論文の内容の要旨

論　文　題　目

Assembly mechanisms of specialized subtypes of the 20S core particle and the lid subcomplex of the proteasome

（プロテアソームの特殊型 20S コア粒子および蓋部の分子集合機構の解明）

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[Introduction]

The 26S proteasome catalyzes degradation of ubiquitinated proteins in eukaryotic cells, thus playing a central role in various cellular processes including cell cycle, transcription, signal transduction, immunity, and protein quality control.

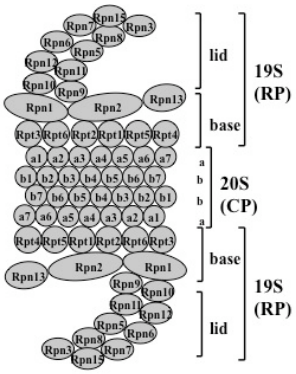


Figure 1. Structure of 26S proteasome

The 26S proteasome has a highly complicated structure and is composed of the catalytic 20S core particle (CP) and the 19S regulatory particle (RP), which are assembled from 14 and 19 different subunits, respectively (Figure 1). The standard CP (sCP) consists of 7 different α-type subunits α1- α7 and 7 different β-type subunits β1- β7, of which β1, β2 and β5 are catalytically active. In vertebrates, two other subtypes of the CP exist. One is the “immunoproteasome (iCP),” in which the catalytic β1, β2 and β5 of the sCP are replaced by another set of catalytic subunits named β1i, β2i and β5i. This iCP efficiently generates antigenic peptides presented on MHC class I molecules. The other is the “thymoproteasome (tCP),” in which β5i of the iCP is replaced by β5t, which is exclusively expressed in cortical thymic epithelial cells. This tCP plays a pivotal role in positive selection of $CD8^+$ T cells. The RP can be divided into a base and a lid subcomplex. The base, which is required for recruiting ubiquitinated proteins and unfolding them, comprises 6 ATPase subunit Rpt1-Rpt6 and four non-ATPase subunit Rpn1, 2, 10 and 13, whereas the lid, which is essential for removing ubiquitin chains from the substrates, comprises 9 non-ATPase subunits Rpn3, 5-9, 11, 12, and 15.

Accurate assembly of the proteasome from the 33 different subunits is a prerequisite for degradation of ubiquitinated proteins. Recent studies have revealed that biogenesis of the sCP and the base subcomplex were assisted by multiple dedicated chaperones. However, little is known about the assembly pathway of the iCP, tCP, and the lid subcomplex. In this study, we investigated the biogenesis of the two specialized types of CPs and the lid subcomplex using combination of RNA interference (RNAi) and mass spectrometry in mammalian cells.

[Results]

1. Dissecting assembly pathways of the 20S immunoproteasome (iCP) and the 20S thymoproteasome (tCP)

1-1. Earlier incorporation of the specialized β-subunits than the standard β-subunits

Making use of HeLa cells treated with IFN-γ and the stable expression of β5t in HEK293T cells, we described a series of biochemical experiments employing RNAi of each β subunit, which resulted in accumulation of distinct intermediates. By characterizing these intermediates, we clarified the order of β subunit incorporation in the iCP and tCP. As we showed previously, β subunits were incorporated strictly and sequentially in the order β2, β3, β4, β5, β6, β1, and β7 in the sCP. However, β5i and β5t could be incorporated immediately after the assembly of β3 even without β4 in the iCP and tCP, respectively. Furthermore, β1i and β2i were incorporated ahead of all the other β subunits (Figure 2).

