In the starfish ovary, maturing oocytes stimulated by 1-methyladenine undergo synchronous germinal vesicle breakdown and then arrest in metaphase of the first meiotic division (metaphase I). 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, whereas it was degraded at pH 7.3. When the MAPK 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 MAPK 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 MAPK pathway, resulting in the degradation of polyubiquitinated cyclin B and the release of the arrest. Thus, the key step of metaphase I arrest in starfish oocytes occurs after the polyubiquitination of cyclin B but before cyclin B proteolysis by the proteasome.

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)1 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 a review, see Ref. 7). Cyclin B is polyubiquitinated by a specific E3 called the anaphase-promoting complex/cyclosome (8) (for a review, see Ref. 9). Once such targeted proteins become polyubiquitinated, they are recognized and degraded by a particle known as the 26 S 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 MAPK 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 MAPK into one blastomere of a two-cell embryo induced metaphase arrest similar to that induced by c-Mos (12). A MAPK 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 MAPK pathway, and depletion of p90Rsk from egg extracts removes CSF activity, which can be restored by readdition of p90Rsk (13, 14). Bub1 acts downstream of p90Rsk and may be an effector of anaphase-promoting complex inhibition and CSF-dependent metaphase arrest by p90Rsk (15). Furthermore, Emi1 acts to prevent cyclin B destruction through anaphase-promoting complex 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 is required for release of MII, and a constitutively active calmodulin-dependent protein kinase II 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 phosphatidylinositol 3-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 phosphatidylinositol 3-kinase and then phosphorylates and inactivates Myt1, the inhibitory kinase of Cdc2. Active MPF eventually induces germinal vesicle breakdown (GVBD).
Degradation of Polyubiquitinated Cyclin B at the MI Arrest

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 (pHt) and active MAPK. Release from the arrest is induced by a rise in the pHt, after spawning (29). In the current study, using cell-free preparations, we show that polyubiquitinated cyclin B remained stable at low pH. When MAPK was inhibited by U0126, degradation of polyubiquitinated cyclin B occurred even at low pH. These results indicate that MI arrest is regulated by the processes that occur after polyubiquitination of cyclin B but before prolyolysis 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. To remove follicle cells, the released oocytes were washed twice in ice-cold calcium-free seawater and stored in artificial seawater (ASW) at 20 °C. To measure fluorescence intensity, an excitation filter at 380 nm and a 450-nm emission filter were used. Ubiquitin was obtained from Sigma.

Sedimented oocytes were transferred to a net of 60-μm nylon mesh (Nylon 6, Osaka, Japan) and washed several times with cold calcium-free seawater, 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 s, 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 the addition of one-fifth 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 were purchased from Peptide Institute (Osaka, Japan). Bestatin (Sigma) was dissolved in distilled water, MG115, ubiquitin aldehyde (Peptide Institute), U0126 (Promega, Madison, WI), and U0124 (Calbiochem) were dissolved in distilled water. MG115, ubiquitin aldehyde (Peptide Institute), U0126 (Promega, Madison, WI), and U0124 (Calbiochem) were dissolved in Me2SO. These solutions were stored at −20 °C.

Release from the arrest is induced by a rise in the pHt, after spawning (29). In the current study, using cell-free preparations, we show that polyubiquitinated cyclin B remained stable at low pH. When MAPK was inhibited by U0126, degradation of polyubiquitinated cyclin B occurred even at low pH. These results indicate that MI arrest is regulated by the processes that occur after polyubiquitination of cyclin B but before prolyolysis by the proteasome.

RESULTS

FIG. 1. Changes in the \( V_0 \) values of the Suc-Phe-Leu-Arg-CAMS hydrolizing activity upon fertilization. To obtain the \( V_0 \) 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 been 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 first cleavage) were indicated.

FIG. 2. Changes in pH during oocyte maturation and fertilization. Immature oocytes in normal seawater were injected with BCECF-dextran before the addition of 1-MA. 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.

Injected was 2% of the total oocyte volume. To estimate pHt, 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). The emitted light passed through a dichroic beam splitter at 510 nm and through a 510–560-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 Heps, and 25 mM Pipes, adjusted to the various pH values with KOH, and with 100 μM dithionitrobenzoic acid (PMS) 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 pHt was measured. In some experiments, oocytes were injected with pH buffers (pH 7.5 or 6.8) containing 300 mM Heps and 300 mM Pipes. To block Na+/H+ antiporter, the oocytes were treated with zero sodium artificial seawater 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 polyvinylidene difluoride membrane (Millipore Corp.). The membrane was blocked with PBS-T (phosphate-buffered saline plus 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 MAPK antibody (Seikagaku Corp.) at a 1:10000 dilution for 1 h 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 1 h. After the membrane was washed, bound antibodies were detected using chemiluminescent substrate (ECL; Amersham Biosciences) and a LAS-1000 Lumino image analyzer (Fuji Photo Film Co., Ltd.).

