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Activation of the Mitogen-activated Protein Kinase ERK1 during Meiotic Progression of Mouse Pachytene Spermatocytes*

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Okadaic acid (OA) causes meiotic progression and chromosome condensation in cultured pachytene spermatocytes and an increase in maturation promoting factor (cyclin B1/cdc2 kinase) activity, as evaluated by H1 phosphorylative activity in anti-cyclin B1 immunoprecipitates. OA also induces a strong increase of phosphorylative activity toward the mitogen-activated protein kinase substrate myelin basic protein (MBP). Immunoprecipitation experiments with anti-extracellular signal-regulated kinase 1 (ERK1) or anti-ERK2 antibodies followed by MBP kinase assays, and direct in-gel kinase assays for MBP, show that p44/ERK1 but not p42/ERK2 is stimulated in OA-treated spermatocytes. OA treatment stimulates phosphorylation of ERK1, but not of ERK2, on a tyrosine residue involved in activation of the enzyme. ERK1 immunoprecipitated from extracts of OA-stimulated spermatocytes induces a stimulation of H1 kinase activity in extracts from control pachytene spermatocytes, whereas immunoprecipitated ERK2 is uneffective. We also show that natural G_2/M transition in spermatocytes is associated to intracellular redistribution of ERKs, and their association with microtubules of the metaphase spindle. Preincubation of cultured pachytene spermatocytes with PD98059 (a selective inhibitor of ERK-activating kinases MEK1/2) completely blocks the ability of OA to induce chromosome condensation and progression to meiotic metaphases. These results suggest that ERK1 is specifically activated during G_2/M transition in mouse spermatocytes, that it contributes to the mechanisms of maturation promoting factor activation, and that it is essential for chromosome condensation associated with progression to meiotic metaphases.

Male meiosis is a process in which a dyploid spermatocyte gives rise to four haploid round spermatids after a single round of DNA replication followed by two subsequent cell divisions. The homologous chromosomes are segregated in the two daughter cells (secondary spermatocytes) during the first meiotic division, whereas the sister chromatids, through a process resembling mitosis, are segregated during the second division to generate haploid cells. The prophase of male first meiotic division is a lengthy process which ensures the correct pairing and the crossing over between homologous chromosomes. In the mouse, this process lasts approximately 10 days, during which the homologous chromosomes become partially condensed, anneal to each other, and are maintained in proximity by a structure called the synaptonemal complex. After genetic exchange (crossing over) has occurred, the synaptonemal complex disappears and chromosome condensation allows the sites of DNA exchange to become visible as chiasmata. As for mitotic division, the nuclear envelope breaks down and microtubules are assembled into a spindle. The chromosomes become aligned to the equator of the meiotic spindle and the two sets of homologous chromosomes are separated during the anaphase. While the meiotic prophase is considerably long, metaphase and anaphase transitions are rather short. Due to the difficulty of isolating homogenous populations of synchronized cells, and the length of meiosis I in spermatocytes, not much is known about the molecular mechanisms controlling the different steps of mammalian male meiosis (for review, see Ref. 1).

Most of the information about cell cycle regulation during meiosis in metazoan cells has been obtained using *Xenopus laevis* oocytes naturally arrested in late G_2 of the first meiotic division. Induction of G_2/M progression by progesterone requires the expression of the proto-oncogene *mos* (2–4), activation of the mitogen-activated protein kinase (MAPK)[1] cascade, and finally activation of the cyclin B·cdc2 complex, also known as mitosis-promoting factor, or maturation-promoting factor (MPF, for review, see Ref. 5). Microinjection of antibodies blocking MAPK kinase are able to block MAPK activation triggered by activated Mos, and to block *Xenopus* oocyte maturation (6) and Mos cytostatic activity at metaphase (7). Furthermore, a dominant negative p42 extracellular signal-regulated kinase 2 (ERK2) is able to block Mos-induced MPF activation in *Xenopus* oocyte extracts (8). Finally, it has been reported that activation of MPF and meiotic progression by progesterone could be bypassed by injection of constitutively active forms of MAPKs (9–10). Although these observations strongly suggest a role for MAPKs in meiotic progression and regulation of MPF activity in *Xenopus* oocytes, the mechanism of this regulation is still unknown. The activity of MPF is regulated at multiple steps, such as cyclin B synthesis, phosphorylation/dephosphorylation events (11, 12), and nuclear export (13, 14). During G_2, the cyclin B·cdc2 complex accumulates in the oocyte, but it is

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[1] The abbreviations ued are: MAPK, mitogen-activated protein kinase; MPF, maturation promoting factor; ERK2, extracellular signal-regulated kinase 2; OA, okadaic acid; PBS, phosphate-buffered saline; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PP-2A, serine-threonine phosphatase 2A; Mops, 4-morpholinepropanesulfonic acid.

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kept inactive through phosphorylation of Thr^{14} and/or Tyr^{15} by the wee1 kinase(s). Inactivation of wee1 kinases and/or activation of the activating phosphatase cdc25 trigger dephosphorylation of Thr^{14} and Tyr^{15} and cause activation of MPF (11, 15). Recently, a possible link between activation of MAPKs and MPF has been proposed on the base of the finding that MAPK-activated $p90^{rsk}$ binds to and phosphorylates the wee1 homologue Myt1 (16). This phosphorylation inhibits Myt1 activity and interferes with inhibition of MPF activity *in vitro*.

In the mouse oocytes, meiotic resumption is under the negative control of cAMP (1). When cAMP levels drop there is an increase in the activity of MPF and MAPKs resulting in the germinal vesicle breakdown and progression through the first meiotic division. On the contrary of what has been observed in *Xenopus* oocyte maturation, activity of Mos is dispensable for the G_2/M transition after prophase I in both female and male mice (17, 18), even though Mos might participate in some aspects of meiotic maturation in mouse oocytes (19, 20). The oocyte proceeds to the second meiotic division and arrests at metaphase II with the sister chromatids aligned on the spindle, due to the concerted action of Mos and MAPKs, which, at this stage, prevent cyclin destruction and consequent MPF inactivation (5). Indeed, Mos-knock out animals showed lack of MII arrest and high rate of parthenogenetic activation of ovulated oocytes (17, 18).

A similar synchronization of events can be artificially reproduced in mouse spermatocytes by treatment with the serine/threonine phosphatase inhibitor okadaic acid (OA) (21–23). OA overcomes the checkpoints that normally delay the progression of the meiotic cycle of mid- and late pachytene spermatocytes and induces nuclear envelope breakdown and chromosome condensation resembling that observed during G_2/M transition after prophase I. The meiotic progression of mouse spermatocytes induced by OA is accompanied by an increase in H1 kinase activity, which is considered a sign of MPF activation (22). The increase in H1 kinase activity during OA-induced G_2/M transition in mouse spermatocytes is due to a concurrent activation of a cyclin/cdk activity detectable in precipitates using $p13^{suc1}$-conjugated agarose (23), suggesting that activation of MPF actually occurs under these experimental conditions.

