# Dissociation of MAP kinase activation and MPF activation in hormone-stimulated maturation of *Xenopus* oocytes

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## SUMMARY

MAP kinase activation occurs during meiotic maturation of oocytes from all animals, but the requirement for MAP kinase activation in reinitiation of meiosis appears to vary between different classes. In particular, it has become accepted that MAP kinase activation is necessary for progesterone-stimulated meiotic maturation of *Xenopus* oocytes, while this is clearly not the case in other systems. In this paper, we demonstrate that MAP kinase activation in *Xenopus* oocytes is an early response to progesterone and can be temporally dissociated from MPF activation. We show that MAP kinase activation can be suppressed by treatment with geldanamycin or by overexpression of the MAP kinase phosphatase Pyst1. A transient and low-level early activation of MAP kinase increases the efficiency of cell cycle activation later on, when MAP kinase activity is no longer essential. Many oocytes can still undergo reinitiation of meiosis in the absence of active MAP kinase. Suppression of MAP kinase activation does not affect the formation or activation of Cdc2-cyclin B complexes, but reduces the level of active Cdc2 kinase. We discuss these findings in the context of a universal mechanism for meiotic maturation in oocytes throughout the animal kingdom.

Key words: MAP kinase, MPF, Oocyte maturation, *Xenopus*

## INTRODUCTION

In oocytes of all species, resumption of the meiotic cell cycle is mediated by activation of MPF, which has been equated to active cyclin B-Cdc2 kinase. As well as Cdc2 kinase, MAP kinase is universally activated around the time of germinal vesicle breakdown (GVBD) and entry into meiotic metaphase. The activity of MAP kinase remains high during meiosis and drops on fertilization and entry into interphase (Abrieu et al., 1997b; Fisher et al., 1998; Haccard et al., 1990; Picard et al., 1996; Verlhac et al., 1993). MAP kinase is activated during oocyte maturation as a consequence of activation of a cascade of upstream kinases, including MAP kinase kinase (MEK) and Mos (in vertebrates). While it is accepted that Cdc2 kinase activation prior to GVBD is essential for meiotic maturation in all species, differences in the timing of, and requirement for, MAP kinase activation occur between different species. By far the best studied species is *Xenopus*, for which several studies have demonstrated that activation of MAP kinase is important or essential for MPF activation in *Xenopus* oocytes. The first suggestions came from the coincidence of its activation with that of Cdc2 kinase (Ferrell et al., 1991; Haccard et al., 1990). It was shown that Mos synthesis was required for hormone-induced GVBD (Sagata et al., 1989), and subsequently that Mos leads to activation of MAP kinase and not Cdc2 (Nebreda and Hunt, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993) in oocyte and cell extracts. Active MAP kinase itself was found to be capable of inducing *Xenopus* oocyte maturation in

the absence of hormone stimulation (Haccard et al., 1995) and use of an in vitro system demonstrated that a deficiency in Mos-induced activation of Cdc2 kinase could be overcome by supplementing the extract with additional MAP kinase (Huang and Ferrell, 1996). Two reports then showed that MAP kinase activation was required for *Xenopus* oocyte meiotic initiation by suppressing MAP kinase activation, in one of two ways. In the first, the authors used an antibody that neutralizes the MAP kinase activating kinase MEK, and demonstrated a block to Cdc2 kinase activation and GVBD (Kosako et al., 1994). However, these authors showed that a subset of injected oocytes managed to activate MAP kinase in response to progesterone but still did not undergo GVBD. This implies that the antibody had other inhibitory effects. The second report used overexpression of the MAP kinase phosphatase CL100 to inhibit progesterone-induced MAP kinase activation and GVBD (Gotoh et al., 1995). Use of the MEK inhibitor PD98059 has been reported to delay oocyte GVBD (Cross and Smythe, 1998). Nevertheless, MAP kinase is dispensable for activation of Cdc2 kinase in oocyte extracts by okadaic acid or by addition of recombinant Cdc25 (Karaiskou et al., 1998).

In equine, bovine and porcine oocytes, MAP kinase and MPF are activated concurrently and may also both be required for GVBD (Fissore et al., 1996; Goudet et al., 1998; Inoue et al., 1996). Inhibition of protein synthesis in porcine oocytes blocks MAP kinase activation and GVBD. However, in starfish, neither protein synthesis nor MAP kinase activation are required for Cdc2 kinase activation and GVBD induction

(Picard et al., 1996) and, in rodents, MAP kinase is only activated after GVBD and Cdc2 kinase activation (Verlhac et al., 1993). In oocytes from $mos^{-/-}$ mice, MAP kinase remains inactive during meiosis, demonstrating that other activators of the MAP kinase cascade cannot replace Mos function, and that MAP kinase activation is unnecessary for MPF activation (Verlhac et al., 1996). It is clear, therefore, that MAP kinase is universally activated during meiosis, while Cdc2 kinase universally controls GVBD and the onset of meiotic maturation. However, whether or not MAP kinase activation is actually necessary for meiotic re-entry varies from species to species. Why this should be so is not clear.

In this study, we show that MAP kinase activation is an early response to progesterone stimulation in *Xenopus* oocytes. We suppressed activation of MAP kinase by using geldanamycin, which inhibits function of the chaperone Hsp90 and prevents it from interacting with protein kinases for which it is a cofactor (Pratt, 1998). We have found conditions in which, as in starfish and rodent oocytes, hormonal stimulation of *Xenopus* oocytes releases the $G_2$/prophase block, and provokes a bona fide $G_2$/M transition accompanied by hyperphosphorylation of Cdc25 and Myt1 in the absence of active MAP kinase. We have corroborated these results by suppressing MAP kinase activation with the specific phosphatase Pyst1. A transient and low-level of MAP kinase activation is sufficient to stimulate initiation of meiotic maturation, thus sustained MAP kinase activation is therefore not essential. We find that while MAP kinase increases the efficiency of Cdc2 activation, it is the intrinsic sensitivity of oocytes to levels of Cdc2 kinase that determines whether or not oocytes undergo initiation of meiotic maturation in the absence of MAP kinase activity.

## MATERIALS AND METHODS

### Recombinant mRNAs and proteins

The GST-Sea-Urchin cyclin B, pMal-mos and pSG5-Pyst1 constructs and antibodies have been described previously (Abrieu et al., 1996; Groom et al., 1996). Capped mRNAs were transcribed by standard procedures (Promega).

### Immunological procedures

The *Xenopus* anti-Raf-1, anti-Mos, anti-ERK1 and anti-active ERK antibodies were obtained from Santa Cruz (sc-133, sc-086, sc-94 and sc-7383 respectively). Other antibodies used were rabbit polyclonal antisera against *Xenopus* full-length recombinant cyclin B1(6x His tag), Cdc25, Cdc2 C-terminus, sea-urchin cyclin B and phosphotyrosine (Abrieu et al., 1997a). Polyclonal antibodies against Myt1 were obtained by immunizing rabbits with a peptide encoding the C-terminal 12 amino acids of Myt1 (plus an N-terminal cysteine) cross-linked to thyroglobulin by the MBS procedure (Pierce) and affinity purifying against the same peptide coupled to albumin.

Immunoprecipitations for kinase assays were performed as described (Fisher et al., 1999).

Western blots were probed with primary antibody at 50 ng/ml and the appropriate secondary antibody-HRP conjugate diluted according to recommendations (Sigma) and revealed by ECL.

### Kinase assays

Histone kinase assays and in-gel MBP kinase assays were performed as described (Labbé et al., 1991; Shibuya et al., 1992).

### *Xenopus* oocytes

Oocyte manipulations and extracts were performed as described

(Fisher et al., 1999). Insulin was used at 10 μM. GVBD was initially scored by appearance of a maturation spot and confirmed by fixation in 4% formaldehyde, or in MMR at 100°C for 5 minutes, and dissection. Geldanamycin was dissolved in DMSO at a concentration of 5 μmol/ml and diluted in MMR to the concentration indicated (usually 5 μM).

