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The kinase Eg2 is a component of the *Xenopus* oocyte progesterone-activated signaling pathway

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Quiescent *Xenopus* oocytes are activated by progesterone, which binds to an unidentified surface-associated receptor. Progesterone activates a poorly understood signaling pathway that results in the translational activation of mRNA encoding Mos, a MAP kinase kinase kinase necessary for the activation of MAP kinase and MPF, the resumption of meiosis, and maturation of the oocyte into the sperm-responsive egg. We have designed a screen to identify early signaling proteins based on the premise that some of these proteins would be phosphorylated or otherwise modified within minutes of progesterone addition. This screen has revealed Eg2, a Ser/Thr kinase. We find that Eg2 is phosphorylated soon after progesterone stimulation and provide evidence that it functions in the signaling pathway. Overexpression of Eg2 via mRNA microinjection shortens the time between progesterone stimulation and the appearance of new Mos protein, accelerates activation of MAP kinase and advances entry into the meiotic cell cycle. Finally, overexpression of Eg2 dramatically reduces the concentration of progesterone needed to trigger oocyte activation. These results argue that the kinase Eg2 is a component of the progesterone-activated signaling pathway that releases frog oocytes from cell cycle arrest.

Keywords: Mos/progesterone/Ser/Thr kinases/small pools screen/*Xenopus* oocyte activation

Introduction

Fully grown *Xenopus* oocytes are arrested at prophase (G_2/M) of meiosis I. The steroid hormone progesterone is the mitogen that breaks this arrest, initiating resumption of the meiotic cell cycles and conversion of the immature oocyte into the mature, fertilizable egg (Masui, 1967; Schuetz, 1967). It has been known for more than 20 years that progesterone acts at or near the surface and activates the oocyte through a pathway that does not depend on transcription (reviewed by Smith, 1989; Cork and Robinson, 1994). Instead, progesterone initiates a poorly understood signaling pathway that leads to the translational recruitment of several stored mRNAs. The protein kinase Mos, one of the first newly made proteins, serves as a key regulator of cell cycle re-entry (Freeman *et al*., 1989; Sagata *et al*., 1989a,b). Mos leads to activation of pre-existing stores of MAP kinase and MPF (cyclin B/cdc2), and to resumption of the meiotic cell cycle (reviewed by Gebauer and Richter, 1997; Sagata, 1997).

In somatic cells, it is well established that steroid hormones regulate transcription: they bind receptors that reside in, or translocate into, the nucleus, where they control the activity of specific target genes (Mangelsdorf *et al*., 1995; Beato and Sanchez-Pacheco, 1996; Katzenellenbogen *et al*., 1996). Rapid, non-transcriptional effects of steroids have been reported in numerous systems (Wehling, 1997), but it is only recently that specific details have emerged. Two conventional steroid receptors, those for estradiol and progesterone, can also rapidly activate cytoplasmic signal transduction pathways in certain circumstances (Migliaccio *et al*., 1996, 1998). Additionally, progesterone can bind and inhibit cytoplasmic signaling from the G-protein-coupled transmembrane receptor that responds to the peptide hormone oxytocin (Grazzini *et al*., 1998).

Early biochemical efforts to identify the *Xenopus* oocyte surface-associated progesterone receptor were promising (Sadler and Maller, 1982; Baulieu *et al*., 1985). However, attempts to isolate the receptor directly have been unsuccessful, and it is not even known whether progesterone signals through a conventional or a novel receptor. Although high concentrations of insulin can induce maturation of *Xenopus* oocytes by binding an IGF-1 type receptor that functions through PI 3-kinase, ras and raf (Maller and Koontz, 1981; Fabian *et al*., 1993; Muslin *et al*., 1993; Liu *et al*., 1995), none of these appears to be part of the physiological, progesterone-regulated pathway (Sadler *et al*., 1986; Deshpande and Kung, 1987; Korn *et al*., 1987; Liu *et al*., 1995; Shibuya *et al*., 1996). Some progress has been made towards identifying some of the earliest changes occurring downstream of the progesterone receptor. Within minutes of exposure to progesterone, there is a rapid decrease in adenylyl cyclase activity, cAMP levels and protein kinase A (PKA) activity, both in whole oocytes and in isolated surface-vesicle preparations (Maller and Krebs, 1977; Finidori-Lepicard *et al*., 1981; Sadler and Maller, 1981). This drop in PKA activity is essential for oocyte activation (Maller and Krebs, 1977; Sadler and Maller, 1981). High PKA levels appear to block oocyte maturation at multiple levels, including inhibition of the synthesis and/or stabilization of new Mos protein and inhibition of MPF activation by the phosphatase cdc25 (Daar *et al*., 1993; Matten *et al*., 1994).

Little is known about the pathway that connects the earliest progesterone-induced events to the translational activation of *mos* and other stored mRNAs, which begins a few hours after hormone stimulation. Most stored mRNAs contain short 3′ oligo(A) tails and progesterone stimulation leads to their adenylation. This polyadenylation is required for their translational activation (reviewed by

Ballantyne *et al*., 1995; Stebbins-Boaz and Richter, 1997). Some of the basal components required for this step are known, including a cytoplasmic polyadenylation element (CPE) in the 3′ UTR and a CPE-binding protein (CPEB) (reviewed in Stebbins-Boaz and Richter, 1997; Wickens *et al*., 1997). However, *mos* mRNA is adenylated and translated much earlier than most other stored mRNAs, and the components responsible for the selective, early adenylation of *mos* mRNA are not known (Ballantyne *et al*., 1997; DeMoor and Richter, 1997).

The work reported in this paper was designed to identify components of the pathway that link the oocyte progesterone receptor to the translational activation of *mos* and other stored mRNAs. Using a variation of the 'small pools' cDNA screening strategy (Stukenberg *et al*., 1997), we have designed a screen to identify proteins that become modified soon after progesterone stimulation. The premise of this approach is that many signaling proteins become phosphorylated or otherwise modified soon after receptor activation, and that such modifications are often detectable as shifts in electrophoretic mobility. In an initial screen, we have identified a 46 kDa kinase that was first reported as a *Xenopus* egg mRNA sequence called Eg2 (Paris and Philippe, 1990). We show that endogenous oocyte Eg2 protein is phosphorylated soon after progesterone stimulation. Overexpression of Eg2 protein via mRNA injection significantly accelerates the progesterone-dependent production of new Mos protein, activation of MAP kinase and MPF, and breakdown of the oocyte nucleus (germinal vesicle, GVBD), the standard morphological indicator of oocyte activation. Overexpression of Eg2 also lowers the concentration of external progesterone needed to induce oocyte activation. These properties suggest that the kinase Eg2 is a component of the pathway that links the progesterone receptor to the translational activation of *mos* mRNA and resumption of the cell cycle in *Xenopus* oocytes.

Results

Design of the screen

To identify proteins that function early in progesterone-activated signaling, we used a small pools cDNA screening protocol. The screen relies on the observation that many signaling proteins become modified soon after receptor activation and that these modifications are often recognizable as shifts in the proteins' electrophoretic mobilities on SDS–polyacrylamide gels (SDS–PAGE). The strategy taken to identify such proteins employs DNAs prepared from small pools of a *Xenopus* oocyte cDNA library. From each pool, a set of DNAs is transcribed and translated in a coupled reaction using reticulocyte lysate to generate a reaction product containing a set of ^{35}S-labeled proteins. The *in vitro* translation products are then incubated with concentrated extract from either unstimulated frog oocytes (OE) or oocytes collected 30 min after progesterone stimulation (PE30). Individual radiolabeled proteins that become differentially modified by one of the extracts are recognized by a difference in their electrophoretic mobility on SDS–PAGE. If such a protein is identified, the pool is subdivided and re-tested until the clone encoding the protein is isolated. The screen is not expected to be saturating, since it will miss proteins that are not modified or do not shift. Also, it may identify proteins such as the kinase p70^{S6K}, which is phosphorylated and activated around 30 min (see below), but is not required for early events of oocyte activation (Morley and Pain, 1995). However, once one or more functional components have been identified, these proteins themselves can then be used to find other proteins in the pathway.

