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The Assembly Pathway of the 19S Regulatory Particle of the Yeast 26S Proteasome[D]

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The 26S proteasome consists of the 20S proteasome (core particle) and the 19S regulatory particle made of the base and lid substructures, and it is mainly localized in the nucleus in yeast. To examine how and where this huge enzyme complex is assembled, we performed biochemical and microscopic characterization of proteasomes produced in two lid mutants, *rpn5-1* and *rpn7-3*, and a base mutant Δ*N rpn2*, of the yeast *Saccharomyces cerevisiae*. We found that, although lid formation was abolished in *rpn5-1* mutant cells at the restrictive temperature, an apparently intact base was produced and localized in the nucleus. In contrast, in Δ*N rpn2* cells, a free lid was formed and localized in the nucleus even at the restrictive temperature. These results indicate that the modules of the 26S proteasome, namely, the core particle, base, and lid, can be formed and imported into the nucleus independently of each other. Based on these observations, we propose a model for the assembly process of the yeast 26S proteasome.

INTRODUCTION

The ubiquitin–proteasome system (UPS) is essential for the degradation of short-lived regulatory proteins that are involved in cell cycle regulation, DNA repair, signal transduction, apoptosis, and metabolic regulation as well as for the elimination of damaged or misfolded proteins (Hershko and Ciechanover, 1998; Schwartz and Ciechanover, 1999). The 26S proteasome acts at the final step of this pathway by degrading poly-ubiquitinated substrates, thereby ensuring the irreversibility of this pathway.

The 26S proteasome is a huge multicatalytic complex consisting of >30 different components that are divided into those of the 20S core particle (CP) and the 19S regulatory particle (RP). The RP can be biochemically further divided into two substructures, the base and the lid. The base consists of six AAA-ATPase subunits, *r*egulatory *p*article *t*riple-A ATPase (Rpt)1p–Rpt6p, and three non-ATPase subunits, *r*egulatory *p*article *n*on-ATPase (Rpn)1p, Rpn2p, and Rpn13p, whereas the lid is made of nine non-ATPases, Rpn3p, Rpn5p–Rpn9p, Rpn11p, Rpn12p, and Sem1p (Rpn15p) (Glickman *et al*., 1998b; Leggett *et al*., 2002; Funakoshi *et al*., 2004; Sone *et al*., 2004). Rpn10p, a non-ATPase subunit that binds polyubiquitin (Ub) chains (van Nocker *et al*., 1996; Saeki *et al*., 2002; Elsasser *et al*., 2004), has been suggested to exist in the interface between the base and the lid (Fu *et al*., 2001).

Interestingly, the composition of the lid is surprisingly similar to that of the COP9/signalosome and the eIF3, from which Glickman *et al*. (1998a) proposed that these protein complexes have diverged from a common ancestor. Most of the components of these complexes share a common proteasome/COP9/Initiation factor (PCI) domain, thought to serve as a scaffold for protein–protein interaction (Hofmann and Bucher, 1998), or a catalytic MPN+ domain (Maytal-Kivity *et al*., 2002). Rpn11p (Verma *et al*., 2002; Yao and Cohen, 2002; Guterman and Glickman, 2004) and CSN5 (Cope *et al*., 2002), which are MPN+ domain proteins of the lid and the COP9, respectively, are the only known components to possess enzymatic activity in these complexes.

It was shown by immunoelectron microscopy (Wilkinson *et al*., 1998) and by fluorescence microscopy of green fluorescent protein (GFP)-tagged proteasomes (Enenkel *et al*., 1999) that in yeast, 26S proteasomes were mainly localized in the nucleus, especially on the inner nuclear membrane. Based on genetic data, the involvement of importin α/β in

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Abbreviations used: CBB, Coomassie brilliant blue; CP, core particle; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; MCA, methycoumaryl-7-amide; NLS, nuclear localization signal; PCI, proteasome/COP9/Initiation factor; mRFP, monomeric red fluorescent protein; RP, regulatory particle; Rpn, regulatory particle non-ATPase; Rpt, regulatory particle triple A ATPase; Suc-LLVY, succinyl-leucyl-leucyl-valyl-tyrosyl; Ub, ubiquitin; UFD, ubiquitin fusion degradation; UPS, ubiquitin–proteasome system.

Table 1. Yeast strains used in this study

Strain	Relevant genotype	Source
W303-1A	MATa *leu2 trp1 his3 ura3 ssd1 can1 ade2*	Our stock
W303-1B	MATα *leu2 trp1 his3 ura3 ssd1 can1 ade2*	Our stock
YEK5	MATa *rpn7::rpn7-3-URA3*	Isono *et al.* (2004)
YEK6	MATα *rpn7::rpn7-3-URA3*	Isono *et al.* (2004)
YEK29	YEK5 *rpn11::RPN11-3×FLAG-HIS3*	Isono *et al.* (2004)
YEK79	MATα *pre6::PRE6-GFP-TRP1*	This study
YEK100	MATa *rpn5::rpn5-1-TRP1*	This study
YEK101	MATα *rpn5::rpn5-1-TRP1*	This study
YEK115	MATa *rpn11::RPN11-YGFP-TRP1*	This study
YEK147	MATa *rpn1::RPN1-YGFP-LEU2*	This study
YEK209	YEK5 *rpn11::RPN11-YGFP-TRP1*	This study
YEK211	YEK5 *pre6::PRE6-YGFP-LEU2*	This study
YEK213	YEK6 *rpn1::RPN1-YGFP-LEU2*	This study
YEK221	MATα *rpn7::RPN7-3×FLAG-KanMX*	This study
YEK225	YEK100 *rpn7::RPN7-3×FLAG-KanMX*	This study
YEK234	YAT2433 *rpn1::RPN1-3×FLAG-HIS3*	This study
YEK235	YAT2433 *rpn1::RPN1-YGFP-LEU2*	This study
YEK236	YAT2433 *rpn11::RPN11-YGFP-TRP1*	This study
YEK246	YAT2433 *srp1::srp1-49-LEU2*	This study
YEK247	YAT2433 *srp1::srp1-49-LEU2 rpn7::RPN7-YGFP-URA3*	This study
YEK248	YAT2433 *srp1::srp1-49-LEU2 rpn1::RPN1-YGFP-URA3*	This study
YKN6	YEK100 *pre1::PRE1-3×FLAG-HIS3*	This study
YKN8	YEK100 *rpn1::RPN1-3×FLAG-HIS3*	This study
YKN16	YEK100 *rpn1::RPN1-YGFP-LEU2*	This study
YKN18	YEK101 *pre6::PRE6-YGFP-TRP1*	This study
YAT2433	MATα Δ*rpn2-TRP1* [Top2612]	This study
YAT3507	YAT2433 *rpn11::RPN11-3×FLAG-LEU2*	This study
YYS37	MATa *pre1::PRE1-3×FLAG-HIS3*	Saeki *et al.* (2002)
YYS39	MATa *rpn1::RPN1-3×FLAG-HIS3*	Saeki *et al.* (2002)
YYS40	MATa *rpn11::RPN11-3×FLAG-HIS3*	Saeki *et al.* (2002)

All strains are in the W303 background.

the nuclear import of the proteasomes was suggested (Tabb *et al*., 2000), and it was shown that in the mutant of importin α *srp1-49*, seclusion of GFP-fused proteasome in the nucleus was no longer observed (Wendler *et al*., 2004). A study in fission yeast has shown that Cut8 is responsible for retaining the proteasome within the nucleus (Tatebe and Yanagida, 2000; Takeda and Yanagida, 2005).

The process of proteasome assembly has been a topic for recent studies and interesting facts have been elucidated. The CP, which is a stack of four seven-membered rings, in the order of $\alpha7\beta7\beta7\alpha7$, is imported into the nucleus as an $\alpha7\beta7$ "half" proteasome (Chen and Hochstrasser, 1996; Ramos *et al*., 1998), and maturation is thought to take place in the nucleus (Fehlker *et al*., 2003). Ump1p, which associates specifically with half-proteasomes, is suggested to function as an accelerator in this process (Ramos *et al*., 1998). Blm10p (formerly registered as Blm3p) was reported to act as an inhibitor of premature dimerization of the half-proteasomes (Fehlker *et al*., 2003), but it is also suggested to be a functional homologue of the mammalian PA200, activator of the CP (Schmidt *et al*., 2005). Recently, a study in mammalian cells showed that PAC1 and PAC2 work during the first step of assembly of the α ring (Hirano *et al*., 2005).

