

Blm10 binds to pre-activated proteasome core particles with open gate conformation

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Blm10, a crucial protease of eukaryotic cells, is bound to yeast proteasome core particles (CPs). Two gates, at both ends of the CP, control the access of protein substrates to the catalytic cavity of the CP. Normally, substrate access is auto-inhibited by a closed gate conformation unless regulatory complexes are bound to the CP and translocate protein substrates in an ATP-dependent manner. Here, we provide evidence that Blm10 recognizes pre-activated open gate CPs, which are assumed to exist in an equilibrium with inactive closed gate CP. Consequently, single-capped Blm10-CP shows peptide hydrolysis activity. Under conditions of disturbed CP assembly, as well as in open gate mutants, pre-activated CP or constitutively active CP, respectively, prevail. Then, Blm10 sequesters disordered and open gate CP by forming double-capped Blm10₂-CP in which peptide hydrolysis activity is repressed. We conclude that Blm10 distinguishes between gate conformations and regulates the activation of CP.

Keywords: Blm10; proteasome; open gate; quality control

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INTRODUCTION

In eukaryotic cells, proteasomes are the principal proteolytic machineries for regulated protein degradation (Wolf, 2000). The proteasome consists of a barrel-shaped 20S core particle (CP) and one adjacent 19S regulatory particle (RP), representing the RP-CP (26S) configuration. The CP with two adjacent RPs constitutes the RP-CP-RP (30S) configuration (Baumeister *et al*, 1998).

The assembly process of this multisubunit protease is coordinated at various levels and involves the regulation of proteasomal gene expression, which is required to supply around 33 subunits in stoichiometric amounts. In yeast, the transcription of proteasomal genes is induced by Rpn4, which, in turn, is degraded by

proteasomal proteolysis, thus allowing a negative feedback circuit on proteasomal gene expression (Xie & Varshavsky, 2001).

The basic concept of CP assembly is known. Seven distinct α - and β -subunits are arranged in a stack of four rings, resulting in an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ -configured CP. Consistent with the dyad symmetry, a CP is assembled from two $\alpha_{1-7}\beta_{1-7}$ -configured half-CPs (Chen & Hochstrasser, 1996). In yeast, the most prominent CP assembly intermediate resembles a half-CP devoid of $\beta 7$ (Lehmann *et al*, 2002; Li *et al*, 2007).

Half-CPs are proteolytically inactive as the β -subunits are not yet matured. The apposition of two half-CPs is driven by propeptides of β -subunits and the final incorporation of $\beta 7$. Buried inside the pre-holo-CP (nascent CP), the active sites are formed by β -propeptide processing (Ramos *et al*, 2004; Li *et al*, 2007).

The mechanisms that control the quality of proteasome assembly are less well understood. Efficient maturation of CP requires accessory chaperones, of which Ump1 has been studied the most. Ump1 facilitates half-CP assembly and functions as a checkpoint on the formation of pre-holo-CP (Ramos *et al*, 1998; Li *et al*, 2007). Additional chaperones, namely Pac1–Pac2 and Pac3–Pac4 heterodimers (alternatively named Pba or Poc), assist in α -ring assembly and act upstream from Ump1 (Le Tallec *et al*, 2007; Kusmierczyk *et al*, 2008).

Access to the catalytic cavity of the CP is controlled by axial channels within both outer α -rings. In particular, interactions between distinct amino-terminal residues of $\alpha 3$ and $\alpha 4$ prevent the entry of substrates by imposing closure on the CP (Groll *et al*, 2000). Thus, CP activity is inhibited or latent under physiological conditions. *In vitro*, latent CP activity is de-repressed by detergent and mild chemical treatments (Wilk & Orłowski, 1983), which influence the equilibrium between the open and closed CP status.

In vivo, activators such as the ATPases of the RP open the axial channels into the CP for the translocation of protein substrates (Rechsteiner & Hill, 2005). Apart from RP, few regulatory proteins are correctly positioned to open the CP channels. The newest member of these regulatory proteins, known as PA200 in mammals and Blm10 in *Saccharomyces cerevisiae* (originally named Blm3), is a nuclear protein with high molecular mass (Ustrell *et al*, 2002). The exact function of PA200/Blm10 has been controversial. Blm10 was found predominantly in Blm10-CP-RP hybrids, a proteasome configuration of unknown function (Schmidt *et al*, 2005). Cryo-electron microscopy showed that

