Wortmannin, an inhibitor of phosphatidyl-inositol 3-kinase, induces oocyte maturation through a MPF-MAPK-dependent pathway

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Abstract Wortmannin has been shown to be a non-competitive and irreversible inhibitor of PI3 kinase. For this reason, it has attracted considerable interest and it has been used, as a selective inhibitor of the PI3 kinase, for the study of signal transduction pathways in different systems including *Xenopus* oocytes. We show here that wortmannin itself is able to induce meiotic maturation at doses slightly higher that those required for complete inhibition of PI3 kinase. This effect was shown to be independent of the ability to inhibit PI3K since another unrelated PI3K inhibitor, LY294002, was unable to induce oocyte maturation at inhibitory concentrations for PI3 kinase. The mechanism for wortmannin-induced maturation involves the activation of maturation promoting factor (MPF) and MAP kinase activities in a time course that preceded the appearance of germinal vesicle breakdown. Thus, the pathway activated by wortmannin directly or indirectly affects other protein or proteins, besides PI3 kinase, responsible for its activity. This new target is placed independently or downstream of the PI3 kinase inhibition and upstream of protein synthesis. Moreover, the inhibition of either MPF or cAMP phosphodiesterase blocks wortmannin-induced maturation. We conclude that wortmannin may be a valuable tool for the study of the pathway leading to mitotic maturation of oocytes, but cannot be used as a specific PI3 kinase inhibitor.

Key words: Oocyte maturation; Wortmannin; Maturation promoting factor; Mitogen-associated protein kinase

1. Introduction

The induction of oocyte maturation by agonists is known to involve an initial action of activation of their receptors. After progesterone or insulin treatment activation of maturation promoting factor (MPF) from an inactive state takes place prior to germinal vesicle breakdown (GVBD). This activation is necessary and sufficient to induce GVBD (for review see [1–4]). The injection of active MPF induces precocious GVBD [5] and the microinjection of $p13^{suc}$ protein, which specifically binds to the cdc2 protein (the kinase component of the MPF complex), inhibits its kinase activity blocking the maturation process [6]. In the case of progesterone, there are two peaks of MPF activity, the first one coincides approximately with metaphase I and needs synthesis of c-Mos protein for induction of activity [7]. The second peak appears in metaphase II and needs the resynthesis of cyclin B (the second component of the MPF complex), which is destroyed at the end of the first mitosis [3,8,9]. Thus, MPF is a complex of cyclin B and cdc2, activation of which leads to a burst of protein phosphorylation 30–60 min prior to GVBD. This pleiotropic effect leads to activation of molecules which are essential steps in the signal transduction pathways of oocyte maturation.

The c-Mos proto-oncogene acts as a meiotic initiator protein. Microinjection of Mos protein or mRNA induces MPF activity, GVBD and progression through meiosis I and II [7,10]. On the other hand, antisense ablation of c-*mos* mRNA blocks oocyte maturation and MPF activity induced by progesterone [7]. The protein synthesis requirement for MPF activation can be ascribed mainly to Mos translation, though recently it has been shown that the de novo synthesis of a cdc2 binding protein also plays an important role [11].

Increased synthesis of Mos kinase leads to mitogen-associated protein (MAP) kinase activation via activation of MEK. MEK is a direct target of Mos, and thus Mos acts as a MAPK kinase kinase [12,13]. Activation of the MAP kinase cascade by injection of a constitutively active MEK mutant may be sufficient for induction of MPF and GVBD [14–16]. Furthermore, using the MAPK phosphatase, CL100, or antibodies that neutralize MAPK activity, it has been shown that MAPK activation is essential for progesterone- and Mos-induced maturation and MPF activation [15]. Finally, injection of purified MPF into immature oocytes or the addition to interphase egg extracts activates endogenous MEK and MAPK [5,17]. Based on these and other indications, it has been proposed that Mos, MAPK and MPF all fall in a feedback loop that promotes maturation of *Xenopus* oocytes (for review see [18,19]).

Wortmannin, a fungal metabolite, has attracted considerable interest since it has been reported to directly inhibit PI3 kinase [20–22]. In fact, it has been used as a selective inhibitor for the study of signal transduction pathways in different systems [23–28]. Wortmannin has been shown to be a non-competitive and irreversible inhibitor of PI3 kinase at nanomolar concentrations with no effect on PI4 kinase, protein kinase C or protein tyrosine kinases, although it has been also reported to be an inhibitor of myosin light chain kinase at micromolar concentrations [21]. However, the specificity of wortmannin has recently been subject to controversy [29,30].

