Fertilization differently affects the levels of cyclin B1 and M-phase promoting factor activity in maturing and metaphase II mouse oocytes

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Abstract

Fertilization affects levels of cyclin B1 and M-phase promoting factor (MPF) activity in maturing and metaphase II mouse oocytes in two distinct ways. In metaphase II oocytes, it leads to a Ca^{2+} -dependent, continuous degradation of cyclin B1 and inactivation of cyclin dependent kinase (CDC2A)–cyclin B1 complex (MPF). In this paper, we show that neither mono- nor polyspermic fertilization of prometaphase I and metaphase I oocytes triggered degradation of cyclin B1. However, polyspermic fertilization of prometaphase I oocytes led to a transient decrease in MPF activity that lasted for 2 h. The inactivation of MPF in polyspermic prometaphase I oocytes did not depend on the fertilization-induced increase in the cytoplasmic concentration of free Ca^{2+} ions, but was caused, at least in part, by dephosphorylation of CDC2A at threonine 161 (Thr161). We found that polyspermic fertilization did not affect glutathione levels in prometaphase I oocytes, and concluded that the decrease in MPF activity and dephosphorylation of CDC2A at Thr161 in polyspermic prometaphase I oocytes were not caused by a change in the redox status of the cell induced by an introduction of excessive amount of sperm protamines. Instead, we propose that inactivation of MPF activity in polyspermic maturing oocytes is caused by a change in nucleocytoplasmic ratio that leads to a 'titration' of kinases and phosphatases responsible for keeping MPF in an active state. This idea is supported by the finding that oocytes fused with thymocytes rather than spermatozoa also showed a transient decrease in MPF activity. *Reproduction* (2008) **136** 741–752

Introduction

The activity of M-phase promoting factor (MPF), a key regulator of both mitotic and meiotic cell cycles, depends upon the formation of a complex consisting of kinase CDC2A (also known as cyclin-dependent kinase 1 or CDK1) and cyclin B. CDC2A serves as a catalytic subunit of MPF, whereas cyclin B plays a crucial role in regulating MPF action. To date, several isoforms of cyclin B have been identified in vertebrates, although only cyclin B1 seems to be essential for the MPF activity (Jackman *et al.* 1995, Brandeis *et al.* 1998, Draviam *et al.* 2001). Degradation of cyclin B1 leads to inactivation of MPF (Murray *et al.* 1989). It also has been reported that the dissociation of CDC2A–cyclin B1 complex, without subsequent proteolysis of cyclin B1, is enough to inactivate MPF (Nishiyama *et al.* 2000, Chesnel *et al.* 2006). Moreover, MPF activity depends upon the phosphorylation status of CDC2A. Phosphorylation of Thr14 and Tyr15 in CDC2A inhibits MPF activity (Krek & Nigg 1991, Solomon *et al.* 1992), whereas phosphorylation of Thr161 is necessary to

maintain MPF in an active state (Solomon *et al.* 1992, De Smedt *et al.* 2002).

During the meiotic cycle of mammalian oocytes (commonly known as meiotic maturation) the cyclin B1 levels, and, as a consequence, the MPF activity, fluctuate in a well-defined manner. They are both low in oocytes arrested in the prophase of the first meiotic division (germinal vesicle or GV oocytes) and rise gradually as oocytes resume meiosis, reaching maximum levels in the metaphase of the first meiotic division (metaphase I). At metaphase I/anaphase I transition, an abrupt degradation of cyclin B1 leads to the inactivation of MPF. Then, as the oocytes progress to metaphase of the second meiotic division (metaphase II), cyclin B1 level and MPF activity rise again and remain high during metaphase II arrest (Winston 1997, Ledan *et al.* 2001, Nixon *et al.* 2002, Hyslop *et al.* 2004, Marangos & Carroll 2004). A trigger necessary to terminate metaphase II arrest is provided by a fertilizing spermatozoon, which introduces an oocyte-activating factor, phospholipase C zeta (PLC zeta, Saunders *et al.* 2002).

PLC zeta, via inositol 1,4,5-trisphosphate production, induces Ca^{2+} oscillations that, in turn, leads to proteosomal degradation of cyclin B1, inactivation of MPF, and completion of meiosis (Nixon *et al.* 2002, Hyslop *et al.* 2004).

Under experimental conditions spermatozoa may also penetrate maturing oocytes, i.e., oocytes that have not yet reached metaphase II (Clarke & Masui 1986, 1987, Pyrzynska *et al.* 1996, Ajduk & Maleszewski 2004). Although it is well known that maturing mouse oocytes react to experimental fertilization or sperm factor injection with attenuated Ca^{2+} oscillations (lower amplitude and/or number of Ca^{2+} transients; Carroll *et al.* 1994, Jones *et al.* 1995, Cheung *et al.* 2000, reviewed in Ajduk *et al.* 2008), the downstream effect of such premature fertilization has not been examined. In the present work, we explore how mono- and polyspermic fertilization of oocytes at different stages of meiotic maturation influences the cyclin B1 level and MPF activity in these cells. Our results confirm that fertilization causes cyclin B1 degradation and long-lasting MPF inactivation only in oocytes that are arrested in metaphase II. We also found that although we did not observe degradation of cyclin B1 in fertilized prometaphase I oocytes, MPF activity transiently decreases in these cells. Therefore, we attempted to elucidate the mechanism that causes the inactivation of MPF in such fertilized prometaphase I oocytes.