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Fig 1 | Pre-activated core particles are stabilized by Blm10 in *ump1Δ* cells. (A) Affinity-purified wild-type proteasomes (Lehmann *et al*, 2002) contain Blm10 and Ecm29, as identified by finger print mass spectroscopy; RP and CP subunits are embraced. (B–E) GFP-labelled CPs of isogenic wild-type (wt; lane 1), *blm10Δ* (lane 2), *ump1Δ* (lane 3) and *blm10Δ ump1Δ* cells (lane 4) were resolved by native PAGE; CP configurations are assigned. (B) CP distributions were visualized by GFP imaging. (C) Blm10-associated CPs were detected by Western blot. (D) By using Suc-LLVY-AMC (Y) as a peptide substrate, in-gel CP activity was detected in the absence and (E) in the presence of 0.02% SDS. The lower part of (D) is shown with high contrast. (F) Blm10 is neither associated with half-CP (Fehlker *et al*, 2003) nor completely recruited to CP. To visualize half-CP by GFP imaging, the contrast below the dotted line is increased. Blm10-associated CPs are detected by immunoblot. (G) GFP-labelled CP were affinity-purified from wild-type (lane 1), *ump1Δ* (lane 2), *blm10Δ* (lane 3) and *blm10Δ ump1Δ* (lane 4) cells, resolved by native PAGE, visualized by GFP imaging, probed for Blm10, and assayed for Y-activity in the absence and presence of SDS. Owing to the strong stimulation of CP activity by 0.02% SDS, the exposure time of the in-gel activity assays had to be reduced. The histogram shows arbitrary AMC/GFP ratios of Blm10₂-CP, Blm10-CP and CP. (H) Blm10₂-CP, Blm10-CP and CP of *ump1Δ* cells were extracted from the native gel, subjected to SDS-PAGE and analysed by Western blot as indicated; lysate acted as a control. Pac1 to Pac4 were not identified in mature CP (data not shown). AMC, 7-amino-4-methyl-coumarin; CP, core particle; GFP, green fluorescent protein; RP, regulatory particle; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

Blm10 binds to both CP gates by forming Blm10-CP-Blm10, abbreviated to Blm10₂-CP. However, the absence of large openings in the surface of Blm10 raised the question of how peptides enter or exit the CP cavity once Blm10 is bound to the α -ring (Iwanczyk *et al*, 2006). As neither PA200 nor Blm10 promote the degradation of selected protein substrates, PA200 and Blm10 were thought to antagonize protein degradation (Ustrell *et al*, 2002; Fehlker *et al*, 2003). Intriguingly, enhanced hydrolysis of small chromogenic peptides was observed for single-capped PA200-CP and Blm10-CP (Ustrell *et al*, 2002; Schmidt *et al*, 2005). Blm10 is also involved in late steps of CP maturation and stabilizes pre-holo-CP (Fehlker *et al*, 2003; Marques *et al*, 2007).

Here, we found that Blm10 distinguishes between the CP gate conformations, and prefers binding of pre-activated CPs with disordered and open gate conformations. Compared with free latent CPs, we confirmed that peptide hydrolysis of Blm10-CP is activated. However, binding of Blm10 to both CP gates, as observed in mutants with constitutively active CPs, repressed peptide hydrolysis.

RESULTS AND DISCUSSION

Pre-activated CPs exist in *blm10Δ* cells

Blm10 is a 240-kDa protein found in substoichiometric amounts in proteasome complexes (Fig 1A). To facilitate the identification of Blm10-associated CP, we used green fluorescent protein (GFP)-tagged β 5-subunits as live reporter proteins of CP populations (abbreviated to GFP-labelled CP; supplementary information online). One reliable method to resolve proteasome populations as closely as possible to the physiological environment is native polyacrylamide gel electrophoresis (PAGE) of total cell extracts. Extracts of wild-type cells, which express GFP-labelled CP, were separated by native PAGE. GFP imaging showed six different CP populations (Fig 1B, lane 1): RP-CP-RP, Blm10-CP-RP, RP-CP, Blm10₂-CP, Blm10-CP and free CP (Fig 1C, lane 1). In-gel activity assays using the chromogenic peptide Suc-LLVY-AMC visualized the activation of CP by RP. The chosen experimental conditions mimicked the physiological environment, as free CPs were preserved as latent enzymes (Fig 1D, lane 1). Compared with free latent CP, peptide hydrolysis of Blm10-CP was activated but to a much lesser extent than RP-activated CP; however, peptide hydrolysis of Blm10₂-CP was hardly detectable (Fig 1D, lane 1). Without GFP imaging and Blm10-specific antibodies, Blm10₂-CP would have been easily missed in the absence of detergent.

