Mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) are activated during Xenopus oocyte maturation concomitant with the activation of maturation promoting factor (MPF). We reported previously that an anti-MAPKK neutralizing antibody inhibited progesterone- or Mos-induced initiation of oocyte maturation. Here, we show that injection of CL100 (also called MAPK phosphatase-1) into immature oocytes inhibited progesterone-induced oocyte maturation as well as MAPK activation and that injection of mRNA encoding a constitutively active MAPKK induced activation of histone H1 kinase and germinal vesicle breakdown in the absence of progesterone. Injection of recombinant STE11 protein (a yeast MAPKK kinase) also induced initiation of oocyte maturation. These data support the idea that the MAPKK/MAPK cascade plays an important role in oocyte maturation. Interestingly, injection of the active MAPKK mRNA or the STE11 protein resulted in induction and accumulation of Mos protein. Furthermore, in the presence of cycloheximide, the STE11-induced activation of MPF as well as the induction and accumulation of Mos was blocked, and the activation of MAPK was greatly reduced. The increase in Mos protein and the activation of MAPK by injecting cyclin A protein into immature oocytes were both blocked also by cycloheximide treatment. These results are consistent with an idea that there may exist a positive feedback loop involving Mos. The results suggest that the protein kinase cascade may integrate several different inputs at the point of MAPKK (5).

Mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine protein kinases that is thought to function in a wide variety of intracellular signaling pathways from yeast to vertebrate (1–5). MAPK activation requires concomitant phosphorylation on specific threonine and tyrosine residues (6, 7), which is catalyzed by a dual specificity kinase, comitant phosphorylation on specific threonine and tyrosine residues (6, 7), which is catalyzed by a dual specificity kinase,
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NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 10 mM Hepes, pH 7.5) for 2–3 h at 22 °C. For each assay point, more than 10 oocytes were homogenized in 20 volumes of buffer (HB, 20 mM Tris, pH 7.5, 12.5 mM β-glycerophosphate, 15 mM NaF, 10 mM EGTA, 2 mM MgCl₂, 50 mM NaCl, 6 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.3% aprotinin) and clarified by centrifugation at 15,000 × g for 20 min.

Immunoblotting—After the extracts were subjected to SDS-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore). Membranes were incubated with anti-MAPK antibody (40) or with anti-Mos antibody (Santa Cruz) in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 3% bovine serum albumin and subsequently with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham). Immunoreactive bands were detected by ECL Western blotting detection system (Amerham).

In Vitro Mutagenesis—An EcoRI fragment of Xenopus MAPK cDNA (41) was subcloned into M13 mp18. The mutagenesis of Ser-218 to Glu and Ser-222 to Glu was performed by the method of Kunkel et al. (42), which yielded SESE–MAPKK. SESE–MAPKK was moderately active as compared to wild-type MAPK. To produce a more active mutant of MAPKK, a deletion (32–51) near the N-terminal was introduced into Xenopus SESE–MAPKK according to the method of Mansour et al. (43) using primers (5′-AGCCTCAGGTGTTCGCTGCTAGG-3′ and 5′-CGTCCTCAGGTTTCTCTCAGAAGC-3′) and was ligated at the XhoI site, yielding dSESE–MAPKK. This Xenopus dSESE–MAPKK can function as a strongly active mutant in Xenopus oocytes (this study) and in COS cells.3

In Vitro Transcription—CL100, wild-type MAPKK, SESE–MAPKK, and dSESE–MAPKK cDNAs were inserted into pSP64T vector (44). Synthesis of capped mRNA was performed with SP6 RNA polymerase essentially as described by Moon and Christian (45). Oocyte Injections—Stage VI oocytes were microinjected with 10–50 nl of samples (0.1–0.7 mg/ml mRNA, 1 mg/ml cyclin A protein, or 5 mg/ml STE11 protein) in 3% Ficoll-0.1 × modified Barth’s solution containing 0.033 mM Ca(NO₃)₂, 0.041 mM CaCl₂, and 10 ng/ml kanamycin using an injector (Eppendorf Transjector 5246). After injection, oocytes were immediately transferred to L15 medium. An N-terminal truncated form of STE11 (38) (residues 370–717 [Ref. 39]) was expressed as a GST-fusion protein in bacteria and purified as described previously (9). Protein A–Sepharose was coupled with GST-tagged fusion protein and immobilized on a column. After washing, unretracted proteins were eluted with 1× saline dene difluoride membrane (Immobilon P, Millipore). Membranes were exposed to film (Amersham) after autoradiography.