Results

The pattern of cyclin B1-luciferase expression in oocytes maturing in vitro

To follow cyclin B1 level in fertilized oocytes we decided to use cyclin B1 tagged with a firefly luciferase. Since the kinetics of expression of this construct has not been described previously in oocytes, we first tested if the expression of cyclin B1-luciferase reflects changes in cyclin B1 level occurring during meiotic maturation. Thus, oocytes that underwent GV breakdown (GVBD) were injected with cRNA encoding cyclin B1 tagged with luciferase (cyclin B1-luc). Next, luminescence, reflecting expression of exogenous cyclin B1, was recorded. In the majority of *in vitro* maturing oocytes (49/72, 68%) expression of cyclin B1-luc precisely imitated previously described changes in cyclin B1-GFP levels (Ledan *et al.* 2001, Nixon *et al.* 2002, Hyslop *et al.* 2004, Marangos & Carroll 2004; Fig. 1). The level of cyclin B1-luc rose after GVBD, in a period hereinafter referred to as prometaphase I, and gained its maximum in metaphase I. The plateau observed in metaphase I lasted for about 3 h, and rapidly decreased during metaphase I/anaphase I transition. Subsequently, cyclin B1-luc levels increased around the metaphase II stage. In the remaining *in vitro* maturing oocytes (23/72, 32%) cyclin B1-luc expression reached maximum in the metaphase I, decreased in metaphase I/anaphase I transition, and then remained low (Fig. 1).

Figure 1 Expression of cyclin B1-luc in maturing mouse oocytes. Luminescence emitted by the oocytes reflected changes in the expression of cyclin B1-luc. In the majority of oocytes, level of cyclin B1-luc rose in prometaphase I (ProMI) and gained its maximum in metaphase I (MI). Then it decreased during metaphase I/anaphase I transition (MI/AI) and rose again in metaphase II (MII) (black line). In one-third of the oocytes, level of cyclin B1-luc expression remained low in metaphase II (grey line).

It was probably caused by a premature degradation of cyclin B1-luc cRNA that precluded a rise in the amount of cyclin B1-luc protein characteristic for metaphase II stage. However, endogenous cyclin B1 seemed to be resynthesized normally, as all these oocytes became arrested in metaphase II stage.

Fertilization of maturing oocytes does not result in cyclin B1 degradation

To examine how cyclin B1 levels are affected in oocytes fertilized in different stages of meiotic maturation we injected oocytes that had undergone GVBD with Oregon green BAPTA (1,2-bis (2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) dextran (OGBD), to monitor Ca^{2+} levels, and cRNA encoding cyclin B1-luc. Such oocytes were placed in a heated chamber under an inverted microscope and inseminated by addition of capacitated spermatozoa. Fluorescence (reflecting concentration of free Ca^{2+} ions in the ooplasm) and luminescence (reflecting expression of cyclin B1-luc) were recorded for the next 12 h. At the end of the recording, oocytes were stained with Hoechst 33342 to visualize and count spermatozoa that penetrated the oocytes.

Spermatozoa were added to the maturing oocytes ~2 h after GVBD; however, fertilization occurred at different times, i.e., either in prometaphase I, or metaphase I or metaphase II. Thus, the precise moment of fertilization was estimated by the time of the first Ca^{2+} transient. Since a membrane block to polyspermy develops in fertilized oocytes in ~1 h (Maluchnik & Borsuk 1994), we assumed that in polyspermic oocytes all spermatozoa fused at the similar time. In all examined oocytes penetrated in prometaphase I by a single spermatozoon (7/7) or several spermatozoa (7/7), Ca^{2+} oscillations caused by sperm penetration did not visibly affect the increase in

Figure 2 Expression of cyclin B1-luc in fertilized mouse oocytes. Luminescence emitted by the oocytes reflected changes in the expression of cyclin B1-luc (black line), whereas fluorescence corresponds to the cytoplasmic concentration of free Ca^{2+} ions (grey line). In oocytes penetrated in prometaphase I by a single spermatozoon (A) or several spermatozoa (B), the rise in cyclin B1-luc level was not visibly disrupted during Ca^{2+} oscillations. The inclination angle of rising slope of the cyclin B1-luc curve is marked as α (A). Similarly, in oocytes fertilized in metaphase I either by a single sperm (C) or by multiple spermatozoa (D), cyclin B1-luc level remained unchanged during Ca^{2+} transients. If fertilization occurred at metaphase II, either in *in vitro* matured (E) or in freshly ovulated (F) oocytes, a continuous decrease in cyclin B1 level was recorded during Ca^{2+} oscillations.

cyclin B1-luc (Fig. 2A and B). This observation was confirmed by the analysis of the rate of cyclin B1-luc increase in mono- and polyspermic oocytes, as well as in unfertilized ones. To this point, we calculated an inclination angle of the rising slope of cyclin B1-luc curve (Fig. 2A). We found that, according to the tangent values of the inclination angles, the cyclin B1-luc level in fertilized (by one or more sperm) and unfertilized oocytes rose at the same rate (Table 1). Similarly, in all oocytes fertilized in metaphase I, either by a single spermatozoon (17/17) or multiple spermatozoa (6/6), cyclin B1-luc level

remained unchanged during Ca^{2+} oscillations (Fig. 2C and D). In comparison, if fertilization occurred in metaphase II, in all examined cases (respectively 6/6 and 8/8), a rapid decrease in cyclin B1 level was recorded during Ca^{2+} oscillations (Fig. 2E and F). Moreover, our recordings clearly indicated that fertilization-induced degradation of cyclin B1 was a continuous, not a stepwise process.

To confirm that the fertilization of prometaphase I oocytes does not initiate an extensive degradation of cyclin B1, which is masked by a continuous synthesis of cyclin B1

Table 1 Comparison of the rates of cyclin B1-luc expression (as measured by an inclination angle) in prometaphase I mouse oocytes fertilized by a different number of spermatozoa.

