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The 26S proteasome degrades mouse and yeast ornithine decarboxylase in yeast cells

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Abstract Eukaryotic cells possess two high-molecular-mass proteases, the 700 kDa, 20S proteasome, as well as the even larger 1,400 kDa, 26S proteasome. It has been demonstrated that ornithine decarboxylase is degraded, in vitro, by the 26S proteasome that contains the 20S protease as its catalytic core, but not by the free 20S proteasome. Recently, by demonstrating severe inhibition of mouse and yeast ODC degradation in a mutant yeast cell line, defective in the chymotripsin-like activity of the yeast 20S proteasome, we implicated the 20S proteasome in the degradation of ODC, in vivo, in yeast cells. Here we show that the degradation of ODC is also severely inhibited in the mutant yeast cell lines, *cim3-1* and *cim5-1*, containing a specific lesion in subunits that are unique to the yeast 26S proteasome. We therefore, conclude, that as illustrated in vitro, also in intact cells, it is the 26S proteasome, not the free 20S proteasome, that degrades ODC. We also demonstrate, that while deficiency in the proteasome chymotrypsine-like activity (in the yeast *pre1-1* mutant) inhibits the degradation of both yeast and mouse ODCs, deficiency in the peptidyl-glutamyl-peptide-hydrolyzing (PGPH) activity inhibits only yeast ODC degradation. Similarly, we have noted that whereas the putative ATPase activity of both the CIM3 and CIM5 subunits is essential for the degradation of mouse ODC, only that of the CIM3 subunit is required for the degradation of yeast ODC. These results suggest differential utilization of individual proteasomal subunits in the recognition and degradation of individual short-lived proteins.

Key words: Ornithine decarboxylase; Protein degradation; 20S and 26S Proteasome; Yeast

1. Introduction

Two high-molecular-mass proteases have been demonstrated in cytoplasmic extracts of a variety of eukaryotic cells [1,2]. The first is a 700 kDa protease, termed 20S proteasome [3–7]. The second is an even larger 1400 kDa protease, termed 26S proteasome. The 20S proteasome which exhibits various proteolytic activities specific to proteins and peptides [3,10,11] was demonstrated, in vitro, as the catalytic core of the 26S proteasome [12–14]. The assembly of the 20S proteasome into the larger 26S proteasome transforms it from an ATP and ubiquitin independent protease into an ATP dependent protease, that displays clear preference towards ubiquitinated proteins [12–14]. Interestingly, it has been demonstrated that the ATP dependent, but ubiquitin independent degradation, in vitro, of ornithine decarboxylase (ODC, a short-lived key enzyme in the biosynthetic pathway of polyamines), is also exerted by the 26S, and not by the 20S proteasome [15,16]. Recently, we have demonstrated that the degradation of both mouse and yeast ODC is inhibited in the mutant yeast cell line, *pre1-1*, which lacks the chymotrypsin-like activity of the yeast 20S proteosome [17]. Based on this result, however, we could not determine whether in cells, the 20S proteasome acts as a free proteolytic particle, or whether as demonstrated in vitro [15,16], it exerts its proteolytic activity as an integral part of the 26S proteasome. By using mutant yeast cells containing specific lesions in two putative ATPase subunits that are unique to the 26S proteasome, we demonstrate that also in vivo, in intact yeast cells, the 20S proteasome is involved in the degradation of ODC as a constituent of the 26S proteasome, and not as an independent free proteolytic particle. We also show differential requirement for proteosomal proteolytic activities in the degradation of mouse and yeast ODCs.

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2. Materials and methods

2.1. Strains

Yeast strains used were: WCG4a (*MATα ura3 leu2-3,112 his3-11,15*); WCG4-1-1a (*MATα ura3 leu2-3,112 his3-11,15 pre1-1*); YHI29-4 (*MATα ura3 leu2-3,112 his3-11,15 pre4-1*); YPH499 (*Matα ura3-52 leu2Δ1 his3Δ-200 1 trp1Δ63 lys2-801 ade2-101*); CMY762 (*Matα cim3-1 ura3-52 leu2Δ1 his3Δ200*); CMY806 (*Matα cim5-1 ura3-52 leu2Δ1 his3Δ-200*)

