Developmental Biology **220,** 392–400 (2000)
doi:10.1006/dbio.2000.9656, available online at http://www.idealibrary.com on IDEAL®

metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Meiotic Maturation of the Mouse Oocyte

Golbahar Pahlavan,* Zbigniew Polanski,*,† Petr Kalab,*,[1] Roy Golsteyn,‡,[2] Erich A. Nigg,‡ and Bernard Maro*,[3]

**Laboratoire de Biologie Cellulaire du Développement UMR 7622, CNRS–Université Paris 6, 9 quai St Bernard, 75005 Paris, France; †Department of Genetics & Evolution, Jagiellonian University, Ingardena 6, 30-060 Krakow, Poland; and ‡Max-Planck-Institute for Biochemistry, D-82152 Martinsried, Germany*

We have characterized plk1 in mouse oocytes during meiotic maturation and after parthenogenetic activation until entry into the first mitotic division. Plk1 protein expression remains unchanged during maturation. However, two different isoforms can be identified by SDS–PAGE. A fast migrating form, present in the germinal vesicle, seems characteristic of interphase. A slower form appears as early as 30 min before germinal vesicle breakdown (GVBD), is maximal at GVBD, and is maintained throughout meiotic maturation. This form gradually disappears after exit from meiosis. The slow form corresponds to a phosphorylation since it disappears after alkaline phosphatase treatment. Plk1 activation, therefore, takes place before GVBD and MAPK activation since plk1 kinase activity correlates with its slow migrating phosphorylated form. However, plk1 phosphorylation is inhibited after treatment with two specific p34^{cdc2} inhibitors, roscovitine and butyrolactone, suggesting plk1 involvement in the MPF autoamplification loop. During meiosis plk1 undergoes a cellular redistribution consistent with its putative targets. At the germinal vesicle stage, plk1 is found diffusely distributed in the cytoplasm and enriched in the nucleus and during prometaphase is localized to the spindle poles. At anaphase it relocates to the equatorial plate and is restricted to the postmitotic bridge at telophase. After parthenogenetic activation, plk1 becomes dephosphorylated and its activity drops progressively. Upon entry into the first mitotic M-phase at nuclear envelope breakdown plk1 is phosphorylated and there is an increase in its kinase activity. At the two-cell stage, the fast migrating form with weak kinase activity is present. In this work we show that plk1 is present in mouse oocytes during meiotic maturation and the first mitotic division. The variation of plk1 activity and subcellular localization during this period suggest its implication in the organization and progression of M-phase. **© 2000 Academic Press**

Key Words: **polo; H1 kinase; p34^{cdc2}; meiosis; mouse oocytes.**

INTRODUCTION

Polo-like kinases (Plks) are serine/threonine protein kinases implicated in the regulation of multiple aspects of mitosis, including entry into and exit from M-phase, spindle assembly, and dynamics as well as cytokinesis (for review see Glover *et al.*, 1998; Mayor *et al.*, 1999; Nigg, 1998). The founding member of this family, polo, was originally identified in *Drosophila* mutants displaying abnormal mitotic spindles and a cell cycle arrest (Sunkel and Glover, 1988). Highly evolutionarily conserved polo homologues have been identified: Cdc5p in *Saccharomyces cerevisiae* (Hartwell *et al.*, 1973), plo1+ in *Schizosaccharmyces pombe* (Ohkura *et al.*, 1995), Plx1 in amphibians (Kumagai and Dunphy, 1996), and Plk1 in mammals (Golsteyn *et al.*, 1994). In addition to the kinase domain, polo kinases have a strikingly conserved sequence in the noncatalytic domain, termed the polo-box, which has been proposed to target plk to its subcellular substrates (Lee and Erikson, 1998).

Numerous studies carried out during these recent years involve plks in crucial aspects of cell cycle regulation. Since

[1] Present address: Laboratory of Molecular Embryology, NICHD, NIH, Building 18, Room 106, Bethesda, MD 20892-5431.
[2] Present address: Morphogenèse et Signalisation Cellulaire, UMR 144, CNRS–Institut Curie, Pavillon Lhomond, 26 rue d'Ulm, 75231 Paris Cedex 5, France.
[3] To whom correspondence should be addressed. E-mail: maro@ccr.jussieu.fr.

0012-1606/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

Plx1 is able to activate Cdc25C by phosphorylation in *Xenopus* extracts (Kumagai and Dunphy, 1996) it has been considered as a potential candidate to "trigger" entry in M-phase. Results obtained in *Xenopus* eggs and extracts indicate that, although Plx1 is involved in the MPF auto amplification loop, other kinases may also function as trigger kinases (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1998; Qian *et al.*, 1998b). In *Xenopus* egg extracts, Plx1 also plays an important role in the activation of the anaphase-promoting complex/cyclosome (APC/C), the proteolytic machinery that controls exit from mitosis (Descombes and Nigg, 1998). Potential Plk substrates have already been identified in the APC/C (Kotani *et al.*, 1998) by which plk itself also seems to be degraded in turn (Shirayama *et al.*, 1998) (Charles *et al.*, 1998).

In HeLa cells microinjection of anti-plk1 antibodies prevents centrosome maturation (Lane and Nigg, 1996). In these cells plk1 is associated with the spindle poles as well as the kinetochore/centromere region (Arnaud *et al.*, 1998). In *Drosophila*, polo immunolocalizes to various components of the mitotic and meiotic apparatus such as the centrosomes, the spindle, and the kinetochores and is required for the phosphorylation of some MPM2 epitopes Logarinho and Sunkel, 1998). Finally, in fission yeast, plo1 associates with the mitotic SPB and is essential for mitotic commitment as well as septum formation (Bahler *et al.*, 1998; Mulvihill *et al.*, 1999; Ohkura *et al.*, 1995). Thus, plks emerge as key regulators of mitosis.

