BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 224–228 (1996)
Article No. 0039

Activation of the Proteasome during *Xenopus* Egg Activation Implies a Link between Proteasome Activation and Intracellular Calcium Release

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Received November 21, 1995

Ubiquitin-dependent proteolysis is required for cell cycle progression. Here, we demonstrate that the proteasome is activated during *in vivo Xenopus* egg activation, induced by treatment with the calcium ionophore A23187. It was found that activation is due to the calcium-induced assembly of the 26 S proteasome from the 20 S proteasome. We propose that proteasome activation is regulated by cell cycle calcium transients, which are controlled upstream by an endogenous cell cycle oscillator that is independent of the cyclin-dependent kinase cycle.

Accumulating evidence indicates that the proteolysis is needed to progress from one stage of the cell cycle to the next. During the metaphase-anaphase transition, cyclin B, a regulatory subunit of M phase-promoting (maturation-promoting) factor, is multiubiquitinated and then degraded by the proteasome (1–2). In addition, the ubiquitin-dependent proteolysis of other unidentified proteins have been reported to be required for the separation of sister chromatids (3). In G1 or G1/S phase of the yeast cell cycle, G1 cyclins and inhibitors of cyclin-dependent kinases (CDKs) are degraded through the ubiquitin-dependent proteolytic pathway (4–7). Recently, in the mammalian cell cycle too, a CDK inhibitor has been reported to be degraded through the same proteolytic pathway (8).

The ubiquitin-dependent proteolytic pathway consists of two systems (for reviews, 9–12); one is the ubiquitinating enzyme system, and the other is the proteolytic machinery (proteasome) system. The fact that ubiquitin-dependent proteolysis of the particular proteins at particular stages of the cell cycle is required for cell cycle progression implies that this pathway and/or the target proteins must be activated/modified at specific points in the cell cycle. In previous studies (13–15), we found that the proteasome undergoes a change in subcellular distribution during the ascidian embryonic cell cycle. The 26 S proteasome is activated during the cell cycle, and proteasome activation, which is due to the assembly of the 26 S proteasome from the 20 S proteasome, is triggered by intracellular calcium release. From these results, we formulated the idea that the proteasome system is regulated by intracellular calcium release, which is thought to regulate the timing of cell cycle progression (16–18). Contrary to our results, Mahaffey *et al.* have reported that the activity of the 26 S proteasome in degrading ubiquitinated proteins remains constant throughout the cell cycle in a cycling cell-free extract of *Xenopus* eggs (19). To determine whether the discrepancy is due to a difference between the animals used, or to a difference between *in vivo* and *in vitro* systems, we measured proteasome activity during *in vivo Xenopus* egg activation, induced by treatment with calcium ionophore. Here, we report that the 26 S proteasome is transiently activated in response to intracellular calcium release in the *in vivo* system of *Xenopus* eggs.

MATERIALS AND METHODS

BAPTA-AM was purchased from Molecular Probes Inc. Polyclonal antibody to *Xenopus* 20 S proteasome was prepared as described previously (20).

The frog, *Xenopus laevis,* was induced to ovulate by injection of human chorionic gonadotropin (500 units). Eggs were

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0006-291X/96 $12.00

collected in De Boer solution (110 mM NaCl, 1.3 mM KCl, and 1.3 mM $CaCl_2$, pH 7.4), dejellied in 2% cysteine (pH 7.8), and washed four times with 2-fold diluted De Boer solution. Washed eggs were activated by 4 μM calcium ionophore A23187 (Sigma) in dimethylsulfoxide, collected at the respective times, and immediately frozen. The eggs were then crushed in buffer A (20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.2 mM ATP, 1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM EGTA, and 1% glycerol) by centrifugation (10,000×g, 15 min) at 4°C, and the resulting supernatant was again centrifuged at 105,000×g for 20 min. To the resulting supernatant was added glycerol to give a final concentration of 10% and the mixture was ultracentrifuged at 105,000×g for 5 h at 4°C. The precipitates were used as the proteasome-containing fraction. Protein concentration was estimated by the method of Bradford (21).

Chymotrypsin-like, trypsin-like, and V8 protease-like activities were assayed as described previously (14). Histone H1 kinase assay was performed according to the procedure described by Felix *et al.* (22).