It is not known whether, in addition to MPF, Mos and MAPKs are also required for G_2/M progression during meiosis in mouse spermatocytes. Targeted gene disruption of Mos did not produce a clear phenotype in the male mice, and did not result in sterile animals. However, it cannot be ruled out that, in the absence of Mos, redundant mechanisms of MAPK activation, *e.g.* via Raf-1 and/or MEKKs (24), substitute for its function. In this study we have examined the activity of MPF and MAPKs during meiotic progression artificially induced in mouse spermatocytes by OA treatment. We found that OA induces a sustained activation of both MAPKs and MPF with similar time courses. OA induces phosphorylation and activation of p44/ERK1, but not of p42/ERK2. Immunoprecipitated p44-ERK1 from OA-stimulated male meiotic germ cells was able to induce MPF activation in extracts from control spermatocytes *in vitro*. Preincubation of cultured pachytene spermatocytes with PD98059 (a selective inhibitor of ERK activating kinases MEK1/2) completely blocked the ability of OA to induce chromosome condensation and progression to meiotic metaphases. Our results suggest an important function for ERK1/MAPK in male mouse meiotic progression, as also indicated by changes in its subcellular distribution during naturally occurring G_2/M transition.

MATERIALS AND METHODS

Preparation of Testicular Cells—Testes of adult CD1 mice (Charles River Italia) were used to prepare germ cells. After dissection of the albuginea membrane, testes were digested for 15 min in 0.25% (w/v) collagenase (type IX, Sigma) at room temperature under constant shaking. Digestion was followed by two washes in minimum essential medium (Life Technologies, Inc.), hence seminiferous tubules were cut in pieces using a sterile blade and further digested in minimum essential medium containing 1 mg/ml trypsin for 30 min at 30 °C. Digestion was stopped by adding 10% fetal calf serum and the released germ cells were collected after sedimentation (10 min at room temperature) of tissue debris. Germ cells were centrifuged for 10 min at 1,500 rpm at 4 °C and the pellet resuspended in 20 ml of elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4(7H_2O)$, 1.3 mM $CaCl_2$, 11 mM glucose, 1 × essential amino acid (Life Technologies, Inc.), penicillin, streptomycin, 0.5% bovine serum albumin). Germ cells at pachytene spermatocyte, round spermatid, and elongated spermatid steps were obtained by elutriation of the unfractionated single cell suspension as described previously (25). Homogeneity of cell populations ranged between 80 and 85% (pachytene spermatocytes) and 95% (round spermatids), and was routinely monitored morphologically. Mature spermatozoa were obtained from mature mice as described previously (26). Spermatogonia (27) and Sertoli cells (27, 28) were obtained from prepuberal mice as previously described.

Cell Culture and Treatments—After the elutriation, pachytene spermatocytes were cultured in minimum essential medium, supplemented with 0.5% bovine serum albumin, 1 mM sodium pyruvate, 2 mM sodium lactate, in 6-well dishes at a density of 10^6 cells/ml at 32 °C in a humified atmosphere containing 95% air and 5% CO_2. After 12 h, cells were treated with 0.5 μM OA, or 5 μM OA, or equal volumes of the solvent dimethyl sulfoxide, and culture was continued for up to 6 h. For time course experiments, aliquots were taken at different time points and processed as described below. In order to test the effect of inhibition of ERK-activating kinases, before OA treatments, cells were preincubated for 12 h with the specific inhibitor of MEK1/2 kinases PD98059 (Calbiochem, catalog number 513000) at the concentration of 50 μM, or equal volumes of the solvent dimethyl sulfoxide. For cytological and immunofluorescence analyses, and kinase assays, aliquots of the same samples were taken and processed accordingly.

Staining of Spermatocyte Nuclei with Giemsa—10^6 cells were collected by centrifugation at 1,000 × *g* for 15 min at 4 °C, pellets were resuspended in 4 ml of KCl, 75 mM hypotonic solution and incubated for 15–20 min at 37 °C. Cell lysates were fixed by adding 0.5 ml of methanol:acetic acid solution (3:1) and incubated at 4 °C for 1 h. After a 10-min centrifugation at 1,000 × *g*, pellets were washed 4 times with 4 ml of methanol:acetic acid solution (3:1), with a 10-min incubation at room temperature between each wash. After the last wash, pellets were resuspended in 200–500 μl of methanol:acetic acid solution (3:1) and the solution was dropped from 10 to 15 cm onto glass slides to allow spreading of the nuclei. Slides were stained in 5% Giemsa dye dissolved in 0.15 mM NaH_2PO_4 containing 3% methanol. Slides were washed with 0.15 mM NaH_2PO_4 containing 3% methanol, allowed to dry and mounted with coverslide. Nuclei were observed by light microscopy.

Immunofluorescence Analysis—Control or OA-treated spermatocytes were spotted on poly-L-lysine-coated glass slides and fixed at room temperature for 15 min in PBS containing 4% paraformaldheyde. Cells were then permeabilized for 5 min in PBS containing 0.1% Triton X-100 and incubated for 30 min at room temperature with 1% fetal calf serum in PBS. After 3 washes in PBS, cells were incubated for 1 h at 37 °C with rabbit polyclonal anti-ERK1 (Santa Cruz Biotechnology, catalog number sc-93-G) and mouse monoclonal anti-β-tubulin (Sigma, catalog number T4026), both at a final concentration of 1 μg/ml, as primary antibodies. Following 5 washes (10 min in PBS), cells were incubated for 1 h at 37 °C with cyanin 3-conjugated anti-rabbit IgGs (Chemicon, catalog number AP132C, 1:400 dilution) and fluoresceinated anti-mouse IgGs (Sigma, catalog number T4026, 1:200 dilution) as secondary antibodies. To stain DNA, 0.1 mg/ml Hoechst dye (Sigma) was added to the solutions containing the secondary antibodies. Control experiments were performed using either rabbit or mouse non-immune IgGs in the first incubation, or the secondary antibodies alone. After 5 more washes in PBS, slides were mounted in 50% glycerol in PBS and immediately examined by fluorescence microscopy.

H1 Kinase Assay—Approximately 2 × 10^5 cells were collected by centrifugation at 2,000 × *g* for 10 min, resuspended in 10 μl of storage solution (10 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 5 mM EGTA, 10 μg/ml leupeptin, and 10

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μg/ml aprotinin) and immediately frozen at −80 °C. Before the assay, cells were thawed on ice and lysed for 10 min on ice in 50 μl of hypotonic H1 kinase buffer (25 mM Mops, pH 7.5, 60 mM β-glycerophosphate, 15 mM EGTA, 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell extracts were centrifuged for 10 min at 10,000 × *g* at 4 °C, and soluble extracts were collected and used for H1 kinase assays. Kinase reactions using 10–15 μl of soluble extracts were carried out for 60 min at 30 °C in a total volume of 25 μl in H1 kinase buffer containing 100 μg/ml H1 (type III-S; Sigma), 1 μg/ml cAMP-dependent protein kinase inhibitor (Sigma), and 0.3 mM [γ-^{32}P]ATP (0.1 μCi/μl). Reactions were stopped spotting 20 μl onto P81 paper squares (Whatman) and immediately immersing them into 0.1% phosphoric acid. Paper squares were washed 5 times for 10 min and air dried. Radioactivity incorporated into H1 was determined by scintillation counting. Values were normalized for protein content, determined according to Bradford (29).

