Degradation of polyubiquitinated cyclin B is blocked by the MAPK pathway at the MI arrest in starfish oocytes

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SUMMARY

In the starfish ovary, maturing oocytes stimulated by 1-methyladenine undergo synchronous germinal vesicle breakdown (GVBD) and then arrest in metaphase of the first meiotic division (metaphase I [MI]). Immediately after spawning, an increase of intracellular pH (pHi) from ~7.0 to ~7.3 is induced by Na⁺/H⁺ antiporter in oocytes, and meiosis reinitiation occurs. Here we show that an endogenous substrate of the proteasome, polyubiquitinated cyclin B, was stable at pH 7.0, while it was degraded at pH 7.3. When the MAP kinase pathway was blocked by MEK inhibitor U0126, degradation of polyubiquitinated cyclin B occurred even at pH 7.0 without an increase of the peptidase activity of the proteasome. These results indicate that the proteasome activity at pH 7.0 is sufficient for degradation of polyubiquitinated cyclin B and that the MAP kinase pathway blocks the degradation of polyubiquitinated cyclin B in the maturing oocytes in the ovary. Immediately after spawning, the increase in pHi mediated by Na⁺/H⁺ antiporter cancels the inhibitory effects of the MAP kinase pathway, resulting in the degradation of polyubiquitinated cyclin B and the release of the arrest. Thus, the key step of MI arrest in starfish oocytes occurs after the polyubiquitination of cyclin B but before cyclin B proteolysis by the proteasome.
INTRODUCTION

In many animals, oocytes are blocked twice in meiosis. Usually, the length of the first arrest at prophase I is extremely long. The release from this arrest is generally triggered by hormonal stimuli that drive the oocyte to the second arrest at metaphase I (MI) in many invertebrates, including ascidians, several molluscan species and Drosophila, or at metaphase II (MII) in vertebrates. The metaphase state is established by the activity of the complex of cyclin B and cdc2 kinase (1-6). The metaphase/anaphase transition is induced by the ubiquitin-dependent degradation of cyclin B. The formation of ubiquitin conjugates requires the concerted activity of a series of enzymes that first activate ubiquitin (E1) and then recognize and transfer ubiquitin (E2 and E3) to proteins destined for turnover (for review, see Ref. 7). Cyclin B is polyubiquitinated by a specific E3 called the anaphase-promoting complex/cyclosome (APC/C, 8); for a review, see Ref. 9. Once such targeted proteins become polyubiquitinated, they are recognized and degraded by a particle known as the 26S proteasome.

Although the mechanisms of metaphase arrest in meiosis I (MI arrest) in invertebrate oocytes are poorly understood, MII arrest in vertebrate unfertilized eggs has been well studied. MII arrest is
mediated by an activity known as cytostatic factor (CSF, 10), which stabilizes cyclin B. The expression of c-Mos, which is a MAP kinase kinase kinase, in one blastomere of a two-cell *Xenopus* embryo leads to CSF arrest (11). It has also been shown that microinjection of thiophosphorylated MAP kinase into one blastomere of a two-cell embryo induced metaphase arrest similar to that induced by c-Mos (12). A MAP kinase target, the protein kinase p90Rsk, was shown to be the sole mediator of CSF arrest: a constitutively active p90Rsk causes CSF arrest in the absence of an active MAP kinase pathway, and depletion of p90Rsk from egg extracts removes CSF activity, which can be restored by re-addition of p90Rsk (13, 14).

Bub1 acts downstream of p90Rsk and may be an effector of APC inhibition and CSF-dependent metaphase arrest by p90Rsk (15). Furthermore, Emi1 acts to prevent cyclin B destruction through APC inhibition in MII and is required for the maintenance of CSF arrest in *Xenopus* eggs (16). Fertilization causes a transient increase in cytoplasmic calcium concentration, leading to CSF inactivation.

Calmodulin-dependent protein kinase II (CaMKII) is required for release of MII, and a constitutively active CaMKII is sufficient to trigger cyclin B destruction and mitotic exit without fertilization or the addition of calcium (17).