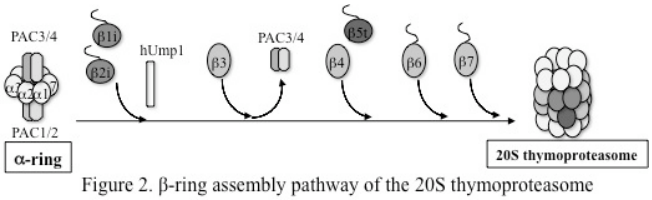


Figure 2. β-ring assembly pathway of the 20S thymoproteasome

To elucidate the mechanism of earlier incorporation of β5i and β5t compared to β5, we focused on their propeptides. Two chimeric β5 subunits were constructed by fusing the propeptide of β5i or β5t to the mature form of β5. Here we refer to these chimeric subunits as β5i(p)+ β5(m) and β5t(p)+ β5(m). We expressed these subunits into cells depleted of endogenous β4 and β5 and observed whether the chimeric β5 subunits were incorporated without β4. β5i(p)+β5(m) was not incorporated in the absence of β4, suggesting that the mature body of β5i, but not the propeptide of β5i, was prerequisite for β5i incorporation (Figure 3). In contrast, β5t(p)+ β5(m) was readily incorporated without β4, indicating that the propeptide of β5t enabled earlier incorporation of β5 to the premature proteasome (Figure 3).

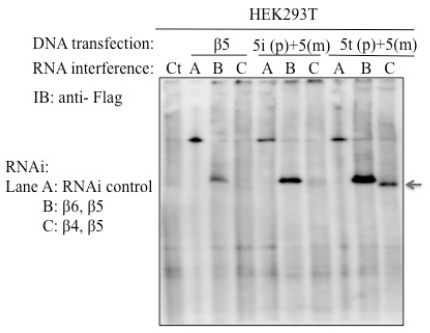


Figure 3. Propeptide of β5t could assist earlier incorporation of β5.

To sum up, the assembly of the iCP and tCP began with the incorporation of β1i and β2i, followed by incorporation of β3, then β4 or β5i/β5t, and β6 and β7 were the last two to be incorporated. In the assembly pathways, the propeptide of β5t is a key factor for its earlier incorporation than β4, while the mature form of β5i is thought to be more crucial to its earlier incorporation. These unique features of β5t and β5i may account for preferential assembly of the iCP and tCP over sCP even when standard and specialized subunits are co-expressed.

1-2. Incorporation of β5t is largely dependent on β1i and β2i

Since β1i and β2i are the common catalytic subunits of iCP and tCP, we examined whether there was any difference in the dependence of β5i and β5t incorporation on the presence of β1i and β2i. β5i and β5t were synthesized with N-terminal propeptides as premature forms. Those propeptides are cleaved upon completion of the CP assembly to expose the catalytic threonine residues, and therefore, processing of the propeptides indicates efficiency of the CP assembly. We expressed β5t or β5i in β5i (-/-) MEF cells. These cells express β1i and β2i only when treated with IFN-γ. Nearly half of the expressed β5t were in premature forms without IFN-γ, but the mature β5t was remarkably increased upon IFN-γ treatment. In contrast, the majority of β5i were already matured in the absence of IFN-γ, and the induction of β5i maturation by IFN-γ was modest (Figure 4). These results indicated that presence of β1i and β2i facilitated incorporation of β5t, whereas β5i could be incorporated efficiently in combination with the standard subunits β1 and β2. This may account for generation of altered TCR repertoire of $CD8^+$ T cells in β1i- and β2i-deficient mice, as reported previously.

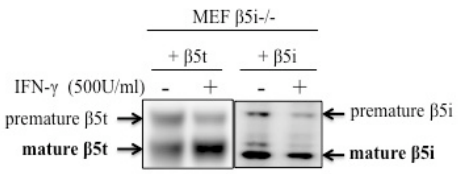
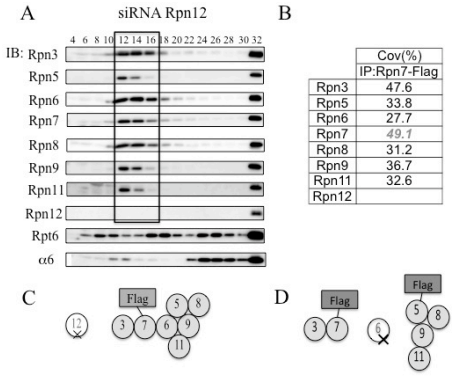


Figure 4. Maturation of β5t was facilitated by β1i and β2i.

