

MAPK interacts with XGef and is required for CPEB activation during meiosis in *Xenopus* oocytes

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Summary

Meiotic progression in *Xenopus* oocytes, and all other oocytes investigated, is dependent on polyadenylation-induced translation of stockpiled maternal mRNAs. Early during meiotic resumption, phosphorylation of CPE-binding protein (CPEB) is required for polyadenylation-induced translation of mRNAs encoding cell cycle regulators. *Xenopus* Gef (XGef), a Rho-family guanine-exchange factor, influences the activating phosphorylation of CPEB. An exchange-deficient version of XGef does not, therefore implicating Rho-family GTPase function in early meiosis. We show here that *Clostridium difficile* Toxin B, a Rho-family GTPase inhibitor, does not impair early CPEB phosphorylation or progression to germinal vesicle breakdown, indicating that XGef does not influence these events through activation of a Toxin-B-sensitive GTPase. Using the inhibitors U0126 for mitogen-activated protein

kinase (MAPK), and ZM447439 for Aurora kinase A and Aurora kinase B, we found that MAPK is required for phosphorylation of CPEB, whereas Aurora kinases are not. Furthermore, we do not detect active Aurora kinase A in early meiosis. By contrast, we observe an early, transient activation of MAPK, independent of Mos protein expression. MAPK directly phosphorylates CPEB on four residues (T22, T164, S184, S248), but not on S174, a key residue for activating CPEB function. Notably, XGef immunoprecipitates contain MAPK, and this complex can phosphorylate CPEB. MAPK may prime CPEB for phosphorylation on S174 by an as-yet-unidentified kinase or may activate this kinase.

Key words: MAPK, GEF, Rho-family G-protein, Polyadenylation, Translation, CPEB, Meiosis, *Xenopus*, Aurora kinase

Introduction

Xenopus laevis oocytes remain quiescent in prophase I of meiosis until induced to re-enter the meiotic cycle by steroids (Haccard and Jessus, 2006; Hammes, 2004). Meiotic resumption involves a cascade of molecular events (meiotic maturation) that proceeds until meiosis is arrested again in metaphase II. The completion of meiosis is subsequently triggered by fertilization (Nebreda and Ferby, 2000). Striking changes in the polyadenylation status of several maternal mRNAs during oocyte maturation were first seen in frog and clam oocytes (Rosenthal et al., 1983; Ruderman et al., 1979). Polyadenylation-induced translation of stockpiled maternal mRNAs is now known to be a major mechanism for regulating meiotic resumption and progression (Wickens et al., 1996). The cytoplasmic polyadenylation element (CPE) is a cis-element in the 3' UTR of certain mRNAs that binds the trans-factor, CPE-binding protein (CPEB) (Hake et al., 1998; Hake and Richter, 1994). Prior to steroid stimulation, CPE-containing mRNAs are translationally masked, and have a short poly(A) tail (de Moor and Richter, 1999). Activation of CPEB by phosphorylation during meiotic resumption results in polyadenylation of CPE-containing mRNAs, which is coupled with their translation (Charlesworth et al., 2004; Mendez et al., 2000a; Mendez et al., 2000b). Phosphorylation of CPEB occurs in at least two phases. (1) Early CPEB phosphorylation occurs after steroid induction and prior to activation of the maturation promoting factor (MPF), i.e. in early meiosis, and

is necessary for normal polyadenylation and temporally early translation of a class of mRNAs, several of which, such as *Mos* mRNA, must be translationally activated for timely meiotic progression (Charlesworth et al., 2004; Mendez et al., 2000a). Early phosphorylation of S174 is particularly important: overexpression of CPEB-AA (a mutant CPEB containing S174A and S180A point mutations) decreases the extent of polyadenylation of early-class mRNAs during meiotic resumption (Charlesworth et al., 2004) and completely blocks 3' UTR polyadenylation of an injected *Mos* mRNA (Mendez et al., 2000a). The RNA-binding-protein Musashi is also involved in polyadenylation-induced translation of the early-class mRNAs (Charlesworth et al., 2006), however, experiments that perturb CPEB function, through injection of dominant-negative CPEB-AA or a peptide inhibitor of S174 phosphorylation, demonstrate that CPEB is required for full polyadenylation-induced translation of early-class mRNAs and meiotic resumption (Charlesworth et al., 2004; Mendez et al., 2000a). (2) Late CPEB hyper-phosphorylation depends upon MPF activation and leads to the ubiquitin-mediated destruction of a subset of the CPEB pool (Mendez et al., 2002; Reverte et al., 2001), which is required for the late polyadenylation-induced translation of a class of mRNAs typified by cyclin B1. Late polyadenylation-induced translation is required for progression from meiosis I to meiosis II (Mendez et al., 2002).

The signaling events that occur between steroid induction and the activation of polyadenylation-induced translation remain

unclear. Steroid signaling is most likely mediated through combinatorial action of classical and novel steroid receptors (Haccard and Jessus, 2006). Within minutes of progesterone addition (the steroid typically used in the laboratory), the levels of cAMP in the oocyte decrease (Speaker and Butcher, 1977), leading to inhibition of the cAMP-dependent protein kinase A (PKA) (Maller and Krebs, 1977). Downstream of PKA inactivation, several proteins have been identified that influence polyadenylation-induced translation during early meiosis, although their mechanism of action is unknown or ambiguous. Aurora kinase A (Aur-A) has been implicated in mediating the early, activating phosphorylation of CPEB (Mendez et al., 2000a; Mendez et al., 2000b), although active Aur-A has not been detected in early meiosis (Frank-Vaillant et al., 2000; Ma et al., 2003; Maton et al., 2003). RINGO, a non-cyclin Cdc2-activating protein, is required during early meiosis (Ferby et al., 1999; Lenormand et al., 1999) perhaps partially because of an influence on early CPEB phosphorylation (Padmanabhan and Richter, 2006); however, the substrates of a RINGO-Cdc2 complex in early meiosis are unknown. Mitogen-activated protein kinase (MAPK) is required for timely meiotic progression (Fisher et al., 1999; Palmer and Nebreda, 2000) and for polyadenylation-induced translation of early-class mRNAs (Charlesworth et al., 2002; Charlesworth et al., 2006; Howard et al., 1999). The MAPK substrates that mediate this influence are unknown.

Our laboratory has examined the role of *Xenopus* Gef (XGef), a Rho-family guanine exchange factor, in early meiotic signaling. When XGef is overexpressed in *Xenopus* oocytes, we observe an increase in the level of phosphorylated CPEB during early meiosis, earlier elongation of poly (A) tails on *Mos* mRNA, earlier accumulation of Mos protein and an acceleration of meiotic progression by ~25% (Martinez et al., 2005; Reverte et al., 2003). A version of XGef that has very reduced interaction with CPEB functions in a dominant-negative manner and substantially impairs these events. In contrast to wild-type XGef, XGef that lacks the DH domain, which is required for guanine-nucleotide exchange factor activity, has no stimulatory influence on early meiotic processes. XGef interacts with CPEB throughout meiosis and is present with CPEB in a *Mos* messenger ribonucleoprotein (mRNP), suggestive of an ongoing role of XGef in either regulation of CPEB activity or CPEB-mediated processes. The stimulatory effect of XGef on meiosis correlates directly with the early, activating phosphorylation of CPEB (Martinez et al., 2005), suggesting that XGef has a role in the signaling mechanism leading to activation of CPEB.

We have explored here how XGef influences early phosphorylation of CPEB and meiotic progression. We have found that early CPEB phosphorylation and normal meiotic progression occur in the presence of a Rho-family GTPase inhibitor, suggesting that the role of XGef in mediating early CPEB phosphorylation is independent of its guanine exchange factor activity. Although our intention was to investigate a potential role for XGef in Aur-A activity towards CPEB, we were unable to detect active Aur-A prior to MPF activation, and found that perturbation of Aur-A levels as well as Aur-A and Aurora kinase B (Aur-B) activity during early meiosis did not impair early phosphorylation of CPEB. We did find that XGef is in a complex containing MAPK and CPEB, and that MAPK activity is necessary for CPEB phosphorylation during

meiosis. These results strongly suggest that MAPK, not Aur-A, is involved in the early meiotic activating phosphorylation of CPEB. In vitro, MAPK phosphorylates several amino acids in CPEB, but not S174. We propose that MAPK primes CPEB for activating phosphorylation on S174 by an as-yet-undefined kinase, although MAPK may instead influence events further upstream of CPEB activation.

