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RAPID COMMUNICATION

Regulation of Nuclear Envelope Assembly/

Jiri Moos,[1] Zhe Xu,† Richard M. Schultz,* and Gregory S. Kopf†

Department of Biology and †Division of Reproductive Biology, Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Mouse eggs arrested in metaphase II display high levels of cdc2/cyclin B1 and MAP protein kinase activities. Following fertilization there is a time-dependent decrease in the activity of each of these protein kinases. The decline in cdc2/cyclin B1 protein kinase correlates with the resumption of meiosis and the emission of the second polar body and precedes the decline in MAP kinase activity, which correlates temporally with the formation of the male and female pronuclear envelopes. These results suggest that high levels of MAP kinase activity are incompatible with the presence of a pronuclear envelope. To test this possibility, we expressed in mouse eggs a constitutively active form of MAP kinase kinase (MEK) whose only known target is p42/p44 MAP kinase. We show that following fertilization cdc2/cyclin B1 kinase activity declines and a second polar body is emitted. The endogenous MAP kinase remains active, however, and no pronuclear envelopes form. Thus, high levels of MAP kinase activity by itself in mouse eggs appear incompatible with the presence of a pronuclear envelope.

INTRODUCTION

We have previously shown that treatment of fertilized mouse eggs that contain pronuclei with the protein phosphatase inhibitor okadaic acid does not active cdc2/cyclin B1 kinase but does activate MAP kinase by maintaining it in its phosphorylated state and results in the precocious breakdown of the pronuclear envelopes (Moos *et al.*, 1995). Moreover, addition of okadaic acid to fertilized mouse eggs at a time subsequent to the decline in cdc2/cyclin B1 kinase activity but prior to the decline in MAP kinase activity and the formation of the pronuclei maintains elevated levels of MAP kinase activity and prevents pronuclear envelope formation (Moos *et al.*, 1995). These results suggest that high levels of MAP kinase activity are incompatible with the presence of a pronuclear envelope. MAP kinase, however, may not be the sole downstream target for the action of okadaic acid.

To circumvent this problem, we expressed in mouse eggs a constitutively activated form of MAP kinase kinase (MEK), called MEKE (Coso *et al.*, 1995), whose only known target is MAP kinase (Marshall, 1994). We report that following fertilization of these MEKE-expressing eggs cdc2/cyclin B1 kinase activity declines and a second polar body is emitted. The endogenous MAP kinase remains active, however, and no pronuclear envelopes form.

MATERIALS AND METHODS

Fully grown, germinal vesicle intact oocytes were collected from PMSG-primed CF-1 mice (Harlan) as previously described (Schultz *et al.*, 1983). The oocytes were microinjected with 10 pl of a solution containing 1.6–2.0 μg/μl of *in vitro* transcribed MEKE mRNA in diethylpyrocarbonate-treated water (Williams *et al.*, 1992). MEKE mRNA was transcribed *in vitro* as follows: Plasmids (pCDNAIIIB) containing the constitutively active MEKE were obtained from Dr. Silvio Gutkind (NIH). The plasmid was transcribed *in vitro* under capping conditions, and the mRNA was isolated as previously described (Moore *et al.*, 1993). The transcript had the anticipated size of 1.3 kb. The injected oocytes were then matured *in vitro* to metaphase II (Williams *et al.*, 1992) and then inseminated and cultured *in vitro* (Moore *et al.*, 1993). At the times indicated, pronuclear formation was examined by Hoffmann modulation optics.

Immunodetection of MAP kinase and ZP2/ZP2$_f$ following SDS–PAGE (10% gel) was performed using enhanced chemilumines-

[1] Present address: Institute of Molecular Genetics, Czech Academy of Sciences, Videnska, 1083, 142 20 Prague 4, Czech Republic.

