

ADD66, a Gene Involved in the Endoplasmic Reticulum-associated Degradation of α -1-Antitrypsin-Z in Yeast, Facilitates Proteasome Activity and Assembly[□]

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Antitrypsin deficiency is a primary cause of juvenile liver disease, and it arises from expression of the "Z" variant of the α -1 protease inhibitor (A1Pi). Whereas A1Pi is secreted from the liver, A1PiZ is retrotranslocated from the endoplasmic reticulum (ER) and degraded by the proteasome, an event that may offset liver damage. To better define the mechanism of A1PiZ degradation, a yeast expression system was developed previously, and a gene, *ADD66*, was identified that facilitates A1PiZ turnover. We report here that *ADD66* encodes an ~30-kDa soluble, cytosolic protein and that the chymotrypsin-like activity of the proteasome is reduced in *add66* Δ mutants. This reduction in activity may arise from the accumulation of 20S proteasome assembly intermediates or from qualitative differences in assembled proteasomes. Add66p also seems to be a proteasome substrate. Consistent with its role in ER-associated degradation (ERAD), synthetic interactions are observed between the genes encoding Add66p and Ire1p, a transducer of the unfolded protein response, and yeast deleted for both *ADD66* and/or *IRE1* accumulate polyubiquitinated proteins. These data identify Add66p as a proteasome assembly chaperone (PAC), and they provide the first link between PAC activity and ERAD.

INTRODUCTION

Newly synthesized secreted proteins must pass a stringent quality control checkpoint in the endoplasmic reticulum (ER), which ensures that only properly folded, assembled, and processed proteins transit the secretory pathway (Ellgaard and Helenius, 2003). Polypeptides that fail to pass this checkpoint may be targeted for ER-associated degradation (ERAD), a process in which aberrant proteins are selected and then delivered—or retrotranslocated—to the cytoplasm and degraded by the 26S proteasome (Fewell *et al.*, 2001; Tsai *et al.*, 2002; Kostova and Wolf, 2003; Meusser *et al.*, 2005; Romisch, 2005; Sayeed and Ng, 2005; Nandi *et al.*, 2006). The 26S proteasome is an ~2.5-MDa multisubunit complex that contains a central 20S proteolytic core particle and two 19S regulatory particles (Voges *et al.*, 1999; Nandi *et al.*, 2006). The 20S core harbors three distinct proteolytic activities—a chymotrypsin-like (CTL), a trypsin-like (TL), and a peptidylglutamyl-peptide hydrolyzing (PGPH) activity—whereas the 19S "cap" (also known as PA700) functions as a gatekeeper at the entrance of the core particle. In addition, the 19S particle contains polyubiquitin-binding sub-

units, enzymes required for polypeptide deubiquitination, and six AAA ATPases that are thought to unfold and feed polypeptides into the core (Glickman *et al.*, 1998; Voges *et al.*, 1999; Leggett *et al.*, 2002; Verma *et al.*, 2002; Guterman and Glickman, 2004; Soboleva and Baker, 2004).

Some ERAD substrates in humans are mutated versions of wild-type proteins, and not surprisingly, their absence may lead to the onset of specific diseases (Aridor and Hannan, 2000, 2002; Coughlan and Brodsky, 2003). One substrate in which the connection between ERAD and loss-of-function disease is very clear is the Z-variant of α -1-antitrypsin, also known as A1PiZ (or α -1 protease inhibitor, Z). Wild-type A1Pi, originally termed the "M" variant, or A1PiM (Myerowitz *et al.*, 1972), is an ~53-kDa serine protease inhibitor that is primarily synthesized in and secreted by hepatocytes (Perlmutter, 2002). Serum A1Pi inhibits neutrophil proteases, which are released during inflammatory responses and can mediate proteolysis of the pulmonary connective tissue matrix (Richmond and Zellner, 2005; Rudnick and Perlmutter, 2005). Although secreted A1PiZ retains partial activity, individuals expressing this protein have lower circulating levels of the protein because the E342K mutation compromises its folding in the ER. The resulting decrease in plasma levels of the protease inhibitor lead to antitrypsin deficiency (ATD), which is exemplified by uninhibited neutrophil elastase activity and destruction of the pulmonary extracellular matrix. However, when the A1PiZ variant accumulates, it can form loop-sheet polymers or aggregates that may trigger cirrhosis (Foreman *et al.*, 1984; Perlmutter *et al.*, 1985; Mornex *et al.*, 1986; Verbanac and Heath, 1986; Brantly *et al.*, 1988; McCracken *et al.*, 1989; Lomas *et al.*, 1992; Mast *et al.*, 1992; Kim *et al.*, 1995; Sidhar *et al.*,

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al., 1995; Carrell and Lomas, 2002; Parfrey *et al.*, 2003) and hepatocellular carcinoma (Carlson *et al.*, 1989; Rudnick and Perlmutter, 2005). Thus, ATD is also a gain-of-function disease.

The reason only ~10% of A1PiZ-homozygotes develop liver disease (Sveger, 1988) remains a mystery. However, this phenomenon may arise because a subset of individuals is unable to efficiently clear this aggregation-prone molecule from the ER. Indeed, A1PiZ-expressing fibroblasts from individuals with liver disease degrade the substrate less efficiently than A1PiZ-expressing fibroblasts from healthy homozygotes (Wu *et al.*, 1994). Therefore, factors that alter the efficiency of A1PiZ ERAD may represent genetic modifiers of ATD.

To identify putative ATD modifiers, we developed an A1PiZ yeast expression system (McCracken and Kruse, 1993) because 1) components of the protein quality control machinery are highly conserved, and 2) yeast expression systems for several human disease-causing proteins have led to a better understanding of the pathological consequences of aberrant protein production (Coughlan and Brodsky, 2003). Notably, the A1PiZ yeast expression system helped establish this protein as a bona fide ERAD substrate (Werner *et al.*, 1996) and identified an Hsp70 molecular chaperone in the lumen of the ER, known as BiP, as an important player in A1PiZ turnover (Brodsky *et al.*, 1999). This result was subsequently confirmed in mammalian cells (Cabral *et al.*, 2002; Schmidt and Perlmutter, 2005). We also identified antitrypsin degradation deficient (*ADD*) mutants by using both a classical genetic (McCracken *et al.*, 1996) and targeted approach (Palmer *et al.*, 2003). One of the genes isolated was *ATG6/VPS30*, which is required for autophagy (Kametaka *et al.*, 1998), and yeast overexpressing A1PiZ deliver the aggregated protein to the autophagic pathway (Kruse *et al.*, 2006). Similarly, autophagic vesicles are abundant in liver biopsies from individuals with late-stage ATD (Teckman and Perlmutter, 2000), and A1PiZ-expressing autophagy-deficient cell lines degrade A1PiZ less efficiently than wild-type cells (Kamimoto *et al.*, 2006).

Another yeast gene that was isolated is *ADD66* (YKL206c). Even though Add66p is required for the degradation of only a subset of ERAD substrates examined (Palmer *et al.*, 2003), the *ADD66* transcript is induced by the unfold protein response (UPR) (Travers *et al.*, 2000), which serves as an indicator of ER stress; moreover, the UPR and ERAD provide complementary mechanisms to lessen the effects of aberrant

protein accumulation in the ER (Fewell *et al.*, 2001; Patil and Walter, 2001; Schroder and Kaufman, 2005). Because of these observations, and because cells deleted for *ADD66* also induce the UPR (Palmer *et al.*, 2003), we suggested that Add66p might play a more general role in ER protein quality control.

In this study, we report that Add66p facilitates the maturation of the 20S proteasome particle. It is vital for maximal proteasome activity and like some other proteasome chaperones (Ramos *et al.*, 1998; Tone and Toh, 2002; Hirano *et al.*, 2005), it can be degraded by the proteasome. Based on these results and on the sequence similarity between *ADD66* and the mammalian proteasome assembly chaperone (PAC)2 (Hirano *et al.*, 2005, 2006), we suggest that Add66p is the *Saccharomyces cerevisiae* PAC2 homologue. Together, these results provide the first direct link between PAC activity and ERAD.

