Low temperature induces oocytes p34^{cdc2} synthesis and accumulation- the acquisition of competence to resume meiosis in toad oocytes[1]

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ABSTRACT

Full grown oocytes derived from *Bufo Bufo gargarizans* rearing at high temperature environment (24℃), never underwent GVBD after progesterone treatment. No p34^{cdc2} H1 kinase activity was detected in the oocytes after progesterone stimulation or OA microinjection; Western blotting analysis showed that the level of p34^{cdc2} and p33 in the oocytes are significantly lower than those in the oocytes derived from the hibernating toads (below 10℃). ^{35}S-Met incorporation analysis showed that when the oocytes were incubated at 6℃, synthesis of about thirty different polypeptides was promoted or induced, including p34^{cdc2} and some other p13^{suc1}-binding proteins. All these results indicated that a low temperature environment is essential for the oocytes of *Bufo Bufo gargarizans* to express and store some cell cycle drivers and its regulators, and to gain the maturation competence. These results will also provide a new clue for explaining the molecular mechanisms why gametogenesis of some organisms depends on a relative low temperature and how to maintain the geographical distribution of some animals.

Key words: *Maturation competence, p34^{cdc2}, low temperature environment.*

INTRODUCTION

Temperature is one of the main factors that effects the reproduction cycle and gametogensis of some organisms. The gametes at different developmental stages of gametogenesis depend on somewhat different tepmperature. For example, the spermatogenesis of some mammals is completed during their hibernation period[l], the spermatogenesis in testes of scrotal mammals needs to have a local temperature in scrotum lower than that of the body temperature[2]. It is also well known that full-grown oocytes derived from *Bufo Bufo gargarizans* rearing at high temperature environment (28℃), designated as HTE-oocytes, or before entering hibernation state never acquire the competence of maturation. In contrast, the full-grown oocytes derived from the toad hibernating at low temperature environment (<10℃), designated as LTE-oocytes, can respond to progesterone stimulation and resume meiotic devision[3, 4]. *In vitro* experiments also have shown that the HTE-oocytes acquired the maturation competence after a certain period of incubation at low temperature environment (below 10℃), and the process is independent to any gonadotropic hormones or sex hormones stimulation[5]. Further analysis revealed that there were some early events of maturation process (such as the decrease of the endogeneous cAMP level, etc.) which took place in HTE-oocytes after progesterone treatment, but neither maturation promoting factor (MPF) activity nor the ability to amplify MPF activity[6] were detectable. These results indicated that there may be some deficiency in components necessary for the late events of oocytes maturation process[6].

A stock of inactive p34^{cdc2}/ cyclin B (inactive pre-MPF) was produced during the course of oogenesis in oocytes, and required only a post-translational modification for the conversion of this inactive form into an active one. At the onset of meiosis, it was the phosphatase (cdc25) which activates MPF and assures its autoamplification by dephosphorylating Tyrl5 and most probably Thrl4 of its catalytic subunit "p34^{cdc2}" [7]. In the present study, we showed that the deficiency in MPF and some of its regulators may be the main cause of the incapability of HTE-oocytes to gain the maturation competence. Moreover, we found that the synthesis of p34^{cdc2} and some other cell cycle regulators are promoted by low temperature in the HTE-oocytes.

MATERIALS AND METHODS

Materials

Bufo Bufo gargarizans were obtained from JiangSu Province in December, some of them were kept in cold room (4℃) to maintain the full -grown oocytes sensitive to progesterone stimulation. The others were reared in a high temperature environment (28℃) all over the year in order to make oocytes inert to progesterone treatment, which means that oocytes of this kind will never undergo germinal vesicle breakdown (GVBD) after progesterone stimulation.

Progesterone, glycerophosphate, PMSF, leupeptin, PKI (20 peptide inhibitor) were purchased from Sigma. γ-^{32}P-ATP (3000ci/mmol) and ECL Western Blotting System were obtained from Amersham. H1 histone, DTT were obtained from Boehringer. p13 agarose was obtained from

Santa Cruz. Okadaic acid (OA) was brought from Life and Technologies.