During meiosis, mutation in the plk gene causes, in addition to cell cycle arrest, spindle abnormalities and chromosomal missegregation both in budding yeast (Sharon and Simchen, 1990; Simchen *et al.*, 1981) and *Drosophila* (Sunkel and Glover, 1988) when plk gene was mutated. This led us to study plk1 in mouse oocytes during meiotic maturation and after parthenogenetic activation to characterize the physiological behavior of plks during meiosis. The mouse system provides a mammalian model in which different stages of meiosis can be easily analyzed allowing the comparison of plk activation to that of MPF and MAPK, the two major meiotic kinases previously characterized (Choi *et al.*, 1991; Kubiak *et al.*, 1992; Verlhac *et al.*, 1994). In this study we have characterized plk1 during meiosis and the first mitotic division in mouse oocytes. Plk1 activity and subcellular localization vary during this period suggesting its involvement in the organization and progression of M-phase as well as an intricate regulatory mechanism.

MATERIAL AND METHODS

Oocyte Culture and Collection

Oocytes blocked at prophase of the first meiotic division (GV stage). To obtain immature oocytes arrested at prophase I of meiosis, the ovaries were removed from 8- to 12-week-old Swiss female mice (Centre National de la Recherche Scientifique, France) and transferred to prewarmed (37°C) M2 medium supplemented with 4 mg/ml bovine serum albumin (BSA; Whittingham, 1971). The ovarian follicles were punctured to release the enclosed oocytes, and immature oocytes displaying a germinal vesicle (GV) were collected and cultured in M2 medium under liquid paraffin oil at 37°C. Oocytes were scored for germinal vesicle breakdown (GVBD) after 1 h of culture and then collected at different time points.

Oocytes blocked at metaphase of the second meiotic division. Metaphase II-arrested oocytes were recovered from mice superovulated by intraperitoneal injections of pregnant mare's gonadotrophin (Intervet) and human chorionic gonadotrophin (hCG; Intervet) 48 h apart. Ovulated oocytes were released from the ampullae of oviducts 14 to 16 h post-hCG. The cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma) and, after careful washing, cultured in M2 medium under liquid paraffin oil at 37°C.

Parthenogenetically Activated Oocytes

Oocytes were activated according to Cuthbertson (1983) by a 6.5-min exposure to a freshly prepared 8% ethanol solution in M2 medium. The oocytes were washed in M2 in order to remove traces of ethanol and cultured in M2 medium under liquid paraffin oil at 37°C.

Oocyte Bisection

The zonae pellucidae were removed by treatment with 0.25% pronase (B grade, Calbiochem) in M2 + BSA containing dbcAMP to block GVBD. The oocytes were preincubated in 1 μg/ml cytochalasin D in M2 + BSA for 10 min and bisected with a glass needle according to the method of Tarkowski (1977). Individual oocytes were bisected into two equivalent halves. After bisection, oocyte fragments were rinsed in M2 + BSA and collected after 2 h.

Culture of HeLa Cells

HeLa cells were grown in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 5% heat-inactivated FCS and penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively) in a 7% CO_2 atmosphere.

Immunocytochemistry

The fixation and labeling of oocytes were performed as described in Maro *et al.* (1984). Oocytes were fixed for 30 min in fresh 3% formaldehyde, 2% sucrose in PBS, and then incubated for 30 min in 0.5% Triton X-100 in 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3, and finally for 5 min at −20°C, in methanol. Samples were then washed with PBS containing 0.1% Tween 20, and incubated with an affinity-purified anti-plk rabbit antibody (AR32; 1:20), followed by a fluorescein-conjugated goat anti-rabbit antibody (KPL; 1:50) and 1:500 propidium iodide. Confocal microscopy was performed with a Leica DMR/TCS-4D instrument.

Immunoblotting

Groups of 50 oocytes were washed in M2 containing 4 mg/ml polyvinylpyrrolidone (PVP), collected in sample buffer (Laemmli, 1970), heated to 100°C for 3 min, and frozen at −20°C. The proteins were separated by electrophoresis in 10% polyacrylamide (ratio acrylamide/bisacrylamide 100/1), containing 0.1% SDS, and elec-

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

trically transferred to nitrocellulose membranes (Schleicher and Schuell, pore size 0.45 μm). Following transfer and blocking for 2 h in 3% skimmed milk in 10 mM Tris (pH 7.5), 140 mM NaCl (TBS) containing 0.1% Tween 20, the membrane was incubated overnight at 4°C with the mouse monoclonal anti-plk, Pl6, or mouse anti-β-tubulin (Amersham). After three washes of 10 min each in 0.1% Tween 20/TBS, the membrane was incubated for 1 h at room temperature with an anti-mouse antibody conjugated to horseradish peroxidase (Amersham) diluted 1:10,000 in 3% skimmed milk in 0.1% Tween 20/TBS. The membrane was washed three times in TBS/Tween and then processed using either the ECL (Amersham) or the Super Signal (Pierce) detection system.

Alkaline Phosphatase Treatment

For the dephosphorylation experiments, samples containing 50 oocytes in phosphatase buffer (Boehringer-Mannheim) and 1% SDS (Sigma) supplemented with anti-proteases were mixed with 1 IU alkaline phosphatase. After a 30-min incubation at 37°C, the reaction was stopped by adding the same volume of twice concentrated Laemmli buffer.