As a control, the addition of detergent stimulated CP activity. Under these conditions, Blm10₂-CP, Blm10-CP and CP showed

activities proportional to the GFP levels (compare Fig 1E,B, lane 1).

In *blm10Δ* cells, only RP-CP-RP, RP-CP and free CP are present. However, compared with wild-type cells, the CP of *blm10Δ* cells showed some cleavage of chromogenic peptides, which argues for a mixture of latent and pre-activated CPs (Fig 1D, compare lanes 1 and 2).

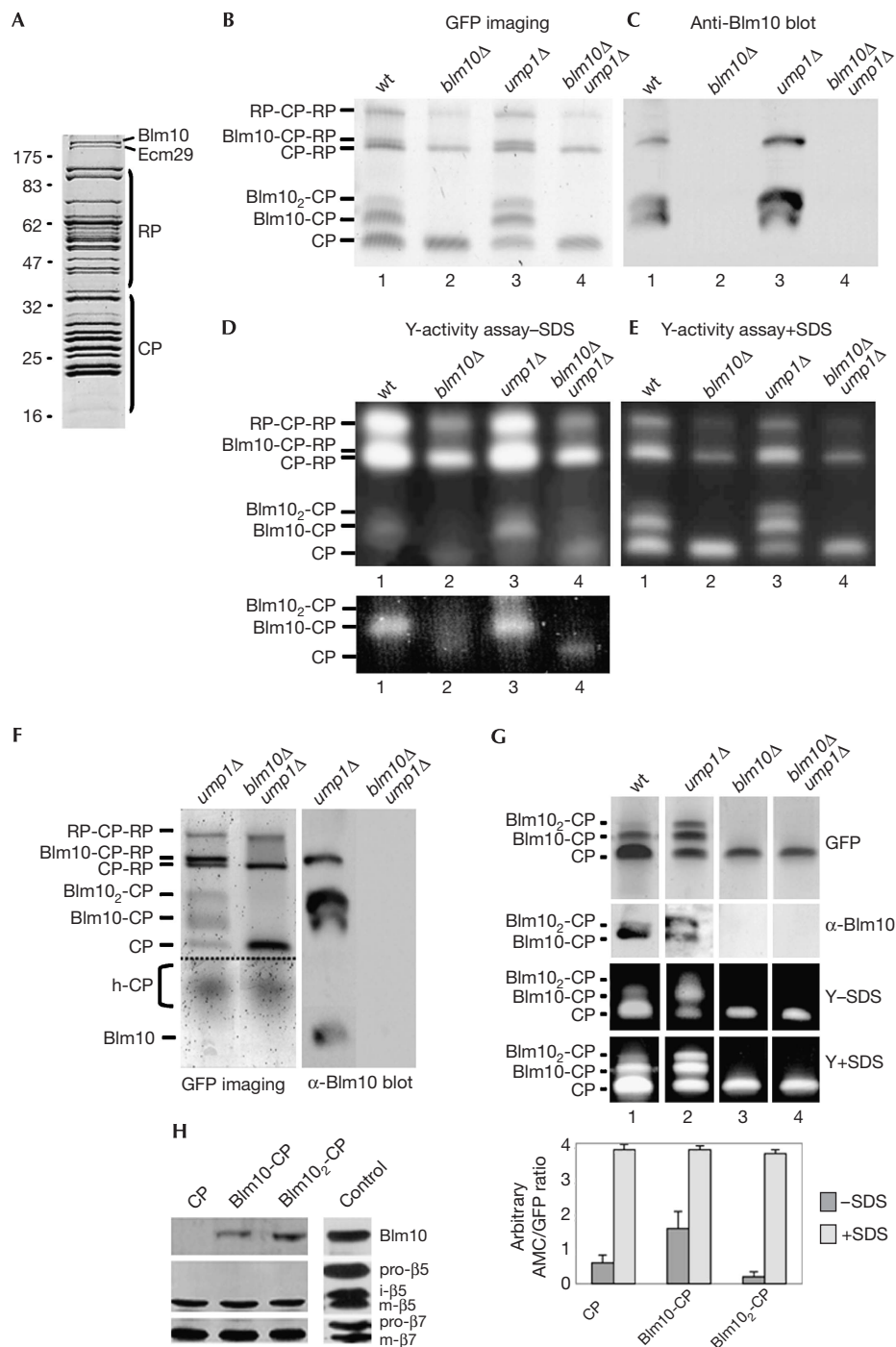
Pre-activated CPs are stabilized by Blm10 in *ump1Δ*

