Activation of p34^{cd}c2 protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos

TAESAENG CHOI¹, FUGAKU AOKI¹, MAKOTO MORI², MASAKANE YAMASHITA³, YOSHITAKA NAGAHAMA³ and KAORU KOHMOTO¹

¹Department of Animal Breeding, Faculty of Agriculture, University of Tokyo, Yayoi 1-1-1, Bunkyo-Ku, Tokyo 113, Japan
²Department of Animal Science, Shizuoka University, Ohya, Shizuoka 422, Japan
³Department of Developmental Biology, National Institute for Basic Biology, Okazaki, Aichi 444, Japan

Summary

p34^{cd}c2 protein kinase is a universal regulator of M-phase in eukaryotic cell cycle. To investigate the regulation of meiotic and mitotic cell cycle in mammals, we examined the changes in phosphorylation states of p34^{cd}c2 and its histone H1 kinase activity in mouse oocytes and embryos.

We showed that p34^{cd}c2 has three different migrating bands (referred to as upper, middle and lower bands) on SDS–PAGE followed by immunoblotting with anti-PSTAIR antibody, and that the upper and middle bands are phosphorylated forms since these two bands shifted to the lower one by alkaline phosphatase treatment.

In meiotic cell cycle, only germinal vesicle (GV) stage oocytes had the three forms. The phosphorylated forms decreased gradually in oocytes up to 2 h after isolation from follicles, and thereafter the phosphorylation states did not change significantly until metaphase II. However, the histone H1 kinase activity oscillated, being activated at the first and second metaphase in meiosis and inactivated at the time of the first polar body extrusion. These results suggest that changes in phosphorylation states of p34^{cd}c2 triggered its activation at the first metaphase, but not inactivation and reactivation at the first and second metaphase, respectively.

In mitotic cell cycle, phosphorylated forms appeared at 4 h after insemination, increased greatly just before metaphase, and were dephosphorylated in metaphase. Histone H1 kinase activity was high only at metaphase. This kinase activation is probably triggered by dephosphorylation of p34^{cd}c2.

Key words: mouse oocyte and embryo, p34^{cd}c2, histone H1 kinase, cell cycle.

Introduction

The cdc2 gene, which encodes p34^{cd}c2 protein kinase, is essential for the transition of G1 to S and G2 to M phase in the cell cycle of fission yeast *Schizosaccharomyces pombe* (Nurse and Thuriaux, 1980; Nurse and Bisset, 1981). Recent studies have shown that p34^{cd}c2 is a universal regulator of M-phase in eukaryotic cell cycle. It has been identified as a component of purified maturation promoting factor (MPF), which induces entry into M-phase in multicellular eukaryotes (Dunphy *et al.* 1988; Gautier *et al.* 1988; Labbe *et al.* 1989). Its protein kinase activity, measured using histone H1 as a substrate, oscillates with its maximum level at M-phase in meiotic and mitotic cell cycles. In addition, the human homologue of cdc2 has been cloned and its protein kinase activity also oscillates during the cell cycle, with a maximum level at M-phase (Lee and Nurse, 1987; Draetta and Beach, 1988; Brizuela *et al.* 1989).

At least two biochemical events necessary for p34^{cd}c2 kinase activation have been elucidated; the dephosphorylation of p34^{cd}c2 and its association with cyclin (Norbury and Nurse, 1989).

Cyclin was first noted in eggs of marine invertebrates by their pronounced synthesis in the interphase followed by specific proteolysis at the end of mitosis (Evans *et al.* 1983). Direct evidence that cyclin plays a role in mitotic regulation came from the finding that the microinjection of mRNA of clam and sea urchin cyclin induced meiotic maturation in *Xenopus* immature oocytes (Swenson *et al.* 1986; Pines and Hunt, 1987). Suggestive evidence for interaction of cyclin with p34^{cd}c2 came from the finding that cyclins were found in p34^{cd}c2 immunoprecipitates and that cyclin antibodies coprecipitated with a histone H1 kinase activity (Draetta *et al.* 1990; Gautier *et al.* 1990; Pines and Hunter, 1989).

p34^{cd}c2 changes in its phosphorylation state and the kinase activity oscillates through the cell cycle. Many papers have shown the relationship between p34^{cd}c2 dephosphorylation and the kinase activation. In starfish
ovulated oocytes, this protein is dephosphorylated shortly after hormonal stimulation and, at the same time, the protein kinase activity increases (Labbe et al. 1989). This dephosphorylation has also been observed during maturation of Xenopus oocytes (Gautier et al. 1989), and the commencement of M-phase of fission yeast (Gould and Nurse, 1989) and cultured cell lines (Morla et al. 1989).

