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Enhanced rate of degradation of basic proteins by 26S immunoproteasomes

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ABSTRACT

Immunoproteasomes are alternative forms of proteasomes specialized in the generation of MHC class I antigenic peptides and important for efficient cytokine production. We have identified a new biochemical property of 26S immunoproteasomes, namely the ability to hydrolyze basic proteins at greatly increased rates compared to constitutive proteasomes. This enhanced degradative capacity is specific for basic polypeptides, since substrates with a lower content in lysine and arginine residues are hydrolyzed at comparable rates by constitutive and immunoproteasomes. Crucially, selective inhibition of the immunoproteasome tryptic subunit β2i strongly reduces degradation of basic proteins. Therefore, our data demonstrate the rate limiting function of the proteasomal trypsin-like activity in controlling turnover rates of basic protein substrates and suggest new biological roles for immunoproteasomes in maintaining cellular homeostasis by rapidly removing a potentially harmful excess of free histones that can build up under different pathophysiological conditions.

1. Introduction

The 26S proteasome is an ATP-dependent protease that is responsible for the degradation of the majority of cellular proteins in eukaryotic cells. This multi-subunit complex consists of the 20S proteasome, in which proteins are degraded, and one or two 19S regulatory particles, which are responsible for recognizing, unfolding, and translocating polyubiquitinated substrates into the 20S internal proteolytic cavity [1]. The 20S proteasome is a barrel-shaped structure composed of four stacked heptameric rings. The two outer rings consist of α-subunits, while the two central rings are made up of β-subunits. Three of the subunits in the β rings (β1, β2, and β5) contain the proteolytic active sites that are positioned on the interior face of the cylinder. Proteolytic activities of proteasomes measured using short fluorogenic substrates have defined three distinct cleavage preferences: β1 has caspase activity (i.e. cleaving after acidic residues); β2 possesses tryptic activity (i.e. cleaving after basic residues); and β5 displays chymotryptic activity (i.e. cleaving after hydrophobic residues). Lymphoid cells and cells exposed to cytokines such as interferon-γ (IFN-γ) or tumor necrosis factor (TNF)-α express three homologous subunits (β1i/LMP2, β2i/MECL-1, β5i/LMP7) that replace the constitutive ones in newly assembled, so-called immunoproteasome particles [2].

Experiments with small fluorogenic substrates have shown that immunoproteasomes have a greater capacity to cleave after hydrophobic and basic residues, and a lower capacity to cleave after acidic residues. Consequently, peptides generated by immunoproteasomes should have a higher percentage of hydrophobic and basic C-termini, both of which favor uptake by TAP transporters and which are essential for tight binding to MHC class I molecules [3]. Furthermore, this altered cleavage specificity may also enhance the production of longer precursors to the MHC-presented peptide without affecting the overall size distribution of proteasomal products [4]. Although there are examples of epitopes that are generated with lower efficiency, or which are not released by immunoproteasomes, the pivotal role of immunoproteasomes in the generation of the vast majority of MHC class I ligands was definitively demonstrated in transgenic mice lacking all three proteasomal catalytic β-immune subunits [5]. Additionally, immunoproteasomes have been shown to be important for efficient cytokine production [6] and have been implicated in a number of pathological disorders such as cancer and neurodegenerative and autoimmune diseases [7–9]. Recently, immunoproteasomes were reported to play a major role in protecting cell viability under cytokine-induced oxidative stress due to their enhanced capacity to degrade nascent, oxidant-damaged polyubiquitinated proteins [10], although subsequent studies failed to confirm these data [11].

Our previous studies have shown that oxidized ovalbumin is degraded in vitro with comparable efficiency by both constitutive and immuno 20S and 26S proteasomes [4]. However, additional data concerning the effects of the INF-γ-induced subunits on the hydrolysis rates of non-ubiquitinated proteins are not available. To address this, we investigated

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the effect of INF-γ-induced β-subunits on the degradation of several loosely folded proteins that are hydrolyzed in vitro by 26S proteasomes in a linear, ATP-dependent manner, without ubiquitination [12]. In this way, we discovered that compared to constitutive proteasomes, 26S immunoproteasomes exclusively degrade at greatly enhanced rates proteins that are characterized by an exceptional high content in basic residues. We further demonstrated that the proteasomal tryptic site has a rate limiting function in controlling turnover rates of basic proteins and suggested potentially new roles of immunoproteasomes in catalyzing the rapid removal of histones.

2. Methods

2.1. Proteasome purification

26S proteasomes and immunoproteasomes were purified from rabbit muscle and spleen, respectively (Pel Freez Biologicals, Rogers, AR, USA), as described previously [4,13] and are free of aminopeptidases that may act on proteasome products.

2.2. Protein degradation and peptide analysis

Protein degradation, analysis of new amino groups using fluorescamine, and HP-SEC analysis were performed as previously described [4,12,14,15]. More details are provided in the Supplementary Materials and Methods.

