Molecular chaperones of the Hsp70 family assist in the assembly of 20S proteasomes

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Abstract

The eukaryotic 26S proteasome is a large protease comprised of two major sub assemblies, the 20S proteasome, or core particle (CP), and the 19S regulatory particle (RP). Assembly of the CP and RP is assisted by an expanding list of dedicated assembly factors. For the CP, this includes Ump1 and the heterodimeric Pba1–Pba2 and Pba3–Pba4 proteins. It is not known how many additional proteins that assist in proteasome biogenesis remain to be discovered. Here, we demonstrate that two members of the Hsp70 family in yeast, Ssa1 and Ssa2, play a direct role in CP assembly. Ssa1 and Ssa2 interact genetically and physically with proteasomal components. Specifically, they associate tightly with known CP assembly intermediates, but not with fully assembled CP, through an extensive purification protocol. And, in yeast lacking both Ssa1 and Ssa2, specific defects in CP assembly are observed.

Key words

Proteasome; protein assembly; molecular chaperone; yeast; heat shock protein; Hsp70

Abbreviations

CP, core particle; RP, regulatory particle; UPS, ubiquitin-proteasome system; MCN, molecular chaperone network; ICAR, immobilized-cobalt affinity resin.

1. Introduction

Protein quality control in eukaryotes is maintained via several evolutionarily conserved machineries, including the ubiquitin-proteasome system (UPS) and the molecular chaperone network (MCN) [1,2,3]. The former provides a major route of protein degradation, removing damaged, misfolded, and no-longer-needed proteins. The latter assists in proteins attaining their native state following synthesis, and in returning misfolded proteins to the native state following stress. How the UPS and MCN interact is a topic of considerable interest.

The MCN is comprised of a number of highly conserved protein families, many of which are nucleotide-driven machines. Perhaps the best studied of these include members of the Hsp60, Hsp70, and Hsp90 protein families [3]. One way in which the MCN interacts with the UPS is via proteins destined for degradation [4]. However, MCN proteins could also function in the biogenesis of the UPS components themselves, such as the 2.5 MDa proteasome, yet this role remains underexplored.

The 26S proteasome is an intricate assembly of 33 distinct proteins in multiple copies [2]. It can be subdivided into two major sub-assemblies, the 19S regulatory particle (RP) and the 20S core particle (CP), also known as the 20S proteasome. The CP consists of four heptameric rings stacked on top of each other. Seven distinct α subunits (α1 to α7) make up each outermost ring and seven distinct β subunits (β1 to β7) make up the two inner rings [5]. Proteolytic activity resides in the β1, β2 and β5 subunits which are synthesized as proproteins [6,7]. CP assembly begins with the formation of an α–ring which becomes a platform for the incorporation of β subunits [8,9]. Addition of β subunits occurs in stages defined by a number of intermediates: the 13S intermediate (which includes β2, β3, and β4); the 15S intermediate (which includes all β subunits except β7); and the half-proteasome (which contains a completed β–ring) [9,10,11]. Two half-proteasomes dimerize to produce the preholoproteasome (PHP), a fleeting species that looks like a CP but whose β subunits retain their propeptides [10]. As the β

subunits undergo autocatalytic processing to reveal their N-terminal threonine nucleophiles, the PHP matures into the fully functional CP.

CP assembly is regulated by features intrinsic to the subunits themselves and by extrinsic factors in the form of dedicated assembly chaperones. The latter include Ump1, Pba1–Pba2, and Pba3–Pba4 (also known as hUmp1, PAC1–PAC2, PAC3–PAC4 in mammals) [2]. Whether any additional proteins participate in CP assembly remains an open question. Using the yeast *Saccharomyces cerevisiae* as a model system, we demonstrate that two members of the Hsp70 family, Ssa1 and Ssa2, are directly involved in CP biogenesis.

2. Materials and Methods

2.1 Strains, Plasmids, and Yeast Techniques

Yeast strains used are listed in Supplementary Table 1. Plasmids used are listed in Supplementary Table 2. Yeast manipulations were carried out according to established protocols [12]. Dilution series were carried out as described [13]. Yeast strain numbers (AKY) are shown in brackets.

