Dissecting β -ring assembly pathway of the mammalian 20S proteasome

Yuko Hirano¹, Takeumi Kaneko²,
Kenta Okamoto³, Minghui Bai²,
Hideki Yashiroda², Kaori Furuyama²,
Koichi Kato^{3,4}, Keiji Tanaka¹
and Shigeo Murata^{2,*}

¹Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, ²Laboratory of Protein Metabolism, Department of Integrated Biology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan, ³Department of Structural Biology and Biomolecular Engineering, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan and ⁴Division of Biomolecular Functions, Department of Life and Coordination-Complex Molecular Science, Institute for Molecular Science, National Institutes of Natural Sciences, Okazaki, Japan

The 20S proteasome is the catalytic core of the 26S proteasome. It comprises four stacked rings of seven subunits each, α_1 – α_7 – β_1 – β_7 – α_1 – α_7 . Recent studies indicated that proteasome-specific chaperones and β -subunit appendages assist in the formation of α -rings and dimerization of half-proteasomes, but the process involved in the assembly of β -rings is poorly understood. Here, we clarify the mechanism of β -ring formation on α -rings by characterizing assembly intermediates accumulated in cells depleted of each β -subunit. Starting from β_2 , incorporation of β -subunits occurs in an orderly manner dependent on the propeptides of β_2 and β_5 , and the C-terminal tail of β_2 . Unexpectedly, hUmp1, a chaperone functioning at the final assembly step, is incorporated as early as β_2 and is required for the structural integrity of early assembly intermediates. We propose a model in which β -ring formation is assisted by both intramolecular and extrinsic chaperones, whose roles are partially different between yeast and mammals.

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Introduction

The ubiquitin–proteasome system is the main pathway for ATP-dependent non-lysosomal degradation of intracellular proteins in eukaryotes (Coux *et al*, 1996; Baumeister *et al*, 1998). Protein degradation achieved by this system is

involved in various cellular processes, including cell cycle regulation, stress response, intracellular signalling, transcription regulation, and acquired immunity (Glickman and Ciechanover, 2002). Proteins involved in such regulatory processes as well as damaged proteins are recognized by the ubiquitin system and are marked for degradation by covalent attachment of polyubiquitin chains. Polyubiquitinated proteins are then recognized and degraded by the 26S proteasome, a 2.5-MDa multisubunit protease.

The 26S proteasome is composed of a catalytic core particle, called the 20S proteasome, bound at one or both ends by a 19S regulatory particle. The 20S proteasome is a cylindrical structure comprised of 28 subunits arranged in four stacked seven-membered rings. Each ring contains seven different subunits, whereby the two outer rings are formed by non-catalytic α -type subunits, named α_1 – α_7 , and the two inner rings are formed by the β -type subunits, β_1 – β_7 , three of which are catalytic (β_1 , β_2 , and β_5) (Baumeister *et al*, 1998). Each of the 14 different proteins occupies a defined position within the 20S proteasome (Groll *et al*, 1997; Unno *et al*, 2002). Vertebrates encode four additional catalytic β -subunits; three interferon- γ -inducible β_{1i} , β_{2i} , and β_{5i} and one thymus-specific β_{5t} , which are incorporated in place of their most closely related β -subunits, thus forming distinct subtypes of proteasomes with altered catalytic activities called immunoproteasome and thymoproteasome (Tanaka and Kasahara, 1998; Murata *et al*, 2007).

The integrity of the 20S proteasome is assured by correct assembly of the 14 α -subunits and 14 β -subunits. All of the active β -subunits as well as non-catalytic β_6 and β_7 are synthesized with N-terminal propeptides, which are removed autocatalytically at the final step of the assembly to expose the catalytic threonine residues of β_1 , β_2 , and β_5 . The N-terminal active sites of β -subunits are on the inner surface of the β -rings, whereas the C termini of β -subunits are on the outer surface of the 20S proteasome (Groll *et al*, 1997). It has been shown that efficient assembly of the 20S proteasome is orchestrated by proteasome-specific chaperones such as PAC1 (Pba1 or POC1 in yeast), PAC2 (Pba2 or POC2 in yeast), PAC3 (Pba3, Dmp2, or POC3 in yeast), PAC4 (Pba4, Dmp1, or POC4 in yeast), hUmp1 (also known as POMP, proteasomblin in mammals and as Ump1 in yeast), the N-terminal propeptides of β -subunits, and C-terminal tails of β -subunits, which provide specific subunit interactions with *cis*- and *trans*- β -rings (Heinemeyer *et al*, 2004; Ramos *et al*, 2004; Hirano *et al*, 2005, 2006; Murata, 2006; Le Tallec *et al*, 2007; Li *et al*, 2007; Kusmierczyk *et al*, 2008; Yashiroda *et al*, 2008).

