In vivo effects of microinjected alkaline phosphatase and its low molecular weight substrates on the first meiotic cell division in Xenopus laevis oocytes

(2-glycerophosphate/phosphorytrosine/maturation-promoting factor/meiosis)

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ABSTRACT  Xenopus laevis oocytes were microinjected with low molecular weight phosphoesters such as 2-glycerophosphate, phosphorytrosine, phosphoserine, phosphothreonine, 4-nitrophenyl phosphate, and orthophosphate. These compounds were able to induce a considerable reduction in the time course of progesterone-induced maturation, with 2-glycerophosphate being the most effective. The basal level of cAMP and its drop during maturation were not affected by the microinjection of 2-glycerophosphate. The injection of alkaline phosphatase (EC 3.1.3.1.) from calf intestine at a low concentration (10 ng per oocyte) was able to decrease or abolish the effect of 2-glycerophosphate. At higher concentration (25 ng per oocyte) this enzyme totally blocked progesterone- or maturation-promoting factor-induced maturation. Alkaline phosphatase might behave in vivo as a phosphoprotein phosphatase active towards phosphorytrosine-containing proteins. In addition, our results indicate that phosphatase or phosphoester-containing buffers should be avoided in the course of maturation-promoting factor purification.

The maturation-promoting factor (MPF) was originally detected as an activity present in the cytoplasm of maturing amphibian oocytes—i.e., oocytes that are resuming the first meiotic cell division (reviewed in ref. 1). When a small amount of mature cytoplasm (50 nl) is transferred into an immature diplotene-stage arrested oocyte, germinal vesicle (oocyte nucleus) breakdown (GVBD) is observed in <2-3 hr. When immature oocytes are treated with progesterone (1 μM), MPF activity is detectable shortly prior to the breakdown of the nuclear envelope. Although existence of MPF has been known for more than a decade, its biochemical nature has not yet been elucidated. MPF can be extracted from mature oocytes and stabilized by low molecular weight phosphate compounds such as 2-glycerophosphate in the presence of MgATP and EGTA (2). It is a polypeptide since proteolytic digestion totally destroys its biological activity. On the other hand, a burst of cAMP-independent protein phosphorylation occurs in vivo when MPF activity becomes detectable in maturing oocytes (3-5). Such indirect evidence suggest that MPF activity is linked to a phosphorylation/dephosphorylation cascade.

The mechanisms leading to the appearance of active MPF in the oocyte cytoplasm are poorly understood. Strong experimental evidence indicates that cAMP, the catalytic (C) and regulatory (R) subunits of the cAMP-dependent protein kinase, and protein phosphatase 1 play a major role in the formation of MPF after progesterone action. Mallar and Krebs (6) have shown that microinjection of R subunit into oocytes provokes the breakdown of the nuclear envelope in the absence of hormonal stimulation, whereas the injection of C subunit inhibits or delays the appearance of MPF in progesterone-treated oocytes. These experiments were confirmed by microinjection of the pure heat-stable protein kinase inhibitor: it also induces the maturation in the absence of hormone (6, 7). More recently, it was shown that the inhibitor 1 (8) and inhibitor 2 (9) of protein phosphatase 1 are able to inhibit the progesterone-induced maturation. It was therefore postulated that a putative phosphoprotein, substrate for both C subunit and protein phosphatase 1, must be dephosphorylated in order to induce the formation of active MPF (7, 8, 10). The increase in protein phosphorylation associated with MPF activity can be clearly distinguished from cAMP-dependent protein phosphorylation (10, 11).

It is therefore of major importance to identify the protein kinases or phosphatases (or both) involved in MPF activity. Recently, in an attempt to purify MPF, we have observed that its activity can be stabilized by adding adenosine 5′-([γ-thio]) triphosphate to the extraction and dissolution buffers (12). It is known that protein kinases, in the presence of 5′-[γ-thio] triphosphate convert their substrates into thiosphorylated forms, which are less accessible to the action of phosphoprotein phosphatases (13). However, addition of 5′-[γ-thio] triphosphate was not sufficient in itself to stabilize MPF activity. The presence of 2-glycerophosphate (50 mM) was always required to maintain in vitro active MPF (12). These results can be interpreted in two ways. Either the MPF extract contains 2-glycerophosphate-inhibited protein phosphatase, which readily hydrolyzes in vitro thiosphorylated proteins, or 2-glycerophosphate itself modifies the oocyte steady-state level of protein phosphorylation when it is microinjected, possibly by inhibiting endogenous protein phosphatases.