	No. of spermatozoa that fertilized oocytes in prometaphase I		
	0 ^a	1	≥2
tgα ^b (±s.d.)	0.0037±0.0022	0.0046±0.0025	0.0054±0.0022

^aTen unfertilized oocytes were analyzed. ^bα is an angle between the X-axis and the tangent to the cyclin B1-luc curve (an inclination angle).

typical for this meiotic stage, we performed an analysis of oocytes treated with cycloheximide (CHX), an inhibitor of protein synthesis. The rationale of this experiment comes from the work of Clarke & Masui (1983) who showed that the inhibition of protein synthesis during metaphase I/anaphase I transition leads to the formation of interphase pronucleus, instead of metaphase II plate and spindle. This observation can be easily explained: cyclin B degrades during metaphase I/anaphase I transition, and in the presence of protein synthesis inhibitor its amount cannot be restored and MPF cannot be reactivated. Therefore, we assumed that if there was any decrease in cyclin B1 level in oocytes fertilized during maturation, CHX would prevent the resynthesis of cyclin B1 and lead to MPF inactivation and decondensation of the oocyte chromatin. To verify this hypothesis, oocytes were incubated with CHX during IVF and the subsequent culture. They were fixed 4 h after fertilization (6.5 h after GVBD), i.e., before metaphase I/anaphase I transition, which in our experiment started not earlier than 7 h after GVBD. This procedure allowed us to follow only the effect of a potential fertilization-induced decrease of cyclin B1 enhanced by the presence of CHX. However, CHX treatment did not cause decondensation of chromatin in any oocytes fertilized either by one ($n=11$) or multiple spermatozoa ($n=45$; Fig. 3). This supports our conclusion that premature fertilization does not initiate cyclin B1 degradation.

Polyspermic fertilization of maturing oocytes transiently decreases MPF activity in a Ca^{2+} -independent manner

It has been reported previously that the MPF activity may be diminished without degradation of cyclin B1 (Nishiyama *et al.* 2000, Josefsberg *et al.* 2001, Chesnel *et al.* 2006). Thus, it is possible that in fertilized prometaphase I oocytes, MPF activity drops despite that cyclin B1 is not degraded. We investigated this hypothesis by analyzing the activity of histone H1 kinase (the biochemical measure of MPF activity) in oocytes fertilized ~2 h after GVBD. To confirm the validity of our assay conditions, we first measured histone H1 kinase activity (hereinafter called MPF activity) in metaphase II oocytes (16–18 h post-human chorionic gonadotropin (hCG)) in which fertilization triggers the proteolysis of cyclin B1. The mean MPF activity 1, 2, 3, and 4.5 h after fertilization of metaphase II oocytes was, as expected, significantly lower than in control, unfertilized oocytes ($P<0.05$; Fig. 4A). Intriguingly, a decrease in MPF activity occurred also in maturing oocytes fertilized 2 h after GVBD, even though cyclin B1 was not degraded in such oocytes. However, the observed decrease in MPF activity was transient and less prominent than that occurring in fertilized metaphase II oocytes. Between 1 and 2 h after fertilization of maturing oocytes the MPF activity was significantly decreased compared with control oocytes (respectively $67.8\pm32.6\%$ and $57.1\pm33.7\%$ of control activity, $P<0.05$), whereas 3 h after fertilization the MPF activity in fertilized oocytes had increased to the same level as in unfertilized counterparts (Fig. 4A).

We noticed that the fertilization of maturing oocytes affected the MPF activity to a variable extent, which was reflected in high s.d. This appeared to correlate with the observation that the number of sperm that penetrated maturing oocytes was also much variable (range from 1 to more than 10). To test whether the decrease in MPF activity depended on the number of spermatozoa that penetrated into maturing oocytes, we assayed the MPF activity

Figure 3 Influence of cycloheximide, an inhibitor of protein synthesis, on condensation of chromatin in mouse oocytes fertilized in prometaphase I. Chromatin of maturing mouse oocytes fertilized by four spermatozoa (A) or a single spermatozoon (B) and subsequently cultured 4 h in the presence of cycloheximide remained condensed (total preparations stained with hematoxylin). To confirm that 4-h incubation with the protein synthesis inhibitor is sufficient for the oocyte chromatin to decondense if degradation of cyclin B1 is initiated, oocytes were isolated at metaphase I/anaphase I transition and incubated with cycloheximide for 4 h. In all cases ($n=19$), oocyte chromatin formed an interphase nucleus with a nucleolus (bright field image; C). Asterisks indicate sperm chromatin and arrowheads indicate chromatin of the oocyte. Nuc, nucleolus; I PB, first polar body; scale bar 10 μ m.

Figure 4 MPF activity in fertilized mouse oocytes. The activity of MPF was assayed as histone H1 kinase. The charts present the MPF activity in fertilized oocytes as a percentage of MPF activity in control unfertilized oocytes (mean values from at least three experiments $\pm$ s.d.). (A) MPF activity in fertilized metaphase II oocytes (light grey line) and fertilized maturing oocytes (dark grey line). (B) MPF activity measured 2 h after fertilization in maturing oocytes penetrated by a different number of spermatozoa. The maturing oocytes were either treated (white column) or not treated (grey columns) with BAPTA prior to the fertilization.

in mono- and polyspermic oocytes 2 h after fertilization. We found out that although one sperm was not able to cause any change in the MPF activity, two and more spermatozoa led to its significant decrease (two spermatozoa – $81.2 \pm 12.6\%$, three to nine spermatozoa – $67.1 \pm 16.8\%$ of control MPF activity, $P < 0.05$; Fig. 4B).

To determine whether inactivation of MPF triggered in fertilized maturing oocytes is Ca^{2+} -dependent, we measured the MPF activity in maturing oocytes that had been incubated with a Ca^{2+} chelator, BAPTA-AM, prior to fertilization. Interestingly, preincubation of maturing oocytes with BAPTA-AM did not prevent the decrease in MPF activity. Two hours after fertilization (three to nine sperms) of BAPTA-AM treated oocytes the MPF activity was $\sim 30\%$ lower than in control, unfertilized maturing oocytes ($P < 0.05$; Fig. 4B). In comparison, in fertilized metaphase II oocytes, which were pretreated with BAPTA, the MPF activity remained at the same level as observed in control oocytes (data not shown).