Xenopus and starfish oocytes have been widely used to study the regulation of cell cycles, because a large amount of synchronized samples can be easily obtained (Maller, 1990; Meijer and Guerrier, 1984). These immature oocytes undergo meiotic maturation following the stimulation of hormones. Mitotic cell cycles of these embryos during the first a few hours following fertilization is composed of S and M phases without any detectable G1 and G2 phases (Newport and Kirschner, 1982). However, the regulation of the cell cycle in mammalian oocytes seems to be different from that in Xenopus and starfish oocytes. Most mammalian oocytes start meiotic maturation spontaneously without hormonal stimulation when they are liberated from follicles (Edwards, 1965), and the mitotic cell cycle after fertilization is composed of four phases, G1, S, G2 and M (Abramzuk and Sawichi, 1975).

In mammals, the mechanisms of regulation of meiotic and mitotic cell cycles seem to be different. In meiotic cell cycle, M-phase occupies almost all the cell cycle and the duration of M-phase is much longer than that in mitosis. For example, in mouse, completion of metaphase I requires 8–10 h and chromatin gradually condenses during this period (Calarco et al. 1972). However, in the first mitotic cell cycle, the duration of M-phase is only 2 h of the 19 h (Howlett, 1986).

To investigate the regulation of meiotic and mitotic cell cycles in mammals, we examined the changes in the phosphorylation state of p34cdc2 and its histone H1 kinase activity in mouse oocytes and embryos. A previous study on mouse embryos has shown that p34cdc2 could be detected with antibody against the conserved peptide EGVPSSTAIREISLLKE (PSTAIR) sequence and its electrophoretic mobility was changed by phosphorylation (McConnell and Lee, 1989). In this paper, we show that p34cdc2 has three different migrating bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and that the two slower migrating bands are phosphorylated forms. We used these differences of electrophoretic mobility for the detection of changes in the phosphorylation state of p34cdc2.

Materials and methods

Collection and culture of oocytes

The mice used in this study were B6C3 F1 females, 21–23 days of age, and mature ICR males. Full-grown oocytes were collected from ovaries 45 h after stimulation with 7.5 IU of pregnant mare serum gonadotrophin (PMSG). Only oocytes with complete cumulus cells were used. To obtain the ovulated oocytes, female mice were injected with 7.5 IU PMSG and then 48 h later with 7.5 IU human chorionic gonadotrophin (hCG). Ovulated oocytes were collected from ampullar oviducts 14–16 h after hCG injection. For in vitro fertilization, spermatozoa were collected from cauda epididymis of mature mice and incubated for 1–2 h at 38.5°C to allow capacitation before insemination. The final concentration of spermatozoa was adjusted to approximately 2 × 10^5 ml⁻¹ for fertilization. The oocytes and embryos were cultured in the modified Whitten's medium (Katsuki, 1984), supplemented with 4 mg ml⁻¹ BSA (fraction V; Boehringer, Mannheim, West Germany) at 38.5°C.

Preparation of antibodies

The anti-PSTAIR antibody was prepared as previously described (Yamaguchi et al. 1991). Anti-serum against C terminus of mouse p34cdc2 was raised against the CDNQIKKM peptide, the C-terminal sequence of mouse homologue of p34cdc2 (Cisek and Corden, 1989) with additional cysteine, as described by Draetta and Bech (1988).

Detection of p34cdc2 by immunoblotting

The p34cdc2 was detected by immunoblotting with anti-PSTAIR antibody. 25 oocytes or embryos were denuded from surrounding cumulus cells by pipetting, collected in 15 μl of a cold extraction buffer (50 mM Tris- HCl pH 7.2, 10 mM MgCl₂, 1 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ pepstatin, 1 μM phenylmethylsulfonfyl fluoride (PMSF), 1% nonidet P-40), and added immediately with an equal volume of 2× SDS sample buffer (Laemmli, 1970). After denaturing by boiling for 3 min, the proteins were separated on 10% polyacrylamide gel (SDS-PAGE) according to the method described by Laemmli (1970) and transferred to nitrocellulose membrane (Millipore, pore size 0.25 μm) with a Milliblott-DE system (Millipore) according to the manufacturer's instruction. The membrane was incubated with a blocking solution (20 mM Tris- HCl, pH 7.6, 137 mM NaCl, 5% dried milk) for 1 h, and then incubated with 1:20 dilution of the antibody adsorbed on the membrane by use of a Blotting Detection Kit (Amersham, UK) according to the manufacturer's instruction.