MATERIALS AND METHODS

Strains and Growth Conditions

The *Escherichia coli* strain used in this study was DH5 α (*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ [*lacZYA-argF*] *U169 deoR* [Φ 80 *dlac* Δ *lacZ* M15]). Bacteria were grown in Luria-Bertani medium supplemented with 50 μ g/ml ampicillin for plasmid selection. An *add66* Δ disruption cassette was obtained by amplifying pRS400 (Brachmann *et al.*, 1998) with the following oligonucleotides: 5'-ACT TCA GGA AAG AAT AGC ACA AAA CCC AAA GGA ACA TAC GCT GTG CCG TAT TTC ACA CCG-3' and 5'-ATA TAT GCA CTT GTA TAG AAA ACA GAT ATA CTT CTC GGT TAG ATT GTA CTG AGA GTG CAC-3'. *ADD66* mutants were obtained as described previously (Brachmann *et al.*, 1998). A *pdr5* Δ haploid strain was isolated by sporulation and dissection of the yeast *pdr5* Δ homozygous diploid (Invitrogen, Carlsbad, CA). All other yeast strains used in this study are detailed in Table 1, and they were grown on yeast extract-peptone (YP)-dextrose (YPD) medium or on synthetic complete (SC) medium lacking the indicated nutrient but supplemented with a carbohydrate source to a final concentration of 2%. Yeast were grown at the indicated temperatures, and all genetic and molecular manipulations followed standard published protocols (Adams *et al.*, 1997).

Detection of Polyubiquitinated Proteins in Yeast

Yeast were transformed with a plasmid engineered for the expression of a ubiquitin-*myc* fusion protein under the transcriptional control of a copper-inducible promoter (Ecker *et al.*, 1987; subcloned and provided by A. Caplan, Mount Sinai School of Medicine), and transformants were isolated on selective medium (SC-HIS) containing glucose. A culture containing 20 optical density units measured at 600 nm (ODs) of cells was grown to mid-log phase (~1.0 OD/ml) at 30°C before CuSO₄ was added at a final concentration of 100 μ M, and the cells were incubated for 1 h. The yeast were harvested and resuspended in 100 μ l of sample buffer (1.0% β -mercaptoethanol, 1% SDS, 5% glycerol, 0.05 mg/ml bromophenol blue, and 65 mM Tris, pH 6.8), 0.2 g of glass beads was added, and lysates were prepared by vigorous agitation on a

Table 1. Yeast strains used in this study

Strain	Relevant genotype	Reference or source
<i>ADD66</i> (BY4742)	MAT α <i>his3</i> Δ 1 <i>leu2</i> Δ 0 <i>lys2</i> Δ 0 <i>ura3</i> Δ 0	Invitrogen
<i>add66</i> Δ	MAT α <i>add66::kanMX his3</i> Δ 1 <i>leu2</i> Δ 0 <i>lys2</i> Δ 0 <i>ura3</i> Δ 0	Invitrogen
<i>ire1</i> Δ	MAT α <i>ire1::kanMX his3</i> Δ 1 <i>leu2</i> Δ 0 <i>lys2</i> Δ 0 <i>ura3</i> Δ 0	Invitrogen
<i>ire1</i> Δ <i>add66</i> Δ	MAT α <i>ire1::kanMX add66::HIS3 his3</i> Δ 1 <i>leu2</i> Δ 0 <i>lys2</i> Δ 0 <i>ura3</i> Δ 0	This study
<i>ADD66</i> (W303)	MAT α <i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1, ura3-1</i>	
<i>add66</i> Δ	MAT α <i>add66::kanMX ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1, ura3-1</i>	This study
CIM5	MAT α <i>ura3-52 lys2-801 ade2-101 his3</i> Δ 200 <i>leu2</i> Δ -1	Ghislain <i>et al.</i> (1993)
<i>cim5-1</i>	MAT α <i>cim5-1 ura3-52 his3</i> Δ 0200 <i>leu2</i> Δ -1	Ghislain <i>et al.</i> (1993)
<i>atg14</i> Δ	MAT α <i>atg14::kanMX his3</i> Δ 1 <i>leu2</i> Δ 0 <i>lys2</i> Δ 0 <i>ura3</i> Δ 0	Invitrogen
<i>pdr5</i> Δ	MAT α <i>pdr5::kanMX his3</i> Δ 1 <i>leu2</i> Δ 0 <i>ura3</i> Δ 0 <i>lys2</i> Δ 0 <i>met15</i> Δ 0	This study
JD133	MAT α <i>his3</i> Δ 200 <i>leu2-3,112 lys2-801 trp1</i> Δ 63 <i>ura3-52 UMP1</i> -ha::YIplac128 PRE2-HA::YIplac211	Ramos <i>et al.</i> (1998)
JD134	MAT α <i>his3</i> Δ 200 <i>leu2-3,112 lys2-801 trp1</i> Δ 63 <i>ura3-52 ump1</i> Δ -1::HIS3 PRE2-HA::YIplac211	Ramos <i>et al.</i> (1998)
RJD1144	MAT α <i>his3</i> Δ 200 <i>leu2-3,112 lys2-801 trp1</i> Δ 63 <i>ura3-52 PRE1</i> -Flag-HIS6::YIplac211 (URA3)	Verma <i>et al.</i> (2000)

Vortex mixer 10 times for 1 min with a 1-min incubation in an ice bath between each treatment. The total protein in a 5- μ l aliquot of each sample was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose blots. The nitrocellulose was incubated for 30 min, while sandwiched in Whatman filter paper, in boiling double-distilled deionized water. Ubiquitin and Sec61p, a component of the translocon that served as a loading control, were identified by Western blotting by using anti-*myc* (kindly provided by G. Apodaca and O. Weisz, University of Pittsburgh School of Medicine) and anti-Sec61p (Stirling *et al.*, 1992). Western blots were developed using enhanced chemiluminescence (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. Images were obtained on a Kodak 440CF Image Station, and the results were quantified using Kodak 1D, version 3.6 imaging software (Eastman Kodak, Rochester, NY).

Expression of Wild Type and the Z Variant of α -1-Antitrypsin and Epitope-tagged Add66p

Yeast were transformed with plasmids containing the genes encoding A1PiM or A1PiZ under the transcriptional control of a galactose-inducible promoter (McCracken *et al.*, 1996), and transformants were isolated on selective medium (SC-URA) containing glucose. As a control, the indicated strains were also transformed with the pYES2 vector (Invitrogen) lacking an insert.

To create an epitope-tagged version of Add66p, a single *myc* epitope was appended onto the C terminus of Add66p by polymerase chain reaction (PCR) amplification of genomic yeast DNA with the following oligonucleotides: 5'-CGC GGA TCC ATG AGC TGC CTG GTG TTG-3' (to construct the pGPD vectors; see below) or 5'-CGC GGA TCC TCC TCG ATT TGA CTG GAA AC-3' (to construct the pRS vector) and 5'-CCC AAG CTT TCA CAG GTC CTC CTC GAT CAG CTT CTC CTC ATT GTA TAA ATC TAC AAA TTT ATC TCT TGC-3' (the underlined portion encodes the 11-amino acid *myc* epitope; Evan *et al.*, 1985). The PCR fragment was inserted into the BamHI and ClaI sites in the pPRS315, pGPD425, and pGPD426 vectors (Sikorski and Hieter, 1989; Mumberg *et al.*, 1995) and the in-frame insertion and integrity of *ADD66* were confirmed by DNA sequence analysis. The corresponding vectors or the vectors lacking an insert were introduced into the indicated strains and transformants were isolated on selective media. Next, yeast were grown to mid-log phase, and 2 ODs of cells were harvested and total cell extracts were prepared as published previously (Brodsky *et al.*, 1998). Add66p-*myc* expression was assessed after SDS-PAGE and by Western blotting, as described above, by using anti-serum against *myc*.

A Yeast Colony Blot Assay for A1PiZ Accumulation

A colony blot immunoassay was performed to assess A1PiZ levels in wild-type and select mutant yeast as described previously (Palmer *et al.*, 2003). In brief, 3 μ l (0.001 OD) of cells from a saturated culture were spotted onto nitrocellulose that had been overlaid onto selective medium containing 2% galactose to induce expression of A1PiZ. After a 36-h incubation at 35°C, the cells were lysed. A1PiZ was detected by immunoblotting, and the results were quantified using the Molecular Analyst program (Bio-Rad, Hercules, CA). The signals corresponded to cell and protein levels in the linear range of detection, and previous work established the validity of using the colony blotting protocol as a means to report on A1PiZ turnover in wild-type and the *add66 Δ* mutant (Palmer *et al.*, 2003).