Inhibitor

To inhibit specifically $p34^{cdc2}$ activation, oocytes were cultured in M2 medium containing roscovitine. In order to determine the appropriate concentration necessary to block GVBD efficiently, dose–response experiments were carried out *in vivo* and *in vitro*. A concentration of 1 μM roscovitine was enough to block H1 kinase activity completely in an *in vitro* assay performed on groups of 18 oocytes (Kubiak *et al.*, 1992) while 100 μM roscovitine was only able to inhibit Plk1 partially in our *in vitro* assay (data not shown). A complete inhibition of GVBD during at least 4 h was observed only at 200 μM when the drug was added to the culture medium. Thus, the drug concentration in the oocyte seemed to be about 200 times lower than in the culture medium, suggesting that roscovitine does not diffuse freely into the oocyte. We used a dose of 200 μM roscovitine in the culture medium (corresponding to about 1 μM in the oocyte) to block totally GVBD for 4 h ruling out any possible leaks of $p34^{cdc2}$ activity at the time points when the samples were collected.

Plk1 Activity Assays

Groups of 30 oocytes were washed in M2/PVP, collected in 1 μl, lysed in 24 μl of NP-40 lysis buffer (50 mM Hepes, pH 7.4; 1% NP-40; 100 mM NaCl; 25 mM NaF; 25 mM sodium β-glycerophosphate; 1 μg/ml each of soybean trypsin inhibitor, leupeptin, and pepstatin; and 30 μg/ml of DNase I and RNase A), frozen immediately on dry ice, and stored at −80°C. Samples were thawed and clarified by a 10-min centrifugation at 10,000*g* at 4°C. Then, the R32 serum was added (1:100) and the samples incubated on ice for 1 h. After a 5-min centrifugation at 10,000*g*, supernatants were transferred to new tubes and incubated with immunoprecipitin (Gibco-BRL) for 30 min at 4°C. Immunoprecipitates were washed once in washing buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, and 5 mM NaF) and stored on ice. The reaction was started by adding 10 μl of assay buffer (washing buffer supplemented with 10 μM ATP, 4 μCi of [γ-^{32}P] ATP, and 0.5 mg/ml of dephosphorylated casein (Sigma)) to the immunoprecipitates and the samples were incubated for 30 min at 30°C. The reaction was stopped by the addition of an equal amount of 2.5× SDS sample buffer. The samples were then heated for 3 min to 100°C before analysis by SDS–PAGE. Quantitation of plk activity was performed using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The Amount of plk1 Protein Does Not Vary during Meiotic Maturation and after Oocyte Activation

In order to detect plk1 protein expression in mouse oocytes samples were taken from the GV stage until 6 h after parthenogenetic activation. We used the Pl6 anti-plk1 monoclonal antibody characterized in HeLa cells by Golsteyn *et al.* (1994, 1995). This antibody detects the same band in HeLa cells and metaphase II-arrested oocytes (Fig 1A). Western blots showed that plk1 was present in mouse oocytes and its quantity seemed stable during meiotic maturation (Fig. 1B) compared to tubulin expression, which is invariable during the same period (Fig. 1C). Constant plk1 expression was also observed after parthenogenetic activation (Fig. 1D). These results are different from those obtained in somatic cells where plk1 protein expression is cell cycle dependent, peaking at M-phase (Golsteyn *et al.*, 1994; Hamanaka *et al.*, 1995; Lee *et al.*, 1995) but in agreement with observations in *Xenopus* oocytes (Qian *et al.*, 1998a).

Changes in plk1 Activity Are Correlated with Changes in Electrophoretic Mobility Due to Phosphorylation

It has already been shown that changes in plk1 activity can be regulated by posttranslational modifications (Hamanaka *et al.*, 1995; Kotani *et al.*, 1998; Mundt *et al.*, 1997; Qian *et al.*, 1998a; Tavares *et al.*, 1996). We therefore looked by SDS–PAGE for posttranslational modifications that could eventually lead to changes in plk1 activity during meiotic maturation and after oocyte activation.

To determine plk1 activity during meiotic maturation, immunoprecipitates were prepared from synchronous oocyte samples at different stages of meiotic maturation and after parthenogenetic activation. Immunoprecipitates were used to perform *in vitro* kinase assays with casein as exogenous substrate and [γ-^{32}P]ATP as phosphate donor according to Golsteyn *et al.* (1995). In parallel, using high-resolution gel conditions, we looked for changes in plk1 electrophoretic mobility (Fig. 2).

At the GV stage only one band could be observed. A slower migrating form appeared as early as 30 min before GVBD. This slow form became predominant at GVBD. An increase in plk1 casein kinase activity could be measured in samples 30 min before GVBD. A peak of activity occurred at GVBD (Fig. 2A). The slow migrating form was predominant throughout maturation. High casein kinase activity was maintained during the same period. No changes could be observed at the time of first polar body extrusion (Fig. 2B).

CSF arrested oocytes had high casein kinase activity

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

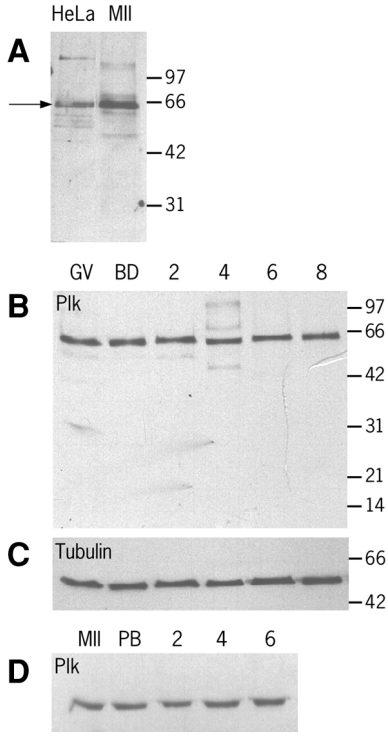

FIG. 1. Plk1 is present in mouse oocytes during meiotic maturation and after parthenogenetic activation. (A) The Pl6 monoclonal antibody detects the same band in HeLa cells (HeLa) and metaphase II-arrested oocytes (MII). (B) Immunoblots were carried out on samples from germinal vesicle stage (GV), germinal vesicle breakdown (BD), and 2, 4, 6, and 8 h after BD which corresponds to first polar body extrusion. (C) β-Tubulin expression, which is stable, was also followed during the same period. (D) MII-arrested eggs were collected after superovulation, and samples were collected after parthenogenetic activation at critical time points: 1 h which corresponds to second polar body extrusion and 2, 4, and 6 h which is pronucleus stage.

