Characterization of Polo-Like Kinase-1 in Rat Oocytes and Early Embryos Implies Its Functional Roles in the Regulation of Meiotic Maturation, Fertilization, and Cleavage

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ABSTRACT Polo-like kinase 1 (Plk1) is a family of serine/threonine protein kinases that play important regulatory roles during mitotic cell cycle progression. In this study, Plk1 expression, subcellular localization, and possible functions during rat oocyte meiotic maturation, fertilization, and embryonic cleavages were studied by using RT-PCR, Western blot, confocal microscopy, drug-treatments, and antibody microinjection. Both the mRNA and protein of this kinase were detected in rat maturing oocytes and developing embryos. Confocal microscopy revealed that Plk1 distributed abundantly in the nucleus at the germinal vesicle (GV) stage, was associated with spindle poles during the formation of M-phase spindle, and was translocated to the spindle mid-zone at anaphase. In fertilized eggs, Plk1 was strongly stained in the cytoplasm between the apposing male and female pronuclei, from where microtubules radiated. Throughout cytokinesis, Plk1 was localized to the division plane, both during oocyte meiosis and embryonic mitosis. The specific subcellular distribution of Plk1 was distorted after disrupting the M-phase spindle, while additional aggregation dots could be induced in the cytoplasm by taxol, suggesting its intimate association with active microtubule assembly. Plk1 antibody microinjection delayed the meiotic resumption and blocked the emission of polar bodies. In conclusion, Plk1 may be a multifunctional kinase that plays pivotal regulatory roles in microtubule assembly during rat oocyte meiotic maturation, fertilization, and early embryonic mitosis. *Mol. Reprod. Dev. 65: 318–329, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: kinases; oocyte; meiosis; fertilization; early development

INTRODUCTION

Fully grown mammalian oocytes are arrested at the G_2/M transition of the first meiotic division until gonadotropin stimulation or released from follicle environment. The resumption of meiotic maturation is manifested by the germinal vesicle breakdown (GVBD), followed by the chromatin condensation and microtubule reorganization. These transformations lead to the formation of the metaphase spindle and subsequent completion of the first meiotic division, after which oocytes become arrested in metaphase II (M II) again. This arrest is maintained until the fertilization or parthenogenetic activation of the oocytes.

Recently a new family of serine/threonine protein kinase, Polo-like kinases (Plks), was found to play important roles at multiple steps in mitotic and meiotic progression. Plks are represent by the *polo* gene product of *Drosophila melanogaster* (Sunkel and Glover, 1988), its putative homologues Cdc5p of *Saccharomyces cerevisiae* (Kitada et al., 1993), plo1$^+$ of *Schizosaccaromyces pombe* (Ohkura et al., 1995), Plx1, Plx2, and Plx3 of *Xenopus* (Kumagai and Dunphy, 1996; Duncan et al., 2001), and polo-like kinase 1 (Plk1) of mouse (Tong et al., 2002) and human (Clay et al., 1993; Golsteyn et al., 1994). The Plks from yeasts, insects, amphibians, and mammals share a similar overall structure, exhibiting a high-degree of sequence conservation both in the N-terminal catalytic domain and the C-terminal regulating domain, suggesting a close evolutionary relationship among these enzymes and their functional importance for the cell.

The functional significance of the Plks was first revealed in *Drosophila* and *S. pombe*. Mutations in the

Drosophila polo gene cause the appearance of abnormal mitotic cells, characterized by monopolar spindles and bipolar spindles with one of the poles broadened (Liamazares et al., 1991). *S. pombe* lacking Plo1$^+$ function exhibits similar abnormalities (Ohkura et al., 1995), suggesting a potential role for Plks in spindle organization. In *Xenopus* oocytes Plx, as an activator of Cdc25, and a repressor of Myt1, is shown to participate in the Cdk1-cyclin B amplification loop in meiotic maturation (Aberieu et al., 1998; Qian et al., 1998). By activating anaphase promoting factor (APC), Plk induces Ca^{2+}-triggered destruction of cyclin B and inactivation of p34^{cdc2} and, consequently, M-phase exit (Donaldson et al., 2001). Plks are also required to promote the onset of cytokinesis by recruiting several key proteins in the establishment of the constriction ring structure (Glover et al., 1998).

Despite the numerous studies on Plks in somatic cells and *Xenopus* oocytes, few reports concerning this enzyme's roles in mammalian meiotic cell cycles were available until now. Recently, the dynamics of localization and enzyme activity of Plk1 was examined during mouse oocyte maturation. At the GV stage, Plk1 diffusely distributed in the cytoplasm, enriched in the nucleus, and concentrated to the spindle poles during prometaphase. It was relocated to the equatorial plate at anaphase and was associated with the midbody at telophase (Wianny et al., 1998). This kinase was activated as early as 30 min before GVBD in mouse oocytes, and the active state was maintained throughout meiotic maturation (Pahlavan et al., 2000). The variation of Plk1 activity and subcellular localization implies its important roles in the meiotic progression of mouse oocytes. However, the characterization of Plk1 in oocytes has not been reported in any mammalian species other than the mouse. We reported recently that the microinjection of Plk1 antibody into mouse oocytes at GV stage prevented the meiotic resumption and the meiotic spindle organization (Tong et al., 2002). This result suggests that Plk1 is a necessary regulator of meiotic cell cycle in mouse oocytes, but it is not known if this kinase plays the same key roles in other mammals. Furthermore, although the Plk1 was observed to concentrate at the mid-zone during anaphase, the necessity of this kinase activity for polar body extrusion is unclear.

The meiotic cell cycle of rat oocytes differs from mouse oocytes in several ways. Released from the follicles, the rat oocytes develop to the M I stage earlier than the mouse oocytes, but MAP kinase activation in rat oocytes is even more delayed than in mouse oocytes (Zernicka-Goetz et al., 1997). Removed from oviducts and cultured in vitro, rat oocytes undergo spontaneous parthenogenetic activation, instead of being arrested at M II stage (Keefer and Schuetz, 1982). The spontaneously activated oocytes extrude their second polar bodies (PB2), but they do not proceed to interphase with the formation of pronuclei. Instead, they enter into a next metaphase-like arrest (Zeilmaker and Verhamme, 1974) termed the third meiotic mataphase (M III). Furthermore, in M III arrested rat oocytes, normal spindles did not form and

dispersed chromosomes surrounded by microtubules were observed by us (Fan et al., 2002). These specificities of the meiotic cycle make the rat oocytes an ideal model for studying the role of Plk1 in mammalian meiotic progression other than mouse oocytes. In this experiment: (1) the expression of Plk1 mRNA and protein in rat oocytes were revealed through RT-PCR and Western blot analysis; (2) the subcellular distribution of Plk1 at different states of meiotic and mitotic cell cycles was detected by confocal microscopy; and (3) the effects of Plk1 antibody microinjection on rat oocytes were observed. All these studies were aimed at elucidating Plk1's important roles in the regulation of mammalian oocyte meiosis and early embryonic mitosis.