In search of conditions for Blm10 requirement, we screened a set of deletion mutants for induced Blm10 expression. A significant upregulation of Blm10 expression was detected in *ump1Δ* cells, an established mutant with defective CP maturation (Ramos *et al*, 1998). Both direct fluorescence microscopy and Western blot analysis confirmed that Blm10 expression is induced in cells lacking Ump1 (supplementary Fig S1A,B online). To compensate for compromised CP maturation in *ump1Δ*, proteasomal gene expression is upregulated owing to the stabilization of Rpn4 (London *et al*, 2004). Similarly, elevated levels of Rpn4 probably account for induced Blm10 expression in *ump1Δ* cells (supplementary Fig S1C online).

To analyse CP populations in *ump1Δ* cell extracts, we separated GFP-labelled CP by native PAGE. Compared with wild type, increased amounts of Blm10₂-CP and Blm10-CP-RP hybrids were observed (Fig 1B,C, compare lanes 1 and 3). Again, in the absence of detergents, hydrolysis of chromogenic peptides was high for RP-associated CP and lower for Blm10-CP (Fig 1D, lane 3). The activity of Blm10₂-CP and CP was observed when stimulating detergent was present (Fig 1E, lane 3). Despite increased levels of Blm10, it is important to note that Blm10 was not completely recruited to CP, suggesting that only a specific CP fraction was bound to Blm10 (Fig 1F).

As a control, RP-CP-RP, RP-CP and free CP were analysed in *blm10Δ ump1Δ* cells (Fig 1B, lane 4). More pre-activated CPs were detected in *blm10Δ ump1Δ* compared with *blm10Δ* cells, suggesting that pre-activated CPs accumulate under conditions of disordered CP assembly (Fig 1D, compare lanes 2 and 4).

In addition to cell extracts, affinity-purified GFP-labelled CPs from wild-type, *ump1Δ*, *blm10Δ* and *blm10Δ ump1Δ* cells were analysed for in-gel activities. In *ump1Δ* cells, Blm10-CP and Blm10₂-CP are the predominant CP configurations, whereas free CPs prevail in wild-type cells (Fig 1G; note that Blm10-CP includes decay products of Blm10-CP-RP hybrids). As shown for *ump1Δ* cells, enhanced peptide hydrolysis of Blm10-CP was



detected, whereas the activity of Blm10₂-CP was reduced. CP is hardly purified as a latent enzyme; its basal activity is due to chemical activation during purification. Nevertheless, peptide hydrolysis of purified CP was stimulated by detergent, as described previously (Groll *et al*, 2000).

Blm10-associated CPs are fully matured

In view of our initial identification of Blm10 in pre-holo-CP (Fehlker *et al*, 2003), we investigated further whether Blm10₂-CP

contains remnants of pre-holo-CP, which could explain the reduced peptide cleavage activity in Blm10₂-CP. For this purpose, the Blm10₂-CP, Blm10-CP and CP bands of *ump1Δ* cells were excised from the native gel, subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and probed for Blm10, β5 and β7. All β5 and β7 subunits were found to be matured independently of Blm10 and despite the lack of Ump1. Thus, inhibition of CP in Blm10₂-CP could not be explained by incomplete CP maturation (Fig 1H). The observation that peptide hydrolysis is impaired in

Blm10₂-CP agreed with our earlier finding that an excess of purified endogenous Blm10 inhibits CP activity against Suc-LLVY-AMC in solution (Fehlker *et al*, 2003). Consistent with our observations in purified samples and cell extracts, in-gel activity assays using Suc-LLVY-AMC also showed increased peptide hydrolysis of reconstituted Blm10-CP and decreased peptide hydrolysis of reconstituted Blm10₂-CP, respectively (supplementary Fig S2A online). However, as described for the mammalian counterpart PA200, the *in vitro* effect of Blm10 on CP activity varied with the peptide substrate, indicating that the chemical nature of the peptide substrate influences the activity assay (Ustrell *et al*, 2002; Fehlker *et al*, 2003).