Full-grown (about 1.8 mm in diameter) oocytes used for microinjection with OA were selected from the toad's ovary fragment, ovarian membrane and follicular membrane around oocytes were divested manually with a pair of microforceps under a stereoscope. The nude oocytes were incubated in Ringer's solution at proper temperature for the experiment. Full-grown oocytes for the extraction of proteins were free from their ovarian and follcular membranes by collagenase treatment.

The rabbit antiserum against the conserved amino acid sequence of p34[cdc2] "EGVPSTAIREISL-LKEC"- keyhole limpet hemocyonin (KLH) complex (anti-PSTAIRE) and antiserum against the conserved amino acid sequence of Y-box binding protein "VRNGYGFINRNDSKEDVC" -KLH complex were prepared by our laboratory according to the methods described in Molecular Cloning (second edition)[8]. The HRP conjugated goat anti-mouse or anti-rabbit lgG were obtained from Promega Company.

Extraction of oocytes proteins and electrophoresis

oocyte proteins were extracted by homogenizing the oocytes in EB (80mM glycerophosphate, 50 mM NaF, 20 mM naphthylphosphate, 20 mM EGTA, 15 mM MgC1$_2$, 1 mM ATP, 1 mM DTT, 300 μM PMSF, 3 μg/ml leupeptin, 20 mM HEPES, pH 7.5), followed by ultracentrifugation (100,000g, 1 h, 4℃). The supernatants were treated with sample buffer containing 10 % mercaptoethanol for 3 min at 100℃, and then analysed by SDS-PAGE in 12.5 % gel.

Western blotting analysis

Proteins separated by SDS-PAGE were transferred to nitrocelleluse membrane by electro-blotting The membrane was rinsed in Tris-buffered saline (TBS: 20mM tris-HC1, 150 mM NaC1, pH7.5), blocked with 5 % non-fat dry milk in TBST (20 mM tris-HC1, 150 mM NaC1, 0.1% Tween 20) for 30 min, and then incubate with primary antibody for 1 h; after washed three times (5 min each) with TBST; the membrane was incubated with a 1:1000 HRP-conjugated goat anti-rabbit IgG or 1:2500 anti-mouse IgG in TBST for 30 min. Following further wash with TBST for three times, drained the excess buffer from the washed membrane, incubated in the ECL detection (the mixture of detection solution 1 and detection solution 2) for 1 min at room temperature, drained off excess detection reagent and wrapped the membrane in Saran Wrap. Then exposed for 15 seconds to 1 h in film cassette on hyperfilm (Amersham).

H1 kinase assays

The 20 μl of protein extracts from oocytes were mixed on ice with equal volume of 40 mM HEPES-NaOH (pH 7.3) consisting 10 mM NaEGTA, 0.2 mg/ml histone H1, 0.2 mM ATP, 0.25 μci γ-^{32}P-ATP and 10 μM cAMP-dependent protein kinase inhibitor. After incubation for 10 min at 22℃, the reactions were terminated by treated with SDS-PAGE sample buffer. Samples were electrophoresed on 12.5 % polyacrylamide gel. After visualization of H1 histone by staining with coomassie blue, ^{32}P incorporation was quantitated by scintillation counting of excised gel pieces or exposed for autoradiography.

Isolation of p34[cdc2] with p13[suc1] agarose beads

The nude HTE-oocytes or LTE-oocytes were incubated at 28℃ or 6℃ respectively in Ringer's solution for 6 h, and then were transferred to the Ringer's solution containing ^{35}S-Met (1 mci/ml) incubated at the respective temperature environment for another 6 h. The oocytes were washed three times in Ringer's solution, and then homogenizing in EB and centrifugated 10 min (20,000 g, 4℃). 50 μl sepharose CL-6B (pre-washed with EB) was added into the tubes containing 1 ml the extracts (2 mg/ml proteins), and were kept under constant rotation at 4℃ for 1 h. After centrifugation at 1000 g for 2 min, the supernatant was collected then 100 μ1 p13 beads (pre-washed with EB) were added and the tubes were kept under constant rotation at 4℃ for another 6 h.