2.2 Protein Purification

The lysis of yeast pellets and subsequent Flag purification was carried out as described [14] except equal amounts of soluble lysate were bound to 150 µl of anti-Flag agarose (Sigma) for 3 hours then eluted. For each sample, the 300 µl Flag eluate was divided as follows: 100 µl aliquot reserved for native PAGE; 50 µl aliquot reserved for SDS-PAGE; and the remaining Flag eluate (150 µl) was subjected to depletion via immobilized-cobalt affinity resin (ICAR). Depletion was carried out as described [14] with the following modifications. The samples were applied to 50 µl of cobalt resin (Talon resin; Clontech) for 1 hour at 4 °C with gentle rocking. The flow through from the first round of ICAR depletion was subjected to a second round of ICAR depletion using a fresh 50 µl aliquot of resin. The flow-through from the second round of ICAR

depletion was split into a 100 μl aliquot reserved for native PAGE and a 50 μl aliquot reserved for SDS-PAGE. The Flag eluate and the second ICAR flow through were analyzed via native PAGE (40 μl), and loading controls were analyzed by reducing SDS-PAGE (15 μl).

2.3 Electrophoresis and Blotting

SDS-PAGE, on 12% gels, and native PAGE, on 4–15% Mini-PROTEAN TGX precast gradient gels (Bio-Rad), were carried out as described except native gels were electrophoresed for 11 hours [14]. Native gels were stained with Imperial Protein Stain (ThermoScientific) and SDS-PAGE gels were stained with GelCode blue (ThermoScientific). Prior to staining, some native gels were subjected to substrate overlay assay carried out as described [15]. The migration of size standards is indicated to the left of each gel. Western blotting was carried out as described [14] using anti-Express antibodies (Invitrogen).

2.4 Proteomic Analysis

Gel slices were submitted to the Indiana University School of Medicine Proteomics Core Facility (IUSM-PCF) on a fee-for-service basis and protein contents identified by LC-MS/MS as described [16]. Summarized and annotated data is presented in the main text and supplementary figures; only proteins identified on the basis of more than one unique peptide are included to decrease the likelihood of false-positives [17]. Data from IUSM-PCF is also permanently archived here [18].

3. Results

Our initial interests lay in determining whether additional extrinsic factors exist that assist in proteasome biogenesis. We focused our search on the CP and employed a depletion scheme [14] to enrich for CP assembly intermediates. We generated a series of yeast strains: “WT”; “PHP”; and “2/3-PHP” (Supplementary Figure 1). The PHP strain is the key experimental strain and should allow the isolation of CP assembly intermediates up until, and including, the

preholoproteasome (PHP). The PHP strain expresses all three mutant β subunits β1(T1A), β (T1A), and β5(T1A), as well as a wild-type β5 to keep the strain alive [6]. The 2/3-PHP strain expresses two mutant β subunits, β1(T1A) and β2(T1A).

We prepared soluble lysates from cultures of all three strains, isolated CP species via the Flag epitope on β4, and analyzed the purified material by native and SDS-PAGE (Figures 1A,B lanes 1 to 3). We've reported the need to overload native gels to visualize assembly intermediates [14] and did so again here. CP was the major species in each sample, migrating near the 670 kDa size standard (Figure 1 and Supplementary Figure 2). Species migrating slower than CP were Blm10-bound CP [19]. More Blm10-bound species were present in the 2/3-PHP sample (Figures 1A,B asterisk). Species migrating faster than CP were likely assembly intermediates (Figure 1A, lanes 1 to 3, bands 1 and 2).

Next, the hexahistidine tag on the β5 subunit (β5-his) enabled a depletion strategy to remove CP via sequential passage of Flag-purified material over immobilized-cobalt affinity resin (ICAR) [14]. Aliquots of the second flow-through were analyzed by native and SDS-PAGE (Figures 1A,B lanes 4 to 6). A number of observations were made. First, in the WT and 2/3-PHP samples, the CP band was almost completely absent suggesting that the depletion strategy was effective. A small amount of CP remained, as judged by a faint residual CP band and weakly detectable activity on the overlay assay (Supplementary Figure 2). However, additional rounds of ICAR depletion did not reduce this residual CP band further and only decreased the overall yield of protein (not shown); we suggest that the residual CP may be due to some β5 subunits losing their his-tag to proteolysis post-lysis. Second, the pair of putative assembly intermediates (bands 1 and 2) was still present following depletion. Finally, the PHP sample retained a considerable band migrating at the position of the CP (band 4). Given that β5(T1A) was not his-tagged, this suggested we were able to isolate the PHP. A fainter band migrating below the putative PHP was also observed (band 3).