In order to test whether wortmannin is a specific inhibitor of PI3 kinase or can have other non-specific biological effects through other proteins we tested the effect of this drug in the *Xenopus laevis* oocyte system. We show that wortmannin itself induces meiotic maturation at slightly higher doses that those required for complete inhibition of PI3 kinase. This activity was associated with MPF and $p42^{MAPK}$ activation. The characterization of the pathway that leads to maturation by wortmannin treatment is presented.

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2. Materials and methods

2.1. Oocyte maturation

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 or the indicated concentrations of wortmannin 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). After 18–20 h of incubation at 18–20°C in Ringer's buffer, 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.

2.2. MPF assays

MPF assays were carried out with total extracts from series of 10 oocytes treated with progesterone or wortmannin. After incubation for 18–20 h oocytes were homogenized in buffer BLO (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 leupeptin, 25 μg/ml aprotinin and 100 μM PMSF). Following centrifugation at $13\,000 \times g$ 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 [^{32}P]γATP (2–5 dpm/fmol), 0.2 μg of PKA inhibitor and 1 mg/ml of type III-S calf thymus histone (Sigma). 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.

2.3. Inmunoblots

Extracts from *Xenopus* oocytes in BLO supplied to 1 mM PMSF were resolved in SDS-10% polyacrylamide gels. Gels were transferred electrophoretically to 0.45 μm nitrocellulose paper (Biotech, SL) and incubated with antibodies specific for MAP kinase. After incubation with the corresponding antibody, the inmunoblots were developed by the ECL system according to the manufacturer (Amersham).

For detection of MAP kinase, an antibody against the peptide KERLKELIFQETAR from the carboxy-terminal region of human MAP-1 kinase was used.

3. Results

3.1. Wortmannin induces maturation of Xenopus laevis oocytes

To study the possible influence of wortmannin on *Xenopus* oocytes we tested different concentrations of the drug from nanomolar to micromolar, and found that wortmannin totally inhibited PI3 kinase in inmunoprecipitates at a concentration of 10–100 nM (data not shown), in agreement with previous publications from other groups [20,28,31]. In fact, Liu and coworkers have recently shown that at 100 nM wortmannin *Xenopus* oocyte PI3 kinase is completely blocked in vivo [28]. We next investigated the effect of the drug on GVBD. Fig. 1A shows that concentrations of wortmannin lower than 50 nM did not induce GVBD. At concentrations of 100 nM, a partial biological activity was observed with a 20–40% induction of GVBD after 20 h of incubation. At concentrations higher than 100 nM, wortmannin treatment induced meiotic maturation in oocytes exposed to the drug. In several experiments 200 nM of wortmannin was able to induce a significant percentage of maturation (70–100%) depending of the batch of oocytes, with a mean value of $85 \pm 15\%$ ($n = 5$), while 1 μM always induces 100% maturation.

In order to study the influence of wortmannin on the time of events leading to GVBD, several concentrations were used (Fig. 1B). All control oocytes as well as those treated with 10 or 50 nM wortmannin showed either no or very little effect on the germinal vesicle after 20 h of incubation. By contrast, 30% of the oocyte population underwent GVBD at 100 nM. In oocytes treated at 200 nM, we found GVBD after 10–12 h of treatment.