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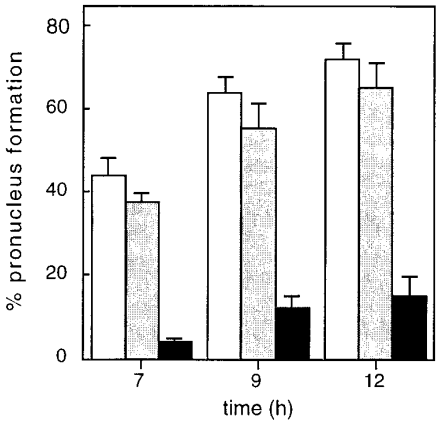

FIG. 1. Effect of MEKE on pronucleus formation following insemination of mouse eggs. Eggs were inseminated and pronucleus formation was examined at the times indicated. Open bars, noninjected eggs; stippled bars, buffer-injected eggs; solid bars, eggs injected with MEKE mRNA. The data are expressed as the mean ± SEM from eight (noninjected, MEKE-injected) or five (buffer-injected) experiments. Typically, 30–40 eggs were scored in each group within each experiment.

cence as previously described (Moos *et al.*, 1995) with groups (30–35) of 1-cell embryos. To detect the ZP2 to $ZP2_f$ conversion a rabbit polyclonal antiserum to mouse ZP2 was used; this antiserum recognizes both ZP2 and $ZP2_f$. The blot was simultaneously probed with a rabbit polyclonal antiserum to MAP kinase (UBI) as previously described (Moos *et al.*, 1995). cdc2/cyclin B1 kinase activity was assayed using a peptide substrate as previously described (Moos *et al.*, 1995).

RESULTS AND DISCUSSION

The constitutively active MEK that was used, MEKE, was derived by mutating the nucleotides that encode for serines 218 and 221 to glutamate resides. Phosphorylation of the serine residues normally leads to MEK activation, and the negative charge of the glutamate residues presumably mimics the negative charge of the phosphate and leads to constitutive activation. Insemination of *in vitro* matured metaphase II-arrested eggs expressing MEKE resulted in a pronounced inhibition of pronuclear envelope formation (Fig. 1) and the maintenance of the endogenous MAP kinase in its active, phosphorylated state (Fig. 2, lane 3). In contrast, insemination of uninjected or buffer-injected eggs resulted in pronuclear envelope formation and the dephosphorylation and inactivation of the endogenous MAP kinase (Figs. 1 and 2, lanes 2 and 4). The inhibition of pronuclear envelope formation and the maintenance of the endogenous MAP kinase in its phosphorylated state in the MEKE-injected eggs were not due to the inability of these eggs to be fertilized. First, these eggs displayed the ZP2 to $ZP2_f$ conversion, a proteolytic modification of one of the components of the *zona pellucida* that occurs following fertilization and constitutes part of the *zona pellucida* block to polyspermy (Fig. 2, lane 3) (Wassarman, 1988). Second, these eggs emitted the second polar body in a time frame similar to that of the control eggs, i.e., within 1–2 hr following insemination (data not shown).

Since several lines of evidence suggest that MAP kinase may activate cdc2/cyclin B1 kinase (Haccard *et al.*, 1993, 1995; Minshull *et al.*, 1994; Kosako *et al.*, 1994), it was formally possible that the inhibition of pronuclear envelope formation in the MEKE-injected eggs was due to maintenance of cdc2/cyclin B1 kinase activity at elevated levels. Although this was unlikely, since the MEKE-injected/inseminated eggs emitted the second polar body, we measured the activity of cdc2/cyclin B1 kinase (as measured by its histone H1 kinase activity) as a function of time following insemination (Fig. 3A). Histone H1 kinase activity declined with a similar initial time course during the first 5 hr following insemination in both untreated and MEKE-injected eggs. This was anticipated since the decline in cdc2/cyclin