Proteasome assembly proceeds through distinct assembly intermediates. The earliest intermediate observed in mammalian cells is an α -ring that is comprised of all seven α -subunits, a PAC1–PAC2 heterodimer, and a PAC3–PAC4 complex (Hirano *et al*, 2005, 2006; Le Tallec *et al*, 2007). Both PAC1–PAC2 and PAC3–PAC4 are involved in the

*Corresponding author. Laboratory of Protein Metabolism, Department of Integrated Biology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 3 5841 4803; Fax: +81 3 5841 4805; E-mail: smurata@mol.f.u-tokyo.ac.jp

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formation of α -rings. Recently, Pba3–Pba4 or Dmp1–Dmp2, yeast orthologues of PAC3–PAC4, has shown to catalyse correct subunit orientation of an α -ring (Kusmierczyk *et al*, 2008; Yashiroda *et al*, 2008). PAC1–PAC2 prevents non-productive dimerization of α -rings. The α -ring serves as a scaffold for the assembly of β -subunits. Another intermediate is the 13S complex composed of one α -ring, unprocessed β 2, β 3, β 4, and (h)Ump1, both in yeast and mammals (Frentzel *et al*, 1994; Nandi *et al*, 1997; Li *et al*, 2007). Recent studies in yeast showed that the addition of the other β -subunits except β 7 form the subsequent intermediate referred to as a ‘half-mer’ precursor complex (Li *et al*, 2007; Marques *et al*, 2007). The 16S complex containing all the subunits and hUmp1 has been described also in mammalian cells (Schmidtke *et al*, 1997; Witt *et al*, 2000). A ‘half-proteasome’ is often used as a general term for assembly intermediates containing unprocessed β -subunits and (h)Ump1. Studies in yeast have shown that dimerization of the half-mer is driven by the propeptide of β 5 and the C-terminal tail of β 7, whose incorporation into the half-proteasome is coupled with the dimerization, where the role of Ump1 is proposed to be an assembly checkpoint factor that inhibits the dimerization until a full set of β -subunits are recruited on the α -ring (Ramos *et al*, 2004; Li *et al*, 2007). Removal of β -propeptides and degradation of Ump1 coincide with completion of proteasome maturation, followed by degradation of PAC1–PAC2 (Ramos *et al*, 1998; Hirano *et al*, 2005). PAC3 is released from the intermediates during the maturation process (Hirano *et al*, 2006).

Several studies in yeast reported that the propeptides and the C-terminal tails of certain β -subunits have important roles in proteasome biogenesis. The propeptide of β 5 is crucial for the incorporation of β 5 during proteasome formation and is thus essential for life (Chen and Hochstrasser, 1996). The propeptides of β 1 and β 2 are dispensable for cell viability but are known to protect the N-terminal catalytic threonine residue against N^ε-acetylation. In addition, mutants lacking these two propeptides displayed modest defects in proteasome biogenesis (Arendt and Hochstrasser, 1999). The C-terminal tail of β 2, which wraps around β 3 within the same β -ring, is also essential for proteasome biogenesis in yeast (Groll *et al*, 1997; Ramos *et al*, 2004). The C-terminal tail of β 7, which is inserted into a groove between β 1 and β 2 in the opposite ring, also has an important function in dimerization of half-proteasomes as well as stabilization of active conformation of β 1 (Groll *et al*, 1997; Ramos *et al*, 2004). In mammals, analysis of propeptides has been mainly conducted in the context of immunoproteasome formation, but there is little or no information on the C-terminal tails of β 2 and β 7, whose location in the mammalian proteasome closely resembles those of yeast β 2 and β 7 in the yeast proteasome (Unno *et al*, 2002).

Here, we describe a series of biochemical experiments employing RNA interference of each β -subunit, which resulted in the accumulation of distinct assembly intermediates. By characterizing these intermediates, we clarified the order of β -subunit incorporation on the α -ring. We also assessed the roles of propeptides and C-terminal tails of β -subunits in mammalian proteasome biogenesis, which revealed that these appendages mostly function in a manner similar to yeast counterparts but also displayed some phenotypes not observed in yeast. Furthermore, we identified

a novel function of hUmp1 in stabilizing assembly intermediate of proteasomes that has not been appreciated in yeast.

Results

Ordered assembly of β -subunits on α -ring

During the assembly pathway from the α -ring through the half-proteasome, each β -subunit assembles on the α -ring. To clarify the order of incorporation of β -subunits, we used the strategy of small interfering RNA (siRNA)-mediated knockdown of each β -subunit, which was expected to result in arrest of the assembly process before the incorporation of the targeted subunit and accumulation of a specific intermediate.

The total level of the different subunits as well as proteasome activity assessed by the peptide-hydrolysing activity of HEK293T cells transfected with siRNA targeting each β -subunit or hUmp1 was markedly reduced compared with those of control cells, suggesting that the biogenesis of proteasomes is severely impaired in each knockdown cell (Supplementary Figure S1). Each cell extract was resolved by native PAGE, followed by active staining or immunoblot analyses for α 6- and all β -subunits (Figure 1). Immunoblot for α 6 revealed accumulation of different complexes (molecular weight, 232–669 kDa) in each knockdown cell, as well as the normal α -ring in control cells, which have been shown to be a distinct assembly intermediate comprising all the seven α -subunits, PAC1–PAC2, and PAC3 (Hirano *et al*, 2006) (Figure 1A). Besides the major, fast-migrating band, a more slowly migrating minor band was observed for each knockdown cell except β 2, β 3, and hUmp1 knockdown (Figure 1A). Both the major and minor species did not show any peptide-hydrolysing activity, which was observed only in the complex of approximately 700-kDa, that is, 20S proteasomes (Figure 1B), indicating that they are assembly intermediates of 20S proteasomes.