The decrease in MPF activity in polyspermic maturing oocytes was caused by a change in phosphorylation status of CDC2A

Since cyclin B1 was not degraded in maturing oocytes that were fertilized in a polyspermic way, we searched for another reason of MPF inactivation. We investigated whether the observed decrease in MPF activity could be caused by a change in the phosphorylation status of

Figure 5 Phosphorylation of CDC2A in maturing mouse oocytes fertilized in a polyspermic manner or treated with diamide. Western blot analysis of the amount of CDC2A phosphorylated on Thr161 (CDC2A-pThr161), total CDC2A and α -tubulin in maturing oocytes fertilized in a polyspermic manner (A) or treated with diamide (B) and their control counterparts. (C) CDC2A phosphorylated on Tyr15 (CDC2A-pTyr15) and total CDC2A in maturing oocytes and embryo lysate: lane 1, control oocytes, untreated with diamide; 2, oocytes treated with diamide; 3, control, unfertilized oocytes; 4, oocytes fertilized by three to nine spermatozoa; 5, lysate prepared from 13.5-day-old mouse embryos ($\sim 5 \mu\text{g}$ total protein); 6, GV oocytes; 7, metaphase II oocytes. The same membranes were used to detect all examined proteins.

CDC2A. Western blot analysis revealed that the amount of CDC2A phosphorylated on Thr161 (CDC2A-pThr161) was significantly lower in maturing oocytes fertilized by several spermatozoa than in control unfertilized maturing oocytes, whereas the level of total CDC2A was not affected (Fig. 5A). On the other hand, CDC2A phosphorylated on Tyr 15 (CDC2A-pTyr15) was absent in both polyspermic maturing oocytes and their control counterparts. However, the same antibody against CDC2A-pTyr15 detected a clear band corresponding to CDC2A-pTyr15 in a sample prepared from similar number of GV oocytes (Fig. 5C).

Both oxidative shock and excessive number of nuclei may induce a decrease in MPF activity in fertilized maturing oocytes

Fertilization of maturing oocytes may lead to the dephosphorylation of Thr161 and a drop in the MPF activity either in a sperm-specific or a sperm-unspecific manner. In the first situation, decrease in MPF activity could be triggered by a rapid change in the redox status of the oocyte induced by an introduction of sperm protamines. Protamines present in sperm chromatin are highly cross-linked by disulphide bonds, which after fertilization are reduced by glutathione stored in the cytoplasm of the oocyte (reviewed in McLay & Clarke 2003). We reasoned that an excessive

amount of protamines introduced into the oocyte during polyspermic fertilization may lead to depletion of glutathione and, as a consequence, change the redox status of the oocyte. This, in turn, may affect enzymes, such as kinases and phosphatases, which are responsible for sustaining high activity of MPF (e.g., CDC25; Savitsky & Finkel 2002, Sohn & Rudolph 2003). Indeed, we showed that the exposure of maturing oocytes to diamide, an agent that oxidizes glutathione in a way similar to protamines (Perreault *et al.* 1984), induced a significant decrease in MPF activity in maturing oocytes (to $57.12 \pm 20.39\%$ of MPF activity in control oocytes, $P < 0.05$; Fig. 6). Moreover, the amount of CDC2A-pThr161, as assayed by western blotting, was also visibly decreased in maturing oocytes treated with diamide compared with the untreated maturing oocytes. On the other hand, we did not detect any CDC2A-pTyr15 either in diamide-treated or control maturing oocytes (Fig. 5C). These results argued for our 'redox hypothesis'. Thus, we tested how polyspermic fertilization affected glutathione level in maturing oocytes. To estimate glutathione concentration in oocytes we used monochlorobimane (MCB), a fluorescent indicator of glutathione (Keelan *et al.* 2001). To validate MCB under our experimental conditions, we first compared glutathione levels in maturing oocytes treated or untreated with diamide, as well as in untreated GV and metaphase II oocytes. The concentration of glutathione was significantly lower in maturing oocytes incubated with diamide than in control maturing oocytes (81.98 ± 13.03 vs 103.0 ± 13.6 a.u., $P < 0.05$). Similarly, glutathione level was lower in GV oocytes than in metaphase II oocytes (68.89 ± 12.24 vs 110.13 ± 6.91 a.u., $P < 0.05$; Fig. 7). These data are consistent with previously published data showing that the glutathione level increases during meiotic maturation of mouse oocytes (Perreault *et al.* 1988, Dumollard *et al.* 2007). However, experiments, in which polyspermic maturing oocytes were incubated in MCB, revealed that 2 h after fertilization the level of glutathione in these cells

Figure 6 MPF activity in maturing mouse oocytes treated with diamide or fused with thymocytes. Activity of MPF was assayed as an activity of histone H1 kinase. MPF activity in experimental oocytes is presented as a percentage of MPF activity in control oocytes (mean values from at least three experiments $\pm$ S.D.). MPF activity was measured 2 h after the incubation with diamide or fusion with thymocytes.

Figure 7 Level of glutathione in mouse oocytes. The level of glutathione is presented as the mean intensity ($\pm$ S.D.) of fluorescence emitted by MCB, a fluorescent indicator of a glutathione concentration. Levels of glutathione were assayed in maturing oocytes 2 h after the experimental treatment, i.e., polyspermic fertilization (dark grey columns) or incubation with diamide (light grey columns). Oocytes that were not fertilized or not treated with diamide served as controls. Levels of glutathione were also assayed in GV and metaphase II oocytes (white columns). ^{a,b}Values marked with the same index are significantly different according to Student's *t*-test ($P < 0.05$).

was similar to the level in their unfertilized counterparts (100.89 ± 18.96 and 107.39 ± 13.82 a.u., respectively, $P > 0.05$; Fig. 7). Thus, these data suggest that polyspermic fertilization does not cause sufficient oxidative stress in maturing oocytes to lead to a decrease in MPF activity.

