Analysis of the mechanism(s) of metaphase I arrest in maturing mouse oocytes

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SUMMARY

Fully grown mouse oocytes are normally competent to progress from prophase I to metaphase II without interruption. However, growing mouse oocytes initially become only partially competent to undergo meiotic maturation. Meiotic maturation in these oocytes does not progress beyond metaphase I. In contrast to the oocytes of most strains of mice, most oocytes of strain LT/Sv mice become arrested at metaphase I even when they are fully grown. The initiation of oocyte maturation is correlated with an increase in p34cdc2 kinase activity that continues to rise until metaphase I. The transition into anaphase I is normally correlated with a decrease in p34cdc2 kinase activity. This study demonstrated that metaphase I arrest in both partially competent growing oocytes and fully grown LT/Sv oocytes is correlated with a sustained elevation of p34cdc2 kinase activity. In fact, p34cdc2 activity continued to increase during the time when activity normally decreased. In normally maturing oocytes, some, but not all, of the cyclin B, the regulatory protein associated with p34cdc2, became degraded in oocytes that entered anaphase I. In contrast, the amount of cyclin B present in the metaphase I-arrested oocytes continued to increase at the time when it was being degraded in normal oocytes progressing to metaphase II. These results suggest that the progression of meiosis is arrested at metaphase I in both groups of oocytes because of continued p34cdc2 kinase activity sustained, at least in part, by restricted degradation of cyclin B. Finally, metaphase I arrest in these oocytes is sustained by a mechanism that differs from that maintaining metaphase II arrest, since an inhibitor of protein phosphorylation, 6-DMAP, induced a dramatic decline in p34cdc2 kinase activity and the resumption of meiosis in metaphase I-arrested oocytes but not in metaphase II-arrested oocytes. Moreover, without 6-DMAP treatment, cyclin B was more stable in the metaphase I-arrested oocytes than in metaphase II-arrested oocytes.

Key words: oocyte, p34cdc2 kinase, cyclin B, metaphase, mouse

INTRODUCTION

The progression of meiosis in mammalian oocytes becomes arrested at the transition from prophase I to M-phase. Initially, this arrest is sustained by factors intrinsic to the oocyte itself, either deficiencies in factors needed to drive progression into M-phase, inhibitory factors, or both. These oocytes are referred to as being incompetent to resume meiosis, a condition that persists until the oocytes become almost fully grown. Oocytes that have become competent to resume meiosis do so spontaneously when liberated from their follicular environment and cultured (Edwards, 1965). Thus, meiotic arrest in meiotically competent oocytes is sustained by follicular somatic cells. Meiotically competent oocytes can complete the first meiotic division, produce a polar body and progress to metaphase II, a stage wherein the progression of meiosis is once again arrested until after sperm penetration. Fully grown oocytes are normally meiotically competent. There is also an intermediate stage of normal oocyte development, between meiotically incompetent and competent, in which oocytes that are not quite fully grown are partially competent (De Smedt et al., 1994; Motlik et al., 1984; Schramm et al., 1993; Sorensen and Wassarman, 1976). These oocytes are able to undergo germinal vesicle breakdown (GVB), the most obvious morphological manifestation of the reinitiation of meiosis, condense their chromosomes, form a metaphase spindle and progress to metaphase I (Wickramasinghe et al., 1991). Partially competent oocytes fail to progress beyond metaphase I even with prolonged culture. Thus, meiotic competence is normally achieved by mammalian oocytes in at least two distinct sequential steps that occur during the final growth phase while the oocytes remain arrested at the germinal vesicle (GV) stage; they first become partially competent, then fully competent (Wickramasinghe et al., 1991).

Oocytes of some strains of mice are atypical in the progression of meiotic maturation. One of the most notable of these is strain LT/Sv. Many fully grown LT/Sv oocytes remain arrested at metaphase I when they mature either in vitro (~95%) or in vivo (~35%) (Eppig and Wigglesworth, 1994; O’Neill and Kaufman, 1987; West et al., 1993). It is not known whether the mechanism that arrests the progression of meiosis at metaphase I in the fully grown LT/Sv oocytes is the same as that occurring in normal, partially competent oocytes.
The mechanism of metaphase I arrest in either case is not known. In all eukaryotic cells, cyclin-dependent kinases orchestrate cell cycle events; see Norbury and Nurse (1992) for review. Entry into M phase is driven by the activation of maturation- or M phase-promoting factor (MPF), a heterodimer composed of p34cdc2 kinase and cyclin B (Draetta et al., 1989; Dunphy et al., 1988; Meijer et al., 1989; Murray and Kirschner, 1989). Exit from M phase in somatic cells is accompanied by the loss of p34cdc2 activity that is associated with the degradation of mitotic cyclins (Draetta et al., 1989; Gallant and Nigg, 1992; Murray and Kirschner, 1989). Anaphase I in mammalian oocytes is also correlated with a decline in p34cdc2 activity (Choi et al., 1991; Fulka et al., 1992; Hashimoto and Kishimoto, 1988), though degradation of cyclin B has, up to now, not been demonstrated. p34cdc2 activity is once again elevated to sustain metaphase II arrest until fertilization (Choi et al., 1991; Hashimoto and Kishimoto, 1988).

