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Differential intra-proteasome interactions involving standard and immunosubunits

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Abstract

Animals with immune systems have two types of proteasomes, “standard proteasomes” and “immunoproteasomes” that respectively contain constitutively expressed catalytic subunits or interferon- γ -inducible catalytic subunits. Interestingly, proteasome assembly is biased against formation of most mixed proteasomes containing combinations of standard subunits and immunosubunits. We previously demonstrated that catalytic subunit propeptide differences contribute to this assembly specificity. In the current study, we investigated the contributions of catalytic subunit propeptides and C-terminal extensions to intra-proteasome protein-protein interactions that are potentially involved in mediating biased assembly of human proteasomes, and we found a number of interactions that differentially depended on these structures. For example, the C-terminal extension of standard subunit $\beta 2$ is required for $\beta 2$'s interaction with adjacent $\beta 3$, whereas the C-terminal extension of immunosubunit $\beta 2i$ is dispensable for $\beta 2i$'s interaction with $\beta 3$. Taken together, our results suggest mechanisms whereby differential intra-proteasome interactions could contribute to proteasome assembly specificity.

Keywords

Proteasome; Immunoproteasome; Proteasome assembly; Propeptide; Protein-protein interaction; Yeast two-hybrid interaction

Proteasomes are multi-subunit multi-catalytic intracellular proteases that are responsible for most non-lysosomal protein degradation in eukaryotic cells. Proteasomes are an integral component of ubiquitin-mediated protein degradation, and as such they are involved in many cellular processes, including cell cycle control, cellular stress responses, intra-cellular signaling, and MHC class I antigen processing [1–3]. The 20S proteasome core contains the proteolytic active sites and consists of four heptameric stacked rings configured $\alpha 7\beta 7\beta 7\alpha 7$. This quaternary structure is conserved from archaea to humans. Proteasomes in the archaebacterium *Thermoplasma acidophilum* are composed of only two different subunits, with all α subunits and β subunits identical. Conversely, eukaryotic proteasomes are composed of seven different α subunits and seven different β subunits. In animals with immune systems, there is increased complexity with two proteasome subtypes, “standard proteasomes” and

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“immunoproteasomes.” Standard proteasomes contain three constitutively expressed catalytic subunits, $\beta 1$ (Delta), $\beta 2$ (Z) and $\beta 5$ (X), that have peptidylglutamyl-peptide hydrolyzing-like, trypsin-like and chymotrypsin-like activities, respectively. Immunoproteasomes contain three interferon- γ -inducible catalytic subunits, $\beta 1i$ (LMP2), $\beta 2i$ (MECL-1) and $\beta 5i$ (LMP7), that are each highly homologous to one of the standard subunits and can substitute for their respective homologues in the proteasome structure [4,5].

As proteasome structural complexity increases, so too does complexity of proteasome assembly. In *Thermoplasma*, β subunits are synthesized as inactive precursor proteins with very short N-terminal propeptides that are removed at the end of the assembly process, freeing N-terminal nucleophile active sites. These propeptides appear to prevent premature enzymatic activation, but they are dispensable for assembly [6]. In contrast, *Rhodococcus* β subunit propeptides are much longer (around 60 amino acid residues), contain conserved residues that interact with adjacent α subunits, and they are required for normal proteasome assembly [7]. Assembly of eukaryotic proteasomes also requires catalytic β subunit propeptides. For example, deletion of the yeast $\beta 5$ propeptide prevents incorporation of $\beta 5$ into assembling proteasomes [8]. Eukaryotic proteasome assembly also involves a number of chaperones, including PAC1/PAC2 heterodimers, PAC3 and proteasemblin (also called POMP or hUMP1) [9]. PAC1/PAC2 heterodimers bind to assembling α -subunits and appear to prevent formation of “dead-end” α -ring dimers [10]. PAC3 promotes α -ring formation and dissociates from α rings during the process of incorporating precursor β -subunits and proteasemblin [11]. The relatively stable assembly intermediates containing one complete α ring, a partial ring of precursor β subunits, and associated chaperones are called “pre-proteasomes.” Proteasemblin coordinates completion of 20S proteasome assembly via the joining of two pre-proteasomes, and then proteasemblin is degraded by proteolytic sites that are activated by autocatalytic removal of propeptides [12].

