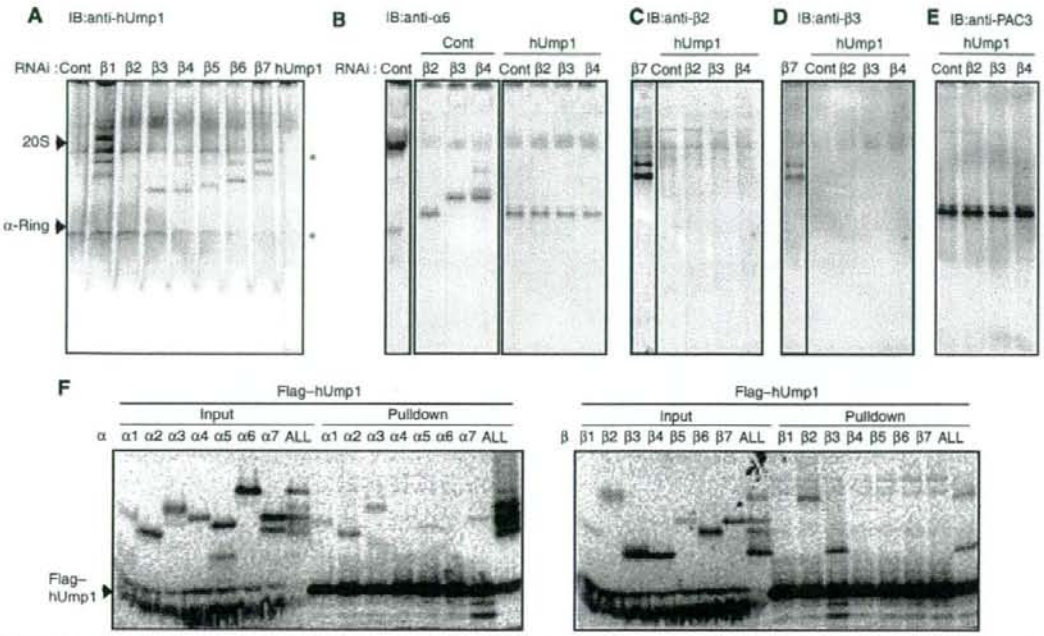

Figure 3 Role of hUmp1 in the structural integrity of early assembly intermediates. (**A**) The same panel in Figure 1 was probed with anti-hUmp1 antibody. (**B–E**) Extracts of HEK293T cells transfected with the indicated combinations of siRNAs were separated by native PAGE. Intermediate complexes were detected by immunoblotting using the indicated antibodies. The left lane representing β7 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 β2, β3, or β4, and the resultant assembly intermediates were compared with those in β2, β3, or β4 single-knockdown cells. Intriguingly, the size of intermediates observed in the simultaneous knockdown of hUmp1 with β3 or β4 was similar to that of the complex in β2 single-knockdown cells (Figure 3B). Notably, the intermediate found in β3–hUmp1 double-knockdown cells lacked β2 (Figure 3C), which was found in the complex of β3 single-knockdown cells (Figure 1D). The β4 and hUmp1 double knockdown was associated with loss of both β2 and β3 in the intermediate (Figure 3C and D), which were clearly detected in the β4 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 β3, 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 β2 and β3 as well as some of the α-subunits (α2, α3, α5, and α7) (Figure 3F). This observation raises the possibility that hUmp1, either alone or as a complex with β2 and β3, is recruited on the α-ring through direct interaction between hUmp1 and certain α-subunits.

Taken together, these results demonstrate that β2 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 β2.

Propeptides of β1, β6, and β7 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 β1, β2, β5, β6, and β7, and the C-terminal tails of β2 and β7 in mammals, we first established cell lines stably transfected with constructs encoding wild-type subunits (β1*, β2*, β5*, β6*, and β7*), mature subunits whose propeptides were replaced with ubiquitin (β1*Δpro, β2*Δpro, β5*Δpro, β6*Δpro, and β7*Δpro), and β2 and β7 lacking their C-terminal tails (β2*Δtail and β7*Δtail) (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 Δtail 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 Δpro constructs