Results

XGef does not influence early CPEB phosphorylation through activation of a Rho-family GTPase

XGef is a Rho-family guanine nucleotide exchange factor that can activate the GTPase Cdc42 in mammalian cells (Reverte et al., 2003). Overexpression in oocytes of a guanine-nucleotide-exchange-deficient version of XGef that is unable to activate small GTPases does not enhance early CPEB phosphorylation, as observed upon overexpression of wild-type XGef (Martinez et al., 2005). We therefore suspected that XGef influences early CPEB phosphorylation through activation of a Rho-family GTPase. We explored this by blocking small Rho-family GTPase function during early meiosis and asking whether this impaired early phosphorylation of CPEB (Fig. 1). We used *Clostridium difficile* Toxin B, which glucosylates T37 of Rho and the analogous residue, T35, of Rac, Ras and Cdc42. This threonine residue is highly conserved among all small Rho-family GTPases and is involved in nucleotide binding and coordination of the Mg²⁺ ion. Glucosylation of this residue prevents GTP-bound GTPase (i.e. activated GTPase) from binding downstream effectors, effectively preventing effector activation (Busch and Aktories, 2000). If the influence of XGef on CPEB phosphorylation during early meiosis is through GTPase activation and subsequent effector activation, preventing GTPase action should block early CPEB phosphorylation. We first examined the ability of Toxin B to inactivate endogenous RhoA, Cdc42 and Rac1 by using an effector-binding-domain pull-down assay (Fig. 1A). Extracts from Toxin-B-treated oocytes were incubated with glutathione-S-transferase (GST)-tagged downstream effectors that would bind Cdc42-GTP and Rac-GTP [Rac/Cdc42 (p21)-binding domain (PBD) of the human p21-activated kinase 1 protein (PAK) protein expressed as GST-PAK-PBD] or RhoA-GTP [GST-Rhotekin-Rho-binding domain (RBD)]. In control samples, as expected, total oocyte Cdc42, Rac1 and RhoA were activated and captured by their respective GST-effector binding domain: glutathione bead complex (Fig. 1A, top three panels, lanes 1-4). Three hours of Toxin B treatment blocked the ability of these small GTPases to interact with their effectors, as indicated by the lack of interaction with the GST-tagged effector domains (Fig. 1A, top three panels, lanes 6-8). Using parallel sets of oocytes for in vitro histidine (His)-CPEB phosphorylation assays (not treated to activate GTPases), we found no difference in the level of CPEB phosphorylation in early meiotic extracts treated with Toxin B, compared with control samples (Fig. 1B, compare lanes 4 and 8). We also found that Toxin-B-injected oocytes proceed to germinal vesicle breakdown (GVBD) normally (Fig. 1C).

These results demonstrate that Toxin B is very effective in blocking Rho-family GTPase function in oocytes. Importantly, this block does not impair either early CPEB phosphorylation or meiotic progression to GVBD. We conclude that XGef does

not influence early CPEB phosphorylation through activation of Toxin-B-sensitive Rho-family GTPases.

Phosphorylation of CPEB on S174 occurs in the absence of Aur-A activity

XGef might influence early CPEB phosphorylation by involvement in either Aur-A activation or Aur-A activity

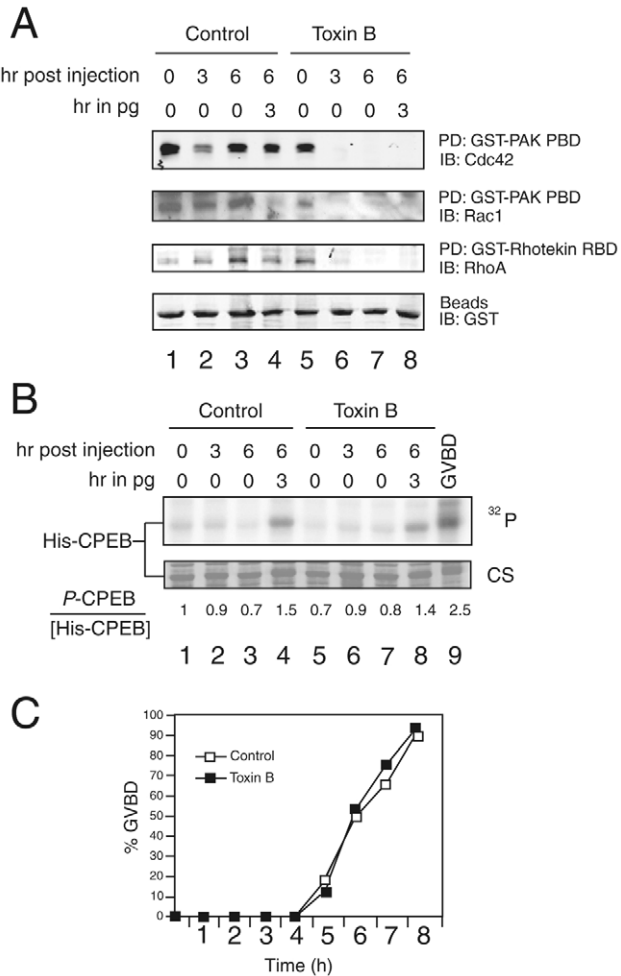


Fig. 1. Blocking Rho-family GTPase function with *Clostridium difficile* Toxin B does not interfere with CPEB phosphorylation during early meiosis. Toxin-B-injected oocytes were incubated for 3 hours prior to stimulation of meiosis with progesterone. PD, pull-down; IB, immunoblotting. (A) Protein extracts from oocytes collected post injection or after incubation in progesterone (hr post injection or hr in pg, respectively) at the indicated times (in hours) were treated to activate GTPases and then incubated with GST-PAK-PBD or GST-Rhotekin-RBD. Samples bound to glutathione-beads were analyzed by immunoblotting with anti-Cdc42, anti-Rac1, anti-RhoA and anti-GST antibodies, as indicated. The bottom panel is a representative immunoblot with anti-GST antibody to confirm equivalent loading of GST-PAK-PBD. Similar results were obtained with beads loaded with GST-Rhotekin-RBD. (B) Extracts from parallel sets of oocytes were incubated with His-CPEB and [γ -³²P]ATP in an in vitro His-CPEB phosphorylation assay. Ni-bead bound samples were analyzed by SDS-PAGE, Coomassie staining (CS) and autoradiography (³²P). (C) Progression to GVBD, monitored by appearance of a white spot at the animal pole, was followed in Toxin-B- and control-injected oocytes, and plotted versus incubation time.

towards CPEB during early meiosis. Since Aur-A has been implicated in the activating phosphorylation of CPEB on S174 (within the sequence SRLDSRSI) (Mendez et al., 2000a) active Aur-A should be present in early meiosis, before MPF activation. However, using Aur-A immunoprecipitation (IP)-phosphorylation assays and a variety of specific substrates, two studies failed to detect active Aur-A in early meiosis (Frank-Vaillant et al., 2000; Maton et al., 2003). Prior to examining a potential role of XGef in early Aur-A activation, we also examined Aur-A activity during early meiosis.

