A MAPK pathway is involved in the control of mitosis after fertilization of the sea urchin egg

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Abstract

Activation and role of mitogen-activated protein (MAP) kinase (MAPK) during mitosis are still matters of controversy in early embryos. We report here that an ERK-like protein is present and highly phosphorylated in unfertilized sea urchin eggs. This MAPK becomes dephosphorylated after fertilization and a small pool of it is transiently reactivated during mitosis. The phosphorylated ERK-like protein is localized to the nuclear region and then to the mitotic poles and the mitotic spindle. Treatment of eggs after fertilization with two different MEK inhibitors, PD 98059 and U0126, at low concentrations capable to selectively induce dephosphorylation of this ERK-like protein, or expression of a dominant-negative MEK1/2, perturbed mitotic progression. Our results suggest that an ERK-like cascade is part of a control mechanism that regulates mitotic spindle formation and the attachment of chromosomes to the spindle during the first mitosis of the sea urchin embryo.

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Introduction

Activation of mitogen-activated protein kinase (MAPK) is generally triggered after exit from a G0 or G2 quiescent state. In somatic cells, stimulation by various extracellular signals that regulate morphology, proliferation, differentiation or survival often leads to the activation of at least one member of the MAPK family, for instance the extracellular signal-regulated kinases (ERK1/2) (reviews by Peysonnaux and Eychéne, 2001; Volmat and Pouysségur, 2001). More than 10 years ago, it was shown that microinjection of the proto- oncogene mos, a MAPK kinase kinase (Sagata et al., 1989), or of thiophosphorylated MAP kinase (Haccard et al., 1993) into one blastomere of a two-cell Xenopus embryo induced cell cycle arrest. This suggested that a MAPK pathway could also regulate the cell cycle in early embryos.

It is well accepted that an ERK cascade, implying mos, acts at different steps during maturation and regulates meiosis in oocytes of various species (Abrieu et al., 2001; Karaiskou et al., 2001). This pathway has been implicated in regulating the meiotic spindle itself (Lefebvre et al., 2002; Verlhac et al., 1996). However, whether MAPK is stimulated and plays a role during the following mitotic cycles in living embryos still remains a matter of controversy. In intact Xenopus oocytes, Gotoh et al. (1991) observed that a 42/44-kDa protein, which phosphorylated preferentially MBP or MAP2 (microtubule associated protein) rather than histone H1, was activated at the time of first mitosis.
Activation of MAPK at time of mitosis was indeed measured in *Xenopus* cycling extracts (Guadagno and Ferrell, 1998), but since the reports by Ferrell et al. (1991), it was admitted for many years that this activity decreased at exit of meiosis II and was not further reactivated in early *Xenopus* embryos (Tunquist and Maller, 2003). This dogma was broken very recently by Yue and Ferrell (2004) who detected, in dividing *Xenopus* embryos, activation of p42 in MAPK that could be due to low levels of Mos remaining after fertilization. In clam oocytes, fertilization that occurs during meiosis I triggers a decrease in MAPK activity, and no further reactivation of MAPK during the following cell cycles has been reported so far (Shibuya et al., 1992). We then set about this work to clarify the situation in sea urchin eggs, where meiosis is completed prior to fertilization, and since activation of MAPK activity during the first mitosis following fertilization has been detected (Chiri et al., 1998; Philipova and Whitaker, 1998) or not (Kumano et al., 2001). We show in the present report that an ERK-like protein, highly phosphorylated in the sea urchin unfertilized egg, is dephosphorylated soon after fertilization. We found that a small proportion of the ERK-like protein is transiently rephosphorylated at the time of mitosis, and that this pool of phosphorylated ERK is localized to the nuclear region and then to the mitotic apparatus.

In various types of somatic cells, several data have shown the association of ERK1/2 and/or of upstream partners such as MEK or RISK with the mitotic spindle (Cha and Shapiro, 2001; Shapiro et al., 1998; Wang et al., 1997; Willard and Crouch, 2001; Zecevic et al., 1998). It has also been suggested that a MAPK pathway plays a role in the spindle checkpoint, a mechanism that delays anaphase onset in eukaryotic somatic cells when all chromosomes are not aligned or under proper tension and that is expressed as a negative signal produced by the Mad2/Bub1 machinery active at unattached kinetochores (Hardwick, 1998; Hoyt, 2001; Sadler and do Carmo Avides, 2000). This hypothesis came from results obtained in somatic cells showing that active MAPK associates with kinetochores (Shapiro et al., 1998; Zecevic et al., 1998) and that this checkpoint does not operate properly after inhibition of the MAPK pathway with PD 98059 (Cross and Smythe, 1998), anti-MAPK antibodies (Takenaka et al., 1997) or injection of XCL100, a MAPK phosphatase (Wang et al., 1997). In cycling *Xenopus* extracts, various data indicate that MAP kinase is required for the spindle assembly checkpoint (Chen, 2004; Chung and Chen, 2003; Minshull et al., 1994; Takenaka et al., 1997). However, it is accepted that such a mechanism depends on the nucleus/cytoplasm ratio, ruling out its existence in early dividing eggs where the ratio nucleus/cytoplasm is too low. The question was then whether the phosphorylated ERK that we detected at time of mitosis in sea urchin early embryos could regulate mitotic events such as the formation of the mitotic spindle. We found that treatment of fertilized eggs with two different MEK inhibitors, PD 98059 and U0126, at low concentrations that selectively trigger dephosphorylation of the ERK-like protein, or expression of a dominant-negative MEK1/2, induced alterations in the formation of the mitotic spindle and of the attachment of chromosomes to the spindle during the first mitosis of the sea urchin embryo. Our results strongly suggest that an ERK-like protein is an important regulator of mitosis and might be part of a spindle checkpoint mechanism in the early sea urchin embryo.