In this study, we investigated the hypothesis that metaphase I arrest is correlated with sustained elevation of p34cdc2 activity in both partially competent growing oocytes and fully grown LT/Sv oocytes. Furthermore, the stability of cyclin B was determined during normal anaphase I in mouse oocytes and during the metaphase I arrest of both partially competent oocytes and LT/Sv oocytes since elevated p34cdc2 activity could be sustained by restricted cyclin B degradation. Finally, as one approach to assessing the similarities and differences between the status of metaphase I and II-arrested oocytes, the effects of an inhibitor of protein phosphorylation, 6-dimethylaminopurine (6-DMPA), on the activity of p34cdc2 kinase and cyclin B stability was determined using both groups of metaphase I-arrested oocytes as well as normal metaphase II oocytes since this inhibitor is known to have differential effects depending on the stage of meiotic maturation (Szőllösi et al., 1991).

**MATERIALS AND METHODS**

**Collection and maturation of oocytes in vitro**

(C57BL/6 × SJL)F1 hybrid mice (hereafter referred to as F1 mice) and strain LT/Sv mice were injected with 5 IU equine chorionic gonadotropin (Diosynth, Oss, Holland) 44-48 hours before the isolation of oocytes. The percentage of partially competent decreases with age in postnatal mice (Sorensen and Wassarman, 1976). Thus, a higher proportion of the oocytes isolated from 18-day-old F1 mice was partially competent (86%; n=165) than oocytes from 24-day-old mice (32%; n=173). Accordingly, 18-day-old mice were usually used as the source of partially competent oocytes and 24-day-old F1 mice were usually used as the source of competent oocytes. Approximately 95% of the oocytes isolated from 24-day-old LT/Sv mice and matured with cumulus cells were partially competent (Eppig and Wigglesworth, 1994). The GV-stage oocyte-cumulus cell complexes were obtained by puncturing the antral follicles with 26-gauge needles and collecting them using glass micropipets. Complexes were cultured in 35 mm polystyrene Petri dishes (Falcon #1008, Becton Dickinson Labware, Lincoln Park, NJ) at 37°C in an atmosphere of 5% CO2, 5% O2 and 90% N2. Complexes were isolated and cultured in medium containing 100 µM 1-isobutyl-3-methylxanthine (IBMX; Aldrich Chemical Co., Milwaukee, WI) to maintain meiotic arrest. Resumption of meiotic maturation was initiated by placing oocytes into IBMX-free culture medium. Whitten’s (1971) culture medium supplemented with crystallized, lyophilized bovine serum albumin (3 g/l; BSA; ICN Immunobiologicals, Lisle, IL) was used in all experiments.

**Immunoreagents**

Rabbit polyclonal antisera G6 to a C-terminal peptide of human p34cdc2 (Draetta and Beach, 1988), which cross-reacts with the mouse homolog (O’Keefe et al., 1991), was generously provided by Dr G. Draetta. Purified monoclonal antibody (mAb) to human cyclin B1, which also cross-reacts with the mouse homolog, was purchased from PharMingen (San Diego, CA). Rabbit polyclonal antisera to human cyclin B, which cross-reacts with the mouse homolog, was generously provided by Dr M. Pagano.

**Immunoblotting**

Oocytes were lysed in Laemmli (1970) sample buffer, boiled for 5 minutes and subjected to separation on 10% SDS/PAGE. Proteins were transferred onto nitrocellulose membrane (Hoefer Scientific Instruments, San Francisco, CA) using a semidry blotting apparatus (OMNI BIO, Brno, Czech Republic). Membrane-bound proteins were probed with the polyclonal serum against cyclin B described above and visualized using the ECL detection system of Amersham Corporation (Arlington Heights, IL).

