Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in *Saccharomyces cerevisiae*

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Nob1p is a nuclear protein that forms a complex with the 19S regulatory particle of the 26S proteasome and with uncharacterized nuclear protein Pno1p. Overexpression of *NOB1* overrode the defects in maturation of the 20S proteasome of *ump1Δ* cells, and temperature-sensitive *nob1* and *pno1* mutants exhibited defects in the processing of the β subunits and in the assembly of the 20S and the 26S proteasomes. A defect in either *NOB1* or *PNO1* caused accumulation of newly formed Pre6p in the cytoplasm, whereas Pre6p of the *ump1Δ* strain accumulated in the nucleus irrespective of the temperature. Here we present a model proposing that (1) Nob1p serves as a chaperone to join the 20S proteasome with the 19S regulatory particle in the nucleus and facilitates the maturation of the 20S proteasome and degradation of Ump1p, and (2) Nob1p is then internalized into the 26S proteasome and degraded to complete 26S proteasome biogenesis.

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The 26S proteasome (>2000 kD) is required specifically for the degradation of ubiquitinated proteins, and consists of the 19S regulatory particle (RP; the RP is also referred to as PA700 in mammals and the μ particle in *D. melanogaster*) and the 20S proteasome (CP; core particle). The 20S proteasome is the catalytic core of the 26S proteasome (Peters et al. 1994; Coux et al. 1996; Baumeister et al. 1998). The 20S proteasome of *S. cerevisiae* contains 14 subunits (Heinemeyer et al. 1994), and the 19S RP contains at least 17 subunits (Glickman et al. 1998a). The 19S RP can be separated into two subcomplexes, that is, the base, consisting of six proteasomal ATPases (Rpt1–Rpt6) and two non-ATPase subunits (Rpn1 and Rpn2) and the lid, consisting of Rpn3, 5, 6, 7, 8, 9, 10, 11, and 12 (Glickman et al. 1998b; Saeki et al. 2000).

The yeast 20S proteasome consists of seven α -type and seven β -type subunits, which are assembled into an $\alpha_7\beta_7\beta_7\alpha_7$ cylinder-like structure. Crystallographic analysis of the 20S proteasome revealed that there is no opening in the outer ring for a substrate polypeptide (Groll et al. 1997). Therefore, a gating system is necessary for digesting polypeptide substrate inside the 20S proteasome (Groll et al. 2000; Kohler et al. 2001). Three of the seven β subunits possess an N-terminal prosequence that is removed upon maturation of the 20S pro-

teasome to give an active site. The yeast counterparts of these β subunits are Pre2p/Doa3p, Pup1p, and Pre3p (Heinemeyer et al. 1993; Chen and Hochstrasser 1996; Arendt and Hochstrasser 1997; Heinemeyer et al. 1997).

Assembly or maturation of multisubunit complexes such as proteasomes is likely to be assisted by a chaperone-like protein(s). One such factor is a short-lived protein of yeast, Ump1p. Ump1p is found inside the immature 20S proteasome containing unprocessed β -subunits and is not detected in the mature 20S proteasome, indicating that it is digested at a particular stage of the assembly of the 20S proteasome (Ramos et al. 1998). Thus Ump1p acts like a chaperone. The yeast Ump1p has homologs in human, mouse, rat, fruit fly, the plant *Arabidopsis thaliana*, and several other eukaryotic organisms. The mammalian Ump1p homolog is copurified with the proteasome precursors and is referred to as POMP (proteasome maturation protein; Witt et al. 2000) or Proteasemblin (Griffin et al. 2000). Therefore, the presence of a factor that facilitates the assembly or maturation of the 20S proteasome is a general phenomenon.

In contrast to the subunits of the 20S proteasome, those of the 19S RP are diverse in amino acid sequences, except for the six ATPases contained in a base. How these divergent polypeptides assemble into a complex is largely unknown. In our previous study, we proposed that Rpn9p plays an important role in the assembly or stabilization of the 26S proteasome and that Rpn9p is needed for accommodation of Rpn10p into the 26S proteasome (Takeuchi et al. 1999). Two amino acid sequence motifs, PCI and MPN, were found in the lid sub-

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units (Glickman et al. 1998b). Since these motifs are present in the subunits consisting of several protein complexes (proteasome, COP9 signalosome, eIF3), they probably play roles in the assembly of each complex (Glickman et al. 1998b). Actually, the PCI motif in the subunit of the COP9 signalosome was shown to be responsible for subunit assembly (Tsuge et al. 2001).

Because the 20S proteasome does not use polyubiquitinated protein as a substrate, the association of the 19S RP with the 20S proteasome is a crucial step for the ubiquitin-proteasome system. Given that the 19S RP and the 20S proteasome are assembled independently, there must be a reaction that interconnects these subcomplexes. The 19S RP and the 20S proteasome can be assembled into the 26S proteasome in the presence of ATP *in vitro* (Chu-Ping et al. 1994; Saeki et al. 2000); however, it is not known whether the same assembly mechanism is functioning *in vivo*.

The main reason for our poor understanding of the maturation/assembly mechanism of the 26S proteasome is our limited information about proteins associated with proteasomes. An obvious approach to break through this problem is a large-scale physical analysis of the proteasome-interacting proteins (Verma et al. 2000). We are attempting to solve the same problem in a different way. In a previous report (Tone et al. 2000), we identified *NOB1*, an essential gene of *S. cerevisiae*, as a gene encoding a protein interacting with Rpn12p, by a two-hybrid assay. We showed that Nob1p is short-lived and is associated with the proteasomes of cells during exponential growth. However, the role of Nob1p in proteasome function is unknown. In this report, we present a functional analysis of Nob1p. Our genetic and biochemical work indicates that Nob1p not only interacts with the components of the 19S RP but also plays a crucial role in the maturation of the 20S proteasome.

Results

The NOB1 gene is required for ubiquitin-mediated proteolysis

We reported previously that Nob1p, an essential protein, is associated with the 26S proteasome (Tone et al. 2000). However, it remains unknown how Nob1p exerts its essential function and whether Nob1p has a functional connection with the 26S proteasome. To obtain a clue for a site of mutation to be introduced into the *NOB1* gene, we aligned the amino acid sequences of Nob1p and Nob1p-like proteins found in other eukaryotes. The alignment reveals the presence of the conserved regions, designated Nob1p box1–Nob1p box3 (Fig. 1A). To explore the function(s) of Nob1p, we introduced mutations to the conserved amino acids by site-directed mutagenesis. The *nob1-4* mutation (*nob1-L280R Q281G M282G*) conferred a temperature-sensitive growth on the cells (Fig. 1B). We found that the *nob1-4* mutation is recessive and that Nob1-4p was present in extract prepared from the *nob1-4* cells grown at various temperatures. The distribution of Nob1-4p across the glycerol density gradient

was the same as that of Nob1p. As shown in Figure 1C, multiubiquitinated proteins accumulated in the *nob1-4* cells incubated at a restrictive temperature. These results indicate that Nob1p function is necessary for ubiquitin-proteasome-dependent proteolysis.

