Molecular Cell 24, 977–984, December 28, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.11.015

Cooperation of Multiple Chaperones Required for the Assembly of Mammalian 20S Proteasomes

Short Article

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Summary

The 20S proteasome is a catalytic core of the 26S proteasome, a central enzyme in the degradation of ubiquitin-conjugated proteins. It is composed of 14 distinct gene products that form four stacked rings of seven subunits each, $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. It is reported that the biogenesis of mammalian 20S proteasomes is assisted by proteasome-specific chaperones, named PAC1, PAC2, and hUmp1, but the details are still unknown. Here, we report the identification of a chaperone, designated PAC3, as a component of α rings. Although it can intrinsically bind directly to both α and β subunits, PAC3 dissociates before the formation of half-proteasomes, a process coupled with the recruitment of β subunits and hUmp1. Knockdown of PAC3 impaired α ring formation. Further, PAC1/2/3 triple knockdown resulted in the accumulation of disorganized half-proteasomes that are incompetent for dimerization. Our results describe a cooperative system of multiple chaperones involved in the correct assembly of mammalian 20S proteasomes.

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Introduction

The ubiquitin-proteasome system is the main nonlysosomal route for intracellular protein degradation in eukaryotes. Short-lived proteins as well as abnormal proteins are recognized by the ubiquitin system and are marked with ubiquitin chains as degradation signals. Polyubiquitinated proteins are then recognized and degraded by 26S proteasomes. The 26S proteasome is composed of one proteolytically active 20S proteasome and two 19S regulatory particles, each attached to one end of the 20S proteasome. The 20S proteasome is a barrel-shaped complex made of two outer α rings and two inner β rings that is a conserved architecture in eukaryotes (Groll et al., 1997, 2005; Unno et al., 2002). The α and β rings are each made up of seven structurally similar subunits, of the α or β type, respectively. The proteolytic activity is exerted by three of the β subunits, namely β1, β2, and β5, which are synthesized in an inactive precursor form and whose propeptides are removed to allow the formation of active sites, accompanied by the assembly of 20S proteasomes.

Our previous work indicated that the assembly of mammalian 20S proteasomes is an ordered multistep process, starting from α ring formation with the help of proteasome-specific chaperones named PAC1 (proteasome assembling chaperone 1) and PAC2 (Hirano et al., 2005). The PAC1-PAC2 heterodimer binds to early α subunit assembly intermediates that contain a restricted subset of α subunits and promotes the formation of heteroheptameric α rings. Moreover, PAC1-PAC2 is responsible for suppressing the formation of off-pathway, nonproductive α ring dimers and thus is important for efficient half-proteasome formation (Hirano et al., 2005). Mammalian half-proteasomes are composed of seven α subunits, seven β subunits, some of which are in precursor forms, and proteasome-dedicated chaperones such as hUmp1 (POMP, Proteassemblin, a homolog of yeast Ump1) (Burri et al., 2000; Griffin et al., 2000; Ramos et al., 1998; Witt et al., 2000) and PAC1-PAC2 (Hirano et al., 2005). Lastly, dimerization of the two half-proteasomes occurs with the help of hUmp1, which completes the maturation of 20S proteasomes, with removal of propeptides of β subunits followed by degradation of hUmp1 and the PAC1-PAC2 heterodimer (Chen and Hochstrasser, 1996; De et al., 2003; Heinemeyer et al., 2004; Hirano et al., 2005; Kingsbury et al., 2000; Nandi et al., 1997; Ramos et al., 1998; Schmidtke et al., 1996). However, the mechanism responsible for half-proteasome formation after the assembly of α rings, i.e., how β subunits and hUmp1 are assembled on α rings, remains elusive. We speculated that another chaperone might be involved in this step.

Results and Discussion

Identification of PAC3 as a Component of α Rings

To identify molecules that are potentially involved in 20S proteasome maturation, we purified α rings from HEK293T cells stably expressing Flag-PAC1 and analyzed them

