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The Regulatory Particle of the *Saccharomyces cerevisiae* Proteasome

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The proteasome is a multisubunit protease responsible for degrading proteins conjugated to ubiquitin. The 670-kDa core particle of the proteasome contains the proteolytic active sites, which face an interior chamber within the particle and are thus protected from the cytoplasm. The entry of substrates into this chamber is thought to be governed by the regulatory particle of the proteasome, which covers the presumed channels leading into the interior of the core particle. We have resolved native yeast proteasomes into two electrophoretic variants and have shown that these represent core particles capped with one or two regulatory particles. To determine the subunit composition of the regulatory particle, yeast proteasomes were purified and analyzed by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Resolution of the individual polypeptides revealed 17 distinct proteins, whose identities were determined by amino acid sequence analysis. Six of the subunits have sequence features of ATPases (Rpt1 to Rpt6). Affinity chromatography was used to purify regulatory particles from various strains, each of which expressed one of the ATPases tagged with hexahistidine. In all cases, multiple untagged ATPases copurified, indicating that the ATPases assembled together into a heteromeric complex. Of the remaining 11 subunits that we have identified (Rpn1 to Rpn3 and Rpn5 to Rpn12), 8 are encoded by previously described genes and 3 are encoded by genes not previously characterized for yeasts. One of the previously unidentified subunits exhibits limited sequence similarity with deubiquitinating enzymes. Overall, regulatory particles from yeasts and mammals are remarkably similar, suggesting that the specific mechanistic features of the proteasome have been closely conserved over the course of evolution.

In eukaryotes, the elimination of many short-lived proteins requires their covalent attachment to ubiquitin (43). This pathway is involved in a wide variety of regulatory mechanisms, with substrates including cyclins and CDK inhibitors (49), membrane proteins such as CFTR (104), p53 (84), NF-κB (71), c-Fos, c-Jun, and luminal components of the endoplasmic reticulum (9). Multiubiquitin chains target proteins for degradation by the proteasome, an ~2-MDa proteolytic complex (reviewed in references 12, 55, 61, and 80).

The mechanism of the proteasome is thought to involve unfolding of a protein substrate and translocation from one subcompartment of the enzyme to another prior to degradation. This model is based primarily on the crystal structure of the proteasomal core particle (CP). The CP has a barrel-like shape, with the proteolytic active sites facing the inner chamber, or lumen. In the proteasomal CP from *Thermoplasma acidophilum*, openings into the lumen are found only at the ends of the barrel (59) and are therefore thought to function as channels for the proteolytic substrate. Because these channels are narrow, it is likely that proteolytic substrates must be unfolded prior to entry into the lumen of the CP. Based on electron micrographs of the proteasome's from various eukaryotes, these channels open out into multisubunit complexes flanking the CP at one or both ends; these complexes have been variously termed PA700, the μ particle, the 19S complex, and the regulatory particle (RP) (15, 72, 73, 99). As the RP confers ATP dependence and ubiquitin dependence on the CP (15, 47), it is presumed to function by recognizing substrates, unfolding them, and directing their translocation through the channel of the CP. The channel observed in the *T. acidophilum* particle, however, is not observed in the crystal structure of the CP from *Saccharomyces cerevisiae* (38). This fact suggests that in eukaryotes the channel is gated, perhaps through the action of the RP. Recent data also indicate that purified PA700, a mammalian RP complex, can deubiquitinate proteolytic substrates (53, 54). Because the subunit composition and crystal structure of the *S. cerevisiae* CP are known and because yeasts are amenable to genetic analysis, yeasts provide a useful system for studies of the proteasome and the role of the RP.

While the RP has been purified from mammals and most of its subunits have been identified, the RP of *S. cerevisiae* has not been characterized biochemically. However, many genetic screens have identified genes suggested to encode components of the RP. For example, such genes were identified in screens for mutations that are synthetically lethal in combination with *cdc28* mutations (30, 50), mutations that suppress recessive alleles of *GAL4* (94), mutations that increase the levels of expression of *SEN1* fusion proteins (14), mutations that are deficient in the degradation of membrane-bound enzyme HMG-coenzyme A reductase (39), mutations that suppress phenotypes associated with a *yme1* deletion mutation (8), and multicopy suppressors of the *nin1-1* mutation (51). While the phenotypic effects of mutations in these genes are remarkably diverse, the paucity of biochemical information about the yeast proteasome has limited the mechanistic insights achievable through analyses of mutants. In this work, we have identified 17 subunits from the RP of the yeast proteasome by amino acid sequence analysis. Together with additional experiments examining the nature of electrophoretic variants of the proteasome, the assembly of multiple ATPases within a single RP, and deletion mutations of RP subunits, these studies define the components of the yeast RP and the complexes that they form.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques. Chromosomal deletions of RP genes were performed with strain DF5 (*MAT***a**/*MAT*α *lys2-801*/*lys2-801 leu2-*

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3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-1/trp1-1) (24). Strain SUB62 (*MAT***a** *his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52*) (24) was used as a wild-type control, as all phenotypic analyses were carried out with *MAT***a** derivatives (31). Yeast cultures were grown at 30°C unless otherwise noted. YPD medium consisted of 1% yeast extract, 2% Bacto Peptone, and 2% glucose. Synthetic medium consisted of 0.7% Difco yeast nitrogen base supplemented with amino acids, uracil, and 2% glucose as described previously (77). Specific nutrients were omitted as necessary for plasmid selections. Standard techniques were used for yeast transformations and tetrad analysis (31, 77).

Construction of strains expressing His$_6$-tagged ATPases. A plasmid designed to express hexahistidine (His$_6$)-tagged versions of the proteasomal ATPases from a single promoter was constructed. Northern blot analysis revealed that *RPT1*, *RPT2*, *RPT3*, *RPT5*, and *RPT6* express comparable levels of RNA at both 30 and 37°C (data not shown). We therefore chose to express recombinant forms of these genes from a single plasmid containing the *RPT1* promoter and a His$_6$ epitope at the N terminus of the gene to be expressed. With a two-step PCR protocol (86), a DNA fragment containing the *RPT1* 5′-untranslated region, a multiple cloning site (MCS), and the *RPT1* 3′-untranslated region was created. In the first step, the oligonucleotide pairs DR27 (5′-CACTGCTTAAGCTTGTCG ACTACCCGCCATTGTTGCAC)-DR29 (5′-CGACGACTGCAGTCGATCTC TAGATTTAATTAAATGGTGATGGTGATGGTGCATTCCGTATAGT TCCTAAC) and DR30 (5′-GATCGACTGCAGTCGTCGGGATCCCCCGGG TACCCATACGACGTCCCAGACTACGC)-DR31 (5′-CTCAGTGGTACC GTCGACGATTATTCCCAATGTCGGTC) were used to amplify DNA from pL44CIM5 (containing wild-type *RPT1* [30]) to create two overlapping fragments. The first fragment contains 500 nucleotides immediately upstream of the start codon, and the second fragment contains 764 nucleotides immediately downstream. To fuse the fragments via the overlapping MCS, a second PCR with DR27 and DR31 was performed. The resulting fragment was digested with *Hin*dIII and *Kpn*I and cloned into YCplac22, a yeast *CEN4* shuttle vector marked with *TRP1* (32); the resulting plasmid was called DP1. All six genes were then amplified by PCR and subcloned into the MCS.

Haploid cells containing a given *rpt* deletion covered by a *URA3*-marked *CEN* plasmid expressing the corresponding wild-type gene under the control of an *RPT1* promoter were generated. Plasmids carrying tagged versions of the ATPase genes were introduced into these strains, and following 5-fluoro-orotic acid selection (77), the following strains were produced: DY17 (His$_6$-*RPT2*), DY19 (His$_6$-*RPT1*), DY40 (His$_6$-*RPT6*), DY41 (His$_6$-*RPT3*), and DY178 (His$_6$-*RPT5*). These strains grow at wild-type rates. His$_6$-*RPT4* was expressed in a wild-type SUB62 haploid strain (DY196).

Deletion of *RPN9*. A strain containing *HIS3* in place of the complete *RPN9* coding region was constructed. The transforming DNA fragment was generated by PCR as described previously (86); the *HIS3* gene was amplified from a plasmid (yDpH) by use of a primer pair with base-pairing sites upstream and downstream of the *RPN9* coding sequence: DR117 (5′-CAAAAAGCAAACAGTG GGCACACGCGAGGAAACCACATTATATTTCGCAAGCTCTTGGCCTC CTCTAGT)-DR118 (5′-TTTATATATATGTGTGCGTGTGTGTTTTATATA TAACTGCCAATGGCCTATCGTTCAGAATGACACG). The resulting fragment contains the *HIS3* gene and, at either end, the 5′ and 3′ sequences that flank the *RPN9* coding sequence. This DNA fragment was transformed into strain DF5, and several transformants were sporulated. His$^+$ segregants displayed a uniform slow-growth phenotype. *MAT***a** *rpn9::HIS3* (MG18) and *MAT*α *rpn9::HIS3* (MG19) segregants were isolated for subsequent studies. The site of integration was confirmed by PCR with a primer for the upstream untranslated region of *RPN9* and a primer internal to *HIS3*: DR123 (5′-AGATCCAAGCT TCAAATTGAAAGATTGTCTATCAATCTGTA) and DR26 (5′-CTGTCATC TTTGCCTTCG). To construct a Δ*rpn9*Δ*rpn10* double mutant, a *MAT***a** *rpn10::LEU2* strain (102) was mated with MG19. His$^+$ segregants displayed a nearly uniform slow-growth phenotype. *MAT***a** haploids with histidine and leucine prototrophy were recovered (MG29).