An antibody that recognizes phosphorylated T288 (*P*-T288) in human Aur-A has recently been shown to recognize recombinant *Xenopus* Aur-A phosphorylated on the analogous residue T295 (Sarkissian et al., 2004). Importantly, *P*-T295 is required for Aur-A activity (Littlepage et al., 2002) and, therefore, this antibody could be used to analyze the appearance of active *P*-T295 Aur-A throughout meiotic maturation. The antibody against *P*-T295 in Aur-A specifically recognizes a protein that appears at GVBD (Fig. 2A, lower panel, lanes 6 and 7, arrow) and migrates at the same position

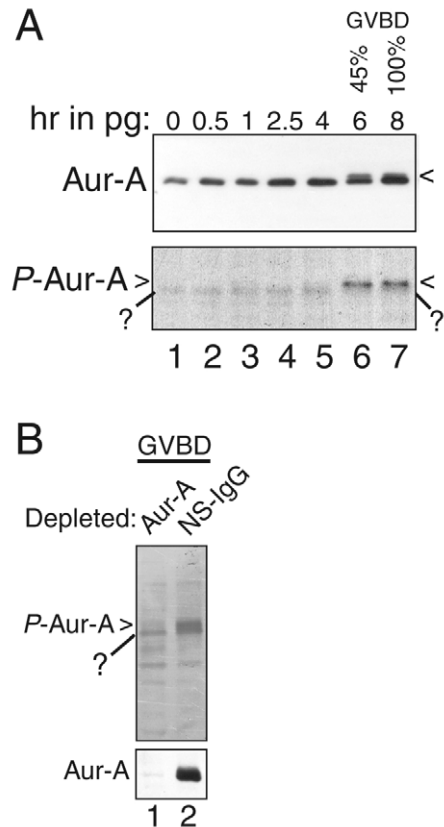


Fig. 2. *P*-T295 of *Xenopus* Aur-A is not detected until GVBD. (A) Protein extracts from oocytes collected after incubation in progesterone (hr in pg) for the indicated times (in hours) were analyzed by SDS-PAGE and immunoblotting with antibodies against total Aur-A (top panel) and *P*-T295 Aur-A (*P*-Aur-A, bottom panel). (B) Protein extracts from oocytes collected at 100% GVBD were immunodepleted with either anti-Aur-A antibody or non-specific IgG. Samples were analyzed by SDS-PAGE and immunoblotting with antibody for either *P*-T295 Aur-A (*P*-Aur-A, top panel) or Aur-A (bottom panel). Arrowheads indicate migration of mobility shifted Aur-A; ? indicates the band of a protein detected non-specifically by the antibody.

as the mobility shifted Aur-A at GVBD, detected using an antibody that recognizes total Aur-A (Fig. 2A upper panel, lanes 6 and 7, arrow). Curiously, a faster migrating band was also detected by the anti-*P*-T295 Aur-A antibody throughout meiosis, prior to GVBD (Fig. 2A, lower panel, lanes 1-5, question mark). Since this antibody does not recognize recombinant Aur-A with T294A and T295A substitutions (Sarkissian et al., 2004), we explored the possibility that this faster migrating band might in fact be a protein that is non-specifically detected. We immunodepleted Aur-A from GVBD-stage oocyte extracts (where both bands are apparent) by using an antibody that recognizes total Aur-A and then probed the extracts with the anti-*P*-T295 Aur-A antibody. The faster migrating band was not depleted from GVBD-stage extracts (Fig. 2B, upper panel, lane 1, question mark), whereas the slower migrating band was depleted (Fig. 2B, upper panel, lane 1, arrow). Notably, the negative control depletion retained the Aur-A band as expected (Fig. 2B, upper panel, lane 2, arrow). Immunoblot analysis with antibody that detects total Aur-A confirmed that the immunodepletion was 99% complete (Fig. 2B, lower panel). Clearly, the faster-migrating band

detected with the anti-*P*-T295 Aur-A antibody from prophase through GVBD is a non-specific band. From this set of experiments, we conclude that phosphorylation of Aur-A T295 cannot be detected until GVBD and, therefore, that Aur-A is most probably inactive in early meiosis.

An alternative explanation of these data and, perhaps, for that of other direct investigations of Aur-A activity is that Aur-A might in fact be activated early during meiosis, but at levels below those detectable in immunoblots (our data) and IP kinase assays (Frank-Vaillant et al., 2000; Maton et al., 2003). We therefore directly investigated the role of Aur-A in early CPEB phosphorylation using two methods to block Aur-A function. First, we assessed the influence of Aur-A depletion on early CPEB phosphorylation (Fig. 3). Interestingly, *in vitro* His-CPEB phosphorylation assays using Aur-A immunodepleted extracts revealed only a slight decrease in the level of early CPEB phosphorylation (3 hours) compared with control extracts (Fig. 3A, upper panel, compare lane 4 with lanes 5 and 6). Immunoblot analysis with anti-Aur-A antibody confirmed the efficiency of the immunodepletion (Fig. 3A, lower panel, lanes 1, 4 and 7). The absence of active MPF in the 3-hour post-progesterone oocyte extracts confirms the oocytes were in early meiosis (Fig. 3B, compare lanes 2 and 3).

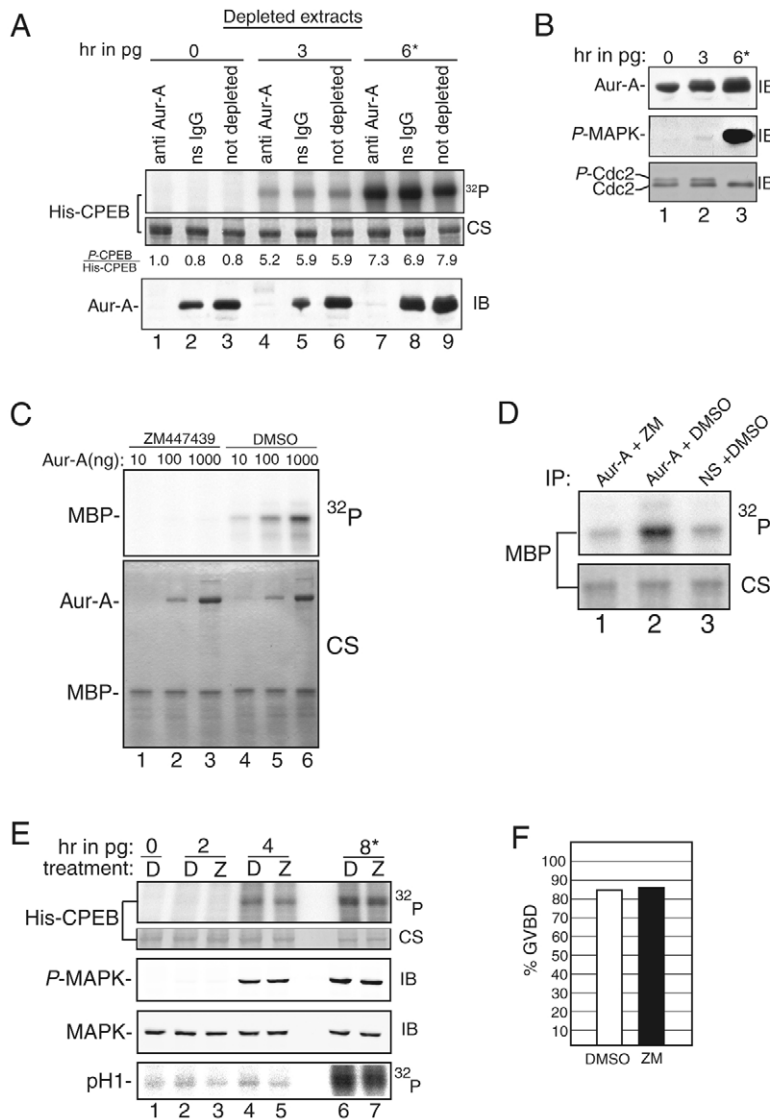


Fig. 3. Early phosphorylation of CPEB occurs in the absence of Aur-A activity. (A) His-CPEB phosphorylation assays using Aur-A immunodepleted extracts. Protein extracts from oocytes collected after incubation in progesterone (hr in pg) for the indicated times (in hours) were immunodepleted with non-specific IgG antibody, antibody against total Aur-A or no antibody and used in His-CPEB phosphorylation assays. Immunodepleted samples were analyzed by SDS-PAGE and immunoblotted with anti-Aur-A antibody. Phosphorylated His-CPEB signal intensity was adjusted relative to the quantity of input His-CPEB. Asterisk indicates 100% GVBD oocytes. (B) Samples from A were immunoblotted with antibodies against Aur-A, phosphorylated (active) MAPK (*P*-MAPK), and unphosphorylated and phosphorylated Cdc2. Asterisk indicates 100% GVBD oocytes. (C) The Aurora kinase inhibitor ZM447439 blocks Aur-A activity *in vitro*. Increasing amounts of recombinant Aur-A were treated with either ZM447439 or DMSO, then incubated with myelin basic protein (MBP) as a substrate in the presence of [γ -³²P]ATP. (D) ZM447439 inhibits endogenous AurA kinase. Affinity-purified Aur-A antibody or NS-IgG immunoprecipitates from GVBD extracts were incubated with MBP and [γ -³²P]ATP in the presence of ZM447439 or DMSO. (E) ZM447439 does not block CPEB phosphorylation. Protein extracts from oocytes collected at the indicated times were treated with either 20 μ M ZM447439 or DMSO and used in His-CPEB phosphorylation assays. Asterisk indicates 100% GVBD oocytes. Input extracts were incubated with histone H1 and [γ -³²P]ATP in an H1 kinase assay (pH1, lower panel). (F) ZM447439 does not inhibit GVBD. Oocytes were injected with either 40 nl of 500 μ M ZM447439 or DMSO two hours prior to the addition of progesterone. After overnight incubation, the number of oocytes that had achieved GVBD was scored. CS, Coomassie staining; ³²P, autoradiography; IB, immunoblotting.

