Crystal Structure of Yeast Rpn14, a Chaperone of the 19 S Regulatory Particle of the Proteasome*

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The ubiquitin-proteasome pathway is a major proteolytic system in eukaryotic cells and regulates various cellular processes. The 26 S proteasome, the central enzyme of this pathway, consists of a proteolytic core particle and two 19 S regulatory particles (RPs) composed of ATPase (Rpt) and non-ATPase (Rpn) subunits. Growing evidence indicates that proteasome assembly is assisted by a variety of chaperones. In particular, it has been reported recently that Nas2, Nas6, Rpn14, and Hsm3 bind specific Rpt subunits, thereby contributing to the formation of 19 S RP. Rpn14 transiently binds to the C-terminal domain of the Rpt6 subunit (Rpt6-C) during maturation of the ATPase ring of 19 S RP. In this study, we determined the crystal structure of yeast Rpn14 at 2.0 Å resolution, which revealed that this chaperone consists of a unique N-terminal domain with unknown function and a C-terminal domain assuming a canonical seven-bladed β-propeller fold. The Rpt6-binding site on Rpn14 was predicted based on structural comparison with the complex formed between Nas6 and Rpt3-C. The top face of Rpn14 exhibits a highly acidic surface area, whereas the putative interacting surface of Rpt6-C is basic. By inspection of structural data along with genetic and biochemical data, we determined the specific residues of Rpn14 and Rpt6 for complementary charge interactions that are required for 19 S RP assembly.

The 26 S proteasome, the major proteolytic machine found in eukaryotic cells, plays a key role in ubiquitin-dependent proteolysis by degrading proteins conjugated to ubiquitin (1, 2). This large machine consists of a proteolytically active 20 S core particle (CP)3 and one or two 19 S regulatory particles (RPs). The 19 S RP is responsible for binding to the ubiquitin-tagged substrate, unfolding, translocating into the 20 S CP, and releasing ubiquitin from the substrate. The 20 S CP is composed of 28 subunits arranged in a cylindrical shape of four heterohexameric rings, α7β7γ7δ7ε7, whereas the 19 S RP is composed of at least 19 different subunits and is divisible into two subcomplexes, the base and the lid. The base is composed of six different homologous AAA+ ATPase subunits (Rpt1–Rpt6) and three additional non-ATPase subunits (Rpn1, Rpn2, and Rpn13). The lid is composed of at least nine non-ATPase subunits. The lid-base association is stabilized by Rpn10 (3, 4).

The molecular mechanisms underlying proteasome assembly have been explored recently. The discovery of the proteasome-specific chaperones (5), UMP1 (6) and PAC1–PAC4 (7, 8) in humans and Umpl (9) and Pba1–Pba4 (Pba1/Poc1, Pba2/Poc2, Pba3/Poc3/Dmp2, and Pba4/Poc4/Dmp1) (10–12) in yeast, revealed that assembly of the 20 S CP is a chaperone-assisted ordered process and not spontaneous self-organization. More recently, four proteasome-interacting proteins, Nas2/p27, Nas6/gankyrin, Rpn14/PAAF1, and Hsm3/S5b, have been shown to contribute to assembly of the 19 S base (13–15). Each base-dedicated chaperone is responsible for the formation of distinct subassemblies of the base and escorts them until the 19 S RP is formed.

Rpn14/PAAF1 was originally identified as a 19 S RP-associated protein that negatively regulates the 26 S proteasome activity in mammalian cells (16, 17). Rpn14 transiently binds to Rpt6 during assembly and consequently is not present in the mature 26 S proteasome (16). A major Rpn14-containing assembly intermediate is Nas6-Rpt3-Rpt6-Rpn14 in both yeast and human cells (13–15, 18, 19). A previous genetic study suggested that Rpn14 and Nas6 function cooperatively in the formation of the subassembly and 19 S base (14).

We reported recently the crystal structure of Pba3/Dmp2-Pba4/Dmp1 complexed with the α7-subunit of the CP and provided a structural basis for mechanisms underlying CP assembly (10). On the other hand, although the crystal structures of Nas6 and its complex with the C-terminal domain

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1, Tables S1 and S2, and an additional reference.

