THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 41, pp. 34400–34409, October 5, 2012

Spg5 Protein Regulates the Proteasome in Quiescence*

Received for publication, June 10, 2012, and in revised form, August 10, 2012 Published, JBC Papers in Press, August 17, 2012, DOI 10.1074/jbc.M112.390294

John Hanna[1,2], **David Waterman**[1], **Monica Boselli, and Daniel Finley**[3]

From the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

Background: The ubiquitin-proteasome system is the major pathway for selective protein degradation in eukaryotes.
Results: Spg5 is induced in quiescence, binds proteasome components, and is required for proteasome function in quiescence.
Conclusion: Spg5 is a novel proteasome regulator, which promotes proteasome function in quiescence.
Significance: Spg5 provides a link between the proliferative state of the cell and the ubiquitin-proteasome pathway.

The ubiquitin-proteasome system is the major pathway for selective protein degradation in eukaryotes. Despite extensive study of this system, the mechanisms by which proteasome function and cell growth are coordinated remain unclear. Here, we identify Spg5 as a novel component of the ubiquitin-proteasome system. Spg5 binds the regulatory particle of the proteasome and the base subassembly in particular, but it is excluded from mature proteasomes. The *SPG5* gene is strongly induced in the stationary phase of budding yeast, and *spg5*Δ mutants show a progressive loss of viability under these conditions. Accordingly, during logarithmic growth, Spg5 appears largely dispensable for proteasome function, but during stationary phase the proteasomes of *spg5*Δ mutants show both structural and functional defects. This loss of proteasome function is reflected in the accumulation of oxidized proteins preferentially in stationary phase in *spg5*Δ mutants. Thus, Spg5 is a positive regulator of the proteasome that is critical for survival of cells that have ceased to proliferate due to nutrient limitation.

All living cells exist in either a state of active proliferation or a state of quiescence. Although proliferation has garnered greater attention, the majority of cells, including most human cells, are quiescent (1). In quiescence, the cessation of growth is accompanied by profound modifications of metabolic and cellular regulatory pathways. The depletion of nutrients from a fixed culture of *Saccharomyces cerevisiae* is marked by a distinct inflection of the growth curve. This transition, the diauxic shift, represents the transition from fermentation to oxidative metabolism. Over the course of days, yeast cells enter a true period of quiescence, referred to as stationary phase, to reflect the complete cessation of net growth. Under these conditions, protein synthesis and degradation continue, but at markedly reduced rates, and remain finely balanced as amino acid precursors are increasingly depleted from the media and must be supplied through the breakdown of endogenous proteins. Autophagy, which provides for nonspecific breakdown of cytoplasmic proteins, as well as selective degradation of organelles such as mitochondria, is induced by nutrient limitation and becomes critical for cell survival under these conditions (2–5). Cells can survive in this quiescent state for extended periods of time and upon nutrient repletion will in time restore growth-associated regulatory circuits. Thus, budding yeast provides a tractable model for studying quiescence, and indeed there are broad parallels between quiescence in yeast and in higher organisms (1, 6).

The ubiquitin-proteasome system is the major pathway of selective protein degradation in eukaryotes (7). Although considerable progress has been made in understanding how protein biosynthesis is coordinated with growth and quiescence (8), little is known about the relationship between cell growth and protein turnover. The proteasome is a 2.5-MDa multisubunit protease whose proteolytic active sites are sequestered inside the cylindrical central portion of the complex known as the core particle (CP)[4] (9). At either end of the CP is a regulatory particle (RP) whose function is to recognize proteins requiring destruction and assist the CP in their disposal. Proteins are designated for proteasome-mediated degradation primarily by the covalent attachment of ubiquitin. Among the functions of the regulatory particle are recognition and binding of this ubiquitin marker, unfolding of the protein substrate, insertion of the unfolded protein into the CP, and removal of the ubiquitin targeting signal (9).

Given the size and complexity of the proteasome, it is perhaps not surprising that there exists a large group of accessory factors, sometimes referred to as proteasome chaperones, which assist in its assembly and maturation. Some of these factors function primarily in CP biogenesis (Ump1 and Pba1–4), whereas others function in RP assembly (Nas6, Hsm3, Rpn14, and Nas2) (10). A common feature of these chaperones is their ability to interact with nascent proteasome species, while being excluded for the most part from mature, fully assembled proteasomes.

We report here the identification of a quiescence-specific regulator of the proteasome, Spg5. This protein directly binds the regulatory particle of the proteasome and is preferentially expressed in stationary phase. Its molecular function appears

* This work was supported, in whole or in part, by National Institutes of Health Grant R37GM043601 (to D. F.).
[1] Both authors contributed equally to this work.
[2] To whom correspondence may be addressed: Dept. of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-388-4925; Fax: 617-432-1144; E-mail: jwhanna@partners.org.
[3] To whom correspondence may be addressed: Dept. of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-3492; Fax: 617-432-1144; E-mail: Daniel_Finley@hms.harvard.edu.

[4] The abbreviations used are: CP, core particle; RP, regulatory particle; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; suc, succinyl; AMC, 7-amino-4-methylcoumarin.

TABLE 1
Yeast strains
The plasmid pEL36 encodes RPT1-TEV-ProA. YCplac33 encodes an empty vector (*URA3*). pJH149 encodes a centromeric yeast expression vector for *SPG5*.

Strain	Genotype	Ref.
SUB62	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1*	33
SDL133	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpn11::RPN11-TEV-ProA* (*HIS3*)	14
SDL135	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEV-ProA* (*HIS3*)	14
SY36	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt1::* (*HIS3*) *pEL36* (*TRP1*)	14
sJH92	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	34
sJH301	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spg5::KAN*	34
sJH303	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPN11::RPN11-Tev-ProA* (*ClonNAT*)	This study
sJH304	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spg5::KAN RPN11::RPN11-Tev-ProA* (*ClonNAT*)	This study
sJH370	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* [YCplac33]	This study
sJH371	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spg5::KAN* [YCplac33]	This study
sJH372	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spg5::KAN* [pJH149]	This study
sJH386	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPN11::RPN11-Tev-ProA* (*ClonNAT*) [YCplac33]	This study
sJH387	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spg5::KAN RPN11::RPN11-Tev-ProA* (*ClonNAT*) [YCplac33]	This study
sJH388	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spg5::KAN RPN11::RPN11-Tev-ProA* (*ClonNAT*) [pJH149]	This study

largely dispensable under conditions of nutrient repletion. By contrast, after nutrient limitation has led to the cessation of growth, the absence of Spg5 results in progressive loss of viability, likely reflecting the essential function of Spg5 in the ubiquitin-proteasome system in quiescence.

EXPERIMENTAL PROCEDURES

Yeast Strains—Yeast strains are listed in Table 1. Standard techniques were used for strain constructions and transformations (12). Yeast were cultured at 30 °C unless otherwise noted. YPD medium consisted of 1% yeast extract, 2% Bacto-peptone, and 2% dextrose.

Plasmids—The coding sequence of *SPG5* was cloned into the vector pGEX-4T-1 to generate plasmid pJH101, which allows for expression and purification of GST-Spg5 from bacteria. The coding sequence of *SPG5*, along with 500 bp prior to the start codon and 200 bp following the termination codon, was cloned into the yeast centromeric vector YCplac33 (*URA3*) to create pJH149.