We therefore decided to investigate the role of the different components of the MPF buffers on MPF activity. We now report that 2-glycerophosphate, as well as other phospho compounds, facilitates the first meiotic cell division and, in some cases, induces it in the absence of hormonal treatment. This effect is abolished by injection of alkaline phosphatase (EC 3.1.3.1) at low concentration. At higher concentrations, alkaline phosphatase totally blocks progesterone- and MPF-induced maturation.

MATERIAL AND METHODS

Animals. Xenopus laevis adult females (Service régional des Amphibiens, Centre National de la Recherche Scientifique, France) were maintained under laboratory conditions (16°C, natural light).

Abbreviations: MPF, maturation-promoting factor; GVBD, germinal vesicle breakdown; GVBD 50, 50% GVBD.

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Materials. [8-3H]cAMP (30 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. Progesterone (pregn-4-ène-3,20-dione), theophylline, cAMP, purified cholera toxin, 2-glycerophosphate (disodium salt), phosphoserine (O-phospho-L-serine), phosphothreonine (O-phospho-DL-threonine), and phosphotyrosine (O-phospho-DL-tyrosine) were from Sigma. Glycerol and sodium phosphate were from Merck and 3-isobutyl-1-methylxanthine from Aldrich. 1-Glycerophosphate (dicyclohexyl ammonium salt) and 4-nitrophenyl phosphate were from Boehringer Mannheim. Collagenase type IA, alkaline phosphatase from Escherichia coli type III R (30–60 units/mg), and bovine serum albumin were from Sigma; dispase grade II and alkaline phosphatase from calf intestine (2500 units/mg) were from Boehringer Mannheim; cAMP-dependent protein kinase inhibitor from bovine brain was a gift from J. G. Demaille (Montpellier, France).

Oocyte Preparation. Ovaries were surgically removed from animals anesthetized with MS 222 (1 g/liter, Sandoz) and transferred to medium A (88 mM NaCl/0.33 mM Ca(NO3)2/1 mM KCl/0.41 mM CaC12/0.82 mM MgSO4/2 mM Tris-HCl, pH 7.4). Ovarian fragments were digested with dispase and collagenase as described (7). After extensive washing, defolliculated, full-grown [1.2–1.4 mm in diameter, stage VI (14)] oocytes were carefully selected under a dissecting microscope. Under these conditions, when defolliculated oocytes were kept at 16°C, they remained responsive to progesterone or MPF treatment for at least 3–4 days.

Oocyte Microinjection. Protein kinase inhibitor was prepared in 5 mM 2-morpholineethanesulfonic acid buffer (pH 7.0) containing albumin (1 mg/ml). Alkaline phosphatase was dialyzed for 18 hr against 5 mM Heps buffer (pH 7.5) containing albumin (1 mg/ml) and diluted in the same buffer. Each oocyte was microinjected at the equator level with 1/10 its volume—i.e., 50 nl in stage VI oocyte. Microinjected solutions yield intracellular concentrations diluted 1/10, assuming a diffusion compartment of about 0.5 μl. All experiments were performed 24 hr after oocytes preparation, unless otherwise indicated. For the MPF microinjections, animal pole cytoplasm taken from oocytes matured in the presence of progesterone (1 μM) was microinjected into recipient oocytes (15 nl per oocyte), at the equator level.

Oocyte Maturation. The criterion for maturation was the appearance of a white maturation spot surrounded by a pigmented ring at the animal pole of the oocyte. GVBD was ascertained by the absence of the germinal vesicle determined by dissection of the oocyte after 5 min of fixation in 10% trichloroacetic acid.

For cytological analysis, oocytes were fixed in Smith’s solution, dehydrated, and embedded in paraffin. Serial sections (7–10 μM) were stained with Feulgen reagent/light green.

cAMP Determination. Usually 4 oocytes were manually homogenized quickly in 500 μl of boiling sodium acetate buffer (50 mM, pH 4) containing 1 mM theophylline. The tubes were spun in a vortex, boiled for 4 min, and then centrifuged at 4°C (24,000 × g, 15 min). The supernatant was used for the cAMP assay. The cAMP content of the extracts was determined by a binding assay method (15). All buffers contained theophylline (1 mM). A standard curve for cAMP was used for each assay (0.2–5 pmol). All of the experimental points were assayed in duplicate and the values reported are the mean of duplicate determinations on oocytes from the same females.