For the assembly of the RP, no external factors are known to date. In previous studies, we have shown that partially assembled lid subcomplexes made up of five (lid$^{rpn7\text{-}3}$: Rpn5p, Rpn6p, Rpn8p, Rpn9p, and Rpn11p) or four (lid$^{rpn6\text{-}1}$: Rpn5p, Rpn8p, Rpn9p, and Rpn11p) components accumulate in lid mutants (Isono *et al*., 2004, 2005). Because Rpn5p, Rpn8p, Rpn9p, and Rpn11p were included in both subcomplexes, we proposed these to be the "core" of lid formation. In this study, based on the analysis of an *rpn5* mutant and an *rpn2* mutant, we show, for the first time, that the formation and nuclear import of both the lid and the base are separable processes and that Rpn5p is indeed one of the core components of the lid.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

Yeast strains used in this work are listed in Table 1, and plasmids used for cloning and subcloning various genes and their fragments are listed in Table 2. Cells were cultured in omission medium prepared by removing appropriate nutrient(s) from synthetic complete (SC) medium, rich medium (YPDAU) (Sherman *et al*., 1986), or SR-U in which 2% glucose of SC was replaced with 2% raffinose and uracil was omitted. *Escherichia coli* strain DH5α (*supE44 ΔlacU169* [*ϕ*80lacZ Δ*M15*] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for construction and propagation of plasmids. Yeast transformations were performed as described previously (Burk *et al*., 2000).

Isolation of Temperature-sensitive Mutants

Temperature-sensitive *rpn5* mutants were screened as described previously (Isono *et al*., 2005). The *RPN5* gene was amplified using primers Rpn5 Mut1 (BglII) 5′-GGCCAAGATTGTAGATCTGCTAGC-3′ and Rpn5 Mut2 (NotI) 5′-GGAAGCGGCCGCAACCAGGCTTGAGTTAAC-3′ and cloned between the BamHI–NotI sites of the YIp vector pRS304. The resulting plasmid pNS101 was used as a template for polymerase chain reaction (PCR) mutagenesis.

Gel Filtration

Total proteins (5 mg) were resolved on a Superose 6 column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) as described previously (Isono *et al*., 2005). For the subsequent fractionation of Superose 6-fractionated samples, 450 μl of the separated samples were applied onto a Superdex 200 gel filtration column (GE Healthcare) connected to a fast-performance liquid

Table 2. Plasmids used in this study

Plasmid	Characteristics	Source
pRS304	*TRP1 Amp^r*	Sikorski and Hieter (1989)
pRS313	*HIS3 CEN Amp^r*	Sikorski and Hieter (1989)
Ub-A-lacZ	*GAL1p-Ub-A-lacZ URA3 Amp^r*	Bachmair *et al.* (1986)
Ub-R-LacZ	*GAL1p-Ub-R-lacZ URA3 Amp^r*	Bachmair *et al.* (1986)
Ub-P-lacZ	*GAL1p-Ub-P-lacZ URA3 Amp^r*	Bachmair *et al.* (1986)
pTS901CL	*5×HA CEN LEU2 Amp^r*	Sasaki *et al.* (2000)
pEK152	*RPN11-YGFP-TRP1 Amp^r*	This study
pEK165	*PRE6-YGFP-TRP1 Amp^r*	This study
pEK221	*RPN5(−400bp ORF∼+1kb ORF) HIS3 CEN Amp^r*	This study
pEK252	*RPN1-YGFP-LEU2 Amp^r*	This study
pEK285	*NUP53-mRFP-LEU2 CEN Amp^r*	This study
pEK291	*RPN7-YGFP-URA3 Amp^r*	This study
pEK296	*RPN1-YGFP-URA3 Amp^r*	This study
pEK297	*RPN3-YGFP-URA3 CEN Amp^r*	This study
pEK298	*RPN7-YGFP-URA3 CEN Amp^r*	This study
pEK299	*RPN12-YGFP-URA3 CEN Amp^r*	This study
pEK300	*RPN15-YGFP-URA3 CEN Amp^r*	This study
pNS101	*RPN5 TRP1 Amp^r*	This study
pNS202	*GST-RPN5 Amp^r*	This study
TOp2612	*ΔN rpn2 (+437∼+2838) HIS3 CEN Amp^r*	This study

chromatograph (GE Healthcare) at a flow rate of 0.5 ml/min, and 500-μl serial fractions were collected using a fraction collector FRAC-100 (GE Healthcare).

Microscopy

Cells harboring GFP- or monomeric red fluorescent protein (mRFP)-fused proteins were photographed by using a BX52 fluorescence microscope (Olympus, Tokyo, Japan) with a UPlanApo 100×/1.45 objective (Olympus) equipped with a confocal scanner unit CSU20 (Yokogawa Electric, Tokyo, Japan) and an EMCCD camera (Hamamatsu Photonics, Bridgewater, NJ). GFP and mRFP were excited using the 488- and 568-nm Ar/Kr laser lines with GFP and RFP filters (Semrock, Rochester, NY), respectively. DNA stained with Hoechst 33342 was photographed using a 405-nm laser line with a UV filter (Semrock). Images were obtained and processed using the IPLab software (Scanalytics, Fairfax, VA) and processed using Photoshop 7.0 (Adobe Systems, Mountain View, CA). For DNA staining, a final concentration of 20 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) was added to the cell suspension and incubated for 10 min at either 25 or 37°C under a light shade.

Fluorescence Recovery after Photobleaching (FRAP)

Samples used for FRAP experiments were embedded in agarose in the following way with modifications of the methods described previously (Hoepfner *et al.*, 2000). First, 100 μl of 1.7% agarose (Takara, Kyoto, Japan) dissolved in filtrated SC media was dropped on a prewarmed holed glass slide (Toshinriko, Tokyo, Japan), and immediately covered with a coverslip (Matsunami Glass, Osaka, Japan). Excess agarose was removed, and the glass slide was cooled until the agarose was set. The coverslip was carefully removed, and 3 μl of freshly cultured cells was dropped on the agarose plane and covered with a new coverslip and sealed. FRAP experiments were performed using a confocal laser-scanning microscope LSM510 META (Carl Zeiss, Jena, Germany) with a Plan-APOCHROMAT 100×/1.4 objective (Carl Zeiss). GFP was excited using the 488-nm laser lines from an Ar ion laser and a GFP filter. Photobleaching was achieved by scanning the selected region with maximal output of the 488-nm laser, and the recovery of the fluorescent signal was observed at the indicated time points under the same recording conditions as at 0 min. The stage was kept at 37°C with a stage-heater MATS-525F (Tokai Hit, Shizuoka, Japan). Fluorescence intensity of the original data was quantified using the LSM510 software, and images were processed with Photoshop 7.0 (Adobe Systems).

Indirect Immunofluorescence Method

For fixation, 420 μl of 37% formaldehyde was added to 3 ml of logarithmically growing cell culture (OD_{600} = 0.8–1.0) and incubated for 30 min at the incubation temperature. Cells were centrifuged and resuspended in 500 μl of phosphate-buffered saline (PBS)-formaldehyde (PBS/formaldehyde, 10:1) and incubated for 30 min at room temperature. For spheroplasting, cells were incubated with 200 μl of PBS-zymolyase (20 μg of zymolyase 100T [Seikagaku America, Rockville, MD]/1 ml of PBS) for 20 min at 30°C. Cells were then incubated in 200 μl of PBS containing 0.5% Triton X-100 for 30 min at room temperature. After washing twice, cells were resuspended in 200 μl of 3% bovine serum albumin in PBS for blocking and incubated for 1 h at room temperature. Mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO; 1/200 dilution) or anti-Rpn5p (1/100 dilution) antibody was used as primary antibody. Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, 1/400 dilution) or Alexa Fluor 546 goat anti-rabbit IgG (1/400 dilution; Invitrogen, Carlsbad, CA) was used as a secondary antibody. DNA was stained with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich).

RESULTS

Isolation of the Temperature-sensitive rpn5-1 Mutant

In our previous study, we found that partially assembled lid subcomplexes accumulated in temperature-sensitive lid mutants. Five (Rpn5p, Rpn6p, Rpn8p, Rpn9p, and Rpn11p) or four (Rpn5p, Rpn8p, Rpn9p, and Rpn11p) out of the nine components of the lid formed a stable complex in lid mutants, *rpn7-3* (Isono *et al.*, 2004) and *rpn6-1/rpn6-2* (Isono *et al.*, 2005), respectively. From these results and the fact that *RPN9* is a nonessential gene, we hypothesized that Rpn5p, Rpn8p, and Rpn11p may play pivotal roles in producing the core of the lid. To examine this hypothesis, we first focused on the function of Rpn5p.

