Rpn7 Is Required for the Structural Integrity of the 26 S Proteasome of Saccharomyces cerevisiae*

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Rpn7 is one of the lid subunits of the 26 S proteasome regulatory particle. The RPN7 gene is known to be essential, but its function remains to be elucidated. To explore the function of Rpn7, we isolated and characterized temperature-sensitive rpn7 mutants. All of the rpn7 mutants obtained accumulated poly-ubiquitinated proteins when grown at the restrictive temperature. The N-end rule substrate (Ub-Arg-β-galactosidase), the UFD pathway substrate (Ub-Pro-β-galactosidase), and cell cycle regulators (Pds1 and Clb2) were found to be stabilized in experiments using one of the rpn7 mutants termed rpn7-3 at the restrictive temperature, indicating its defect in the ubiquitin-proteasome pathway. Subsequent analysis of the structure of the 26 S proteasome in rpn7-3 cells suggested that the defect was in the assembly of the 26 S holoenzyme. The most striking characteristic of the proteasome of the rpn7-3 mutant was that a lid subcomplex affinity-purified from the rpn7-3 cells grown at the restrictive temperature contained only 5 of the 8 lid components, a phenomenon that has not been reported in the previously isolated lid mutants. From these results, we concluded that Rpn7 is required for the integrity of the 26 S complex by establishing a correct lid structure.

The 26 S proteasome is a protein complex with a molecular mass of ~2000 kDa and is highly conserved among eukaryotic organisms. It is essential not only for eliminating damaged or misfolded proteins but also for degrading short-lived regulatory proteins involved in cell cycle regulation, DNA repair, signal transduction, apoptosis, and metabolic regulation (1, 2). The 26 S proteasome is a 2600 kDa protein complex with two kinds of subcomplexes, a 20 S core particle (CP) and a 19 S regulatory particle (RP). The CP has a cylinder-like structure composed of a stack of two α rings and two β rings in the order of αβαβα, each of which consists of seven related subunits. The active sites are sequestered in the cavity of the CP (7). Crystallographic study revealed that the gate of the CP allows the entry of substrate proteins only upon binding with the RP (8). The RP contains at least 17 subunits and is responsible for the ubiquitin-dependent proteolytic pathway. The 26 S proteasome has the RP attached on either end of the CP (RP1CP) or at both ends of the CP (RP2CP). The RP can be further divided into two subcomplexes, the base and the lid (9). The base consists of six AAA-ATPase subunits (Rpt1–Rpt6) and two non-ATPase subunits (Rpn1 and Rpn2), whereas the lid is made of eight non-ATPase subunits (Rpn3, Rpn5–Rpn9, Rpn11, and Rpn12) (9, 10). One of the non-ATPase subunit, Rpn10, has been suggested to exist in the interface between the base and the lid (9). The base ATPases are required for unfolding a substrate protein before delivering it into the CP (11).

To understand further the biochemical processes conducted by the 26 S RP, it is necessary to uncover how individual components of the RP are functioning. At present, some components have been studied in detail. Of the 19 S components, Rpn10 and Rpt5 have been suggested to be responsible for the recognition of substrate through its poly-ubiquitin chain (5, 12–15). Multiple deubiquitinating enzymes are thought to be involved in ubiquitin recycling, and Rpn11 metalloisopeptidase, one of the lid components, is shown to play an essential role in cleaving off the Ub chain from poly-ubiquitinated substrate proteins (16). Although functional characterization of the RP has progressed considerably, little is known about the mechanism of its assembly. Interactions among lid (17) and base components (18) were studied by using yeast two-hybrid analysis. Obviously more information concerning individual subunits of the lid is necessary for a more detailed understanding of the function of the lid as well as the 26 S proteasome in relation to various phenomena.

In this study, we focused on Rpn7, whose function has only been poorly understood, by isolating and characterizing...
Mutations were introduced into the was cloned between the SalI and NotI site of the YIp vector pRS306 GCAATTATGTAACTTTC, and genomic DNA of W303-1A as template GACGTAGACGTGGAAGAGAAAAG-3′-CCTTGTC-

1501) amplified by PCR using a pair of primers, 5′/H11001

1 nucleotide. In brief, the method described previously (21). The adenine residue of ATG corre-

mutants were screened by the integration-disruption/replacement rpn7

subcloning various genes and their fragments are listed in Table II.

Rpn7-6XHis recombinant protein. Yeast transformations were per-

E. coli thi-1 relA1 strain or 1/H9262

20°C. After induction, 1 ml of each cell suspension was harves-

Temperature-sensitive Mutants—Isolation of Temperature-sensitive Mutants—

Yeast strains used in this study table Genotype Source

rpn7-3PG-GBD TRP1

See Table I. Cells were cultured in the synthetic complete (SC) medium, rich medium (YPD) (19), or the synthetic medium with

10 mM, respectively, and a 1 lysate was prepared by adding a-factor to a final concentration of 10 μg/ml to the BARI strain or 1 μl/ml to the bar1 strain. Escherichia coli strain DH5α (supE44 ΔlacU169 [Δ80lacZ ΔM15] hisD1 recA1 endA1 gyr96 thi-1 relA1) was used for construction and propagation of plasmids. E. coli Rosetta (DE3) strain (Novagen) was used for production of the Rpn7-6XHis recombinant protein. Yeast transformations were performed as described previously (20). Plasmids used for cloning and subcloning various genes and their fragments are listed in Table II.