Finally, we tested the possibility that decrease in MPF activity in maturing oocytes fertilized in a polyspermic manner was not sperm specific but simply caused by an introduction of an excessive number of interphase nuclei. The additional nuclei might have provided an excessive amount of substrates for kinases and phosphatases responsible for sustaining a CDC2A in an active state. To check this hypothesis, the nuclei of somatic cells (thymocytes) were introduced into maturing oocytes by the means of cell fusion induced by polyethylene glycol (PEG). We found that, similar to what happened in polyspermic oocytes, MPF activity dropped in the oocytes that fused with at least two thymocytes (two to nine thymocytes) to $76.93 \pm 26.5\%$ of MPF activity in control oocytes that were treated with PEG but did not fuse with thymocytes ($P < 0.05$; Fig. 6).

Discussion

Changes in the cyclin B1-luciferase level accurately mimic changes in endogenous cyclin B1

In the present study, we have shown that cyclin B1 tagged with luciferase can accurately mimic changes in endogenous cyclin B1 occurring during meiotic maturation of mouse oocytes (Winston 1997). The level of cyclin B1-luc rose after GVBD to gain its maximum at metaphase I, then it decreased at the transition from metaphase I to anaphase I and rose again at metaphase II. We also observed that cyclin B1-luc was rapidly degraded in fertilized metaphase II oocytes. However, the sperm-induced decrease in cyclin B1-luc was continuous instead of stepwise, which was

reported previously (Nixon *et al.* 2002, Hyslop *et al.* 2004). In these previous reports, exogenous cyclin B1 was tagged with a fluorescent marker GFP, which was used to follow fertilization-triggered changes in the levels of cyclin B1. Since GFP is excited at the same wavelength as fluorescence emitted by oxidized flavoproteins (FAD^{++}), it was proposed that the stepwise fashion of the decrease in cyclin B1 level was an artifact of autofluorescence changes in fertilized oocytes (Marangos & Carroll 2004). This assumption is supported by the observations of Dumollard *et al.* (2004), who showed that fertilization induced oscillations of FAD^{++} concentration, concomitant with Ca^{2+} oscillations: each Ca^{2+} transient is associated with a decrease in FAD^{++} autofluorescence. Thus, imposition of these two fluorescent signals, i.e., fluorescence emitted by GFP and autofluorescence emitted by FAD^{++} might lead to the false conclusion that fertilization-induced degradation of cyclin B1 is a stepwise process. In our experiments, expression of cyclin B1 was reflected by the intensity of luminescence instead of fluorescence, so autofluorescence could not interfere with our recordings. It is also noteworthy that oocytes do not show any luminescence signals at all in the absence of luciferase. Thus, it is more probable that cyclin B1 is degraded upon fertilization in a continuous way, as reflected by our cyclin B1 tagged with luciferase.

Fertilization-induced degradation of cyclin B1 requires metaphase arrest of oocytes

We found that Ca^{2+} oscillations caused by fertilization of maturing oocytes did not lead to a decrease in cyclin B1 level, whereas fertilization of metaphase II oocytes induces rapid degradation of cyclin B1. This result agrees with the data obtained by Marangos & Carroll (2004), who showed that introduction of inositol 1,4,5-trisphosphate triggers proteolysis of cyclin B1 in metaphase II oocytes, but not in maturing ones. Similarly, Hyslop *et al.* (2004) revealed that ethanol-induced Ca^{2+} increase was able to affect cyclin B1 levels only in oocytes arrested in metaphase II stage or, in case of abnormal meiosis, in oocytes prematurely arrested in metaphase I. It seems that maturing oocytes lack a mechanism linking fertilization-induced Ca^{2+} release with degradation of cyclin B1. This mechanism depends on cytosolic factor (CSF) activity, which appears for the first time in oocytes arrested in metaphase II (Kubiak *et al.* 1993, Nixon *et al.* 2002, Hyslop *et al.* 2004, Marangos & Carroll 2004), or, in some rare cases, in oocytes arrested prematurely in metaphase I (Eppig *et al.* 1994, Ciemerych & Kubiak 1998, Hyslop *et al.* 2004). CSF stabilizes MPF activity by inhibiting anaphase promoting complex/cyclosome (APC/C)-mediated ubiquitination of cyclin B and in turn, its proteolysis. The level of Emi2, a key element of the CSF activity (Rauh *et al.* 2005, Tung *et al.* 2005, Shoji *et al.* 2006), increases during meiotic maturation of mouse oocytes and reaches its maximum in metaphase II oocytes (Madgwick *et al.* 2006), i.e., when CSF starts functioning.

Emi2 binds to CDC20, the APC/C activator, and prevents activation of APC/C (Shoji *et al.* 2006). Ca^{2+} transients caused by fertilization of metaphase II oocytes lead to the degradation of Emi2 (Rauh *et al.* 2005, Hansen *et al.* 2006) and in consequence of the activation of APC/C and degradation of cyclin B1 (Madgwick *et al.* 2006). In maturing oocytes, in which CSF activity is absent, this mechanism does not function.

It also appears that both dissociation of cyclin B1 from CDC2A and its subsequent proteolysis may be prevented in fertilized maturing oocytes by an active spindle assembly checkpoint (SAC). SAC is inactivated when all kinetochores of all chromosomes become stably connected to the spindle microtubules, i.e., just before first meiotic division (Brunet *et al.* 1999). Until this moment proteins engaged in SAC, such as MAD2L1 (Homer *et al.* 2005) or BUB1B (Tsurumi *et al.* 2004), inhibit the APC/C and prevent ubiquitination of cyclin B1 and other crucial M-phase related factors (Nishiyama *et al.* 2000, Sudakin *et al.* 2001, Tang *et al.* 2001, Fang 2002). In the absence of ubiquitination, cyclin B1 can be neither detached from CDC2A nor degraded.