Recombinant Proteins—Recombinant GST-Spg5 was expressed from plasmid pJH101 in *Escherichia coli* Rosetta 2 (DE3) cells (Novagen). To prepare partially purified lysates, cells were harvested and resuspended in phosphate-buffered saline (PBS) supplemented with protease inhibitors, lysed by a French press, and clarified by centrifugation at 21,000 × *g* at 4 °C for 25 min. The protease inhibitors were aprotinin solution (Sigma), leupeptin (2 μg/ml), antipain (4 μg/ml), benzamidine (20 μg/ml), chymostatin (2 μg/ml), pepstatin (2 μg/ml), and 4-(2-aminoethyl)benzenesulfonyl fluoride (1 μM).

To prepare purified protein, *GST-SPG5* was expressed in Rosetta 2 (DE3) cells. Cells were harvested and resuspended in PBS supplemented with an additional 750 mM NaCl and protease inhibitors (as above), lysed by a French press, and clarified by centrifugation at 21,000 × *g* at 4 °C for 25 min. Clarified lysate was incubated with glutathione-Sepharose 4B resin (GE Healthcare) at 4 °C for 1.5 h. The resin was then washed with 100 bed volumes of PBS containing 750 mM NaCl, followed by 10 bed volumes of GST elution buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, and 150 mM NaCl. The recombinant protein was then eluted by incubating the resin with elution buffer containing 15 mM reduced glutathione for 20 min at room temperature.

Proteasome Purification—Proteasome purification was carried out by affinity methods as described previously (13, 14). Briefly, the appropriate strains were grown in YPD or minimal media for the indicated amounts of time. For standard purified proteasomes, cells were grown overnight (16–20 h) and were in logarithmic phase growth (doubling time of ~2.0 h) at the time of harvesting. For experiments in stationary phase, cells were cultured for 6 days (YPD) or 10 days (minimal medium). Clarified lysates were prepared, and proteasome complexes were immobilized on IgG resin. Proteasome holoenzyme was prepared by washing with purification buffer (50 mM Tris-HCl [pH 7.5], 1 mM ATP, and 5 mM $MgCl_2$) supplemented with 50 mM NaCl. RP and CP were prepared by incubating immobilized proteasomes for 1 h at room temperature in purification buffer containing 500 mM NaCl and then washing with the same buffer. Lid and base were prepared by incubating immobilized proteasomes for 1 h at room temperature in purification buffer containing 1 M NaCl, and then washing with the same buffer. For Spg5 binding assays, GST-Spg5 lysate or purified protein was added to the immobilized complexes as indicated, incubated at 4 °C for 1 h, washed with 100 bed volumes of purification buffer containing 50 mM NaCl, and eluted via tobacco etch virus protease.

Native Gel Analysis—Native gel analysis was performed by nondenaturing PAGE (4%) as described previously (15). In-gel proteasome activity assays were performed by incubating gels with 100 μM suc-LLVY-AMC at 30 °C for 5–15 min in proteasome purification buffer. LLVY hydrolyzing activity was visualized by ultraviolet transillumination. For Western blotting, gels were transferred to PVDF membranes and processed by standard immunohistochemical techniques.

Quantitative Real Time PCR—Strains were grown in YPD for the indicated length of time. Cells were lysed using acid-washed

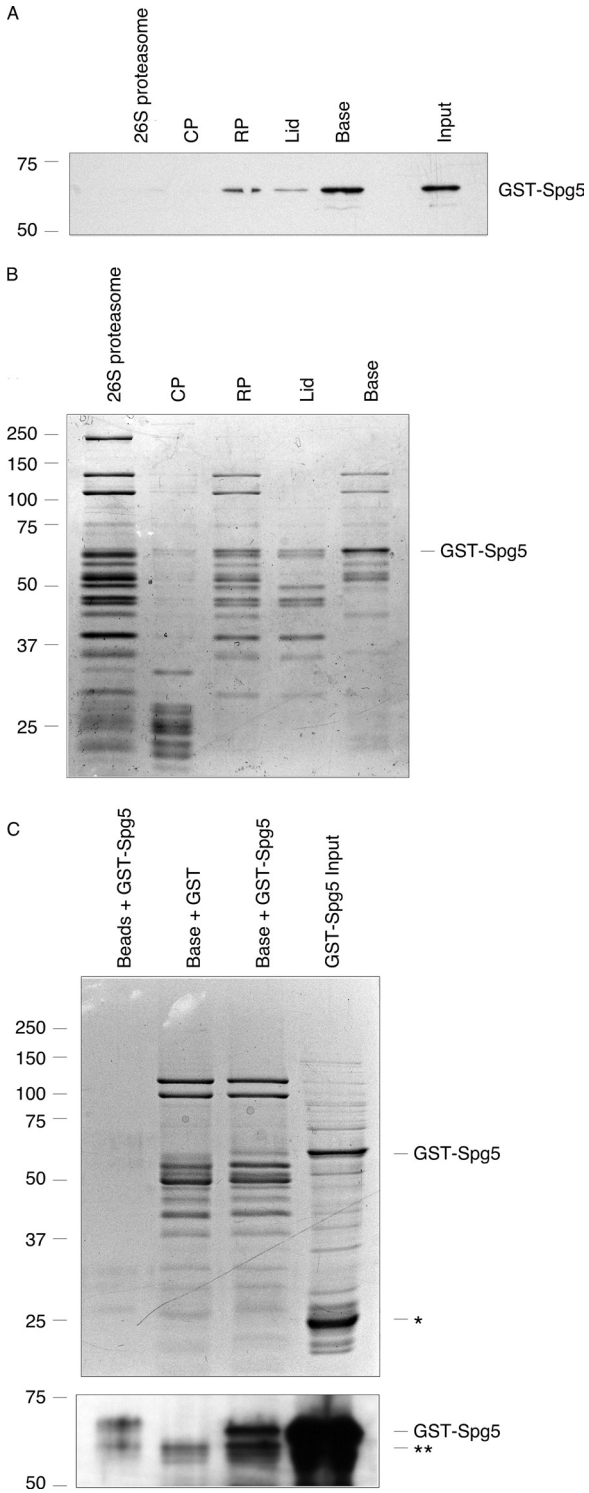

FIGURE 1. **Spg5 binds to the RP and base but not mature proteasomes.** *A,* binding of bacterially expressed GST-Spg5 (pJH101) to purified proteasome or proteasome subcomplexes. Visualization is by anti-GST antibody. *Input* represents ~3% of the total GST-Spg5 present in each binding assay. GST-Spg5 was added in excess of the proteasome species. *B,* electrophoretic profile of the proteasome and proteasome subcomplexes from *A*, as visualized by Coomassie staining. (*26 S*, *RP*, and *lid* were generated from strain SDL133; *base* from SY36; *CP* from SDL135). Note that our CP preparations have a minor contaminant that comigrates with GST-Spg5, which may account for the weak band of this mobility in the *2nd lane*, because this band is not detected by antibody to GST (*A*). *C,* binding of purified GST-Spg5 expressed in bacteria to the base subcomplex, as visualized by Coomassie stain (*upper panel*) or Western blot with ant-GST antibody (*lower panel*). The *asterisk* indicates a fragment of GST-Spg5 that comigrates with *bona fide* GST. The *double asterisk* indicates a nonspecific band.

glass beads, and RNA was isolated by phenol/chloroform extraction. Total RNA was purified using the RNeasy method (Qiagen) according to the manufacturer's instructions, and RNA integrity was verified by evaluation of ribosomal RNA by agarose gel electrophoresis. Two hundred nanograms of RNA were reverse-transcribed and assayed in quadruplicate using TaqMan Gene Expression Assay and analyzed as described previously (16).