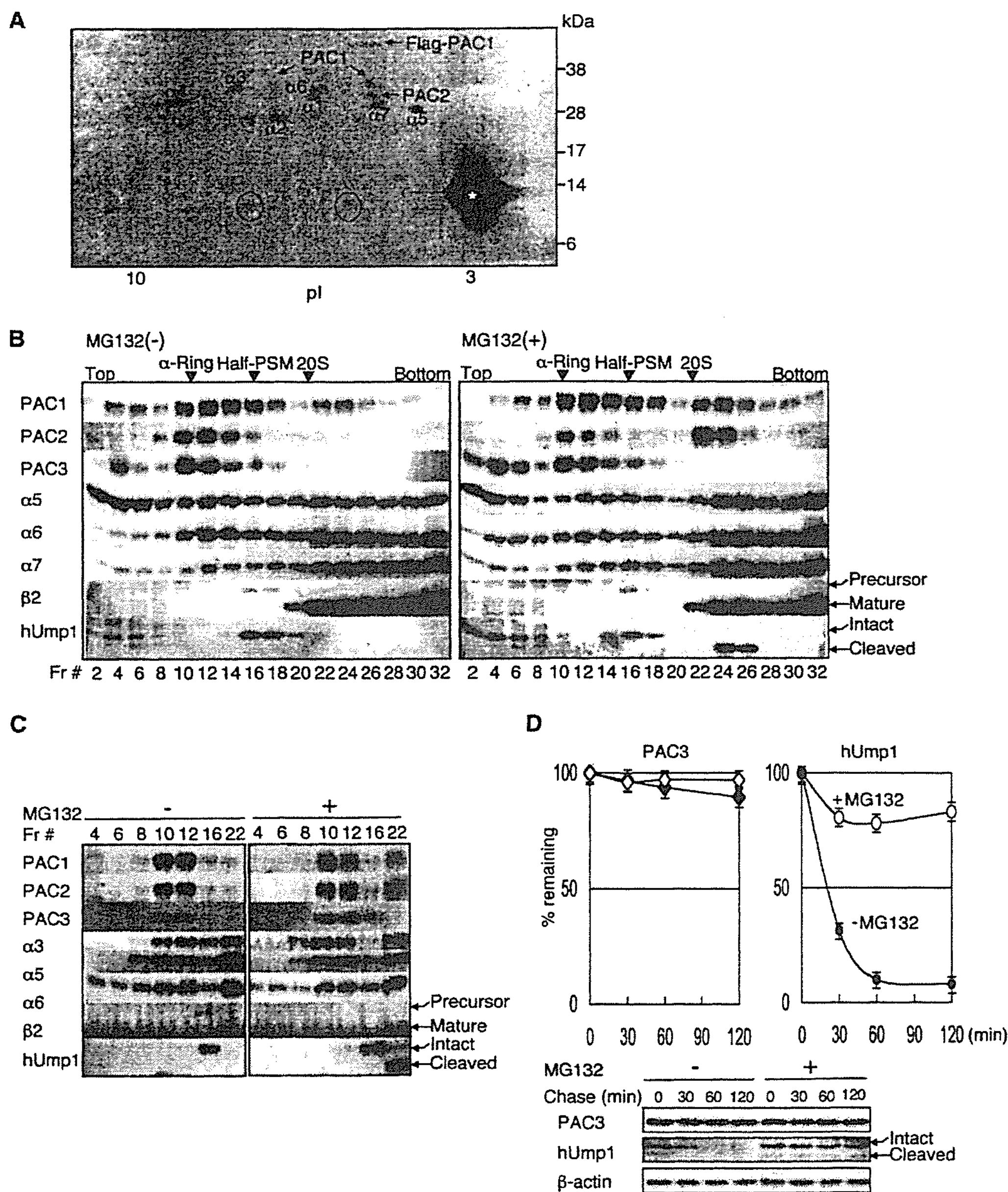

Figure 1. Identification of PAC3 as a Component of α Rings

(A) 2D-PAGE and CBB staining of purified α rings. The α rings were purified from HEK293T cells stably expressing Flag-PAC1 by glycerol gradient centrifugation followed by immunoprecipitation of α ring fractions with M2 agarose. All the spots were identified by MS/MS. The two spots indicated by circles represent hypothetical protein MGC10911. Asterisks indicate nonspecific spots.

(B) 4%–24% glycerol gradient centrifugation of the extracts of HEK293T cells treated with or without MG132. Fractions were immunoblotted as indicated. Arrowheads depict the locations of subcomplexes of proteasomes. Half-PSM, half-proteasomes; 20S, 20S proteasomes. Note that 26S proteasomes sediment near the bottom fraction.

(C) Fractions from (B) were immunoprecipitated with anti-α6 antibody, followed by immunoblotting.

(D) The half-life of PAC3 and hUmp1. Cycloheximide was added to HEK293T cells pretreated with or without 20 μM MG132 for 20 min, and the cells were chased for the indicated time points in the presence or absence of MG132, respectively. The cell lysates were subjected to immunoblotting for PAC3, hUmp1, and β-actin (loading control, bottom). The decay curves of PAC3 (top, left) and hUmp1 (top, right) were generated from the band quantification of the bottom panels. Data are mean ± SEM values of three independent experiments.

by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In addition to the spots of α subunits and PAC1-PAC2 heterodimer, we found two spots of ~14 kDa. Using tandem mass spectrometry (MS/MS), we identified the two spots as a protein called MGC10911 (Figure 1A). We renamed it PAC3 (for proteasome assembling chaperone-3). PAC3 is a small protein of 122 amino acids with no distinct domains or homology to any other known proteins, and its function was entirely unknown. We found genes with significant similarity in metazoans, plants, and fungi, as for PAC2, PAC3, and hUmp1, but in metazoans and fungi as for PAC1 (Figure S1 in the Supplemental Data available with this article online).