MATERIALS AND METHODS
Chemicals

All chemicals used in this experiment were purchased from Sigma Chemical Company unless otherwise mentioned. Stock solutions of cycloheximide (CHX, 2 mg/ml), calcium ionophore A23187 (5 mM), cytochalasin B (CB, 1 mM), taxol (1 mM), colchicine (10 mg/ml), staurosporine (Stau, 3 mM), isobutylmethyxanthine (IBMX, 500 mM) were prepared with dimethyl sulfoxide and stored frozen at $-20°$C in the dark. They were diluted with M2 medium just prior to use.

Collection and Culture of Oocytes

Wister strain rats of 20−30-days old were primed with 20 IU pregnant mare's serum gonadotropin (PMSG). Approximately 48−50 hr later, fully grown GV intact oocytes were collected from the ovaries in M2 medium (Sigma) containing 4 mg/ml bovine serum albumin (BSA) and cultured in the same medium under paraffin oil at 37°C, 5% CO$_2$ in air. The cumulus cells surrounding the oocytes were removed by repeated pipetting before maturation culture.

To obtain ovulated oocytes, 25−30-days old Wister rats were superovulated by intraperitoneal injections with 20 IU PMSG and 20 IU human chorionic gonadotropin (hCG) 54 hr apart. Animals were sacrificed 15 h after hCG injection and cumulus-enclosed metaphase II (M II) oocytes were recovered from the oviducts. Cumulus cells were dispersed by a brief exposure to 300 IU/ml hyaluronidase (Sigma). All animal experimentations were conducted in conformity with the Guiding Principles for Research Involving Animals and Human Beings.

Fertilization In Vitro

ZP-free M II oocytes were used to achieve synchronous fertilization and to minimize the lag period of sperm-egg interaction. Zona pellucida (ZP) was removed with acid Tyrode's solution (pH 2.5). On the day of collecting oocytes, male rats were sacrificed, and the cauda epididymides were dissected from animal, and minced slightly into 2 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 20 mg/ml BSA, and then placed in a 5% CO$_2$ air incubator for 10 min at 37°C to allow

spermatozoa to swim up. Top sperm suspension showing vigorously progressive motility and containing about 1.5×10^7 cells/ml were collected. For capacitation, the sperm suspension was kept in the concentrated form in a 5% CO_2 air incubator for three hours. Capacitated spermatozoa were diluted at 1:10 before insemination. The emission of the second polar body and the formation of the pronuclei were observed with an inverted microscope. The fertilized eggs were collected at different stages for confocal microscopy or Western blot analysis.

RT-PCR

Oocytes at GV or M II stage, zygotes, and 2-cell embryos were collected. A total of 100 oocytes or embryos at each stage were treated with TRIZOL reagent (Gibco-BRL, Gaithersburg, MD) with an addition of 50 µg yeast tRNA (Boehringer Mannheim) into each sample as a carrier RNA. The total RNA was extracted from samples according to the manufacturer's instructions. Final RNA was dissolved in DEPC-dH$_2$O and digested with RQ1 DNase I (Promega Corp., Madison, WI). Compared to the supplemented yeast tRNA, the RNA quantitation from the embryos was much lower. The RNA concentration from each sample was measured to calculate the recovering rate of sample RNA only based on the concentration of supplemented yeast tRNA. Extracted RNA was then diluted to contain 7 embryos in each microliter sample prior to use.

The primers for rat Plk1 were used to amplify Plk1 mRNA in rat oocytes and embryos. Rat Plk1 primers designed by rat Plk1 cDNA (NCBI ACCESSION L06144) were 5′-GCTTCGCCAAATGCTTCG-3′ and 5′-TGATGCACCCAATGGACC-3′. cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for the quality of cDNA preparations. Mouse GAPDH primers were 5′-ACCACAGTCCATGC-CATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for a 452 bp fragment. The RNA samples were reversely transcribed and amplified by PCR using Takara BcaB-EST RNA PCR kit (Takara Biotechniques, Dalian, China) according to the manufacturer's protocol. The amplification of Plk1 cDNA was conducted for 36 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The condition for GAPDH amplification was done for 32 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. To check the specificity of RT-PCR, three controls were set for testing the contamination of genomic DNA: (1) RNA samples were directly amplified without reverse transcription; (2) reverse transcription was performed without adding reverse transcriptase followed by PCR amplification; (3) RNA samples were replaced by DEPC-dH$_2$O in RT-PCR. All the RT-PCR reactions were carried out only after no DNA contamination was detected in all of three controls. All the RT-PCR reactions were repeated three times.

Western Blot Analysis

Western blot analysis was conducted as previously described by us with minor modifications (Sun et al., 1999). Proteins extracted from 50 cells were separated by 10% SDS–PAGE and electrophoretically transferred onto PVDF membrane (Millihore, poresize 0.45 µm). The membrane was immersed in methanol for 1 min and dried overnight at room temperature. The membrane was then incubated for 2 hr at 37°C with monoclonal mouse anti-Plk1 antibody (Zymed Laboratories Inc., South San Francisco, CA) diluted 1:300 in TBST (TBS with 0.1% Tween-20, pH 7.4) containing 5% skimmed milk. After three washes in TBST, the membrane was incubated for 1 hr at 37°C with rabbit anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1,000. Finally, the membrane was thoroughly washed and processed by using the ECL detection system (Amersham International).

Confocal Microscopy

The zona pellucida was removed by a short exposure of the oocytes to the acid M2 medium (pH 2.5). Denuded oocytes were fixed for 30 min in 3% formaldehyde, 2% sucrose in PBS at room temperature, and then incubated for 30 min in 0.5% Triton X-100 in 20 mM HEPES, 3 mM MgCl$_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN$_3$, and finally for 5 min in methanol at -20°C. Then the oocytes were washed three times in washing buffer (PBS containing 0.1% Tween-20 and 0.01% Triton X-100) and then incubated with 1:100 monoclonal mouse anti-Plk1 antibody for 1 hr. The oocytes were rinsed three times and incubated with 1:100 FITC-conjugated goat anti-mouse IgG for 1 hr, followed by staining with 10 µg/ml propidium iodide (PI). Finally, the oocytes were mounted on glass slides and examined using a TCS-NT laser scanning confocal microscope (Leica Microsystems).