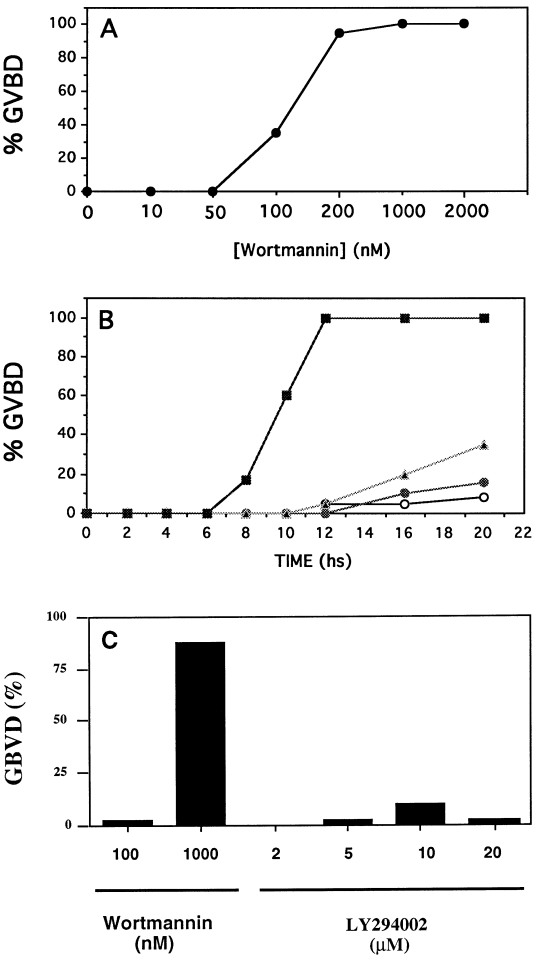

Fig. 1. Induction of GVBD by wortmannin. A: Dose response of wortmannin-induced GVBD. Oocytes were incubated in Ringer's buffer with different concentrations of wortmannin. GVBD was analyzed 18 h after treatment as described in Section 2 by fixing in 10% TCA. B: Time course of wortmannin-induced GVBD. Oocytes were incubated in 10 (○), 50 (●), 100 (△) or 200 (■) nM wortmannin. GVBD was analyzed at different times after fixation in 10% TCA as indicated in A. C: Effect of PI3 kinase inhibition on GVBD. Batches of 40 oocytes were treated with the indicated concentrations of either wortmannin (100 nM or 1000 nM) or LY294002 (2, 5, 10 or 20 μM) and GVBD scored at 20 h as indicated in A.

The above results may be explained if inhibition of PI3 kinase was sufficient to trigger GVBD. Therefore, wortmannin would be inducing GVBD by its ability to inhibit PI3 kinase. We then investigated whether inhibition of PI3 kinase by an alternative, unrelated compound such as LY294002, was also able to induce GVBD at concentrations that drastically inhibit PI3 kinase. As shown in Fig. 1C, LY294002 was unable to induce GVBD at concentrations of up to 20 μM, previously shown to completely inhibit PI3 kinase in *Xenopus laevis* oocytes [32]. Thus the effects observed after treatment of the oocytes with wortmannin were not a consequence of inhibition of PI3 kinase.

3.2. MPF activation can be detected after wortmannin treatment

To assess the functional importance of wortmannin-induced GVBD we analyzed the activation of the MPF complex, which can serve as a biochemical marker of maturation. We

determined first the activation of MPF after 20 h of exposure to different concentrations of wortmannin. As shown in Fig. 2A, treatment with 10 or 50 nM does not induce MPF activation, correlating with the lack of GVBD induction. At 100 nM, wortmannin induced partial activation of MPF (Fig. 2) and partial induction of GVBD, with about 20–40% of mature oocytes (Fig. 1). Incubation with over 100 nM wortmannin induced MPF activation at a comparable level to that observed after progesterone treatment (Fig. 2).

We next characterized the sequence of events leading to GVBD induced by wortmannin. We analyzed the time course of activation of H1 kinase activity. Oocytes were exposed to wortmannin and at indicated times, samples were frozen at −70°C. Oocytes were processed and H1 kinase activity assayed as described in Section 2. As shown in Fig. 2B, 100 nM of wortmannin induced a partial MPF activity (170–190%) only at late times, in agreement with the partial GVBD (30%) observed after 16–20 h of exposure to the drug. In 200 nM wortmannin-treated oocytes, MPF was induced with a kinetics slightly preceding GVBD. Partial MPF activity was observed after 8 h of exposure (160%) when only 15% GVBD was observed. At 10 h of treatment, 60% GVBD