2.2. Expression of mouse and yeast ODC proteins in yeast cells and Pulse-chase experiments

Mouse and yeast ODC cDNAs were cloned into the yeast expression vector pKV49, as described [17]. The resulting constructs were introduced into the above mentioned strains by electroporation. A 5 ml portion of yeast culture grown in a minimal medium to $O.D._{600} = 0.5$ in the presence of galactose was harvested by centrifugation and labeled with [^{35}S]methionine (200 μCi/ml). Following 10 min of labeling, cells were harvested either immediately, or following a chase period in minimal medium containing glucose and unlabeled methionine (2%). The cells were disrupted by vortexing with glass beads in immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.1% Triton X-100, 0.1% SDS). Portions containing equal amounts of incorporated radioactivity were subjected to immunoprecipitation with anti-mouse, or anti-yeast ODC sera. The immunoprecipitated material was fractionated by electrophoresis in a 10% SDS-polyacrylamide gel, and visualized by autoradiography. Radioactivity, in individual bands, was determined using the Fujix Bas1000 bioimager. Each experiment was performed at least three times.

3. Results

In order to express mouse and yeast ODCs in yeast cells, the corresponding cDNAs were cloned into the 2μ based yeast expression vector, pKV49 [18]. Using this expression system, we have recently demonstrated that wild-type mouse and yeast ODCs are rapidly degraded in wild-type yeast cells [17]. This degradation is severely inhibited in the *pre1-1* mutant cells that lack the chymotrypsin-like activity of the yeast 20S proteasome [17], and (Fig. 1).

In order to determine whether the degradation of these two

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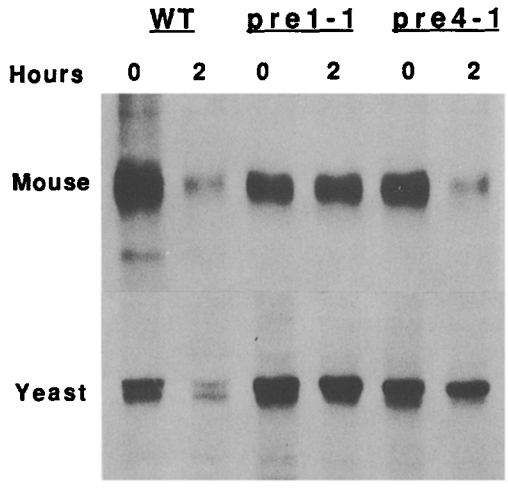

Fig. 1. Degradation of mouse and yeast ODC in wild-type yeast and in the *pre1-1* and *pre4-1* mutants. Cells were grown in medium containing galactose, and labeled for 10 min with [^{35}S]methionine. Cellular extracts were prepared immediately, or after 2 h of chase in glucose and unlabeled methionine containing medium. The extracts were subjected to immunoprecipitation analysis, using highly specific anti-mouse or anti-yeast ODC sera. Immunoprecipitated material was fractionated by electrophoresis in a 10% SDS-polyacrylamide gel, and visualized by autoradiography.

ODC proteins also requires another proteolytic activities of the yeast proteasome, the expression constructs were introduced into wild type yeast and the mutant *pre4-1* cells that lack the peptidyl-glutamyl-peptide-hydrolyzing (PGPH) activity of the yeast proteasome. In contrast to its stabilization in the *pre1-1* mutant, mouse ODC was efficiently degraded in the *pre4-1* mutant cells (Fig. 1). Interestingly, while mouse ODC was rapidly degraded in the *pre4-1* mutant cells, the degradation of yeast ODC was severely inhibited (Fig. 1). Overall, while our present results further emphasize the involvement of the 20S proteasome in the process of ODC degradation in vivo, they also suggest that different proteosomal proteolytic activities may be involved in the degradation of mouse and yeast ODC proteins. These results however, could not determine whether the 20S proteasome degrades ODC as a free proteolytic particle, or whether, as demonstrated in vitro, it is involved in this proteolytic process as a constituent of the 26S proteasome.

In order to distinguish between these two possibilities, we capitalized on the recent isolation of two mutant yeast strains (*cim3-1* and *cim5-1*), that contain specific lesions in ATPase-like subunits that are unique to the 26S proteasome [19]. Wild-type yeast cells, and the *cim3-1* and *cim5-1* mutant cells were transformed with the expression constructs and the stability of the two ODC proteins was determined by pulse-chase analysis. As shown in Fig. 2, mouse ODC was rapidly degraded in wild-type yeast cells, but was stable in the two *cim* mutant strains. In contrast, yeast ODC was rapidly degraded in *cim5-1* cells, but its degradation was inhibited in *cim3-1* cells (Fig. 2). Our results demonstrate that a lesion in each of the two subunits that are specific to the 26S protease severely inhibit mouse ODC degradation, and that the function of one of these subunits (CIM3) is required for the degradation of yeast ODC. These results, therefore, suggest that, in intact yeast cells, the 20S proteasome exerts its role in the degradation of ODC as an integral part of the larger 26S proteasome and not as a free particle. Moreover, the efficient degradation of yeast ODC in *cim5-1* cells which fail to degrade mouse ODC, suggests that different ATPase activities may be required for the degradation of these two ODC proteins.