Yeast Spot Assays—Cultures were grown in YPD for the indicated amount of time, diluted in sterile water, normalized to an A_{600} of 0.2, and spotted in 3-fold serial dilutions onto the indicated plates, which were grown at the indicated temperatures for 2–5 days.

Detection of Oxidized Proteins—The presence of protein carbonyls was assayed by Western blot analysis according to the manufacturer's instructions (OxyBlot protein oxidation detection kit, Millipore). Briefly, cultures were grown in YPD or synthetic media lacking the appropriate nutrient for the indicated amount of time. Cells were harvested and washed with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, and 1 mM ATP, then resuspended in 10 ml of the same buffer. Resuspended cells were lysed using a French press; the lysate was clarified by centrifugation at 21,000 × *g* for 25 min and filtered through cheesecloth. Fifteen μg of total protein was reacted with 2,4-dinitrophenylhydrazine and separated on a 4–12% BisTris gel (Invitrogen). After transferring to a PVDF membrane, protein carbonyls were visualized by the chemiluminescence of a secondary antibody attached to a primary antibody specific to dinitrophenylhydrazone-derivatized residues (OxyBlot).

RESULTS

Spg5 Binds the Regulatory Particle but Not Proteasome Holoenzyme—Proteome-wide protein-protein interaction studies suggested that Spg5 might interact with several proteasome subunits (17, 18). To test for an interaction between Spg5 and the proteasome, we used an affinity purification method to isolate yeast proteasomes (14), and we challenged them with recombinant GST-tagged Spg5 expressed in bacteria. However, we were unable to detect any binding of Spg5 to the proteasome (Fig. 1 and data not shown). Concerned that endogenous Spg5 might have blocked recognition of GST-Spg5, we repeated the experiment with proteasomes purified from an *spg5*Δ strain, but we still failed to detect any binding of GST-Spg5 (data not shown).

The proteasome comprises two subcomplexes, the 28-subunit core particle (CP) and the 19-subunit RP, the latter itself consisting of two subcomplexes, the lid and the base (9). Reasoning that Spg5, although not binding to fully assembled proteasomes, might recognize a proteasome subcomplex, we next tested binding of Spg5 to the CP and the RP. We found that Spg5 bound to the RP, but not the CP, and within the RP, the major binding determinant for Spg5 proved to be the base (Fig. 1*A*). In fact, Spg5 bound to the base more strongly than to the RP, a binding pattern that has been seen for other base-interacting proteins (14). The purified proteasome complexes were

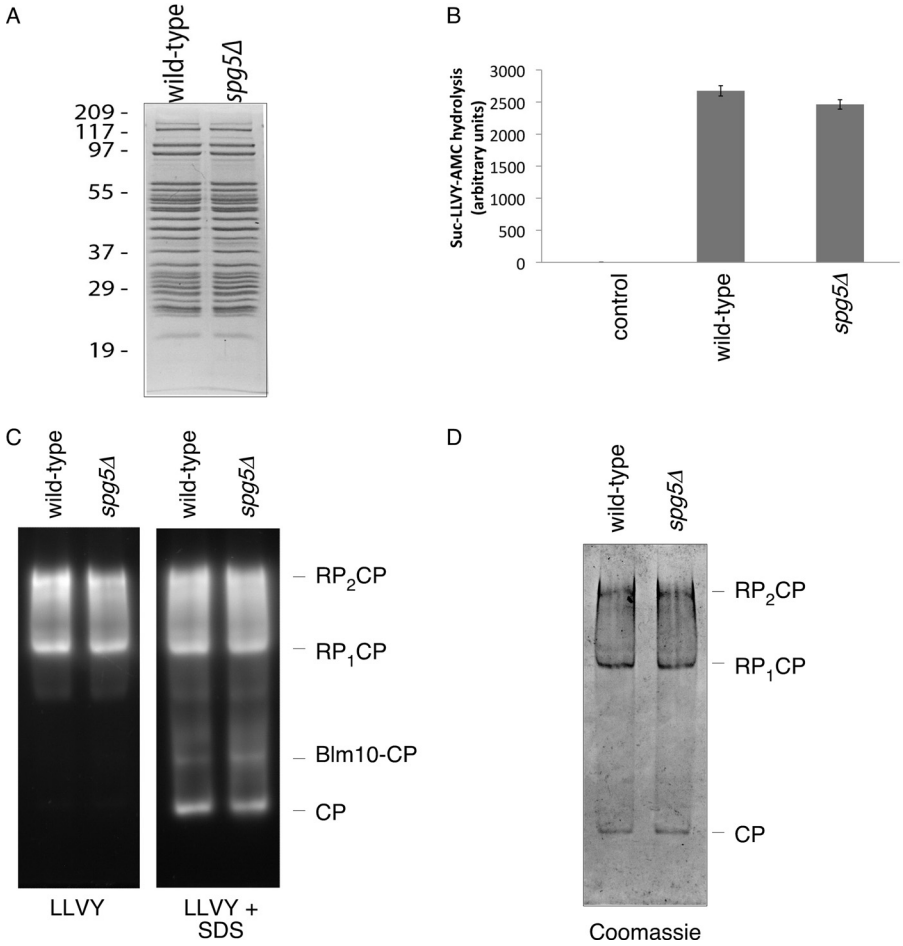

FIGURE 2. ***spg5*Δ proteasomes are similar to those of wild type in logarithmic or late-logarithmic phase cultures.** *A,* electrophoretic profile of proteasomes purified from wild-type (SJH303) and *spg5*Δ strains (SJH304), visualized by Coomassie staining. *B,* suc-LLVY-AMC hydrolyzing activity of proteasomes (5 μg) from *A*. *Control*, buffer only. *C,* native gel electrophoresis coupled with in-gel suc-LLVY-AMC assay highlights proteolytically active proteasome species, as indicated, for the purified proteasomes from *A*. *D,* electrophoretic profile of the proteasome species, separated by native gel electrophoresis as in *C*, and visualized by Coomassie staining.

also visualized by Coomassie staining (Fig. 1*B*), which, in addition to confirming the purity and uniform loading of the proteasome species, highlighted the abundance of bound Spg5. By visual inspection, Spg5 appeared to approach 1:1 stoichiometry with integral subunits of the base. Binding proved reversible, as bound Spg5 could be liberated from the base by high salt concentrations (500 mM NaCl; data not shown).

The binding studies described above utilized partially purified bacterial lysates expressing GST-Spg5. Further purification of GST-Spg5 initially proved difficult under standard conditions. However, when high salt concentrations were included during purification, higher yields of purified GST-Spg5 were obtained (Fig. 1*C*, *4th lane*). Using this purified GST-Spg5, we next tested whether the binding of Spg5 to proteasome subassemblies was in fact direct. We were able to detect significant binding of Spg5 to the base, as demonstrated by Coomassie staining and Western blotting with anti-GST antibodies (Fig. 1*C*). There was no binding of Spg5 to beads alone (Fig. 1*C*), and free GST showed no binding to the base subcomplex (Fig. 1*C* and data not shown).

*Proteasomes from spg5*Δ *Mutants Are Similar to Wild-type under Nutrient-rich Conditions*—We next sought to determine the effects of deleting the *SPG5* gene on proteasomes. To do this, we purified proteasomes from wild-type and *spg5*Δ strains. The electrophoretic profiles of these proteasomes were similar (Fig. 2*A*). Succinyl-LLVY-AMC, a tetrapeptide substrate of the proteasome, allowed us to assay the proteolytic activity of the purified proteasomes; again, we were unable to detect a significant difference (Fig. 2*B*). The purified proteasome, analyzed by nondenaturing gel electrophoresis, appeared to consist of predominantly RP_2CP and RP_1CP forms, with a small amount of CP, which likely dissociated after purification. However, we did not see significant differences in either the enzymatic activity (Fig. 2*C*) or the composition of these species in the *spg5*Δ mutant (Fig. 2*D*).