## RESULTS

### Suppression of MAP kinase activation by geldanamycin

It has been accepted that MAP kinase activation is necessary for oocyte maturation in *Xenopus*, and probably other species. The aim of these experiments was to define which components of the cell cycle machinery are suppressed when oocytes are stimulated by progesterone while preventing the activation of MAP kinase.

Our first approach was to target Raf-1. Indeed, previous reports suggest that Raf-1 is necessary for MAP kinase activation in *Xenopus* oocytes, although whether or not it is essential for oocyte maturation and whether it is involved in the Mos pathway remains controversial (Fabian et al., 1993; Muslin et al., 1993; Shibuya et al., 1996). Using novel dominant-negative mutants of *Xenopus raf-1*, we did not find evidence to support the view that Raf-1 is essential for transducing the progesterone signal.

Cross and Smythe (1998) have reported that the MEK inhibitor PD 098059 inhibits entry into meiosis in *Xenopus* oocytes, again reinforcing the idea that MAP kinase activation is required for this process. We repeated these experiments and found that, in fact, all oocytes underwent GVBD, although delayed with respect to controls, and neither MAP kinase activation nor Cdc25 hyperphosphorylation was prevented by PD 098059 (data not shown) used at a concentration sufficient to strongly delay GVBD. Thus, PD98059 is only sufficient to temporarily retard MAP kinase activation and oocyte GVBD.

We then tested the Hsp90 binding inhibitor geldanamycin for its ability to inhibit MAP kinase activation in oocytes, since Hsp90 is a cofactor for a variety of protein kinases (Pratt, 1998). We found that pretreatment of oocytes with 10 μM geldanamycin blocked maturation and Cdc2 kinase activation induced by progesterone as well as insulin (Fig. 1A). Treatment of mammalian cells with geldanamycin causes a destruction of Raf-1 protein (Pratt, 1998), but this was not the case in oocytes, although MAP kinase was not activated (Fig. 1B lower panel). To eliminate the possibility that geldanamycin was blocking progesterone-induced maturation simply at the level of the progesterone receptor, we injected recombinant Mos protein, which induces synthesis of endogenous Mos and activation of the MAP kinase cascade. Geldanamycin prevented this activation of MAP kinase as well as the phosphorylation-induced shift of MEK and of Raf-1, dephosphorylation (activation) of Cdc2 and accumulation of cyclin B1 protein (Fig. 1C panels 1-5). Injection of high concentrations of Mos can cause activation of MAP kinase in the absence of protein synthesis, but not in the presence of geldanamycin (Fig. 1D). This shows that geldanamycin is acting specifically to inhibit Mos-induced activation of MAP kinase.

## Effects of geldanamycin on oocyte maturation induced by progesterone and insulin

We reasoned that some aspects of the maturation process might be more or less dependent on Hsp90 and potentially be inhibited at different concentrations of geldanamycin, and so we undertook a dose-response to progesterone from 0.5 μM to 10 μM geldanamycin. At the lowest concentration, oocyte GVBD was retarded, while at 1.67 μM upwards the progesterone response was virtually abolished, within the time frame of normal oocyte maturation. On leaving for extended periods of time, however, a subset of oocytes underwent GVBD at all concentrations tested (Fig. 2A), implying that oocytes can 'leak' past the block due to geldanamycin. However, no oocytes matured in response to insulin, if treated with geldanamycin, even after extended periods of time (Fig. 2A). We therefore asked what happened to MAP kinase. To our surprise, we found that at 1.67-10 μM geldanamycin, MAP kinase did not undergo the mobility shift reflecting activation, whether or not the oocytes underwent GVBD (Fig. 2B lower panel and data not shown). Even at 0.5 μM both inactive and active MAP kinase bands are present at about equal amounts: only a subset of oocytes had activated MAP kinase on GVBD.

This demonstrates two points: firstly, that progesterone can stimulate meiotic maturation in a majority of oocytes in the absence of MAP kinase activity at the time of GVBD. Secondly, the longer the incubation in the presence of progesterone, even without MAP kinase, the more oocytes undergo GVBD. This suggests the possibility that MAP kinase might simply act to increase the efficiency of Cdc2 kinase activation, but if a sufficient level of Cdc2 kinase can be attained in the absence of MAP kinase activity, oocytes can still escape from $G_2$ arrest.

Indeed, Cdc2 kinase was only activated to a high level by either progesterone or insulin if MAP kinase was activated, even if a subset of progesterone-treated oocytes underwent GVBD in the absence of MAP kinase activation (Fig. 2B upper panel). One possibility could be that, in the latter cases, Cdc2 is activated transiently to a high level which then drops in the absence of MAP kinase, but we have never observed such a profile.

## Molecular analysis of progesterone-stimulated oocyte maturation in the absence of MAP kinase activity

Since progesterone can indeed stimulate the onset of maturation in the absence of active MAP kinase, at least a subset of the events that are normally induced must occur. We wished to know what differences exist at the molecular level, in this subset of oocytes. To achieve this on a physiological time scale, we first incubated oocytes in the presence of progesterone and, either 1 or 2 hours later, added geldanamycin at 5 μM to the medium – i.e. well before normal oocyte maturation. This reproducibly blocks MAP kinase activation, as determined by the mobility shift on SDS-PAGE, but allows a subset, around 50%, of oocytes to undergo GVBD (Fig. 4 and data not shown). We confirmed that this was a genuine breakdown of the germinal vesicle by

fixation of the individual oocyte and dissection (Fig. 3), as well as by a molecular marker for oocyte GVBD, Cdc25 hyperphosphorylation (Fig. 4 panel 2). Nevertheless, this GVBD was often significantly delayed. Again, Cdc2 kinase activity was lower (Fig. 7A and data not shown), although Cdc25 was hyperphosphorylated to the same extent as in control maturing oocytes (Fig. 4 panel 2), implying that phosphorylation of Cdc25 may not need either MAP kinase or Raf-1 function.