To start with, we generated a temperature-sensitive mutant allele of *RPN5* by PCR-based random mutagenesis (Cadwell and Joyce, 1992; Toh-e and Oguchi, 2000) and named it *rpn5-1*. The *rpn5-1* mutant grew normally at 25°C, but it stopped growth after 6–8 h at 37°C (data not shown). The growth defect of the *rpn5-1* mutant at 37°C was complemented by a single copy of the wild-type *RPN5* gene, proving that the temperature sensitivity is due to a mutation within the *RPN5* gene and that the *rpn5-1* mutation is not dominant (Figure 1A). Sequencing analysis revealed that the *rpn5-1* open reading frame (ORF) possessed three mutations (Figure 1B), of which I180L or R344G alone did not lead to temperature-sensitive growth when introduced into the wild-type background. The nucleotide substitution from G to A at the 1245th nucleotide, leading to a nonsense mutation at the 415th tryptophan was found to be responsible for the temperature sensitivity (data not shown). Amounts of Rpn5-1p along with other proteasomal components were not significantly changed during incubation at the restrictive temperature, whereas a slight mobility shift of Rpn5-1p was

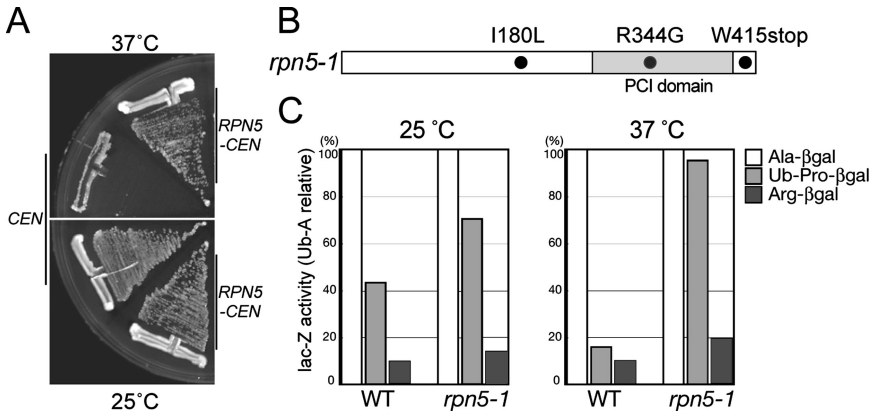

Figure 1. Characterization of the temperature-sensitive *rpn5* mutant. (A) *rpn5-1* (YEK100) cells carrying either a *CEN* vector (pRS314) or *RPN5-CEN* plasmid (pEK221) were streaked on YPDAU plates and photographed after incubating for 2 d at either 25 or 37°C. (B) Amino acid substitution in *rpn5-1*. The nucleotide sequence of the *rpn5-1* ORF was determined by the dideoxychain termination method and compared with that of the wild-type *RPN5* ORF. Gray, PCI domain. (C) Degradation of N-end rule pathway- and UFD pathway-substrates. Wild-type (W303-1A) and *rpn5-1* (YEK100) cells were transformed with plasmids expressing an N-end rule model substrate Ub-Ala-βgal or Ub-Arg-βgal, or a UFD pathway substrate Ub-Pro-βgal. Production of the model substrates was induced by adding 2% galactose to SR-U medium. Cells were harvested after 4 h of induction at either 25 or 37°C, and steady-state levels of β-galactosidase activity were assayed. The amounts of Ub-Pro-βgal and Arg-βgal are indicated relative to that of Ala-βgal. Average of three independent experiments is shown (open, Ala-βgal; light gray, Ub-Pro-βgal; and solid, Arg-βgal).

observed due to the C-terminal truncation (Supplemental Figure 1).

To examine the effect of the *rpn5-1* mutation on the UPS, we evaluated the stability of three model substrates of the ubiquitin–proteasome pathway, namely, Ala-βgal, Arg-βgal, and Ub-Pro-βgal (Bachmair *et al*., 1986). Whereas Ala-βgal is stable, Arg-βgal, and Ub-Pro-βgal are short-lived substrates of the N-end rule- and Ub-fusion degradation (UFD) pathway, respectively. Wild-type and *rpn5-1* mutant cells, transformed with plasmids expressing one of these substrates under a galactose inducible promoter were cultured at either 25 or 37°C. After 3 h, galactose was added to the culture to induce the production of the substrates, and cells were incubated for further 4 h. Total extract was prepared from each culture, and steady-state levels of the substrates were estimated by β-galactosidase assay. Compared with the wild-type cells, *rpn5-1* cells maintained the normally short-lived Arg-βgal and Ub-Pro-βgal at a higher level, especially at 37°C, indicating that the *rpn5-1* mutation caused a defect in the UPS at the restrictive temperature (Figure 1C).

Assembly of the Lid in rpn5-1 Mutants

In a previous report on a Δ*rpn5* mutant in fission yeast, the state of assembly of each substructure of the 26S proteasome was ambiguous, because Rpn10p that is not stably associated with the base or the lid was used for the affinity purification of proteasomes (Yen *et al*., 2003a). To examine the assembly of the 26S proteasome in the *rpn5-1* mutant, we compared the gel filtration profile of proteasomes in wild-type and *rpn5-1* extracts. Wild-type and *rpn5-1* cells were cultured for 7 h at 37°C in YPDAU, and total lysates were prepared in the presence of ATP and $MgCl_2$. Extract equivalent to 5 mg of protein was resolved on a Superose 6 gel filtration column, and the eluate was collected into 500 μl of sequential fractions. Relevant fractions (16~32) were subjected to peptidase activity measurement by using succinyl-leucyl-leucyl-valyl-tyrosyl-methycoumaryl-7-amide (Suc-LLVY-MCA), a fluorogenic peptide substrate. In the wild-type sample, the most enzymatically active fraction was at 22, showing this fraction to be the peak fraction of the 26S holoenzyme (Figure 2A, top).

In contrast, in gel filtration of extract prepared from 37°C-grown *rpn5-1* cells, the peak at fraction 22 has almost vanished, suggesting that the assembly of the 26S proteasome is disturbed. On SDS treatment, which activates free CPs, a high enzyme activity occurred at fractions 26 and 27, indicating that there is a large pool of free CPs in *rpn5-1* cells grown at 37°C (Figure 2A, bottom). There was also a small peak in fraction 23, the identity of which will be discussed later. *rpn5-1* cells cultured at 25°C had almost the same profile as the wild-type, except that the peak of enzyme activity at fraction 26 was higher than that in the wild-type sample (Figure 2A, middle).

Fractions were then subjected to Western blotting by using antibodies against proteasome components. CP signals were detected around fraction 22 in the wild type sample, whereas they were detected around fraction 26 in the sample derived from *rpn5-1* cells grown at 37°C, in accordance with the result of activity measurement (Figure 2B, top and bottom). Interestingly, all lid components of *rpn5-1* cells grown at 37°C, but not at 25°C, were detected in low-molecular-mass fractions (Figure 2B, middle and bottom). To investigate whether they are monomers or forming a complex, we further resolved fractions 32 and 34 by Superdex 200, and fractions were subjected to Western blotting (Figure 2C). Comparison with marker proteins indicated that at least Rpn5p (52 kDa), Rpn8p (38 kDa), and Rpn9p (46 kDa) existed in a free form. Rpn7p (49 kDa) was found to move slower than expected from its molecular mass.

To see whether Rpn7p forms a stable complex with any other components in *rpn5-1* cells, we generated *rpn7::RPN7-3xFLAG* strains with or without the *rpn5-1* mutation and performed affinity purification. *RPN7-3xFLAG RPN5* and *RPN7-3xFLAG rpn5-1* strains were cultured at either 25 or 37°C for 7 h and proteasomes were affinity purified by anti-FLAG antibody immobilized on agarose from total lysates. Purified proteasomes were resolved by SDS-PAGE and stained with Coomassie brilliant blue (CBB), and the band patterns were compared with purified authentic proteasomes (Saeki *et al*., 2005). All components of the 26S proteasome were copurified with Rpn7p-3xFLAG in wild-type cells and *rpn5-1* cells cultured at 25°C (Figure 2D, lanes 2 and 3; data for wild-type 25°C not shown). However, no protein was copurified with Rpn7p-3xFlag from *rpn5-1* cells cultured at 37°C. Because Rpn7p-3xFLAG was present in the total extract at both 37 and 25°C (Supplemental Figure 2), Rpn7p is probably not forming a soluble and stable complex with other components in *rpn5-1* cells under the restrictive conditions.