Our second approach involved addition of ZM447439, an inhibitor of the Aurora kinase family of proteins (Ditchfield et al., 2003; Yang et al., 2005), to the His-CPEB phosphorylation assay. In control experiments, ZM447439 prevented recombinant Aur-A from incorporating ^{32}P into myelin basic protein (MBP; Fig. 3C, top panel, compare lane 3 with 6). ZM447439 also inhibited endogenous Aur-A activity (Fig. 3D), when Aur-A was immunoprecipitated and incubated with MBP in the presence (lane 1) or absence (lane 2) of ZM447439. To test the effect of ZM447439 on early CPEB phosphorylation, staged oocyte protein extracts were incubated with either ZM447439 (Z) or DMSO (D) and used in in-vitro phosphorylation assays (Fig. 3E). Neither ZM447439 nor DMSO had a discernible effect on the level of early CPEB phosphorylation (Fig. 3E, upper panel, compare lanes 4 and 5) prior to MPF activation (assessed using an H1 kinase assay; Fig. 3E, lower panel). Notably, phosphopeptide analysis by liquid-chromatography tandem mass spectrometry (LC-MS/MS; data not shown) revealed that oocyte extracts treated with either ZM447439 or DMSO still phosphorylated CPEB on S174. In addition, injected ZM447439 did not impede progression to GVBD (Fig. 3F).

These experiments strongly suggest that Aur-A is not active during early meiosis, and indicate that S174 phosphorylation of CPEB occurs under conditions that interfere with Aur-A activity. We conclude that the kinase involved in the activating phosphorylation of CPEB in early meiosis is unlikely to be Aur-A.

MAPK activation is required for early CPEB phosphorylation

The MAPK signaling pathway is necessary for normal *Mos* mRNA cytoplasmic polyadenylation and protein accumulation during oocyte maturation (Charlesworth et al., 2002; Charlesworth et al., 2006; Howard et al., 1999). Potential targets for MAPK activity include proteins involved in polyadenylation-induced translation of early-class mRNAs. Musashi and CPEB are the only RNA-binding proteins identified to date that have roles in determining specificity in polyadenylation-induced translation early during meiotic progression (Charlesworth et al., 2006; Hake and Richter, 1994). Musashi-mediated cytoplasmic polyadenylation is not controlled by the MAPK pathway (Charlesworth et al., 2006). We therefore tested whether the requirement for MAPK activity in early meiosis was due to an influence on early CPEB activation. We used the chemical inhibitor U0126, which prevents MAPK activation by blocking the activity of the MAPK activator MEK (Favata et al., 1998). Oocytes were incubated in medium containing U0126, stimulated with progesterone and then extracts from selected oocytes were tested in a His-CPEB phosphorylation assay (Fig. 4A). As expected, treatment with U0126 effectively blocked activation of MAPK, assessed with an antibody specific for phosphorylated activated MAPK (*P*-MAPK, Fig. 4A third panel, lanes 3 and 6). Interestingly, U0126 completely abolished phosphorylation of CPEB during meiosis (Fig. 4A top panel, compare lanes 3 and 2, 6 and 5). We confirmed this result by phosphopeptide analysis using LC-MS/MS. These analyses indicate that MAPK activation is required for CPEB phosphorylation during meiosis.

Since MAPK activation is dependent on protein synthesis

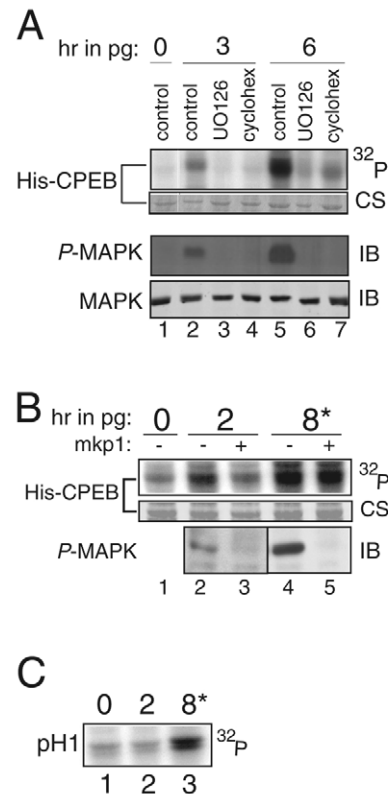


Fig. 4. MAPK activation and protein synthesis are required for early CPEB phosphorylation. (A) U0126 blocks CPEB phosphorylation. Oocytes were cultured in the presence of U0126, cycloheximide (cyclohex), or no additive (control) for 1 hour prior to the addition of progesterone (pg). Extracts were prepared from oocytes collected at the indicated times (hr in pg) and used in His-CPEB phosphorylation assays. Samples were analyzed by immunoblotting with anti-*P*-MAPK and MAPK antibodies (lower two panels). (B) Inactivation of MAPK reduces CPEB phosphorylation. Protein extracts from oocytes collected at the indicated times (hr in pg) were treated first with either Mkp1 or buffer, and then His-CPEB and [γ - ^{32}P]ATP were added. Samples were analyzed as in A. Asterisk indicates 100% GVBD oocytes. (C) The extracts used in B were used in an H1 kinase assay. Asterisk indicates 100% GVBD oocytes. CS, Coomassie staining; ^{32}P , autoradiography; IB, immunoblotting.

(Fisher et al., 2000), we also tested the effect of the protein synthesis inhibitor cycloheximide on early CPEB phosphorylation. Cycloheximide blocked activation of MAPK (Fig. 4A, third panel, lanes 4 and 7). Indeed, cycloheximide also blocked early CPEB phosphorylation (Fig. 4A, top panel, lane 4). This result was not surprising given that early CPEB phosphorylation is blocked when RINGO synthesis is prevented by antisense oligonucleotide injection (Padmanabhan and Richter, 2006). Therefore, this cycloheximide-induced block in early CPEB phosphorylation could be due to the block in synthesis of RINGO protein.

As a second approach, we tested the effect of MKP1, a MAPK-specific phosphatase, on early CPEB phosphorylation (Fig. 4B). MKP1 inhibits MAPK activity by removing the activating phosphorylation (Gotoh et al., 1995). Treatment with MKP1 diminished active MAPK as expected (Fig. 4B, lower panel, compare lane 2 with 3, and lane 4 with 5). Notably, early

meiotic oocyte extracts treated with MKP1 had significantly decreased kinase activity towards His-CPEB compared with non-MKP1-treated extracts (Fig. 4B, top panel, compare lane 3 with lane 2). These data agree with the previous result from the U0126 experiment and provide additional evidence for the involvement of MAPK in CPEB phosphorylation during early meiosis. In GVBD-stage oocyte extracts, MKP-1 had little effect on overall CPEB phosphorylation (Fig. 4B, lanes 4 and 5), probably due to hyperphosphorylation of CPEB by active MPF (Martinez et al., 2005; Mendez et al., 2002; Paris et al., 1991), present at this time (Fig. 4C, lane 3).