and the slow migrating form was the major form observed. After parthenogenetic activation, the activity slowly decreased and was minimal from 4 to 10 h when only the fast migrating form could be detected (Fig. 2C). The slow migrating form became predominant during the first embryonic mitosis (12 h after activation). At that time, the activity rose again and then decreased at the second embryonic interphase (Fig. 2D). Thus, casein kinase activity correlated with the slow migrating form in SDS–PAGE.

We then investigated the nature of this posttranslational modification. When samples were treated with alkaline phosphatase the slow migrating form of plk1 was greatly reduced suggesting that this posttranslational modification corresponded to phosphorylation (Fig. 3).

Plk1 Activation Is Related to the MPF Autoamplification Loop

It has been shown that Plx1 is involved in the MPF autoamplification loop (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1998; Qian *et al.*, 1998a). In order to investigate whether in mouse oocytes plk1 also played a role in MPF autoamplification we treated samples with roscovitine (Meijer *et al.*, 1997), a specific $p34^{cdc2}$ inhibitor. Plk1 slower migrating form could not be observed after this treatment (Fig. 4). Similar results were obtained when samples were treated

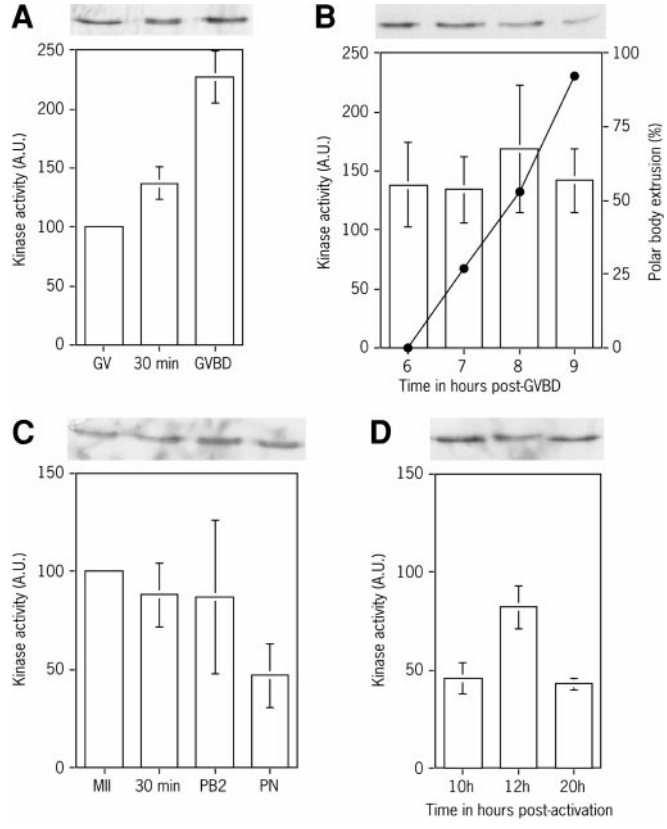

FIG. 2. Plk1 undergoes early posttranslational modification which correlates with its kinase activity. (A) At germinal vesicle stage (GV) a fast migrating form is present with weak kinase activity. Plk1 activity increases as early as 30 min before GVBD when the slower migrating form appears. Activity peaks at GVBD and is maintained after first polar body extrusion. (B) The slow migrating form persists and activity remains high at the time of polar body extrusion, between 7 and 8 h post-GVBD. (C) In MII-arrested eggs only the slow migrating form is present and casein kinase activity is high. After parthenogenetic activation, plk1 activity decreases slowly until pronucleus stage (PN; 6 h postactivation) when the fast migrating form reappears. (D) High plk1 activity and the slow migrating form can be observed at nuclear envelope breakdown upon entry into first mitotic M-phase. Activity decreases again as eggs are in interphase 20 h postactivation.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

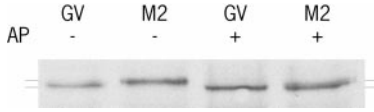

FIG. 3. Plk1 is phosphorylated during meiosis in mouse oocytes. When samples are treated with alkaline phosphatase (lanes 3 and 4), the slow form is no longer present in the MII sample (lane 4) compared to the control sample (lane 2).

with butyrolactone (Kitagawa *et al.*, 1993), another specific $p34^{cdc2}$ inhibitor. According to these results, plk1 phosphorylation depends on the presence of active $p34^{cdc2}$. Plk1 activity was also inhibited in roscovitine-treated samples. These results suggest that $p34^{cdc2}$ activity is necessary for plk1 phosphorylation and activation although direct activation would be unlikely (Hamanaka *et al.*, 1995; Qian *et al.*, 1998b).