Myc$_6$ tagging of *RPN9*. To create a plasmid carrying *Myc$_6$-Rpn9* under the control of its own promoter, genomic DNA from SUB62 was amplified with the following primers: DR123 (5′-AGATCCAAGCTTCAAATTGAAAGATTGTC TATCAATCTGTA) and DR116 (5′-GCTGTATCCCTAAACCCAGATGGA TTGGCCA). The resulting fragment was cloned into a *LEU2*-marked *CEN* plasmid containing a downstream Myc$_6$ sequence followed by a stop codon (pNU119). This plasmid was transformed into MG18 to generate strain MG26.

Purification of the proteasome by conventional chromatography. Purification of the proteasome by conventional chromatography was modified from that described previously (79). The proteasome was purified from a yeast cell lysate by the protocol shown in Fig. 1. SUB62 was grown to the stationary phase on YPD in a 12-liter fermentor. After centrifugation, the cell pellet was resuspended in a threefold volume of buffer A, containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 10% glycerol, 1 mM ATP (grade 1; Sigma), and 1 mM dithiothreitol (DTT). For cell resuspension and lysis, buffer A was supplemented with an additional 4 mM ATP. Cells were lysed with a French press, and the extract was clarified by centrifugation at 30,000 × *g* for 20 min and passage through cheesecloth. The extract was fractionated on a 100-ml DEAE–Affi-Gel Blue column (Bio-Rad), followed by anion-exchange chromatography and gel filtration chromatography. Anion exchange was performed by use of an XK26 column packed with 50 ml of Resource Q resin (Pharmacia). Proteins were resolved on a 500-ml gradient of 100 to 500 mM NaCl at 4 ml/min. Fractions (8 ml) were collected and screened

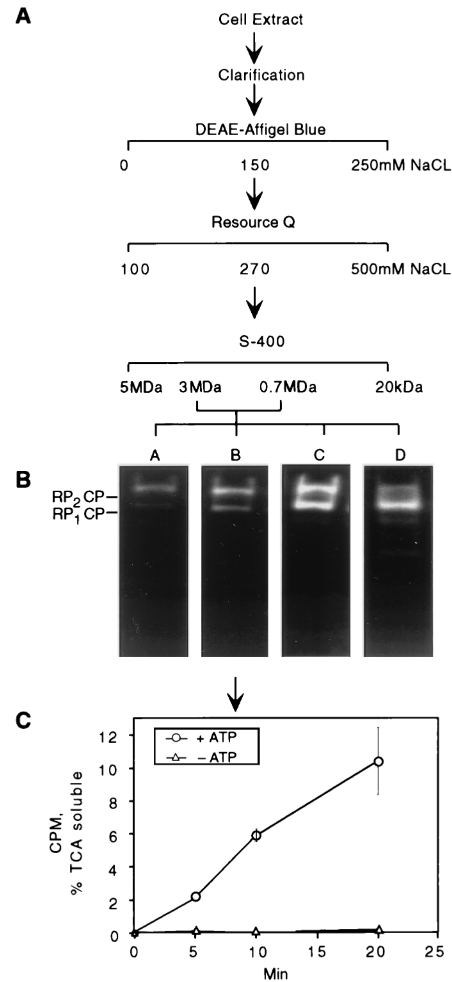

FIG. 1. Proteasome purification procedure. (A) A yeast lysate was fractionated on a series of columns containing DEAE–Affi-Gel Blue, Resource Q resin, or S-400 resin (see Materials and Methods for details). Fractions containing peptidase activity were combined into four pools (A to D) in descending molecular mass order. Protein content and specific peptidase activity at each step are shown in Table 1. (B) Proteasomes from each pool were visualized by nondenaturing PAGE and fluorogenic peptide overlay. In pool D, two faster-migrating species were observed in addition to RP_2CP and RP_1CP. The fastest-migrating species was the CP, and the other contained the CP and a subset of RP subunits (data not shown). (C) Proteasomes from pool B were tested for the ability to proteolyse multiubiquitinated ^{125}I-labeled lysozyme in the presence or absence of ATP. Degradation was measured as trichloroacetic acid-soluble ^{125}I counts per minute at a given time point. Background radioactivity was subtracted from all readings. Error bars indicate standard deviations.

for the ability to hydrolyze Suc-LLVY-AMC (Bachem). Fractions containing the peak of activity, eluting at 270 to 330 mM NaCl, were pooled, desalted, concentrated to 1 ml by use of Ultrafree concentrators with a molecular weight cutoff of 30 kDa (Millipore), and further resolved by gel filtration. For gel filtration, 100 ml of S-400 resin (Pharmacia) was packed in an XK16 column. Samples were run isocratically in buffer A with 100 mM NaCl at a flow rate of 1 ml/min. Fractions (2 ml) were collected and screened for peptidase activity. Fractions from a broad peak of peptidase activity were pooled into separate aliquots (pools A to D).

Assays of proteasome activity and concentration. Aliquots from column fractions were incubated in buffer A with 0.1 mM Suc-LLVY-AMC for 10 min at 30°C. The reaction was quenched by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%. Fluorescence readings of released 7-amido-4-methylcoumarin (AMC) were taken at an excitation wavelength of 380 nm and an emission wavelength of 440 nm and were recorded as arbitrary (fluorescence) units per milligram of protein. Ubiquitin-lysozyme conjugate breakdown assays were performed essentially as described previously (79) by incubating proteasome with ubiquitinated, ^{125}I-labeled lysozyme for 30 min and recording the trichloroacetic acid-soluble radioactivity released from the proteo-

lysed substrate compared to the background. Protein concentrations in the different fractions were determined by using Coomassie Plus (Pierce) with bovine serum albumin as a standard.

Proteasome purification by Ni-NTA affinity chromatography. Purification of the proteasome by Ni-nitrilotriacetic acid (NTA) affinity chromatography was performed as described previously (79). Briefly, eluates from the DEAE–Affi-Gel Blue column were loaded onto Ni-NTA–agarose columns (Qiagen). Nonspecifically bound proteins were eluted with 100 mM NaCl and 15 mM imidazole in buffer A. His_6-tagged proteasome was eluted with 100 mM NaCl and 100 mM imidazole in buffer A. Column fractions were tested for the presence of proteasomal subunits by immunoblotting with appropriate antibodies. Protein samples were also assayed for the ability to hydrolyze Suc-LLVY-AMC.

Ni-NTA affinity purification of the RP. Purified proteasome preparations were dissociated to reveal uncapped CPs after incubation for 30 min at 30°C in buffer A without ATP and supplemented with 500 mM NaCl. Dissociation of the proteasome was confirmed by comparing the migration of the sample before and after NaCl incubation on nondenaturing polyacrylamide gels. Partial purification of the RP was performed by first-depleting cells of ATP; the cells were then incubated after harvesting in 1 volume of buffer A without ATP and supplemented with 0.2 nM dinitrobenzene and 200 mM deoxyglucose for 30 min at 30°C. Subsequent cell lysis and protein purification steps were performed in the absence of ATP. Clarified cell extract was applied to DEAE–CL-6B anion-exchange resin (Sigma), rinsed with 100 mM NaCl, and eluted with 500 mM NaCl in buffer A. Ni-NTA chromatography was performed as mentioned above for the intact proteasome but in the absence of ATP. Column fractions were tested for the presence of Rpt1, Rpt6, and Rpn10 by immunoblotting with specific antibodies.

Denaturing and nondenaturing PAGE. Except for purposes of sequence analysis (see below), purified proteasome polypeptides were resolved by SDS–12% polyacrylamide gel electrophoresis (PAGE) by standard techniques (52). Proteins were either stained in the gel with Coomassie blue or transferred to nitrocellulose for immunoblotting. For identification of proteins by immunoblotting, samples were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes (Whatman) with a semidry transfer system (Owl Scientific). Immunoblotting was performed with antisera to Rpn10 (generously provided by Steve van Nocker and Richard Vierstra) and to Rpt1 and Rpt6 (generously provided by Carl Mann). Primary antibodies were visualized with alkaline phosphatase-labeled goat anti-rabbit immunoglobulins (Promega) and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Promega).

Protein samples were resolved by nondenaturing PAGE by a modification of the method of Hoffman et al. (44). We used a single gel layer consisting of 0.18 M Tris-borate (pH 8.3), 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 4% acrylamide-bisacrylamide (at a ratio of 37.5:1) and polymerized with 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate. The running buffer was the same as the gel buffer but without acrylamide. Xylene cyanol was added to protein samples prior to loading onto the gels. Nondenaturing minigels were run at 100 to 150 mV until the Xylene cyanol eluted from the gels (approximately 2 h). The gels were then incubated in 10 ml of 0.1 mM Suc-LLVY-AMC in buffer A for 10 min. Proteasome bands were visualized upon exposure to UV light (360 nm) and photographed with a Polaroid camera.

For extracting intact proteasome from a gel, nondenaturing PAGE was performed as described above except that the usual cross-linker bisacrylamide mixture in the gel was replaced with the reversible cross-linker N,N'-bisacrylylcystamine (Bio-Rad). Gels containing 4% acrylamide–N,N'-bisacrylylcystamine at a ratio of 22:1 were polymerized with 0.1% TEMED and 0.1% ammonium persulfate. After the standard peptidase overlay assay, the proteolytically active bands were cut out of the gels and incubated with 40 μl of 2 M DTT per 100 μl of gel slice for 30 min at 30°C. Laemmli loading buffer (52) was added, and the samples were heated to 80°C for 3 min and loaded onto SDS-PAGE minigels. The gels were then stained with Coomassie blue, and the resulting protein banding pattern was quantitated by densitometry with NIH Image and LabGel software. The same methods were used for the quantitation of immunoblots.