Fully grown starfish oocytes are arrested at the G2/M-phase border of meiosis I. Meiosis is reinitiated in response to 1-methyladenine (1-MA) which is released from surrounding follicle cells (18).
The receptor of 1-MA on the plasma membrane is coupled to the αβγ trimeric G protein (19-22). The hormonal stimulation dissociates Gβγ from Gα, and the dissociated Gβγ activates PI3-kinase and forms a maturation-promoting factor (MPF) in the cytoplasm (23-27). Activation of MPF is achieved via the activation of cdc2. Recently, Okumura et al. (28) showed that Akt is a downstream signaling molecule of PI3K, and then phosphorylates and inactivates Myt1, the inhibitory kinase of cdc2. Active MPF eventually induces germinal vesicle breakdown (GVBD).

It has been widely believed that in starfish oocytes the meiotic cycles are completed without MI or MII arrest. Recently, however, we found that maturing oocytes after GVBD undergo arrest again at MI in the starfish ovary. The MI arrest is dependent on low intracellular pH (pHi) and active MAP kinase. Release from the arrest is induced by a rise in the pHi after spawning (29). In the current study, using cell-free preparations, we show that polyubiquitinated cyclin B remained stable at low pH. When MAP kinase was inhibited by U0126, degradation of polyubiquitinated cyclin B occurred even at low pH. These results indicate that MI arrest is regulated by the process that occur after polyubiquitination of cyclin B but before proteolysis by the proteasome.
EXPERIMENTAL PROCEDURES

Animals and Oocytes

Starfish Asterina pectinifera were collected on the Pacific coast of Honshu Island and kept in laboratory aquaria supplied with circulating seawater at 15°C. Isolated ovaries were incubated in ice-cold calcium-free seawater (CFSW). To remove follicle cells, the released oocytes were washed twice in ice-cold CFSW and stored in artificial seawater (ASW) at 20°C. To remove jelly and vitelline envelopes, oocytes were treated with artificial seawater containing 0.1 mg/ml pronase (Kaken Seiyaku) for 10 min at 20°C, washed several times with cold CFSW and kept in ASW at 20°C. These oocytes were at the first meiotic prophase and are referred to as "immature". Oocyte maturation was induced by the addition of 1 μM 1-MA. We refer to the oocytes that are undergoing GVBD (about 13 min after 1-MA addition) and that have MPF activity as "maturing oocytes".

Preparation of the Oocyte Supernatant
The oocyte supernatant was prepared as described previously (30). Briefly, immature or maturing oocytes (1 ml) were washed twice in 10 ml of ice-cold buffer P (150 mM glycine, 100 mM EGTA, 200 mM Hepes buffer, pH 7.0). After the oocytes were sedimented by gravity, as much buffer P was removed as possible. Sedimented oocytes were transferred to a net of 60-µm mesh in the neck of the microtube and pressed onto the net with the cap of the tube. When the tube was centrifuged at 1,400×g for 3 sec, these oocytes were homogenized by passage through the net. The homogenate was centrifuged at 20,000×g for 15 min. The supernatant was transferred to a microtube, frozen in liquid nitrogen and kept at -80°C. Before use, the frozen supernatant was thawed at 20°C and kept on ice.

The cell-free preparation at pH 7.2 or 7.3 was generated by addition of 1/5 volume of buffer P at pH 7.7 or 8.4, respectively, to the cell-free preparation at pH 7.0.

Materials

Succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) and 7-amino-4-methyl-coumarin (AMC) were purchased from Peptide Institute, Osaka. Bestatin (Sigma) was dissolved in distilled water. MG115, ubiquitin aldehyde (Peptide Institute, Inc), U0126 (Promega, Madison) and
U0124 (Calbiochem, La Jolla) were dissolved in dimethyl sulfoxide (DMSO). These solutions were stored at -20°C and thawed immediately prior to use. Ubiquitin was obtained from Sigma.