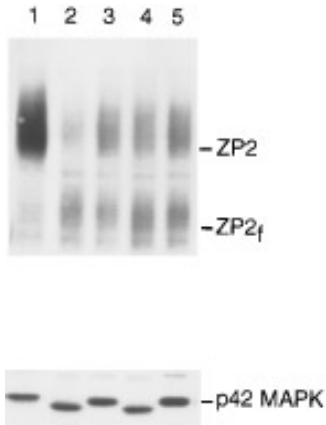

FIG. 2. Effect of MEKE on the ZP2 conversion and phosphorylation state of endogenous MAP kinase. The oocytes were injected, matured, inseminated, and cultured for 12 hr. Lane 1, metaphase II-arrested eggs; lane 2, noninjected inseminated eggs; lane 3, MEKE-injected and inseminated eggs; lane 4, buffer-injected and inseminated eggs; lane 5, MEKE-injected and inseminated eggs that were then treated with cycloheximide (20 μg/ml) starting 5 hr following insemination. Note that in the metaphase II-arrested egg (lane 1) little $ZP2_f$ is present and MAP kinase exists in the active and phosphorylated form of lower electrophoretic mobility, whereas in the inseminated egg (lane 2) a significant amount of $ZP2_f$ is present and MAP kinase exists in the inactive, dephosphorylated form of higher electrophoretic mobility. Each experimental group was analyzed in 3–5 experiments and similar results were obtained in each experiment. Shown are the results of a representative experiment. The "apparent" difference in the extent of ZP2 to $ZP2_f$ conversion observed in the MEKE-injected and inseminated eggs (lane 3), compared to either noninjected inseminated eggs (lane 2) or buffer-injected and inseminated eggs (lane 4), seen in this experiment is not observed consistently and is likely due to experimental variation.

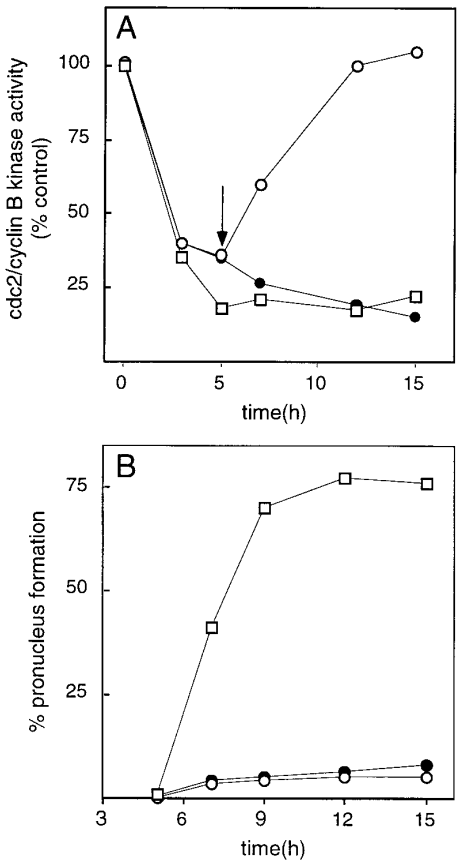

FIG. 3. (A) Effect of MEKE on cdc2/cyclin B1 kinase activity following insemination and incubation in the absence or presence of cycloheximide. The oocytes were injected, matured, inseminated, and cultured as described under Materials and Methods. At the indicated times following insemination, three 1-cell embryos were removed and cdc2/cyclin B1 kinase activity, as assessed by its histone H1 kinase activity, was assayed. (□) Control inseminated eggs; (○) MEKE-injected and inseminated eggs; (●) MEKE-injected and inseminated eggs that were then cultured in medium containing cycloheximide (20 μg/ml) starting 5 hr following insemination (arrow). Shown are the cumulative data from three experiments in which the amount of activity in the metaphase II-arrested egg was set as 100%. It should be noted that in a separate set of experiments in which cdc2/cyclin B1 kinase activity was assayed in MEKE-injected eggs 12 hr after insemination, the cdc2/cyclin B1 kinase activity was 114 ± 9% (mean ± SEM) that of the activity in the metaphase II-arrested egg; this compares favorably with the results shown at the 12-hr time point in A. (B) Effect of MEKE on pronucleus formation following insemination and incubation in the absence or presence of cycloheximide. The oocytes were injected, matured, inseminated, and cultured as described under Materials and Methods. At the indicated times following insemination, 15–40 eggs were scored for pronucleus formation. (□) Control inseminated eggs; (○) MEKE-injected and inseminated eggs; (●) MEKE-injected and inseminated eggs that were then cultured in medium containing cycloheximide (20 μg/ml) starting 5 hr following insemination (arrow). Shown are the cumulative data from three experiments.

B1 activity that occurs during this time period occurs while MAP kinase activity remained elevated (Verlhac *et al.*, 1994; Moos *et al.*, 1995). Surprisingly, cdc2/cyclin B1 activity started to increase in these MEKE-injected and inseminated eggs by 7 hr and reached levels similar to those found in the metaphase II-arrested egg (Fig. 3A). This was in contrast to control inseminated eggs in which cdc2/cyclin B1 activity remained low throughout this period.