Add66p Localization

The residence of Add66p-*myc* was assessed by biochemical fractionation using a previously published method, with minor modifications (Kabani *et al.*, 2002). Specifically, detection of Add66p-*myc* by Western blotting required 1000 ODs of mid-log phase yeast that contained the pGPD425-Add66p-*myc* expression vector and that had been grown at 30°C.

To assess Add66p-*myc* localization by indirect immunofluorescence microscopy, a previously described protocol was used (Coughlan *et al.*, 2004) that involved yeast containing either the Add66p-*myc* expression vector under the transcriptional control of a constitutive promoter (pGPD) or the vector lacking an insert (as a negative control). Images were captured on an Olympus BX60 microscope (Olympus, Tokyo, Japan) fitted with a Hamamatsu C4742-95 digital camera (Hamamatsu, Bridgewater, NJ), and they were analyzed using QED Imaging software (Media Cybernetics, Silver Spring, MD).

Purification of Yeast 26S Proteasomes

FLAG-tagged 26S proteasomes were purified from RJD1144 as described previously (Verma *et al.*, 2000; Saeki *et al.*, 2005; Verma and Deshaies, 2005) with minor modifications. In brief, cells were grown to an OD of \sim 3, frozen in liquid nitrogen and ground in a Waring blender. The ground powder (\sim 20 ml) was thawed with 9 ml of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, and 5 mM MgCl₂) plus 5 mM ATP, 2 \times ATP regenerating system (0.02 mg/ml creatine phosphokinase and 20 mM creatine phosphate), 5 mM MgCl₂, and 1 mM dithiothreitol (DTT). After centrifugation at 15,000 rpm for 20 min at 4°C in a SS34 rotor (Sorvall, Newton, CT), the supernatant (\sim 13 ml) was supplemented with 5 mM ATP, 2 \times ATP regenerating system, and 5 mM MgCl₂, and it was incubated with 300 μ l of 50% (vol/vol) washed FLAG agarose beads (Sigma-Aldrich, St. Louis, MO) at 4°C for 1.5 h with rocking.

The agarose beads were washed twice with 10 ml of lysis buffer plus 2 mM ATP and 1 mM DTT; once with 5 ml of lysis buffer plus 2 mM ATP, 1 mM DTT, and 0.2% Triton X-100; once with 5 ml of lysis buffer plus 2 mM ATP and 1 mM DTT; once with 800 μ l of lysis buffer plus 2 mM ATP and 1 mM DTT; and once with 1 ml of 26S elution buffer (25 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, and 15% glycerol) plus 2 mM ATP. The bound proteins were eluted with 400 μ l of 26S elution buffer plus 2 mM ATP supplemented with 1/50 volume of 5 mg/ml 3xFLAG peptide (Biotechnology Center, University of Pittsburgh) by incubating the solution for 3 h at 4°C with rocking. The eluted proteasomes were enriched to \sim 0.9 mg/ml by using a Centricon-30 microconcentrator (Millipore, Billerica, MA), and they were stored at -80°C .

Proteasome Activity Assays and Glycerol Gradient Analysis

Proteasome activity was assessed in clarified yeast cytosols that were obtained from 2 l of the indicated strains grown in selective medium to mid-log phase at 30°C. The cell pellet was washed with water and resuspended in 500 μ l of buffer 88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 5 mM MgOAc, and 250 mM sorbitol), and the cell-slurry was frozen in liquid nitrogen. Frozen cells were lysed by grinding with a mortar and pestle in the presence of liquid nitrogen for 12 min, and the cells were thawed in the presence of a minimal amount of buffer 88 containing 1 mM DTT. Unbroken cells and debris were removed by centrifugation in a Sorvall SS34 rotor at 9000 \times g for 10 min at 4°C, and the supernatant was clarified by centrifugation at 300,000 \times g for 1 h at 4°C. The clarified cytosol was then aliquoted and snap-frozen in liquid nitrogen and stored at -80°C . Total protein was quantified using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Proteasome activity was determined by modifying a previously described protocol (Glickman *et al.*, 1998). First, a total of 100 μ g of clarified cytosol was diluted into 1.8 ml of buffer A (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 10% glycerol, 1 mM ATP, and 1 mM DTT) and incubated on ice for 30 min in either the presence or absence of 100 μ M MG-132 or leupeptin. Next, a fluorescent substrate to detect each proteasome activity (CTL: Suc-LLVY-AMC [Sigma-Aldrich]; TL: Cbz-AAR-AMC [Calbiochem, San Diego, CA]; PGPH: Cbz-LLE-AMC [Calbiochem]) was added to a final concentration of 100 μ M, and the reactions were shifted to 30°C for the indicated times and quenched by the addition of 1% SDS (final concentration). Where noted, a total of 0.5 μ g of purified 26S proteasomes was used and treated identically. Fluorescence was determined on an Aminco-Bowman Series 2 luminescence spectrometer (Thermo Electron Corp., Madison, WI) (excitation, 380 nm; emission, 436 nm). The CTL, TL, and PGPH activities were confirmed using the following inhibitors at a final concentration of 100 μ M: MG-132 (for CTL and PGPH) and leupeptin (for TL) (Savory and Rivett, 1993; Gaczynska and Osmulski, 2005), and the activity in each reaction and at each time point was obtained after the background fluorescence in the presence of each inhibitor was subtracted from the net fluorescence (Figure 4). When extracts were examined, the data were then normalizing to the activities observed in lysates from the respective isogenic wild-type strains at reaction times of 10 min for the CTL activity, and at 60 min for the TL and PGPH activities (Figure 4). When highly enriched proteasomes were examined, the data were normalized to the activities observed in the absence of inhibitors at 180 min (Supplemental Figure 2, third column), and when lysates were examined in this figure (first and second columns) the CTL, TL, and PGPH activities were normalized to the wild-type activities observed at 180 min in the absence of inhibitors. Maximal, normalized activities are denoted as 100%.

To assess the integrity of the proteasome using glycerol gradient centrifugation, the indicated cells expressing Add66p-*myc* under its endogenous promoter (Figure 5) or when overexpressed (Supplemental Figure 6) or containing the expression vector lacking an insert were grown in SC-LEU and 2% glucose at 30°C. A total of 100 ODs of mid-log phase cells were harvested and washed once with water and resuspended in 2 ml of buffer A lacking glycerol. (The *pr5 Δ* strain was incubated with 100 μ g of MG-132 for 1 h at 30°C before harvesting.) Cells were lysed as described above, and unbroken cells were removed by centrifugation in a Sorvall SS34 rotor at 9000 \times g for 30 min at 4°C. Extracted proteins (5 mg total, as assessed using the Bio-Rad protein assay kit with BSA as the standard) were fractionated on a 30 ml 4–25% linear glycerol gradient in an SW28 rotor (Beckman Coulter, Fullerton, CA) at 83,000 \times g for 24 h at 4°C. Molecular mass markers (Sigma-Aldrich) were examined in parallel. One-milliliter fractions were removed, and the refractive index was examined to verify the establishment of a linear gradient. Fractionated proteins were precipitated with trichloroacetic acid (25% final concentration), and they were resolved by SDS-PAGE and analyzed as described above by Western blotting with anti-*myc*, anti-20S (BIOMOL Research Laboratories, Plymouth Meeting, PA), anti-hemagglutinin (HA) (Roche Molecular Biochemicals, Indianapolis, IN), and anti-Cim5p (Ghislain *et al.*, 1993) antisera.

Add66p-*myc* Degradation Assay

The *pr5 Δ* yeast strain expressing Add66p-*myc* was grown in SC-LEU to mid-log phase at 30°C, and protein synthesis was arrested by the addition of cycloheximide to a final concentration of 100 μ g/ml. Four ODs of cells were removed at the indicated times. The cells were washed, and total protein was

isolated by glass bead lysis as detailed above. Proteins were resolved on either 12.5 or 18% polyacrylamide gels, and they were analyzed and quantified as described above by Western blotting with anti-*myc* and anti-Sec61p antisera.