Phosphorylation of CPEB by MAPK

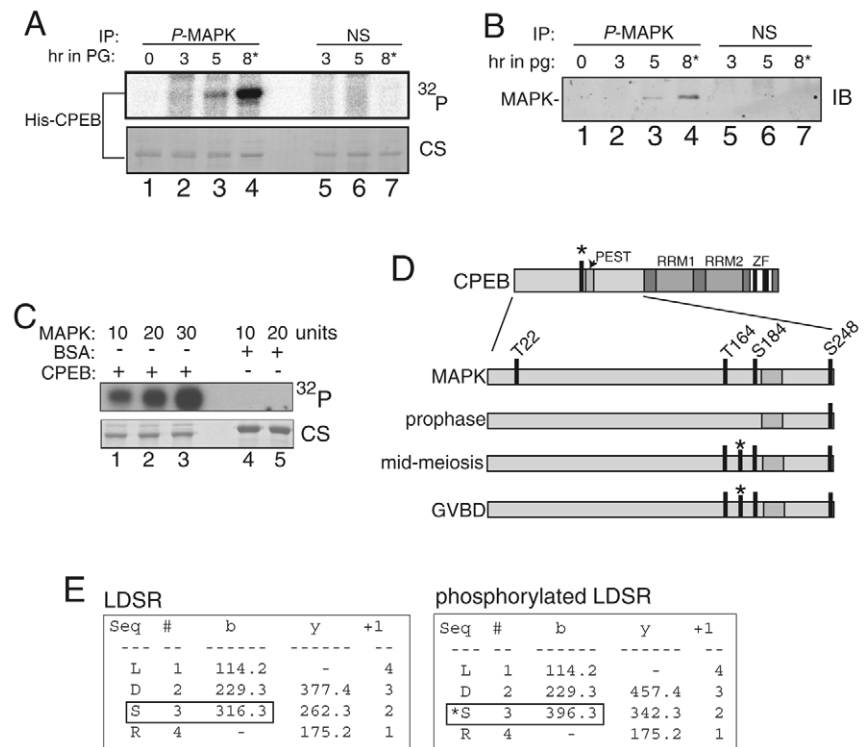
We next examined the ability of endogenous MAPK to phosphorylate CPEB. Endogenous active MAPK was immunoprecipitated from extracts prepared from oocytes that were collected throughout meiotic progression and tested for kinase activity towards His-CPEB (Fig. 5). Phosphorylation of CPEB was observed in the MAPK immunoprecipitates (Fig. 5A, lanes 3 and 4) and no phosphorylation was detected in negative control immunoprecipitates (Fig. 5A, lanes 6 and 7). MAPK activity was only seen in immunoprecipitates that phosphorylated CPEB (Fig. 5B, lanes 3 and 4). The 3- and 5-hour post-progesterone samples are from oocytes in early meiosis (data not shown). Moreover, we did not detect any Cdc2 in the MAPK immunoprecipitates (data not shown).

We then determined those residues in CPEB that were phosphorylated by MAPK. Since the amount of endogenous

active MAPK immunoprecipitated from oocytes was too low (Fig. 5B, lanes 3 and 4) to generate a sufficient quantity of phosphorylated CPEB for phosphopeptide mapping, we used recombinant MAPK. His-CPEB was readily phosphorylated by MAPK, whereas phosphorylation on a BSA-negative control was negligible (Fig. 5C). Phosphopeptide mapping using LC-MS/MS revealed that MAPK phosphorylated CPEB on residues T22, T164, S184 and S248 that are present in the minimal MAPK consensus sites SDTPAL, YPTPLL, SSSPSD and TVSPLG, respectively (Fig. 5D). We did not observe phosphorylation of S174 (which is not within a MAPK consensus site) by MAPK *in vitro* but – as expected – S174 was phosphorylated in extracts from oocytes in early meiosis (Fig. 5E).

If MAPK phosphorylation of CPEB were involved in activating CPEB in cytoplasmic polyadenylation of *Mos* mRNA, we would expect CPEB to be phosphorylated on T22, T164, S184 and S248 after meiotic resumption, i.e. during early meiosis and not in prophase. We examined the appearance of these phosphorylated residues throughout meiosis by phosphopeptide analysis of His-CPEB incubated with extracts from prophase oocytes, mid-meiosis oocytes or GVBD-stage oocytes (Fig. 5D). Although S248 was occasionally phosphorylated in extracts prepared from prophase oocytes, P-S248 was much more frequently first observed in early meiotic and GVBD-stage oocyte extracts. Phosphorylated T164 and S184 were first observed in early meiosis, and GVBD-stage oocyte extracts retained the

Fig. 5. CPEB is phosphorylated by endogenous MAPK during early meiosis. (A) Protein extracts from oocytes collected at the indicated times were used in IP assays with either anti-P-MAPK antibody or non-specific rabbit antibody (NS). His-CPEB tethered to Ni beads were added to the immunoprecipitates and incubated in the presence of [γ - 32 P]ATP. Ni-bead bound samples were analyzed by SDS-PAGE, Coomassie staining and autoradiography. Asterisks indicate 100% GVBD oocytes. (B) Bead-bound samples from A were immunoblotted with anti-MAPK antibody to confirm efficiency of immunoprecipitation. Asterisks indicate 100% GVBD oocytes. (C) *In vitro* phosphorylation of His-CPEB using recombinant MAPK. His-CPEB or BSA were incubated with the indicated units of MAPK, together with [γ - 32 P]ATP. Samples were analyzed by SDS-PAGE, Coomassie staining (CS) and autoradiography (32 P). (D) Schema of His-CPEB protein showing positions of MAPK phosphorylation sites determined by LC-MS/MS phosphopeptide mapping after using either recombinant MAPK from experiments described in C or staged oocyte extracts (bottom three lines) as the kinase source. RRM, RNA-recognition motif; ZF, zinc-finger motif; *, S174; PEST, degradation-targeting domain rich in proline, glutamate, serine and threonine. (E) Table of average charge-to-mass values for ionized peptides (b and y) generated after secondary fragmentation of the CPEB tryptic peptide LDSR. (Left) His-CPEB phosphorylated *in vitro* using MAPK (as shown in D). (Right) His-CPEB phosphorylated by incubation with extracts from early-maturing oocytes. A difference of 80 in the mass-to-charge values of the S174 phosphorylated versus non-phosphorylated peptide ions indicates the presence of the phosphate group. *, S174; CS, Coomassie staining; 32 P, autoradiography; IB, immunoblotting; IP, immunoprecipitation.



peptides (b and y) generated after secondary fragmentation of the CPEB tryptic peptide LDSR. (Left) His-CPEB phosphorylated *in vitro* using MAPK (as shown in D). (Right) His-CPEB phosphorylated by incubation with extracts from early-maturing oocytes. A difference of 80 in the mass-to-charge values of the S174 phosphorylated versus non-phosphorylated peptide ions indicates the presence of the phosphate group. *, S174; CS, Coomassie staining; 32 P, autoradiography; IB, immunoblotting; IP, immunoprecipitation.

capability to phosphorylate T164, S184 and S248. We have only observed phosphorylation of T22 in the in vitro MAPK phosphorylation assays and, therefore, suspect that T22 of CPEB may not be phosphorylated in the context of complete oocyte extract. Notably, roscovitine, which inhibits Cdc2 (Meijer et al., 1997), did not block the ability of early meiotic extracts to phosphorylate these residues or S174 (LC-MS/MS; data not shown). Based on these data and the timing of endogenous MAPK activation, we conclude that T164, S184 and S248 in endogenous CPEB are probably phosphorylated by MAPK.

Low level of MAPK activation before Mos synthesis

Early CPEB phosphorylation correlates with *Mos* polyadenylation and protein accumulation during early meiosis (Martinez et al., 2005; Mendez et al., 2000a). In our classic understanding of meiotic progression, *Mos* activates MEK, which then activates MAPK (Posada et al., 1993). Our data, showing that early CPEB phosphorylation is perhaps dependent upon MAPK activation, therefore appears to contradict previous reports on the timing of these events. Interestingly, however, some groups have reported that MAPK is activated in a biphasic manner during meiosis (Fisher et al., 1999; Fisher et al., 2000). The first transient activation is detectable at a very low level as early as 15 minutes after the addition of progesterone, before detectable levels of *Mos* protein. The second activation occurs to a much higher level, approximately 2 hours after progesterone addition, coincident with the accumulation of *Mos* protein. The level of MAPK activation increases again with activation of MPF at GVBD, through positive feedback (Fisher et al., 2000), in a protein-synthesis-dependent manner (Gotoh et al., 1995).