Measurement of Proteasome Activity

Determination of the initial rate of hydrolysis of Suc-Phe-Leu-Arg-CAMS (V₀) and estimation of pH increase were performed as described previously (31, 32). Briefly, the fluorescent products (ACMS) of hydrolysis at a concentration of 6.3 µM were injected into oocyte cytoplasm, along with the exopeptidase inhibitor bestatin (800 µM). Next, the fluorogenic substrate Suc-Phe-Leu-Arg-CAMS (63 µM) was injected at the indicated time to obtain the values of V₀. Fluorescence from an oocyte injected with ACMS or Suc-Phe-Leu-Arg-CAMS was collected with a 20 x, 0.5 N.A. objective and focused onto a photomultiplier (Nikon, P1) mounted on an inverted fluorescence microscope TMD with a xenon lamp (Nikon). The photomultiplier was connected to a pen recorder Type 3066 (Tosoh). To measure fluorescence intensity, an excitation filter at 380 ± 10 nm, a dichroic beam splitter at 400 nm, and a 450 nm emission filter were used. V₀ was expressed as µmol liberated ACMS/liter cytoplasm/min (µM/min).
The cell-free preparation was treated with 200 µM bestatin for 10 min at 20°C. Then proteolytic reactions were carried out in the cell-free preparation (pH 7.0, 7.2 and 7.3) using 100 µM Suc-Leu-Leu-Val-Tyr-MCA at 20°C. Fluorescence was measured (excitation, 380 nm; emission, 460 nm) using a fluorescence spectrophotometer (650-10S; Hitachi).

*Determination of pH with BCECF-dextran*

A dextran (10 kDa)-conjugate of 2', 7'-bis[2-carboxyethyl]-5-[and-6]-carboxyfluorescein (BCECF) (Molecular Probes) was dissolved at 2 mM in aspartate buffer (100 mM potassium aspartate, 20 mM Hepes, pH 7.2). The volume injected was 2% of the total oocyte volume. To estimate pH, an inverted light microscope (DMIRB; Leica) was connected via an adapter tube to the HiSCA CCD camera (C6790) of the ARGUS/HiSCA image processing system (Hamamatsu Photonics K. K.). Excitation light from a xenon lamp was alternated between 450 and 490 nm under computer control (C6789; Hamamatsu Photonics K. K.). The emitted light passed through a dichroic beam splitter at 510 nm and through a 515-to 560-nm emission filter (Leica). The ratios of the emission intensities at 490/450 nm were calculated
using the ARGUS/HiSCA image processing system. For calibration, oocytes injected with BCECF were treated with model intracellular medium containing 300 mM glycine, 175 mM KCl, 185 mM mannitol, 20 mM NaCl, 5 mM MgCl₂, 25 mM HEPES, and 25 mM Pipes, adjusted to the various pHs with KOH, and with 100 µM digitonin to permeabilize the oocytes. The ratio of emission intensities from alternate excitation with 490- and 450-nm light increased linearly with increasing pH from 6.5 to 7.7. Using these intracellular calibration data, the change of pHi was measured. In some experiments, oocytes were injected with at different pHs buffers (pH 7.5 or 6.8) containing 300 mM HEPES and 300mM Pipes. To block Na⁺/H⁺ antiporter, the oocytes were treated with zero-sodium artificial SW (0NaSW) containing 480 mM choline chloride, 55 mM MgCl₂, 10 mM CaCl₂, 5 mM KCl, 2.5 mM KHCO₃, pH 8.0 adjusted with KOH.

**SDS-PAGE and Western Blot Analysis**

Oocytes and the cell-free preparations were boiled for 5 min in sample buffer, subjected to electrophoresis using 10% SDS-polyacrylamide gels and transferred to a PVDF membrane (Millipore).
The membrane was blocked with PBS-T (phosphate-buffered saline/0.05% Tween-20) containing 5% skim milk, and incubated with an anti-starfish cyclin B antibody at 1:1000 dilution or an anti-rat MAP kinase R2 antibody (Seikagaku Corp) at 1:1000 dilution for 1h at room temperature. After the membrane was washed with PBS-T, it was incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1000) for 1h. After the membrane was washed, bound antibodies was detected using chemiluminescent substrate (ECL; Amersham Pharmacia Biotech) and a LAS-1000 lumino image analyzer (Fuji Photo Film Co., Ltd).

