

Involvement of Protein Phosphatases 1 and 2A in the Control of M Phase-promoting Factor Activity in Starfish

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Abstract. Specific inhibition of types 1 and 2A protein phosphatases by microinjection of okadaic acid (OA) into starfish oocytes induced germinal vesicle breakdown and activation of M phase-promoting factor (MPF) and histone H1 kinase. The effects were evident in immature oocytes arrested at first meiotic prophase as well as in fully mature oocytes arrested at the pronucleus stage. In addition, MPF and histone H1 kinase were stabilized for several hours and protected from inactivation by inhibition of type 1 protein phosphatases with either OA or specific anti-phosphatase antibodies. Microinjection of okadaic acid was associated with unusual changes of the microtubule network, including the disappearance of spindles and extension

of the cytoplasmic array of microtubules. MPF activation after OA injection was associated with dephosphorylation of phosphothreonine and phosphoserine residues in *cdc2*, showing that neither type 1 nor 2A protein phosphatases catalyzes these dephosphorylations. The effects of OA on MPF activation and inactivation appeared to involve the cyclin subunit. OA did not induce MPF activation in the absence of protein synthesis and it prevented degradation of cyclin. Therefore protein phosphatases types 1 and 2A appear to be involved in activation and inactivation of MPF involving mechanisms that operate after cyclin synthesis and before its degradation.

ENTRY into and exit from M phase of the cell cycle are controlled by changes in protein phosphorylation. These changes are attributed to fluctuations in the activity of a mitotic kinase, also called MPF¹ (maturation-promoting factor, see Lohka, 1989 for review). Homologues of a yeast mitotic kinase encoded by the *cdc2* gene were shown to be components of MPF (Gautier et al., 1988; Dunphy et al., 1988; Arion et al., 1988; Labbé et al., 1988a, 1989b). More recently, MPF was shown to be a heterodimer containing one molecule of *cdc2* and one molecule of cyclin (Labbé et al., 1989a,b).

It has been known for a long time that protection against protein phosphatases is helpful to maintain MPF activity in crude extracts (Drury et al., 1978; Wu and Gerhart, 1980; Hermann et al., 1985). Microinjection into starfish oocytes of α -naphthyl-phosphate, a broad spectrum phosphatase inhibitor, was found to increase by ~50-fold the activity of the mitotic kinase (Labbé et al., 1988b) and to trigger entry into M phase (Pondaven and Meijer, 1986). It was shown that okadaic acid (OA), a tumor promoter that acts as a specific inhibitor of protein phosphatase types 1 and 2A (Bialojan and Takai, 1988; Hescheler et al., 1988; Haystead et al.,

1989) induced MPF activation when injected into *Xenopus* oocytes (Goris et al., 1989).

However, an inhibitor of type 1 phosphatase was found to slow posttranslational activation of MPF that accompanies the first meiotic division in *Xenopus* oocytes (Cyert and Kirschner, 1988). Moreover, both in vivo and in vitro experiments have shown that MPF is deactivated by phosphorylation and activated by dephosphorylation of its *cdc2* subunit (Labbé et al., 1988a, c, 1989b; Dorée et al., 1989; Gautier et al., 1989).

These results were of great interest because they suggested that phosphorylation could both increase and decrease MPF activity. This led us to investigate carefully the effects of microinjecting OA at various stages of meiotic maturation and early embryogenesis on the cell cycle progression in starfish.

When used at a concentration sufficient to block protein phosphatases, OA was found to have two effects on MPF: it both induced its activation and prevented its inactivation. The specific effects of OA were confirmed by microinjecting specific antibodies directed against type 1 phosphatase. The results fit together nicely and extend to higher eukaryotes recent genetic investigations that show genes encoding type 1 protein phosphatase act pleiotropically upstream and downstream of mitosis in fission yeast and in *Aspergillus* (Ohkura et al., 1989; Booher and Beach, 1989; Doonan and Morris, 1989).

1. *Abbreviations used in this paper:* a.h.a., time after hormone addition; GVBD, germinal vesicle breakdown; MPF, M phase-promoting factor; OA, okadaic acid; 1-MeAde, 1-methyladenine.

Materials and Methods

Animals and Oocytes

The starfishes *Marthasterias glacialis* and *Astropecten aranciatus* and the sea cucumber *Holothuria tubulosa* were collected near Banyuls, France. Prophase-arrested oocytes were prepared free of follicle cells by washing them several times in artificial Ca^{2+} -free sea water and finally transferred to natural sea water. In starfish, meiotic maturation was induced with $1 \mu\text{M}$ 1-methyladenine (1-MeAde; Kanatani et al., 1969); in the sea cucumber, it was induced with 5 mM DTT applied for 10 min.

Antibodies

Polyclonal antibodies raised against the peptide EGVPTSTAIRESLLKE, corresponding to the conserved region of cdc2 and against a fragment of the catalytic subunit of rabbit skeletal muscle type-1 phosphatase, have been described previously (Lee and Nurse, 1987; Brautigam et al., 1986).

Microinjections

Microinjections were performed according to Hiramoto (1974). MPF activities were monitored by transferring cytoplasm (usually 150-200 pl) into G2-arrested oocytes of the starfish *Marthasterias glacialis*.

