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Cdc2-Cyclin B–Induced G2 to M Transition in Perch Oocyte Is Dependent on Cdc25[1]

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ABSTRACT

The G2 to M phase transition in perch oocytes is regulated by maturation promoting factor (MPF), a complex of Cdc2 and cyclin B. In *Anabas testudineus*, a fresh water perch, 17α,20β-dihydroxy-4-pregnen-3-one, the maturation inducing hormone (MIH), induced complete germinal vesicle breakdown (GVBD) of oocytes at 21 h. An unusual cyclin, p30 cyclin B, has been identified in oocyte extract using both monoclonal and polyclonal antibodies. Surprisingly, Cdc2 could not be identified, although a Northern blot with *Cdc2* cDNA demonstrated expression of the gene. Purification of MPF through an immunoaffinity column followed by SDS-PAGE showed three proteins, Cdc2, cyclin B, and a 20 kDa fragment, indicating earlier failure in immunodetection may be due to the interference by this fragment. In uninduced oocytes, p30 cyclin B was present, and its expression was increased by MIH. MIH increased p30 cyclin B accumulation at 3 h, a high level which was maintained between 9 and 21 h, but an effective increase in GVBD and H1 kinase activation could only be observed between 15 and 21 h. This delay in active MPF formation was found to be related to the activation of Cdc25, phosphorylation of which was detected at 12 h, and a substantial increase occurred during 15–18 h. Sodium orthovanadate, a tyrosine phosphatase inhibitor, inhibited H1 kinase activity and GVBD, suggesting the requirement of Cdc25 activity in MPF activation. Our results show occurrence of pre-MPF in uninduced oocytes and its conversion to active MPF requires dephosphorylation by Cdc25, the existence of which has not yet been shown in fish.

meiosis, oocyte development

INTRODUCTION

Oocytes, once their full growth is attained, get arrested at prophase of first meiosis. Maturation-inducing hormone (MIH) relieves oocytes from this arrest. Progesterone has been shown to function as MIH in frogs and higher vertebrates, whereas in fish 17α,20β-dihydroxy-pregnen-3-one (17α,20β-DP) is the MIH [1, 2]. MIH promotes the formation of a dimeric protein kinase known as MPF [3, 4], which in turn triggers meiotic maturation events like germinal vesicle breakdown (GVBD) through dispersion of nuclear lamina, chromosome condensation, and formation of metaphase spindles [5–9]. Maturation-promoting factor (MPF) is also shown to phosphorylate and activate EF1, an eukaryotic polypeptide chain elongation factor, resulting in protein synthesis associated with oocyte maturation in fish [10]. MPF has been purified from many vertebrates and invertebrates, all of which share a strikingly common molecular structure [11]. This is a complex of two proteins, a regulatory component called cyclin B and the catalytic component protein kinase known as cyclin-dependent kinase, Cdc2 [12–15]. Several investigators attempted to explain molecular mechanisms of MPF formation and activation during oocyte maturation in different species [16–21] and found that despite the common molecular structure of MPF in eukaryotes, mechanisms involved in active MPF formation differ [22–27].

In the *Xenopus* model, even before MIH activity, preformed cyclin B remains dimerized with Cdc2 to form pre-MPF or inactive MPF. This complex is phosphorylated at Thr161 but still remains inactive as the result of the inhibitory phosphorylation at Thr14 and Tyr15 [17, 28, 29]. Progesterone, the MIH in amphibians, stimulates Cdc25 to dephosphorylate Thr14 and Tyr15, which convert pre-MPF to active MPF having only Thr161 phosphorylation [30, 31]. Injection of small amounts of MPF has been shown to autoamplify MPF through Cdc25-mediated dephosphorylation, even in the presence of translation inhibitors [32, 33]. Cdc25 is inevitable for the progression of G2 to M phase not only in *Xenopus*, but also in other vertebrates, including mammals [34–38]. In contrast, pre-MPF is absent in immature bovine and pig oocytes [39, 40]. Pre-MPF also does not exist in fish [41–43]. Cell cycle is further controlled by the subcellular localization effecting physical separation of components [36, 37]. This model has been found valid for most higher animals, including mammals [38, 39].

MPF has been purified and characterized from various fish [41]. MIH induces de novo synthesis of cyclin B protein from its maternal mRNA, which in association with Cdc2 form active MPF in fish [23, 42, 43]. According to this model, preformed CDK1, a 34 kDa homologue of *Saccharomyces pombe* protein Cdc2, associates with newly synthesized 46 kDa cyclin B protein to form MPF. MPF is immediately phosphorylated at Thr161 of Cdc2 by cyclin-dependent kinase activating kinase (CAK) resulting in its activation [19]. Here, cyclin B translation, under MIH induction, is the key factor regulating oocyte maturation. There is no existence of Cdc25 because tyrosine phosphatase activity is not a requirement [41]. Mechanisms of MPF activation in pig are similar to those in fish [40]. Cyclin B1 could not be detected in fully grown pig oocytes, and activity of Cdc2 kinase is low. Following gonadotropin stimulation, oocytes start to accumulate cyclin B1 with greater Cdc2 kinase activity [44].

In this paper, we report the existence of pre-MPF in a freshwater perch, *Anabus testudineus*, where a unique p30 cyclin B could be detected in uninduced oocytes and its accumulation increased by MIH coinciding with histone H1

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kinase activity. Conversion of pre-MPF to MPF depends on Cdc25 activation and a tyrosine phosphatase inhibitor that blocks both Cdc25 activation and histone H1 phosphorylation. All of these indicate a different type of situation in perch than what has so far been reported for fish.

MATERIALS AND METHODS

Animals and Oocytes

Climbing perch (*A. testudineus*, Bloch.) were collected from in and around Santiniketan, West Bengal, India, during the prespawning and spawning stages (mid-April to August) of the annual reproductive cycle [45] and were maintained under controlled laboratory conditions until used. Fish with gravid ovary were dissected to collect full-grown immature ovarian follicles in ice-cold M199 medium (Sigma-Aldrich, St. Louis, MO). These oocytes were manually defolliculated, denuded, and washed several times with medium. Denudation effected GVBD or lysis in 5%–7% of the oocytes. Healthy oocytes were selected after 1 h preincubation prior to the addition of MIH. Denuded oocytes were subjected to MIH induction in vitro by incubating oocytes at 30° ± 2°C in Medium199 with 0.6% salinity supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) with or without (control) 1 μg/ml 17α,20β-DP (Sigma-Aldrich). Samples were collected in 3-h intervals up to the 21st hour of MIH induction. One microgram per milligram cycloheximide (SRL, Mumbai, India) or 1 mM sodium orthovanadate (Na_3VO_4; Sigma-Aldrich) was used along with MIH in parallel incubations as negative control. Migration of germinal vesicle (GV) and GVBD were determined by placing oocytes in a clearing solution (ethanol, formaldehyde, acetic acid, 3:6:1; modified from [46]) followed by microscopic examination. Viability of oocytes was examined by trypan blue dye exclusion. Concentration of inhibitors (i.e., cycloheximide and Na_3VO_4) used for experiments were chosen after testing for viability with trypan blue. All animal experiments were performed in accordance with the guidance of the Animal Ethics Committee of the Indian Institute of Chemical Biology.