The screen depends on the ability of extracts from unstimulated oocytes and progesterone-activated oocytes to maintain their stage-specific modifying activities toward radiolabeled *in vitro* translation products. Extracts were prepared using a slight modification of the methods described by Shibuya *et al*. (1992). Two previously characterized proteins, p70^{S6K} and MAP kinase, were chosen to test the stage-specific activity of the extracts. *In vivo*, p70^{S6K} is activated within 30 min of progesterone stimulation and its activation is accompanied by an electrophoretic shift (Lane *et al*., 1992; Morley and Pain, 1995). In contrast, the major detectable MAP kinase of *Xenopus* oocytes does not become activated or shifted until much later, typically a few hours after progesterone stimulation (Ferrell *et al*., 1991; Gotoh *et al*., 1991; Posada *et al*., 1991).

Radiolabeled p70^{S6K} and MAP kinase were produced by *in vitro* translation and incubated with buffer alone or with extract from either unstimulated oocytes or oocytes collected 15 or 30 min after progesterone stimulation. Following incubation at 20°C for 40 min, the proteins were analyzed by SDS–PAGE followed by autoradiography. The initial p70^{S6K} *in vitro* translation product migrates as a tight band at ~70 kDa (Figure 1, left panel, lane IVT). Following incubation with unstimulated oocyte extract (lane OE), p70^{S6K} shifts to a faster mobility, probably because the p70^{S6K} made in reticulocyte lysate carries some basal phosphorylations that are removed by the oocyte extract. After incubation with the progesterone-stimulated extracts (lanes PE15 or PE30), the mobility of p70^{S6K} is somewhat retarded. As shown in the right panel of Figure 1, MAP kinase *in vitro* translation product (IVT) migrates as a single band around 42 kDa. Its electrophoretic mobility is essentially unchanged by incubation with either oocyte extract (OE) or extracts of progesterone-stimulated oocytes taken 15 or 30 min after hormone addition (PE15 and PE30, respectively). When mixed with extract prepared from mature eggs (ME), in which endogenous MAP kinase is known to be phosphorylated and active, the tracer MAP kinase shows its characteristic shift in mobility. These results indicate that the extracts retain at least some of their stage-specific modifying activities and are suitable for screening.

Identification of two proteins that are modified soon after progesterone stimulation

We constructed a new *Xenopus* oocyte cDNA library using poly(A)+ RNA from the entire developmental range (stages 1–6) of defolliculated oocytes. An initial screen of 90 pools, each containing about 100 clones per pool, yielded two (and only two) clones that were shifted by extracts of progesterone-activated oocytes. Figure 2A shows the results obtained by screening of one such pool. As experienced by others (Stukenberg *et al*., 1997), many fewer than the expected 100 reticulocyte lysate translation products were apparent. Of the detectable translation

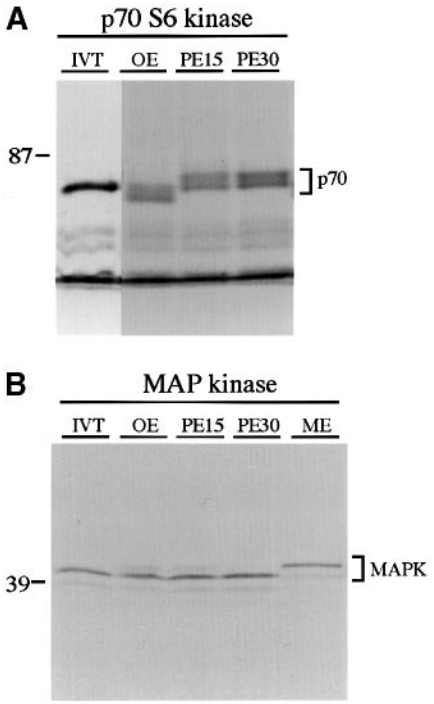


Fig. 1. Extracts of unstimulated and progesterone-stimulated oocytes maintain their stage-specific modifying activities towards two test proteins, (**A**) $p70^{S6K}$ kinase and (**B**) MAP kinase. ^{35}S-methionine-labeled $p70^{S6K}$ and MAP kinases were synthesized *in vitro* using a coupled transcription–translation reaction in reticulocyte lysate as described in Materials and methods. *In vitro* translation products were incubated with either buffer (IVT), extract of unstimulated oocytes (OE), extracts of progesterone-stimulated oocytes that were collected 15 or 30 min after hormone addition (PE15 and PE30, respectively), or extracts of mature eggs (ME). Reaction products were analyzed by SDS–PAGE followed by autoradiography.

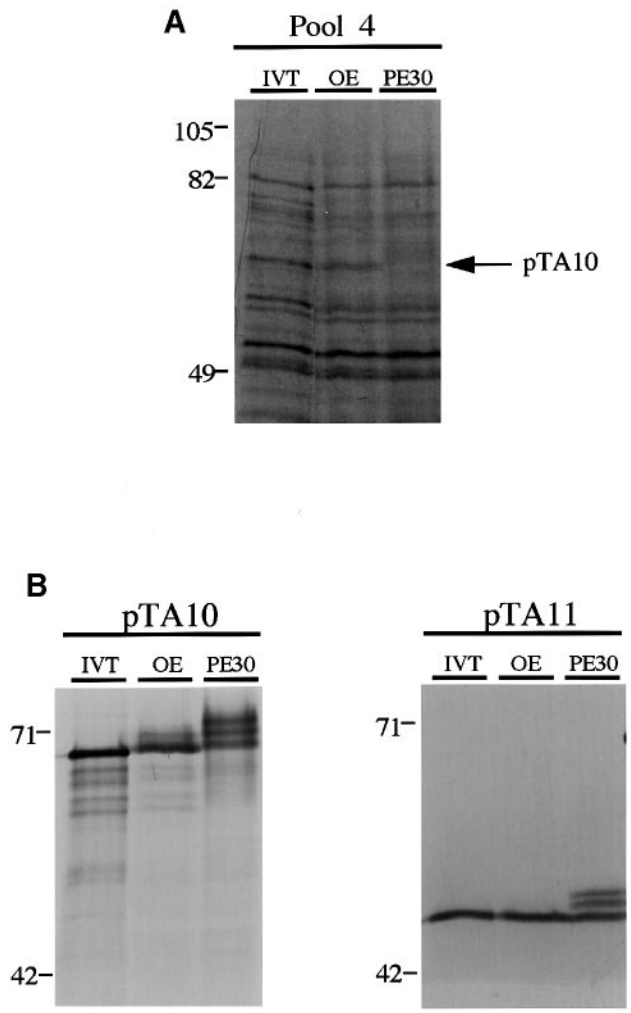


Fig. 2. Identification of two clones, pTA10 and pTA11, encoding proteins that are modified after progesterone stimulation. (**A**) ^{35}S-methionine-labeled proteins encoded by DNA from library pool 4 were produced by *in vitro* translation and incubated with either buffer (IVT), oocyte extract (OE) or extract from progesterone-stimulated oocytes (PE30), as described in Figure 1. Arrow indicates a 69 kDa protein that shifts and was subsequently identified as the product encoded by clone pTA10. (**B**) Stage-specific modifications of two individual clones, pTA10 and pTA11. Left panel: pool 4 was subdivided and retested as above until a single clone encoding the shifting 69 kDa protein (pTA10) was isolated. Right panel: the clone pTA11 was identified and isolated using the same approach. pTA11 encodes the kinase Eg2 (see text and Figure 3).