**Immunoprecipitation**

180 to 200 oocytes metabolically labeled with L-[35S]methionine (specific activity >1,000 Ci/mmol; Amersham, Arlington Heights, IL) were washed in phosphate-buffered saline (PBS) containing 3 mg/ml polyvinylpyrrolidone (PVP, 40,000; Sigma Chemical Co.) and lysed by freezing and thawing in ice-cold lysis buffer (50 mM Tris/HCl (pH 7.4), 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, 1 mM dithiothreitol (DTT) and 100 µM Na3VO4). The following protease inhibitors were included: phenylmethyl-sulfonyl fluoride (PMSF; 100 µM), leupeptin (1 µg/ml), aprotinin (1 µg/ml), soybean trypsin inhibitor (10 µg/ml) and tosyl-L-phenylalanine chloromethyl ketone (TPCK; 10 µg/ml). Disruption of oocytes was accomplished by freezing at −70°C. After thawing, lysates were cleared by centrifugation in an Eppendorf microcentrifuge at 15,000 revs/minute for 10 minutes at 4°C. Lysates were then subjected to Protein A Sepharose CL-4B (PAS; Pharmacia) preabsorption and immunoprecipitation with 3 µl of anti-p34cdc2 serum and/or with 2 µg of anti-cyclin B1 mAb for 1 hour in an ice bath. Immunoprecipitates were collected using PAS, washed three times with lysis buffer, resuspended in 2× Laemmli sample buffer and subjected to SDS/PAGE. Polyacrylamide gels were fixed, treated with Enfintensify (DuPont NEN Products, Boston, MA), dried and exposed to Fuji RX film at −70°C or to a Fuji Imaging Plate (Type BAS-III; Fuji Photo Film Co. Japan).

**Histone H1 kinase assay**

Histone H1 kinase activity was used as a measure of the p34cdc2 kinase activity. Crude oocyte lysates and/or anti-p34cdc2 or anti-cyclin B immunoprecipitates were used in this assay. The lysates were prepared by freezing oocytes in H1 kinase buffer (4 oocytes/µl) containing 25 mM MOPS (pH 7.2), 60 mM β-glycerophosphate, 30 mM p-nitrophenylphosphate, 15 mM EGTA, 15 mM MgCl2, 100 µM Na3VO4, 1 mM DTT, 1 mM PMSF and BSA (1 mg/ml). Lysates were stored at −70°C until use. Thawed lysates were cleared before use by centrifugation in an Eppendorf microcentrifuge at 15,000 revs/minute for 10 minutes at 4°C. Kinase reactions were carried out for 1 hour at 30°C in a total volume of 25 µl of H1 kinase buffer supplemented with 100 µg/ml of histone H1 (type III-S; Sigma Chemical Co.), 1 µg/ml of cAMP-dependent protein kinase inhibitor peptide (Sigma) and 40 µCi/µl of [γ-32P]ATP using lysates of 20 oocytes. Incorporation of 32P into histone H1 was quantified either by spotting 20 µl onto Whatman paper, followed by 5x washing in 1% phosphoric acid and scintillation spectrometry, or by mixing 20 µl of the reaction mixture with the same volume of 2× concentrated Laemmli sample buffer, followed by 10% SDS/PAGE and by autoradiography.
Immunoprecipitates for the histone H1 kinase assay were prepared essentially as described for metabolically labeled oocytes, except that lysates of 20 unlabeled oocytes were immunoprecipitated and H1 kinase buffer was used for final washing of PAS beads. Immediately after washing, histone H1 kinase buffer containing histone H1, cAMP-dependent kinase inhibitor and $[^\gamma-32P]ATP$ was added and kinase reaction was carried out as above. The histone H1 kinase activity is presented as the fold increase over the basal level detected in prophase-arrested oocytes.

**Treatment with 6-dimethylaminopurine**

6-dimethylaminopurine (6-DMAP) was dissolved directly in culture medium to a final concentration of 5 mM immediately before use. One hour treatment of oocytes with 6-DMAP was carried out in borosilicate glass tubes (Fisher Scientific) under standard culture conditions as above.

**Data presentation**

In all cases, a typical representative of at least three independent experiments is presented.

**RESULTS**

**Metaphase I arrest is associated with sustained p34$^{cdc2}$ kinase activity**

In fully competent mouse oocytes, p34$^{cdc2}$ kinase activity is down-regulated concomitantly with the exit from the first metaphase I of meiosis (after ~8 hours maturation) (Choi et al., 1991; Fulka et al., 1992; Hashimoto and Kishimoto, 1988). In order to measure the p34$^{cdc2}$ kinase activity in both partially competent F1 and fully grown LT/Sv MI-arrested oocytes, samples of in vitro-maturing oocytes were taken before (GV-stage), and 3.5 hours after the resumption of meiosis, and then every hour from the 7th to the 13th hour of culture. Only oocytes that did not produce the first polar body were selected for p34$^{cdc2}$ kinase assay. It was determined in preliminary experiments that, compared to the number of oocytes at either metaphase I or II, the proportion at anaphase is low when examining chromosome preparations made at various times between the 8th and 12th hour of maturation. Thus, only a short time period is necessary to produce the first polar body once the exit from metaphase I is initiated (data not shown). Therefore, the absence of the first polar body was used as an indicator of oocytes arrested at metaphase I. As a control, fully grown meiotically competent oocytes from 24-day-old F1 females were matured simultaneously with experimental groups of oocytes in each experiment, and samples of nonselected oocytes were used for p34$^{cdc2}$ kinase assays. Activity in control oocytes reached its highest point by 8 hours of culture when the oocytes were at metaphase I, declined to its lowest point by 11 hours and was elevated once again by 13 hours (Fig. 1) as reported by others (Choi et al., 1991; Fulka et al., 1992; Hashimoto and Kishimoto, 1988). The p34$^{cdc2}$ kinase activity in the LT/Sv oocytes and the partially competent F1 oocytes increased with the same kinetics as the control up to the time of normal metaphase I (Fig. 1A,B). In contrast, there was no decline in p34$^{cdc2}$ kinase activity in the metaphase I-arrested oocytes up to 13 hours of culture. In fact, the level of p34$^{cdc2}$ kinase activity in both partially competent F1 and fully grown LT/Sv metaphase I-arrested oocytes eventually reached levels above the maximum observed in the control oocytes passing through metaphase I (Fig. 1).

