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Pba3–Pba4 heterodimer acts as a molecular matchmaker in proteasome α-ring formation

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ABSTRACT

Eukaryotic proteasome assembly is assisted by multiple dedicated chaperones. In yeast, formation of the heteroheptameric ring composed of α1–α7 subunits is promoted by the heterodimeric chaperone Pba3–Pba4. Here we reveal that in the absence of this dimeric chaperone, α2 replaces α4 during α-ring assembly, thereby giving rise to a non-productive complex that lacks α4, β1, β5, β6, and β7 subunits and aggregates of α4. Furthermore, our structure-guided mutational data demonstrate that the Pba3–Pba4 heterodimer acts as molecular matchmaker reinforcing the interaction between α4 and α5, which is the crucial step in the α-ring formation.

1. Introduction

Ubiquitin/proteasome-mediated proteolysis plays a regulatory role in a number of diverse cellular processes, including cell-cycle progression, DNA repair, apoptosis, immune response, signal transduction, transcription, metabolism, and protein quality control. The 26S proteasome, which is responsible for the degradation of ubiquitinated substrates, is a highly conserved proteolytic machine consisting of a proteolytically active 20S core particle (CP) and one or two 19S regulatory particles [1–3]. In eukaryotic cells, the 20S CP is composed of 28 subunits arranged in a twofold symmetric cylindrical shape of four stacked heteroheptameric rings. Each outer ring is composed of seven different but homologous α subunits, i.e., α1–α7, whereas each inner ring is constructed from seven different but homologous β subunits, i.e., β1–β7. These subunits are located at specific positions in the quaternary structure of the 20S CP. Assembly of the eukaryotic 20S CP begins with α-ring formation; subsequently, β subunits are recruited at a specific position on the α-ring in the following order: β2, β3, β4, β5, β6, β1, and β7 [4]. The C-terminal 15 residues of β7 subunit bind to the cleft formed between β1 and β2 subunits in a *trans* manner, thereby promoting the formation of the 20S CP [5]. Completion of 20S CP assembly is accompanied by autocatalytic removal of the propeptides, unsealing the catalytic sites of the β subunits.

Accumulating evidence indicates that assembly of the proteasomal subunits is not a spontaneous process, but is assisted by a series of specific chaperones. It has been shown that human UMP1 and PAC1-4 and yeast Ump1 and Pba1–4 (Pba1/Poc1, Pba2/Poc2, Pba3/Poc3/Dmp2, and Pba4/Poc4/Dmp1) serve as assembly chaperones contributing to the formation of the 20S CP [6–8]. While Pba1 and Pba2 form a heterodimer and bind the α-ring of the 20S CP through their C-terminal HbYX motifs [9,10], a heterodimeric complex formed between Pba3 and Pba4 interacts with the α5 subunit [9,11]. In contrast, Ump1 has been shown to be an intrinsically unstructured protein that forms a complex with assembly intermediates containing β subunit(s) and remains associated with the preholo-20S proteasome [12,13]. Elimination of these assembly chaperones results in malformation of the proteasome. For example, a complex smaller than 20S CP has been identified in *Δpba3* and *Δpba4* cells [11]. This complex contains the β2 subunit and all α subunits, except for α4. This abnormal

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complex was induced by the shut off of *PBA4* (or *PBA3*) expression but did not diminish promptly even when *PBA4* (or *PBA3*) expression resumed after complex formation. This suggested that the complex was not a normal on-pathway intermediate.

Despite the progress in structural studies, the mechanism by which assembly chaperones assist in the correct arrangement of the proteasome subunits is not well understood. Herein we address this issue by focusing on the functional mechanisms of the Pba3–Pba4 heterodimer.

2. Materials and methods

2.1. Strains and genetic manipulations

The *Escherichia coli* strain DH5α was used for propagating plasmids. BL21 (DE3) cells were used for expression and purification of recombinant proteins. The methods used for manipulation of yeast have been described previously [14]. The following strains of *Saccharomyces cerevisiae* were used: YYS1297 (*MATa Δlys2::TRP1 pre5::PRE5-3xFLAG-LEU2*); YYS1299 (*MATa Δlys2::TRP1 pre5::PRE5-3xFLAG-LEU2: Δpba4*); YT478 (*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 PRE6-EGFP-kanMX4 Δpba4::HIS3MX6*); YT480 (*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 PRE6-EGFP-kanMX4*); and YT931 (*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 PRE10-EGFP-kanMX4:: Δpba4::HIS3MX6*).