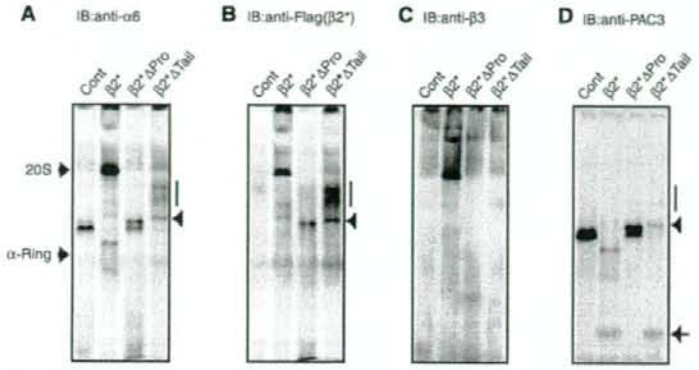

Figure 4 Both the propeptide and C-terminal tail of β2 are indispensable for β3 incorporation. Stable cell lines expressing the indicated mutant β2-subunits were treated with the siRNA targeting endogenous β2. Intermediate complexes were detected by immunoblotting using the indicated antibodies following native PAGE (**A–D**). Intermediates observed in β2*ΔTail 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).

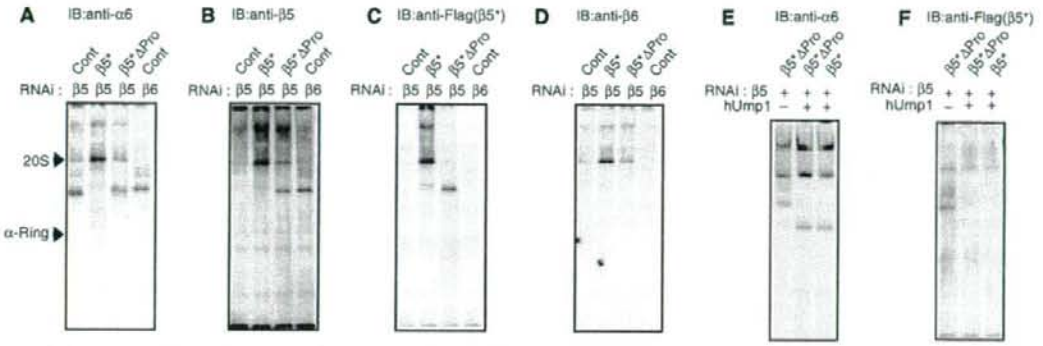

Figure 5 β5 propeptide is required for β6 incorporation. Stable cell lines expressing the indicated mutant β5-subunits were treated with the siRNA(s) for endogenous β5 or β6 (**A–D**), or for the indicated combinations (**E, F**). Cell extracts were resolved by native PAGE, followed by immunoblot analysis 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 (β1*, β2*, β5*, β6*, and β7*) 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 β1*Δpro, β6*Δpro, and β7*Δpro 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 β1, β6, and β7 are not

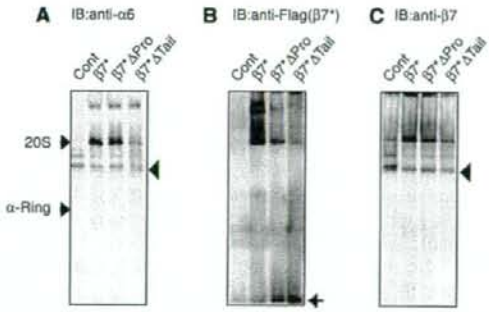

Figure 6 C-terminal tail of β7 is essential for the incorporation of β7 and dimerization of half-mers. Stable cell lines expressing the indicated mutant β7-subunits were treated with siRNA targeting endogenous β7. Cell extracts were resolved by native PAGE, followed by immunoblot analysis using the indicated antibodies. The free form of β7*Δtail 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 β2*Δpro, β5*Δpro, β2*Δtail, and β7*Δtail 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 β2 are required for incorporation of β3