Bands 1 to 4 were excised and analyzed by LC-MS/MS (Supplementary Figures 3 to 10). Band 4 was actually a series of three closely spaced bands (Figure 1A, enlarged). It was not possible cut these bands apart, thus the analysis of band 4 reflects material from all three bands. Band 4 contained all α and β subunits, as well as the assembly factors Ump1 and Pba1–Pba2 (Table 1). We also recovered peptides from the β5 propeptide (not shown). This composition is consistent with band 4 containing PHP. Interestingly, band 4 exhibited some catalytic activity (Supplementary Figure 2) but the resolution was not sufficient to determine which of the three bands was active. Consequently, band 4 must also contain some species with wild-type β5. We offer a plausible explanation for the three bands in Supplementary Figure 11. Band 3 had identical composition to band 4 (Table 1), including peptides from the β5 propeptide (not shown), but it displayed no activity (Supplementary Figure 2). Band 3 likely represents a half-proteasome.

Band 2 migrated similarly across all three samples, as did band 1, suggesting two identical species were present. Indeed, band 2 from each sample returned considerable peptide spectral matches (PSMs) from all α subunits plus β2, β3, β4 and the assembly factors Ump1 and Pba1–Pba2 (Table 1). Some peptides for other β subunits were also recovered. However, these were either not uniformly present in all bands, or were present in considerably lower PSMs, suggesting they were not stoichiometric components of band 2 – which most likely contained the 13S intermediate. Band 1 was similar to band 2 except that we observed comparatively fewer PSMs for α5 and α6 in all three samples, and fewer PSMs for α7 in the 2/3-PHP and WT samples (Table 1). As above, this suggests that these subunits may not be stoichiometric components of the complex in band 1. Furthermore, we recovered no peptides for Pba2. We conclude that band 1 likely contains a species we refer to as “sub-13S” (i.e. a 13S intermediate lacking Pba2, α5, α6, and probably α7).

Each of the bands analyzed gave rise to peptides from a number of additional proteins (Supplementary Figures 3 to 10). Most of these were not likely candidates for assembly factors because they were recovered with only a few peptides, and/or were derived from highly abundant metabolic enzymes (e.g. Eno2, Pfk26, Adh1) which are likely contaminants. However, two proteins from the Hsp70 family, Ssa1 and Ssa2, emerged as possible candidates. We shall refer to these proteins as Ssa1/2 because they share 97% amino acid identity, making it difficult to definitively assign all observed peptides to either isoform. Ssa1/2 were identified with considerable PSMs in bands 1 and 2 from all three strains, but not in the more highly abundant band 4, arguing that they were not just binding to CP subunits non-specifically.

Physical association of Hsp70 proteins with proteasomes has been observed [20,21,22]. But these high-throughput studies only considered chaperone binding to intact proteasomes (see Supplementary Notes). To strengthen the case for a role in assembly, we first carried out a series of genetic experiments. When yeast were grown at 36º C (Figure 2), we observed synthetic sick interactions between deletion of *SSA1*, or *SSA2*, and proteasome mutants that affect assembly of CP (*pre9*Δ) and the lid subcomplex of the RP (*sem1*Δ or *rpn12-234*Δ) [13,23,24]. This was not due to a general decrease in cytoplasmic Hsp70 activity because no effects were observed when another Hsp70 gene, *SSB1*, was deleted (Supplementary Figure 12A). No obvious synthetic sick interaction was seen with a mutant affecting assembly of the base subcomplex of the RP (*nas2*Δ*nas6*Δ*rpn14*Δ) [25]. However, this mutant already grows quite weakly at 36º C (Figure 2A); repeating the experiment at 33 º C, we instead observed a slight suppression when *SSA1* was deleted (Supplementary Figure 12B).

Overexpression of Ssa1 suppressed growth defects of CP, but not RP, assembly mutants in the presence of the amino acid analog canavanine (Figure 3 and Supplementary Notes). Western blots verified that protein was expressed from the introduced overexpression plasmids (Supplementary Figure 12C and not shown). The pattern of suppression with

overexpression of Ssa1 differed from that observed with overexpression of Ssb1, a known suppressor of CP mutants [26]. Here, Ssb1 suppressed both CP and RP base defects. Taken together, the genetic results support the link between Ssa1/2 and CP assembly.