Isolation of Temperature-sensitive Mutants—Temperature-sensitive rpn7 mutants were screened by the integration-disruption/replacement method described previously (21). The adenine residue of ATG corre-

responding to the putative translation initiation codon was defined as the +1 nucleotide. In brief, the RPn7 ORF and the 3′-translated region ( +1 to +1501) amplified by PCR using a pair of primers, 5′-CTTGTG-

GACGTAGACGTGGAAGAGAAAAG-3′ and 5′-CTTGTGGCGCTT-GCAAATATGTAACCTTC, and genomic DNA of W303-1A as template was cloned between the SalI and NotI site of the YIp vector pRS306 (22). A BamHI site was introduced at +132–137 in the RPn7 ORF. Mutations were introduced into the RPn7 coding region ( +1 to +1501) by random PCR mutagenesis (23) using this plasmid as a template with the same pair of primers shown above. The resulting PCR products were inserted into the SalI-NotI site of pRS306 and used to transform E. coli strain DH5α. Colonies were harvested en masse, and the plasmid DNA recovered from them was stored as the mutation library of RPN7.

The library DNA was digested at the BamHI site and was introduced into W303-1B cells to replace the RPN7 gene with the mutated rpn7 gene in the library. About 1,200 colonies were screened on the basis of a growth defect at 37°C, and six colonies were obtained. These candidates were crossed with W303-1A and were subjected to tetrad analysis. Thus, the linkage between temperature-sensitive growth and the plasmid marker URA3 was established, indicating that the temper-

ature sensitivity was because of the rpn7 mutations incorporated.

Cytological Methods—Microscopic photo was taken using an Olympus BX52 with an UPlanApo 100X/1.35 objective and a CCD camera (Hamamatsu photonics) attached to CSU21 (Yokogawa). Images were analyzed by the IPLab (Scanalytics) software. DNA was stained with 20 μg/ml Hoechst 33342 (Sigma) for 10 min at room temperature.

DNA Manipulation—DNA engineering and agaroose gel electrophore-

sis were performed as described elsewhere (24). Nucleotide sequences were determined by the dyeode dye terminating method (25) using automated DNA sequencer ABI 310 (Applied Biosystems).

Anti-Rpn7 Rabbit Antibody—The ORF of RPn7 without the stop codon was cloned into the BamHI-Xhol site of pET21a (Novagen), and the resulting plasmid, pEK106, was introduced into the E. coli Rosetta (DE3) strain (Novagen). Production of Rpn7-6XHis was induced by adding 0.2 mg isopropylthio-β-galactoside to the culture. Cells were harvested from 500 ml of culture after 10 h of induction at 25°C, and the Rpn7 protein was purified using nickel-nitrirotioc acid-agaroose beads (Qiagen) under a denaturing condition. 2 mg of the purified protein was used to raise antibodies in rabbit. The serum was first purified using the HiTrap Protein G HP (Amersham Biosciences) and further by Rpn7-coupled HiTrap N-hydroxysuccinimide-activated HP column (Amersham Biosciences).

Protein Extraction, SDS-PAGE, and Western Blotting—Yeast total proteins were extracted as follows. Cells were grown to late log phase, harvested by centrifugation, and broken with inner diameter, 0.5-mm, glass beads in cold Buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol) supplemented with Protease Complete Inhibitor (Roche Applied Science) using a multibeads shocker (MB501, YASUI KIKAI Corp.). ATP and MgCl2 were added at the final concentrations of 2 and 10 mM, respectively, and a 1× ATP regeneration system was added to

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Methods—Yeast strains used in this work are listed in Table I. Cells were cultured in the synthetic complete (SC) medium, rich medium (YPD) (19), or the synthetic medium with 2% raffinose and without uracil (SR-U). Synchronous cultures were prepared by adding a-factor to a final concentration of 10 μg/ml to the BARI strain or 1 μl/ml to the bar1 strain. Escherichia coli strain DH5α (supE44 ΔlacU169 [Δ80lacZ ΔM15] hisD1 recA1 endA1 gyr96 thi-1 relA1) was used for construction and propagation of plasmids. E. coli Rosetta (DE3) strain (Novagen) was used for production of the Rpn7-6XHis recombinant protein. Yeast transformations were performed as described previously (20). Plasmids used for cloning and subcloning various genes and their fragments are listed in Table II.