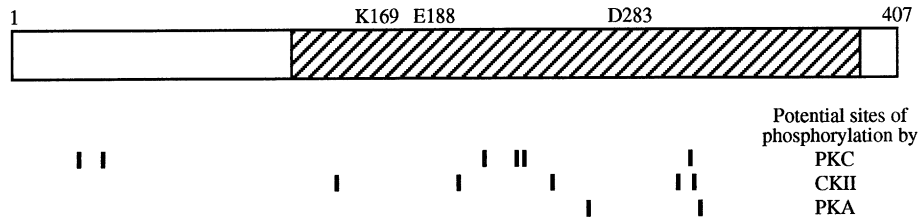
products, one showed a difference in mobility after incubation in extracts made from unstimulated oocytes (OE) versus progesterone-stimulated cells (PE30). Subdivision and further testing yielded a single clone, pTA10. A second clone, initially designated pTA11, was similarly identified (Figure 2B).

Sequencing revealed that both clones encode proteins with properties consistent with their involvement in a signaling pathway. pTA10 encodes a 69 kDa protein with five N-terminal WD40 repeats, domains that are often involved in protein–protein interactions (data not shown). No obviously related proteins were found in available databases. At this time, pTA10 is in the early stages of analysis, and we have no information on its function.

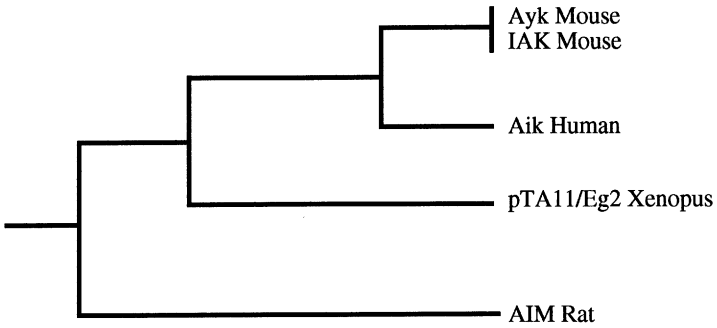
As shown in Figure 3, pTA11 encodes a 46 kDa protein whose sequence predicts it to be a Ser/Thr kinase consisting of an N-terminal noncatalytic domain (residues 1–142), a central catalytic domain (residues 143–390) and a short C-terminal domain (residues 391–407). pTA11 is identical to the DDBJ/EMBL/GenBank sequence Eg2 (Xlp46BPK), which was first identified as an mRNA that is deadenylated and degraded after fertilization (Paris and Philippe, 1990). We thus refer to pTA11 as Eg2. The Eg2 protein contains potential sites of phosphorylation by PKA, protein kinase C and casein kinase I (Figure 3A), consistent with the possibility that it is regulated by phosphorylation by these or other kinases. It also contains a potential mitotic destruction box sequence near the C-terminus (R^{378} LPLKGV, Figure 3C).

Eg2 shows highest sequence homology with a small group of kinases with known or suspected cell cycle roles (Figure 3B and C). It is most similar to human Aik (Kimura *et al.*, 1997), mouse Ayk (Yanai *et al.*, 1996), mouse IAK-1 (Gopalan *et al.*, 1997) and rat AIM-1 (Terada *et al.*, 1998). These kinases show highest similarities in their catalytic domains and are more divergent in their N-terminal non-catalytic domains. All contain a sequence near the end of kinase domain XI that weakly resembles the mitotic destruction box (Figure 3C, underlined). Eg2 is more distantly related (not shown) to the *Drosophila* kinase Aurora, which is required for proper separation of duplicated centrosomes (Glover *et al.*, 1995), and the budding yeast kinase Ipl1, mutations in which produce a high rate of chromosome loss (Chan and Botstein, 1993).

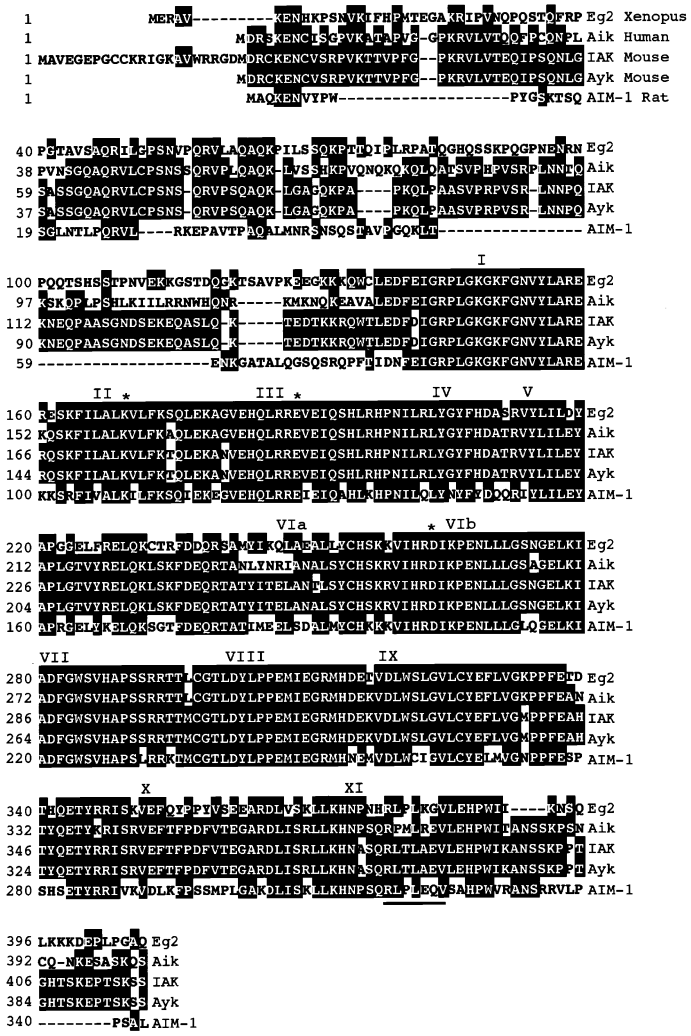
A


1
K169 E188
D283
407
Potential sites of phosphorylation by
PKC
CKII
PKA

B


Ayk Mouse
IAK Mouse
Aik Human
pTA11/Eg2 Xenopus
AIM Rat

C


Eg2 Xenopus
Aik Human
IAK Mouse
Ayk Mouse
AIM-1 Rat
I
II
III
IV
V
VIa
VIb
VII
VIII
IX
X
XI

The functional relationships among these kinases are considered later, in the Discussion.

Endogenous Eg2 protein is modified in response to progesterone

An important first step in testing the relevance of any candidate identified in the screen is to determine whether the endogenous protein exists in the intact oocyte and if it is modified soon after progesterone stimulation. We were fortunate to obtain Eg2 antibody from Prigent and colleagues (Roghi *et al*., 1998), which allowed us to monitor Eg2 directly on immunoblots. As shown in Figure 4, endogenous Eg2 migrates as a single band in unstimulated oocytes and as two electrophoretically shifted bands in oocytes collected 30 min after progesterone stimulation. Phosphatase treatment reverses the shift (data not shown), indicating that Eg2 is modified by phosphorylation. Thus, endogenous Eg2 protein is present in the quiescent *Xenopus* oocyte and is phosphorylated within 30 min of progesterone stimulation. No significant oscillations in the level of Eg2 protein during the meiotic cell cycles were seen (not shown). Unfortunately, the small amount of antibodies currently available have precluded a detailed analysis of changes in Eg2 modification and Eg2 kinase activity during oocyte maturation.

