic face of the ER membrane. HA-Ub–conjugated CPY* was glycosylated: Treatment of the protein with endoglycosidase F resulted in deglycosylation, apparent as an increase in the mobility of the protein bands (Fig. 4C). Furthermore, HA-Ub–conjugated CPY* bound to concanavalin A (Con A)–Sepharose in an endoglycosidase F–sensitive manner (Fig. 4D). Thus, CPY* must have entered the ER in order to undergo glycosylation.

Our data are consistent with the following sequence of events: After import and glycosylation in the ER, CPY*, or fragments thereof, undergoes retrograde transport out of the ER, becomes ubiquitinated, remains associated with the ER membrane, and is subsequently degraded by the 26S proteasome. ER-associated degradation of misfolded proteins on the cytoplasmic side of the ER is supported by the observation that degradation in vitro of the yeast secretory protein pro–α-factor, mutated so as to be incapable of undergoing glycosylation, required cytosol and adenosine triphosphate (28). The ER-associated degradation of CFTR is likely facilitated by the large (~60%) cytoplasmic domain of this protein, which should be readily accessible to the ubiquitin-proteasome system (5). Degradation of major histocompatibility complex (MHC) class I heavy chains by the proteasome as a result of the action of the ER-resident human cytomegalovirus *US11* gene product has been described (29). This protein is thought to mediate the dislocation of glycosylated MHC class I heavy chains from the ER membrane to the cytosol (29), indicating the possibility of translocation of a glycosylated membrane protein from the ER lumen to the cytosol.

The CPY* protein is transported into the lumen of the ER, where it accumulates. Its degradation must require machinery responsible for recognition inside the ER and ret-

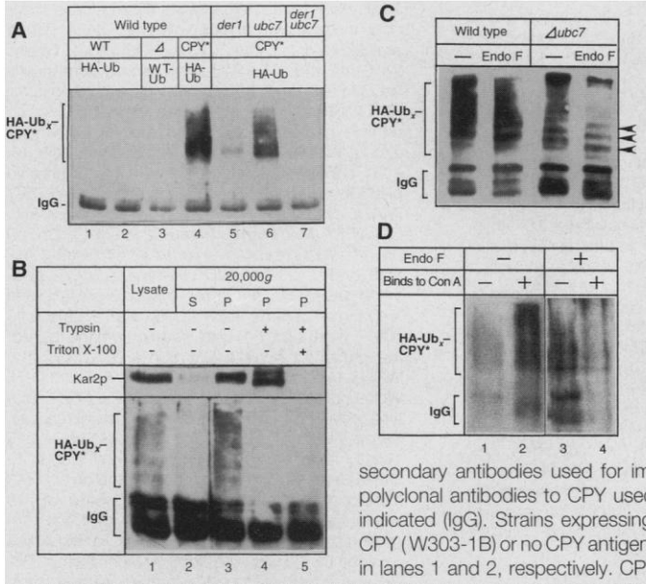

Fig. 4. Ubiquitination and glycosylation of CPY*. Expression of CPY* was induced in cells of isogenic strains expressing wild-type (WT-Ub) or HA epitope–tagged (HA-Ub) ubiquitin (31). (**A**) After cell lysis under alkaline conditions, CPY* was immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose. HA-Ub–conjugated CPY* (HA-Ub$_x$–CPY*) was visualized with HA epitope–specific antibodies (32). Nonspecific cross-reactions of the secondary antibodies used for immunoblot detection with the polyclonal antibodies to CPY used for immunoprecipitation are indicated (IgG). Strains expressing HA-Ub and either wild-type CPY (W303-1B) or no CPY antigenic material (CB018) are shown in lanes 1 and 2, respectively. CPY* was present in a wild-type genetic background (W303-1C) and WT-Ub or HA-Ub was overexpressed (lanes 3 and 4). CPY* and HA-Ub were present in a Δ*der1* deletion strain (W303-CD) (lane 5), a Δ*ubc7* deletion strain (W303-CQ) (lane 6), or a Δ*der1* Δ*ubc7* double-deletion strain (W303-CDQ) (lane 7). (**B**) Wild-type (W303-1C) cells expressing CPY* and HA-Ub (31) were deprived of their cell wall and gently lysed (33), and HA-Ub$_x$–CPY* was immunoprecipitated and subjected to immunoblot analysis as in (A). Lanes 2 and 3, the distribution of HA-Ub$_x$–CPY* in the supernatant (S) and pellet (P) after centrifugation at 20,000*g* (33) before immunoprecipitation, as compared with the unfractionated lysate (lane 1). Equal portions of the pellet fraction were treated with trypsin alone (lane 4) or together with Triton X-100 (lane 5), as compared with an untreated sample (lane 3). As a control, the yeast Bip analog Kar2p in samples taken before immunoprecipitation with the CPY-specific antibodies was subjected to immunoblot analysis. The aberrant mobility of the Kar2p band in lane 4 is due to the high concentration of trypsin in the sample, as was shown in an independent experiment (8). (**C**) CPY* antigenic material immunoprecipitated from cells of the wild-type strain W303-1C and the isogenic Δ*ubc7* strain W303-CQ (31) overexpressing HA-Ub was incubated in the absence or presence of endoglycosidase F (Endo F) (32) as indicated. Immunoblot analysis with antibodies to HA revealed that enzyme treatment resulted in a shift of bands to lower molecular mass. The Δ*ubc7* strain showed more specific bands (arrowheads) than the wild type. (**D**) CPY* immunoprecipitated from cells of strain W303-CQ (31) overexpressing HA-Ub was incubated with Con A–Sepharose (34) and separated into a nonbinding supernatant (lane 1) and a Con A–Sepharose–bound fraction (lane 2), both of which were then subjected to immunoblot analysis with antibodies to HA. The experiment was repeated but the immunoprecipitated material was treated with endoglycosidase F (32) before Con A–Sepharose fractionation (lanes 3 and 4).