Immunofluorescence and DNA Staining

Immunofluorescence and DNA staining as previously described (Picard et al., 1988).

Histone H1 Kinase Activities

Groups of microinjected oocytes (usually five oocytes) were rapidly washed (<10 s) in an excess of buffer containing 50 mM beta-glycerophosphate, 15 mM EGTA, 10 mM MgCl_2 , and 0.7 mM DTT at pH 7.3; then the groups of oocytes were taken in a small volume of buffer (5 oocytes in 2 μl) and frozen in liquid nitrogen. Immediately after thawing, which disrupted the oocytes, an identical volume of a mixture containing 100 μM gamma- ^{32}P ATP (1,200 cpm/pmol; Amersham International, Amersham, UK) 10 mM MgCl_2 , and 2 mg/ml H1 histone (Boehringer, Mannheim, FRG) was added. After a 5-min incubation at 21°C, the reaction was stopped by addition of a mixture containing 30% urea-8% DTT-5% SDS (wt/vol) in 0.5 Tris-HCl, pH 6.8, and then the proteins were separated by SDS-PAGE (Laemmli, 1970). Finally parts of the gels corresponding to H1 histones were cut and counted by liquid scintillation.

Pulse-labeling of Cyclin

Oocytes of the starfish *Astropecten aranciatus* were induced to mature with 1-MeAde. At the time of germinal vesicle breakdown (GVBD), the oocytes were pulse-labeled for 20 min in 10 mCi/ml carrier-free ^{35}S methionine (Amersham International). Then a chase was performed with 10 mM cold methionine. At the selected times, groups of oocytes were fixed in Laemmli's buffer and proteins separated by SDS-PAGE. Autoradiography was performed with dried gels, using X-OMAT-AR films (Eastman Kodak Co., Rochester, NY).

^{32}P -labeling and Immunoprecipitations

Oocytes of the starfish *Astropecten aranciatus* were preloaded for 3 h with 5 mCi/ml carrier-free ^{32}P (Amersham International) then rinsed before hormonal stimulation or OA microinjection. Groups of 20 oocytes were suspended in 1 ml of immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1 mg/ml soybean trypsin inhibitor, 1 mM PMSF, 50 mM NaF, 10 mM Na pyrophosphate, and 0.1 mM Na orthovanadate) and frozen in liquid nitrogen. After thawing, which disrupted the oocytes, samples were centrifuged for 30 min at $10^5 g$. Then 10 μl of an antiserum directed against cdc2 was added to the supernatant. After 2 h incubation at 4°C, protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) was added and, after 1 h, the immune complexes were collected by centrifugation, washed four times with immunoprecipitation buffer and twice with 50 mM Tris 7.5, solubilized in sample buffer, and heated at 95°C for 4 min. Then proteins were separated by SDS-PAGE. Gels were stained, dried, and processed for autoradiography.

Analysis of Labeled Phosphoaminoacids

This was carried out as described by Cooper et al. (1983). Briefly, the gel band was cut out after SDS-PAGE and hydrolyzed for 1 h in 6 N HCl at 110°C, and the extract was dried under vacuum. The amino acids were extracted by ion exchange chromatography on Dowex AGI-X8, eluted with 0.1 N HCl, and concentrated by lyophilization. After addition of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine, they were analyzed by two-dimensional thin-layer electrophoresis on a 100- μm cellulose plate (E. Merck, Darmstadt, FRG) with migration at pH 1.9 (1,500 V for 25 min) in the first dimension and at pH 3.5 (1,500 V for 25 min) in the second dimension. The position of standards was determined by ninhydrin staining and radioactivity was scored by autoradiography.

Results

Microinjection of OA in Starfish Oocytes Arrested at First Meiotic Prophase

When OA was microinjected to give an estimated intracellular concentration $<0.4 \mu\text{M}$, no obvious cytological events were observed and the oocytes remained arrested with an intact germinal vesicle. No increase in histone H1 kinase activity was detected. In contrast, in oocytes microinjected with OA to an intracellular concentration of $1.2 \mu\text{M}$, histone H1 kinase was activated ~ 30 -fold and MPF activity was produced (Table I). Recipient oocytes underwent GVBD 15 min after microinjection of OA (Fig. 1 A), as observed when meiosis reinitiation is induced by hormonal stimulation under physiological conditions. However, when release from prophase block was due to OA microinjection rather than hormonal stimulation, there were major differences in other responses.

In 1-MeAde-stimulated oocytes, an increased microtubule-nucleating activity associated with the formation of the meiotic asters was observed starting ~ 5 min after hormone addition (5 min a.h.a) and the cytoplasmic network of microtubules faded away progressively. By 45 min a.h.a., the condensed chromosomes had aligned on the metaphase plate of the first meiotic spindle (Fig. 1, D and E), then the spindle rotated and chromosomes separated. By 70 min a.h.a., the first meiotic cleavage had occurred, at which time MPF activity was no longer detected. Finally, the unfertilized, hormone-stimulated oocytes arrested without MPF activity after running the second meiotic cell cycle and forming the female pronucleus (data not shown; see Picard et al., 1988).