The earliest phase of MAPK activation might be involved in early phosphorylation of CPEB. Since early CPEB phosphorylation is then involved in the polyadenylation and translation of *Mos* mRNA, early MAPK activation should occur upstream of *Mos* synthesis, as suggested by Fisher and co-workers (Fisher et al., 2000). To test this possibility, we used *Mos* antisense oligonucleotides (Sagata et al., 1988), which block meiotic maturation by preventing the second phase of MAPK activation but do not block early phosphorylation of CPEB (Mendez et al., 2000a). As previously reported, oocytes injected with *Mos* antisense oligonucleotides never achieved appreciable levels of MAPK activation (Fig. 6A, third panel, lanes 4-9) and did not achieve GVBD (data not shown). Interestingly, however, after only 30 minutes in progesterone, oocytes injected with *Mos* antisense oligonucleotides still displayed an early, transient and low level of MAPK activation, which disappeared after one hour (Fig. 6A, third panel, lanes 2 and 3). Importantly, we can detect very early His-CPEB phosphorylation (Fig. 6B, lane 2), which is sensitive to inhibition with U0126 (Fig. 6B, lane 5), at times that correlate with early MAPK activity. These data indicate that a low level of transient MAPK activation occurs prior to *Mos* synthesis, providing an early source of *P*-MAPK at the appropriate time for early CPEB phosphorylation.

XGef is present in a complex with activated MAPK

To investigate whether XGef is present in a signaling complex that contains a kinase recognizing CPEB, we immunoprecipitated XGef from prophase-arrested oocytes and

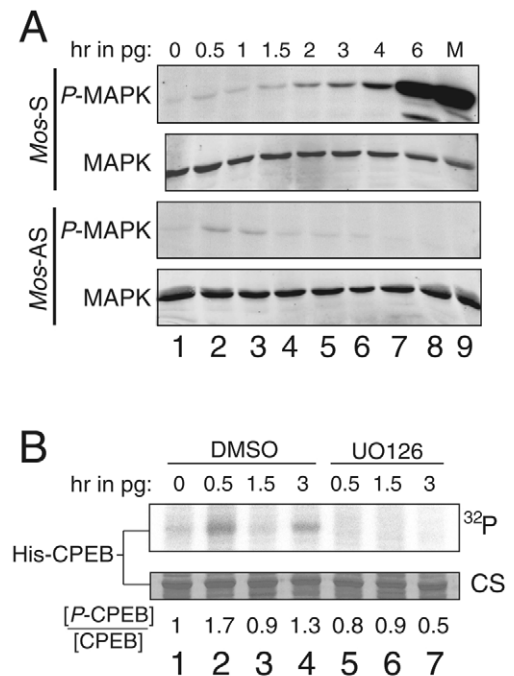


Fig. 6. Phosphorylation of CPEB in early meiosis correlates with transient, low level MAPK activation, which is independent of *Mos* synthesis. (A) Oocytes were injected with either *Mos* sense oligonucleotides (*Mos-S*) or *Mos* antisense oligonucleotides (*Mos-AS*). After 1 hour, progesterone was added and oocytes were collected at times indicated (hr in pg). Protein extracts were analyzed by SDS-PAGE and immunoblotted with anti-*P*-MAPK and anti-MAPK antibodies. (B) In vitro phosphorylation assay of His-CPEB. Oocytes were cultured in the presence of DMSO or U0126 for 1 hour prior to progesterone addition. Extracts from 90 oocytes selected at the indicated times (hr in pg) were used in His-CPEB phosphorylation assays. CS, Coomassie staining; ^{32}P , autoradiography.

from oocytes in the early phase of meiosis, and tested these complexes in a His-CPEB phosphorylation assay (Fig. 7A). Extracts from oocytes collected 3 hours after treatment with progesterone were in early meiosis, apparent by the lack of GVBD (data not shown) and background H1 kinase activity (Fig. 7C, lane 2). His-CPEB was phosphorylated when added to the XGef immunoprecipitate from early meiotic extracts (Fig. 7A, lane 3), but not prophase oocyte extracts (Fig. 7A, lane 1) or immunoprecipitates using nonspecific IgG (Fig. 7A, lanes 2 and 4). Notably, the kinase in this complex that phosphorylates CPEB is inhibited by incubation of oocytes with U0126, 1 hour before addition of progesterone. These results demonstrate that XGef, during early meiosis, is present in a complex containing U0126-sensitive kinase activity towards CPEB.

We then tested whether this kinase was MAPK. Overexpressed HA-XGef was immunoprecipitated from prophase and early meiotic-oocyte extracts (Fig. 7D). Importantly, immunoprecipitated HA-XGef protein complexes also contained kinase activity towards CPEB (data not shown). MAPK was present specifically in HA-XGef immunoprecipitates from both prophase and early meiotic oocytes (Fig. 7D, top panel). Immunoprecipitates were also

immunoblotted with anti-XGef antibody to confirm the effectiveness of the immunoprecipitation, and input samples were immunoblotted with anti-MAPK antibody to ensure equal loading (Fig. 7D, lower 2 panels). The presence of XGef and MAPK in the same complex suggests that XGef mediates early CPEB phosphorylation by recruiting MAPK to CPEB.

Discussion

We have explored how XGef influences the early, activating phosphorylation of CPEB. By using specific inhibitors of MAPK, immunoprecipitation and *in vitro* phosphorylation assays, we have strongly implicated MAPK as a kinase acting upstream of CPEB activation. Recombinant MAPK phosphorylated CPEB on four of nine minimal MAPK consensus sites (Sharrocks et al., 2000) T22, T164, S184 and S248 but did not phosphorylate S174 (within sequence LDSR, a non S/TP motif), which is involved in activation of CPEB (Mendez et al., 2000a). Importantly, T164 and S184 are first found phosphorylated during early meiosis, suggesting that MAPK directly phosphorylates these sites *in vivo* and that their phosphorylation is involved in the function of CPEB during early meiosis. Very early MAPK activation might be necessary for a priming phosphorylation of CPEB, to prepare it for the subsequent activating phosphorylation on S174 by a different kinase, but probably not Aur-A (see below). Alternatively, although MAPK directly phosphorylates CPEB *in vitro*, the influence of MAPK on CPEB activation *in vivo* may be indirect: MAPK activation may instead be required for activation of a kinase upstream of CPEB. Our proposal that MAPK activity is necessary to activate CPEB in polyadenylation during early meiosis is supported by several studies. MAPK activity is required for normal *Mos* mRNA polyadenylation and protein accumulation, and the polyadenylation of other early-class mRNAs (Charlesworth et al., 2002; Charlesworth et al., 2006; Howard et al., 1999). Since early CPEB activation is also required for normal polyadenylation of early-class mRNAs (Mendez et al., 2000a), CPEB is a likely target of MAPK in this process. Furthermore, in clam oocytes, MAPK generates an early CPEB phosphorylation before hyperphosphorylation by Cdc2 (Katsu et al., 1999). MAPK has also been implicated in translational control mechanisms for synaptic plasticity and memory (Kelleher, 3rd et al., 2004), some of which are mediated by CPEB-regulated polyadenylation-induced translation.