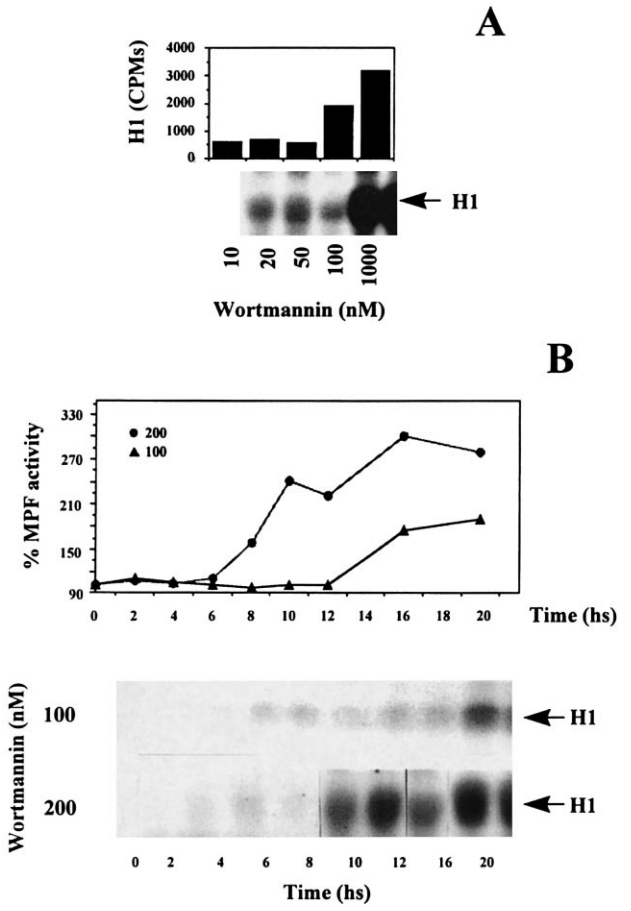

Fig. 2. MPF activation by wortmannin treatment. A: MPF activation at different concentrations of wortmannin. Oocytes were treated with indicated concentrations of wortmannin and after 18 h processed for MPF activity as described in Section 2. B: Time course of MPF activation induced by wortmannin. Oocytes were treated with 100 nM (▲) or 200 nM (●) wortmannin. At indicated times, oocytes were placed on ice and processed for the MPF assay as described in Section 2. The phosphorylated band corresponding to the H1 protein was cut and counted.

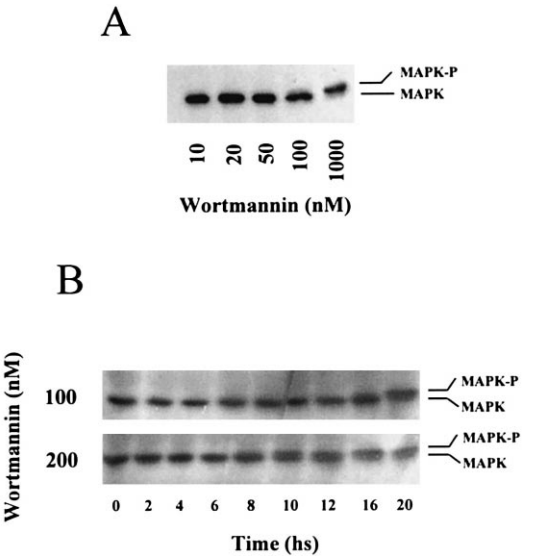

Fig. 3. MAP kinase activation by wortmannin treatment. Activation of the $p42^{MAPK}$ was measured by Western blot as its mobility shift after electrophoresis in polyacrylamide gels. Total extracts of unstimulated or wortmannin-stimulated oocytes were run in a 10% PAGE, transferred to nitrocellulose and incubated in the presence of an α-MAP kinase (polyclonal) antibody, followed by a biotinylated anti-rabbit antibody and the streptavidin-peroxidase-conjugated protein. Blots were developed with the ECL system as recommended by the manufacturer (Amersham). A: MPF activation at different concentrations of wortmannin. Oocytes were treated with different concentrations of wortmannin and after 18 h were processed as described in Section 2. B: Time course of MPF activation induced by wortmannin. Oocytes were treated with 100 or 200 nM wortmannin. At indicated times, oocytes were placed on ice and processed for the mobility shift assay as described in Section 2.

was observed along with 240% MPF activity, near to maximal MPF activity observed after 16 h of exposure. Thus, MPF activation preceded and correlated with the observed GVBD.

3.3. Activation of MAP kinase follows wortmannin treatment

In *Xenopus* oocytes p42MAPK can be activated as a consequence of MPF activation (see Section 1). Moreover, activation of the MAP kinase pathway seems to be necessary for progesterone-induced maturation [14,33]. Thus, we analyzed the phosphorylated state of MAP kinase. As previously demonstrated, phosphorylation of this protein leads to its activation and this process can be followed by its mobility shift on bisacrylamide-polyacrylamide gel electrophoresis [34]. As shown in Fig. 3A, treatment with doses lower than 100 nM wortmannin, which did not induce GVBD, did not alter MAPK mobility. However, a dose of 1 μM wortmannin, which induced oocyte entry into GVBD, also induced a shift in the mobility of MAPK, indicating that most of the protein is phosphorylated and activated. Finally, in keeping with this observation, treatment with 100 nM wortmannin was able to induce a partial MPF activity and a partial activation of MAP kinase, most likely a consequence of the small percentage of oocytes that underwent GVBD (Fig. 3A).