Another interesting structural aspect of several β subunits is the presence of C-terminal segments of varying length that extend away from the core β subunit structure and are potentially involved in intra-proteasome protein-protein interactions. For example, $\beta 2$ subunits have long C-terminal extensions that wrap around adjacent $\beta 3$ subunits, as delineated in the crystal structures of both yeast [13] and bovine proteasomes [14]. Likewise, C-terminal extensions of yeast $\beta 7$ subunits project across the β ring interface to associate with subunits of the opposite β ring, while in bovine proteasomes C-terminal extensions of both $\beta 7$ and $\beta 1$ similarly project across the β ring interface [14]. A role for C-terminal extensions in the assembly process is suggested by impaired assembly when these structures were removed from yeast $\beta 2$ and $\beta 7$ [15].

In animals with immune systems, immunoproteasomes and standard proteasomes are typically expressed together in the same cell, but they assemble by alternative pathways that bias against the assembly of mixed proteasomes containing most combinations of standard subunits and immunosubunits. Differences between immunosubunit and standard subunit propeptides contribute to this assembly specificity [16,17], and interestingly, these alternative assembly pathways appear to have different sequences for incorporating catalytic β subunit precursors. Given the role of propeptides and C-terminal extensions in the assembly process, we investigated the possibility that these structures mediate differential subunit-subunit interactions that lead to alternative assembly pathways. We demonstrate a number of differential interactions involving human immunosubunits and standard subunits and offer potential mechanisms whereby these differential intra-proteasome interactions could contribute to assembly specificity.

Materials and methods

Strains and plasmids

Saccharomyces cerevisiae haploid strains AH109 and Y187, and vectors pGBKT7 and pGADT7, were obtained from Clontech (Palo Alto, CA) and used in the yeast two-hybrid Gal4 interaction trap assay system, as previously described [18]. Human precursor proteasome subunit cDNA's were also previously described [18]. Truncated $\beta 2/\beta 2i$, $\beta 1i/\beta 1$, and $\beta 7$ cDNA's lacking coding sequence for 41, 9 and 14 amino acid residues, respectively, from their C-termini were generated from complete cDNA's using PCR cloning methods. The length of each C-terminal deletion was determined by identifying the segment that extends beyond the core structure of each β subunit in the bovine crystal structure [14]. Mature proteasome subunit cDNA's lacking propeptide coding sequences were also generated from complete cDNA's using PCR methods. Each cDNA was PCR amplified using primers containing restriction sites for cloning into pGBKT7 and pGADT7. Sequences of cDNA's were verified by DNA sequence analysis.

Yeast two-hybrid interaction trap assay

Vectors encoding proteasome subunits fused with the Gal4 binding domain (BD) or Gal4 activation domain (AD) were transformed into either AH109 or Y187 yeast strains, respectively, or co-transformed into AH109 strain. Constructs included complete precursor proteasome subunits, subunits lacking N-terminal propeptides, and subunits lacking C-terminal extensions. Transformants were selected on synthetic complete (SC) media lacking the appropriate nutritional selection marker (tryptophan and/or leucine). Haploid transformants were mated according to the Clontech Yeast Protocol Handbook and selected on SC media lacking both tryptophan and leucine. Protein-protein interactions were detected by replica plating of at least six colonies on SC media lacking tryptophan, leucine and histidine, and containing various concentrations of 3-aminotriazole (3-AT), a competitive inhibitor of histidine synthesis that reduces background growth. Yeast growth at 30°C was assessed at 3 weeks, with scoring of growth as strong (+++) for confluent growth, moderate (++) for more than five colonies on average per clone but not confluent, and weak (+) for one to five colonies per clone. Interaction between p53 and SV40 T-antigen (T-ag) served as a positive control. Negative controls included interactions between proteasome subunits and BD alone or AD alone.