Peptide sequence analysis. Samples from different proteasome preparations, containing about 30 μg of purified protein, were precipitated with methanol-chloroform (105), denatured with SDS, reduced, and alkylated with iodoacetamide (96). Samples were then reprecipitated and resuspended in SDS-PAGE loading buffer (16) for resolution of subunits on an acrylamide gradient (10 to 20%) minigel (0.75 mm) with a standard Laemmli buffer system (52). No more than 10 μg of reduced and alkylated protein was loaded per gel lane to achieve acceptable resolution. After electrophoresis, the gel was stained with Coomassie blue and destained by standard protocols (25).

The Coomassie blue-stained subunits of the proteasome were cut from the gel with a scalpel, digested with trypsin in situ, and extracted as described previously (78). The extracts were concentrated under vacuum, resuspended in 0.1% trifluoroacetic acid, and fractionated by reverse-phase high-pressure liquid chromatography (HPLC) on a C_{18} column. Proteins were eluted with a gradient of acetonitrile containing 0.1% trifluoroacetic acid. The isolated peptides were sequenced by automated Edman degradation on a model 470/900/120A gas-phase sequencer (Applied Biosystems) with standard chemistry.

TABLE 1. Proteasome purification procedure

Fraction[a]	Total mg	Total activity[b]	Sp act[c]
Clarified cell extract	7,500	4,860	0.648
Affi-Gel Blue	600	3,260	5.43
Resource Q	18.4	1,346	73.2
S-400			
A	0.24	62	256
B	1.2	266	222
C[d]	1.8	370	205
D[d]	2.4	348	145

[a] See Fig. 1.
[b] Peptidase activity in arbitrary units, as described in Materials and Methods.
[c] Units per milligram.
[d] The lower specific activities of pools C and D largely reflect the presence of free CPs, which have lower specific peptidase activities (see Fig. 9).

RESULTS

Purification and characterization of the proteasome. The proteasome was purified from cell lysates by the protocol described in Fig. 1A and Table 1. In the final step of gel filtration, a broad peak of peptidase activity eluted between 3 and 0.7 MDa. Peak fractions were collected into four pools in descending molecular mass order as they eluted from the gel filtration column (pools A to D; Fig. 1B). The proteasome pools were active in the degradation of ubiquitin-protein conjugates in the presence of ATP (Fig. 1C and data not shown). Peptidase activity coincided with proteolytic activity, as assayed with ubiquitin-lysozyme conjugates (Fig. 1B and C and data not shown).

Each pool was analyzed by nondenaturing PAGE, with proteasome bands visualized by their ability to hydrolyze the fluorogenic peptide Suc-LLVY-AMC (Fig. 1B). Two major electrophoretic bands whose relative abundance was characteristic from pool to pool were observed (Fig. 1B). Mammalian proteasome preparations have similarly been resolved into multiple forms by nondenaturing PAGE (44, 47, 100). To define the compositions of the two forms, they were resolved by nondenaturing PAGE, using a gel that had been polymerized with a reversible cross-linker, N,N'-bisacrylylcystamine. The two bands were excised, the gel matrix was dissolved, and the protein components of the particle were resolved by SDS-PAGE.

Similar polypeptide patterns were observed for the two electrophoretic forms of the proteasome (Fig. 2). The molecular masses of the CP subunits in yeast are all between 15 and 30 kDa (12), while most RP subunits are larger than 30 kDa (see below). We therefore compared the integrated intensity of the protein bands in the 30- to 120-kDa region to that in the 15- to 25-kDa region for the slower-migrating form of the proteasome and then compared this ratio to that for the faster-migrating form. The ratio of the integrated intensity of the RP subunits to that of the CP subunits for the slower-migrating form of the proteasome was approximately double that for the faster-migrating form (Fig. 2). The same results were obtained for the ratio of the cumulative intensity of all the RP bands to the cumulative intensity of all the CP bands and for the ratio of individual RP components to individual CP bands (Fig. 2). We conclude that the slowest-migrating form on nondenaturing PAGE represents doubly-capped proteasomes (RP_2CP), that the faster-migrating form (of the two present in pool B) represents singly-capped proteasomes (RP_1CP), and that the protein compositions of the two forms are otherwise equivalent. Electron micrographs confirmed the presence of dumb-bell-shaped, doubly-capped proteasomes and mushroom-

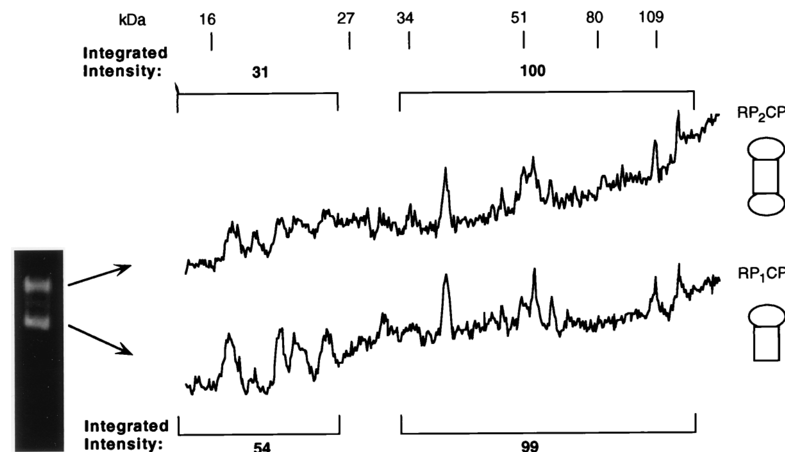

FIG. 2. The proteasome migrates as singly and doubly capped forms on nondenaturing PAGE. Purified proteasomes from pool B were resolved by nondenaturing PAGE with a reversible cross-linker, *N,N′*-bisacrylylcystamine. The fast- and slow-migrating forms were excised. Their proteins were extracted from the gels and resolved by SDS–12% PAGE. The gels were then stained with Coomassie blue. Densitometric quantitation of the resulting protein banding pattern is shown for each form. The protein banding pattern can be compared to that shown in Fig. 3 prior to nondenaturing PAGE. However, as the two gel systems are different, the comparison cannot be made on a one-to-one basis. Based on the data in Table 2 (and data not shown), proteins below 25 kDa were assumed to be CP subunits, and those above 30 kDa were assigned as RP subunits. The integrated intensities of CP and RP subunits are displayed over the corresponding region of the gel. The ratio of intensity of RP subunits to that of CP subunits in the slower-migrating form of the proteasome is approximately double that in the faster-migrating form.

shaped, singly-capped proteasomes in these preparations (data not shown). Whether there are significant functional differences between singly- and doubly-capped forms of the proteasome is unknown.

Subunit composition of the proteasome. Amino acid sequence analysis was used to identify the subunits of the RP of the proteasome. Purified proteasomes were resolved by gradient SDS-PAGE, and the protein bands were stained with Coomassie blue (Fig. 3). The protein pattern in the 20- to 30-kDa region resembled that of independently purified CP (data not shown). Protein bands in the higher-molecular-mass region (30 to 120 kDa) with strong or intermediate staining intensities were numbered in descending molecular mass order from 1 to 17 (Fig. 3). The bands were excised from the gel and treated with trypsin. Representative peptides sequenced from each protein are shown in Table 2. The sequence of each peptide matched completely the deduced sequence from a yeast open reading frame (ORF) present in the SGD database, allowing the assignment of each protein as the product of a specific chromosomal locus (Table 2). Given that the *S. cerevisiae* genome is entirely known, the peptide sequence data are sufficient to assign each RP subunit as the product of a single gene. Although a few residues were not unambiguously assigned by the sequencer, no amino acid that was assigned differed from the corresponding yeast ORF-encoded sequence. In summary, seventeen RP proteins were resolved into 15 electrophoretic bands of comparable intensities (1 to 6, 8 to 15, and 17) and one more intense band (band 7), which contained peptides from two different RP subunits (Table 2). The RP proteins were named as follows: six of the proteins, which are putative ATPases of the AAA family (11), were designated Rpt1 to Rpt6 (for RP triple-A protein), and the other proteins were designated Rpn1 to Rpn12 (for RP non-ATPase). We note that the new nomenclature was arrived at in consultation with other yeast proteasome researchers and that nomenclatural conventions will be discussed in more detail in a separate communication.

Among bands 1 to 17, only one was found not to be an RP subunit; band 16 was identified as Pre10, a subunit of the CP (12, 43). Band 14 yielded peptides from two different proteins: Rpn10, which we have previously identified as Mcb1, a proteasome subunit that can bind multiubiquitin chains in vitro (102), and, in some preparations, Cdc10 (58). However, Cdc10 is not a component of the proteasome but rather is a substochiometric contaminant, since Cdc10 failed to comigrate with the proteasome upon nondenaturing PAGE, as determined by immu-

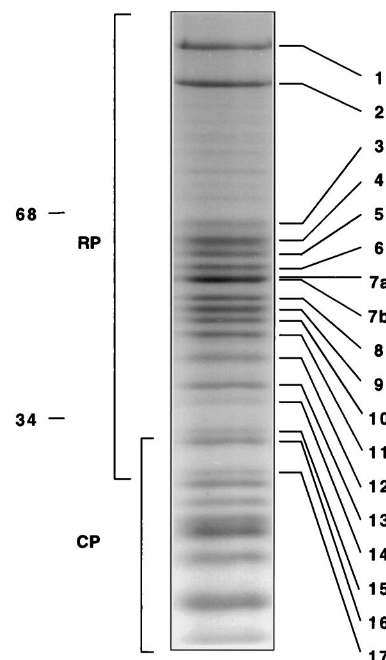

FIG. 3. Subunit composition of the proteasome determined by gradient SDS-PAGE. Proteins from pool B (Fig. 1) were resolved on a 10 to 20% polyacrylamide gradient gel. Protein bands were stained with Coomassie blue. Seventeen protein bands in the 120- to 30-kDa region were numbered in descending molecular mass order (masses are shown on the left). Proteins were excised from the gel and digested with trypsin. The resulting peptides were separated by reverse-phase HPLC and subjected to sequence analysis. Peptides sequenced from each band are shown in Table 2.