### Materials and methods

#### Handling of gametes

Gametes collected from the sea urchin *Paracentrotus lividus* were prepared and fertilized in artificial sea water (ASW, Reef Crystals Instant Ocean) as described in our previous papers (Chiri et al., 1998; De Nadai et al., 1998).

Eggs were treated or not before or after fertilization with PD 98059 (Calbiochem, 15 mM stock in DMSO), U0124 or U0126 (Calbiochem, 15 mM stock in DMSO).

#### Western blot analysis

Samples of eggs were prepared as previously described (Chiri et al., 1998; De Nadai et al., 1998). In all experiments, 10 μg of proteins was separated by 12.5% SDS–PAGE and transferred on PVDF membranes. After transfer, the membrane was rinsed in distilled water for 5 min, dried in air for 15 min in order to fix proteins and then incubated for one h in blocking buffer (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20, 3% BSA). The membrane was incubated with primary antibody for 2 h at room temperature and then washed three times for 5 min in washing buffer (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 0.5% NP40). The membrane was incubated with secondary antibody for 1 h, rinsed three times for 5 min in washing buffer, and proteins were revealed by enhanced chemiluminescence (ECL, Amersham).

Whenever necessary, band intensities were quantified by scanning radiographic films followed by densitometry analysis using Adobe Photoshop.

All the antibodies were diluted in blocking buffer. Primary antibodies and dilutions were as following: mouse anti-phospho-MAPK42/44 (Thr202/Tyr204, Cell Signalling) at 1/2000, rabbit anti-phospho-MEK1/2 (ser217/221, Cell Signalling, 9121) at 1/1000, rabbit anti-phospho-cdc2 (Tyr15, Cell Signalling) at 1/500 and rabbit anti-ckd1/ckd2 (PSTAIR, UBI) at 1/1000. The anti-mouse HRP-conjugated goat IgG and the anti-rabbit HRP-conjugated goat IgG secondary antibodies (ICN) were diluted 1/1000 and 1/10,000, respectively.

In order to verify that the anti-phospho-MAPK42/44 antibody recognized only the active form of a MAPK-like protein, samples of unfertilized eggs were treated by
alkaline phosphatase before SDS–PAGE analysis as following. A dry pellet of eggs (Chiri et al., 1998) containing 250 μg proteins was incubated for 2 h at 37°C in 65 μl bicarbonate buffer (100 mM NaHCO₃, pH 10.5) containing 84 U/ml alkaline phosphatase. Incubation was stopped by adding 65 μl denaturation buffer (Chiri et al., 1998) and boiling for 10 min at 95°C. 10 μg of proteins was then separated on SDS–PAGE and analyzed by Western blot.

**Cytochemistry**

Eggs were fertilized in the presence of 1 mM ATAZ (3-amino-1,2,4-triazole, Sigma) to prevent hardening of the fertilization membrane. 2 min after fertilization, eggs were diluted 10 times in normal filtered sea water (NFSW) and quickly filtered several times through a 85-μm filter to remove fertilization membranes. Decanted eggs were rinsed twice in NFSW and cultured in NFSW, treated or not with PD 98059 or U0126 and taken for fixation and labeling at various times post-fertilization.

At a given time point, an aliquot of eggs (typically 1 ml) was quickly decanted twice in an Eppendorf tube, and NFSW was replaced by Ca²⁺-free artificial sea water (CaFSW: 484 mM NaCl, 10 mM KCl, 27 MgCl₂, 29 mM MgSO₄, 2.4 mM NaHCO₃, pH 8.2) just before fixation.

Actin filaments and chromatin were labeled using a method described by Mabuchi (1994) that was modified as follows. Embryos were fixed for 0.5–1 h at room temperature with 5% (v/v) formaldehyde in F-buffer (0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 10 mM MOPS buffer, pH 7.4). They were then incubated for 30 min in F-buffer containing 5% formalin, 0.8 M glucose and 0.2% NP40. Eggs were then washed with F-buffer containing 0.8 M glycerol (glycerol-F-buffer) and stuck on 0.1% polylysine or protamine-treated slide. Eggs were incubated for 30 min in blocking buffer (1% BSA in PBS–0.1% Triton X-100 (PBS-T)), and then stained for 3 min with 0.3 μM rhodamine-conjugated phalloidin (Rh-ph, Sigma) and 0.5 μg/ml Hoechst 33258 dissolved in PBS-T containing 0.1 mM β-mercaptoethanol in order to label actin filaments and chromatin.
chromosomes, respectively. Eggs were finally washed three times in PBS-T and mounted in Immuno-Mount (Shandon).