First, to examine the behavior of endogenous PAC3, extracts from HEK293T cells were separated by glycerol

gradient centrifugation. PAC3 was located in the α ring fractions (fractions 10–12), i.e., in fractions without β subunits but with α subunits and PAC1-PAC2 (Figure 1B, left). The association of PAC3 with α subunits and PAC1-PAC2 was confirmed by immunoprecipitation with anti-α6 antibody followed by immunoblotting, indicating that PAC3 is a component of α rings, similar to PAC1-PAC2 (Figure 1C, left). Interestingly, when the cells were treated with a proteasome inhibitor, MG132, PAC3 was increased in the light fractions (fractions 2–6), whereas PAC1-PAC2 accumulated in the 20S proteasome fractions (fractions 22–24) as reported previously by our group (Figure 1B, right panels) (Hirano et al., 2005). This suggests that PAC3 dissociates from precursor proteasomes during the maturation pathway, unlike PAC1-PAC2 and hUmp1 (Hirano et al., 2005; Ramos et al., 1998). The increase in PAC3 in the light fractions does not represent an increase in newly synthesized PAC3 upon MG132 treatment, because PAC3 messenger RNA was not increased at this point of time (data not shown). To examine whether the stability of PAC3 is also regulated differently from that of PAC1-PAC2 and hUmp1, which have been shown to be short-lived proteins (Hirano et al., 2005; Ramos et al., 1998), we measured the half-life of PAC3 as well as that of hUmp1 by determining the protein levels at various time points after treatment with cycloheximide. As shown in Figure 1D, hUmp1 had a short half-life of about 20 min, which was greatly prolonged by MG132. This observation is consistent with the previous report (Ramos et al., 1998). As for hUmp1, we noted accumulation of its free forms (Figure 1B, right, fractions 2–6) as well as its cleaved forms in 20S proteasome fractions upon MG132 treatment (Figures 1B–1D). In contrast, PAC3 had a much longer half-life, which was not affected by MG132. These results suggest that PAC3 is involved in the maturation of 20S proteasomes and behaves differently from PAC1-PAC2 and hUmp1.

Knockdown of PAC3 Attenuates α Ring Formation

To elucidate the role of PAC3 in the assembly of the 20S proteasome in vivo, we performed small interfering RNA (siRNA)-mediated knockdown of PAC3 as well as PAC1+PAC2 (PAC1/2), PAC1+PAC2+PAC3 (PAC1/2/3), and hUmp1 to specify their distinct roles. In PAC3 knockdown cells, where we achieved a 75% reduction of PAC3 mRNA (data not shown), polyubiquitin-conjugated proteins accumulated to a level comparable to that in PAC1/2 knockdown cells (Figure 2A). In PAC1/2/3 knockdown cells, the accumulation of polyubiquitinated proteins was enhanced, and the effect of such knockdown was as large as with hUmp1 knockdown (Figure 2A). Consistent with these observations, the decrease in chymotrypsin-like activity of proteasomes was comparable between PAC3 and PAC1/2 knockdown, and the activity was profoundly reduced in PAC1/2/3 knockdown, similar to that in hUmp1 knockdown. These results suggest that PAC1-PAC2 and PAC3 are not epistatic with each other but rather work differently or compensate each other.

To determine the role of PAC3 in the assembly of 20S proteasomes, extracts of knockdown cells were subjected to glycerol gradient analysis (Figure 2C and Figure S2). To compare the quantity of relevant components in each fraction, fractions corresponding to α ring, half-proteasome, and 20S proteasomes in each knockdown experiment were electrophoresed in the same gel (Figure 2D). PAC1/2 knockdown resulted in reduction of α ring peak and emergence of α ring dimers in the half-proteasome fractions (fractions 14–16) as we reported previously (Hirano et al., 2005), and it turned out that PAC3 was a component of this abnormal structure (Figure 2C, bottom left, and Figure 2D, lane 7), indicating that PAC3 plays no role in inhibiting the formation of α ring dimers. The accumulation of PAC3 in light fractions (Figure 2C, bottom left, fractions 4–6) was probably due to ineffective α ring formation in PAC1/2 knockdown cells. Ectopic expression of PAC3 in PAC1/2 knockdown cells did not complement the phenotypes in regard to the formation of α ring dimers and reduction in proteasome activity (Figure S3), indicating that PAC3 and PAC1-PAC2 play distinct roles in α ring formation and do not function redundantly.