Plk1 Localizes to MTOCs and the Midbody during Meiosis

In order to study plk1 subcellular localization during meiosis we used rabbit affinity-purified anti-plk1 AR32. Oocytes were collected and fixed according to Golsteyn *et al.* (1995) at critical time points. In GV stage oocytes, which are arrested in prophase of the first meiotic division, plk1 was diffusely distributed in the oocyte and enriched in the nucleus (Fig. 5a). At metaphase it localized to the spindle poles (Fig. 5c). Staining of the spindle could also be observed. During meiotic anaphase plk1 staining disappeared completely from the spindle poles and became concentrated in the equatorial region of the spindle (Fig. 5b). Plk1 then persisted in a region corresponding to the cleavage plane throughout telophase and concentrated close to the midbody in the bridge connecting the oocyte and the polar body (Fig. 5d). In order to quantify the amount of plk1 found in the germinal vesicle, Western blots were performed on nuclear and cytoplasmic halves. They showed that both halves contained similar amounts of plk1 (Fig. 6), suggesting that there is only a slight increase in the nuclear concentration (see Discussion). During meiosis, plk1 there-

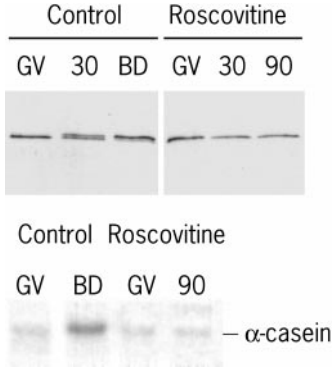

FIG. 4. Plk1 is phosphorylated and activated by the MPF amplification loop. Samples treated with a specific $p34^{cdc2}$ inhibitor, roscovitine (top, lanes 4–6), do not present the plk1 slow migrating form compared with untreated samples (top, lanes 1–3). GV, germinal vesicle stage; 30, after 30 min of culture; BD, germinal vesicle breakdown stage; 90, after 90 min of culture, 30 min after GVBD in control oocytes. Samples treated with roscovitine (bottom, lanes 3 and 4) do not phosphorylate α-casein. GV, germinal vesicle stage; BD, germinal vesicle breakdown stage; 90, after 90 min of culture.

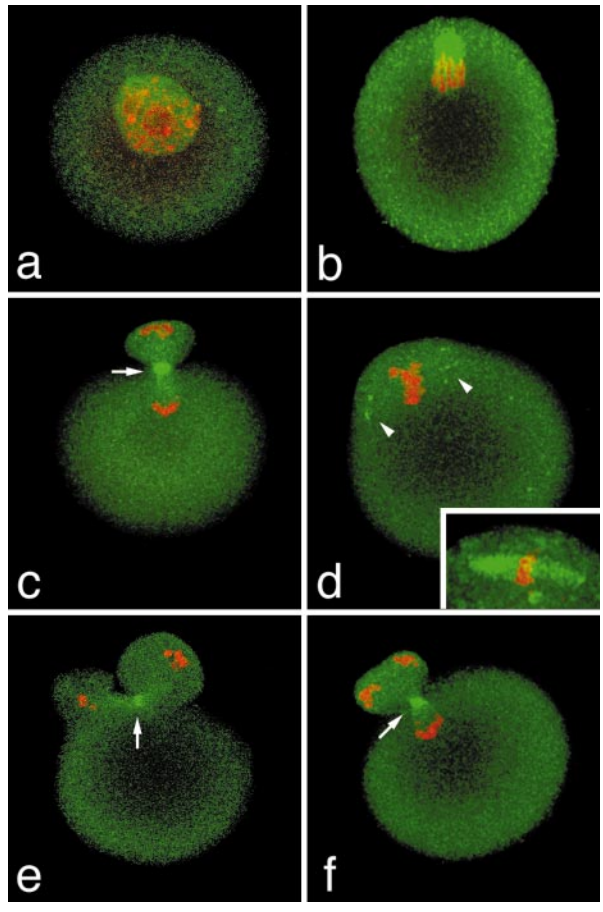

FIG. 5. During meiosis plk1 undergoes subcellular redistribution consistent with its putative targets. In these figures Plk1 is labeled with an FITC-conjugated anti-rabbit (green) and the chromosomes are stained with propidium iodide (red). (a) At GV stage, plk1 is localized diffusely in the cytoplasm and enriched in the nucleus. (b) Plk1 is associated with the spindle during late prometaphase I. (c) During first polar body (PB1) extrusion, 7–8 h post-GVBD, it localizes to the cleavage plane. (d) During the metaphase II arrest, the staining can be seen at the poles and on the spindle. (e and f) Plk1 is concentrated at the midbody during second polar body (PB2) extrusion.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

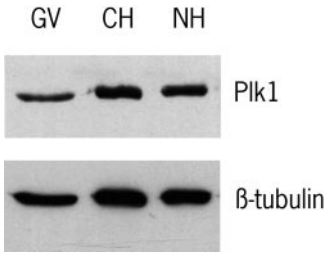

FIG. 6. Plk1 is present in nuclear and cytoplasmic halves. The difference between the Plk1/β-tubulin ratios in nuclear and cytoplasmic halves (0.64 and 0.66, respectively) is not significant.

fore seems to undergo a subcellular redistribution consistent with its putative targets.

DISCUSSION

Two Different Electrophoretic Forms of plk1 Are Present in Mouse Oocytes

We have shown in this work, using an antibody directed against the C-terminal region of human plk1, that plk1 is present in mouse oocytes during meiosis until the first mitotic division. Plk1 protein level is invariable during this period, like β-tubulin, contrarily to the mitotic cell cycle in yeast (Cheng *et al.*, 1998), murine, and human cell lines (Golsteyn *et al.*, 1995; Hamanaka *et al.*, 1995) where it is shown to be cell cycle dependent, peaking at M-phase. Our results are in contradiction with the recent characterization of plk1 protein expression in mouse oocytes published by Wianny *et al.* (1998) using an antibody generated against the internal residues of *Xenopus* Plx1. This contradiction may in part be due to the use of different antibodies. However, the possibility that the latter antibody recognizes only the slow migrating form observed by us can be excluded since this form appears before GVBD.