Myelin Basic Protein (MBP) Kinase Assay—Samples were collected and frozen as described for the H1 kinase assay. Cells were thawed on ice and lysed for 10 min on ice in 50 μl of MBP kinase buffer (20 mM Hepes, pH 7.4, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 2 mM EGTA, 20 mM $MgCl_2$, 5 μg/ml leupeptin, 5 μg/ml aprotinin). Reactions were carried out for 30 min at 30 °C in 25 μl of MBP kinase buffer containing 0.3 mM [γ-^{32}P]ATP (0.2 μCi/μl) (Amersham Pharmacia Biotech) and 0.5 mM MBP-derived peptide (Santa Cruz Biotechnology, catalog number sc-3011) as a specific substrate for MAPKs. Reactions were stopped by adding 1 volume of 20% trichloroacetic acid, and proteins were allowed to precipitate on ice for 10 min using 2 mg/ml bovine serum albumin as carrier. After centrifugation at 8,000 × *g* for 10 min, aliquots of the supernatant fractions were spotted onto P81 phosphocellulose paper and processed as described for the H1 kinase assay (26).

Immunoprecipitation and Immunokinase Assays—Control or OA-treated spermatocytes (approximately 2 × 10^6 cell/sample) were collected by centrifugation at 2,000 × *g* for 10 min, and washed twice in ice-cold PBS. Cells were homogenized in either H1 kinase buffer or MBP kinase buffer and cytosolic fractions were collected after centrifugation at 10,000 × *g* for 10 min at 4 °C. For immunoprecipitation, 1 μg of mouse monoclonal anti-cyclin B1 antibody (Santa Cruz Biotechnology, catalog number sc-245) or rabbit polyclonal anti-ERK1 (Santa Cruz Biotechnology, catalog number sc-93-G) or anti-ERK2 antibody (Santa Cruz Biotechnology, catalog number sc-154-G) were preincubated for 60 min with a mixture of protein A- and protein G-Sepharose beads (Sigma) under constant shaking at 4 °C. At the end of the incubation, the beads were washed once with 20 mM Tris-HCl, pH 7.8, containing 0.5 M NaCl, twice with 20 mM Tris-HCl, pH 7.8, and then incubated for 90 min at 4 °C with the soluble spermatocyte cell extracts (0.5 mg of protein) under constant shaking. Sepharose bead-bound immunocomplexes were rinsed three times with PBS containing 0.05% bovine serum albumin, and twice with either H1 kinase buffer or MBP kinase buffer. Anti-cyclin B1 pellets were then incubated with 1 μg of H1, 1 μg of cAMP-dependent protein kinase inhibitor, and 0.3 mM [γ-^{32}P]ATP (0.1 μCi/μl) in H1 kinase buffer (total volume 30 μl) for 30 min at 30 °C. Anti-ERKs pellets were incubated with either 0.5 mM MBP-derived peptide or 1 μg of full-length MBP (Sigma, catalog number M1891) and 0.3 mM [γ-^{32}P]ATP (0.1 μCi/μl) in MBP kinase buffer (total volume 30 μl) for 30 min at 30 °C. Samples incubated with the MBP-derived peptide were processed as described in the paragraph describing the MBP kinase assay. As for the samples incubated with H1 or with the full-length MBP protein, at the end of the incubation, pellets were separated by centrifugation (3,000 × *g* for 5 min) and the supernatants were diluted in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (w/v) bromphenol blue). Radioactivity incorporated into H1 or MBP was analyzed by autoradiography after separation of the proteins on SDS-PAGE gel. In some experiments, immunoprecipitation pellets were washed two additional times with PBS, immunocomplexes were then eluted in SDS-PAGE sample buffer for Western blot analysis.

Kinase Assays in MBP-containing SDS-PAGE Gels—Control or OA-treated spermatocytes were collected as described above for MBP kinase assays. Soluble extracts were diluted in SDS-PAGE sample buffer and separated on polyacrylamide gels containing 0.1 mg/ml full-length MBP. After the run, SDS was removed by washing the gel twice in 20% 2-propanol in 50 mM Tris-HCl, pH 8.0, for 1 h at room temperature. Proteins were then denatured by incubation of the gel in 6 M guanidine HCl at room temperature for 1 h, and renatured overnight with five washes in 50 mM Tris-HCl, pH 8.0, containing 0.04% Tween 20 and 5 mM 2-mercaptoethanol. The gel was then preincubated for 1 h at 25 °C in 50 mM Hepes, pH 8.0, containing 2 mM dithiothreitol and 10 mM $MgCl_2$. Phosphorylation of MBP was carried on for 1 h at 25 °C in 10 ml of preincubation buffer adding 0.5 mM EGTA, 2 μM cAMP-dependent protein kinase inhibitor, 40 μM ATP, and 100 μCi of [γ-^{32}P]ATP. The reaction was stopped by immersing the gel in 5% trichloroacetic acid solution containing 10 mM sodium pyrophosphate. The gel was washed for 5–10 times in the same solution, dried, and subjected to autoradiography.

Activation of MPF by Immunoprecipitated Activated ERK1—Spermatocyte cell extracts from control or OA-treated cells were immunoprecipitated with either anti-ERK1 antibody or anti-ERK2 antibody essentially as described for the MBP-immunokinase assay. After the washes with MBP kinase buffer, immunocomplexes were incubated for 30 min with a soluble cell extracts from untreated spermatocytes (approximately 0.2 mg/sample) at 30 °C under constant shaking. At the end of the incubation, soluble cell extracts and immunocomplexes were separated by centrifugation at 3,000 × *g* for 5 min, and H1 kinase activity was measured in the supernatant fractions as described above.

Western Blot Analyses—Spermatocyte cell extracts were separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech) and subjected to Western blot analysis with anti-ERK1 (100 ng/ml) (Santa Cruz Biotechnology, catalog number sc-93-G), anti-ERK2 (100 ng/ml) (Santa Cruz Biotechnology, catalog number sc-154-G), or PanERK (300 ng/ml) (Transduction Laboratories, catalog number E17120) rabbit polyclonal antibodies, or the mouse monoclonal antibody anti-p-ERK (400 ng/ml) (Santa Cruz Biotechnology, sc-7383). The first antibody incubation was carried on for 90 min at room temperature. Second antibody incubation was carried out with either 1:10,000 dilution of anti-rabbit-IgGs antibody, or with 1:5,000 dilution of anti-mouse IgGs antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Immunostained bands were detected by the ECL chemiluminescent method (Amersham Pharmacia Biotech). The anti-p-ERK monoclonal antibody specifically recognizes tyrosine-phosphorylated active ERKs, and we tested its specificity by treatment of cell extracts with calf intestine alkaline phosphatase, which completely suppressed recognition of the relative bands, unless calf intestine alkaline phosphatase treatment was performed in the presence of phosphatase inhibitors (data not shown).

RESULTS

Activation of Cyclin B1/cdc2 (MPF) during the OA-induced G_2/M Transition in Mouse Spermatocytes—It has been previously reported that treatment of mouse spermatocytes with the serine/threonine phosphatase inhibitor OA, in the presence of 5% fetal calf serum, is able to induce G_2/M progression in approximately 90% of the cells within 4–6 h (21, 22). OA triggered nuclear envelope breakdown and chromosome condensation and this phenomenon was accompanied by a 2–4-fold increase in H1 kinase activity (22, 23), which was interpreted to reflect activation of MPF, since it was also detected in $p13^{suc1}$-agarose precipitates (23). In our experimental conditions, treatment with 5 μM OA in the absence of fetal calf serum induced a dramatic chromatin condensation which was observed in about 90% of the cells (Fig. 1*A, right side*), suggesting that serum is not required for G_2/M transition induced by OA treatment. Activation of H1 kinase activity was accompanied by a rapid (2-fold activation after 1 h) and sustained increase in H1 kinase activity that reached a maximum (5-fold) between 4 and 6 h (Fig. 1*B*). To investigate whether the increase in H1 kinase activity was indeed due to activation of MPF, we immunoprecipitated MPF activity from control and treated cells using a monoclonal antibody that recognizes cyclin B1 (see "Materials and Methods"), the major cyclin component of MPF in these cells (30). Immunoprecipitates from control and OA-treated cells were incubated with purified H1 and [γ-^{32}P]ATP, and the soluble fractions were then analyzed by SDS-PAGE and autoradiography for phosphorylation of the H1 substrate (Fig. 1*C*). Densitometric analysis indicates that 4-h treatment of spermatocytes with OA induced a 4-fold increase in cyclin B1/cdc2 kinase activity (Fig. 1*D*), demonstrating that the increase in H1 kinase activity observed in the cytosolic extracts (Fig. 1*B*) is actually due to activation of MPF.