2.2. Microscopic analysis

Cells harboring GFP-fused proteins were photographed using a Leica DM4000B microscope equipped with a HCX PL FLUOTAR 100×/1.30 objective (Leica) and a Leica DFC300 FX camera. Images were obtained and processed using LAS AF6000 software (Leica) and processed using Adobe Photoshop CS4.

2.3. MS analysis

Affinity purification of the proteasome and MS analysis were performed as described previously [14]. To quantify each CP subunit contained in the complex, we performed quantitative MS using stable isotope labeling in culture (SILAC); the WT background strains were grown in SILAC medium supplemented with "light" lysine, ^{12}C-Lys. To prepare the 20S CP that contained all the subunits stoichiometrically, we cultured the *PRE5-3xFLAG* cells, in which the CP α6 subunit was tagged with 3xFLAG tag, in SILAC medium. The cells were lysed using glass beads in 50 mM Tris–HCl buffer (pH7.5) containing 100 mM NaCl, 10% glycerol, and Complete Protease Inhibitors (-EDTA, Roche). Then, the FLAG-tagged protein was recovered by adsorption to an antiFLAG M2 agarose (Sigma), followed by elution with 3xFLAG peptide in 50 mM Tris–HCl buffer (pH 7.5) containing 100 mM NaCl and 10% glycerol. Purified α6-containing complexes were then separated by BN-PAGE using a NativePAGE™ Novex® 3–12% Bis–Tris Protein Gel (Life Technologies). The specific bands corresponding to the 20S CP were extracted. The same experimental steps were repeated for purification of isotope-labeled abnormal complex using a SILAC medium supplemented with "heavy" lysine, ^{13}C-Lys.

For quantitative MS analysis, the protein bands were excised, denatured with 0.5% RapiGest (Waters) in 50 mM Tris–HCl (pH 8.8), and digested with 20 ng/ml lysyl endopeptidase (Wako) for 16 h at 37 °C. The resulting peptides were combined and subjected to LC-MALDI TOF/TOF (AB SCIEX, 4800 MALDI TOF/TOF analyzer). MS and MS/MS data were processed using ProteinPilot software with Paragon Algorithm (AB SCIEX).

2.4. Protein expression and purification

Pba3, Pba4, and Pba4 mutants were expressed as described previously [11]. Proteasome subunits α2, α4, and α5 were expressed from the pGEX4T plasmid (GE Healthcare) as GST fusion proteins. GST-α2, GST-α4, and GST-α5 were purified using glutathione Sepharose 4B affinity chromatography. The GST moiety was cleaved from the α5 by thrombin treatment. The protein was further purified using anion exchange chromatography on a HiTrap Q HP column (GE Healthcare) and gel filtration chromatography on a Superdex 75 column (GE Healthcare).

2.5. Pull-down assay

GST-α4 and its mutant forms immobilized on glutathione-Sepharose resin were washed extensively with 20 mM Tris–HCl, pH 7.5 containing 150 mM NaCl. The Sepharose resins containing approximately 10 μg of GST fusion proteins were incubated with 1 ml of the buffer containing 150 μg of Pba3, 130 μg of Pba4, and 220 μg of α5 for 1 h at 4 °C, and then washed three times with 1 ml of the buffer. Proteins bound to the glutathione-Sepharose resin were subjected to SDS–PAGE followed by CBB staining. Formation of protein complex was also confirmed by size-exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare) equilibrated with 20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and 10 mM 2-mercaptoethanol, followed by SDS–PAGE and Western blotting against α4.

2.6. Structural modeling

3D structural model of the proteasome α-ring interacting with Pba3–Pba4 was built on the basis of crystal structures of yeast 20S proteasome (PDB ID code 1RYP) [15] and the α5 subunit complexed with the heterodimer of Pba3 and Pba4 Δloop (PDB ID code 2Z5C) [11], by superimposing the Cα atoms of the α5 subunit using LSQKAB program from CCP4 suite [16,17]. The model was subjected to energy minimization process using CNS [18].