Taken together, Blm10 binding affects the peptide cleavage activity of CP depending on the Blm10:CP stoichiometry: activation in the case of Blm10-CP and inhibition in the case of Blm10₂-CP. One possibility to solve this paradox is that Blm10 prefers binding to open gate CPs, which are in an equilibrium with closed gate CPs. Despite sufficient amounts of Blm10, only a few CPs are bound to Blm10 in wild-type cells, which is consistent with the assumption that the equilibrium between the open and closed states of the CP is shifted towards the closed state. *In vitro*, CPs are opened and thus pre-activated during purification, which facilitates the reconstitution of Blm10-CP and Blm10₂-CP (supplementary Fig S2A online). Under these conditions, as well as on Blm10 overexpression, the equilibrium between the closed and open gate states of CP is shifted towards the open gate state, which is sustained by binding of Blm10 (supplementary Fig S2B online).

To control access into the proteolytic chamber of the CP, the appropriate closure of the axial channels is required at the β -propeptide processing stage (Groll *et al*, 2000). At this juncture, Blm10 joins the pre-holo-CP (Fehlker *et al*, 2003; Li *et al*, 2007; Marques *et al*, 2007), suggesting that Blm10 distinguishes between gate conformations already at the last step of CP maturation. The lack of Ump1 might induce a build-up of disordered gates and hence pre-activated CP, which promotes the interaction with Blm10. If Blm10 checks the status of the gate during the last step of CP maturation and releases those with a closed gate, it is also possible that Blm10 has a higher affinity for open gate CP, as known from other regulatory proteins that exert their functions by stabilizing certain conformations of the binding partner.

Constitutively active CPs are capped by Blm10 or RP

If Blm10 prefers binding to pre-activated CPs, constitutively active CPs should be sequestered by Blm10 because the energetic costs for gate opening are saved. To test this, we analysed the distribution of GFP-labelled CP in cell extracts of α 3 Δ N, α 7 Δ N and α 3 Δ N/ α 7 Δ N mutants with disordered or open gate CPs (Groll *et al*, 2000). Strikingly, CPs of α 3 Δ N, α 7 Δ N and α 3 Δ N/ α 7 Δ N mutants were found primarily as Blm10₂-CP, Blm10-CP-RP and RP-CP-RP (Fig 2A,B, lanes 2–4; note the shift from RP-CP to Blm10-CP-RP) showing that open gate CPs are occupied either by Blm10 or by RP. Uncapped α 3 Δ N CP, which is beyond the detection limit of GFP imaging, showed peptide cleavage activity as there is no hindrance for the entry of substrate into the CP (Groll *et al*, 2000; Fig 2C,D, lane 2). The activity of α 3 Δ N/ α 7 Δ N Blm10₂-CP seemed to be repressed under these conditions (Fig 2D, compare lanes 1 and 4). Both α -rings of α 3 Δ N/ α 7 Δ N CP are bound to Blm10, resulting in blocked entrance for peptide substrates or product release. The inhibition of CP

activity in α 3 Δ N/ α 7 Δ N Blm10₂-CP was not caused by defective CP maturation and comparable amounts of proteasomal subunits and Blm10 were detected in wild-type and open gate mutants (Fig 2E).

As the interaction between N-terminal residues of α 3 and α 4 imposes gate closure on CP, we also tested the open gate mutant α 4 Δ N. Consistent with our observations above, both open α -rings of the α 4 Δ N CP are capped either by Blm10 or by RP. Again, Blm10 is an abundant protein and only a few CPs are bound to Blm10 in wild-type cells. In α 4 Δ N mutants, all open gate CPs occurred as Blm10₂-CP but not as free enzymes, providing further evidence that Blm10 is responsible for the sequestration of open gate CP (Fig 2F).

Furthermore, we were interested as to whether the heterodimeric chaperones Pac1–Pac2 and Pac3–Pac4, which assist in α -ring assembly, interfere with the function of Blm10. In particular, Pac3–Pac4 coordinate the incorporation of α 3 and α 4, which are mainly responsible for α -ring closure. Although the loss of Pac1–Pac2 has mild effects on CP assembly, the loss of Pac3–Pac4 leads to alternatively configured CP with disordered gate conformations (Kusmierczyk *et al*, 2008). To test whether CPs with altered α -ring configurations are also recruited by Blm10, CP populations of *pac1 Δ pac2 Δ* and *pac3 Δ pac4 Δ* cell lysates were analysed (Fig 2G). As expected, *pac1 Δ pac2 Δ* cells showed CP distributions similar to wild-type cells. Again, Blm10-CP was active, whereas free CP and Blm10₂-CP hardly hydrolysed chromogenic peptides (for histogram, see supplementary Fig S3 online). In contrast to *pac1 Δ pac2 Δ* cells, free CP, Blm10-CP and RP-CP nearly disappeared in *pac3 Δ pac4 Δ* cells. Instead, CPs were found primarily in RP-CP-RP, Blm10-CP-RP and Blm10₂-CP, as observed above for open gate mutants with constitutively active CP.