Polyspermic fertilization of maturing oocytes induces dephosphorylation of CDC2A at Thr161 and leads to a transient decrease in MPF activity

Although cyclin B1 was not degraded in fertilized maturing oocytes, we found that the MPF activity in such oocytes was decreased 1 and 2 h after fertilization. A transient decrease in MPF activity is also observed during transition between metaphase I and II, or, in case of suboptimal activation conditions, in transition between metaphase II and III (Kubiak 1989, Kubiak *et al.* 1992). In both these situations, inactivation of MPF is triggered by degradation of cyclin B1, although separase has been reported recently to act as an additional factor that inhibits CDC2A at the first meiotic division (Gorr *et al.* 2006). Our results suggest, however, that the decrease in MPF activity in polyspermic maturing oocytes depends on a different mechanism and may be related to the dephosphorylation of CDC2A at Thr161. We did not detect any CDC2A phosphorylated on Tyr15 either in fertilized or control maturing oocytes, but we cannot completely exclude the possibility that the inactivation of MPF was partially caused by an increase in phosphorylation of Tyr15 that was too small to be detected by immunoblotting. Experiments performed on meiotic and mitotic *Xenopus* extracts confirm that both dephosphorylation of Thr161 and phosphorylation of Tyr15 and Thr14 participate in the process of inactivation of MPF (Lorca *et al.* 1992, Chesnel *et al.* 2005, 2007, D'Angiolella *et al.* 2007).

Our data indicate that the decrease in MPF activity in polyspermic maturing oocytes was an effect of an introduction of additional nuclei that changed the nucleo-cytoplasmic ratio of the oocytes. We showed

that, similarly as it happens in polyspermic oocytes, the MPF activity was decreased in prometaphase I oocytes fused with thymocytes. We hypothesize that the additional interphase nuclei provide new substrates for CDC2A–cyclin B1 complex, such as lamins, nucleoporins, etc. (reviewed in [Margalit et al. 2005](#)). This in turn may lead to a decrease in the phosphorylation of other CDC2A–cyclin B1 substrates, including cyclin dependent kinase 7 (CDK7), which phosphorylates CDC2A at Thr161 ([Solomon et al. 1992](#), [Poon et al. 1993](#)). Decreased phosphorylation compromises the activity of CDK7 ([Martinez et al. 1997](#), [Garrett et al. 2001](#)) and, in consequence, may result in lower phosphorylation of CDC2A at Thr161 and inactivation of MPF. CDC25 (a phosphatase responsible for dephosphorylation of CDC2A at Thr14 and Tyr15; [Strausfeld et al. 1991](#)) and WEE1 (a kinase that phosphorylates CDC2A on Tyr15; [Parker & Piwnicka-Worms 1992](#)) are other CDC2A–cyclin B1 substrates. Phosphorylation catalyzed by CDC2A–cyclin B1 activates CDC25 ([Hoffmann et al. 1993](#)) and inhibits WEE1 ([Mueller et al. 1995](#)). Thus, a decrease in the phosphorylation level of either CDC25 or WEE1 may lead to increased phosphorylation of CDC2A at Tyr15 and the inactivation of MPF. It is probable that the inactivation of MPF can be reversed after some time, when nuclear envelopes of the additional nuclei disassemble and their chromatin condenses.

Oxidative stress leads to dephosphorylation of CDC2A at Thr161 and a decrease in MPF activity

It was tempting to speculate that polyspermic fertilization, which involves introduction of an excessive amount of highly oxidized sperm-specific protamines (reviewed in [Balhorn 1982](#)), might have caused a change in the redox state of the maturing oocyte and consequently led to an inhibition of kinases and phosphatases responsible for keeping MPF active ([Savitsky & Finkel 2002](#), [Sohn & Rudolph 2003](#)). However, we did not register any decrease in glutathione level in polyspermic maturing oocytes compared with their unfertilized counterparts. Glutathione is the main guardian of a reductive environment in oocytes (reviewed in [Luberda 2005](#)) and is responsible for the reduction of protamines introduced by sperm, so its concentration should have been affected if polyspermic fertilization actually led to an oxidative stress.

Even though oxidative stress is not responsible for the inactivation of MPF caused by the polyspermic fertilization, we found that a thiol-oxidizing agent, diamide, inhibited MPF activity in maturing oocytes. Inactivation of MPF caused by an oxidative stress induced by selenium deficiency was reported previously in male germ cells ([Kaushal & Bansal 2007](#)). We also determined that diamide treatment led to dephosphorylation of CDC2A at Thr161. Thus, we can speculate that oxidative stress can affect, either in direct or indirect manner, enzymes

regulating phosphorylation of CDC2A at Thr161, i.e., CDK7 ([Solomon et al. 1992](#), [Poon et al. 1993](#)) and/or protein phosphatase 2C ([Cheng et al. 1999](#), [De Smedt et al. 2002](#)). In addition, we cannot completely exclude that diamide affects phosphorylation of CDC2A at Tyr15 in maturing oocytes, but our results do not confirm this possibility. However, it has been shown that an active site of CDC25 contains a cysteine residue, which is highly susceptible to oxidation. Oxidative stress therefore leads to inactivation of CDC25 and consequently may cause an increase in the amount of CDC2A phosphorylated on Thr14 and Tyr15, and a decrease in MPF activity ([Savitsky & Finkel 2002](#), [Sohn & Rudolph 2003](#)).

In summary, our results provided new insights into the mechanism of regulation of MPF activity in mammalian oocytes. We have shown, using cyclin B1 tagged with a firefly luciferase, that degradation of cyclin B1 in fertilized metaphase II oocytes was a continuous, not a stepwise, process. Moreover, we have demonstrated that polyspermic fertilization of prometaphase I oocytes triggers a transient decrease in MPF activity that does not involve proteolysis of cyclin B1. It seems that the observed MPF inactivation was caused, at least in part, by dephosphorylation of CDC2A at Thr161. Our results also indicate that the MPF activity in maturing mouse oocytes can be effectively affected by the nucleo-cytoplasmic ratio.

Materials and Methods

Reagents

All reagents were purchased from Sigma–Aldrich unless stated otherwise.