SPG5 Is Induced in Stationary Phase—SPG5 was first identified in a genome-wide, microarray-based evaluation of stationary phase transcription (19). Given our inability to detect an effect of the *spg5*Δ mutation on proteasome function, we sought to examine the dependence of *SPG5* transcription on the growth state of the culture in greater detail. Using quantitative real time PCR, we found that *SPG5* is progressively induced in stationary phase (Fig. 3*A*), with an ~10-fold induction over logarithmic phase growth after 5 days of culture, and with statistically significant induction seen as early as after 2 days of culture (Fig. 3*A*). In contrast, an unrelated species, the

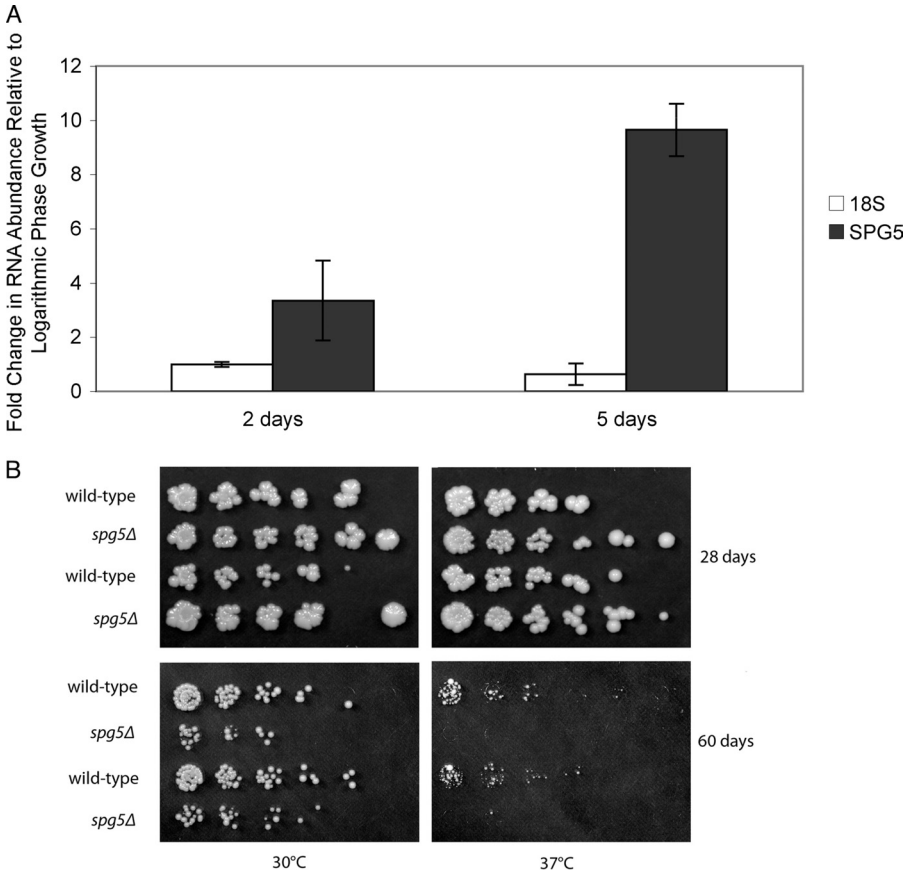

FIGURE 3. **Stationary phase transcriptional induction of *SPG5*.** *A,* quantitative real time PCR analysis of *SPG5* transcription after 2 and 5 days of culture, expressed as fold-induction relative to exponential phase growth. The 18 S ribosomal RNA serves as a control. *Error bars* indicate standard deviations of experiments carried out in triplicate. RNA was generated from strain SUB62. *White bars,* 18 S RNA; *black bars, SPG5* RNA. *B,* growth of wild-type (SJH92) and *spg5*Δ (SJH301) strains on rich medium at the indicated temperatures after growth for 28 or 60 days in rich medium at 30 °C.

18 S ribosomal RNA (rRNA), showed no time-dependent increase in abundance.

To evaluate the physiologic significance of *SPG5* transcriptional induction, we monitored survival of yeast cells after prolonged periods of culture in liquid medium. To assay survival, cells were spotted onto fresh plates, which were then incubated further. After 28 days of culture at 30 °C, we were unable to identify a survival difference relative to wild type (Fig. 3*B*). However, after 60 days of culture at 30 °C, the *spg5*Δ mutant showed a decrease in survival when plated onto fresh media at 30 °C, which was more pronounced at 37 °C (Fig. 3*B*). Previous studies, which involved initially culturing cells at 37 °C, yielded a much quicker loss of viability; under these conditions, *spg5*Δ mutants remained viable after 9 days of growth but showed a 1000-fold loss in viability at day 16 (19). These findings suggest that induction of *SPG5* during stationary phase is part of an adaptive response to quiescence. It is worth noting that many proteasome hypomorphic mutants show decreased viability at 37 °C, presumably due to increased demand on the ubiquitin-proteasome system at that temperature. Furthermore, these results could provide a physiologic basis for the lack of overt functional defects in proteasomes purified from the *spg5*Δ mutant (Fig. 2), because these proteasomes were purified after overnight culture (*i.e.* 16–20 h of growth). These cells show a doubling time of approximately 2 h (data not shown) and thus are in the logarithmic to late logarithmic phase of growth and are unlikely to exhibit the stationary phase induction of *SPG5*.

Spg5 Is Required for Proteasome Structure and Function in Quiescence—We next purified proteasomes from wild-type and *spg5*Δ cells that had been cultured for 6 days and thus were in true quiescence, with a doubling time that was so long as to be difficult to quantify. The profiles of the wild-type and mutant purified proteasomes were strikingly different (Fig. 4*A*). Upon nondenaturing gel electrophoresis followed by an in-gel LLVY activity assay, wild-type proteasomes appeared similar to those seen in conventional preparations (*i.e.* those prepared from logarithmic or late logarithmic cultures), showing predominantly RP_2CP and RP_1CP forms, with a small amount of free CP and Blm10-CP (see Fig. 2*C*). By contrast, proteasomes purified from *spg5*Δ cells showed only RP_2CP, with a near-complete absence of the other active species (Fig. 4*A*). Furthermore, despite having loaded an equivalent amount of purified material (see Fig. 4*C*), the enzymatic activity of RP_2CP was diminished in the *spg5*Δ mutant (Fig. 4*A*). Evaluation of this same gel with Coomassie staining confirmed these findings (Fig. 4*B*). Remarkably, when these same materials were analyzed by denaturing electrophoresis, the overall proteasome profiles were similar between the wild-type and the *spg5*Δ mutant (Fig. 4*C*), implying that the defects seen in the *spg5*Δ mutant are likely related to a failure of assembly or stability of the proteasome, rather than to