Nob1p is required for the assembly of the 26S proteasome

To explore Nob1p function in the ubiquitin-proteasome system, we first attempted to compare proteasomes produced by *nob1-4* cells with those of wild-type cells. W303-1A (wild-type) and Y138 (*nob1-4*) cells grown in YPD medium to mid-logarithmic phase were diluted to 0.5×10^7 cells/mL in YPD and incubated at 37°C for 2, 4, or 6 h. Crude extracts from these cells were analyzed by glycerol density gradient centrifugation (Fig. 2A). One peak of activity hydrolyzing Suc-LLVY-MCA was centered at fraction 15, corresponding to the peak of the 20S proteasome, and the other peak was centered at fraction 21 in wild-type cells, corresponding to the peak of the 26S proteasome. The 20S proteasome peak and the 26S proteasome peak in the wild-type extract persisted throughout the incubation at 37°C (Fig. 2A, left panel). In contrast, two peaks corresponding to the 20S and 26S proteasomes were seen in the 0-h sample of *nob1-4* extract, whereas the peak of the 20S proteasome gradually disappeared and the peak of the 26S proteasome decreased (Fig. 2A, right panel). Immunoblot analysis of fractions separated by glycerol density gradient centrifugation revealed prominent changes in the distribution of proteasome subunits in the *nob1-4* extract; the 20S proteasome signal disappeared from its normal position and appeared at lighter fractions during incubation at 37°C (Fig. 2B, right panel). The components of the 19S RP also showed a characteristic change in their distribution in that they seemed to dissociate from the 26S proteasome and to accumulate at the position of the 19S RP (Fig. 2B, right panel, fractions 13, 15). Denser material reacting with anti-20S antibody accumulated during incubation at 37°C in both wild-type and *nob1-4* cells, indicating production of aggregates of proteasomes.

To compare the molecular species of proteasomes in *nob1-4* cells and those in the wild-type cells, we analyzed crude extract prepared from either the wild-type cells or the *nob1-4* cells by native PAGE. Wild-type cells and *nob1-4* cells grown to logarithmic phase at 25°C were shifted to 37°C. At the indicated time after the shift, extracts were prepared and separated by native PAGE followed by peptidase assay and Western blotting using anti-20S proteasome antibody. Peptidase activity in the *nob1-4* extract was extinguished during incubation at 37°C (Fig. 2C, left panel). During this period, fast-moving proteins reacting with anti-20S proteasome antibody accumulated in the *nob1-4* extracts (Fig. 2C, right panel). Next, we examined whether Nob1p participates in the stabilization of the 20S proteasome or in the maturation of the 20S proteasomes. These possibilities can be discriminated by detecting precursor forms of the β subunits. At the indicated time after shift up, extracts were

A

B

C

Figure 1. Characterization of the *nob1-4* mutant. (A) Alignment of amino acid sequences of Nob1p and its putative homologs. Identical residues are highlighted. Conservative changes are shadowed. Asterisks indicate the site of the *nob1-4* mutations. Three highly conserved regions designated box1, box2, and box3 are shown at the bottom. Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Ce, *Caenorhabditis elegans*; Dr, *Drosophila melanogaster*; h, *Homo sapiens*; At, *Arabidopsis thaliana*. (B) W303-1A and Y138 cells were streaked across two YPD plates; one was incubated at 25°C and the other at 37°C for 3 d. (C) W303-1A (wild-type), Y138 (*nob1-4*), and YK109 (*rpn12-1*) were grown in YPD and then shifted to 37°C. At the indicated times after the shift, extracts were prepared by the hot extraction method and separated by SDS-PAGE, followed by Western blotting using anti-polyubiquitin and anti-actin antibodies.

prepared from the cells of test strains expressing *PRE2*-HA or *PUP1*-HA, followed by Western blotting analysis using anti-HA antibody (Fig. 2D). Precursors of the β -subunits were hardly detected in the wild-type culture, whereas a large amount of the precursors accumulated in the *nob1-4* extract. Crude extracts from cultures incu-

bated for 6 h at 37°C were fractionated by glycerol density gradient centrifugation. A substantial amount of precursor forms was seen in the lighter fractions derived from the *nob1-4* extract (Fig. 2E). These phenotypes are similar to those shown by *ump1 Δ* cells (Ramos et al. 1998). These results indicate that Nob1p plays an impor-

Figure 2. Nob1p is required for the formation of 20S proteasomes. (A) Extracts were prepared from the wild-type or *nob1-4* strain incubated at 37°C for the indicated periods and subjected to glycerol density gradient centrifugation. Peptidase activity was assayed using Suc-LLVY-MCA as substrate. (○) Activity without SDS; (●) activity with 0.05% SDS. (B) Proteins in each fraction of the above glycerol density gradient centrifugation were analyzed by Western blotting using anti-20S, anti-Rpt1p, and anti-Rpn12p antibodies. (C) Extracts prepared from wild-type cells and *nob1-4* cells incubated at 37°C for the indicated periods were separated by native PAGE. (Left) An in-gel peptidase assay. (Right) Western blotting using anti-20S antibody. (D) Extracts from Y148 (*PUP1-HA*), Y151 (*nob1-4 PUP1-HA*), Y153 (*PRE2-HA*), and Y154 (*nob1-4 PRE2-HA*) cells incubated for the indicated periods were analyzed by Western blotting using anti-HA antibody and anti-actin antibody. (E) Extracts were prepared from Y148, Y151, Y153, and Y154 cells incubated at 37°C for 6 h and subjected to fractionation by glycerol density gradient centrifugation. Each fraction was subjected to SDS-PAGE followed by Western blotting using anti-HA antibody. p, precursor form of Pre2p or Pup1p; m, mature form of Pre2p or Pup1p.

tant role in the maturation of the 20S proteasome. The precursor form of Pup1p was seen at the 0 time after shift-up, whereas the 26S proteasome did not contain the precursor form of Pup1p, indicating that an excess amount of Pup1p precursor may be produced, and these excess Pup1p precursors may remain unincorporated into the proteasomes.