**Immunoprecipitation**

An anti-ubiquitin (Ub) monoclonal antibody (Santa Cruz Biotechnology) was mixed with protein A-cellulofine (Chisso Corporation), and incubated overnight at 4°C. The antibody-protein A-cellulofine complex was recovered by centrifugation at 2,500 \( \times \) g for 10 sec, and washed three times with PBS (phosphate-buffered saline) containing 1% BSA and 0.5% Tween-20. After the cell-free preparation was incubated for 30 min at 20°C, it was treated with 200 \( \mu \)M MG115 for 20 min on ice. The cell-free preparation was centrifuged at 50,000 \( \times \) g for 40 min and the supernatant was diluted 10-fold in buffer A.
(0.5% Tween-20, 1% Triton X-100 and 0.5% cholic acid in PBS) containing 200 µM MG115. It was incubated for 2 h at 4°C with an anti-Ub monoclonal antibody bound to protein A-cellulofine. In the control experiment, protein A-cellulofine without the anti-Ub monoclonal antibody was used. The immunocomplexes were washed two times with buffer A, boiled for 5 min in sample buffer, and analyzed by 10% SDS-PAGE. The membrane was immunoblotted with the anti-cyclin B antibody or anti-Ub antibody, and the bound antibodies were detected using ECL.
RESULTS

**pHi-dependent Proteasome Activity**

In the living starfish oocyte, *in vivo* proteasome activity, which can be measured by the microinjection of the fluorogenic substrate Suc-Phe-Leu-Arg-CAMS, increases gradually after 1-MA treatment and reaches a sub-maximal level just before the first polar body formation (31). To determine whether fertilization also causes an increase in the proteasome activity, maturing oocytes were inseminated and injected with the substrate. As shown in Fig. 1, fertilization as well as 1-MA treatment induced an increase in the proteasome activity. This increase in the activity may be due to a rise in pHi, since fertilization of sand dollar eggs induces the activation of the proteasome via a rise in pHi (32). Also, pHi of starfish oocytes is increased by treatment with 1-MA (29). To test whether a rise in pHi occurs at fertilization, we injected the oocytes with the pH-sensitive fluorescent dye BCECF. As shown in Fig. 2, pHi increased transiently after fertilization.

To examine if a rise of pHi is sufficient for proteasome activation *in vivo*, buffers with different
pHs were microinjected into immature or maturing oocytes and then the proteasome activity was measured. When pH 7.5 buffer was injected into immature oocytes, the rate of Suc-Phe-Leu-Arg-CAMS hydrolysis was increased (Fig. 3A). On the other hand, injection of pH 6.8 buffer into maturing oocytes decreased the substrate hydrolysis significantly (Fig. 3C). An increase or decrease in pH by the injection of pH buffers was confirmed by measurement of pH using pH-sensitive dye BCECF (Fig. 3B and D).

Thus, the increase in the proteasome activity in starfish oocytes is most likely to be induced by both 1-MA- and fertilization-dependent pH increases.

Na⁺/H⁺ antiporters are a family of plasma membrane proteins catalyzing the electroneutral exchange of intracellular H⁺ for extracellular Na⁺. Since the rise in pH after 1-MA treatment occurs via Na⁺/H⁺ exchange and a lack of extracellular Na⁺ inhibits Na⁺/H⁺ antiporters, resulting in blockage of the pH increase (29), the activity of the proteasome was expected to be low in the maturing oocytes treated with SW lacking Na⁺. As shown in Fig. 3E, 1-MA treatment in the absence of external Na⁺ did not stimulate proteasome substrate hydrolysis, while injection of pH 7.5 buffer caused an increase the substrate hydrolysis. Also, an increase of pH by injection of pH 7.5 buffer was confirmed by measurement of pH (Fig. 3F). Thus, we concluded that the increase in pH by 1-MA or fertilization induces the activation of the proteasome in vivo.
Similarly, we could detect pH-dependent proteasome activation in the cell-free preparation obtained from immature or maturing oocytes using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-MCA. In the cell-free preparation from maturing oocytes, the proteasome was activated up to about 2-fold at pH 7.3, as compared to that at pH 7.0 (Fig. 3G). Similar results were obtained in the cell-free preparation from immature oocytes. These results clearly indicate that the proteasome is activated directly by a rise in pHi.

