

The 26 S proteasome is activated at two points in the ascidian cell cycle

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The proteasome undergoes cell cycle-dependent changes in its subcellular distribution during ascidian embryonic development [(1992) *Dev. Biol.* 151, 27–33]. In this study, we demonstrate that the 26 S proteasome is markedly activated in both prophase and metaphase of the mitotic cell cycle in the ascidian embryos in comparison with the case of the 20 S proteasome. These results suggest that the 26 S proteasome is activated and participates in proteolysis at the restricted stages of the cell cycle.

Proteasome; Cell cycle; Cell division; Mitosis; Ascidian cell cycle

1. INTRODUCTION

It is well known that proteolysis plays an essential role in the progression of the eukaryotic cell cycle. In metaphase of the mitosis, cyclin, a regulatory subunit of M-phase promoting factor (MPF), is degraded, resulting in an inactivation of MPF. Studies using proteolysis-resistant cyclin mutants indicate that the degradation of cyclin is indispensable for the exit from mitosis [1,2]. Furthermore, several lines of evidence that cyclin is degraded by ubiquitin-dependent proteolytic system have been presented [3,4].

The proteasome, a high-molecular-weight (HMW) multicatalytic proteinase complex that comprises several nonidentical subunits [5,6], is thought to be a crucial component in the ubiquitin-dependent proteolytic system [7]. In the system, the proteasome functions in an ATP-dependent manner as a 26 S complex, which is assembled from a 20 S complex and other several regulatory subunits in the presence of ATP [8–10]. Thus it can be thought that cyclin is periodically ubiquitinated and degraded by the 26 S proteasome in an ATP-dependent manner in metaphase of the mitosis. However, whether the activity of the 26 S proteasome is periodically activated during the cell cycle has not yet been demonstrated.

In a previous immunocytochemical study [11], we have shown that the proteasome undergoes changes in its subcellular distribution, depending on the mitotic cell cycle, during ascidian embryonic development; it is localized at the condensed chromosomes in early metaphase and at the mitotic apparatus in late metaphase, and then it disappears around the mitotic apparatus in

anaphase. This observation suggests that the proteasome appears accumulated and functions at localized sites in metaphase. Since chymotrypsin-like activity, among several catalytic activities of the proteasome, appears most important for the ubiquitin-dependent proteolysis [12], we attempted to examine whether chymotrypsin-like activity of the proteasome is activated during the mitosis.

In this report, we present evidence for the first time that chymotrypsin-like activity of the 26 S proteasome assayed in the presence of ATP is activated at two points, i.e. in prophase and metaphase, during the mitotic cell cycle of the synchronously-dividing ascidian embryos. This result prompts us to propose that proteolysis of cell cycle-related proteins by the 26 S proteasome is required at least twice during the cell cycle.

2. MATERIALS AND METHODS

2.1. Gametes and blastomeres

Unfertilized eggs and spermatozoa of the ascidian *Halocynthia roretzi* were collected and fertilization between strictly self-sterile gametes was carried out as described previously [13]. The eggs derived from each individual animal were used in a series of experiments in order to ensure the synchronous division of the fertilized eggs. After insemination, follicle cells were removed from the vitelline coats of fertilized eggs with 0.55 M NaCl containing 0.1% EDTA, and then the eggs were cultured at 13°C. Synchronously-dividing blastomeres in the process of the third cleavage were harvested at 5 min intervals. One part of blastomeres for assay of proteasome activity and for gel filtration was immediately frozen at –40°C and was stored at –40°C until use, while the other for cytochemistry immediately was fixed with absolute methanol and ethanol at –20°C as described previously [11].

Immature eggs of *H. roretzi* were collected from the gonads excised from each individual.

2.2. Preparation of proteasome-containing HMW fraction

Immature eggs or blastomeres (0.3 g each) were homogenized in 2 ml of ice-cold buffer A (50 mM Tris-HCl, pH 8.0, containing 0.2 mM ATP, 1 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA and 1% glycerol). The homogenate was centrifuged at 10,000 × g for 25 min

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and then at 105,000 \times g for 10 min in a Hitachi RP100AT2 rotor. To the resulting supernatant was added glycerol to give a final concentration of 10% and then the mixture was again ultracentrifuged at 105,000 \times g for 5 h [14]. The resulting precipitate was dissolved in buffer B (buffer A except for 10% glycerol) and proteasome activity was assayed as described below.