In PAC3 knockdown cells, we also observed a reduction of the α ring peak, but no α ring dimers (Figure 2C, top right). Consequently, half-proteasomes, which included α subunits, pro-β subunits, and hUmp1 in proportions like those observed in control cells, were formed to a lesser extent, resulting in decreased formation of 20S proteasomes (Figure 2D, lanes 8 and 13). In addition, PAC1-PAC2 accumulated in light fractions, and free forms of α subunits were increased in PAC3 knockdown cells as well as PAC1/2 and PAC1/2/3 knockdown cells (Figure 2C, top right, and Figure S4). These results suggest that PAC3 plays an important role in α ring assembly and that poor α ring formation resulted in surplus PAC1-PAC2 heterodimer and free α subunits in light fractions.

Simultaneous Loss of PAC1-PAC2 and PAC3 Causes Accumulation of Disorganized Half-Proteasomes

Intriguingly, in PAC1/2/3 knockdown cells, several α subunits and β subunits, including pro-β2 and hUmp1, cosedimented in the half-proteasome fractions to levels comparable to, or even higher (for example, hUmp1, pro-β1, and pro-β2) than, those in control cells, but still the formation of 20S proteasomes was severely impaired (Figure 2C, bottom right, and Figure 2D, lanes 9 and 14). Specifically, the amount of pro-β5, whose propeptide is essential for 20S proteasome formation in yeast (Chen and Hochstrasser, 1996), was much smaller in the complex observed in the half-proteasome fraction of PAC1/2/3 knockdown cells than control cells (Figure 2D, lanes 6 and 9, and Figure 2E). Considering that α ring formation is attenuated by knockdown of both PAC1/2 and PAC3, these results suggest that this complex of abnormal half-proteasomes, observed in PAC1/2/3 knockdown cells, accumulated because it could not dimerize to form mature 20S proteasomes, at least due to a shortage of pro-β5, which should accompany a disorganized constitution of this abnormal half-proteasomes.

Taken together, the knockdown experiments suggest that both PAC1-PAC2 and PAC3 contribute to α ring formation by separate mechanisms, and thus, the effects of knockdowns are additive. In addition, our results suggest that PAC1-PAC2 and PAC3 act cooperatively on the correct formation of half-proteasomes. On the other hand, knockdown of hUmp1 did not influence