*The Destruction of the Ubiquitinated Cyclin B*

One of the natural substrates of the proteasome is cyclin B. To determine whether the degradation of this endogenous substrate is also affected by pH, the quantity of cyclin B *in vivo* and *in vitro* was analyzed by immunoblotting with an anti-cyclin B antibody. In agreement with the findings of Ookata *et al.* (33), the 48-kDa cyclin B in oocytes decreased significantly at 67-87 min after 1-MA treatment (Fig. 4). Interestingly, in the same experiment, an unexpected 250-kDa band that also reacted with the anti-cyclin B antibody appeared from 5 min after 1-MA treatment and disappeared at 87 min (Fig. 4).
4. Glotzer et al. (8) found that a small amount of cyclin is apparently converted to a higher molecular mass form just before the onset of cyclin degradation. Thus, we suspect that the band of 250-kDa is cyclin B and this shift to a higher mass form is due to polyubiquitination occurring during oocyte maturation. Similarly, as shown in Fig. 5, a band of 250-kDa was clearly detected in the cell-free preparation from maturing oocytes at metaphase. At pH 7.0, the band of 250-kDa was stable. At higher pHs (7.2 or 7.3) corresponding to pH of maturing oocytes (Fig. 2), it disappeared within 40-60 min. The length of the period during which the 250-kDa band remained stable (about 40 min at pH 7.3) supports the above hypothesis, since MPF activity is stable for about 40 min after GVBD, and MPF activates the APC/C catalyzing the polyubiquitination of cyclin B (34-38).

The polyubiquitinated proteins are degraded by the proteasome, when ubiquitin monomers are simultaneously deconjugated from the polyubiquitin chain by deubiquitinating enzymes (DUBs) (39, 40). It is reported that deubiquitination of the substrates occurs even in the presence of proteasome inhibitors such as MG132 or MG115 (41, 42). Such deubiquitination causes a decrease of the polyubiquitinated substrates without proteolysis of the substrates, resulting in an accumulation of the deubiquitinated substrates. If the 250-kDa protein is polyubiquitinated cyclin B, MG115 treatment would be expected to cause a decrease in 250-kDa protein. As shown in Fig. 6A, 250-kDa protein decreased in the cell-free
preparation treated with MG115, supporting the hypothesis that 250-kDa protein is polyubiquitinated cyclin B.

Deubiquitination is catalyzed by the DUBs, which have been found within the regulatory complex of the proteasome (39, 40). The activity of DUBs can be blocked by the isopeptidase inhibitor ubiquitin-aldehyde (Ub-al), blocking the degradation of the polyubiquitinated substrate (43, 44). When Ub-al was added to the cell-free preparation, the 250-kDa protein was stable (Fig. 6A, Ub-al), further supporting the hypothesis that the 250-kDa protein was polyubiquitinated cyclin B. Ub-al and MG115 treatment also inhibited degradation of the 250-kDa protein as shown in Fig. 6A.

When we used 3% stacking and 5% separating gels of SDS-PAGE to resolve proteins more finely, we could detect ladder and smear bands near 250-kDa in the western blot, as shown in Fig. 6B. Since it is well reported that ubiquitinated proteins show a ladder pattern, these results again support the idea that a 250-kDa band is ubiquitinated cyclin B.

Ubiquitin as well as ubiquitinated proteins were detected in the cell-free preparation when it was analyzed using western blot probed with anti-ubiquitin antibody (Fig. 6C, lane 2). To confirm that the 250-kDa band was polyubiquitinated cyclin B, an anti-Ub antibody was used to immunoprecipitate polyubiquitinated proteins in the cell-free preparation. When the immunoprecipitated samples were
subjected to SDS-PAGE followed by immunoblotting using the anti-cyclin B antibody, the 250-kDa band was stained (Fig. 6D, lane 1). The presence of ubiquitinated proteins in the immunoprecipitated sample was confirmed by western blot using anti-ubiquitin antibody (Fig. 6D lane 3). In the control experiments, neither cyclin B (Fig. 6D, lane 2) nor ubiquitinated proteins (Fig. 6D, lane 4) were stained. We therefore concluded that the 250-kDa band is polyubiquitinated cyclin B.

Activation of MAP Kinase Prevents Degradation of Polyubiquitinated Cyclin B at pH 7.0

Polyubiquitinated cyclin B remained stable for >120 min after the start of the in vitro incubation at pH 7.0, whereas it disappeared within 40-60 min at pH 7.3 (Fig. 5A, B). Thus, polyubiquitinated cyclin B was not destroyed by the proteasome at pH 7.0. The proteasome activity, however, was not especially low at pH 7.0, as shown in Fig. 3G: the difference of the activity between pH 7.0 and pH 7.3 was about only 2-fold. Thus, the blockage of the destruction of polyubiquitinated cyclin B at pH 7.0 may not be due to the lower activity of the proteasome.