rograde transport of the glycosylated protein into the cytoplasmic environment, in order to render it accessible to the ubiquitin-proteasome system. Der1p in the ER membrane (*7*) may be a component of this machinery. Given that PrA* is degraded with similar kinetics to CPY* (*6*) and the misfolded protein also accumulates in *der2-1* (*ubc7*) mutants (*7*, *8*), we suggest that its breakdown also depends on the ubiquitin-proteasome system. Indeed, it is possible that this cytosolic system is responsible for most ER-associated protein degradation.

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30. For pulse-chase experiments, cells corresponding to 2 units of optical density at 600 nm (OD_{600}) (0.6×10^8 to 0.8×10^8 cells) were taken from a logarithmically growing culture for each time point and labeled with [^{35}S]methionine. Growth, labeling, and chase conditions, as well as all other experimental procedures (cell breakage, immunoprecipitation, and SDS-PAGE), were as described (*6*).
31. The wild-type strains used in this study were W303-1B (*MATα ade2-1^{oc} ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*) [H. L. Chiang and R. Schekman, *Nature* **350**, 313 (1991)], WCG4a (*MATα his3-11,15 leu2-3,112 ura3*) (*21*), and YPH499 (*MAT**a** ura3-52 leu2Δ1 his3Δ200 trp1Δ63 lys2-801 ade2-101*) (*22*). Strain *der2-1* was derived from ethylmethane sulfonate mutagenesis of cells of strain YAF6 (*MAT**a** pra1ΔSS prc1-1 leu2-3,112 URA3 HIS3*). Strains WCGY4a and YPH499Y were derived from strains WCG4a and YPH499 by replacing the chromosomal *PRC1* allele with the *prc1-1* allele (encoding CPY*) by the two-step gene replacement method [S. Scherer and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4951 (1979)] with Bgl II–digested DNA of plasmid pRS306*prc1-1* (*7*). The mutant strains CMY762Y (*MAT**a** cim3-1 ura3-52 leu2Δ1 his3Δ200 prc1-1*), CMY806Y (*MAT**a** cim5-1 ura3-52 leu2Δ1 his3Δ200 prc1-1*), WCGY4-11a (*MATα his3-11,15 leu2-3,112 ura3 pre1-1 prc1-1*), WCGY4-11/22a (*MATα his3-11,15 leu2-3,112 ura3 pre1-1 pre2-2 prc1-1*), and YHIY29/14 (*MATα his3-11,15 leu2-3,112 ura3 pre1-1 pre4-1 prc1-1*) were derived from the respective mutants CMY762, CMY806 (*22*), WCG4-11a, WCG4-11/22a (*21*), and YHI29/14 (W. Hilt and D. H. Wolf, unpublished data) by replacing the *PRC1* wild-type allele with the *prc1-1* allele, as described above. Strains W303-1C (*MATα ade2-1^{oc} ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) and W303-CD, derived from W303-1C but harboring the *der1::URA3* mutation, were provided by M. Knop (*7*). Strain CB018 (*MAT**a** ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1^{oc} can1-100 pep4Δ::HIS3 prb1::hisG prc1Δ::hisG*) was provided by S. Fuller. The *ubc6* deletion strain W303-CP was constructed by transforming cells of strain W303-1C with Hind III–digested DNA of plasmid pTX33 (*ubc6::LEU2*) (*13*) followed by selection for Leu$^+$ transformants. The *ubc7* deletion strain W303-CQ was constructed by transforming cells with Pst I– and Bam HI–digested DNA of plasmid pGR172 (*ubc7::LEU2*) (*10*) followed by selection for Leu$^+$ transformants. In all instances, the homologous recombination events were confirmed by Southern (DNA) blot analysis. W303-CQa was derived from W303-CQ through mating type switching, with the use of the plasmid pGalHO [J. B. Hicks and I. Herskowitz, *Genetics* **83**, 245 (1976)], and subsequent screening for the **a**-mating type. W303-CPQ and W303-CDQ were derived from crosses of W303-CQa with W303-CP and W303-CD, respectively. For all strain constructions, standard yeast genetic techniques were used [C. Guthrie and G. R. Fink, Eds., *Methods Enzymol.* **194** (1991)]. The 1.1-kb Eco RI–Pst I genomic fragment containing the *UBC7* gene, made blunt at the Pst I end, was subcloned into the Eco RI– and Eco RV–digested CEN plasmid pRS313 [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)] or 2μ plasmid pRS423 [T. W. Christianson, R. S. Sikorski, M. Dante, J. H. Shero, P. Hieter, *Gene* **110**, 119 (1992)] to yield the plasmids pRS313-*UBC7* and pRS423-*UBC7*, respectively. The plasmids Yep96, Yep110, and Yep112 contain WT-Ub, Ub-R48 and HA-Ub under the control of the *CUP1* promoter on a 2μ plasmid (*17*). The plasmids containing Ub-R29 and Ub-R63 are derivatives of Yep96 (*16*).