Genetic interaction between NOB1 and UMP1

As mentioned above, the function of Nob1p seems similar to that of Ump1p in that either *nob1* or *ump1* mutation resulted in a defect in the maturation of the 20S proteasome. This finding prompted us to seek evidence

of genetic interactions between *NOB1* and *UMP1*. We found that overexpression of *NOB1* suppressed the temperature sensitivity shown by *ump1Δ* cells (Fig. 3A), whereas overexpression of *UMP1* did not suppress the temperature sensitivity of the *nob1-4* strain. *ump1Δ* cells overexpressing *NOB1* produced more 26S proteasome than did the *ump1Δ* cells (Y. Tone and A. Toh-e, unpubl.).

Next we examined the distribution of Ump1p in *nob1-4* cells. Extracts were prepared from the wild-type or *nob1-4* cells expressing *UMP1-HA*. Crude extracts from cultures incubated for 6 h at 37°C were fractionated by glycerol density gradient centrifugation. Ump1p-HA was detected in fractions 9–13 in wild-type extract,

Figure 3. *NOB1* genetically interacts with *UMP1* and *RPT1*. (A) *NOB1* as a multicopy suppressor of *ump1Δ*. YCp*UMP1* (pYT460), YCp*NOB1* (pYT464), *P_{TDH3}*-*NOB1* (pYT465), and pRS314 (vector) were separately introduced into JD59 (*ump1Δ*), and a representative transformant of each experiment was streaked across YPD plates, one of which was incubated at 25°C or 36.5°C for 3 d. (B) Ump1p accumulated in *nob1-4* cells. Extract from Y168 (*UMP1*-HA) or Y169 (*nob1-4 UMP1*-HA) cells incubated at 37°C for 6 h was fractionated by glycerol density gradient centrifugation. Proteins in each fraction were separated by SDS-PAGE, followed by Western blotting using anti-HA antibody. *, cross-reacting protein. (C) Ump1p remains in the proteasomes in *nob1-4* cells. Pooled fractions (18–20) from Y168 (lanes 1,3) and Y169 (lanes 2,4) cells were pooled and mixed with anti-20S antibody (Total, lanes 1,2). Antibody was precipitated with protein A-Sepharose beads. After the beads were washed, bound proteins were eluted (ppt, lanes 3,4). Proteins were detected using anti-20S, anti-Nob1p, and anti-HA antibodies. (D) *NOB1* as a multicopy suppressor of *cim5-1* (*rpt1-1*). YCp*RPT1* (pJUN290), YCp-*NOB1* (pYT53), *P_{TDH3}*-*NOB1* (pYT82), and pKT10 (vector) were separately introduced into J88 (*cim5-1*), and a representative transformant from each experiment was streaked across YPD plates, one of which was incubated at 25°C or 35°C for 3 d. (E) Nob1p forms a complex with the 19S RP. Fractions (13–15) obtained by glycerol density gradient centrifugation from J106 (*RPT1*-6×His; lanes 1,3) or W303-1A (lanes 2,4) cell extracts (data not shown; note that these fractions do not contain the 26S proteasome) were treated with Ni-NTA agarose beads to precipitate His-tagged Rpt1p (Total, lanes 1,2; Eluate, lanes 3,4). Proteins were detected using anti-Nob1p, anti-Rpt1p, anti-Rpn12p, and anti-20S antibodies.

whereas Ump1p-HA distribution in *nob1-4* extract was extended to fraction 17 (Fig. 3B). To compare the amount of Ump1p in 18–20 fractions derived from wild-type extract with those from *nob1-4* extract, a 100-μL portion was collected from each fraction (18–20), pooled, precipitated by acetone, and separated by SDS-PAGE. Ump1p-

HA was detected in the *nob1-4* sample, whereas Ump1p was hardly detected in the comparable fraction of wild-type sample (Fig. 3C, left). Next, the pooled fractions were treated with Sepharose A agarose conjugated with anti-20S antibody to pull down proteasomes, and the precipitates were analyzed by immunoblotting. The result

demonstrated that Ump1p was effectively coprecipitated with 20S proteasome in *nob1-4* cells, although the 20S proteasome in the *nob1-4* fraction seemed inefficiently precipitated with anti-20S antibody (Fig. 3C, right). This result, along with the genetic interaction between *NOB1* and *UMP1*, indicates that Nob1p facilitates the degradation of Ump1p and that Nob1p may function after Ump1p in the maturation of the 20S proteasome.

Nob1p is localized to the nucleus and exists as a complex with Pno1p

In glycerol density gradient centrifugation, the distribution of Nob1p overlaps with that of the 20S proteasome. Because Nob1p, like Ump1p, is required for the maturation of the 20S proteasome, we examined whether Nob1p is associated with proteasomes. Pre1p-FH was pulled down by Ni-NTA beads from the pooled fraction (fractions 12–15 in Fig. 4B) containing both Nob1p and the 20S proteasome, and the resultant precipitates were analyzed by Western blotting using anti-Flag antibody. The result shown in Figure 4C (lanes 2,5) indicates that Nob1p in this fraction is not associated with the 20S proteasome. The finding that Nob1p is distributed in fractions containing higher-molecular-weight proteins than the Nob1p monomer suggests that Nob1p is associated with a large protein complex. One candidate for Nob1p-interacting protein is Yor145cp, which we identified by a two-hybrid screening (Fig. 4A). We designated it *YOR145c PNO1* (partner of *Nob1p*). The 14th–274th amino acid residue of the *PNO1* open reading frame (ORF) was fused with the Gal4 activation domain in pNOT9.

To examine whether Nob1p forms a complex with Pno1p, we fractionated extract of a strain expressing Pno1p-His6-Flag by glycerol density gradient centrifugation. In this gradient, Nob1p was detected in fractions 9–23 and Pno1p was detected in fractions 13–31 (Fig. 4B). Fractions 12–15, which contained the 20S proteasome, were pooled and treated with Ni-NTA agarose to pull down Pno1p-FH. The result demonstrated that Nob1p was coprecipitated with Pno1p (Fig. 4C, lane 6). These data show that Nob1p and Pno1p form a complex. Interestingly, it has been reported that Pno1p, an essential protein, was localized to the nucleolus (Grava et al. 2000) and was copurified with the nuclear pore complex (Rout et al. 2000).