2.3. Assay of proteasome activity

Chymotrypsin-like activity was assayed at 25°C in 50 mM Tris-HCl, pH 7.0, containing 0.5 mM ATP, 3.3 mM MgCl₂ and 0.1 mM bestatin (Peptide Institute, Osaka) using 0.02 mM succinyl(Suc)-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) (Peptide Institute) as a substrate as described previously [13].

Trypsin-like activity toward *t*-butyloxycarbonyl-Phe-Ser-Arg-MCA (Peptide Institute) and V8 protease-like activity toward benzoyloxycarbonyl-Leu-Leu-Glu- β -naphthylamide (Bachem) were assayed under the same conditions as described above. The appearance of fluorescence due to β -naphthylamine was monitored with excitation at 355 nm and emission at 410 nm.

2.4. Superose 6 gel filtration

Proteasome-containing HMW fractions were subjected to FPLC (Pharmacia) on Superose 6 (Pharmacia) column previously equilibrated with buffer C (buffer B containing 100 mM NaCl). The effluent was collected in 0.3 ml fractions at 4°C and then proteasome activity of each fraction was assayed in the presence of 0.5 mM ATP as described above.

2.5. Immunochemical analysis

Anti-proteasome rabbit serum was prepared as follows: ascidian 20 S proteasome was purified from eggs of *H. roretzi* as described previously [13]. The proteasome (0.03 mg) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously. An initial injection was followed by subcutaneous boosters of the same amount each with Freund's incomplete adjuvant at the 10th and the subsequent 10 days. The antibody was purified by 50% ammonium sulfate precipitation and protein A-Sepharose (Sigma) chromatography.

SDS-polyacrylamide gel electrophoresis was carried out in a slab gel containing 12% polyacrylamide according to the method of Laemmli [15]. Immunoblotting was performed according to Towbin et al. [16].

Immunoprecipitation experiments were performed as follows: purified anti-proteasome antibody (100 mg) was preincubated for 30 min at 4°C with protein A-Sepharose gel (0.1 ml) and the complex formed was washed several times with buffer C. To the proteasome-containing HMW fraction was added the complex and the suspension was allowed to stand for 2 h at 4°C. After centrifugation, proteasome activity in the resulting supernatant was assayed as described above.

2.6. Cytochemistry

Sections of fixed blastomeres were prepared and stained with 4',6-diamidino-2-phenylindole (DAPI, Nacalai Tesque, Kyoto), a DNA-labeling reagent, as described previously [11].

3. RESULTS

3.1. 660 kDa and 1000 kDa proteasomes are present in ascidian eggs and both are recognized by anti-20 S proteasome antibody

Several lines of evidence suggest that the proteasome is present as 20 S and 26 S complexes [8–10]: the 26 S complex is formed from the 20 S complex and other regulatory subunits, and its formation is stimulated by ATP.

To examine whether the 26 S proteasome, in addition to the 20 S proteasome [13], is present in ascidian eggs,

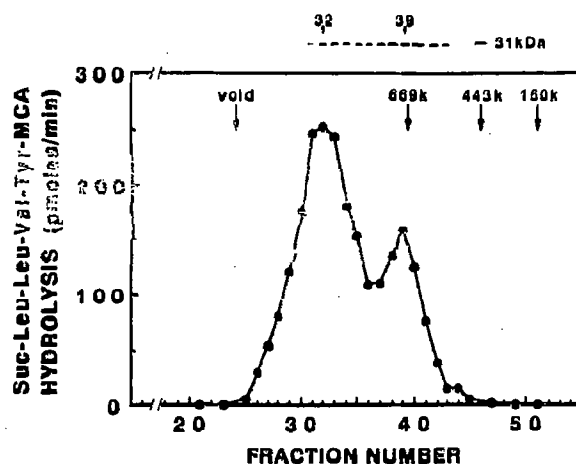


Fig. 1. Gel filtration on Superose 6 FPLC of proteasome-containing HMW fraction prepared from ascidian eggs. The activity toward Suc-Leu-Leu-Val-Tyr-MCA was assayed in the presence of 0.5 mM ATP. The upper panel shows immunoblots of gels containing samples in individual fractions. Molecular weight markers used were thyroglobulin (669 kDa), apoferritin (443 kDa) and alcohol dehydrogenase (150 kDa).

we prepared the proteasome-containing HMW fraction from immature eggs in the presence of ATP and subjected it to Superose 6 gel filtration under the conditions in which ATP was included (Fig. 1). Chymotrypsin-like activity toward Suc-Leu-Leu-Val-Tyr-MCA assayed in the presence of ATP was detected in two peaks, the molecular masses of which were 660 kDa and 1000 kDa. The molecular mass of the smaller chymotrypsin-like enzyme coincides with that of the 20 S proteasome isolated previously [13]. Anti-20 S proteasome antibody, which mainly recognizes the 31 kDa proteasome subunit, reacted with the same 31 kDa component contained in the 1000 kDa and 660 kDa protease complexes (Fig. 1, upper panel), indicating that the 1000 kDa protease complex contains the subunit common to that of the 660 kDa proteasome. These results led us to conclude that both the 20 S and 26 S proteasomes are present in ascidian eggs and that conventional procedures including ultracentrifugation [14] for preparation of the proteasome-containing HMW fraction are useful to collect both the proteasomes at the same time.