If Ssa1/2 proteins have a direct role in CP assembly, one should observe assembly defects biochemically if these proteins are absent. Since Ssa1 and Ssa2 likely have a redundant role in CP assembly (see Discussion), we looked for assembly defects in an *ssa1*Δ*ssa2*Δ strain. We purified proteasomes from wild-type and mutant strains, via a Flag-tag on α4, and analyzed them by native PAGE (Figure 4A). CP was again the major species and two faster migrating bands were observed in the WT sample; again, they were easier to visualize when the gel was overloaded (Figure 4B). We excised the indicated bands and analyzed them by LC-MS/MS. Because this experiment did not involve a depletion, bands 5 and 6 each likely contained a mixture of assembly intermediates [14]. Nevertheless, the relevant result is that considerable PSMs were observed for Ssa1/2 in bands 5 and 6 but none in bands 7 and 8, corresponding to fully assembled CP (Supplementary Figures 13 to 16). This is consistent with our previous results showing Ssa1/2 associating with assembly intermediates (Figure 1). Most importantly, bands 5 and 6 were greatly reduced in the *ssa1*Δ*ssa2*Δ mutant, as would be expected for an assembly defect.

We also observed what appeared to be more doubly-Blm10 capped CP in the *ssa1*Δ*ssa2*Δ mutant (Figure 4B asterisk). Blm10 plays an undefined role in CP maturation and increased binding of Blm10 to CP can indicate a maturation defect [11]. Consistent with this, we recovered peptides derived from the β2 propeptide from CP in the *ssa1*Δ*ssa2*Δ strain (band 8) but not CP in the wild-type strain (band 7). The lack of β2 propeptide-derived peptides in band 7 was not due to a detection sensitivity issue; we readily recovered β2 propeptide-derived peptides from bands 5 and 6 (as expected for CP assembly intermediates) despite these two bands being much less abundant than band 7 (Supplementary Figure 17).

Finally, the Ssa1/2 band observed by SDS-PAGE in the purified material from the wild-type strain (Figure 4A) was replaced by a slightly slower migrating doublet in the *ssa1*Δ*ssa2*Δ mutant. LC-MS/MS analysis identified this doublet as containing Ssa3, Ssa4 and Kar2 (not shown but see Supplementary Notes).

4. Discussion

We present evidence that Ssa1/2 proteins function as CP assembly factors. First, these chaperones associate with assembly intermediates in purifications from four independent yeast strains utilizing two different Flag-tagged CP subunits (Table 1 and Figure 4B). Depletion analysis confirms association with the 13S intermediate, and a novel species we refer to as sub-13S, though Ssa1/2 likely associate with intermediates up to the half-proteasome (Supplementary Figures 3–5, 13, 14). Second, the physical association of Ssa1/2 with CP intermediates is corroborated by genetic association between *ssa* mutants and proteasome assembly mutants. Moreover, phenotypic suppression often indicates physical interaction, and the suppression by *SSA1* of CP (but not RP) assembly mutants is entirely consistent with a physical role for this chaperone in CP biogenesis. Third, our depletion strategy involves multiple purification steps with extensive washing. The ability of Ssa1/2 to remain bound to CP assembly intermediates throughout our depletion protocol, and their absence from bands corresponding to the PHP and fully assembled CP (Table 1, Supplementary Figures 15, 16), argues that they dissociate prior to completion of assembly; this is a key component of the definition of an assembly factor. Finally, strains lacking Ssa1/2 have greatly reduced levels of assembly intermediates and likely exhibit a weak defect in β2 propeptide processing (Figure 4). Two previous observations are consistent with our findings. First, upregulation of *RPN4*, encoding the transcription factor that regulates expression of proteasome subunits [27], has been observed in an *ssa1*Δ*ssa2*Δ double mutant [28]. A greater need for proteasomes would be

expected if assembly is impacted by a loss of Ssa1/2. Second, the *rpn4*Δ*ssa1*Δ*ssa2*Δ triple mutant grows worse than the *ssa1*Δ*ssa2*Δ double mutant [29]. This is reminiscent of the synthetic sick (or lethal) phenotypes that result when deletion of *RPN4* is combined with a deletion of any CP assembly factor [30].