A conditional *pno1* mutant is needed for characterization of the *PNO1* gene. We noted that Pno1p contains the KH-domain that is conserved in RNA-binding and several other proteins (Tollervey et al. 1991; Siomi et al. 1993, 1994). Since it is known that the mutation at the glycine residue in the conserved domain causes temperature-sensitivity of *FMR1* (Verkerk et al. 1991) and *GLD-1* (Jones and Schedl 1995), we introduced a G203D substitution in Pno1p by in vitro mutagenesis to generate a *pno1-1* mutant. This mutant showed temperature-sensitive growth (data not shown). We found that Nob1p is localized normally in the *pno1-1* mutant (Y. Tone and A. Toh-e, unpubl.). Crude extracts from wild-type and

pno1-1 cultures expressing *PRE2-HA* (Y189) or *PUP1-HA* (Y190) for 4 h at 37°C were fractionated by glycerol density centrifugation. In contrast to wild-type extract (Fig. 4D, left), a substantial amount of precursor forms of Pre2p and Pup1p was seen in lighter fractions derived from the *pno1-1* extract (Fig. 4D, right). Thus, Pno1p plays some roles in the maturation of the 20S proteasome, as does Nob1p.

To investigate the localization of Nob1p, we attempted to express Nob1p-GFP fusion protein. The *NOB1* gene was replaced with the GFP-tagged *NOB1* gene. Strains expressing *NOB1-GFP* were cultured to mid-log phase, and the cells were observed under a fluorescence microscope. The green fluorescence signal of Nob1p-GFP was localized in the nucleus and coincided with Hoechst 33342 staining of the nucleus (Fig. 5A). The localization of Nob1p in the nucleus is consistent with the result that Nob1p formed a complex with nuclear protein Pno1p.

Nob1p and Pno1p, but not Ump1p, are required for incorporation of the newly synthesized Pre6p

Because proteasomes are assembled in the nucleus (Lehmann et al. 2002), it is plausible that precursors of proteasomes may be accumulated outside of the nucleus under conditions in which the assembly processes are malfunctioning. To test this possibility, we constructed the strain containing *PRE6* and *P_{GAL1}-PRE6-GFP*. This construct enabled us to follow newly synthesized Pre6p after induction of the *P_{GAL1}*-driven *PRE6-GFP*. A strain containing the *GAL1*-driven *PRE6-GFP* gene in the background of wild-type, *nob1-4*, *pno1-1*, or *ump1Δ* was cultured to mid-log phase at 25°C, and transferred to medium containing galactose at 37°C. Before the addition of galactose, green fluorescent signals were not seen in any of the strains. During the first h of the induction, the green fluorescent signal of Pre6p-GFP was localized in the nucleus and the nuclear envelope in wild-type cells, whereas the GFP signals were delocalized in *nob1-4* cells and *pno1-1* cells at a restrictive temperature. In contrast, GFP signals were localized to the nucleus in *ump1Δ* cells at the restrictive temperature (Fig. 5B). These results indicate that Nob1p and Pno1p, but not Ump1p, are required for nuclear transfer of the 20S proteasome.

Nob1p attaches on the surface of the proteasomes

We previously showed that Nob1p is an unstable protein existing in growing cells that can be stabilized by MG132 and that Nob1p was coprecipitated with Rpt1p (Tone et al. 2000). As described above, Nob1p participates in the maturation of the 20S proteasome, as does Ump1p. Because Ump1p is degraded by the 20S proteasome upon completion of the assembly, the functional similarity between Nob1p and Ump1p in the maturation process of the 20S proteasome led us to examine whether Nob1p is incorporated into the proteasomes like Ump1p. Given that Nob1p is trapped within the proteasome and

(Figure 4 legend on facing page)

subsequently degraded by it, Nob1p is expected to be present in fractions containing the 26S proteasome where proteasome activity is inhibited by MG132.

We prepared wild-type extract to which MG132 or DMSO was added. Extract with or without MG132 was fractionated by glycerol density gradient centrifugation,

followed by Western blotting using anti-Nob1p and anti-20S antibodies. Peptidase activity (Fig. 6A) was at a similar level irrespective of the presence of MG132 in glycerol gradient. This is likely due to dilution of the inhibitor in the assay mixture. Nob1p was detected in fractions 9–25, peaking at fraction 21 of each extract (Fig. 6A). In fractions 21 and 23, both RP₁CP and RP₂CP were present in a similar ratio (Fig. 6B). To examine whether Nob1p is inside the lumen of the 26S proteasome, proteasomes in fraction 23 derived from the MG132-treated extract were exposed to graded amounts of trypsin. As shown in Figure 6C,b (middle panel), Nob1p in fraction 23 was partly protected from digestion by trypsin, whereas Nob1p was degraded in the presence of 0.01% SDS (Fig. 6C,b, right panel). Nob1p inside the proteasomes was degraded by incubation in the absence of MG132 in the reaction mixture (Fig. 6C,b, left panel). In contrast, Nob1p in fraction 17 derived from MG132-treated extract and in fraction 23 derived from control extract was degraded by trypsin (Fig. 6C,a,c). Similar experiments using fraction 21 gave rise to the same results (data not shown). These results indicate that Nob1p exists outside of the 20S proteasome in a trypsin-accessible state and that Nob1p is internalized into the 26S proteasome when subcomplex assembly is completed. This is in clear contrast to the behavior of Ump1p, which is sequestered in the lumen of the immature 20S proteasome and degraded upon maturation of the 20S proteasome (Ramos et al. 1998).

NOB1 suppresses the growth defect of cim5-1/rpt1-1 cells

Originally, *NOB1* was identified as a gene interacting with *RPN12* encoding one of the lid components. We further looked for genetic interactions between *NOB1* and the 19S RP genes by testing suppression of proteasome mutants by the *NOB1* gene on a multicopy vector. Plasmid pYT465 (*NOB1* on pKT10) was introduced into the *pre1-1*, *rpn12-1*, *rpn3-1*, *rpn9Δ*, *rpt3-1*, and *cim5-1* strains. Only *cim5-1* cells carrying this plasmid grew well at 35°C (Fig. 3D). To examine a physical interaction between Rpt1p and Nob1p, we pulled down Rpt1p with Ni-NTA beads from pooled fractions containing the 19S RP and the 20S proteasome but not the 26S proteasome (gradient profile not shown). Coprecipitated proteins were eluted and separated by SDS-PAGE followed by

Western blotting using anti-Nob1p, anti-Rpt1p, anti-20S proteasome, and anti-Rpn12p antibodies. As shown in Figure 3E, Nob1p exists in a complex containing Rpt1p and Rpn12p but not the 20S proteasome, most probably the 19S RP.

Discussion

Nob1p, an essential and short-lived protein, was identified as a protein interacting with Rpn12p by a two-hybrid screening (Tone et al. 2000). By exploiting a *nob1* mutant, we showed in the present study that Nob1p plays pivotal roles in biogenesis of the proteasomes. A Basic Local Alignment Search Tool (BLAST) search using Nob1p as the query sequence revealed the presence of putative homologs in various organisms, none of which has been studied with regard to its function. Here, we propose that Nob1p acts on the formation of the 26S proteasome in eukaryotes.