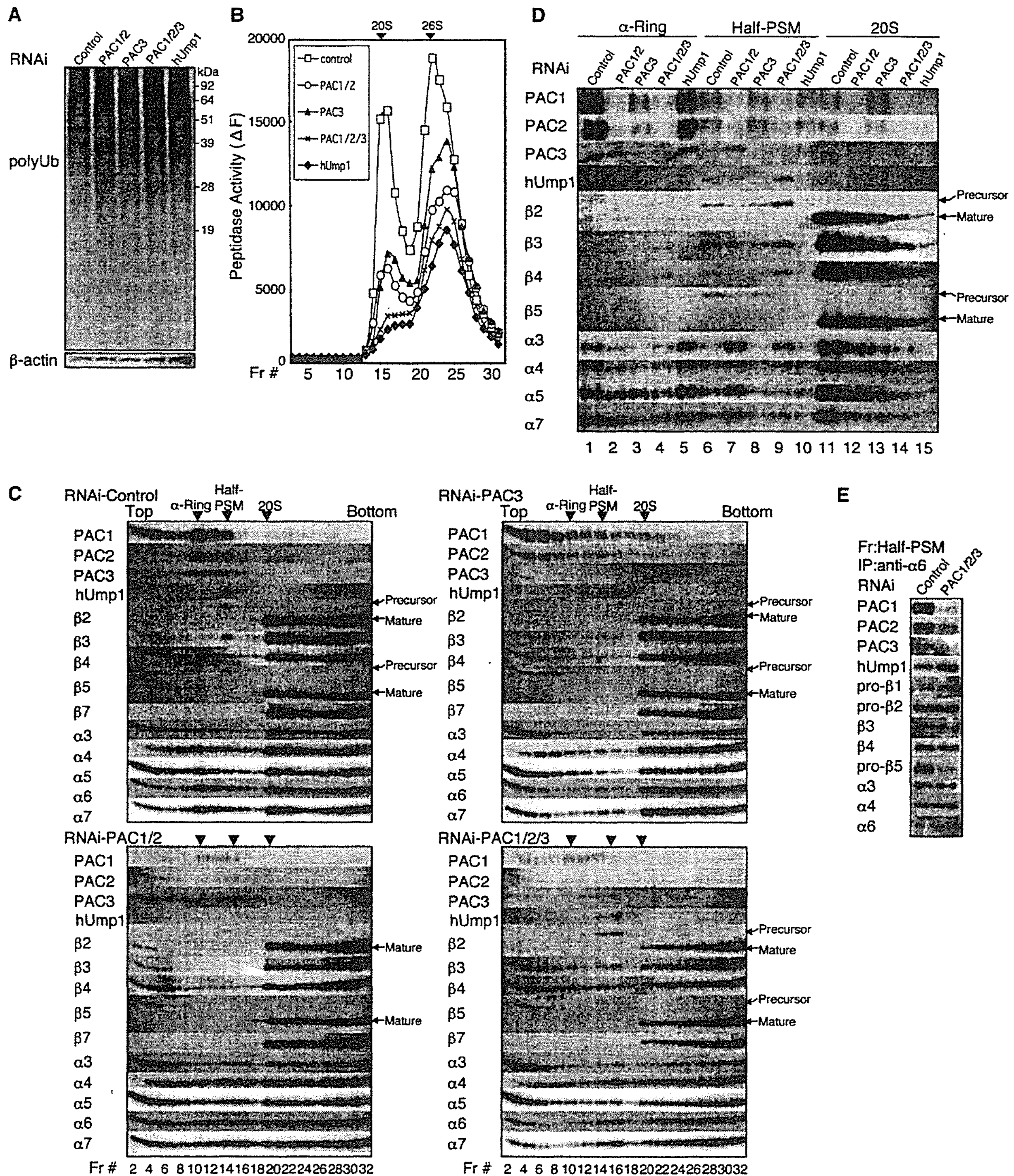

Figure 2. siRNA-Mediated Knockdown of PAC3 Causes Defect in Proteasome Assembly

siRNAs targeting PAC1/2, PAC3, PAC1/2/3, hUmp1, or control were transfected into HEK293T cells. The whole-cell extracts (A) and fractions separated by 8%–32% (B) or 4%–24% (C–E) glycerol gradient centrifugation were immunoblotted (A, C, D, and E) or assayed for Suc-LLVY-MCA hydrolyzing activity of proteasomes (B). In (D), the peak fractions of the indicated subcomplexes from (C) (α ring, fraction 12; Half-PSM, fraction 16; and 20S, fraction 22) were subjected to SDS-PAGE in the same gel to compare the quantity of subunits. (E) Fraction 16 of control or PAC1/2/3 knockdown cells from (C) was immunoprecipitated with anti-α6 antibody, followed by immunoblotting. Data are representative of four experiments.

the sedimentation pattern of PAC3 or PAC1-PAC2 (Figure S2), consistent with the notion that hUmp1 is involved in the last step of the assembly, i.e., dimerization of half-proteasomes, and not in α ring and half-proteasome formation (Hirano et al., 2005; Ramos et al., 1998).

PAC3 Directly Associates with Both α and β Subunits

To gain mechanistic insight into the action of PAC3 and the differences between PAC3 and PAC1-PAC2, we set up in vitro binding experiments. First, we examined direct interactions between PACs. PAC3 did not bind to

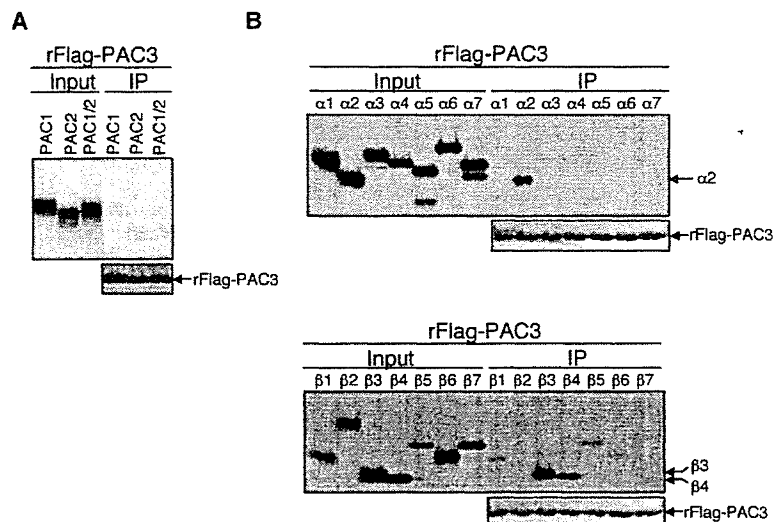

Figure 3. PAC3 Directly Binds to Both α2 and Several β Subunits

(A) Interactions of PAC3 with PAC1 and PAC2. Recombinant Flag-tagged PAC3 (rFlag-PAC3) was incubated with the indicated products translated and radiolabeled in reticulocyte lysates, immunoprecipitated with M2 agarose, and analyzed by SDS-PAGE and autoradiography.

(B) Interactions of PAC3 with individual α and β subunits. Interactions between rFlag-PAC3 and α or β subunits were analyzed as in (A).