TABLE 2. Peptide sequences of RP subunits

Band	Protein	Peptide sequence[a]	Chromosomal locus[b]	Residue no.	No. of matches to genomic sequence[c]
1	Rpn1	RLKEDDSSLYE	YHR027c	55	11
2	Rpn2	KLALGIALEGYR	YIL075c	151	12
		RYGGAFTIALAYA		588	13
3	Rpn3	KFANQLXDEYL	YER021w	566	10
4	Rpt1[d]	RELFEMAR	YKL145w	292	8
		KVMFATNRPNTLD		350	13
5	Rpt2	KVAGENAPSIVFIDE	YDL007w	269	15
6	Rpt3	RYVILQSDLEEAY	YDR394w	399	13
7a	Rpt4	REVIELPLK	YOR259c	194	9
		RNCATEAGFFAIR		392	13
7b	Rpt5	KDSYLILDTLPSE	YOR117w	150	13
		KLAAPQLVQMYI		245	12
8	Rpn5	KSLDLNTR	YDL147w	108	8
		KTYEPVLNEDDLA		316	13
		RVIEXNLR		344	7
9	Rpn6	KIMLNLIDDV	YDL097c	269	10
		KIIEPFEXVEI		349	10
10	Rpn7	KAFLLTQSK	YPR108w	29	9
		RXADFFVR		324	7
11	Rpt6	RLDILDPALLR	YGL048c	296	11
12	Rpn8	RSIIAFDDLIENK	YOR261c	283	13
13	Rpn9	RDLLDDLEK	YDR427w	149	9
		KIPILAQHESF		316	11
14[e]	Rpn10	RVLSTFTAEFG	YHR200w	60	10
		KLXMATALQI		87	9
15	Rpn11	KVGSADTG	YFR004w	12	8
		KXYDYEEK		226	7
16	Pre10[f]	PIPIPAFAD	YOR326c	106	9
17	Rpn12	KNTELSYDFLP	YFR052w	191	11

[a] Bands were resolved by SDS-PAGE as shown in Fig. 3, excised from the gel, and digested with trypsin. Resulting peptides were separated by HPLC, and peptides were sequenced. Unassigned residues are indicated with an X. For details, see Materials and Methods.
[b] Determined by a Blastp search of the yeast genome (GenBank) with the peptide sequence shown.
[c] For all peptides that yielded high-confidence sequence data, assigned residues were in complete agreement with deduced amino acid sequences based on the DNA sequence of the yeast genome.
[d] Five additional peptides were sequenced from this band; all were derived from Rpt1.
[e] In some proteasome preparations, band 14 was contaminated with Cdc10 (see the text).
[f] Pre10 is a CP subunit; therefore, the Rpt/Rpn notation does not apply.

noblotting with anti-Cdc10 and anti-Rpt1 antibodies (data not shown). Also, in the final, gel filtration step of purification, Cdc10 peak fractions did not coincide with the proteasome peak.

Fujimuro et al. (27) recently showed that the Son1 protein cofractionates with the proteasome by gel filtration and that antibodies to Son1 precipitate proteasome subunits. Consistent with Son1 being a component of the proteasome, a subset of proteasome substrates were stabilized in *son1* mutants (48). However, we were unable to detect Son1/Rpn4 in our purified proteasome preparation by direct sequencing or by using antibodies to Son1. Son1 homologs have not been found in purified mammalian proteasomes either. Unlike most proteasome subunits, Son1 is nonessential (69). It is perhaps a loosely associated component.

Characterization of novel RP subunits. Table 3 lists the known subunits of the RP of the proteasome in yeast, their properties, and their homologs in other species. Two proteins did not have homologs previously identified as proteasomal subunits in other organisms: Rpn9 is encoded by ORF YDR427w, which has not been characterized, and Rpn11 is the product of the *MPR1* gene. The *mpr1* mutant was isolated as a suppressor of a defect in mitochondrial tRNA processing; deletion of the *MPR1* gene is lethal (76). After submission of this manuscript, Rpn11 was identified as a subunit of human and *Schizosaccharomyces pombe* proteasomes (91). Like other Rpn subunits, Rpn9 has clear homologs in other species (Table 3 and Fig. 4). Rpn9 is 29% identical to a protein encoded by a hypothetical ORF in *Caenorhabditis elegans* (Fig. 4), and in various mammals there are expressed sequence tag (EST) fragments that are 42% identical to the C terminus of Rpn9 (Fig. 4). The Rpn9 homologs represented by these EST fragments are most likely RP subunits. An additional four of the non-ATPase subunits (Rpn5 to Rpn8), which have not been characterized biochemically in *S. cerevisiae*, are homologs of known mammalian subunits (Table 3). The *RPN5/NAS5* and *RPN6/NAS4* genes were recently found to be essential by Saito and colleagues (82). The six ATPases (Rpt1 to Rpt6), as well as Rpn1 to Rpn4, Rpn10, and Rpn12, were previously proposed to be RP subunits, with the nature of the evidence varying from case to case (Table 3 and references therein).

A precise deletion of the *rpn9* coding sequence resulted in a slow-growth defect at 30°C (Fig. 5). After 48 h at 37°C, Δ*rpn9* mutants failed to form colonies. In a large-scale analysis of expressed genes in *S. cerevisiae*, the ORF corresponding to *RPN9* was reported to be nonessential (7). *RPN9* is apparently the only proteasome subunit gene that confers a temperature-sensitive phenotype when deleted, although this phenotype has been observed for point mutations in a number of other subunits. Temperature-sensitive phenotypes have been observed for *rpn1* and *rpn2* disruptions (98, 108), but complete deletions of these genes appear to be lethal (14, 54a, 57a). Aside from *RPN9*, the only other RP genes that are known to be nonessential are *RPN10/MCB1* (102) and *RPN4/SON1* (27). Δ*rpn9* Δ*rpn10* double mutants did not display a marked synthetic phenotype for vegetative growth (Fig. 5B and C).

To confirm that Rpn9 is a subunit of the proteasome, Rpn9 was tagged at its C terminus with six copies of the Myc epitope. Expression of tagged Rpn9 in a Δ*rpn9* mutant background resulted in full complementation (Fig. 5A). Proteasomes from wild-type and *myc$_6$-RPN9* strains were partially purified on a DEAE–Affi-Gel Blue column and further resolved by nondenaturing gel electrophoresis. The gel was immunoblotted, and the filter was probed with anti-Myc antibodies. Two bands which comigrated with singly- and doubly-capped proteasomes were detected (Fig. 6). We conclude that Rpn9 is a subunit of the proteasome.

Interestingly, Rpn11/Mpr1 contains a single conserved cysteine which is flanked by a highly conserved sequence with similarities to the active-site "Cys box" seen in many deubiquitinating enzymes (Tables 4 and 5). The catalytic triad of many deubiquitinating enzymes is thought to be made up of a

TABLE 3. Subunit composition of the *S. cerevisiae* RP

Band	Yeast subunits					Homologs			% Yeast-human identity[f]
	Proposed nomenclature	Existing nomenclature (or ORF)[a]	MM[b]	AA[b]	pI[b]	Human[c]	Bovine and human[d]	Other[e]	
1	Rpn1	Hrd2/Nas1	109.4	993	4.32	S2/Trap-2	p97	mts4	41
2	Rpn2	Sen3	104.3	945	5.92	S1	p112		41
3	Rpn3	Sun2[h]	60.4	523	5.37	S3	p58		21.5[f]
	Rpn4	Son1/Ufd5[i]	60.1	531	5.20				
4	Rpt1	Cim5/Yta3[h]	52.0	467	5.32	S7/Mss1			76
5	Rpt2	Yta5	48.8	437	5.78	S4	p56	mts2	71
6	Rpt3	Yta2/Ynt1	48.0	428	5.38	S6/Tbp7	p48	MS73	66
7a	Rpt4	Crl13/Sug2/Pcs1	49.4	437	5.53	S10b	p42	CADp44	67
7b	Rpt5	Yta1	48.2	434	4.93	S6′/Tbp1			68
8	Rpn5	Nas5	51.8	445	5.79		p55		40
9	Rpn6	Nas4	49.8	434	5.90	S9	p44.5		41
10	Rpn7	ORF u32445	49.0	429	5.16	S10	p44		36
11	Rpt6	Sug1/Cim3/Crl3[h]	45.2	405	9.09	S8/Trip1	p45	m56	74
12	Rpn8	ORF z75169	38.3	338	5.43	S12	p40	Mov-34	47
13	Rpn9	ORF u33007	45.9	394	5.51			dbEST[g]	42[g]
14	Rpn10	Mcb1/Sun1[h]	29.7	268	4.73	S5a		Mbp1/p54	34
15	Rpn11	Mpr1	34.4	306	5.66	Poh1		pad1	65.5
17	Rpn12	Nin1[h]	31.9	274	4.8	S14	p31	mts3	32

[a] Existing nomenclature for *S. cerevisiae* in GenBank database [and reference(s)]: Nas1 (98), Hrd2 (39), Sen3 (14, 108), Sun2 (51), Cim5 (30, 85), Yta5 (85), Yta2 (85), Ynt1 (8), Crl13 (65), Sug2 (81), Pcs1 (66), Yta1 (85), Nas5 (82), Nas4 (82), (Sug1 (79, 94), Cim3 (30), Crl3 (29), Mcb1 (102), Sun1 (51), Mpr1 (76), and Nin1 (50). RP subunit genes that have not yet been named in yeast are designated by their ORF site in GenBank.

[b] Molecular mass (MM) (in kilodaltons), number of amino acids (AA), and calculated pI were determined by use of the gene sequence and MegAlign (DNASTAR, Inc.).