Isolation of Temperature-sensitive Mutants—Temperature-sensitive rpn7 mutants were screened by the integration-disruption/replacement method described previously (21). The adenine residue of ATG corre-
the extract for nonadentating PAGE, gel filtration chromatography, and affinity purification. Protein samples for Western blotting were prepared by the alkali method as described previously (26). SDS-PAGE, Coomassie Brilliant Blue staining, and Western blotting were performed according to the standard protocol. Protein concentration was determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Antibodies used for Western blotting were anti-Cdc2 (Santa Cruz Biotechnology), anti-c-Myc Ab-1 (Oncogene), anti-HA 16B12 (Babco), anti-Rpn9 (27), anti-Rpn12 (28), anti-Rpn5 (29), anti-20 S (29), anti-Rpt5 (Affiniti Research Products Ltd.), anti-polyubiquitin, FK2 (30), and anti-Rpn7 antibodies.

β-Galactosidase Assay—Cells carrying plasmid encoding Ub-Ala-β-galactosidase, Ub-Pro-β-galactosidase, or Ub-Arg-β-galactosidase were grown in SC-U medium to mid-logarithmic phase at 37 °C supplemented with 2% galactose and incubated for 5 h at 37 °C. Cell extracts were prepared as described above and subjected to β-galactosidase assay using o-nitrophenyl-β-galactoside as substrate as described previously (31).

Nonadentating PAGE—Protein samples were resolved by nonadentating PAGE as described previously (9). Total proteins were extracted as described above, and 5 × loading dye (75% glycerol with xylene cyanol) was added to the protein samples prior to loading onto the gels. Electrophoresis was run at 15 mA for 4 h at 4 °C. The gels were overlaid with 0.1 ml Suc-LLVY-MCA in the running buffer with or without 0.05% SDS for 30 min at room temperature, and proteasome bands were visualized upon exposure to 360 nm of UV light and photographed using Polaroid T667 films.

Gel Filtration—Yeast total lysates were prepared as described above and filtrated through an inner diameter 0.2-mm syringe filter (Millipore). Samples (4 mg of protein) were applied onto a Superose 6 column (1.0 × 30 cm) (Amersham Biosciences) and eluted by running Buffer A supplemented with 1 mM ATP and 5 mM MgCl₂ at 0.25 ml/min using the fast protein liquid chromatography system (Amersham Biosciences). Fractions (500 μl) were collected and subjected to measurement of peptidease activity using Suc-LLVY-MCA as a substrate by a fluorophotometer (F-2000 Hitachi) (excitation at 380 nm; emission at 460 nm). Proteasomal components contained in the fractions were detected by Western blotting using the indicated antibodies.

Affinity Purification of Proteasomes—Yeast strains with one of the following tagged genes, PRE1-3xFLAG, RPN1-3xFLAG, and RPN11-3xFLAG, were constructed as described previously (32). Yeast total lysate was prepared from the indicated strain as described above, and 10 μl of anti-FLAG antibody immobilized M2 beads washed with Buffer A were added to 200 μl of the lysate (20 mg of protein) and incubated for 1 h at 4 °C. The beads were washed extensively with Buffer A, and the purified proteasomes were eluted with 30 μl of 0.1 M glycine-HCl buffer, pH 3.0, and immediately neutralized with 1 μl of 1.5 M Tris-HCl buffer, pH 8.8. For the affinity purification of the wild type lid, the 26 S proteasome was washed with buffer containing a high concentration of salt as described previously (33) with the following modifications. The 26 S proteasome was first purified from a Rpn11-3xFL 26 S proteasome strain, and the beads were treated with 1 μl NaCl containing Buffer A for 20 min at room temperature before eluting with 0.2 mg/ml 3xFLAG peptides (Sigma). Coomassie Brilliant Blue-stained gels were photographed using an image analyzer (LAS-3000, Fujiﬁlm), and protein bands were quantiﬁed with the Image Gauge (Fujiﬁlm) software.

Mass Spectrometry—Protein bands were excised from the SDS-polyacrylamide gel, destained, and digested in the gel with 15 μl of 10 μg/ml modiﬁed trypsin (Promega) in 20 mM ammonium bicarbonate. The resulting peptides were desalted using ZipTip-C₁₈ (Millipore) and mixed with 5 mM α-cyano-4-hydroxycinnamic acid (Sigma) in a 0.3% triﬂuoroacetic acid, 50% acetonitrile mixture. Peptide mass fingerprints of proteins were obtained by a matrix-assisted laser desorption ionization/time of ﬂight (MALDI/TOF) mass spectrometry (MS), Voyager DE-PRO (Applied Biosystems). Data base search was performed using the MS-Fit program (prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

RESULTS

Isolation of Temperature-sensitive rpn7 Mutants—We isolated six temperature-sensitive rpn7 mutants as described under “Experimental Procedures,” which were named rpn7-1–rpn7-6 (Fig. 1A). All the mutations were linked to the RPN7 locus. To conﬁrm that their temperature-sensitive growth was because of the mutations in RPN7, a single copy plasmid carrying the wild type allele was introduced into each of the rpn7 mutants, and the ability of a representative transformant to grow at 37 °C was tested. The single copy RPN7 gene complemented the temperature sensitivity of the rpn7 mutants, whereas the vector alone did not restore their growth (Fig. 1B).