3 The abbreviations used are: CP, core particle; RP, regulatory particle; MAD, multiwavelength anomalous dispersion; GST, glutathione S-transferase; yEGFP1F, yeast enhanced green fluorescent protein 1 with a FLAG tag.
of Rpt3 have been reported (20, 21), detailed mechanisms regarding how the 19 S RP is formed remain unclear. For a better understanding of assembly mechanisms, it is necessary to elucidate the three-dimensional structure of the other chaperones, i.e. Rpn14, Hsm3, and Nas2. In this study, we report the crystal structure of yeast Rpn14 and predict a structural model for the Rpn14-Rpt6 interaction. Using yeast genetics and biochemical studies, we show that a specific interaction between Rpt6 and Rpn14 is required for the proper assembly of the 19 S RP.

EXPERIMENTAL PROCEDURES

Plasmid Construction—We amplified the Saccharomyces cerevisiae Rpn14 open reading frame by PCR from yeast genomic DNA of BY4741 and cloned it into the pET28(+) vector (Novagen). Site-directed mutants were generated with the QuickChange site-directed mutagenesis kit (Stratagene) using pET28-Rpn14 as the template. We subcloned wild-type and mutant RPN14 genes into the pGEX6P1 vector (GE Healthcare) and the pYF5 vector (22), which express N-terminally FLAG-tagged Rpn14 under the control of the GAL1 promoter. We also constructed the pET28(+)--Rpt6-C vector, which expresses the C-terminal region (amino acids 320–405) of Rpt6. Details of the expression plasmids and the primer sequences used for plasmid construction are given in supplemental Tables S1 and S2, respectively.

Protein Expression and Purification—We cultured Escherichia coli BL21(DE3) cells (Novagen) harboring pET28-Rpn14

## TABLE 1
Data collection, phasing, and refinement statistics

|                      | Native | SeMet |
|----------------------|--------|-------|
| X-ray source         | Spring-8 BL44XU |
| Space group          | P6_4   | P6_4  |
| Unit cell parameters |         |       |
| a (Å)                | 78.58  | 78.39 |
| b (Å)                | 78.58  | 78.39 |
| c (Å)                | 110.12 | 110.06|
| Wavelength (Å)       | 0.9    | 0.97914|
| Distance (mm)        | 400    | 500    |
| Exposure time (s)    | 1      | 1      |
| Resolution range (Å) | 39.28–2.00 (2.11–2.00) | 67.88–2.70 (2.80–2.70) |
| Completeness (%)     | 100.0  | 96.3  |
| Rmerge (%)           | 4.6 (40.5) | 9.2 (39.7) |
| Rfree (%)            | 5.7 (49.0) | 10.7 (46.4) |
| I/σ(I)               | 10.1 (2.1) | 23.2 (6.9) |
| Wilson B-factors (Å²)| 32.09  | 10.1 (2.1) |

### Refinement

|                | Native | SeMet |
|----------------|--------|-------|
| Resolution (Å) | 68.04–2.00 | 67.88–2.70 |
| No. of working reflections | 24720 | 223156 |
| No. reserved to evaluate R_free | 1325 | 19932 |
| R_factor (%)/R_free (%) | 20.0/25.5 | 9.2 (39.7) |
| r.m.s.d. bond lengths (Å) | 0.021 | 0.021 |
| r.m.s.d. bond angles | 1.96° | 1.96° |
| No. of protein atoms | 3348 | 3348 |
| B-factors of all atoms (Å²) | 40.07 | 40.07 |
| Ramachandran plot (%) | Most favored regions 85.0 | 85.0 |
|                 | Additionally allowed regions 14.7 | 14.7 |
|                 | Generously allowed regions 0.3 | 0.3 |
|                 | MolProbity score | 95.2 |

Values in parentheses are for the outer shell.