Immunoprecipitation and protein kinase assay

50 cumulus-free oocytes or embryos were lysed by freezing and thawing in 100 μl of RIPA buffer (150 mM NaCl, 1.0% nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid and 50 mM Tris- HCl, pH 8.0) supplemented with 15 mM EGTA, 100 μM Na₃VO₄, 50 mM NaF and following protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM benzamidine HCl, 1 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ leupeptin and 1 μg ml⁻¹ pepstatin A. The lysed cells were added with 10 μl anti-mouse cdc2 C-terminal peptide antibody and incubated at 4°C overnight. The immune complexes were incubated with 30 μl of a 50% (v/v) slurry of protein A-Sepharose (Pharmacia) in kinase assay buffer (50 mM Tris-HCl, 10 mM MgCl₂ and 1 mM dithiothreitol, pH 7.4) for 1 h at 4°C and precipitated by centrifugation at 5000 g for 1 min. The pellets were washed three times with 1 ml each of the kinase assay buffer.

Kinase reaction was carried out in 30 μl of kinase assay buffer containing 100 μg ml⁻¹ of histone H1, 50 μM ATP, 555 kBq of [γ-³²P]ATP (NEN) and 50 nM cAMP-dependent protein kinase inhibitor peptide (Sigma, catalog number P3294). The kinase reaction was started by the addition of radioactive ATP and continued for 15 min at 30°C. The reaction was terminated by spotting 10 μl aliquotes onto 2×2.5 cm pieces of Whatman p81 phosphocellulose paper and, after 30 s, the filters were washed five times (at least
5 min each time) in a solution of 1% phosphoric acid. Incorporation of $^{32}$P into histone H1 was determined by counting the radioactivities on filter paper by Cerenkov counter. The incorporation in the absence of enzyme was also determined and subtracted from each determination to control for the nonenzymatic transfer of $^{32}$P into the substrate.

Alkaline phosphatase treatment

100 embryos were lysed in 100 µl RIPA buffer supplemented with protease inhibitors and immunoprecipitated with protein A-Sepharose as described above. The immune complex was washed four times with alkaline phosphatase buffer (100 mM Tris–HCl, pH 8.0, 5 mM MgCl$_2$, 100 mM NaCl, 1 mM dithiothreitol and protease inhibitors), and resuspended with the same buffer to make a total volume of 30 µl. The suspension was added with 1 µl of an alkaline phosphatase (Sigma, type XXX-A, catalog number p0780) and incubated for 15 min at 37°C. In the control experiment, 100 µM p-nitrophenyl phosphate was included in the incubation to prevent the phosphatase reaction. The samples thus treated with alkaline phosphatase were subjected to SDS–PAGE and immunoblotting with anti-PSTAIR antibody as described above.

Results

Detection of phosphorylated form of p34$^{cdc2}$

Immunoblotting of the extracts of mouse embryos collected 10 h after insemination with PSTAIR antibody revealed that p34$^{cdc2}$ comprises three different migrating forms on SDS–PAGE, referred to as upper, middle and lower bands (Fig. 1A). In the previous study with human culture cells, three different bands of p34$^{cdc2}$ were also detected with anti-C-terminal peptide antiserum.

Fig. 1. Detection of phosphorylated form of p34$^{cdc2}$.
(A) The extracts of embryos collected 10 h after insemination were immunoprecipitated with anti-C-terminal peptide serum. In some cases, the extracts were denatured with 1% SDS in 50 mM Tris buffer, pH 7.4 by boiling for 5 min before immunoprecipitation (lane 4, 5). The immunoprecipitates were treated with alkaline phosphatase (lane 2, 5), or with phosphatase plus p-nitrophenyl phosphate, a phosphatase inhibitor (lane 3), or with phosphatase buffer alone (lane 1, 4). After electrophoresis, the gel was immunoblotted with anti-PSTAIR antibody. (B) The embryos were cultured with 20 µg ml$^{-1}$ genistein (lane 3) or without genistein (lane 1, 2) from 2 h after insemination, and collected at 2 h (lane 1) and 6 h (lane 2, 3). The whole cell lysates were immunoblotted with anti-PSTAIR antibody.