32. Cells expressing the different ubiquitin variants from plasmids (*31*) were grown at 30°C in complete synthetic medium (CM) into log phase until an OD_{600} of 2 units was achieved. They were then transferred into CM containing 0.1% glucose and 100 μM copper sulfate (OD_{600}, 10) and grown for 4 hours to allow for induction of CPY* and the synthesis of the respective ubiquitin variant. Cells (OD_{600}, 100 units) (3×10^9 to 4×10^9 cells) were subjected to alkaline lysis (0.25 M NaOH, 1% mercaptoethanol) for 10 min on ice, followed by precipitation with 10% (w/v) trichloroacetic acid (TCA) for 10 min on ice. After centrifugation (10 min, 20,000*g*), the resulting pellet was washed once with ice-cold ethanol, resuspended in 500 μl of UREA buffer [5% SDS, 8 M urea, 200 mM tris-HCl (pH 6.8), 0.1 mM EDTA, bromophenol blue], diluted 1 to 20, and subjected to immunoprecipitation with antibodies to CPY essentially as described previously (*6*). Deglycosylation of samples was performed as described [D. O. Spormann, J. Heim, D. H. Wolf, *J. Biol. Chem.* **267**, 8021 (1992)]. Immunoprecipitated material was boiled in 60 μl of UREA buffer before SDS-PAGE. CPY* antigenic material corresponding to 15 OD_{600} units of cells (4.5×10^8 cells) was separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes according to standard methods. HA-Ub was detected with the 12CA5 antibody to HA and enhanced chemiluminescence (ECL; Amersham). Kar2p was similarly detected with specific polyclonal antibodies.
33. Cells were grown as described (*32*). Spheroplast generation and cell breakage were performed essentially as described [R. Egner, Y. Mahe, R. Pandjaitan, K. Kuchler, *Mol. Cell. Biol.* **15**, 5879 (1995)] with an additional treatment with 20 mM *N*-ethylmaleimide before spheroplast generation. Spheroplasts were generated from 300 OD_{600} units of cells and gently lysed with a tissue grinder. The lysate was cleared of remaining cells and debris by repeated centrifugation for 5 min at 3000*g*, and the final supernatant was divided into portions corresponding to 60 OD_{600} units of cells. From the time of lysis, all material was maintained on ice. Membranes were separated from the soluble fraction by centrifugation at 20,000*g* for 30 min at 4°C. For protease treatment, the pellet was resuspended and trypsin was added to a concentration of 0.5 mg/ml. The sample was incubated for 30 min on ice (if added, Triton X-100 was present at 1% during this incubation). All treatments were terminated by precipitation of proteins with 10% TCA for 10 min on ice. Solubilization of the TCA pellet, immunoprecipitation of CPY* antigenic material, SDS-PAGE, and detection of HA-Ub–conjugated CPY* were performed as described (*32*).
34. The separation of unglycosylated from glycosylated proteins by Con A–Sepharose (Sigma) was performed in Con A buffer [0.01 M tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$]. Immunoprecipitated CPY* antigenic material was solubilized in UREA buffer (*32*), diluted 1:20 in Con A buffer, and incubated with Con A–Sepharose (10 to 16 mg of Con A per milliliter of gel; 1.1 μl of gel per OD_{600} unit of cells) for 3 hours at room temperature with gentle agitation. The Con A–Sepharose was separated by centrifugation (1500*g*, 3 min) and washed twice with Con A buffer. Proteins of the combined supernatant and washes were precipitated with 10% TCA for 10 min on ice and solubilized in UREA buffer (*32*), as were the proteins bound to Con A–Sepharose. SDS-PAGE and detection of HA-Ub–conjugated CPY* were performed as described (*32*).

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