For staining of microtubules in rat oocytes, fixed and permeabilized samples were incubated with FITC-conjugated anti-α-tubulin antibody (Sigma) 1:50 diluted for 1 hr at room temperature, stained with PI, and mounted on a slide as mentioned above.

Experimental Design

Experiment 1. The expression and localization of Plk1 during rat oocyte meiotic maturation and fertilization were studied. Oocytes at different developmental stage were collected for RT-PCR, Western blot analysis, or confocal microscopy.

Experiment 2. To investigate the Plk1 distribution of rat oocytes after spontaneous pathenogenetic activation, M II oocytes were collected from oviduct and cultured in M2 medium for 8 hr before staining with α-tubulin, and the PB2 emission rate was recorded after observation with an inverted phase-contrast microscope.

Experiment 3. To induce the parthenogenetic activation of rat oocytes, cumulus-free M II oocytes were treated with 5 µM calcium ionophore A23187 for 5 min, and than cultured in M2 medium containing 10 µg/ml CHX. The nuclear status and Plk1 distribution were examined at 2 or 6 hr of incubation.

Experiment 4. Induction of pseudo-cleavage was performed as reported in mouse (Wassarman et al.,

1976). Briefly, cumulus-free and GV intact oocytes were cultured in M2 medium containing 0.2 mM IBMX and 5μM CB overnight. The microtubule organization and Plk1 localization were detected in pseudo-cleaved oocytes.

Experiment 5. The correlation between Plk1 localization and microtubule assembly/disassembly in M II oocytes were investigated. Oocytes were treated with 10 μg/ml microtubule assembly inhibitor colchicine for 1 hr, or 1 μM microtubule disassembly inhibitor taxol for 10 min. In another experiment, M II oocytes were treated with 30 μM protein kinase inhibitor staurosporine for 30 min to disturb the meiotic spindle. Some oocytes treated with staurosporine were further exposed to 1 μM taxol for 10 min. After each treatment, oocytes were collected for confocal microscopy.

Experiment 6. To clarify the functional roles of Plk1 in meiotic maturation of rat oocytes, approximately 10 pl antibody against mouse and rat Plk1 diluted 200 μg/ml in PBS were injected into the ooplasm of GV oocytes using an Eppendorff microinjector. As the control, GV oocytes were injected with mouse IgG of the same concentration and volume as Plk1 antibody. The oocytes were kept in M2 medium supplemented with 0.2 μM IBMX during the injection period to prevent GVBD. The injected oocytes were thoroughly washed with IBMX-free M2 medium and cultured in this medium. GVBD and PB1 emission were observed 2, 3, and 10 hr after maturation culture, respectively. The oocytes that underwent GVBD were fixed 10 hr after antibody injection and subjected to confocal microscopy for microtubule observation. This experiment was repeated three times and 30−40 oocytes were injected each time.

Statistical Analysis

All percentages from three repeated experiments were expressed as mean ± SEM and the number of oocytes observed was labeled in brackets as (n=). The rates of GVBD were subjected to arcsin transformation. The transformed data were analyzed by ANOVA followed by Student−Newman-Keuls test. Differences at $P < 0.05$ were considered to be statistically significant. In Western blot, the relative Plk1 quantity in different developmental stages was determined by Plk1 intensity obtained by densitometric scan of the band and the values were analyzed by Student's t-test.

RESULTS

Expression of Plk1 mRNA and Protein in Rat Oocytes and Embryos

RT-PCR was performed with Plk1 and GAPDH specific primers for rat oocytes at GV and M II stage, zygotes, and 2-cell embryos, respectively. GAPDH was used to verify the integrity of cDNA preparations. The predicted 452 bp fragment was observed with GAPDH primers (Fig. 1A), and the predicted 534 bp fragment was obtained with rat Plk1 primers. Plk1 mRNA was expressed at all stages examined (Fig. 1B). Neither fragment was detected in negative control experiments described above (Fig. 1C). No quantification was per-
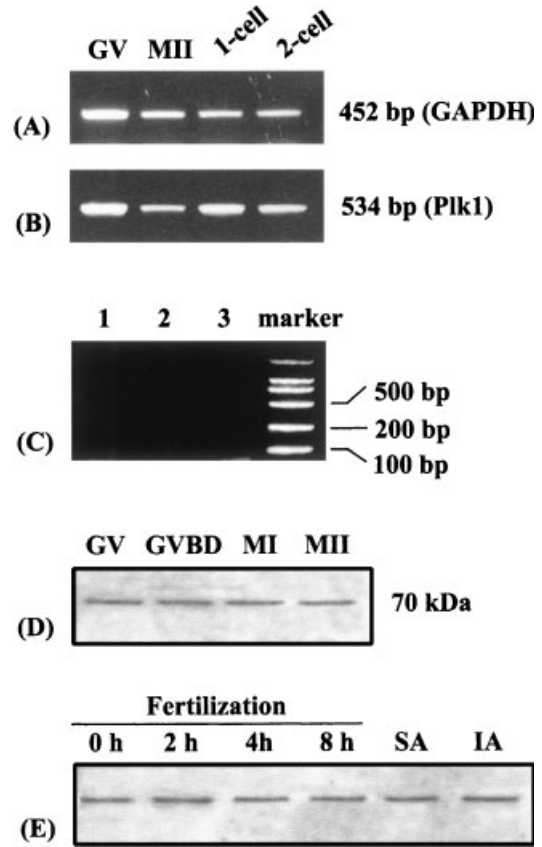

Fig. 1. Expression of Plk1 mRNA and protein in rat oocytes and early embryos. GAPDH (**A**) and Plk1(**B**) mRNAs are expressed in rat oocytes at GV and M II stage, 1-cell and 2-cell embryos. Reverse transcription and PCR using rat Plk1-specific oligonucleotides was performed to amplify Plk1 mRNA. The amplified cDNA products were analyzed by agarose gel electrophoresis and ethidium bromide staining. **C**: negative controls of RT-PCR using Plk1 primers. **Lane 1**: RNA samples were directly amplified without reverse transcription; (**lane 2**) reverse transcription was performed without adding reverse transcriptase followed by PCR amplification; and (**lane 3**) RNA samples were replaced by DEPC-dH$_2$O in RT-PCR. Expression of Plk1 protein in maturing rat oocytes (**D**) and activated eggs (**E**) was detected by Western blotting. SA, spontaneous parthenogenetic activation; IA, induced parthenogenetic activation.

formed, but based on our data, the levels of Plk1 mRNA at M II stage were lower than those at other stages.

The expression of Plk1 protein was detected in maturing rat oocytes (Fig. 1D) and fertilized eggs (Fig. 1E). The quantity of Plk1 protein remained stable during meiotic maturation, and this pattern of expression was unchanged after fertilization, spontaneous pathenogenetic activation, or activation induced by A23187 and CHX. The stable expression of Plk1 protein in rat eggs was further proven by densitomatric scans (data not shown).