Our finding that inhibition of MAPK activation blocked early CPEB phosphorylation was surprising considering that normal *Mos* synthesis is regulated by CPE-mediated polyadenylation-induced translation (Mendez et al., 2000a; Stebbins-Boaz et al., 1996) and that *Mos* is a MAPKKK that activates MAPK during meiotic resumption (Nebreda and Hunt, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993). How can MAPK be involved in CPEB activation, if CPEB activation is needed upstream of MAPK activation? We looked more closely at the time course of MAPK activation

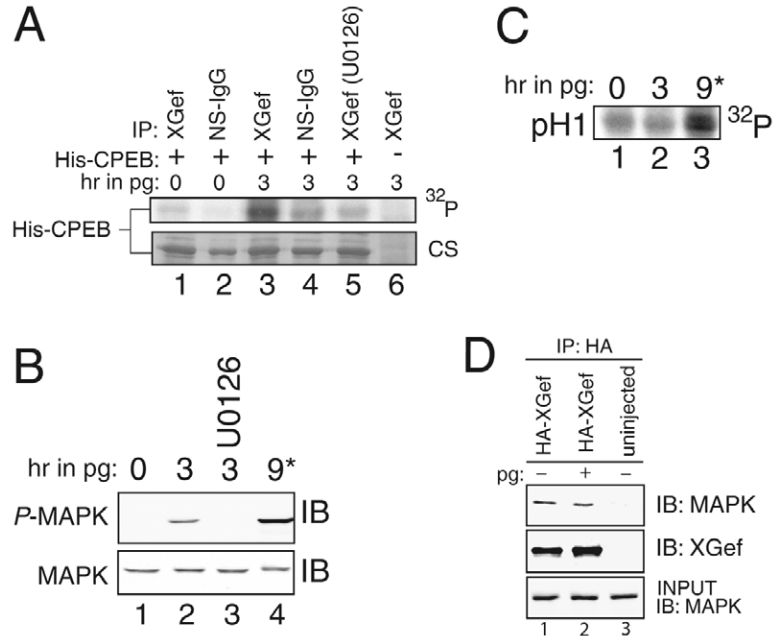


Fig. 7. XGef is present in a complex with MAPK. (A) Endogenous XGef associates with a U0126 sensitive kinase that can phosphorylate CPEB. Some oocytes were treated with U0126 for 1 hour prior to progesterone addition; protein extracts were prepared from oocytes collected at the indicated times (hr in pg). Endogenous protein complexes were immunoprecipitated with anti-XGef antibody or non-specific IgG. Either recombinant His-CPEB tethered to Ni beads or blank beads alone were added to the immunoprecipitated complexes and incubated in the presence of [γ - 32 P]ATP. Ni-bead-bound samples were analyzed by SDS-PAGE, Coomassie staining and autoradiography. (B) Immunoblot analysis for the samples used in A with anti-P-MAPK and anti-MAPK antibodies. Asterisk indicates 100% GVBD oocytes. (C) Extracts from A were used in an H1 kinase assay. Asterisk indicates 100% GVBD oocytes. (D) MAPK is present in HA-XGef immunoprecipitates. Protein extracts from prophase oocytes (–pg, not exposed to progesterone) and early meiotic oocytes (+pg, exposed to progesterone for 3 hours), expressing HA-XGef or not were used in immunoprecipitations with anti-HA antibody conjugated to Protein-G- agarose. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-MAPK (top panel) and anti-XGef antibodies (middle panel). Input samples were analyzed with MAPK antibody (bottom panel). CS, Coomassie staining; 32 P, autoradiography; IB, immunoblotting; IP, immunoprecipitation.

during early meiosis and observed a very early, transient, low-level MAPK activation within 30 minutes of progesterone addition, confirming earlier reports (Fisher et al., 1999; Fisher et al., 2000). Notably, we demonstrate that this very early MAPK activation is independent of *Mos*, as proposed by Fisher and co-workers (Fisher et al., 2000), which would be consistent with the requirement for CPEB activation in polyadenylation-induced translation of *Mos* mRNA.

Our data indicate that Toxin-B-sensitive Rho-family GTPases are not required for progesterone-induced early CPEB phosphorylation or progression to GVBD. This finding implies that the influence of XGef on early meiosis is not mediated by XGef exchange factor activity. XGef might facilitate nucleotide exchange for a GTPase outside the Rho-family, although this is unlikely given its ability to exchange for Cdc42 in mammalian cells (Reverte et al., 2003). Interestingly, some GEFs exert physiological effects in the

absence of GEF activity. Kal-GEF1 (Kalirin-Gef), an efficient GEF for Rac1 and RhoG, can induce the formation of lamellopodia in cells in culture (the normal phenotype of Rac activation) in the absence of GEF activity (Schiller et al., 2005). Tiam1, a Rac1 GEF, influences the association of MAPK signaling components to the scaffold protein IB2/JIP2 in a pancreatic cell line and rat brain extracts (Buchsbbaum et al., 2002). Notably, XGef contains several putative D-domains and an FxF motif, regions identified in other proteins to act as MAPK docking sites (Sharrocks et al., 2000). This raises the possibility that XGef functions as a scaffold by binding both MAPK and the MAPK substrate CPEB. This is supported by the presence of XGef in protein complexes competent for CPEB phosphorylation that contain MAPK. The fact that XGef associates with MAPK in prophase as well as early meiotic oocytes – as observed for the association of XGef and CPEB in the *Mos* mRNP (Martinez et al., 2005) – suggests that it is not the mere association of these proteins that stimulates the activating phosphorylation of CPEB. As discussed previously (Martinez et al., 2005), a progesterone-stimulated event is required for the influence of XGef on early CPEB phosphorylation. This study provides a new possibility: the progesterone-stimulated event could be the very early activation of MAPK. Further work is needed to determine the mechanism for how XGef and MAPK mediate early CPEB phosphorylation.

We have not detected *P*-T295 Aur-A in early meiosis and also have no evidence to support a role for Aur-A in the activating phosphorylation of CPEB that occurs prior to activation of MPF. Using an antibody specific for the phosphorylated activation-loop residue T295 in Aur-A, combined with Aur-A immunodepletion and Anderson gel analysis, we first detect *P*-T295 Aur-A coincident with GVBD. Phosphorylation of T295 correlates with active Aur-A, and mutation to either alanine or aspartic acid abolishes activity (Littlepage et al., 2002). Our data contradict a recent study proposing that Aur-A is phosphorylated on T295 from prophase through meiotic progression, with Aur-A activity in prophase oocytes held in check by inhibitory phosphorylation on S349 that is potentially released during early meiosis by inhibition of GSK-3 (Sarkissian et al., 2004). Although phosphorylation of an activation-loop residue occurs in a number of kinases causing conformational changes that facilitate enzymatic activity (Nolen et al., 2004), it is possible for Aur-A that *P*-T295 is not a reliable indicator of kinase activity. However, additional support for Aur-A activation later in meiosis, at GVBD, comes from reports showing Aur-A kinase activity coinciding with GVBD – not earlier in meiosis (Frank-Vaillant et al., 2000; Maton et al., 2003) – and the finding that active Cdc2 (i.e. active MPF) is involved in activation of Aur-A (Maton et al., 2005; Maton et al., 2003). Recent reports have also uncovered the new Aur-A-interacting protein Bora, which is integral to Aur-A activation at mitosis onset, but only when excluded from the nucleus upon mitotic entry in a Cdc2-dependent manner (Hutterer et al., 2006).

Aur-A has been implicated in the activating phosphorylation of S174 in CPEB (Mendez et al., 2000a). Although undetectably low levels of active Aur-A may be present in early meiosis, our experiments using Aur-A immunodepletion and the Aur-A and Aur-B kinase inhibitor ZM447439 strongly

suggest that Aur-A activity is not required for the activating phosphorylation of CPEB on S174. In fact, the recently determined consensus Aur-A phosphorylation site, [R/K/N]Rx[S/T] B (in which B stands for any hydrophobic residue except proline) is distinct from the sequence RLDSR surrounding S174 of CPEB. Although this site contains a suitable arginine at position –3, there is no arginine at position –2 and the arginine at position +1 is not hydrophobic; both an arginine at position –2 and a hydrophobic amino acid at position +1 are crucial for phosphorylation by Aur-A (Ferrari et al., 2005). This new information on the Aur-A consensus site makes it unlikely that CPEB is functionally phosphorylated by Aur-A in vivo. The fact that GVBD was not delayed in oocytes injected with ZM447439 also suggests that Aur-A and Aur-B are not required for early meiotic events. However, recent data (Yang et al., 2005) show that during mitosis, Aur-B can compensate for the absence of Aur-A activity and, in the absence of both kinases, the need for Aur-A is completely bypassed. Thus inhibition of both Aur-A and Aur-B in early meiosis might simply allow a bypass of Aur-A activity, perhaps resulting in activation of a redundant pathway including a kinase that can phosphorylate S174 of CPEB. Alternatively, although ZM447439 clearly inhibits Aur-A activity in the in vitro phosphorylation assay, it may not effectively inhibit Aur-A activity when injected into oocytes (Girdler et al., 2006). In cultured cells, ZM447439 more effectively inhibits Aur-B, whereas in the in vitro systems Aur-A is also targeted (Ditchfield et al., 2003; Girdler et al., 2006). Therefore, although our findings with ZM447439 are consistent with our immunodepletion data, more specific inhibitors and in vivo markers for active Aur-A will be necessary for further analysis of Aur-A function during meiosis. In summary, consideration of the accumulated data on timing of Aur-A activity, dispensability of Aur-A with regard to CPEB S174 phosphorylation in early meiotic extracts and the lack of a consensus Aur-A phosphorylation motif in CPEB lead us to strongly suggest that Aur-A is not involved in CPEB activation during early meiosis.

