Journal of Cellular Biochemistry 55:465–476 (1994)

Progesterone but not *ras* Requires MPF for In Vivo Activation of MAPK and S6 KII: MAPK Is an Essential Conexion Point of Both Signaling Pathways

Amancio Carnero, Benilde Jiménez, and Juan Carlos Lacal

Instituto de Investigaciones Biomédicas, CSIC (A.C., B.J., J.C.L.) and Departamento de Bioquímica, Facultad de Medicina, Universidad Autónoma de Madrid (B.J.), 28029 Madrid, Spain

Abstract Induction of mitosis in *Xenopus laevis* oocytes by hormones and the oncogenic *ras*-p21 protein has been shown to correlate with a cascade of phosphorylations of the Ser/Thr family of kinases. However, the exact hierarchy of enzymes and their mutual interdependency has not been fully elucidated yet. We have used the *Xenopus laevis* system to investigate the mechanism of activation of the Ser/Thr kinases cascade and their relationship. Comparison between progesterone-induced germinal vesicle breakdown (GVBD), a hallmark of mitosis in oocytes, to that triggered by *ras*-p21, revealed the existence of at least two independent mechanisms to activate the MAP kinase enzyme in vivo. While progesterone function is dependent of cdc2 protein kinase activity, *ras*-p21 is independent of this enzyme. However, both progesterone and *ras*-p21 converge at the MAP kinase level, and depletion of MAP kinase activity inhibits the GVBD and S6 kinase II activation induced by both progesterone and *ras*-p21. These results provides further evidence that MAP kinase is a critical step for regulation of the cell cycle in oocytes and a critical point where *ras* and progesterone signaling converge.

Key words: *Xenopus laevis*, *ras*-p21, Ser/Thr kinases, GVBD, MAP kinase

Regulation of cell proliferation involves events initiated at the plasma membrane that control reentry into the cell cycle as well as intracellular biochemical changes that direct the process of cell division itself. Both of these aspects of cell growth control can be studied in *Xenopus* oocytes undergoing meiotic maturation in response to mitogenic stimulation by hormones and *ras* proteins [Maller, 1990; Jacobs, 1992]. Unlike most vertebrate somatic cells, which are arrested at the G0 phase, *Xenopus* oocytes are arrested at the G2/M border of the first meiotic prophase. Among several signals, progesterone stimulation and injection of oncogenic *ras* proteins seem to activate a preexisting factor designed as pre-MPF (Maturation Promoting Factor) which is actually a complex of cyclin B and the cdc2 protein kinase. As a consequence, after activation of pre-MPF, resumption of the cell cycle takes place [reviewed in Maller, 1990; Nurse, 1990; Jacobs, 1992].

Received January 25, 1994; accepted March 1, 1994.

Address reprint requests to Juan Carlos Lacal, Instituto de Investigaciones Biomédicas, CSIC, Calle Arturo Duperier 4, 28029 Madrid, Spain.

Mammalian MAP kinase is a serine/threonine kinase whose activation and phosphorylation on Tyr and Thr residues is rapidly induced by a variety of mitogens [Ray and Sturgill, 1987; Hoshi et al., 1988a,b; Ray and Sturgill, 1988; Rossomando et al., 1989; Boulton et al., 1991]. This kinase is considered to have a critical role in a network of protein kinases in mitogenic signal transduction [Ray and Sturgill, 1987; Hoshi et al., 1988a,b; Ray and Sturgill, 1988; Rossomando et al., 1989; Sturgill et al., 1988; Boulton et al., 1991; Pagés et al., 1993]. *Xenopus* $p42^{MAPK}$ is also a Ser/Thr kinase closely related to the mammalian MAP kinase [Gotoh et al., 1991a]. It is phosphorylated on Tyr and Thr residues, a mechanism for its activation which takes place during entry into the M phase of the cell cycle in *Xenopus* oocytes [Ferrel et al., 1991; Matsuda et al., 1992].

In *Xenopus*, $p42^{MAPK}$ becomes Tyr phosphorylated during oocyte maturation concomitantly with the dephosphorylation of cdc2 [Ferrel et al., 1991]. Moreover, $p42^{MAPK}$ is activated after microinjection of MPF into inmature oocytes, and the addition of MPF to cell-free extracts of interphase eggs can induce MAP kinase activa-

tion via phosphorylation [Gotoh et al., 1991a]. Thus, MAP kinase is activated during the M phase under the control of MPF and may constitute part of the kinase cascade downstream of MPF. Moreover, the interphase-M phase transition of microtubule dynamics can be induced in vitro by addition of the purified M phase MAP kinase to interphase extracts [Gotoh et al., 1991b]. However, Shibuya et al. [1992] and Itoh et al. [1993] using extracts prepared from oocytes arrested at interphase by cycloheximide treatment, reported that the addition of *ras*-p21 to cell extracts induces activation of MAP kinase. Thus, in vitro activation of $p42^{MAPK}$ induced by *ras* occurs in the absence of cdc2 activation, since cycloheximide-treated oocytes contain inactive MPF [Murray and Kirschner, 1989; Roy et al., 1991]. Although $p42^{MAPK}$ is activated by *ras* both in vivo and in vitro, activation of MPF in vitro has not been observed, and the normal pathway for $p42^{MAPK}$ activation in vivo is not clear.

In this report we show that oncogenic *ras*-p21 but not progesterone, activates $p42^{MAPK}$ in the absence of MPF activity in vivo, suggesting a MPF-independent pathway for activation of $p42^{MAPK}$ during *Xenopus laevis* oocyte maturation. Moreover, in vivo inhibition of $p42^{MAPK}$ activity blocks S6 kinase (S6 KII) activation and GVBD induced by both progesterone and *ras* proteins.

METHODS

Oocyte Maturation and Microinjection

Stage VI oocytes were selected by manual dissection. Series of 30–50 oocytes were treated for hormonal induction of maturation with 1 μg/ml progesterone in Ringer's buffer (100 mM NaCl, 1.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM $NaHCO_3$, pH 7.8). Microinjection of 25 ng of the *ras*-p21 protein was performed as previously described [Lacal, 1990]. After incubation at 18–20°C in Ringer's buffer for 18–20 h, oocytes were lysed for biochemical characterization or fixed in 16% TCA. Visual verification of nuclear vesicle breakdown was performed by open splitting the oocytes after fixation.

Protein Purification

The v-H-*ras* p21 protein was purified as described previously [Lacal et al., 1986]. After protein induction, 7 M urea extracts were subjected to further purification by chromatography through a Sephadex G-100 column (90 × 2.5 cm) in 7 M urea-20 mM MES, pH 7.0. Fractions of 3 ml were collected and analyzed by SDS PAGE to estimate purity and by a GTP-binding (Bio-Rad, Munich, Germany) assay to determine the activity of *ras*-p21. Fractions containing up to 95% purified *ras*-p21 were pooled and dialyzed against 20 mM MES, pH 7.0, and the concentrations were estimated by the Bradford assay system.

$p13^{suc}$ was purified as described by Brizuela et al. [1987]. Shortly, the *E. coli* line BL21.DE3 containing the pRK172.suc1$^+$ plasmid, a generous gift of Dr. S. Moreno, was grown in LB-broth containing 100 μg/ml ampicilin, and $p13^{suc}$ expression induced by 0.4 mM IPTG. The soluble fraction of a 400 ml bacterial culture lyzed in buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10% glycerol) was loaded on a 1.5 × 80 cm Sepharose CL6B column and eluted in the same buffer without glycerol, and increasing concentrations of NaCl to a final concentration of 500 mM. Two milliliter fractions were collected and analyzed by SDS-PAGE. $p13^{suc}$ was linked to sheparose CNBr-4B according to the manufacturer (Pharmacia, Sweden).