## TABLE 2
Yeast strains used in this study

| Strain     | Genotype                                      | Ref./source     |
|------------|-----------------------------------------------|-----------------|
| W303-1A   | MATα ura3-1, trp1-1, leu2-3,112, his3-11,15, ade2-1, can1-100 | Our stock      |
| YYS1201   | MATα Δnast::HIS3                               | Ref. 14        |
| YYS1203   | MATα Δrpn14::TRP1                              | Ref. 14        |
| YYS1206   | MATα Δnast::HIS3 Δrpn14::TRP1                  | Ref. 14        |
| YYS1644   | MATα rpn1::pPT6R373A/R374A-TRP1                | This study      |
| YYS1648   | MATα Δnast::HIS3 rpn6::pPT6R373A/R374A-TRP1     | This study      |
| YYS1255   | MATα rpn1::RPN1::yEGFP1F-LEU2                 | Ref. 14        |
| YYS1482   | MATα Δnast::HIS3 rpn1::RPN1::yEGFP1F-LEU2      | This study      |
| YYS1484   | MATα Δrpn14::TRP1 rpn1::RPN1::yEGFP1F-LEU2     | This study      |
| YYS1487   | MATα Δnast::HIS3 Δrpn14::TRP1 rpn1::RPN1::yEGFP1F-LEU2 | This study |
| YYS1693   | MATα rpn1::RPN1::yEGFP1F-LEU2                 | This study      |
| YYS1694   | MATα Δnast::HIS3 rpn6::pPT6R373A/R374A-TRP1 rpn1::RPN1::yEGFP1F-LEU2 | This study |
| YYS1693   | MATα rpn1::RPN1::yEGFP1F-LEU2                 | This study      |
| YYS1694   | MATα Δnast::HIS3 rpn6::pPT6R373A/R374A-TRP1 rpn1::RPN1::yEGFP1F-LEU2 | This study |

This study
FIGURE 1. Three-dimensional structure of yeast Rpn14. A, ribbon diagram of Rpn14. The N-terminal domain and the C-terminal β-propeller are colored green and cyan, respectively. The secondary structural elements are labeled. B, topological diagram of the secondary structural elements of Rpn14. The α-helices and β-strands are represented as cylinders and arrows, respectively. C, surface potential representation of Rpn14. Red, blue, and white represent acidic, basic, and neutral, respectively. Mutations are depicted via black boxes around acidic residues. D, molecular surface conservation of Rpn14. Mutation positions of conserved residues are shown by arrows.
in LB medium containing kanamycin (50 μg/ml) at 37 °C. Protein expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside at 18 °C for 20 h. Cells were lysed in buffer A (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl) by sonication. Cell supernatants were obtained by centrifugation at 15,000 g for 10 min at 4 °C. We purified the recombinant protein by affinity, anion exchange, and gel filtration chromatographies and then concentrated it to 10 mg/ml. The selenomethionine derivative of Rpn14 was expressed in E. coli B834(DE3) (Novagen). The preparation and purification of the selenomethionine derivative of Rpn14 were carried out by the same procedures as described for the wild-type protein.

**Crystallization and Data Collection**—Crystals of Rpn14 were obtained under the condition of 200 mM magnesium chloride, 50 mM Tris-HCl (pH 8.0), and 25% (w/v) polyethylene glycol 3350 by the hanging-drop vapor diffusion method using Hampton screening kits. We obtained single crystals of the selenomethionine derivative of Rpn14 in a reservoir buffer containing 8% (w/v) polyethylene glycol 3350 by microseeding at 293 K. Crystals (0.2 × 0.2 × 0.8 mm) were equilibrated in cryoprotectant buffer containing reservoir buffer plus 30% (v/v) glycerol and then cryogenized in a cold nitrogen stream at 100 K. Diffraction data sets were collected at beamline BL44XU of SPring-8 (Hyogo, Japan). These data were indexed and integrated with the program MOSFLM (23) and scaled using the program SCALA (24). Data collection statistics are given in Table 1. Crystals belonged to the space group P6_3, with cell dimensions of a = 73.3, b = 73.3, and c = 102.4 Å. The value of the Matthews coefficient was 2.13 Å³/Da for one molecule in the asymmetric unit, corresponding to a solvent content of 42.4%.

**Structure Determination and Refinement**—The structure of Rpn14 was determined by the multiwavelength anomalous dispersion (MAD) method using a selenomethionine derivative. The positions of the heavy atoms were obtained using SHELXD (25) and refined using SHARP (26). Initial MAD phases were extended to a higher resolution with the diffraction data collected from a native crystal to 2.0 Å resolution with solvent flattening using PIRATE (24) and DM (27). The initial model was constructed with the program ARP/wARP (28). This program automatically constructs a structural model containing 365 amino acid residues. The remaining parts of the structure were built manually using the program COOT (29). The model was refined at 2.0 Å resolution with the program REFMAC5 (30). The final Rpn14 model contained residues 1–414. There were no residues in disallowed regions of the Ramachandran plot. The final refinement statistics are summarized in Table 1.

**Glutathione S-Transferase (GST) Pulldown Assay**—GST-Rpn14 or the mutants expressed in Rosetta 2(DE3) cells (Novagen) were prebound to glutathione-Sepharose 4B and mixed with the E. coli lysate that expressed Rpt6-C at 4 °C. After centrifugation, beads were washed five times with buffer A and subjected to SDS-PAGE.