Our data does not preclude a role for Aur-A later in meiotic progression. Studies in both meiosis and mitosis demonstrate that Aur-A is involved in M-phase entry. Overexpression of Aur-A in *Xenopus* oocytes can accelerate meiotic progression and reduces the concentration of progesterone required to stimulate meiotic resumption (Andresson and Ruderman, 1998). Interference with Aur-A function impairs mitotic entry in mammalian cells (Hirota et al., 2003; Sasayama et al., 2005) and in cycling *Xenopus* egg extracts (Liu and Ruderman, 2006). Precisely how Aur-A influences M-phase entry is unclear: overexpression of Aur-A leads to enhanced polyadenylation of Cdk1 and cyclin B1 mRNAs in mitotic cells (Sasayama et al., 2005), but does not cause obvious changes in cyclin B1 protein levels in cycling egg extracts (Liu and Ruderman, 2006). Recent reports indicate that Aur-A can also be activated independently of Cdc2 (Krystyniak et al., 2006). The ability of myristoylated, constitutively active Aur-A to induce GVBD (Ma et al., 2003) might be through an influence of Aur-A on MPF activity (Dutertre et al., 2004), thus activating the positive feedback loop to *Mos* synthesis. Although no connection between constitutively active Aur-A and phosphorylation of S174 in CPEB was shown, the observed hyperphosphorylation of CPEB (Ma et al., 2003) is

reminiscent in scope and timing to hyperphosphorylation of CPEB by MPF. Endogenous Aur-A may, in fact, influence CPEB activity, either directly when active Aur-A is detected at GVBD or indirectly by influencing MPF activity, which is clearly required for CPEB function late during meiotic progression (Charlesworth et al., 2004; Charlesworth et al., 2002; Howard et al., 1999; Mendez et al., 2002; Paris et al., 1991).

One intriguing possibility is that RINGO/Spy is involved in the early transient phase of MAPK activation. RINGO/Spy is a new Cdc2-interacting protein that can associate with and activate Cdc2 (Ferby et al., 1999). Interestingly, a recent report demonstrated that early CPEB phosphorylation requires the synthesis of RINGO protein that occurs prior to Mos synthesis (Padmanabhan and Richter, 2006). Since an early and transient low level of MAPK activation occurs independently of Mos accumulation but is blocked by cycloheximide (Fisher et al., 2000), it is tempting to speculate that this low level of MAPK activation perhaps depends instead on RINGO synthesis. This would also agree with previous work (Lenormand et al., 1999), proposing that RINGO works through a MAPK-dependent but Mos-independent pathway because injection of RINGO into *Xenopus oocytes* was (1) able to induce GVBD in the presence of *Mos* antisense oligonucleotides but (2) not able to induce GVBD in the presence of an anti-MEK antibody that blocks MAPK activation (Lenormand et al., 1999). Perhaps RINGO protein synthesis is required for the early transient MAPK activation potentially involved in priming CPEB for activation by phosphorylation on S174.

Materials and Methods

Routine assays, immunoblotting, antibody preparation

Culturing of oocytes, in vitro transcription, microinjection, protein extraction, SDS-PAGE, in vitro phosphorylation assay, H1 kinase assay and phosphopeptide analysis were carried out as described (Martinez et al., 2005). Immunoblot analysis was performed using either chemiluminescence (Perkin Elmer) or the LiCor Odyssey. Antibodies anti-XGef, anti-Cdc2, anti-MAPK and anti-P-MAPK were used as described (Martinez et al., 2005). Rabbit polyclonal antibody against Aur-A (1:1000 dilution), *Xenopus* His-tagged Aur-A plasmid and protocols for protein expression, purification and antibody affinity purification were generously provided by Joan Ruderman (Harvard Medical School, Boston, MA). Anti-P-T288 human Aur-A antibody (Cell Signaling Technology) was used at a 1:5000 dilution. For LiCor Odyssey blots, IR-dye-linked secondary antibodies (Molecular Probes) were used at a 1:10,000 dilution.

Immunoprecipitation

Immunoprecipitations were performed as described (Martinez et al., 2005) using Protein G agarose beads (Invitrogen). For XGef immunoprecipitation (IP) and CPEB phosphorylation assay, precleared extract from 130 oocytes was used with 3 μ l of rabbit anti-XGef polyclonal serum (Reverte et al., 2003), XGef preimmune serum, or normal rabbit IgG (Santa Cruz). Washed immunoprecipitates were resuspended in extraction buffer (EB) (Martinez et al., 2005) and incubated with 2.5 μ g of His-CPEB and 6 μ Ci of [γ - 32 P]ATP in the CPEB phosphorylation assay. A similar approach was used for MAPK IP and CPEB phosphorylation assay with anti-P-MAPK antibody (Cell Signaling Technologies).

GTPase assay

Oocytes were microinjected with 46 nl of *Clostridium difficile* Toxin B (CalBiochem; reconstituted in 50 mM NaCl, 50 mM Tris pH 7.5) to a final concentration of 12 nM (Just et al., 1994). Clarified oocyte cytoplasmic extracts were loaded with the non-hydrolyzable GTP analog GMPPNP by treatment with 10 mM EDTA pH 8.0 and 0.1 mM GMPPNP (Cytoskeleton Inc., Denver, CO) and incubation for 15 minutes at 30°C (adapted from Pierce Biotechnology). Reactions were stopped with 60 mM MgCl₂. *E. coli* BL21-DE cells (Stratagene) were transformed with pGEX2T-GST-PAK PBD or pGEX2T-GST-Rhotekin RBD for protein expression and purification (Buchsbaum et al., 2003). GMPPNP-loaded lysates were incubated with 20 μ g of glutathione bead bound GST-PAK PBD or GST-Rhotekin RBD for 1 hour at 4°C. Beads were boiled in 2 \times SDS loading buffer and analyzed by 10% or 12.5% SDS-PAGE and immunoblotting with antibodies

against Cdc42 (BD Biosciences 1:750 dilution), RhoA (Cytoskeleton, 1:750 dilution), and Rac1 (Abcam, 1:750 dilution). Alexa Fluor-680-conjugated anti-mouse IgG was used to detect anti-Cdc42 and anti-RhoA antibodies (Molecular Probes, 1:10,000 dilution). Horseradish peroxidase-conjugated anti-chicken IgY was used to detect the anti-Rac1 antibody (AbCam, 1:7000 dilution).

Inhibitor treatment

U0126 (Cell Signaling Technology) in DMSO (50 μ M), cycloheximide (Sigma) in water (35 μ M) or roscovitine (Sigma, 50 μ M) was added 1 hour prior to addition of progesterone. Protein extracts prepared in EB buffer without phosphatase inhibitors were treated with MKP1 (40 units/50 oocytes; Upstate Cell Signaling Systems) for 30 minutes at 30°C. His-CPEB substrate was then added with phosphatase inhibitors (10 mM β -glycerophosphate, 20 mM NaF, and 2 mM NaVanadate). *Xenopus* His-Aur-A was purified after overexpression in BL21 cells and used in kinase assays with myelin basic protein (Sigma) in kinase buffer (20 mM Tris pH 7.5, 10 mM MgCl₂, 50 mM KCl, 1 mM DTT, 30 μ M ATP) with or without 20 μ M ZM447439 (Astra Zeneca, provided by Joan Ruderman) for 20 minutes at room temperature.

MAPK phosphorylation assay

Murine p42MAPK (Erk2) (New England Biolabs) was incubated at 30°C for 30 minutes with 3 μ g of either His-CPEB or BSA. For autoradiography, 10 μ Ci of [γ - 32 P]ATP was included. For LC-MS/MS, the Coomassie-stained protein band corresponding to His-CPEB was excised and analyzed by the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA) as described (Martinez et al., 2005).

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