MPF Assays

MPF assays were carried out with total extracts from series of ten oocytes treated with progesterone or microinjected with *ras*-p21 proteins. After incubation for 18–20 h oocytes were homogeneized in buffer BLO containing 20 mM Hepes, pH 7.0, 10 mM β-glycerophosphate, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 2 mM DTT, 10 μg/ml leupeptine, 25 μg/ml aprotinin, and 100 μM PMSF. Following centrifugation at 13,000*g* for 15 min, extracts were assayed for 15 min at 30°C in a final reaction volume of 50 μl containing 20 mM Hepes, pH 7.0, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 μM γ-^{32}P-ATP (2–5 dpm–fmol), 0.2 μg of PKA inhibitor, and 1 mg/ml of type III-S calf thymus histone (Sigma Chemical Co., St. Louis, MO). Reactions were stopped by addition of PAGE-sample buffer and boiling for 5 min. Samples were run in a 15% PAGE, dried and exposed at −70°C. The band corresponding to H1 was excised from the gel and counted in a scintillation counter.

Alternatively, MPF assay was also performed after precipitation using p13-sepharose beads. In this case series of 20 oocytes were treated with progesterone or microinjected with indicated amounts of the *ras*-p21 protein. Oocytes were lyzed in BLO, centrifuged at 13,000*g* and the supernatant incubated for 2 h under con-

stant rotation at 4°C with 50 μl of p13-sepharose in a final volume of 1 ml. The p13-Sephasore pellets were washed once with BLO and twice with 20 mM Hepes, pH 7.0, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$. The kinase assays using the p13-Sepharose precipitates were performed as described above for total extracts.

S6 Kinase Assay

To determine S6 KII activity in total extracts, series of 20 oocytes were homogeneized in BLO in a final volume of 200 μl. Extracts were centrifuged at 13,000 rpm 15 min and 30 μl of supernatant were assayed with S6 KII substrate peptide. The reaction mixture in a final volume of 50 μl, contained 250 μM rsk substrate peptide (UBI), 50 mM glycerophosphate, pH 7.3, 7 mM NaF, 0.3 mM EDTA, 150 nM $MgCl_2$, 2 mM DTT, 50 μM ^{32}P-γATP (3,000 Ci/mmol, Amersham, Madrid, Spain), and 7 μM PKA inhibitor peptide (Sigma Chemical Co., St. Louis, MO). The assays were incubated at 30°C for 20 min and stopped with ice-cold TCA to a final concentration of 16% TCA. Samples were maintained 15 min at 4°C and centrifuged at 13,000 rpm for 15 min. The supernatants were spotted onto Watman p81 phosphocellulose paper filters, washed extensively with 1% ortophosphoric acid, and once with 95% ethanol. The radioactivity retained on the filters was quantified in a scintillation counter.

Chromatography of MAP K on Mono Q Column

Oocytes were lyzed in 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM DTT, 400 μM PMSF, 2 μM leupeptin, 25 μg/ml aprotinin, 5 mM NaPPi, and 1 mM NaF. Extracts were centrifuged at 100,000*g* in a TL100 centrifuge and filtered in 0.2 μm filters. Two milligrams of total proteins were applied to Mono Q columns that had been equilibrated in the same buffer without NaF. The columns were washed in the equilibrating buffer and proteins were eluted with a linear gradient of NaCl (0 to 500 mM); 2 ml fractions were collected and assayed for protein kinase activity.

MAP K Assay

Oocyte extracts were prepared as indicated above and 30 μl per fraction were assayed with 0.25 mg/ml MBP in 50 mM Tris, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 50 μM ^{32}P-γATP (3,000 Ci/mmol), and 2.5 μM PKA inhibitor in a final volume of 50 μl. After 15 min at 30°C samples were spotted onto Watman p81 phosphocellulose paper filters, washed extensivelly with 1% ortophosphoric acid, and once with 95% ethanol. The radiactivity retained on the filters was quantified in a scintillation counter. All results shown are representatives from at least two independent experiments.

RESULTS

Activation of Cell Cycle Kinases in Oocytes After Stimulation by Progesterone and *ras*-p21

Mitogenic stimulation of *Xenopus laevis* oocytes can be followed by monitoring post-translational modification of the key enzymes involved in the process. Progesterone *treatment* or *ras*-p21 microinjection induce similar overall phosphorylation patterns of proteins [Maller and Smith, 1985; Nebreda et al., 1993], suggesting that both agents could activate a similar pathway of intracellular events. Quiescent stage VI oocytes contain inactive MPF which correlates with its Tyr phosphorylated stage of cdc2 [reviewed in Maller, 1990; Nurse, 1990]. Upon stimulation with progesterone or oncogenic *ras,* dephosphorylation of cdc2, and activation of MPF complex (Fig. 1A,B) take place. As a consequence of MPF activation there is a burst in phosphorylation of intracellular substrates.

$p42^{MAPK}$ and S6 KII are two of the best characterized phosphorylated proteins after MPF activation [Maller, 1990]. After progesterone or *ras*-p21-induced stimulation, both $p42^{MAPK}$ and S6 KII become activated, as indicated respectively by Tyr phosphorylation and a net shift on the electrophoretic mobility observed in a SDS-PAGE for $p42^{MAPK}$ (Fig. 1C) or phosphorylation of the S6 KII (rsk) peptide substrate for S6 kinase II (Fig. 1D).

ras-Induced $p42^{MAPK}$ Activation Occurs in the Absence of Protein Synthesis

Induction of *Xenopus* oocyte meiotic maturation by hormones depends on protein synthesis [Maller, 1990]. Microinjection of MPF can bypass this requirement, suggesting that protein synthesis is actually needed for activation of MPF. It has been recently shown that GVBD of *Xenopus* oocytes does not require de novo cyclin synthesis [Minshull et al., 1991]. In contrast, synthesis of the *mos* proto-oncogene product, a serine-threonine kinase, is necessary for initiation of progesterone-induced GVBD [Sagata et