We find that Myt1 undergoes a mobility shift typical of hyperphosphorylation, on normal meiotic maturation (Fig. 4, panel 3), as reported recently (Palmer et al., 1998). Phosphorylation of Myt1 by p90[rsk] has recently been proposed
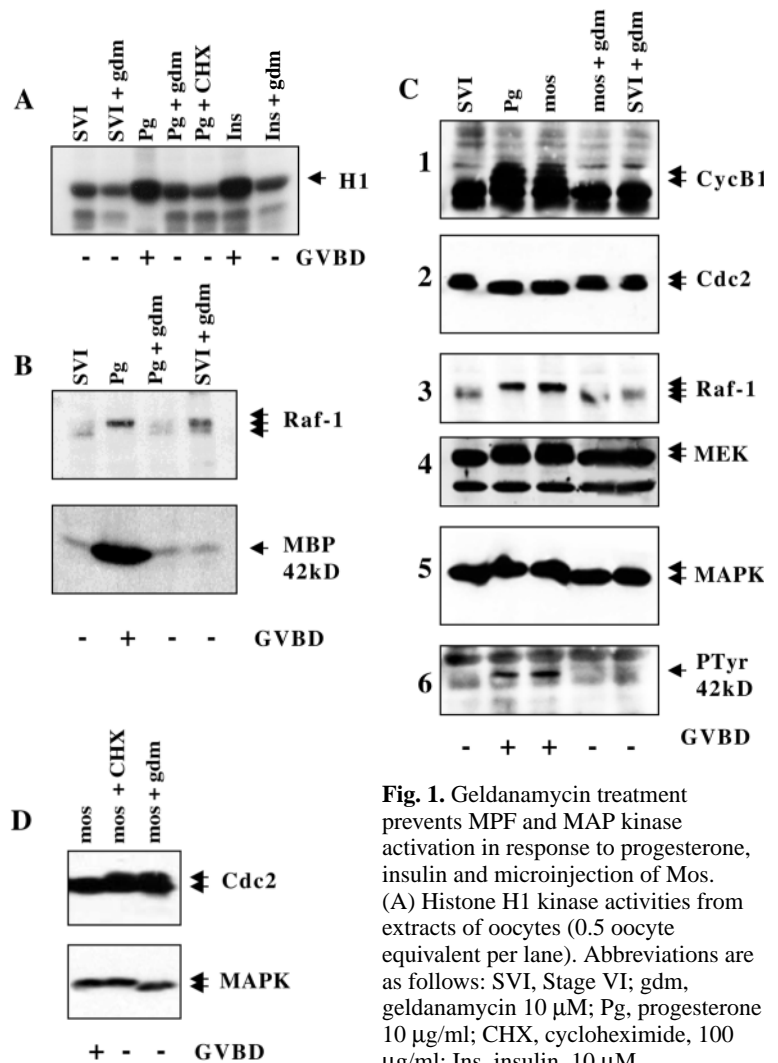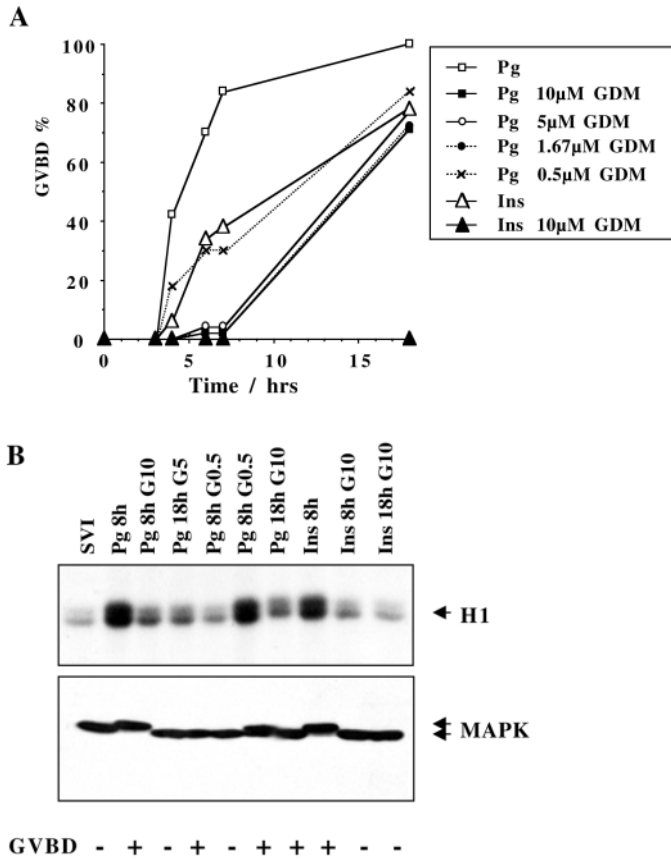


**Fig. 1.** Geldanamycin treatment prevents MPF and MAP kinase activation in response to progesterone, insulin and microinjection of Mos. (A) Histone H1 kinase activities from extracts of oocytes (0.5 oocyte equivalent per lane). Abbreviations are as follows: SVI, Stage VI; gdm, geldanamycin 10 μM; Pg, progesterone 10 μg/ml; CHX, cycloheximide, 100 μg/ml; Ins, insulin, 10 μM.
(B) Western blot of Raf-1 (upper panel, equivalent of 4 oocytes per lane) and in-gel MBP kinase assay (lower panel, equivalent of 2 oocytes per lane) from oocytes treated according to the legend in A. (C) Western blot of the indicated proteins, from oocytes treated according to the legend in A, as well as Mal-BP-mos microinjection (mos). The equivalent of 4 oocytes was loaded per lane. We, and others, (Kobayashi et al., 1991) find that two new bands reacting to anti-cyclin B1 antibodies appear on progesterone treatment, while a lower major band is already present in stage VI oocytes and does not change on maturation. The lower band is therefore probably not cyclin B1.
(D) Western blot of cdc2 and MAP kinase, from oocytes injected with higher activity mos; legend as in A.

**A**



**B**



**Fig. 2.** Time course and dose-response of hormone-treated oocytes to geldanamycin. (A) Germinal vesicle breakdown (GVBD) was scored as a function of time of hormone treatment of oocytes treated in the indicated way. (Ins, Insulin, 10 μM). (B) Histone H1 kinase assays (0.5 oocyte equivalent per lane, upper panel) and Western blots (4 oocytes / lane) MAP kinase (lower panel), from oocytes of the experiment in A, treated and either non-mature or mature oocytes (as indicated) selected as follows: (1) SVI, Stage VI; (2) Pg (8 hours), GVBD; (3) Pg (8 hours) + 10 μM GDM; (4) Pg (18 hours) + 5 μM GDM, GVBD; (5) Pg (8 hours) + 0.5 μM GDM, non-mature; (6) Pg (8 hours) + 0.5 μM GDM, GVBD; (7) Pg (18 hours) + 10 μM GDM, GVBD; (8) Insulin (Ins) (8 hours), GVBD; (9) Ins (8 hours) + 10 μM GDM; (10) Ins (18 hours) + 10 μM GDM.



**Fig. 3.** Complete germinal breakdown in the absence of active MAP kinase. Oocytes were fixed and dissected to reveal the germinal vesicle (GV, arrow) in stage VI oocytes (SVI); while it is absent in progesterone-treated oocytes either without, or a subpopulation of those with, geldanamycin (GDM) treatment. Bar, 200 μm.

**Fig. 4.** Regulatory changes of Cdc25 and Myt1 proteins on progesterone-induced maturation with or without MAP kinase. Western blot of the indicated proteins (4 oocytes/lane) from oocytes treated and selected as follows. (1) Stage VI; (2) Pg (8 hours), GVBD; (3) Pg (36 hours), GVBD; (4) Pg (8 hours) + 5 μM GDM after 90 minutes, non-mature; (5) Pg (8 hours) + 5 μM GDM after 90 minutes, GVBD.



as a mechanism of inactivation of Myt1 linking MAP kinase to the progesterone response (Palmer et al., 1998). In the presence of geldanamycin, and the absence of MAP kinase activity, Myt1 is still highly electrophoretically retarded (Fig. 4, panel 3), though not quite to the same extent as in the presence of active MAP kinase. This is, however, still consistent with its phosphorylation and inactivation (Palmer et al., 1998). A priori, p90[rsk] cannot be responsible for this partial phosphorylation since p90[rsk] activation is mediated by MAP kinase. Nevertheless it may well be that MAP kinase and p90[rsk] activation are necessary for the complete phosphorylation of Myt1, although this is dispensable for GVBD.