To clarify the role of the propeptide and C-terminal tail of β2, extracts resolved by native PAGE were probed with several antibodies (Figure 4). β2*Δpro- and β2*Δtail-expressing cells showed accumulation of intermediates that included α6 and β2, indicating that β2 propeptide and β2 tail are not required for the incorporation of β2 itself (Figure 4A and B). However, these intermediates did not contain β3 (Figure 4C), suggesting that the assembly pathway is arrested before β3 incorporation and that both β2 propeptide and β2 tail are required for the incorporation of β3. PAC3 was also found in the intermediates in β2*Δpro cells and the faster migrating intermediates in β2*Δtail cells (indicated by an arrowhead), consistent with lack of β3 (Figure 4D). However, the slower migrating intermediates in β2*Δtail cells (indicated by the bar) did not include PAC3 (Figure 4D). These species did not contain any β-subunits other than β2Δtail (Supplementary Figure S6), precluding the possibility that loss of β2 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 β2 tail or PAC3.

β5 propeptide is required for incorporation of β6 but not hUmp1-dependent proteasome maturation

Next, we examined the role of β5 propeptide. As expected from its lethality, β5*Δpro-expressing cells could not produce mature 20S proteasomes but showed accumulation of an intermediate that included α6 and β5, indicating that β5 propeptide is not essential for the incorporation of β5 itself (Figure 5A–C). This intermediate did not contain the next subunit β6 (Figure 5D) and represented the same size as the intermediate observed in β6-knockdown cells (Figure 5A and B), suggesting that β5 propeptide is required for the incorporation of β6 in mammalian cells.

In yeast, deletion of β5 propeptide, which is fatal, was rescued by concomitant loss of Ump1, suggesting the role of β5 propeptide in Ump1-dependent maturation of the yeast proteasome (Ramos *et al*, 1998). In the next series of experiment, siRNAs for both hUmp1 and β5 were transfected into β5*Δpro-expressing mammalian cells. Unlike the observation in yeast, however, simultaneous loss of β5 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 β5* (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 β5 propeptide.

Importance of C-terminal tail of β7 for stable incorporation of β7

β7*Δpro-expressing cells grew normal, incorporated β7*, and produced 20S proteasomes similar to wild-type β7*-expressing cells (Figure 6A–C). However, deletion of the C-terminal tail could not rescue loss of endogenous β7, and the cells could hardly produce 20S proteasomes (Figure 6A–C). In these cells, β7*Δtail failed to be incorporated in the assembly intermediates, presumably half-mers (Li *et al*, 2007), as suggested by the similar size as those in β7-knockdown cells and by the presence of β6 (Figure 6A and C, arrowheads) and accumulation of β7*Δtail 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 β7 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 β2 and hUmp1 on the α-ring, the adjacent β-subunit within the same β-ring, except β1, appears to assemble one after another, β7 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; β2, β3, and β4 as 'early' subunits and others as 'late' subunits (Heinemeyer *et al*, 2004). In both yeast and mammals, the 13S complex containing β2, β3, β4, and (h)Ump1 was observed as a distinct assembly intermediate, suggesting that the following step, that is, incorporation of β5, is one of the rate-limiting steps in proteasome biogenesis. This may account for the intriguing observation that sole overexpression of β5 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 β2–β3–β4–hUmp1 complex, under normal conditions as we observed intermediate species that can be seen only when a certain β-subunit is depleted.

β1 appears to be the last but one incorporated during proteasome assembly, which is a prerequisite for β7 incorporation. However, β1 can also be incorporated earlier than β4, β5, and β6 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 β1-containing intermediates might be artefacts under non-physiological situations, this recalls the early assembly intermediate observed during immunoproteasome biogenesis, which contains β1i, β2i, β3, and β4 (Nandi *et al*, 1997). β1i is incorporated into the precursor earlier than β1,