The precise role of Ssa1/2 in CP assembly remains to be determined. They may help stabilize assembly intermediates. For instance, the 13S intermediate contains a full α-ring and three out of seven β subunits (β2, β3, β4). Recently we reported that Ssa1/2 bind to high molecular weight complexes of α4 in vivo, which are most likely rings [14]. Thus, perhaps Ssa1/2 assist the formation of α subunit rings, or stabilize them once formed. Also, Ssa1/2 appear to be involved in the formation (or stabilization) of a novel complex we call the sub-13S species. This complex probably contains a subset of α and β subunits (most likely α1–α4, and β2–β4) but whether or not it is a true assembly intermediate remains to be determined. On the one hand, it could be the result of a 13S intermediate falling apart during purification and/or electrophoresis. On the other hand, it could be a precursor to the 13S intermediate, suggesting the existence of an assembly pathway that does not involve an isolated α-ring. We've demonstrated such α-ring independent pathways for archaeal proteasomes, which serve as models for eukaryotic proteasome assembly [31].

Regardless of the status of the sub-13S species, it is likely that CP assembly has multiple redundancies built in. There is the obvious redundancy between Ssa1 and Ssa2, as evidenced by the recovery of peptides for both Ssa1 and Ssa2 from assembly intermediates and by the similar synthetic phenotypes observed when deletion of either *SSA1* or *SSA2* is combined with assembly mutants. Consistent with this redundancy, whereas the *rpn4*Δ*ssa1*Δ*ssa2*Δ triple mutant grows worse than the *ssa1*Δ*ssa2*Δ double mutant [29], we observe no obvious phenotypes when *rpn4*Δ is combined with only *ssa1*Δ or *ssa2*Δ (Supplementary Figure 12D). Redundancy is also seen in the *ssa1*Δ*ssa2*Δ mutant where levels

of assembly intermediates (bands 5 and 6) are reduced but overall CP levels are not appreciably affected. Clearly, the subunits are still capable of coming together to form CP species, even if a small fraction of them has incompletely matured β2 subunits. Taking a broader view, the simple observation that none of the CP or RP assembly factor genes are essential [2] is strongly suggestive of assembly redundancy.

Although our purification/depletion analysis did not identify additional candidates beyond Ssa1/2, the stringency of our method means that weakly/transiently bound assembly factors could have been washed away. The existence of redundancy in assembly, which implies multiple assembly pathways, makes it more probable that additional factors assisting proteasome assembly await discovery. That some of these are likely to be members of the MCN now has considerable precedent given our results here and the reported, though incompletely understood, role of Hsp90 in proteasome biogenesis [32].

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Figure Legends

Fig. 1. Depletion analysis to isolate CP assembly intermediates. (A) Native PAGE analysis of Flag-purified CP (β4-Flag) from the indicated yeast strains (lanes 1 to 3). The Flag-purified material was subjected to two rounds of depletion by ICAR to remove his-tagged proteins. Aliquots of the flow through from the second ICAR round were analyzed on the same native PAGE gel (lanes 4 to 6). Arrowheads denote CP and other bands of interest. Asterisk denotes position of Blm10-bound CP. (B) Aliquots in (A) analyzed by SDS-PAGE; asterisk denotes position of Blm10.

Fig. 2. Phenotype analysis of *ssa* mutants. Dilution series were prepared and the yeast spotted onto SD plates (top). Alternatively, yeast from individual colonies were struck out onto YPD plates (bottom). The temperature and duration of plate incubation are indicated.