Full-grown oocytes isolated from the follicles resume meiotic maturation spontaneously. Under our experimental conditions, germinal vesicle break down (GVBD) occurred within 1 h of isolation from follicles (88%, 216/245, Fig. 2B), and the first polar body extrusion occurred between 8 and 10 h (88%, 172/195, Fig. 2B). To obtain synchronized oocytes, only the oocytes that had lost their GV within 1 h and extruded their first polar body within 10 h were collected. The changes in phosphorylation states of p34$^{cdc2}$ in the meiotic cell cycle were examined by immunoblotting with anti-PSTAIR antibody. Fig. 2A shows that both of the phosphorylated forms (upper and middle bands) were detected only in GV stage oocytes. The upper band disappeared and the middle band decreased by 2 h after isolation from follicles. Thereafter, the upper band was not detected and a small amount of middle band was detected until 14 h after isolation from follicles. The lower band was detected throughout the meiotic cell cycle. The protein kinase activity of p34$^{cdc2}$ in these stages of oocytes was measured using histone H1 as a substrate. As shown in Fig. 2B, the activity oscillated in the cell cycle: it increased gradually from 0 h to 8 h after isolation from follicle and was abruptly decreased at 10 h. At this time, the first polar body was extruded. The kinase activity increased again from 10 h to 14 h.

The meiotic maturation of mouse oocytes is blocked with cAMP via cAMP-dependent protein kinase (Cho et al. 1974; Elayne et al. 1986). To investigate whether this inhibitory effect of cAMP is due to the inhibition of dephosphorylation of p34$^{cdc2}$, oocytes whose meiosis was inhibited with 3-isobutyl-methylxanthine (IBMX) were immunoblotted. Fig. 3 shows that the phosphorylated forms were detected in such oocytes. They were dephosphorylated after the oocytes were liberated from IBMX.

p34$^{cdc2}$ in meiotic cell cycle

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In the mitotic cell cycle, pronuclear membranes were formed within 5 h after insemination (85%, 99/116), disappearance of pronuclear membrane occurred at 14 h after insemination (76%, 90/118, Fig. 4A), and cleavage to two cells occurred at 15 h after insemination (87%, 97/111, Fig. 4A). To obtain synchronized embryos, only those that had formed two pronuclear membranes within 5 h after insemination and that had lost their two pronuclear membranes within 14 h after insemination were collected. The results of immunoblotting with anti-PSTAIR antibody show that the upper and the middle bands appeared at 4 h after insemination, greatly increased at 13 h, and decreased at 14 h (Fig. 4B). In contrast, the lower band existed until 12 h after insemination, abruptly decreased at 13 h and increased at 14 h. Fig. 4B shows that the kinase activity oscillated during the mitotic cell cycle. It was kept at high level in ovulated oocytes. After the oocytes were fertilized, the kinase activity decreased and thereafter remained at low level during interphase. At the time of entry into mitosis, the activity increased abruptly.

Fig. 2. Changes in phosphorylation states and H1 kinase activity of p34cdc2 in the meiotic cell cycle. (A) Immunoblots of whole cell lysates with anti-PSTAIR antibody. Numbers below each lane indicate the hours after isolation from follicles. 25 oocytes were used per lane. (B) Time course of germinal vesicle breakdown, first polar body extrusion and histone H1 kinase activity during meiotic cell cycle. 50 oocytes were immunoprecipitated and examined for histone H1 kinase activity as described in Materials and methods.

Fig. 3. Effect of IBMX on p34cdc2 dephosphorylation. The phosphorylation state was examined by immunoblotting with anti-PSTAIR antibody. Oocytes were collected for immunoblotting immediately after isolation from the follicles (lane 1). The isolated oocytes were incubated for 2 h and 4 h without (lane 2 and lane 4) or with (lane 3 and lane 5) 0.2 mM IBMX. Oocytes were preincubated with IBMX for 2 h and then incubated for 2 h without IBMX (lane 6).