**Fig. 1.** Comparison of histone H1 kinase activity in (A) partially and fully competent F1 oocytes, and (B) fully grown LT/Sv oocytes and fully competent F1 oocytes. (A) Oocytes isolated from 18-day-old F1 mice (circles; ~86% partially competent oocytes) and 24-day-old F1 mice (squares; control, ~32% partially competent oocytes) were simultaneously matured in vitro and samples of oocytes were taken at the time points as indicated. Only oocytes without polar bodies were used in the assays of 18-day-old F1. Polar body emission in competent oocytes begins at approximately 10 hours, but these were excluded from the assay of the 18-day-old group since the objective was to determine the histone H1 kinase activity in partially competent oocytes. Oocytes from the 24-day-old group were not selected and were a mixture of partially competent and competent oocytes; ~86% of these oocytes were competent to progress to metaphase II. (B) Oocytes from 24-day-old F1 mice (squares, control) and 24-day-old LT/Sv mice (circles; ~95% partially competent oocytes) were simultaneously matured in vitro and samples of oocytes were taken at the time points as indicated. Only oocytes without polar bodies were used in the assays of LT/Sv oocytes. Histone H1 kinase was measured in crude oocyte lysates and it was expressed as the fold increase over the basal level detected in GV-stage oocytes.
It is possible for p34<sup>cdc2</sup> kinase to retain its activity even after degradation of its regulatory subunit, cyclin B (Lorca et al., 1992; Moreno et al., 1989). Thus, since the data presented above were obtained using crude oocyte lysates, there was a possibility that monomeric p34<sup>cdc2</sup> kinase might be responsible for all or just the certain portion of p34<sup>cdc2</sup> kinase activity maintained beyond its normal point of decline. Therefore, histone H1 kinase activities of immunoprecipitates prepared using anti-p34<sup>cdc2</sup> and anti-cyclin B antibodies were determined. Both antibodies were used in concentrations that recover the maximum precipitable histone H1 kinase activity. At 12 hours of meiotic maturation, the histone H1 kinase activity was equal in the anti-p34<sup>cdc2</sup> and anti-cyclin B precipitates in both groups of metaphase I-arrested oocytes (Fig. 2). The same results were obtained at other times between 8 and 16 hours of maturation (data not shown). Thus, all of the histone H1 kinase activity measured in metaphase I-arrested oocytes represents the activity of p34<sup>cdc2</sup>-cyclin B complex and not monomeric p34<sup>cdc2</sup>.

**Cyclin B degradation occurs concomitantly with the inactivation of p34<sup>cdc2</sup> kinase during the exit from metaphase I in normally maturing oocytes but it is restricted in oocytes arrested at metaphase I**

Although studies using other experimental systems show that cyclin B is degraded in concert with entry into anaphase (Pines, 1991), cyclin B degradation has not yet been demonstrated during the exit from the first metaphase in mouse oocytes. Therefore, to test the hypothesis that the lack of decline of p34<sup>cdc2</sup> activity in metaphase I-arrested oocytes is correlated with a failure to degrade cyclin B, it was first necessary to demonstrate that cyclin B is, in fact, degraded during the normal exit from metaphase I. A pulse-chase labeling strategy was employed to address this question.

In initial experiments, control oocytes from 24-day-old F<sub>1</sub> mice were labeled for 2 hours at different times during the G2 to metaphase I transition with 0.5 µCi/µl of [35S]methionine. This treatment, however, invariably resulted in the failure of the labeled oocytes to produce polar bodies. Since irradiation produces perturbations in the progression of somatic cells through the mitotic cell cycle (Muschel et al., 1993), experiments were conducted to determine the optimal duration of labeling and the amount of radioactivity used for labeling the oocytes that would minimize possible radiation effects on the ability to produce polar bodies. Oocytes labeled for only 15 minutes with 0.25 µCi/µl were able to complete the first meiotic division in 11 to 12 hours as demonstrated by the production of the first polar body (data not shown). This represents only a slight delay in polar body emission compared with unlabeled oocytes. The use of a sensitive phosphor imaging system allowed us to detect the low levels of radioactivity in the immunoprecipitated cyclin B resulting from using this labeling procedure.