Immunoprecipitation experiments were performed as described previously (14). The proteasome-containing fraction was subjected to gel filtration on a column of Superose 6 (Pharmacia) in buffer B (20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.2 mM ATP, 1 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol) at 4°C. The eluate was collected in 0.3 ml fractions at a flow rate of 0.3 ml/min, and used for enzymatic assay and for SDS-polyacrylamide gel electrophoresis (23), followed by western blot analysis; blots were reacted with a 1:2000 dilution of the anti-*Xenopus* 20 S proteasome antibody. A horseradish peroxidase-labeled goat anti-rabbit secondary antibody was used for detection. For signal detection, the ECL western blotting detection system (Amersham) was employed.

RESULTS

We have previously reported that the proteasome is activated during the meiotic and mitotic division cycles of ascidian eggs (14, 15). First, to define whether such a change in proteasome activity is detected in other systems than ascidian eggs, we examined the change during the activation of *Xenopus* eggs because there is little apparent change of proteasome activity in a cycling *Xenopus* egg extract (19). Calcium ionophore A23187 can activate unfertilized frog eggs and induce the metaphase-anaphase transition, which can be monitored by the appearance of pigment contraction. As shown in Fig. 1 (A), a chymotrypsin-like activity in the proteasome-containing fraction was transiently activated around 2 min after egg activation induced by treatment with calcium ionophore A23187. In controls, there was little significant change of activity when dimethylsulfoxide alone was added. Trypsin-like and V8-protease-like activities also increased during egg activation (data not shown). To confirm that cell cycle progression occurred in the eggs, in which we measured changes in proteasome activity, we checked for alterations in histone H1 kinase activity during egg activation. As shown in Fig. 1 (B), histone H1 kinase activity decreased after the A23187 treatment, which is characteristic of the metaphase-anaphase transition of meiosis II.

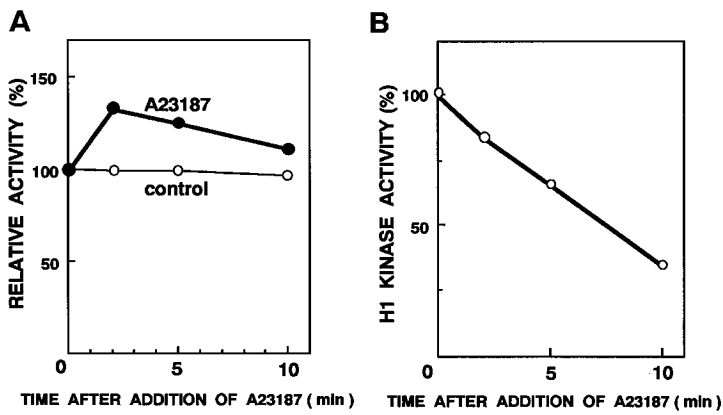

FIG. 1. Changes in the activities of the proteasome and histone H1 kinase during *Xenopus* egg activation. (A) Chymotrypsin-like activity of the proteasome-containing fraction prepared from eggs, which had been treated with 4 μM A23187 in 0.01% dimethylsulfoxide (–●–) or with 0.01% dimethylsulfoxide only (–○–) and harvested at the times indicated. (B) Histone H1 kinase activity of the 10,000xg supernatant prepared at the times indicated. The activity in unfertilized eggs was defined as 100%.

To test whether the change of chymotrypsin-like activity was due to proteasome activity, we immunoprecipitated the proteasome using the antibody to *Xenopus* 20 S proteasome and measured the activity of the resulting supernatant. The supernatant immunodepleted by treatment with anti-proteasome antibody had little chymotrypsin-like activity (less than 5% of the original activity), while the activity after treatment with control γ-immunoglobulin remained unchanged. These results indicate that the activity observed was completely due to the proteasome. We then measured total proteasome levels, i.e., the sum of the 20 S and the 26 S proteasomes, by western blotting using anti-*Xenopus* 20 S proteasome antibody. The total amount of proteasome remained constant throughout egg activation (data not shown).

Next, the proteasome-containing fractions were subjected to Superose 6 gel filtration, to determine the relative proportion of the 20 S and the 26 S proteasomes. Chromatographic patterns at various times after egg activation showed that the activity of the 26 S proteasome, but not that of 20 S proteasome, was changed during egg activation; the highest activity was observed at 2 min after egg activation and then the activity decreased. In addition to the changes in activity, the amount of the 26 S proteasome was also found to be increased at 2 min after egg activation, as revealed by western blotting of each of the fractions obtained from the gel filtration column (Fig. 2). Furthermore, the amount of the 26 S proteasome varied as inversely with that of the 20 S proteasome, which indicates that the activation of 26 S proteasome is due to the assembly of the 26 S proteasome from the 20 S proteasome.