Among the seven β -subunits, β 2 was the first assembled on the α -ring based on the finding that β 2 was detected in all the intermediates except for that in its own knockdown (Figure 1D) and the intermediate that accumulated in β 2-knockdown cells did not contain any β -subunit (Figure 1C–I, lanes for β 2 RNAi). The assembly of β 3 followed that of β 2; β 3 was detected in the intermediates observed in β 1-, β 4-, β 5-, β 6-, and β 7-knockdown cells, and thus the incorporation of β 3 should precede these subunits (Figure 1E). This view was further confirmed by the fact that the intermediate in β 3-knockdown cells contained only β 2 among the β -subunits (Figure 1C–I, lanes for β 3 RNAi). β 3 assembly was followed by β 4 incorporation, which would result in the formation of the 13S complex, comprising the α -ring plus β 2, β 3, and β 4, as suggested by the presence of β 4 in the intermediate in β 1, β 5, β 6, and β 7 knockdown (Figure 1F), consistent with the previous reports that identified the 13S complex as a distinct entity of proteasome precursors (Frentzel *et al*, 1994; Nandi *et al*, 1997; Li *et al*, 2007).

β 5 was the next β -subunit incorporated into the 13S complex because β 5 was detected only in the intermediates in β 1-, β 6-, and β 7-knockdown cells (Figure 1G). The assembly of β 6 followed that of β 5, as evidenced by the presence of β 6 in the intermediates of β 1 and β 7 knockdown

Figure 1 Accumulation of distinct assembly intermediates in each β -subunit knockdown cells. The cell extracts (40 μ g) used in Supplementary Figure S1 were separated by native PAGE. Assembly intermediates were detected by immunoblotting using the indicated antibodies (**A**, **C–I**). The bands corresponding to α -ring and the 20S proteasome as well as the locations of molecular size markers are depicted by arrowheads. (**B**) The peptide-hydrolyzing activity was assayed by active staining of the gel using Suc-LLVY-MCA in the presence of SDS. Note that the 26S proteasome did not move inside the native PAGE gel.

(Figure 1H). $\beta 7$ was likely the last β -subunit incorporated in the precursor proteasomes because $\beta 7$ was not found in any of the intermediate complexes (Figure 1I) and because the intermediate observed in $\beta 7$ -knockdown cells contained all the β -subunits with the exception of $\beta 7$ (Figure 1C–I, lanes for $\beta 7$ RNAi). The behaviour of $\beta 1$ was rather elusive. The intermediate in $\beta 1$ -knockdown cells contained $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, and $\beta 6$ (Figure 1C–I, lanes for $\beta 1$ RNAi), whereas $\beta 1$ was already included in the intermediates of $\beta 4$, $\beta 5$, $\beta 6$, and $\beta 7$ knockdown (Figure 1C). The former observation suggests that $\beta 1$ was incorporated following $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, and $\beta 6$, and that $\beta 1$ is required for $\beta 7$ incorporation.

The latter observation suggests that the presence of $\beta 2$ and $\beta 3$ is sufficient for the incorporation of $\beta 1$ and that $\beta 1$ can be incorporated anytime during the maturation pathway from the complex containing both $\beta 2$ and $\beta 3$ through the half-mer.

Association of PA28, Hsp90, and Hsc70 with 20S proteasome precursors

When the same panel was probed for PAC1, the major assembly intermediate bands were associated with PAC1 (Figure 2A), which has been shown to be retained in the proteasome precursor until the completion of the assembly

Figure 2 Release of PAC3 is coupled with β 3 incorporation. The same panels in Figure 1 were probed with anti-PAC1 (A) and -PAC3 (B) antibodies. The arrow indicates PAC3 species dissociated from proteasome precursors (B).

(Hirano *et al*, 2005). However, the slowly migrating minor bands above the major bands did not contain PAC1, whereas the composition of each major and minor bands in terms of α - and β -subunits is identical (Figures 1 and 2A). It is also curious that the intermediate in β 2-knockdown cells was apparently larger than the α -ring (Figure 1A), although the subunit composition is supposed to be identical to that of the α -ring. To address the identity of these bands, we tested whether PA28, PA200, Hsp90 α , and Hsc70, which have been reported to be involved in proteasome biogenesis (Schmidtke *et al*, 1997; Preckel *et al*, 1999; Fehlker *et al*, 2003; Imai *et al*, 2003; Marques *et al*, 2007), associate with the intermediates.

