Inhibition of mos-induced Oocyte Maturation by Protein Kinase A

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Abstract. The relationship between the mos protooncogene protein and cAMP-dependent protein kinase (PKA) during the maturation of Xenopus oocytes was investigated. Microinjection of the PKA catalytic subunit (PKA) into Xenopus oocytes inhibited oocyte maturation induced by the mos product but did not markedly affect the autophosphorylation activity of injected mos protein. By contrast, PKA did not inhibit maturation-promoting factor (MPF) activation or germinal vesicle breakdown (GVBD) that was initiated by injecting crude MPF preparations. In addition, inhibiting endogenous PKA activity by microinjecting the PKA regulatory subunit (PKAr) induced oocyte maturation that was dependent upon the presence of the endogenous mos product. Moreover, PKA potentiated mos protein-induced MPF activation in the absence of progesterone and protein synthesis. These data are consistent with the hypothesis that progesterone-induced release from G2/M is regulated via PKA and that PKA negatively regulates a downstream target that is positively regulated by mos.

Fully grown Xenopus oocytes are arrested in prophase of meiosis I and are induced to mature upon exposure to progesterone (29). Synthesis of the mos protooncogene product, pp39reno, is required for the activation of maturation-promoting factor (MPF), an activity responsible for coordinating the biochemical events of meiosis I and II (9, 22, 39, 41). Injecting the mos product into a two-cell embryo arrests the injected cell at metaphase, which led to the identification of mos as an active component of cytoskeletal factor (CSF) (41). CSF is a calcium-sensitive activity believed to be responsible for the arrest of an unfertilized egg at metaphase of meiosis II and for the stabilization of MPF (29, 31, 32, 41).

The observation that mos is synthesized prior to MPF activation during meiosis I, led us to propose that mos is an "initiator" and is required to activate MPF from pre-MPF (40). Furthermore, pp39reno is required at all stages during oocyte maturation (9, 22). mos RNA or protein can initiate MPF activation when microinjected into fully grown oocytes (16, 40, 50). Recently, using recombinant mos protein (MBP-mos), we have shown that the protooncogene product is both necessary and sufficient to initiate meiosis I (50). However, injected oocytes do not progress to meiosis II in the absence of protein synthesis (50), even though mos is required during this period (9, 22). This suggests that additional proteins synthesized de novo are required for meiosis II and CSF arrest (50).

Progesterone markedly enhances MBP-mos-induced germ vesicle breakdown (GVBD) in the absence of protein synthesis (50), suggesting that the hormone removes a biochemical block to MPF activation that pre-exists in the oocyte. This system provided a means for testing the biochemical events involved in oocyte maturation in the absence of protein synthesis requirements. cAMP-dependent protein kinase (PKA) has been implicated as a negative regulator of G2/M transition and it is generally believed that progesterone stimulation of oocytes causes a transient decrease in cAMP levels. This, in turn, leads to a decrease in PKA activity and results in the dephosphorylation of a presumptive maturation-inhibiting phosphoprotein (44). While the role of cAMP and PKA in oocyte maturation is unclear (44) the following observations support the above hypothesis: progesterone inhibits adenylate cyclase activity in frog oocytes (13, 20, 34, 36); cAMP levels decrease during oocyte maturation in several organisms including frog (24, 25, 42), starfish (30), and mammals (43); the injection of phosphodiesterase induces maturation (7; Foerder, C. A., T. J. Martins, J. A. Beavo, and E. G. Krebs. 1982. J. Cell Biol. 95:304a), while inhibitors of phosphodiesterase prevent maturation (7, 37, 43); activators of adenylate cyclase cause an increase in cAMP levels, resulting in inhibition of Xenopus and mammalian oocyte maturation (17, 34, 49); and the injection of either the PKA regulatory subunit (PKAr), which binds to and inactivates the catalytic subunit, or the PKA inhibitory peptide (PKI) induces maturation (26), while the catalytic subunit of PKA (PKAc) inhibits oocyte maturation in amphibians (26) and mammals (7).

Since progesterone influences the PKA pathway and...
potentiates mos-induced MPF activation in the absence of protein synthesis, we examined the effects of the PKA subunits on mos function. We show that the PKA does not affect MPF-induced maturation, but markedly inhibits MBP-mos*-induced GVBD. By contrast, in the absence of protein synthesis the regulatory subunit (PKA,) potentiates MBP-mos* promotion of GVBD almost as efficiently as progesterone.