Rpn7 Protein Levels in rpn7 Mutants—Next we examined whether the temperature-sensitive growth was caused by a reduction in the protein level of Rpn7 in the mutant cells. To test this, total proteins were extracted by the alkaline extraction method from wild type and rpn7-1–rpn7-6 mutant cells cultured either at the permissive (25 °C) or restrictive (37 °C) temperature and subjected to Western blotting using an anti-Rpn7 antibody (Fig. 1C). A slight reduction of the Rpn7 protein level was indeed observed in mutants like rpn7-1 even at the permissive temperature. However, notably rpn7-3 retained a protein level as high as wild type cells both at the permissive and restrictive temperatures. None of the mutants showed the reduction of the amounts of Rpn7 at the restrictive temperature compared with the permissive temperature.

Poly-ubiquitinated Proteins Are Accumulated in the rpn7 Mutants at the Restrictive Temperature—The 26 S proteasome is a key mechanism for the degradation of poly-ubiquitinated proteins in the ubiquitin-proteasome system. Therefore, poly-ubiquitinated proteins accumulate in mutants defective in the proteasome function in yeast (13, 34–38), Trypanosoma (39), and Arabidopsis (40). To investigate the effect of the rpn7 mutation upon the proteasomal function, we performed Western blotting against total cell extracts using FK2 antibody (30). For this purpose, overnight cultures of wild type and rpn7-1–rpn7-6 mutants in YPD were diluted into fresh YPD. Each culture was incubated at 25 °C to early log phase, divided into two portions, and grown further at either 25 or 37 °C for 6 h. Total cell lysates were then prepared and subjected to Western blotting (Fig. 1D). Accumulation of poly-ubiquitinated proteins was observed in samples prepared from rpn7 mutants grown at 37 °C, whereas it was not the case in wild type extracts as well as in rpn7 mutant cells grown at 25 °C. This suggests that proteolysis by the ubiquitin-26 S proteasome system is not properly functioning in the rpn7 mutants under the restrictive conditions.

To investigate further the deficiency of rpn7 mutants in protein degradation, we performed two experiments by using the rpn7-3 strain. We chose this allele for detailed analysis because the abundance of Rpn7-3 was comparable with that of Rpn7 (Fig. 1C). First we tested the degradation of model substrates, Ub-Ala-β-galactosidase, Ub-Arg-β-galactosidase, and Ub-Pro-β-galactosidase (41). Ub-β-galactosidase is a stable protein, whereas Arg-β-galactosidase and Ub-Pro-β-galactosidase are degraded by the N-end rule pathway and the UDP pathway, respectively. In wild type cells, it was reported although the deubiquitinated Ala-β-galactosidase was stable, Arg-β-galactosidase and Ub-Pro-β-galactosidase had relatively short half-lives of less than 5 min (42). Wild type and rpn7-3 cells were transformed with plasmids carrying one of the Ub-X-lacZ (X = Ala, Arg, or Pro) constructs, and representatives of each transformation were used to measure the steady state levels of β-galactosidase activity (Fig. 1E). Both Arg-β-galactosidase and Ub-Pro-β-galactosidase were significantly more stable in rpn7-3 cells than in wild type cells. Although neither of the substrates was stabilized to the level shown by Ala-β-galactosidase, stabilization of Ub-Pro-β-galactosidase was more prominent than that of Arg-β-galactosidase.

Next, we examined the ability of rpn7-3 cells to degrade substrates of the ubiquitin-proteasome pathway during cell cycle progression. Proteasomal lid mutants reported so far all show a cell cycle delay or arrest under restrictive conditions. Most of them are arrested in G₂/M phase (35–38), whereas
The rpn7 Temperature-sensitive Mutants of Budding Yeast

Fig. 1. rpn7-1–rpn7-6 mutants show temperature-sensitive growth at 37 °C. A, spot test. Cell cultures of wild type (WT) (W303-1B) and rpn7-1–rpn7-6 strains (YEK2, YEK4, YEK6, YEK9, YEK10, and YEK12, respectively) were grown to -E left (YEK50) strain. Plasmid possessing Ub-A-lacZ,Ub-P-lacZ, or Ub-R-lacZ, or Ub-P-lacZ was introduced into W303-1B and YEK6 and the plasmid born Ub-X-β-galactosidase (X = Ala (A), Arg (R), or Pro (P)) was induced. Steady state levels of β-galactosidase (β-gal) activity were assayed as described under “Experimental Procedures.”