3.2. 26 S proteasome activity is activated at certain stages of the mitotic cell cycle

We have previously reported that the proteasome undergoes changes in its subcellular distribution, depending on the mitotic cell cycle [11]. To investigate whether the activity of proteasome also oscillates in a cell cycle-dependent manner, we measured the chymotrypsin-like activity in the presence of ATP of the proteasome-containing HMW fraction obtained from embryos at various stages during progress from the second cleavage to the third (Fig. 2). The chymotrypsin-like activity was

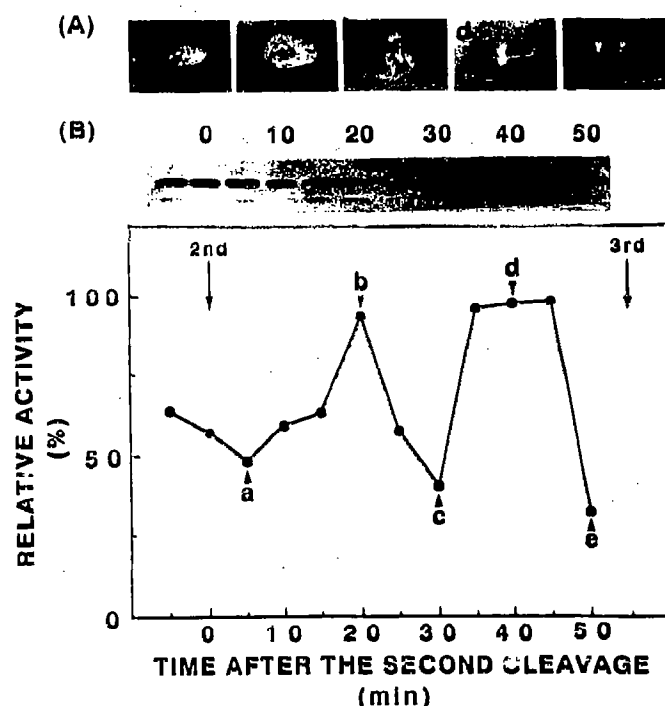


Fig. 2. Chymotrypsin-like activity of proteasome in the mitotic cell cycle from the second cleavage to the third. The highest activity was defined as 100%. The upper panel (A) shows photographs of samples fixed at the indicated stages (a-e) and stained with DAPI. The upper panel (B) shows immunoblots of gels containing samples at individual times. The second cleavage begins at 183 min and ceases at 237 min after insemination.

found to be activated twice in one mitotic cell cycle, i.e. around 20 and 40 min after the beginning of the second cleavage. The trypsin-like and V8 protease-like activities were also periodically activated in a similar manner (data not shown). The two activation stages can be defined as prophase, a stage when the nuclear envelope begins to break down, and metaphase, a stage when the condensed chromosomes align at the metaphase plate (Fig. 2, upper panel (A), b and d). Immunoblot analysis using anti-20 S proteasome antibody (Fig. 2, upper panel (B)) indicates that at least the 31 kDa proteasome

subunit remains constant during progress throughout the cycle. Thus, the sum of the 20 S and 26 S proteasomes appears unchanged during the mitotic cell cycle.

Next, to define whether the proteasome is responsible for the chymotrypsin-like activity assayed in the presence of ATP, we immunoprecipitated the proteasome from the proteasome-containing HMW fraction, which had been obtained from embryos at various stages of the cycle, by using anti-20 S proteasome antibody. This treatment caused a large reduction in the chymotrypsin-like activity in any embryo (the residual activity was less than 5% of the original one), whereas the treatment with control IgG scarcely inhibited the activity.