Since the fertilization-induced decline in cdc2/cyclin B1 activity is due to cyclin B1 proteolysis (Moos *et al.*, 1995), the reactivation of cdc2/cyclin B1 kinase observed in the MEKE-injected/inseminated eggs could be due to the stimulation of cyclin B1 synthesis by the MAP kinase that would ordinarily not be active at this time. If such were the case, this reactivation would be abolished by inhibitors of protein synthesis. Transfer of the MEKE-injected eggs to medium containing cycloheximide 5 hr following insemination blocked the subsequent time-dependent increase in cdc2/cyclin B1 kinase activity seen in the absence of this protein synthesis inhibitor (Fig. 3A). This inhibition could not be ascribed to a decrease in the amount or the activity of endogenous MAP kinase, since neither the mass nor the phosphorylation state of MAP kinase changed compared to those of MEKE-injected/inseminated eggs that were cultured in the absence of cycloheximide (Fig. 2, compare lanes 3 and 5).

The treatment of MEKE-injected/inseminated eggs with cycloheximide following the fertilization-induced decline in cdc2/cyclin B1 activity permitted us to explore further the incompatibility of a nuclear envelope in the presence of MAP kinase activity but in the absence of cdc2/cyclin B1 activity; cycloheximide does not prevent the formation of pronuclei following fertilization (Moore *et al.*, 1993). Normally, following fertilization pronuclear envelope formation occurs under conditions in which both cdc2/cyclin B1 and MAP kinase activities are low (Fig. 3B) (Verlhac *et al.*, 1994; Moos *et al.*, 1995). However, if MAP kinase activity was maintained at high levels by MEKE injection in either the presence (no cycloheximide) or absence (presence of cycloheximide) of high levels of cdc2/cyclin B1 kinase activity, pronuclear envelope formation did not occur (Fig. 3B).

The results presented here provide strong evidence that elevated levels of MAP kinase activity, in the absence of cdc2/cyclin B1 kinase activity, regulate events that lead to nuclear envelope assembly and disassembly. In contrast to somatic cells that enter interphase shortly after the decline in cdc2/cyclin B1 kinase activity, pronuclei do not form in the fertilized mouse egg for some 5–6 hr following the observed drop in cdc2/cyclin B1 kinase activity, a time during which MAP kinase activity remains elevated (Verlhac *et al.*, 1994; Moos *et al.*, 1995). This proposed function of MAP kinase may also explain why the metaphase I to metaphase II transition during mouse oocyte maturation occurs in the absence of an intervening interphase. The activity of cdc2/cyclin B1 kinase is low in the germinal vesicle-intact oocyte, increases to a peak level by metaphase I, transiently decreases following exit from metaphase I, and then in-

creases and remains elevated by metaphase II (Hashimoto and Kishimoto, 1988; Choi *et al.*, 1991). In contrast, MAP kinase activity increases subsequent to the increase in cdc2/cyclin B1 kinase, remains elevated during the transition from metaphase I to metaphase II, and remains elevated in the metaphase II-arrested egg (Verlhac *et al.*, 1993; Gavin *et al.*, 1994). The elevated level of MAP kinase activity that is present between metaphase I and metaphase II may therefore prevent the formation of an interphase nucleus.

Our results also suggest that MAP kinase regulates cdc2/cyclin B1 activity by stimulating cyclin B1 mRNA translation, since the reactivation of cdc2/cyclin B1 kinase activity in the MEKE-injected/inseminated eggs is cycloheximide-sensitive. This stimulatory effect could possibly be mediated by PHAS-1, which is involved in protein synthesis (Pause *et al.*, 1994). The binding of PHAS-1 to the mRNA cap-binding protein eIF-4E inhibits eIF-4E function (Lin *et al.*, 1994), which is usually rate-limiting in protein synthesis (Hershey, 1991). Phosphorylation of PHAS-1 by MAP kinase results in the dissociation of PHAS-1 from eIF-4E and thereby relieves this inhibition (Lin *et al.*, 1994). Thus, the presence of elevated levels of MAP kinase activity during oocyte maturation could explain how the transient decrease in cdc2/cyclin B1 kinase activity following exit from metaphase I is followed by an increase in cdc2/cyclin B1 kinase activity during the transition to metaphase II.

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