Control F<sub>1</sub> oocytes were then labeled using this protocol during the last 15 minutes of the 8th hour of meiotic maturation and samples of 200 oocytes were taken immediately and 1, 2, 3 and 4 hours after the labeling. These time points correspond to 8, 9, 10, 11 and 12 hours of maturation. In the first 4 groups (8, 9, 10, 11 hours), a random population of oocytes was lysed for immunoprecipitation because the oocytes that may actually produce a polar body after further incubation could not be identified, while metaphase I-arrested oocytes and oocytes that progressed beyond this point and produced a polar body were lysed separately at 12 hours. There was no significant decrease in the amount of radiolabeled cyclin B immunoprecipitated unless the oocytes progressed through metaphase I and produced a polar body (Fig. 3A). Thus, cyclin B is degraded in oocytes progressing from metaphase I to anaphase I. There was no loss of radiolabeled cyclin B in oocytes not progressing to metaphase II (Fig. 3A, lane ‘12 MI’), suggesting that degradation of cyclin B is restricted in the metaphase I-arrested oocytes. The same restriction in cyclin B degradation was observed in partially competent metaphase I-arrested oocytes from 18-day-old F<sub>1</sub> mice (data not shown). Likewise,
the metaphase I arrest of LT/Sv oocytes was also associated with restricted cyclin B degradation (Fig. 3B).

**Only a fraction of the total amount of cyclin B undergoes degradation during the metaphase I to anaphase transition**

The data presented above show that cyclin B degradation occurs concomitantly with the exit from metaphase I in mouse oocytes. However, the fate of only a fraction of total amount of cyclin B present in oocyte was followed due to the extremely short labeling period. The extent of cyclin B degradation during the exit from metaphase I was therefore addressed by immunoblotting analysis. Control oocytes were allowed to mature in vitro and samples were taken and prepared for immunoblotting at 8 (metaphase I) and 10 hours. Most of the oocytes had progressed beyond metaphase I at 10 hours, as evident from the presence of the first polar body and from the significant decrease in p34cdc2 kinase activity compared to 8 hours (see Fig. 1). However, even at 10 hours, when the p34cdc2 kinase activity reached its lowest point, the total disappearance of cyclin B was never observed (Fig. 4A). The amount of cyclin B was always between the metaphase I and GV levels, depending on the time and individual experiments (Fig. 4A). Several factors might contribute to this result, including (1) maturational asynchrony between oocytes, (2) degradation of only the specific portion of cyclin B present in oocytes at this stage of meiosis, and (3) a shorter degradation window combined with its continuous synthesis. The synthesis of cyclin B reaches its plateau at about the 3rd hour of meiotic maturation and then remains constant until metaphase I (Hampl and Eppig, 1994). This plateau level of cyclin B synthesis then remains unchanged during the exit from the metaphase I (Fig. 5A). Since the same amount of radiolabeled cyclin B was precipitated with both anti-cyclin B and anti-p34cdc2 antibodies (used at concentrations that recover all precipitable protein), all cyclin B newly synthesized during this period is immediately incorporated into the complexes with the p34cdc2 kinase (Fig. 5A). When protein synthesis (including the synthesis of cyclin B) was prevented from the 8th hour of meiotic maturation by the addition of cycloheximide to the culture medium, oocytes completed the first meiotic division as indicated by the production of polar bodies. Simultaneously, p34cdc2 kinase activity decreased to its basal (GV) level and did not increase again (not shown). Under these conditions, the amount of cyclin B detectable at 10 hours was similar to that present in prophase-arrested oocytes (Fig. 4A, lane “10C”). Thus, without treatment with cycloheximide, the level of cyclin B present in oocytes at 10 hours of maturation is due, at least in part, to the continued cyclin B synthesis simultaneous with cyclin B degradation. However, even in the presence of cycloheximide, the amount of cyclin B does not decrease below that found in GV-stage oocytes. Thus, some cyclin B must be excluded from degradation.

**Cyclin B hyperaccumulates in metaphase I-arrested oocytes**

As shown above, the p34cdc2 kinase activity in metaphase I-arrested oocytes exceeds the normal metaphase I level instead of undergoing down-regulation following 8 hours of meiotic maturation. The hypothesis that this hyperactivation of p34cdc2 kinase is associated with the hyperaccumulation of cyclin B was tested using metaphase I-arrested LT/Sv oocytes. Samples of 300 oocytes, either at the GV-stage, or matured for 8 or 10 hours, were immunoblotted using anti-cyclin B polyclonal serum. As shown in Fig. 4B, the relative amount of cyclin B present in oocytes at 10 hours (lane “10”) was approximately 45% higher than the amount at 8 hours (lane “8”). Cyclin B synthesis, determined by metabolic labeling, continued in metaphase I-arrested LT/Sv oocytes (Fig. 5B). Moreover, the activity of p34cdc2 kinase in metaphase I-arrested LT/Sv oocytes increased 45 ± 7% (n=3) between 8th and 10th hours of maturation. The measurements of cyclin synthesis and p34cdc2 kinase were made using the same groups of oocytes.