Induction of Autophagy

The indicated strains were grown overnight in YPD, and 2.5 ODs of cells were diluted into 10 ml of rich medium (YP with 2% galactose) or nitrogen-depleted media (SC lacking ammonium sulfate but supplemented with 2% galactose) to induce autophagy. After incubation for 5 h at 30°C, equal numbers of cells were harvested, and lysates were prepared by glass bead lysis as described above. Equal amounts of protein (as assessed above) in each sample were resolved by SDS-PAGE and analyzed by Western blotting by using anti-Ape1p (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Sec61p antisera.

RESULTS

Genetic Interactions between *ADD66* and *IRE1*, a Transducer of the UPR

We reported previously on the identification of UPR-target genes in yeast that—when deleted—result in A1PiZ stabilization (Palmer *et al.*, 2003). In turn, the deletion of one gene, *ADD66*, induced the UPR, suggesting that the corresponding protein might be involved in more general aspects of ER quality control. To further explore the connection between Add66p and the UPR pathway, the following strains were constructed. One strain lacked Ire1p, an ER-resident transmembrane protein that senses a rise in the concentration of misfolded ER luminal proteins and initiates the transcription of genes whose products lessen ER stress (Cox *et al.*, 1993; Mori *et al.*, 1993; Shamu and Walter, 1996; Credle *et al.*, 2005; Zhou *et al.*, 2006). The *ire1Δ* strain was examined along with *add66Δ* yeast and an *ire1Δadd66Δ* strain. Next, these cells and an isogenic wild-type strain (Table 1) were transformed with either a control plasmid or with a plasmid that expresses a ubiquitin-*myc* fusion protein under the transcriptional control of a copper inducible promoter. As shown in Figure 1A (right half of figure), we first observed that strains lacking Add66p showed a modest increase in polyubiquitinated proteins compared with the *ADD66* strain (~2-fold in this experiment), and yeast deleted for *IRE1*—regardless of whether *ADD66* was present—accumulated somewhat greater amounts of polyubiquitinated protein. Furthermore, we noted that yeast lacking both *ADD66* and *IRE1* exhibited a strong, synthetic growth defect when incubated on media containing DTT, a reducing agent that induces the UPR (Figure 1B). These data support the hypothesis that Add66p plays a role in the turnover of polyubiquitinated proteins and ER quality control, and they are consistent with reports indicating that mutations in genes required for both ERAD and the UPR exhibit synthetic phenotypes (Ng *et al.*, 2000; Travers *et al.*, 2000).

In recently published work, autophagy has been shown to play a significant role in mediating A1PiZ degradation in yeast, mammalian cell culture, and mouse models (Teckman and Perlmutter, 2000; Kamimoto *et al.*, 2006; Kruse *et al.*, 2006). Therefore, it was possible that deletion of *ADD66* leads to the accumulation of A1PiZ and exhibits synthetic interactions with *IRE1* because the autophagic pathway, which is induced by ER stress (Bernales *et al.*, 2006; He *et al.*, 2006; Ogata *et al.*, 2006; Yorimitsu *et al.*, 2006), is compromised. To examine this hypothesis, the maturation of Ape1p, a protease that is targeted to the vacuole during autophagy, was assessed in both *ADD66* and *add66Δ* strains and in a well-characterized autophagy-deficient strain, *atg14Δ* (Kametaka *et al.*, 1998). As Ape1p enters the vacuole it is proteolytically cleaved, and thus the conversion of immature-Ape1p (“pre-Ape1p”) to mature Ape1p (“m-Ape1p”) can be used to assess induction of autophagy (Su-

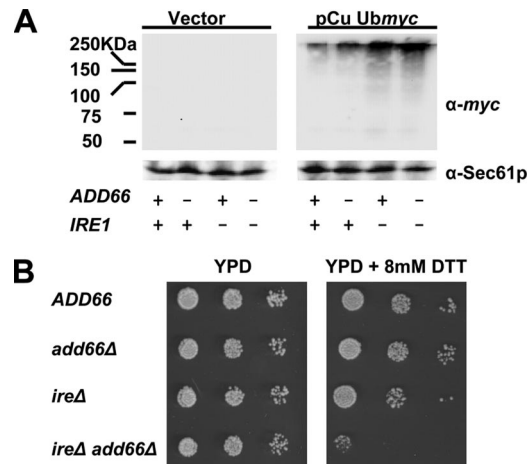


Figure 1. *ADD66* and *IRE1* synthetically interact. *ADD66*, *add66Δ*, *ire1Δ*, and *ire1Δ add66Δ* strains were transformed with a control plasmid or a plasmid expressing a ubiquitin-*myc* fusion protein under the transcriptional control of a copper inducible promoter (pCu Ubmyc). (A) Representative Western blots of extracts from cells containing a vector control or the ubiquitin expression vector are shown. Extracts were prepared after cells had been treated with 100 μ M CuSO₄ for 1 h at 30°C. Blots were probed with anti-*myc* and anti-Sec61p (as a loading control). (B) Serial dilutions of the indicated strains (Table 1) were grown on YPD in the presence or absence of 8 mM DTT, as indicated, for 48 h at 30°C. Of note, we chose to examine DTT sensitivity on YPD medium at pH 6.5 to reduce alternate stresses, although previously published work demonstrated a greater sensitivity to DTT in the *ire1Δ* strain when grown using other conditions (Frand and Kaiser, 1998; Pollard *et al.*, 1998).

zuki *et al.*, 2002). As anticipated, we found that Ape1p failed to mature in the *atg14* mutant, regardless of whether autophagy was induced upon nutrient starvation. In contrast, greater amounts of mature Ape1p were evident in both the wild-type and *add66Δ* strains upon starvation (Supplemental Figure 1). These data indicate that autophagy is proficient in yeast lacking *ADD66*.

Add66p Is a Cytosolic, Soluble Protein

To determine why cells deleted for *ADD66* accumulate A1PiZ and polyubiquitinated proteins, we wanted to characterize the gene product. To this end, a sequence encoding the *myc* epitope was appended onto the C terminus of Add66p, and the construct was cloned into a vector designed for the strong, constitutive expression of the desired protein (pGPD425) (Mumberg *et al.*, 1995) and into a vector in which *ADD66* expression was driven by the endogenous promoter (pRS315; see *Materials and Methods*). To determine whether the tagged protein was active, we assessed whether A1PiZ degradation was restored to wild-type levels in Add66p-*myc*-expressing *add66Δ* yeast. To this end, we used a quantitative colony blot assay that reports on A1PiZ expression levels and ERAD, and in fact was used to isolate the *ADD* mutants (McCracken *et al.*, 1996; Palmer *et al.*, 2003). As shown in Figure 2, A and B, we found that A1PiZ accumulated to wild-type levels when *add66Δ* cells contained the Add66p-*myc* expression vector. As expected, Add66p-*myc* expression was observed only in *ADD66* and *add66Δ* strains that had been transformed with the expression vector (Figure 2C).

