Fully grown G2-arrested Xenopus oocytes resume meiosis in vitro upon exposure to hormonal stimulation. Progesterone triggers oocyte meiosis resumption through a Ras-independent pathway that involves a p39Mos-dependent activation of the mitogen-activated protein (MAP) kinases. Insulin also triggers meiosis resumption through a tyrosine kinase receptor that activates a Ras-dependent pathway leading to the MAP kinases activation. Antisense phosphorothioate oligonucleotides were used to prevent p39Mos accumulation and Erk-like Xp42Mpk1 activation during insulin-induced Xenopus oocytes maturation. In contrast to previous works, prevention of p39Mos-induced activation of Xp42Mpk1 in insulin-treated oocytes did not inhibit but delayed meiotic resumption, like in progesterone-stimulated oocytes. Activations of Xp42Mpk1, the unique Erk of the oocyte, and of its downstream target p90Rsk, were impaired and phosphorylation of the MAPKK kinase Raf was partially inhibited. Similarly, oocytes treated with the MEK inhibitor U0126, stimulated by insulin exhibited delayed germinal vesicle breakdown, absence of Xp42Mpk1 activation, and partial phosphorylation of Raf. To summarize, whereas p39Mos-induced activation of MEK/MAPK pathway is dispensable for insulin-induced germinal vesicle breakdown, Xp42Mpk1 activation induced by insulin is dependent upon p39Mos synthesis. Raf complete phosphorylation appears to require the MEK/MAPK pathway activation both in progesterone and insulin-stimulated oocytes.

Immature Xenopus oocytes are physiologically arrested at the G2 stage of the first meiotic division. Meiosis resumes after stimulation by the steroid hormone progesterone and is marked by dissolution or breakdown of the germinal vesicle (GVBD), resulting in the formation of a white spot at the animal pole. At the biochemical level, GVBD is initiated through the activation of maturation-M phase promoting factor (MPF), a protein complex made up of p34Cdc2 kinase and cyclin B (1). Progesterone induces mRNA polyadenylation (2, 3) and synthesis of the Ser/Thr kinase p39Mos. Consequently, the mitogen-activated protein kinase (MAPK) pathway, including the MAPK/Erk kinases MEK 1 and 2 and the Erk Xp42Mpk1, is activated. Xp42Mpk1 phosphorylates and activates ribosomal S6 kinase (p90Rsk) that in turn inactivates Myt1, a negative regulator of MPF (4).

p39Mos and its downstream targets induce meiotic resumption in the absence of progesterone when microinjected into prophase-arrested Xenopus oocytes (5–8). There are conflicting reports regarding the necessity of p39Mos in progesterone-induced GVBD. Whereas injection of phosphodiester antisense oligonucleotides against p39Mos mRNA has been shown to not only inhibit p39Mos accumulation but also progesterone-induced GVBD (9, 10), inhibition of p39Mos synthesis by morpholino antisense oligonucleotides has been shown recently to impair neither resumption of meiosis nor activation of MPF into progesterone-stimulated Xenopus oocytes (11). Moreover, other reports show that MEK 1/2 activity is not required for MPF activation induced by progesterone (12, 13).

Insulin and insulin growth factor can also trigger meiotic resumption (14, 15), through a tyrosine kinase receptor (16), inducing the activation of the MEK/MAPK pathway via the GTP-binding protein Ras (17, 18). Xp42Mpk1 activation induced by Ras can be impaired by dominant-negative forms of Raf in Xenopus oocytes and in oocytes extracts (19, 20), leading to the conclusion that activation of MEK in these cases occurs through Raf activity, like in somatic cells (21). However, p39Mos synthesis and accumulation has also been reported after fibroblast growth factor 1 stimulation in Xenopus oocytes expressing fibroblast growth factor receptors (22) and in oocytes treated with insulin (23).

Raf activation is also known to stimulate Raf-independent pathways (for a review in somatic cells, see Ref. 24). In Xenopus oocytes, the phosphoinositide 3-kinase pathway (25–27), which leads to activation of protein kinase B/Akt, has been well described because recent data demonstrate a crucial role for protein kinase B/Akt in the insulin- but not progesterone-stimulated resumption of meiosis (28, 29).