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After centrifugation at 1000 g for 2 min of EB after removed of the supernatant. The beads were treated with SDS-PAGE sample buffer and the solubilized proteins were electrophoresed on 12.5 % polyacrylamide gel, stained with coomassie blue, dried, and then detected by autoradiography.

Microinjection of okadaic acid

75 nl of different dilutions of OA were prepared from a stock solution of 2.5 mM OA/DMSO in 10 mM HEPES (pH 7.4) buffer containing 1 mg/ml BSA and 5 mM DTT, and were microinjected into the oocytes at the animal hemisphere near the equator and incubated at 18℃ in Ringer's solution for further analysis.

RESULTS AND DISCUSSION

No p34^{cdc2} H1 kinase activity were detected in the HTE-oocytes after progesterone or OA stimulation

It has been verified that the HTE -oocytes can not undergo GVBD after progesterone treatment and do not manifest any activity of MPF autoamplification[5, 6]. Results from the H1 kinase activity assay revealed that a high level of p34^{cdc2} H1 kinase activity was induced in the progesterone treated LTE-oocytes, but no trace of p34^{cdc2} H1 kinase activity was detected in the HTE-oocytes before or after progesterone treatment (Fig 1). So this may be the reason why GVBD could not take place in the HTE-oocytes after the hormone stimulation.


Fig 1. The p34^{cdc2} H1 kinase activity in HTE- and LTE- toad oocytes. HTE-oocytes before (lane 1) and after (lane 2) progesterone treatment; LTE-oocytes before (lane 3) and after (lane 4) progesterone treatment.
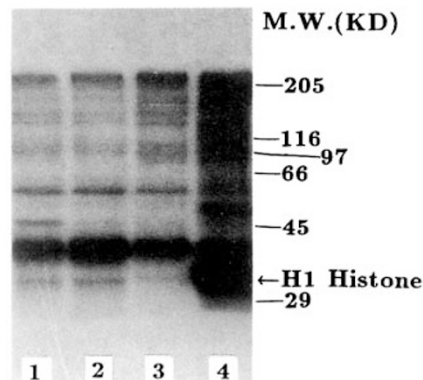
It is well known that the p34^{cdc2} H1 kinase is activated by a phosphatase (cdc25), which is negatively regulated by another phosphatase PP2A. Moreover, the activity of PP2A can be strongly inhibited by OA. So, cdc25 can be activated by suppressing PP2A with OA, which in turn can activate p34^{cdc2} H1 kinase[7, 9]. As OA can activate MPF indirectly, OA can undoubtly induce GVBD of HTE-oocytes if their MPF subunits(p34^{cdc2} and cyclin B) and cdc25 are present normally.

We found that 50 % effective dose (ED$_{50}$) OA to induce GVBD in LTE-oocytes by microinjection was 47.5 femtomoles (Fig 2a), and it took about 6 h to induce 100 % of LTE-oocytes maturation by injecting 74 femtomoles of OA (Fig 2b). While the HTE-oocytes never underwent GVBD even injecting 75 femtomoles of OA, and