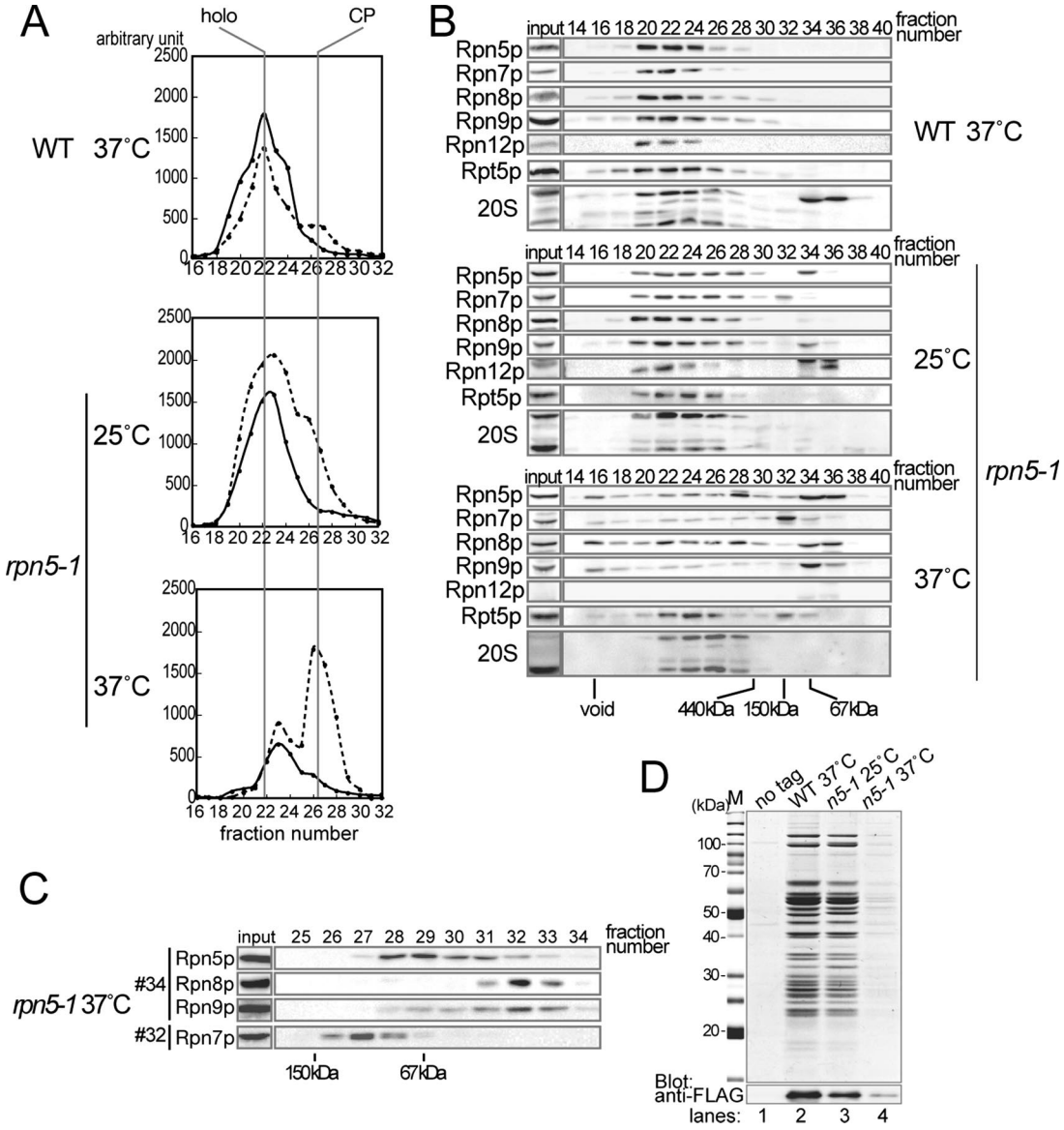

Figure 2. Lid formation in *rpn5-1* cells. Wild-type (W303-1A) and *rpn5-1* (YEK100) cells were cultured for 7 h at the indicated temperature, and total cell extracts were prepared by breaking the cells by glass-beads under the existence of ATP and $MgCl_2$. (A) Gel filtration. Peptidase activity toward the fluorogenic substrate Suc-LLVY-MCA was measured in relevant fractions (16–32). Positions of the 26S holoenzyme and the CP are indicated at the top of the graph. Solid line, without SDS; and dotted line, with 0.02% SDS. (B) Western blotting. Twenty microliters of each of the even numbered fractions was mixed with SDS-PAGE loading buffer and resolved by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and proteasome subunits were detected by Western blotting by using the indicated antibodies (Rpn5p, Rpn7p, Rpn8p, Rpn9p, and Rpn12p, lid; and Rpt5p, base) Positions of the void fraction and marker proteins (ferritin [440 kDa], aldolase [150 kDa], and bovine serum albumin [67 kDa]) are indicated at the bottom of the panels. (C) Second gel filtration. Fractions 32 and 34 in Superose 6 gel filtration of *rpn5-1* extracts (37°C) in A were subsequently resolved by a Superdex 200 gel filtration column. Five hundred microliters sequential fractions were collected, and relevant fractions were subjected to Western blotting as in B. Antibodies used are indicated on the left. Positions of marker proteins (aldolase [150 kDa] and bovine serum albumin [67 kDa]) are indicated on the bottom of the panels. (D) Wild-type and *rpn5-1* strains expressing *RPN7-3xFLAG* (YEK221 and YEK225, respectively) along with the untagged wild-type strain (W303-1A) were cultured for 7 h at 25 or 37°C as indicated, and extract was prepared from each culture. Proteasomes were affinity purified using anti-FLAG agarose. Purified products were run on a 12.5% SDS-PAGE gel, and protein bands were stained with CBB (M, marker).

Base-CP Complexes in rpn5-1

The base and the CP of the extract prepared from *rpn5-1* cells incubated at 37°C seemed to comigrate (Figure 2B). Results of nondenaturing PAGE of total lysates of wild-type or *rpn5-1* cells grown at 37°C showed the existence of base-CP complexes, B1CP, and B2CP, in *rpn5-1* cells (Figure 3A). The peak of peptidase activity observed in fraction 23 in the *rpn5-1* sample grown at 37°C (Figure 2A, bottom) was likely due to these base–CP complexes. We next tried to affinity-purify these base–CP complexes by using CP- or base-tagged strains. However, as shown in Figure 3B, no base–CP complex was obtained from *rpn5-1* cells regardless of the tagged subunit used, suggesting that the base-CP interaction in *rpn5-1* cells is unstable.

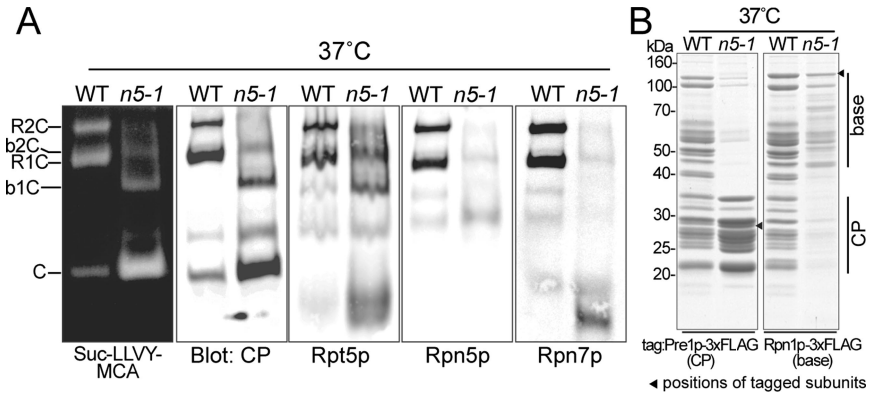

Figure 3. Proteasome species in wild-type extract and *rpn5-1* extract. (A) Extracts were prepared from wild-type (W303-1B) or *rpn5-1* (YEK101) cells incubated for 7 h at 37°C, and extract equivalent to 50 μg of protein was resolved by nondenaturing PAGE. Proteasomes were visualized by overlaying buffer containing 0.1 mM Suc-LLVY-MCA and 0.05% SDS on the gel (far left). The gels were subsequently subjected to Western blotting by using antibodies indicated on the bottom of the panels (Rpt5p, base; and Rpn5p and Rpn7p, lid). Bands corresponding to various proteasome species are indicated on the far left of the panels (R, RP; C, CP; and b, base). (B) Affinity purification of proteasomes from CP- and base-tagged stains. YYS37 (*PRE1-3xFLAG*) and YYS39 (*RPN1-3xFLAG*), YKN6 (*rpn5-1 PRE1-3xFLAG*) and YKN8 (*rpn5-1 RPN1-3xFLAG*) cells were cultured for 7 h at 37°C and proteasomes were affinity purified from 2 mg of total proteins using anti-FLAG agarose. The purified proteasomes were resolved on a 12.5% SDS-polyacrylamide gel and stained with CBB (left, CP tagged; and right, base tagged). Bands corresponding to the tagged components are indicated with solid arrowheads. The approximate migrating positions of the base and the CP components are indicated by bars on the right side of the panel.

Assembly of the Lid in a Base Mutant ΔN rpn2

In the previous section, we showed that the base was produced independently of the assembly of the lid. Next, we ask the following question: Are the lid and the base assembled independently to each other? To address this issue, we examined the status of lid assembly in a temperature-sensitive base mutant of *RPN2* termed Δ*N rpn2* (equivalent to *rpn2*Δ described by Yokota *et al*. 1996). Δ*N rpn2* is a mutant that carries an N-terminal truncated (Δ1-220aa) version of *RPN2* in a null *rpn2* strain. It stopped growth after 4–6 h at 37°C (data not shown). To see the assembly state in this mutant, total lysates were prepared from Δ*N rpn2* cells grown for 6 h at either 25 or 37°C, and 5 mg of total proteins was resolved on a Superose 6 gel filtration column.