Injections in Xenopus oocytes of V12 H-Ras (30) and Raf (31) can trigger meiotic resumption without progesterone stimulation, independently of p39Mos. Moreover, dominant-negative forms of Raf can prevent Mos injection-induced GVBD (31). Nevertheless, other results showed that dominant-negative forms of Raf do not block Mos-induced MAPK activation in oocyte extracts (32). In fact, Raf activation subsequently to Mos injection would be under the control of the MAPK pathway.
itself (32). Despite all of these results, the hypothesis that p39Mos might be dispensable to GVBD and MAP kinase activation induced by insulin has not yet been tested in Xenopus oocytes. Therefore, we assessed the role of p39Mos and activation of the Erk-Rsk pathway in maturation induced by insulin stimulation of immature Xenopus oocytes using phosphorothioate antisense oligodeoxynucleotides (PS-AS) to prevent p39Mos synthesis and accumulation. Here, we show that activation of Xp42Mpk1 induced by p39Mos is not essential for MPF activation and GVBD induced by insulin. These observations also highlight that the active MEK1/2-Xp42Mpk1 pathway is necessary for full activation of Raf, independently of p39Mos accumulation.

MATERIALS AND METHODS

Handling of Oocytes—Adult Xenopus females were purchased from the University of Rennes I, France. After anesthesia with 1 g/liter MS222 (tricaine methanesulfonate, Sandoz), ovarian lobes were surgically removed and placed in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5 (NaOH)). Full-grown stage VI oocytes (33) were isolated and follicles were removed by collagenase treatment for 30 min (1 mg/ml collagenase A, Roche Applied Science), followed by manual microdissection. Oocytes were stored at 14 °C in ND96 medium until experiments. Experimental Conditions—Phosphorothioate deoxyoligonucleotides were purchased from Eurogentec. The sequence of the antisense against p39Mos mRNA (PS-AS) was AAGGCATTGCTGTGTGACTCGCTGAAC. As a control we used the inverted sequence GTTTCAGCGAGTC-
ACACAGCAATGCCTT designed as sense oligonucleotides (PS-S) or 20 nl of RNase-free water. 10 ng (20 nl) were microinjected into each oocyte by the use of a positive displacement digital micropipette (Nichiryo). Oocytes were then incubated in OR2 medium (82 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.5) overnight at 20°C before insulin (1/1000M) or progesterone (4/1000 g/ml; Sigma) treatment. Purified murine Mos protein (mu-Mos) was kindly provided by Dr. Vande Woude and Dr. Ahn. 37.5 ng (50 nl) were injected into PS-AS-injected oocytes just before hormonal treatment. Control oocytes were injected with 50 nl of water.

U0126 (Promega) was made soluble in Me₂SO to obtain a stock solution at 50 mM and was used at a final concentration of 50 μM. Treatment began 1 h before insulin or progesterone addition. Control oocytes were treated with Me₂SO, 1/1000.

Electrophoresis and Western Blotting—Oocytes were taken off and homogenized in homogenization buffer (34) and then centrifuged for 5 min at 10,000 x g (4 °C) to eliminate yolk platelets. Proteins were then