Recently, we found that starfish oocytes are arrested at MI in the ovary where the pH of oocytes is around 7.0. When arrested oocytes are spawned, pH increases to 7.2-7.3 and the MI arrest is
released. Since MEK inhibitor U0126 enhances cyclin B degradation at pH 7.0, MAP kinase is necessary to establish the MI arrest (29). To test whether polyubiquitinated cyclin B degradation is blocked by the activity of MAP kinase, we preincubated a cell-free preparation with MEK inhibitor U0126 at pH 7.0. As shown in Fig. 7A, U0126 treatment released the block of polyubiquitinated cyclin B degradation. At pH 7.3, U0126 treatment did not affect the degradation of polyubiquitinated cyclin B (Fig. 7B). Immunoblots using an anti-MAP kinase antibody showed that the MAP kinase was inactivated by U0126 (Fig. 7C). To confirm the role of MAP kinase in regulation of cyclin B stability, we performed further experiments using U0124, which is an inactive analogue of U0126 without inhibitory effects on MAP kinase. As shown in Fig. 7D, cyclin B destruction did not occur when cell-free preparation at pH 7.0 was treated with U0124. Thus, these results strongly support the hypothesis that MAP kinase blocks cyclin destruction at pH 7.0. Immunoblots using an anti-MAP kinase antibody confirmed that MAP kinase inactivation was not induced by U0124 (Fig. 7E).

To eliminate the possibility that the MAP kinase inactivation triggers an increase of the proteasome activity, the cell-free preparation at pH 7.0 was treated with U0126 and the proteasome activity was measured by assessing the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA. As shown in Fig. 8, we could not detect an increase in the proteasome activity in the U0126-treated preparation. Thus, a key step
for degradation of cyclin B should occur between polyubiquitination and proteolysis by the proteasome.

MAP kinase blocks this step at pH 7.0. To our surprise, the block of polyubiquitinated cyclin B degradation was released at pH 7.2-7.3 even in the presence of MAP kinase activity (Figs. 5 and 7). This step is the checkpoint at the MI arrest in the starfish oocyte.
DISCUSSION

In this study, we show that the proteasome activity cleaving artificial substrates at pH 7.3 was about 2 times higher than that at pH 7.0 \textit{in vivo} as well as \textit{in vitro}. An endogenous substrate of the proteasome, polyubiquitinated cyclin B, was also degraded within 40 min after a shift to pH 7.3, while it was stable at pH 7.0 for over 120 min. Thus, during an early phase of this study, we wondered whether the lower activity of the proteasome at pH 7.0 caused inhibition of the degradation of polyubiquitinated cyclin B. However, our finding that the U0126 induced the degradation of polyubiquitinated cyclin B at pH 7.0 without an increase in the peptidase activity of the proteasome made us change our hypothesis to the following: There should be a rate limiting step for the degradation of cyclin B after polyubiquitination but before the proteolysis by the proteasome. This step is blocked by the MAP kinase pathway at pH 7.0. Although the MAP kinase is still active at pH 7.3, the rate-limiting step disappears at this pH and the degradation of cyclin B occurs. We believe that this step causes the MI arrest of starfish oocytes in the ovary, where the pH of oocytes is lower than 7.0 (29). Immediately after spawning, the pH increase by Na"/H" antiporter causes cancelation of the rate-limiting step, resulting in the release of the arrest.

In starfish, the spawning period continues for 2-3 h after synchronous GVBD in the ovary,
while meiosis ends within 1.5 h after GVBD in seawater. It is also well known that fertilization during meiosis I is important for normal fertilization of starfish oocytes, since polyspermy block is lost gradually after meiosis I (45). Thus, most oocytes would lose the best period for fertilization if MI arrest did not work. Also, during the breeding season for starfish, congregating animals release an enormous number of gametes at the same time. Therefore, fertilization is expected to occur immediately after spawning in the field. Thus, the occurrence of MI arrest and resumption of meiosis in response to spawning ensure normal fertilization and development in starfish.