We have characterized two forms of plk1 which differ by their electrophoretic mobility. The fast migrating form can be observed at all stages and is predominant at GV and during interphase in one- and two-cell embryos. The slow migrating form appears before GVBD and persists throughout meiosis, disappearing at the pronucleus stage, about 5 h after parthenogenetic activation. The slow migrating form, which disappears after an alkaline phosphatase treatment, corresponds to phosphorylation. Plk activation by phosphorylation at M-phase has already been shown in *Drosophila, Xenopus,* and human cells (Hamanaka *et al.*, 1995; Kotani *et al.*, 1998; Mundt *et al.*, 1997; Qian *et al.*, 1998a; Tavares *et al.*, 1996). In mouse oocytes, the slow timing of meiotic events allows an assessment of the relative activation of different kinases *in vivo.*

Plk1 Activity Precedes GVBD and Correlates with Protein Phosphorylation

In mouse oocytes an increase in plk1 activity can be observed 30 min before GVBD when the slow migrating phosphorylated form first appears and 2.5 h before MAPK activation. Kinase activity peaks at GVBD and high levels are maintained during the metaphase I–metaphase II transition and until 5 hours after parthenogenetic activation. The kinase activity of plk1 correlates with the presence of its phosphorylated form. This is also true during the first mitotic divisions. At the pronucleus stage when kinase activity is weak, the dephosphorylated form is predominant. At nuclear envelope breakdown (NEBD) plk1 is phosphorylated and its activity increases. Activity decreases again at the two-cell stage when plk1 is dephosphorylated.

Plk1 activity therefore precedes GVBD and is stable at the metaphase–anaphase transition as well as during polar body extrusion. The early activation of plk1 is in agreement with its involvement particularly in the organization of the spindle, since in mouse oocytes this structure is in place as early as 2 h after GVBD (Brunet *et al.*, 1999; Verlhac *et al.*, 1994).

Plk1 Participates in the MPF Autoamplification Loop in Mouse Oocytes

Compelling data (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1998; Qian *et al.*, 1998a) implicate plk in the MPF autoamplification loop. Using two different specific $p34^{cdc2}$ inhibitors, roscovitine and butyrolactone, we observed that plk1 phosphorylation was inhibited. Also, plk1 remains inactive in samples incubated with roscovitine. Plk1 phosphorylation and activation seem to be strongly $p34^{cdc2}$ dependent, although it is unlikely that $p34^{cdc2}$ should directly phosphorylate plk1 (Hamanaka *et al.*, 1995). Furthermore $p34^{cdc2}$ activity is required to activate Plkk1 (Karaiskou *et al.*, 1999), the major kinase able to phosphorylate and activate Plx1 (Qian *et al.*, 1998b). Alternatively, plk1 activation could be controlled by an unknown kinase, different from $p34^{cdc2}$ and sensitive to roscovitine and butyrolactone, but this seems unlikely.

Plk1 is activated before GVBD in mouse oocytes. This could suggest that very low levels of MPF, insufficient to trigger GVBD, could activate plk1. After GVBD meiotic phosphatases PP2a are activated (Winston and Maro, 1999). High levels of phosphatases could modify the equilibrium leading to the activated form of plk1 and thus reduce its activity either directly or indirectly (Qian *et al.*, 1998b). Moreover, in our kinase assays, we have observed plk1 autophosphorylation during maturation and after parthenogenetic activation (data not shown). This could maintain plk activity even when MPF activity is low like during the MI–MII transition.

The early activation of plk1 in mouse oocytes could suggest that plk acts as a trigger kinase for M-phase entry as had been suggested from studies in which initial activation of Cdc25C occurred in the absence of cyclinB-$p34^{cdc2}$ (Izumi

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

and Maller, 1995). However, the absence of plk1 activation in the presence of $p34^{cdc2}$ inhibitors (this paper) and the observation in *Xenopus* oocyte extracts that complete inhibition of cyclinB-$p34^{cdc2}$ by $p21^{cip1}$ blocks completely Plx1 activation (Karaiskou *et al.*, 1998) suggest that a small amount of $p34^{cdc2}$ kinase, insufficient for M-phase entry, must activated first. It has been shown that Plx1 is absolutely required for full MPF activation in cycling egg extracts through the MPF autoamplification loop (Abrieu *et al.*, 1998) while in prophase egg extracts full $p34^{cdc2}$ activation depends on the two-step activation of Cdc25 in which Plx1 participates but is not the limiting factor (Karaiskou *et al.*, 1998). Finally, *in vivo*, Plx1 inactivation only delays the onset of MPF full activation (Qian *et al.*, 1998a).These results argue against plk being the only trigger for MPF activation leading to M-phase entry, even though it is clearly involved in the MPF autoamplification loop (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1998; Qian *et al.*, 1998a).

Plk1 Subcellular Redistribution during Mouse Meiosis Is Consistent with Its Putative Targets

In HeLa cells (Golsteyn *et al.*, 1994, 1995), as well as during mitosis (Logarinho and Sunkel, 1998) and recently male meiosis in *Drosophila* (Herrmann *et al.*, 1998), plks have been shown to localize to different components of the M-phase spindle apparatus.

In GV-stage oocytes, which are arrested in prophase of the first meiotic division, plk1 is found in the nucleus. According to Wianny *et al.* (1998) plk is cytoplasmic in GV-stage oocytes and at GVBD concentrates around the chromosomes. Western blots performed on nuclear and cytoplasmic halves of GV stage oocytes (Fig. 6) showed that both halves contained similar amounts of plk1 (there is only a 3% increase in the plk1/β-tubulin ratio in the nuclear halves). Since the GV represents such a small percentage of the total oocyte volume (about 3%, 25 μm in diameter for the GV, and 80 μm for the oocyte), to observe a significant difference in the quantity of plk1 between the two halves, it would have to be at least four to five times more concentrated in the GV. This is not the case. Plk nuclear localization at interphase has already been observed during spermatogenesis (Herrmann *et al.*, 1998). Conversely, plk in somatic cells seems to be predominantly cytoplasmic. This nuclear localization, which is also observed for Cdc25C, could be involved in the precise timing of MPF activation, when cyclin B1 enters the nucleus at the beginning of mitosis, before nuclear lamina breakdown (Pines and Hunter, 1991).