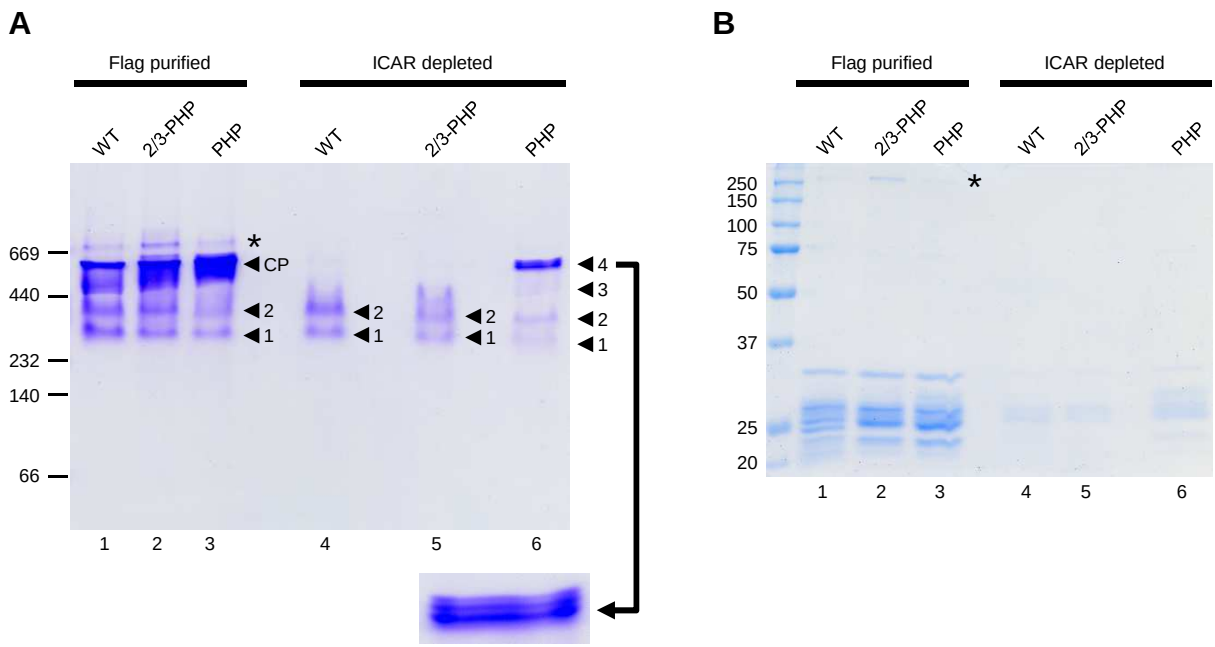
Fig. 3. Suppressor analysis. Dilution series of CP mutants (A), and RP mutants (B), transformed with Hsp70-containing plasmids, or vector-only controls. Plates were incubated for 3 days.

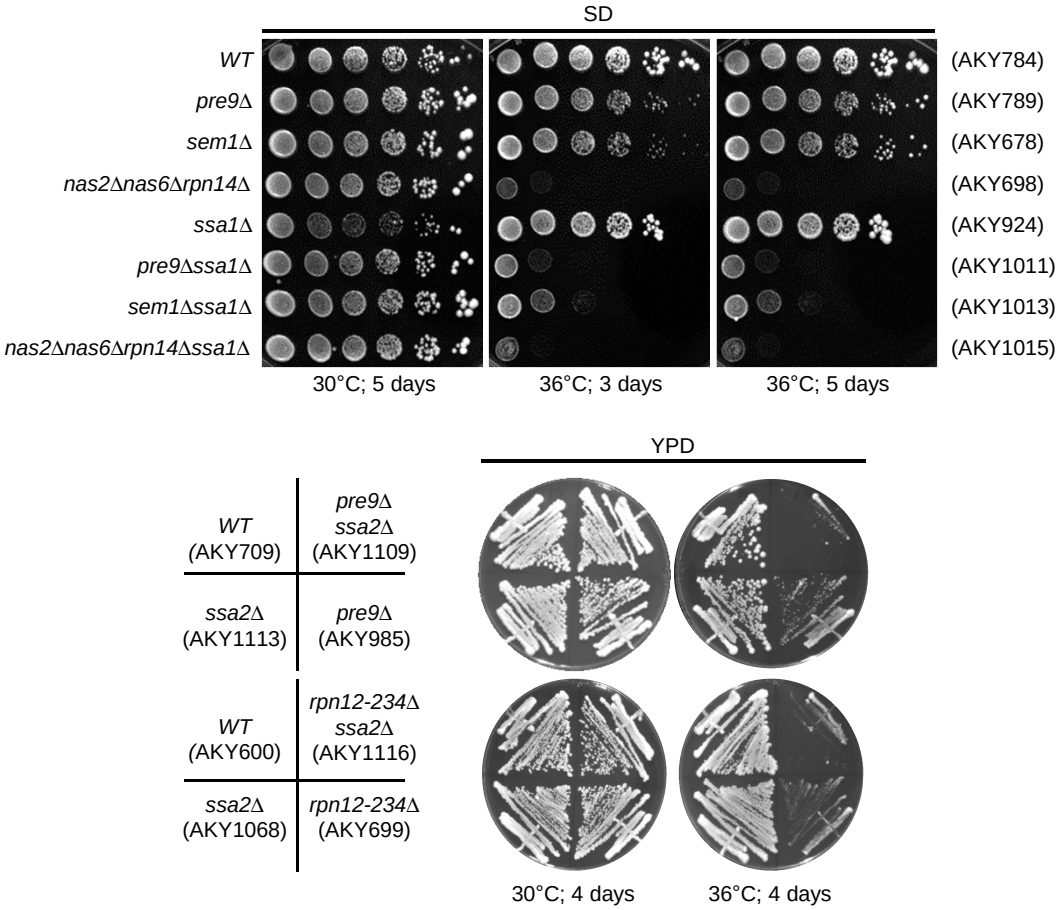
Fig. 4. Assembly defects in a mutant lacking Ssa1 and Ssa2. (A) Equal amounts (8 μg) of Flag-purified CP material (α4-Flag) from the indicated yeast strains were analyzed by native PAGE (left). An SDS-PAGE loading control is also shown (right). (B) Same native PAGE analysis as in (A) except a larger amount of protein (25 μg) was loaded. In all gels, arrowheads denote bands of interest. Asterisk denotes migration of Blm10-bound CP (singly and doubly capped).