no p34^{cdc2} H1 kinase activity was detected in HTE-oocytes (Fig 3), indicating that at least one of the subunits of MPF (P34^{cdc2} and cyclin B) or cdc25 may be in a deficient state.
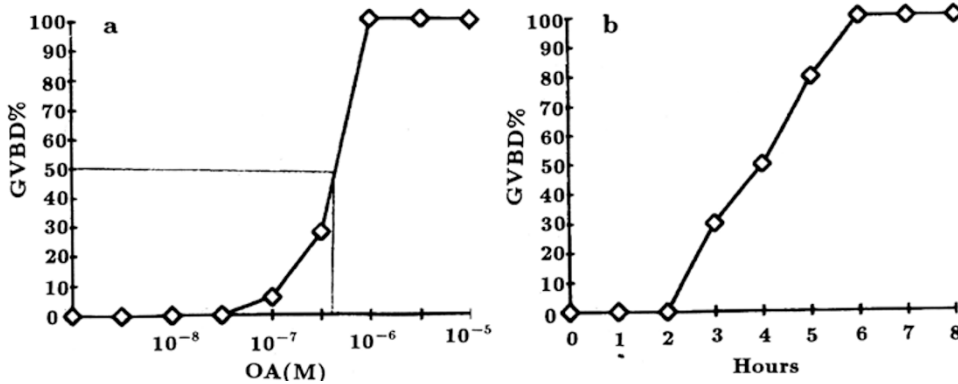
**Fig 2a.** Dose-response curve of okadaic acid induction of LTE-oocytes maturation.

   b. Time-response curve of okadaic acid (75 femtomoles) to induce LTE-oocytes maturation.

The levels of p34^{cdc2} and its homologues are different in LTE- and HTE-oocytes

Western blotting analysis with monoclonal antibody specifically against p34^{cdc2} and antiserum against PSTAIRE peptide showed that the amounts of p34^{cdc2} and its homologue "p33" in LTE-oocytes are five and ten times more than those in HTE-oocytes (Fig 4, data of densitometric analysis not shown). It was reported that the low level of p34^{cdc2} may account for their inability to resume meiosis upon exposure to OA in GVBD-incompetent mouse oocytes. After two days *in vitro* culture, the granulosa cell-free GVBD- incompetent mouse oocytes accumulate p34^{cdc2} autonomously and become progressively more responsive to OA[10].

Enormous p34^{cdc2} and the other cell cycle control factors are expressed and stored during oogenesis in oocytes which will be necessary for meiosis and early embryonic cell division[11]. No report has mentioned about the proper amount of p34^{cdc2} required for oocytes to resume in meiosis. Even there was little amount of p34^{cdc2} in HTE-oocytes, nor any p34^{cdc2} H1 kinase activity could be detected in HTE-oocytes treated with progesterone or OA. Our further experiments showed that the enriched p34^{cdc2} -cyclin B complexes (after precipitation with the p13^{suc1} beads) from both

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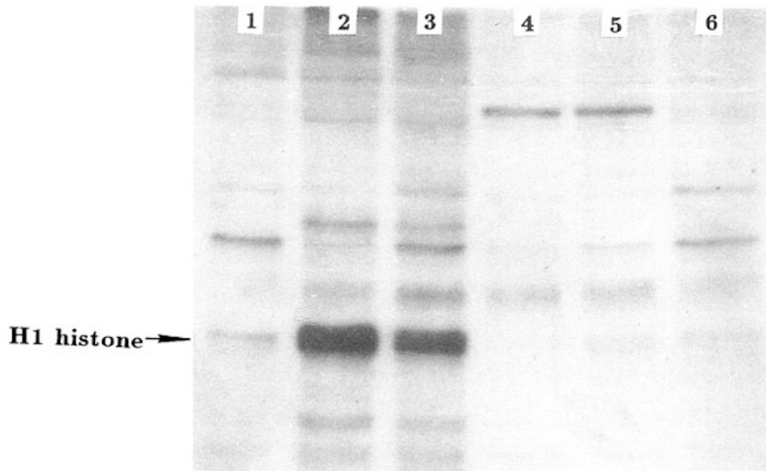
Fig 3. The levels of p34^{cdc2} H1 kinase activity in oocytes after OA microinjection
Lane 1, control, LTE-oocytes injected with HEPES buffer without OA;
Lane 2, LTE-oocytes injected with HEPES buffer containing OA;
Lane 3, LTE-oocytes treated with progesterone;
Lane 4, control, HTE-oocytes injected with HEPES buffer without OA;
Lane 5, HTE-oocytes injected with HEPES buffer containing OA;
Lane 6, HTE-oocytes treated with progesterone.