### Suppression of MAP kinase activation by Pyst1 delays but does not prevent GVBD

We find that at least a subset of oocytes can undergo GVBD in the absence of active MAP kinase. As such, suppression of MAP kinase activation by methods other than using
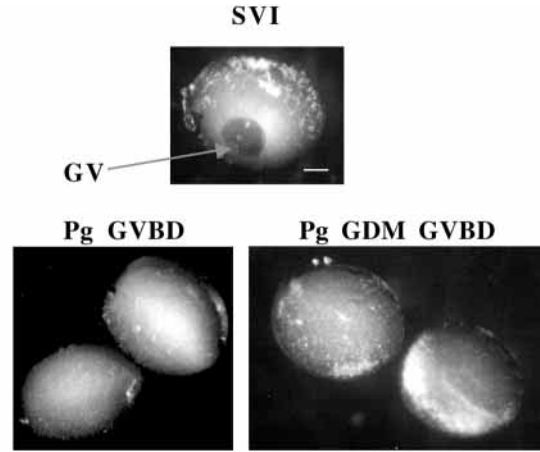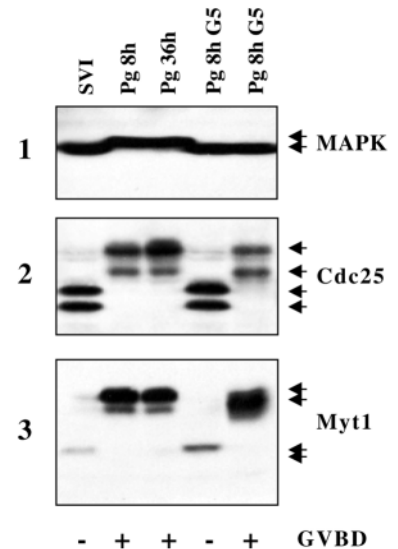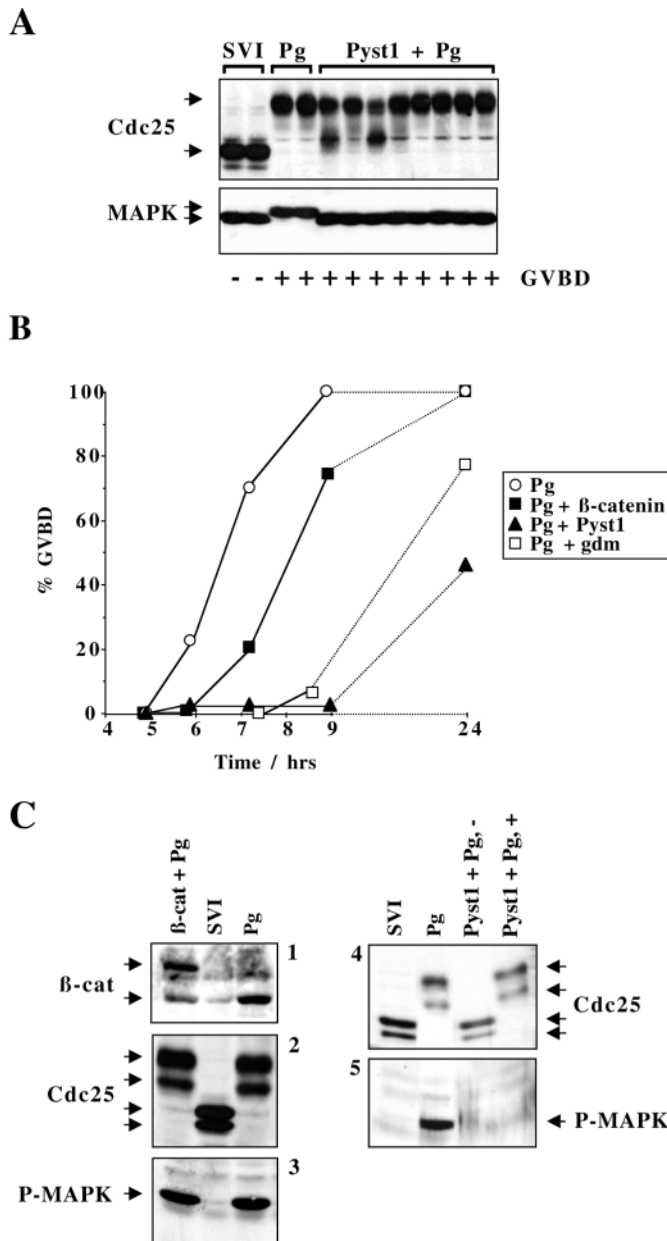
geldanamycin should have a similar effect. A number of MAP kinase phosphatases have been cloned and characterized, and shown to inactivate MAP kinase (Groom et al., 1996). It has been reported that overexpression of one of these, CL100/MKP-1, blocks progesterone-induced MAP kinase activation and GVBD in *Xenopus* oocytes (Gotoh et al., 1995). Since Pyst1/MKP-3 is a more specific MAP kinase phosphatase than CL100/MKP-1 (Camps et al., 1998; Groom et al., 1996), we microinjected mRNA encoding Pyst1 into *Xenopus* oocytes and treated them with progesterone. We found that, as predicted, Pyst1 overexpression delayed but did not prevent GVBD (Fig. 5). In individual oocytes undergoing GVBD, MAP kinase remained in the inactive form, but Cdc25 was hyperphosphorylated, confirming that GVBD had occurred (Fig. 5A). We have previously observed that overexpression of other unrelated cell cycle proteins such as cyclin H or Cdk7 does not cause such a delay in oocyte maturation (unpublished observations), but it could be argued
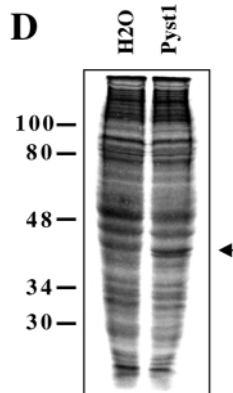
# A



# B



# C



# D



**Fig. 5.** Suppression of MAP kinase activation by Pyst1 delays but does not prevent initiation of meiotic maturation. (A) In vitro synthesized capped mRNA of Pyst1 was injected into oocytes (about 10 ng/oocyte). These and control oocytes were treated with progesterone (Pg, 10 μg/ml) and individual oocytes were selected before (SVI, stage VI) or at GVBD. A Western blot of Cdc25 and MAP kinase from individual oocytes is shown, as follows: SVI, Stage VI; Pg, progesterone treated; Pyst1 + Pg, microinjected with Pyst1 mRNA and treated with progesterone. (B) Comparison of delay over progesterone (pg)-induced GVBD by Pyst1 and geldanamycin (gdm) treatment with a non-specific delay induced by overexpression of the unrelated mRNA for β-catenin. (C) Pyst1 but not β-catenin overexpression blocks MAP kinase but has no effects on Cdc25 hyperphosphorylation. Western blots of the equivalent of two oocytes per lane for the indicated proteins. Endogenous β-catenin (β-cat, lower arrow) accumulates during oocyte maturation, and migrates at a different molecular weight from overexpressed myc tagged β-catenin (upper arrow) (Fisher et al., 1999). P-MAPK: Western blot for the active form of MAP kinase. For Pyst1, – and + refers to oocytes not having or having undergone GVBD respectively. (D) In vivo ^{35}S-labelling to confirm that Pyst1 mRNA injection indeed leads to overexpression of the protein (arrow): oocytes were microinjected with about 10 ng capped mRNA or water and labelled in 1 ml MMR with 1 mCi [^{35}S]methionine for 4 hours, before homogenization and treatment. 4 oocyte equivalents were loaded per lane.

predicted size (doublet at 43 kDa, Fig. 5D lane 2, arrow) which was not produced in oocytes injected with water (lane 1).

## Low-level MAP kinase activation is an early response to progesterone and is temporally distinct from MPF activation

It has been reported that MAP kinase acts as a 'molecular switch' at around the time of MPF activation, and that the response is essentially simultaneous due to a highly cooperative feedback loop (Ferrell and Machleder, 1998). This implies that MAP kinase might participate directly in the MPF activation loop. However, Nebreda et al. (1995) showed by inhibition of Cdc2, that detectable MAP kinase activation is dependent on activation of Cdc2 kinase and is thus downstream. How then does MAP kinase contribute to the efficiency of GVBD? We hypothesized that these results could be reconciled by a general model, consistent with other animal models, in which MAP kinase is not directly involved in activation of cell cycle machinery, but can increase the efficiency by stimulation of a related event, such as protein translation. As such, it could be that a low-level or a transient activation of MAP kinase would be sufficient for this stimulation, and that this had previously gone unnoticed due to the relative insensitivity of the western-blot mobility shift assay.