[c] Existing nomenclature in GenBank database [and reference(s)]: S1, S2, S3, S4, S6, S7, S8, S10, S12, and S14 (19–22); S9 (46); S6′ and S10b (75); S5a (17, 18); Trap-2 (6, 90); Tbp1 (68); Tbp7 (70); Mss1 (88); Trip1 (56); and Poh1 (91).

[d] Existing nomenclature in GenBank database and references 2, 15, 28, 51, 82, 97, and 108.

[e] Existing nomenclature in GenBank [and reference(s)]: mts4 (106), mts3 and mts3 (87, 106), Mov-34 (37), Mbp1 (101), p54 (40), pad1 (89), MS73 (13), CADp44 (5), and m56 (92).

[f] Homologies were determined with MegAlign by the Jotun Hein method (gap penalty, 11; gap length, 3), except for the Sun2/p58 homology, which was taken from reference 51.

[g] See the text and the legend to Fig. 4 for details. The sequence was found in the dbEST database of the NCBI.

[h] In addition to sequence analysis, the identities of these five bands were corroborated by immunoblotting with appropriate specific antibodies.

[i] From reference 96a; see also references 27, 48, and 69.

nucleophile (Cys), a general base (His), and an acidic residue (Asp) (4, 23, 107). In Rpn11, a number of conserved aspartates and histidines are present downstream of the conserved Cys box and could potentially serve these functions.

Evolutionary conservation of RP subunits. All RP subunits identified in the purified preparation from yeast (Fig. 3) have clear homologs in other eukaryotes (Table 3). The Rpt subfamily of putative ATPases shows a greater degree of homol-

```
228 Y N F G E L L H H P I M E T I V N D S N Y D W L F Q L L N A L T V G D F D K F D   S. cerevisiae
220 H N F G E L L A H P I L K S L E G T R E - R W I V D V L L A F N S G D L T R F F   C. elegans
    F N F G E L L M H P V L E S L R D T D R - Q W L I D T L Y A F N S G A V D R F Q   mouse
    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   human

    S L I K V Q I S K I P I L A Q H E S F L R Q K I C L M T L I E T V F V K - - N I   S. cerevisiae
    S L - E G D W G G W D D L K K Q K D F L T A K I R L M A V M E L A V S R P T K A   C. elegans
    T L - K C A W G Q Q P D L A A N E A Q L L R K I Q L L C L M E M T F T R P A N H   mouse
    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   human

    R M L S F E D I S K A T H L P K D N V E H L V M R A I S L G L L K G S I D Q V N   S. cerevisiae
    R S V S F K E I A T K C Q I P F D E V E F L V M K A L S K D L I R G D I N Q V E   C. elegans
    R Q L T F E E I A K S A K I T V N K V E L L V M K A L S V G L V R G S I E L C G   mouse
    - - - - - - E I A K S A K I T V N E V E L L V M K A L S V G L V K G S I D E V D   human

    E L V T I S W V Q P R I I S G D Q I T K M K D R L V E W N D Q V E K L G K K M E   S. cerevisiae
    Q V V Y V T W V Q P R V L D N P Q I M Q M A T R I S A W R N D V N S M E G I V S   C. elegans
    Q A G S H D V G A A R V L D L Q Q I K G M K D R L E L W C T D V K S M E M L V D   mouse
    K R V H M T W V Q P R V L D L Q Q I K G M K D R L E F W C T D V K S M E M L V E   human

    A R G Q S I W V     393                                                           S. cerevisiae
    K E A R E I L T Q N 387                                                           C. elegans
    T G - - - - L R                                                                   mouse
    H Q A H D I L T                                                                   human
```

FIG. 4. Rpn9 is homologous to putative proteasome subunits in other eukaryotes. Homology of the C terminus of Rpn9 to deduced protein sequences of the product of a hypothetical ORF in *C. elegans* (z49130) and mouse (w34049) and human (aa122133) EST fragments is shown. The alignment was obtained by the Jotun Hein method with MegAlign (gap penalty, 11; gap length, 3). The C terminus of Rpn9 was 35, 36, and 42% identical to the *C. elegans*, mouse, and human sequences, respectively. Boxes indicate amino acid identities; dashes indicate gaps in the alignment.

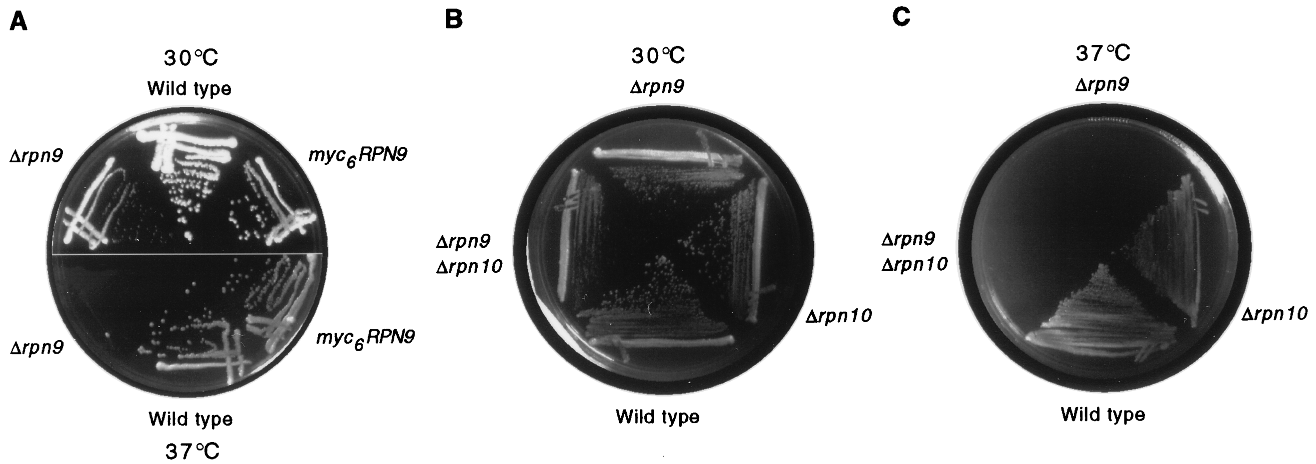

FIG. 5. Temperature-sensitive phenotype caused by *Δrpn9* deletion mutation. (A) Wild-type (SUB62), *myc*$_6$*-RPN9* (MG26), and *Δrpn9* (MG18) strains were grown on YPD at 30°C (top panel) or 37°C (bottom panel). The *Δrpn9* strain was temperature sensitive, showing no detectable growth after 48 h at 37°C. (After 1 week, a few small colonies were observed.) The *myc*$_6$-tagged version of *RPN9* fully complemented the deletion. (B and C) The *Δrpn9Δrpn10* double mutant did not display a marked synthetic phenotype. Wild-type (SUB62), *Δrpn9* (MG18), *Δrpn10* (102), and *Δrpn9Δrpn10* double mutant (MG29) strains were grown for 48 h on YPD at either 30°C (B) or 37°C (C).

ogy between species than the Rpn subunits (Fig. 7). The Rpt subunits are 66 to 76% identical between yeasts and humans, whereas the non-ATPase subunits show a lower yet significant amount of sequence identity, varying between 22 and 47% between species. Alone among the Rpn subunits, Rpn11 is 65% identical to its human counterpart, a level of identity similar to that observed for the ATPase subunits, consistent with the suggestion above that it could serve an enzymatic function within the RP.

A number of RP subunits show homology to each other. The six ATPase subunits (Rpt1 to Rpt6) are roughly 40% identical to each other. Among the non-ATPase subunits, three pairs show close to 20% identity to each other: Rpn1 with Rpn2 (6, 60), Rpn5 with Rpn7, and Rpn8 with Rpn11. The same relationship is maintained among their mammalian counterparts. Despite the similarity between Rpn11 and Rpn8, Rpn8 lacks candidates for a conserved active-site cysteine. However, another set of homologous proteins with a greater level of identity to Rpn11 (~30%) is found in numerous eukaryotes and contains the Cys consensus region (Table 5, group II). There is no evidence that these proteins are proteasome subunits; however, the significant level of homology to both Rpn11 and Rpn8 suggests that they might play a role in the ubiquitin-proteasome pathway. The sequence similarities among different Rpt and Rpn subunits raises the possibility that gene duplication played a major role in the evolution of the RP. The RP subunits may have diverged from a small number of subunits in an evolutionary precursor to the proteasome, similar to the apparent divergence of the 14 subunits of the CP from two precursors (12, 43).

TABLE 4. Alignment of Rpn11 to the conserved active-site cysteine in Ubp enzymes

Protein	GenBank accession no.	Cys box[a]
Rpn11	X79561/P43588	113 GFG**C**WLSSV 121
hUBP3	Q92995	343 GNS**C**YLSSV 350
yUBP14	P38237	351 GNS**C**YLNSV 359
hIsoT	U35116	332 GNS**C**YLNSV 340
yUBP8	P50102	143 GST**C**FMSSI 151
yUBP2	Q01476	742 GNT**C**YLNSL 750

[a] The putative active-site cysteine is marked in boldface.