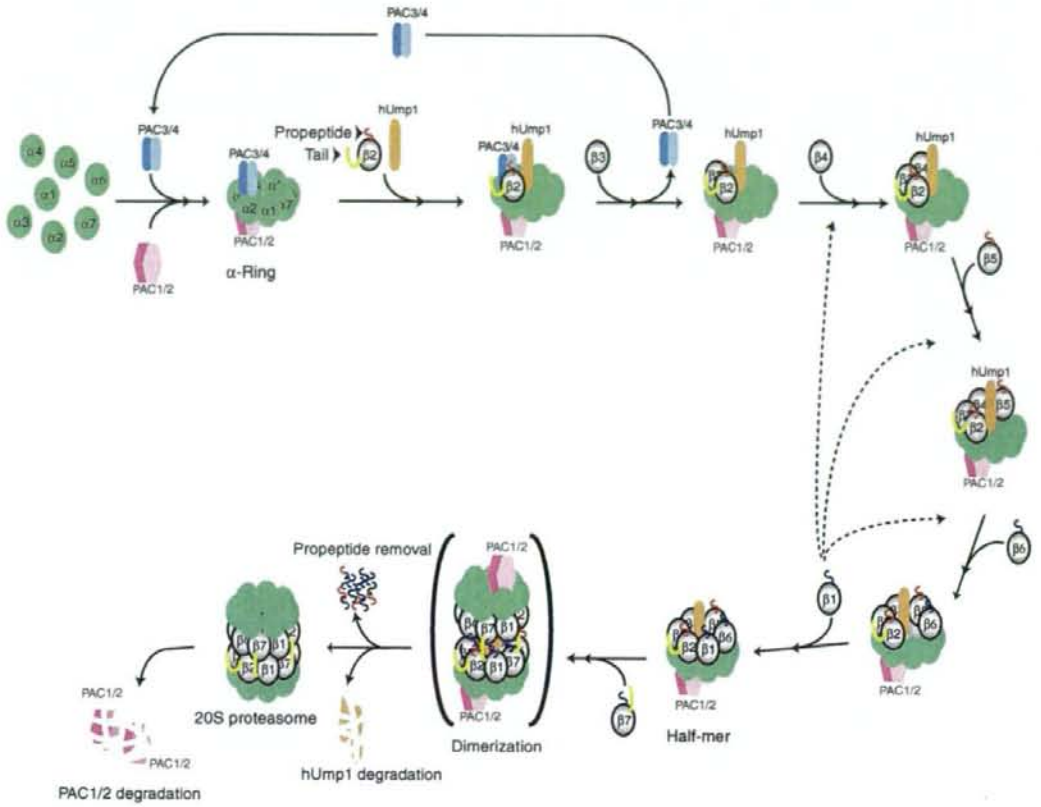

Figure 7 A model for β-ring formation in mammalian 20S proteasome assembly. The roles of PAC1–PAC2 and PAC3 in the formation of α-rings were described previously (Hirano *et al*, 2005, 2006). PAC4 was recently identified as a heterodimeric partner of PAC3 (Le Tallec *et al*, 2007). Sequential incorporation of β-subunits starts from the association of β2 and hUmp1 on the α-ring. hUmp1 is required for the association of β2 in the early assembly intermediates. PAC3–PAC4, whose release is coupled with association of β3, holds the structural integrity of the intermediates until β3 is incorporated on the α-ring. Subsequent orderly incorporation of other β-subunits is also assisted by intramolecular chaperones such as the propeptides of β2 and β5 and the C-terminal tail of β2. Although β1 can be incorporated at various steps (dotted lines), such incorporation most likely follows that of β6. Dimerization of half-mers is assisted by the C-terminal tail of β7. This is followed by removal of β-subunit propeptides (β1, β2, β5, β6, and β7) 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 β2 is dispensable for efficient proteasome biogenesis (Arendt and Hochstrasser, 1999), but that of mammalian β2 contributes to the recruitment of its neighbouring subunit β3 (Figure 4). The propeptide of yeast β5 is reported to be required for its own incorporation (Chen and Hochstrasser, 1996), whereas that of mammalian β5 was not; instead, its loss caused failure of β6 recruitment (Figure 5). The role of the C-terminal tail of mammalian β7 appears to be quite similar to that of yeast β7, but it proved to be an essential component in proteasome biogenesis, unlike yeast β7 (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 β2, the first β-subunit assembled on the α-ring. In the early assembly intermediates, hUmp1 unexpectedly had an important function in the association of β2 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 β3 with the assembly intermediate. This observation is consistent with