Materials and Methods

Frogs and Oocytes

* Xenopus laevis oocytes were surgically removed and defolliculated by incubation in modified Barth solution (MBS; 88 mM NaCl, 1 mM KCl, 2.5 mM NaHCO3, 10 mM HEPES, pH 7.5, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2) containing collagenase A (1.5 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 2 h (18). After several washes, oocytes were cultured overnight in 50% Leibovitz-15 media (Gibco Laboratories, Grand Island, NY).

Injections

18 h after oocytes isolation, microinjections were performed by using an Attocine injector (ATTO Instruments, Rockville, MD) with 30 nl of one of several of the following: MBP-mos*-protein produced in Escherichia coli and isolated as described (50), protein kinase A catalytic subunit (Promega, Madison, Wisc.), PKAγ RNA transcripts synthesized in vitro as described (10), protein kinase A regulatory subunit (Promega, Madison, WI), and mos specific oligonucleotides (39) (60 ng/oocyte). The H-Ras lys12 p21 protein was expressed in cells and purified from E. coli (a gift of Angel Nebreda, National Institute of Allergy and Infectious Diseases). Oocytes were injected with MBP-mos* alone (9 ng per oocyte) or MPB-mos* with 10 ng of PKAc (42 ng per oocyte; Promega Biotec, Madison, Wisc.), PKAc RNA transcripts synthesized in vitro as described (18). After several washes, oocytes were cultured overnight in 50% Leibovitz-15 media (Gibco Laboratories, Grand Island, NY).

Immunoprecipitations and In Vitro Kinase Assays

Groups of 10 oocytes were homogenized in lysis buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 1 mM EDTA, 0.1% NP-40) containing 1% deoxycholate, 1% Triton X-100. Extracts were clarified by centrifugation at 14,000 g for 16 h at 4°C. The ribosome pellets were suspended in 2 x sample buffer, then resuspended in 2 x sample buffer, analyzed by SDS-PAGE on a 12% gel, and then visualized by autoradiography. The ribosomal S6 subunit bands were excised and counted.

Histone H1 Kinase Assays

Crude MPF extracts were prepared by homogenizing groups of 10 to 20 oocytes in 20 to 40 µl of extraction buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 20 mM HEPES, pH 7.2, 1 mM ATP, 1 mM DTT, and 5 mM NaF). Homogenates were clarified by centrifugation at 14,000 g for 5 min at 4°C. 2 µl of the supernatant were added to 50 µl of stabilization buffer (80 mM β-glycerophosphate, 20 mM EGTA, 50 mM MgCl2, 2.5 mM PMSF, 10 µg of leupentin per ml, 10 µM protein kinase A inhibitor). The histone H1 kinase assay was performed by adding 10 µl of stabilized extract to 6 µl of a mixture containing 2 µg of histone H1 (Sigma, St. Louis, MO), 1 mM ATP, and 1.5 µCi of [γ-32P]ATP. The reaction was incubated 15 min at room temperature, and then stopped with an equal volume of 2 x sample buffer. Samples were resolved by SDS-PAGE on a 10% gel, and then fixed and autoradiographed.

Results

Inhibition of mos-induced Oocyte Maturation by the PKA Catalytic Subunit

PKA, injection into Xenopus oocytes blocks progesterone-induced meiotic maturation (26). We tested the influence of PKA activity on MBP-mos*-induced maturation. MBP-mos* plus increasing units of PKA, protein were co-injected into fully grown oocytes (Fig. 1). At 1 ng per oocyte, PKA, prevented MBP-mos* induction of maturation in 50% of the oocytes (Fig. 1). Maximum inhibition was observed when 4 ng of PKA, was injected per oocyte. Similar levels of PKA, also inhibit progesterone induced GVBD (data not shown). When MBP-mos* was injected with PKA, and PKAc, GVBD occurred in 92% of oocytes, showing that the PKA, inhibition was specifically abrogated by the regulatory subunit. PKA, also prevented the appearance of MPF and its associated histone H1 kinase activity (Figs. 1 and 2). Moreover, the ras oncoprotein (raswt) (which also efficiently induces GVBD (4, 8) and has been shown to be antagonistic to the PKA pathway (4, 38), was also blocked by injection of PKA, (Fish, S., D. Grieco, V. E. Arredimento, and M. E. Gottesman, unpublished data) (Fig. 2). By contrast, PKA, did not inhibit crude MPF preparations from efficiently inducing meiotic maturation (Fig. 2), even when PKA, was injected 1 h before MPF injection (data not shown). These results show that PKA activity inhibits both ras or mos maturation either directly or indirectly by negatively regulating a downstream substrate. They also suggest that PKA, acts as an early inhibitor of maturation, upstream from MPF.