**Yeast Strains and Media**—Standard techniques were used for strain constructions and transformations (31). YPD medium consisted of 1% yeast extract, 2% Bacto-peptone, 2% glucose, 400 mg/liter adenine, and 20 mg/liter uracil. SGal-Ura medium consisted of 0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 2% galactose, 20 mg/liter tryptophan, 400 mg/liter adenine, and 10 mM phosphate buffer (pH 7.5). All strains listed in Table 2 are congenic to strain W303. To construct an rpt6 mutant, a DNA fragment encoding 5’-truncated RPT6 (137 to 1563) was inserted into the YIp vector pRS304 (32), and the residues corresponding to Arg373 and Arg374 of Rpt6 were mutated to Ala. The resulting plasmid, pRS304-rpt6R373A/R374A, was digested at the ClaI site and transformed into wild-type diploid cells (strain W303D). The positive transformant, in which correct integration was confirmed by DNA sequenc-
ing, was subjected to tetrad analysis, and YYS1644 was obtained. The other yeast strains, YYS1482, YYS1484, YYS1487, YYS1648, YYS1693, and YYS1694, were constructed by genetic crossing.

Native PAGE and Fluorescence Imaging—We analyzed proteasome assembly as described previously (14). In brief, the cell lysate of Rpn1-YEGFP1F-expressing cells was loaded onto 3.5% native polyacrylamide gel. After electrophoresis, the fluorescence of Rpn1-YEGFP1F on the gel was imaged using a Typhoon 9400 scanner (GE Healthcare). Using the same gel, proteasome peptidase activities against succinyl-LLVY-7-amido-4-methylcoumarin (Peptide Inc.) were imaged using an LAS-4000 IR system (Fujifilm).

Western Blotting—Total cell extracts were prepared by the mild alkali method (33). Antibodies used for Western blotting were horseradish peroxidase-conjugated anti-FLAG antibody M2 (Sigma), anti-Rpt6 antibody (a generous gift from Dr. Dan Finley), anti-Nas6 antibody (a generous gift from Dr. Junko Takeuchi), and anti-Rpn14 antibody. In this study, we raised anti-Rpn14 polyclonal antibody against rabbit using a full-length Rpn14 recombinant protein.

RESULTS AND DISCUSSION

Overall Structure of Rpn14—The crystal structure of S. cerevisiae Rpn14 was determined by the MAD method and refined to 2.0 Å resolution (Table 1). Rpn14 consists of a small N-terminal domain (residues 1–75) and a C-terminal WD40 repeat domain (residues 76–417) (Fig. 1, A and B). The N-terminal domain is composed of two antiparallel β-sheets (S1/S2/S3 and S4/S5/S6) comprising six β-strands and a short α-helix (H1). The C-terminal WD40 repeats assume a β-propeller domain with seven blades, each of which adopts a β-sheet conformation composed of four antiparallel β-strands. Thus, Rpn14 exhibits a canonical β-propeller structure, although a 23-residue loop is inserted between SD1 and SD2. This insertion is not strictly conserved in sequence or in length among organisms (supplementary Fig. S1). The top face of the propeller is defined by loops 2-3 and 4-1, which joins S4 (A–F) and S1 (A–F) of consecutive blades, and the bottom face by loops 1-2 and 3-4. The width of Rpn14 from the top face to the bottom face is ~22 Å, and the diameter is ~43 Å. The central channel of the β-propeller is ~7 Å in diameter.

Structural homologs of Rpn14 were searched in the Protein Data Bank using the Dali server (34). Rpn14 shares a significant degree of structural similarity with protein-binding proteins to proteins with a known structure. The C-terminal WD40 repeats assume a β-propeller domain, with seven blades, each of which adopts a β-sheet conformation composed of four antiparallel β-strands. Thus, Rpn14 exhibits a canonical β-propeller structure, although a 23-residue loop is inserted between SD1 and SD2. This insertion is not strictly conserved in sequence or in length among organisms (supplementary Fig. S1). The top face of the propeller is defined by loops 2-3 and 4-1, which joins S4 (A–F) and S1 (A–F) of consecutive blades, and the bottom face by loops 1-2 and 3-4. The width of Rpn14 from the top face to the bottom face is ~22 Å, and the diameter is ~43 Å. The central channel of the β-propeller is ~7 Å in diameter.