RP can substitute for Blm10 in CP binding

To determine the specificity of Blm10 for open gate CP, we analysed whether the deletion of *BLM10* is tolerated in our set of open gate mutants. Tetrad analysis yielded viable mutants. As exemplified for α 4 Δ N *blm10 Δ* and α 3 Δ N/ α 7 Δ N *blm10 Δ* cells, respectively, most CPs occur in RP-CP-RP configuration, indicating that sufficient RPs are available to substitute for Blm10 (Fig 2H). Recruitment of RP to CP in α 3 Δ N/ α 7 Δ N *blm10 Δ* cells (Fig 2I) supports the observation that RP can substitute for Blm10 during CP maturation (Marques *et al*, 2007). However, recent cryo-electron microscopy analysis of RP-CP structures shows that in contrast to PA200/Blm10, RP induces a radial movement in the α -ring, leading to a wider and more prominent channel into the proteolytic chamber of the CP, which is required for the translocation of protein substrates (Fonseca & Morris, 2008).

Speculation

On the basis of our findings, we propose the following model for the function of Blm10 (Fig 3). Two half-CP precursors assemble into pre-holo-CP, which triggers the processing of β -propeptide and the degradation of Ump1 (marked by dotted red ellipses). The final maturation yields CP with closed gate conformation and latent enzyme activity, as the axial channels are sealed by N-terminal regions of α -subunits (marked by filled red ellipses). RP opens CP by forming active RP-CP and RP-CP-RP. Blm10 distinguishes between the CP gate conformations in pre-holo-CP, the last intermediate of CP maturation. The energetic costs of gate