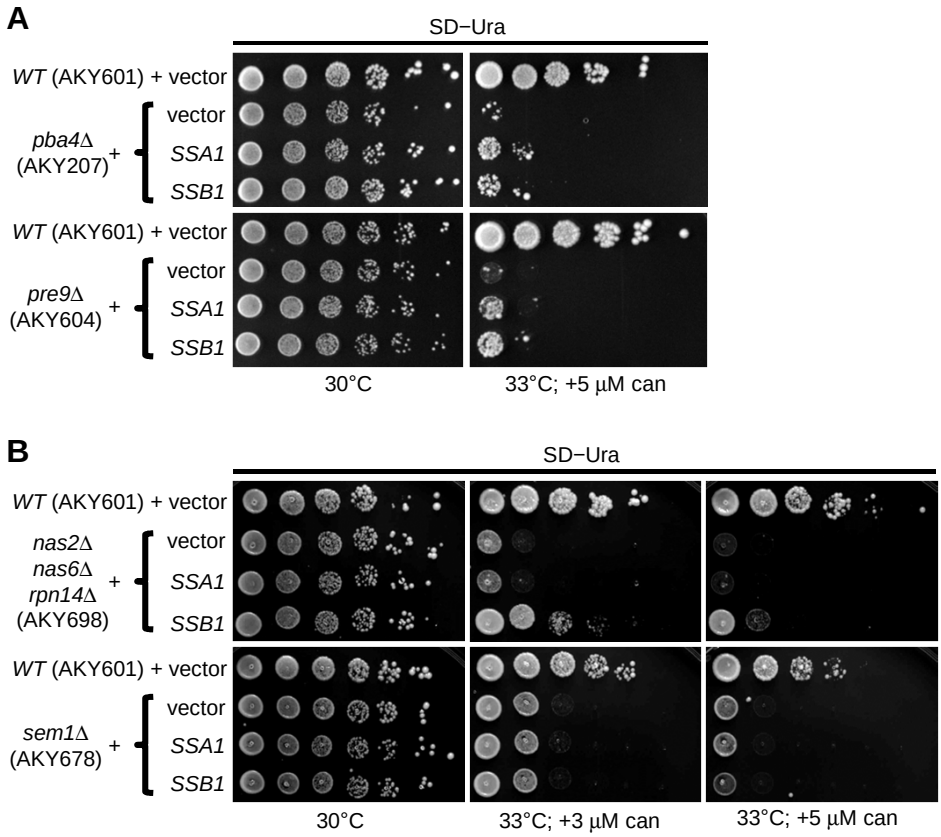
Table 1. **Select composition of excised bands.** Excised native PAGE bands from Figure 1 were analyzed by LC-MS/MS. For each protein, the number of peptide spectral matches (PSMs) is shown.