3. Results and discussions

3.1. The composition of abnormal complex in Δpba4 cells

To define the functional role of the Pba3–Pba4 heterodimer precisely, we first characterized the abnormal complex observed in *Δpba4* cells. The α6-containing complexes were purified and then separated using BN-PAGE (Fig. 1A). The specific bands for the ^{13}C-Lys-labeled abnormal complexes and unlabeled 20S CP were excised and digested into peptides. The resultant peptides were jointly subjected to LC–MS/MS analysis to quantify subunit composition. Although our previous western blot analysis identified the β2 subunit and all the α subunits (except for α4) in the abnormal complex [11], the quantitative composition has not been determined. The quantitative MS data presented here revealed that the subunit ratios of this abnormal complex were stoichiometric for α1, α3, α5, α6, α7, β2, β3, and β4. Intriguingly, the ratio of the α2 subunit in this complex was twice as high in a normal complex (Fig. 1B, Supplementary Fig. 1), suggesting that the α4 subunit was replaced by α2.

We have previously reported that, among the α subunits, α5 binds to Pba3–Pba4 most strongly [11]. This raises a possibility that Pba3–Pba4 controls the proper interaction between α4 and α5 during the α-ring formation. To test this possibility, we examined interactions of α5 with α2 and α4 *in vitro* in the presence and absence of Pba3–Pba4 (Fig. 1C). We found that the interaction between α4 and α5 depends on Pba3–Pba4, while α2 binds α5

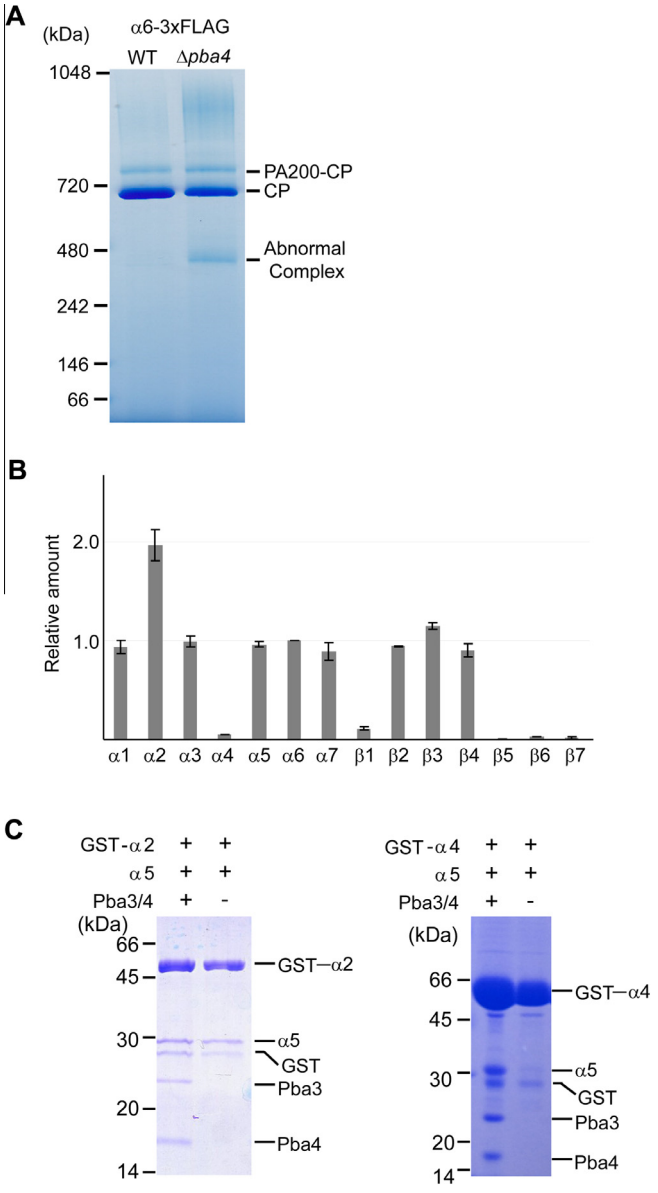

Fig. 1. Characterization of impaired α-ring formation in *Δpba4* cells (A) Blue native (BN)-PAGE analysis of the α6-containing complex in *Δpba4* cells. Crude lysate from PRE5(α6)-3xFLAG (YYS1297) and PRE5(α6)-3xFLAG/ΔPba4 (YYS1299) strains were affinity-purified using AntiFLAG M2 beads and resolved using 3–12% BN-PAGE followed by CBB staining. Major protein bands were assigned to PA200/Blm10-CP, CP, and the abnormal complex (by LC–MS/MS analysis). (B) Quantitative MS analysis of subunit composition of the abnormal complex in *Δpba4* cells. The amount of each subunit is shown in a bar graph, relative to the amount of α6 subunit. Values are mean ± S.D., $n = 4$. (C) Protein–protein pull-down assay of interaction of α5 with GST-α4 or GST-α2 immobilized on glutathione-Sepharose gel in the presence or absence of Pba3–Pba4.

directly in a chaperone-independent manner. The formation of Pba3/4-α5-α4 ternary complex was confirmed by size-exclusion chromatography (Supplementary Fig. 2). These data strongly suggest that the process of joining α4 and α5 is the hardest part of the α-ring formation, and α2 replaces α4 in the absence of the Pba3–Pba4 heterodimer.