In contrast, although MPF activity was produced, no meiotic asters were formed in oocytes injected with OA. The network of microtubules did not fade away, but rather seemed to extend further. No meiotic spindle was formed, and the condensed chromosomes remained dispersed (Fig. 1, B and C). Polar bodies were not emitted and both histone H1 kinase and MPF activities kept a high level for at least 5 h (Table I). Therefore MPF activity was induced by either OA or 1-MeAde, and GVBD resulted, but the further consequences of the inducing agents were different. In particular, microinjection of OA to inhibit protein phosphatases prevented reorganization of microtubules into a spindle and blocked MPF inactivation.

Microinjection of OA into Hormone-stimulated Oocytes and Early Embryos

In other experiments, oocytes were released from prophase

Table I. Effect of OA Microinjection at Various Stages of Meiotic Maturation on H1 Histone Kinase and MPF Activities of Starfish Oocytes

Stage of OA injection	OA concentration μM	H1 histone kinase*		MPF†	
		After 15 min	After 5 h	After 15 min	After 5 h
Prophase I	0	1	1	Not detected	Not detected
	0.4	1	1	Not detected	Not detected
	1.2	30	32	+++	+++
Metaphase I	0	25	1.2	+++	Not detected
	0.4	28	1.5	+++	Not detected
	1.2	30	29	+++	+++
Pronucleus	0.4	1.3	1.3	Not detected	Not detected
	1.2	32	30	+++	+++

* H1 histone kinase activities are normalized to activity in unstimulated oocytes arrested at first meiotic prophase = 1. (The actual activity in such oocytes was ~ 2 fmol P transferred per min and per oocyte).

† Successful induction of meiotic maturation occurring in 100% of the recipient oocytes injected with 200 pl of cytoplasm (volume of an oocyte: ~ 2 nl) is indicated by +++.

block by hormone (1-MeAde) stimulation and then OA was microinjected at various times after GVBD.

At first meiotic metaphase, microinjection of OA induced the disassembly of the meiotic spindle, and oocytes arrested despite having high histone H1 kinase and MPF activities (Table I). Several hours after microinjection a starburst array of microtubules was observed around the condensed chromosomes (Fig. 2).

When OA was injected after the second meiotic cleavage (either just after the female pronucleus had formed or after its migration towards the center of the egg), it induced a rapid nuclear envelope disassembly (Fig. 3) and the activation of both histone H1 kinase and MPF (~ 10 min after the microinjection). Nuclear envelope never reformed subsequently, and both MPF and histone H1 kinase activities remained at high level for hours (Table I). This implicates the *in vivo* action of protein phosphatases type 1 and 2A in nuclear envelope assembly and MPF inactivation.

Finally, OA was microinjected into a single blastomere at the four-cell stage during early development. The microinjected blastomere arrested with condensed chromosomes and with microtubules polymerized in a similar way as in unfertilized microinjected eggs (Fig. 4) and MPF activity was kept at a constant high level (data not shown).

In all these experiments, OA was microinjected at an intracellular concentration calculated to be $1.2 \mu\text{M}$. In parallel experiments no effects were observed when the intracellular concentration was $0.4 \mu\text{M}$.

Microinjection of Antibodies Affinity-purified against Type 1 Protein Phosphatase

The catalytic subunit of type 1 protein phosphatase is one of the most conserved enzymes in eukaryotes, as recently emphasized by the near identity of the *Aspergillus* phosphatase to the mammalian protein (Doonan and Morris, 1989). Types 1 and 2A phosphatases are homologous, but distinct, with only $\sim 50\%$ identity in sequence. Previously, a trypsin-resistant fragment of the rabbit skeletal muscle type-1 phosphatase ($M_r = 33$ kD), the most highly conserved portion of the subunit, was used to immunize sheep, then used to

affinity purify the antibodies from hyperimmune sera (Brautigan et al., 1985). The antibodies neutralized only type 1, not 2A, phosphatase activity and also were type 1 specific in Western immunoblotting (Brautigan et al., 1986). Because starfish have both type 1 protein phosphatase and its specific inhibitory protein, each of which show full cross-reactivity with their mammalian counterparts (Pondaven and Cohen, 1987), we expected the anti-type 1 antibodies to neutralize the starfish type 1 phosphatase. Indeed, when these antibodies were microinjected either into G2-arrested starfish oocytes or into maturing oocytes at metaphase I they delayed MPF inactivation considerably and increased duration of meiotic metaphase from 30 min to several hours. Thus, neutralizing only type 1 phosphatase was sufficient to delay or prevent exit from M phase. However, microinjection of these antibodies did not release oocytes from prophase block, so neutralization of only type 1 phosphatase was not sufficient to drive starfish oocytes into M phase. Presumably both types 1 and 2A phosphatases must be inhibited, e.g., by OA. Nonetheless, microinjecting the same antibodies into oocytes of the sea cucumber *Holothuria tubulosa* not only inhibited MPF inactivation, but readily drove them into M phase (Table II). This shows that at least in some species, simply blocking type 1 phosphatase is sufficient to activate MPF.