Following GVBD, when a bipolar spindle forms, up to 2 h after GVBD, plk1 is active. At that time, it may be involved in the reorganization of the numerous cytoplasmic MTOCs that will form the two poles (Maro *et al.*, 1985; Verlhac *et al.*, 1994). At metaphase, plk1 is localized to the spindle poles and staining of the whole spindle can also be observed. Kinetochore staining was observed in mouse oocytes at metaphase (Wianny *et al.*, 1998), in *Drosophila* larvae (Logarinho and Sunkel, 1998), and in tissue culture cells (Arnaud *et al.*, 1998). Using our antibody, we were also able to detect such staining in cultured cells (data not shown) but not in oocytes. During meiotic anaphase plk1 staining disappears completely from the spindle poles and is concentrated in the equatorial region of the spindle. Plk1 then persists in a region corresponding to the cleavage plane throughout telophase and concentrates close to the midbody in the bridge connecting the oocyte and the polar body. Even though no obvious changes in Plk1 activity are observed during this period at the oocyte level, the concentration of plk1 in the cleavage plane area may lead to a local increased activity that could participate in cytokinesis.

Taken together, these results suggest that plk1 has multiple functions during meiosis, possibly involved in M-phase entry and exit, as well as targeting elements that mediate meiotic organization and progression. The identification of these substrates and plk1s exact role and position in the transduction cascade leading to MPF activation and inactivation in the mouse oocyte remain to be elucidated.

ACKNOWLEDGMENTS

We thank Laurent Meijer for the generous gift of Roscovitine and Ricardo Bastos for careful reading of the manuscript. This work was supported by grants from CNRS, ARC, and FRM to B.M. G.P. is the recipient of a FRM fellowship.

REFERENCES

Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J. C., and Doree, M. (1998). The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in *Xenopus* eggs. *J. Cell Sci.* **111,** 1751–1757.

Arnaud, L., Pines, J., and Nigg, E. A. (1998). GFP tagging reveals human Polo-like kinase 1 at the kinetochore/centromere region of mitotic chromosomes. *Chromosoma* **107,** 424–429.

Bahler, J., Steever, A. B., Wheatley, S., Wang, Y., Pringle, J. R., Gould, K. L., and McCollum, D. (1998). Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. *J. Cell Biol.* **143,** 1603–1616.

Brunet, S., Santa Maria, A., Guillaud, P., Dujardin, D., Kubiak, J. Z., and Maro, B. (1999). Kinetochore fibers are not involved in the formation of the first meiotic spindle in mouse oocytes, but control the exit from the first meiotic metaphase. *J. Cell Biol.* **146,** 1–12.

Charles, J. F., Jaspersen, S. L., Tinker-Kulberg, R. L., Hwang, L., Szidon, A., and Morgan, D. O. (1998). The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr. Biol.* **8,** 497–507.

Cheng, L., Hunke, L., and Hardy, C. F. J. (1998). Cell cycle regulation of the *Saccharomyces cerevisiae* polo-like kinase cdc5p. Mol. *Cell. Biol.* **18,** 7360–7370.

Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y., and Kohmoto, K. (1991). Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. *Development* **113,** 789–795.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

Cuthbertson, K. S. R. (1983). Parthenogenetic activation of mouse oocytes in vitro with ethanol and benzyl alcohol. *J. Exp. Zool.* **226,** 311–314.

Descombes, P., and Nigg, E. A. (1998). The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in *Xenopus* egg extracts. *EMBO J.* **17,** 1328–1335.

Glover, D. M., Hagan, I. M., and Tavares, A. A. (1998). Polo-like kinases: A team that plays throughout mitosis. *Genes Dev.* **12,** 3777–3787.

Golsteyn, R. M., Mundt, K. E., Fry, A. M., and Nigg, E. A. (1995). Cell cycle regulation of the activity and subcellular localization of PLK1, a human protein kinase implicated in mitotic spindle function. *J. Cell Biol.* **129,** 1617–1628.

Golsteyn, R. M., Schultz, S. J., Bartek, J., Ziemiecki, A., Ried, T., and Nigg, E. A. (1994). Cell cycle analysis and chromosomal localization of human Plk1, a putative homologue of the mitotic kinases *Drosophila* polo and *Saccharomyces cerevisiae* Cdc5. *J. Cell Sci.* **107,** 1509–1517.

Hamanaka, R., Smith, M. R., O'Connor, P. M., Maloid, S., Mihalic, K., Spivak, J. L., Longo, D. L., and Ferris, D. K. (1995). Polo-like kinase is a cell cycle-regulated kinase activated during mitosis. *J. Biol. Chem.* **270,** 21086–21091.

Hartwell, L. H., Mortimer, R. K., Culotti, J., and Culotti, M. (1973). Genetic control of the cell division cycle in yeast: V. genetic analysis of cdc mutants. *Genetics* **74,** 267–286.

Herrmann, S., Amorim, I., and Sunkel, C. E. (1998). The POLO kinase is required at multiple stages during spermatogenesis in *Drosophila melanogaster. Chromosoma* **107,** 440–151.

Izumi, T., and Maller, J. L. (1995). Phosphorylation and activation of the *Xenopus* Cdc25 phosphatase in the absence of Cdc2 and Cdk2 kinase activity. *Mol. Biol. Cell* **6,** 215–226.