For microtubule labeling (De Nadai et al., 1998), fertilized eggs deprived of fertilization membrane were taken at different times after sperm addition and fixed for 1 h at room temperature with 2% (v/v) formaldehyde, 20 mM PIPES, 5 mM EGTA, 0.5 mM MgSO4 and 0.1% Triton X-100. Eggs were then rinsed in TBS-T, stuck on 0.1% polylysine coated slide, incubated for 1 h in blocking buffer and then overnight with the monoclonal rat anti-tubulin antibody YL1/2 (Chemicon) diluted 1/1000 in blocking buffer containing 1% donkey serum. After three rinses in TBS-T, eggs were incubated for 1 h with FITC- or TRITC-conjugated-donkey anti-rat IgG 1/250 (Jackson ImmunoResearch), rinsed again three times and treated with 0.5 Ag/ml Hoechst 33258. The eggs were finally rinsed three times in PBS-T and mounted between slide and coverslip in a homemade mounting medium containing an anti-bleach reagent.

A similar protocol was followed for anti-phospho-MAPK42/44 labeling, except that 20 mM beta-glycerophosphate, 2 mM Sodium Ortho-Vanadate and 4 mM NaF were added in the fixation buffer.

For conventional fluorescence microscopy, all microtubule and actin filaments observations were performed on a Nikon Eclipse TE300, using a ×20 plan fluor or a ×40 S fluor Nikon objectives. Pictures were taken on Kodak Gold 100 Asa films that were developed and then scanned using an Epson Perfection 2400 scanner. Final images were treated using Adobe Photoshop. Confocal microscopy was done on a Leica Laser Scanning Confocal Microscope (SP2) using a ×63 lens. 1024 × 1024 images were captured using the Leica Lite Software and edited with Adobe Photoshop.

**H1 kinase activity**

H1 kinase activity was measured on egg extracts following the same protocol as that described previously (Chiri et al., 1998; De Nadai et al., 1998). Histone H1 (Sigma type III-S) was used as substrate.

**Video microscopy**

In order to estimate the timing of mitosis, fertilized eggs treated or not with U0126 or PD 98059 were observed under bright-field illumination on an inverted microscope Leica DM IRB and images taken every 30 s. Eggs were maintained at 18°C under a slight and constant flow of refrigerated ASW. The time from nuclear envelope breakdown (NEB) to beginning of cytokinesis was referred as the duration of mitosis.

Fig. 2. Effect of U0126 and PD 98059 on ERK-like phosphorylation. Western blot analysis was performed after SDS – PAGE using the anti-phospho-MAPK42/44 antibody. (A) Effect of increasing concentrations of U0126 in unfertilized eggs after different treatment duration. The inhibitor was added at time zero. (B) Effect of 1 or 10 μM U0124 or U0126 on ERK-like phosphorylation. Unfertilized eggs were treated during 25 or 60 min with the drug. (C) Effect of 1 μM U0126 on ERK-like reactivation during mitosis. Eggs were treated or not (control) 30 min after fertilization with U0126 and arrested at various times after fertilization. (D) Effect of increasing concentrations of PD 98059 in unfertilized eggs treated for 30 min with the drug. (E) Effect of increasing concentrations of PD 98059 in fertilized eggs analyzed 6 min after fertilization or at time of mitosis (62 min). The inhibitor was added prior to sperm addition.
**MEK constructs, mRNA preparation and microinjection**

We used a Mek\textsuperscript{neg} expression plasmid containing the entire coding sequence of the K96M human MEK mutant (Mansour et al., 1994). The plasmid was constructed, and mRNA purified and injected in the presence of 0.12 M KCl as previously described (Fernandez-Serra et al., 2004).

**Results**

**A MEK/ERK pathway is transiently activated at time of mitosis**

In order to detect changes after fertilization in the active form of MAPK, we used a commercial monoclonal antibody against phospho-MAPK42/44 and performed Western blotting and immunofluorescence analysis.