ERK Kinases Expression in Testicular Cells—To investigate

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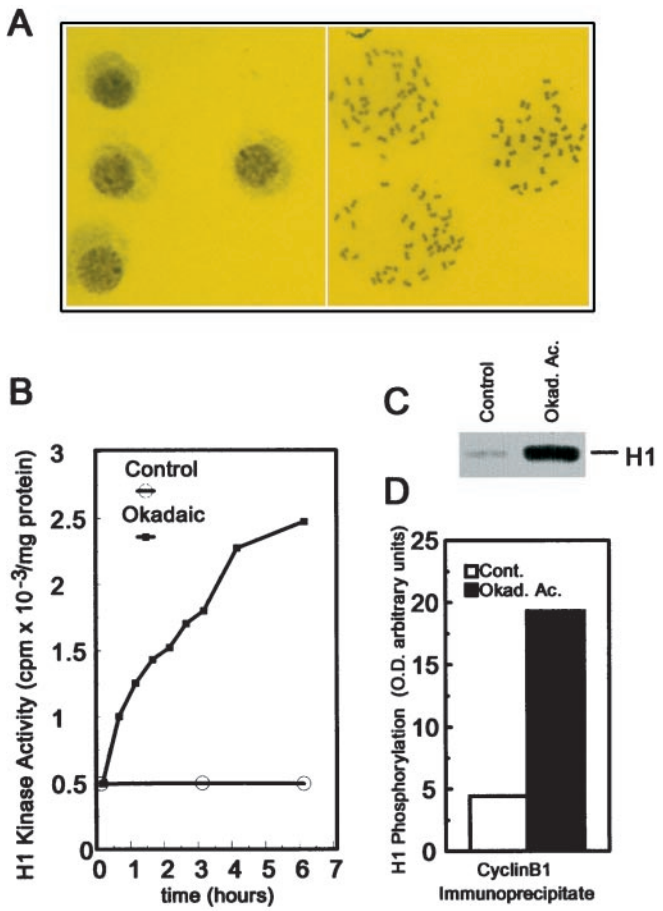

FIG. 1. **Activation of cyclin B1/cdc2 (MPF) during the OA-induced G_2/M transition in mouse spermatocytes.** *A*, Giemsa staining of nuclei from control spermatocytes (*left side*) and spermatocytes treated for 4 h with 5 μM OA (*right side*). Magnification: × 420. *B,* H1 kinase assay on cell extracts from middle-late pachytene mouse spermatocytes treated with 5 μM OA for the indicated times. *C,* phosphorylation of H1 by anti-cyclin B1 immunoprecipitates from control and OA-treated spermatocytes. Immunoprecipitated cyclin B1·cdc2 complexes from 2×10^6 cells were assayed for kinase activity using H1 as substrate. After separation by centrifugation and several washes, immunocomplexes were resuspended in H1 kinase reaction buffer and incubated under constant shaking for 30 min at 30 °C. After separation by centrifugation, the soluble fraction was analyzed by SDS-PAGE and autoradiography. This experiment was repeated three times with similar results. *D,* densitometric analysis of the bands shown in *C* to evaluate the degree of H1 phosphorylation by MPF in control and OA-treated spermatocytes.

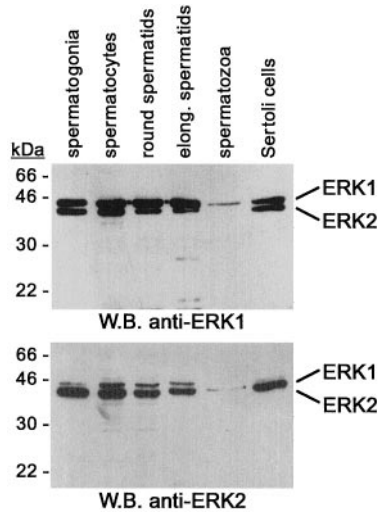

FIG. 2. **ERK kinase expression in mouse testicular cells.** Immunoblot analysis of protein extracts (50 μg) from the indicated cell types using affinity purified anti-ERK1 or anti-ERK2 polyclonal antibodies.

the role of MAPKs in the male meiosis, we analyzed the expression of ERK1 and ERK2 in germ cells and somatic cells of the testis by Western blot analysis. Spermatogonia were isolated from 8-day-old mice, Sertoli cells were isolated from 17-day-old mice, and spermatocytes, round and elongated spermatids, and spermatozoa were isolated from adult mice (see "Materials and Methods"). As shown in Fig. 2, both ERK1 and ERK2 were expressed in all the cells examined. The anti-ERK1 antibody recognized two major bands in cytosolic extracts, a 44-kDa band which corresponds to ERK1 and a 42-kDa band that corresponds to ERK2. Although this antibody recognized both MAPK isoforms, it showed some specificity toward ERK1, as shown by the staining of only the 44-kDa band in mouse spermatozoa, where the levels of MAPKs are much lower than in the other cells. The anti-ERK2 antibody mainly recognized the p42 ERK2 isoform, even though a fainter band corresponding to p44 ERK1 was also detected in most samples (Fig. 2). These data indicate that these antibodies recognize only p44 ERK1 and p42 ERK2, although with variable degree of cross-reactivity, and not other MAPK isoforms. They also indicate that both ERKs are expressed in pre-meiotic, meiotic, and post-meiotic germ cells as well as in Sertoli cells. Mature spermatozoa have lower amounts of both MAPKs.

Activation of MAPKs during the OA-induced G_2/M Transition in Mouse Spermatocytes—In mouse oocytes, a parallel activation of MPF and MAPKs occurs during G_2/M transition of both meiotic divisions (31) and MAPK activation is necessary for chromosome condensation in these cells (32). To test whether G_2/M transition induced by OA in mouse spermatocytes also involves activation of MAPKs, we measured kinase activity in soluble cell extracts using a peptide derived from MBP as a specific substrate for MAPKs. OA triggered activation of MAPKs in pachytene spermatocytes with a time course similar to that seen for MPF (Fig. 3). An increase in MAPK activity was detectable as early as 60 min after OA stimulation (75% increase) and reached a maximum (5-fold increase) after 4 h of treatment.