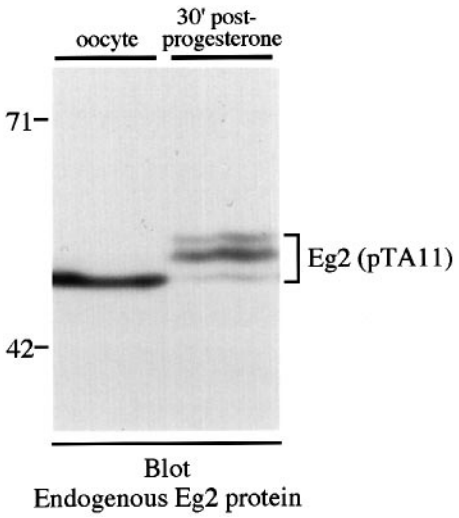


Fig. 4. Endogenous Eg2 protein is modified in response to progesterone stimulation. Homogenates of unstimulated oocytes or oocytes collected 30 min after progesterone stimulation were separated by SDS–PAGE and immunoblotted with an Eg2 monoclonal antibody generously provided by C.Prigent (Rennes).

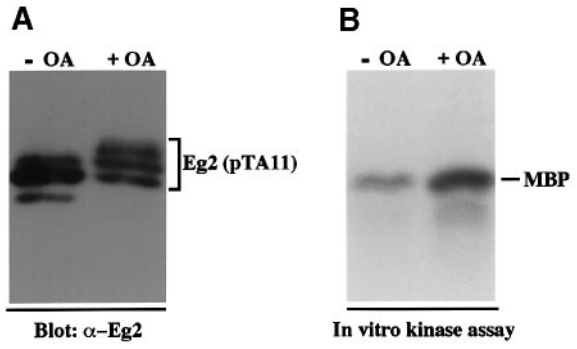


Fig. 5. *In vitro* kinase activity of baculovirus-expressed Eg2. Wild-type Eg2 cloned into a baculovirus vector was expressed in Sf9 cells. During preparation of the kinase, okadaic acid was added (or omitted) to block phosphatase activity. Purified Eg2 was analyzed (**A**) for electrophoretic mobility on immunoblots and (**B**) for its kinase activity toward the substrate myelin basic protein (MBP) as idicated in the autoradiogram.

A different approach, however, allowed us to equate the activated form of Eg2 with the phosphorylated, shifted form (Figure 5). We used baculovirus to express Eg2 in Sf9 cells. When okadaic acid was included during preparation of Eg2 to inhibit phosphatases that might remove activating phosphorylations, the Eg2 recovered is shifted, phosphorylated and highly active. When okadaic acid was omitted, Eg2 was found mainly in the unshifted, unphosphorylated and inactive form. These results argue that Eg2 becomes activated in response to progesterone.

Overexpression of Eg2 accelerates progesterone-induced GVBD

To test for a functional role, we asked if overexpression of Eg2 could induce or accelerate the process of oocyte activation. Oocytes were injected with either Eg2 mRNA or buffer and incubated overnight to allow the accumulation of newly made proteins. Injection of Eg2 mRNA did not trigger GVBD. It did, however, accelerate progesterone-induced GVBD, with injected oocytes reaching $GVBD_{50}$ about 2 h ahead of control-injected oocytes (Figure 6). By contrast, oocytes injected with a mutated version of Eg2, in which the presumptive catalytic lysine residue was changed to arginine (K169R), did not show accelerated entry into GVBD. Comparable results were obtained in two additional independent experiments (not shown). These results provide the first evidence that Eg2 might function in the pathway of progesterone-initiated oocyte activation.

Kinase-inactivating mutants can sometimes function as dominant negatives. Injection of higher levels of Eg2 (K169R) mRNA, which generally lead to expression of recombinant Eg2 at levels a few-fold higher than levels of endogenous Eg2 protein, did not inhibit GVBD (not shown). At this point, we cannot say whether the lack of inhibition is due to expression of the mutant in amounts that are insufficient to have a detectable effect on GVBD, or to the inability of this mutant to act as a dominant negative.

Fig. 3. pTA11 encodes Eg2, a 46 kDa Ser/Thr kinase that is related to a small family of kinases implicated in cell cycle control. (**A**) Diagram of pTA11/Eg2 protein. The kinase domain is shaded, and the positions of the catalytic triad residues (lysine 169, glutamic acid 188 and aspartic acid 283) are indicated. The sites of potential phosphorylation by PKC, CKII and PKA are indicated by vertical bars. (**B**) Hypothetical phylogenetic tree generated, using MegAlign. (**C**) The predicted protein sequence (single-letter amino acid code) of TA11/Eg2 was aligned with those of human Aik, mouse Ayk, mouse IAK and rat AIM-1 using MegAlign. Eg2 is most similar to Aik, showing 62% identity overall and 80% identity within the catalytic domain. The other sequences show <50% sequence identity. The positions of the catalytic residues K169, E188 and D283 are indicated by asterisks, and the kinase subdomains are marked above the sequences. The position of a potential mitotic destruction box sequence (RxxLxxV) starting in Eg2 at position R378 and shared by the other kinases is marked by a horizontal bar below the sequences.

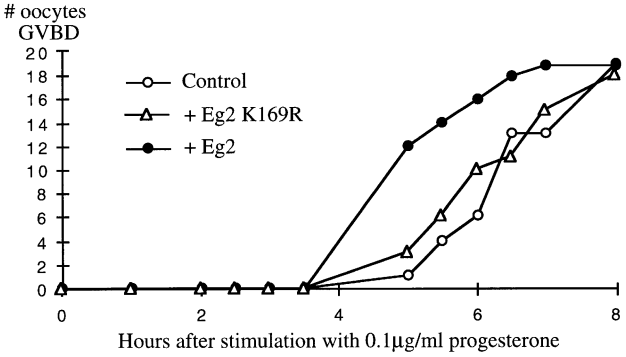


Fig. 6. Overexpression of wild-type Eg2 accelerates GVBD, whereas a presumptive kinase-dead mutant (K169R) does not. Oocytes were injected either with buffer (○), wild-type Eg2 RNA (●) or RNA encoding Eg2 mutated at the presumptive catalytic lysine residue (Eg2 K169R, △). Injected oocytes (20 in each set) were incubated overnight to allow newly made Eg2 to accumulate. All oocytes were then stimulated with 0.1 μg/ml progesterone and scored for GVBD at the indicated times. The results of this experiment are representative of three separate experiments.

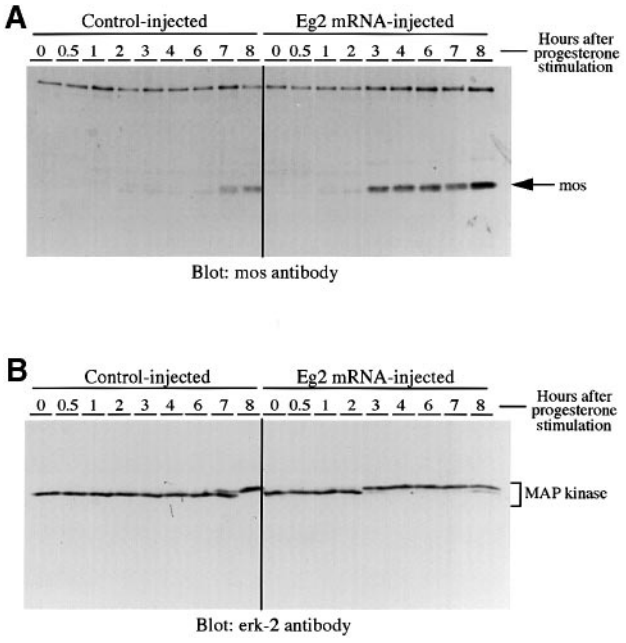


Fig. 7. Eg2 overexpression leads to premature activation of MAP kinase and premature appearance of Mos protein. Oocytes were injected with either water or wild-type Eg2 RNA, incubated overnight to allow accumulation of newly made Eg2 protein and stimulated with 0.1 μg/ml progesterone. Samples were collected at the indicated times, separated by SDS–PAGE and blotted with (**A**) Mos and (**B**) MAP kinase antibodies, respectively.