3.2. Suppression of α4 aggregation by Pba3–Pba4

We next examined subcellular localization of the dislocated α4 subunit in the *Δpba4* cells (Fig. 2). The α4-GFP fusion protein was primarily localized in the nuclei of both *Δpba4* and wild type cells grown at 37 °C for 3 h, although the signal intensity of the GFP tag was slightly weakened in the absence of Pba4. Strikingly, in *Δpba4* cells, α4-GFP aggregated in the cytoplasm showing as a large dot-like structure. Such dots were not observed for α7-GFP. Aggregates of α4-GFP in *Δpba4* cells were not observed at 27 °C, suggesting that the high temperature promoted the aggregation of α4-GFP.

These data suggest that the deletion of *pba3* and *pba4* results in the formation of the non-productive complex containing an abnormal α-ring, in which a α2 subunit is embedded instead of α4, leaving the dislocated α4 subunits to accumulate as aggregates. Thus, the Pba3–Pba4 heterodimer contributes to suppression of intracellular accumulation of the α4 aggregates.

3.3. α4–α5 matchmaking by Pba3–Pba4

To gain an insight into the mechanisms underlying the chaperone-dependent interaction between α4 and α5, we generated a 3D model of Pba3–Pba4 interactions with the α-ring based on superimposing the crystal structure of the α5 subunit to form complexed with Pba3–Pba4 Δloop and the 20S CP (Fig. 3A). In the refined model, we found that Pba3–Pba4 interacts not only with α5 but also with α4 and α6 subunits. In particular, α4 makes contact exclusively with Pba4; Ser78, Lys89, Asp91, Asp95, His99, and Leu132 of Pba4 and Asp5, Ser95, Thr99, Leu100, Arg119, and Tyr120 of α4 form a binding interface. In this interface, Lys89 and His99 of Pba4 play central roles by multiple hydrogen bond formation and van der Waals interaction with α4, and the complementary charge interactions are mediated between Asp91 of Pba4 and Arg119 of α4 (Fig. 3B). Alanine substitutions of these amino acid residues resulted in significantly impaired interaction of GST-fused α4 with Pba3–Pba4 and α5 (Fig. 3C). These results suggest that the direct interaction between Pba4 and α4 is a prerequisite for its proper recruitment adjacent to α5.

3.4. Proteasome assembly assisted by Pba3–Pba4

We unexpectedly found that α5 has an intrinsically higher affinity for α2 than for α4, the inherent neighbor in the native α-ring. Our findings indicated that the Pba3–Pba4 heterodimer not

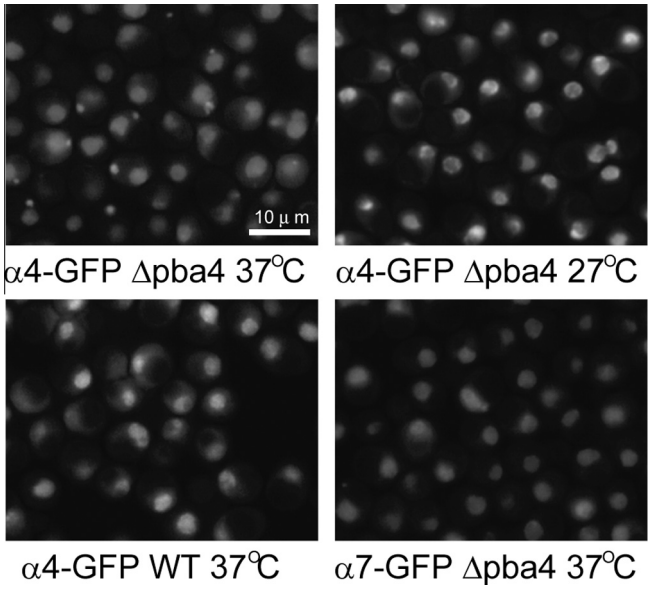

Fig. 2. Suppression of α4 aggregation by Pba3–Pba4 α4-GFP aggregates in the cytoplasm of *Δpba4* cells at high temperature. Cells expressing α4-GFP or α7-GFP were grown in YPD medium at 27 °C or 37 °C for 3 h.