Specific Activation of ERK1 during the OA-induced Meiotic G_2/M Transition—To investigate whether the increase in MAPK activity was attributable to ERK1 and/or ERK2, MAPKs were immunoprecipitated with either anti-ERK1 or anti-ERK2 antibodies and MBP kinase activity of the immunoprecipitates was measured. Both antibodies immunoprecipitated MBP kinase activity from control spermatocytes, however, while OA induced a 2-fold activation of MBP kinase immunoprecipitated with anti-ERK1, it induced only a small increase in the activity precipitated with anti-ERK2 (Fig. 4*A*). Since the two antibodies have a certain degree of cross-reactivity between p44 ERK1 and p42 ERK2 (Fig. 2), it is likely that the small increase in activity measured in the anti-ERK2 immunoprecipitates is due to ERK1 co-precipitating with ERK2. On the other hand, the actual degree of specific ERK1 stimulation might be masked by co-immunoprecipitating ERK2 in anti-ERK1 immunoprecipitates. Furthermore, when the immunoprecipitates were incubated with the full-length MBP protein and [γ-^{32}P]ATP, followed by SDS-PAGE and autoradiography, we observed a more evident phosphorylation of MBP after OA treatment in anti-ERK1 than in anti-ERK2 immunoprecipitates (Fig. 4*B*). These data suggest that OA essentially induces activation of ERK1, rather than of ERK2 in mouse spermatocytes. To test this assumption, we utilized an in-gel kinase assay of spermatocyte extracts with the full-length MBP protein, which is a substrate also for other protein

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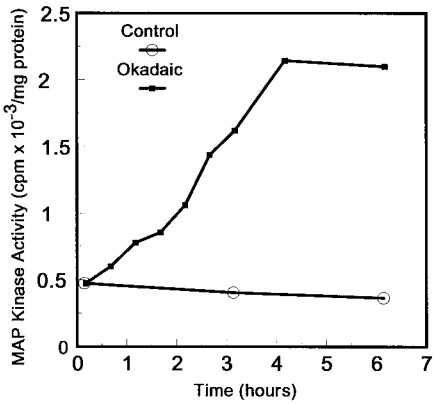

FIG. 3. **Activation of MAPKs during the OA-induced G_2/M transition in mouse spermatocytes.** MBP kinase assay on cell extracts from middle-late pachytene mouse spermatocytes treated with 5 μM OA for the indicated times, using a MBP-derived peptide as a specific substrate for MAPKs and cell extracts from 2×10^6 cells (see "Materials and Methods" for details).

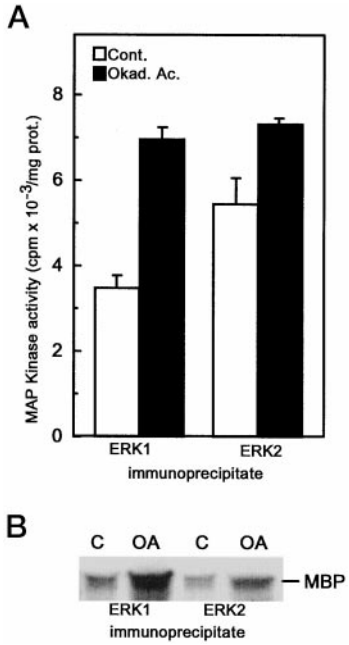

FIG. 4. **OA treatment activates ERK1 but not ERK2 in mouse spermatocytes.** *A,* MBP kinase assay in anti-ERK1 and anti-ERK2 immunoprecipitates from control and OA-treated spermatocytes (5 μM, 4 h). After ERKs were immunoprecipited from 2×10^6 cells, a kinase assay was performed using the same MBP-derived peptide used in Fig. 3 as a specific MAPK substrate. Data represent the mean ± S.D. of triplicate determinations from three separate experiments. *B,* phosphorylation of the full-length MBP protein by anti-ERK1 and anti-ERK2 immunoprecipitates from control and OA-treated spermatocytes. After immunoprecipitation of ERKs, a kinase assay was performed using the full-length MBP protein as a substrate, and the soluble fraction was analyzed by SDS-PAGE and autoradiography.

kinases. OA induced a large increase in MBP kinase activity of two major polypeptide bands, of approximately 55 and 44 kDa (Fig. 5*A*). Control in-gel kinase assays performed without addition of the MBP substrate showed that the p55 and p44 bands do not represent OA-induced autophosphorylating kinases (data not shown). Only the 44-kDa polypeptide band showed a time course of activation comparable to the time course of MAPK activation measured in whole cell extracts using the MBP-derived peptide shown in Fig. 3, with a maximum of activation between 4 and 6 h. The ~55-kDa band appeared to be maximally stimulated after just 1 h of OA treatment, suggesting that it corresponds to a OA-activated protein kinase

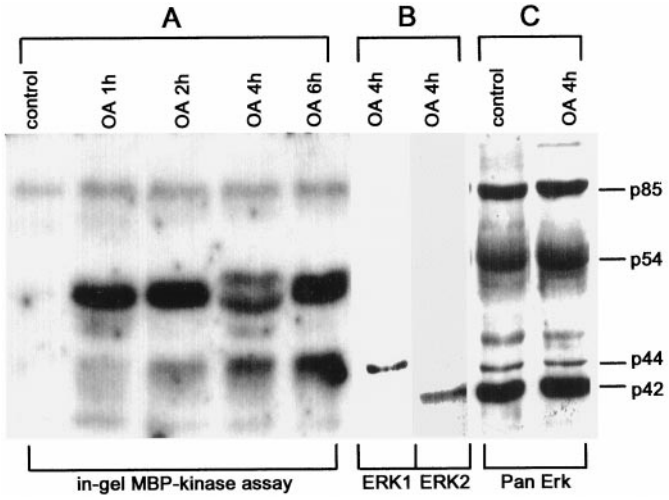

FIG. 5. **OA treatment activates different MBP kinases in mouse spermatocytes, but specifically activates ERK1 and not other MAPKs.** *A,* kinase assay in SDS-PAGE gels containing 0.1 mg/ml of the full-length MBP protein. Soluble extracts (50 μg) from spermatocytes treated with 5 μM OA for the indicated times were separated by SDS-PAGE. After removal of SDS and a cycle of protein denaturation and renaturation, a direct in-gel kinase assays was performed by adding [γ-^{32}P]ATP (see "Materials and Methods" for details). This experiment was repeated four times with similar results. *B,* parallel immunoblot analysis using specific anti-ERK1 and anti-ERK2 antibodies of cell extracts (50 μg) from spermatocytes treated for 4 h with 5 μM OA. *C,* parallel immunoblot analysis using the pan-ERK antibody of cell extracts (50 μg) from control or OA-treated spermatocytes.

which phosphorylates MBP in a region different from the fragment specifically phosphorylated by MAPKs. Moreover, the 44-kDa band co-migrated with ERK1, as shown by parallel Western blot analysis of spermatocyte extracts using either specific anti-ERK1 and anti-ERK2 antibodies (Fig. 5*B*), or an anti-MAPKs antibody (Pan-ERK) which recognize several other isoforms of this family (Fig. 5*C*). Densitometric analysis of the 44-kDa band detected in the MBP in-gel kinase assay shown in Fig. 5*A* revealed a 4-fold increase after 4 h of treatment with OA. This degree of activation is similar to MAPK activation measured in whole cell extracts using the MBP-derived peptide shown in Fig. 3. These results confirm that the actual increase in ERK1 activity induced by OA treatment is higher than the 2-fold increase detected with the immunokinase assay shown in Fig. 4, and that ERK2 is not activated by OA treatment.