To determine Add66p's residence, lysates were prepared from cells constitutively expressing epitope-tagged Add66p

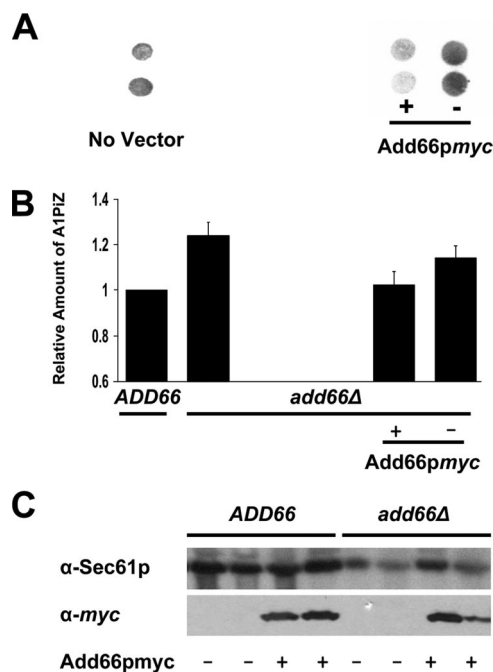


Figure 2. The A1PiZ degradation defect is rescued in *add66Δ* strains expressing *Add66p-myc*. (A) A colony-blot immunoassay was performed with anti-antitrypsin antiserum on *add66Δ* strains expressing A1PiZ and that lacked a vector or that were transformed with an empty vector (-) or with an *ADD66-myc* expression plasmid (+). (B) The results from three independent colony-blot immunoassays were quantified for wild-type yeast (*ADD66*) and *add66Δ* yeast that lacked a vector or that were transformed with the *ADD66-myc* expression vector (+) or a vector control (-). Data were quantified from signals detected in the linear range of the analysis. (C) Proteins extracts were prepared from *ADD66* and *add66Δ* yeast transformed with a vector control (-) or with the *ADD66myc* expression plasmid (+) and total proteins were resolved by SDS-PAGE. The blots were probed with anti-*myc* and anti-Sec61 antisera. Duplicate colonies were analyzed and are shown here.

or containing a vector control, and the lysates were analyzed by differential centrifugation. Add66p was found exclusively in a high-speed supernatant (Figure 3A, S2), suggesting cytoplasmic residence. Similar results were observed in strains expressing *Add66p-myc* expressed under the endogenous promoter (our unpublished data; but see Figure 5). Cytoplasmic residence was further supported through the use of indirect immunofluorescence microscopy: The anti-*myc* fluorescent signal exhibited a diffuse, cytoplasmic staining, unlike a marker for the ER, BiP, which was evident in perinuclear and peripheral patterns (Figure 3B).

Add66p Is Required for Maximal Proteasome Activity

A clue to Add66p's function was provided by a large-scale yeast proteomic analysis in which the gene product was found in a multiprotein complex that included Pre1p (Ho *et al.*, 2002). Pre1p is a subunit in the 20S proteasome core that is one of two subunits that facilitates CTL activity (Heinemeyer *et al.*, 1991; Hilt *et al.*, 1993). More recently, Add66p was identified in a multiprotein complex with Pre5p (Krogan *et al.*, 2006), a nonproteolytic subunit of the 20S proteasome (Heinemeyer *et al.*, 1994), and with Ump1p, a protein required for the maturation of the proteasome core particle (Ramos *et al.*, 1998). These data suggested to us why *add66Δ* yeast accumulated polyubiquitinated proteins and why synthetic growth

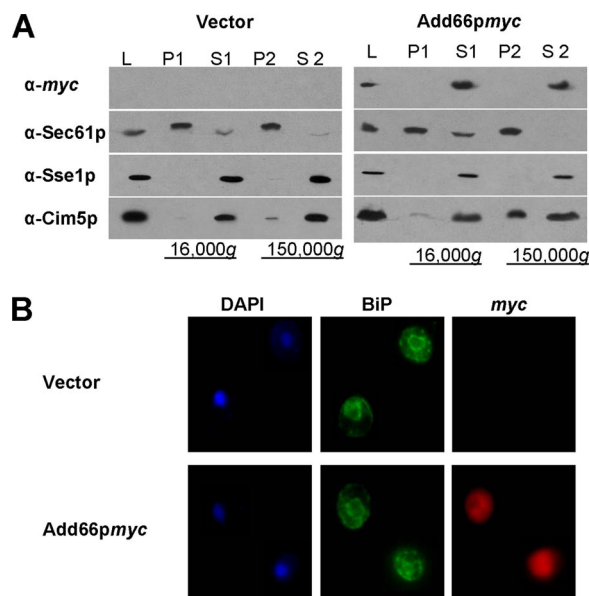


Figure 3. Add66p is cytosolic. (A) *add66Δ* strains were transformed with a control plasmid or with a plasmid engineered for the constitutive expression of *Add66p-myc*. Cell lysates (L) were subjected to 16,000 × *g* and 150,000 × *g* centrifugations. Total proteins in the pellets (P1 and P2) and supernatants (S1 and S2) were resolved by SDS-PAGE, and then they were analyzed by Western blot analysis with anti-*myc*, anti-Sec61p (ER membrane protein), anti-Sse1p (a primarily cytosolic protein; Goekeler *et al.*, 2002), and anti-Cim5p (a regulatory subunit of the 26S proteasome with cytosolic and ER membrane subcellular localizations) antisera. (B) Indirect immunofluorescence of *add66Δ* strains transformed with the plasmids described in A were stained with 4,6-diamidino-2-phenylindole (nuclear staining), and then they were probed with anti-BiP (ER perinuclear and peripheral staining) and with anti-*myc* antisera, and signals were detected as described in *Materials and Methods*.

defects were observed when mutations in *ADD66* and *IRE1* were combined and ER stress was induced (Figure 1).

To test directly whether the deletion of *ADD66* affected proteasome function, the three proteolytic activities of the proteasome were measured in clarified cytosolic extracts derived from *ADD66* and *add66Δ* strains by modifying an established fluorescence assay (see *Materials and Methods*). In each set of assays, the background (spontaneous) hydrolysis of the fluorogenic substrate was subtracted, and the resulting values for activity in the mutant lysates were normalized to the activity in the wild-type lysates. As shown in Figure 4A, we observed that the proteasome's CTL activity—which constitutes most of the proteasome's activity—was markedly reduced in extracts from *add66Δ* yeast (BY4742). To ensure that this effect was not strain specific, the *ADD66* gene was ablated in another strain background (W303) and a similar reduction in CTL activity was observed. As a control for these assays, we noted that all three activities were compromised in extracts prepared from a *cim5-1* strain (Figure 4A, gray bars), which is known to exhibit delayed protein turnover, an elongated G2/M cell cycle transition, and growth arrest under various stress conditions (Ghislain *et al.*, 1993; Rubin *et al.*, 1998). As another set of controls for this assay, the time dependence of the CTL, TL, and PGPH activity in each extract was measured and compared with the activity in highly enriched 26S proteasomes (Supplemental Figure 2).