We have found, by the very sensitive active-MAP kinase blot assay, that Pyst1 and geldanamycin both block all detectable MAP kinase activity but still allow GVBD (Figs 5C, 6, 8; data not shown). We therefore undertook a comprehensive time-course experiment to determine the timing and level of MAP kinase activation in progesterone-treated oocytes, either in the
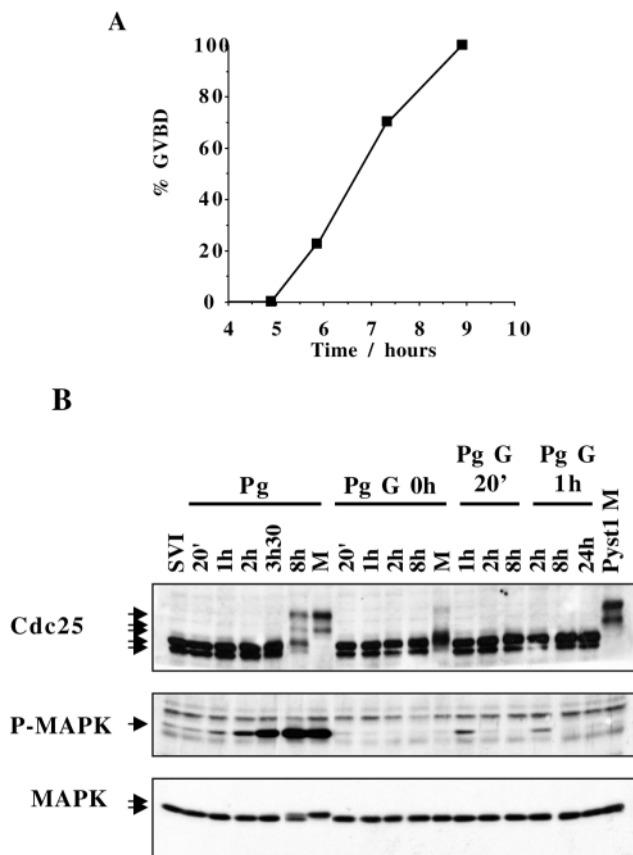
that a potential nonspecific delay might have been neutralized by a positive effect of such proteins on the cell cycle. We therefore compared overexpression of an unrelated mRNA, encoding β-catenin, to overexpression of Pyst1 in the same batch of oocytes. β-catenin overexpression (Fig. 5C panel 1) did cause a slight delay in progesterone-stimulated maturation, although this delay was nowhere near that obtained by inhibition of MAP kinase by Pyst1 overexpression or geldanamycin treatment (Fig. 5B). By using a very sensitive western blot assay for active MAP kinase, we confirmed that both Pyst1 (Fig. 5C panel 5) and geldanamycin (see below) completely inhibited MAP kinase activation, while β-catenin had no effect (Fig. 5C panel 3). Finally, we confirmed that the effect of Pyst1 mRNA was indeed due to production of the protein. Although we do not possess Pyst1 antibodies, we showed by in vivo ^{35}S-labelling of translated proteins that Pyst1 mRNA injection gave rise to synthesis of a protein of the

**A**



**B**



**Fig. 6.** Low level MAP kinase activation is an early response to progesterone treatment. (A) Time course of GVBD in progesterone-treated oocytes for whom the time course is shown in B. (B) Western blots showing a time course of MPF activation reflected by Cdc25 hyperphosphorylation (upper panel) and MAP kinase activation (lower two panels). Middle panel, anti-active MAP kinase; Lower panel, anti-total MAP kinase on the same blot rehybridized after stripping the blot in 100 mM glycine, 1% SDS, pH 2.8 for 10 minutes. SVI, stage VI; Pg, progesterone treated; geldanamycin added at the same time (Pg G 0 hours) as, or 20 minutes (Pg G 20′) or 1 hour (Pg G 1h) later than progesterone. M, M-phase, i.e. GVBD having occurred. Pyst1, pyst1 mRNA microinjected. The time in minutes or hours after progesterone addition is indicated.

absence of geldanamycin, or with geldanamycin added at zero-time or at various times after progesterone. The results were reproducible, and one such experiment is shown in Fig. 6. The time course of GVBD of progesterone-treated oocytes is shown in Fig. 6A. By the mobility-shift assay, we reproduce the result that the majority of MAP kinase activation occurs concurrently with MPF activation, visualized by the progressive hyperphosphorylation of Cdc25 (Fig. 6B lower and upper panels). However, we stripped and reprobed the same blot with an anti-active MAP kinase antibody, with surprising results (Fig. 6B middle panel). In this experiment, we found that in fact a low level of activation of MAP kinase occurred already 1 hour after progesterone addition, active MAP kinase steadily accumulated and, by 3 hours 30 minutes, well before GVBD, there was a relatively high level of active MAP kinase, even though by mobility shift this was undetectable. We found that, if we added geldanamycin at the same time as progesterone, MAP kinase was undetectable throughout the time course even

though some oocytes underwent GVBD (M, 3 out of 40). However, we cannot rule out that, had it been possible to examine these same three oocytes at an early point, they might have shown a low-level transient MAP kinase activation. If we added geldanamycin after 20 minutes or 1 hour of progesterone treatment, there was a very slight and transient activation of MAP kinase, not associated with Cdc25 hyperphosphorylation: from 2-24 hours no more MAP kinase activity could be detected (middle panel), and some oocytes underwent GVBD between 9 and 24 hours (data not shown). The level of MAP kinase activity attained in these two cases did not significantly increase the percentage of oocytes undergoing GVBD (0 out of 40 and 5 out of 48, respectively). Adding geldanamycin 2 hours after progesterone allowed a significant but still transient activation of MAP kinase, still at a level below the threshold of detection by mobility shift (data not shown), and greatly stimulated the percentage of oocytes undergoing GVBD (41 out of 54). Thus, low-level MAP kinase activation occurs early; it is temporally dissociable from activation of MPF; some oocytes can undergo GVBD without any apparent activation of MAP kinase; while even a low-level transient early activation of MAP kinase stimulates efficiency of the response. All of these observations are consistent with a model in which sustained or high level MAP kinase is neither essential nor directly involved in the MPF amplification loop, but rather that it can enhance the process, possibly by a more general mechanism. We wanted to further investigate this hypothesis.

## Formation of cyclin B-Cdc2 kinase in the absence of MAP kinase

These results suggest that the criterion for oocyte maturation is activation of Cdc2 kinase to a 'sufficient' level, which reflects the intrinsic sensitivity of individual oocytes to Cdc2 kinase, and that the role of MAP kinase is to increase the efficiency of this process. This can be seen from the data shown in Fig. 7: inhibition of MAP kinase either by Pyst1 microinjection or geldanamycin treatment reduces the Cdc2 kinase activity found at GVBD. Our model explains the previous apparently contradictory observations that progesterone can induce oocyte maturation but not MAP kinase activation in the presence of certain dominant negative mutants of Raf-1 (Fabian et al., 1993) and that microinjection of cyclin A causes GVBD in the absence of protein synthesis and MAP kinase activation (Kosako et al., 1994) and that inhibition of Cdc2 kinase activation prevents MAP kinase activation by progesterone (Nebreda et al., 1995), while MAP kinase is apparently necessary for progesterone-induced oocyte maturation (Gotoh et al., 1995; Kosako et al., 1994). It may be that, in the latter case, cyclin-Cdc2 kinase had not been activated to a sufficient level to promote GVBD in these oocytes.

Cdc25 is apparently regulated normally in the absence of MAP kinase, and Myt1 is at least phosphorylated to an intermediate degree and probably inhibited. We therefore investigated the possibility that formation or activation of cyclin B-Cdc2 complexes might be impaired in the absence of MAP kinase. We predicted that geldanamycin would not inhibit oocyte maturation induced by cyclin B protein microinjection, because a sufficient level of Cdc2 kinase activity should be generated even if MAP kinase remained blocked. This was indeed the case: although the MAP kinase
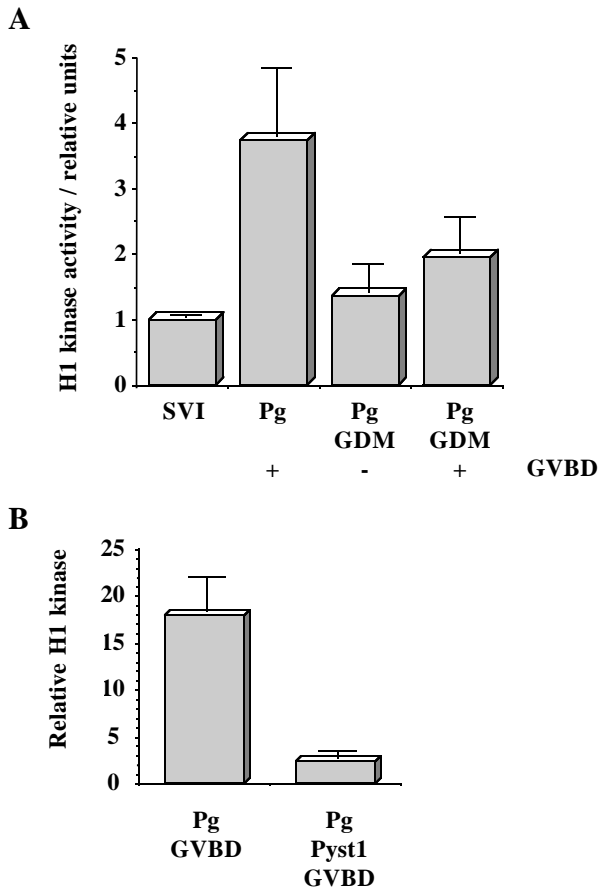
**A**