Finally, we compared gel filtration patterns between the proteasome-containing HMW fraction at the activation stage and that at the resting stage in order to define which is activated, the 20 S proteasome (660 kDa) or the 26 S proteasome (1000 kDa). Comparison of the proteasomes in prophase (Fig. 3a) with those in prometaphase (Fig. 3b) is shown in Fig. 3. The large difference was detected in the 26 S proteasome: the chymotrypsin-like activity of the 26 S proteasome was markedly activated at the activation stage in comparison with the case of the 20 S proteasome. The result at the other activation stage, i.e. in metaphase, was the same as that in prophase (data not shown). Taking account of the constant level of the 31 kDa proteasome subunit during the cell cycle, we conclude that the 26 S proteasome is activated with or without assembly from the 20 S proteasome in prophase and metaphase of the mitotic cell cycle.

4. DISCUSSION

In this report, we have demonstrated that the 26 S proteasome activity is activated at least in prophase and metaphase of the mitotic cell cycle. Although the extent of activation depends on the time to harvest embryos and also on the batch of embryos, the above observation is reproducible in different batches of embryos. Thus it is likely that the 26 S proteasome is transiently

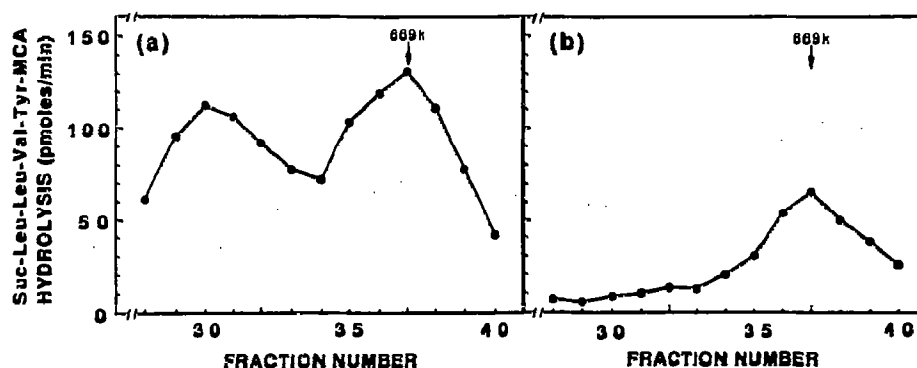


Fig. 3. Comparison in gel filtration patterns of proteasomes between prophase (a) and prometaphase (b).

activated at two stages of the cell cycle and functions in ATP-dependent proteolysis. The present observation that the proteasome activity oscillates during the cell cycle appears closely related to our previous finding that the proteasome undergoes cell cycle-dependent changes in its distribution [11]. This is the first report on the cell cycle-dependent activation of the proteasome.

It has been proposed that chymotrypsin-like activity of proteasome is essential for the ubiquitin-dependent proteolysis [12] and that the 26 S proteasome assembled from the 20 S proteasome in the presence of ATP functions in the ATP-dependent degradation of ubiquitin-conjugated proteins [8–10]. Based on these proposals, we analyzed in this study cell cycle-dependent changes of the 26 S proteasome activity by measuring mainly chymotrypsin-like activity in the presence of ATP. As a result, analysis by gel filtration clearly indicates that the 26 S proteasome, measurable by this assay, is markedly activated in comparison with the case of the 20 S proteasome. The following mechanisms can be considered for the activation of the 26 S proteasome; the latent 26 S proteasome preformed is activated through its conformational change induced by some signals (for example, phosphorylation [17]), or through association of activators [18] or dissociation of inhibitors [19]. Another possibility is that the assembly of the 26 S proteasome from the 20 S proteasome and other regulatory components is a key event. To test such possibilities, further studies on the analyses of subunit compositions in the activated and resting forms of the 26 S proteasome are now in progress.

In the eukaryotic cell cycle, proteolysis has been reported to occur at least in mitosis [1–4]; i.e. cyclin A and B are degraded during the prophase–metaphase transition and the metaphase–anaphase transition, respectively [20–22]. Our present demonstration on the activation of the proteasome in metaphase is well consistent with the above observations. On the other hand, our result on the activation in prophase implies that additional proteolysis is required in prophase of the mitotic cell cycle. In this study, we used the blastomeres which divide in a biphasic cell cycle between the S state and the M state. Therefore, the prophase in this case can be considered as not only the beginning of the M state but also the end of the S state (or interphase). The demonstration that several DNA-binding regulatory proteins are degraded by the ubiquitin-dependent proteolytic system [23,24], together with the above considerations, led us to propose that the proteolysis mediated by the

26 S proteasome is also required for the exit from the S state (or interphase) in a cell cycle common to somatic cells. Alternatively, such an event is required for the initiation of mitosis. Further studies to search natural substrates degraded prior to mitosis, as well as in mitosis, are necessary to elucidate the physiological roles of the proteasome in the progression of the cell cycle.

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