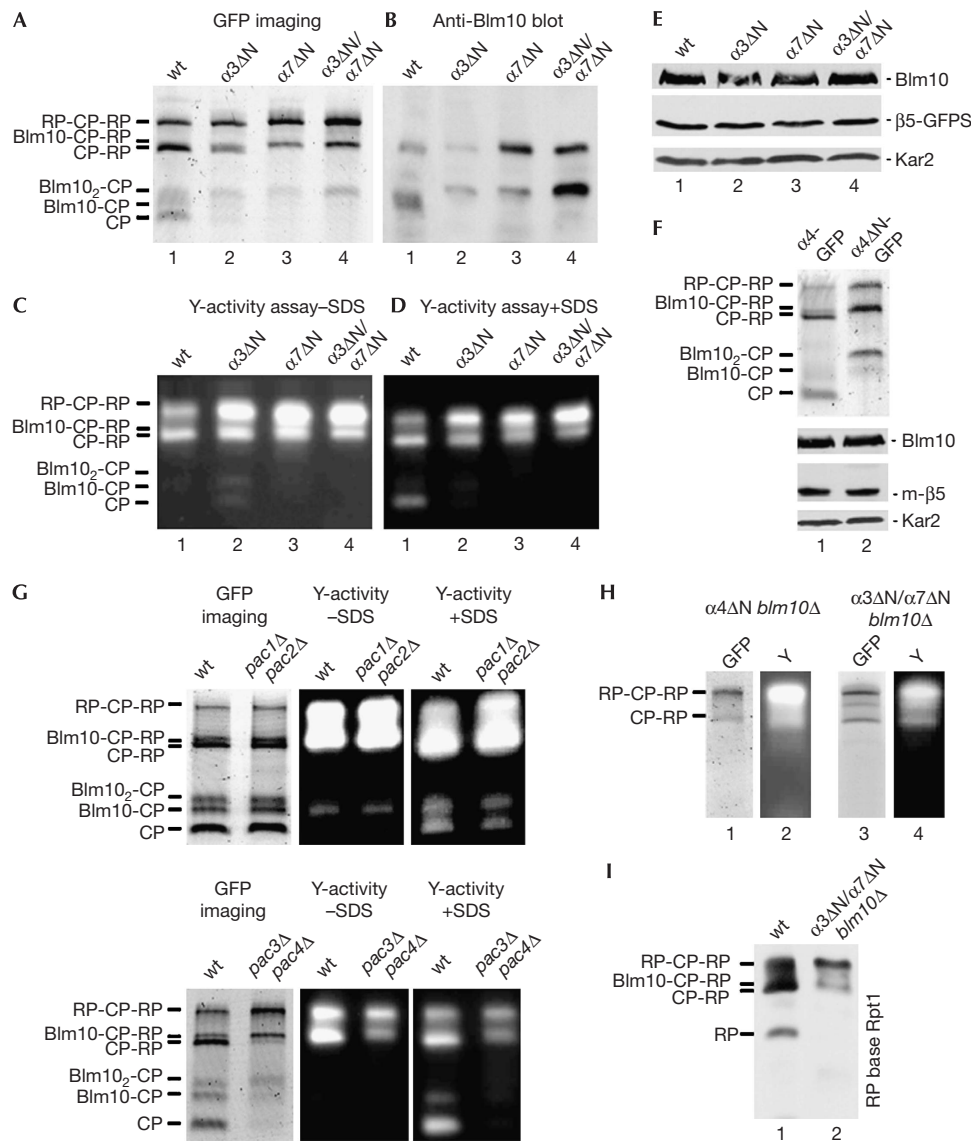


Fig 2 | Constitutively active core particles are capped by Blm10 or RP. (A) Extracts of wild-type (wt; lane 1), $\alpha 3\Delta N$ (lane 2), $\alpha 7\Delta N$ (lane 3) and $\alpha 3\Delta N/\alpha 7\Delta N$ (lane 4) cells were analysed by native PAGE and GFP imaging. Equal amounts of protein were loaded. (B) Blm10-bound CPs were identified by Western blot. (C) In-gel assays for Y-activity were performed in the absence of SDS or (D) in the presence of SDS. (E) Blm10 and $\beta 5$ levels of wild type (lane 1), $\alpha 3\Delta N$ (lane 2), $\alpha 7\Delta N$ (lane 3) and $\alpha 3\Delta N/\alpha 7\Delta N$ (lane 4) were determined by Western blot. (F) Extracts of wild-type cells (lane 1) and $\alpha 4\Delta N$ mutants (lane 2) expressing GFP-tagged $\alpha 4$ were analysed by native PAGE and GFP imaging. Blm10 and $\beta 5$ levels were determined by Western blot. (G) Extracts of *pac1* Δ *pac2* Δ (upper panels), *pac3* Δ *pac4* Δ (lower panels) and isogenic wild-type cells, respectively, were analysed by native PAGE and GFP imaging. Y-activity assays were performed in the absence and presence of SDS. To visualize Blm10-CP activity, the upper in-gel activity assay was overexposed. Equal amounts of protein were loaded. (H) Native PAGE of $\alpha 4\Delta N$ *blm10* Δ cell extracts (GFP-tagged $\alpha 4$) were analysed by GFP imaging (lane 1) and Y-activity assay (lane 2). Native PAGE of $\alpha 3\Delta N/\alpha 7\Delta N$ *blm10* Δ cell extracts (GFP-tagged $\beta 5$) were analysed by GFP imaging (lane 3) and Y-activity assay (lane 4). (I) Extracts from wild-type (lane 1) and $\alpha 3\Delta N/\alpha 7\Delta N$ *blm10* Δ (lane 2) cells were subjected to native PAGE, blotted and probed for Rpt1 (RP base). CP, core particle; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; RP, regulatory particle.

opening limit the formation and stability of Blm10-bound CP. Thus, Blm10 preferentially binds to open gate CP and sustains the activated state once it is bound to the α -ring. Binding of Blm10 to the *cis* gate might promote the opening of the *trans* gate. Thus, enhanced peptide hydrolysis of Blm10-CP might owe to the *trans* gate effect; in Blm10₂-CP, CP activity is repressed. Finally,

Blm10-CP-RP hybrids might account for slower protein degradation, as substrate translocation or product release is regulated by Blm10.

METHODS

Yeast strains and plasmids are described in the supplementary information online.