Overexpression of Eg2 accelerates the appearance of Mos protein and activation of MAP kinase

As a test of whether Eg2 functions upstream or downstream of the point regulating translational activation of *mos* mRNA, we asked if overexpression of Eg2 would lead to premature appearance of Mos protein. Oocytes were injected with water or Eg2 mRNA, incubated overnight and then stimulated with progesterone. Four oocytes were collected at each of the indicated times, pooled and homogenized. A portion of each homogenate was analyzed by blotting with either Mos or MAP kinase antibodies. As shown in Figure 7, overexpression of Eg2 dramatically accelerates both the appearance of new Mos protein and the activation of MAP kinase. This result further supports the idea that Eg2 functions in the progesterone-signaling pathway and that it positively regulates the translational activation of *mos* mRNA.

Overexpression of Eg2 lowers the level of external progesterone needed to trigger oocyte activation

If Eg2 acts early in the progesterone-activated signaling pathway, increasing the amount of Eg2 protein might amplify an incoming signal. We thus compared the response of control and Eg2-injected oocytes to decreasing concentrations of progesterone. In all of the preceding experiments, progesterone was provided at 0.1 μg/ml, a standard concentration that is 5–10 times the amount needed to induce 100% GVBD. As shown in Figure 8, ~90% of control oocytes exposed to 0.01 μg/ml progesterone, a 10-fold dilution of the standard concentration, underwent GVBD (panel A). At a 25-fold dilution, only half of the control oocytes were able to undergo GVBD (panel B). At 50- or 100-fold dilution, none of the control oocytes underwent GVBD (panels C and D). By contrast, 100% of the oocytes that had been injected with Eg2 mRNA reached GVBD when stimulated with 10-, 25- or 50-fold dilutions of progesterone. Even when presented with a 100-fold dilution of progesterone, ~20% of the these oocytes were able to undergo GVBD. Furthermore, the population of Eg2-supplemented oocytes reached GVBD with the same accelerated kinetics (Figure 8A–D). By contrast, oocytes injected with the Eg2 K169R mutant did not respond to subthreshold concentrations of progesterone (data not shown). These results further argue that Eg2 is a component of the progesterone-activated pathway that breaks cell cycle arrest, that this effect depends on Eg2's kinase activity, and that Eg2 might function at a relatively early point in a signal amplification pathway.

Discussion

In a screen for proteins that are modified soon after oocytes are exposed to progesterone, we identified Eg2, which was first reported as a cDNA cloned from *Xenopus* eggs (Paris and Philippe, 1990). Here we provide evidence that Eg2 is a component of the progesterone-activated signaling pathway. First, endogenous Eg2 protein is phosphorylated soon after progesterone stimulation. Secondly, overproduction of Eg2 in oocytes leads to the premature appearance of new Mos protein, premature activation of MAP kinase and the premature occurrence of GVBD, the standard morphological marker of cell cycle re-entry in this system. Thirdly, the presence of additional Eg2 lowers the concentration of progesterone needed to induce GVBD. Finally, Eg2(K169R) does not have these effects, suggesting that progesterone-dependent activation of Eg2 is important in this pathway.

Where in the signaling pathway does Eg2 act? In the simplest scheme, Eg2 would function early, somewhere between the binding of progesterone to its receptor and the translational activation of mos mRNA. Mos and other stored mRNAs contain short 3′ oligo(A) tails and progesterone stimulation leads to adenylation of these mRNAs by cytoplasmic poly(A) polymerase (reviewed by Ballantyne *et al*., 1995; Stebbins-Boaz and Richter, 1997). This polyadenylation is absolutely required for the

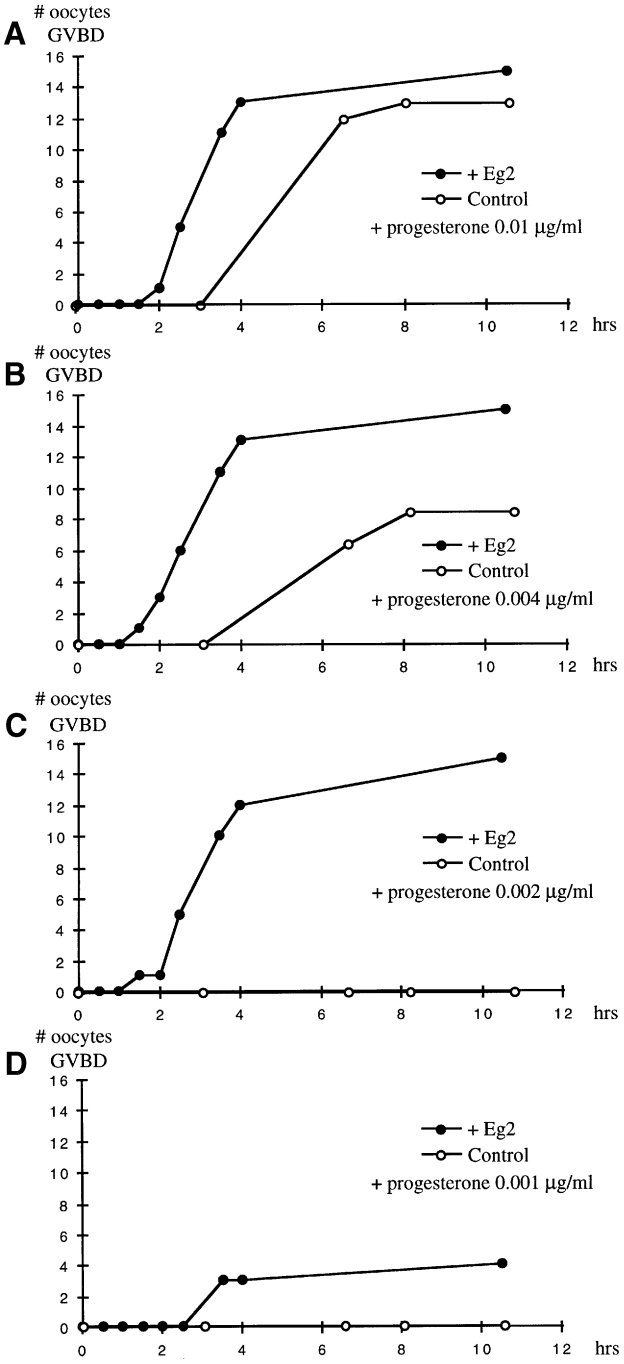


Fig. 8. Overexpression of wild-type Eg2 lowers the concentration of progesterone needed to induce GVBD. Oocytes were injected with wild-type Eg2 RNA and incubated overnight to allow accumulation of newly made Eg2 protein. Injected and control oocytes (16 in each set) were then stimulated with (**A**) 0.01 μg/ml progesterone (1/10th the progesterone concentration normally used), (**B**) 0.004 μg/ml, (**C**) 0.002 μg/ml or (**D**) 0.001 μg/ml progesterone as indicated on the panels, and scored for GVBD. The results of this experiment were confirmed in a second, independent experiment (data not shown).

translational activation of *mos* mRNA and several other mRNAs (McGrew and Richter, 1990; Sheets *et al*., 1994, 1995; Ballantyne *et al*., 1997; DeMoor and Richter, 1997). Newly made Mos serves, at least in part, as a MAP kinase kinase kinase, leading to activation of a pre-existing MAP kinase, which initiates activation of pre-MPF. MPF then catalyzes entry into the first meiotic M phase. The facts that (i) endogenous Eg2 protein is phosphorylated soon after progesterone stimulation and (ii) Eg2 overexpression both reduces the amount of progesterone needed to activate oocytes and advances the time of GVBD argue that Eg2 functions early in the pathway.