Karaiskou, A., Cayla, X., Haccard, O., Jessus, C., and Ozon, R. (1998). MPF amplification in *Xenopus* oocyte extracts depends on a two-step activation of cdc25 phosphatase. *Exp. Cell Res.* **244,** 491–500.

Karaiskou, A., Jessus, C., Brassac, T., and Ozon, R. (1999). Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J. Cell Sci.* **112,** 3747–3756.

Kitagawa, M., *et al.* (1993). Butyrolactone I, a selective inhibitor of cdk2 and cdc2 kinase. *Oncogene* **8,** 2425–2432.

Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P. M., Watanabe, N., Hoog, C., Hieter, P., and Todokoro, K. (1998). PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. *Mol. Cell.* **1,** 371–380.

Kubiak, J. Z., Weber, M., Géraud, G., and Maro, B. (1992). Cell cycle modification during the transition between meiotic M-phases in mouse oocytes. *J. Cell Sci.* **102,** 457–467.

Kumagai, A., and Dunphy, W. G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science* **273,** 1377–1380.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lane, H. A., and Nigg, E. A. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J. Cell Biol.* **135,** 1701–1713.

Lee, K. S., and Erikson, R. L. (1997). Plk is a functional homolog of Saccharomyces cerevisiae Cdc5, and elevated Plk activity induces multiple septation structures. *Mol. Cell. Biol.* **17,** 3408–3417.

Lee, K. S., Grenfell, T. S., Yarm, F. R., and Erikson, R. L. (1998). Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. *Proc. Natl. Acad. Sci. USA* **95,** 9301–9306.

Logarinho, E., and Sunkel, C. E. (1998). The *Drosophila* POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J. Cell Sci.* **111,** 2897–2909.

Maro, B., Howlett, S. K., and Webb, M. (1985). Non-spindle microtubule organizing centers in metaphase II-arrested mouse oocytes. *J. Cell Biol.* **101,** 1665–1672.

Maro, B., Johnson, M. H., Pickering, S. J., and Flach, G. (1984). Changes in the actin distribution during fertilisation of the mouse egg. *J. Embryol. Exp. Morphol.* **81,** 211–237.

Mayor, T., Meraldi, P., Stierhof, Y. D., Nigg, E. A., and Fry, A. M. (1999). Protein kinases in control of the centrosome cycle. *FEBS Lett.* **452,** 92–95.

Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G., and Moulinoux, J. P. (1997). Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **243,** 527–536.

Mulvihill, D. P., Petersen, J., Ohkura, H., Glover, D. M., and Hagan, I. M. (1999). Plo1 kinase recruitment to the spindle pole body and its role in cell division in *Schizosaccharomyces pombe. Mol. Biol. Cell* **10,** 2771–2785.

Mundt, K. E., Golsteyn, R. M., Lane, H. A., and Nigg, E. A. (1997). On the regulation and function of human polo-like kinase 1 (PLK1): Effects of overexpression on cell cycle progression. *Biochem. Biophys. Res. Commun.* **239,** 377–385.

Nigg, E. A. (1998). Polo-like kinases: Positive regulators of cell division from start to finish. *Curr. Opin. Cell Biol.* **10,** 776–783.

Ohkura, H., Hagan, I. M., and Glover, D. M. (1995). The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G(1) and G(2) cells. *Genes Dev.* **9,** 1059–1073.

Pines, J., and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* **115,** 1–17.

Qian, Y. W., Erikson, E., Li, C., and Maller, J. L. (1998a). Activated polo-like kinase Plx1 is required at multiple points during mitosis in *Xenopus laevis. Mol. Cell. Biol.* **18,** 4262–4271.

Qian, Y. W., Erikson, E., and Maller, J. L. (1998b). Purification and cloning of a protein kinase that phosphorylates and activates the polo-like kinase Plx1. *Science* **282,** 1701–1704.

Sharon, G., and Simchen, G. (1990). Mixed segregation of chromosomes during single-division meiosis of *Saccharomyces cerevisiae* [published erratum appears in *Genetics* 1991 Mar; 127(3): 630]. *Genetics* **125,** 475–485.

Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae. EMBO J.* **17,** 1336–1349.

Simchen, G., Kassir, Y., Horesh Cabilly, O., and Friedmann, A. (1981). Elevated recombination and pairing structures during meiotic arrest in yeast of the nuclear division mutant cdc5. *Mol. Gen. Genet.* **184,** 46–51.

Sunkel, C. E., and Glover, D. M. (1988). polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J. Cell Sci.* **89,** 25–38.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

Tarkowski, A. K. (1977). In vitro development of haploid mouse embryos produced by bisection of one-cell fertilized eggs. *J. Embryol. Exp. Morphol.* **38,** 187–202.

Tavares, A. A., Glover, D. M., and Sunkel, C. E. (1996). The conserved mitotic kinase polo is regulated by phosphorylation and has preferred microtubule-associated substrates in *Drosophila* embryo extracts. *EMBO J.* **15,** 4873–4883.

Verlhac, M.-H., Kubiak, J. Z., Clarke, H. J., and Maro, B. (1994). Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development* **120,** 1017–1025.

Whittingham, D. G. (1971). Culture of mouse ova. *J. Reprod. Fertil.* **14**(Suppl)., 7–21.

Wianny, F., Tavares, A., Evans, M. J., Glover, D. M., and Zernicka-Goetz, M. (1998). Mouse polo-like kinase 1 associates with the acentriolar spindle poles, meiotic chromosomes and spindle midzone during oocyte maturation. *Chromosoma* **107,** 430–439.

Winston, N. J., and Maro, B. (1999). Changes in the activity of type 2A protein phosphatases during meiotic maturation and the first mitotic cell cycle in mouse oocytes. *Biol. Cell* **91,** 175–183.

Received for publication December 30, 1999
Revised February 2, 2000
Accepted February 2, 2000

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.