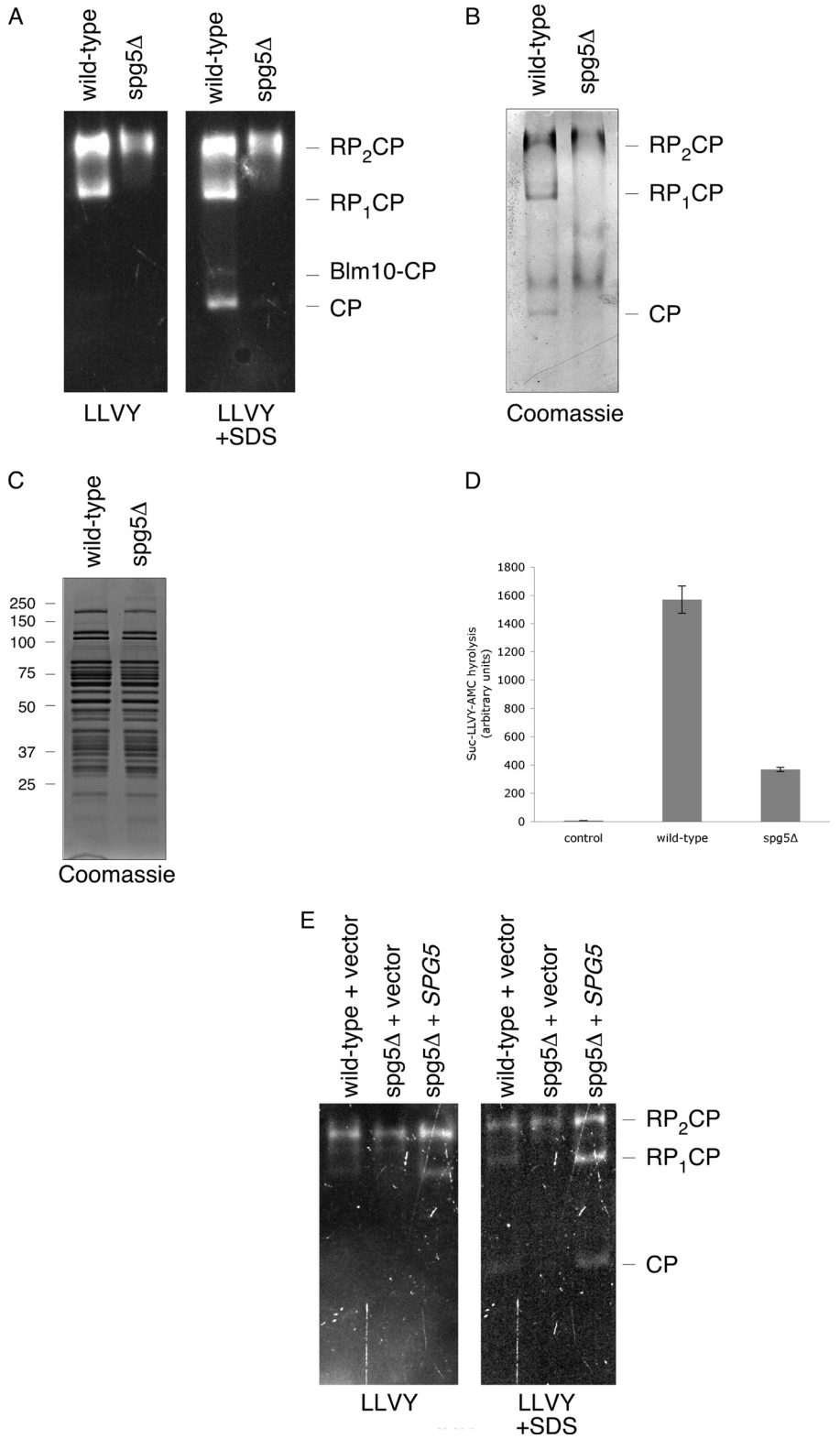

FIGURE 4. ***spg5*Δ proteasomes from stationary phase cells are defective.** *A,* native gel electrophoresis coupled with in-gel suc-LLVY-AMC assay highlights proteolytically active proteasome species, as indicated, for wild-type (SJH303) and *spg5*Δ (SJH304) proteasomes. *B,* electrophoretic profile of the proteasome species, separated by native gel electrophoresis as in *A*, and visualized by Coomassie staining. *C,* denaturing gel electrophoresis of the proteasome species as in *A*, visualized by Coomassie staining. *D,* suc-LLVY-AMC hydrolyzing activity of proteasomes (3.5 μg) from *A*. *Control*, buffer only. *E,* native gel electrophoresis coupled with in-gel suc-LLVY-AMC assay shows that restoration of *SPG5* expression corrects the proteasome defects of the *spg5*Δ mutant. Strains: wild type with empty vector (SJH386), *spg5*Δ with empty vector (SJH387), and *spg5*Δ with *SPG5* CEN plasmid (SJH388).

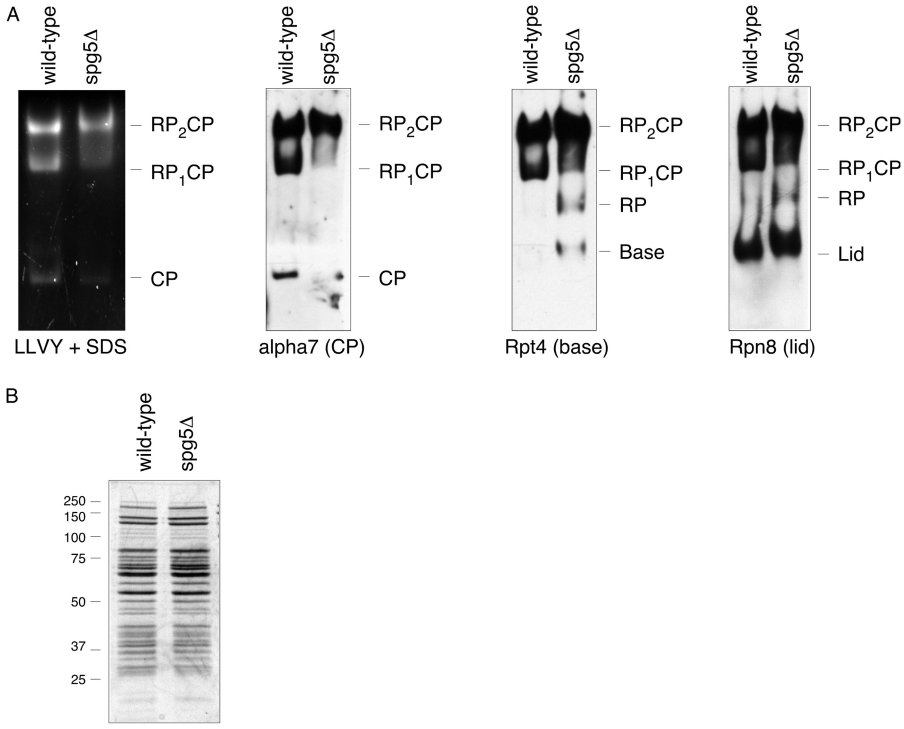

FIGURE 5. ***spg5*Δ proteasomes from stationary phase cells show an altered proteasome subcomplex profile.** *A,* wild-type and *spg5*Δ proteasomes as in Fig. 4, analyzed by native gel electrophoresis and visualized by suc-LLVY-AMC in-gel activity assay or immunoblotting with antibodies to the CP subunit α7, the base subunit Rpt4, and the lid subunit Rpn8, as indicated. *B,* denaturing gel electrophoresis of the proteasome species, visualized by Coomassie staining.

the loss or gain of individual subunits in the *spg5*Δ proteasomes. Finally, we evaluated the enzymatic activity of the purified proteasomes in a solution phase LLVY hydrolysis assay. The proteasomes purified from the *spg5*Δ mutant showed a strong defect in the chymotrypsin-like activity of the CP as compared with those of wild type (Fig. 4*D*). This defect is unlikely to represent direct modulation of the chymotrypsin-like activity of the proteasome; rather this defect likely reflects broader structural and functional defects of the proteasome holoenzyme, as observed in Fig. 4*B*.