Preparation of Oocyte Extracts

Fifty denuded oocytes were used per incubation. Oocytes were washed with extraction buffer (EB) containing 100 mM sodium β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 100 μM 4-amidinophenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.5 [47]. One microliter of EB/oocyte was added and homogenized with a glass homogenizer. The homogenate was centrifuged at 17 500 × *g* for 30 min at 4°C and the supernatant was stored at −20°C until further use.

Electrophoresis and Western Blot Analysis

Protein content in oocyte extract was determined according to the method described by Lowry et al. [48] and run on a 12.5% SDS-PAGE according to Laemmli [49]. Briefly, 10-μl samples were dissolved in 40 μl of 1× sample buffer containing 0.5 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.1% bromophenol blue; heated for 3 min; cooled; and 10-μl aliquots loaded on the stacking gel (4%), which was layered over a 12.5% running gel. Electrophoresis was carried out at 80 V constant voltage, and after completion of the run, the gel was either stained with 0.1% Coomassie blue (overnight) for visualization or transferred to Immobilon P membrane (Millipore Corp., Bradford, MA) for immunoblot. Membranes were incubated with 5% blocking buffer (Tris buffered saline with 0.1% Tween 20 and 5% nonfat milk) for 1 h followed by incubation with primary antibody overnight at 4°C. Rat polyclonal anti–cyclin B1 antibody; mouse monoclonal anti–cyclin B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); and goldfish anti–cyclin B monoclonal antibody (a kind gift from Dr. S. Haider, Banaras Hindu University, India, who received it from Prof. Y. Nagahama, NIBB, Japan) were used at 1:1000 dilution. Mouse anti-p34 Cdc2 monoclonal antibody and rabbit anti-p34 Cdc2-PSTAIRE polyclonal antibody (Santa Cruz Biotechnology) were used at 1:1000 dilution. Rabbit anti-phospho Cdc25C (Ser216) antibody (Cell Signaling Technology, Beverly, MA) was used at 1:1000 dilution. Bound primary antibodies were visualized using corresponding secondary antibodies at 1:1000 dilution, which were tagged with either alkaline phosphatase or horseradish peroxidase and were developed with corresponding substrates nitro blue tetrazolium/5-bromo 4-chloro 3-indoyl phosphate or tetra methyl benzidine/H_2O_2 (SRL, Mumbai, India), respectively.

H1 Kinase Activity

H1 kinase activity was measured according to Yamashita et al. [41]. Briefly, batches of 50 oocytes incubated with 1 μg/ml MIH containing Medium199 at 30°C for the indicated time intervals were homogenized in 50 μl EB using a glass homogenizer. The homogenate was centrifuged at 17 500 × *g*, 4°C for 30 min. Twenty microliters each of supernatants were incubated at 30°C for 2 min in the presence of the following: 100 μM histone H1 (Type-III-S; Sigma-Aldrich); 500 μM ATP; 1.5 μCi γ^{32}P-ATP (6000 Ci/mmol; Amersham Biosciences, Little Chalfont, UK); 1 mM EGTA, 10 mM $MgCl_2$; 4.5 mM 2-mercaptoethanol; and 20 mM Tris HCl (pH 7.4). After terminating the reaction with 20 μl of 300 mM phosphoric acid, 80 μl of reaction mixture was spotted on Whatman P81 phosphocellulose paper (Whatman, Brentford, UK), washed three times with 1% phosphoric acid, and dried, and radioactivity was measured in a liquid scintillation counter (LS 6000 SC, Beckman Coulter, Fullerton, CA).

To measure H1 kinase activity by autoradiography, the same procedure was followed with the exception that kinase reaction was stopped by adding 40 μl 1× SDS sample buffer, and 15 μl aliquots were analyzed on 15% SDS-PAGE, transferred to PVDF membrane, and autoradiographed on Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY).

Cloning of Cdc2 and Northern Analysis

Total RNA was extracted from full-grown denuded oocytes using Trireagent (Sigma-Aldrich). Two micrograms of total RNA was reverse transcribed with MuLV-reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) primed with oligo (deoxythymidine; 5′-GGAAGCTTTTTTTTTT TTTTT-3′). Reaction was carried out in 20 μl total volume containing 10 μM dithiotreitol (DTT), 0.5 mM of each deoxynucleotide triphosphate, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ for 1 h at 42°C. Two microliters of first-strand cDNA was amplified in 50 μl PCR reaction mixture containing 2.5 U Taq polymerase (Advantage2 RT-PCR kit, BD Biosciences, Palo Alto, CA); 1.5 mM $MgCl_2$; and 200 μM each of deoxy-NTP (Sigma-Aldrich) and the buffer supplied with the enzyme along with 2 μl each of sense and antisense primers diluted to 10 μM. For designing the primer, p34 *Cdc2* kinase mRNA sequences from mouse (accession U58633), frog (accession AF159158), and fish (accession D17758) were aligned using ClustalW software (http://WWW.ch.embnet.org/ software/ clustalW.html). The following degenerate primers, 5′-TA(T/C)GG(T/G)G TGGTATATAAGGG-3′ (sense) and 5′-GCTGGTC(T/A)AT(T/C)TCTG AGTC-3′ (antisense), designed from consensus regions on multiple alignment, were used for amplifying an approximately 600 bp segment of perch *Cdc2* homologue. The PCR was performed for 30 cycles of denaturation at 94°C for 30 sec (5 min in the first cycle), annealing at 54°C for 30 sec and extension at 72°C for 1 min (10 min last cycle; Perkin Elmer 9700; PE Applied Biosystems, Foster City, CA) with a manual hot-start protocol. PCR product was directly cloned into pTZ57R/T plasmid using InsT/A clone PCR cloning kit (MBI Fermentas) following the manufacturer's instructions. The cloned product was sequenced using ABI PRISM 3100 (Perkin Elmer; PE Applied Biosystems) automated sequencer. Sequence analysis was done by BLAST software available on the NCBI web site and submitted to the GenBank with the accession number AY533308. The amino acid sequence derived from the perch *Cdc2* cDNA matched a corresponding segment of *Oryzias javanicus* Cdc2 homologue accession number (accession BAB17220) over 99% (182/183). (See the supplementary file for the details on primer designing, perch *Cdc2* sequence, and multiple alignment.)

