Cell

Multiple Assembly Chaperones Govern Biogenesis of the Proteasome Regulatory Particle Base

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DOI 10.1016/j.cell.2009.04.061

SUMMARY

The central protease of eukaryotes, the 26S proteasome, has a 20S proteolytic core particle (CP) and an attached 19S regulatory particle (RP). The RP is further subdivided into lid and base subcomplexes. Little is known about RP assembly. Here, we show that four conserved assembly factors govern biogenesis of the yeast RP base. Nas2 forms a complex with the Rpt4 and Rpt5 ATPases and enhances 26S proteasome formation in vivo and in vitro. Other RP subcomplexes contain Hsm3, which is related to mammalian proteasome subunit S5b. Hsm3 also contributes to base assembly. Larger Hsm3-containing complexes include two additional proteins, Nas6 and Rpn14, which function as assembly chaperones as well. Specific deletion combinations affecting these four factors cause severe perturbations to RP assembly. Our results demonstrate that proteasomal RP biogenesis requires multiple, functionally overlapping chaperones and suggest a model in which subunits form specific subcomplexes that then assemble into the base.

INTRODUCTION

In eukaryotes, short-lived proteins are degraded primarily by the ubiquitin-proteasome system (Hochstrasser, 1996; Heinemeyer et al., 2004). Defects in the system are linked to a variety of human diseases, and proteasomal inhibitors are used to treat several cancers (Goldberg, 2007; Schwartz and Ciechanover, 2009). Most proteasome substrates are first modified by polyubiquitin chains, allowing recognition by the proteasome and degradation of the substrate. The 26S proteasome consists of a proteolytically active 20S proteasome core particle (CP) bound at one or both ends by a 19S regulatory particle (RP) (Schmidt et al., 2005). In addition to substrate binding, the RP is responsible for substrate unfolding, substrate translocation into the proteolytic chamber of the CP, and release of ubiquitin from the substrate.

High-resolution structural information is available for the CP, and it has been subject to extensive biochemical and genetic analysis (reviewed in Marques et al., 2009). The CP forms a cylinder of four stacked heptameric rings. Two structurally related classes of subunits make up the rings. The outer rings have α-type subunits, and the inner rings β-type subunits, with the protease sites formed by specific β subunits.

The structure and activities of the RP are less well understood. The lid and base each contain at least nine different subunits, with additional polypeptides, such as Rpn10, associated more loosely or only under specific conditions (Figure 1A) (Schmidt et al., 2005). The base includes six different AAA+ ATPase subunits, Rpt1–Rpt6, as well as the two largest subunits, Rpn1 and Rpn2. Recent structural analysis suggests that Rpn1 and Rpn2 form toroids, with Rpn1 stacked on Rpn2 and Rpn2 in direct contact with the CP α ring (Rosenzweig et al., 2008). The ATPases are thought to form a hexameric ring that encircles the Rpn1-Rpn2 stack and also contacts the α ring (Ferrell et al., 2000; Hartmann-Petersen et al., 2001).

Rpn10 and the base subunit Rpn13 are proteasomal polyubiquitin receptors (Husnjak et al., 2008). Additional proteins function as mobile receptors that bring polyubiquitin-modified substrates to the proteasome. In yeast, the related Rad23, Dsk2, and Ddi1 proteins use their ubiquitin-like domains to dock onto Rpn1 and their UBA domains to bind substrate polyubiquitin chains (reviewed in Hurley et al., 2006). There is considerable redundancy for substrate binding among these receptors, and genetic data indicate additional receptors must exist (Diaz-Martinez et al., 2006; Husnjak et al., 2008).

Although much is known about how the proteasome recognizes and degrades its substrates, investigations have just begun into how this highly abundant complex of ~2500 kD and at least 33 different subunits is assembled in the first place. Understanding proteasome assembly should provide general insight into strategies of multisubunit protein assembly in vivo. It will also be crucial for finding ways to inhibit assembly as part of emerging therapies that target the proteasome (Goldberg, 2007). Analysis of CP assembly is far more advanced than for the RP (Marques et al., 2009; Murata et al., 2009). Eukaryotic CP assembly initiates with formation of an α ring followed by ordered addition of β subunits to the α ring heteroheptamer (Hirano et al., 2005; Li et al., 2007). Joining of two half-proteasomes triggers autocatalytic processing of active-subunit propeptides and CP maturation (Chen and Hochstrasser, 1996). Moreover, a least three phylogenetically conserved CP-specific assembly chaperones facilitate proteasome biogenesis, and one of these