Media and culture conditions

Medium M2 (medium 16 buffered with HEPES; [Fulton & Whittingham 1978](#)) containing BSA (4 mg/ml) was used for collection of oocytes and for culture of maturing and ovulated oocytes. Spermatozoa were capacitated in T6 medium ([Quinn et al. 1982](#)) or fertilization medium ([Fraser 1982](#)) containing BSA (4 mg/ml, Pentex, Bovine Albumin Crystallized; Miles Inc., Kankakee, IL, USA). *In vitro* fertilization was performed in the fertilization medium or, for the imaging experiment, in HEPES-buffered KSOM (see below). Fertilization medium, as well as T6 medium, was preincubated before use for about 24 h at 37.5 °C in a humidified atmosphere of 5% CO₂ in the air. All incubations were carried out in droplets of medium under mineral oil at 37.5 °C in a humidified atmosphere of 5% CO₂ in the air.

Oocyte collection and handling

Follicular development was stimulated in 4- to 8-week-old F1 (CBA/H × C₅₇Bl₆ or C₅₇Bl₆ × CBA/H) or MF1 female mice by an i.p. injection of 10 IU of pregnant mares' serum gonadotropin (PMSG, 'Folligon'; Intervet, Warsaw, Poland). After 46–52 h, females were killed by cervical dislocation. Fully grown oocytes were released from ovarian antral follicles into M2

medium. Oocytes were freed from cumulus cells by pipetting and were cultured in M2 for 1.5 h. After that time, only oocytes that underwent GVBD, i.e., resumed meiosis, were selected and subjected to the experimental procedure.

To induce ovulation, female F1 or MF1 mice were injected with 10 IU of PMSG followed by 10 IU of hCG ('Chorulon', Intervet) 46–52 h later. Metaphase II oocytes were collected 16–17 h after hCG injection. Ovulated oocytes surrounded by follicular cells were released from the oviducts into a solution of hyaluronidase (150 IU/ml) in PBS (Biomed, Lublin, Poland). After dispersing of follicular cells, oocytes were washed in M2 and cultured until beginning of the experimental procedure, i.e., for ~1.5–2 h.

Depending on the experiment, both maturing and metaphase II oocytes were subjected either to IVF (see below), injection of Ca^{2+} indicator and cRNA (see below), fusions with thymocytes (see below) or 1 h incubation in 100 μM diamide in M2.

In vitro fertilization

Spermatozoa released from *caudae epididymides* of a mature F1 (CBA/H $\times$ C₅₇Bl₆ or C₅₇Bl₆ $\times$ CBA/H) male mouse were suspended in 0.5 ml fertilization medium and incubated for 1.5 h to allow capacitation and spontaneous acrosome reaction. Sperm concentration was $\sim 2 \times 10^7$ spermatozoa/ml. Prior to fertilization, *zonae pellucidae* were removed by exposure of oocytes to acidic Tyrode's solution (pH 2.5; Nicolson *et al.* 1975). In some experiments, oocytes were preincubated 30 min with 1, 2-bis (2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetylester) (BAPTA-AM; 30 μM in M2) before insemination. Oocytes were placed in 100 μl droplets of fertilization medium and 1 μl of the preincubated sperm suspension was added (final concentration of sperm suspension was $\sim 2 \times 10^5$ spermatozoa/ml). After 1 h, the oocytes were gently pipetted several times to remove loosely attached spermatozoa and then cultured in M2 for 1–4.5 h. In some experiments insemination, as well as a subsequent culture, were performed in a medium supplemented with cycloheximide (CHX; 10 $\mu\text{g}/\text{ml}$). Moreover, in some experiments fertilized oocytes were stained with a chromatin dye, Hoechst 33342 (100 ng/ml M2), and examined under an inverted fluorescence microscope in order to count the number of spermatozoa inside.

Microinjections and fluorescence and luminescence imaging

Microinjection was carried out as described previously (Saunders *et al.* 2002). Briefly, maturing or metaphase II oocytes were microinjected using pressure pulses applied to the back of micropipettes inserted into eggs using over-compensation of the negative capacitance of a serially connected electrical amplifier. The volume of solution injected was ~3–5% of an oocyte volume. Oocytes were injected with a solution containing 0.5 mM Oregon green BAPTA dextran (OGBD; Invitrogen Ltd) and 0.75 $\mu\text{g}/\mu\text{l}$ cRNA of cyclin B1 tagged with firefly luciferase, (pipette concentrations). Human cyclin B1 from cyclin B1-GFP construct (Nixon *et al.* 2002) was linked to N-terminus of firefly luciferase (pGL3) and placed into

the pCS2+ vector. Polyadenylated cRNA was then generated from the SP6 promoter as described previously (Nixon *et al.* 2002). Before injection, *zonae pellucidae* were removed by treatment with acidic Tyrode's solution and then oocytes were attached to a polylysine-coated cover slip that formed the bottom of a heated (37 °C) chamber on an inverted microscope. Oocytes were maintained in HEPES-buffered KSOM medium (potassium simplex optimization medium; Lawitts & Biggers 1993) containing 100 μM luciferin (Dumollard *et al.* 2004). Oocytes were inseminated by adding 10 μl capacitated sperm suspension. (For these experiments, sperm isolated from two *caudae epididymides* were incubated in T6 medium for 2 h before use).

Fluorescence (reflecting cytoplasmic concentration of free Ca^{2+} ions) and luminescence (reflecting cyclin B1-luciferase expression) were both imaged in the same sets of oocytes using a Nikon TE2000 microscope equipped with a 20 $\times$ 0.65NA Fluor objective lens as was described previously (Campbell & Swann 2006). After the measurements, oocytes were stained with Hoechst 33342 (100 ng/ml HEPES-buffered KSOM) and the number of spermatozoa inside the oocytes was counted.