Twenty micrograms of total RNA isolated from perch oocytes was electrophoresed on 1% agarose-formaldehyde gel; transferred to a nylon membrane (Hybond-N^+, Amersham) by capillary suction method; and cross-linked. A random labeled probe was prepared using an *Eco*RI digested pTZ57R/T-*Cdc2* insert and gel eluted using Quiaquick gel extraction kit (QIAGEN, Hilden, Germany). [α^{32}P]d-ATP (Amersham) random labeling was carried out with Hexa Label Plus DNA labeling system (MBI Fermentas). Prehybridization was allowed for 2 h in the buffer containing 6× standard saline citrate (SSC) with 50% formamide, 1× Denhardt solution, and 0.5% SDS at 42°C. Hybridization was carried out under the same conditions along with labeled *Cdc2* cDNA for 18 h. The membrane was washed for 30 min at 55°C in 2× SSC and 0.1% SDS with three repeats. The hybridized membrane was exposed to Kodak Biomax MR film (Eastman Kodak).

Purification of MPF

Oocytes undergoing GVBD in vivo were collected and used as starting material for purification of MPF. For this purpose, gravid perch were in-

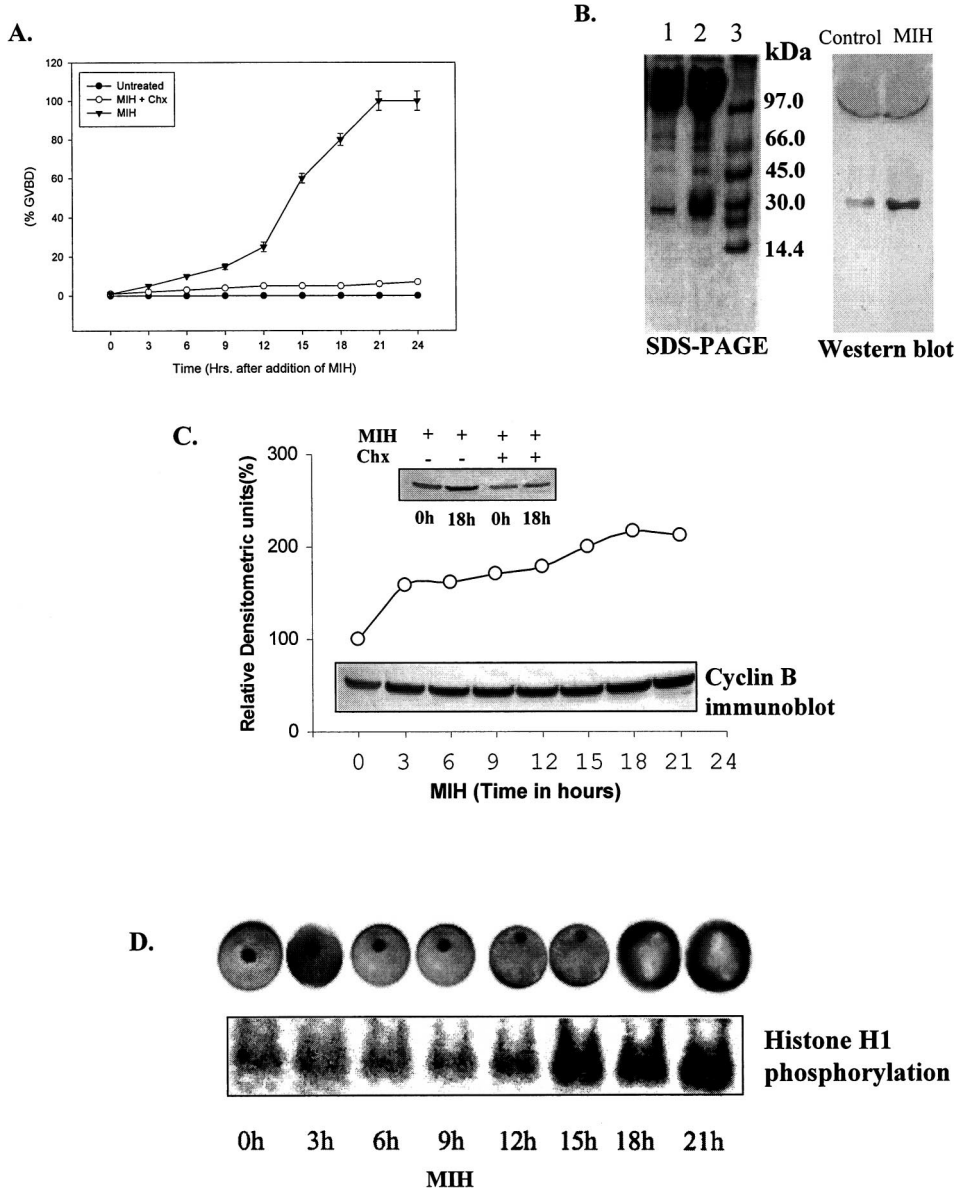

FIG. 1. MIH-induced maturation of perch oocyte. **A**) Percentage GVBD (closed triangles) of oocytes treated with MIH increased sharply from 12 to 21 h. Untreated oocytes (closed circles) showed no change. Cycloheximide (Chx) blocks MIH-induced GVBD (open circles). All data are mean ± SEM of five independent observations. **B**) Left panel: SDS-PAGE of oocyte extract from untreated (lane 1) and 18-h, MIH-treated oocytes (lane 2) with low-range molecular size markers in lane 3 (Amersham). Right panel demonstrates immunoblot of a denaturing gel with polyclonal anti–cyclin B antibody, which cross-reacted with 30 kDa protein. This protein showed a higher expression in response to MIH. Note that this protein expresses in uninduced oocytes as well. **C**) Time-dependent increase of p30 cyclin B in MIH-incubated oocytes was examined by Western blot analysis using rat polyclonal anti-cyclin B antibody followed by densitometric analysis (performed by using a software Image Master 1D Elite from Pharmacia Biotech). Perch oocyte extracts, previously incubated with MIH for the indicated time periods, were prepared in EB. Fifty micrograms of protein were loaded in each lane of a 12.5% SDS-PAGE and transblotted to a PVDF membrane before detection with rat polyclonal anti-cyclin B antibody. Addition of Chx during incubation of oocytes prevented MIH-induced accumulation of cyclin B detected by mouse monoclonal anti-cyclin B antibody (Inset). **D**) Histone H1 kinase activity of MIH-incubated oocyte extracts; incubations were terminated at indicated time points, and cytosolic extracts were subjected to H1 kinase assay followed by SDS-PAGE and autoradiography. Accompanying photographs show migration of GV and subsequent GVBD of oocytes of parallel incubations coinciding with H1 kinase activity (magnification 100×).

jected with carp gonadotropic hormone (purified by us [50]; 50 μg/fish) intraperitoneally and oocytes were stripped out just before ovulation. Nearly all (97%–99%) of these oocytes exhibited GVBD. Approximately 10^5 oocytes were denuded and homogenized in EB and centrifuged at 17 500 × g as mentioned above. The supernatant was subjected to ultracentrifugation at $10^5 \times g$ in Beckman Ultracentrifuge for 1 h; supernatant was applied to a DEAE-cellulose column (10 × 2 cm) equilibrated with EB containing 15 mM NaCl. After washing the column, proteins were eluted with discontinuous NaCl gradient of 50 and 100 mM in the same buffer. Two milliliters of fractions were collected and subjected to histone H1 kinase determination.