PA28 was associated with the slow-migrating minor bands but not with the primary bands, different from PAC1 and Hsp90 α , which were detected only in the major bands (Supplementary Figure S2A and B). Hsc70 was observed in both the major and the minor bands (Supplementary Figure 2C). Neither Hsp90 α nor Hsc70 was detected in the α -ring. By contrast, PA200, whose yeast orthologue Blm10 was shown to associate with nascent proteasomes (Fehlker *et al*, 2003; Marques *et al*, 2007), was not observed in the intermediates, whereas its association with 20S proteasomes was detected (Supplementary Figure S2D, arrowhead). However, we cannot conclude that PA200 is not bound to assembly intermediates as free forms of PA200, which probably dissociated from 20S or nascent proteasomes during native PAGE analysis, were found (Supplementary Figure S2D, arrow).

The association of Hsp90 and Hsc70 with the assembly intermediates accounts for the increased size of the intermediate in β 2-knockdown cells and suggests that recruitment of these chaperones precedes β 2 and hUmp1 incorporation. The minor bands are characterized by the association of PA28, a 200-kDa heterohexameric complex. At present, we do not know whether these molecules really have some functions in the proteasome biogenesis or are associated with the intermediates as an experimental artefact. Further studies are needed to answer this question.

Release of PAC3 upon incorporation of β 3

We previously showed that precursor proteasomes purified with tagged hUmp1 did not contain PAC3 and demonstrated

that PAC3 is released during the maturation pathway of the mammalian proteasome (Hirano *et al*, 2006). To elucidate the step where PAC3 was released, we took advantage of the knockdown experiments in which distinct assembly intermediates accumulated depending on which β -subunit was targeted (Figure 1).

The same panel in Figure 1 was probed with anti-PAC1 and -PAC3 antibody. All the assembly intermediates as well as the α -ring were associated with PAC1 (Figure 2A) (Hirano *et al*, 2005). PAC3 is also associated with the α -ring in control cells as reported previously (Hirano *et al*, 2006). However, PAC3 was associated with intermediates of β 2-, β 3-, and hUmp1-knockdown cells but not with those of others, where PAC3 was found as fast migrating species, presumably as a free complex (Figure 2B, arrow). Considering the order of incorporation of β -subunits shown in Figure 1, the release of PAC3 is apparently coupled with the incorporation of β 3.

A new role of hUmp1 in the assembly pathway

One intriguing difference in the phenotypes of loss of Ump1 orthologues between yeast and mammals is that knockdown of hUmp1 in mammalian cells did not result in the accumulation of intermediates containing unprocessed β -subunits (Figure 1, lanes for hUmp1 RNAi), whereas deletion of Ump1 in yeast caused apparent accumulation of such intermediates (Ramos *et al*, 1998). This observation in hUmp1-knockdown cells has also been shown in previous studies (Hirano *et al*, 2005, 2006). This finding raises the possibility that the role of Ump1 orthologues is different between yeast and mammals.

To determine the step at which hUmp1 is incorporated, the same panel in Figure 1 was probed with anti-hUmp1 antibody. hUmp1 was included in a complex other than that in β 2-knockdown cells (Figure 3A), indicating that the incorporation of hUmp1 precedes that of β 3. On the other hand, the intermediate in the hUmp1 knockdown complex did not contain any of the β -subunits, including β 2, a finding closely resembling that in β 2-knockdown cells with regard to size and composition (Figures 1 and 2; compare lanes for β 2 RNAi to lanes for hUmp1 RNAi). These results suggest that incorporations of β 2 and hUmp1 are coupled with each other and that loss of either result in dissociation of the other.

Figure 3 Role of hUmp1 in the structural integrity of early assembly intermediates. (A) The same panel in Figure 1 was probed with anti-hUmp1 antibody. (B–E) Extracts of HEK293T cells transfected with the indicated combinations of siRNAs were separated by native PAGE. Intermediate complexes were detected by immunoblotting using the indicated antibodies. The left lane representing β 7 RNAi serves as a positive control for immunoblotting (C, D). Asterisks indicate nonspecific bands (A). (F) Flag-hUmp1 and each 20S subunit were co-translated and radio-labelled in reticulocyte lysates, immunoprecipitated with M2 agarose, and analysed by SDS-PAGE and autoradiography. ‘ALL’ represents co-translation of all β -subunits together with hUmp1.

To confirm this concept, hUmp1 was knocked down concurrently with β 2, β 3, or β 4, and the resultant assembly intermediates were compared with those in β 2, β 3, or β 4 single-knockdown cells. Intriguingly, the size of intermediates observed in the simultaneous knockdown of hUmp1 with β 3 or β 4 was similar to that of the complex in β 2 single-knockdown cells (Figure 3B). Notably, the intermediate found in β 3-hUmp1 double-knockdown cells lacked β 2 (Figure 3C), which was found in the complex of β 3 single-knockdown cells (Figure 1D). The β 4 and hUmp1 double knockdown was associated with loss of both β 2 and β 3 in the intermediate (Figure 3C and D), which were clearly detected in the β 4 single knockdown complex (Figure 1D and E). Furthermore, the complexes manifested in the double knockdown cells were associated with PAC3, consistent with the absence of β 3, whose incorporation would detach PAC3 from the precursor proteasome (Figures 2B and 3E).