rpn12-1 (nin1-1-1) was reported to show a G1/S phase arrest in addition to a G2/M phase arrest (34). To establish the function of RPN7 in cell cycle, we examined the cell cycle phenotype of the rpn7-3 mutant under restrictive conditions. Early log phase cells of a wild type strain and rpn7-3 mutant were incubated at either 25 or 35 °C for 6 h and observed under the microscope. When grown at 35 °C, large budded cells with one nucleus containing a short spindle became frequent in rpn7-3 cells, which was not the case in the wild type cells, implying that rpn7-3 cells are defective in the G1/M progression (Fig. 2A). To examine the degradation of regulators of G1/M progression such as the securin Pds1 or the B-type cyclin Clb2, we performed the following experiment. Logarithmically growing wild type and rpn7-3 cells containing either Myc-tagged Pds1 or Myc-tagged Clb2 were shifted to 37 °C and incubated for 2 h and then arrested in the G1 phase by incubating for 3 h with α-factor at 37 °C. Cells were released from the G1 phase arrest by washing out the α-factor with prewarmed YPD and cultured further at 37 °C. Samples were withdrawn every 20 min after the release and subjected to Western blotting using anti-Myc antibody to check the protein levels of Pds1 and Clb2 (Fig. 2C). At 40 min after the release, α-factor was added to the culture to prevent cells from progressing to the next round of the cell cycle. Indeed, in rpn7-3 cells both Pds1 and Clb2 protein levels remained high for a longer period than in wild type cells in which both proteins were rapidly degraded.

rpn7 Mutants Have a Defect in Formation of the 26 S Proteasome—The observed deficiency in the degradation of proteasomal substrate proteins may be due to an imperfect formation of the 26 S proteasome. To distinguish these two possibilities, we first examined whether the proteasomes are formed normally in rpn7-3 mutant 26 S proteasome. To distinguish these two possibilities, we first examined whether the proteasomes are formed normally in rpn7-3 mutant 26 S proteasome. In contrast, it is still possible that the deficiency in degrading substrate proteins is because of an enzymatic dysfunction of the mutant 26 S proteasome. To distinguish these two possibilities, we first examined whether the proteasomes are formed normally in rpn7-3 mutant cells or not. After resolving the total cell extract by nondenaturing PAGE, proteasome bands were visualized by peptidase activity against a fluorogenic substrate Suc-LLVY-MCA in the presence or absence of 0.05% SDS (Fig. 3). Although RP2CP and RP1CP forms of the 26 S proteasome were clearly detected in wild type extracts irrespective of the growing temperature, the amounts of the two forms of the 26 S proteasome were reduced in mutants especially in the extract prepared from cells grown at 37 °C (Fig. 3, top two panels). Instead, faster migrating bands appeared in mutant extracts (Fig. 3, bottom panel), suggesting...
FIG. 2. The rpn7-3 mutant is deficient in degrading Clb2 and Pds1. A, Tub1-GFP containing wild type (WT) (YK1268) and rpn7-3 (YK171) cells were cultured for 6 h at 35 °C, and DNA was stained with Hoechst 33342. B, degradation of Pds1 and Clb2 was delayed in rpn7-3 cells. The Clb2-myc strains (SAY873 and YEK86) and the Pds1-myc strains (TMY201 and YEK88) were arrested in late G1 phase by treatment with a-factor at 37 °C for 3 h and then released from a-factor arrest at 37 °C. To prevent the cells from entering the next cell cycle, a-factor was added after 40 min of release. Cells were collected at 20-min intervals and extracts prepared from these cells were subjected to Western blotting by using anti-Myc antibody. Cdc28 detected by an anti-Cdc2 antibody was used for loading control.

The incompleteness of the 26 S proteasome is obvious in the rpn7-3 mutant grown at 37 °C. Most mutants. An RP Subcomplex Exists in the rpn7-3 Strain—The incomplete assembly of the 26 S proteasome observed in rpn7 mutants raised a question as to which step of the assembling process the 26 S proteasome is affected by the rpn7 mutations. To investigate this, we performed gel filtration chromatography of wild type and rpn7-3 cell extracts, and the peptidase activity of each fraction was measured using a fluorogenic substrate (Fig. 4A). The profile of peptidase activity derived from the rpn7-3 mutant extract was shifted to lower molecular weight fractions compared with that of the wild type extract. Fractions were also subjected to SDS-PAGE followed by Western blotting analysis, using antibodies against lid components Rpn3, Rpn7, Rpn9, a base component Rpt5, and the 20 S proteasome (CP) (Fig. 4B). Comparison between the blotting patterns of wild type and rpn7-3 fractions along with the marker protein showed that a complete 26 S proteasome (eluting around fraction 20) is almost absent in the rpn7-3 extract. Instead, the base and CP were found to be co-eluted (around fraction 22), which matches the shift of the peptidase activity peak. Among the other two lid components, the 32-kDa Rpn12 seems to exist as a free subunit (around fraction 34), whereas Rpn9 seemed to form some kind of a complex because it was detected in a higher molecular weight fraction (around fraction 28) than that expected from its molecular mass of 46 kDa. Neither free Rpn9 nor free Rpn7-3 was detected in the rpn7-3 extract prepared by breaking cells with glass beads.