Fig. 4. Changes in phosphorylation state and H1 kinase activity of p34cdc2 in the mitotic cell cycle. (A) Immunoblots of whole cell lysates with anti-PSTAIR antibody. Numbers below each lane indicate hours after insemination. 25 embryos were used per lane. (B) Time course of nuclear envelope breakdown (NEBD), cell division and histone H1 kinase activity during mitotic cell cycle.
Discussion

Recent studies have shown that p34\(^{cd2}\) protein kinase is a universal regulator of M-phase in eukaryotic cells and its activity is regulated by its phosphorylation. In a previous study, McConnell and Lee (1989) demonstrated that it also exists in mouse embryos. In this study, we could detect p34\(^{cd2}\) protein kinase by immunoblotting with anti-PSTAIR antibody in mouse oocytes as well as embryos and discriminate the phosphorylated forms of p34\(^{cd2}\) from the dephosphorylated one by differences in migration on SDS-PAGE. The possibility that anti-PSTAIR antibody reacts with other proteins (Toh-e et al. 1988; Paris and Philippe, 1990) can be excluded because the antibody against C-terminal peptide of mouse homologue p34\(^{cd2}\) also immunoprecipitated all three bands (Fig. 1A, lane 1).

Although the quantity of p34\(^{cd2}\) did not show appreciable changes during the cell cycle, its phosphorylation states oscillated. Furthermore, the phosphorylation states were different between meiotic and mitotic cell cycles. The considerable differences in phosphorylation states of p34\(^{cd2}\) during meiotic and mitotic cell cycles are as follow. (1) Phosphorylated forms abruptly decreased at M-phase in the first meiotic and mitotic cell cycles, but not in the second meiotic cell cycle. (2) Phosphorylated forms increased before M-phase in mitotic cell cycle but not in the first and second meiotic cell cycles. These differences in phosphorylation states of p34\(^{cd2}\) during meiotic and mitotic cell cycles seem to indicate that the mechanisms operating for activation of p34\(^{cd2}\) are different in the two cycles.

Although the activation of p34\(^{cd2}\) protein kinase requires both phosphorylation and association with cyclin (Norbury and Nurse, 1989), only one of them seems to trigger the activation; dephosphorylation triggers the activation in the first meiotic and mitotic cell cycles and association with cyclin triggers that in the second meiotic cell cycle. In the meiotic cell cycle of mouse oocytes, activity of histone H1 kinase was gradually increased from 0 to 8h after isolation from follicle, decreased at 10h, at which time the first polar body was extruded, and again increased from 10 to 14h. These changes in activity are coincident with those of MPF measured by Hashimoto and Kishimoto (1988). These changes in activity do not seem to be regulated only by phosphorylation, since the phosphorylation state of p34\(^{cd2}\) did not show any changes after dephosphorylation from 0 to 2h. Although there is no evidence for the requirement of cyclin synthesis in mouse meiotic cell cycle, the requirement of protein synthesis for MPF activation has been examined (Hashimoto and Kishimoto, 1988). In mouse oocytes, protein synthesis is not required for GVBD and subsequent chromatin condensation (Wassarman and Letourneau, 1976; Golbus and Stein, 1976; Schultz and Wassarman, 1977). Activation of MPF in this phase takes place even in the absence of protein synthesis. In contrast, after first polar body extrusion, reactivation of MPF required protein synthesis (Hashimoto and Kishimoto, 1988). Therefore, we suggest that activation of p34\(^{cd2}\) protein kinase during metaphase I is triggered only by dephosphorylation of p34\(^{cd2}\), while the inactivation and reactivation after metaphase I is triggered not by its phosphorylation and dephosphorylation but by degradation and synthesis of cyclin, respectively. However, in the mitotic cell cycle, the activation of p34\(^{cd2}\) protein kinase may be triggered by its dephosphorylation. p34\(^{cd2}\) was abruptly dephosphorylated and the kinase activity increased at 14h after insemination. When the dephosphorylation was blocked by the treatment of sodium orthovanadate, an inhibitor of tyrosine phosphatase, the embryos did not enter to M-phase (data not shown). Rime and Ozon (1990) showed that the chromatin condensation was induced in the mouse embryos by the treatment of okadaic acid, which induced dephosphorylation of p34\(^{cd2}\) in starfish oocytes (Picard et al. 1989), even in the absence of protein synthesis.