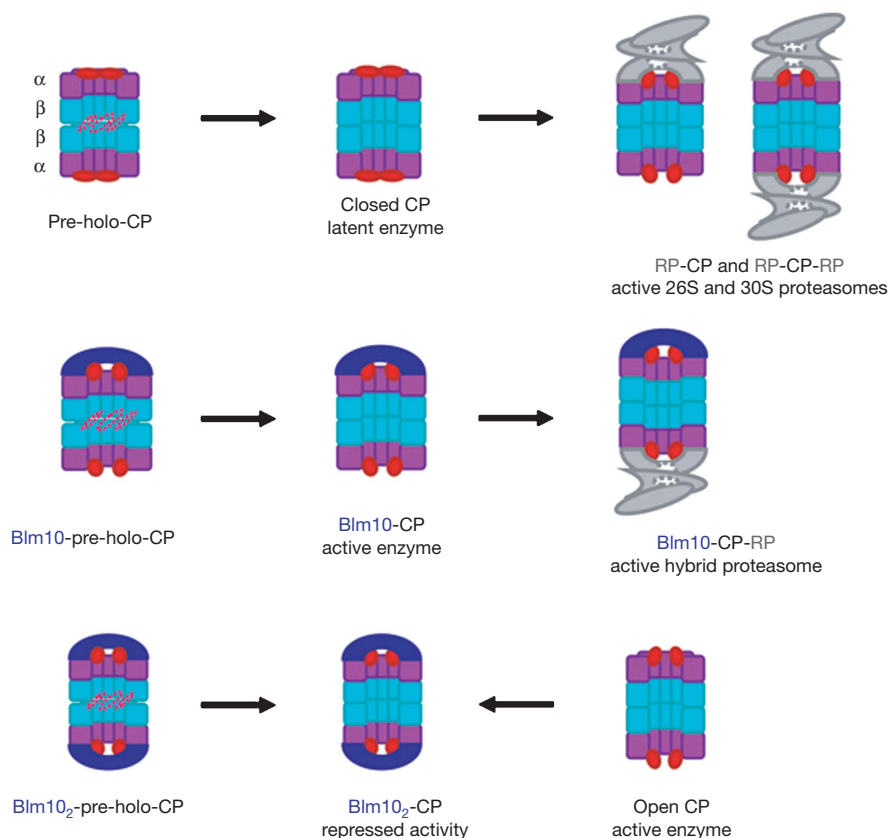


Fig 3 | Model of Blm10 function. For a description of the model, see the Speculation section. CP, core particle; RP, regulatory particle.

Protein chemistry. Yeast cells were grown in rich medium (YPD) to logarithmic phase ($OD_{600} = 1$). Affinity purifications under native conditions were performed using Strep-Tactin matrices (IBA, Göttingen, Germany). Cells were disintegrated by French press in two cell volumes of buffer W (100 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA, pH 8). Affinity-bound CPs were eluted by 5 mM desthiobiotin in buffer W. To analyse CP distributions in total cell lysates, cells were disintegrated by glass beads at 4 °C in two cell volumes of buffer TB (20 mM HEPES/KOH pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol and 2 mM ATP). The lysates were cleared by centrifugation and immediately subjected to native PAGE (3.5–6.0% polyacrylamide). Blm10 was purified according to Fehlker *et al* (2003).

Blm10-specific antibodies were raised in rabbit against N-terminal amino-acid residues 1–157 (Pineda, Berlin, Germany). In-gel activity assays were performed for 30 min at 37 °C using 100 μ M substrate Suc-LLVY-AMC in 20 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$ and 100 mM KCl. The released chromophores were visualized by transillumination using the short wave band pass of the Syngene G Box equipped with Gene Snap software (Synoptics, Cambridge, UK).

Immunodetection of endogenous or tagged versions of Ump1, $\beta 5$ (Pre2), Rpn2, Rpn11 and Kar2 have been described previously (Fehlker *et al*, 2003).

GFP monitoring. Direct fluorescence microscopy of living yeast cells was performed with a Leica DMR microscope. Digital images were taken with a charge-coupled device camera

(C5985-10; Hamamatsu, Herrsching, Germany) and processed in Adobe Photoshop. Pixel intensities were quantified by MetaVue software/Visitron System (Wendler *et al*, 2004). GFP fluorescence of CP separated by native PAGE was visualized by the BASreader and the Advanced Image Data Analysis (raytest; Straubenhardt, Germany) installed on a Phospho-Fluoro-Imager FLA3000 (Fuji-film, Tokyo, Japan). The fluorescent imager uses a solid-state laser SHG (second harmonic generation light source) with an excitation wavelength of 473 nm and a light recipient filter of 520 nm.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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