RESULTS

2-Glycerophosphate Shortens the Kinetics of Oocyte Maturation. Oocytes were microinjected with 50 nl of a 50 mM solution of 2-glycerophosphate in distilled water adjusted to pH 7.5. They were treated 0, 30, 60, and 120 min later with progesterone (1 μM). In all cases, the times necessary to obtain 50% GVBD (GVBD 50) were shortened as compared to the uninjected control oocytes; a 60-min preincubation period was sufficient to obtain an optimal response (Fig. 1A). The same effect was observed when 48 hr–cultured oocytes were microinjected with 50 mM 2-glycerophosphate (Fig. 1B). Microinjection of 50 nl of NaCl (100 mM) solution had no effect. When 2-glycerophosphate solutions in Tris, 2-morpholineethanesulfonic acid, and Hepes buffer (5 mM) were used, the kinetics of maturation were again shortened in a similar manner. The maximal advance for GVBD 50 was obtained when the pH was adjusted in the range of pH 7.5–8. The addition of bovine serum albumin (1 mg/ml) to the 2-glycerophosphate solution did not significantly modify the kinetics of maturation. These experiments were repeated by using oocytes >16 different females. In all instances, the microinjection of 2-glycerophosphate accelerated the progress of progesterone-induced maturation. As shown in Fig. 1 Inset, 2-glycerophosphate is more efficient on slowly maturing oocytes than on rapidly maturing oocytes. The effect of 2-glycerophosphate is dose-dependent (Fig. 2). A cytological analysis was performed on 2-glycerophosphate- and progesterone-treated oocytes. It showed that the breakdown of the germinal vesicle and the condensation of the chromosomes had occurred normally. Furthermore, 2 hr after the forma-

![FIG. 1. Effect of 2-glycerophosphate microinjection. Thirty oocytes from the same female were microinjected (50 nl per oocyte) with 2-glycerophosphate (50 mM) dissolved in distilled water and adjusted to pH 7.5. After 1 hr of incubation in medium A, oocytes were exposed to progesterone (1 μM) until maturation. The kinetics of maturation determined in control (a) and 2-glycerophosphate-microinjected (c) oocytes. (A) Experiments performed 24 hr after oocyte preparation; (B) experiments performed 48 hr after oocyte preparation. (Inset) The ratio (R, time for GVBD 50 in microinjected oocytes versus time for GVBD 50 in control oocytes) was plotted as a function of GVBD 50 in control oocytes prepared from 16 different females. Experiments were performed 24 hr after oocyte preparation.](image-url)
Fig. 2. Dose-dependent effect of 2-glycerophosphate. Groups of 10 oocytes were microinjected with 2-glycerophosphate at the indicated concentrations. After 1 hr of incubation, maturation was induced by 1 μM progesterone. The time corresponding to GVBD 50 was determined for each group from the kinetic curves of maturation.

Fig. 3. Kinetics of MPF appearance. One hundred oocytes were microinjected (50 nl per oocyte) with 2-glycerophosphate solution (50 mM). After 1 hr of incubation, maturation was induced by addition of 1 μM progesterone. The kinetics of maturation of control (○—○) and 2-glycerophosphate-microinjected (■—■) oocytes were determined. The amount of transferable MPF in control (●—●) and 2-glycerophosphate-microinjected (□—□) oocytes was determined by transfer experiment: at the indicated time, 10 oocytes were punctured and their cytoplasm (≈15 nl) was microinjected into 10 untreated recipient oocytes. MPF activity was expressed as the number of recipient oocytes that underwent GVBD.

The possibility that 2-glycerophosphate directly or indirectly modifies the cAMP level in the oocytes was tested. The basal level of cAMP was identical to 2-glycerophosphate-treated and control oocytes. In a typical experiment, the cAMP content was 1.4 pmol per oocyte in untreated oocytes versus 1.5 and 1.7 pmol per oocyte 1 and 2 hr, respectively, after 2-glycerophosphate microinjection. The drop in the level of cAMP induced by progesterone was similar in 2-glycerophosphate-treated and in control oocytes (Table 1).