Kinetics of PKA, Inhibition during Maturation

The period of PKA, sensitivity was determined in maturing oocytes. Oocytes were either treated with progesterone or in-
Inhibition of MBP-mos* and other inducers of GVBD by the PKA catalytic subunit. Oocytes were either treated or injected as indicated along with the coinjection of PKA~ (42 ng) (+) or in its absence (−). GVBD was examined 12-18 h later. The ratio of the number of oocytes with GVBD to the total number injected is displayed above each bar. Histone H1 kinase assays were performed on extracts from 10 appropriately injected oocytes and the autoradiograph is displayed above each bar.

Figure 2. Inhibition of MBP-mos* and other inducers of GVBD by the PKA catalytic subunit. Oocytes were either treated or injected as indicated along with the coinjection of PKA~ (42 ng) (+) or in its absence (−). GVBD was examined 12-18 h later. The ratio of the number of oocytes with GVBD to the total number injected is displayed above each bar. Histone H1 kinase assays were performed on extracts from 10 appropriately injected oocytes and the autoradiograph is displayed above each bar.

Jected with MBP-mos* protein and followed by PKA injection at various times (Fig. 3 A). 50% of the oocytes resisted the inhibitory effect of PKA when the subunit was injected 2.5 to 3 h after progesterone treatment (Fig. 3, A and B). This period represents 0.56 to 0.67 GVBD (a time which corresponds to MPF activation and protein synthesis independence). 50% of the MBP-mos*-injected oocytes became PKA resistant by 3.0 to 3.5 h after mos injection, or at 0.67 to 0.77 GVBD (Fig. 3, A and B). The difference observed in the times of PKA resistance between the two groups may be the result of the more synchronous nature of progesterone exposure when compared to the time required for microinjection of MBP-mos* protein as well as the time required for this protein to be activated (50). These data are consistent with those previously reported (26), but suggest that the PKA inhibitory effect persists in meiosis I until maturation becomes protein synthesis independent at ~0.6 GVBD (40).

PKA Does Not Inhibit MBP-mos* Activation In Vivo
To determine whether PKA inhibition of maturation results from inhibition of mos activity, we injected MBP-mos* either alone or with the PKA subunit into fully grown oocytes. We performed mos immune complex kinase assays on extracts prepared at various times after injection and measured MBP-mos* autophosphorylation activity (50). These results show that MBP-mos* autophosphorylation was not blocked by PKA and there was only a slight delay in the appearance of maximal activity (~0.5 h) (Fig. 4). Moreover, phosphorylation of MBP-mos* by PKA in vitro did not diminish its autophosphorylation activity (data not shown) even though PKA prevents GVBD induced by MBP-mos* protein (Figs. 1 and 2). These results suggest that the target of PKA inhibition may not be the mos product but, rather, a substrate downstream in the meiotic initiation pathway, prior to GVBD.

Inhibition of S6 Phosphorylation by PKA
The ribosomal subunit S6 is phosphorylated during oocyte maturation induced by insulin or progesterone (33) and requires endogenous mos protein function (3). We determined whether PKA has an effect on MBP-mos*-induced S6 phosphorylation. Oocytes were pre-labeled with 32P, for 3.5 h and subsequently injected with MBP-mos* protein either alone or with the PKA protein. Over a period of 9 h, ribosomes were isolated and phosphoproteins were analyzed by SDS-PAGE. Again, the injection of PKA inhibited MBP-mos*-induced GVBD (Fig. 5) and S6 protein phos-
 phosphorylation was ~50% lower than in oocytes injected with MBP-mos alone or PKA alone (Fig. 5).