Localization of Plk1 in Rat Oocytes During Meiotic Maturation, Fertilization, and Embryonic Cleavage

In GV oocytes, Plk1 was distributed evenly in the cytoplasm, but a strong staining was detected in the germinal vesicles (Fig. 2A). Plk1 concentrated to the region near the condensed chromosomes shortly
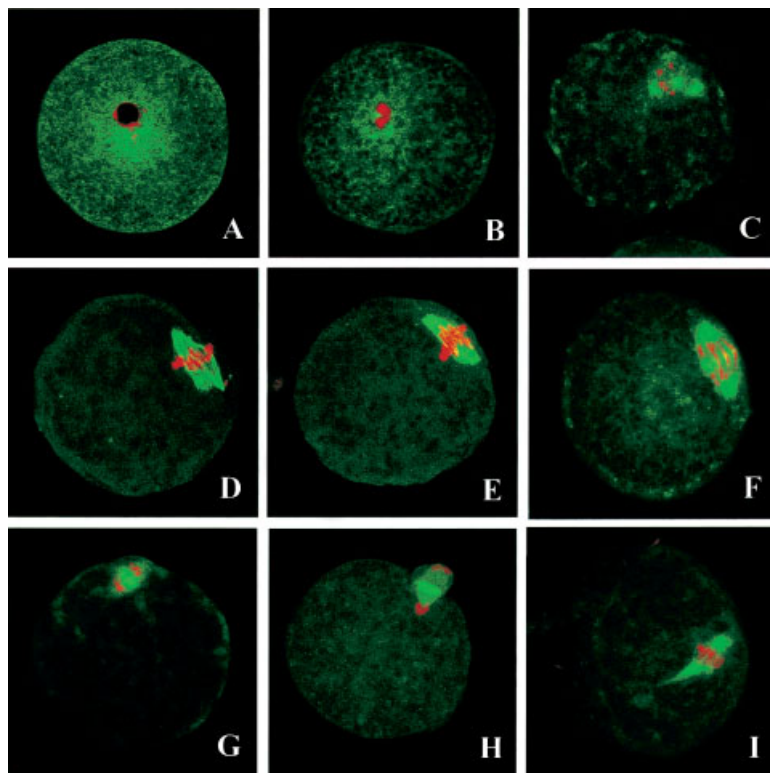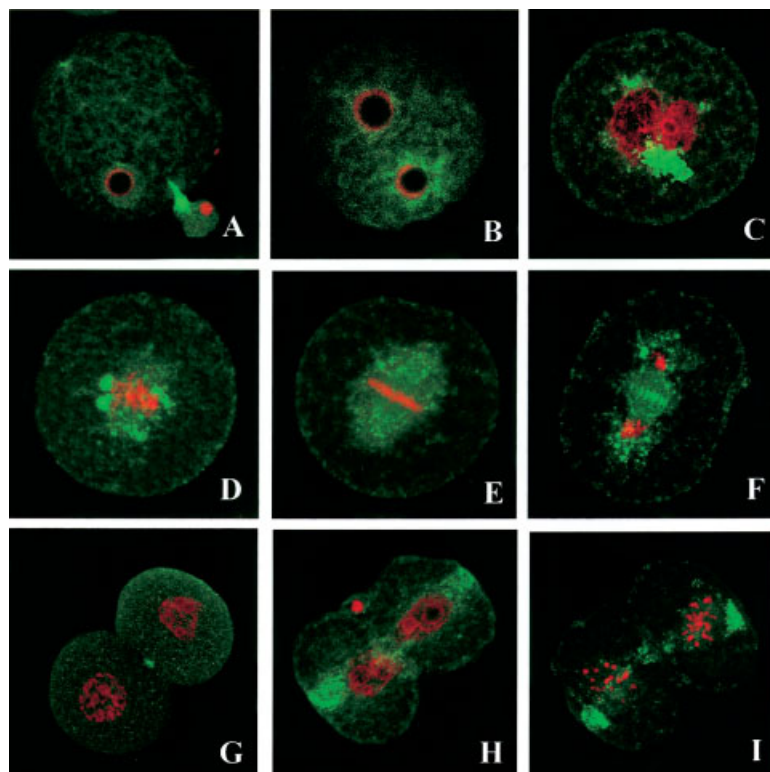
Fig. 2.

Fig. 3.

after GVBD and aggregated as several dots 2 hr after (Fig. 2B,C). At pre-M I or M I stage, Plk1 was detected in the entire spindle except for the equatorial region, with the most prominent staining at the spindle poles (Fig. 2D,E). Following the metaphase–telophase transition and the separation of homologues chromosomes, Plk1 translocated from the spindle poles to the mid-zone of the elongating spindle (Fig. 2F,G), and persisted at the division plane throughout telophase (Fig. 2H). After extrusion of PB1, a characteristic taper-shape M II spindle was formed as described (Zernicka-Goetz et al., 1993). Correspondingly, Plk1 was present in the entire M II spindle except for the equator zone (Fig. 2I).

After fertilization in vitro, 78.31% (65/83) of the eggs released their PB2 within 2 hr and 69.88% (58/83) of them formed pronuclei 6 hr following insemination. The subcellular distribution of Plk1 during PB2 extrusion was similar to that observed in the release of PB1 (data not shown). Concentration of this kinase was detected in both the male and female pronuclei, as well as the remnants of the cytoplasmic bridge between the zygote and PB2 (Fig. 3A,B). Furthermore, Plk1 was strongly stained in the cytoplasm between the two pronuclei when they were closely apposed, which is a phenomenon normally observed 18 hr after fertilization (Fig. 3C). Shortly after, the zygotes underwent nuclear envelope breakdown (NEBD) and entered first mitosis. Plk1 was concentrated in multiple foci surrounding the condensed chromosomes after NEBD (Fig. 3D), at the spindle region at mitotic metaphase (Fig. 3E), and migrated to the mid-zone at anaphase (Fig. 3F). This protein distributed evenly except for the remnants in the intercellular bridge in 2-cell interphase embryos (Fig. 3G). However, before the entering second cleavage, Plk1 redistributed to a zone forming the longitudinal axis of the 2-cell embryos (Fig. 3H). Following nuclear membrane breakdown, Plk1 was concentrated in two dots localized opposite from each other in the embryos (Fig. 3I).