Table 1. Effect of 2-glycerophosphate on the progesterone-induced drop in the level of cAMP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP content, % of control</th>
<th>Experiments, no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>74.1 ± 5.8</td>
<td>5</td>
</tr>
<tr>
<td>2-Glycerophosphate</td>
<td>99.7 ± 3.3</td>
<td>7</td>
</tr>
<tr>
<td>2-Glycerophosphate with progesterone</td>
<td>76.3 ± 4.4</td>
<td>4</td>
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</table>

Oocytes were preincubated for 4 hr in the presence of 6 mM cholera toxin and 1 mM 3-isobutyl-1-methylxanthine to magnify cAMP changes. They were then incubated for 1 hr under the following conditions: without treatment; in the presence of 1 μM progesterone; after microinjection of 50 nl of a 50 mM 2-glycerophosphate solution; and in the presence of progesterone just at the time of 2-glycerophosphate injection. cAMP content is given as the mean ± SEM; results are expressed as the % of control value without treatment, except in the case of 2-glycerophosphate with progesterone, in which control refers to 2-glycerophosphate alone.
progesterone stimulation. Under these conditions, the enzyme injection was found to inhibit maturation. As shown in Fig. 5, a dose of 10 ng of microinjected enzyme (=200 nM intracellular concentration, assuming an uniform diffusion in the water volume of the oocyte) inhibits about 50% maturation. A similar result was obtained by using oocytes of 3 different females. The oocytes that did not resume meiosis remained apparently healthy during >10 hr following alkaline phosphatase and progesterone treatment as judged by the presence of a normal germinal vesicle observed after cyto logical analysis. This inhibition was dose dependent (Fig. 6).

When alkaline phosphatase was microinjected 4 hr before progesterone treatment, the dose of enzyme necessary to inhibit 50% of maturation was decreased by one-half (Fig. 6). This result indicates that the in vivo action of the enzyme is time dependent.

At higher concentration (25 ng), alkaline phosphatase totally blocked progesterone-induced maturation; however, in some cases, the migration of the germinal vesicle toward the animal pole was observed.

Alkaline phosphatase from E. coli was tested also at the same activity as calf intestine phosphatase (assayed at 20°C, pH 7.5, with 4-nitrophenyl phosphate as substrate). When 55 ng (activity corresponding to 5 ng of calf enzyme) was microinjected 4 hr before progesterone treatment, we have observed 50% inhibition.

Alkaline phosphatase was also efficient in blocking MPF-induced maturation. Oocytes were microinjected with 25 ng of enzyme and incubated for 1 hr at room temperature. They then were microinjected with cytoplasm of mature donor oocytes. No maturation occurred, in contrast to control injected oocytes.

A MPF-containing cytoplasmic extract was prepared in the absence of 2-glycerophosphate as described by Wasserman and Masui (16). It was treated with alkaline phosphatase (5 μM) at 0°C for 30 min and injected into oocytes. No maturation was observed.

**DISCUSSION**

When alkaline phosphatase is microinjected into the Xenopus oocyte at a concentration of about 0.2 μM (final intracellular concentration), the breakdown of the nuclear envelope is blocked. Conversely, 2-glycerophosphate, phosphotyro sine, and other low molecular weight substrates of alkaline phosphatase facilitate or even induce GVBD. These results raise at least three questions: (i) What are the endogenous substrates of alkaline phosphatase? (ii) Does an endogenous phosphatase with a substrate specificity similar to that of al-

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**Fig. 4.** Effect of the microinjection of different low molecular weight phosphate esters. For each compound, 30 oocytes were microinjected [50 nl of a 25 mM solution (pH 7.5) containing albumin at 1 mg/ml]. After 1 hr of incubation (arrow), maturation was induced by progesterone (1 μM). A progesterone control was obtained in each case; the kinetics of control maturations were similar. 
•, Control; ○, 2-glycerophosphate; □, phosphotyrosine; △, phosphoserine; △, phosphothreonine; △, 4-nitrophenyl phosphate; and ◇, orthophosphate.

**Fig. 5.** Effect of alkaline phosphatase on the kinetics of maturation of 2-glycerophosphate-injected and control oocytes. Kinetics of maturation were followed in untreated oocytes (●), oocytes microinjected with 50 mM 2-glycerophosphate (○), oocytes microinjected with 2 μM alkaline phosphatase (▲), and oocytes microinjected with 2 μM alkaline phosphatase and 50 mM 2-glycerophosphate (△). Batches of 10 oocytes were used. Progesterone (1 μM) was added 1 hr after microinjection.

**Fig. 6.** Dose-dependent inhibition of maturation after microinjection of alkaline phosphatase. Groups of 10 oocytes were microinjected with different concentrations of alkaline phosphatase. Progesterone (1 μM) was added after 1 hr (○) or 4 hr later (▲). GVBD was scored after incubation for 18 hr.
In vivo function of alkaline phosphatase remains unknown [a wide variety of low molecular weight phosphate esters can serve, in vitro, as substrates (17)]; its activity is known to change dramatically during altered cell growth. It was recently reported that an alkaline phosphatase, at low concentrations (50 nM) and neutral pH, dephosphorylates *in vitro* proteins containing phosphotyrosines (18). Much higher concentrations of this alkaline phosphatase are needed to dephosphorylate phosphoserine or phosphothreonine residues. Therefore, microinjected alkaline phosphatase might hydrolyze either low molecular weight phosphate esters present in the oocyte or phosphorylated proteins (or both).