Results and discussion

We previously conducted a detailed investigation of protein-protein interactions among human 20S proteasome subunits and found a number of differential interactions involving standard subunits vs. immunosubunits that potentially impact on the mechanism of biased proteasome assembly [18]. In the current study, we investigated the contributions of propeptides and C-terminal extensions to these yeast two-hybrid interactions, and we have uncovered additional differences that may contribute to assembly specificity. The approach of using yeast two-hybrid interaction trap assays to assess the strength of specific protein-protein interactions among 20S proteasome subunits has been used to study interactions within proteasomes from *S. cerevisiae* [19], *C. elegans* [20], and *H. sapiens* [18,21]. In the current study, we compared interactions involving intact subunits with those involving mature subunits lacking propeptides ("m" subunits), and truncated subunits lacking C-terminal extensions ("t" subunits). Each construct was fused to the C-terminus of either the Gal4 binding domain (BD) or Gal4 activation domain (AD). Protein-protein interactions were detected by yeast growth on histidine-deficient media. Scoring of interaction strength is described in *Materials and methods*. Growth was assayed in the presence of 3-aminotriazole (3-AT) typically at three concentrations (3 mM, 10 mM and 25 mM), though we found that $\beta 1$ and $\beta 1i$ subunits, as well

as their mature and truncated forms, are strong self-activators when expressed as BD fusions, and thus interactions with these subunits were assayed at higher concentrations of 3-AT (50 mM, 75 mM and 100 mM) to reduce background.

Interactions involving $\beta 2$ subunits

$\beta 2$ (or Z) and $\beta 2i$ (or MECL-1) are catalytic subunits that possess trypsin-like activity [22, 23]. We previously demonstrated that both $\beta 2$ and $\beta 2i$ interact with adjacent $\alpha 1$ subunits [18], which we postulated may be involved in initiating β subunit loading onto α rings, particularly noting that both $\beta 2$ and $\beta 2i$ are incorporated relatively early into pre-proteasomes during assembly [24]. The $\beta 2/\alpha 1$ interaction is stronger than the $\beta 2i/\alpha 1$ interaction, which may account for the fact that $\beta 2i$ requires $\beta 1i$ for efficient co-incorporation into immunoproteasomes, whereas the more strongly-interacting $\beta 2$ is incorporated into standard proteasomes independently of $\beta 1$ [25]. Figure 1 and Table 1 show the results for interactions of $\alpha 1$ with mature and truncated forms of $\beta 2$ and $\beta 2i$. Removing either propeptide or C-terminal extension from $\beta 2$ does not adversely affect its interaction with $\alpha 1$, and in fact appears to strengthen it. Conversely, the weaker interaction of $\beta 2i$ with $\alpha 1$ is significantly strengthened by removing $\beta 2i$'s C-terminal extension. These results imply that the C-terminal extension of $\beta 2i$ interferes with the $\beta 2i/\alpha 1$ interaction to a greater extent than the C-terminal extension of $\beta 2$, which may underlie the ability of $\beta 2$, but not $\beta 2i$, to be incorporated early and independently into assembling pre-proteasomes.