**B**



**Fig. 7.** MAP kinase inhibition reduces Cdc2 kinase activity at GVBD. (A,B) Oocyte extracts from stage VI (SVI), progesterone (Pg)-treated oocytes, with or without geldanamycin treatment (GDM) or Pyst1 microinjection. Extracts from individual oocytes selected at GVBD were assayed for histone H1 kinase; phosphorylated histone was quantified by phosphor-imager, and the level present in stage VI oocytes was either represented as 1 (A) or subtracted from these values (B). Represented are mean values from oocytes from 5 different experiments (A) or different individual oocytes (B). Error bars represent standard deviation.

cascade was still inhibited by geldanamycin treatment, microinjection of recombinant GST-sea urchin cyclin B protein nevertheless caused Cdc2 dephosphorylation (data not shown) and activation, and entry of oocytes into meiotic M-phase (Fig. 8).

Injected cyclin B forms an active kinase complex with endogenous Cdc2, and this in turn activates a positive-feedback loop to activate synthesis of cyclin B1, formation and activation of Cdc2-endogenous cyclin B complexes. We assayed Cdc2 kinase from these two sources, in the absence and presence of geldanamycin, to inhibit MAP kinase.

Fig. 8 shows that, on injection of recombinant cyclin B protein, total Cdc2 kinase is generated from exogenous and endogenous cyclin complexes, and is at a level similar to that activated by progesterone. The endogenous cyclin B1-kinase that is activated by injection of cyclin B is less than that induced by progesterone (Fig. 8A,C), while geldanamycin may further reduce this activity. In the majority of experiments, such as that shown in Fig. 8A, less cyclin B1 was synthesized in the

presence of geldanamycin and immunoprecipitated cyclin B1 kinase activity was reduced (Fig. 8A, middle and lower panels). However, in some experiments, such as that shown in Fig. 8B, the exogenous cyclin stimulated enough Cdc2 kinase activity to generate full cyclin B1 synthesis, even in the absence of MAP kinase activity (Fig. 8B, middle and lower panels), and the absence or presence of MAP kinase no longer affected endogenous cyclin B1 kinase activity (Fig. 8C). In the presence of geldanamycin and the absence of MAP kinase activation, exogenous cyclin forms a complex with Cdc2 and is activated to the same extent as in control oocytes (Fig. 8C). This shows that either Cdc2-inhibitory kinases can be inactivated even in the absence of MAP kinase or that they are bypassed.

It is therefore clear that MAP kinase has no effect on the formation or activation of cyclin-B-Cdc2 kinase complexes, but that one potential contribution of MAP kinase is the stimulation of cyclin B1 synthesis. Nevertheless, MAP kinase is clearly not essential for this cyclin B1 synthesis induced by microinjection of exogenous cyclin. We then reinvestigated the synthesis of cyclin B1 induced by progesterone in the absence or presence of MAP kinase (Fig. 8D). Surprisingly, we found that there is no absolute correlation between the ability to synthesize cyclin B1 and the ability to undergo GVBD, other than that all GVBD events were associated with cyclin B1 synthesis. Indeed, a relatively high level of cyclin B1 was attained in oocytes that never matured in the absence of MAP kinase, although levels of cyclin B1 were lower than in the presence of active MAP kinase. It therefore seems that a transient early level of MAP kinase could be sufficient to stimulate cyclin B1 synthesis, while further high levels of MAP kinase stimulate the efficiency of GVBD by an unknown mechanism.

## DISCUSSION

Given the conservation of mechanisms of cell cycle control in general, it has been somewhat of an enigma that controls regulating the initiation of meiotic maturation in oocytes from different animal species operate in apparently different ways. The role of MAP kinase activation in the activation of MPF and re-entry into meiosis has exemplified this problem. Although it is dispensable for meiotic reinitiation in other oocyte systems, MAP kinase activation has been reported to be essential in *Xenopus* oocytes. Because it is apparently activated simultaneously with MPF activation and displays switch-like behaviour (either undetectable or maximal), MAP kinase has been proposed to be the basis of a 'cell-fate switch' (Ferrell and Machleder, 1998).

Using a recently developed sensitive method of detection of active MAP kinase, we demonstrate that, in fact, MAP kinase activation is an early event in response to progesterone. Active MAP kinase gradually accumulates in the absence of MPF activity, and an early, transient and very low level of MAP kinase, temporally dissociable from MPF activation, appears to be sufficient to initiate meiotic maturation. Indeed, we report that a subpopulation of *Xenopus* oocytes can undergo GVBD and activation of cell cycle machinery in the absence of any detectable MAP kinase activity. Such oocytes are therefore not an exception to a universal model for oocyte meiotic reinitiation controlled by the activation of cyclin B-Cdc2 kinase, and it may
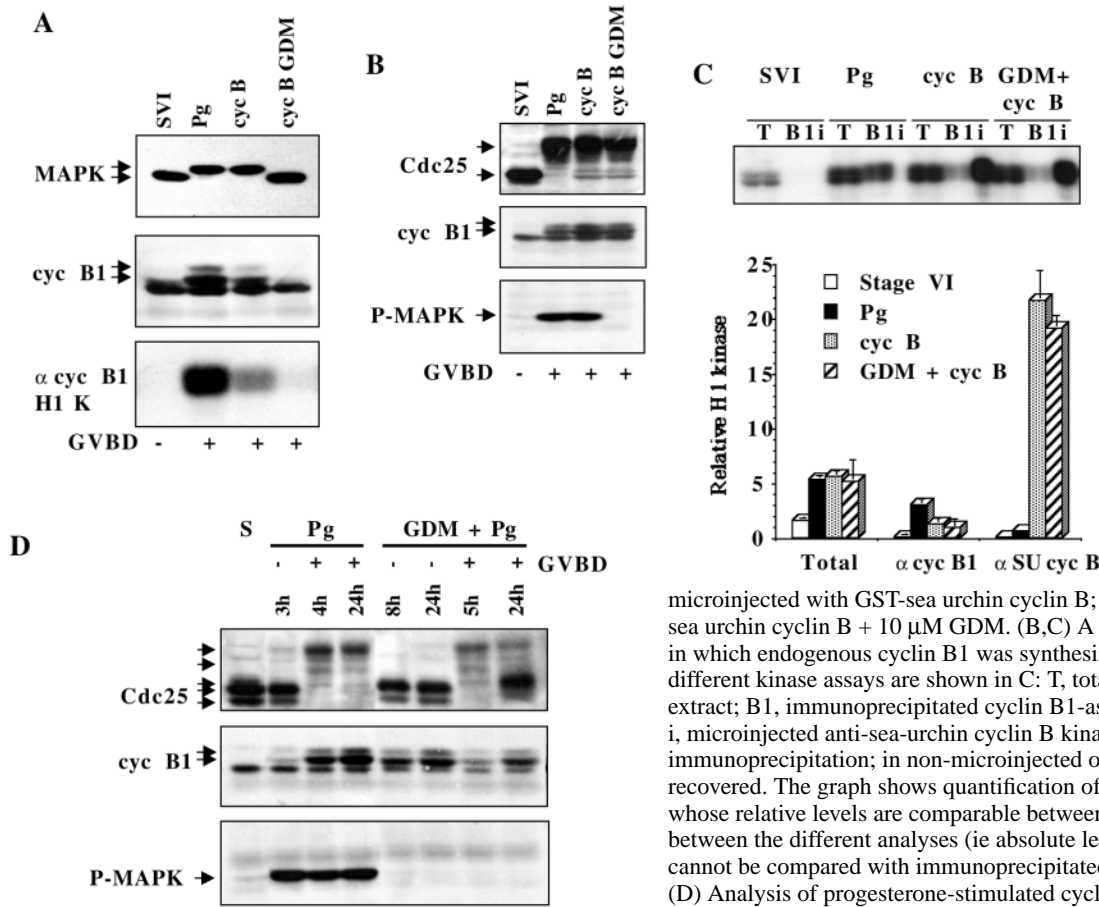
**Fig. 8.** Oocyte maturation induced by microinjection of cyclin B demonstrates that cyclin B-Cdc2 kinase complexes are formed and activated in the absence of MAP kinase. (A) Western blots (equivalent of 2 oocytes / lane) of MAP kinase (upper panel) and cyclin B1 (middle panel), histone H1 kinase activity of immunoprecipitated (from extract equivalent to 8 oocytes / lane) cyclin B1 (lower panel) from extracts of oocytes treated as follows (lane, column). (1) Stage VI; (2) Pg; (3) microinjected with GST-sea urchin cyclin B; (4) microinjected with GST-sea urchin cyclin B + 10 μM GDM. (B,C) A similar experiment to A, but in which endogenous cyclin B1 was synthesized and activated. The different kinase assays are shown in C: T, total H1 kinase assay from the extract; B1, immunoprecipitated cyclin B1-associated H1 kinase activity; i, microinjected anti-sea-urchin cyclin B kinase activity recovered by immunoprecipitation; in non-microinjected oocytes no activity is recovered. The graph shows quantification of these kinase activities, whose relative levels are comparable between different samples but not between the different analyses (ie absolute levels of total Cdc2 kinase cannot be compared with immunoprecipitated cyclin B1 kinase, etc). (D) Analysis of progesterone-stimulated cyclin B1 synthesis in the absence or presence of MAP kinase activity. Western blot for the indicated proteins, the equivalent of three oocytes was loaded per lane. Times indicated are times after progesterone addition. S, Stage VI oocytes; Pg, progesterone; Pg + GDM, progesterone and geldanamycin; cyc B1, cyclin B1; P-MAPK, active MAP kinase.