To verify that that the proteasome defects seen in the *spg5*Δ mutant were truly due to loss of Spg5, we generated a low copy centromeric (*CEN*) plasmid that expresses *SPG5* under the control of its own promoter, and we transformed proteasome-tagged wild-type and *spg5*Δ cells with either an empty vector or the *SPG5*-expressing plasmid. We then cultured cells for 10 days in minimal media to ensure expression of the plasmids and purified proteasomes. We again visualized the proteasome species by native gel electrophoresis followed by an in-gel LLVY hydrolysis assay. As in Fig. 4*A*, we detected a strong difference in the proteasome profiles between wild-type and *spg5*Δ proteasomes (Fig. 4*E*). Moreover, when we restored *SPG5* expression to *spg5*Δ cells, the proteasome profile was comparable with that seen in wild-type cells (Fig. 4*E*). Specifically, expression of *SPG5* restored the presence of RP_1CP and free CP bands. These results indicate that the proteasome defects seen in *spg5*Δ mutants specifically reflect loss of the Spg5 protein.

In addition to the major enzymatically active species we could detect (RP_2CP, RP_1CP, Blm10-CP, and CP), native gel analysis followed by Coomassie staining showed a small number of faint bands that might represent additional proteasome species. We therefore pursued immunoblotting to further analyze these species. Evaluation with an antibody to a core particle subunit (α7) showed a profile comparable with that of the LLVY-based in-gel activity assay (Fig. 5*A*). In contrast, when we examined Rpt4, a subunit of the base of the regulatory particle, we detected two new species predominantly present in the *spg5*Δ proteasomes, with near absence in those of wild type (Fig. 5*A*). These two species, by their composition and electrophoretic properties (27), appear to represent free RP and free base (Fig. 5*A*). Examination of a lid subunit (Rpn8) also identified the free RP, as expected, which again predominated in the mutant proteasomes (Fig. 5*A*). A second band was also detected, running just below that of the free base, which appears to represent the free lid (Fig. 5*A*). In contrast to the RP and the base, the free lid was present in both the wild-type and *spg5*Δ proteasome samples, and its increased abundance may be related to preferential purification given that the proteasome affinity tag is present on the lid subunit Rpn11.

Spg5 Is Distinct from Known RP Chaperones—Our data identify Spg5 as novel proteasome-interacting protein with some features reminiscent of several of the recently described RP chaperones: Nas6, Hsm3, and Rpn14. Like those proteins, Spg5 binds to the RP and specifically the base but appears to be excluded from mature proteasomes. Transcriptional regulation of the genes encoding known RP chaperones has not previously been evaluated. Examination of Nas6, Hsm3, Rpn14, as well as a fourth RP chaperone, Nas2, showed no transcriptional induction in stationary phase, in contrast to *SPG5* (Fig. 6). Thus, Spg5 is distinct from the known RP chaperones in its pattern of gene expression.

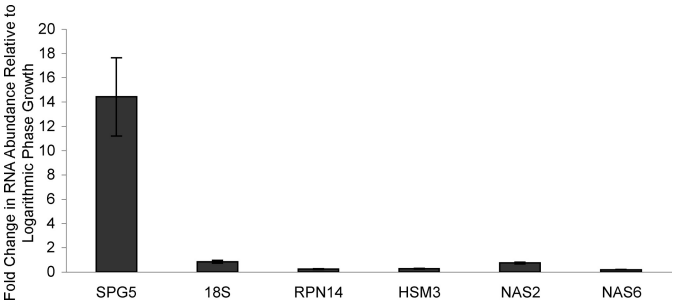

FIGURE 6. **Regulation of *SPG5* expression is distinct from that of known RP chaperones.** Quantitative real time PCR analysis of transcription of the indicated genes after 5 days of culture, expressed as fold-induction relative to exponential phase growth. The 18 S ribosomal RNA serves as an endogenous control. *Error bars* indicate standard deviations of experiments carried out in triplicate. RNA was generated from strain SUB62.

Accumulation of Oxidized Proteins in Stationary Phase in spg5Δ Mutants—To generate metabolic energy, yeast cells growing under conditions of nutrient limitation rely heavily on mitochondrial respiration, a process that generates reactive oxygen species. Oxidized proteins are subject to varying degrees of misfolding and dysfunction and are generally good substrates of the proteasome (20). We therefore investigated the status of oxidized proteins in *spg5*Δ mutants using a detection system that is specific for carbonyl modifications, which are generated by oxidation.

When cells were evaluated under conditions of logarithmic growth, the abundance of oxidized species in the *spg5*Δ mutant relative to wild type was comparable (Fig. 7*A*). However, in stationary phase, there was a marked increase in total oxidized proteins in the *spg5*Δ mutant relative to wild type. A Pgk1 loading control was similar under all conditions (Fig. 7*A*).

To show that the increase in oxidized proteins was due to loss of Spg5 function, we again utilized the low copy *CEN* plasmid that expresses *SPG5* under the control of its own promoter, and we transformed *spg5*Δ cells with either an empty vector or the *SPG5*-expressing plasmid. When cells were evaluated in stationary phase, we again observed an increase in oxidized proteins in the *spg5*Δ mutant transformed with an empty vector, and this decrease was reversed in the *spg5*Δ mutant complemented the *SPG5* plasmid (Fig. 7*B*). Thus, *spg5*Δ cells show an accumulation of oxidized proteins during quiescence, further indicating that *spg5*Δ proteasomes are functionally defective in stationary phase.

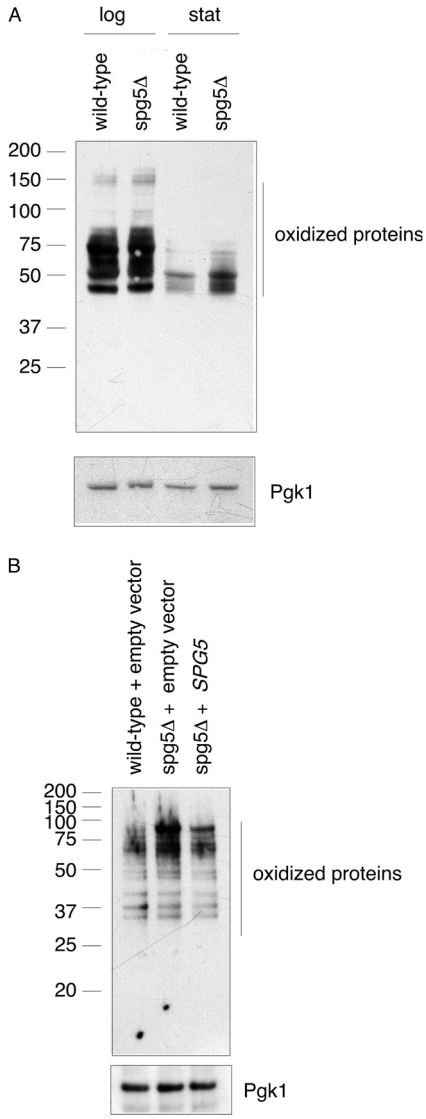

FIGURE 7. **Accumulation of oxidized proteins in stationary phase in *spg5*Δ mutants.** *A,* total cellular abundance of oxidized proteins in wild-type (SJH92) and *spg5*Δ (SJH301) strains in logarithmic and stationary phase growth, as indicated. *Pgk1*, loading control. Comparable results were obtained in four independent experiments. *B,* total cellular abundance of oxidized protein in stationary phase. Strains: wild-type with empty vector (sJH370), *spg5*Δ with empty vector (sJH371), and *spg5*Δ complemented with a plasmid-borne *SPG5* gene (sJH372). *Pgk1*, loading control.