PAC1 or PAC2 (Figure 3A). Next, we tested the interactions between PAC3 and each 20S proteasome subunit. PAC3 could directly bind to not only an α subunit (α2) but also to several β subunits, strongly to β3 and β4 but weakly to β1 and β5 (Figure 3B). This is in contrast to the property of PAC1-PAC2, which directly associated with α5 and α7, but not with any of the β subunits (Hirano et al., 2005). These results indicate that PAC1-PAC2 heterodimer and PAC3 are distinct entities that work at different aspects in the maturation of 20S proteasomes.

PAC3 Is Released from Precursor Proteasomes during Half-Proteasome Formation

Because PAC3 seemed to be released from precursor proteasomes during the maturation pathway (Figure 1B), we analyzed the precursor proteasomes that contain PAC3. An extract of HEK293T cells stably expressing Flag-PAC3, -hUmp1, or -PAC1 was separated by glycerol gradient centrifugation, and fractions 8–20, which included α rings (fraction 12) and half-proteasomes (fractions 16–18) were immunoprecipitated with anti-Flag antibody, followed by immunoblotting (Figure 4A). Flag-PAC3 did not precipitate hUmp1 or β subunits in half-proteasome fractions, and Flag-hUmp1 did not precipitate PAC3 at all (Figure 4A). Subsequently, we purified α rings and half-proteasomes from Flag-PAC2 and Flag-hUmp1 expressing cells, respectively, and subjected them to immunoblotting and CBB staining. Although half-proteasomes were loaded in much larger molar amounts, a band corresponding to PAC3, which was clearly visible in α rings, was not observed in half-proteasomes (Figure 4B and Figure S5). These results clearly show that the release of PAC3 from precursor proteasomes is coupled to the recruitment of hUmp1 and β subunits. Considering that PAC3 can directly bind to several β subunits in vitro (Figure 3B) and that PAC3 knockdown together with PAC1/2 knockdown resulted in production of disorganized half-proteasomes that were not competent for 20S proteasome formation (Figure 2), it is suggested that the association between PAC3 and β subunits is either intrinsically unstable in vivo or destabilized upon half-proteasome formation and that the release of PAC3 from precursor proteasomes is an obligatory step for the correct assembly of half-proteasomes by mediating interactions between α rings and β subunits.

Our present work provides a model (Figure 4C) where the chaperone PAC3 assists in the formation of α rings, together with PAC1-PAC2 heterodimer, and mediates correct formation of half-proteasomes in cooperation with PAC1-PAC2. PAC3 itself is then released and recycled in further rounds of proteasome assembly. The unique feature of PAC3 is its ability to interact with various β subunits, raising the possibility that it plays a role in the assembly of β subunits on α rings. In the present model, we emphasize that correct assembly of mammalian 20S proteasomes is achieved by the cooperative actions of multiple proteasome-specific chaperones.

Experimental Procedures

DNA Constructs

The cDNA encoding PAC3 was synthesized from total RNA isolated from HeLa cells using Superscript II (Invitrogen). PCR was carried out on the cDNA by using Phusion DNA polymerase (FINNZYMES). The cDNAs encoding PAC1, PAC2, PAC3, hUmp1, and proteasome α and β subunits were cloned into pcDNA3.1 (Invitrogen) and/or pIR-ESpuro3 (Clontech). All constructs were confirmed by sequencing. For expression of Flag-fusion protein, the cDNA was subcloned into pET22b (Novgen) in frame with a C-terminal Flag tag. For expression of GST and MBP-fusion proteins, the cDNAs were subcloned into pGEX6P-1 (Amersham) and pMAL (NEB), respectively.

Cell Culture

HEK293T cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate (all from Gibco-Invitrogen). Transfections of plasmids into HEK293T cells were performed with Fugene 6 (Roche). To generate stable cell lines, transfected HEK293T cells were selected with 5 μg/ml of puromycin. We used 20 μM MG132 (Peptide Institute) to inhibit proteasome activities for 2 hr before harvest. For cycloheximide-chase experiments, HEK293T cells were treated with 100 μg/ml cycloheximide (Sigma).

Protein Extracts, Immunological Analysis, and Antibodies

Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 0.5% [v/v] NP-40, and 1 mM dithiothreitol [DTT]) with 2 mM ATP and 5 mM $MgCl_2$, and the extracts were clarified by centrifugation at 20,000 × g for 10 min at 4°C. The supernatants were mixed with