**Metaphase I-arrested oocytes differ from metaphase II-arrested oocytes in their sensitivity to 6-DMAP**

These experiments were conducted to determine whether there are differences in the mechanisms that sustain metaphase I and

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**Fig. 4.** (A) The change in relative amounts of cyclin B during the exit from metaphase I in fully grown meiotically competent F1 oocytes. Lysates of 300 oocytes were subjected to SDS-PAGE followed by immunoblotting using anti-cyclin B polyclonal antibody. Lane GV, GV-stage oocytes; lane 8 and 10, oocytes matured for 8 and 10 hours, respectively; lane 10C, oocytes matured for 10 hours in the presence of cycloheximide from 8th hour of maturation. (B) The relative amounts of cyclin B in metaphase II-arrested LT/Sv oocytes. Lysates of 300 oocytes were subjected to SDS-PAGE followed by immunoblotting using anti-cyclin B polyclonal antibody. Lane GV, GV-stage oocytes; lanes 8, 10 and 11, oocytes matured for 8 and 10 and 11 hours, respectively; lane 11D, oocytes treated with 6-DMAP between 10th and 11th hour of maturation. The curve above the blot represents the densitometric scan.
metaphase II arrest. Treatment of mouse oocytes with 6-DMAP, an inhibitor of protein phosphorylation, has different effects on the progression of oocyte maturation, depending upon the stage at which the treatment occurs (Rime et al., 1989; Szöllösi et al., 1993). Treatment of oocytes with 6-DMAP before the time of metaphase I causes chromosome decondensation and the formation of a nucleus, but treatment at metaphase I does not prevent the further progression of meiosis. In contrast, treating metaphase II oocytes with 6-DMAP does not affect metaphase II arrest (Rime et al., 1989; Szöllösi et al., 1993). These results are consistent with the idea that 6-DMAP promotes p34\(^{cdc2}\) inactivation before, but not at metaphase II. Here, we have determined whether metaphase I-arrested oocytes respond to 6-DMAP similarly to normal metaphase II-arrested oocytes. p34\(^{cdc2}\) kinase activity was determined in metaphase I-arrested, partially competent F1 oocytes and LT/Sv oocytes and metaphase II-arrested F1 oocytes before and 1 hour after treatment with 5 mM 6-DMAP. Although metaphase II-arrested oocytes were refractory to 6-DMAP as expected, treatment of metaphase I-arrested oocytes with 6-DMAP invariably led to the rapid inactivation of p34\(^{cdc2}\) kinase (Fig. 6). The experiment depicted in Fig. 6 used metaphase I-arrested oocytes cultured for 14 hours, but the same result occurred when the oocytes were cultured for different times from 10 to 16 hours as well as those that matured in vivo (data not shown). In addition, when oocytes were treated with 6-DMAP at 8 hours of culture, the time of normal metaphase I, both a decline of p34\(^{cdc2}\) kinase activity and entry into anaphase within 1 hour of treatment were observed (data not shown). Thus, metaphase I and metaphase I-arrested oocytes respond differently to 6-DMAP than normal metaphase II-arrested oocytes.

The effect of treating oocytes with 6-DMAP on the stability of cyclin B was determined by metabolically labeling the oocytes with \(^{35}\text{S}\)methionine and treating them with 6-DMAP

Fig. 5. (A) The synthesis of cyclin B during the exit from metaphase I in fully grown meiotically competent F1 oocytes. Maturing oocytes were labeled for 60 minutes during 6th, 8th and 10th hours (lanes 5-6, 7-8, 9-10), immediately lysed and lysates were divided into 2 equal parts and immunoprecipitated using anti-cyclin B (p34) and anti-p34\(^{cdc2}\) (cycB) antibodies, respectively. Thus, the ‘cycB’ lanes represent directly precipitated cyclin B and the ‘p34’ lanes show cyclin B coprecipitated by anti-p34\(^{cdc2}\) antibodies. (B) The synthesis of cyclin B in metaphase I-arrested LT/Sv oocytes. Oocytes were labeled for 60 minutes during 8th, 10th, 12th and 14th hours after the resumption of meiotic maturation (lanes 7-8, 9-10, 11-12, 13-14), immediately lysed and lysates were subjected to immunoprecipitation using anti-cyclin B antibody.