Coassembly of Rpt subunits within the proteasome. The ATPase subunits of the RP are distinct from the non-ATPase subunits in that they show a high level of similarity to one another (Fig. 7). This fact raises the question of whether interchangeability among the ATPases during assembly may yield proteasomes with different subunit compositions and functional properties, as suggested, for example, by studies with *Manduca sexta* (13, 95). In *Escherichia coli*, the ClpP protease can assemble with multiple ATPase-containing RPs, but each ATPase is found in a different, homomeric assembly (36). In vitro, the mammalian proteasomal ATPases can interact in a pairwise manner, but assemblies of more than two ATPases have not been observed in such experiments (75). We tested whether multiple Rpt subunits are present in the same proteasome molecule by using Ni-NTA–chelate affinity chromatography of proteasomes bearing His$_6$-tagged Rpt subunits. Wild-type and His$_6$-Rpt2-containing proteasomes were par-

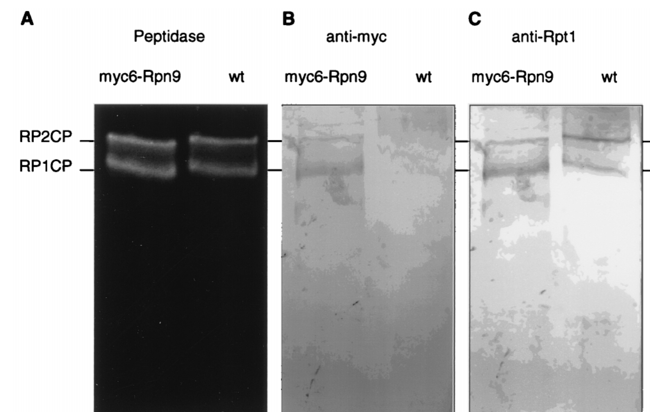

FIG. 6. Rpn9 is an RP subunit. Proteasomes from wild-type (wt) and *myc*$_6$*-RPN9* strains were partially purified on a DEAE–Affi-Gel Blue column and further resolved by nondenaturing PAGE. (A) Proteasome bands visualized in situ by peptidase activity against Suc-LLVY-AMC. (B and C) Immunoblots probed with the indicated antibodies.

TABLE 5. Conservation of putative Cys boxes of Rpn11 homologs

Group	Species	Locus or name[d]	Cys box[a]	Total amino acids	% Identity to Rpn11[b]
I[c]	*Saccharomyces cerevisiae*	YFR004w (Rpn11)	104 VVGWYHSHPGFG**C**WLSSVDVNTQ 126	306	100
	Caenorhabditis elegans	U00032	106 ----------------------- 128	319	52
	Discoides discoideum	U96916	108 -I--------------------- 130	306	58
	Schizosaccharomyces pombe	D31731 (pad1)	107 -----N-------------I--- 129	308	64
	Homo sapiens	U86782 (Poh1)	108 ----------------G--I--- 130	310	65
	Mus musculus	Y13071	107 ----------------G--I--- 129	309	65
	S. mansoni	AF014465	111 ----------------G--M--- 133	313	65
II[c]	*C. elegans*	U80814	134 ----------Y-----GI--S-- 156	368	27
	Arabidopsis thaliana	AF000657	137 ----------Y-----GI--S-- 159	357	29
	H. sapiens	U70734 (Jab1)	133 AI--------Y-----GI--S-- 155	334	29
	M. musculus	U70736	133 AI--------Y-----GI--S-- 155	334	29
	S. cerevisiae	YDL216c (Z74264)	174 ----F-----YD----NI-IQ-- 196	455	23

[a] The putative active-site cysteine is marked in boldface. Dashes represent identities to Rpn11.
[b] The identity of the product of each hypothetical ORF to Rpn11 was determined by the Jotun Hein method (gap penalty, 11; gap length, 3) with MegAlign. The identity within the putative Cys box was greater than that over the entire ORF.
[c] A pairwise comparison of all proteins indicated that the proteasomal subunits in group I were 52 to 65% identical to each other. Similarly, putative members of group II from different phyla were up to 60% identical to each other. Identity between proteins from different groups was slightly less than 30%.
[d] pad1 and Poh1 (89, 91) and Jab1 (10) were previously characterized.

tially purified on DEAE–Affi-Gel Blue columns and then affinity purified with Ni-NTA. The presence of proteasomes in the His_6-Rpt2 eluate was shown by peptidase activity as well as the copurification of multiple proteasome subunits (Fig. 8A and B). Importantly, during affinity purification, the ratios of Rpt1 to Rpt6 in the column load, flowthrough, and eluate remained essentially constant (1.35, 1.35, and 1.25, respectively; Fig. 8A), indicating that the purification procedure did not select a specific subset of proteasomes. Thus, Rpt1 and Rpt6 are both present in Rpt2-containing proteasomes, and the ratio of Rpt1 to Rpt6 in Rpt2-containing proteasomes is indistinguishable from that in total proteasomes. Similar experiments were done with strains expressing His_6-tagged versions of all six ATPases (Fig. 8C). Tagging of Rpt1, Rpt2, Rpt3, or Rpt4 allowed affinity purification of proteasomes without significantly altering the relative ratios of Rpt1 and Rpt6 in the column load and eluate (Fig. 8C). These data demonstrated that an individual proteasome contains multiple ATPases and that affinity purification of proteasomes from individually tagged ATPases yields proteasomes with similar compositions. The difficulty in purifying intact proteasomes from His_6-Rpt5- and His_6-Rpt6-expressing strains with Ni-NTA was probably due to the His_6 tag being occluded when the RP was complexed to the CP (see below).

Because the proteasome can contain one or two RPs (Fig. 3 and 4), different ATPases may copurify even though they are not present in the same RP complex. To test whether distinct ATPases assembled into a single RP, we found conditions in which the RP can be dissociated from the CP (Fig. 9). The CP is visualized as a faster-migrating complex on nondenaturing PAGE after dissociation of the proteasome components in the absence of ATP (Fig. 9A). The peptidase activity of the proteasome is greater by 1 order of magnitude than that of the CP alone (Fig. 9B). However, the peptidase activity of the CP is stimulated by SDS to levels similar to that of the proteasome holoenzyme (Fig. 9B).

Components of the RP were affinity purified after dissociation from the CP; column profiles of wild-type and His_6-Rpt1-expressing strains are shown in Fig. 10A and B. By tagging Rpt1 with His_6 we purified a complex that contained Rpt6 and Rpn10, as shown by immunoblotting of the column fractions (Fig. 10A). That the CP was not retained on the column was shown by the absence of peptidase activity in the eluate (Fig. 10B). The ratios of Rpt1 to Rpt6 in the column load, flowthrough, and eluate remained essentially constant for the His_6-Rpt1-containing cell extract (1.25, 1.40, and 1.20, respectively; Fig. 10A), indicating that the purification procedure did not select a specific subset of RPs. Indistinguishable results were obtained for His_6-tagged versions of each Rpt subunit (Fig. 10C). Thus, each Rpt subunit coassembles with multiple Rpt subunits into complexes with similar ratios of Rpt1 to Rpt6. These data strongly suggest that each RP contains all six ATPases.

DISCUSSION

Previous work on the proteasome of *S. cerevisiae* focused on the CP, culminating in the solution of its crystal structure (38). However, CPs fail to degrade physiological substrates of the proteasome, and their activity is not stimulated by ubiquitin or ATP. Thus, substrate selection and other key early steps in protein breakdown by the proteasome must be studied with the holoenzyme form of the complex. Here we report the biochemical characterization of the proteasome holoenzyme from *S. cerevisiae*. By amino acid sequence analysis, we directly identified 17 subunits that form the RP of the yeast proteasome. Genes encoding a number of these subunits or their homologs were originally identified through a wide variety of genetic screens (8, 14, 30, 35, 39, 50, 51, 76, 89, 94), only a few of which

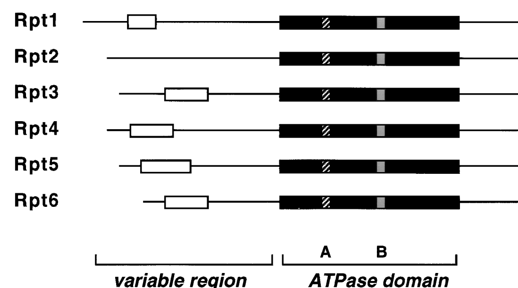

FIG. 7. Structural alignment of six proteasomal ATPases. Comparison of the six Rpt subunits based on their primary structure shows a highly conserved ATPase module (black box) containing the A and B loops which form the predicted ATP binding domain (33, 34, 67, 103). The N termini are variable, in some genes containing a predicted (62) coiled-coil domain (open box).