Microinjection of OA in Emetine-arrested Starfish Oocytes and Unfertilized Sea Urchin Eggs

Under physiological conditions, accumulation of newly synthesized cyclin after its proteolysis at anaphase is required for entry into M phase (Murray and Kirschner, 1989; Minshull et al., 1989; Picard et al., 1985, 1987). We investigated whether cyclin synthesis also was required for OA to induce entry into M phase. Maturing starfish oocytes were treated with 0.1 mM emetine, an inhibitor of protein synthesis that arrested them after the first meiotic cleavage. At this point the oocytes had a well-formed nucleus that contained condensed chromosomes, which suggests that protein synthesis is required in starfish for chromosome decondensation, as already reported for mouse oocytes (Hashimoto and Kishi-

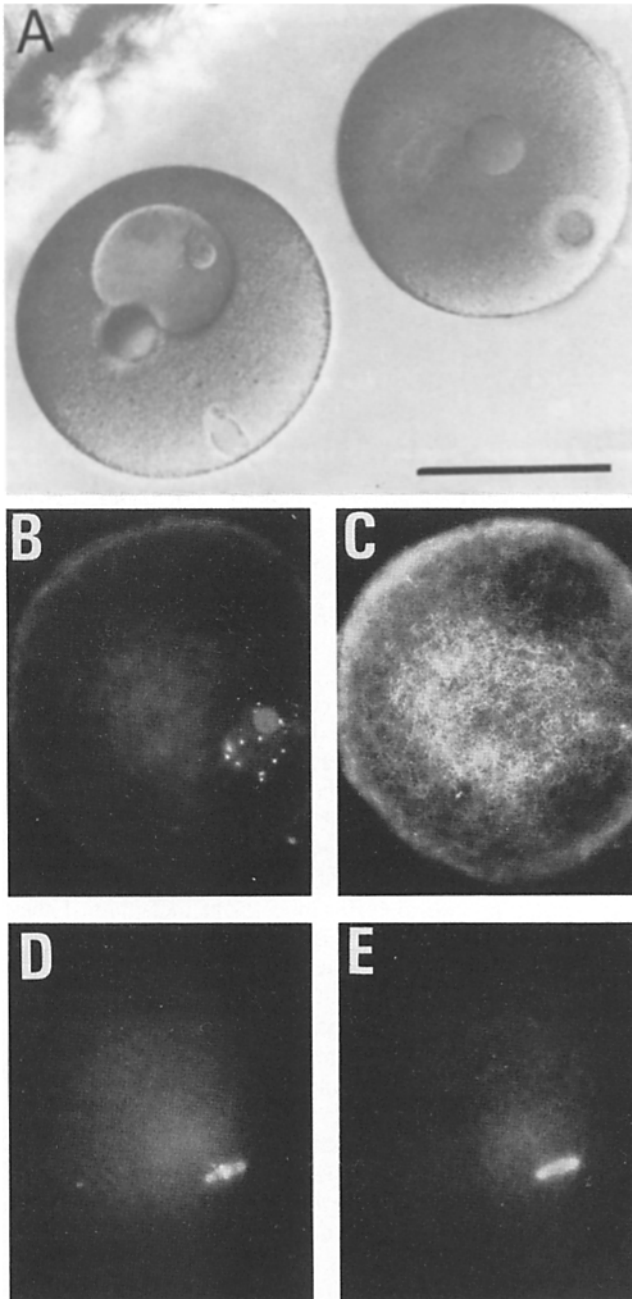


Figure 1. Effects of OA microinjection on prophase-arrested starfish oocytes. (A) Oocytes were microinjected with OA to a concentration of $1.2 \mu\text{M}$ (right) or with its vehicle (10% DMSO, left). The picture was taken 30 min after microinjection: GVBD obviously has occurred only in the oocyte on the right, with OA. Samples were microinjected together with a droplet of oil to avoid contact with sea water. (B) The OA injected oocyte contained dispersed chromosomes 30 min after GVBD, as shown by staining DNA with Hoechst dye. (C) A dense network of cytoplasmic microtubules in the OA-injected oocytes was shown by immunofluorescence with anti-tubulin antibodies. In contrast, when oocytes were released from prophase block by hormonal stimulation and fixed 30 min after GVBD, chromosomes were found to be aligned on the metaphase plate (D, stained with Hoechst dye) of a typical spindle (E, stained with anti-tubulin). Bar, $100 \mu\text{m}$.

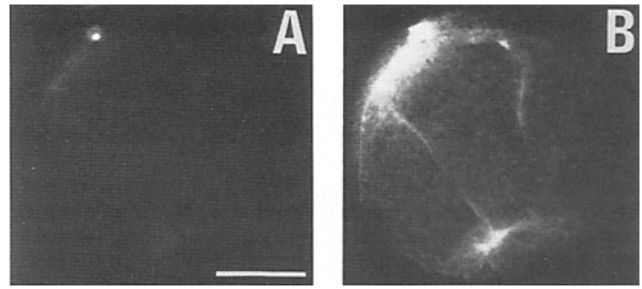


Figure 2. Long term effects of OA microinjected at first meiotic metaphase in hormone-stimulated oocytes. Oocytes were fixed 2 h after microinjection, stained with Hoechst dye (A) and the network of microtubules observed by immunofluorescence (B, same oocyte as in A). The chromosomes did not separate and remained clustered superficially. The spindle disappeared and the microtubules formed an array that seems to be mainly superficial in a lateral view (B). Bar, $50 \mu\text{m}$.

moto, 1988). Then the oocytes were microinjected with OA (to $1.2 \mu\text{M}$). No MPF activation was induced and the microinjected oocytes remained arrested (Fig. 5).