The Δ*N rpn2* mutant was found to have a more severe defect in the structure of proteasomes than the *rpn5-1* mutant, because peptidase assays and Western blotting both showed reduced amount of the 26S holoenzyme even at the permissive temperature (Figure 4, A and B, left). The defects were strongly enhanced when cells were grown at the restrictive temperature. Peptidase activity measurement showed that in Δ*N rpn2* cells incubated at 37°C, the peak corresponding to the 26S proteasome was almost completely lost, and a single high peak of free CPs was detected at fraction no.26 (Figure 4A, right).

Western blotting of chromatographic fractions (Figure 4B, right) revealed that all lid components tested were detected in fraction no.28, which is comparable with the eluting position of a free lid (ca. 500 kDa). These results suggest that in Δ*N rpn2* under the restrictive condition, the lid exists in a free form, unbound to the base. Indeed, this was confirmed by native PAGE (Figure 4C) and affinity purification by using Rpn11p-3xFLAG (lid), by which a complete lid was affinity purified from Δ*N rpn2* cells (Figure 4D, lane 6). The incorporation of all of the nine lid components was verified by band comparison with a wild-type lid and liquid chromatography tandem mass spectrometry (Supplemental Figure 2). It was also shown that a ΔN Rpn2p-less base was formed in Δ*N rpn2* cells at the restrictive temperature (Figure 4D, lane 3). Together with the results of the *rpn5-1* extract, we conclude that the base formation and the lid formation can be independent of each other and are separable processes.

Localization of the Base and the CP in Lid Mutants

In yeast, the 26S proteasome is known to be highly enriched in the nucleus (Wilkinson *et al*., 1998; Enenkel *et al*., 1999). Biochemical analysis described above has shown that the lid formation was independent of the base formation. It is not known to date whether the assembly of the base and the lid into an RP is a prerequisite for their nuclear localization. To observe the localization of proteasomes in lid mutants, we generated GFP (Cormack *et al*., 1996)-fused proteasomes, *PRE6-GFP* (CP), *RPN1-GFP* (base), and *RPN11-GFP* (lid) strains, in which one of the chromosomal *PRE6*, *RPN1*, and *RPN11* genes had been replaced with a C-terminally GFP-tagged gene, and the incorporation of the GFP-fused proteins into the proteasome were verified (Supplemental Figure 3).

Rpn1p-GFP and Pre6p-GFP showed strong nuclear localization in wild-type cells regardless of the incubating temperature (Figure 5A, left; data for 25°C not shown) as reported previously (Enenkel *et al*., 1999; Wendler *et al*., 2004). The localization was also examined in two lid mutants, *rpn5-1* and *rpn7-3*, in which the base was not associated with the lid under restrictive conditions. Both base and CP signals were observed in the nucleus regardless of the cultivation conditions (Figure 5A, middle and right), indicating that the *rpn5-1* and *rpn7-3* mutations that perturb the interaction between the lid and the base do not affect the nuclear localization of the base and the CP and that the nuclear localization of the base and the CP is independent of the binding of the lid to the base.

FRAP Experiments

One concern was that the base and CP signals detected at the restrictive temperature might be those of the remaining proteins synthesized and imported under the permissive temperature. To test this possibility, we observed the FRAP, by bleaching the nuclear region with intense laser and observing the recovery of fluorescence after 120 min.

We first carried out an experiment using a wild-type strain producing Rpn1p-GFP instead of Rpn1p. The GFP signals indeed vanished after photobleaching the region of the nucleus (Figure 5B, left and middle). When observed at 120 min after photobleaching, the GFP fluorescence occurred in the nucleus again, proving that nuclear import of the base has occurred (Figure 5B, right). Next, the recovery from photobleaching in *rpn7-3* cells was similarly examined using

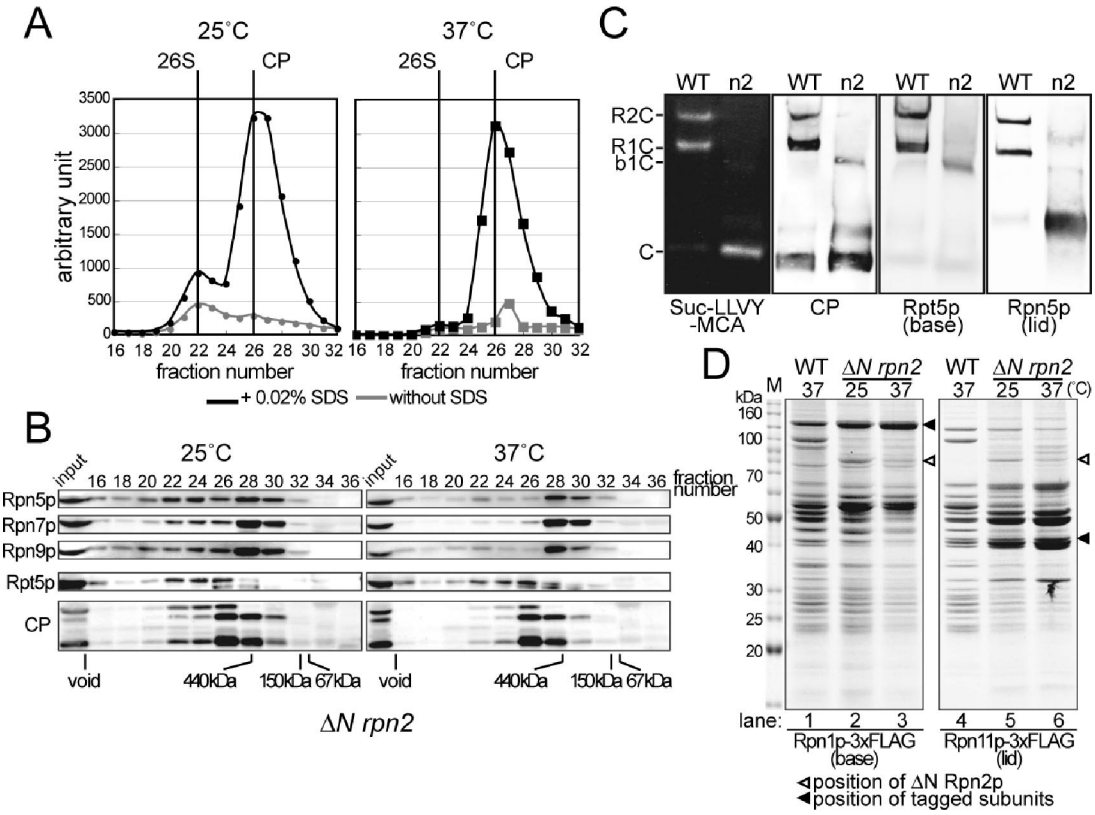

Figure 4. Lid formation does not depend on the binding of the lid to the base. (A) Extract of Δ*N rpn2* (YAT2433) cells cultured for 6 h at 25 or 37°C was resolved on a Superose 6 column, and peptidase activity was measured as described in Figure 2A. Positions of the 26S holoenzyme and the CP are indicated at the top of the graph (black lines, with 0.02% SDS; and gray lines, without SDS). (B) Fractions were subjected to Western blotting as described in Figure 2B. Antibodies used are indicated on the left of the panel (Rpn5p, Rpn7p, and Rpn9p, lid; and Rpt5, base). Positions of the void fraction and marker proteins (ferritin [440 kDa], aldolase [150 kDa], and bovine serum albumin [67 kDa]) are indicated at the bottom of the panels. Note that all lid components examined comigrated. (C) Wild-type (W303-1A) or Δ*N rpn2* (YAT2433) cells were cultured for 6 h at 37°C, and extract was prepared as described above. Extract equivalent to 50 μg of protein was resolved by nondenaturing PAGE. Proteasomes were visualized by overlaying buffer containing 0.1 mM Suc-LLVY-MCA and 0.05% SDS on the gels (far left panel). The same gels were subsequently subjected to Western blotting by using antibodies indicated on the bottom of the panels (Rpt5p, base; and Rpn5p, lid). Bands corresponding to various proteasome species are indicated on the far left of the panels (R, RP; C, CP; and b, base). (D) Affinity purification of proteasomes from base- and lid-tagged strains YYS39 (*RPN1-3xFLAG*), YYS40 (*RPN11-3xFLAG*), YEK234 (Δ*N rpn2 RPN1-3xFLAG*), and YAT3507 (Δ*N rpn2 RPN11-3xFLAG*) cells were cultured for 6 h at 25 or 37°C as indicated, and proteasomes were affinity-purified using anti-FLAG agarose. The purified proteasomes were resolved on a 12.5% SDS-polyacrylamide gel and stained with CBB (left, base tagged; and right, lid tagged). Protein bands were cut out and identified by mass spectrometry (see Supplemental Figure 2). The approximate migrating positions of base and lid components are indicated on the right of the panel (solid arrowhead, tagged component; open arrowhead, ΔN Rpn2p; and M, marker).

rpn7-3 strains expressing *RPN1-YGFP* and *PRE6-YGFP*. Cells were held under the restrictive condition by keeping the glass slides at 37°C by using a stage heater. In *rpn7-3* cells, recovery of both base and CP signals were observed even at the restrictive temperature (Figure 5, C and D). The fluorescence intensity in the nucleus was quantified and the degree of fluorescence recovery in *rpn7-3* cells was found to be comparable with that in the wild-type cells (Figure 5, C and D).