In-gel Kinase Assay—For detecting MAPK activity, samples (8 μl of extract each) were subjected to SDS-polyacrylamide gel electrophoresis in a 10% acrylamide gel containing 5 mg/ml gelatin as substrate. Purified MAPK (data not shown) was used as a substrate for detecting MAPK activity (Fig. 1C, upper panel). Activation of MAPK was accompanied by activation of histone H1 kinase activity (a measure of MPF) (Fig. 1B) and germinal vesicle breakdown (GVBD) (Fig. 1A). In the CL100-injected oocytes, no activation of MAPK was observed until 24 h after progesterone treatment (Fig. 1, C and D, lower panels). At the same time, progesterone-induced activation of MPF and GVBD were almost completely inhibited in the CL100-injected oocytes for 2 days (Fig. 1, A and B, and data not shown). Injection of the same amount of control mRNA (such as luciferase) had little effect on the timing of progesterone-induced oocyte maturation and MPF activation (data not shown), suggesting that the observed effects of CL100 were not due to possible nonspecific, toxic side effects resulting from injection of a large amount of mRNA. When the reduced amount of CL100 mRNA was injected into oocytes, progesterone-induced MAPK activation and GVBD were both delayed markedly but took place (data not shown), and the delay in MAPK activation correlated well with the delay in GVBD (data not shown). These results suggest that activation of MAPK is required for the activation of MPF and the occurrence of GVBD induced by progesterone. Interestingly, the amount of Mos protein whose synthesis is induced by progesterone treatment was greatly diminished by expressing CL100 (Fig. 1E). Injection of CL100 did not alter markedly total protein synthesis (data not shown). Then, the activation of MAPK might be involved in the up-regulation of the protein level of Mos (see below).

To examine whether or not activation of the MAPK cascade is capable of initiating oocyte maturation, we injected immature oocytes with a constitutively active mutant of MAPKK mRNA (dSESE–MAPKK, see “Experimental Procedures”). Full activation of MAPK occurred without hormonal stimulation in the dSESE–MAPKK-injected oocytes (Fig. 2C), and almost all the oocytes underwent GVBD (Fig. 2A). In control oocytes (uninjected oocytes or water-injected oocytes), no activation of MAPK occurred without progesterone (Fig. 2C), and no GVBD took place (Fig. 2A). Injection of wild-type MAPKK mRNA or a moderately active mutant of MAPKK (SESE–MAPKK) mRNA did not induce GVBD within 15 h of the injection (data not shown). Histone H1 kinase activity, a measure of MPF, was also activated by injection of dSESE–MAPKK mRNA (Fig. 2B). Thus, the constitutively active MAPKK that induces activation of MAPK is capable of initiating Xenopus oocyte maturation.

To confirm the above observation, we next injected immature oocytes with an active mutant of STE11 protein. We have previously shown that a N-terminal truncated form of STE11 (39), one of the yeast MAPKK kinases (38), can phosphorylate and activate MAPKK in Xenopus oocyte extracts (9). In the STE11-injected oocytes, full activation of MAPK occurred (Fig. 3B and C), and almost all the oocytes underwent GVBD (Fig. 3A). Histone H1 kinase activity (Fig. 3E) was also increased in the STE11-injected oocytes. Taken together, activation of the MAPK cascade is apparently sufficient for initiating maturation in Xenopus immature oocytes.