To gain mechanistic insight into the early function of hUmp1, we tested the interactions between hUmp1 and each 20S proteasome subunit. hUmp1 could directly bind to β 2 and β 3 as well as some of the α -subunits (α 2, α 3, α 5, and α 7) (Figure 3F). This observation raises the possibility that hUmp1, either alone or as a complex with β 2 and β 3, is recruited on the α -ring through direct interaction between hUmp1 and certain α -subunits.

Taken together, these results demonstrate that β 2 is unable to associate with the α -ring without hUmp1 and suggest the important role of hUmp1 in promoting the maturation

process beyond the α -ring, either by stabilizing the complex or by recruiting β 2.

Propeptides of β 1, β 6, and β 7 are dispensable for proteasome maturation

It has been reported that the propeptides and C-terminal tails of β -subunits have important roles in proteasome biogenesis in yeast (Chen and Hochstrasser, 1996; Ramos *et al*, 2004; Li *et al*, 2007; Marques *et al*, 2007), but little is known about their roles in the maturation of proteasomes in mammals.

To elucidate the role of propeptides of β 1, β 2, β 5, β 6, and β 7, and the C-terminal tails of β 2 and β 7 in mammals, we first established cell lines stably transfected with constructs encoding wild-type subunits (β 1*, β 2*, β 5*, β 6*, and β 7*), mature subunits whose propeptides were replaced with ubiquitin (β 1* Δ pro, β 2* Δ pro, β 5* Δ pro, β 6* Δ pro, and β 7* Δ pro), and β 2 and β 7 lacking their C-terminal tails (β 2* Δ tail and β 7* Δ tail) (Supplementary Figure S3A). Synonymous mutations were introduced into these constructs so that they were not sensitive to siRNAs against each β -subunit used in Figure 1. These constructs were attached with C-terminal Flag tag to distinguish the expressed proteins from endogenous proteins. We first confirmed the expression of both precursor and mature forms of β -subunits in HEK293T cells transfected with constructs encoding wild-type and Δ tail β -subunits (Supplementary Figure S3B). On the other hand, as expected, only mature forms of β -subunits were detected in cells transfected with ubiquitin-fused Δ pro constructs

Figure 4 Both the propeptide and C-terminal tail of $\beta 2$ are indispensable for $\beta 3$ incorporation. Stable cell lines expressing the indicated mutant $\beta 2$ -subunits were treated with the siRNA targeting endogenous $\beta 2$. Intermediate complexes were detected by immunoblotting using the indicated antibodies following native PAGE (A–D). Intermediates observed in $\beta 2^* \Delta Tail$ cells can be divided into two species; faster migrating ones (arrowheads) and slower migrating ones (vertical bars). The free complex of PAC3 is depicted by an arrow (D).

Figure 5 $\beta 5$ propeptide is required for $\beta 6$ incorporation. Stable cell lines expressing the indicated mutant $\beta 5$ -subunits were treated with the siRNA(s) for endogenous $\beta 5$ or $\beta 6$ (A–D), or for the indicated combinations (E, F). Cell extracts were resolved by native PAGE, followed by immunoblot analysis for the indicated antibodies.

(Supplementary Figure S3B). These ubiquitin-fused proteins are known to be cleaved rapidly by cellular deubiquitinating enzymes to generate free ubiquitin and the mature moiety of the proteasome subunit, so that the exposure and integrity of the N-terminal residue are ensured (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1999; Jager *et al*, 1999). siRNA-mediated knockdown of endogenous subunits in these cells allowed us to determine the precise roles of propeptides and C-terminal tails.

Expression of constructs encoding each wild-type subunit ($\beta 1^*$, $\beta 2^*$, $\beta 5^*$, $\beta 6^*$, and $\beta 7^*$) restored production of 20S proteasomes and rescued cells from death caused by siRNA treatment (data not shown), verifying that exogenously expressed constructs worked appropriately (Figures 4A, 5A and 6A; Supplementary Figures S4 and S5). Among the Δpro constructs, cells expressing $\beta 1^* \Delta pro$, $\beta 6^* \Delta pro$, and $\beta 7^* \Delta pro$ grew apparently normal and produced 20S proteasomes at an amount comparable to wild-type expressing cells (Supplementary Figures S4 and S5; Figure 6A). These findings indicate that the propeptides of $\beta 1$, $\beta 6$, and $\beta 7$ are not

Figure 6 C-terminal tail of $\beta 7$ is essential for the incorporation of $\beta 7$ and dimerization of half-mers. Stable cell lines expressing the indicated mutant $\beta 7$ -subunits were treated with siRNA targeting endogenous $\beta 7$. Cell extracts were resolved by native PAGE, followed by immunoblot analysis using the indicated antibodies. The free form of $\beta 7^* \Delta Tail$ is depicted by an arrow (B). The ‘half-mer’ assembly intermediates are depicted by arrowheads (A, C).

prerequisite for proteasome maturation in mammals, similar to the results in yeast. On the other hand, cells expressing $\beta 2^*\Delta$ pro, $\beta 5^*\Delta$ pro, $\beta 2^*\Delta$ tail, and $\beta 7^*\Delta$ tail were non-viable in the absence of their endogenous counterparts (data not shown), suggesting the indispensable roles of these propeptides and C-terminal tails in proteasome biogenesis in mammals.