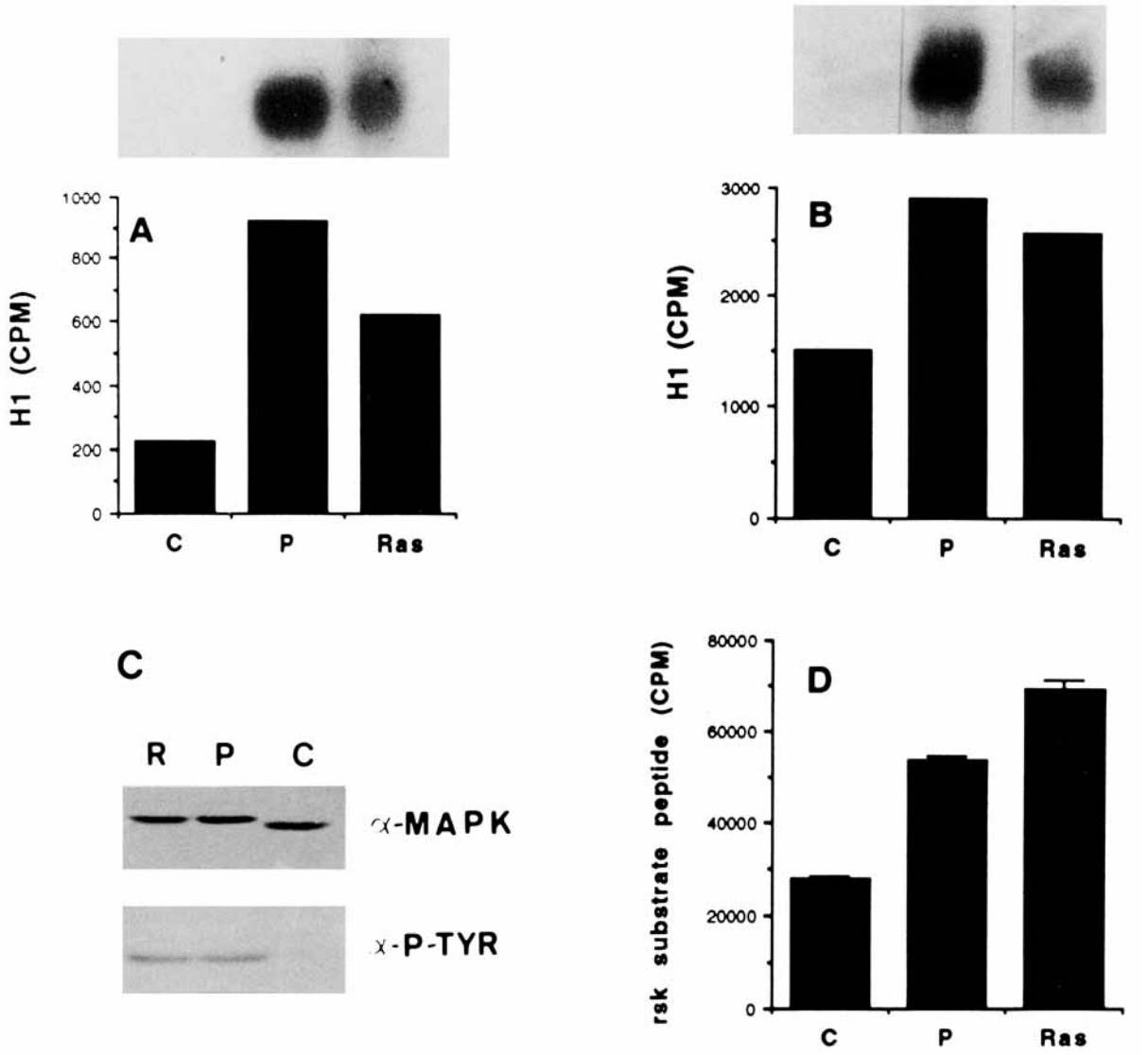


Fig. 1. In vivo activation of MPF, $p42^{MAPK}$, and S 6 K II by progesterone and *ras*-p21. **A, B:** Phosphorylation of H1 by MPF, performed as described in Methods, was carried out after progesterone treatment or *ras*-p21 protein microinjection, using either total oocyte extracts (A) or p13-agarose precipitates (B). Unstimulated oocytes (C), oocytes treated with 1 μg/ml progesterone (P) or oocytes microinjected with 25 ng of v-H-*ras*-p21 (Ras). **C:** Activation of $p42^{MAPK}$, measured as mobility shift and Tyr phospohorylation. Total extracts of unstimulated (C), progesterone treated (P) or *ras*-p21 microinjected oocytes (R), were run in a 10% PAGE, transfered to nitrocellulose and incubated in the presence of an anti-MAP-kinase (polyclonal) or anti-P-Tyr (monoclonal) antibodies, followed by a biotinilated antirabbit or antimouse antibodies and the streptavidin-peroxidase conjugated protein. Blots were revealed by the ECL system (Amersham International, England). **D:** Phosphorylation of the rsk substrate peptide in total oocyte extracts from unstimulated oocytes (C), oocytes treated with 1 μg/ml progesterone (P), or oocytes microinjected with 25 ng of v-H-*ras*-p21 (Ras).

al., 1988]. However, *ras*-p21 can induce meiotic maturation and MPF activation independently of endogenous *mos* [Daar et al., 1991], in agreement with a previous report indicating that *ras*-p21 can induce GVBD in cycloheximide (CHX)-treated oocytes [Allende et al., 1988].

In the presence of CHX, both progesterone and *ras*-p21 showed a reduced MPF activity comparable to those of untreated oocytes (Fig. 2A). A similar result was obtained by the MPF assay in p13-agarose precipitates, since p13 specifically bind to cdc2 protein kinase (Fig. 2B). However, while in progesterone-treated oocytes there is no detectable activation of $p42^{MAPK}$ in the absence of protein synthesis, oocytes microinjected with the *ras*-p21 protein showed $p42^{MAPK}$ phoshorylated at Tyr residues and the shift in mobility associated to activation of the $p42^{MAPK}$

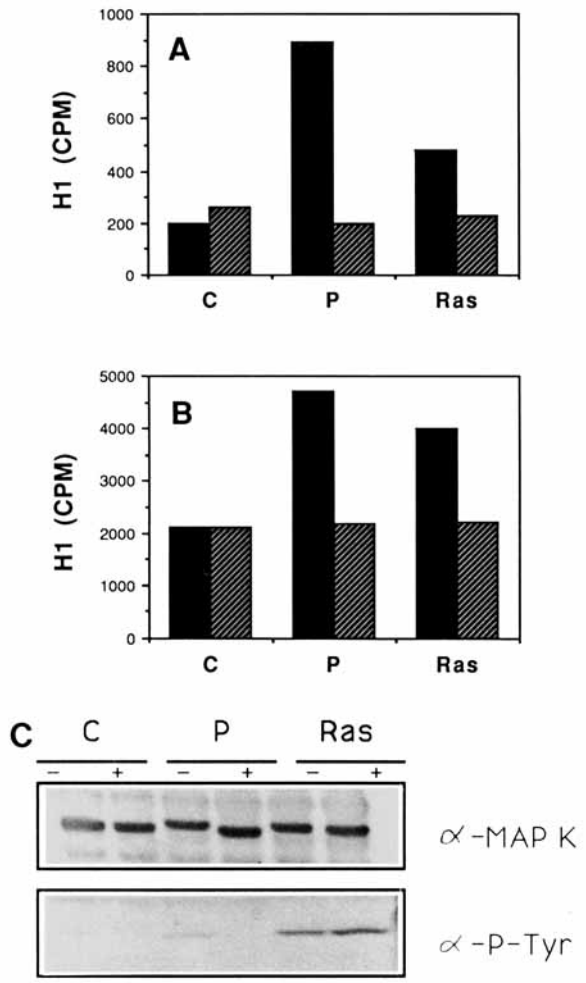


Fig. 2. Effect of cycloheximide treatment in the in vivo activation of MPF and $p42^{MAPK}$. **A,B:** MPF activity in total oocyte extracts (A) and in p13-agarose precipitates (B). Oocytes were treated with progesterone or microinjected with *ras*-p21 proteins in the absence (■) or presence of 2 μM CHX (▨). **C:** Western blots from total extracts of oocytes treated (+) or not (−) with 2 μM CHX. Western blots were performed as described in the legend to Figure 1. C, untreated; P, Progesterone 1 μg/ml; Ras, 25 ng of v-H-*ras*-p21.

enzyme (Fig. 2C). These results suggest that MPF activation is not necessary for activation of $p42^{MAPK}$ induced by *ras*-p21 proteins.

To confirm further these results, a time course of cdc2 protein kinase activity was performed along with the determination of GVBD induced after progesterone treatment or *ras*-p21 microinjection. Series of 20 oocytes/point were followed for GVBD by TCA-fixation and microscopic determination of the germinal vesicle. As shown in Figure 3A, oocytes underwent maximal GVBD at around 10 h of progesterone addition or *ras* microinjection. However, while progesterone induced cdc2 protein kinase activation was fully achieved at 6 h (Fig. 3B), *ras*-p21-induced activation of this enzyme was achieved at 12 h (Fig. 3C), after full GVBD incubation was observed. These results indicate that in progesterone-treated oocytes, the cdc2 protein kinase was activated right before GVBD. By contrast, in *ras*-p21-injected oocytes, cdc2 protein kinase activation was observed after GVBD.

6-DMAP Inhibits MPF Both In Vitro and In Vivo, but not *ras*-Induced $p42^{MAPK}$ Activation

6-DMAP (6 dimethyl-aminopurine) is a puromycin analog that inhibits protein kinases but which does not inhibit protein synthesis. 6-DMAP has been previously shown to be able to block oocyte maturation in several species (patella, starfish, or mouse) and progesterone-induced maturation of *Xenopus* oocytes [Neant and Guerrier, 1988; Rime et al., 1989; Jessus et al., 1991], probably by acting directly at the level of MPF activation. Jessus et al. [1991] reported that 6-DMAP inhibit maturation of *X. laevis* oocytes induced by microinjection of active MPF.