Nob1p is required for the formation of the 20S proteasome

Early studies on the mechanism of proteasomal maturation were carried out by pulse-chase experiments using animal cells. These studies revealed that a precursor consisting of one α -ring and one β -ring containing unprocessed β -subunits is first assembled as a 15S half-proteasome complex. The assembly of two 15S complexes results in processing of the β subunit N termini and thus formation of the mature 20S proteasome (Yang et al. 1995; Nandi et al. 1997). By exploiting the yeast system, Chen and Hochstrasser (1996) devised a model of assembly of the yeast 20S proteasome, in which cleaving off the propeptides from unprocessed subunits generates the active sites of the proteasomes when two half-proteasome precursors are assembled. More recently, Ramos et al. (1998) found a chaperone, Ump1p, inside of the immature 20S proteasome, which is degraded upon the maturation of the 20S proteasome.

The temperature-sensitive *nob1-4* mutant displayed defects in the maturation of the 20S proteasome and accumulation of unprocessed β -subunits. These phenotypes are quite similar to those of the *ump1Δ* mutant. These observations led us to conclude that Ump1p and Nob1p both function in the formation of the 20S proteasome.

Figure 4. Nob1p forms a complex with Pno1p. (A) Two-hybrid interaction between Nob1p and Pno1p. pNOT9: amino acid residues from 31 to 274 of Pno1p was fused to Gal4AD. pYT66: amino acid residues from 1 to 459 of Nob1p was fused to LexA. (B) Profile of proteasomes. Y184 (*PNO1-FH*) cell extract was separated by glycerol density gradient centrifugation and analyzed by Western blotting using anti-Nob1p, anti-Flag, anti-Rpn12p, and anti-20S antibodies. Fraction numbers of the lower panel correspond to those in the upper panels. (C) Nob1p forms a complex with Pno1p. Fractions (12–15) from W303-1A (gradient profile not shown; lanes 1,4), Y166 (*PRE1-FH*; lanes 2,5, gradient profile not shown), and Y184 (lanes 3,6; panel B) cells were treated with Ni-NTA agarose beads. After the beads were washed, bound proteins were eluted with 0.2 M imidazole (Total, lanes 1–3; Eluate, lanes 4–6). Proteins were detected using anti-Nob1p and anti-Flag antibodies. (D) Profile of proteasomes in *pno1-1* cells. Y148 (*PUP1-HA*), Y153 (*PRE2-HA*), Y189 (*pno1-1 PUP1-HA*), and Y190 (*pno1-1 PRE2-HA*) strains were grown to mid-logarithmic phase at 25°C and then shifted to 37°C. At 4 h after the shift, extract was prepared from each culture and subjected to fractionation by glycerol density gradient centrifugation. Each fraction was subjected to SDS-PAGE, followed by Western blotting using anti-HA antibody.

Figure 5. Cytological analyses. (A) Localization of Nob1p-GFP. Y178 (Nob1p-8×GFP) was monitored by fluorescence microscopy. Cells were viewed by Nomarski optics, and the Hoechst 33342-stained nuclei were viewed by the UV channel. (B) Localization of Pre6p-GFP. Y199 ($PRE6 P_{GALI}-PRE6-8\times GFP$), Y201 ($nob1-4 PRE6 P_{GALI}-PRE6-8\times GFP$), Y202 ($pno1-1 PRE6 P_{GALI}-PRE6-8\times GFP$), and Y208 ($ump1\Delta PRE6 P_{GALI}-PRE6-8\times GFP$) were incubated in SC-Raff medium at 25°C and transferred to SC-Gal medium at 25°C or 37°C. At 1 h after the shift, cells were monitored by fluorescence microscopy. Bars, 10 μ m.

Nob1p and Ump1p play different roles in proteasome formation

In glycerol density gradient, Ump1p exists in the fractions containing the half-proteasome, whereas Nob1p is present in broad fractions, from the half-proteasome to the singly capped 26S proteasome (Tone et al. 2000). This result is likely to reflect the fact that Ump1p is degraded just after the assembly of the 20S proteasome (Ramos et al. 1998), whereas Nob1p seems to be degraded just after the doubly capped 26S proteasome is completed (Fig. 6C; Tone et al. 2000). Cellular localization of these proteins

should provide useful information about their function. We demonstrated that Nob1p is localized to the nucleus (Fig. 5A), most probably as a complex either with the 19S RP or with Pno1p. Lehmann et al. (2002) showed that Ump1p localizes in the nucleus. From the fact that Ump1p makes a complex with a half proteasome, they concluded that the maturation of 20S proteasomes are completed in the nucleus. This idea is supported by the findings that (1) the 20S proteasome subunits are localized in the cytoplasm and the nucleus (Reits et al. 1997), (2) the nuclear localization signals (NLSs) are found in the α subunits of 20S proteasomes (Tanaka et al. 1990),

Figure 6. Nob1p is internalized inside the proteasomes upon maturation of the 26S proteasome. (A) Distribution of Nob1p. Extract was prepared from wild-type cells. After addition of MG132 or dimethyl sulfoxide (DMSO) to extract, the extract was separated by glycerol density gradient centrifugation. Peptidase activity (top panel) was assayed using SUC-LLVY-MCA as substrate, and Nob1p and the 20S proteasome were detected by Western blotting (bottom panel). *, cross-reacting proteins. (B) Native PAGE analysis of fractions 21 and 23. Rpn12p was detected by Western blotting. (C) Trypsin sensitivity of Nob1p. Assays of trypsin sensitivity are described in Materials and Methods. Reaction products were separated by SDS-PAGE and detected using anti-Nob1p antibody. A reagent (shown above each panel) was included in the reaction mixture for trypsin digestion. (a) Fraction no. 23 from extract treated with DMSO. (b) Fraction no. 23 from extract treated with MG132. (c) Fraction no. 17 from extract treated with MG132.

(3) these NLSs are required for their nuclear localization (Nederlof et al. 1995; Knuehl et al. 1996) and for assembly of the 20S proteasome (Wang et al. 1997), and (4) the precursor of 20S proteasomes were accumulated in *srp1-49* cells carrying a defect in importin α (Lehmann et al. 2002).