Recently, Solomon et al. (1990) showed that the phosphorylation of p34\(^{cd2}\) is a prerequisite for the kinase activation. In the cell-free system constructed with *Xenopus* egg extract, p34\(^{cd2}\) was first phosphorylated, which was induced by binding to cyclin, and then dephosphorylated and activated as a protein kinase. Indeed, in the mitotic cell cycle of mouse embryo, phosphorylation of p34\(^{cd2}\) abruptly increased at 13h after insemination, just before M-phase. Thereafter, the phosphorylated p34\(^{cd2}\) was abruptly dephosphorylated and p34\(^{cd2}\) kinase was activated. However, the phosphorylation of p34\(^{cd2}\) preceding its activation may not be required at least in the second meiotic cell cycle. Although the phosphorylation states of p34\(^{cd2}\) did not change, the kinase activity gradually increased. During this period, dephosphorylated p34\(^{cd2}\) protein may be activated as a kinase as soon as it associates with cyclin newly synthesized. The kinase activity gradually increased along with cyclin synthesis. Another explanation could be proposed for our results in the second meiotic cell cycle. The phosphorylation and dephosphorylation might occur simultaneously during this period. In this case, the phosphorylated form may not have been accumulated. As soon as p34\(^{cd2}\) was phosphorylated, it was dephosphorylated and activated as a protein kinase. The cycles of phosphorylation and dephosphorylation were continuous and thus the kinase activity was gradually increased. A small amount of phosphorylated form detected constantly might be a temporary phosphorylated form of p34\(^{cd2}\).

In fission yeast, the mutations in cdc2 gene cause cell cycle arrest at G\(_1\) phase as well as G\(_2\) phase (Nurse and Thuriaux, 1980; Nurse and Bisset, 1981). Recent studies have also demonstrated that p34\(^{cd2}\) is involved in the regulation of G\(_1\)–S transition (Furukawa et al. 1990; D'Urso et al. 1990; Broek et al. 1991), although it remains to be elucidated how the activity of p34\(^{cd2}\) is regulated as an initiator of G\(_1\)–S transition. p34\(^{cd2}\) may be activated by phosphorylation at G\(_1\)–S boundary. Lee et al. (1989) reported that p34\(^{cd2}\) was phosphorylated just before G\(_1\)–S transition in serum-stimulated mam-
malian fibroblasts, which had been arrested in G_1 phase by serum starvation. In our results on the mitotic cell cycle, p34^{cdc2} was phosphorylated before the S-phase, 4h after insemination at which time embryos have no pronucleus. It is known that DNA synthesis occurs after pronucleus formation (Luthardt and Donahue, 1973; Howlett and Bolton, 1985). In the meiotic cell cycle during which DNA synthesis does not occur, p34^{cdc2} was not phosphorylated although a small amount of middle band was detected constantly. Study on the phosphorylation site(s) of p34^{cdc2} in the upper and middle bands is now being undertaken.

The phosphorylation states of p34^{cdc2} through the first mitotic cell cycle were different in the report of McConnel and Lee (1989) and in our study. Although the cause of this difference is unclear, we suppose that it may be due to the differences of experimental procedures such as the temperature for the culture of embryos, electrophoresis, antibody used and strain of mice. Finally, in mouse oocytes, meiotic arrest is maintained by the treatment with dibutyryl cyclic adenosine monophosphate (dbcAMP) or IBMX via cAMP-dependent protein kinase (Cho et al. 1974; Elayne et al. 1986). Also, in Xenopus and starfish, hormone-induced maturation is inhibited when the intracellular levels of cAMP are increased by cholaer toxin or IBMX (Doree et al. 1981; Schorderet and Baulieu, 1982; Schorderet et al. 1982). From our results that p34^{ccc2} in the meiotic arrested oocytes by IBMX is not dephosphorylated, we suggest that cAMP-dependent protein kinase inhibits p34^{ccc2} kinase activation by keeping p34^{ccc2} in phosphorylated state.

We thank Dr Takeo Kishimoto, Tokyo Institute of Technology, for his critical advice. We would like to thank Dr Paul Nurse, University of Oxford, for reading this manuscript and helpful advice. We wish to express our acknowledgement to Dr Hiromichi Nagasawa, University of Tokyo for his helpful advice for synthesis of peptide and production of antibody. This work was supported in part by grants-in-aid for synthesis of peptide and production of antibody used and strain of mice. This work was supported in part by grants-in-aid for synthesis of peptide and production of antibody used and strain of mice.

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(Accepted 17 July 1991)