We have analyzed the effect of 6-DMAP treatment in *Xenopus* oocytes both in vitro and in vivo. As shown in Figure 4A, indeed, 6-DMAP inhibited the in vitro phosphorylation of H1 by progesterone-activated extracts of *Xenopus* oocytes. Thus, we then studied whether this inhibitor had any effect on the in vivo activation of MPF. Oocytes treated with 1 μM progesterone or microinjected with *ras*-p21 were incubated for 18–20 h in the presence of 0.5 mM 6-DMAP, and then oocyte extracts were assayed for MPF activity. As shown in Figure 4B and 4C, progesterone-treated and *ras*-p21 microinjected oocytes showed a reduction of MPF activity to the basal levels found in unstimulated oocytes. Thus, 6-DMAP also blocked the activation of MPF by progesterone or *ras*-p21 in vivo.

Addition of cycloheximide (CHX) blocks activation of $p42^{MAPK}$ in vivo in progesterone-treated but not in *ras*-injected oocytes (Fig. 2). This result suggests that progesterone and *ras* may use alternative pathways for induction of GVBD. We have also used the cdc2 protein kinase inhibitor 6-DMAP to study whether progesterone- and *ras*-induced $p42^{MAPK}$ activation require MPF activity. Oocytes were treated with progesterone or injected with *ras*-p21 and incubated in the presence of the inhibitor. After 18–20 h of incubation, oocyte extracts were run in 10% SDS-PAGE, transfered to nitrocellulose paper, and incubated with α-P-Tyr and α-MAPK antibod-

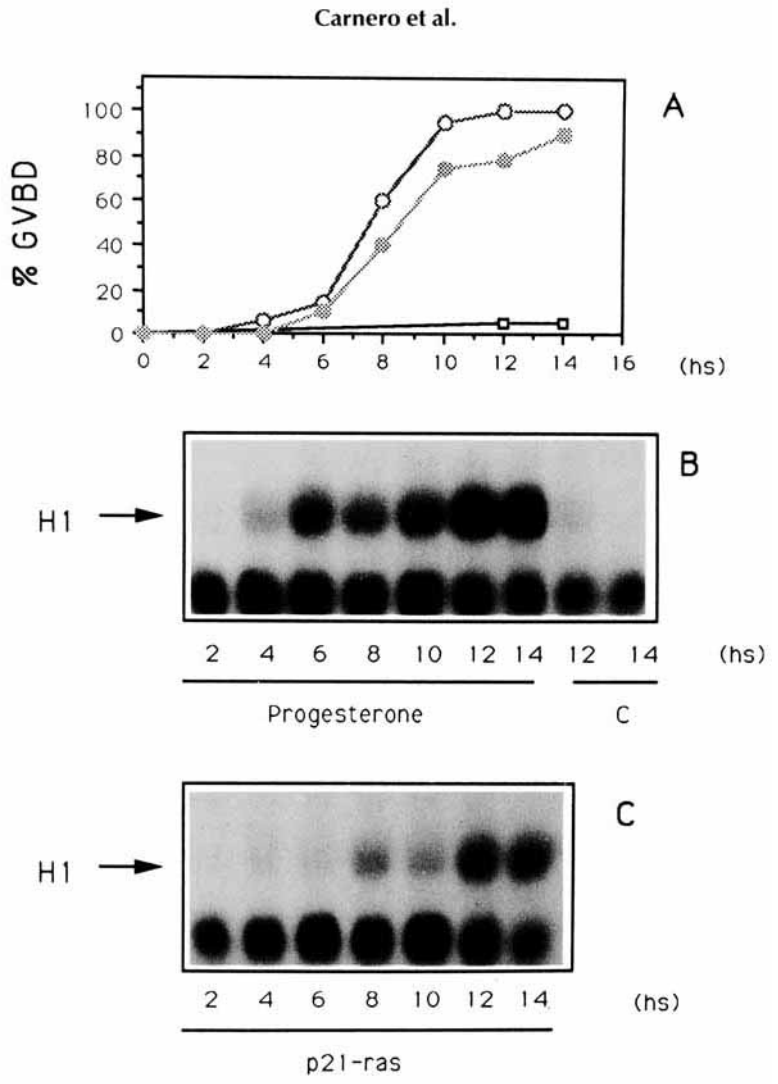


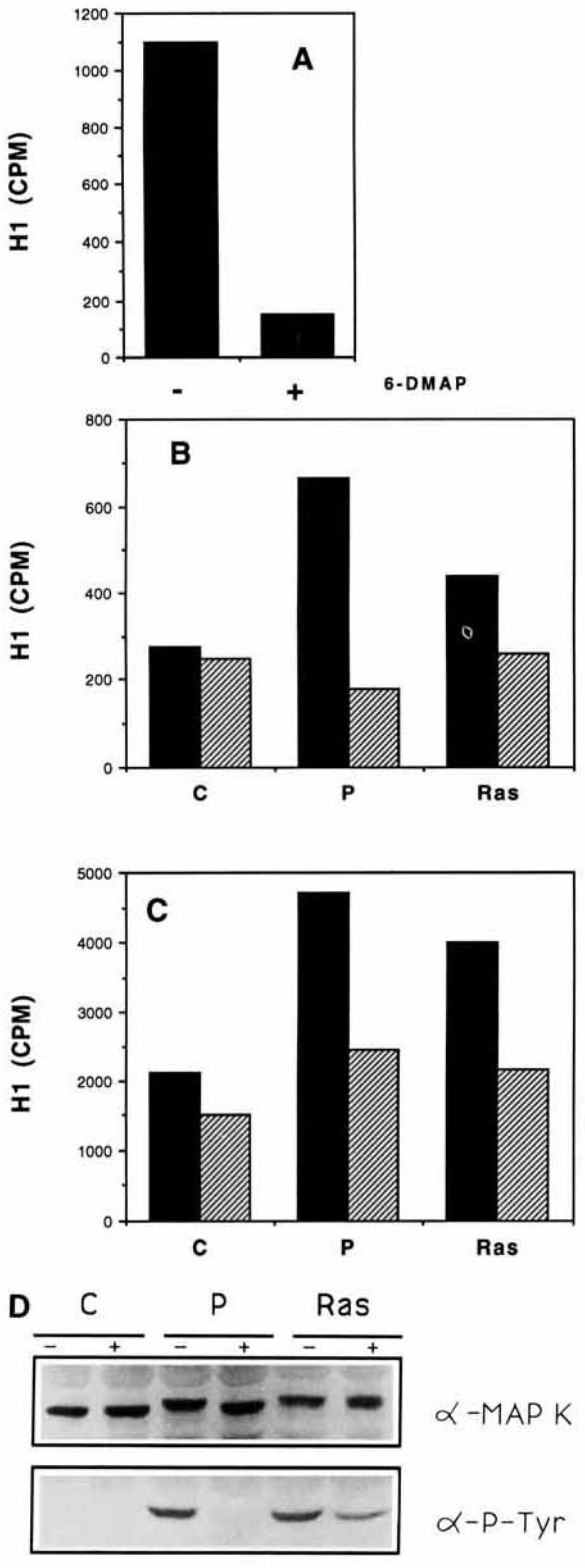
Fig. 3. Correlation of MPF activation and GVBD induction. **A:** GVBD was analyzed as described in Methods at the indicated times after treatment with (○) progesterone (1 μg/ml) or microinjection of *ras*-p21 protein (●) (25 ng/oocyte). **B,C:** Phosphorylation of H1 by MPF, performed as described in Material and Methods, was carried out at indicated times after progesterone treatment (B) or *ras*-p21 protein microinjection (C), using total oocyte extracts. Unstimulated oocytes (C), oocytes treated with 1 μg/ml progesterone or oocytes microinjected with 25 ng of v-H-*ras*-p21.

ies. Under the presence of 6-DMAP, progesterone-treated oocytes showed no $p42^{MAPK}$ phosphorylated in Tyr and showed no shift of mobility (Fig. 4D). However, in *ras*-p21 injected oocytes, $p42^{MAPK}$ was phosphorylated at Tyr and showed a mobility shift even in the presence of the inhibitor. Thus, the *ras*-p21 protein does not require active MPF to bring the mitogenic signal up to the $p42^{MAPK}$ step.