Both the propeptide and C-terminal tail of $\beta 2$ are required for incorporation of $\beta 3$

To clarify the role of the propeptide and C-terminal tail of $\beta 2$, extracts resolved by native PAGE were probed with several antibodies (Figure 4). $\beta 2^*\Delta$ pro- and $\beta 2^*\Delta$ tail-expressing cells showed accumulation of intermediates that included $\alpha 6$ and $\beta 2$, indicating that $\beta 2$ propeptide and $\beta 2$ tail are not required for the incorporation of $\beta 2$ itself (Figure 4A and B). However, these intermediates did not contain $\beta 3$ (Figure 4C), suggesting that the assembly pathway is arrested before $\beta 3$ incorporation and that both $\beta 2$ propeptide and $\beta 2$ tail are required for the incorporation of $\beta 3$. PAC3 was also found in the intermediates in $\beta 2^*\Delta$ pro cells and the faster migrating intermediates in $\beta 2^*\Delta$ tail cells (indicated by an arrowhead), consistent with lack of $\beta 3$ (Figure 4D). However, the slower migrating intermediates in $\beta 2^*\Delta$ tail cells (indicated by the bar) did not include PAC3 (Figure 4D). These species did not contain any β -subunits other than $\beta 2\Delta$ tail (Supplementary Figure S6), precluding the possibility that loss of $\beta 2$ tail and PAC3 causes disordered incorporation of β -subunits. Rather, it is likely that they represent either aggregation of intermediates or association of other molecules, which would be prevented in the presence of $\beta 2$ tail or PAC3.

$\beta 5$ propeptide is required for incorporation of $\beta 6$ but not hUmp1-dependent proteasome maturation

Next, we examined the role of $\beta 5$ propeptide. As expected from its lethality, $\beta 5^*\Delta$ pro-expressing cells could not produce mature 20S proteasomes but showed accumulation of an intermediate that included $\alpha 6$ and $\beta 5$, indicating that $\beta 5$ propeptide is not essential for the incorporation of $\beta 5$ itself (Figure 5A–C). This intermediate did not contain the next subunit $\beta 6$ (Figure 5D) and represented the same size as the intermediate observed in $\beta 6$ -knockdown cells (Figure 5A and B), suggesting that $\beta 5$ propeptide is required for the incorporation of $\beta 6$ in mammalian cells.

In yeast, deletion of $\beta 5$ propeptide, which is fatal, was rescued by concomitant loss of Ump1, suggesting the role of $\beta 5$ propeptide in Ump1-dependent maturation of the yeast proteasome (Ramos *et al*, 1998). In the next series of experiment, siRNAs for both hUmp1 and $\beta 5$ were transfected into $\beta 5^*\Delta$ pro-expressing mammalian cells. Unlike the observation in yeast, however, simultaneous loss of $\beta 5$ propeptide and hUmp1 was still lethal in mammalian cells, with the accumulation of an intermediate closely resembling to that observed in hUmp1 single-knockdown cells that did not contain $\beta 5^*$ (Figure 5E and F). Although these results do not exclude the checkpoint function of hUmp1 as proposed in yeast Ump1 (Li *et al*, 2007), they confirm the important role of hUmp1 in the integrity of early assembly intermediates, which is a function independent of $\beta 5$ propeptide.

Importance of C-terminal tail of $\beta 7$ for stable incorporation of $\beta 7$

$\beta 7^*\Delta$ pro-expressing cells grew normal, incorporated $\beta 7^*$, and produced 20S proteasomes similar to wild-type $\beta 7^*$ -expressing cells (Figure 6A–C). However, deletion of the C-terminal tail could not rescue loss of endogenous $\beta 7$, and the cells could hardly produce 20S proteasomes (Figure 6A–C). In these cells, $\beta 7^*\Delta$ tail failed to be incorporated in the assembly intermediates, presumably half-mers (Li *et al*, 2007), as suggested by the similar size as those in $\beta 7$ -knockdown cells and by the presence of $\beta 6$ (Figure 6A and C, arrowheads) and accumulation of $\beta 7^*\Delta$ tail as a free subunit (Figure 6B, arrow). These observations indicate that the C-terminal tail, which directly associates with the trans- β -ring, is essential for its stable incorporation into proteasomes and supports the model where $\beta 7$ incorporation is tightly coupled with dimerization of half-mers, thus forming 20S proteasomes, as proposed in previous reports in yeast (Li *et al*, 2007; Marques *et al*, 2007).