In vertebrates, the MII arrest is caused by CSF and MAP kinase is the key engine of CSF. The MAP kinase pathway inhibits APC-dependent synthesis of polyubiquitinated cyclin B, resulting in blockage of the cyclin B degradation during CSF arrest (46). Thus, a key step for cyclin B degradation exists before polyubiquitination. Interestingly, the MI arrest does not occur in vertebrates even in the presence of the active MAP kinase after GVBD, and cyclin B degradation as well as polyubiquitination occurs. Cyclin E was reported to be involved in the MII arrest in Xenopus oocytes (47). It would be of interest to study the fate of cyclin E during oocyte maturation in starfish.

How is the degradation of polyubiquitinated cyclin B inhibited by the MAP kinase in starfish oocytes? Although we do not have an answer to this question, it is possible that the 19S proteasome is
phosphorylated in the MAP kinase pathway, causing the inhibition of polyubiquitinated cyclin B degradation. Indeed, the 19S proteasome recognizes polyubiquitin chains on the substrate and has the deubiquitinating activity (40, 48-50). Another possibility is that adaptor proteins may be involved in the process of access of substrates to the catalytic sites located within a hollow cavity of the 20S proteasome (51). Such functions of adaptor proteins may be affected by MAP kinase, resulting in the block of proteolysis.
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FIGURE LEGENDS

Fig. 1. Changes in the $V_o$ values of the Suc-Phe-Leu-Arg-CAMS hydrolyzing activity upon fertilization.

To obtain the $V_o$ values, the proteasome substrate, Suc-Phe-Leu-Arg-CAMS was injected into unfertilized (open circles) and fertilized oocytes (closed circles). Each data point represents the result obtained from a single oocyte that had collected from the same animal. The occurrence of GVBD and the formation of polar bodies were indicated. The timings of nuclear fusion of male and female pronuclei, and cell division (the 1st cleavage) were indicated.

Fig. 2. Changes in pH during oocyte maturation and fertilization. Immature oocytes in normal seawater were injected with BCECF-dextran before 1-MA addition. The changes in pH were measured after 1-MA treatment (open circles) and during fertilization (closed circles). The BCECF-dextran fluorescence ratio (left y axis) and the calibrated pH values (right y axis) are shown.
Fig. 3. Effects of buffers with different pHs on in vivo and in vitro proteasome activity. A and C, for the in vivo assays, Suc-Phe-Leu-Arg-CAMS was injected into an immature oocyte (A) or maturing oocyte after GVBD (C) at time 0, and then buffer at pH 7.5 (A) or pH 6.8 (C) was injected at the time indicated. E, for the in vivo assay in the zero-sodium artificial sea water (0NaSW), Suc-Phe-Leu-Arg-CAMS was injected into a maturing oocyte after GVBD at time 0. Then the oocyte was injected with the buffer at pH 7.5. The fluorescence intensity of the oocyte was normalized by injection of ACMS. To confirm that pH buffer injection in A, C and E caused significant changes in pHi, BCECF-dextran was preinjected into oocytes to measure pHi, and then pH buffers were injected at the time indicated into immature (B), maturing (D), and 0NaSW-treated maturing oocytes (F), respectively.

G, for the in vitro assay, the V₀ values of proteasome activity towards an artificial substrate, Suc-Leu-Leu-Val-Tyr-MCA were measured at pH 7.0 and 7.3 using the cell-free preparations from immature (white bars) or maturing (gray bars) oocytes. Data were presented as the mean ± S.E obtained from three independent experiments.
Fig. 4. Destruction of 250-kDa protein in vivo. Samples from oocytes were prepared at the indicated times after 1-MA treatment. The samples were analyzed by 10% SDS-PAGE and immunoblotted with an anti-cyclin B antibody. Molecular size markers are indicated on the left side of the picture.