Localization of Plk1 in Rat Oocytes After Spontaneous and Induced Activation

Most M II oocytes (93.68%, 89/95) underwent spontaneous activation cultured in vitro for 2 hr, marked by PB2 emission. Confocal microscopy revealed that the chromosomes dispersed irregularly in these oocytes (Fig. 4A'). No pronucleus formation was observed 8 hr after culture (Fig. 4B'). Instead, oocytes entered a metaphase-like arrest (M III). Plk1 was observed to concentrate around the dispersed chromosomes in these oocytes, displaying a cloud-like pattern of distribution (Fig. 4A,B).

Rat M II oocytes could be activated by the treatment of calcium ionophore A23187 plus CHX. Most activated oocytes released PB2 within 2 hr and formed a pronucleus 6 hr after activation. Plk1 staining was detected in the cytokinetic ring during PB2 extrusion (Fig. 4C). Plk1 concentrated as a mass near the pronucleus 6 hr after activation (Fig. 4D).

Correlation Between Plk1 Localization and Microtubule Assembly/Disassembly

After treatment of eggs with colchicine, a microtubule polymerization inhibitor, the spindle disappeared, α-tubulin scattered evenly in the eggs (Fig. 5A'). Plk1 protein also diffused into the cytoplasm (Fig. 5A). In contrast, after treating the oocytes with taxol, the spindle was significantly enlarged due to the excessive microtubule polymerization, and multiple microtubule asters formed in the cytoplasm (Fig. 5B'). Correspondingly, the distribution of Plk1 on the spindle was reinforced, and dots of Plk1 aggregation were detected in the cytoplasm (Fig. 5B). The spindle disassembled when the oocytes were treated with broad-spectrum protein kinase inhibitor staurosporine (Fig. 5C'). Furthermore, taxol did not up-regulate the microtubule organizing ability of the spindle after pretreatment with staurosporine, although some short microtubule networks were detected in the cytoplasm (Fig. 5D'). In these two cases, no aggregation of Plk1 around chromosomes or in the cytoplasm was observed (Fig. 5C,D).

Plk1 Distribution in Pseudo-Cleaved GV Oocytes

Nearly half (48.24%, 41/85) rat oocytes cultured overnight in the presence of both IBMX and CB underwent pseudo-cleavage, which involved the division of

Fig. 2. Localization of Plk1 in rat oocytes during meiotic maturation. Green, Plk1; Red, chromatin; yellow, overlapping of green and red. In GV oocytes, Plk1 distributed evenly in the cytoplasm, but a strong staining of Plk1 expression was detected in the germinal vesicles (**A**). Plk1 concentrated to the area near the condensed chromosomes shortly after GVBD and aggregated as several dots 2 hr after (**B** and **C**). In rat oocytes at pre-M I (**D**) or M I (**E**) stage, Plk1 could be detected over the entire spindle except for the equatorial region, with the most prominent staining at the spindle poles. During the metaphase–anaphase transition and the separation of homologues chromosomes, Plk1 translocated from the spindle poles to the mid-zone of the spindle (**F** and **G**), and persisted at the division plane throughout the telophase (**H**). At M II stage, Plk1 was distributed over the entire spindle except for the equator zone (**I**). Original magnification ×630 (the same is applied in the following figures).

Fig. 3. Localization of Plk1 in rat fertilized eggs and early embryos. Concentration of this kinase was detected in both the male and female pronuclei, as well as the remnants of the cytoplasmic connection between the zygote and PB2 (**A** and **B**). Furthermore, Plk1 was strongly stained in the cytoplasm between the two closely apposed pronuclei (**C**). Plk1 was found to concentrate at multiple foci surrounding the condensed chromosomes around the time of nuclear envelope breakdown (**D**), present at the spindle region at mitotic metaphase (**E**), and migrated to the mid-zone at anaphase (**F**). This protein distributed evenly except for the remnants in the intercellular bridge in rat 2-cell interphase embryos (**G**). However, before entering of second cleavage, Plk1 redistributed to a zone forming the longitudinal axis of the 2-cell embryos (**H**). Following nuclear membrane breakdown, Plk1 became concentrated in two dots localized opposite from each other in the embryos (**I**).
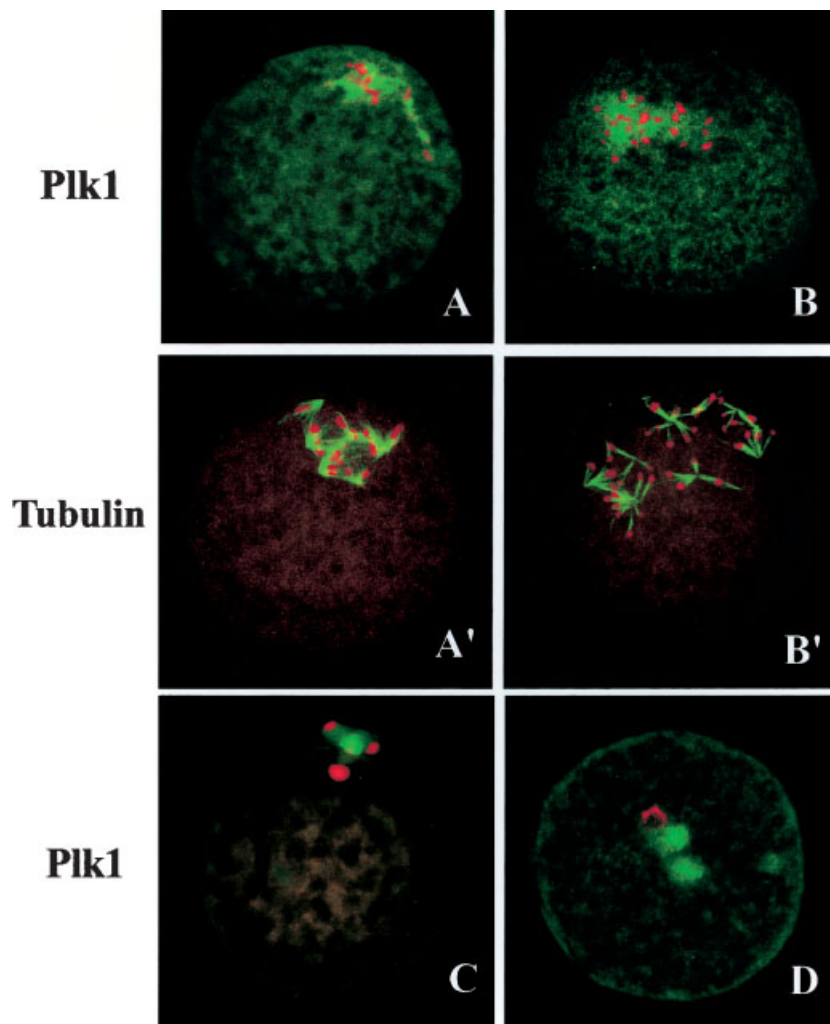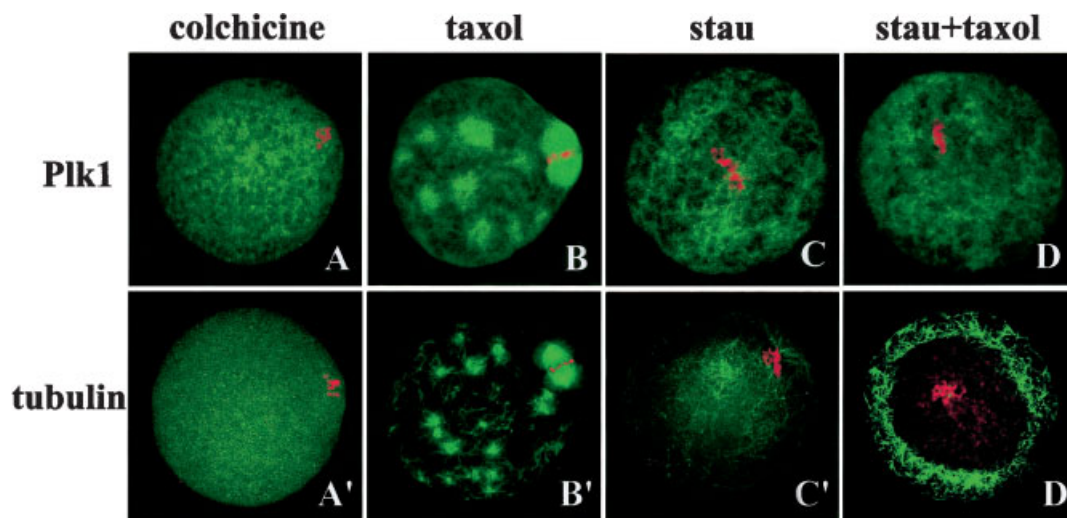
Fig. 4.