Specific Phosphorylation of ERK1 on a Tyrosine Residue Involved in Enzyme Activation during the OA-induced Meiotic G_2/M Transition—We also performed Western blot analysis on spermatocyte cell extracts using a mouse monoclonal anti-phospho-ERK (anti-p-ERK) antibody, which specifically recognizes an epitope corresponding to amino acids 196–209 of ERK1 of human origin phosphorylated on Tyr^{204} (identical to the corresponding ERK2 sequence). This antibody specifically recognize both mouse ERK isoforms only when they are tyrosine phosphorylated in the corresponding mouse epitopes, which reflects the activated state of these enzymes (33). Fig. 6 shows that OA treatment clearly increases the phosphorylation of ERK1, but not of ERK2, on the tyrosine residue present in the epitope recognized by this antibody. These data confirm that OA induces activation of ERK1, rather than of ERK2 in mouse spermatocytes.

Subcellular Redistribution of ERKs in Mouse Spermatocytes during the G_2/M Transition—The localization of ERKs in mouse spermatocytes was investigated by immunofluorescence analysis, using the anti-ERK1 polyclonal antibody (Fig. 7). In untreated cells, mostly represented by mid-late pachytene

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spermatocytes, and a few contaminating round spermatids, anti-ERK1 immunostaining was uniformely observed in the cytoplasm and nucleus, with no evident localization in particular subcellular compartments; however, the rare cells in metaphase showed a peculiar distribution of anti-ERK1 staining (Fig. 7, *third row, right panel*). Indeed, a higher level of ERK1 immunopositivity was associated with the microtubules of the meiotic spindle, as demonstrated by the double staining with anti-β-tubulin antibody (Fig. 7, *third row, middle panel*). On the other hand, anti-ERK1 and anti-tubulin staining was negative where the condensed chromosomes are localized. In OA-treated cells, in which formation of the meiotic spindle is impaired (34) and the chromosomes condense to form a ring structure, both anti-tubulin and anti-ERK1 stainings maintain a diffuse distribution, whereas they are absent in the area were the condensed chromatin is found (Fig. 7, *fourth row, middle* and *right panels*). Considering the degree of ERK2 cross-reactivity (see Fig. 2) of the anti-ERK1 antibody used for immunofluorescence experiments, we cannot rule out that the subcellular localization seen for ERK1 reflects, at least in part, also the localization of ERK2. This result shows that ERKs concentrate in the meiotic spindles during physiological G_2/M transition in mouse spermatocytes.

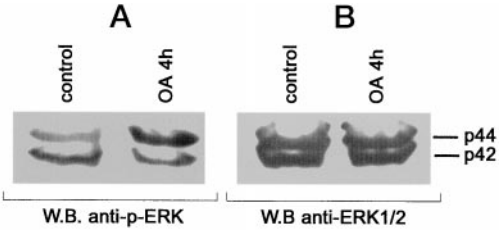

FIG. 6. **OA treatment specifically stimulates phosphorylation of ERK1, but not ERK2, on a tyrosine residue required for enzyme activation.** *A,* immunoblot analysis of protein extracts (70 μg) from control or OA-treated (5 μM, 4 h) mouse pachytene spermatocytes using the monoclonal anti-p-ERK antibody, which specifically recognizes an epitope corresponding to amino acids 196–209 of ERK1 of human origin phosphorylated on Tyr^{204} (identical to the corresponding ERK2 sequence, and cross-reactive with mouse ERKs). This experiment was repeated three times with similar results. *B,* the same samples shown in *A* were probed with a mixture of anti-ERK1 and anti-ERK2 polyclonal antibodies to show that OA treatment does not affect the expression levels of the two ERK isoforms.

OA-activated ERK1 Induces MPF Activity in Mouse Spermatocyte Cell Extracts—It has been reported that in *Xenopus* oocytes activation of ERK2 is required for Mos-dependent activation of MPF (8). We investigated whether ERK1, which appears to be specifically activated during the OA-induced G_2/M transition in mouse spermatocytes, is able to play a similar role. To test this hypothesis, we immunoprecipitated cell extracts from spermatocytes incubated for 4 h in the absence or presence of 5 μM OA with either the anti-ERK1 or anti-ERK2 antibody. The immunoprecipitated ERKs were incubated for 30 min with a cytosolic extract from untreated spermatocytes to test the eventual ability of the immunoprecipitated ERKs to activate endogenous MPF in these extracts. After separation from the immunoprecipitates, H1 kinase activity was measured in the cell extracts. As shown in Fig. 8, a 2-fold increase in H1 kinase activity was measured in cytosolic extracts incubated with anti-ERK1 immunoprecipitates from OA-treated cells as compared with anti-ERK1 immunoprecipitates from control cells. ERK2 immunoprecipitated from OA-

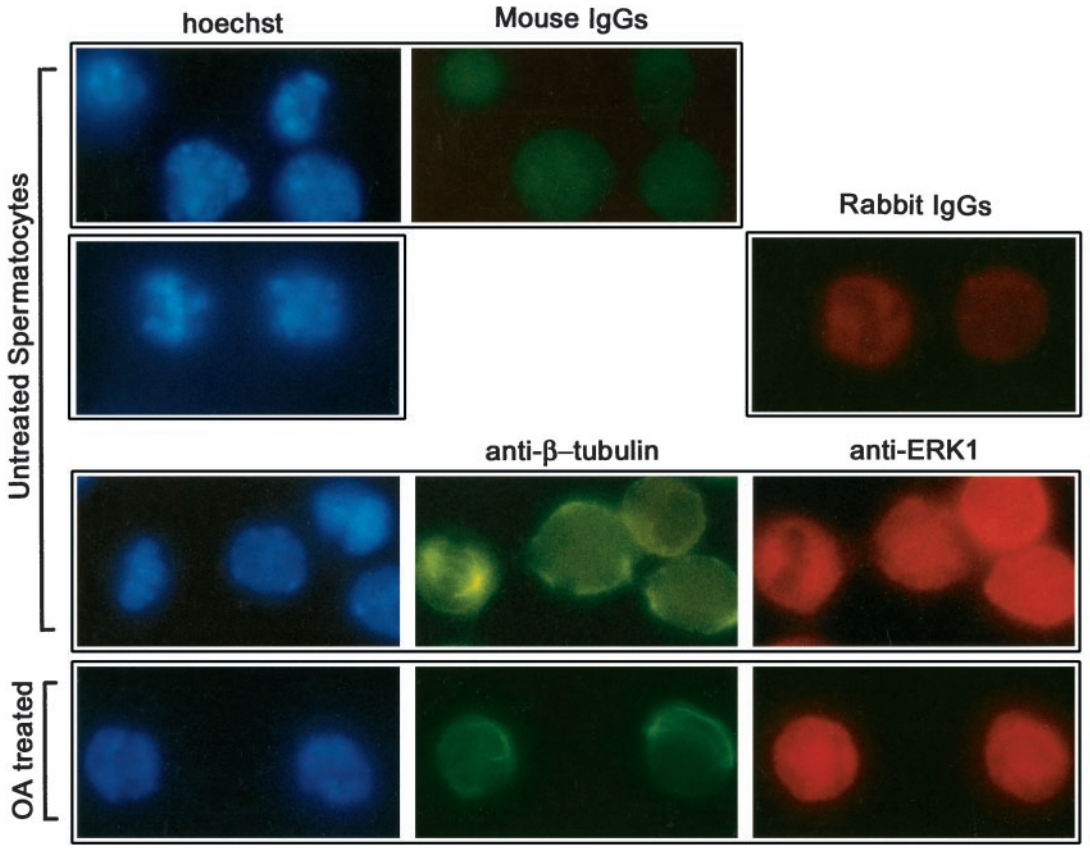

FIG. 7. **Subcellular redistribution of ERKs in mouse spermatocytes during the G_2/M transition.** Fluorescence microphotographs of untreated (*first, second,* and *third row*) and OA-treated (*fourth row*) mouse spermatocytes subjected to double indirect immunofluorescence analysis with mouse monoclonal anti-β-tubulin (*middle panels*) and rabbit polyclonal anti-ERK1 (*right panels*) antibodies, or to immunofluorescence analysis with non-immune mouse or rabbit IgGs as a control. All samples were also stained with Hoechst for the visualization of chromatin (*left panels*). Magnification: × 1050.