4. Discussion

Here we provide strong evidence that the 26S proteasome, and not the free 20S proteasome, is the protease that degrades ODC in intact yeast cells. In a recent study we demonstrated that the 20S proteasome is involved in the degradation of both yeast and mouse ODC in yeast cells [17]. These degradations are severely inhibited in *pre1-1* mutant yeast cells which lack the chymotrypsin-like activity of the yeast 20S proteasome [17]. Studies in vitro have demonstrated that the ATP independent 20S proteasome alone is incapable of degrading ODC; its role in ODC degradation is manifested when it serves as a catalytic core of the ATP dependent 26S proteasome [15,16]. It was, therefore, important to determine whether also in intact cells the 20S proteasome is involved in the degradation of ODC as an integral part of the 26S protease. Inhibition of mouse ODC degradation in the *cim3* and *cim5* yeast strains, mutated in putative ATPase subunits that are part of the 26S proteasome, not of the 20S proteasome, demonstrated that it is the 26S proteasome, not the free 20S proteasome that degrades ODC. It was recently shown that the degradation of Ub-Pro-βgal, but not of Leu-βgal, is inhibited in the *cim3* and *cim5* mutants [19]. It was, therefore, concluded that although CIM3 and CIM5 may participate in the process of ubiquitin dependent degradation, they are not involved in the degradation of N-end rule substrates [19]. Accumulation of two types of B-type cyclins, CLB2 and CLB3, in *cim3* and *cim5* cells suggested the involvement of CIM3 and CIM5 in the degradation of these proteins as well [19].

As mentioned above, we demonstrate here, that while the function of the CIM3 and CIM5 subunits of the yeast 26S proteasome is required for the degradation of mouse ODC, only the CIM3 subunit is required for the degradation of yeast

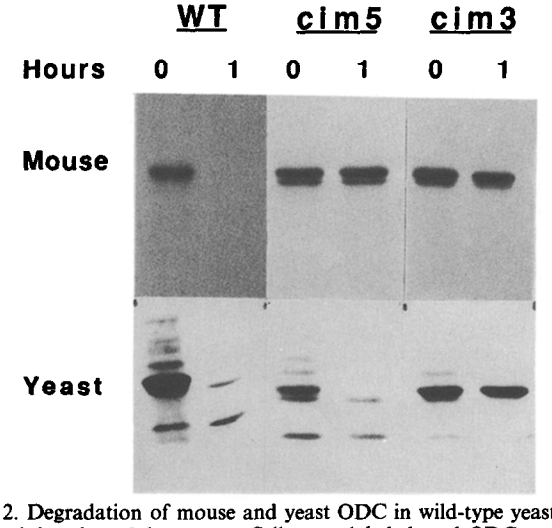

Fig. 2. Degradation of mouse and yeast ODC in wild-type yeast and in *cim3-1* and *cim5-1* mutants. Cells were labeled, and ODC proteins visualized as described in the legend to Fig. 1.

ODC. Differential requirement for the PGPH activity encoded by the PRE4 subunit of the 20S proteasome was also observed. In this case, lack of PGPH activity did not affect the degradation of mouse ODC, but prevented the degradation of yeast ODC. From in vitro studies it was suggested that the PGPH activity could be the major proteosomal activity responsible for the degradation of proteins [20–22]. Surprisingly however, studies in vivo in the *pre4-1* mutants could not detect any defect in protein degradation activity [22]. Here, we demonstrate that the degradation of yeast, but not of mouse ODC, requires the PRE4 encoded PGPH activity, and that the degradation of mouse, and not of yeast ODC, requires the putative ATPase activity of the CIM5 subunit. It is, therefore, possible that proteolytic and regulatory activities encoded by individual proteosomal subunits may be differentially required for the degradation of different short-lived proteins. It was recently shown that eukaryotic cells possess several 26S proteases that differ in their subunit composition and proteolytic capabilities [23]. Hence, it is also possible that different short-lived proteins may be degraded by different sub-types of the 26S proteasome.

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