Fig. 5.

an oocyte into two approximately equal-sized compartments, one of which contained an intact GV and nucleolus. Confocal microscopy indicated that long microtubules existed in the pseudo-cleaved oocytes, and most of them extended parallel to the axis of pseudo-cleavage (Fig. 6A). No condensation of Plk1 in the vicinity of the division furrow was observed (Fig. 6B).

Effects of Plk1 Antibody Microinjection

The effect of Plk1 antibody microinjection on meiotic resumption in rat oocytes was shown in Figure 7. In oocytes injected with Plk1 antibody, $39.23 \pm 8.07\%$ ($n = 94$) of them underwent GVBD within 2 hr, but $68.64 \pm 3.92\%$ ($n = 84$) of the oocytes injected with mouse IgG underwent GVBD at the same time. The GVBD rate increased to $72.20 \pm 4.45\%$ ($n = 94$) and $84.49 \pm 5.93\%$ ($n = 84$) in oocytes injected with Plk1 antibody or mouse IgG 3 hr after injection, respectively. When the GVBD oocytes were further cultured, PB1 emission rate in Plk1 antibody-injected oocytes (13.95%, $n = 43$) was significantly lower when compared to the control (62.22%, $n = 45$). Results from confocal microscopy revealed that normal M II spindles were formed in oocytes with PB1 emission, both in the Plk1 antibody-injected group ($n = 6$) and in the control group ($n = 26$, Fig. 8D). However, three types of abnormalities were observed in oocytes that failed to extrude a PB1 after Plk1 antibody microinjection. Some oocytes arrested at M I stage with an abnormal spindle (Fig. 8A); and the meiosis in other oocytes were blocked at anaphase I, containing an improperly organized anaphase spindle characterized by the lack of a mid-zone (Fig. 8B) or asymmetric-shaped spindles (Fig. 8C). Despite of the existence of three patterns of abnormalities, precise calculation of the frequency of specific defects was difficult, since there were oocytes showed intermediate phenotypes and were hard to be classified.

DISCUSSIONS

In this work, both Plk1 mRNA and protein were detected in rat oocytes and early embryos. The Plk1 mRNA accumulated in rat oocytes may be stored as maternal messages and translated into functional enzymes during the subsequent meiosis and cleavage, since there is no gene transcription activity in meiotic oocytes and early embryos. Plk1 protein level is stable during meiotic maturation, fertilization, and parthenogenetic activation, in agreement with the report in mouse eggs by Pahlavan et al. (2000). However, no Plk1 forms with different electrophoretic mobility are detected in our system. This may be due to the insensitivity of our SDS–PAGE system to distinguish the phosphorylation of Plk1.

The spatial and temporal separation of several specific phases of the meiotic cycle makes the maturing rat oocytes and activating eggs an attractive model for studying the role of Plk1 in meiotic progression of mammals. In rat oocytes at the GV stage and zygotes at the pronucleus stage, extensive Plk1 staining was observed in the nucleus. The nuclear accumulation of active M-phase promoting factor (MPF) during prophase is essential for coordinating M-phase events in vertebrate cells (Kanatsu-Shinohara et al., 2000; Ledan et al., 2001). Phosphorylation of cyclin B1 is central to its nuclear translocation (Ohashi et al., 2001). In addition, the protein phosphatase Cdc25C, an activator of MPF, enters the nucleus to keep MPF active during prophase. Recent reports showed that Plk1 was responsible for the phosphorylation and nuclear migration of cyclin B1 and Cdc25C (Toyoshima-Morimoto et al., 2001, 2002).

In rat oocytes, as in somatic cell lines, Plk was localized to different components of the spindle apparatus. Following GVBD, when the bipolar spindle formed, Plks concentrated to several foci around the condensed chromosomes. These foci may be involved in the merging of the multiple cytoplasmic MTOCs that will form the two spindle poles (Maro et al., 1985). At metaphase, Plk1 was localized to the spindle poles, the principle MTOCs at this stage, suggesting that Plk1 may regulate microtubule organizing activity of spindle poles. In human cells, Plk appears to stimulate the centrosome's microtubule-nucleating activity upon entry into mitosis (Lane and Nigg, 1996). Furthermore, it appears to facilitate recruitment of γ-tubulin and activates Asp at the centrosome. Asp is a microtubule-associated protein that accumulates at the minus ends of microtubules and helps focus the microtubule ends to maintain their proximaty to the chromosome (Donaldson et al., 2001).