The fractions containing histone H1 kinase activity were pooled; the volume was reduced by lyophilization and chromatographed on a Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column and equilibrated with EB using a low-pressure Biologic LP chromatography system (Biorad Laboratories Pvt. Ltd., Hercules, CA). Peak fractions were pooled, lyophilized, dialyzed, and checked for H1 kinase activity.

Active peak was further purified by an immuno-affinity chromatography using mouse anti–cyclin B1 monoclonal antibody linked to Sepharose 4B matrix (Amersham). Antibody was tagged to the matrix according to the instructions from the manufacturer. SG 200 active peak was loaded onto an affinity matrix and incubated overnight at 4°C. The column was washed with EB to remove unbound proteins. Bound protein(s) was eluted with EB containing 2 M KI as chaotropic reagent, dialyzed, and lyophilized to use in subsequent experiments.

Purified p13 Suc1 protein was a kind donation from Prof. Tim Hunt (Cancer Research, UK, London, UK; Clare Hall Laboratories). The p13 Suc1 was conjugated to Cyanogen Bromide (CnBr) activated Sepharose 4B (Amersham) according to the instructions of the manufacturer. Unreacted groups of the gel were quenched with 1 M ethanolamine (pH 8.0). The concentration of coupled Suc1 was approximately 3.5 mg/ml of gel. A column (4 × 1 cm) was prepared with the matrix, and active peak from S-200 was allowed to bind to the column overnight. The column was washed consecutively with five column volumes of EB, 4M NaCl in EB, and ultimately a 3 mM solution of Suc1 without NaCl [12] to elute the unbound and bound proteins, respectively; optical density was measured at 280 nm.

Microinjection of Oocytes

Affinity-eluted fractions from 6- or 18-h MIH-induced oocytes (6hAII or 18hAII fraction, respectively) were pooled, dialyzed against water, and concentrated by lyophilization, and approximately 7 pg protein was injected into full-grown oocytes collected from control fish. With the help of a local manufacturer, John Seal & Co. (Kolkata, India), we prepared injection pipettes (outer diameter, 5 μm; inner diameter, 2 μm) and holding pipettes (outer diameter, 18 μm; inner diameter, 15 μm). One hundred oocytes were microinjected on an inverted phase-contrast microscope (Olympus Optical Ltd., Tokyo, Japan) either with 6hAII or 18hAII fraction or EB. About 15%–20% 18hAII injected oocytes underwent GVBD synchronously when visualized with clearing solution after 1 h, whereas 6hAII or EB (served as control) injected oocytes showed no GVBD.

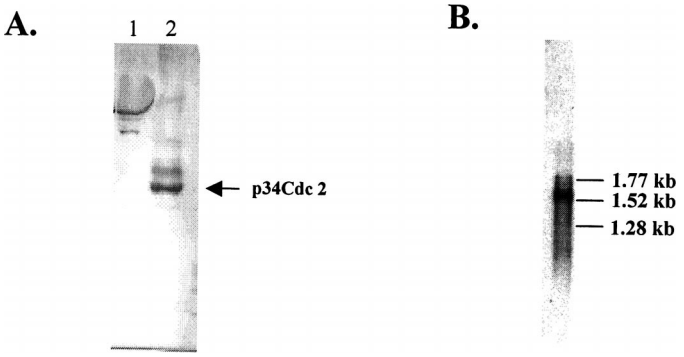

FIG. 2. Identification of Cdc2. **A**) Cdc2 could be detected in rat testis extract by PSTAIRE antibody (lane 2), but could not be detected in perch oocyte extract (lane 1). **B**) Northern hybridization of perch oocyte RNA using perch *Cdc2* cDNA (accession AY533308) detects mRNA of 1.5 kb long, indicating *Cdc2* gene expression.

RESULTS

MIH-induced GVBD in perch oocytes increased sharply from 12 h onward, which was blocked by cycloheximide (Fig. 1A) indicating the requirement of new protein synthesis. Extract of 18-h, MIH-incubated oocytes in SDS-PAGE showed overexpression of 30 kDa protein (Fig. 1B, left panel). Western blot analysis of control and MIH-induced oocyte extracts showed cross-reaction of 30 kDa protein with rat polyclonal anti-cyclin B antibody, indicating it to be a cyclin B. However, we confirmed this 30 kDa protein as cyclin B by using mouse and goldfish anti-cyclin B monoclonal antibodies. In perch, cyclin B could be detected in uninduced oocytes, but its expression was increased in response to MIH (Fig. 1B, right panel). The densitometric analysis of Western blot shows an increased cyclin B content due to MIH from 3 to 12 h—which remained almost in the same level during this period—and then a rise between 15 and 21 h (Fig. 1C). The rise during 15 and 21 h appears to be critical as maximum GVBD occurred during this period. The lag phase between 3 and 12 h in perch oocyte maturation (Fig. 1A) was difficult to understand since an MIH-induced increase in p30 cyclin B could be detected during this period (Fig. 1C). Cycloheximide (Chx), a translation inhibitor, blocked an MIH-induced increase of cyclin B and also GVBD (Fig. 1, A and inset of C). Detection of cyclin B in uninduced oocytes of fish seems to be unusual (Fig. 1, B and C), but that resembles the situation observed in *Xenopus* [17, 29, 51]. Although a higher level of cyclin B was maintained between 3 and 12 h, it did not coincide with histone H1 kinase activation or GVBD. This suggests a critical requirement of cyclin B content in oocytes to form an optimum mass of MPF, which appears to be satisfied during 15–21 h of MIH induction as both histone H1 phosphorylation and oocyte GVBD occurred during this period (Fig. 1, A and D). However, we failed to comprehend why a considerable level of cyclin B between 3 and 12 h was unable to activate histone H1 kinase or cause GVBD.