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treated cells was not able to induce such an increase. These data show that, following OA treatment, ERK1, but not ERK2, is able to trigger MPF activation in cytosolic extracts of unstimulated spermatocytes.

Inhibition of ERK Activating Kinases (MEKs) Prevents OA-induced Chromosome Condensation—In order to establish the contribution of the MAPK/ERK pathway in chromosome condensation during OA-induced meiotic male G_2/M transition, we preincubated cultured mouse spermatocytes with the specific MEK1/2 inhibitor PD98059 (35), which was predicted to impair selectively ERK activation by OA. We found that treatment with 0.5 μM OA, instead than 5 μM, was sufficient to induce nuclear envelope breakdown and chromosome condensation typical of G_2/M transition (Fig. 9*A*). Overnight preincubation with 50 μM PD98059 completely blocked the massive induction of metaphase by subsequent treatment with 0.5 μM OA (Fig. 9*A*). Under these conditions, PD98059 inhibited OA-induced ERK1 activation, as measured by MBP phosphorylation by anti-ERK1 immunoprecipitates (Fig. 9*B*). Interestingly, PD98059 pretreatment did not affect OA-induced MPF activation, measured through phosphorylation of H1 by either total spermatocyte cell extracts or anti-cyclin B1 immunoprecipitates (data not shown). Thus, ERK1 induction by OA is essential for chromosome condensation in spermatocytes undergoing the first meiotic division, independently from its ability to participate in MPF activation.

DISCUSSION

The present data, in addition to confirming that the increase in H1 kinase activity previously observed in the OA-induced G_2/M transition in mouse pachytene spermatocytes (22, 23) is actually due to activation of MPF (the cyclin B1·cdc2 complex), also demonstrate that the OA-induced G_2/M transition is associated with an increase in MAPK activity, with a timing parallel to that of MPF activation and to that of chromosome condensation. The increase of MAPK activity is due to selective activation of the ERK1 isoform, and it is associated with increased phosphorylation of a tyrosine residue involved in enzyme activation. MPF activation might be directly responsible for the further chromosome condensation observed during the OA-induced G_2/M meiotic transition. Indeed, it has been recently shown that cdc2 can phosphorylate and activate the 13 S condensin multisubunit protein complex which is essential for mitotic chromosome condensation (36). However, studies in mouse oocytes also suggest that MAPKs can play a direct role in chromosome condensation independently from the state of MPF activity (19, 32). Indeed we found that blocking OA-induced ERK1 activation through preincubation of spermatocytes with PD98059, a selective inhibitor of ERK-activating MEK1/2 kinases, is able to prevent the formation of metaphase chromosomes. Since this treatment did not block OA-induced MPF activation, it can be concluded that activation of ERK1, rather than of MPF, is strictly required for chromosome condensation during male meiotic methaphase I.

We also observed that the natural G_2/M transition of spermatocytes is associated to changes in the subcellular localization of ERKs, which reorganize from a diffuse cytoplasmic distribution in pachytene spermatocytes to an apparent association with the meiotic spindle in metaphase spermatocytes. The redistribution of ERKs at metaphase appears to be dependent on spindle assembly, since in OA-treated cells, where the spindle formation is impaired (34), ERKs remain diffused in the cytoplasm.

The effects of OA on the cell cycle are thought to be mediated by inhibition of serine-threonine phosphatase 2A (PP-2A) with the consequent activation of the cdc2 kinase (37). The observation that purified PP-2A dephosphorylates *in vitro* activating

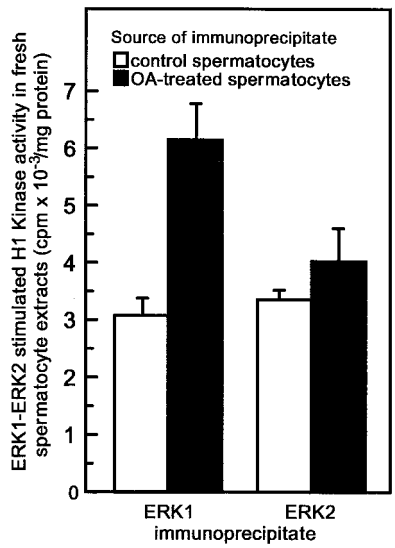

FIG. 8. **OA-activated ERK1 induces MPF activity in mouse spermatocyte cell extracts.** ERKs were imunoprecipitated from control spermatocytes or spermatocytes treated with 5 μM OA for 4 h using either anti-ERK1 or anti-ERK2 antibodies. Immunocomplexes were washed thoroughly and 200 μg of cytosolic extracts from untreated pachytene spermatocytes were added and incubated with the immunocomplexes for 30 min at 30 °C under constant shaking. After separation by centrifugation, an H1 kinase assay was performed on the soluble cell extracts that had been incubated with either anti-ERK1 or anti-ERK2 immunoprecipitates from control and OA-treated cells (see "Materials and Methods" for details). Data represent the mean ± S.D. of triplicate determinations from three separate experiments.

residues of cdc2 kinase, such as Thr^{161}, would suggest a direct regulation of MPF activity by PP-2A (38, 39), whereas experiments performed with *Xenopus* egg extracts indicate that PP-2A acts by inhibiting the activity of the dual specificity phosphatase cdc25 (40). Dephosphorylation of Thr^{14} and Tyr^{15} of cdc2 by cdc25 results in activation of MPF (12). This mechanism could be part of a positive feedback, since activated MPF is able to phosphorylate and activate cdc25 (41, 42). In addition to cdc2, other protein kinases are able to phosphorylate and regulate cdc25 (43). For instance, it has been shown that cdc25 associates with Raf-1 in both mammalian somatic cells and frog meiotic oocytes, and it can be activated *in vitro* in a Raf-1-dependent manner (44). Raf-1 is a well known upstream activator of ERK1/ERK2 and it is present in mouse spermatocytes (45).[2] We now show that OA-activated ERK1 is able to induce activation of MPF in spermatocyte cell extracts, suggesting that OA might regulate MPF activity by downstream activation of multiple Raf-dependent pathways (cdc25 and/or ERK1), and that the Ras/Raf pathway might be functional in male meiotic cells.