Structural homologs of Rpn14 were searched in the Protein Data Bank using the Dali server (34). Rpn14 shares a significant degree of structural similarity with protein-binding proteins such as the signal transduction regulator RACK1 (35) and the G protein β-subunit, Gβ (36), with root mean square deviations for related elements ranging from 2.1 to 3.2 Å. Rpn14 has 16–19% amino acid identity with these proteins. The core structure of β-propellers superimposes well, but the structure of the N-terminal domain of Rpn14 has no significant similarity to proteins with a known structure.

Electrostatic surface potential analysis of Rpn14 showed that the region of blades A, F, and G at the top of the propeller and the N-terminal region exhibit negatively charged surfaces, which are composed of Asp13, Glu19, Glu20, Asp22, Glu23, Asp25, Asp26, Asp27, Asp31, Glu38, Glu115, Asp117, Asp132, Asp157, Asp182, Asp183, and Glu184 (Fig. 1C). The remaining regions of the molecule have mixed electrostatic potentials. Mapping of the conserved residues on the surface of Rpn14 was performed using a ClustalW (37) multiple-sequence alignment of Rpn14 proteins from eight different species and the ConSurf program (38). The conserved patches of residues are located at the top of the propeller, around blades A and B and the N-terminal region (Fig. 1D), whereas the bottom and perimeter surfaces are noticeably less conserved. The conserved surface at the top face of the molecule is relatively abundant in negatively charged residues, i.e. Asp13, Asp22, Glu23, Glu27, Glu115, and Asp157.

The C-terminal domain of Rpn14 shows overall sequence and structural similarity to Gβ (19% sequence identity and root mean square deviation of 2.1 Å) (Fig. 2A). Gβ exhibits a β-propeller fold composed of seven four-stranded β-sheets and an N-terminal extended helix and interacts with many binding partners. The crystal structure of Gβ complexed with an α-helical peptide (Protein Data Bank code 1XMH) has been reported previously (39). Because the C-terminal domain of Rpt6 is predicted to form an α-helical structure, one might expect that Rpn14 binds to this domain in a manner similar to the binding of the β-propeller of Gβ to the helical peptide exclusively at its top
surface. This interaction site is separated into two regions: one bearing acidic residues (Asp228, Asn230, and Asp246) and one bearing hydrophobic residues (Met101 and Met188) (Fig. 2B).

These residues are less conserved between GB and Rpn14. In Rpn14, the corresponding residues form a negatively charged area at the top face. Therefore, this unique surface property is likely required for binding to Rpt6.

Structural Features and Functional Similarity between Rpn14 and Nas6—Although the tertiary structures of Rpn14 and Nas6 are quite different, Rpn14 contains WD40 motifs, and Nas6 contains ankyrin repeats, and these chaperones bind to the C-terminal regions of specific Rpt subunits (13–15, 19). Nas6 was shown to interact specifically with the C-terminal region of Rpt3, Rpt3-C (residues 348–428), through surface charge complementarity (Fig. 3, A and B), i.e. one positively and two negatively charged patches in Nas6 and one negatively and two positively charged patches in Rpt3-C (20, 21). On the basis of the above results, we hypothesized that the Rpn14-Rpt6 interaction may also occur through the complementary charge interaction, with the acidic surface at the top of Rpn14 accommodating positively charged residues of Rpt6-C. To investigate this possibility, a homology model of Rpt6-C (Fig. 3C) was built from the R333A/R334A and R344A/R345A mutants had no effect on the interaction. This result suggested that Arg373 and Arg374 in Rpt6-C are critical residues for Rpn14 binding. To determine the physiological relevance of the interaction, we constructed a yeast rpt6 mutant strain in which Arg373 and Arg374 of the Rpt6 gene were mutated to Ala. The rpt6R373A/R374A cells grew normally even when cultured at the high temperature of 37 °C (Fig. 4B). Previously, we found that the Δnas6 Δrpn14 double deletion cells show severe growth defects at high temperatures, whereas cells with single deletions do not show such defects (14). This genetic interaction suggests that the Nas6-Rpt3 and Rpn14-Rpt6 interactions are not indispensable for 19 S RP assembly but lethal when both interactions are absent. If Arg373 and Arg374 of Rpt6 are critical residues for Rpn14 binding, genetic interaction between rutp6R373A/R374A and Δnas6 should be observed. As anticipated, the rutp6R373A/R374A Δnas6 double mutant cells showed a temperature-sensitive phenotype, as did the Δnas6 Δrpn14 cells (Fig. 4B). The protein levels of Nas6, Rpn14, and Rpt6 were not affected by deletion or mutation (Fig. 4C).