Surprisingly, Cdc2 could not be detected in perch oocytes (Fig. 2A). A parallel experiment with rat testis extract using PSTAIRE antibody in Western blot analysis clearly identified Cdc2; this indicates that failure to detect Cdc2 with PSTAIRE antibody in perch oocyte extract is not the result of experimental error. We have also used rabbit anti-Cdc2 polyclonal antibody but could not detect Cdc2 (data not shown). Presuming association of other proteins with Cdc2, which may interfere with its immunodetection, p13

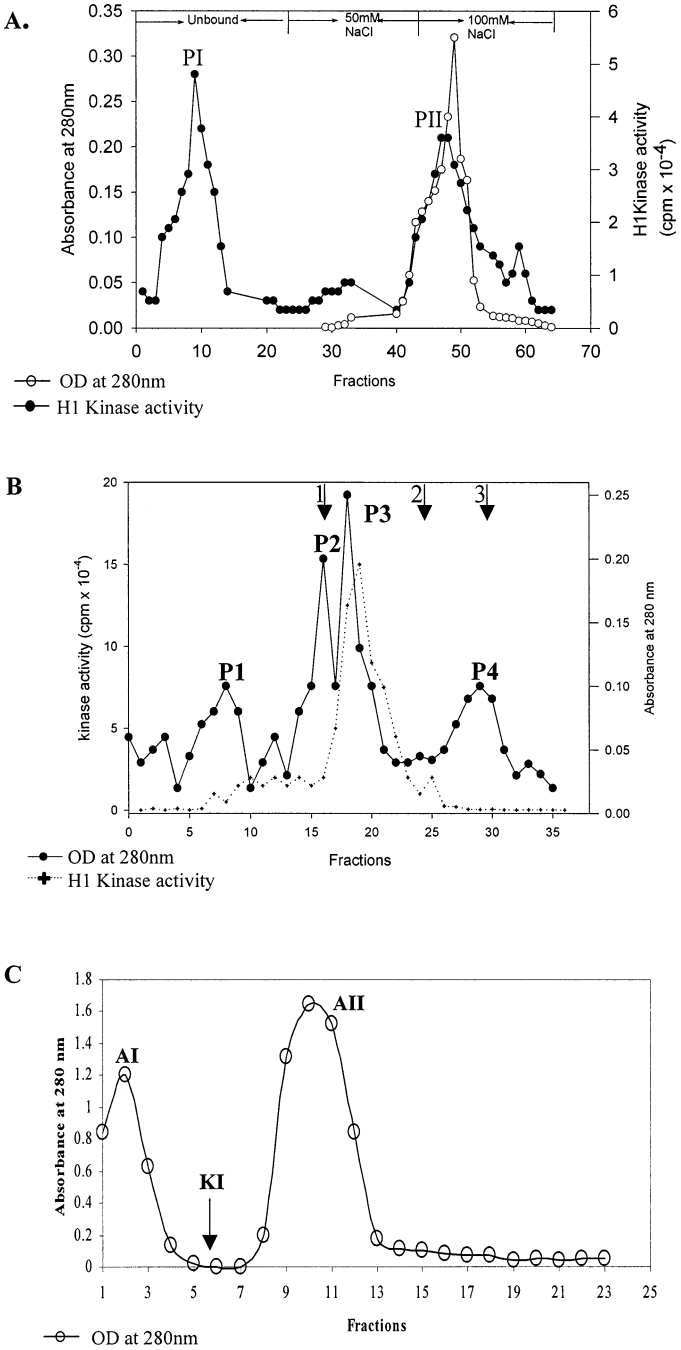

FIG. 3. Purification of MPF from perch oocyte extract. **A**) Oocyte extracts were subjected to DEAE-cellulose chromatography. DEAE-bound fractions eluted with 100 mM NaCl (closed circles) showed histone H1 kinase activity with 75 μg of protein (open circles). **B**) Further purification of MPF on a Sephadex G200 column. The closed circle shows elution of proteins in four major peaks of which SG-P3 fractions (10 μg protein) demonstrated H1 kinase activity (crossed hair). **C**) SG-P3 was allowed to bind overnight to the immuno-affinity matrix containing mouse monoclonal anti-cyclin B antibody and Sepharose 4B. Column was washed with extraction buffer to remove the unbound proteins (AI), and bound protein was eluted with 2M KI (AII).

Suc1 a Sepharose column was used to isolate Cdc2. It was intriguing to find all protein eluted without binding to the matrix, and eluted material showed MPF activity, which again did not exhibit Cdc2 in Western blot with anti-PSTAIRE antibody (data not shown). We therefore attempted to observe *Cdc2* gene expression in perch oocytes. An RT-PCR with primers designed from the consensus se-

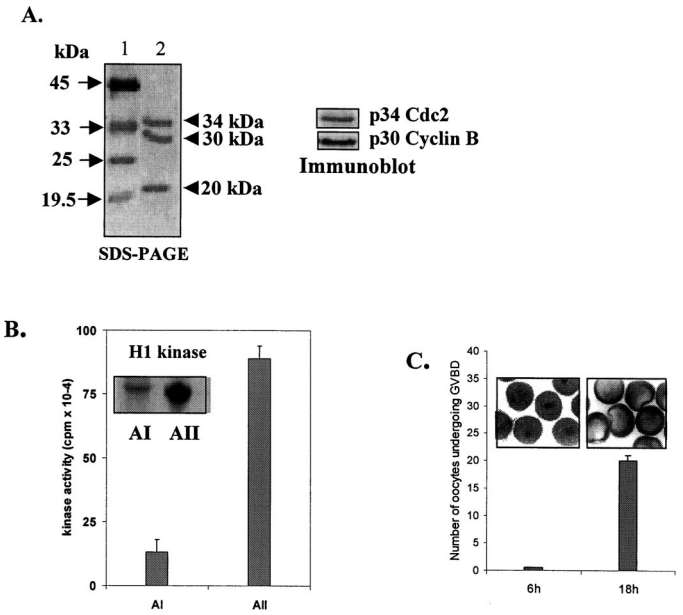

FIG. 4. Identification of Cdc2 in the eluate from immunoaffinity column. **A**) Immunoaffinity bound fraction, AII sample was specially treated by using 5% SDS followed by boiling for 15 min and then subjected to 15% SDS-PAGE. Gel was stained with silver showing three bands having 34, 30, and 20 kDa molecular sizes. Another similarly treated AII sample was electrophoresed in 15% SDS-PAGE, and the gel was immunoblotted with mouse monoclonal anti-cyclin B1 and anti-PSTAIRE antibodies. This time 34 kDa as Cdc2 could be identified by PSTAIRE antibody and 30 kDa as cyclin B. **B**) AI and AII peaks were pooled separately and dialyzed, and volume was reduced by lyophilization; 1.5 μg protein was added to the incubation mixture to examine histone H1 kinase activity. **C**) Injection of AII protein into the perch oocyte–effected GVBD. The representative figures on top were taken at 50× magnification.

quence of *Cdc2* of different animals including goldfish gave a product, which showed homology to known fish *Cdc2* sequences, and was cloned. *Cdc2* cDNA hybridized with perch oocyte total RNA (Fig. 2B), transcript size (~1.6 kb), was close to the known *Cdc2* genes as evident from GenBank.