Localization of the Free Lid in ΔN rpn2 Cells

One of the striking features of the Δ*N rpn2* mutant is that its lid exists as a free complex. Because in no known mutant was the lid separated from the base in vivo, it has been difficult to examine whether the lid and the base could be imported into the nucleus independent of each other. We used the Δ*N rpn2* strain to observe the localization of the free lid along with the base by creating its *RPN11-GFP* (lid) or *RPN1-GFP* (base) derivative (Figure 6). Surprisingly, not only the base but also the lid was localized in the nucleus even at 37°C, suggesting that the import of the lid and the base does occur independently. It was verified by gel filtration (Figure 6B) and subsequent immunoprecipitaiton (Figure 6C) that the GFP signals corresponded to the respective complexes. The import of the lid into the nucleus under the restrictive condition was further corroborated by performing FRAP experiments (Figure 6D).

Localization of lid$^{rpn7\text{-}3}$ in rpn7-3 Cells

In the previously characterized *rpn7-3* mutant, it was shown that five of the nine lid components formed a subcomplex termed lid$^{rpn7\text{-}3}$ (Isono *et al*., 2004). To see whether this partially assembled lid$^{rpn7\text{-}3}$ could be imported into the nucleus, we attempted to analyze the localization of lid$^{rpn7\text{-}3}$ in the *RPN11p-GFP rpn7-3* strain. However, because Rpn11p-GFP was not incorporated into lid$^{rpn7\text{-}3}$ at the restrictive temperature (data not shown), we observed its localization by using the indirect immunofluorescence method. *RPN11-3xFLAG* wild-type and *RPN11-3xFLAG rpn7-3* cells were

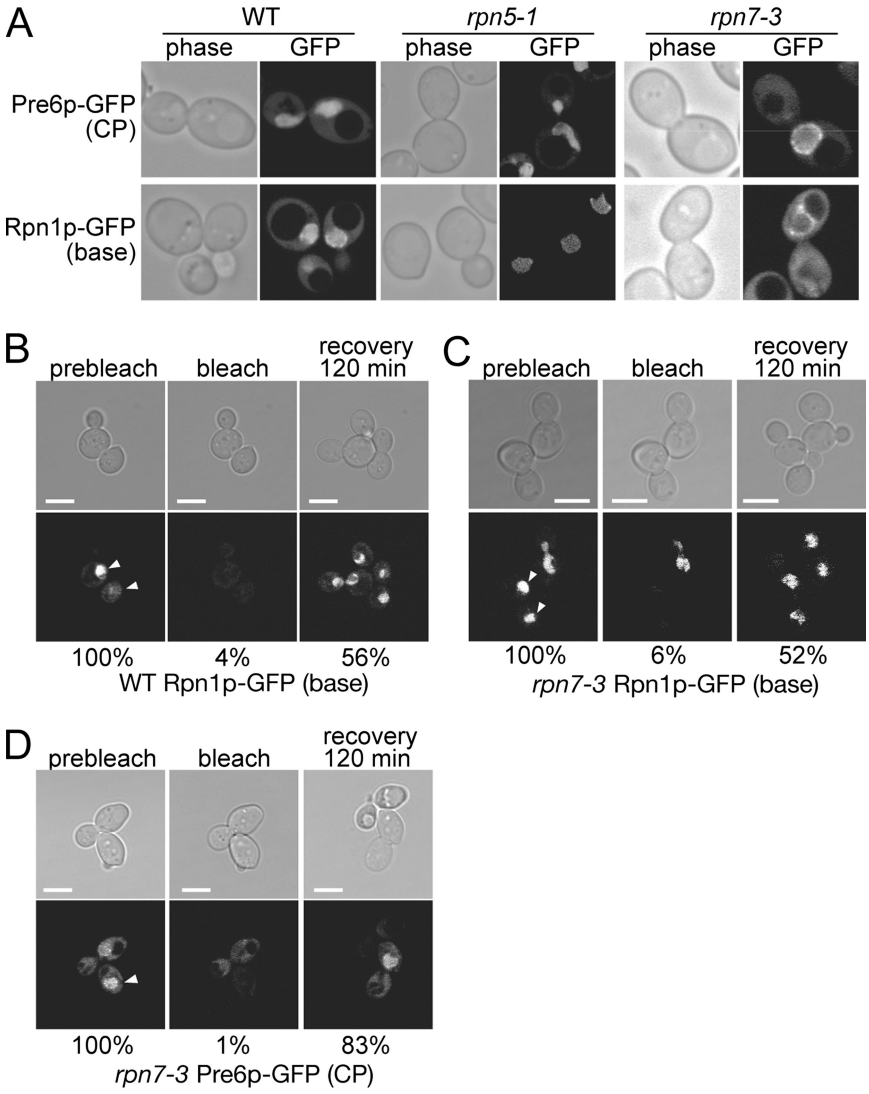

Figure 5. The base and the CP are localized in the nucleus in lid mutants even at the restrictive temperature. (A) Wild-type, *rpn5-1* and *rpn7-3* cells producing Pre6p-GFP (CP) or Rpn1p-GFP (base) instead of the authentic Pre6p and Rpn1p, respectively, were cultured for 7 h at 37°C and photographed under a confocal microscope. Strains used were *PRE6-GFP* (CP), wild type (YEK79), *rpn5-1* (YKN18), *rpn7-3* (YEK211), and *RPN1-GFP* (base) wild type (YEK147), *rpn5-1* (YKN16), *rpn7-3* (YEK213). (B–D) The base and the CP are imported into the nucleus after shift to the restrictive temperature. Rpn1p-GFP (base) producing wild-type (YEK147) and Rpn1p-GFP or Pre6p-GFP (CP) producing *rpn7-3* (YEK213 and YEK211, respectively) cells were cultured for 6 h at 37°C and embedded in agarose as described in *Materials and Methods*. GFP signals in the nucleus (prebleach, left) were photobleached with intense laser (bleach, middle), and FRAP was observed and photographed after 120 min (recovery, right). The stage was kept at 37°C throughout the experiment. Fluorescence intensity ([/mm^2] − background [/mm^2]) was quantified and shown as a relative value to the prebleach intensity at the bottom of each panel. The mean value of two independent experiments is shown. Bar; 5 μm.

cultured for 7 h at 37°C, fixed, and stained. In wild-type cells and *rpn7-3* cells cultured at 25°C, the signal corresponding to the 26S proteasome was clearly nuclear localized, and strong signals at the nuclear periphery was observed (Figure 7A, second and third panels from the top; data for 25°C wild-type samples not shown). On the contrary, in *rpn7-3* cells cultured at 37°C, the nuclear localization was not seen, and signals of DAPI-stained DNA did not merge with the FLAG signals any more (Figure 7A, lowermost panel), showing that the lid$^{rpn7\text{-}3}$ was not localized in the nucleus.

To confirm this observation, immunostaining was also performed using an antibody against Rpn5p, one of the components of lid$^{rpn7\text{-}3}$. Again, the nuclear localization seen in wild type cells could not be observed in the *rpn7-3* cells, in which signals of Rpn5p were detected dispersed in the cytosol (Figure 7B). This result suggests that the lid is built up in the cytosol and then imported into the nucleus to be assembled into the 26S complex, although we cannot exclude the possibility that the lid$^{rpn7\text{-}3}$ is reexported to the cytosol after carried into the nucleus. The structure of the nucleus itself was not damaged in *rpn7-3* cells under the restrictive condition, which was verified by the normal localization of mRFP (Campbell *et al*., 2002) fused Nup53p, a component of the nuclear pore complex, in wild-type and also in *rpn7-3* cells at 37°C (Figure 7C).

From the above-mentioned results, we anticipated that the components not included in the lid$^{rpn7\text{-}3}$, namely, Rpn3p, Rpn7p, Rpn12p, and Rpn15p, might be responsible for the import of the lid. To test this possibility, we expressed one of the GFP-fused constructs of *RPN3*, *RPN7*, *RPN12*, or *RPN15* under their native promoters in *rpn5-1* cells. The cells were cultured for 7 h at 37°C and GFP signals were observed under a confocal microscope (Figure 7D). Rpn3p, Rpn7p, and Rpn12p showed nuclear localization, whereas Rpn15p did not, showing that Rpn3p, Rpn7p, and Rpn12p can be localized in the nucleus as monomers under these conditions.