Next, we investigated the impact of the basic residues of Rpt6 on 26 S proteasome assembly. To monitor proteasome assembly, we constructed green fluorescent protein-tagged

Effects of Rpt6 Mutations on Complex Formation—To test the hypothesis that surface charges are involved in the interaction, we mutated the residues in the charged patches of Rpt6 and examined Rpn14 binding in vitro. Although the expression of the Rpt6-C protein alone yielded an aggregated form in E. coli, Rpt6-C was soluble when coexpressed with Rpn14. We then mutated basic residues (R333A/R334A, R344A/R345A, and R373A/R374A) in Rpt6-C and coexpressed them with Rpn14. As shown in Fig. 4A, the R373A/R374A mutation reduced binding to Rpn14, whereas
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Genetic data indicated that the specific residue Asp\(^{157}\) plays an important role in the function of Rpn14. We confirmed that both Rpn14 mutants D157A and D157K were expressed as well as wild-type Rpn14 in yeast cells (data not shown). Finally, we conducted in vitro pulldown assays using GST-Rpn14 and *E. coli* lysates expressing Rpt6-C. Compared with wild-type Rpn14, the D157A and D157K mutants resulted in reduced binding to Rpt6-C (Fig. 5C). On inspection of these data, we concluded that Asp\(^{157}\) of Rpn14 contributes to the interaction with Rpt6 and the function of RP assembly.

In conclusion, we determined the crystal structure of yeast Rpn14 at 2.0Å resolution. The refined structure in conjunction with mutational and genetic data provided further insight into the interaction between Rpn14 and Rpt6. In particular, our findings suggest that the complex formation between Rpn14 and Rpt6 occurs through electrostatic interactions in a manner similar to that between Nas6 and Rpt3. Although mechanistic actions of how the base-dedicated chaperones regulate RP assembly remain unclear, our structural study of the Rpn14-Rpt6 interaction provides a framework for future studies of RP assembly.

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**FIGURE 5. Impact of negatively charged residues on Rpn14 function.** A and B, genetic analyses of Rpn14 surface mutants. The Δnas6 Δrpn14 cells harboring a plasmid that expresses RPN14 or mutants under the control of the galactose-inducible promoter were streaked onto SGal-Ura plates and incubated for 5 days at 37 °C. We tested charge-neutralizing (A) and charge-reversal (B) mutations. C, GST pulldown assay of Rpn14 mutants. Affinity-purified GST, GST-Rpn14, GST-Rpn14D157A, and GST-Rpn14D157K were analyzed by SDS-PAGE (left panel). Resin-bound GST or GST fusion proteins were incubated with the *E. coli* lysate expressing Rpt6-C (+) or not (−). After washing extensively, the resin-bound proteins were eluted and analyzed by SDS-PAGE, followed by silver staining (right panel). WT, wild-type protein; M, molecular mass markers; CBB, Coomassie Brilliant Blue.

**RPN1** strains through which RP subassemblies can be detected directly on native polyacrylamide gel (14). Similar to previous reports, 26 S proteasome levels were decreased in the Δnas6 Δrpn14 mutant due to a defect in RP formation (Fig. 4D). In the rpt6R373A/R374A single mutant cells, a slight decrease in 26 S proteasomes was observed, presumably because of a defect in the RP-CP interaction rather than RP assembly. Alternatively, severe impairment of RP assembly was observed in the rpt6R373A/R374A Δnas6 double mutant cells and in the Δnas6 Δrpn14 mutant cells. Thus, Arg\(^{373}\) and Arg\(^{374}\) in Rpt6 are critical residues for Rpn14 binding in vivo.

**Role of Negatively Charged Amino Acids of Rpn14**—The surface of Rpn14 has multiple acidic patches (Fig. 1C). To determine which acidic residues in Rpn14 are important for the chaperone function, i.e. Rpt6 binding, we performed genetic analysis using Δnas6 Δrpn14 cells. Each of the Rpn14 mutants (E19A/E20A, D22A/E23A, D25A/D26A/E27A, E115A, E138A, D157A, D382A/D383A/E384A, E384A, D27K, D25K/D26K/E27K, E115K, D157K, E384K, and D382K/D383K/E384K) was expressed in yeast (Fig. 5A and B).
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