Fig. 6. The differential effect of 6-DMAP on the histone H1 kinase activity in metaphase I- and II-arrested oocytes. Metaphase I-arrested F1 and LT/Sv oocytes and metaphase II-arrested F1 oocytes were treated with 5 mM 6-DMAP at 14 hours after resumption of meiosis. Histone H1 kinase was determined using crude lysates prepared from oocytes of all three groups both before (solid bars) and 1 hour after treatment with 6-DMAP (empty bars) and it is expressed as the fold increase over the basal level detected in GV-stage oocytes.
for 1 hour as above. The amount of radiolabeled cyclin B before and after the treatment was determined by immunoprecipitation using anti-cyclin B antibody. As documented in Fig. 7A,B, the treatment of both F1 and LT/Sv metaphase I-arrested oocytes with 6-DMAP led to the disappearance of all radiolabeled cyclin B. The relative amount of cyclin decreased to the level of GV-stage oocytes (Fig. 4B, lane ‘11D’). In contrast, no significant change in the amount of radiolabeled cyclin B was observed in metaphase II-arrested F1 oocytes subjected to the same 6-DMAP treatment (Fig. 7C) as well as in all nontreated controls.

**DISCUSSION**

These studies demonstrated that metaphase I arrest in both partially competent growing (F1) oocytes and in fully grown LT/Sv oocytes is correlated with a sustained elevation of p34\(^{cdcl2}\) kinase activity. In fact, p34\(^{cdcl2}\) kinase activity continued to increase during the time when activity normally decreased. Although the decline in p34\(^{cdcl2}\) kinase activity correlated with the exit from metaphase I in mouse oocytes is now well described (Choi et al., 1991; Fulka et al., 1992; Hampel and Eppig, 1994; Hashimoto and Kishimoto, 1988), the degradation of cyclin B at this time had not been demonstrated in mammalian oocytes. We show here that degradation of cyclin B is indeed correlated with progression beyond metaphase I. In sharp contrast, the amount of cyclin B present in the metaphase I-arrested oocytes continued to increase at the time when it was being degraded in normal oocytes progressing to metaphase II. These results suggest that the progression of meiosis in partially competent, growing (F1) oocytes and in fully grown LT/Sv oocytes is arrested at metaphase I by continued p34\(^{cdcl2}\) kinase activity sustained, at least in part, by restricted degradation of cyclin B. Finally, metaphase I arrest in these oocytes is sustained by a mechanism different from that maintaining metaphase II arrest, since an inhibitor of protein phosphorylation, 6-DMAP, induces a dramatic decline in p34\(^{cdcl2}\) kinase activity and the resumption of meiosis in metaphase I-arrested oocytes but not in metaphase II-arrested oocytes. In addition, cyclin B was more stable in metaphase I-arrested oocytes than in metaphase II-arrested oocytes.

Cyclin B degradation occurred coincidentally with the inactivation of p34\(^{cdcl2}\) kinase during the normal exit from metaphase I in mouse oocytes. Nevertheless, even when the p34\(^{cdcl2}\) kinase activity declined to its lowest point between the meiotic metaphases, a significant amount of cyclin B was still detected in oocytes matured for 10 hours. Cyclin B was continuously synthesized during the exit from metaphase I, in a manner similar to that described by Hunt et al. (1992) in clam embryos. But when the continuous synthesis of cyclin B was prevented by treatment of the oocytes with cycloheximide, the lowest level of cyclin B reached was approximately the same as that detected in GV-stage oocytes (Fig. 4A). These results, together with the finding that there is only a partial destruction of cyclin B during the exit from metaphase I in frog oocytes (Kobayashi et al., 1991), indicate that a substantial pool of cyclin B survives the metaphase I to metaphase II transition.

The inactivation of p34\(^{cdcl2}\) kinase is normally coupled to the degradation of cyclins in both mitosis and meiosis. Preventing the destruction of cyclin B with a truncated nondegradable mutant form of cyclin B results in the inability to inactivate p34\(^{cdcl2}\) kinase (Gallant and Nigg, 1992; Luca et al., 1991; Murray et al., 1989). It was, therefore, reasonable to hypothesize that the failure of partially competent oocytes, or LT/Sv oocytes, to exit metaphase I was due to a restriction of cyclin B degradation (Wickramasinghe et al., 1991). Unlike the disappearance of radiolabeled cyclin B observed in normally maturing oocytes, there was no disappearance of radiolabeled cyclin B detected in either group of metaphase I-arrested oocytes. Moreover, as shown in LT/Sv oocytes, the total amount of cyclin B actually increased about 45% above the metaphase I level (8 hours). This increase was probably the net result of the lack of cyclin B degradation combined with its persistent synthesis. Consistent with our findings, the introduction of new cyclin proteins into oocytes results in activation of p34\(^{cdcl2}\) kinase to the level that exceeds the level detectable in normal metaphase (Solomon et al., 1990). The hyperactivation of p34\(^{cdcl2}\) kinase that coincides with the hyperaccumulation of cyclin B shown here most likely causes the spontaneous arrest of the cell cycle at metaphase I in mouse oocytes.