DISCUSSION

Spg5 Is a Novel Component of the Ubiquitin-Proteasome System—The results presented here identify both a novel proteasome-interacting protein, Spg5, and an unexpected link between quiescence and the proteasome. Yeast cells grow logarithmically under nutrient-rich conditions, but as nutrients are depleted, the growth rate slows and eventually ceases, culminating in quiescence. Spg5 is progressively induced in stationary phase, and, reflecting the physiologic relevance of this induction, *spg5*Δ mutants show increasing loss of viability as stationary phase progresses. Spg5 binds the proteasome regulatory particle and its subcomplex, the base, but appears to be excluded from mature, fully assembled proteasomes. Proteasomes from *spg5*Δ mutants show defects in proteasome structure and function, which are supported by *in vivo* phenotypes that appear to reflect deficient proteasome function.

There are deep parallels between the states of quiescence experienced by yeast cells and those of higher organisms. In yeast, quiescence is characterized by a complete absence of growth, increasing reliance on oxidative metabolism, induction of autophagy, the selective repression of ribosomal genes, a strong decrease in protein synthesis, and the specific induction of quiescence-related genes (1, 6). Regulation of the proteasome may represent another important feature of quiescence. Although data are lacking from mammalian cells, studies in budding yeast as well as fission yeast, an organism considerably diverged from budding yeast, suggest a critical role as the proteasome is strictly required for cells to maintain viability in quiescence (21–23). Further supporting this idea, other studies

highlight tight control over subcellular proteasome localization in quiescence (24), with nuclear proteasomes migrating to discrete cytoplasmic foci. The physiologic basis for the requirement of the proteasome in quiescence remains unclear, but one might speculate that it relates to the function of the proteasome in protein quality control or possibly a role in generating amino acids to serve as biosynthetic precursors under conditions of nutrient limitation (25). Indeed, there is evidence that pathways of protein synthesis and degradation might regulate one another under conditions of nutrient limitation (26).

An important question concerns the evolutionary conservation of Spg5. To date, a clear mammalian ortholog of Spg5 has not been identified by sequence-based algorithms, nor have we identified any functional motifs within *SPG5* which might inform its cellular role.[5] Nevertheless, a functional ortholog may exist as the challenges posed by quiescence in yeast are likely to be similar in higher organisms. Indeed, there is precedent for such functional conservation within the ubiquitin-proteasome system. The mammalian form of securin, an anaphase-promoting complex substrate that regulates mitotic spindle function, performs the same function as the budding yeast protein Esp1, but it shows no significant sequence homology (11).

Role of Spg5 in Proteasome Function—The precise role that Spg5 plays in proteasome function remains to be elucidated. At a minimum, the *in vitro* and *in vivo* data support a positive role for Spg5 in the ubiquitin-proteasome system. Loss of Spg5 results in defects in both the structure and function of the proteasome *in vitro*. At least two models could account for such findings. In the first model, Spg5 might be dispensable for initial biosynthetic proteasome assembly but necessary for the stability and integrity of the mature proteasome holoenzyme. That Spg5 fails to bind fully assembled proteasomes argues against but does not fully exclude this model. In a second model, Spg5 promotes the assembly of newly synthesized proteasomes, assuming that new proteasomes are in fact generated in deep stationary phase. Indeed, the binding pattern of Spg5 to the base and RP, but not the intact 26 S proteasome, is similar to that of most known RP chaperones. That notwithstanding, Spg5 is clearly distinguished from the known RP chaperones by virtue of its unique transcriptional regulation.

Despite the differences in transcriptional regulation between *SPG5* and the genes encoding the four known RP chaperones, there are suggestions of a close relationship at a mechanistic level. Like the RP chaperones, Spg5 binds the base but does not do so when the base is incorporated into the proteasome holoenzyme. In the case of the RP chaperones, this specificity is thought to be related to their binding proximally to the RP-CP interface (27–30), which puts them in competition with CP for RP binding. The CP-proximal binding sites of the chaperones are the C-terminal domains (known as C-domains) of the proteasomal ATPases (Rpt1–Rpt6) (27, 31). Whether Spg5 binds similarly to the C-domains is currently under study. A second suggestion of similarity between Spg5 and the chaperones is that an accumulation of proteasome subassemblies can be observed in both types of mutant. As chaperones function to assist in the generation of mature protein complex, this phenotype is consistent with a chaperone function for Spg5. However, further work is required to define the nature of this defect in detail. For example, it is unclear whether *de novo* proteasome formation is affected in the mutant over prolonged periods of quiescence. Finally, although *spg5*Δ mutant proteasomes are markedly unstable, it is unclear to what extent the observed defects represent *in vivo* or *in vitro* instability.

Cellular Regulation of Spg5—Transcriptional induction of *SPG5* is best understood as an adaptive response to the cellular conditions associated with quiescence. Yet why a specific adaptation of the ubiquitin-proteasome system has evolved in quiescence remains unclear. Central to this uncertainty is the fact that the proteasome and protein quality control have not been well studied under these conditions. In one model, the stresses associated with stationary phase growth might create an increased or unique set of substrates, such as oxidized proteins, requiring modification of the proteasome. In another model, the stresses associated with stationary phase might compromise the proteasome itself.

The transcriptional machinery that orchestrates *SPG5* induction remains to be discovered. Equally important will be an exploration of how the need for *SPG5* induction is recognized by the cell. A number of cellular alterations associated with quiescence, most notably carbon depletion, nitrogen depletion, increasing oxidation, and increasing acidity of the culture medium, are potential candidate inputs. Available data indicate that induction of *SPG5* occurs in response to multiple nutrient deficiencies, including glucose, nitrogen, and phosphorus deprivation, with perhaps a more robust response in the context of glucose starvation (32). The full delineation of the stimuli resulting in *SPG5* induction will be key to understanding how cells coordinate proteasome function in quiescent states.

The present results provide strong evidence that the proteasome is subject to growth phase-dependent regulation, similarly to protein biosynthetic pathways. Indeed, the concept of protein homeostasis, which involves not only the elimination of aberrant or dysfunctional proteins but an overall balance between protein synthesis and degradation, implies just this sort of proteasomal regulation. In recent years, our understanding of the complexity of the pathways integrating growth and protein synthesis has expanded tremendously. The results presented here provide a framework to help elucidate what may be an important parallel complex pathway integrating cell growth and proteasome function.

Acknowledgments—We thank Suzanne Elsasser, Brad Pearse, Soyeon Park, and the members of the Finley laboratory for helpful suggestions. We also thank Margaret Werner-Washburne for helpful discussion, and Suzanne Elsasser and Marion Schmidt for critical reading of the manuscript.

[5] J. Hanna, D. Waterman, M. Boselli, and D. Finley, unpublished data.