FIG. 2. p39Mos inhibition by antisense phosphorothioate oligonucleotides during insulin-induced maturation. A, time course occurrence of GVBD. Control oocytes were microinjected with water (H₂O/Ins; open circles) or sense phosphorothioate oligodeoxynucleotides (PS-S/Ins; black circles). A batch of oocytes were microinjected with antisense phosphorothioate oligodeoxynucleotides (PS-AS/Ins; black squares). After overnight incubation, oocytes were stimulated with insulin. Appearance of the white spot was monitored every 30 min. B, Western blot analysis. At the end of the maturation, 3 oocytes were taken off, homogenized, and immunoblotted with antibodies against p39Mos, Xp42Mpk1, p90Rsk, Raf, and cyclin B2. Arrows show phosphorylation states. In immature oocytes (prophase), p39Mos was not detected, p42Mpk1, p90Rsk, and Raf were found under their non-phosphorylated isoforms (down arrows) and cyclin B2 was found as a doublet of two isoforms. In control mature oocytes injected with water (H₂O/Ins), p39Mos was synthesized and Xp42Mpk1, p90Rsk, Raf, and cyclin B2 were found under their completely phosphorylated isoforms (up arrows). PS-S injection (PS-S/Ins) had no effect compared with water-injected oocytes (H₂O/Ins). PS-AS injection abolished p39Mos synthesis, resulting in total inhibition of Xp42Mpk1 and p90Rsk phosphorylations and partial inhibition of Raf phosphorylation. It did not prevent cyclin B2 phosphorylation but we observed lower amounts of cyclin B2. Ability of a purified mu-Mos to overcome the effects of inhibition of p39Mos synthesis was tested by injecting mu-Mos in PS-AS-treated oocytes just before hormonal stimulation (PS-AS/μMos/Ins). Injection of mu-Mos restored normal phosphorylation of Raf, Xp42Mpk1, and p90Rsk and normal amounts of cyclin B2 compared with control mature oocytes.
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FIG. 3. Insulin never activates the MAP kinase pathway in the absence of p39Mos synthesis. Oocytes were microinjected with PS-S or PS-AS, incubated overnight in OR2 medium and then, stimulated by insulin. Each hour, 3 oocytes were taken off, homogenized, and immunoblotted with antibodies against p39Mos, Xp42Mpk1, and p90Rsk as described under “Materials and Methods.” Time at which GVBD50 had occurred is illustrated. A, 4 h after hormonal stimulation, phosphorylated forms of both Xp42Mpk1 and p90Rsk appeared. Only phosphorylated forms are observed around GVBD50 time. B, 4 h after GVBD50, no phosphorylated forms of both Xp42Mpk1 and p90Rsk could be detected. The last well (H2O/Ins) shows control oocytes injected with water, incubated overnight, and then stimulated by insulin. Oocytes were taken off 13 h after stimulation.

RESULTS

p39Mos Inhibition by Antisense Phosphorothioate Oligonucleotides during Progesterone-induced Maturation—We have first investigated the effects of PS-AS oligodeoxynucleotides against p39Mos mRNA on progesterone-induced GVBD. Compared with classical phosphodiester oligodeoxynucleotides (PO), phosphorothioate oligodeoxynucleotides (PS) have a sulfur atom instead of an oxygen in the phosphate group, resulting in better nuclease resistance. This extends in vivo longevity of PS and allows better specificity. 10 ng of PS-AS were injected in immature oocytes under its non-phosphorylated form. In mature oocytes injected with water or with PS-S, Raf was detected under a shifted up phosphorylated form. But in PS-AS-injected oocytes, progesterone stimulation resulted in detection of Raf as an intermediary shift between non-phosphorylated and completely phosphorylated forms (Fig. 1B). To verify on Western blot the activation of the MAP kinase pathway by detecting p39Mos synthesis, the phosphorylation of Xp42Mpk1, and the hyperphosphorylation of p90Rsk. Phosphorylation of Xp42Mpk1 is a marker for its activation, which is confirmed by the hyperphosphorylation of p90Rsk. In PS-AS-injected oocytes, p39Mos was minimally detectable and Xp42Mpk1 and p90Rsk remained unactivated as demonstrated by the absence of an electrophoretic shift, whereas water or sense injection had no or little effect (Fig. 1B). In parallel, shifting up of Raf was also partially inhibited following PS-AS treatment (Fig. 1B). Indeed, on Western blot, Raf was detected in immature oocytes under its non-phosphorylated form. In mature oocytes injected with water or with PS-S, Raf was detected under a shifted up phosphorylated form. But in PS-AS-treated oocytes, progesterone stimulation resulted in detection of Raf as an intermediary shift between non-phosphorylated and completely phosphorylated forms (Fig. 1B). To control for eventual nonspecific effects of PS-AS, purified mu-Mos protein was injected before progesterone stimulation into PS-AS-injected oocytes. We observed that mu-Mos was able to revert all the effects of PS-AS on progesterone treatment (Fig. 1B). Although PS-AS injection appeared to strongly prevent p39Mos accumulation, a small amount of Mos may still accumulate in PS-AS-injected oocytes either stimulated by insulin or progesterone (Figs. 1 and 2). However, it failed to activate Xp42Mpk1 and p90Rsk because we were unable to detect activated isoforms of Xp42Mpk1 and p90Rsk.