To understand why Cdc2 protein could not be detected in immunoblots using anti-Cdc2 or anti-PSTAIRE antibodies, we partially purified MPF by using a DEAE cellulose column (Fig. 3A) and SG200 gel chromatography (Fig. 3B). We then prepared an immunoaffinity column with anti-cyclin B1-Sepharose 4B matrix to isolate MPF. Anti-cyclin B antibody bound material was eluted in AII fractions (Fig. 3C). AII fraction resolved into three bands in SDS-PAGE of molecular masses 34, 30, and 20 kDa (Fig. 4A). The 34 kDa protein clearly cross-reacted with anti-PSTAIRE antibody, thus showing it as Cdc2. The 30 kDa protein cross-reacted with mouse monoclonal anti-cyclin B antibody, whereas the 20 kDa fragment appears to be a contamination that probably masked immunodetection of Cdc2. Immunoaffinity eluate, AII, showed strong H1 kinase activity (Fig. 4B). Injection of 18hAII caused GVBD, whereas 6hAII had no effect (Fig. 4C). This shows the difference between pre-MPF and MPF activity. Oocytes injected with EB did not produce any effect (photograph not shown).

Although cyclin B and Cdc2 were present in uninduced oocyte, why MPF remained inactive until 15 h after MIH treatment required an answer. During this period, pre-MPF possibly started to convert to active MPF and reached the peak at 18–21 h. This indicates the appearance of a factor at about 15 h. We suspected this to be Cdc25. Therefore, we used specific phospho-antibody against Cdc25C to monitor its phosphorylation at different time intervals. Figure 5A shows that activation of Cdc25 could be detected from 12 h onward, which intensified at 18 h. Involvement of Cdc25 in perch oocyte maturation was further substantiated by the observations that sodium orthovanadate (Na_3VO_4),

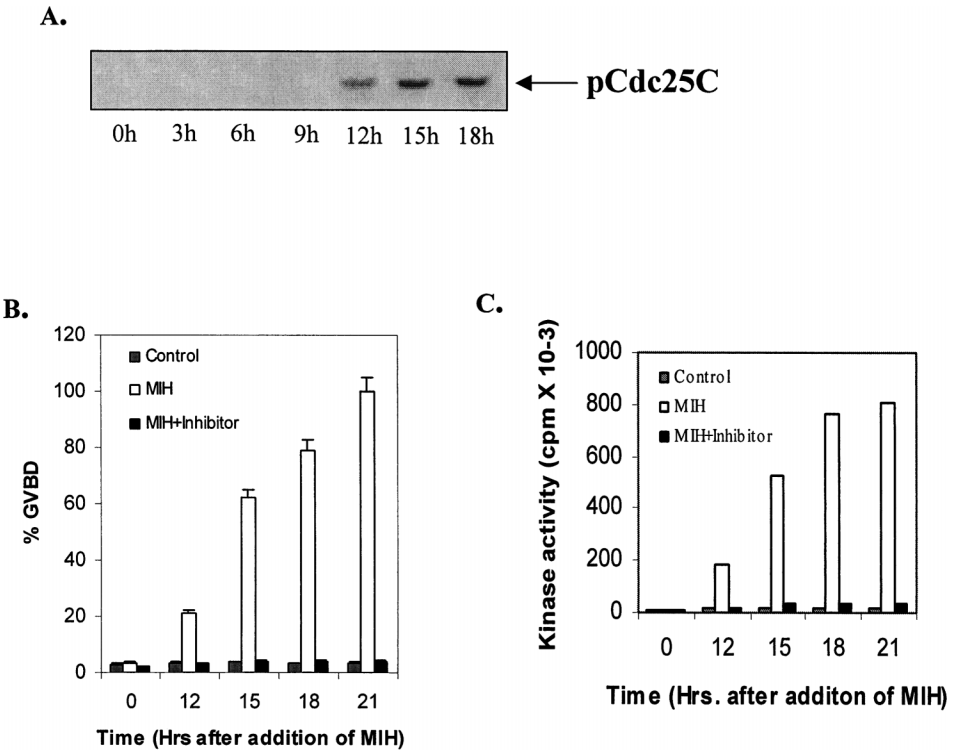

FIG. 5. Final maturation of perch oocyte closely follows Cdc25 phosphorylation pattern. **A**) Extract from MIH-induced perch oocyte at different time levels was immunoblotted by using rabbit anti-pCdc25C antibody. **B**) MIH-induced GVBD was blocked by tyrosine phosphatase inhibitor, Na_3VO_4. **C**) Histone H1 kinase activity of oocyte extract induced by MIH is also ablated by Cdc25C inactivation.

an inhibitor of tyrosine phosphatase activity, blocked MIH-induced GVBD (Fig. 5B) and histone H1 phosphorylation (Fig. 5C). These experiments clearly show the importance of activation of Cdc25 in the final maturation of perch oocytes.

DISCUSSION

In this paper we report that in *A. testudineus*, a freshwater perch, cyclin B exists in uninduced oocytes, which is a deviation from what has so far been reported in fish. Cyclin B is de novo synthesized in fish oocyte from maternal mRNA by MIH induction, and by associating with Cdc2 it forms active MPF [23, 24, 31–33]. In contrast, MIH induces greater accumulation of cyclin B in perch oocytes. This 30 kDa protein cross-reacted with both monoclonal and polyclonal cyclin B antibodies from goldfish, mouse, and rat. Because cycloheximide blocks MPF activity, there may be an optimum requirement of MIH-induced, newly synthesized cyclin B that on association with Cdc2 forms a critical mass of MPF to achieve the peak of GVBD during 15–21 h. Perch oocytes have an unusual cyclin B in terms of molecular mass (i.e., 30 kDa, which is much smaller than the 46–55 kDa cyclin B reported in other animals [52–54]). This immunoreactive cyclin B as a constituent of MPF is reflected from its overexpression by MIH at 18 h when GVBD is in peak; its inhibition by cycloheximide at 18 h then coincides with inhibition of GVBD. Immunoaffinity eluate through anti-cyclin B matrix, containing p30 cyclin B and p34 Cdc2, showed strong MPF activity both in terms of H1 kinase activity and GVBD. Moreover, injection of immunoaffinity eluate to uninduced perch oocyte caused GVBD. MIH activity in perch oocyte followed by MPF activation has similarity with the *Xenopus* model. In *Xenopus*, preformed cyclin B remains dimerized with Cdc2 to form pre-MPF, and MIH induces new synthesis of cyclin B for Cdc2 kinase activation [51].