Composition of Lid Components in Wild Type and the rpn7-3 Mutant—To analyze the proteasome assembly in the rpn7-3 mutant at the level of individual components, we constructed derivatives of the rpn7-3 mutant whose PRE1, RPN1, or RPN11 had been tagged with 3xFLAG at the 3′-end of each ORF in-frame. Pre1, Rpn1, and Rpn11 are CP, base, and lid components respectively. Extracts were prepared from these strains incubated at either 25 or 37 °C, and proteasomes were affinity-purified from the extracts using anti-FLAG antibody immobilized beads. Subsequently, the affinity-purified proteasomes were eluted by treating the beads with low pH buffer (0.1 M glycine-HCl, pH 3.0) followed by immediate neutralization with 1.5 M Tris-HCl, pH 8.8. They were then resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 5B). Fig. 5A shows the subunit composition of the 26 S proteasome thus separated by SDS-PAGE. In the wild type extract, the subunits of the lid, the base, and the CP were detected in a similar extent irrespective of the tagged subunit that had been used for purification of the 26 S proteasome. In contrast, affinity-purified proteasomes from Pre1–3xFLAG-tagged rpn7-3
cell extracts did not contain the lid subunits, and a smaller amount of the base subunits and proteasomes obtained with Rpn1–3xFLAG did not contain the lid subunits and fewer amounts of the CP. When immunoprecipitates from the Rpn11–3xFLAG-tagged rpn7-3 cells were analyzed by SDS-PAGE, only some of the lid components were detected. Subsequent Western blot analysis of the immunoprecipitates using antibodies against Rpn3, Rpn7, Rpn12, Rpt5, and the 20 S proteasome showed that the precipitated complex lacked Rpn3, Rpn7, and Rpn12 (Fig. 5C). We named this subcomplex lid rpn7-3. These results indicate that the rpn7-3 strain is defective in the assembly of the 26 S proteasome and that some lid subunits form a lid rpn7-3 mutant cells incubated at 37 °C.

From the migration pattern, the components consisting the lid rpn7-3 were likely to be Rpn5, Rpn6, Rpn8, Rpn9 and Rpn11, and it was further confirmed by MALDI/TOF-MS analysis (Fig. 5D). The gel bands were quantified using an image analyzer, and each band was found to exist in an equal molar amount to each other (data not shown). A subcomplex like the lid rpn7-3 consisting of only part of the lid components has not been reported before. It became apparent that lid rpn7-3 lacked Rpn3, Rpn7, and Rpn12 and that neither the base nor the 20 S proteasome was co-precipitated with the lid rpn7-3. Affinity purification by using an RPN12–3xFLAG-tagged strain revealed that Rpn3, Rpn7-3, and Rpn12 did not form any complex on their own (Fig. 5E). Altogether these results indicate that the dysfunction of Rpn7 leads to a weaker association of the base and the lid and that Rpn7 is necessary for the incorporation of Rpn3 and Rpn12 into the lid subunit or anchoring them to the lid.

**Rpn7-3 Is Defective in the Interaction with Rpn3**—According to the two-hybrid analysis reported previously (17), Rpn7 binds only Rpn3 among the lid components, and the full-length of Rpn7 was necessary for binding Rpn3 whose PCI domain was necessary and sufficient for the interaction. Mutations introduced in Rpn7 may lead to a defect in the binding between Rpn7 and Rpn3 and therefore to an imperfect assembly of the 26 S proteasome. To test this hypothesis, we first determined the mutation points with amino acid substitution of all six mutants (Fig. 6A). All mutants have multiple amino acid substitutions in addition to an S240P mutation that had been introduced in the template plasmid used for the mutagenesis PCR. We have confirmed that this S240P mutation alone does not cause temperature-sensitive growth (data not shown). We then analyzed the interaction between Rpn7-3 and Rpn3 by yeast two-hybrid analysis. Rpn7-3 has five amino acid substitutions (Fig. 6A), three of which are outside the PCI domain and the rest are in the PCI domain. Rpn7-3 was unable to bind Rpn3 (Fig. 6B). To examine whether the mutations in the PCI domain were responsible for this inability of two-hybrid interaction, we constructed rpn7-3GRP that had reverted the mutations inside the PCI domain and rpn7-3PG that had reverted the mutations outside the PCI domain. In contrast to the rpn7-3 strain, either rpn7-3GRP strain or rpn7-3PG strain did not show temperature-sensitive growth. The GBD-Rpn7-3GRP fusion and the GBD-Rpn7-3PG fusion were expressed in the PJ69-4A cells containing the GAD-Rpn3 fusion. Although it was confirmed that GBD-Rpn7-3GRP and GBD-Rpn7-3PG fusion proteins were produced in a comparable level of wild type Rpn7, they both failed to interact with Rpn3 even at 25 °C (Fig. 6C).