Our results show that phosphorylation of Xp42Mpk1 and of its downstream target p90Rsk induced by progesterone are dependent upon p39Mos synthesis. Moreover, progesterone-induced GVBD occurs even when activation of these pathways are almost completely suppressed.
Inhibition by Antisense Phosphorothioate Oligonucleotides during Insulin-induced Maturation—To test the effects of the inhibition of p39\textsuperscript{Mos} accumulation on GVBD and Raf phosphorylation induced by insulin, oocytes were injected with 10 ng of PS-AS and incubated overnight before stimulation by insulin. Minimal accumulation of p39\textsuperscript{Mos} but no activation of Xp42\textsuperscript{Mpk1} or p90\textsuperscript{Rsk}, even transitory, was observed (Figs. 2B and 3). Injection of PS-S had little or no effect on the rate or extent of GVBD in comparison to control oocytes injected with deionized water. In contrast, injection of PS-AS significantly delayed GVBD (Fig. 2A). GVBD\textsubscript{50} of PS-S-injected oocytes was 1.12 ± 0.02-fold of GVBD\textsubscript{50} of control oocytes, whereas GVBD\textsubscript{50} of PS-AS-injected oocytes happened almost two times later than in control water-injected oocytes (1.92 ± 0.09 GVBD\textsubscript{50}).

Inhibition of p39\textsuperscript{Mos} synthesis by PS-AS did not prevent phosphorylation of Xp42\textsuperscript{Mpk1} and p90\textsuperscript{Rsk} phosphorylations and partial inhibition of Raf phosphorylation. It did not prevent cyclin B2 phosphorylation but we observed lower amounts of cyclin B2. At last, U0126 did not prevent synthesis of p39\textsuperscript{Mos}.

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**Fig. 4. Effects of MEK inhibitor U0126 on insulin-induced maturation.** Control oocytes were incubated in OR2 medium containing 1/1000 Me\textsubscript{SO} (DMSO/Ins; open circles). U0126-inhibited oocytes were incubated in OR2 medium containing 50 μM U0126 (U0126/Ins; black circles). 1 h after beginning the incubation, insulin was added to the medium. A, time course occurrence of GVBD. Appearance of the white spot was monitored every hour. B, Western blot analysis. At the end of maturation, 3 oocytes were taken off, homogenized, and immunoblotted with antibodies against p39\textsuperscript{Mos}, Xp42\textsuperscript{Mpk1}, p90\textsuperscript{Rsk}, Raf, and cyclin B2. Arrows show phosphorylation states. In immature oocytes (prophase), p39\textsuperscript{Mos} was not detected, Xp42\textsuperscript{Mpk1}, p90\textsuperscript{Rsk}, and Raf were found under their non-phosphorylated isoforms (down arrows) and cyclin B2 was found as a doublet of two isoforms. In control mature oocytes incubated in Me\textsubscript{SO} (DMSO/Ins), p39\textsuperscript{Mos} was synthesized and Xp42\textsuperscript{Mpk1}, p90\textsuperscript{Rsk}, Raf, and cyclin B2 were found under their completely phosphorylated isoforms (up arrows). U0126 treatment resulted in total inhibition of Xp42\textsuperscript{Mpk1} and p90\textsuperscript{Rsk} phosphorylations and partial inhibition of Raf phosphorylation. It did not prevent cyclin B2 phosphorylation but we observed lower amounts of cyclin B2. At last, U0126 did not prevent synthesis of p39\textsuperscript{Mos}.
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DISCUSSION

Two contradictory approaches have been used before ours to prevent p39Mos synthesis and accumulation during progesterone-induced maturation of Xenopus oocytes: first, PO-AS against p39Mos mRNA were used, which completely inhibited GVBD (9, 10). Second, morpholino strategy revealed that p39Mos-Xp42Mpk1 pathway was not required for progesterone-induced GVBD and that PO-AS inhibition of maturation resulted from a nonspecific effect (11). In our hands, injection of PS-AS against p39Mos mRNA, followed by progesterone stimulation, greatly diminished p39Mos accumulation and Xp42Mpk1 activation as shown by the absence of phosphorylation of both Xp42Mpk1 and p90Rsk. However, PS-AS were unable to block GVBD, according to results obtained with morpholinos (Ref. 11 and data not shown) and suggesting that, like morpholino oligonucleotides, PS-AS do not have nonspecific effects. We cannot exclude the possibility that the minimal amount of p39Mos may influence GVBD as has been shown to occur previously (38).