The Nuclear Import of the Lid Does Not Depend on Srp1p

Srp1p, karyopherin α, was reported to be involved in the import of proteasomes into the nucleus (Tabb *et al*., 2000; Lehmann *et al*., 2002; Wendler *et al*., 2004). In accordance with previous reports, in a temperature-sensitive mutant of *SRP1* termed *srp1-49*, Rpn11p-GFP (lid) and Rpn1p-GFP (base) were delocalized (Supplemental Figure 4). However, because no method has been available to form the lid and

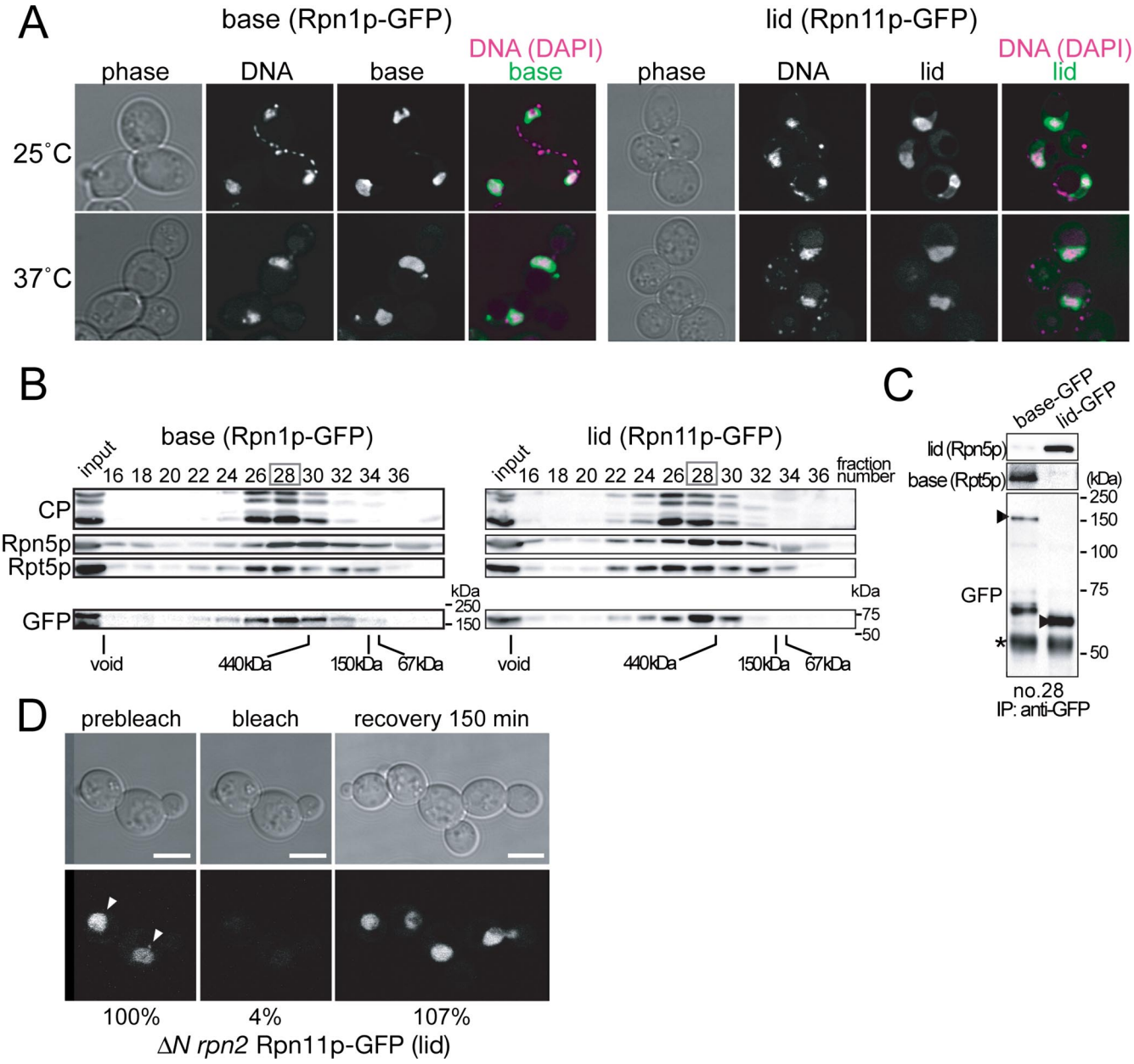

Figure 6. The nuclear import of the base and the lid are independent of each other. (A) Δ*N rpn2* cells producing Rpn1p-GFP (base, YEK235) or Rpn11p-GFP (lid, YEK236) instead of the authentic Rpn1p or Rpn11p, respectively, were cultured for 6 h at either 25 or 37°C and photographed under a confocal microscope. DNA was stained with Hoechst 33342. (B) Total proteins were extracted from the cells cultured at 37°C as described in A and subjected to gel filtration by using a Superose 6 column. Fractions were subsequently subjected to Western blotting by using the antibodies indicated on the left of the panels. (Rpn5p; lid, Rpt5p; base) Positions of the void fraction and marker proteins (ferritin [440 kDa], aldolase [150 kDa], and bovine serum albumin [67 kDa]) are indicated at the bottom of the panels. For the GFP blot, positions of molecular mass markers are shown on the right of each of the panels. (C) Immunoprecipitation was performed against fraction 28 in B by using anti-GFP antibody and analyzed by Western blotting by using anti-Rpn5p (lid), anti-Rpt5p (base), and anti-GFP antibodies. Asterisks indicate nonspecific bands. Arrowheads indicate the GFP-fused components. (D) *RPN11-GFP*–expressing Δ*N rpn2* (YEK256) cells were cultured for 6 h at 37°C, and FRAP experiments were performed as in Figure 5, except that the recovery was observed after 150 min. Fluorescence intensity ([/mm^2] − background [/mm^2]) was quantified and shown as relative values to the prebleach intensity at the bottom of each panel. The mean value of two independent experiments is shown. Arrowheads indicate the cell that was photobleached. Bar; 5 μm.

the base separately in vivo, it had been impossible to examine whether their nuclear import would be dependent on Srp1p.

To address this issue, we took advantage of the Δ*N rpn2* mutant, in which the lid exists as a free complex at the restrictive temperature. Either *RPN7-GFP* or *RPN1-GFP* was introduced into the Δ*N rpn2 srp1-49* double mutant to replace each of the authentic genes. Cells were cultured for 8 h at 37°C and observed under a confocal microscope. Although the base lost its strong nuclear localization seen at 25°C, and a part of the signals was dispersed in the cytosol, the lid signals remained strongly in the nucleus at 37°C (Figure 8A), which was corroborated by the quantification of the fluorescence signals (Figure 8B). These results suggest

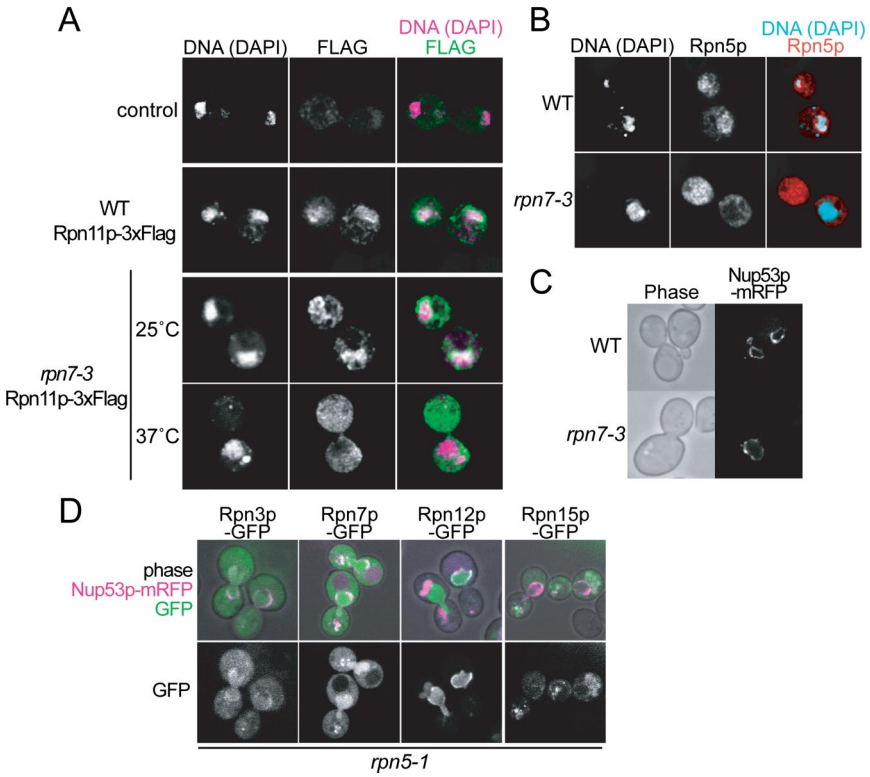

Figure 7. Localization of the partially assembled lid$^{rpn7\text{-}3}$. (A) *RPN11-3xFLAG* (YYS40) and *rpn7-3 RPN11-3xFLAG* (YEK29) cells, along with untagged wild-type (W303-1A) cells, were cultured for 6 h at 25 or 37°C as indicated, and localization of lid$^{rpn7\text{-}3}$ was detected by the indirect immunofluorescence method by using anti-FLAG M2 antibody. Photographs were taken under a confocal microscope. DNA was stained with DAPI. (B) Wild-type (W303-1B) and *rpn7-3* (YEK6) cells were incubated for 6 h at 37°C, and localization of Rpn5p was detected as described in A by the indirect immunofluorescence method except that an anti-Rpn5p antibody was used. DNA was stained with DAPI. (C) The nuclear envelope is normal in *rpn7-3* cells under the restrictive condition. Nup53p-mRFP (pEK285) was produced in wild-type (W303-1A) and *rpn7-3* (YEK6) cells cultured at the same condition as described in B and photographed under a confocal microscope. (D) Localization of Rpn3p-GFP (pEK297), Rpn7p-GFP (pEK298), Rpn12p-GFP (pEK299), or Rpn15p-GFP (pEK300) in *rpn5-1* (YEK100) cells. Cells were cultured for 8 h at 37°C, and GFP signals were photographed under a confocal microscope. Nup53p-mRFP was used as a marker for the nuclear envelope.

that the nuclear import of the base is dependent on Srp1p, whereas that of the lid does not.