We cannot, however, exclude the possibility that Eg2 acts later on in the pathway. Eg2 could act downstream of *mos* mRNA translation by stabilizing newly made Mos. Mos made before GVBD has a rapid turnover and becomes more stable around the time of GVBD. This stabilization is probably due to phosphorylation of serine 3, which protects it from ubiquitin-mediated proteolysis, although this level of regulation has been investigated more extensively in somatic cells than in oocytes (Nishizawa *et al*., 1992, 1993). Phosphorylation of Mos at serine 3 also enhances its physical interaction with and stimulation of MAP kinase kinase (Chen and Cooper, 1995). There is a lag between the earliest appearance of new Mos protein and the later, abrupt activation of MAP kinase and MPF. Work of Ferrell and colleagues argues compellingly that the MAP kinase cascade can convert graded inputs, such as the gradual accumulation of newly translated Mos, into a switch-like output, such as MAP kinase/MPF activation in oocytes (Huang and Ferrell, 1996; Ferrell and Machleder, 1998). Presumably, this property serves to filter out the noise of low-level input signals while maintaining the ability of the system to respond to high-level stimuli. If Eg2 led to stabilization of Mos, either directly or indirectly, Mos would accumulate faster and trip the MAP kinase/MPF activation switch earlier.

A second mechanism for suppressing subthreshold signals involves the non-catalytic β subunit of casein kinase II (CKIIβ), which can bind and inactivate mos *in vitro*. In the intact oocyte, raising the level of CKIIβ inhibits Mos-dependent activation of MAP kinase, MPF and GVBD. Reduction of endogenous CKIIβ by antisense RNA injection lowers the amount of progesterone needed to trigger GVBD (Chen and Cooper, 1997; Chen *et al*., 1997). Eg2 could act to reduce the threshold set by CKIIβ.

Finally, there is compelling evidence for several positive feedback loops connecting MPF, MAP kinase and translational activation of *mos* mRNA, and it is possible that Eg2 functions in one or more of these loops. For example, introduction of active MPF itself, which can be viewed in functional terms as the most downstream component of the regulatory pathway, can lead to MAP kinase activation *in vitro* and to both MAP kinase activation and the appearance of mos protein *in vivo* (Nebreda and Hunt, 1993; Posada *et al*., 1993; Shibuya and Ruderman, 1993; Gotoh *et al*., 1995; Matten *et al*., 1996; Roy *et al*., 1996). It is believed that these positive feedback loops, and others like the one involving MPF and its activating phosphatase cdc25 (Coleman and Dunphy, 1994), contribute to the rapid, step-like activation of MPF, committing the cell irreversibly to the resumption of the meiotic cell cycle and development into a fertilizable egg (Ferrell and Machleder, 1998).

At first glance, the idea that Eg2 might act downstream of MAP kinase or MPF, i.e. at or after entry into meiotic M phase, might fit more with a recent set of observations on Eg2 and a small number of other kinases, some of which appear to be involved in mitotic M phase events: human Aik (Kimura *et al*., 1997), mouse IAK-1 (Gopalan

et al., 1997), mouse Ayk (Yanai *et al*., 1996) and rat AIM-1 (Terada *et al*., 1998). When analyzed in somatic tissue culture cells, these kinases have two general properties in common. First, mRNA and/or protein levels are low in G_1- and S-phase cells, and rise during G_2 and mitosis. The drop in protein levels may be attributable to the fact that all of these kinases contain a C-terminal domain that weakly resembles the mitotic destruction box found in mitotic cyclins and other proteins that are targeted for proteolysis by a specialized ubiquitination system (reviewed by Hershko, 1997). Secondly, each is found associated with one or more mitotic structures, including the spindle pole, spindle microtubules, the spindle midzone and/or the spindle microtubules remaining in the midbody during cytokinesis.

A very recent study shows that, in somatic cells, Eg2 itself is localized at the spindle poles, where it appears to be in close proximity to γ-tubulin (Roghi *et al*., 1998). As mitosis progresses, it spreads into the adjacent spindle microtubules. In somatic cells, the spindle pole contains a centrosome, composed of a pair of centrioles and surrounding pericentriolar material (PCM). γ-tubulin is associated with this region, which serves as the microtubule-organizing center for nucleation of spindle microtubules (Oakley *et al*., 1990; Moritz *et al*., 1995; Zheng *et al*., 1995). Roghi *et al*. (1998) also provide biochemical evidence for a physical association of Eg2 with microtubules, and report that a kinase-inactive mutant can interfere with the process of spindle formation *in vitro*. By sequence, Eg2 is most closely related to human Aik and its pattern of localization during the cell cycle is also very similar (Kimura *et al*., 1997). More distantly related in terms of both sequence and localization are mouse IAK-1 and Ayk. Like Eg2, IAK-1 has been found at the spindle poles early in mitosis and then relocalizes to adjacent spindle microtubules during anaphase; unlike Eg2, IAK-1 is also found associated with the microtubules in the midbody during telophase and cytokinesis, along with α-tubulin (Gopalan *et al*., 1997). Rat AIM-1, which is most divergent in sequence from Eg2, shows a different pattern of localization: it is found at the equator of the spindle (the midzone) during anaphase and then moves to the midbody during telophase and cytokinesis. Strikingly, introduction of a kinase-inactive AIM-1 blocks cytokinesis without affecting nuclear division (Terada *et al*., 1998). The general similarities of these kinases suggest that they are part of a group that has evolved to carry out diverse functions during mitosis. However, since each is from a different species and functional information is limited, it is impossible at this point to sort out which, if any, might be true functional homologs.

Partial co-localization of Eg2 with γ-tubulin at the spindle poles is especially intriguing in light of the unusual distribution of γ-tubulin in quiescent oocytes and the atypical morphology of microtubules in oocytes going through meiosis I. *Xenopus* oocytes lack recognizable centrioles or centrosomes, but do contain a pool of centrosome components, including γ-tubulin (Gard *et al*., 1990; Gard, 1991; Stearns *et al*., 1991; Verde *et al*., 1991). Confocal studies show that γ-tubulin is associated with the periphery of the oocyte nucleus (GV), supporting the idea that the GV functions as a microtubule-organizing center in these cells (Gard, 1991). Surprisingly, a significant amount of γ-tubulin is also found at the oocyte cortex, the presumed site of the progesterone receptor. Work is currently underway to determine the localization of Eg2 in oocytes and the proteins with which it interacts.

It is important to emphasize that kinases typically regulate more than a single target, and that oocytes and somatic cells probably contain different subsets of Eg2 target proteins. Even within one cell type, a single kinase can regulate multiple events. An especially relevant example comes from Mos, which functions at least four times during conversion of the quiescent oocyte into the mature egg (reviewed by Gebauer and Richter, 1997; Sagata, 1997). First, Mos initiates cell cycle re-entry. Secondly, it is required for entry into meiosis II. Thirdly, it is required to suppress DNA replication between meiosis I and II. Finally, Mos is required to keep the mature egg arrested at meiosis metaphase II until fertilization. It would not be surprising to find that Eg2 functions at multiple points as well.