2. Dissecting assembly pathway of the lid subcomplex

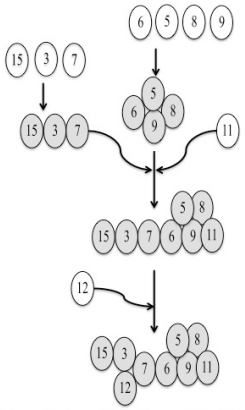
In order to dissect the assembly pathway of the lid subcomplex, we analyzed specific intermediates observed during knockdown of each lid subunit in HEK293T cells by immunoblot and mass spectrometry analysis. When treated with siRNA targeting Rpn12, a complex comprising all the lid subunits except Rpn12 was detected, indicating that Rpn12 was the last subunit incorporated during the lid formation (Figure 5C). When we knocked down Rpn6, two intermediate complexes Rpn3-7 and Rpn5-8-9-11 were observed, suggesting that these two intermediates were formed independently and bound to each other via Rpn6 (Figure 5D). While the association between Rpn3 and Rpn7 was not essential for the stability of either subunit, Rpn5, Rpn8 and Rpn9 were not able to form an intermediate complex without any one of the other subunits, indicating that these three subunits assembled as a set. Additionally, Rpn11 was markedly decreased by knockdown of Rpn5 and Rpn8, indicating that stability of Rpn11 was dependent on the Rpn5-8-9 complex.



Figrue 5. Two intermediates was formed during the assembly, and Rpn12 was incorporated at the final step of the lid formation.

Since no effective antibody against human Rpn15 was available and it was impossible to detect fragmented peptides derived from Rpn15 by our MS analysis due to the detection limit after trypsin digestion, we constructed HEK293T cells stably expressing Rpn15-Venus. An intermediate comprising the lid subunits except Rpn3 and Rpn12 was identified in Rpn15-knockdown cells, and no subunit was co-immunoprecipitated with Rpn15 when we knocked down Rpn3, suggesting that Rpn3 and Rpn15 were assembled into the lid in an interdependent manner.

In brief, an intermediate comprising Rpn5-6-8-9 served as a core module that was prerequisite for assembly of the essential deubiquitinase Rpn11, which then associated with Rpn15-3-7 complex. Rpn12 was the last subunit to be incorporated (Figure 6). The lid formation did not seem to be assisted by any specific chaperones, like CP and the base assembly, because we did not detect molecules other than the lid subunits in the intermediates of lid subcomplex by MS analysis.



Figrue 6. Assembly pathway of the lid subcomplex

[Discussion]

1. It has been known that pre-CPs containing β1i and β2i favored the incorporation of β5i because β5i mediated efficient processing of β1i and enhanced the effectiveness of CP assembly. We showed that incorporation of β5t was dependent on β1i and β2i more than that of β5i (Result 1-2), and the propeptide of β5t played an essential role in its incorporation. This feature of β5t might explain the fact that more than 90% of the CPs is the tCP in cTECs while β5t and β5i are transcriptionally co-expressed.
2. Recent research has discovered that overexpression of Rpn6 subunit in *C. elegans* and Rpn11 in fruit fly prolongs the lifespan. It is also shown that FOXO4-mediated upregulation of Rpn6 is a prerequisite for maintaining pluripotency in embryonic stem cells. Overexpression of Rpn11 or Rpn7 is also known to be involved in DNA damage response. However, the mechanisms are still unidentified. Elucidating the assembly pathway of the lid subcomplex should be of help to understand the mechanism in which high expression of a single lid subunit plays such significant roles.