Intracellular calcium release plays an important role in the egg activation and the progression of cell cycle (16–18). To determine whether intracellular calcium release was essential for proteasome activation, we examined the effect of pretreatment with a cell-permeable calcium chelating agent, BAPTA-AM, on the activation of the proteasome in the absence of extracellular calcium. Pretreatment with BAPTA-AM completely blocked the activation of proteasome, while proteasome activity was transiently increased in the control experiment, as before (Fig. 3). This result suggests that proteasome activity is regulated by intracellular calcium release during *Xenopus* egg activation.

DISCUSSION

In this report, we have shown that the proteasome is activated during *in vivo Xenopus* egg activation and that activation of the proteasome is due to the interconversion between the 20 S and the 26 S forms, triggered by intracellular calcium release. This event in the *Xenopus* system, in which the resumption of the meiosis II is accompanied by the egg activation, is almost equivalent to the ascidian system, in which the meiosis I resumes upon egg activation due to calcium (15). In addition, the same event also occurs during the metaphase-anaphase transition, as well as at prophase, in the ascidian egg mitotic cleavage cycle (14). It should be noted that intracellular

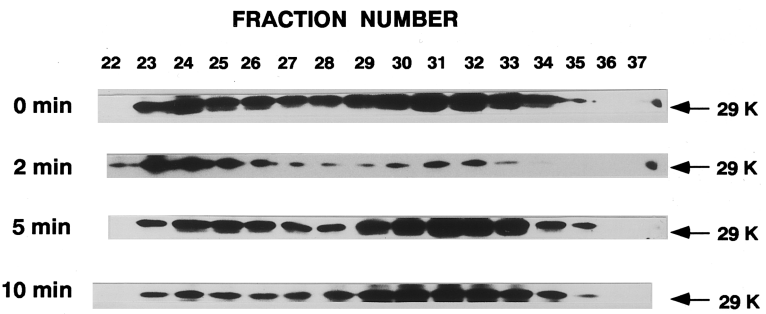

FIG. 2. Western blotting of the 20 S and 26 S proteasome-containing fractions prepared at the times indicated and separated by Superose 6 gel filtration. The 26 S and the 20 S proteasomes emerged in fractions 22–27 and 28–34, respectively. Note that anti-20 S proteasome antibody mainly recognized the 29K proteasome subunit.

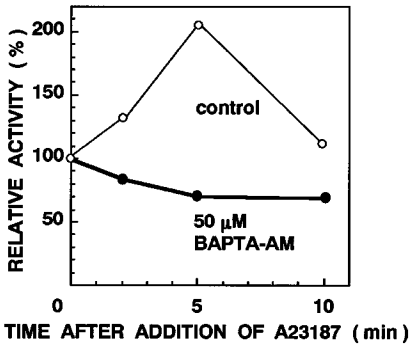

FIG. 3. Effect of pretreatment with BAPTA-AM on the change in the activity of the proteasome during *Xenopus* egg activation. The proteasome-containing fractions were prepared at the times indicated from A23187-activated eggs, which had been pretreated with 0.05 mM BAPTA-AM in 0.1% dimethylsulfoxide (–●–) or with 0.1% dimethylsulfoxide only (–○–) for 3 h in calcium-free De Boer solution. Their chymotrypsin-like activity was assayed and the activity in unfertilized eggs was defined as 100%.

calcium transients are observed at metaphase, as well as at prophase, in the sea urchin egg mitotic cleavage cycle (18). Thus, *in vivo* proteasome activation triggered by intracellular calcium release is a common event at least in embryonic meiotic and mitotic cell cycles.