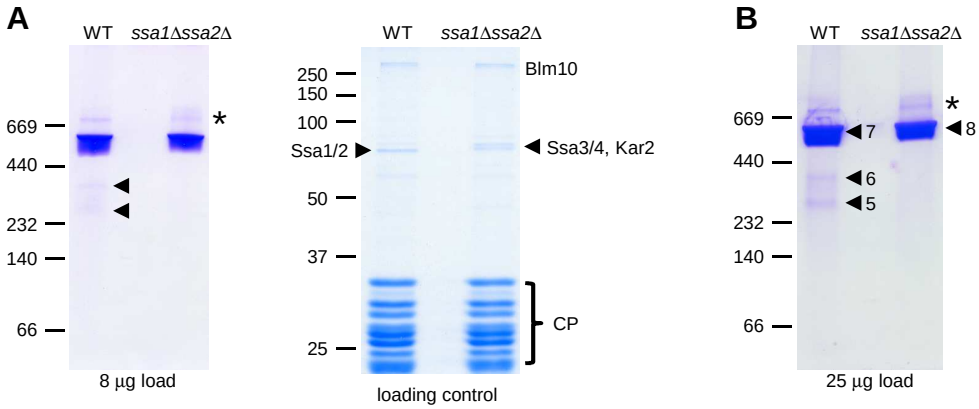
Table 1: Select composition of excised bands.

	PHP				2/3-PHP		WT	
	4	3	2	1	2	1	2	1
α1	328	66	95	109	112	134	201	195
α2	124	28	39	36	58	60	72	78
α3	170	42	57	59	85	74	110	104
α4	223	35	72	98	92	131	558	314
α5	124	20	26	8	34	13	64	15
α6	222	40	54	6	56	12	132	5
α7	85	21	37	67	50	19	74	11
β1	109	13	4					5
β2	157	42	49	58	75	94	99	125
β3	62	24	31	37	44	54	57	51
β4	53	17	20	26	33	35	36	39
β5	253	47	6	6	5		7	6
β6	209	28						
β7	277	74	10					
Ump1	80	5	7	30	36	31	34	54
Pba1	78	19	25	7	34	9	114	9
Pba2	38	4	11		17		44	
Ssa1/2		6	63	29	56	16	40	11


A
Flag purified
ICAR depleted
WT
2/3-PHP
PHP
WT
2/3-PHP
PHP
669
440
232
140
66
*
CP
2
1
2
1
2
1
4
3
2
1
1
2
3
4
5
6
B
Flag purified
ICAR depleted
WT
2/3-PHP
PHP
WT
2/3-PHP
PHP
250
150
100
75
50
37
25
20
*
1
2
3
4
5
6


SD
WT
pre9Δ
sem1Δ
nas2Δnas6Δrpn14Δ
ssa1Δ
pre9Δssa1Δ
sem1Δssa1Δ
nas2Δnas6Δrpn14Δssa1Δ
(AKY784)
(AKY789)
(AKY678)
(AKY698)
(AKY924)
(AKY1011)
(AKY1013)
(AKY1015)
30°C; 5 days
36°C; 3 days
36°C; 5 days
YPD
WT (AKY709)
pre9Δ ssa2Δ (AKY1109)
ssa2Δ (AKY1113)
pre9Δ (AKY985)
WT (AKY600)
rpn12-234Δ ssa2Δ (AKY1116)
ssa2Δ (AKY1068)
rpn12-234Δ (AKY699)
30°C; 4 days
36°C; 4 days


A
SD−Ura
WT (AKY601) + vector
pba4Δ (AKY207) +
vector
SSA1
SSB1
WT (AKY601) + vector
pre9Δ (AKY604) +
vector
SSA1
SSB1
30°C
33°C; +5 μM can
B
SD−Ura
WT (AKY601) + vector
nas2Δ nas6Δ rpn14Δ (AKY698) +
vector
SSA1
SSB1
WT (AKY601) + vector
sem1Δ (AKY678) +
vector
SSA1
SSB1
30°C
33°C; +3 μM can
33°C; +5 μM can


A
WT
ssa1Δssa2Δ
669
440
232
140
66
*
8 µg load
WT
ssa1Δssa2Δ
250
150
100
Ssa1/2
50
37
25
Blm10
Ssa3/4, Kar2
CP
loading control
B
WT
ssa1Δssa2Δ
669
440
232
140
66
*
7
8
6
5
25 µg load

Highlights

- CP assembly is assisted by Hsp70 molecular chaperones, Ssa1 and Ssa2
- Ssa1/2 are genetically linked to CP assembly
- Ssa1/2 are physically linked to specific assembly intermediates
- Expands known interactions between chaperone machinery and the proteasome
- Suggests list of known factors assisting in proteasome assembly is not complete