The PKA Regulatory Subunit Enhances MBP-mos-induced Maturation

Progestosterone potentiates the ability of MBP-mos to induce GVBD in the presence of cycloheximide. Since progesterone treatment of oocytes leads to reduced cAMP levels and presumably reduced PKA activity, we determined whether PKA enhances MBP-mos-induced maturation in the absence of protein synthesis and progesterone. The concentrations of MBP-mos or PKA were titrated for their ability, to induce GVBD in cycloheximide-treated oocytes. The injection of 2.0 ng of MBP-mos into cycloheximide-treated oocytes failed to cause GVBD (Fig. 6, bar 2). 1–3 U of PKA-induced GVBD (26) in 60 to 88% of untreated oocytes, respectively, while PKA did not cause GVBD in the presence of cycloheximide (Fig. 6, bars 7–9) even with progestosterone present (data not shown). As previously shown, progestosterone potentiates the induction of GVBD by MBP-mos in cycloheximide-treated oocytes (50) (Fig. 6, bar 3). When levels of PKA, suboptimal for inducing maturation were injected with MBP-mos in the presence of cycloheximide, a substantial increase in the percentage of oocytes undergoing GVBD was observed. Thus, from a baseline of 2% for MBP-mos alone, 63, 81, and 88% GVBD was observed for 1.0, 2.0, and 3.0 U of PKA, respectively (Fig. 6, bars 4–6). These data show that PKA, potentiates MBP-mos-induced GVBD almost as effectively as progestosterone in the absence of protein synthesis.

PKA-induced Maturation Requires pp39mos Synthesis

To determine whether the synergy between MBP-mos and the PKA regulatory subunit is due to PKA activity functioning downstream of mos or through a parallel (mutually dependent) pathway, we metabolically labeled oocytes with [35S]methionine and microinjected PKA. After 4 h, the mos product was precipitated from oocytes using a mos specific antibody. Oocytes treated with either progesterone or PKA, expressed mos product indicating that mos synthesis was induced, while untreated oocytes did not express pp39mos (Fig. 7 A). We tested whether pp39mos is required for PKA, induced oocyte maturation by blocking mos protein formation with antisense oligonucleotides (39). Only 3% of the antisense injected oocytes underwent GVBD, while 87% of control sense oligonucleotide injected oocytes matured with PKA, (Fig. 7 B). These data show that PKA and mos function through a mutually dependent pathway.

Figure 6. Synergy between MBP-mos and the PKA regulatory subunit in the absence of protein synthesis. Between 20 and 80 oocytes were injected with the indicated amounts of MBP-mos and/or PKA regulatory subunit and scored for GVBD 8 to 12 h later. Mos*, MBP-mos protein; PKA, regulatory subunit; C, cycloheximide (10 μg/ml); P, progesterone.

Figure 7. (A) The induction of pp39mos synthesis in PKA-injected oocytes. 50 oocytes were metabolically labeled for 3 h in MBS containing 35S-translabel (0.5 Mci/ml), and then either injected with PKA, (R) (3.0 U), or treated with progesterone (P) (10 μg/ml) or left untreated (U). After 4 h, the oocytes were subjected to immunoprecipitation with a mos specific antibody (5S) and analyzed by 10% SDS-PAGE. (B) The requirement for pp39mos synthesis in PKA, injected oocytes. Fully grown oocytes were injected with either antisense mos oligonucleotides (39) (60 ng/oocyte) or sense oligonucleotides (60 ng/oocyte). After one hour, these oocytes were either treated with progesterone or injected with PKA, (3.0 U). GVBD was examined externally and internally after 12–14 h. The ratio of oocytes with GVBD to the number injected is displayed over each bar.
**Discussion**

In *Xenopus* oocytes, the introduction of 1–4 ng of PKA<sub>e</sub> blocks MPF activation initiated by progesterone or the mos product. However, crude MPF preparations can induce GVBD (Fig. 2) and meiotic progression through meiosis II in the presence of PKA<sub>e</sub> (data not shown). Since the MPF preparations are crude, they contain many other proteins in addition to the active cdc2–cyclin complex, and thus cannot be excluded from influencing this result. The above results suggested that PKA inhibits, while mos positively regulates, a downstream target that is upstream of MPF during meiosis I, but this target is either inactive or absent between meiosis I and II, a period requiring mos function (9, 22). Consistent with this idea, PKA<sub>e</sub> prevents maturation initiated by the ras oncprotein, which can induce GVBD when mos translation is blocked (8) and in the presence of cycloheximide (1).