Fig. 4. Localization of Plk1 in rat eggs following spontaneously or induced parthenogenetic activation. Chromosomes dispersed irregularly in oocytes undergoing spontaneous activation after culture in vitro for 2 hr ($\mathbf{A}'$). No pronucleus formation was observed 8 hr after culture. Instead, oocytes entered a metaphase-like arrest (M III), with short microtubules radiating from individual chromosome ($\mathbf{B}'$). Consistent with the configuration of microtubules, Plk1 was observed to concentrate around the dispersed chromosomes in these oocytes, displaying a cloud-like pattern of distribution ($\mathbf{A}$ and $\mathbf{B}$). In rat oocytes activated by calcium ionophore A23187 plus CHX, Plk1 staining was detected in the cytokinetic ring during PB2 extrusion occurring 2 hr after activation ($\mathbf{C}$), and concentrated as a mass near the pronucleus 6 hr after activation ($\mathbf{D}$).

Fig. 5. Changes of Plk1 distribution in rat eggs after disturbing of microtubule assembly/disassembly. After treatment of eggs with colchicine, the spindle disappeared, α-tubulin scattered evenly in the eggs ($\mathbf{A}'$). Plk1 protein also diffused into the cytoplasm ($\mathbf{A}$). In contrast, after treating the eggs with taxol, the spindle was significantly enlarged due to the excessive microtubule polymerization, and multiple microtubule asters formed in the cytoplasm ($\mathbf{B}'$). Correspondingly, the distribution of Plk1 on the spindle was reinforced, and dots of Plk1 aggregation were detected in the cytoplasm ($\mathbf{B}$). The spindle was destructed when the oocytes were treated with staurosporine ($\mathbf{C}'$). Taxol did not up-regulate the microtubule organizing ability of the spindle after pretreatment with staurosporine, although some short microtubule networks were detected in the cytoplasm after taxol treatment ($\mathbf{D}'$). In these two cases, no aggregation of Plk1 near chromosomes or in the cytoplasm was observed ($\mathbf{C}$ and $\mathbf{D}$).
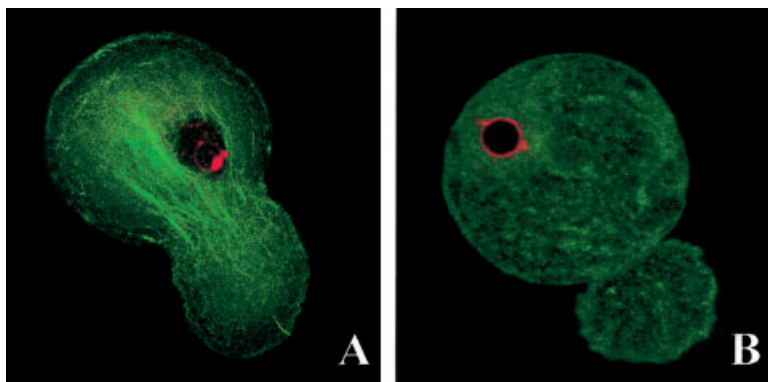
Fig. 6. Localization of Plk1 after pseudo-cleavage induced by IBMX plus CB. Long microtubules existed in the pseudo-cleaved oocytes, and most of them extend parallel to the axis of pseudo-cleavage (**A**). No concentration of Plk1 was observed in the vicinity of the division furrow (**B**).

The association of Plk1 with spindle construction was further proved by disturbing the microtubule organization. Plk1 lost its specific intracellular localization when the spindle was destructed by colchicine or staurosporine. In mouse and rat M II-arrested oocytes, 16 latent MTOCs exist in the cytoplasm (Longo, 1997), but Plk1 concentrated exclusively to spindle poles. Taxol rein-

Fig. 7. Effects of Plk1 antibody microinjection on meiotic resumption of rat oocytes. GVBD rates in injected oocytes 2 and 3 hr after maturation culture were shown in (**A**) and (**B**). In oocytes injected with Plk1 antibody, $39.23 \pm 8.07\%$ (n = 94) of them underwent GVBD within 2 hr, but $68.64 \pm 3.92\%$ (n = 84) of the oocytes injected with mouse IgG underwent GVBD at the same time. The GVBD rate increased to $72.20 \pm 4.45\%$ (n = 94) and $84.49 \pm 5.93\%$ (n = 84) at 3 hr in oocytes injected with Plk1 antibody or mouse IgG, respectively.

forced microtubule assembly and induced multiple cytoplasmic asters in oocytes. At the same time, accumulation of Plk1 on these asters was observed. All these data suggest that MTOCs with active microtubule assembly could recruit Plk1 via an unknown mechanism, so as to enhance its microtubule nucleating ability. However, the association between Plk1 and tubulin might be indirect and mediated by other molecules. We found that the microtubule nucleation and Plk1 aggregation induced by taxol were inhibited by staurosporine, a broad-spectrum protein kinase inhibitor (Sun et al., 1997; Green et al., 1999). It is possible that a staursporine-sensitive protein kinase is responsible for the microtubule-associated localization of Plk1.

During meiotic anaphase in rat oocytes, Plk1 disappeared completely from the spindle poles and redistributed to the equatorial region of the spindle. It is well documented that anaphase-promoting complex (APC) is responsible for the degradation of M-phase cyclins and multiple anaphase inhibitors, as well as the metaphase−anaphase transition in both mitosis and meiosis (Kotani et al., 1999). Plks phosphorylate at least three APC subunits, APC1, APC3, and APC6, and activate APC (Charles et al., 1998; Descombes and Nigg, 1998; Kotani et al., 1998; Shirayama et al., 1998). In both *Drosophila* (Sunkel and Glover, 1988) and budding yeast (Schild and Byers, 1980), Plk is required to
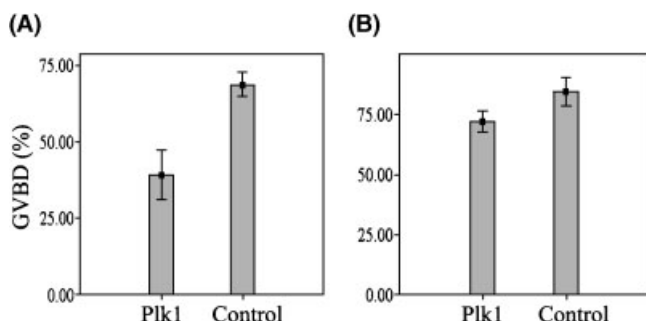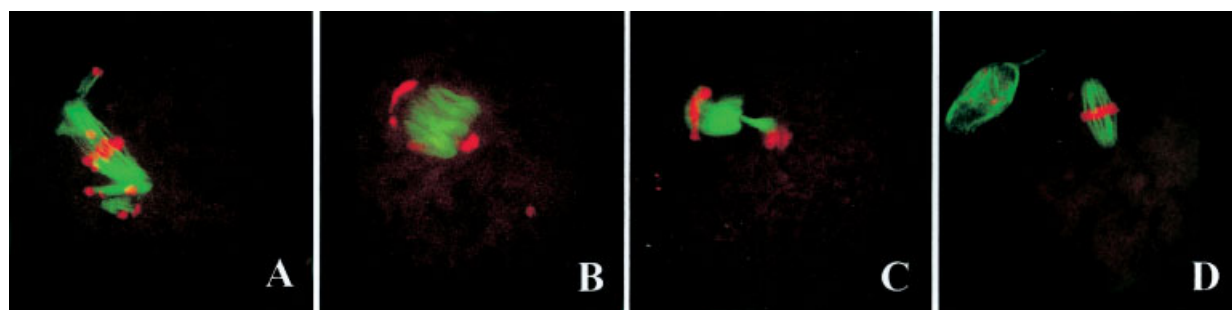