The other subunit with which $\beta 2$ subunits have been shown to strongly interact is adjacent $\beta 3$. Crystal structure data demonstrate that the C-terminal extension of $\beta 2$ wraps around the outside of $\beta 3$, which likely yields a strong protein-protein interaction [13,14]. Additionally, C-terminal deletion of $\beta 2$ is lethal in yeast, which suggests that the C-terminal extension of $\beta 2$ is essential for proper proteasome assembly [15]. Thus, we expected $\beta 2/\beta 3$ interactions to depend on the presence of $\beta 2$ C-terminal extensions. Indeed, removal of the C-terminal segment from $\beta 2$ completely abolished its interaction with $\beta 3$ (Table 1), whereas removal of the $\beta 2$ propeptide had no effect, as expected. Surprisingly, interaction of $\beta 2i$ with $\beta 3$ persisted in the absence of $\beta 2i$'s C-terminal extension, which suggests a stronger interaction between the core structure of $\beta 2i$ with $\beta 3$ that was not apparent when comparing intact subunits.

Interactions involving $\beta 1$ subunits

$\beta 1$ (or Delta) and $\beta 1i$ (or LMP2) are catalytic subunits that possess peptidylglutamyl-peptide hydrolyzing-like activity [23]. Differential interactions involving these subunits that we previously identified include a strong interaction between $\beta 1i$ and $\alpha 7$, but no detectable interaction between $\beta 1$ and $\alpha 7$ [18]. We also found strong interactions between $\beta 1$ and $\beta 7$ and between $\beta 1$ and $\beta 3$, whereas these interactions were not found with $\beta 1i$ [18]. Our results shown in Table 2 indicate that the $\beta 1i/\alpha 7$ interaction is unaffected by removal of either $\beta 1i$'s propeptide or its C-terminal extension, and thus this interaction appears to involve the core structure of $\beta 1i$. None of the forms of $\beta 1$ interacted with $\alpha 7$. We suggest that this differential interaction with $\alpha 7$ may involve an amino acid residue difference at position 106 in the $\beta 1/\beta 1i$ subunits. This residue is an arginine in $\beta 1i$ and a glutamine in $\beta 1$. The arginine residue in $\beta 1i$, but not the glutamine residue in $\beta 1$, is postulated to form a salt bridge with aspartic acid 144 of $\alpha 7$ and thereby mediate the strong interaction between $\beta 1i$ and $\alpha 7$ [14]. The results in Table 2 also demonstrate that the interactions between $\beta 1$ and either $\beta 7$ or $\beta 3$ are dependent on the propeptide of $\beta 1$, as these interactions are completely abolished with deletion of this propeptide. This suggests that the $\beta 1$ propeptide may facilitate $\beta 1$ incorporation into partially assembled β rings via interactions with these neighboring β subunits, whereas this function is not required of the $\beta 1i$ propeptide, since $\beta 1i$ is incorporated earlier in conjunction with $\beta 2i$, and this early loading of $\beta 1i$ onto the α ring could be facilitated by its strong interaction with $\alpha 7$.

We have also previously demonstrated strong interactions between both $\beta 1$ and $\beta 1i$ and the assembly chaperone, proteasemblin [18]. The data in Table 2 demonstrate that these interactions do not depend on either propeptides or C-terminal extensions of $\beta 1/\beta 1i$ subunits. Proteasemblin also interacts with several other β subunits ($\beta 5$, $\beta 6$ and $\beta 7$) [18], and in the current study, deletion of the $\beta 5$ propeptide and removal of the $\beta 7$ C-terminal extension had no effect on these interactions (data not shown), which taken together suggests that proteasemblin interacts with the core structures of each of these adjoining β subunits. This contrasts with the observation that $\beta 5$ lacking a propeptide fails to incorporate into proteasomes in normal yeast, whereas it readily incorporates when ump1p, the yeast homologue of proteasemblin, is absent [8]. This observation suggests that an interaction occurs between the $\beta 5$ propeptide and ump1p that regulates $\beta 5$ incorporation, but in the current study we do not find evidence for this interaction using mammalian subunits in our yeast two-hybrid assay system, but cannot discount the possibility of such an interaction occurring but not being detected in our system [8].