The screen described here, in which 3% of the library (~9000 clones) was examined, yielded two and only two candidate clones, both with properties consistent with signaling roles. One is a novel protein containing WD40 repeats, a motif found in G proteins and other signaling proteins. The other is Eg2, a kinase with demonstrated biological activity in the progesterone pathway. Whatever the ultimate molecular explanation for Eg2's ability to accelerate appearance of Mos, MAP kinase activation and cell cycle re-entry, as well as reducing the requirement for progesterone, these abilities demonstrate the potential of the screen for identifying components in the progesterone-activated signaling pathway in oocytes.

Materials and methods

Xenopus oocyte cDNA library construction

Whole ovaries were removed from an adult female. Oocytes were dissociated from connective tissue and surrounding follicle cells by gentle stirring for 1.5–2 h in calcium-free MBS (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES pH 7.6) containing 2 mg/ml type 1A collagenase (Sigma) and 0.5 g/l polyvinyl pyrrolidone (Shibuya *et al*., 1992). Oocytes were washed ×10 in calcium-free MBS. Stage 1–6 oocytes were collected and total RNA isolated as described by Evans and Kay (1991).

Poly(A)$^+$ RNA was isolated using PolyA Ttract system (Promega). Five micrograms of poly(A)$^+$ RNA was used as template for cDNA synthesis using a cDNA synthesis kit (Stratagene). *Xho*I poly(T) primer was used for first-strand synthesis, RNase H nicking for the second-strand synthesis and *Eco*RI adapters were ligated to the 5′ ends. The cDNA preparation was size-fractionated on Sephacryl S-500 spin columns (Stratagene) by collecting three fractions at 400 *g* for 2 min. Following digestion with *Xho*I and *Eco*RI, the cDNAs were cloned into the *Xho*I/*Eco*RI site of the vector pBK-CMV and transformed into XL1-Blue bacteria (Stratagene). Eighty percent of the colonies were estimated to contain inserts >1 kb.

The library was first split into 80 large pools of 2500–10 000 colonies per pool. To create pools containing ~100 cDNA clones, the glycerol stock of a large pool was plated onto LB/kanamycin (40 mg/ml) plates in amounts that gave about 100 colonies per plate. Following overnight incubation at 37°C, colonies were collected and mixed; half of the mix was frozen as a glycerol stock and the other half was used to make DNA.

Oocyte extracts

Young female frogs were primed with 50 U pregnant mare serum gonadotropin (PMSG, Calbiochem) 2 days before oocyte isolation. One ovary was removed, cut into small fragments and incubated in 5 ml of calcium-free MBS containing 10 mg/ml Type 1A collagenase, 5 mg/ml soybean trypsin inhibitor (Sigma) and 5 mg/ml bovine serum albumin (BSA) (Sigma), and agitated using an orbital shaker for 2–3 h. Oocytes

were washed ×3 in 0.1 M KH_2PO_4/K_2HPO_4, ×3 in calcium- free MBS and ×5 in MBS. Stage VI oocytes were collected manually and allowed to recover overnight in MBS. One group of oocytes was left unstimulated, and another group was treated with 0.1 μg/ml progesterone for either 15 or 30 min, as indicated in the text. Extracts were made using the method described by Shibuya *et al*. (1992), with minor modifications. Briefly, oocytes were washed twice with extract buffer (0.25 M sucrose, 0.1 M NaCl, 2.5 mM $MgCl_2$, 20 mM HEPES pH 7.2). Approximately 4 ml of packed oocytes were transferred to a 5 ml centrifuge tube containing 1 ml of extract buffer supplemented with 10 μg/ml each of leupeptin, chymostatin and pepstatin. The tube was inverted once, oocytes were allowed to settle and excess buffer was removed. Oocytes were crushed by centrifugation at 15 000 r.p.m. for 15 min at 2°C using a Sorvall AH 650 swinging-bucket rotor. The supernatant between the lipid cap and the pellet was collected, brought to 50 μg/ml cytochalasin B, and centrifuged again. The supernatant was mixed with 100% glycerol to give 10% final glycerol. Extracts were aliquoted into 14 μl portions, frozen in liquid nitrogen and stored at –70°C.

Metaphase II-arrested egg extracts

Females were primed with 50 U PMSG and 2 days later induced to lay eggs by injection with 50 U human chorionic gonadotropin. Eggs were dejellied by swirling for 10 min with an equal volume of 2% L-cysteine hydrochloride, rinsed in MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES pH 7.8) until the wash solution was clear, and washed ×3 in extract buffer (100 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM potassium HEPES pH 7.7, 50 mM sucrose, 5 mM EGTA pH 7.7). Eggs were settled and buffer was removed. Eggs were homogenized by disruption through a 25 ml pipette in the presence of 1 μM okadaic acid (Calbiochem), followed by centrifugation at 50 000 r.p.m. for 1 h at 4°C using a Ti70 rotor (Beckman). The supernatant was collected, aliquoted, frozen in liquid nitrogen and stored at –70°C.

Electrophoretic shift assay

DNA from individual clones or small pools was transcribed and translated *in vitro* using coupled transcription and translation reactions in reticulocyte lysate (TnT, Promega) to yield ^{35}S-methionine-labeled proteins. Reaction mixes contained 1 μl (0.3–0.4 μg) of DNA, 5 μl reticulocyte lysate, 0.2 μl TnT buffer, 0.2 μl amino acid mix minus methionine, 0.2 μl RNasin, 0.2 μl RNA polymerase (T3 or SP6 depending on the vector), 0.8 μl translation grade ^{35}S-methionine (New England Nuclear, 1000 Ci/mmol) and 2.6 μl water. Reactions were incubated at 30°C for 1.5 h. Two microliters of *in vitro* translation product were mixed with 2 μl extract made from either unstimulated oocytes or progesterone-stimulated oocytes and supplemented with an ATP regeneration system (Shibuya, 1992), 0.1 μg/ml cycloheximide (Sigma) and 0.3 μM okadaic acid (Calbiochem). Reactions were incubated for 40 min at 20°C and stopped by the addition of 2× sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol).

cDNA clones encoding rat $p70^{S6K}$ (pS6KB.1-HA; Cheatham *et al*., 1995) and *Xenopus* MAP kinase ($Xe^{MAPK–WT}$; Waskiewicz and Cooper, 1993) were used initially to establish assay conditions. Other clones were used as indicated in the Results. For screening small pools of cDNAs, reactions were performed in the same way, except that 1.2 μl of translation product was mixed with 1.2 μl of extract from unstimulated or progesterone-stimulated oocytes. Reaction products were analyzed by electrophoresis on a 12.5% polyacrylamide gel (Anderson, 1973) followed by autoradiography against X-ray film (Kodak or Marsh). Exposure times typically were overnight for single-clone-shift assays and 7–10 days for pool-shift assays. To isolate an individual clone, miniprep DNA from the small pool was transformed into bacteria; 96 individual colonies were picked into a 96-well plate and grown overnight. Twelve subpools were prepared from each of the 12 columns, and eight subpools from each of the eight rows. Plasmid DNA was prepared from each subpool and assayed as above. The individual clone encoding the desired protein was thus located by the presence of its product in both a specific column and a row.

Subcloning and mutagenesis

pTA11/Eg2 was isolated as a 2.7 kb cDNA insert containing 800 bp 5′ UTR, 1221 bp coding sequence and 760 bp 3′ UTR. For *in vitro* translation, the coding region was subcloned by PCR-based techniques into $pCS2^+$. Four gene-specific primers were made, two corresponding to the 5′ (pr11-3 and pr11-5) and two corresponding to the 3′ end (pr11-4 and pr11-6) of the clone.