Although the data here demonstrate a restriction of cyclin B degradation in metaphase I-arrested oocytes, it is possible that there are other factors, not necessarily the same in the two types of oocytes arrested at metaphase I, that underlie the failure to enter anaphase. Thus, it is possible that the failure to enter anaphase does not reflect a deficiency of the p34\(^{cdcl2}\) kinase inactivating system per se, but rather a deficiency in an upstream event or system that is recognized at a metaphase I checkpoint and consequently the triggering of p34\(^{cdcl2}\) kinase inactivating system is prevented.

The mechanism(s) for sustaining metaphase I arrest in partially competent (F1) oocytes and in fully grown LT/Sv oocytes are different from those that sustain metaphase II arrest. This conclusion is based upon the differential response of metaphase I- and II-arrested oocytes to treatment with an inhibitor of protein phosphorylation, 6-DMAP. In metaphase I-arrested oocytes, this inhibitor rapidly induced cyclin B degradation and a dramatic decrease in p34\(^{cdcl2}\) kinase activity. In contrast, the inhibitor did not have these effects on metaphase II-arrested oocytes in agreement with the observations of others (Rime et al., 1989; Szöllösi et al., 1993). The difference in sensitivity between metaphase I- and metaphase II-arrested mouse oocytes to 6-DMAP is not due to the culture conditions since ovulated oocytes arrested at the metaphase I or II displayed a similar sensitivity to 6-DMAP. The possibility of differential uptake of 6-DMAP into metaphase I- and metaphase II-arrested oocytes cannot be completely excluded, but this inhibitor affects the organization of the metaphase II spindle in about 50% of the metaphase II oocytes (Rime et al., 1989), induces the formation of microtubule asters and decreases phosphate incorporation into phosphoproteins (Szöllösi et al. 1993).

Treatment of metaphase I-arrested oocytes with 6-DMAP resulted in cyclin B degradation. In contrast, there was no detectable degradation of cyclin B in 6-DMAP treated metaphase II oocytes. Thus, when metaphase I-arrested oocytes were treated with 6-DMAP there was a loss of cyclin B stability. The opposite was observed in oocytes not treated with 6-DMAP; there was greater stability of cyclin B in the metaphase I-arrested oocytes than in oocytes arrested at metaphase II. Synthesis and degradation of cyclin B take place simul-
taneously in mouse oocytes arrested at metaphase II, and a steady level responsible for the maintenance of the constant p34cdc2 kinase activity is established (Kubiak et al., 1993). We have confirmed this turnover at metaphase II using our experimental conditions (data not shown). In contrast, as discussed above, there was a restricted degradation of cyclin B in metaphase I-arrested oocytes that resulted in cyclin B hyperaccumulation. These differences in the stability of cyclin B in either metaphase I or II arrest, or upon treatment with 6-DMAP distinguish the mechanism(s) involved in metaphase I from metaphase II arrest.

Despite these differences in the mechanism(s) sustaining metaphase I and II arrest, there is a common response of both groups of oocytes to insemination. Partially competent F1 oocytes arrested at metaphase I respond to insemination by producing a polar body, followed by pronuclear formation and preimplantation embryo development. Most of these embryos are triploid (Eppig et al., 1994). Similarly, triploid embryos are produced as a result of insemination of metaphase I-arrested LT/Sv oocytes (Kaufman and Speirs, 1987). In addition, both metaphase I- and II-arrested oocytes are activated by treatment with calcium ionophore A23187 (Eppig et al., 1994). Sperm penetration initiates the cascade of events that lowers MPF activity and promotes entry of metaphase II-arrested oocytes into anaphase II (Hashimoto and Kishimoto, 1988; Weber et al., 1991) and it appears that sperm penetration and calcium ionophore treatment have the same effect on metaphase I-arrested oocytes. Most likely, the anaphase I and anaphase II triggers are not the same, but the triggers for entry into anaphase II are sufficient to launch entry into anaphase I. This implies that anaphase II-initiating mechanisms, such as the inositol trisphosphate-induced calcium release mechanisms (Ducibella et al., 1993; Fujiiwara et al., 1993; Fulton and Whittingham, 1978), can develop in partially competent oocytes that are incapable of producing the systems sufficient to drive normal entry into anaphase I and that this anaphase II-initiating system can also initiate anaphase I. Thus, although there are clearly differences between the metaphase I- and II-arresting mechanisms, there must also be much in common between them.

In summary, this study shows, using two model systems of oocytes that fail to progress beyond the first metaphase, that metaphase I arrest is associated with the lack of p34cdc2 kinase inactivation and a restriction of the degradation of cyclin B that is normally observed during the maturation of oocytes that are competent to complete meiotic maturation. In addition, the results presented here show that some aspects of the mechanisms that arrest the progression of maturation at metaphase I and II are different because of their differing response to an inhibitor of protein phosphorylation, 6-DMAP, and the differences in cyclin B turnover.

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