Results obtained with PO-AS strategy that were used to prevent p39Mos accumulation in insulin-stimulated oocytes have to be reconsidered. Injection of such PO-AS resulted in complete inhibition of GVBD (9, 10). We assessed the effects of the more specific PS-AS on insulin-induced maturation. In contrast to PO-AS (9, 10), PS-AS injection did not block insulin-induced GVBD. However, insulin failed to activate Xp42Mpk1 independently of p39Mos. This observation was surprising because p39Mos and Raf have been shown in cell-free extracts to independently act to stimulate the Xp42Mpk1 pathway (32). Moreover, dominant-negative forms of Raf inhibit Xp42Mpk1 activation induced by oncogenic Ras protein both in Xenopus oocytes and in oocyte extracts (19, 32, 39, 40). All these observations led to the conclusion that, during Ras-induced meiotic resumption, Xp42Mpk1 is under control of Raf. In opposite, following progesterone stimulation, which is independent of Ras, Xp42Mpk1 is under the control of p39Mos (11). In contrast to these views, our results indicate that p39Mos is required for Xp42Mpk1 activation following stimulation of the tyrosine kinase receptor by insulin (Fig. 5). Results obtained by inhibition of Raf with dominant-negative forms might be explained by the fact that dominant-negative forms were truncated from their catalytic domains but still contained the Ras-binding domain. Also, these dominant-negative forms may have an inhibitory effect directly on Ras (32).

Most strikingly, Xp42Mpk1 activation by p39Mos but not p39Mos itself appeared to be necessary for complete phosphorylation of Raf because PS-AS partially prevented Raf phosphorylation. Total phosphorylation of Raf was rescued by injection of exogenous mu-Mos protein. Raf has been shown to be activated in response to Ras activity after growth factor stimulation in somatic cells (for a review, see Ref. 21) and it has been proposed that the human oncogenic pathway involved between Ras and Raf have been demonstrated to be insufficient to stimulate Raf activity in vitro (43, 44). Our results in Xenopus oocytes suggest that Raf activation is a multistep process involving activation of Xp42Mpk1 activity (Fig. 5).

In PS-AS-treated oocytes, MPF activation as assessed by cyclin B2 phosphorylation, and GVBD were both delayed but not prevented in contrast to results obtained with PO-AS (9, 10). As well, U0126 treatment resulted in such a delay in GVBD and did not prevent MPF activation. p90Rsk and its
upstream activator Xp42<sup>Mplk1</sup> have been suggested to indirectly activate pre-MPF by inhibiting Myt1 activity (4), and their activities, if not necessary for GVBD, are required for timely activation of MPF. Consequently, Myt1 activity is involved in the inhibition of pre-MPF by phosphorylating and stabilizing the p39Mos oncoprotein (49) or by phosphorylating directly or indirectly the cytoplasmic polyadenylation element-binding protein whose activation is necessary for p39Mos mRNA polyadenylation. Indeed, p39Mos mRNA polyadenylation has been shown to be a crucial step in p39Mos synthesis (3). Then, insulin is able to induce p39 Mos synthesis (3). Thus, insulin is able to indirectly activate pre-MPF stored in prophase-arrested oocytes by the above mentioned mechanisms.

To conclude, our results showed that Xenopus oocyte provides us the first known model of the tyrosine kinase receptor signaling pathway implying the proto-oncogene p39Mos in the MAP kinase pathway activation. It gives us unique opportunities for analyzing linear pathways and interdependencies of complex signal transduction of Ras-dependent and independent pathways.

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