Discrimination between ubiquitin-dependent and ubiquitin-independent proteolytic pathways by the 26S proteasome subunit 5a

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Abstract The 26S proteasome subunit 5a binds polyubiquitin chains and has previously been shown to inhibit the degradation of mitotic cyclins. Presumably inhibition results from S5a binding and preventing recognition of Ub-cyclin conjugates by the 26S proteasome. Here we show that S5a does not inhibit the degradation of full-length ornithine decarboxylase (ODC) consistent with previous reports that the enzyme is degraded in an antizyme-dependent, but ubiquitin-independent reaction. S5a does, however, inhibit degradation of short ODC translation products generated by internal initiation events. Because in vitro translation often produces some shortened products, the existence of ubiquitin conjugated to a ^{35}S-labeled protein is not necessarily evidence that the full-length protein is a substrate of the Ub-dependent proteolytic pathway.

Key words: Ornithine; Decarboxylase; Polyubiquitin chain; Proteolysis inhibitor

1. Introduction

The ubiquitin-dependent proteolytic pathway is responsible for the degradation of a variety of important regulatory proteins [1]. Ubiquitin (Ub) is a small protein that becomes covalently attached to protein substrates and marks them for destruction (see [2] for a recent review). Ub conjugation is catalyzed by a series of enzymes. E1 activates the carboxy terminus of Ub in an ATP-dependent reaction; the activated Ub is transferred to one of a family of E2 enzymes; the E2s in conjunction with an E3 or Ub ligase transfer Ub to the target protein [2]. Ubiquitin is usually covalently attached to epsilon amino groups on lysine residues, but it has recently been shown to modify also the N-terminal methionine of Myo D [3]. Since Ub itself contains several lysines that can act as acceptor sites for other Ub molecules, multi-Ub chains are often produced [4,5]. Proteins conjugated to Ub chains are bound and degraded by the 26S proteasome [6]. A subunit of the 26S proteasome, S5a, has been shown to bind Ub chains [7]. cDNAs for human, plant and yeast S5a proteins have been identified [8–10], and plant S5a has been shown to inhibit proteolysis of ubiquitinated proteins in vitro [11]. S5a-dependent inhibition of Ub conjugate degradation is presumably due to excess free S5a molecules binding Ub chains and preventing their recognition by the 26S proteasome. In principle, S5a should only inhibit the proteolysis of *ubiquitinated* proteins by the 26S enzyme.

Ornithine decarboxylase (ODC), a key enzyme in the polyamine biosynthetic pathway, is rapidly degraded with half-lives ranging from several min to 1 h depending upon physiological conditions [12,13]. Although rapid ODC proteolysis is not Ub-dependent, it does require ATP and a polyamine inducible protein called antizyme, which binds ODC and targets it for proteolysis by the 26S proteasome [14–18]. To date, ODC is the only protein known to be degraded in an antizyme-dependent manner. Here, we show that S5a does not inhibit the degradation of full-length ODC providing further evidence that proteolysis of the enzyme does not require attachment of polyUb chains. S5a does, however, inhibit proteolysis of lower molecular weight ODC translation products indicating that they are degraded by the Ub system. Thus, S5a can be used to discriminate between Ub-dependent and antizyme-mediated proteolytic pathways.

2. Materials and methods

Histidine tagged human S5a was prepared as described [9]. ODC and AZ DNAs with T7 promoters, produced by PCR, were a generous gift from Phil Coffino. Reticulocyte lysate for translation, T7 RNA polymerase and polymerase buffer, rATP, rGTP, rCTP, rUTP, RNase free DTT and RNasin were from Promega. [^{35}S] Methionine was from DuPont-NEN. Cycloheximide, creatine phosphokinase, ATP, and phosphocreatine were from Sigma. Rainbow markers were from Amersham; lactacystin was from Calbiochem.

2.1. In vitro transcription and translation

ODC and AZ mRNAs were transcribed from PCR products for 1 h at 30°C, in a 27 μl reaction containing: 5 μl PCR DNA, T7 polymerase buffer, 0.93 mM rNTP, 3.7 mM RNase free DTT, 1 μl RNasin and 10–20 units of T7 RNA polymerase. For the experiment shown in Fig. 1, ODC mRNA was translated in Promega reticulocyte lysate containing 1.6 μCi/μl [^{35}S]-methionine for 1 h at 30°C. For the experiment shown in Fig. 2, ODC mRNA was translated in Promega reticulocyte lysate containing 6.2 μCi/μl [^{35}S]-methionine for 30 min at 30°C, then lactacystin was added to 0.5 mM (or an equal volume of H_2O) and incubated for 1 h. AZ mRNA was translated in Promega reticulocyte lysate with cold methionine.