even be the case that MAP kinase activation is completely dispensable for GVBD in a subpopulation of oocytes.

We find that prevention of sustained and high level activation of MAP kinase by either treatment with geldanamycin, or by overexpression of the specific MAP kinase phosphatase Pyst1/MKP-3, delays but does not prevent GVBD. We cannot be sure why Gotoh et al. (1995) did not find that at least a subset of oocytes can undergo GVBD in the absence of MAP kinase, but we favour the idea that different batches of oocytes have different sensitivities of GVBD to levels of Cdc2 kinase. In other words, in some batches of oocytes, sustained MAP kinase activation might be necessary to generate a sufficient level of Cdc2 kinase to enter meiosis. We show in this paper that this is not an absolute requirement for progesterone-induced meiotic initiation in *Xenopus* oocytes.

The fact that some oocytes can undergo GVBD at low levels of Cdc2 kinase and in the absence of MAP kinase activity, while others do not, suggests that in fact it is the sensitivity of oocytes to Cdc2 kinase, and not to MAP kinase, that determines the cell fate. The biochemical basis for this sensitivity therefore remains unknown.

We also examined the state of several key cell cycle proteins in oocytes that undergo GVBD in the absence or presence of MAP kinase activity. Specifically, it has recently been proposed (Palmer et al., 1998) that a kinase acting downstream of MAP kinase, p90[rsk], inactivates the Cdc2 inhibitory kinase

Myt1 by phosphorylation and thereby links MAP kinase activation to progesterone-induced MPF activation. This may explain why premature MAP kinase activation in both *Xenopus* and starfish oocytes prevents inactivation of microinjected Cdc2-cyclin B kinase (Abrieu et al., 1997a), even though starfish do not require MAP kinase activation for GVBD. However, we show that, in oocytes that undergo GVBD in the absence of MAP kinase, Myt1 still undergoes SDS-PAGE mobility shift characteristic of phosphorylation and inactivation. In other words, MAP kinase may activate p90[rsk] and inhibit Myt1 even though a MAP kinase-independent enzyme may normally accomplish this task.

We further show that the Cdc25 hyperphosphorylation SDS-PAGE mobility shift is unaffected in oocytes that mature in the presence of geldanamycin or overexpression of Pyst1, demonstrating that Cdc25 does not require MAP kinase nor, presumably, Raf-1 kinase function in this system.

In many species, including starfish, Spisula and mice, protein synthesis is not required for fully grown oocytes to undergo GVBD in response to the natural maturation-inducing signals. In such species, conversion of inactive cyclin B-Cdc2 kinase to an active form is sufficient to trigger the $G_2$-M transition, and no further cyclin B synthesis is required before GVBD, even though it still occurs after GVBD. In *Xenopus*, however, protein synthesis is required for the $G_2$-M transition, and one protein whose synthesis is required in at least the majority of oocytes

is Mos. Could it be that this requirement for Mos, and thus MAP kinase activation, is to stimulate cyclin B synthesis?

We find, by suppressing the activation of MAP kinase using geldanamycin, that Cdc2 kinase activation occurs much slower and to a lower level in response to progesterone. We show that this defect in activation is not due to an impaired ability of cyclin B to associate with Cdc2, nor for such complexes to be activated. MAP kinase activation is no longer necessary for GVBD if exogenous cyclin B is injected, and sustained MAP kinase activation is unnecessary for progesterone-induced maturation. These results can be partly interpreted in terms of a regulation of the rate of formation of active cyclin B-Cdc2 complexes by the control of cyclin B1 accumulation. MAP kinase may well regulate this process (de Moor and Richter, 1997), and a low-level, transient MAP kinase activation may be sufficient to trigger cyclin B mRNA polyadenylation and thus protein synthesis and GVBD.

In apparent contradiction with an earlier report by Minshull et al. (1991), dominant negative Cdc2 mutants inhibit MPF activation in *Xenopus* oocytes (Nebreda et al., 1995), probably by blocking assembly of the active kinase from free Cdc2, which is in excess, and newly synthesized cyclin B. As expected, these mutants do not suppress MPF activation in oocytes that do not require protein synthesis for GVBD (Picard et al., 1996). This suggests that, in *Xenopus* oocytes, cyclin B synthesis is required for Cdc2 kinase activation. Since GVBD was suppressed in 80% of oocytes in which antisense cyclin oligonucleotides were microinjected in the experiments of Minshull et al. (1991), it may well be that, at least the majority of oocytes, in *Xenopus*, do require cyclin B synthesis for GVBD.

Even in species that do not require cyclin B synthesis for GVBD, MAP kinase regulates cyclin B synthesis after GVBD. For example, oocytes from $mos^{-/-}$ mice can undergo initiation of meiotic maturation but do not activate MAP kinase, indicating that other activators of the MAP kinase cascade, such as Raf-1, cannot replace Mos function (Verlhac et al., 1996). The rate of formation of Cdc2 kinase activity after GVBD in mouse oocytes is controlled by cyclin B1 synthesis (Polanski et al., 1998), while cyclin B does not accumulate in mouse oocytes injected with Mos antisense oligonucleotides (O'Keefe et al., 1991). Our results in *Xenopus* oocytes (this paper) show striking parallels to these results and lead us to suggest that oocyte meiotic initiation might indeed be controlled in a universally similar way. Quantitative differences may well exist between different oocyte systems, such as the sensitivity of GVBD to Cdc2 kinase levels, which means that meiosis is organized along the same principles but quantitatively different ways.

## REFERENCES

**Abrieu, A., Lorca, T., Labbé, J. C., Morin, N., Keyse, S. and Dorée, M.** (1996) MAP kinase does not inactivate, but rather prevents the cyclin degradation pathway from being turned on in *Xenopus* egg extracts *J. Cell Sci.*, **109**, 239-246.

**Abrieu, A., Doree, M. and Picard, A.** (1997a) Mitogen-activated protein kinase activation down-regulates a mechanism that inactivates cyclin B-cdc2 kinase in G2-arrested oocytes *Mol. Biol. Cell*, **8**, 249-261.