Interactions involving $\beta 5$ subunits

$\beta 5$ (or X) and $\beta 5i$ (or LMP7) are catalytic subunits that possess chymotrypsin-like activity [23,26]. We previously found that $\beta 5i$ exhibited stronger interactions than $\beta 5$ with $\beta 1$ and $\beta 1i$, whereas $\beta 5$ exhibited stronger interactions than $\beta 5i$ with $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 3$ and proteasemblin [18]. The effects of removing propeptides from either $\beta 1$ or $\beta 5/\beta 5i$ propeptides abolished interactions of $\beta 1$ with either $\beta 5i$ or $\beta 5$. Conversely, removing the $\beta 1i$ propeptide had little effect on the $\beta 1i/\beta 5i$ interaction, whereas this interaction depended on the $\beta 5i$ propeptide, similar to the $\beta 1/\beta 5i$ interaction. These effects contrast with the finding that C-terminal truncation of $\beta 1$ had little effect on interaction with $\beta 5i$, whereas C-terminal truncation of $\beta 1i$ significantly impaired interaction with $\beta 5i$. These results suggest that while the $\beta 5/\beta 5i$ propeptide is involved in interactions with the $\beta 1/\beta 1i$ subunits, the $\beta 1/\beta 1i$ subunits have different requirements for interaction with $\beta 5$ subunits, with important structures being the $\beta 1$ propeptide and the C-terminal extension of $\beta 1i$. These interactions are of particular interest because pre-proteasomes containing $\beta 1i$ preferentially incorporate $\beta 5i$ over $\beta 5$ [25], and we previously demonstrated that differences in the $\beta 5$ propeptides contribute to assembly specificity. Our data suggest that assembly specificity may involve not only these propeptides but also C-terminal segment differences between $\beta 1$ and $\beta 1i$.

With regard to interactions of $\beta 5$ with $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\beta 3$, we found that all of these interactions depended on the presence of $\beta 5$'s propeptide (data not shown), which suggests that they may also contribute to the essential assembly role of the $\beta 5$ propeptide. Conversely, the $\beta 5$ propeptide was not required for $\beta 5$'s interaction with proteasemblin (data not shown), and in fact, removal of the $\beta 5i$ propeptide strengthened $\beta 5i$'s interaction with proteasemblin to be equivalent with the $\beta 5/\text{proteasemblin}$ interaction, which is consistent with the observations of Heink, *et al.*, regarding comparable interactions of $\beta 5/\beta 5i$ subunits with proteasemblin [27]. Additionally, these results reinforce the conclusion that proteasemblin interacts with core structures of β subunits and not their propeptides.

Interactions involving $\beta 7$ subunits

$\beta 7$ (or N3) is a non-catalytic β subunit whose C-terminal extension crosses the β ring interface and may contribute to the joining of two pre-proteasomes during the formation of 20S proteasomes [14]. We previously found that $\beta 7$ not only interacts with $\beta 1$ and proteasemblin (discussed above), but also interacts with itself, $\beta 3$, $\beta 5$ and $\beta 6$ [18]. We constructed truncated $\beta 7$ lacking its C-terminal extension and found that this C-terminal extension was not required for interactions with $\beta 1$, $\beta 7$ or proteasemblin (Table 2 and data not shown). This truncation did reduce interactions with $\beta 5$, $\beta 5i$, and $\beta 3$, though notably these interactions with intact $\beta 7$

were already relatively weak (data not shown), and furthermore they are of uncertain biological significance since these subunits are not adjacent to $\beta 7$ in the proteasome structure.

Conclusions

Interpretation of the results of yeast two-hybrid interaction assay studies must keep in mind the limitations of this approach, including a number of possibilities for false positive and negative results. We have discussed a number of these limitations previously [18]. Nevertheless, we have used this method to demonstrate a number of differential interactions involving propeptides or C-terminal extensions of catalytic standard subunits vs. immunosubunits that suggest mechanisms by which these structures could contribute to the biased assembly process that favors homogeneity of these two proteasome subtypes.

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Fig. 1.