Fig. 3. Localization of phospho-MAPK during mitosis by confocal imaging. (A) Control eggs. Eggs were observed before fertilization (a), at early prophase (b), later prophase (c), metaphase (d) or telophase (e). Eggs were double labeled with Hoechst 33258 (a\textsubscript{1}, a\textsubscript{1}', a\textsubscript{2}, a\textsubscript{2}') and with either an anti-tubulin antibody (a\textsubscript{V}1, b\textsubscript{V}1, c\textsubscript{V}1, d\textsubscript{V}1, e\textsubscript{V}1) or an anti-phospho-MAPK42/44 (a\textsubscript{V}2, b\textsubscript{V}2, c\textsubscript{V}2, c\textsubscript{V}3, d\textsubscript{V}2, e\textsubscript{V}2). PhosphoMAPK42/44 labeling was intense in the unfertilized egg (a\textsubscript{V}2), very weak at early prophase (b\textsubscript{V}2), then localized in the nuclear region (c\textsubscript{V}2), to centrosomes (c\textsubscript{V}3) and finally to the spindle at mitosis (d\textsubscript{V}2). Labeling disappeared before telophase (e\textsubscript{V}2). Scale bar: 50 \textmu m. (B) Effect of U0126. Unfertilized eggs (a) were treated by 5 \textmu M U0126 for 30 min, and fertilized eggs (b and c) were treated at 20 min post-fertilization and observed at metaphase (b) or at telophase (c), respectively. Eggs were labeled with Hoechst (a, b, c) or with the anti-phospho-MAPK42/44 (a\textsubscript{V}', b\textsubscript{V}', c\textsubscript{V}').
antibody raised against the dual phosphorylated form of human ERK1 and ERK2. On a Western blot of sea urchin extracts, it labeled a single band of 42 kDa that likely corresponds to the phosphorylated form of an ERK-like protein, since this signal was lost after treatment of the egg extracts either by alkaline phosphatase or by PD 98059, an inhibitor of MEK (Fig. 1A). The high level of this phosphorylated protein contained in the unfertilized egg progressively decreased after fertilization to become undetectable 20 min after fertilization (Figs. 1B and C). A slight increase in the amount of the phosphorylated form of this ERK-like protein occurred at the time of first and second mitosis (Fig. 1C). Results obtained from several experiments (Fig. 1D) establish that the level of transient phosphorylation of ERK at mitosis could reach in some experiments 20% of the level observed in the unfertilized egg, the mean increase ± SEM of 18 values obtained between 55 and 68 min was 10.0 ± 1.9%.

We investigated whether the activity of MEK, the MAPK kinase that is the ERK1/2 activator in most systems, was subject to the same variations after fertilization as that of the ERK-like protein described above. The anti-phospho-MEK antibody raised against the dual phosphorylated form of a mammalian MEK1/2 recognized a single band migrating around 45 kDa (Fig. 1E). This MEK-like protein was progressively dephosphorylated after fertilization to become poorly phosphorylated 20 min after fertilization (Fig. 1E). An increase of this signal then observed 50 min after fertilization, that remained elevated during mitosis and then decreased again after cleavage (Fig. 1E). The quantification of the MEK signal (Fig. 1E) shows that these variations correspond to those observed for the ERK-like protein (Fig. 1D).

**Effect of MEK inhibitors on ERK activation during the first cell cycle**

In order to analyze the role of the MEK-MAPK cascade during mitosis, we used two different MEK inhibitors, U0126 which inhibits both the active and the inactive forms of MEK1/2, and PD 98059 which only inhibits activation of inactive MEK (Davies et al., 2000). We determined the concentration necessary to cancel ERK-like phosphorylation. 1 μM U0126 was sufficient to rapidly turn this activity off in unfertilized eggs (Fig. 2A) and during mitosis (Fig. 2C). The inactive analog U0124 did not trigger dephosphorylation of the ERK-like protein, even when used at 10 μM, suggesting that the effects observed with U0126 are specific (Fig. 2B). 20-min treatment with 0.5 μM PD 98059 could also abolish this activity in unfertilized eggs (Fig. 2D). However, 15 μM (Fig. 2E) of PD 98059, depending on the batch of eggs, was necessary to obtain a complete loss of the activity in fertilized eggs.

**Localized activation of ERK in the fertilized sea urchin egg**

We then checked whether ERK activation by phosphorylation occurred homogeneously throughout the sea urchin embryo. Eggs were fixed at different times following fertilization and immunostained using the anti-phospho-MAPK42/44 antibody (Fig. 3A). Unfertilized eggs (Fig. 3Aa) were highly fluorescent, the cytoplasmic labeling appearing granular and the intranuclear space brightly stained (Fig. 3Aa). 30 min after fertilization, eggs reached prophase (Figs. 3Ab and Ac). Control eggs started to polymerize microtubules (Figs. 3Ab1 and c1) and to condense chromatin in the nucleus (Figs. 3Ab1, b2, c1, c2 and c3). The anti-phospho-MAPK signal was highly reduced at early prophase (Fig. 3Ab2) compared to that observed in unfertilized eggs. At later prophase (Fig. 3Ac), the cytoplasmic signal appeared less granular and faint, while a higher labeling accumulated close to nuclei (Fig. 3Ac2α and β) and then in the vicinity of the centrosomes (Figs. 3Ac2β and Ac3γ).