**Abrieu, A., Fisher, D., Simon, M. N., Dorée, M. and Picard, A.** (1997b) MAPK inactivation is required for the G2 to M-phase transition of the first mitotic cell-cycle *EMBO J.* **16**, 6407-6413.

**Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S.** (1998) Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase *Science* **280**, 1262-1265.

**Cross, D. A. E. and Smythe, C.** (1998) PD 98059 prevents establishment of the spindle assembly checkpoint and inhibits the G2-M transition in meiotic but not mitotic cell cycles in *Xenopus. Exp. Cell Res.* **241**, 12-22.

**de Moor, C. H. and Richter, J. D. (1997)** The mos pathway regulates cytoplasmic polyadenylation in Xenopus oocytes. *Mol. Cell. Biol.* **17**, 6419-6426

**Fabian, J. R., Morrison, D. K. and Daar, I. O.** (1993) Requirement for Raf and MAP kinase function during the meiotic maturation of Xenopus oocytes *J. Cell Biol.* **122**, 645-652.

**Ferrell, J. E. J. and Machleder, E. M.** (1998) The biochemical basis of an all-or-none cell fate switch in Xenopus oocytes *Science* **280**, 895-898.

**Ferrell, J. J., Wu, M., Gerhart, J. C. and Martin, G. S.** (1991) Cell cycle phosphorylation of p34cdc2 and a microtubule associated protein kinase homolog in Xenopus oocytes and eggs *Mol. Cell. Biol.* **11**, 1965-1971.

**Fisher, D., Abrieu, A., Simon, M. N., Keyse, S., Vergé, V., Dorée, M. and Picard, A.** (1998) MAP kinase inactivation is required only for G2–M phase transition in early embryogenesis cell cycles of the starfishes *Marthasterias glacialis* and *Astropectens aranciacus Dev. Biol.* **202**, 1-13.

**Fisher, D.L., Morin, N. and Dorée, M.** (1999) A novel role for glycogen synthase kinase-3 in *Xenopus* development: maintenance of oocyte cell cycle arrest by a ß-catenin independent mechanism *Development* **126**, 567-576.

**Fissore, R. A., He, C. L. and Vande Woude, G. F.** (1996) Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes *Biol. Reprod.* **55**, 1261-1270.

**Gotoh, Y., Masuyama, N., Dell, K., Shirakabe, K. and Nishida, E.** (1995) Initiation of Xenopus oocyte maturation by activation of the mitogen-activated protein kinase cascade *J. Biol. Chem.* **270**, 25898-25904.

**Goudet, G., Belin, F., Bezard, J. and Gerard, N.** (1998) Maturation-promoting factor (MPF) and mitogen activated protein kinase (MAPK) expression in relation to oocyte competence for in-vitro maturation in the mare *Mol. Hum. Reprod.* **4**, 563-570.

**Groom, L. A., Sneddon, A. A., Alessi, D. R., Dowd, S. and Keyse, S. M.** (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase *EMBO J.* **15**, 3621-3632.

**Haccard, O., Jessus, C., Cayla, X., Goris, J., Merlevede, W. and Ozon, R.** (1990) In vivo activation of a microtubule-associated protein kinase during meiotic maturation of the Xenopus oocyte *Eur. J. Biochem.* **192**, 633-642.

**Haccard, O., Lewellyn, A., Hartley, R. S., Erikson, E. and Maller, J. L.** (1995) Induction of *Xenopus* oocyte maturation by MAP kinase *Dev. Biol.* **168**, 677-682.

**Huang, C. Y. F. and Ferrell, J. E.** (1996) Dependence of Mos-induced Cdc2 activation on MAP kinase function in a cell-free system *EMBO J.* **15**, 2169-2173.

**Inoue, M., Naito, K., Nakayama, T. and Sato, E.** (1996) Mitogen-activated protein kinase activity and microtubule organisation are altered by protein synthesis inhibition in maturing porcine oocytes *Zygote* **4**, 191-198.

**Karaiskou, A., Cayla, X., Haccard, O., Jessus, C., Ozon, R.** (1998) MPF amplification in Xenopus oocyte extracts depends on a two-step activation of cdc25 phosphatase *Exp. Cell Res.* **244**, 491-500

**Kobayashi, H., Minshull, J., Ford, C., Golsteyn, R., Poon, R. and Hunt, T.** (1991) On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation in Xenopus laevis *J. Cell Biol.* **114**, 755-765.

**Kosako, H., Gotoh, Y. and Nishida, E.** (1994) Requirement for the MAP kinase kinase/MAP kinase cascade in xenopus oocyte maturation *EMBO J.* **13**, 2131-2138.

**Labbé, J. C., Cavadore, J. C. and Dorée, M.** (1991) M phase-specific cdc2 kinase: preparation from starfish oocytes and properties *Methods Enzymol.* **200**, 291-301.

**Minshull, J., Murray, A., Colman, A. and Hunt, T.** (1991) Xenopus oocyte

maturation does not require new cyclin synthesis *J. Cell Biol.* **114**, 767-772.

**Muslin, A. J., MacNicol, A. M. and Williams, L. T.** (1993) Raf-1 protein kinase is important for progesterone-induced Xenopus oocyte maturation and acts downstream of mos *Mol. Cell. Biol.* **13**, 4197-4202.

**Nebreda, A. R. and Hunt, T.** (1993) The c-mos proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of Xenopus oocytes and eggs *EMBO J.* **12**, 1979-1986.

**Nebreda, A. R., Gannon, J. V. and Hunt, T.** (1995). Newly synthesized protein(s) must associate with p34cdc2 to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes *EMBO J.* **14**, 5597-5607.

**O'Keefe, S. J., Kiessling, A. A. and Cooper, G. M.** (1991) The c-mos gene product is required for cyclin B accumulation during meiosis of mouse eggs *Proc. Natl. Acad. Sci. USA* **88**, 7869-7872.

**Palmer, A., Gavin, A. C. and Nebreda, A. R.** (1998) A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1 *EMBO J.* **17**, 5037-5047.

**Picard, A., Galas, S., Peaucellier, G. and Dorée, M.** (1996) Newly assembled cyclin B-cdc2 kinase is required to suppress DNA replication between meiosis I and meiosis II in starfish oocytes *EMBO J.* **15**, 3590-3598.

**Polanski, Z., Ledan, E., Brunet, S., Louvet, S., Verlhac, M. -H., Kubiak, J. Z. and Maro, B.** (1998) Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes *Development* **125**, 4989-4997.

**Posada, J., Yew, N., Ahn, N. G., Vande Woude, G. F. and Cooper, J. A.** (1993) Mos stimulates MAP kinase in Xenopus oocytes and activates a MAP kinase kinase in vitro *Mol. Cell. Biol.* **13**, 2546-2553

**Pratt, W. B.** (1998) The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors *Proc. Soc. Exp. Biol. Med.* **217**, 420-34.

**Sagata, N., Daar, I., Oskarsson, M., Showalter, S. D. and Vande Woude, G. F.** (1989) The product of the mos proto-oncogene as a candidate 'initiator' for oocyte maturation *Science* **245**, 643-6.

**Shibuya, E. K., Boulton, T. G., Cobb, M. H. and Ruderman, J. V.** (1992) Activation of p42 MAP kinase and the release of oocytes from cell cycle arrest *EMBO J.* **11**, 3963-3975.

**Shibuya, E. K. and Ruderman, J. V.** (1993) Mos induces the in vitro activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. *Mol. Biol. Cell* **4**, 781-790

**Shibuya, E. K., Morris, J., Rapp, U. and Ruderman, J. V.** (1996) Activation of the Xenopus oocyte MAP kinase pathway by mos is independent of raf. *Cell Growth Differ.* **7**, 235-241

**Verlhac, M. H., de Pennart, H., Maro, B., Cobb, M. H. and Clarke, H. J.** (1993) MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes *Dev. Biol.* **158**, 330-340.

**Verlhac, M. H., Kubiak, J. Z., Weber, M., Geraud, G., Colledge, W. H., Evans, M. J. and Maro, B.** (1996) Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse *Development* **122**, 815-822.