Inhibition of $p42^{MAPK}$ by 2-Aminopurine Blocks Progesterone and *ras* Induced GVBD

The purine analog 2-aminopurine (2-AP) has been described as a rather selective inhibitor of certain growth factors-activated protein kinases [Volonté et al., 1989; Tsao and Greene, 1991]. Moreover, Qiu and Green [1992] have recently shown that 2-AP is a potent inhibitor of the myelin basic protein kinase activity of p44 ERK 1 in PC12 cells, and that inhibition of MAP K activity blocks the neurite outgrowth induced by the oncogenic N-*ras* in the dexamethasone-inducible cell line, UR61 [Qiu and Green, 1992].

We have investigated the effect of 2-AP treatment on the signaling pathways of *Xenopus laevis* oocytes after activation by either progesterone or *ras* proteins. As shown in Figure 5A, incubation with 10 mM 2-AP previous to progesterone treatment or *ras*-p21 microinjection blocks the mobility shift of $p42^{MAPK}$ and its Tyr phosphorylation, an indication of the inhibition



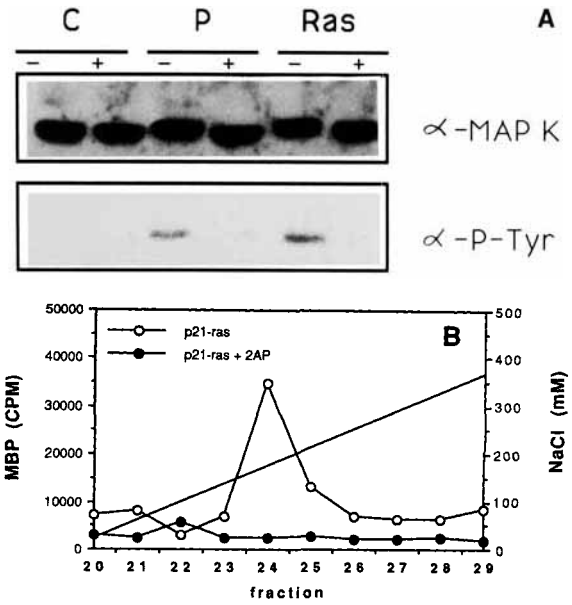


Fig. 5. Effect of 2-aminopurine treatment in the in vivo activation of MPF and $p42^{MAPK}$. **A:** Gel electrophoresis mobility (upper panel) and tyrosine phosphorylation state (lower panel) of $p42^{MAPK}$ after progesterone or *ras*-stimulation, in the presence (+) or absence (−) of 10 mM 2-AP Western blots from total oocyte extracts were performed as described in the legend to Figure 1. C, untreated; P, Progesterone 1 μg/ml; Ras, 25 ng of v-H-*ras*-p21. **B:** Analysis of MAP kinase activity after partial purification of the enzyme by chromatography on a Mono-Q column. Oocytes treated (●) or not (○) with 10 mM 2-AP were microinjected with 25 ng of v-H-*ras*-p21; 20 h after microinjection, oocytes were homogenized and processed as described in Methods, and fractions assayed for MAP kinase activity with MBP as substrate.

of the $p42^{MAPK}$ activity. Moreover, 2-AP treatment blocks the increase in MAP K activity induced by microinjected *ras*-p21 protein analyzed by purification on Mono Q columns (Fig. 5B), confirming the full inhibition of *ras*-induced $p42^{MAPK}$ activity by this drug.

Inhibition of *ras*- and progesterone-induced $p42^{MAPK}$ activation by 2-AP leads to inhibition of GVBD in *Xenopus* oocytes (Fig. 6). However, inhibition of MPF activity by CHX only blocks

Fig. 4. Effect of 6-DMAP treatment on the in vivo activation of MPF and $p42^{MAPK}$. **A:** MPF activity was analyzed as in vitro as described in Methods under the presence (+) or absence (−) of 0.5 mM 6-DMAP after treatment with 1 μg/ml progesterone. **B,C:** MPF activity in total oocyte extracts (B) and p13-agarose precipitates (C). Control, untreated oocytes (■) and oocytes treated with 0.5 mM 6-DMAP (▨). **D:** Gel electrophoresis mobility and tyrosine phosphorylation state of $p42^{MAPK}$ after progesterone or *ras*-stimulation. Western blots from total extracts of oocytes treated (+) or not (−) with 0.5 mM 6-DMAP were performed as described in the legend to Figure 1. C, untreated; P, Progesterone 1 μg/ml; Ras, 25 ng of v-H-*ras*-p21.

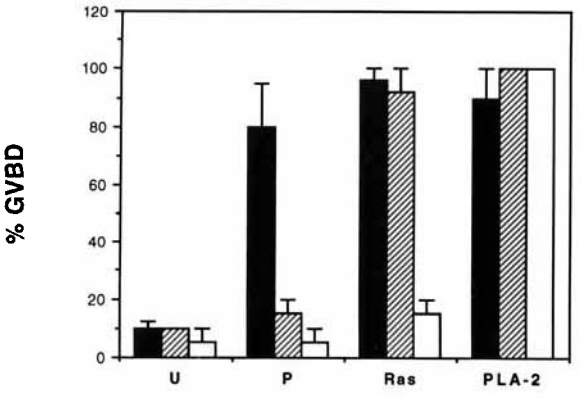


Fig. 6. Lack of effect of 2-aminopurine in the biological activity of phospholipase A2. Oocytes were microinjected with either 25 μU of PLA2 or 25 ng of *ras*-p21 protein, or treated with 1 μg/ml of progesterone. Data represent percentage of oocyte maturation measured as GVBD at 20 h of treatment. Oocytes were either incubated in Ringer's buffer alone (■), or in Ringer's buffer containing 2 μM CHX (▨) or 10 mM 2-AP (□). The results shown are the average from three independent experiments. Bars indicate the standart desviation of the three experiments.

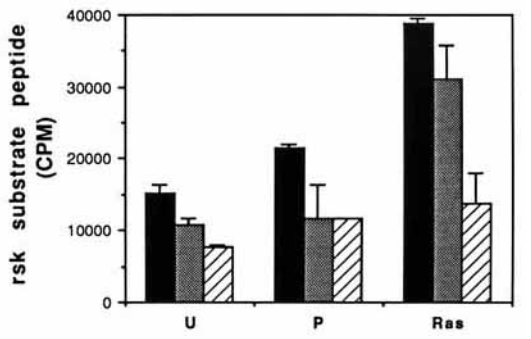


Fig. 7. Effect of inhibition of MPF or p42^{MAPK} on S6 kinase activity. Oocytes were treated with 1 μg/ml of progesterone (P), or microinjected with 25 ng of v-H-*ras*-p21 (Ras) or were left untreated (U) in the presence of CHX (A) or 2-AP (B). Ringer's buffer alone (■), in Ringer's buffer containing 2 μM CHX (▨) or 10 mM 2-AP (▨).

progesterone- but not *ras*-induced GVBD. Since CHX treatment blocks efficiently the cdc2 protein kinase activation, these results indicate that in vivo, *ras*-p21 requires active MAP K but not active cdc2 protein kinase for induction of GVBD of *X. laevis* oocytes. In contrast, progesterone requires protein synthesis, as well as MPF and MAP kinase activation.