We also analyzed the time course for MAP kinase activation after wortmannin treatment. Oocytes were exposed to wortmannin and every 2 h kept frozen at −70°C. Oocytes were processed and p42MAPK activation estimated by its mobility shift on PAGE. As shown in Fig. 3B, 100 nM of wortmannin induced a partial MAPK activation only at late

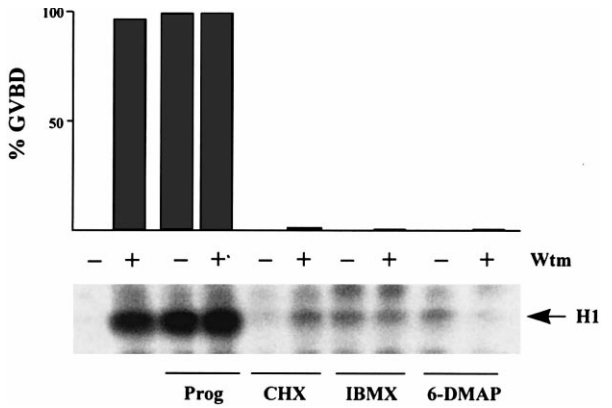

Fig. 4. Effect of CHX, IBMX and 6-DMAP on wortmannin-induced GVBD and MPF activity. Oocytes were prepared as described in Section 2. Batches of 20–30 oocytes were treated with 200 nM of wortmannin (+) or buffer alone (−) in the presence or absence of 10 μM progesterone, 20 μM CHX, 10 mM IBMX or 0.5 mM 6-DMAP. Induction of GVBD was analyzed 18 h after treatment by fixing in 10% TCA. Data represent percentage of GVBD. MPF activity was analyzed as described in Section 2.

times, in agreement with the observed partial GVBD induction and the partial MPF activation after 16–20 h of exposure to the drug. In 200 nM wortmannin-treated oocytes, MAP kinase was activated with a kinetics slightly preceding the appearance of GVBD. A partial MAP kinase activation was observed after 8 h of exposure to the drug, and activation of MAP kinase correlated with the MPF activity and GVBD observed. Thus, MAP kinase activation preceded GVBD, and parallelled with MPF activity.

3.4. Inhibition of wortmannin-induced meiotic maturation by 6-DMAP, IBMX and cycloheximide (CHX)

6-Dimethyl-aminopurine (6-DMAP) is a serine/threonine kinase inhibitor that induces MPF inactivation when applied to *Xenopus* oocytes [33,35]. We tested whether the activation of MPF is necessary for wortmannin-induced GVBD. Oocytes were treated with different concentrations of wortmannin and incubated in the presence of 0.5 mM 6-DMAP. After 20 h incubation, none of the oocytes showed GVBD (Fig. 4). Thus, 6-DMAP blocks the meiotic maturation induced by wortmannin, as previously reported for progesterone treatment [33,35].

In *Xenopus* oocytes, initiation of maturation has been reported to rely on the reduction of cAMP-dependent protein kinase activity [36] and the synthesis of the c-Mos protein [10]. We tested whether maturation induced by wortmannin is able to bypass these requirements. Oocytes were treated with 1 mM IBMX, which inhibits the cAMP phosphodiesterase activity induced by maturation inducers [36,37], or 20 μM CHX, which inhibits protein synthesis more than 90% [38]. Oocytes were incubated in the presence of the inhibitors and then treated with different concentrations of wortmannin. High concentrations of wortmannin did not induce GVBD in the presence of either inhibitor (Fig. 4).

To assess further whether GVBD induced by wortmannin depends upon the pathway blocked by these inhibitors we analyzed MPF activation, as a biochemical marker, in the presence of the inhibitors. As shown in Fig. 4, CHX, IBMX and 6-DMAP inhibited MPF activation induced by wortmannin, indicating that activation of the maturation pathway by wortmannin precedes protein synthesis. Furthermore, these results also provide evidence that both cAMP phosphodiesterase and MPF activation are required.