Discussion

In the present study, we investigated in detail the assembly pathway of mammalian 20S proteasome and found a strictly ordered β -subunit incorporation, which was supported by propeptides of certain β -subunits. Starting from recruitment of $\beta 2$ and hUmp1 on the α -ring, the adjacent β -subunit within the same β -ring, except $\beta 1$, appears to assemble one after another, $\beta 7$ being the last subunit whose incorporation is tightly coupled with dimerization of half-mers (see the model illustrated in Figure 7). This view is also supported by the observation that the size of the assembly intermediate in each knockdown cells increased as the maturation process proceeded (Figure 1A). The order of the β -subunit recruitment clarified in this study is entirely consistent with previous findings where β -subunits were separated into two categories; $\beta 2$, $\beta 3$, and $\beta 4$ as 'early' subunits and others as 'late' subunits (Heinemeyer *et al*, 2004). In both yeast and mammals, the 13S complex containing $\beta 2$, $\beta 3$, $\beta 4$, and (h)Ump1 was observed as a distinct assembly intermediate, suggesting that the following step, that is, incorporation of $\beta 5$, is one of the rate-limiting steps in proteasome biogenesis. This may account for the intriguing observation that sole overexpression of $\beta 5$ in mammalian cells increased the amount of assembled proteasomes (Chondrogianni *et al*, 2005). Although our data imply a stepwise addition of β -subunits on the α -ring, we cannot preclude the possibility that some β -subunits are incorporated as a group, for example as a $\beta 2$ – $\beta 3$ – $\beta 4$ –hUmp1 complex, under normal conditions as we observed intermediate species that can be seen only when a certain β -subunit is depleted.

$\beta 1$ appears to be the last but one incorporated during proteasome assembly, which is a prerequisite for $\beta 7$ incorporation. However, $\beta 1$ can also be incorporated earlier than $\beta 4$, $\beta 5$, and $\beta 6$ in the absence of these subunits, whereas the other subunits are assembled in a manner strictly dependent on the preceding incorporation of the neighbouring subunit. Although such $\beta 1$ -containing intermediates might be artefacts under non-physiological situations, this recalls the early assembly intermediate observed during immunoproteasome biogenesis, which contains $\beta 1i$, $\beta 2i$, $\beta 3$, and $\beta 4$ (Nandi *et al*, 1997). $\beta 1i$ is incorporated into the precursor earlier than $\beta 1$,

Figure 7 A model for β -ring formation in mammalian 20S proteasome assembly. The roles of PAC1–PAC2 and PAC3 in the formation of α -rings were described previously (Hirano *et al*, 2005, 2006). PAC4 was recently identified as a heterodimeric partner of PAC3 (Le Tallec *et al*, 2007). Sequential incorporation of β -subunits starts from the association of $\beta 2$ and hUmp1 on the α -ring. hUmp1 is required for the association of $\beta 2$ in the early assembly intermediates. PAC3–PAC4, whose release is coupled with association of $\beta 3$, holds the structural integrity of the intermediates until $\beta 3$ is incorporated on the α -ring. Subsequent orderly incorporation of other β -subunits is also assisted by intramolecular chaperones such as the propeptides of $\beta 2$ and $\beta 5$ and the C-terminal tail of $\beta 2$. Although $\beta 1$ can be incorporated at various steps (dotted lines), such incorporation most likely follows that of $\beta 6$. Dimerization of half-mers is assisted by the C-terminal tail of $\beta 7$. This is followed by removal of β -subunit propeptides ($\beta 1$, $\beta 2$, $\beta 5$, $\beta 6$, and $\beta 7$) and hUmp1 degradation. Essential propeptides, non-essential propeptides, and essential C-terminal tails of β -subunits for mammalian 20S proteasome biogenesis are depicted in red, blue, and yellow, respectively. See text for more details.

which has an important function in the immunoproteasome assembly (Griffin *et al*, 1998).

In the present study, we identified several new roles for the propeptides and the C-terminal tails of mammalian β -subunits that have not been appreciated in those of yeast β -subunits. The propeptide of yeast $\beta 2$ is dispensable for efficient proteasome biogenesis (Arendt and Hochstrasser, 1999), but that of mammalian $\beta 2$ contributes to the recruitment of its neighbouring subunit $\beta 3$ (Figure 4). The propeptide of yeast $\beta 5$ is reported to be required for its own incorporation (Chen and Hochstrasser, 1996), whereas that of mammalian $\beta 5$ was not; instead, its loss caused failure of $\beta 6$ recruitment (Figure 5). The role of the C-terminal tail of mammalian $\beta 7$ appears to be quite similar to that of yeast $\beta 7$, but it proved to be an essential component in proteasome biogenesis, unlike yeast $\beta 7$ (Figure 6).

Our study also clarified the sequences leading to hUmp1 incorporation and release of PAC3 from proteasome

precursors. Incorporation of hUmp1 was as early as that of $\beta 2$, the first β -subunit assembled on the α -ring. In the early assembly intermediates, hUmp1 unexpectedly had an important function in the association of $\beta 2$ with the precursor proteasomes and proved to be a prerequisite for the assembly of β -subunits on the α -ring (Figures 3 and 7). This function is not appreciated for yeast Ump1, which regulates dimerization of half-proteasomes and maturation of 20S proteasomes, presumably by acting as a checkpoint protein (Ramos *et al*, 1998; Li *et al*, 2007). As the phenotype of loss of hUmp1 emerged at a stage upstream of the dimerization, we are uncertain whether hUmp1 in mammalian cells also has a function similar to that in yeast.