Fig. 5. Destruction of cyclin B in the cell-free preparation at various pH values. A, the cell-free preparations at pH 7.0, 7.2 or 7.3 were incubated at 20°C. At the indicated times, the samples were quenched with sample buffer and analyzed by 10% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody. Molecular size markers are indicated on the left. The arrows indicate 48-kDa and 250-kDa protein. B, the percentages of remaining 250-kDa protein (filled circles) and 48-kDa protein (open squares) were quantitated.
Fig. 6. The 250-kDa protein is cyclin B-ubiquitin conjugate. A, effects of MG115 or Ub-al on the destruction of the 250-kDa protein. The cell-free preparation at pH 7.3 was incubated with 200 µM MG115, 12 µM Ub-al, or 200 µM MG115 plus 12 µM Ub-al at 0°C for 10 min. Then samples were incubated at 20°C for 0, 10, 20, 30, 40, 60, 80 or 100 min and subsequently analyzed by 10% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody. Molecular size markers are indicated on the left. Vehicle; the cell-free preparation treated with DMSO. Cont; the cell-free preparation without DMSO. B, the cell-free preparations at pH 7.3 were incubated at 20°C. At the indicated times, the samples were quenched with sample buffer and analyzed by 5% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody. The 250-kDa protein is indicated by the arrow. Smear and ladder bands are indicated by the arrowhead. C, ubiquitin (lane 1) and the cell-free preparations at pH 7.0 (lane 2 and 3) were analyzed by 15% SDS-PAGE followed by immunoblotting with anti-ubiquitin antibody (lane 1 and 2) and anti-cyclin B antibody (lane 3). D, the cell-free preparation was immunoprecipitated with (lane 1 and 3) or without (lane 2 and 4) an anti-Ub antibody. The samples were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody (lane 1 and 2) and anti-ubiquitin antibody (lane 3 and 4).
Fig. 7. Effects of MAP kinase inactivation on cyclin B degradation. A, the cell-free preparation at pH 7.0 was incubated with 100 µM U0126 or DMSO (as a control) at 0°C for 10 min. Then samples were incubated at 20°C for the times indicated. The samples were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody. Molecular size markers are indicated on the left. B, the cell-free preparation at pH 7.3 was incubated with 100 µM U0126 or DMSO (as a control) at 0°C for 10 min. Then samples were incubated at 20°C for the times indicated. The samples were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody. Molecular size markers are indicated on the left. C, activation of MAP kinase was inhibited by U0126. The cell-free preparations at pH7.3, or at pH7.0 with U0126 or with DMSO (as a control) were resolved by 12.5% SDS-PAGE and analyzed by western blotting probed with an antibody against MAP kinase (ERK1). The arrow and the arrowhead indicate the active and inactive forms of MAP kinase, respectively. D, cell-free preparation at pH 7.0 was incubated with 20 µM U0126, 20 µM U0124 or DMSO at 0°C for 10 min. Then samples were incubated at the indicated times. The samples were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-cyclin B antibody. Molecular size markers are indicated on the left. E, effects of U0124 on the
phosphorylation state of MAP kinase. Cell-free preparations at pH 7.0 with 20 \( \mu \text{M} \) U0126, 20 \( \mu \text{M} \) U0124 or with DMSO were resolved by 12.5% SDS-PAGE and analyzed by western blotting probed with an antibody against MAP kinase (ERK1). The *arrow* and the *arrowhead* indicate the active and inactive forms of MAP kinase, respectively.

Fig. 8. Proteasome activity was not blocked by the MAP kinase pathway. The cell-free preparation at pH 7.0 from maturing oocytes was pretreated with 100 \( \mu \text{M} \) U0126 or with DMSO (as a control) at 0°C for 10 min. Proteolytic reactions were carried out with the cell-free preparations at pH 7.0 or pH 7.2 using Suc-Leu-Leu-Val-Tyr-MCA at 20°C. Data were presented as the mean ± S.E. obtained from three independent experiments.
ABBREVIATIONS

1 The abbreviations used are: MI, metaphase of the first meiotic division; MII, metaphase of the second meiotic division; APC/C, anaphase-promoting complex/cyclosome; CSF, cytostatic factor; MAP, mitogen-activated protein; 1-MA, 1-methyladenine; MPF, maturation-promoting factor; GVBD, germinal vesicle breakdown; CSFW, calcium-free seawater; ASW, artificial seawater; DMSO, dimethyl sulfoxide; ACMS, 7-amino-coumarin-4-methanesulfonic acid; BCECF, 2', 7'-bis[2-carboxyethyl]-5-[and-6]-carboxyfluorescein; DUBs, deubiquitinating enzymes; MEK, mitogen-activated protein kinase kinase.
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Fig. 4. Oita et al.
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Degradation of polyubiquitinated cyclin B is blocked by the MAPK pathway at the MI arrest in starfish oocytes

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