2.2. ODC degradation assay

For the experiment shown in Fig. 1, 4 μl of [^{35}S]-ODC translation mix was added to the following degradation mix: 10 μl reticulocyte lysate (prepared as described previously [19]), 0.1 mg/ml cycloheximide, 2 μl reticulocyte lysate (Promega), 0.5 mg/ml S5a (or an equal volume of buffer; 25 mM Tris pH 7.5, 10 mM NaCl) and an ATP regenerating system (0.5 mM ATP, 60 μg/ml creatine phosphokinase, 6.6 mM phosphocreatine, 10 mM Tris-HCl, 0.5 mM $MgCl_2$, 1 mM KCl, 0.05 mM DTT). The reaction was incubated at 30°C and at the indicated times 3 μl of the reaction was removed and stopped in 30 μl of 1×SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.05% Bromophenol blue). For the experiment shown in Fig. 2, 10 μl of proteolysis optimized reticulocyte lysate [19], 0.1 mg/ml cycloheximide, the ATP regeneration system, 2 μl AZ translation mix and 0.5 mM lactacystin (or H_2O) was incubated for 2 h at 30°C. Then 2 μl of [^{35}S]-ODC translation mix containing either lactacystin or H_2O was added and incubated at 30°C for another hour before the reaction was stopped as

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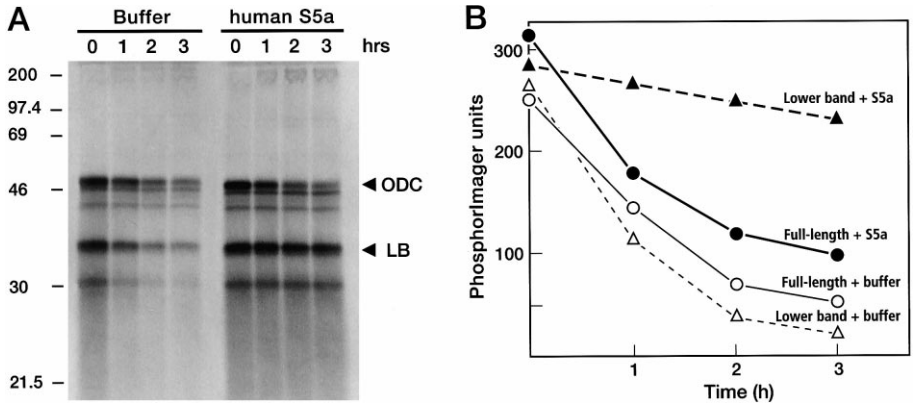

Fig. 1. Degradation of ODC in the presence or absence of the polyUb binding protein S5a. Panel A is an autoradiogram of an SDS-PAGE gel used to analyze ODC degradation in reticulocyte lysate. Translation of ODC mRNA in the presence of [^{35}S]-methionine produces the full-length enzyme (ODC) and four lower molecular weight species. The most prominent of these is designated LB for lower band. The four lanes at the left show that all four species are degraded in the absence of S5a. The four lanes on the right demonstrate that the four lower molecular weight species are stabilized in the presence of S5a, but full-length ODC is still rapidly degraded. Panel B: The lanes in panel A were subjected to phosphorImage analysis and the data are presented in graphical form.

described above. Samples were analyzed on 12.5% SDS-polyacrylamide gels imaged by autoradiography and quantitated by phosphorImager analysis.

2.3. [^{125}I]-lysozyme-ubiquitin conjugate degradation assay

[^{125}I]-lysozyme-ubiquitin conjugates were prepared as described previously [20]. To monitor 26S proteasome activity, 10 μl of reticulocyte lysate, 0.1 mg/ml cycloheximide, the ATP regenerating system, 2 μl of AZ translation mix and 0.5 mM lactacystin (or H_2O) was incubated for 2 h at 30°C. To start the reaction, 60 μl of [^{125}I]-lysozyme-ubiquitin conjugates (136 cpm/μl) containing the ATP regenerating system were added to 12 μl of reticulocyte lysate. At the indicated times 20 μl was removed and precipitated with trichloroacetic acid (TCA). Precipitated samples were centrifuged for 10 min at 16 000×*g*, the supernatants were counted for 10 min in a Beckman Gamma 4000 counter, and degradation was calculated by the appearance of soluble ^{125}I. For reactions containing S5a, 0.5 mg/ml S5a (or an equal volume of buffer: 20 mM MOPS pH 7.5, 1 mM DTT, 20 mM NaCl, 5 mM Na acetate) was added to the [^{125}I]-lysozyme-ubiquitin conjugates immediately before the reaction was started.

3. Results and discussion

Radiolabeled ODC was produced by translation in reticulocyte lysate containing [^{35}S]-methionine; approximately 20% of the translated ODC was the full-length product of 53 K (Fig. 1A, 0 min lanes). Several smaller fragments that result from internal initiations (P. Coffino, personal communication) were also produced. ODC degradation was measured after adding the translation products to proteolytically competent reticulocyte lysate in the presence or absence of 0.5 mg/ml S5a (Fig. 1). In the absence of S5a, full-length ODC and a smaller fragment, designated the lower band, were degraded with half-lives of about 60 min. In the presence of S5a, degradation of full-length ODC was barely affected. By contrast, S5a markedly inhibited proteolysis of the lower band. These results indicate that shortened ODC is degraded by the Ub-dependent pathway whereas proteolysis of full-length ODC is Ub-independent.