Contrary findings have been reported that indicate that 26 S proteasome activity remains unchanged in a cycling *Xenopus* cell-free extract, in which the metaphase-anaphase transition occurs (19). With respect to the discrepancy between these, it should be noted that cell cycle progression in the above *in vitro* system is controlled by activation and inactivation of CDKs, whose activities are regulated by synthesis and degradation of cyclin, together with by phosphorylation and dephosphorylation of CDKs (24). In our *in vivo* system, however, progression of the cell cycle is triggered by intracellular calcium release, which also induces activation of the proteasome. In connection with this, Whitaker and his colleagues have reported that inositol 1, 4, 5-trisphosphate oscillates even in the absence of the CDK cycle, and have proposed that an endogenous oscillator, which is independent of the cyclin oscillator, controls the timing of the cell cycle through inositol 1,4,5-triphosphate production and subsequent intracellular calcium release (17). On the basis of their proposal, it can be inferred that proteasome activation may be regulated by the putative endogenous oscillator rather than by the CDK cycle.

Two mechanisms for the regulation of cell cycle progression through the ubiquitin-dependent proteolytic pathway have been presented; one mechanism works at the level of multiubiquitination at M phase, in which a cyclin B-specific ubiquitin protein ligase (E3) that exists in a large protein complex called the cyclosome/anaphase-promoting complex is activated by CDC 2 kinase (25–27). The other mechanism involves the modification of substrates destined to be multiubiquitinated; the phosphorylation of G1 cyclins and CDK inhibitors targets these proteins for degradation (4–7). Our finding that activation of the proteasome is triggered by intracellular calcium release suggests the presence of the third regulatory mechanism at the level of proteolysis. In summary, we propose that the third regulatory mechanism plays an important role *in vivo* in the determination of the timing of the embryonic meiotic and mitotic cell cycles. Further studies are necessary to clarify the detailed mechanisms functioning upstream of the proteasome.

ACKNOWLEDGMENTS

We thank Professor Michael Whitaker for helpful discussion and correction of our manuscript. This study was in part supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan, from the Takeda Science Foundation, and from the Japan Foundation for Applied Enzymology.

REFERENCES

1. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) *Nature* **349,** 132–138.
2. Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R. E., and Cohen, L. H. (1991) *J. Biol. Chem.* **266,** 16376–16379.
3. Holloway, S. L., Glotzer, M., King, R. W., and Murray, A. W. (1993) *Cell* **73,** 1393–1402.
4. Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992) *EMBO J.* **11,** 1773–1784.
5. McKinney, J. D., Chang, F., Heintz, N., and Cross, F. R. (1993) *Genes & Dev.* **7,** 833–843.
6. Schwob, E., Bohm, T., Mendehnall, M. D., and Nasmyth, K. (1994) *Cell* **79,** 233–244.
7. Deshaies, R. J., Chau, V., and Kirschner, M. (1995) *EMBO J.* **14,** 303–312.
8. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) *Science* **269,** 682–685.
9. Hershko, A., and Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61,** 791–807.
10. Tanaka, K., Tamura, T., Yoshimura, T., and Ichihara, A. (1992) *New Biol.* **4,** 173–187.
11. Ciechanover, A. (1994) *Cell* **79,** 13–21.
12. Peters, J.-M. (1994) *Trends Biochem. Sci.* **19,** 377–382.
13. Kawahara, H., and Yokosawa, H. (1992) *Dev. Biol.* **151,** 27–33.
14. Kawahara, H., Sawada, H., and Yokosawa, H. (1992) *FEBS Lett.* **310,** 119–122.
15. Kawahara, H., and Yokosawa, H. (1994) *Dev. Biol.* **166,** 623–633.
16. Whitaker, M., and Patel, R. (1990) *Development* **108,** 525–542.
17. Ciapa, B., Pesando, D., Wilding, M., and Whitaker, M. (1994) *Nature* **368,** 875–878.
18. Whitaker, M. (1995) *Adv. Second Messenger and Phosphoprotein Res.* **30,** 299–310.
19. Mahaffey, D., Yoo, Y., and Rechsteiner, M. (1993) *J. Biol. Chem.* **268,** 21205–21211.
20. Tanaka, K., Yoshimura, T., Kumatori, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K., and Takagi, T. (1988) *J. Biol.Chem.* **2638,** 16209–16217.
21. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
22. Felix, M. A., Pines, J., Hunt, T., and Karsenti, E. (1989) *EMBO J.* **8,** 3059–3069.
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
24. Pines, J. (1995) *Biochem. J.* **308,** 697–711.
25. Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L. H., Luca, F. C., Ruderman, J. V., and Eytan, E. (1994) *J. Biol. Chem.* **269,** 4940–4946.
26. Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. (1995) *Mol. Biol. Cell* **6,** 185–198.
27. King, R. W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. W. (1995) *Cell* **81,** 279–288.