We are confronted with two aspects in our findings: 1) why we could not detect Cdc2 in perch oocyte and 2) why the presence of appreciable amounts of cyclin B during 6–12 h in response to MIH could not augment H1 kinase activity and GVBD. To address the first aspect, we attempted purification of perch oocyte MPF presuming that Cdc2 may partially remain blocked with contaminating protein, which probably disallowed cross-reaction with PSTAIRE antibody or Cdc2 polyclonal antibody. To detect Cdc2, we prepared an anti-cyclin B antibody-Sepharose 4B matrix (using mouse monoclonal anti-cyclin B) so that Cdc2 could be co-eluted with anti-cyclin B bound protein. To get the separation of Cdc2 in SDS-PAGE, we had to use 15% gel and 10 min boiling with sample buffer, and this treatment attributed Cdc2 separation and then identification by the PSTAIRE antibody. SDS-PAGE of immunoaffinity eluate showed three protein bands: 34, 30, and 20 kDa. The 34 kDa protein was identified as Cdc2, and 30 kDa as cyclin B. Association of p20 with the cyclin B–Cdc2 complex probably interferes with Cdc2 immunodetection. MPF association with p20 opens new possibilities. A regulatory protein RINGO has recently been shown to be associated with Cdc2 in *Xenopus* oocyte [55]. The p20 is smaller than RINGO [22], and its regulatory role is not known. However, future investigation with p20 is indeed very necessary.

The second aspect, where accumulation of appreciable amounts of cyclin B induced by MIH from 3–12 h did not show stimulation of H1 kinase activity or GVBD, suggests an increase in pre-MPF during this span of time. This is a situation very similar to the mechanisms involved in *Xenopus* oocyte maturation [29]. There, Cdc2 and cyclin B remain dimerized as pre-MPF; phosphorylation of Thr161 could not activate MPF because of Thr14 and Tyr15 phosphorylation, which inhibits MPF activity [17, 41, 42]. Activation of pre-MPF requires inhibition of wee 1 protein kinase that phosphorylates Tyr15 of p34 Cdc2, as well as activation of Cdc25, a phosphatase that removes this inhibitory phosphate [56]. The lag phase until 12 h of MIH induction and a sharp rise of MPF activity from 12 h onward indicates the appearance of a factor that can rapidly convert pre-MPF to active MPF. We thought that a suitable candidate for such rapid conversion is Cdc25 because inhibitory phospho-Tyr15 is already remaining with the inactive MPF; hence wee 1 is not a candidate. With this idea in mind, we have used specific anti-phospho-Cdc25C, which is activated by phosphorylation at Ser216 residue. Detection of phosphorylated Cdc25 at 12 h and its increase at 15–18 h showed that MPF activation in perch oocytes requires Cdc25 phosphatase activity. The pattern of Cdc25 activation coincides with the H1 kinase phosphorylation and GVBD. Requirement of Cdc25 in MPF activation in perch oocyte is further supported from the experiment where sodium orthovanadate, a tyrosine phosphatase inhibitor, inhibited histone H1 kinase activation and GVBD.

Reports on the mechanisms of oocyte maturation in teleosts show that phosphorylation of Thr161 residue on Cdc2 (after it binds to cyclin B) by CAK results in the activation of MPF [19, 43] and does not include phosphorylation and subsequent dephosphorylation on Tyr15. Contrary to these reports, MPF activation in perch requires Cdc25 phosphorylation, indicating existence of a different pathway in this teleost. How Cdc25 is activated in *A. testudineus* is yet to be investigated, but the presence of this phosphatase in a fish and its relationship with MPF in regulating final maturation is new information. Further investigation with perch oocytes would be interesting and may provide meaningful insight in terms of the evolution of regulatory mechanisms involved in oocyte maturation.

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REFERENCES

1. Nagahama Y, Adachi S. Identification of maturation-inducing steroid in teleost, the amgo salmon (*Onycorhynchus rhodurus*). Dev Biol 1985; 109:428–435.
2. Nagahama Y. 17α,20β-dihydroxy-4-pregnen-3-one: a teleost maturation-inducing hormone. Dev Growth Differ 1987; 29:1–12.
3. Masui Y, Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool 1971; 177:129–145.
4. Masui Y, Clarke HJ. Oocyte maturation. Int Rev Cytol 1979; 57:185–282.
5. Lewin B. Driving the cell cycle: M phase kinase, its partners and substrates. Cell 1990; 61:743–752.
6. Peter M, Nakagawa J, Dorée M, Labbé JC, Nigg EA. In vitro disassembly of the nuclear lamina and M-phase specific phosphorylation of lamins by cdc2 kinase. Cell 1990; 61:591–602.
7. Pfaller R, Smythe C, Newport JW. Assembly/disassembly of the nuclear envelop membrane: cell-cycle dependent binding of nuclear membrane vesicles to chromatin in vitro. Cell 1991; 65:209–217.
8. Collas P. Sequential PKC- and Cdc2-mediated phosphorylation events