4. Discussion

Wortmannin has been reported to be a potent inhibitor of PI3 kinase activity with ID_{50} values in the range of 10–100 nM. In *Xenopus laevis* oocytes wortmannin inhibits the PI3 kinase activity in vitro at concentrations as low as 10 nM under experimental conditions of 0.1–1 nM concentrations of ATP (results not shown) and at 100 nM *Xenopus* PI3 kinase is fully inhibited in vivo [28]. We report here that wortmannin itself is able to induce meiotic maturation in oocytes, an effect that is not mediated by its ability to inhibit PI3 kinase, since another inhibitor of this enzyme, LY294002, was not able to induce a similar effect. The concentrations needed for oocyte maturation were similar to those previously reported for inhibition of serine/threonine kinases by wortmannin such as myosin light chain kinase ($IC_{50} = 200$ nM). Moreover, it has been reported that other known inhibitors of signalling molecules such as okadaic acid or propanol can also induce oocyte maturation [39], making more complex the proper evaluation of these results. Thus, our study suggests that wortmannin has other cellular targets besides PI3 kinase that may be important for signal transduction in eukaryotes.

We have also characterized the pathway induced by wortmannin that leads to GVBD. Wortmannin induced MPF activation with a time course that precedes the appearance of GVBD, indicating that activation of the complex cdc2-cyclin B can be a requirement for wortmannin-induced GVBD. Moreover, wortmannin induced MAP kinase activation. These two processes showed a parallel kinetics after oocyte stimulation by wortmannin, suggesting that both events may be functionally linked.

We have further analyzed the pathway that leads to MPF and MAPK activation by using different inhibitors which have been previously characterized for their effects on the progesterone-induced pathway. 6-DMAP is a purine analogue that inhibits oocyte maturation in several species, including *Xenopus* oocytes [35,40,41], acting directly at the MPF activation level at the concentrations used in this study. Treatment with 6-DMAP was able to inhibit in vitro and in vivo the MPF activity [33], and completely inhibited maturation of *Xenopus* oocytes induced by microinjection of active MPF [35]. We found that 6-DMAP blocks both MPF activation as well as GVBD induced by wortmannin.

Induction of *Xenopus* oocyte maturation by hormones depends on protein synthesis [2]. Microinjection of active MPF can bypass this requirement, suggesting that protein synthesis is needed for activation of MPF. We analyzed the requirement of protein synthesis for the GVBD induced by wortmannin. The effect was blocked in the presence of CHX, indicating that wortmannin requires protein synthesis, and it activates the GVBD pathway at a level upstream of the protein synthesis step.

Inhibition of PKA is a requirement for both hormone and oncogenic *ras*-p21 induced GVBD [36]. It has been found recently that PKA acts at multiple points to inhibit *Xenopus* oocyte maturation by preventing both Mos translation and MPF activation [42]. It has been shown that progesterone, insulin and oncogenic *ras* induce the activation of cAMP phosphodiesterase [36,37] reducing the endogenous pool of

cAMP, the activator of PKA. IBMX is an inhibitor of cAMP phosphodiesterase which maintains high levels of cAMP, blocking the meiotic maturation of hormones and *ras* [36]. IBMX also inhibited MAPK phosphorylation and GVBD induced by wortmannin, supporting the results shown before. Moreover, as expected from these results, wortmannin was unable to activate MPF in extracts of unstimulated oocytes (data not shown). Thus, activation of the MPF-MAPK pathway by wortmannin most likely will induce a number of signals ending in the induction of GVBD.

Activation of the MPF-MAPK pathway is not a direct consequence of the inhibition of the PI3K activity. Concentrations of wortmannin where PI3K is fully inhibited induce entry of just a small percentage of oocytes into GVBD. Moreover, it has been reported that the microinjection of the SH2 region of the p85 regulatory subunit that also blocks PI3K activity in *Xenopus* oocytes did not induce maturation by itself [43,44]. A more likely explanation is that wortmannin is acting through another target molecule, sustained inhibition/activation of which induces the MPF/MAPK pathway. This new wortmannin target will be placed before protein synthesis.

In summary, wortmannin induces meiotic maturation of *Xenopus* oocytes through a mechanism that is independent of PI3 kinase inhibition but through the activation of a pathway involving MPF and MAPK. Thus, this pathway directly or indirectly affects other proteins, besides PI3 kinase, involved in cell signalling. Wortmannin-dependent activation of this pathway is placed upstream of protein synthesis. Inhibition of protein synthesis, MPF activation or a decrease of cAMP levels are all able to block wortmannin-induced maturation. Thus, wortmannin may be a valuable tool for the study of the pathway leading to mitotic maturation of oocytes, but it cannot be used as a specific PI3 kinase inhibitor. The identification of the novel targets for wortmannin action may be of great interest for a better understanding of signal transduction pathways in eukaryotic cells.

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