**Immunoprecipitation**—An anti-ubiquitin (anti-Ub) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was mixed with protein A-cellulofine (Chisso Corp.) and incubated overnight at 4°C. The antibody-protein A-cellulofine complex was recovered by centrifugation at 2,500 × g for 10 s and washed three times with phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Tween 20. After the cell-free preparation was incubated for 30 min at 20°C, it was treated with 200 μM MG115 for 20 min on ice. The cell-free preparation was centrifuged at 50,000 × 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 phosphate-buffered saline) 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**

**pH-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 submaximal 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 pH, since fertilization of sand dollar eggs
induces the activation of the proteasome via a rise in pH (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 whether a rise of pHi is sufficient for proteasome
activation in vivo, buffers with different pH values were micro-
injected into immature or maturing oocytes, and then the pro-
easome activity was measured. When pH 7.5 buffer was injected
into immature oocytes, the rate of Suc-Phe-Leu-Arg-CAMS hy-
teasome activity was measured. When pH 7.5 buffer was injected
in the absence of external Na+/H11001,

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). 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 degrada-
tion. 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 pH values (7.2 or 7.3)
corresponding to the pHi of maturing oocytes (Fig. 2), it disap-
peared 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 anaphase-
promoting complex/cyclosome catalyzing the polyubiquitina-
tion of cyclin B (34–38).

The polyubiquitinated proteins are degraded by the protea-
some, when ubiquitin monomers are simultaneously deconju-
gated from the polyubiquitin chain by deubiquitinating en-
zymes (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 poly-
ubiquitinated 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 deubiquitinating en-
zymes, which have been found within the regulatory complex
of the proteasome (39, 40). The activity of deubiquitinating en-
zymes can be blocked by the isopeptidase inhibitor ubiquitin
aldehyde (Ub-al), blocking the degradation of the polyubiquiti-
nated substrate (43, 44). When Ub-al was added to the cell-free
preparation, the 250-kDa protein was stable (Fig. 6A, Ub-al),
confirming the hypothesis that the 250-kDa protein
is 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 SDSL
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 polyubiq-
titinated proteins in the cell-free preparation. When the im-

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.
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- 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 Me2SO. Cont, the cell-free preparation without Me2SO. 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 (lanes 2 and 3) were analyzed by 15% SDS-PAGE followed by immunoblotting with anti-ubiquitin antibody (lanes 1 and 2) and anti-cyclin B antibody (lane 3). D, the cell-free preparation was immunoprecipitated with (lanes 1 and 3) or without (lanes 2 and 4) an anti-Ub antibody. The samples were analyzed by 10% SDS-PAGE followed by immunoblotting (WB) with anti-cyclin B antibody (lanes 1 and 2) and anti-ubiquitin antibody (lanes 3 and 4).
Degradation of Polyubiquitinated Cyclin B at the MI Arrest

Activation of MAPK 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. 5). 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. 5C; the difference in the activity between pH 7.0 and 7.3 was only about 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, MAPK is necessary to establish the MI arrest (29). To test whether polyubiquitinated cyclin B degradation is blocked by the activity of MAPK, 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-MAPK antibody showed that the MAPK was inactivated by U0126 (Fig. 7C). To confirm the role of MAPK in regulation of cyclin B stability, we performed further experiments using U0124, which is an inactive analogue of U0126 without inhibitory effects on MAPK. As shown in Fig. 1D, 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 MAPK blocks cyclin destruction at pH 7.0. Immunoblots using an anti-MAPK antibody confirmed that MAPK inactivation was not induced by U0124 (Fig. 7E).

To eliminate the possibility that the MAPK 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 proteasome activity 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 MeSO (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 MAPK was inhibited by U0126. The cell-free preparations at pH 7.3 or at pH 7.0 with U0126 or with MeSO (as a control) were resolved by 12.5% SDS-PAGE and analyzed by Western blotting probed with an antibody against MAPK (ERK1). The arrow and the arrowhead indicate the active and inactive forms of MAPK, respectively. D, cell-free preparation at pH 7.0 was incubated with 20 μM U0126, 20 μM U0124, or MeSO 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 MAPK. Cell-free preparations at pH 7.0 with 20 μM U0126, 20 μM U0124 or with MeSO were resolved by 12.5% SDS-PAGE and analyzed by Western blotting probed with an antibody against MAPK (ERK1). The arrow and the arrowhead indicate the active and inactive forms of MAPK, respectively.
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. U0126 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 MAPK 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 in *vivo* as well as in *vitro*. An endogenous substrate of the proteasome, polyubiquitinated cyclin B, was also degraded within 40 min after a shift to pH 7.3, whereas 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 MAPK pathway at pH 7.0. Although the MAPK 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+ antipporter causes cancellation 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, whereas 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 MI arrest is caused by CSF and MAPK is the key engine of CSF. The MAPK pathway inhibits anaphase-promoting complex-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 MAPK after GVBD, and cyclin B degradation as well as polyubiquitination occurs. Cyclin E was reported to be involved in the MI 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 MAPK in starfish oocytes? Although we do not have an answer to this question, it is possible that the 19 S proteasome is phosphorylated in the MAPK pathway, causing the inhibition of polyubiquitinated cyclin B degradation. Indeed, the 19 S 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 20 S proteasome (51). Such functions of adaptor proteins may be affected by MAPK, resulting in the block of proteolysis.

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