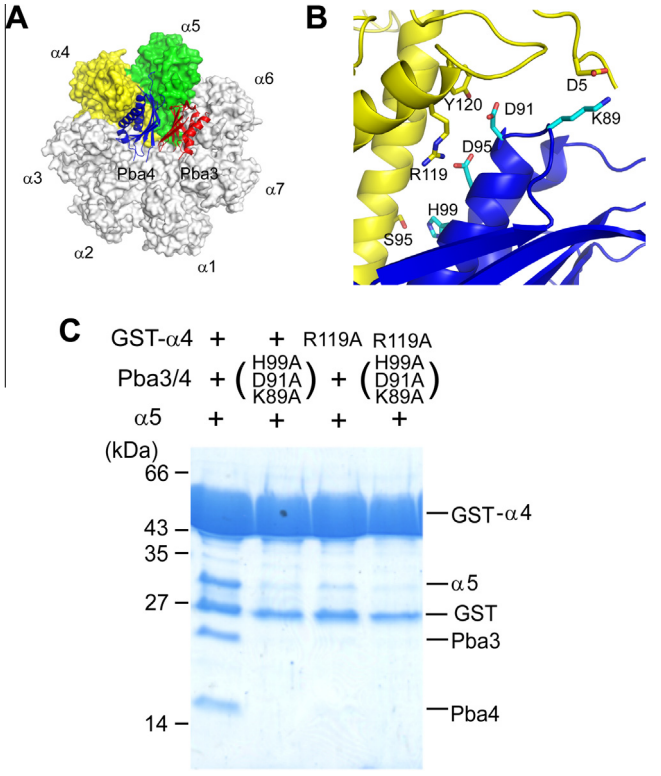

Fig. 3. Effects of the mutations of α4 and/or Pba4 on the Pba3–Pba4-dependent interaction (A) 3D model of the proteasome α-ring complexed with the Pba3–Pba4 heterodimer. Pba3 (red) and Pba4 (blue) are shown as a ribbon model, while the α subunits are shown as a surface model highlighting α4 (yellow) and α5 (green). (B) Close-up view of the interface between Pba4 and α4 is displayed in the right panel; residues involved in the interaction are shown in a stick representation. (C) Protein–protein pull-down assays of interaction of α5 with GST-α4 immobilized on glutathione–Sepharose gel in the presence of Pba3–Pba4 were performed for wild type and α4-Pba4 binding-interface mutations.

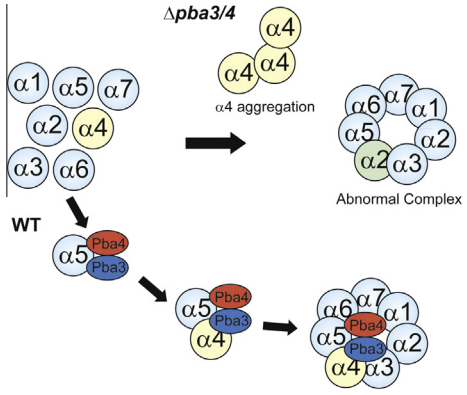

Fig. 4. Schematic model for the Pba3–Pba4-assisted α-ring formation. The Pba3-Pba4 heterodimer mediates the appropriate interaction between α4 and α5, facilitating the formation of α-ring and suppressing the aggregation of α4.

only captures α5 but also interacts with α4 through the charged residues of Pba4. On the basis of these data, we propose a mechanistic model in which Pba3–Pba4 acts as a molecular matchmaker that enhances the interaction between α5 and α4, thereby suppressing α4 aggregation, and contributes to the assembly of α-ring (Fig. 4).

Our structural inspection has suggested that the Pba3–Pba4 heterodimer is released from the assembly intermediate on recruitment of the β4 subunit in avoidance of steric crush [11]. Hence, it is conceivable that the subsequent subunit recruitments are achieved in the absence of this assembly chaperone complex and is primarily based on the complementary properties of the proteasomal subunits. In the intact 20S CP, the β5 subunit is placed in contact with both α4 and α5 [15]. It is noteworthy that the α4–β5 interface is mediated by charge complementarities that are not conserved in α2. Thus, the misassembly of α-ring in the absence of Pba3 and/or Pba4 may result in the termination of β-ring formation, which is supported by our MS data. The proteasome assembly chaperones could become targets of novel anticancer drugs which antagonize functional proteasome formation [19]. Our findings also provide new clues for the design of inhibitors against proteasome biogenesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.119.

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