3. Results

3.1. Enhanced rates of breakdown of basic proteins by the 26S immunoproteasome

Incorporation of INF-γ-induced β subunits significantly modifies proteasome peptidase activities [2]. Accordingly, 26S immunoproteasomes show an enhanced capacity to cleave short fluorogenic peptides on the carboxyl side of both basic (Fig. S1A) and hydrophobic (Fig. S1B) residues and a reduced ability to cleave after acidic amino acids (Fig. S1C). Specifically, the incorporation of INF-γ-induced subunits increases the maximal rate (V_{max}) at which proteasomes hydrolyze the basic substrates Z-ARR-amc, Boc-LRR-amc, and Bz-VGR-amc by two to threefold, and the hydrophobic substrate AFF-amc by more than sevenfold, while it reduces the V_{max} of the degradation of the acidic peptide Suc-YVAD-amc by about one-half (Table 1). Notably, in the case of the caspase site of immunoproteasomes, at a reduced maximum velocity the K_m value increases by nearly fourfold (Table 1). In contrast, the difference in K_m between constitutive and immunoproteasomes is much lower for the chymotrypsin-like activity, while for the trypsin-like activity it seems to mainly depend on the substrate utilized (Table 1).

Table 1
Kinetics parameters for the degradation of different fluorogenic peptides by 26S proteasomes and immunoproteasomes.

Substrate	26S proteasomes			
	Immuno		Constitutive	
	V_{max} (nmol/mg∗min)	K_m (μM)	V_{max} (nmol/mg∗min)	K_m (μM)
Bz-VGR-amc	379 ± 33	1801 ± 319	120 ± 11	665 ± 171
Z-ARR-amc	115 ± 3	668 ± 33	51 ± 3	493 ± 61
Boc-LRR-amc	247 ± 17	573 ± 65	127 ± 10	697 ± 82
AAF-amc	116 ± 25	170 ± 53	15 ± 4	139 ± 57
Suc-YVAD-amc	12 ± 3	503 ± 167	23 ± 2	127 ± 33

Maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) were calculated as described in Materials and Methods from the data shown in Supplementary Fig. S1. Values are mean ± SE.Maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) were calculated as described in Materials and Methods from the data shown in Supplementary Fig. S1. Values are mean ± SE.

A more relevant question, however, is to understand how these differences in peptidase activity, unveiled by the use of short fluorogenic peptides, relate to the true process of protein degradation and, specifically, whether they influence the overall rates of protein breakdown by proteasomes. To address this point, we studied in vitro degradation by 26S constitutive and immunoproteasomes of IGF-1, casein and histones by measuring the appearance of new amino groups generated as a consequence of hydrolysis of the substrate with fluorescamine. Casein and histones have little tertiary structure and are degraded by purified 26S proteasomes without ubiquitylation at linear rates for several hours in the presence of ATP [12,15]. On the contrary, IGF-1 requires preliminary denaturation by reduction of disulfide bonds and carboxymethylation of the cysteins in order to be hydrolyzed in vitro by 26S proteasomes in an ATP-dependent but ubiquitin-independent manner [15]. As shown in Fig. 1a, IGF-1 and casein were hydrolyzed by 26S constitutive and immunoparticles at linear, identical rates, thus confirming findings previously reported for ovalbumin with other two model proteasome substrates [4]. In contrast, the results obtained for the hydrolysis of H1, the linker histone in chromatin protects internucleosomal DNA, were unexpected. In fact, this extremely basic substrate was degraded six times faster by immuno- than by constitutive 26S proteasomes (Fig. 1b). Importantly, a four-fold increase in histone concentration did not modify the rates of H1 degradation, thus demonstrating that in these experiments both proteasomal species were catalyzing the hydrolysis reaction at maximum velocity (i.e. in conditions of substrate saturation) (Fig. S2).

These results were subsequently confirmed by directly comparing the rates of substrate consumption. Towards this end, histone H1 was incubated with 26S constitutive or immunoproteasomes and the amount of undegraded protein present at different time points was quantified. In agreement with the fluorescamine data, these experiments clearly revealed the greatly increased rates of histone H1 hydrolysis by 26S proteasomes containing INF-γ-induced β-subunits (Fig. 1c). The enhanced capacity of immunoproteasomes to hydrolyze basic proteins was subsequently confirmed by assessing the rates of degradation of the core histones H2A, H2B, and H3. Similarly to histone H1, these substrates were also degraded at rates that were about four-fold higher by proteasome containing immune β-subunits compared to regular 26S particles (Figs. 2a, b and S3). This enhanced capacity of immunoproteasomes to hydrolyze proteins rich in lysine and arginine was subsequently confirmed by assessing degradation of another completely unrelated basic substrate, namely myelin basic protein (MBP). Similar to histones and casein, MBP has very little tertiary structures and therefore can be degraded by proteasomes without the need for ubiquitination [16,17]. As shown in Figs. 3 and S4, MBP was also degraded about four-fold faster by 26S immuno- than by 26S constitutive proteasomes. Taken together, these results clearly demonstrate that highly basic proteins are hydrolyzed with higher efficiency by immunoproteosomes than by constitutive proteasomes.

The rate limiting role of immunoproteasome tryptic activity in determining the rate of hydrolysis of basic proteins was subsequently investigated using leupeptin, a competitive inhibitor that was reported to specifically inactivate the β2 subunit of constitutive proteasomes [12]. Preliminary experiments using fluorogenic peptides indeed showed that leupeptin is able to block the β2i subunit of 26S immunoproteasome with high efficiency without effecting β1i and β5i sites (Table 2). Having established conditions that allow selective inhibition of the tryptic site of the immunoproteasome in the absence of any detectable effects on the other two peptidase activities, we analyzed the effect of leupeptin on hydrolysis of histones and MBP. As shown in Fig. 4, leupeptin consistently decreased the degradation of these basic proteins by about 40%. Although incomplete, the inhibition obtained with a competitive inhibitor that specifically targets only the β2i subunit unambiguously demonstrates the rate limiting role of immunoproteasome tryptic activity in controlling the turnover rates of basic proteins.

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