Fig. 8. Microtubule configurations in rat oocytes 10 hr after Plk1 antibody microinjection. Three types of abnormalities were observed in oocytes that failed to extrude a PB1 after Plk1 antibody microinjection. Some oocytes arrested at M I stage with an abnormal spindle (**A**); the meiosis in other oocytes was blocked at anaphase I, containing an improperly organized anaphase spindle characterized by the lack of a mid-zone (**B**) or asymmetric-shaped spindles (**C**). Normal M II spindle was formed in oocytes with PB1 emission in control group (**D**).

mediate the appropriate pattern of chromosome segregation at anaphase in the meiotic divisions. Our results, together with these findings, suggest a role for Plks in regulating the APC, which directs the degradation of chromosomal proteins responsible for maintaining the cohesion of sister chromatids. Throughout meiotic telophase, Plk1 was present in a region corresponding to the cleavage plane and then concentrated in the bridge connecting the egg and the polar body. In *Drosophila*, Polo appears to co-localize with a microtubule motor protein Pav-KLP, at the midbody during cytokinesis (Adams et al., 1998). Although the substrates of Plks during cytokinesis in mammalian cell lines are scarcely known, similar mechanism might be preserved in the process of polar body emission in mammalian oocytes.

For the first time, we showed that GV-intact rat oocytes could be induced to undergo pseudo-cleavage by CB. The mechanisms of pseudo-cleavage have not been fully understood until now. It was hypothesized that CB induced pseudo-cleavage of mouse oocytes as a consequence of the interaction between the drug and the contractile components of the cell cortex (Wassarman et al., 1977). In our results, no accumulation of Plk1 near the division plane was observed. It is suggested that in animal cells a signal originated from the spindle midzone may dictate the position of the cleavage furrow (Cao and Wang, 1996). Thus, the concentration of Plk1 to the region of the contractile ring in meiosis might be also mediated by signals from the anaphase spindle. In the case of pseudo-cleavage, Plk1 failed to migrate to the division plane since cytokinesis was artificially separated from karyokinesis and no signals from the spindle were available.

Mouse and rat sperm lack centrosomes, while the egg possesses 16 MTOCs. Following sperm incorporation, each of the egg MTOCs organizes an aster and the asters become associated with the developing male and female pronuclei during their movements to the center of the zygote (Schatten et al., 1985, 1986; Schatten and Schatten, 1986). Concurrently, a prominent Plk1 concentration was detected in the area between the two apposing pronuclei, where the MTOCs are located and from where the microtubules radiate, as illustrated by many othors. This kinase may be involved in the regulation of MTOC function during syngamy as well as in cell division. The change of Plk1 distribution in the following embryonic cleavages was similar to that observed in meiosis. Thus, Plk1 may be a multifunctional kinase taking part in the regulation of microtubule organization in both oocyte meiosis and embryonic mitosis.

Some novel patterns of Plk1 distribution were observed in this experiment. In 2-cell embryos and CHX-induced activated eggs, Plk1 is localized to a region near the nucleus. Although no reports about these phenomena are available, we suggest that the Plk1 concentration at these stages may also be MTOC associated, since the centrosomes present near the nucleus, just the same as the localization of Plk1 at that time. In spontaneously activated eggs, Plk1 concentrated near the randomly distributed chromosomes. Bundles of short microtubules radiated from the chromosomes. Since no spindle poles are formed in this case, the kinetochores of individual chromosomes may be responsible for the microtubule assembly (Zernicka-Goetz et al., 1993). Plk1 may take part in the regulation of microtubule assembly by kinetochores as well.

The importance of Plk1 in meiotic maturation of rat oocytes was further proven by antibody microinjection. The GVBD rate 2 hr after maturation culture was decreased in oocytes injected with Plk1 antibody, but most injected oocytes could undergo GVBD if further cultured. We may infer from this result that although Plk1 facilitates GVBD, it may not be an indispensable factor for meiotic resumption in rat oocytes. Although the Plk1 antibody-injected oocytes underwent GVBD, the meiotic cell cycle was blocked at metaphase or anaphase of meiosis I and abnormalities of spindle organization was observed. Similar results were also obtained in the mitosis and meiosis of yeast, *Drosophila*, *C. elegans* (Chase et al., 2000), and HeLa cells using gene mutation, RNA-mediated interference, or antibody microinjection. All these data suggest that Plk1 activity is crucial for the correct microtubule organization during cell division, both in meiosis and in mitosis. There are both similarities and differences when compare the results from antibody microinjection in mouse and rat oocytes. In mouse oocytes, Plk1 antibody microinjection leads to the block of GVBD, but the GVBD is only retarded in rat oocytes after the same treatment. This may reflect the different extent of dependence on Plk1 activity during meiotic resumption between the two species. We reported in mouse oocytes that the organization of meiotic spindles was interfered after Plk1 antibody microinjection, but no further culture was tried. Here we showed in rat oocytes that the extrusion of polar bodies was prevented after the same treatment as expected, much likely being the results of microtubule organization defects. However, we should analyze the results of Plk1 antibody microinjection with caution, since the function of Plk1 was only tested in oocytes underwent spontaneous meiotic resumption, which is mechanically different from the gonodotropin-induced meiotic resumption occurred in physiological conditions. For example, others (Su et al., 2001, 2002) and we (Fan et al., 2003) proved that MAP kinase activity is essential for induced meiotic resumption but not the spontaneous meiotic resumption in mouse and pig oocytes. If the physiological meiotic resumption of mammalian oocytes is also Plk1 activity-dependent is waiting to be elucidated.

Taken together, Plk1 mRNA and protein are expressed in rat oocytes and early embryos, and the subcellular distribution of this kinase is highly relevant to microtubule formation. Plk1 may be an important regulator of microtubule organization during rat oocyte meiotic maturation, fertilization, and embryonic cleavage. In further research, the study of physiological upstream activators and target molecules of Plk1 should be stressed, so as to deepen our understanding of its roles in cell cycle regulation.

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