We have analyzed the sensitivity to 2-AP treatment of a variety of agents able to induce GVBD to eliminate the possibility of a nonspecific effect. We have previously reported that a number of phospholipases and lipid metabolites can induce oocyte maturation [Carnero and Lacal, 1993]. Among these, we have found that oocyte maturation induced by microinjection of phospholipase A2 is insensitive to 2-AP treatment (Fig. 6), excluding nonspecific or toxic effects of this drug.

Inhibition of MAP K by 2-AP Blocks *ras*- and Progesterone-Induced S6 Kinase II Activation

Phosphorylation of the ribosomal S6 protein is one of the common biochemical events for all known oocyte maturation inducers. In *Xenopus laevis* oocytes, phosphorylation of the S6 is carried out by the S6 Kinase II (S6KII), the *Xenopus* homologue of mammalian rsk [Erikson, 1991]. This enzyme is activated by phosphorylation on Ser and Thr residues by p42^{MAPK}. This process is one of the last known events in the kinase pathway that leads to the reentry into mitosis in oocytes [Maller, 1990].

We have studied whether S6 KII activation in oocytes after stimulation with both progesterone or microinjection with the *ras*-p21 protein follow similar pathways by using the CHX and 2-AP inhibitors. As shown in Figure 7A, when oocytes were treated with CHX and then stimulated with progesterone, no activation of the S6 KII enzyme could be detected, indicating that S6 KII activation requires protein synthesis. In contrast, when the oocytes were microinjected with the *ras*-p21 protein, the S6 KII enzymatic activity could be detected at a similar rate as that of control, CHX-untreated oocytes. These results indicate that S6 KII activation induced by *ras* follows an independent route to that followed by progesterone induction. In addition, *ras*-mediated activation of S6 KII does not require protein synthesis or MPF, since CHX-treated oocytes have been shown previously to lack MPF activity.

When oocytes were treated with the MAP kinase inhibitor 2-AP, and then stimulated with progesterone, a complete inhibition of S6 KII activity was observed (Fig. 7B), indicating that S6 KII activation by progesterone is dependent on MAP kinase activity. Similar results were also observed in *ras*-microinjected oocytes, indicating that *ras*-induced activation of S6 KII is also mediated by the MAP kinase.

DISCUSSION

Protein phosphorylation appears to play a pivotal role in the regulation of cell cycle progression during meiosis and mitosis. During meiotic

maturation of *X. laevis* oocytes there is a burst in net protein phosphorylation prior to germinal vesicle breakdown (GVBD), the hallmark of the mitosis I transition [Tsao, 1985]. During GVBD, activation of MPF and a complex network of phosphorylation at both Tyr and Ser/Thr residues can be detected [Ferrell et al., 1991; Barret et al., 1990; Daar et al., 1991; Pomerance et al., 1992]. Although there has been intensive research in this process during the last few years, a clear picture of the cascade of events involved in the regulation of this mitogenic pathway has not emerged. Both progesterone and oncogenic *ras*-p21 proteins induce GVBD [Birchmeier et al., 1985]. As a model, we have studied the mechanism of phosphorylation induced by the oncogenic *ras*-p21 protein and compared the cascade of alterations to that of progesterone.

In *X. laevis* oocytes, both progesterone and oncogenic *ras*-p21 activate MPF through a pathway dependent on protein synthesis, since CHX treatment prevents MPF activation (Fig. 2). The Mos protein kinase is one of the best characterized proteins involved in this pathway that needs to be synthesized de novo [Sagata et al., 1988]. Previous reports have shown that progesterone but not *ras* needs Mos protein for induction of GVBD and for MPF activation [Daar et al., 1991]. This result suggests that other protein/s different to Mos is/are implicated in *ras*-induced MPF activation.

We have shown in this study that *ras* microinjection can induce MAPK activation and GVBD in the absence of MPF activity. GVBD occurs during mitosis I, and MPF can be activated both in mitosis I and in mitosis II, before and after GVBD takes place. Our results suggest that the H1 kinase activity observed in *ras*-injected oocytes is associated with mitosis II rather than to the induction of GVBD. Abundant evidence indicates that MPF is inactivated during the M phase of mitosis I, through degradation of cyclin B [Nurse, 1990; Jacobs, 1992]. Furthermore, it has been shown that CHX blocks progression to mitosis II even in the presence of the active Mos protein, indicating the involvement of another protein for mitosis II [Yew et al., 1992]. In fact, resynthesis of cyclin B is the only requirement for MPF activity during mitosis II, since the cdc2 protein kinase, the other component of the MPF factor, is present through both mitosis I and mitosis II [Murray and Kirschner, 1989]. Thus, a first round of MPF activation takes place by activation of Mos (around 2 h of progesterone addition), and cyclin B then becomes degraded during the M phase transition (around 10–12 h of progesterone addition), leading to GVBD and completion of the first mitosis. Mitosis II can only proceed after resynthesis of cyclin B, which will activate MPF again and terminate the maturation process.

The differential effect of CHX treatment on the in vivo activation of $p42^{MAPK}$ mediated by progesterone or *ras*-p21 suggests that they use different pathways. Indeed, CHX treatment not only inhibits de novo synthesis of Mos and MPF activation but also inhibits $p42^{MAPK}$ activation by progesterone. In contrast, we provide evidence that activation of $p42^{MAPK}$ induced by *ras*-p21 is independent of protein synthesis and MPF activity. Moreover, biological activity of *ras,* measured by its ability to induce GVBD, is not affected by CHX treatment. In fact, we show that GVBD takes place before activation of the cdc2 protein kinase, in agreement with recent observations by others [Nebreda et al., 1993]. Finally, we also present evidence that MPF is not necessary for *ras*-induced $p42^{MAPK}$ and S6 KII activation in vivo. These results are in full agreement with recent observations that MPF-induced activation of MAPK is achieved by a novel MAPK activator [Matsuda et al., 1992] and that *ras*-p21 can induce the in vitro activation of MAPK in a MPF-independent manner [Shibuya et al., 1992; Itoh et al., 1993].

S6 phosphorylation is a common event to all known inducers of *Xenopus laevis* oocyte maturation, mostly mediated by activation of the S6 KII [Erikson, 1991]. Thus, it is used as a biochemical marker for GVBD. In the present study, we show that inhibition of MPF blocks S6 KII activation under progesterone treatment, but not by microinjection of oncogenic *ras*-p21, confirming the existence of different pathways for *ras* and progesterone maturation.

We have observed that activation of $p42^{MAPK}$ seems to be essential to induce GVBD by both progesterone and *ras*-p21 pathways. This idea is supported by the fact that inhibition of $p42^{MAPK}$ by 2-AP blocked oocyte maturation by both progesterone and *ras*-p21, but had no effect on PLA2-induced GVBD. Furthermore, inhibition of $p42^{MAPK}$ also blocked S6 KII activation in both pathways, most likely due to the fact that S6 kinase is phosphorylated and activated by $p42^{MAPK}$ [Sturgill et al., 1988]. These results are also supported by recent evidence indicating that in specific cell types, inhibition of $p42^{MAPK}$ and

$p44^{MAPK}$ blocks proliferation of somatic cells induced by growth factors [Pagés et al., 1993].