Control eggs were at metaphase 40 min after fertilization, showing alignment of condensed chromosomes (Figs. 3Ad1 and d2) on the bipolar mitotic spindle (Fig. 3Ad1). The central spindle and asters were labeled by the anti-phospho-MAPK antibody, whereas the periphery of the central spindle, which also contains a high density of microtubules in sea urchin embryos, was negative (Fig. 3Ad2). The cytoplasm surrounding the spindle at that stage still contained a low but significant granular staining.

Finally, control eggs reached the telophase stage 60 min after fertilization (Fig. 3Ae). A very low and diffuse
staining was obtained with the anti-phospho-MAPK antibody (Fig. 3e’2).

Unfertilized eggs treated for 30 min by U0126 showed a much reduced and diffuse fluorescent signal throughout the eggs even though some faintly stained granules could still be seen (Fig. 3Ba’). Only a very low and diffuse phospho-MAPK signal was seen in eggs treated by U0126 and observed 30 min (Fig. 3Bb’) or 60 min after fertilization (Fig. 3Bc’), times when eggs were at prophase (Fig. 3Bb) and telophase (Fig. 3Bc), respectively. Similar results were obtained using U0126 or PD 98059 (not shown). It is important to note that the intensity of general staining obtained in control eggs with the anti-phospho-MAPK antibody followed that obtained after Western blotting using this same antibody (Figs. 1B and C), that is, high in unfertilized eggs, very low at prophase, slightly increased during mitosis and undetectable at telophase. Taken together, our results show that ERK is transiently activated during sea urchin egg mitosis and that this activation mostly takes place at the spindle poles during prophase and prometaphase.

Effect of MEK inhibition on MPF activation

We analyzed whether the MEK inhibitors affected MPF activity. The MPF activity due to cdc2/cyclin B is often referred to as histone H1 kinase, since MPF shows a pronounced activity toward this substrate and is the main source of histone H1 kinase activity in eggs during mitosis (Meijer et al., 1989). We observed that H1 kinase activity during the first two mitosis was slightly increased when eggs were treated with 15 μM PD 98059 or with 5 μM U0126, concentrations that inhibited the ERK-like activity (Fig. 4). This increase in H1 activity corresponded to a greater decrease in the tyrosine phosphorylation status of cdc2 during mitosis (Fig. 4 inset), a decrease in phosphorylation of cdc2 corresponding to an increase in MPF activity (Ohi and Gould, 1999).

Role of MEK/ERK on mitotic spindle formation and cell division

The role of MAPK cascade during the first two division cycles was tested by treating eggs 30 min after sperm addition with concentrations of U0126 or PD 98059 that induce ERK-like dephosphorylation (Fig. 2). Similar altered morphologies were observed after both treatments. Compared to controls, treated embryos showed abnormal metaphase plates or incomplete cytokinesis, and formed two or three daughter cells of different sizes or with altered shapes (Fig. 5). Proportions of altered embryos are reported in Table 1. The proportion of altered embryos increased with the concentration of PD 98059. A very high proportion of embryos were abnormal after treatment with 15 μM PD 98059; almost all eggs divided (Fig. 5A) and formed morula, although with very altered morphologies, that finally died before reaching the gastrula stage (not shown). Treatment with 1 μM U0126 induced abnormal divisions (Fig. 5B) or mitotic arrest in a number of eggs (Table 1). Increasing U0126 concentration to 5 μM led to the increase in the proportion of non-divided eggs (Fig. 5B and Table 1). Video microscopy experiments allowed us to determine the timing of the first cell cycle. We observed that 15 μM PD 98059 or 5 μM U0126 significantly delayed entry in mitosis but slightly accelerated exit of mitosis (Table 2).

The abnormal divisions described above led us to investigate whether U0126 and PD 98059 altered the formation of the cleavage furrow. Examples of alterations are shown in Fig. 6. We observed that treated eggs normally formed a contractile ring of actin filaments between two sets of chromosomes that were either not as widely separated (Fig. 6B) as in the control experiment (Fig. 6A) or not separated at all (Figs. 6C and D). This probably led to the situation where chromosomes remained close enough to the cleavage plane and did not separate properly in a number of cases. Several furrows were also observed in a number of treated eggs that formed three or four sets of chromosomes. Those eggs with multiple furrows seemed to form lobes or cells of different sizes (Figs. 6E–G). No clear difference in the timing of formation of the actin ring was observed, suggesting that these alterations were not due to an accelerated or premature formation of the cleavage furrow.

We tested whether the smaller gap between the two sets of chromosomes seen at the time of furrow formation in PD 98159 or U0126 treated eggs (Fig. 6) was due to an altered mitotic spindle. Eggs were treated in the presence of these inhibitors 30 min after fertilization and fixed for microtubule and chromatin staining at the normal time of anaphase in control eggs. In those conditions, we observed the same percentage of treated eggs as that reported above showing chromosomes that were not equally distributed in two sets and several types of aberrant mitotic spindles (Fig.
Some eggs showed two distinct sets of chromosomes, but one of the two sets appeared separated (Figs. 7B1 and C1), which could lead to the formation of a tripolar spindle (Fig. 7C2). Some other eggs contained four sets of chromosomes (Fig. 7D1), separated on a tetrapolar mitotic spindle (Fig. 7D2). Finally, a number of eggs showed one or a few chromosomes lagging out of the normal sets of chromosomes (Figs. 7D1, E1, F1 and G1). In some cases, bipolar spindles were formed but were made either of unequal half spindles and with a poorly defined main axis, suggesting a loose connection between half spindles (Figs. 7E2 and F2), or with an extra lobe containing extra microtubules (Fig. 7G2). Similar morphological alterations were obtained with U0126 and PD 98059, at a higher frequency in PD 98059-treated eggs, and no alteration was observed when eggs were treated with the inactive analog U0124 (not shown).