LTE-and HTE-oocytes (from a total number of 40 oocytes) contained only low level of H1 kinase activity (unpublished data), indicating that the quality of p34^{cdc2}-cyclin B complexes existed in HTE-oocytes is similar to that in LTE-oocytes. Thus, the low level of p34^{cdc2} and the deficiency of cdc25 (a phosphatase to activate pre-MPF[7] seem to be the main causes that HTE-oocytes were unable to gain the maturation competence.

It is also interesting to note that a p34^{cdc2} homologue with a molecular mass of 33.5 kD (called p33.5) was only expressed in HTE-oocytes, and was not recognized by monoclonal antibody specific to p34^{cdc2} (Fig 5), but its functions is still unclear. On the other hand, it was reported that there are some faster migrating forms of p34^{cdc2} or p33 as recognized by the antiserum to PSTAIRE in the mature oocytes of goldfish and starfish[12, 13]. In the similar manner, we found that there are two faster migrating forms (p32 and p31) appeared in the mature oocytes of *Bufo bufo gargarizans* (Fig 4). These results indicated that the appearance of different p34^{cdc2} homologues are affected by different external environmental conditions.

Fig 4. The levels of p34^{cdc2} and its homologues in LTE- and HTE-oocytes. Western blotting results obtained by using the antiserum specific to the PSTAIRE peptide. Lane 1, extract of intact LTE-oocytes and lane 2, extract from LTE-oocytes treated with progesterone; lane 3, extract of intact HTE-oocytes.
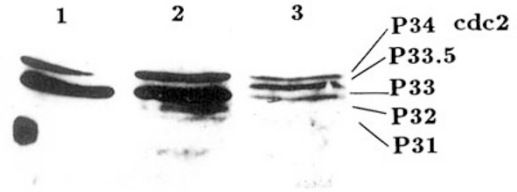
Fig 5. The level of p34^{cdc2} in LTE- and HTE-oocytes as revealed by Western blotting with monoclonal antibody against to C-terminal of p34^{cdc2}.
Extract of intact LTE-oocytes (lane 1) and extract from LTE-oocytes treated with progesterone (lane 2), extract of intact HTE-oocytes (lane 3).
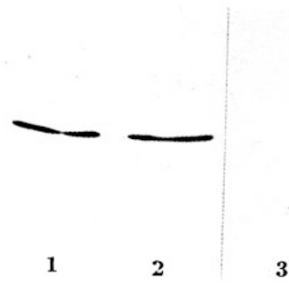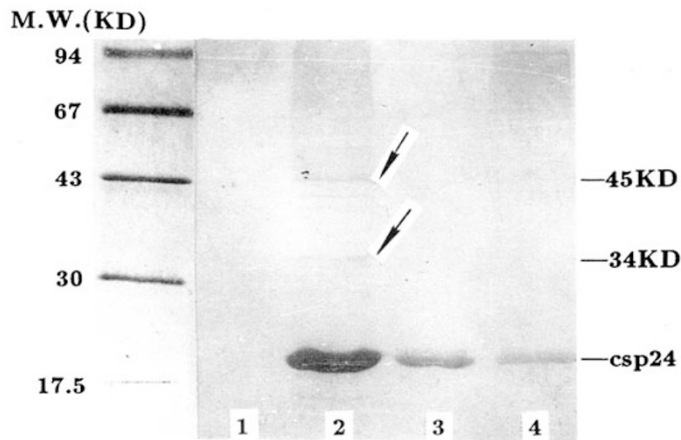

The synthesis of p34^{cdc2} and some p13^{suc1} binding proteins are promoted by low temperature in HTE-oocytes