The unfertilized sea urchin egg stops synthesizing proteins, due to its acidic intracellular pH (Grainger et al., 1979), and as a consequence it lacks cyclin (Meijer et al., 1989), which is synthesized only after fertilization. This contrasts with the unfertilized starfish egg whose intracellular pH remains high after completion of meiotic maturation (Peaucellier et al., 1987) and thus continues to synthesize cyclin (Standart et al., 1987). Okadaic acid failed to induce entry into M phase when microinjected into unfertilized sea urchin eggs (data not shown). This at first appears at variance with successful induction of entry into M phase in starfish eggs arrested at the same cytological state. However, neither emetine-arrested starfish oocytes nor unfertilized sea urchin eggs synthesize cyclin and both failed to respond to microinjection of OA. Therefore, cyclin synthesis appeared to be required for a response to OA.

OA Induces *cdc2* Dephosphorylation and Prevents Cyclin Degradation

MPF activation has been shown to be associated with net dephosphorylation of *cdc2* under physiological conditions in both starfish and *Xenopus* oocytes (Labbé et al., 1989b;

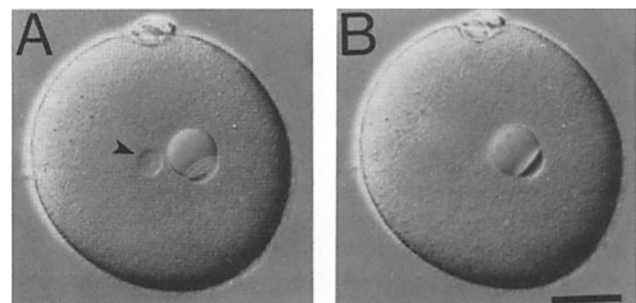


Figure 3. Nuclear envelope breakdown and entry into M phase of unfertilized starfish eggs at the pronucleus stage is triggered by OA. The pronucleus (A, arrowhead) is still intact 5 min after microinjection of OA but by 20 min (B) after microinjection, nuclear envelope breakdown occurred. Bar, $40 \mu\text{m}$.

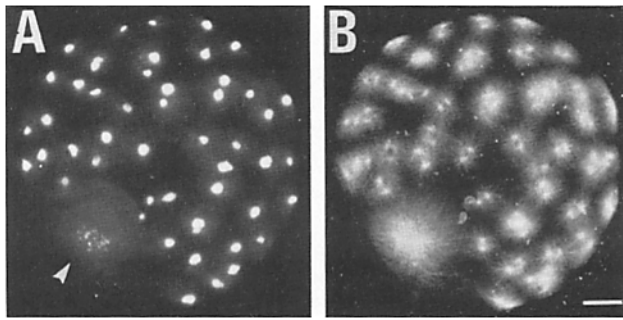


Figure 4. Effect of OA on a blastomere during early embryogenesis. A blastomere at the four-cell stage was microinjected with 1.2 μ M OA and 3 h later the embryo was fixed and processed either for Hoechst staining (A) or antitubulin immunofluorescence (B). The microinjected blastomere (arrowhead) remained arrested with condensed chromosomes and a cytoplasmic array of microtubules. There are 48 other nuclei seen with normal patterns of staining, showing that the 3 noninjected blastomeres had divided four times each. The embryo was fixed when control (noninjected) blastomeres were in prophase. Bar, 20 μ m.

Doré et al., 1989; Gautier et al., 1989). To investigate whether the same correlation was observed when MPF activation was triggered by microinjecting OA, 20 oocytes of the starfish *Astropecten aranciacus* (this species was selected because of large (300 μ m) diameter of the oocytes) were labeled with 32 P and microinjected with OA. At the time of GVBD, they were homogenized and immunoprecipitation was performed using anti-cdc2 antibodies.

As already reported, cdc2 was phosphorylated in control, noninjected oocytes. The radioactivity was found to be associated with threonine and serine residues after acid hydrolysis. No phosphotyrosine was detected (Fig. 6 c). As shown on Fig. 6 a, OA microinjection triggered cdc2 dephosphorylation, as observed under physiological conditions. OA was also found to induce cdc2 dephosphorylation when injected at the pronucleus stage (Fig. 6 b). This presents quite a paradox, where microinjection of a potent phosphatase inhibitor results in enhanced dephosphorylation of a target protein.

We wondered whether cyclin degradation was affected in oocytes microinjected with OA, because MPF activity was