Higher resolution micrographs, obtained after confocal imaging, of some alterations seen after fertilization in U0126 treated eggs, are shown in Fig. 8. Two very altered


Table 1

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<th>PD 98059</th>
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<tr>
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<td>2.5 μM, n = 6</td>
<td>1 μM, n = 5</td>
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<td></td>
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<td>5 μM, n = 6</td>
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<tr>
<td>Abnormal divisions (%)</td>
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<tr>
<td>Non-divided eggs (%)</td>
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<td>Mortality (%)</td>
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<td>Total (%)</td>
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Eggs were treated with MEK inhibitors 30 min after fertilization and observed under light microscopy. Results are mean ± SEM; 15–57 eggs were counted in each experiment, n representing the number of experiments.

Discussion

The data provided here in sea urchin embryos show that a MEK/ERK-like cascade is transiently activated at the time of mitosis. Moreover, we show that the activated ERK pool is located close to the nucleus then to the mitotic spindle. The specific inhibition of this cascade leads to mitotic defects that strongly implicates a MEK-ERK cascade in the formation of the mitotic spindle and in the attachment of chromosomes during the early embryo mitotic cell cycles.

The unfertilized sea urchin egg contains a highly phosphorylated ERK-like protein

The fact that unfertilized eggs contain a highly phosphorylated ERK-like protein that was gradually dephosphorylated during the first minutes following fertilization (this paper and Carroll et al., 2000; Chiri et al., 1998; Kumano et al., 2001) fits with the idea that maintaining MAPK activity at a high level in the unfertilized egg blocks DNA synthesis. Indeed, a decrease in this activity with MEK inhibitors leads to progression into S phase in starfish oocytes (Abrieu et al., 1997; Fisher et al., 1998; Tachibana et al., 1997) and in the sea urchin eggs of *S. purpuratus* (Carroll et al., 2000). In contrast, Philipova and Whitaker (1998) observed a low MAPK activity in unfertilized *L. pectus* eggs that was followed by an increase after fertilization. These authors may not have detected MAPK contained in the nucleus (Carroll et al., 2000) or in the cortex (Walker et al., 1997) of sea urchin eggs since they followed a protocol described by Shibuya et al. (1992) and used soluble fractions that may lack nuclei and insoluble cytoskeleton elements for Western blots and immunoprecipitations. This could also be explained if these authors have detected another MAPK in their study. As a matter of fact, these authors reported very recently that a ERK1-like MAPK, which should then be different from the ERK that we detect here, was required for S phase onset in sea urchin embryos (Philipova et al., 2005).

A small pool of ERK-like protein is activated during mitosis

Only a minor fraction of total ERK-like activity is transiently activated at time of mitosis and is located in close vicinity to the mitotic spindle in the sea urchin egg of *P. lividus* (this paper) and in some other cell types (Cha and Shapiro, 2001; Shapiro et al., 1998; Wang et al., 1997; Willard and Crouch, 2001; Zecevic et al., 1998). This could explain why MAPK activity has not been detected at the time of mitosis in oocytes of clam (Shibuya et al., 1992) or even other species of sea urchin (Carroll et al., 2000; Kumano et al., 2001).
al., 2001). The changes in levels of MAPK activity just before or during mitosis could be a way to control exit from mitosis during the first cell cycles of the early embryo, at least in sea urchin, starfish and *Xenopus*. We observed that treatment of fertilized eggs with 20 μM wortmannin, which leads to arrest in prometaphase-like state (De Nadai et al., 1998), inhibited ERK-like dephosphorylation normally seen at exit of mitosis (results not shown and Chiri et al., 1998). This sustained MAPK phosphorylation can then be correlated with arrest at mitosis during the first embryonic cell cycle in sea urchin as reported in *Xenopus* (Chau and Shibuya, 1998; Haccard et al., 1993; MacNicol et al., 1995; Sagata et al., 1989) and starfish (Abrieu et al., 1997).

