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 MPF 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 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.

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*
(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 G2/prophase block, and provokes a bona fide G2/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. 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 G2 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 p90rsk has recently been proposed
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, p90rsk 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 p90rsk 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...
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 35S-labelling of translated proteins that Pyst1 mRNA injection gave rise to synthesis of a protein of the 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

**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 35S-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 [35S]methionine for 4 hours, before homogenization and treatment. 4 oocyte equivalents were loaded per lane.
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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
Further reduce this activity. In the majority of experiments, such induced by progesterone (Fig. 8A,C), while geldanamycin may that is activated by injection of cyclin B is less than that endogenous cyclin B1-kinase and presence of geldanamycin, to inhibit MAP kinase. We assayed Cdc2 kinase from these two sources, in the absence activation of Cdc2-endogenous cyclin B complexes. We microinjection of recombinant GST-sea urchin cyclin B protein microinjected 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.

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.

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 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, endogenous 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 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-Čdc2 kinase, and it may
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, p90rsk, 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 p90rsk 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 G2-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 G2-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 B was suppressed in 80% of oocytes in which antisense cyclin B was not expressed in relation to oocyte competence for in-vitro maturation in the mare Mol. Reprod. 4, 563-570.


REFERENCES