Fusion of oocytes with thymocytes

Maturing oocytes (2–3 h after GVBD) were fused with thymocytes by a polyethylene glycol (PEG) method as described previously (Czolowska *et al.* 1984). Thymocytes were isolated from thymuses of 1- to 5-day-old mice. Maturing oocytes were exposed to 0.5% solution of pronase in Ringer's saline in order to remove *zonae pellucidae* and washed in M2. Then oocytes were stained with Hoechst 33342 (100 ng/ml) for 30 min in order to visualize the chromatin. Subsequently, oocytes were washed carefully in M2 without BSA and transferred into phytohemagglutinin (PHA) solution (300 mg/ml in M2 without BSA) for ~5 min. Then, they were transferred to M2 without BSA and agglutinated with thymocytes. The oocytes coated with thymocytes were exposed to 40% (w/v) PEG solution (MW2000; Fluka, Poznan, Poland) in BSA-free M2 for 30–45 seconds to induce fusion. Then, the cells were washed in M2 with BSA and cultured *in vitro* for 1–1.5 h in M2. Dishes containing M2 without BSA, PHA solution, and M2 with BSA were coated with agar (Bacto-Agar, Difco, Lawrence, KS, USA, 1% (w/v) solution in 0.9% (w/v) solution of NaCl). Fusion usually occurred within 15–30 min after exposure to PEG. After 1 h culture in M2, hybrids were examined under a fluorescence microscope in order to calculate the number of thymocyte nuclei inside the oocytes. Since oocytes were incubated in Hoechst 33342 before fusion with thymocytes, only chromatin of thymocytes that fused with oocytes was stained.

Glutathione measurements

To estimate the glutathione concentration in oocytes we used a fluorescent indicator of glutathione, monochlorobimane (MCB; Keelan *et al.* 2001). Oocytes were incubated with 50 μM MCB in M2 medium for 45 min and then fluorescence of MCB was registered at 390 nm by a digital camera (Pixelfly, PCO, Kelheim, Germany). Intensity of fluorescence was

analyzed by Image J software (<http://rsb.info.nih.gov/ij/>). When glutathione concentration was measured in fertilized oocytes, they were subsequently fixed in 4% paraformaldehyde for 20 min and stained with chromomycin (0.01 mg/ml PBS supplemented with 5 mM MgCl₂). Oocytes were then examined under a fluorescence microscope at 450 nm, the chromomycin excitation wavelength, to calculate the number of sperm inside.

Histone H1 kinase assay

The activity of MPF was measured as an activity of histone H1 kinase, according to the method described by Verlhac *et al.* (1994). Oocytes were washed in PBS and pooled in groups of 5 in 1.5 µl drops of PBS. Samples were frozen and stored in –80 °C until further analysis. Three microliters of a lysing buffer (containing 0.16 M glycerophosphate, 40 mM EGTA (pH 7.3), 30 mM MgCl₂, 2 mM dithiothreitol, protease inhibitors (diluted 1:20, Complete Protease Inhibitor Cocktail, Roche), and BSA (11.3 mg/ml); final concentrations) was added to each sample. Oocytes were then lysed by freezing and thawing, and subsequently 1.5 µl reaction buffer (containing 0.5 mg/ml histone H1, 5 mM ATP, and 1.67 µCi/µl [32P]-ATP (MP Biomedicals, Illkirch, France); final concentrations) was added. Samples were incubated for 30 min in 30 °C. The reaction was stopped by an addition of 4 µl Laemmli buffer (Laemmli 1970) per sample. Samples were boiled for 10 min and proceeded for 12% SDS-PAGE. Gels were exposed to autoradiography films (BioMax MS Film, Kodak) at –80 °C for 24–72 h. Intensity of bands on autoradiography films was measured with GelDoc using software Quantity One 4.2.2 (Bio-Rad). The intensity of the bands reflected the intensity of histone H1 phosphorylation and enabled us to quantify histone H1 kinase (MPF) activity.

Immunoblotting

The levels of total CDC2A, CDC2A phosphorylated at Thr 161 and Tyr 15, and α -tubulin were examined in maturing oocytes that were fertilized in a polyspermic way or treated with diamid. Cell lysates from 180 oocytes were mixed with 4× NuPage LDS sample Buffer and 10× NuPage Sample Reducing Agent (Invitrogen) and were heated for 10 min in 70 °C. The samples were subjected to electrophoresis on NuPage Novex 10% Bis-Tris gels in NuPage MOPS SDS Running Buffer (Invitrogen) and separated proteins were transferred onto PVDF membranes (Hyperbond-P, Amersham Biosciences). PVDF membranes were probed overnight at 4 °C with: 1) a mouse monoclonal antibody (MAB) against total CDC2A (diluted 1:100, Abcam, Cambridge, UK, cat. no. ab18); 2) a rabbit polyclonal antibody anti-CDC2A phosphorylated at pTyr15 (anti-CDC2A-pTyr15, 1:100, Abcam, cat. no. ab10533); 3) a rabbit polyclonal antibody against CDC2A phosphorylated at Thr161 (anti-CDC2A-pThr161, 1:50, Cell Signaling Technology, Danvers, MA, USA, cat. no. 9114); 4) a mouse MAB against α -tubulin (1:1000, Sigma-Aldrich, cat. no. T5168). Antibodies against total CDC2A, CDC2A-pTyr15, and α -tubulin were diluted in 5% nonfat milk in TBS containing 0.05% Tween 20 (Bio-Rad). Antibody against CDC2A-pThr161

was diluted in 5% BSA in TBS containing 0.1% Tween 20. Subsequently, the membranes were incubated for 1 h with secondary antibodies (goat anti-rabbit or anti-mouse antibodies (Pierce, Rockford, IL, USA) dilution 1:6000) conjugated with HRP. Proteins were detected by the ECL technique using SuperSignal West Dura Extended Duration Substrate reagents (Pierce) according to the manufacturer's instruction.

Cytological examination

For cytological examination maturing oocytes were fixed with Heidenhain's fixative. Whole-mount preparations were stained with hematoxylin according to the method of Tarkowski & Wroblewska (1967).

Statistical analysis

Statistical analysis of the results was performed using the Student's *t*-test. Results were considered statistically significant when $P \leq 0.05$.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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