Microinjection of either the Mos protein or active MPF into oocytes induces activation of $p42^{MAPK}$ [Posada et al., 1993; Ferrel et al., 1991; Gotoh et al., 1991a]. Mos-mediated activation of MPF is probably mediated by cyclin B, since this protein is a substrate for the Mos protein kinase [Roy et al., 1990], suggesting the existence of a linear pathway Mos → MPF → MAPK. However, other alternatives may also coexist, since recent evidence indicates that the Raf-1 kinase is necessary for *ras*-induced GVBD and MAPK activation [Fabian et al., 1993], and Mos can also activate MAPK in vitro by phosphorylation of the MAP kinase kinase, Mek [Posada et al., 1993; Nebreda and Hunt, 1993]. These results, along with those demonstrating a physical interaction between *ras*-p21 and Raf-1 in mammalian cells and yeast [Moodie et al., 1993; Zhang et al., 1993; Vojtek et al., 1993; Warne et al., 1993], suggest another independent linear pathway to activate MAPK such as Ras → Raf-1 → MAPK. These results suggest that MAP kinase may be a universal step for cell cycle regulation. However we have shown that MAPK is not the only crosspoint for GVBD in *Xenopus laevis* oocytes since we have observed GVBD in the absence of MAPK activity by microinjection of PLA2. Furthermore, this result implies that 2-AP may be an important tool to unveal MAPK-independent pathways in this system.

The results obtained after in vivo treatment with CHX, 6-DMAP, and 2-AP lead us to consider the model described in Figure 8. Progesterone seems to follow a linear pathway for induction of GVBD, with the sequential activation of Mos, MPF, and $p42^{MAPK}$. Both MPF and $p42^{MAPK}$ activation by progesterone are sensitive to CHX, most likely by inhibition of the synthesis of Mos. In contrast, *ras*-p21 may be involved in an alternative pathway since GVBD preceeds MPF activation. The late activation of MPF induced by *ras*-p21 is sensitive to CHX. It is achieved through a protein that needs de novo protein synthesis, and this is taking place most likely during MPF activation of mitosis II, which requires cyclin B synthesis. Inhibition of this branch by CHX would allow *ras* to induce GVBD by an alternative pathway to that used by progesterone, involving a cascade of different kinases such as Raf-1 and/or Mek, which also leads to $p42^{MAPK}$ activation.

Our results with 6-DMAP treatment indicate that MPF activity is required for progesterone-induced but not for *ras*-induced $p42^{MAPK}$ activation. However, 6-DMAP blocked GVBD induced by either progesterone or *ras* (data not shown), suggesting that this drug may affect either MAP kinase itself or other kinase(s) essential for

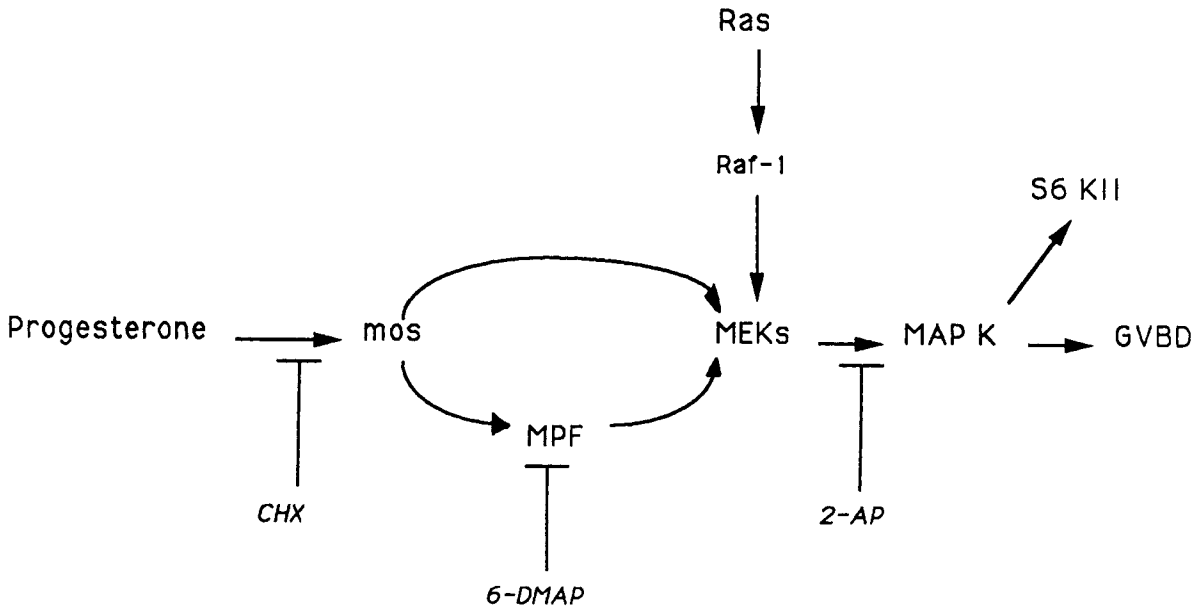


Fig. 8. Suggested model for progesterone and *ras*-dependent signalling pathways during oocyte maturation. See text for details.

GVBD, in agreement with the alternative pathways described for *ras* [Shibuya et al., 1992; Itoh et al., 1993]. We have also observed that, similar to the results with 2-AP, PLA_2-induced GVBD is insensitive to 6-DMAP (data not shown), suggesting that neither of these two drugs is toxic to the oocytes. This could be explained considering the recent observation that $p42^{MAPK}$ phosphorylates and activates PLA_2, suggesting that PLA_2 is placed downstream to $p42^{MAPK}$ [Lin et al., 1993]. Furthermore, we have recently published that generation of lipid metabolites by PLA2 (araquidonic acid, Lyso-PC, and Lyso-PI) are potent biological agents in this system. Thus, further characterization of the signaling pathways used by these lipid metabolites will be essential to define more precisely all the components involved in the regulation of the cell cycle. The results presented in our study indicate that $p42^{MAPK}$ can be considered as an essential convergence point between progesterone and *ras* pathways in the oocyte system. Whether MAPK activation is the only requirement to achieve GVBD or not is still an important issue to be investigated. Our study suggest the existence of at least one alternative pathway.

ACKNOWLEDGMENTS

We thank the expert technical assistance of M.A. Ramos, S. Moreno for the *E. coli* strain BL21.DE3, M. Fernández, and A. Núñez for help in the purification of MAPK. This work was supported by Grant PB89-0020, Dirección General de Investigación Científica y Técnica (DGICYT) from the Spanish Department of Education and Science and Fundación Salud 2000.

REFERENCES

Allende CC, Hinrichs MV, Santos E, Allende JE (1988): Oncogenic ras protein induces meiotic maturation of amphibian oocytes in the presence of protein synthesis inhibitors. FEBS Lett 234:426–430.

Barret CB, Schroetke RM, Van der Hoorn FA, Nordeen SK, Maller JL (1990): Ha-$ras^{Val\text{-}12,Thr\text{-}59}$ activates S6 kinase and $p34^{cdc2}$ kinase in *Xenopus* oocytes: Evidence for $c\text{-}mos^{xe}$-dependent and -independent pathways. Mol Cell Biol 10:310–315.

Birchmeier C, Broek D, Wigler M (1985): Ras proteins can induce meiosis in *Xenopus* oocytes. Cell 43:615–621.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser DD, DePinho RA, Panayotatos N, Coob MH, Yancopoulos GD (1991): ERKs: A family of protein serine/threonine kinases that are activated ant tyrosine phosphorylated in response to insulin and NGF. Cell 65:663–675.

Brizuela L, Draeta G, Beach D (1987): $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. EMBO J 6:3507–3514.

Carnero A, Lacal JC (1993): Phospholipase induced maturation of *Xenopus laevis* oocyte maturation: Mitogenic activity of generated metabolites. J Cell Biochem 52:440–448.

Daar I, Nebreda AR, Yew N, Sass P, Paules R, Santos E, Wigler M, Vande Woude GF (1991): The *ras* oncoprotein an M-phase activity. Science, 253:74–76.

Erickson E (1991): Structure, expression and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. J Biol Chem 266:6007–6010.

Fabian JR, Morrison DK, Daar IO (1993): Requirement for Raf and MAP kinase function during the meiotic maturation of *Xenopus* oocytes: J Cell Biol 122:645–652.

Ferrell JE, Wu M, Gerhart J, Martin GS (1991): Cell cycle tyrosine phosphorylation of $p34^{cdc2}$ and *microtubule associated* protein kinase homolog in xenopus and eggs. Mol Cell Biol 11:1965–1971.