REFERENCES

1. Gray, J. V., Petsko, G. A., Johnston, G. C., Ringe, D., Singer, R. A., and Werner-Washburne, M. (2004) "Sleeping beauty." Quiescence in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **68,** 187–206
2. Devenish, R. J., and Klionsky, D. J. (2012) Autophagy: Mechanism and

physiological relevance "brewed" from yeast studies. *Front. Biosci.* **4,** 1354–1363
3. Takeshige, K., Baba, M., Tsuboi, S., Noda, T., and Ohsumi, Y. (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* **119,** 301–311
4. Teichert, U., Mechler, B., Müller, H., and Wolf, D. H. (1989) Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. *J. Biol. Chem.* **264,** 16037–16045
5. Gresham, D., Boer, V. M., Caudy, A., Ziv, N., Brandt, N. J., Storey, J. D., and Botstein, D. (2011) System-level analysis of genes and functions affecting survival during nutrient starvation in *Saccharomyces cerevisiae*. *Genetics* **187,** 299–317
6. Kaeberlein, M. (2010) Lessons on longevity from budding yeast. *Nature* **464,** 513–519
7. Grabbe, C., Husnjak, K., and Dikic, I. (2011) The spatial and temporal organization of ubiquitin networks. *Nat. Rev. Mol. Cell Biol.* **12,** 295–307
8. Loewith, R., and Hall, M. N. (2011) Target of rapamycin (TOR) in nutrient signaling and growth control. *Genetics* **189,** 1177–1201
9. Finley, D. (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* **78,** 477–513
10. Tomko, R. J., Jr., and Hochstrasser, M. (2011) Order of the proteasomal ATPases and eukaryotic proteasome assembly. *Cell Biochem. Biophys.* **60,** 13–20
11. Zou, H., McGarry, T. J., Bernal, T., and Kirschner, M. W. (1999) Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* **285,** 418–422
12. Burke, D. J., Amberg, D. C., and Strathern, J. N. (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, 5th Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Hanna, J., Hathaway, N. A., Tone, Y., Crosas, B., Elsasser, S., Kirkpatrick, D. S., Leggett, D. S., Gygi, S. P., King, R. W., and Finley, D. (2006) Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell* **127,** 99–111
14. Leggett, D. S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R. T., Walz, T., Ploegh, H., and Finley, D. (2002) Multiple associated proteins regulate proteasome structure and function. *Mol. Cell* **10,** 495–507
15. Elsasser, S., Schmidt, M., and Finley, D. (2005) Characterization of the proteasome using native gel electrophoresis. *Methods Enzymol.* **398,** 353–363
16. Schmittgen, T. D., and Livak, K. J. (2008) Analyzing real time PCR data by the comparative *CT* method. *Nat. Protoc.* **3,** 1101–1108
17. Krogan, N. J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., Li, J., Pu, S., Datta, N., Tikuisis, A. P., Punna, T., Peregrín-Alvarez, J. M., Shales, M., Zhang, X., Davey, M., Robinson, M. D., Paccanaro, A., Bray, J. E., Sheung, A., Beattie, B., Richards, D. P., Canadien, V., Lalev, A., Mena, F., Wong, P., Starostine, A., Canete, M. M., Vlasblom, J., Wu, S., Orsi, C., Collins, S. R., Chandran, S., Haw, R., Rilstone, J. J., Gandi, K., Thompson, N. J., Musso, G., St Onge, P., Ghanny, S., Lam, M. H., Butland, G., Altaf-Ul, A. M., Kanaya, S., Shilatifard, A., O'Shea, E., Weissman, J. S., Ingles, C. J., Hughes, T. R., Parkinson, J., Gerstein, M., Wodak, S. J., Emili, A., and Greenblatt, J. F. (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* **440,** 637–643
18. Collins, S. R., Miller, K. M., Maas, N. L., Roguev, A., Fillingham, J., Chu, C. S., Schuldiner, M., Gebbia, M., Recht, J., Shales, M., Ding, H., Xu, H., Han, J., Ingvarsdottir, K., Cheng, B., Andrews, B., Boone, C., Berger, S. L., Hieter, P., Zhang, Z., Brown, G. W., Ingles, C. J., Emili, A., Allis, C. D., Toczyski, D. P., Weissman, J. S., Greenblatt, J. F., and Krogan, N. J. (2007) Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map. *Nature* **446,** 806–810
19. Martinez, M. J., Roy, S., Archuletta, A. B., Wentzell, P. D., Anna-Arriola, S. S., Rodriguez, A. L., Aragon A. D., Quiñones, G. A., Allen, C., and Werner-Washburne, M. (2004) Genomic analysis of stationary-phase and exit in *Saccharomyces cerevisiae*. Gene expression and identification of novel essential genes. *Mol. Biol. Cell* **15,** 5295–5305
20. Medicherla, B., and Goldberg, A. L. (2008) Heat shock and oxygen radicals stimulate ubiquitin-dependent degradation mainly of newly synthesized proteins. *J. Cell Biol.* **182,** 663–673
21. Takeda, K., Yoshida, T., Kikuchi, S., Nagao, K., Kokubu, A., Pluskal, T., Villar-Briones, A., Nakamura, T., and Yanagida, M. (2010) Synergistic roles of the proteasome and autophagy for mitochondrial maintenance and chronological life span in fission yeast. *Proc. Natl. Acad. Sci.* **107,** 3540–3545
22. Bajorek, M., Finley, D., and Glickman M. H. (2003) Proteasome disassembly and down-regulation are correlated with viability during stationary phase. *Curr. Biol.* **13,** 1140–1144
23. Fujimuro, M., Takada, H., Saeki, Y., Toh-e, A., Tanaka, K., and Yokosawa, H. (1998) Growth-dependent change of the 26 S proteasome in budding yeast. *Biochem. Biophys. Res. Commun.* **251,** 818–823
24. Laporte, D., Salin, B., Daignan-Fornier, B., and Sagot, I. (2008) Reversible cytoplasmic localization of the proteasome in quiescent yeast cells. *J. Cell Biol.* **181,** 737–745
25. Vabulas, R. M., and Hartl, F. U. (2005) Protein synthesis upon acute nutrient restriction relies on proteasome function. *Science* **310,** 1960–1963
26. Lopez, A. D., Tar, K., Krügel, U., Dange, T., Ros, I. G., and Schmidt, M. (2011) Proteasomal degradation of Sfp1 contributes to the repression of ribosome biogenesis during starvation and is mediated by the proteasome activator Blm10. *Mol. Biol. Cell* **22,** 528–540
27. Roelofs, J., Park, S., Haas, W., Tian, G., McAllister, F. E., Huo, Y., Lee, B. H., Zhang, F., Shi, Y., Gygi, S. P., and Finley, D. (2009) Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature* **459,** 861–865
28. Lee, S. Y., De la Mota-Peynado, A., and Roelofs, J. (2011) Loss of Rpt5 protein interactions with the core particle and Nas2 protein causes the formation of faulty proteasomes that are inhibited by Ecm29 protein. *J. Biol. Chem.* **286,** 36641–36651
29. Barrault, M. B., Richet, N., Godard, C., Murciano, B., Le Tallec, B., Rousseau, E., Legrand, P., Charbonnier, J. B., Le Du, M. H., Guérois, R., Ochsenbein, F., and Peyroche, A. (2012) Dual functions of the Hsm3 protein in chaperoning and scaffolding regulatory particle subunits during the proteasome assembly. *Proc. Natl. Acad. Sci. U.S.A.* **109,** E1001–E1010
30. Park, S., Roelofs, J., Kim, W., Robert, J., Schmidt, M., Gygi, S. P., and Finley, D. (2009) Hexameric assembly of the proteasomal ATPases is templated through their C termini. *Nature* **459,** 866–870
31. Saeki, Y., Toh-E, A., Kudo, T., Kawamura, H., and Tanaka, K. (2009) Multiple proteasome-interacting proteins assist the assembly of the yeast 19 S regulatory particle. *Cell* **137,** 900–913
32. Klosinska, M. M., Crutchfield, C. A., Bradley, P. H., Rabinowitz, J. D., and Broach, J. R. (2011) Yeast cells can access distinct quiescent states. *Genes Dev.* **25,** 336–349
33. Finley, D., Ozkaynak, E., and Varshavsky, A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell* **48,** 1035–1046
34. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C. A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14,** 115–132