PP-2A has been reported to dephosphorylate critical threonine residues of MAPKs required for enzyme activation (46–48), and OA stimulates MAPK activity in several cell systems (8, 49). In agreement with a direct role of PP-2A on both MPF and MAPK regulation, we found that OA treatment induced a concomitant increase of MPF and MAPK activity in mouse spermatocytes. Furthermore, inhibition of OA-induced ERK1 activation by preincubation of mouse spermatocytes with PD98059 did not prevent MPF activation, confirming that activation of cdc25 by OA is sufficient to trigger activation of the cyclin B·cdc2 complex during the male meiotic G_2/M transition. Thus, our data suggest that OA overcomes the physiological regulation of the cell cycle events in spermatocytes by inhibiting PP-2A activity. However, since activation of ERK1 leads to

[2] C. Sette and A. Bianchini unpublished observation.

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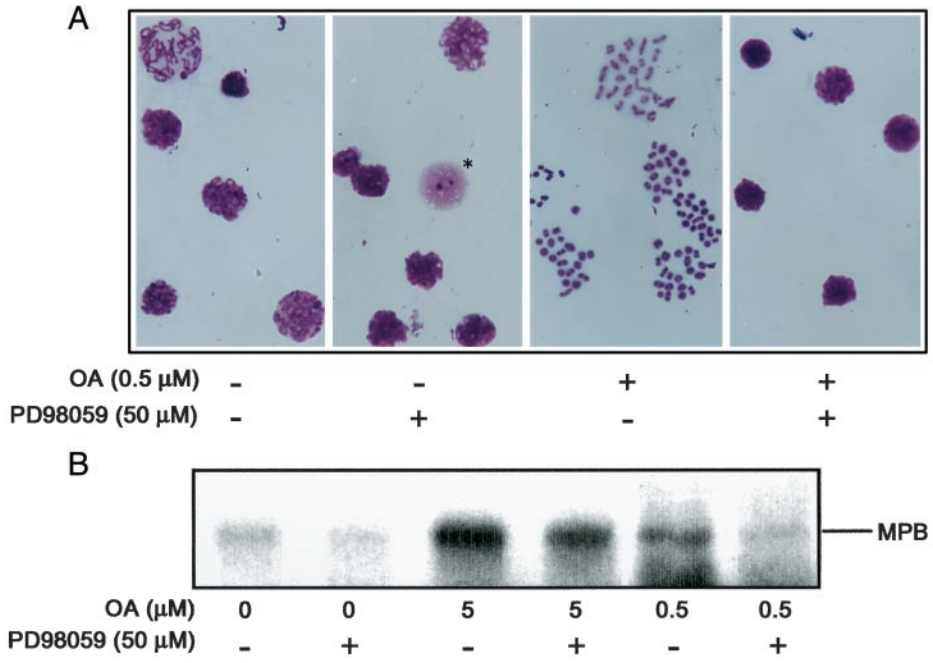

FIG. 9. **Preincubation with the MEK1/2 inhibitor PD98059 prevents the ability of OA to trigger ERK1 activation and chromosome condensation associated with meiotic progression in mouse spermatocytes.** *A,* Giemsa staining of nuclei from control spermatocytes preincubated for 12 h with or without 50 μM PD98059, and subsequently treated for 5 h with or without 0.5 μM OA. All panels show spermatocyte nuclei with different degrees of chromosome condensation, with the exception a Sertoli cell nucleus in the second panel (indicated by an *asterisk*). Magnification: × 160. *B,* phosphorylation of the full-length MBP protein by anti-ERK1 immunoprecipitates from control spermatocytes preincubated for 12 h with or without 50 μM PD98059, and subsequently treated for 5 h with or without 5 μM or 0.5 μM OA. After immunoprecipitation of ERK1, a kinase assay was performed using the full-length MBP protein as a substrate, and the soluble fraction was analyzed by SDS-PAGE and autoradiography.

activation of MPF in spematocyte cell extracts, it is conceivable that a differential temporal activation of ERK1 and MPF occurs during physiological meiotic progression in these cells. Furthermore, we report here that OA induces a 5-fold increase in MPF activity in the absence of serum, whereas the increase in H1 kinase activity in spermatocytes cultured in the presence of serum varies between 2- and 4-fold (22, 23). Although these differences could be simply due to culture conditions, it is also possible that growth factors present in serum are able to trigger partial activation of MAPKs (and consequently of MPF), lowering the apparent effect of OA. In agreement with this hypothesis, we observed that MAPK activity was higher in spermatocytes cultured in the presence of serum, and that OA-induced MAPK activation was less evident under these conditions.[2]

In *Xenopus* oocytes, Mos-dependent activation of MPF requires ERK2 (8, 10), whereas we found that OA selectively activates ERK1, but not ERK2, during meiotic progression of primary spermatocytes. This is a further example of dimorphism in the regulation of cell cycle events between male and female meiosis in mammals (1). Activated ERK1 immunoprecipitated from OA-treated spermatocytes can activate H1 kinase activity in extracts from control pachytene spermatocytes. Since we found that the increase in H1 kinase activity induced by OA measured in the spermatocyte cell extracts corresponds to that immunoprecipitated by the anti-cyclin B1 antibody, these results indicate that ERK1 might be directly involved in MPF activation in these cells. Recently, it has been shown that MAPKs, via activation of $p90^{rsk}$ kinase (50) and the consequent inactivation of the cdc2 inhibitory kinase Myt1, can regulate MPF activity in *Xenopus* oocytes (16). It is, therefore, possible that similar mechanisms are also functional in male meiosis.

MPF is also a major target of endogenous checkpoints controlling the timing of meiotic events (1). Genomic integrity is a prerequisite for the progression of the cell cycle, as shown by targeted disruption of genes involved in DNA mismatch repair, such as Pms2 (51) and Mlh1 (52–53), or in DNA recombination, such as Dmc1 (54, 55), which leads to a meiotic arrest resulting in sterility in male and female mice. A possible link between the DNA damage checkpoint and the progression of the meiotic cell cycle is represented by serine-threonine kinases such as ATM and chk1. Targeted disruption of ATM, a phosphatidylinositol 3-kinase-related protein kinase homologous to the yeast DNA-damage checkpoint protein Rad3 (56), causes mouse infertility due to meiotic arrest (57–60) in the early prophase I (61). A protein kinase that acts downstream to ATM is chk1, which is associated with meiotic chromosomes in mouse pachytene spermatocytes (62) and has been shown to phosphorylate and indirectly inhibit the cdc25 in yeast and mammalian mitotic cells (63–65). MPF activity and meiotic progression in spermatocytes is also regulated by temporal expression of germ cell-specific genes (66). For instance, HSP70-2, a heat-shock protein specifically expressed in pachytene spermatocytes and associated with synaptonemal complexes, seems to function as a molecular chaperone required for the assembly of cyclin B1·cdc2 complex formation and activity in pachytene spermatocytes (67). Mice carrying targeted disruption of HSP70-2 display a spermatogenic block in late prophase I, due to failure of chromosomes to desynapse (68, 69). A similar phenotype, *i.e.* arrest in late prophase I coupled to low cyclin B1·cdc2 kinase activity, was also observed in mice carrying targeted disruption of another spermatocyte-specific gene, cyclin A1 (70).

Activation of ERK1 during OA-induced meiotic progression, its association with the metaphase spindle, its ability to activate MPF in extracts from mouse spermatocytes, and the observation that it is required for OA-induced chromosome condensation at metaphase (independently from its ability to induce MPF activation) indicate that ERK1 might play a role in the G_2/M transition in male meiosis. It is known that ERK1 is activated by extracellular signals (24), and this might imply that the environment surrounding spermatocytes within the seminiferous epithelium acts in concert with intracellular mechanisms to regulate the ordered progression of male meiosis through prophase I.

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