The 5′ primer pr11-5 (5′-TTACATGGATCCATGGACACCTATCGCTATATA**GAGCGGGCTGTTAAGGAGAAC**-3′) encoded a *Bam*HI restriction site, ATG, six codons for the AU1 epitope (underlined) (Lim *et al*., 1990) and the first seven codons of pTA11/Eg2 (bold). The 5′ primer pr11-3 (5′-TTACATGGATC-CGCCACCATG**GAGCGGGCTGTTAAG**-3′) encoded a *Bam*HI site, Kozak sequence, ATG and the first five codons of pTA11/Eg2 (in bold). The 3′ primer pr11-6 (5′-CGAGGCCTCGAGCTA**TTGGGCGCCTGGAAG**-3′) encoded the last six codons of pTA11/Eg2 (in bold), a stop codon and an *Xho*I site. The 3′ primer pr11-4 (5′-GGGCCTCGAGCTATATATAGCGATAGGTGTC-**TTGGGCGCCTGGAAGGG**-3′) endcoded the last 17 bases from pTA11/Eg2 (bold), six codons for the AU1 epitope (underlined), a stop codon and the *Xho*I site. Primers pr11-3 and pr11-4 or pr11-5 and pr11-6 were then used in a PCR reaction using the original pTA11/Eg2 cDNA clone as template, creating wild-type clones with either an N- or C-terminal tag. A presumptive kinase dead mutant was made by two-step PCR. Two internal primers pr11-8 (5′-CTGGCGCTG**AGA**GT-CCTGTTT-3′) and pr11-9 (5′-AAACAGGAC**TCT**CAGCGCCAG-3′) were made encoding a point mutation in codon 169, changing the catalytic lysine to arginine (bold). The first PCR reaction was performed using primers pr11-5 and pr11-8 together, and pr11-9 and pr11-6, generating two PCR products. The second PCR reaction was carried out using the two PCR products as a template and primers pr11-5 and pr11-6. The PCR product was digested with *Bam*HI and *Xho*I and cloned into $pCS2^+$.

The wild-type *Xenopus* p42 map kinase was subcloned into $pCS2^+$ using PCR. The 5′ primer (Xep42 *Bam*HI 5′: 5′-GCCGGATCC-CC**ATGGCGGCGGCAGCGGCCTCGTCT**-3′′) encodes a *Bam*HI site, ATG and 21 bases from the 5′ end of *Xenopus* map kinase (in bold). The 3′ primer (Xep42 *Bam*HI 3′; 5′-CCAATGCATGGATC-CTGCAGTCA**GTACCCTGGCTGGAATCTAGC**-3′ encodes *Bam*HI and *Pst*I sites, a stop codon and the last 21 bases of *Xenopus* map kinase (bold). The primers were used to generate a PCR fragment using pAD4delta (Xp42 MT-WT) (Waskiewicz and Cooper, 1993) as a template. The PCR fragment was digested with *Bam*HI and cloned into $pCS2^+$.

In vitro transcription and oocyte injection

pTA11/Eg2 DNA was linearized by digestion overnight with *Not*I, extracted with phenol/chloroform and precipitated with ethanol. Two micrograms of DNA were transcribed *in vitro* (MegaScript kit, Ambion), purified and stored in 1 μl aliquots at –70°C. For RNA injection, fully grown stage 6 oocytes were collected and allowed to recover overnight in MBS, as described above. Oocytes were injected with 30 nl of either injection buffer (88 mM NaCl, 5 mM Tris pH 7.5), H_2O, or 50 ng/μl RNA encoding the wild type or the K169R mutant. Injected oocytes were incubated for 12–16 h to allow accumulation of newly made protein.

Immunoblotting

One microliter of oocyte- or progesterone-stimulated oocyte extract (or homogenate equivalent to 0.5–1 oocyte) was separated by SDS–PAGE. Proteins were transferred to an Immobilon membrane (Millipore). Membranes were incubated for 1 h in TBS-T (10 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing either 1.5% BSA or 5% powdered skimmed milk. Membranes were incubated overnight at 4°C with a 1:100 dilution of an Eg2 monoclonal antibody generously provided by Claude Prigent (University of Rennes, France), 1 μg/ml mos antibody (Santa Cruz) or 0.1 μg/ml Erk2 antibody (Santa Cruz). Membranes were washed ×3 in TBS-T, incubated with a 1:5000 dilution of horseradish peroxidase conjugated mouse or rabbit secondary antibody (Amersham), washed ×3 in TBS-T, developed using ECL (Amersham) and visualized by autoradiography.

Expression of Eg2 in Sf9 cells

Wild-type Eg2 cDNA was inserted into the *Bam*HI/*Xho*I sites of the vector pFastBac (Babco BRL) and recombinant virus was generated. Briefly, plasmid DNA was transformed into DH10Bac cells containing a baculovirus shuttle vector. Following site-specific transposition, high-molecular-weight DNA was isolated and transfected into Sf9 cells using Cellfectin (Babco BRL) according to the manufacturer's instructions. Culture supernatant was collected after 2 days and evaluated for expression of Eg2 by Western blotting. To prepare active Eg2, Sf9 cells were infected with recombinant virus and cultured for 44 h; cells were then cultured for an additional 4 h in the presence (or absence) of 0.5 μM okadaic acid to block removal of potentially activating phosphorylations. Cells were harvested by centrifugation at 1100 r.p.m. for 10 min in a GSA rotor (Sorvall) and washed twice in PBS. Cell pellets were lysed by addition of 50 ml 20 mM β-glycerolphosphate, 10 mM HEPES–KOH pH 7.7, 5 mM EGTA, 5 mM β-mercaptoethanol, 150 mM NaCl,

1% Chaps, 1 mM PMSF and 1× complete protease inhibitors (EDTA-free, Boehringer Mannheim) to the cell pellet, followed by centrifugation at 30 000 r.p.m. for 30 min in a Ti70 rotor (Beckman). The supernatant was filtered through a 0.45 μ filter and incubated with 6 ml of 50% slurry Ni–NTA agarose (Qiagen) for 2 h at 4°C. The resin was washed with 100 ml of 20 mM β-glycerolphosphate, 10 mM HEPES–KOH pH 7.7, 5 mM EGTA, 5 mM β-mercaptoethanol, 500 mM NaCl, 0.1% Chaps, 1 mM PMSF and protease inhibitors. Eg2 was eluted with 20 mM β-glycerolphosphate, 10 mM HEPES–KOH pH 7.7, 5 mM EGTA, 5 mM β-mercaptoethanol, 25 mM NaCl, 200 mM imidizole, 1 mM DTT, 1 mM EDTA, 1 mM PMSF and protease inhibitors, and dialyzed overnight against 500 ml 10 mM HEPES–KOH pH 7.7, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 10% glycerol, followed by a second dialysis for 2 h. The protein was aliquoted, frozen in liquid nitrogen and stored at –70°C.

In vitro kinase assay of baculovirus-expressed Eg2

Baculovirus-expressed Eg2 was added to 10 μl of 20 mM Tris pH 7.7, 10 mM MgCl, 50 mM KCl, 1 mM DTT, 30 μM ATP, 2 mg/ml BSA, 0.3 μg MBP and 10 mCi [γ-^{32}P]. Incubations were carried out for 15 min at 30°C and stopped by the addition 10 μl 2× SDS-gel sample buffer. Five microliters were analyzed by electrophoresis by SDS–PAGE followed by autoradiography.

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