**Specificity of PD 98059 or U0126**

We believe that PD 98059 or U0126, used at concentrations as low as 1 μM and never exceeding 15 μM or 5 μM, respectively, inhibited specifically the MEK/ERK-like pathway while U0124 had no effect, considering the data reported by Davies et al. (2000). These concentrations are in the same range as those used by Carroll et al. (2000), and very low if compared to the 50-μM U0126 used in somatic cells (Horne and Guadagno, 2003) or the 200-μM PD 98059 used in *Xenopus* extracts (Guadagno and Ferrell, 1998). The fact that only a very low percentage of cells was blocked in mitosis by PD 98059 or U0126 corroborates the data reported by Kumano et al. (2001). Increasing the concentrations of PD 98059 or U0126 at levels as high as 50 μM could indeed lead either to eggs arrested at pseudometaphase showing one or a few unaligned chromosomes and an unusually small mitotic spindle, or to divided eggs showing alterations very similar to our present observations (Pesando et al., 1998, 1999). Indeed, 100 μM U0126 was necessary to inhibit DNA synthesis in sea urchin embryos (Philipova et al., 2005). However, such high concentrations of PD 98059 or U0126 might act on other MAPK cascades or act non-specifically on other proteins that may have an MBP kinase activity, since several myc or MBP kinases can be detected in the sea urchin egg and are activated during mitosis (Chiri et al., 1998; Walker et al., 1997). Finally, we observed that dominant-negative MEK human constructs had a greater effect than the inhibitors, comparing the percentage of altered eggs. This could be due to the fact that MEK inhibitors were added 30 min after fertilization to a population of eggs, where diffusion in eggs, access to MEK, and sensitivity to the drugs may differ between eggs. These limiting factors cannot be taken into account in dominant-negative MEK experiments, where all eggs were injected before fertilization. However, we cannot rule out non-specific effects of human proteins in the sea urchin embryo.

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**Fig. 6.** Effect U0126 or PD 98059 on chromosomes separation and actin ring formation. 5 μM U0126 or 15 μM PD 98059 was added 30 min after fertilization and eggs were taken 30 min later for fixation and labeling as described in Materials and methods. Chromosomes (upper picture) and actin (lower picture) were labeled with Hoechst and rhodamine phalloidin, respectively. (A) An anaphase in control eggs. (B–G) Different phenotypes that were commonly observed in U0126 (B, C, E) or PD 98059 (D, F, G) treated eggs. Scale bar: 100 μm.
Fig. 7. Effect of PD 98059 or U0126 on mitotic spindle formation and chromosome separation. A1 to A6 show control eggs that were taken at time of early anaphase (1,2), late anaphase (3,4) and early telophase (5,6), labeled with Hoechst (1,3,5) or with an anti-tubulin antibody (2,4,6), and observed with a light microscope. Eggs were treated 30 min after fertilization with either 10 μM PD 98059 (B, F, G) or with 5 μM U0126 (C, D, E) and taken at different times of mitosis for labeling with Hoechst (1) or with anti-tubulin antibody (2). Scale bar: 50 μm.
Fig. 8. Examples of alterations in U0126 treated eggs observed by confocal imaging. 5 μM U0126 was added 20 min after fertilization and eggs taken for fixation at metaphase (a/a' and b/b') or at telophase (c/c'). Eggs were labeled with Hoechst (a, b, c) or with an anti-tubulin antibody (a', b' and c') and observed by confocal imaging. Very altered spindles were seen at metaphase (a' and b'), while the two daughter cells observed at telophase showed lots of decondensed and dispersed chromosomes (c). Scale bar: 50 μm.

Fig. 9. Effect of injection of Mek<sup>neg</sup> on first mitosis. Embryos are observed 2 or 3 h after injection with Mek<sup>neg</sup> or not (control eggs non-injected or injected with KCl only). (A) Different types of altered divisions seen in eggs injected with MEK mutants. (B) The percentage of altered embryos. 27, 43 and 56 embryos were counted after 2 h in control embryos, embryos injected with KCl buffer, or injected with Mek<sup>neg</sup>, respectively. 22, 21 and 62 embryos were counted after 3 h in control embryos, embryos injected with KCl or injected with Mek<sup>neg</sup>, respectively.
Role of ERK-like in mitotic events

The association of the phosphorylated ERK-like with the centrosomes and the mitotic spindle in the early sea urchin embryo corroborates various data obtained in somatic cells (Shapiro et al., 1998; Wang et al., 1997; Willard and Crouch, 2001). The different defects induced by the MEK inhibitors, such as poorly defined mitotic axis, anarchic microtubule organization within the spindle, extra arrays of microtubules and poor chromosome separation, suggest that an ERK-like cascade acts at different levels of mitotic spindle formation. By being associated with the cortex (Walker et al., 1997), this ERK-like could regulate the mitotic spindle–cortex interactions important for spindle orientation and anaphase B. This could explain why we frequently observed spindles that were not properly centered and formation of two daughter cells of different sizes in eggs treated by MEK inhibitors. Moreover, daughter cells might contain an abnormal number of chromosomes, since a few chromosomes were not properly aligned on the metaphase plate in many cases, which could lead to the delay of cell division that accumulated as the embryos developed and to asynchronous divisions within embryos, giving rise to stages three or five (Fig. 5). The occurrence of multipolar spindles (Fig. 6) also contributed to the formation of three or four cells of unequal sizes and of unequal content in chromosomes. The association of phosphorylated ERK-like with the centrosomes suggests a role of this MAPK in the regulation of centrosome activity. MEK inhibitors could induce centrosome/spindle poles to split, which would explain the formation of tri- or tetrapolar spindles in these conditions. Finally, almost all eggs divided, even though the number of actin rings was variable and a function of the number of microtubule asters (spindle poles or extra microtubule arrays). These results suggest that the ERK-like activity is not involved in the induction of the cleavage furrow or of the actin ring formation. Other proteins that also have an MBP kinase activity and have been identified in the cortex of the sea urchin egg (Walker et al., 1997) could be involved in the triggering of cleavage furrow formation (Shuster and Burgess, 2002a,b).