To assess the effect of temperature on the rates of synthesis of p34^{cdc2} and its homologue in oocytes, HTE-oocytes or LTE-oocytes were incubated at 28℃ or 6℃ in Ringer's solutions with ^{35}S-Met (1 mci/ml) for 6 h, and then performed the precipitation analysis with p13^{suc1} agarose respectively. It was found that the synthesis of some kind of peptides, including a peptide with the molecular mass around 34 kD which we strongly suspected to be corresponded to p34^{cdc2} or its homologue, and two other peptides with the molecular mass of 45 kD and 24 kD (cold shock protein 24, csp24), were promoted by low temperature only in HTE-oocytes (Fig 6). We also found that csp24 was a protein which bound to p13^{suc1}, but did not bind to p34^{cdc2}/cyclin B complex (unpublished data), indicating csp24 may play an important role in MPF activation or in MPF localization in oocytes.

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Fig 6. The synthesis rates of p34^{cdc2} and some p13^{suc1} binding proteins in HTE-oocytes and LTE-oocytes incubated at different temperature.
^{35}S-Met incorporated proteins of oocytes were precipitated with p13^{suc1} agarose, and then subjected to SDS-PAGE, and exposed for autoradiography. The extracts of HTE-oocytes were incubated at 28℃ (lane 1) and 6℃ (lane 2), and those of LTE-oocytes were incubated at 28℃ (lane 3) and at 6℃ (lane 4).

It was reported that there are some new and enhanced synthesis of cold shock proteins in cultured amphibian cells[15], in winter wheat[14] and Escherichia coli etc.. One of the major cold shock proteins in E.coli, called cspA, shares a sequence similarity with the nucleic acid binding domain (cold shock domain) of Y-box binding proteins (such as FRGY1 etc.) in eukaryotic cells[16]. Similar to Y-box binding proteins, cspA can promote the expression of the genes which possess CCAAT element in their promoters. In addition, most of the low temperature inducible promoters contained CCAAT element in the E.coli[17, 18]. We were surprised to know that there are two copies of CCAAT elements in the promoters of human cdkl gene and Xenopus cdk2 gene[19, 20]. Meanwhile, we found that one of the Y-box binding proteins called "p54" was highly expressed in LTE-oocytes (Fig 7) and positively correlated with the high level of p34^{cdc2} and p33 in these oocytes, suggesting that p54 may be one of the factors involved in the process of the synthesis of the p34^{cdc2} and its homologue induced by low temperature.

Fig 7. The Y-box binding protein p54 is expressed mainly in LTE-oocytes. Results obtained from immunoblotting with antiserum against VRNGYGFINRNDSKEDVC peptide (a conserved sequence in the cold shock domain of Y-box binding protein). The extract of HTE-oocytes (lane 1) and the extract of LTE-oocytes (lane 2).
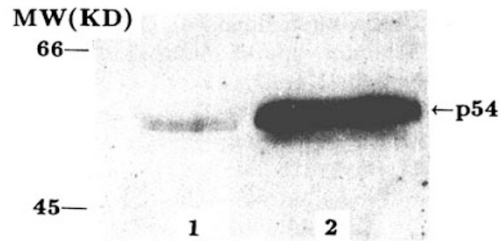
All these findings make a more reasonable explanation on the mechanism concerning the acquisition of maturation competence in oocytes which depends on hibernation: the low temperature as a external signal induces or enhances the expression of some cold shock proteins (such as p54 and csp24), and then the Y-box binding proteins as transcriptional or translational regulators, promoting the oocytes to express and store some proteins (such as $p34^{cdc2}$ and p33) related to oocytes maturation competence. Furthermeve, these results will also help us to understand the dependence of spermatogenesis in scrotal mammals on a relative low temperature, the mechanism of vernalization of plants for sprouting and flowering, and also the molecular base of the geographical distribution of the toad which is restricted to the region north to the 23° north latitude and east to the 100° east longitude in China.

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