the recent study showing that Dmp1–Dmp2, yeast orthologue of PAC3–PAC4, was copurified with β2, but not with other β-subunits (Yashiroda *et al*, 2008). Therefore, a complex containing both hUmp1 and PAC3, together with β2, is assumed to be present during the maturation pathway, and such a complex was indeed observed in β3-knockdown cells (Figure 1). As PAC3 is known to bind directly to β3 *in vitro* (Hirano *et al*, 2006), it may be argued that PAC3 facilitates the incorporation of β3, 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 β3 in PAC3-knockdown cells, such as a complex comprising α-ring and β2, which would be expected to occur on the assumption that PAC3 is required for β3 incorporation (Supplementary Figure S7). Although biochemical data both in yeast and mammals indicated that PAC3 is replaced when β3 enter, a model generated by superimposing Dmp1–Dmp2–α5 complex on the yeast 20S proteasome suggested steric hindrance of β4-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 β1*Δpro, β2*Δpro, β5*Δpro, β6*Δpro, and β7*Δpro were constructed by fusing human ubiquitin cDNA to the 5′ end of the cDNAs encoding mature forms of the corresponding subunits. In β2*Δtail and β7*Δtail, 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 μg/ml streptomycin sulphate (all from Invitrogen). Stable transfection of HEK293T cells was performed using Fugene 6 (Roche), and the cells were selected with 5 μg/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 $MgCl_2$) 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, β4 (55F8), β5 (P93250), β6 (P93199), and PA28α polyclonal antibodies were described previously (Tanahashi *et al*, 2000; Hirano *et al*, 2006). Antibodies against proteasome α6 (MCP20), β1 (MCP421), β2 (MCP168), β3 (MCP102), β7 (MCP205), and PA200 were purchased from BioMol. Anti-Hsp90α 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).

Acknowledgements

This study was supported by grants to SM and KT from the Ministry of Education, Science and Culture of Japan (MEXT), Target Protein Project of MEXT (to KT and KK), and the Takeda Science Foundation (KT).

References

Adams J (2004) The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* **4:** 349–360

Arendt CS, Hochstrasser M (1999) Eukaryotic 20S proteasome catalytic subunit propeptides prevent active site inactivation by N-terminal acetylation and promote particle assembly. *EMBO J* **18:** 3575–3585

Baumeister W, Walz J, Zuhl F, Seemuller E (1998) The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92:** 367–380

Chen P, Hochstrasser M (1996) Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. *Cell* **86:** 961–972

Chondrogianni N, Tzavelas C, Pemberton AJ, Nezis IP, Rivett AJ, Gonos ES (2005) Overexpression of proteasome beta5 assembled subunit increases the amount of proteasome and confers ameliorated response to oxidative stress and higher survival rates. *J Biol Chem* **280:** 11840–11850

Coux O, Tanaka K, Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* **65:** 801–847

Fehlker M, Wendler P, Lehmann A, Enenkel C (2003) Blm3 is part of nascent proteasomes and is involved in a late stage of nuclear proteasome assembly. *EMBO Rep* **4:** 959–963

Frentzel S, Pesold-Hurt B, Seelig A, Kloetzel PM (1994) 20S proteasomes are assembled via distinct precursor complexes. Processing of LMP2 and LMP7 proproteins takes place in 13–16S preproteasome complexes. *J Mol Biol* **236:** 975–981

Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82:** 373–428

Griffin TA, Nandi D, Cruz M, Fehling HJ, Kaer LV, Monaco JJ, Colbert RA (1998) Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *J Exp Med* **187:** 97–104

Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386:** 463–471

Heinemeyer W, Ramos PC, Dohmen RJ (2004) The ultimate nanoscale mincer: assembly, structure and active sites of the 20S proteasome core. *Cell Mol Life Sci* **61:** 1562–1578

Hirano Y, Hayashi H, Iemura S, Hendil KB, Niwa S, Kishimoto T, Kasahara M, Natsume T, Tanaka K, Murata S (2006) Cooperation of multiple chaperones required for the assembly of mammalian 20S proteasomes. *Mol Cell* **24:** 977–984

Hirano Y, Hendil KB, Yashiroda H, Iemura S, Nagane R, Hioki Y, Natsume T, Tanaka K, Murata S (2005) A heterodimeric complex that promotes the assembly of mammalian 20S proteasomes. *Nature* **437:** 1381–1385

Imai J, Maruya M, Yashiroda H, Yahara I, Tanaka K (2003) The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. *EMBO J* **22:** 3557–3567