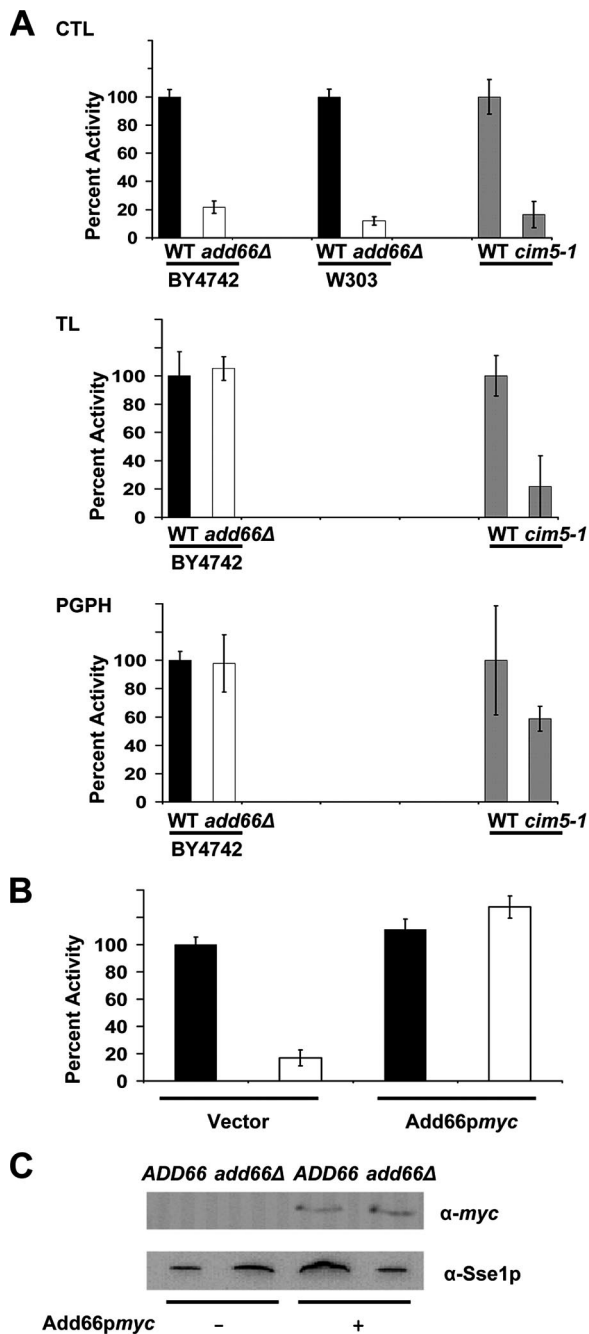


Figure 4. The chymotrypsin-like activity of the 26S proteasome is reduced in extracts prepared from the *add66Δ* strain. The CTL, TL, and PGPH activities in clarified extracts from *ADD66* (black bar) or *add66Δ* (white bar) yeast in two different strain backgrounds (BY4742 and W303) were determined. Proteasome activities in *CIM5* and *cim5-1* (gray bar) strains were used as a positive control. The relative activity was determined by normalizing the fluorescent signals to the levels corresponding to the wild-type strains, as described in *Materials and Methods*. (B) Constitutive expression of *Add66p-myc* restores the CTL activity in the *add66Δ* strain. Wild-type and *add66Δ* strains were transformed with an empty vector (–) or a vector engineered for the expression of *Add66p-myc* (+), and the CTL activity was analyzed as described in A. (C) Cytosolic proteins from the strains in B were resolved by SDS-PAGE, and then they were probed for *Add66p-myc* and *Sse1p* expression by Western blot analysis. *Sse1p* served as a loading control.

We found that the constitutive, strong expression of *Add66p-myc* in the *add66Δ* strain complemented the CTL defect and restored activity to wild-type levels (Figure 4, B and C). However, it was unknown whether overexpression of *Add66p-myc* would present some unforeseen secondary affect on proteasome activity. Therefore, *Add66p-myc* was also expressed in strains under the control of its endogenous promoter (see *Materials and Methods*), and lysates were prepared from *add66Δ* yeast containing a vector control and the expression vector. Here, too, we noted that the CTL defect was complemented (Supplemental Figure 2, *ADD66myc*).

Combined with the fact that *Add66p-myc* rescues the A1PiZ degradation defect in *add66Δ* yeast, these data indicate that *Add66p* is required for maximal CTL activity, and they strongly suggest that the A1PiZ degradation defect in *add66Δ* yeast arises from defects in proteasome function.

20S Precursors Accumulate in Yeast Deleted for *ADD66*

The simplest explanation for the reduced CTL activity in extracts derived from *add66Δ* yeast is that the number of proteasomes is decreased in this strain. To address this possibility, quantitative immunoblotting was used to measure the levels of six 20S subunits—through the use of a nonspecific polyclonal antiserum—and one 19S subunit (*Cim5p*) in the *add66Δ* and wild-type strains (Supplemental Figure 3). However, no significant difference in the relative levels of these subunits was detected.

Pre1p is one of the two essential 20S subunits required for the CTL activity of the proteasome (Heinemeyer *et al.*, 1991; Hilt *et al.*, 1993). Because reduced CTL activity was observed in extracts from *ADD66*-deleted cells (Figure 4A), it was possible that the absence of the corresponding protein might grossly alter the conformation of Pre1p's substrate binding site. To test this hypothesis, the CTL activity was assayed in extracts from wild-type and *add66Δ* strains in the presence of increasing concentrations of MG-132 and epoximycin. MG-132 and epoximycin specifically block the CTL activities in nearly irreversible noncovalent and covalent manners, respectively (Gaczynska and Osmulski, 2005). As shown in Supplemental Figure 4, there was no difference in the apparent K_1 values for these inhibitors ($\sim 5 \times 10^{-7}$ M) when titrated into cytosols prepared from wild-type or *add66Δ* yeast. This result suggests that the conformation of the Pre1p substrate-binding site was not radically altered.

The 26S proteasome is made up of at least 31 different subunits that combine in a spatially and temporally defined manner in various intermediate complexes (Ramos *et al.*, 1998; Voges *et al.*, 1999; Tone *et al.*, 2000; Hirano *et al.*, 2005; Li *et al.*, 2007). Although little is known about the exact mechanism by which the proteins in the cap and the seven distinct α and seven β subunits in the core assemble (but see *Discussion*), two proteins were identified previously that are required for the proper maturation of the core particle in yeast: *Nob1p* and *Ump1p* (Ramos *et al.*, 1998; Tone *et al.*, 2000). Yeast with mutations in *NOB1* exhibit defects in the processing of 20S β subunits, which are the central, proteolytic subunits, and in the assembly of the 20S and 26S particles (Tone and Toh, 2002). In addition, as described above, *Ump1p* coprecipitates with *Add66p* in a multiprotein complex (Krogan *et al.*, 2006), and mutations in *UMP1* affect the function of all three proteolytic activities due to defects in β subunit maturation (Ramos *et al.*, 1998). Thus, it was possible that *Add66p* participates in proteasome subunit maturation and/or assembly. Moreover, we found that *ADD66* is $\sim 20\%$ identical to *PAC2* (Supplemental Figure 5), which facilitates the assembly of the 26S proteasome in mammals (Hirano *et al.*, 2005). *PAC2* is also known as

CLAST3 and HCCA3, a gene that is up-regulated in hepatic cancers (Wang *et al.*, 2001; Bahar *et al.*, 2002).

Based on these observations and our data presented above, we examined whether 20S proteasome assembly intermediates accumulate in yeast lacking Add66p. To this end, extracts derived from the *ADD66* or the *add66Δ* strains transformed with a control plasmid or with the *Add66p-myc* expression plasmid were fractionated on a glycerol density gradient. A low percentage (4–25%) glycerol gradient was used in this experiment to better resolve early proteasome intermediates, and it was used previously to note assembly intermediates in mammalian cells when PAC2 expression was silenced (Hirano *et al.*, 2005). We first observed that the

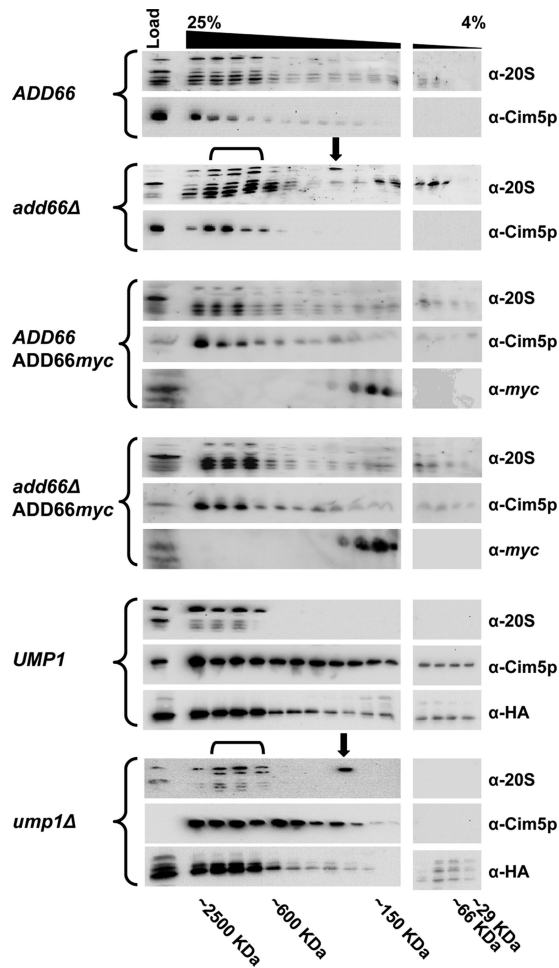


Figure 5. Yeast deleted for *ADD66* accumulate a 20S intermediate and unprocessed 20S subunits. Cell extracts were prepared from an *ADD66* and *add66Δ* strain containing either a control plasmid or a plasmid engineered for the endogenous expression of *Add66p-myc*, and from a *UMP1* (JD133) and *ump1Δ* (JD134) strain. In total, 5 mg of protein was then resolved on a linear glycerol gradient (4–25%), and fractions were collected. Proteins in every other fraction were examined for the presence of 20S subunits, a component of the 19S subunit (Cim5p), and *Add66p-myc* by Western blot analysis. The migrations of molecular mass markers, which were analyzed in parallel, are indicated below the gel, the black downward bracket indicates fractions containing immature 20S subunits (a slower migrating doublet), and the black downward arrow indicates the migration of a 20S assembly intermediate. Note that the later two were observed only in extracts prepared from *add66Δ* and *ump1Δ* cells. The immunoreactive HA species in the *UMP1* and *ump1Δ* gradients represents Pre2p (Table 1).