Gotoh Y, Moriyama K, Matsuda S, Okumura E, Kishimoto T, Kawasaki H, Suzuki K, Yahara I, Sakai H, Nishida E (1991a): *Xenopus* M-phase MAP kinase: Isolation of its cDNA and activation by MPF. EMBO J 10:2661–2668.

Gotoh Y, Nishida E, Matsuda S, Shiina N, Kosako H, Shiokawa K, Akiyama T, Ohta K, Sakai H (1991b): In vitro effects on microtubule dynamics of purified *Xenopus* M-phase activated MAP kinase. Nature 349:251–254.

Hoshi M, Nishida E, Sakai H (1988a): Activation of a Ca^{+2} inhibitable protein kinase that phosphorylates microtubule associated protein 2 in vitro by growth factors, phorbol esters and serum in quiescent cultured human fibroblast. J Biol Chem 263:5396–5401.

Hoshi M, Nishida E, Sakai H (1988b): Characterization of a mitogen activated, Ca^{+2} sensitive microtubule associated protein-2 kinase. Eur J Biochem 184:477–486.

Itoh T, Kaibuchi K, Masuda T, Yamamoto T, Matsuura Y, Maeda A, Shimizu K, Takai Y (1993): A protein factor for *ras* p21-dependent activation of mitogen-activated protein (MAP) kinase through MAP kinase kinase. Proc Natl Acad Sci USA 90:975–979.

Jacobs T (1992): Control of the cell cycle. Dev biol 153:1–15.

Jessus C, Rime H, Haccard O, van Lint J, Goris J, Merlevede W, Ozon R (1991): Tyrosine phosphorylation of $p34^{cdc2}$ and p42 during meiotic maturation of *Xenopus* oocyte: Antagonistic action of okadaic acid and 6-DMAP. Development 111:813–820.

Lacal JC (1990): Diacylglycerol production in *Xenopus laevis* oocytes after microinjection of $p21^{ras}$ proteins is a consequence of activation of phosphatidylcholine metabolism. Mol Cell Biol 10:333–340.

Lacal JC, Srivastava SK, Anderson PS, Aaronson SA (1986): Ras p21 proteins with high or low GTPase activity can enficiently transform NIH 3T3 cells. Cell 44:609–617.

Lin L-L, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ (1993): cPLA2 is phosphorylated and activated by MAP kinase. Cell 72:269–278.

Maller JL (1990): *Xenopus* oocytes and the biochemistry of cell division. Biochemistry 29:3157–3166.

Maller JL, Smith DS (1985): Two dimensional polyacrylamide gel analysis of changes in protein phosphorylation during maturation of Xenopus oocytes. Dev Biol 109:150–156.

Matsuda S, Kosako H, Takenaka K, Moriyama K, Sakai H, Akiyama T, Gotoh Y, Nishida E (1992): *Xenopus* MAP kinase activator: Identification and function as a key intermediate in the phosphorylation cascade. EMBO J 11:973–982.

Minshull J, Murray A, Colman A, Hunt T (1991): *Xenopus* oocyte maturation does not require new cyclin sinthesis. J Cell Biol 114:767–772.

Moodie SA, Willumsen BM, Weber MJ, Wolfman A (1993): Complexes of Ras-GTP with Raf-1 and Mitogen-Activated Proteín kinase kinase. Science 260:1658–1661.

Murray AW, Kirschner MW (1989): Cyclin sinthesys drives the early embrionic cell cycle. Nature 339:275–280.

Neant I, Guerrier P (1988): 6-Dimethil-aminopurine blocks starfish oocyte maturation by inhibiting a lelevant protein kinase activity. Exp Cell Res 176:68–79.

Nebreda AR, Porras A, Santos E (1993): p21-induced meiotic maturation of *Xenopus* oocytes in the absence of protein synthesis: MPF activation is preceded by activation of MAP and S6 kinases. Oncogene 8:467–477.

Nebreda AR, 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.

Nurse P (1990): Universal control mechanism regulating onset of M-phase. Nature 344:503–508.

Pagés G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, and Pouysségur J (1993): Mitogen-activated protein kinases $p42^{MAPK}$ and $p44^{MAPK}$ are required for fibroblast proliferation. Proc Natl Acad Sci USA 90:8319–8323.

Pomerance M, Schweighoffer F, Tocque B, Pierre M (1992): Stimulation of mitogen-activated protein kinase by oncogenic *ras*-p21 in *Xenopus* oocytes. J Biol Chem 267:16155–16160.

Posada J, Yew N, Ahn NG, Vande Woude GF, Cooper JA (1993): Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase in vitro. Mol Cell Biol 13:2546–2553.

Qiu M-S, Green SH (1992): PC12 cell neuronal differentiation is associated with prolongued p21*ras* activity and consequent prolonged ERK activity. Neuron 9:705–717.

Ray LB, Sturgill TW (1987): Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc Natl Acad Sci USA 84:1502–1506.

Ray LB, Sturgill TW (1988): Characterization of insulin-stimulated microtubule-associated protein kinase. Rapid isolation and stabilization of a novel serin/thronin kinase from 3T3-L1 cells. J Biol Chem 263:12721–12727.

Rime H, Néant I, Guerrier P, Ozon R (1989): 6-Dimethilaminopurine (6-DMAP) a reversible inhibitor of the transition to metaphase during the first meiotic cell division of the mouse oocyte. Dev Biol 141:169–179.

Rossomando AJ, Payne DM, Weber MJ, Sturgill TW (1989): Evidence that pp42, a mayor tyrosin kinase target protein is a mitogen-activated serin/threonin protein kinase. Proc Natl Acad Sci USA 86:6940–6943.

Roy LM, Singh B, Gautier J, Arlinghaus RB, Nordeen SK, Maller JL (1990): The cyclin B2 component of MPF is a substrate for the $c\text{-}mos^{Xe}$ proto-oncogene product. Cell 61:825–831.

Roy LM, Swenson KI, Walker DH, Gabrielli BG, Li RS, Piwnica-Worms H, Maller JL (1991): Activation of $p34^{cdc2}$ by cyclin A. J Cell Biol 113:507–514.

Sagata N, Oskarsson M, Copeland T, Brumbaugh J, Vande Woude GF (1988): Function of c-*mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. Nature 335:519–525.

Shibuya EK, Polverino AJ, Chang E, Wigler M, Ruderman JV (1992): Oncogenic *Ras* tiggers the activation of 42 kda mitogen-activated protein kinase in extracts of quiescent *Xenopus* oocytes. Proc Natl Acad Sci USA 89:9831–9835.

Sturgill TW, Ray LB, Erickson E, Maller JL (1988): Insulin stimulated MAP2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 334:715–718.

Tsao H, Greene Ll A (1991): Roles of macromolecular synthesis and phosphorylation in the regulation of a protein kinase activity transiently stimulated by Nerve growth factor. J Biol Chem 266:12981–12988.

Vojtek AB, Hollenberg MS, Cooper JA (1993): Mammalian Ras interacts directly with the serine/threonine kinasa Raf. Cell 74:204–214.

Volonté C, Rukenstein A, Loeb DM, Greene LA (1989): Differential inhibition of NGF responses by purine analoges: Correlation with inhibition of a NGF activated protein. J Cell Biol 109:2395–2403.

Warne PH, Rodríguez-Viciana P, Downward J (1993): Direct interaction of Ras and the aminoterminal region of Raf-1 in vitro. Nature 364:352–355.

Yew N, Mellini ML, van de Woud GF (1992): Meiotic initiation by the *mos* protein in *Xenopus*. Nature 355:645–652.

Zhang X-F, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledges J, Marhall MS, Bruder JT, Rapp UR, Avruch J (1993): Normal and oncogenic p21-*ras* proteins bind to the aminoterminal regulatory domain of c-*raf*-1. Nature 364:308–313.