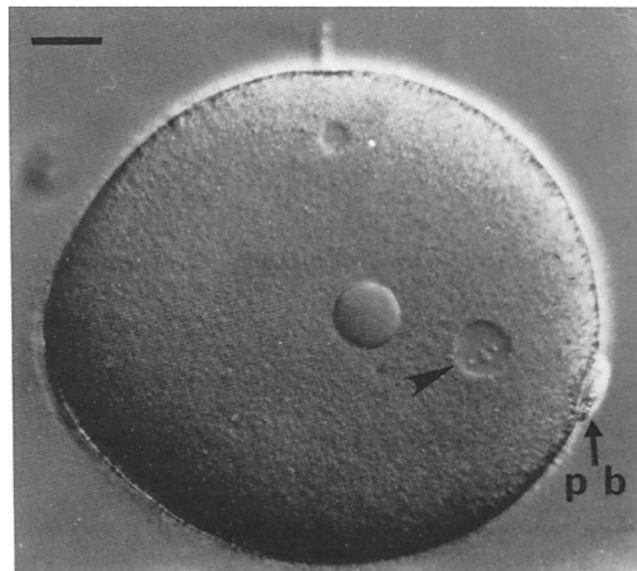


Figure 5. Microinjection of OA fails to induce entry into M phase when oocytes are arrested after first meiotic cleavage by blocking protein synthesis. Oocytes were induced to reinitiate meiosis by hormonal stimulation and treated with 10^{-4} M emetine at the time of GVBD. After first polar body (pb) emission, a nuclear envelope reformed around the still condensed chromosomes (arrowhead). Microinjection of OA at a concentration of 4 μ M (together with an oil droplet) 10 min after the nucleus had formed failed to drive the cell into M phase, even after 65 min, at which time this picture was taken. Bar, 20 μ m.

stabilized. As shown in Fig. 7, pulse-labeled cyclin escaped proteolysis and remained undegraded for hours in oocytes injected with OA at the time of GVBD. The prolonged activation of MPF could be correlated to the retarded degradation of cyclin.

Discussion

Our results show that microinjection of OA at various stages of oocyte meiotic maturation and early starfish embryogenesis has two principal effects: it induces MPF activation and

Table II. Effect of Microinjecting Affinity-purified Antibodies Directed against Type 1 Phosphatase (PP1) on MPF Activation and MPF Stabilization

Species	Stage at injection	Injection solution	MPF activity at the indicated times from microinjection (min)							
			15	30	45	75	120	180	210	240
<i>Marthasterias glacialis</i>	Prophase 1	(Anti-PP1)	-	-	-	-	-	-	-	-
	Metaphase 1	Anti-PP1	+	+	+	+	+	+	+	-
		Control	+	+	+	-	-	-	-	-
<i>Holothuria tubulosa</i>	Prophase 1	Anti-PP1	+	+	+	+	+	+	+	-
		Control	-	-	-	-	-	-	-	-
	Metaphase 1	Anti-PP1	+	+	+	+	+	+	+	-
		Control	+	+	+	+	-	-	-	-

Oocytes of the starfish *Marthasterias glacialis* or of the sea cucumber *Holothuria tubulosa* were injected either with 120 μ l of a 1-mg/ml solution in PBS of affinity-purified sheep immunoglobulins against type-1 phosphatase (anti-PP1) or with 120 μ l of PBS (*Marthasterias* control) or PBS containing or not 1 mg/ml unspecific sheep anti-rabbit immunoglobulins (*Holothuria* control). Injections were performed either in oocytes arrested at prophase 1 or 40 min after stimulation with 1 μ M 1-MeAde (*Marthasterias*) or 5 mM DTT (*Holothuria*), when oocytes had reached metaphase 1. Groups of three to five oocytes were checked for MPF activity at the indicated times after microinjection. Successful and unsuccessful induction of GVBD in recipient oocytes are respectively indicated (+) and (-).

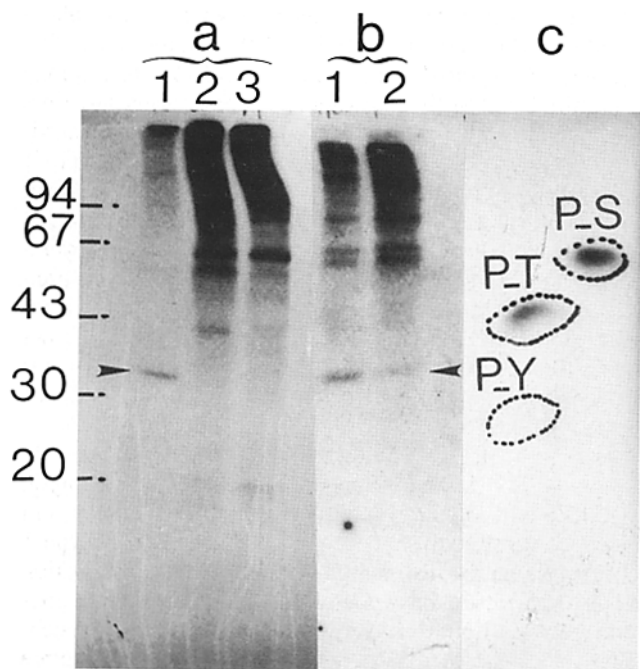


Figure 6. Changes in *cdc2* phosphorylation after OA microinjection. For each experiment 20 oocytes of the starfish *Astropecten aranciatus* were preloaded for 3 h with 5 mCi/ml ^{32}P inorganic phosphate, then rinsed before hormonal stimulation or OA microinjection. Oocytes were processed for *cdc2* immunoprecipitation, gel electrophoresis, and autoradiography as described under Materials and Methods. In *a* (lane 1) *cdc2* (arrow at 34 kD) was phosphorylated in phosphatase-blocked oocytes and dephosphorylated at GVBD (lane 3) in hormone-stimulated oocytes. Injection of OA did not prevent dephosphorylation of *cdc2* at GVBD (lane 2). The same amount of *cdc2* was present in each lane, based on Western immunoblotting (not shown) so the samples differed in the extent of *cdc2* dephosphorylation. In hormone-stimulated oocytes (*b*) that were arrested at the pronucleus stage *cdc2* was again phosphorylated (lane 1), but was dephosphorylated within 15 min after OA was microinjected at that stage (lane 2). The background of labeled proteins coimmunoprecipitated with *cdc2* increases when the M phase-specific kinase is activated (when *cdc2* is dephosphorylated). Phosphoamino acid residues in *cdc2* that was immunoprecipitated from prophase-arrested oocytes (as in lane 1 in *a*) were analyzed after acid hydrolysis (*c*). Dotted circles show the position of the phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) standards visualized by ninhydrin staining. Only ^{32}P -labeled P-S and P-T were detected.