**DISCUSSION**

RPN7 encodes one of the components of the lid of the 26 S proteasome and is a counterpart of the human proteasome subunit S10/p44 (43). However, its biological function is not known except it is essential. To uncover the function of Rpn7, we isolated and characterized temperature-sensitive rpn7 mutants. All of the six mutants obtained were found to accumulate poly-ubiquitinated proteins when cultured under the restrictive conditions (Fig. 1D), and N-end rule and UFD substrates were stabilized in rpn7-3 cells (Fig. 1E). The UFD pathway...
substrate was more efficiently stabilized in \( rpn7-3 \) cells than the N-end rule substrate, which was also the case in the \( \Delta rpn10 \) strain (17).

In addition to the proteasome mutants as reported previously (27, 37, 38), the \( rpn-7-3 \) mutants displayed a defect in cell cycle progression. The terminal phenotype of the \( rpn7-3 \) cells incubated at the restrictive temperature was dumbbell-shaped cells with one nucleus at the isthmus (Fig. 2A), and they showed an increased ratio of 2C against 1C cells (data not shown) supporting the observed G2/M delay phenotype. In accordance with this phenotype of the \( rpn7-3 \) mutant, two cell cycle regulator proteins, Pds1 and Clb2, were shown to be stabilized in \( rpn7-3 \) cells under the restrictive conditions (Fig. 2B). Altogether, the \( rpn7-3 \) mutant, and probably other \( rpn7 \) mutants too, has a defect in proteolysis by way of the ubiquitin-proteasome pathway. The leakiness of this mutant, not showing a complete G2/M arrest at the examined time point, is probably due to the activity of the complete 26 S proteasome complex, remaining in small amounts even after 6 h of incubation at the restrictive temperature.

Because all six \( rpn7 \) mutants showed a defect in the assembly/maintenance of the 26 S proteasome under the restrictive conditions, we performed a detailed analysis in this dysfunc-

![Image 1](https://via.placeholder.com/150)

**Fig. 5. Subunit composition of affinity-purified proteasomes.** A, the affinity-purified 26 S proteasome. Rpn11–3xFLAG-tagged proteasome of the wild type strain (YYS40) was purified by using anti-FLAG antibody-coated M2 beads and resolved on a 12.5% SDS-polyacrylamide gel followed by Coomassie Brilliant Blue staining. Each band was cut out from the gel and subjected to MALDI/TOF-MS for identification. Base and lid components are indicated on the left and right side of the panel, respectively. B, SDS-PAGE analysis of affinity-purified proteasomes. Wild type (WT) (YYS77, -39, and -40) and \( rpn7-3 \) (YEK27, -28, and -29) cells containing one of the indicated 3xFLAG-tagged components were cultured at 37 °C for 6 h in YPD, and immunoprecipitation was performed by using anti-FLAG antibody-immobilized beads. Samples were resolved and stained as in A. Solid arrowheads indicate the tagged components, and open arrowheads indicate the bands identified by MS analysis. C, immunoblot of affinity-purified proteasomes. Part of the samples used in B were resolved on a 12.5% polyacrylamide gel and subjected to Western blotting using the antibodies indicated on the left side of the panel. D, comparison of the wild type lid and lid\(^{rpn7-3}\) (left panel). The wild type lid was purified as described under “Experimental Procedures.” Protein bands of lid\(^{rpn7-3}\) indicated as bands 1–4 were cut out from the gel, digested with trypsin, and subjected to MALDI/TOF-MS analysis. Bands 1–4 were identified as indicated in the table. The numbers of peptides that found a match in the data base and the ratio of coverage against the full-length sequence are shown. E, affinity purification of proteasomes containing Rpn12–3xFLAG. Rpn12–3xFLAG containing wild type (YEK69) and \( rpn7-3 \) (YEK70) cells were grown for 6 h at either 25 or 37 °C, and proteasomes were purified as described in B.

The \( Rpn7 \) protein possesses a purely \( \alpha \)-helical PCI domain of about 90 amino acid residues at its C terminus, which is shared by components of the COP9 signalosome and eukaryotic initiation factor 3 (44). The COP9 signalosome is proved to be involved in intracellular signaling pathways (45) and is found in Arabidopsis, mammals, Drosophila, and Schizosaccharomyces pombe but not in Saccharomyces cerevisiae (reviewed in Ref. 46). Components of the lid of the 26 S proteasome and the COP9 signalosome, both of which are octameric complexes, have a one-to-one sequence correspondence, which is the reason why these complexes are thought to share common ancestry (9).