majority of 20S subunits and a component of the 19S cap resolved at fractions in the gradient that corresponded to particles with a molecular mass of ~2.5 MDa (Figure 5). This value is in good agreement with the native size of the 26S proteasome. Second, we observed a 20S immunoreactive protein in a distinct, lighter fraction when extracts from the *add66Δ* strain were examined (see downward arrow, *add66Δ*, α-20S). This species, which migrated at ~300 kDa, was absent when extracts from wild-type cells or from *add66Δ* yeast expressing endogenous levels of *Add66p-myc* were examined. Third, we found that the same 20S immunoreactive species was present when extracts were resolved from yeast lacking *Ump1p* (*ump1Δ*, α-20S), a factor required for 20S maturation (Ramos *et al.*, 1998). Fourth, when extracts were examined from *add66Δ* or *ump1Δ* yeast, a 20S, slower-migrating doublet was observed that fractionated at the native size for 26S proteasomes (see downward bracket). Previous work demonstrated that one of the species in the doublet represents an unprocessed β subunit in the 20S proteasome (Ramos *et al.*, 1998). This result suggests similar defects during 20S subunit processing in the *add66Δ* and *ump1Δ* strains. And fifth, we found that *Add66p* resided at a position consistent with a molecular mass of ~150–300 kDa, suggesting that this ~30-kDa protein is a component of a multiprotein complex and/or forms higher order oligomers (see *Discussion*; Li *et al.*, 2007). Overexpression of *Add66p-myc* was also able to decrease the amount of the immature 20S doublet and of the ~300-kDa assembly intermediate (Supplemental Figure 6), although the overexpressed *Add66p-myc* instead resolved in fractions corresponding to a molecular mass of ~66–150 kDa. Together, these data indicate that yeast deleted for *ADD66* accumulate an intermediate in the 20S assembly pathway, as observed in *ump1Δ* and *nob1Δ* strains (Ramos *et al.*, 1998; Tone *et al.*, 2000).

Add66p Is Degraded by the 26S Proteasome

Other proteasome chaperones interact transiently with early proteasome assembly intermediates, and in some cases, they are then degraded by the proteasome (Ramos *et al.*, 1998; Tone *et al.*, 2000; Tone and Toh, 2002; Hirano *et al.*, 2005). We therefore incubated a *pdr5Δ* *Add66p-myc* expressing strain with either dimethyl sulfoxide (DMSO) or MG-132. The *pdr5Δ* strain was used based on the fact that this mutation, like other similarly used mutations, disables a plasma membrane drug efflux pump; therefore, the effect of proteasome inhibitors is magnified (Balzi *et al.*, 1994; Lee and Goldberg, 1996). As shown in Figure 6A, *Add66p-myc* shifts to fractions containing complexes of greater molecular masses, which contain 20S subunits, when extracts were prepared from cells treated with MG-132. Treatment with MG-132 also results in the accumulation of the slower migrating, 20S “doublets,” as seen when extracts were examined from *add66Δ* and *ump1Δ* strains (Figure 5) when the blots were overexposed (our unpublished data). Next, to examine whether *Add66p*—like several other proteasome chaperones—is degraded by the proteasome, a cycloheximide chase was performed in the presence or absence of MG-132. Once again, the *pdr5Δ* strain was transformed with a plasmid engineered for the constitutive expression of *Add66p-myc*, and the cells were incubated with either DMSO or MG-132 for 1 h before the chase (Figure 6B). Although *Add66p* was rapidly degraded in cells treated with DMSO, we discovered that MG-132 addition resulted in a profound, reproducible stabilization of the protein. To better visualize *Add66p-myc* in this experiment, it was necessary to overexpress the protein; thus, these data should be interpreted with

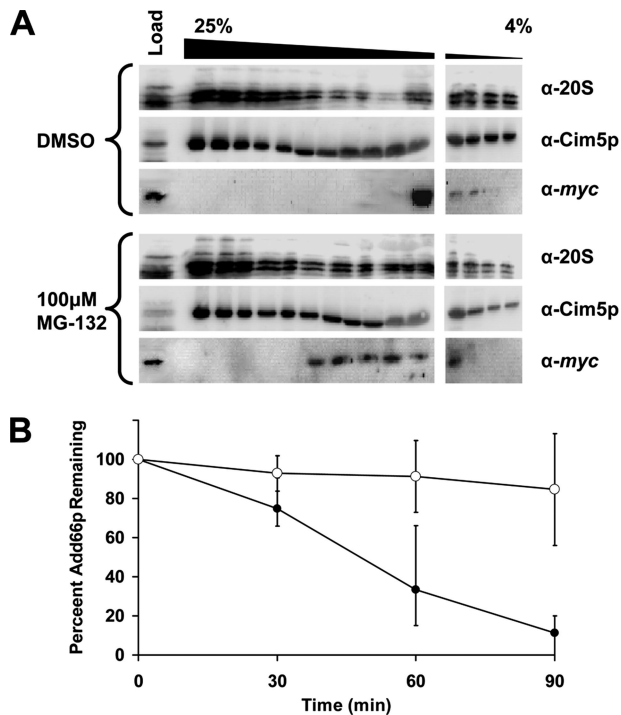


Figure 6. Add66p is degraded by the 26S proteasome. A *ptr5Δ* strain transformed with a plasmid designed for the constitutive expression of *ADD66-myc* was incubated either with 100 μM MG-132 or the equivalent volume of DMSO for 1 h at 30°C. (A) Cell extracts from each strain were prepared, and 5 mg of protein was resolved on a linear glycerol gradient (4–25%), and fractions were collected. Proteins in every other fraction were immunoblotted for 20S subunits, a component of the 19S subunit (Cim5p), and Add66p-myc. Molecular mass markers, which were analyzed in parallel, are indicated below the gel. Note that these blots were purposely overexposed (compared with those in Figure 5). (B) The strains described in A were harvested at the indicated time points after the addition of cycloheximide, and cell extracts were prepared and subjected to SDS-PAGE and immunoblotted for Add66p-myc and Sec61p (as a loading control). The amount of Add66p-myc at the start of the chase in each strain, after standardization to the amount of Sec61p at each time point, was set to 100%. ○, MG-132; ●, DMSO control.

caution. Nevertheless, the results strongly suggest that Add66p is a proteasome substrate.

DISCUSSION

In this report, we present the characterization of Add66p, a protein previously implicated in the ERAD of A1PiZ in yeast (Palmer *et al.*, 2003). We found that strains deleted for *ADD66* accumulate polyubiquitinated proteins and grow poorly when they are unable to mount a UPR and challenged with a UPR-inducing agent. These data may be explained by our discovery that the CTL activity of the proteasome is compromised in *add66Δ* yeast. Yeast deleted for *ADD66* also accumulate some of the same proteasome assembly intermediates as those observed when the gene encoding the Ump1p proteasome assembly factor is disabled. Our observations are consistent with the fact that Add66p is found in a multiprotein complex that includes Pre1p and Pre5p—proteins embedded within the 20S core particle—as well as Ump1p itself (Ho *et al.*, 2002; Krogan *et al.*, 2006). Based on these data, we propose that Add66p is a yeast PAC and that it is vital for maximal proteasome function.