Our observations that immature 20S proteasomes were accumulated in the *nob1-4* mutant cells and that Ump1p was stabilized in the *nob1-4* mutant cells (Fig. 3B) strongly suggest that Nob1p facilitates the maturation of the 20S proteasome by Ump1p. Nob1p, at least some fraction of it, forms a complex with Pno1p that was reported to be copurified with the nuclear pore complex (Rout et al. 2000), suggesting that Nob1p meets with immature 20S proteasomes containing Ump1p in the nucleus. It should be noted that Ump1p was coprecipitated with the proteasomes in *nob1-4* cells grown at a restrictive temperature (Fig. 3C) and that Pre6p was not transported into the nucleus in the *nob1-4* mutant at a restrictive temperature (Fig. 5B). These observations suggest that Ump1p can be incorporated into 20S proteasome precursors in the cytoplasm, which are then transported into the nucleus to be matured. This sequence of maturation is consistent with the results of genetic suppression of *ump1 Δ* by *NOB1*: a multicopy of *NOB1* suppressed the temperature sensitivity of *ump1 Δ* (Fig. 3A), whereas a multicopy of *UMP1* did not suppress the temperature sensitivity of *nob1-4*. The observation that the nuclear transfer of the newly synthesized Pre6p was inhibited in *nob1-4* cells and in *pno1-1* cells at their restrictive temperature whereas *ump1 Δ* cells incorporated newly synthesized Pre6p into the nucleus suggests that immature 20S proteasomes are delivered into the nucleus with the aid of the nuclear import machinery in the cytoplasm and the Nob1p–Pno1p complex in the nucleus. Ump1p does not seem to play a role in this transportation process.

A possible function of Nob1p in maturation of the 26S proteasome

We demonstrated that *NOB1* overexpression suppressed the temperature sensitivity of *cim5-1* (Fig. 3D). In addition to genetic evidence of interaction between Nob1p and the 19S RP, we demonstrated the physical interaction between them by a coprecipitation experiment (Fig. 3E). It is attractive to assume that the immature 20S proteasome containing Ump1p is transferred to the Nob1p–19S RP complex and then the maturation of the 20S proceeds. At this stage, Nob1p may exist at the interface between the 19S RP and the 20S proteasome. This process is inferred from the following lines of evidence (Fig. 6): When extract was prepared in the presence of MG132, an inhibitor of the proteasome activity, remnants of Nob1p were detected after treatment of the 26S proteasome fraction with trypsin. In contrast, trypsin-resistant Nob1p was not seen in the 26S proteasome fraction prepared in the absence of MG132. These results, along with our previous finding that Nob1p coexisted with the RP₁CP proteasome (Tone et al. 2000), suggest

that Nob1p is internalized into the 26S proteasome when the attachment of the 19S RP at the end of the 20S proteasome is completed. When the MG132-treated 26S proteasome was attacked by trypsin, the size of Nob1p decreased (Fig. 6C, middle). We believe that the size difference after the trypsin attack occurred because a part of Nob1p that protrudes from the 26S proteasome was cleaved off by trypsin.

Model of the formation of the 26S proteasome

We propose a model, illustrated in Figure 7, for biogenesis of the 26S proteasome. The process of assembly and maturation of the 20S proteasome is based on the models proposed by Chen and Hochstrasser (1996) and Ramos et al. (1998). The earliest precursor of the 20S proteasome identified thus far is the 15S half-proteasome ($\alpha 7\beta 7$) in which pro- β subunits are present (Fig. 7, step 1). Ramos et al. (1998) discovered Ump1p, which functions as a chaperone to facilitate maturation steps of the 20S proteasome. Lehmann et al. (2002) showed that importin transports the precursor of the 20S proteasome containing Ump1p into the nucleus (Fig. 7, step 2). Nob1p is localized in the nucleus and forms a complex with Pno1p and with the 19S RP. It is possible to assume that Nob1p–19S RP complex binds the precursor of the 20S proteasome (Fig. 7, step 3). Step 1 and step 2 can proceed without Ump1p, although less efficiently, because the *ump1 Δ* strain is viable. Next, Ump1p is degraded by the 20S proteasome containing Nob1p (Fig. 7, step 4). When the 19S RP makes a tight contact with the 20S proteasome, Nob1p is internalized inside the 26S proteasome, followed by degradation (Fig. 7, step 5). Finally, the doubly capped 26S proteasome is produced and, by the time it is completed, Nob1p vanishes (Fig. 7, step 6). It is unclear how the singly capped 26S proteasome that has Nob1p in a trypsin-accessible state is derived. We believe there may be multiple pathways leading to this form of the 26S proteasome.

Our model implies that there are two different types of the 26S proteasome; one is synthesized de novo, and the other is the recycling 26S proteasome. Because Nob1p is present only in the growing phase, de novo synthesis of the proteasomes may be restricted during this period. Cells in the stationary phase do not produce the proteasomes de novo, but these cells do not suffer from a shortage of proteasomes, because they have a large amount of the 26S proteasome. The 26S proteasome may dissociate into the 20S proteasome and the 19S RP, and in turn, the 20S proteasome may associate with the 19S RP in an energy-dependent manner, as seen in in vitro experiments (Chu-Ping et al. 1994; Saeki et al. 2000). Nob1p-depleted stationary phase cells can begin growing without expressing the *NOB1* gene, and transcription of the *NOB1* gene begins when the cells enter logarithmic phase to produce new proteasomes (Y. Tone and A. Toh-e, unpubl.). *NOB1* is a key factor linking the proteasome and cellular growth, and therefore investigation of the *NOB1* function will shed some light on the mechanism of growth control by the proteasomes.

Figure 7. A model of Nob1p function in proteasome biogenesis. Subunits of the 20S proteasome assemble into a proteasome precursor (step 1). Structure A is a proteasome precursor complex containing Ump1p and unprocessed β subunits. In step 2, precursor complexes are transported from the cytoplasm to the nucleus. In step 3, precursor complexes and the 19S regulatory complex join as a result of interaction between premature 20S proteasome and Nob1p–19S RP complex (structure B). During construction of structure C, Ump1p is degraded and pro-sequences of the β -subunits are processed (step 4). Only after making a tight complex between the 20S proteasome and the 19S RP is Nob1p internalized in the 26S proteasome (step 5) and degraded inside the newly formed proteasome (step 6). Only structures A, B, and D are stable enough to be detected in wild-type cells. Under the conditions where proteasome activity is inhibited, Nob1p can survive in structure C.