Relations MPF/MAPK during mitosis

All these results strongly suggest that an ERK-like contributes to regulate mitotic spindle structure and function in the early embryo similarly to somatic cells. These effects cannot be due to inhibition of MPF activity normally stimulated during mitosis, since treatment with U0126 or PD 98059 did not inhibit MPF but on the contrary led to a slight increase in this activity during mitosis. This is in agreement with the results obtained by Murakami et al. (1999) and Walter et al. (2000) who showed that the MAPK pathway regulates cdc2 phosphorylation and thus MPF activation through Wee1. The activation of ERK-like seems then to be necessary to ensure normal progression through mitosis in the early embryo, but not essential to entry or exit mitosis. This fits with results obtained in Xenopus extracts where MAPK activity is not needed to enter or exit mitosis but is required for normal mitotic progression (Guadagno and Ferrell, 1998; Takenaka et al., 1997). Finally, similar alteration of the length of mitosis and no arrest of mitosis were observed in tissue culture cells where M phase ERK activity was blocked (Roberts et al., 2002).

Is an ERK-like part of a spindle checkpoint mechanism in the early sea urchin embryo?

Our results strongly suggest that an ERK-like cascade is part of a mechanism that controls the formation of the mitotic spindle and the attachment of chromosomes to the mitotic spindle during the first mitosis of the sea urchin embryo. In somatic cells and in cycling Xenopus extracts, various data show a role of a MEK/ERK pathway in the spindle checkpoint mechanism (review by Maller et al., 2002). Several points argue for the existence of a similar mechanism in the early sea urchin embryo. Firstly, phosphorylated ERK-like associates with the centrosomes and the spindle at mitosis. Secondly, alterations of the mitotic spindle and chromosomes attachment in PD 98059 or U0126-treated eggs that we report here look like those observed in somatic cells lacking the mitotic checkpoint proteins Mad2 (Dobles et al., 2000) or Bub1 (Taylor and McKeon, 1997). Preliminary Western blotting analysis using anti-Bub1 and -Bub3 polyclonal antibodies showed that these proteins are present in P. lividus embryos (unpublished results), and it would be interesting to investigate whether these proteins are active during cleavage stages or not, and whether other checkpoint proteins are expressed in the early sea urchin embryo. Thirdly, only a minority of mitoses were abnormal when ERK-like was inhibited, which indicates that this MAPK might only be required in case of mitosis defects, as opposed to being an intrinsic part of mitosis. However, the existence itself of a spindle checkpoint in the sea urchin early embryo has been questioned. Mitosis is prolonged when the zygotes are induced to form monopolar spindles or when the spindle is cut into two half spindles (Sluder and Begg, 1983), which is in favor of this hypothesis. On the contrary, unattached or misoriented chromosomes do not induce arrest of division (Rieder et al., 1995; Sluder et al., 1994), and duration of second mitosis is not altered in embryos containing tripolar or tetrapolar spindles (Sluder et al., 1997), which rather suggests that no spindle checkpoint was functional. One possibility is that an ERK-like is part of a mechanism that is not a “spindle checkpoint” stricto sensus, since it is not able to stop division as in somatic cells in case of a single misoriented chromosome or other...
spindle defects, and some component of this checkpoint could be either absent or inactive. Another possibility is that a real spindle checkpoint mechanism exists in the early sea urchin embryo, but the inhibitory activity produced by an unattached kinetochore either remains local or diffuses in a large cytoplasmic volume containing large asters and a high microtubule density, which would render inefficient this activity. This is in agreement with the hypothesis where the spindle checkpoint is regulated by a mechanism that depends on the nucleo-cytoplasmic ratio (Maller et al., 2002), ratio that then allows an active mechanism in somatic cells or in Xenopus egg extracts supplemented with a high concentration of nuclei per microliter of cytoplasm (Guadagno and Ferrell, 1998; Minshull et al., 1994; Takenaka et al., 1997). It would be interesting to investigate whether the inhibition of the mos/MEK/ERK pathway leads in dividing Xenopus embryos to the same alterations of mitotic events than those we report here. However, the dependency on the nucleo-cytoplasmic ratio of the spindle checkpoint mechanism in Xenopus embryos was questioned a few years ago by Clute and Masui (1997).

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References