DISCUSSION

The *RPN5* gene, originally named *n*on-*A*TPase *s*ubunit 5 (*NAS5*), is an essential gene in *S. cerevisiae*, in contrast to *Schizosaccharomyces pombe*, and is a homologue of human p55 (Finley *et al*., 1998). In this study, we have shown that in *rpn5-1* cells, not even a partially assembled subcomplex of the lid was detected at the restrictive temperature (Figure 2, B–D). This result, together with our previous reports, indicates that the mutation in Rpn5p inhibits the complex formation of the lid, and thus Rpn5p is a key component in the core formation of the lid. A very recently published report has shown the interaction between subunits of the RP by tandem MS analysis (Sharon *et al*., 2006). Using this novel approach, it was demonstrated that Rpn5p, Rpn8p, Rpn9p, and Rpn11p is forming a stable soluble subcomplex and a subunit interaction map was proposed, which is in good accordance with our results that Rpn5p is a core component in lid formation.

Interestingly, the addition of a 3xFLAG tag at the C terminus to another lid component Rpn11p rescued the temperature-sensitivity of *rpn5-1* and at the same time its defect in assembling the 26S proteasome (Supplemental Figure 6). Probably, the C terminus of Rpn11p is located near to the C terminus of Rpn5p so that its extension suppresses the structural alternation of the proteasome caused by the incorporation of the truncated Rpn5-1p. Because the structural recovery of the 26S proteasome led to a complete growth recovery at the restrictive temperature, the *rpn5-1* mutation was probably causing purely structural defects.

The fact that the base-CP interaction in mutant cells is unstable indicates the possibility that the lid functions to strengthen the base-CP binding by allosterically affecting the base-CP interface. This result is in contrast to a previous report in which a base-CP complex was purified from a mutant of *RPN11* termed *mpr1–1* in the absence of the lid

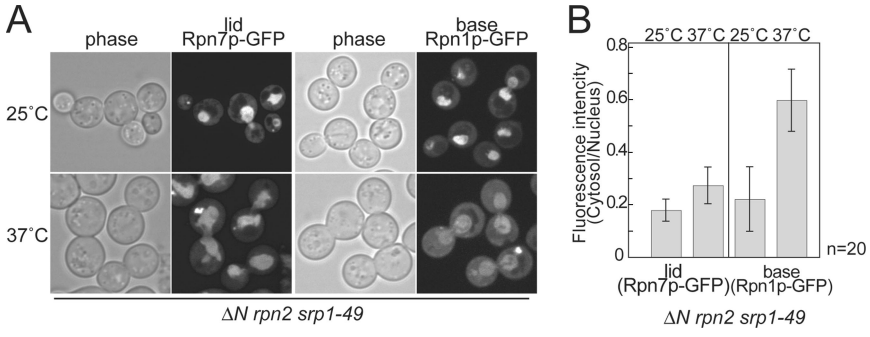

Figure 8. Nuclear localization of the base, but not the lid, is affected by *srp1-49*. (A) Δ*N rpn2 srp1-49* cells expressing Rpn7p-GFP or Rpn1p-GFP (YEK247 and YEK258, respectively) were cultured for 8 h at either 25 or 37°C, and localization of the GFP-fused components was observed under a confocal microscope. (B) Signals of A were quantified (fluorescence intensity per area) using the IPLab software, and ratio of the nuclear and cytosolic signals is shown. Error bars represent SD (n = 20).

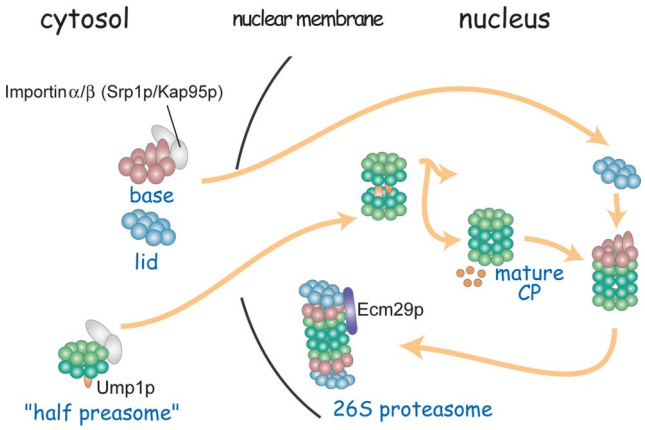

Figure 9. Model for the assembling process of the 26S proteasome in budding yeast. The base and the lid are made in the cytosol and are imported into the nucleus independently. On the dimerization of half-proteasomes into a mature CP, the base binds the CP. The immediate binding of the lid to the base–CP complex stabilizes the whole complex. Additional interacting proteins are bound to the 26S proteasome.

(Verma *et al*., 2002). We have no explanation for this discrepancy at present.

In fission yeast, it was proposed that SpRpn5, together with the human breast cancer related gene Int6/Yin6, serves for the nuclear localization of the lid (Yen *et al*., 2003b), although our results showed that the lid$^{rpn7\text{-}3}$ containing Rpn5p was not localized in the nucleus (Figure 7, A and B). One explanation of these seemingly controversial results may be that there is no Int6/Yin6 homologue in budding yeast and hence the Int6/Yin6 associated function of Rpn5p is not conserved in the two yeast species.

The CP and an interacting ATPase exist already in prokaryotic organisms. Given that the lid shares its origin with COP9/signalosme and eIF3, both of which are functioning as a single complex, it is reasonable that the formation and the nuclear localization of the base and the lid are independent to each other. However, although the base is imported into the nucleus via the Srp1p-dependent importin α/β pathway, the lid seems to be carried into the nucleus via another system that is independent of Srp1p (Figure 8, A and B). As for the base, two of the base components, Rpn2p and Rpt2p, were shown to possess functional nuclear localization signal (NLS) sequences, and because the simultaneous deletion of these sequences is lethal, it was suggested that the nuclear import of the base probably depends on its own NLS(s) (Wendler *et al*., 2004). No component of the lid was yet proved to be responsible for the nuclear import of the lid. Our results suggest that Rpn3p, Rpn7p, and Rpn12, each of which is localized into nucleus by itself, may serve for the nuclear import of the lid (Figure 7D).

We have shown in this study that the lid, a substructure of the 26S proteasome, can be formed and imported into the nucleus independently of the other subcomplexes of the 26S proteasome. Together with the result that a base–CP complex is formed in the analyzed lid mutants, we propose the following scenario of the assembling pathway of the 26S proteasome (Figure 9). The half-proteasome as well as base and the lid are formed independently in the cytosol, and they are imported into the nucleus. Then, the base binds the mature CP, and finally the lid binds the base–CP complex to form a mature 26S proteasome. In the course of the formation of the lid, Rpn5p, together with its interacting components forms the core of the lid, into which Rpn6p then the rest of the components are sequentially incorporated to become a complete lid. It should be noted that the scenario of the assembly pathway described above had been drawn using mutants, and there might be a different pathway in wild-type cells. In wild-type cells, the assembly processes are probably too rapid to be detected biochemically, because the apparent intermediates of the 26S proteasome such as the base–CP complex, lid$^{rpn6\text{-}1}$ and lid$^{rpn7\text{-}3}$ existed in lid mutants under the restrictive condition (Isono *et al*., 2004, 2005) cannot be detected.

This consideration casts another question as to whether there are any factors that facilitate or regulate the interaction between the base, lid and CP. It should be noted that a recent report with mammalian cells has shown that a protein interacting with the ATPase subunits of the base, named PAAF1, inhibits the binding of the RP and the CP (Park *et al*., 2005). Whether external factors are involved in the formation of the lid and the base is also a question that remains to be answered.

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