The results also indicate a dual role for pp39<sup>mos</sup> as “initiator” and as CSF, since this inhibition only affects mos “initiator” function and not mos activity during the latter part of meiosis. The time period when MBP-mos<sup>pp39mos</sup>-induced GVBD becomes resistant to PKA activity (0.67–0.77 GVBD<sub>50</sub>) in 50% of oocytes corresponds to the time when GVBD becomes independent of protein synthesis and MPF becomes activated. These results are not significantly different than those reported in an earlier study by Maller and Krebs (26), where 50% of GVBD was insensitive to PKA<sub>e</sub> at 2.5 h (75 GVBDs). Consistent with these findings, it has recently been reported that when oocytes are treated with known elevators of intracellular cAMP (IBMX and cholaer toxin), the nondegradable Δ90 cyclin cannot induce MPF activation, and in oocytes depleted of endogenous cyclins, p34<sup>cdc2</sup> kinase is inactivated by phosphorylation on tyrosine 15 (35). Although the detectable decrease in cAMP levels occurs early in progesterone stimulated oocytes, this does not rule out the possibility that the target of PKA activity is involved in the activation of the MPF complex or possibly a component of the MPF complex which is already present in an inactive form. Although PKA<sub>e</sub> inhibited MBP-mos<sup>pp39mos</sup>-induced GVBD, it did not prevent the activation of the *E. coli*-expressed recombinant protein in vivo. It is possible that PKA activity inhibits the ability of pp39<sup>mos</sup> to phosphorylate an important substrate required for MPF activation, but this would have to be accomplished without suppressing its autokinase activity.

Ribosomal S6 protein phosphorylation is implicated in cell proliferation and transformation (5, 6, 46, 48). In *Xenopus* oocytes, the ribosomal subunit S6 is hyperphosphorylated after the initiation of meiotic maturation by progesterone (12, 33), insulin (11, 25), and several oncogene products including ras (3, 21), v-src (45), v-abl (28), and tpr-met (10). While hormonal stimulation of S6 phosphorylation is inhibited by mos-depletion (3), S6 kinase activity induced by activated ras or tpr-met appears to be only partially affected by the lack of mos product (3, 10). In this study, nearly 50% of the ribosomal subunit S6 phosphorylation induced by MBP-mos<sup>pp39mos</sup> was suppressed by PKA activity, however, the remaining 50% appears to be the result of the injected PKA<sub>e</sub>. Thus, PKA<sub>e</sub> inhibits S6 phosphorylation induced by mos.

The cAMP-dependent protein kinase has been implicated as a negative regulator of meiosis and early mitosis in several organisms. Recently, inhibition of PKA has also been reported to play a pivotal role in the G<sub>S</sub>/M transition of mammalian fibroblasts (23). We have shown that MBP-mos<sup>pp39mos</sup>-induction of the G<sub>S</sub>/M transition in cycloheximide-treated oocytes was potentiated by progesterone exposure, suggesting a possible role for the inhibition of PKA in this event (50). Here, we report a synergistic effect between PKA<sub>e</sub> and MBP-mos<sup>pp39mos</sup> that enhanced the number of oocytes undergoing the G<sub>S</sub>/M transition in the absence of protein synthesis, thereby mimicking the effect of progesterone. It has been previously shown that during oocyte maturation certain substrates are hyperphosphorylated, while other proteins are dephosphorylated (27), and it has been suggested that these dephosphorylation events may be the result of PKA inactivation (23). It is possible that mos and PKA<sub>e</sub> are antagonists, where mos may inactivate an inhibitor of MPF activation, while PKA<sub>e</sub> phosphorylates this inhibitor or an activator of this inhibitor, and thereby causes its activation. However, it is also possible that mos may activate, while PKA<sub>e</sub> inactivates, an activator of pre-MPF. We have shown here that PKA<sub>e</sub> requires mos synthesis to activate MPF. Perhaps, as has been suggested (19), progesterone acts by releasing the brakes (in this case, inhibiting PKA activity) and stepping on the accelerator (mos synthesis). Curiously, both PKA<sub>e</sub> and pp39<sup>mos</sup>, two proteins with significant homology (2), have been shown to associate with microtubules and phosphorylate tubulin in vitro (47, 51). Perhaps the antagonism between mos and PKA involves microtubule modification. Collectively, our results suggest that the inhibitory effect of PKA is a late step in the initiation of MPF activation and that inactivation of PKA, along with the promoting activities of the mos product, leads to MPF activation.

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