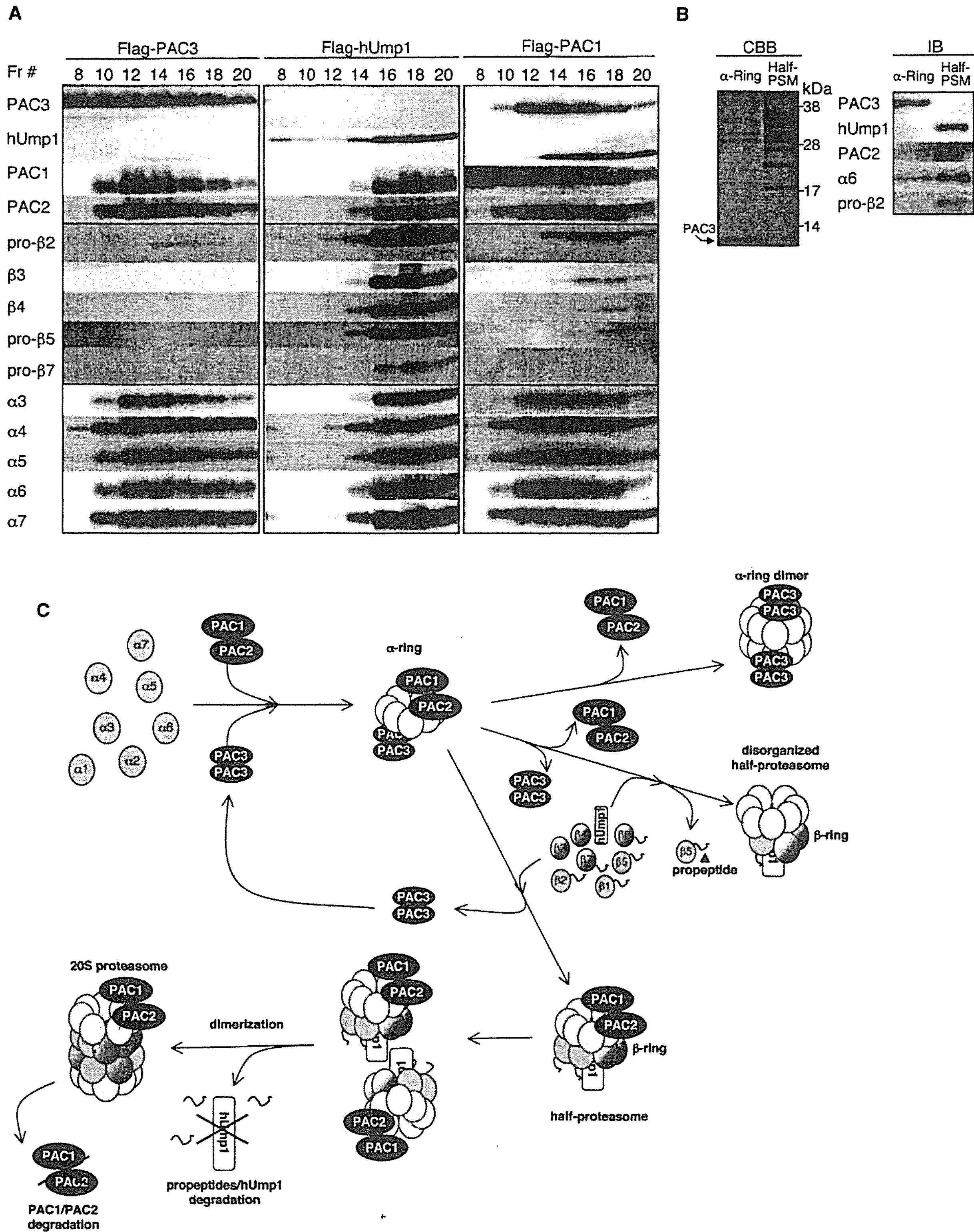

Figure 4. Mutually Exclusive Incorporation of PAC3 and hUmp1 into Precursor Proteasomes

(A) HEK293T cells stably expressing Flag-PAC3, -hUmp1, or -PAC1 were fractionated as in Figure 1B. The indicated fractions were immunoprecipitated with M2 agarose, resolved, and analyzed by SDS-PAGE and immunoblotting.

(B) SDS-PAGE followed by CBB staining (left) and immunoblotting (right) of purified α rings and half-proteasomes. α rings were purified from fraction 12 of Flag-PAC2 expressing cells. Half-proteasomes were purified from fraction 16 of Flag-hUmp1 expressing cells in (A). The band for PAC3 was identified by MS/MS. The 2D-PAGE analyses of these complexes are shown in Figure S5.

(C) A model for proteasome assembly assisted by multiple chaperones. PAC1-PAC2 heterodimer and PAC3, which probably forms homodimers based on the analysis of molecular sieve chromatography of the recombinant PAC3 (data not shown), assist α ring formation. Whereas PAC1-PAC2 suppresses off-pathway aggregation of α subunits and keeps α rings competent for half-proteasome formation, PAC3, which can bind

SDS sample buffer. SDS-PAGE (12% gel or 4%–12% gradient Bis-Tris gel [Invitrogen]) was performed according to the instructions provided by the manufacturer. The separated proteins were transferred onto polyvinylidene difluoride membrane and reacted with the indicated antibody. Development was performed with Western Lighting reagent (PerkinElmer). 2D-PAGE was performed as described previously (Murata et al., 2001).