The recent characterization of PACs in both yeast and mammals have yielded new insight into the pathway of proteasome biogenesis (Chen and Hochstrasser, 1995, 1996; Heinemeyer *et al.*, 1997; Ramos *et al.*, 1998, 2004; Arendt and Hochstrasser, 1999; Burri *et al.*, 2000; Griffin *et al.*, 2000; Tone *et al.*, 2000; Witt *et al.*, 2000; Tone and Toh, 2002; Hirano *et al.*, 2005, 2006; Li *et al.*, 2007). The symmetrical, 20S barrel-shaped core particle (CP) of the proteasome is made up of two half-proteasome (15S) complexes (Nandi *et al.*, 1997). These half-proteasomes contain a ring of α subunits and a ring of β subunits, three of which are responsible for the CP's proteolytic activity and must be processed. In mammals, after the two 15S complexes combine, the “pro” regions in the β subunits are cleaved and a cohort of PACs—including hUmp1 (also known as POMP or Proteasembilin), PAC1, PAC2, and PAC3—participate in complex formation. PAC1 and PAC2 have been proposed to maintain the assembly competence of the α ring, and they are degraded after assembly of the two half-proteasomes; in contrast, PAC3 assists in the assembly of the β ring before dissociating. PAC3 also recruits hUmp1, which later catalyzes the dimerization of the two half-proteasomes. In yeast, Nob1p facilitates the association of the 19S particle to the CP (Tone and Toh, 2002; Hirano *et al.*, 2005, 2006). Based on our results and data derived from studies of PAC2 function in mammalian cells (Hirano *et al.*, 2005, 2006), we propose that Add66p is the *S. cerevisiae* homologue of PAC2: 1) the proteins are ~20% identical throughout their sequence (Supplemental Figure 5), and several conserved residues are present in PAC2 homologues in all species (our unpublished data); 2) both factors are proteasome substrates; 3) lowering protein levels (by RNA interference in mammalian cells) or completely ablating the gene (in yeast) leads to the accumulation of CP precursors, increases the concentration of polyubiquitinated proteins, and decreases the proteasome's CTL activity; and 4) MG-132 treatment in each cell type results in the accumulation of the protein in denser fractions after glycerol gradient centrifugation.

While this article was in revision, Hochstrasser and colleagues reported that Add66p (which they named Pba2, for proteasome biogenesis-associated polypeptide 2) associates with another protein (Pba1) to form a stable complex (Li *et al.*, 2007); the Add66p–Pba1 complex resolves with distinct proteasome assembly intermediates, and it was found that deletion of *ADD66* restores the growth of an *ump1Δ* mutant, which is consistent with antagonistic action between Ump1p and the PACs. They also reported that the concentration of a pro- $\beta 5$ assembly intermediate increased in the *add66Δ* mutant. Combined with the results reported here and with studies in mammalian cells (Hirano *et al.*, 2005, 2006), these data provide support that Pba1 and Add66p are the yeast homologues of PAC1 and PAC2, respectively.

In contrast to prior studies of PAC function in mammalian cells and yeast, we have determined the importance of Add66p on the degradation of a distinct class of proteins. More specifically, we have discovered a link between PAC activity and ERAD. In previous work, we noted that Add66p facilitates the degradation of some ERAD substrates (A1PiZ and the cystic fibrosis transmembrane conductance regulator) but not others (carboxypeptidase Y* and pro- α factor) (Palmer *et al.*, 2003). At first glance, these data and the results presented in this article may seem at-odds, i.e., if the proteasome is partially disabled in *add66Δ* mutants, why is the turnover of all ERAD substrates not affected similarly, especially because any one of the proteasome's three activities may be sufficient to remove an ERAD substrate (Oberdorf *et*

al., 2001)? Consistent with data from other laboratories, we suggest two possible answers to this question.

First, we propose that the rate-limiting step during ERAD differs for unique classes of substrates. Notably, ERAD can be envisaged as a five-step temporal and—for some substrates—spatial processes, consisting of substrate identification, retrotranslocation, polyubiquitination, deubiquitination, and degradation. In reality, each of these steps is likely further broken down into multiple, discrete kinetic events. Regardless, the overall rate of substrate degradation, as for any multistep process, is established by the rate-limiting step. During the degradation of some proteasome substrates, it has been shown that deubiquitination is rate limiting (Yao and Cohen, 2002; Guterman and Glickman, 2004; Hanna *et al.*, 2006). During ERAD, the removal of a specific substrate class (proteins in the “ERAD-L” family) takes significantly longer than the removal of other substrates (proteins in the “ERAD-C” family), possibly because only ERAD-L substrates can transit to the Golgi apparatus before ER retrieval and degradation (Vashist *et al.*, 2001). Thus, effects arising from impaired proteasome function (Figure 4) might be masked by the robust activity of factors acting at an alternative, rate-limiting step.

Second, it has been proposed that proteasome isoforms exist within cells: Those that are stable and those that recycle (Tone and Toh, 2002). In fact, it was reported later that the proteasome disassembles after ATP hydrolysis and substrate degradation (Babbitt *et al.*, 2005). Combined with the concept that specific proteasome subpopulations may recognize distinct classes of substrates (Fujimuro *et al.*, 1998), it is possible that A1PiZ is degraded by only one of these “classes.” Formally, then, deletion of *ADD66* might compromise the assembly of a unique proteasome subset that is required for the degradation of some but not all substrates. Alternatively, the absence of Add66p might alter the proteasome’s interaction with the ER membrane. Different ERAD substrates might be delivered from the ER in different conformations, only some of which may require close apposition of the proteasome with the ER and possibly Sec61p (Kalies *et al.*, 2005).

A surprising aspect of this study was that deletion of *ADD66* seemed to affect the CTL activity of the 26S proteasome, whereas there were no obvious defects in the TL and PGPH activities. This is in contrast to the fact that all three activities decrease in an *ump1Δ* strain (Ramos *et al.*, 1998). We propose two explanations for this phenomenon. First, Ump1p may have a global effect on proteasome activity due to its role as an assembly checkpoint during the dimerization of half proteasomes (Li *et al.*, 2007). Thus, deletion of *UMP1* may result in a significant accumulation of defective dimers or other intermediates, thereby reducing the level of functional 26S proteasomes. In contrast, the Add66p dependence during proteasome maturation may give rise to subtle changes in proteasome assembly or even in the conformations of individual, catalytic subunits, or associated subunits. Thus, there might be qualitative, perhaps minor differences in the architecture of CTL-requiring subunits in proteasomes between wild-type and *add66Δ* cells. Consistent with this model, more severe proteasome assembly defects were noted in *ump1Δ* versus *add66Δ* (*pba2Δ*) mutants when intermediates were resolved by gel filtration chromatography (Ramos *et al.*, 1998; Li *et al.*, 2007). Second, more trivially, we note that neither the TL nor PGPH activity was measured when specific proteasome assembly factors were ablated (Tone and Toh, 2002; Hirano *et al.*, 2005, 2006). Therefore, it remains possible that unique effects on proteolytic

activities do exist when related PACs are disabled, at least in some cell types or under specific conditions.

One goal of our long-term studies is to identify conserved yeast genes that impact the ERAD of distinct substrates. This undertaking is particularly relevant for A1PiZ, given that only a small percentage of *A1PiZ* homozygotes develop severe liver disease (Sveger, 1988). It is thought that both environmental and genetic modifiers play a role in determining the onset and severity of liver disease (Wu *et al.*, 1994; Perlmutter, 2002). Therefore, it is critical that genetic polymorphisms or secondary mutations that alter A1PiZ quality control are identified. Intriguingly, we found that ~15% of *add66Δ* cells expressing A1PiZ are inviable (data not shown; but see Supplemental Figure 7 for an example). In principle, one might be able to co-opt this phenotype to screen for second-site or suppressor mutations. It is also possible that the observed genetic penetrance arises from stochastic variability in some cells relative to others: Recent insight into phenotypic variation have led to a greater understanding of the “noise” that accounts for the stochastic nature of protein production and phenotypic variation, especially in single-cell organisms (Raser and O’Shea, 2004; Newman *et al.*, 2006; Samoilov *et al.*, 2006).

Another explanation for why a subpopulation of cells exhibits growth defects (and why a subpopulation of individuals with ATD develops liver disease) is that alternate mechanisms exist to clear the ER of A1PiZ. Indeed, it is now established that the autophagic pathway can degrade A1PiZ in mammalian cells (Teckman and Perlmutter, 2000; Kamimoto *et al.*, 2006) and in yeast (Kruse *et al.*, 2006) when the protein is overexpressed. However, we found that autophagy is active in *add66Δ* cells (Supplemental Figure 1), suggesting that variations in this alternate mode of protein quality control do not contribute to the toxic effects of A1PiZ, at least in yeast. In the future, it will be important to measure how variations in the efficiency of autophagy—as well as variations in the relative steady-state levels of A1PiZ—impact A1PiZ clearance and liver disease in individuals afflicted with ATD.

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