As an alternative means of inhibiting ODC proteolysis, we used lactacystin which specifically inhibits proteasomes [21]. We treated protease optimized reticulocyte lysate with 0.5 mM lactacystin for 2 h before assaying ODC degradation. As shown in Fig. 2, lactacystin equally inhibited proteolysis of both full-length ODC and N-terminally truncated ODC each to about 80%. Complete inhibition of the 26S proteasome was not achieved as shown by approximately 20% [^{125}I]-lysozyme-ubiquitin conjugate degrading activity that remained in the lactacystin treated lysate (Fig. 3). Unfortunately, longer pre-treatments with lactacystin were not possible because the lysate lost ODC degrading activity with time.

Our results show that addition of S5a molecules to reticulocyte lysate had little effect on degradation of full-length ODC in vitro, thus confirming previous reports that ODC proteolysis does not require ubiquitin [15,16]. However, S5a did inhibit the proteolysis of short translation products of ODC indicating that they are substrates of the Ub system. The most likely explanation for this result is that the missing N-terminal sequences of ODC fulfill two functions. First, they provide the AZ binding site, and in fact, the AZ binding site has been localized to the amino terminal, 120 amino acids

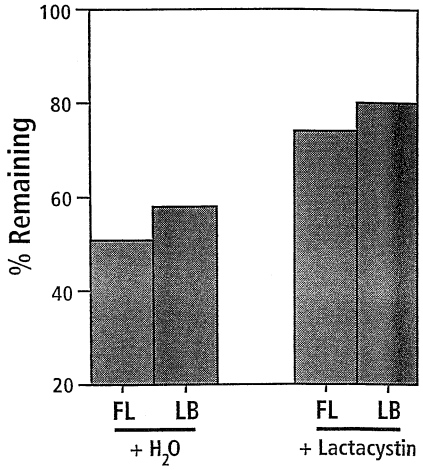

Fig. 2. Inhibition of ODC degradation by lactacystin. [^{35}S]-ODC translation products were incubated in reticulocyte lysate in the presence or absence of 500 μM lactacystin. After 1 h samples were separated on an SDS-PAGE gel and analyzed by phosphorImaging. The relative amounts of full-length (FL) ODC and the lower band (LB) species were quantitated. The results are presented as the amount of each species remaining at 1 h divided by the amount of each species at the start of the incubation.

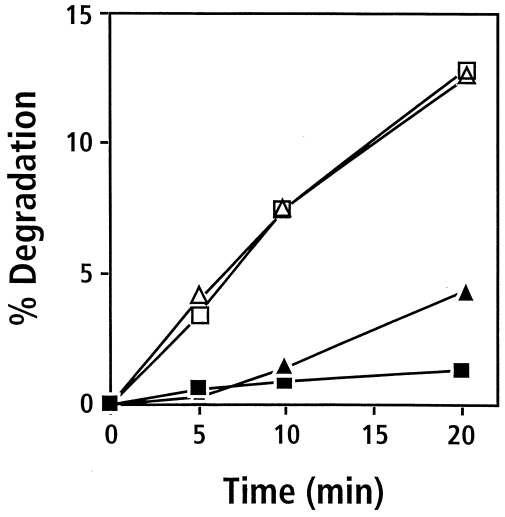

Fig. 3. Degradation of Ub-[^{125}I]-lysozyme conjugates in the presence or absence of lactacystin or S5a. [^{125}I]-lysozyme-Ub conjugates were incubated in reticulocyte lysate in the absence of lactacystin or S5a and degradation was measured by acid precipitation at the times shown (open triangles, open squares). Degradation in the presence of 500 μg/ml of S5a is denoted by the filled squares, and degradation in the presence of 500 μM lactacystin is represented by filled triangles.

[13], which would be absent from the 35 K ODC shortened by internal initiation. Second, the N-terminal sequences prevent the formation of a recognition site for Ub conjugation presumably generated by misfolding of the truncated ODC molecules.

The experiments presented above also show that one should exercise caution when Ub conjugates are detected from in vitro translations that contain multiple radiolabeled products. The existence of Ub conjugated to shortened translation products could lead to an incorrect conclusion that the full-length protein is a normal physiological substrate of the Ub pathway. Finally, this report demonstrates that the 26S proteasome is not directly inhibited by S5a as shown by the unimpaired degradation of full-length ODC in the presence of S5a. This finding is consistent with the idea that excess S5a binds Ub chains masking their recognition by the 26S proteasome. Because S5a only inhibited proteolysis of the lower molecular weight ODC translation products, its use permits discrimination between Ub-dependent and other proteolytic pathways. Thus, this proteasome subunit can be a valuable reagent for studying intracellular proteolysis.

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