Materials and Methods

Microbiological methods

Yeast strains and plasmids used in this study are listed in Table 1. For yeast culture, YPD and SD were prepared as described (Sherman 1991). SC-Ura was prepared by supplementing 0.5% casamino acid (Difco), 10 μ g/mL tryptophan, and 400 μ g/mL adenine sulfate to SD. SC-Trp was prepared by adding 0.5% casamino acid, 10 μ g/mL uracil, and 400 μ g/mL adenine sulfate to SD. SC-Raff was prepared by adding 2% raffinose in place of glucose to SC-U. SC-Gal was prepared by adding 0.5% galactose in place of glucose to SC-U. Methods for yeast genetics were described previously (Sherman 1991). Yeast transformation was performed by the method described by Ito et al. (1983). The permissive temperature for yeast temperature-sensitive mutants was 25°C, and the restrictive temperature for yeast temperature-sensitive mutants was 35°C–37°C. Ump1p, Nob1p, Pno1p, and proteasome subunits (Pre1p, Pup1p, and Pre2p) were tagged at their C terminus as follows. The N-terminally truncated version of the respective gene was amplified by PCR, and the resulting fragments were inserted in-frame just upstream of the indicated tag on pRS303 or pRS306 (Sikorski and Hieter 1989). Each of the resulting plasmids was linearized within the coding sequence of the yeast gene and targeted into the *S. cerevisiae* genome, yielding strains with one copy of the respective tagged gene expressing from its natural promoter. The epitope

tag used was triple HA (designated HA), Flag-His₆ (designated FH), or GFP.

Construction of the nob1-4 mutant and pno1-1 mutant by site-directed mutagenesis

Construction of the nob1-4 mutant: The method of PCR-aided site-directed mutagenesis was described by Clackson and Wells (1994). The first reaction was performed using genomic DNA of the wild-type strain as a template and two pairs of convergent primers; one pair is 56c-6 (5'-AAAAGTCGACCTAACTTCTCCTTTTGGAAGTGT-3') and 56c-LQM-RGG-F (5'-AATGTAGCGCGGGGGGGAATCTAAATCT-3'), and the other pair is 56c-45 (5'-CCCCCCCCCGCGCCGCTTTCAAGAAATTAAGGATGCTCAA-3') and 56c-LQM-RGG-R (5'-AGATTTAGATTCCCCCCCCCGCGCTACATT-3'). The resulting fragments were mixed and amplified by using two convergent primers, 56c-6 and 56c-45. The amplified DNA fragments were digested with *NotI* and *Sall*, and the resulting segments were inserted between the *NotI* and *Sall* gap of pRS305 to generate pYT410. pYT410 digested with *BamHI* was integrated at the *NOB1* locus of the wild-type cells.

Construction of the pno1-1 mutant: The first reaction was performed using genomic DNA of the wild-type strain as a template and two pairs of convergent primers; one pair is 145c-6 (5'-AAAGTCGACTTAGTAGCGTTCTTTTAATCTAGA-3') and 145c-21 (5'-AGAGCCATCGATCGTATTGCCG-3'), and the other pair is 145c-13 (5'-GGGGGAATCCGATGAGGAC

Table 1. Yeast strains and plasmids

Strain	Genotype	Source/comments
W303-1A	<i>MATa leu2 his3 trp1 ade2 ura3 can1</i>	Our stock
YK109	<i>MATa leu2 his3 trp1 ade2 ura3 can1 rpn12-1</i>	Kominami et al. 1994
J88	<i>MATa leu2 his3 trp1 ade2 ura3 can1 cim5-1</i>	Our stock
J106	<i>MATα leu2 his3 trp1 ade2 can1 RPT1-6xHis::URA3</i>	Takeuchi et al. 1999
JD59	<i>MATa ump1-Δ1::HIS3 leu2 trp1 ade2 ura3 lys2</i>	Ramos et al. 1998
L40	<i>MATa leu2 his3 trp1 ura3 URA3::lexA-lacZ LYS2::lexA-HIS3</i>	Fields and Song 1989
Y138	<i>MATa nob1-4::LEU2</i>	This study
Y148	<i>MATa PUP1-HA::HIS3</i>	This study
Y151	<i>MATa nob1-4::LEU2 PUP1-HA::HIS3</i>	This study
Y153	<i>MATa PRE2-HA::HIS3</i>	This study
Y154	<i>MATa nob1-4::LEU2 PRE2-HA::HIS3</i>	This study
Y166	<i>MATa PRE1-Flag-6xHis::URA3</i>	This study
Y168	<i>MATα UMP1-HA::HIS3</i>	This study
Y169	<i>MATa UMP1-HA::HIS3 nob1-4::LEU2</i>	This study
Y178	<i>MATa NOB1-8xGFP::URA3</i>	This study
Y184	<i>MATa PNO1-Flag-6xHis::URA3</i>	This study
Y189	<i>MATα pno1-1::URA3 PUP1-HA::HIS3</i>	This study
Y190	<i>MATα pno1-1::URA3 PRE2-HA::HIS3</i>	This study
Y199	<i>MATa PRE6-8xGFP::URA3::TRP1</i>	This study
Y201	<i>MATα nob1-4::LEU2 P_{GAL1}-PRE6-8xGFP::URA3::TRP1</i>	This study
Y202	<i>MATa pno1-1::LEU2 P_{GAL1}-PRE6-8xGFP::URA3::TRP1</i>	This study
Y208	<i>MATa ump1Δ::LEU2 P_{GAL1}-PRE6-8xGFP::URA3::TRP1</i>	This study
Plasmids		
pACTII	<i>LEU2 2μ ori Amp^r</i>	gift from Elledge S.
pBTM116	<i>lexA TRP1 2μ ori Amp^r</i>	Fields and Song 1989
pKT10	<i>P_{TDH3}-URA3 2μ ori Amp^r</i>	Our stock
pNOT9	<i>GAL4-AD PNO1-ΔN</i>	This study
pRS303	<i>HIS3 Amp^r</i>	Sikorski and Hieter 1989
pRS305	<i>LEU2 Amp^r</i>	Sikorski and Hieter 1989
pRS306	<i>URA3 Amp^r</i>	Sikorski and Hieter 1989
pRS314	<i>TRP1 CEN Amp^r</i>	Sikorski and Hieter 1989
pJUN290	<i>RPT1 URA3 CEN Amp^r</i>	Our stock
pYT53	<i>NOB1 URA3 CEN Amp^r</i>	Tone et al. 2000
pYT66	<i>lexA-NOB1 TRP1 2μ ori Amp^r</i>	This study
pYT82	<i>P_{TDH3}-NOB1 URA3 2μ ori Amp^r</i>	This study
pYT272	<i>pno1-1-ΔN URA3 Amp^r</i>	This study
pYT410	<i>nob1-4-ΔN LEU2 Amp^r</i>	This study
pYT460	<i>UMP1 TRP1 CEN Amp^r</i>	This study
pYT464	<i>NOB1 TRP1 CEN Amp^r</i>	This study
pYT465	<i>P_{TDH3}-NOB1 TRP1 2μ ori Amp^r</i>	This study

GATGATGACGAC-3') and 145c-20 (5'-CGGCAATACGATC GATGGCTCT-3'). The resulting fragments were mixed and amplified by using two convergent primers, 145c-6 and 145c-13. The amplified DNA fragments were digested with *Eco*RI and *Sal*I, and the resulting segments were inserted between the *Eco*RI-*Sal*I gap of pRS306 to generate pYT272. pYT272 digested with *Nhe*I was integrated at the *PNO1* locus in wild-type cells. The *pno1-1* mutation was found to be recessive, and the authentic *PNO1* gene complemented the temperature-sensitivity of *pno1-1* mutation.