Jager S, Groll M, Huber R, Wolf DH, Heinemeyer W (1999) Proteasome beta-type subunits: unequal roles of propeptides in core particle maturation and a hierarchy of active site function. *J Mol Biol* **291:** 997–1013

Kusmierczyk AR, Kunjappu MJ, Funakoshi M, Hochstrasser M (2008) A multimeric assembly factor controls the formation of alternative 20S proteasomes. *Nat Struct Mol Biol* **15:** 237–244

Le Tallec B, Barrault MB, Courbeyrette R, Guerois R, Marsolier-Kergoat MC, Peyroche A (2007) 20S proteasome assembly is orchestrated by two distinct pairs of chaperones in yeast and in mammals. *Mol Cell* **27:** 660–674

Li X, Kusmierczyk AR, Wong P, Emili A, Hochstrasser M (2007) Beta-subunit appendages promote 20S proteasome assembly by overcoming an Ump1-dependent checkpoint. *EMBO J* **26:** 2339–2349

Marques AJ, Glanemann C, Ramos PC, Dohmen RJ (2007) The C-terminal extension of the beta 7 subunit and activator complexes stabilize nascent 20s proteasomes and promote their maturation. *J Biol Chem* **282:** 34869–34876

Murata S (2006) Multiple chaperone-assisted formation of mammalian 20S proteasomes. *IUBMB Life* **58:** 344–348

Murata S, Sasaki K, Kishimoto T, Niwa S, Hayashi H, Takahama Y, Tanaka K (2007) Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* **316:** 1349–1353

Murata S, Udono H, Tanahashi N, Hamada N, Watanabe K, Adachi K, Yamano T, Yui K, Kobayashi N, Kasahara M, Tanaka K, Chiba T (2001) Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. *EMBO J* **20:** 5898–5907

Nandi D, Woodward E, Ginsburg DB, Monaco JJ (1997) Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor beta subunits. *EMBO J* **16:** 5363–5375

Preckel T, Fung-Leung WP, Cai Z, Vitiello A, Salter-Cid L, Winqvist O, Wolfe TG, Von Herrath M, Angulo A, Ghazal P, Lee JD, Fourie AM, Wu Y, Pang J, Ngo K, Peterson PA, Fruh K, Yang Y (1999) Impaired immunoproteasome assembly and immune responses in PA28−/− mice. *Science* **286:** 2162–2165

Ramos PC, Hockendorff J, Johnson ES, Varshavsky A, Dohmen RJ (1998) Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. *Cell* **92:** 489–499

Ramos PC, Marques AJ, London MK, Dohmen RJ (2004) Role of C-terminal extensions of subunits beta2 and beta7 in assembly and activity of eukaryotic proteasomes. *J Biol Chem* **279:** 14323–14330

Schmidtke G, Schmidt M, Kloetzel PM (1997) Maturation of mammalian 20S proteasome: purification and characterization of 13S and 16S proteasome precursor complexes. *J Mol Biol* **268:** 95–106

Tanahashi N, Murakami Y, Minami Y, Shimbara N, Hendil KB, Tanaka K (2000) Hybrid proteasomes. Induction by interferon-gamma and contribution to ATP-dependent proteolysis. *J Biol Chem* **275:** 14336–14345

Tanaka K, Kasahara M (1998) The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. *Immunol Rev* **163:** 161–176

Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T (2002) The structure of the mammalian 20S proteasome at 2.75 Å resolution. *Structure (Camb)* **10:** 609–618

Witt E, Zantopf D, Schmidt M, Kraft R, Kloetzel PM, Kruger E (2000) Characterisation of the newly identified human Ump1 homologue POMP and analysis of LMP7(beta 5i) incorporation into 20S proteasomes. *J Mol Biol* **301:** 1–9

Yashiroda H, Mizushima T, Okamoto K, Kameyama T, Hayashi H, Kishimoto T, Niwa S, Kasahara M, Kurimoto E, Sakata E, Takagi K, Suzuki A, Hirano Y, Murata S, Kato K, Yamane T, Tanaka K (2008) Crystal structure of a chaperone complex that contributes to the assembly of yeast 20S proteasomes. *Nat Struct Mol Biol* **15:** 228–236