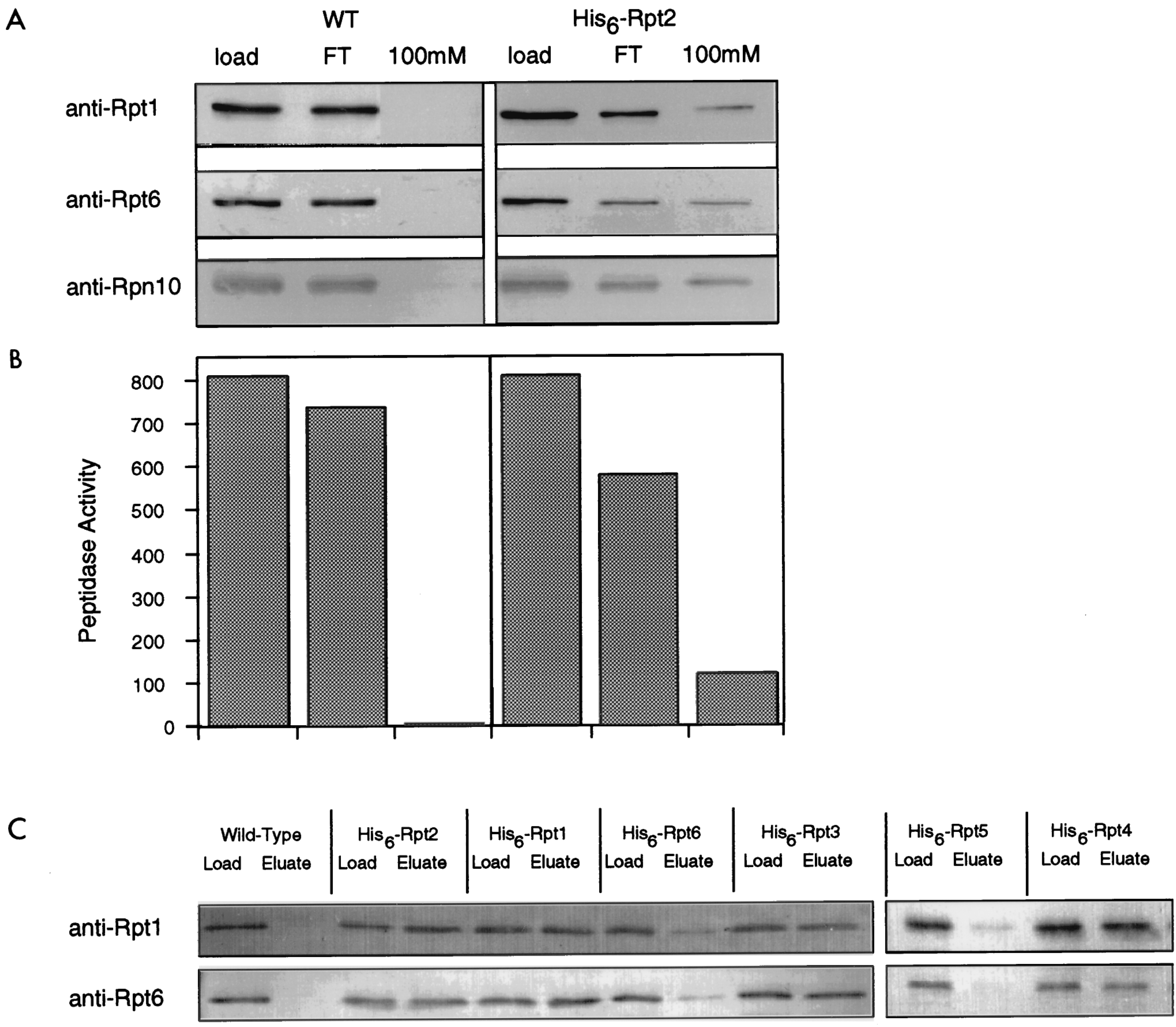

FIG. 8. The proteasome is a heteromeric complex of ATPases. His$_6$-Rpt2 was expressed in a Δ*rpt2* background (DY17). Extracts from His$_6$-Rpt2-expressing and wild-type (WT) control strains were partially purified by DEAE–Affi-Gel Blue chromatography in the presence of 1 mM Mg-ATP. The 150 mM NaCl eluate was subjected to Ni-NTA affinity chromatography. Column fractions were immunoblotted (A) and tested for peptidase activity against Suc-LLVY-AMC (B). The epitope-tagged complex eluted at 100 mM imidazole, as indicated by immunoblotting against Rpt1, Rpt6, and Rpn10 (A) and by peptidase activity (B). The wild-type proteasome eluted during low-imidazole rinses. (C) Extracts from strains expressing His$_6$-tagged versions of each of the six ATPases were individually purified by Ni-NTA chromatography. Fractions loaded onto the Ni-NTA column (Load) were compared to fractions from the 100 mM imidazole eluate (Eluate) by immunoblotting with anti-Rpt1 and anti-Rpt6 antibodies.

were designed to detect proteolysis mutants (14, 39). These data point to the breadth of the regulatory functions of the proteasome. The assembly of these proteins into the same complex provides a common explanation for the disparate and in many cases unexpected phenotypes. These genetic studies also suggest that substrate-specific effects on protein turnover can result from mutations in any of a large number of RP subunit genes, a suggestion which has interesting mechanistic implications.

Of the known mammalian RP subunits, only S5b/p50.5 (17, 18) appears absent from yeast. We found no evidence for an S5b homolog in purified yeast proteasomes; in agreement with this result, no clear S5b homologs are identifiable in the yeast genome database. Our survey of yeast proteasome components also did not identify proteins homologous to the proteasome activator PA28 (63), a result which is similarly supported by the lack of close PA28 homologs in the yeast genome. Yeast proteasomes appear to be more uniform than those of mammals in several ways: they do not appear to associate with PA28-like activator proteins that can replace the RP complex, and each of the 32 known subunits is apparently encoded by a single gene. The heterogeneity of the mammalian proteasome appears to regulate the nature of peptide end products of degradation rather than substrate selection and may be linked to the role of the proteasome in antigen processing (12). The relevance of subunit interchangeability to antigen processing is best exemplified by the LMP proteins, which interchange with other proteolytically active β subunits of the CP to alter the cleavage site specificity of the proteasome (12).

Possible interchangeability among the ATPases is suggested both by their strong sequence similarity to one another and by evidence that the ratio of one ATPase to another in the pro-

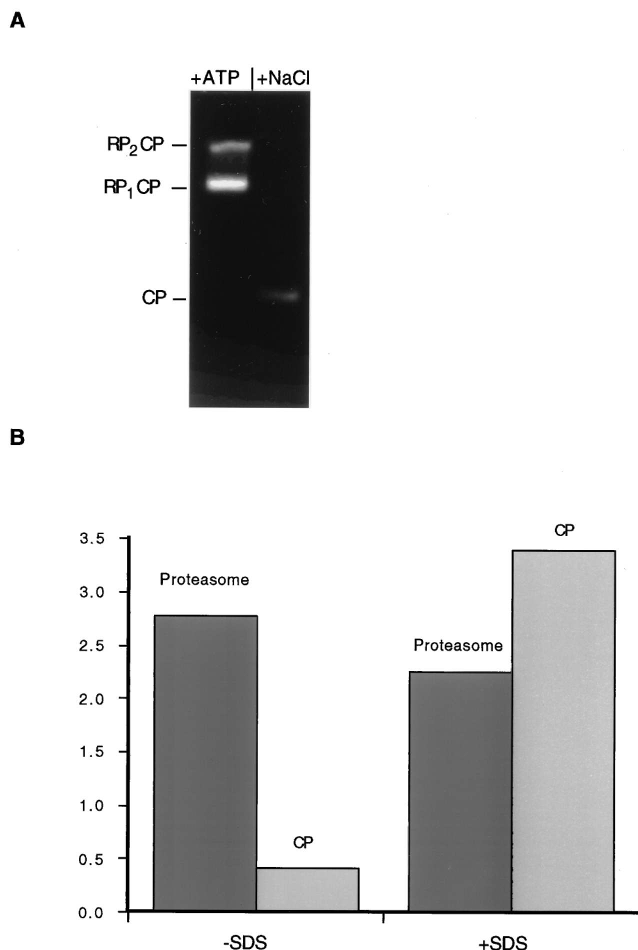

FIG. 9. Dissociation of the RP from the CP inhibits peptidase activity. Equal amounts of purified proteasome were incubated for 30 min at 30°C in buffer A or in buffer A without Mg-ATP but with 500 mM NaCl. (A) The two samples were resolved by nondenaturing PAGE and visualized by activity against the fluorogenic peptide substrate Suc-LLVY-AMC. After incubation in 500 mM NaCl, both singly- and doubly-capped forms of the proteasome (RP_2CP and RP_1CP, respectively; left lane) disassembled, giving rise to free CPs (right lane). It is apparent that the CP had lower peptidase activity than the proteasome on a molar level. The RP was not visualized by this method, as it contains no intrinsic peptidase activity. (B) To quantify the difference in peptidase activities between the proteasome and the CP, approximately equimolar quantities of the two samples were incubated for 10 min at 30°C in buffer A with 0.1 mM fluorogenic peptide Suc-LLVY-AMC; the fluorescence of released AMC is shown in the left columns. The two samples were also incubated for 10 min at 30°C in buffer A with 0.1 mM Suc-LLVY-AMC and 0.02% SDS (right columns). The CP exhibited a lower level of peptidase activity and a higher level of SDS stimulation than the intact proteasome.

teasome may change during the course of programmed cell death in *M. sexta*, with possible replacement of one ATPase subunit for another (13, 95). The simplest model for interchangeability among the ATPases, which has a precedent in prokaryotic ATP-dependent proteases (36), is that each RP contains a single type of ATPase and thus that the various ATPases define distinct proteasome populations. The results of the His_6 tagging experiments shown in Fig. 10 exclude this and related models. The data indicate that the six ATPases of the proteasome are present in the same complex, further suggesting that the subunit composition of the yeast proteasome may be uniform from particle to particle. The presence of six ATPases within a given proteasome is consistent with their assembly into a six-member ATPase ring structure analogous to those found in the simple ATP-dependent proteases of prokaryotes (36, 93). The same analogy suggests that this ring is situated in contact with the CP and that substrates pass through the center of this ring as they translocate into the CP. This model is consistent with the ATP dependence of proteasome assembly from the RP and CP complexes (3, 15, 45;unpublished data). A strictly determined site of assembly for each ATPase is suggested both by the coassembly of ATPases into a single particle and by the requirement for each ATPase in yeast (30, 81, 85).

The 17 subunit assignments proposed here all have a high degree of confidence. For example, for 29 peptides sequenced, all amino acids assigned were in agreement with predictions from the sequence of the yeast genome. Moreover, most of the subunits identified were homologs of known subunits of the mammalian RP (PA700). However, the existence of additional RP subunits in yeast remains a distinct possibility, which could best be addressed by two-dimensional isoelectric focusing and SDS-PAGE. In particular, the low-molecular-mass region of the one-dimensional gels that we used contained many CP-derived bands, which could comigrate with as-yet-unidentified RP subunits. In mammals, PA700 is a stable complex which has been purified and found to associate with the CP to produce a complex that is competent for the degradation of ubiquitin-protein conjugates (1, 15, 64). We have also partially purified a particle from yeast that can, when added to CPs, similarly reconstitute the degradation of ubiquitin-protein conjugates (unpublished data). However, it has yet to be established that PA700 is identical to the RP dissociated from purified proteasomes (83). As suggested above, certain components of the proteasome may be loosely associated and thus underrepresented in purified preparations.