In a previous study, we showed that PAC3 was scarcely included in the complex purified by a tag attached to hUmp1 (Hirano *et al*, 2006). In the present study, we clarified that PAC3 release was coupled with association of $\beta 3$ with the assembly intermediate. This observation is consistent with

the recent study showing that Dmp1–Dmp2, yeast orthologue of PAC3–PAC4, was copurified with β 2, but not with other β -subunits (Yashiroda *et al*, 2008). Therefore, a complex containing both hUmp1 and PAC3, together with β 2, is assumed to be present during the maturation pathway, and such a complex was indeed observed in β 3-knockdown cells (Figure 1). As PAC3 is known to bind directly to β 3 *in vitro* (Hirano *et al*, 2006), it may be argued that PAC3 facilitates the incorporation of β 3, which might induce a conformational alteration of the assembly intermediate to release PAC3. Because PAC3 is also required for efficient α -ring formation (Hirano *et al*, 2006), we could not detect accumulation of an intermediate before the incorporation of β 3 in PAC3-knockdown cells, such as a complex comprising α -ring and β 2, which would be expected to occur on the assumption that PAC3 is required for β 3 incorporation (Supplementary Figure S7). Although biochemical data both in yeast and mammals indicated that PAC3 is replaced when β 3 enter, a model generated by superimposing Dmp1–Dmp2– α 5 complex on the yeast 20S proteasome suggested steric hindrance of β 4-subunit to Dmp1 (Yashiroda *et al*, 2008). The precise reason for this discrepancy is not clear at present. This may be simply because the model, which was based on the structure of Dmp1–Dmp2 determined in the absence of α -ring, does not represent bona fide structure of assembly intermediates. Further studies should be required for understanding the role of PAC3 in β -subunit incorporation.

Recently, the proteasome-specific inhibitor bortezomib was used clinically in refractory multiple myeloma, and a clinical trial of this agent in other malignant neoplasms is currently underway (Adams, 2004). Accordingly, the proteasome is now recognized as a potent target for cancer therapy (Adams, 2004). Assuming that inhibition of proteasome biogenesis is also beneficial in cancer therapy, it is important to understand the detailed mechanism of mammalian proteasome biogenesis. Our results could provide the foundation for the design and development of novel anticancer drugs that target proteasome biogenesis.

Materials and methods

DNA constructs

The cDNAs encoding β -subunits and those derivatives were cloned into pIRESpuro3 (Clontech) in frame with a C-terminal Flag tag. To make constructs for β -subunits resistant to siRNAs, synonymous mutagenesis in RNAi targeting sequences was performed using the

QuikChange site-directed mutagenesis kit (Stratagene). Plasmids encoding β 1* Δ pro, β 2* Δ pro, β 5* Δ pro, β 6* Δ pro, and β 7* Δ pro were constructed by fusing human ubiquitin cDNA to the 5' end of the cDNAs encoding mature forms of the corresponding subunits. In β 2* Δ tail and β 7* Δ tail, sequences encoding amino acids 244–277 and 248–264 were deleted, respectively. PCR was performed using Phusion DNA polymerase (Finnzymes), and all constructs were confirmed by sequencing.

Cell culture

HEK293T cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal calf serum, 100 IU/ml penicillin G, 100 μ g/ml streptomycin sulphate (all from Invitrogen). Stable transfection of HEK293T cells was performed using Fugene 6 (Roche), and the cells were selected with 5 μ g/ml of puromycin (Sigma).

Protein extracts, immunological analysis, and antibodies

Cells were lysed in an ice-cold lysis buffer (50 mM Tris–HCl (pH 7.5), 0.5% (v/v) NP-40, 1 mM dithiothreitol, 2 mM ATP, and 5 mM MgCl₂) and the extracts were clarified by centrifugation at 20 000 g for 15 min at 4°C. The supernatants were subjected to glycerol gradient analysis or native PAGE. Glycerol gradient analysis and assay of proteasome activity were described previously (Murata *et al*, 2001; Hirano *et al*, 2006). *In vitro* binding assay was described previously (Hirano *et al*, 2006). SDS–PAGE (12% Bis-Tris gel (Invitrogen)) and native PAGE (7% Tris–acetate gel (Invitrogen)) were performed according to the instructions provided by the manufacturer. The separated proteins were transferred onto polyvinylidene difluoride membrane and reacted with the indicated antibody. Anti-PAC1, PAC2, PAC3, hUmp1, β 4 (55F8), β 5 (P93250), β 6 (P93199), and PA28 α polyclonal antibodies were described previously (Tanahashi *et al*, 2000; Hirano *et al*, 2006). Antibodies against proteasome α 6 (MCP20), β 1 (MCP421), β 2 (MCP168), β 3 (MCP102), β 7 (MCP205), and PA200 were purchased from BioMol. Anti-Hsp90 α and anti-Hsc70 were obtained from MBL. Anti-FLAG M2 antibody was from Sigma.

RNA interference

The siRNAs targeting human β -subunits and hUmp1 (Supplementary Table S1) were transfected into HEK293T cells using Lipofectamine RNAi MAX (Invitrogen) at a final concentration of 50 nM in six-well dishes twice at a 12-h interval. The cells were analysed 24 h after the second transfection.

Supplementary information

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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