The presence of free phosphorylated amino acids has not been reported in *Xenopus* oocytes, although their amino acid pool has been studied extensively (19). The possibility that a free phospho amino acid plays a key role in the mechanism of maturation cannot be totally excluded. Recently, Fukami and Lipmann (20) reported that a high level of phosphotyrosine is present in *Drosophila* larvae and that a phosphotyrosine phosphatase controls its hydrolysis. We have, to date, no evidence supporting such a possibility in *Xenopus* oocytes.

The most logical and simple explanation of our findings is that alkaline phosphatase dephosphorylates one or more endogenous phosphoproteins of the *Xenopus* oocytes. If this assumption is correct, one may postulate that the putative endogenous protein(s) substrate of exogenous alkaline phosphatase must be maintained under its phosphorylated form during the phosphorylation cascade involved in the formation of MPF; this phosphorylated protein might be MPF itself. On the other hand, after microinjection, alkaline phosphatase is diluted inside the oocyte to a low concentration (0.1–0.2 μM) at physiological pH. Under these conditions, it may act as a phosphotyrosine protein phosphatase (18).

The hypothesis that a phosphoprotein, perhaps a protein phosphorylated on tyrosine, is involved in MPF activity is reinforced by the phosphotyrosine and 2-glycerophosphate microinjection experiments (Fig. 4). The simplest explanation of these findings is that all of the microinjected phosphoesters inhibit more or less completely the same endogenous phosphoprotein phosphatase. The inhibition of this phosphatase activity would be necessary to generate transferable MPF activity. Little is known about endogenous phosphoprotein phosphatases of the *Xenopus* oocyte. Braquet has reported the presence of an alkaline phosphatase in both the cytoplasm and germinal vesicle of *Rana* oocytes (21). It was shown recently that "P-labeled P-histones are dephosphorylated when microinjected into *Xenopus* oocytes (22). This indicates the presence of active protein phosphatases in the living oocyte. However, these *in vivo* experiments do not provide information about the specificity of the phosphatase activities. Nevertheless, the phosphatase inhibited by 2-glycerophosphate and other phosphoesters can be distinguished from protein phosphatase 1. In fact, when protein phosphatase 1 is inhibited by the proteic inhibitor 1 or 2 (8, 9), the formation of MPF and, consequently, the maturation are blocked, whereas 2-glycerophosphate facilitates maturation. Our results indicate that two different phosphatase activities are involved during oocyte maturation: (i) protein phosphatase 1, which dephosphorylates a putative maturation protein substrate of CAMP-dependent protein kinase (6, 7, 10), and (ii) a phosphoprotein phosphatase with a substrate specificity similar to that of alkaline phosphatase at neutral pH. The hypothesis that it might be a phosphotyrosine phosphatase must be tested since we have found that proteins containing phosphotyrosine were present in the membranes of mature oocytes (unpublished results).

The present results raise an important technical point concerning the purification of MPF. In fact, we demonstrate in this article that all of the buffers used to prepare MPF, containing either 2-glycerophosphate or orthophosphate, interfere *in vivo* with the mechanism of MPF formation. Therefore, the claim for MPF enrichment would be more convincing if 2-glycerophosphate was omitted from the microinjection buffers. This does not mean that "purified" MPF does not contain active protein factor(s), as shown by dilution curves (2, 12, 23), but these factors, when tested in the presence of 2-glycerophosphate, may only be related indirectly to MPF.

In conclusion, we have shown that purified alkaline phosphatase exhibits an in vivo biological activity; it probably acts as a phosphoprotein phosphatase to block the first meiotic cell division of the *Xenopus* oocyte. Our microinjection experiments have established that alkaline phosphatase inhibits both *in vitro* and in vivo MPF activity. MPF activity has been reported to be present not only in mature oocytes of vertebrates and invertebrates (1) but also to exist in the G2 → M transition of cycling somatic cells (24). The finding that exogenous alkaline phosphatase blocks nuclear envelope breakdown in *Xenopus* oocytes may be of general significance for the elucidation of an essential step in the cell cycle.

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