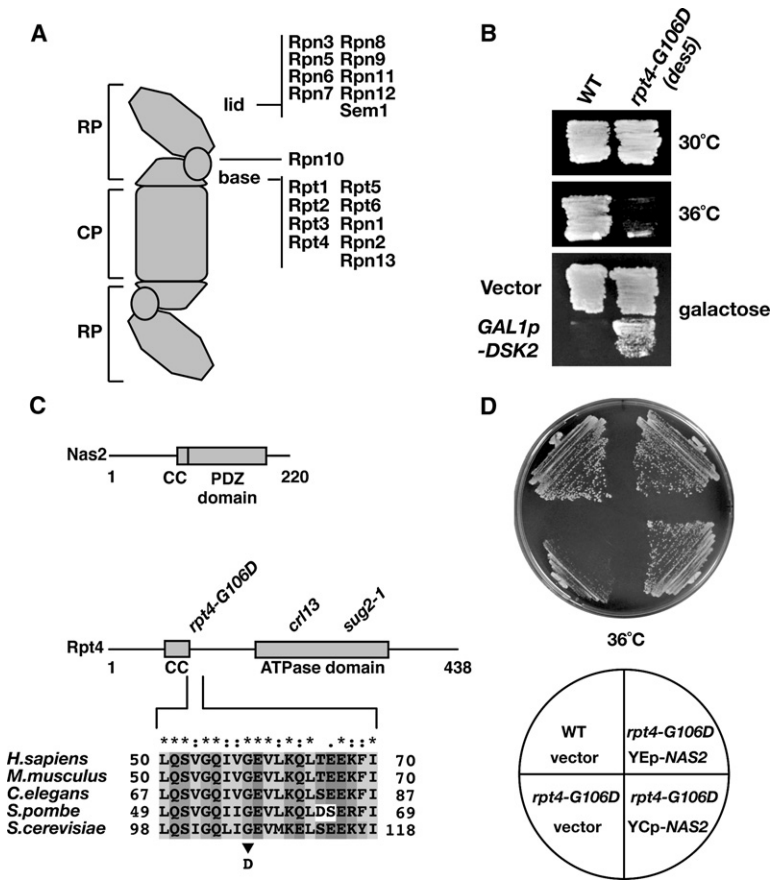

Figure 1. *NAS2* Is a Dosage Suppressor of the *rpt4-G106D* ATPase Mutant

(A) Illustration of the doubly capped form of the 26S proteasome. The composition of the yeast 19S regulatory particle (RP) base and lid subcomplexes is given.

(B) The *des5* mutation (*rpt4-G106D*) suppresses the lethality caused by galactose-dependent overexpression of the Dsk2 polyubiquitin-binding protein (bottom panel) but causes temperature-sensitive growth.

(C) Domain organization of the Nas2 and Rpt4 proteins and the position of the rpt4-G106D mutation (and two other tested mutations). CC, putative coiled coil. Rpt4-G106 is at the end of the CC.

(D) Low-copy (YCp-NAS2) and high-copy (YEp-NAS2) plasmids expressing *NAS2* suppress the temperature-sensitive growth of the *rpt4-G106D* mutant.

is known to control CP composition as well (Kusmierczyk et al., 2008).

Whether de novo proteasomal RP assembly also requires dedicated assembly chaperones has been uncertain (Heinemeyer et al., 2004). Recent evidence suggests that the CP can enhance RP base biogenesis in the cell and might therefore be an RP assembly factor (Kusmierczyk et al., 2008). Additional proteins are known to associate with the proteasome, but the functional significance of most of these associations is unclear (Guerrero et al., 2008). Here, we show that assembly of the RP base in yeast is orchestrated by at least four distinct assembly chaperones. One of these factors, Nas2, was identified as a dosage suppressor of an *rpt4* mutant. The remaining three, Hsm3, Nas6, and Rpn14, were identified biochemically in subcomplexes with specific base subunits. None of them associates detectably with the mature 26S proteasome. Genetic and biochemical data suggest that Nas2 (orthologous to human p27/Bridge-1) and Hsm3 (human S5b) overlap more closely in function, as do Nas6 (human gankyrin) and Rpn14 (human PAAF1). These factors are conserved from yeast to human and were until now either unconnected to the proteasome or thought to be subunits or inhibitors of the proteasome. Shortly before submission of this paper, Le Tallec et al. (2009) also reported that Hsm3 has properties of an RP assembly factor. Our data unify this previously disconnected set of factors, which all function specifically in the assembly of the RP base and are not components of the mature 26S proteasome. The results lead to a model in which the RP base assembles from a set of discrete chaperone-associated base subunit complexes; once assembled, the base binds to the lid and all chaperones are released prior to or during RP-CP association.

RESULTS

Identification of *NAS2* as a Dosage Suppressor of *rpt4-G106D*

High levels of the Dsk2 polyubiquitin receptor are toxic to *S. cerevisiae* (Figure 1B, bottom), and a previous genetic screen identified a series of suppressors of this toxicity (Funakoshi et al., 2002, 2004). All of the characterized suppressors were found to be recessive proteasome mutants. One previously uncharacterized mutant, designated *des5*, was found to be temperature sensitive for growth, but multiple attempts to clone the affected gene failed, instead repeatedly yielding the unlinked *NAS2* gene (Figure 1C). We therefore identified the gene mutated in the *des5* strain by chromosomal mapping and linkage analysis (see the Supplemental Data). The *des5* strain had a mutation in *RPT4*, which encodes one of the six ATPases of the proteasomal RP (Figure 1A). The mutant allele encodes a protein with a single amino acid change, G106D, mapping to a conserved region of Rpt4 at the end of a predicted coiled coil (CC) (Figure 1C). Nas2 is also predicted to have a coiled-coil domain. Both low-copy and high-copy plasmids bearing *NAS2* were strong suppressors of the *rpt4-G106D* mutant (Figure 1D).

Previous analysis of *nas2Δ* mutants had not uncovered any abnormalities (Watanabe et al., 1998; Russell et al., 1999). Nas2 had been assessed for a potential role in proteasome function because of its ~35% sequence identity to mammalian p27/Bridge-1, which is a component of the "modulator" complex that also contains the Rpt4 and Rpt5 proteasomal ATPases (DeMartino et al., 1996; Watanabe et al., 1998). Addition of purified mammalian modulator to purified RP and CP complexes leads to enhanced association of RP complexes with the CP (Adams et al., 1998). The mechanism of how this enhanced 26S formation occurs through the modulator has remained obscure.