The percentages of identities between sequences of yeast and human homologs of the various RP subunits are given in Table 3. The ATPases are exceptionally conserved, showing 66 to 76% identity, while identity scores for the non-ATPase Rpn subunits are much lower. The only exception is Rpn11/Mpr1, which is 65% identical between yeast and humans. Interestingly, the amino acid sequence surrounding Cys-117 within Rpn11 shows similarity to sequences flanking the active-site cysteine which serves as the nucleophile in deubiquitinating enzymes (Table 4). No other RP subunit thus far identified shows significant similarity to known deubiquitinating enzymes. All known Rpn11 homologs contain extended regions of identity to one another surrounding Cys-117 in Rpn11 (Table 5). It is plausible that Rpn11 and its homologs from other species function as a new class of deubiquitinating enzymes (Table 5, group I), potentially accounting for the deubiquitinating activity detected in preparations of the mammalian PA700 complex (53, 54). The predicted molecular masses of Rpn11 and its homologs are consistent with estimates based on active-site labeling of the bovine PA700 deubiquitinating factor (54). However, our proteasome preparations had low activity in several deubiquitination assays (data not shown) (52a), despite containing apparently intact Rpn11. It is possible that the ubiquitin conjugates tested thus far are not the preferred substrates of Rpn11 and that other substrates will allow the detection of Rpn11-dependent isopeptidase activity in yeast proteasomes.

Lam and coworkers have suggested that isopeptidase activity within the proteasome may serve to inhibit the degradation of certain conjugates by progressively trimming their ubiquitin chains from the distal end (53). We suggest that proteasomal isopeptidase activity may also, depending on the substrate, accelerate conjugate breakdown by removing ubiquitin groups that prevent translocation of the proteolytic substrate through

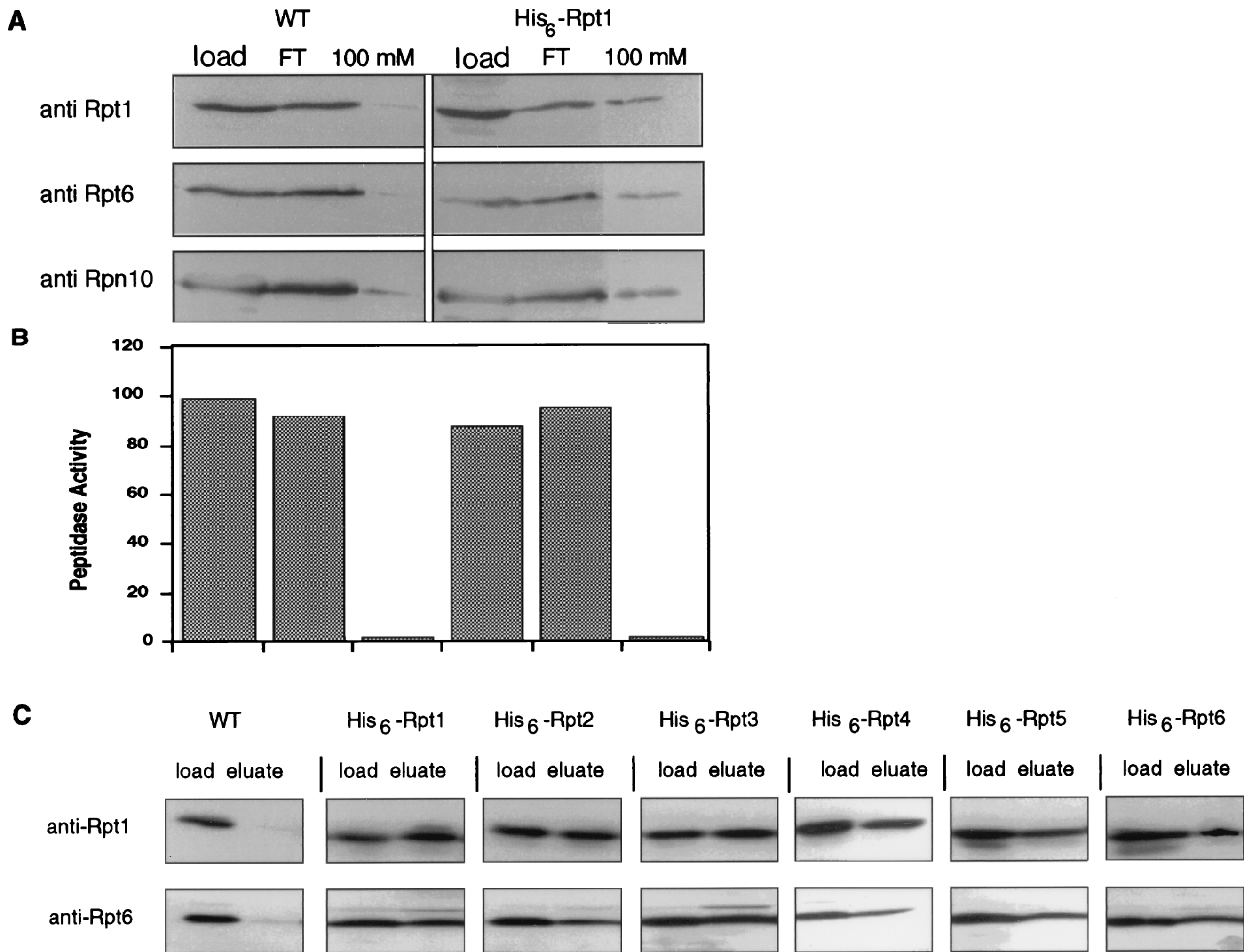

FIG. 10. Proteasomal ATPases associate into a heteromeric complex. His_6-Rpt1 was expressed in a Δ*rpt1* background (DY19). Extracts from His_6-Rpt1-expressing and wild-type (WT) control strains were partially purified on DEAE–CL-6B resin in the absence of ATP. The 500 mM NaCl eluate was fractionated on Ni-NTA affinity columns. Column fractions were subjected to immunoblotting (A) and tested for peptidase activity against Suc-LLVY-AMC (B). The epitope-tagged complex eluting at 100 mM imidazole contained a number of RP subunits (Rpt1, Rpt6, and Rpn10) (A) but lacked peptidase activity (B). The wild-type complex eluted during low-imidazole rinses. (C) Extracts from strains expressing His_6-tagged versions of each of the six ATPases were also purified by Ni-NTA chromatography. Fractions loaded onto the Ni-NTA column (Load) were compared to fractions from the 100 mM imidazole eluate (Eluate) by immunoblotting with anti-Rpt1 and anti-Rpt6 antibodies.

the channel of the CP. Stimulatory effects of removing ubiquitin groups from the substrate may be particularly dramatic for substrates in which ubiquitin groups are bound to multiple lysine residues within the target protein, rather than being assembled into a single chain. Assuming that the tertiary structure of ubiquitin is too stable to be unfolded by the proteasome, as suggested by structural studies (57), every ubiquitin group that is directly bound to the substrate is expected to prevent access of the substrate polypeptide to the CP in the region surrounding the ubiquitination site. Deubiquitinating enzymes that reverse such linkages would be expected to facilitate degradation, perhaps accounting for the observed simulatory effects of the deubiquitinating enzyme UCH-3 on in vitro ubiquitin-protein conjugate degradation (42). Consistent with this hypothesis, such results were obtained with a UbK48R derivative of ubiquitin which is deficient in chain formation.

It is presently unclear whether any of the remaining Rpn subunits possess enzymatic activity, since they lack sequence similarities to known enzymes. They are likely to function in the binding of proteolytic substrates, in the binding of soluble cofactors of the proteasome, or as scaffolding proteins that maintain the architecture of the RP complex. Another possible function is to target the proteasome to specific subcellular sites, although recent photobleaching studies with green fluorescent protein-tagged proteasomes indicated that >90% of proteasomes are freely diffusible in both the nucleus and the cytoplasm (74). Among Rpn subunits other than Rpn11, the only significant sequence motif identified thus far is a ninefold repeat covering approximately 400 residues in both Rpn1 and Rpn2 (60). The repeat motif is similar to previously described leucine-rich repeats which have been implicated in specific protein binding. One possible role for these repeats therefore may be binding of the proteolytic substrate, as suggested by Lupas and Baumeister (60).

The only Rpn subunit that has been extensively studied is Rpn10/Mcb1 and its homologs in *Arabidopsis thaliana* (Mbp1), *Drosophila melanogaster* (p54), and humans (S5a). Rpn10 homologs from all of these species are capable of binding multiubiquitin chains in vitro. The universality of this binding interaction strongly suggests its functional significance, and Rpn10/

Mcb1/S5a has consequently been proposed to be the multiubiquitin chain receptor of the proteasome (17, 18). However, yeast mutants in which the *RPN10/MCB1* gene has been deleted are viable and competent for the degradation of many ubiquitin conjugates (102). Only the model substrate ubiquitin–Pro–β-galactosidase has been found to be stabilized in the *rpn10* deletion strain. To test whether Rpn10 functions as a ubiquitin receptor, the in vitro ubiquitin chain binding site of Rpn10 was localized. When mutants in which the in vitro ubiquitin chain binding site was deleted were assayed for ubiquitin–Pro–β-galactosidase degradation in vivo, they were found to be fully competent (26). These data indicate that the role of Rpn10 in protein degradation is probably independent of its ability to bind multiubiquitin chains, at least in *S. cerevisiae*. Nonetheless, a comparison of the sequences of Rpn10 and its homologs across eukaryotes indicates that the in vitro ubiquitin chain binding site is stringently conserved evolutionarily (26, 41, 109), suggesting that it may have a role in proteasome function that has yet to be identified. The mechanistic role of Rpn10/Mcb1 in protein breakdown remains problematic but should emerge from additional genetic analysis.

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