Example of two-hybrid interaction trap assays by yeast co-transformation for interactions between $\alpha 1$ -BD and AD fusions to the indicated $\beta 2$ subunits. Results are included in Table 1, with the scoring system defined in *Materials and methods*. The first row is a negative control involving $\alpha 1$ -BD and empty vector-AD. The bottom row is a positive control involving p53-BD and T-ag-AD. Yeast were grown on SC media lacking tryptophan, leucine, and histidine, and containing 3-AT at the three indicated concentrations.

Table 1Effects of propeptide deletion or C-terminal truncation on interactions involving $\beta 2$ subunits

BD fusion	AD fusion	Strength of interaction		
		3 mM 3-AT	10 mM 3-AT	25 mM 3-AT
$\alpha 1$	$\beta 2$	+++	++	+
	m $\beta 2$	+++	+++	+++
	t $\beta 2$	+++	+++	+++
$\alpha 1$	$\beta 2i$	+		
	m $\beta 2i$	—		
	t $\beta 2i$	+++	++	+
$\beta 2$	$\beta 3$	+++	++	++
m $\beta 2$		+++	+++	++
t $\beta 2$		—		
$\beta 2i$		+++	+++	+++
m $\beta 2i$		++	+	
t $\beta 2i$		+++	++	

Abbreviations: m, mature subunit lacking propeptide; t, truncated subunit lacking C-terminal extension.

Table 2Effects of propeptide deletion or C-terminal truncation on interactions involving $\beta 1$ subunits

BD fusion	AD fusion	Strength of interaction		
		50 mM 3-AT	75 mM 3-AT	100 mM 3-AT
$\beta 1$	$\alpha 7$	—		
m $\beta 1$		—		
t $\beta 1$		—		
$\beta 1i$	$\alpha 7$	+++	+++	+++
m $\beta 1i$		+++	+++	+++
t $\beta 1i$		+++	+++	++
$\beta 1$	$\beta 7$	+++	+++	++
m $\beta 1$		—		
t $\beta 1$		+++	++	+
$\beta 1i$	$\beta 7$	—		
m $\beta 1i$		—		
t $\beta 1i$		—		
$\beta 1$	t $\beta 7$	+++	+++	+
m $\beta 1$		—		
t $\beta 1$		+++	++	
$\beta 1$	$\beta 3$	+++	++	+
m $\beta 1$		—		
t $\beta 1$		++	++	+
$\beta 1i$	$\beta 3$	—		
m $\beta 1i$		—		
t $\beta 1i$		—		
$\beta 1$	PA	+++	+++	+++
m $\beta 1$		+++	+++	+++
t $\beta 1$		+++	+++	++
$\beta 1i$	PA	+++	+++	+++
m $\beta 1i$		+++	+++	+++
t $\beta 1i$		+++	+++	+++

Abbreviations: m, mature subunit lacking propeptide; t, truncated subunit lacking C-terminal extension; PA, proteasassemblin.

Table 3Effects of propeptide deletion or C-terminal truncation on interactions involving both $\beta 1$ and $\beta 5$ subunits

BD fusion	AD fusion	Strength of interaction		
		50 mM 3-AT	75 mM 3-AT	100 mM 3-AT
$\beta 1$	$\beta 5$	+		
m $\beta 1$		—		
t $\beta 1$		—		
$\beta 1i$	$\beta 5$	—		
m $\beta 1i$		—		
t $\beta 1i$		—		
$\beta 1$	$\beta 5i$	+++	++	+
m $\beta 1$		—		
t $\beta 1$		+++	++	
$\beta 1i$	$\beta 5i$	+++	++	+
m $\beta 1i$		++	+	+
t $\beta 1i$		+		
$\beta 1$	m $\beta 5$	—		
	m $\beta 5i$	—		
$\beta 1i$	m $\beta 5$	—		
	m $\beta 5i$	—		

Abbreviations: m, mature subunit lacking propeptide; t, truncated subunit lacking C-terminal extension.