Two-hybrid screening

pYT66 and the yeast two-hybrid cDNA library (in pACTII, *LEU2* marker, a gift from S. Elledge, Houston, TX) were simultaneously introduced into the reporter strain L40 by selecting *Leu*⁺ *Trp*⁺ transformants. The *Leu*⁺ *Trp*⁺ *His*⁺ colonies are candidates containing a cDNA clone encoding a Nob1p-interacting protein. Library plasmid was recovered from each candidate and

reintroduced into L40 along with either pYT66 or pBTM116. Only those plasmids that reproducibly gave rise to a positive interaction with Nob1p bait protein were studied further. β -galactosidase activity was measured using permeabilized mid-log phase cells as an enzyme source (Ozcan and Johnston 1995).

DNA manipulation

The methods for yeast DNA engineering, such as isolation of plasmids and construction of plasmids, were essentially those described by Sambrook et al. (1989).

Biochemical methods

Yeast protein was extracted by disrupting yeast cells in lysis buffer [0.1 M Tris-HCl at pH 7.5, 0.2 M NaCl, 1 mM ethylenediaminetetraacetate (EDTA), and 5% glycerol] with glass beads. After centrifugation at 12,000 g for 15 min, the resulting supernatant was used as crude cell extract. Protein concentration was

determined by the BCA Protein Assay Reagent Kit (Pierce) using bovine serum albumin as the standard.

Proteins (50 µg) were separated by SDS-PAGE according to the method described by Laemmli (1970). Anti-20S proteasome antibody (Tanaka et al. 1988), anti-Rpt1p antibody (Shibuya et al. 1992), Anti-Rpn12p antibody (Nisogi et al. 1992), anti-polyubiquitin antibody (Fujimuro et al. 1994), and anti-Nob1p antibody (Tone et al. 2000) were described previously. Anti-Flag monoclonal antibody (Sigma), anti-HA monoclonal antibody (Babco), and anti-actin antibody (Boehringer) were used as primary antibody. Anti-rabbit IgG goat antibody conjugated with horseradish peroxidase (HRP; Promega) or anti-mouse IgG goat antibody conjugated with HRP (Promega) was used as the secondary antibody.

Glycerol density gradient centrifugation

The methods for glycerol density gradient centrifugation and peptidase assay using Succinyl-L-Leucyl-L-Leucyl-L-Valyl-L-Tyrosine 4-Methyl-Coumaryl-7-Amide (Suc-LLVY MCA; Peptide Institute, Osaka) were described previously (Tone et al. 2000).

Pull down experiment using Ni-NTA agarose beads

Pno1p-Flag-6×His and Pre1p-Flag-6×His were pulled down with Ni-nitrilotriacetic acid (NTA) agarose beads (QIAGEN) from the fractions separated by glycerol density gradient centrifugation. A fraction (300 µL) containing 0.15 M NaCl was gently mixed with 80 µL of slurry of 50% Ni-NTA agarose beads and rotated at 4°C for 1 h. The beads were washed five times with buffer A [0.1 M Tris-HCl at pH 7.5, 0.15 M NaCl, 0.5 mM EDTA, 0.1 mM DTT, 2% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 15 µg/mL PepstatinA, 2 mM MgCl₂, and 2 mM ATP] containing 10 mM imidazole. Then, proteins bound with the Ni-NTA agarose were eluted with 60 µL of buffer containing 0.2 M imidazole. The resulting eluate was analyzed by SDS-PAGE followed by immunoblotting.

Immunoprecipitation experiment

Polyclonal antibody against the 20S proteasome (20 µg in 60 µL of PBS buffer) was mixed with protein A-Sepharose beads, and the mixture was rotated at 4°C for 2 h. The beads were treated with 3% skim milk in PBS buffer at 4°C for 20 min, and washed two times with PBS buffer and two times with buffer A. A fraction (300 µL) was gently mixed with 80 µL of protein A-Sepharose beads. After the mixture was rotated at 4°C for 2 h, the beads were washed four times with buffer A, mixed with sample buffer (100 µL), and heated at 95°C for 10 min. Insoluble material was removed by centrifugation at 12,000 g for 5 min, and resulting supernatant was analyzed by SDS-PAGE followed by immunoblotting. To avoid cross-reaction, monoclonal anti-rabbit antibody conjugated with HRP (Sigma) was used as the secondary antibody for the detection of Nob1p and the 20S proteasome.

Nondenaturing PAGE

Native PAGE was carried out according to the method described by Glickman et al. (1998b). Peptidase activity in gel was assayed using 0.1 mM Suc-LLVY-MCA as a substrate with or without 0.05% SDS in 100 mM Tris-HCl, at pH 8.0. Proteasome bands were visualized upon exposure to UV light (360 nm) and photographed with a Polaroid camera.

Fluorescence microscopy

Strains bearing Nob1p-GFP were grown in SC-Ura medium to mid-logarithmic phase. Cells were incubated for 15 min with 1 µg/mL Hoechst 33342. The cells were mounted and observed by epifluorescence-photomicroscopy. Images were captured using an Olympus photomicroscope Olympus IX70, a CCD camera (Sensys, Photometrics), and Signal Analytics IP Lab Spectrum capture software. Photos were analyzed using Adobe Photoshop.

Assay of trypsin sensitivity

Trypsin sensitivity was assayed using fractions that were obtained from wild-type extract with or without 50 µM MG132 (Peptide Institute) by glycerol density gradient centrifugation. First, 0–1.0 µg/mL trypsin (Sigma) was added to fractions (50 µL) with or without 50 µM MG132 in 25 mM Tris-HCl, at pH 7.5 (total reaction volume, 100 µL), and the mixture was incubated for 1 h at 37°C. The reactions were stopped by adding five volumes of cold acetone. Proteins were dried up, dissolved in 15 µL of sample buffer, and separated by SDS-PAGE.

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Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in *Saccharomyces cerevisiae*

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