The \( Rpn7 \) protein possesses a purely \( \alpha \)-helical PCI domain of about 90 amino acid residues at its C terminus, which is shared by components of the COP9 signalosome and eukaryotic initiation factor 3 and is thought to play a role in the binding between components within each complex (17, 44, 47). Mutational analysis of Csn1/Fus1, a component of the COP9 signalosome in Arabidopsis thaliana and a protein with 20% amino acid sequence homology with \( Rpn7 \), demonstrated that the PCI domain of Csn1 plays an essential role for the integrity of the complex (48). We found that the mutant protein \( Rpn7-3 \) did not interact with Rpn3 in a two-hybrid assay (Fig. 6B), and \( Rpn7-3\)GRP and \( Rpn7-3\)PG proteins with mutations in the N-terminal half and the PCI domain, respectively, both lost the interaction with Rpn3 indicating that the region outside the PCI domain of \( Rpn7 \) is also important in this interaction. This is in accordance with a previous report (17) showing that the full
length of Rpn7 is necessary for the interaction with Rpn3. Most interesting, neither rpn7-3GRF nor rpn7-3PG mutant displayed temperature-sensitive growth (data not shown), although they did not bind the Rpn3 protein even at the permissive temperature. One possible explanation for this discrepancy is that protein interaction in the assembling process of the lid, where multiple subunits are involved, is different from that in the artificial two-hybrid system. The assembly of the lid may be able to proceed under conditions where the interaction between Rpn7-3 and Rpn3 is so weak that it could not be detected by the two-hybrid assay. It is also possible that the loss of interaction between Rpn7-3 and Rpn3 is so weak that it could not be detected by the two-hybrid assay. It is also possible that the temperature sensitivity of interaction between Rpn7-3 and Rpn3 may not be the only reason leading to the temperature sensitivity of rpn7-3 cells.

Affinity purification of proteasomes using Rpn11-3xFLAG, Rpn1-3xFLAG, or Pre1-3xFLAG-tagged rpn7-3 strains grown under restrictive conditions revealed the presence of the lid consisting of five of the eight lid components, Rpn5, Rpn6, Rpn8, Rpn9, and Rpn11 (Fig. 5C). In previously reported mutants of lid components, no such a lid subcomplex containing several subunits has been reported. Santamaria et al. (37) purified proteasomes by affinity chromatography using the Pre1-Tev-ProA fusion from the rpn6Δ-GAL1-RPN6 cells depleted with Rpn6. When proteasomes thus purified were resolved by SDS-PAGE, they found that most of the lid components were lost, which is consistent with the results presented in this study. However, it remains unknown whether a subcomplex of the lid such as that found in this study was produced in rpn6-depleted cells. Unexpectedly, a less amount of the base was co-purified with the CP (Pre1-3xFLAG) in rpn7-3 cells grown at the restrictive temperature, leading us to speculate that a correct spatial organization of Rpn7 in the lid is essential not only for a stable lid-base association but also for a stable base-CP association. It is an interesting question how the mutant lid component affects the base-CP interaction.

The composition of the lid found in the rpn7-3 extract is in accordance with the results of the subunit interaction map proposed by Fu et al. (17), where Rpn5, Rpn6, Rpn8, Rpn9, and Rpn11 are included in one group and Rpn3, Rpn7, and Rpn12 are in the other group. In the present study we could not detect a complex consisting of Rpn3, Rpn12, and Rpn7 (Fig. 5E). It seems controversial that although Rpn7-3 does exist in the total protein extracted from rpn7-3 cells (Fig. 1C), it is detected only slightly in the input lane of gel filtration along with Rpn3 (Fig. 4B, right panel, input lane). This is probably because of the difference in the methods of protein extraction. Because both Rpn3 and Rpn7-3 were extracted with the alkali method in which cells were boiled with an SDS-containing buffer after a brief alkali treatment (Fig. 1C, data of Rpn3 not shown), while breaking the cells with glass beads could only poorly do so, they may exist mainly in the insoluble fraction in the mutant cell. From the results obtained in Fig. 1C, we think it more likely that the formation of lid consistent with the results presented in this study was produced in rpn6-depleted cells. Unexpectedly, a less amount of the base was co-purified with the CP (Pre1-3xFLAG) in rpn7-3 cells grown at the restrictive temperature, leading us to speculate that a correct spatial organization of Rpn7 in the lid is essential not only for a stable lid-base association but also for a stable base-CP association. It is an interesting question how the mutant lid component affects the base-CP interaction.

In summary, we showed that Rpn7 is essential for the integrity of the 26 S proteasome, and we found for the first time a partially assembled lid in the rpn7-3 cells. Most important, it was shown in vitro that Rpn7 is necessary for the incorporation/anchoring of Rpn3 and Rpn12 to the lid (Fig. 6D). These results obtained by using the temperature-sensitive rpn7 mutant pro-
vide us with new knowledge regarding the spatial organization of the lid subcomplex of the 26 S proteasome, which until now could only be analyzed by two-hybrid analysis.

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