elicit zebrafish nuclear envelope disassembly. J Cell Sci 1999; 112: 977–987.
9. Contreras A, Hale TK, Stenoien DL, Rosen JM, Mancini MA, Herrera RE. The dynamic mobility of histone H1 is regulated by cyclin/CDK phosphorylation. Mol Cell Biol 2003; 23:8626–8636.
10. Tokumoto M, Nagahama Y, Tokumoto T. A major substrate for MPF: cDNA cloning and expression of polypeptide chain elongation factor 1 gamma from goldfish (*Carassius auratus*). DNA Seq 2002; 13:27–31.
11. Yamashita N, Mita K, Yoshida N, Kondo T. Molecular mechanisms of the initiation of oocyte maturation: general and species specific aspects. In: Meijer I, Jezequel A, Ducommun B (eds.), Progress in Cell Cycle Research. New York: Kluwer; 2000:115–129.
12. Lohka MJ, Hayes MK, Maller JL. Purification of maturation-promoting factor, an intracellular regulator of early miotic events. Proc Natl Acad Sci U S A 1988; 85:3009–3013.
13. Labbé JC, Capony JP, Caput D, Cavadore JC, Derancourt J, Kaghdad M, Lelias JM, Picard A, Dorée M. MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc^2 and one molecule of cyclin B. EMBO J 1989; 8:3053–3058.
14. Labbé JC, Picard A, Peaucellier G, Cavadore JC, Nurse P, Dorée M. Purification of MPF from starfish: identification as the H1 histone kinase $p34^{cdc2}$ and a possible mechanism for its periodic activation. Cell 1989; 57:253–263.
15. Yamashita M, Fukada S, Yoshikuni M, Bulet P, Hirai T, Yamaguchi A, Lou YH, Zhao Z, Nagahama Y. Purification and characterization of maturation promoting factor in fish. Dev Biol 1992; 148:8–15.
16. Kishimoto T. Regulation of metaphase by a maturation-promoting factor. Dev Growth Differ 1988; 30:105–115.
17. Kobayashi H, Minshull J, Ford C, Golsteyn R, Poon R, Hunt T. On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation of *Xenopus levis*. J Cell Biol 1991; 114: 755–765.
18. Katsu Y, Yamashita M, Kajiura H, Nagahama Y. Behavior of the components of maturation-promoting factor, cdc2 kinase and cyclin B, during oocyte maturation of goldfish. Dev Biol 1993; 160:99–107.
19. Yamashita M, Kajiura H, Tanaka T, Onoe S, Nagahama Y. Molecular mechanisms of the activation of maturation-promoting factor during goldfish oocyte maturation. Dev Biol 1995; 168:62–75.
20. Palmer A, Nebreda AR. The activation of MAP kinase and p34cdc2/cyclin B during the meiotic maturation of *Xenopus* oocytes. Prog Cell Cycle Res 2000; 4:131–143.
21. Tachibana K, Yanagishima N, Kishimoto T. Preliminary characterization of maturation-promoting factor from yeast *Saccharomyces cerevisiae*. J Cell Sci 1987; 88:273–281.
22. Ferby I, Blazquez M, Palmer A, Eritja R, Nebreda AR. A novel $p34^{cdc2}$ binding and activating protein that is necessary and sufficient to trigger G2/M progression in *Xenopus* oocytes. Gene Dev 1999; 13: 2177–2189.
23. Katsu Y, Yamashita M, Nagahama Y. Translational regulation of cyclin B mRNA by 17α,20β-dihydroxy-4-pregnen-3-one (maturation-inducing hormone) during oocyte maturation in a teleost fish, the goldfish (*Carassius auratus*). Mol Cell Endocrinol 1999; 158:79–85.
24. Ihara J, Yoshida N, Tanaka T, Mita K, Yamashida M. Either cyclin B1 or B2 is necessary and sufficient for inducing germinal vesicle breakdown during frog (*Rana japonicus*) oocyte maturation. Mol Reprod Dev 1998; 50:499–509.
25. Kishimoto T. Activation of MPF at meiosis reinitiation in starfish oocytes. Dev Biol 1999; 214:1–8.
26. McGowan CH. Regulation of the eukaryotic cell cycle. Prog Cell Cycle Res 2003; 5:1–4.
27. Ye J, Flint AP, Luck MR, Campbell KH. Independent activation of MAP kinase and MPF during the initiation of meiotic maturation in pig oocytes. Reproduction 2003; 125:645–656.
28. Cyert MS, Kirschner MW. Regulation of MPF activity in vitro. Cell 1988; 53:185–195.
29. Gautier J, Maller JL. Cyclin B in *Xenopus* oocytes: implications for the mechanism of pre-MPF activation. EMBO J 1991; 10:177–182.
30. Strausfeld U, Labbé JC, Fesquet D, Cavadore JC, Picard A, Sadhu K, Russel P, Dorée M. Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein. Nature 1991; 351:242–245.
31. Jessus C, Ozon R. Function and regulation of cdc25 protein phosphate through mitosis and meiosis. Prog Cell Cycle Res 1995; 1:215–228.
32. Wasserman WJ, Masui Y. Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. Exp Cell Res 1975; 91:381–388.
33. Drury K, Schorderet-Slatkine S. Effects of cycloheximide on the autocatalytic nature of maturation-promoting factor (MPF) in oocytes of *Xenopus laevis*. Cell 1975; 11:269–274.
34. Shibuya EK. G2 cell cycle arrest—a direct link between PKA and Cdc25C. Cell Cycle 2003; 2:39–41.
35. Donzelli M, Draetta GF. Regulating mammalian checkpoints through Cdc25 inactivation. EMBO Rep 2003; 4:671–677.
36. Obaya AJ, Sedivy JM. Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci 2002; 59:126–142.
37. Porter LA, Donoghue DJ. Cyclin B1 and CDK1: nuclear localization and upstream regulators. Prog Cell Cycle Res 2003; 5:335–347.
38. Kanatsu-Shinohara M, Schultz RM, Kopf GS. Acquisition of meiotic competence in mouse oocytes: absolute amounts of $p34^{Cdc2}$, cyclin B1, Cdc25C, and wee 1 in meiotically incompetent and competent oocytes. Biol Reprod 2000; 63:1610–1616.
39. Lévesque JT, Sirard MA. Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. Biol Reprod 1996; 55: 1427–1436.
40. Kanayama N, Miyano T, Lee J. Acquisition of meiotic competence in growing pig oocytes correlates with their ability to activate Cdc2 kinase and MAP kinase. Zygote 2002; 10:261–270.
41. Yamashita M, Fukada S, Yoshikuni M, Bulet P, Hirai T, Yamaguchi A, Lou YH, Zhao Z, Nagahama Y. M-phase specific histone H1 kinase in fish oocytes: its purification components and biological properties. Eur J Biochem 1992; 205:537–543.
42. Tanaka T, Yamashita M. Pre-MPF is absent in immature oocytes of fishes and amphibians except *Xenopus*. Dev Growth Differ 1995; 37: 387–393.
43. Kondo T, Kotani T, Yamashita M. Dispersion of cyclin B mRNA aggregation is coupled with translational activation of the mRNA during zebrafish oocyte maturation. Dev Biol 2001; 229:421–431.
44. Miyano T, Lee J, Fulka J Jr. G2/M transition of pig oocytes: how do oocytes initiate maturation? Reprod Med Biol 2003; 2:91–99.
45. Chakraborti P, Bhattacharya S. Plasma thyroxine level in freshwater perch: influence of season, gonadotropins and gonadal hormones. Gen Comp Endocrinol 1984; 53:179–186.
46. Lessman CA, Kavumpurath S. Cytological analysis of nuclear migration and dissolution during steroid-induced mitotic maturation in vitro of follicle-enclosed oocytes of goldfish (*Carassius auratus*). Gamete Res 1984; 10:21–29.
47. Hirai T, Yamashita M, Yoshikuni M, Tokumoto T, Kajiura H, Sakai N, Nagahama Y. Isolation and characterization of gold fish cdk2, a cognate variant of the cell cycle regulator cdc2. Dev Biol 1992; 152: 113–120.
48. Lowry OH, Rosebrough NJ, Farr AE, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193:265–275.
49. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 27:680–685.
50. Banerjee PP, Bhattacharya S, Nath P. Purification and properties of pituitary gonadotropic hormone from Indian teleosts: fresh water murrel (*Channa punctatus*) and carp (*Catla catla*). Gen Comp Endocrinol. 1989; 73:118–128.
51. Nebreda AR, Gannon JV, Hunt T. Newly synthesized protein(s) must associate with $p34^{cdc2}$ to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes. EMBO J 1995; 14: 5597–5607.
52. Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. Nature 1989; 339:275–280.
53. Nagahama Y. Molecular biology of oocyte maturation in fish. Perspect Comp Endocrinol 1994: 193–198.
54. Hochegger H, Klotzbücher A, Kirk J, Howell M, Guellec K, Fletcher K, Duncan T, Sohail M, Hunt T. New B type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation. Development 2001; 128:3795–3807.
55. Karaiskou A, Perez LH, Ferby I, Ozon R, Jesus C, Nebreda AR. Differential regulation of Cdc2 and Cdk2 by RINGO and cyclins. J Biol Chem 2001; 276:36028–36034.
56. Coleman TR, Dunphy WG. Cdc2 regulatory factors. Curr Opin Cell Biol 1994; 6:877–882.