prevents MPF inactivation. Furthermore, OA had profound effects on the dynamics of microtubules, preventing formation of meiotic or mitotic asters and spindles and instead stabilizing a cytoplasmic network.

OA is known as a potent inhibitor of protein phosphatases types 1 and 2A, and both of these enzymes have been detected in starfish oocytes (Pondaven and Cohen, 1987). Various reports have indicated that OA inhibits both these protein phosphatases with ID_{50} of 1–500 nM. Reportedly phosphatase type 2A is inhibited at lower concentrations of OA than phosphatase type 1 (Bialojan and Takai, 1988; Hescheler et al., 1988; Goris et al., 1989; Haystead et al., 1989; Ishihara et al., 1988). Furthermore, the concentration of OA required to inhibit phosphatase 2A is dependent on the concentration of enzyme (Cohen et al., 1989). Therefore, it appears that

OA may act as a tightly bound inhibitor that combines with phosphatase type 2A in a stoichiometric complex. If this is the case, then one explanation of our experiments is an intracellular concentration of protein phosphatase 2A in oocytes of >0.4 , but $<1.2 \mu\text{M}$. However, inhibition of phosphatase 2A alone is probably not sufficient to induce MPF activation. At micromolar concentrations, OA will inhibit type 1 as well as type 2A phosphatases. Involvement of type 1 phosphatase in MPF activation is evidenced by our observation that antibodies specific for neutralizing type-1 phosphatase could activate MPF in the sea cucumber. Because MPF activation in starfish was induced by OA, but not by anti-type 1 phosphatase antibodies, we conclude that both type 1 and 2A phosphatases need to be inhibited in this species. It is not known whether this is also the case for *Xenopus* oocytes, in which OA induces MPF activation at first meiotic prophase (Goris et al., 1989). Regardless, inhibition of only the type 1 protein phosphatase, either with OA or with anti-phosphatase antibodies, appeared sufficient to block inactivation of MPF.

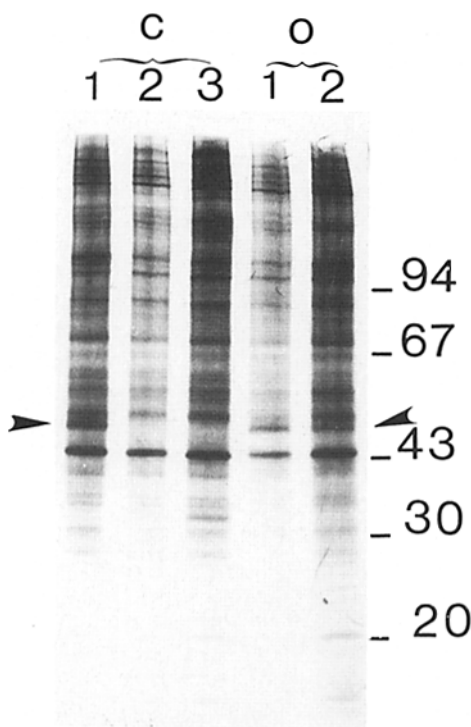


Figure 7. OA microinjection prevents cyclin B from degradation. Each experiment used five oocytes of the starfish *Astropecten aranciatus*, that were induced to mature by hormone stimulation. At GVBD, the oocytes were pulse-labeled for 20 min in 10 mCi/ml carrier-free ^{35}S methionine. Then a chase was performed by adding 10 mM unlabeled methionine. Oocytes were immediately injected either with okadaic acid (lanes *O1* and *O2*) or its solvent (10% DMSO : lanes *C1*, *C2*, and *C3*). In this experiment, GVBD, first polar body emission, second polar body emission, and pronucleus formation occurred 30, 100, 150, and 180 min after hormonal stimulation, respectively. At 80 min, *C1* and *O1* were fixed and ^{35}S -labeled proteins were separated by electrophoresis. The arrow points to cyclin B that appears as the lower band of a doublet, just above $M_r = 43$ kD. At 105 min, the cyclin in sample *C2* had disappeared and at 240 min cyclin was missing from *C3* but was still present in OA-injected oocytes (lanes *O2*) long after its degradation in control oocytes.

These results agree nicely with genetic evidence that a gene encoding a type 1 phosphatase was able to induce G2 arrest when expressed at a high copy number in the fission yeast (Booher and Beach, 1989), whereas mutants of *Aspergillus* that is defective in type 1 phosphatase are unable to complete mitosis (Doonan and Morris, 1989). Thus, protein phosphatases serve to prevent MPF formation and cause its inactivation. Therefore, phosphatase inhibitors like OA can induce MPF formation and prolong its activation.