For immunoprecipitation, we used antibodies MCP20 bound to protein G Sepharose (Amersham) in Figure 1C, antibodies MCP20 crosslinked to NHS-activated Sepharose (Amersham) in Figure 2E, or M2 Agarose (Sigma) in Figures 1A, 4A, and 4B. These beads were added to the extracts, mixed under constant rotation for 2 hr at 4°C, washed four times with lysis buffer with 30 mM NaCl, and boiled in SDS sample buffer. Otherwise, these washed samples were eluted with 100 μg/ml Flag peptides (Sigma) or with 0.2 M glycine-HCl (pH 2.8).

Anti-PAC1 and PAC2 polyclonal antibodies were described previously (Hirano et al., 2005). Anti-PAC3 polyclonal antibodies were raised in rabbits by using recombinant PAC3 (full-length) proteins, which were produced by cleavage of GST by PreScission protease (Amersham) after purification of GST-fused PAC3 proteins. Anti-hUmp1 polyclonal antibodies were raised in rabbits by using recombinant MBP-hUmp1 (full-length) proteins. Antibodies against proteasome α2 subunit (MCP21), α3 (MCP257), α4 (MCP34), α5 (MCP196), α6 (MCP20), α7 (MCP72), β1 (MCP421), β2 (MCP168), β3 (MCP102), and β7 (MCP205) were purchased from BioMol. Anti-β5 (P93250) and β4 (55F8) were prepared as described previously (Tanahashi et al., 2000). Anti-ubiquitin antibodies were obtained from Dako. Anti-β-actin antibodies were from Chemicon.

Glycerol Gradient Analysis

Cell extracts (1 mg of protein) were separated in 32 fractions by centrifugation (22 hr, 100,000 × g) in 4%–24% [v/v] or 8%–32% [v/v] linear gradients, as described previously (Hirano et al., 2005).

Binding Assay

In vitro labeling was performed by using TNT T7 Quick for PCR DNA system (Promega) with ^{35}S-labeled methionine, according to the procedure supplied by the manufacturer. Recombinant Flag-PAC3 proteins were expressed in *E. coli* and purified with M2 Agarose. Binding assay was performed in lysis buffer, and the resulting product was washed with lysis buffer with 150 mM NaCl before elution with Flag peptides. The eluates were separated by SDS-PAGE and visualized by autoradiography.

RNA Interference

siRNA targeting human PAC1, PAC2, PAC3, and hUmp1 with the following 19 nucleotide sequences were designed by B-Bridge and synthesized by Dharmacon. The targeting sequences of PAC1, PAC2, and hUmp1 were described previously (Hirano et al., 2005). These of PAC3 are 5′-CCGUGAAGGACAAAAGCAU-3′ and 5′-GAUCAAUUGUAGGAGGAAA-3′. Control siRNA (Non-specific Control Duplex VIII) was purchased from B-Bridge. Transfections of siRNAs into HEK293T cells were performed by using Lipofectamine 2000 at a final concentration of 50 nM. It was performed three times at intervals of 24 hr. The cells were analyzed 96 hr after first transfection.

Assay of Proteasome Activity

Peptidase activity was measured by using a fluorescent peptide substrate, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-MCA), as described previously (Murata et al., 2001). Note that the assay was carried out in the presence of 0.03% SDS, which is a potent artificial activator of the latent 20S proteasome, as previously reported (Tanaka et al., 1989).

Supplemental Data

Supplemental Data include five figures and can be found with this article online at http://www.molecule.org/cgi/content/full/24/6/977/DC1/.

Acknowledgments

We thank K. Furuyama for technical support. This work was supported by grants from Japan Science and Technology Agency (to S.M.), the Ministry of Education, Science and Culture of Japan (to S.M. and K.T.), and NEDO (to T.N.). Y.H. was supported by Japan Society for the Promotion of Science.

Received: July 7, 2006
Revised: October 10, 2006
Accepted: November 15, 2006
Published: December 28, 2006

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directly to several β subunits, dissociates before the formation of half-proteasomes, a process coupled to the recruitment of β subunits and hUmp1. Loss of both PAC1-PAC2 and PAC3 before β subunit incorporation causes formation of disorganized half-proteasomes that lack pro-β5 almost completely. Released PAC3 is recycled. Two half-proteasomes dimerize with the help of hUmp1, and propeptides of β subunits (β1, β2, β5, β6, and β7) were cleaved. hUmp1 and PAC1-PAC2 are subsequently degraded by the newly formed active 20S proteasomes.

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