OA also caused distinctive alterations in microtubule reorganization in maturing oocytes and early starfish embryos. The complete absence of meiotic or mitotic asters and spindles implicates the action of type 1 and 2A protein phosphatases in the assembly of these structures. Anomalous spindles have also been described in *Aspergillus* mutants defective in a type 1 phosphatase (Doonan and Morris, 1989). Additionally, one might speculate that protein phosphatases (acting on MAPs?) drive depolymerization of cytoplasmic microtubules. Inhibition of the phosphatases then tips the dynamic balance of polymerization and depolymerization and a network of elongated microtubules accumulates.

Current knowledge about the structure and activation of MPF and our results here present a fundamental paradox: inhibition of protein phosphatases induces formation of active MPF, a process that appears now to clearly involve dephosphorylation. The *cdc2* homologue in higher eukaryotes is an integral part of MPF. In both starfish and amphibian oocytes, *cdc2* undergoes dephosphorylation at the time of its activation after hormonal stimulation, and becomes phosphorylated again after completion of meiotic maturation when cells enter G1 (Labbé et al., 1989b; Dorée et al., 1989; Gautier et al., 1989). These phosphorylation-dephosphorylation reactions occur at the level of threonine and serine residues, as shown here. MPF can also be activated in vitro simply by removing ATP from crude extracts in both starfish and amphibian (Labbé et al., 1988a, c, 1989b). These results led us to propose that the key event in MPF activation is dephosphorylation of its *cdc2* subunit. Our results with OA show that *cdc2* dephosphorylation involves neither the typical phosphatase type 1 nor phosphatase 2A. MPF activation readily occurs in starfish oocytes injected either with 2 mM EGTA (Picard and Dorée, 1983) or with various anti-calmodulin antibodies or drugs (Dorée et al., 1982), so it is unlikely that the MPF-activating phosphatase is phosphatase 2B (calcineurin). Thus, one cannot avoid the conclusion that a protein phosphatase reactive with Ser(P) and Thr(P) residues, but immune to OA, is responsible for *cdc2* dephosphorylation. One possibility is protein phosphatase 2C, which is not inhibited by OA (Bialojan and Takai, 1988) and has a structure quite distinct from type 1 and 2A phosphatases (Tamura et al., 1989).

Although the purified *cdc2* subunit has MPF activity by itself in its dephosphorylated form (Labbé et al., 1989b; Hutchinson et al., 1989), MPF can be purified as a heterodimer containing cyclin in association with *cdc2* (Labbé et al., 1989a). Cyclin is absolutely required for the activation of the mitotic kinase in vivo (Picard et al., 1985, 1987) as well as in vitro, in the presence (Murray and Kirschner, 1989; Minshull et al., 1989) or in the absence of ATP (Labbé et al., 1988a,c). Cyclin also appeared, in this work, to be required for okadaic acid to trigger MPF activation.

Newly synthesized cyclin has been shown to undergo phosphorylation (Standart et al., 1987). Although the role of this posttranslational modification remains far from clear, it may be required for MPF activation, as both events are time related (Pines and Hunt, 1987; Meijer et al., 1989). According to our view MPF is subject to both negative control (at the level of its *cdc2* subunit) and positive control (at the level of its cyclin subunit) by phosphorylation. Microinjection of OA would thus induce MPF activation by preventing cyclin dephosphorylation. Should this be the case, both phosphatase 1 and phosphatase 2A would be expected to catalyze dephosphorylation of cyclin in starfish (probably at different sites). Differences between starfish and sea cucumber cyclins would account for the involvement of both type 1 and 2A or only a type 1 phosphatase in MPF activation.

Under physiological conditions, the drop of MPF activity when cells exit from M phase depends on the proteolytic degradation of cyclin (Picard et al., 1985; Murray and Kirschner, 1989; Minshull et al., 1989). Both anti-type 1 phosphatase antibodies and OA blocked MPF inactivation and OA prevented disappearance of cyclin. One might conclude that type 1 phosphatase action is involved in the mechanism that controls MPF inactivation at some step before cyclin degradation. We believe that phosphatase action is required either to make cyclin accessible to proteases or to activate the cyclin-degrading protease, although there is no evidence for cyclin dephosphorylation before its proteolysis. The involvement of type 1 phosphatase in the completion of mitosis may be related to the increased phosphatase activity observed during mitosis in frog (Karsenti et al., 1987) and the rapid dephosphorylation of proteins observed at anaphase of the first meiotic and mitotic cell cycles in starfish (Dorée et al., 1983; Picard et al., 1987).

Besides revealing a role for protein phosphatases both in the activation and in the inactivation of MPF, presumably involving modification of the cyclin component, these studies have exposed a phosphatase activity that dephosphorylates Ser(P) and Thr(P) residues in *cdc2* itself, thereby activating its kinase function. This phosphatase is not blocked by OA and therefore is functionally different from type 1 and 2A enzymes reported to date. The *cdc2* phosphatase may prove to be a protein with a sequence distinct from known phosphatases, representing a separate line of descent within this family of enzymes.

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Note Added in Proof. Phosphotyrosine was also detected in *cdc2* from prophase-blocked oocytes when it was immunoprecipitated in the presence of 10 mM phenylphosphate and 0.1 mM ZnCl₂.

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