Immunoblotting with anti-Mos antibody revealed that injection of the active MAPKK mRNA (Fig. 2D) or that of STE11 protein (Fig. 3D) induced an increased protein level of Mos. This unexpected finding, together with the observation described above that ectopic expression of CL100 inhibited the induction and accumulation of Mos protein by progesterone, may suggest a requirement of the MAPK cascade for the induction and accumulation of Mos protein during oocyte maturation. The increased translation of c-mos mRNA was reported to require polyadenylation (47). The maturation-induced polyadenylation of maternal mRNA(s) could be regulated by MPF, as is the case with cyclin B mRNA (48). Then, it is possible that the MAPK cascade-induced increase in the protein level of Mos is mediated by MPF activation. To see whether protein synthesis is required for the STE11-induced oocyte maturation, oo-
cytes were injected with the STE11 protein in the presence of cycloheximide and incubated without progesterone. Neither GVBD, H1 kinase activation nor Mos induction occurred by STE11 in the cycloheximide-treated oocytes (Fig. 3, A, D, and E). Therefore, synthesis of some protein, most probably Mos, is required for the STE11-induced oocyte maturation. This result may be interpreted in two ways. One possibility is that the positive feedback loop consisting of MAPK, MPF, and Mos is essential for oocyte maturation. The other is that Mos (or some other cycloheximide-sensitive protein) has another function independent of the MAPK cascade, which is essential for oocyte maturation. Consistent with the former possibility, the activation of MAPK by injecting STE11 was markedly suppressed in the presence of cycloheximide (Fig. 3, B and C). When individual oocytes injected with STE11 in the presence of cycloheximide were analyzed, full activation of MAPK occurred in the absence of cycloheximide, and only partial activation of MAPK occurred in any of the cycloheximide-treated oocytes (Fig. 4). These results may suggest that cycloheximide treatment blocks the presumptive positive feedback loop by which MAPK becomes fully active. In other words, synthesis and accumulation of Mos, which may be supported by activated MPF, might be necessary for sustained, full activation of MAPK. Then, full activation of MAPK may be necessary and sufficient for inducing MPF activation, and the low level activation of MAPK may be insufficient for oocyte maturation.

Using the cell-free extracts prepared from immature oocytes, addition of cyclin A was shown to be capable of activating

**Fig. 1. Inhibition by CL100 of progesterone-induced GVBD, MPF activation, MAPK activation, and Mos accumulation.** Immature Xenopus oocytes were injected with CL100 mRNA (25 ng) or with H2O as a control, and 5 h after injection, the oocytes were exposed to 4 μM progesterone (PG). Various times (0–24 h) after the exposure to progesterone, more than 50 oocytes were scored for appearance of a white spot in the animal pole to judge GVBD (A). Oocytes were homogenized at each time point, and the resulting extracts were assayed for histone H1-phosphorylating activity (B), subjected to in-gel assay for detecting MAPK activity in MBP-containing gels (C), or subjected to immunoblotting with anti-MAPK antibody (D) and anti-Mos antibody (E). In B, 32P incorporation into H1 is shown by autoradiography. In D, the electrophoretically retarded band represents an active form of 42-kDa MAPK.
MAPK even in the presence of cycloheximide (49). This suggests that some MAPKK kinase other than Mos could be activated in vitro, downstream of MPF. Thus, it was not clear whether Mos is responsible for MAPK activation by MPF during oocyte maturation. Rime et al. (50) reported that sea urchin cyclin B can induce MPF activation without tyrosine phosphorylation of MAPK in cycloheximide-treated oocytes. We obtained similar results by having shown that cycloheximide treatment inhibits cyclin A-induced activation of MAPK but not cyclin A-induced activation of MPF (data not shown). Thus, during oocyte maturation, MPF may activate MAPK mainly through induction and accumulation of Mos protein.

**DISCUSSION**

In this paper, we have shown that injection of CL100 mRNA, depending on the amount of the mRNA, caused marked delay or complete suppression of both MAPK activation and oocyte maturation (GVBD) in response to progesterone. CL100 is a dual specificity phosphatase that is thought to act specifically on members of the MAPK superfamily including the MAPK family, the MPK2 (HOG1)/p38 family, and the SAPK/J NK family (51). In our preliminary experiments, the activity of SAPK and MPK2 could not be detected during oocyte maturation. Moreover, the extent of inhibition of MAPK activity correlated well with the inhibition of GVBD in the CL100-injected oocytes. Thus, it is likely that CL100 inhibits oocyte maturation by inhibiting MAPK activation. The result presented here, together with our previous report showing that an anti-MAPKK neutralizing antibody inhibits oocyte maturation, suggests that activation of MAPKK and MAPK is required for initiating Xenopus oocyte maturation.

We have shown here that activation of the MAPK cascade alone is capable of initiating oocyte maturation, since a constitutively active mutant of MAPKK or STE11 induced MPF activation and GVBD when injected into oocytes in the absence of hormonal stimulation. This result further supports the importance of the MAPK cascade in Xenopus oocyte maturation, although the possibility cannot be ruled out that the MAPK cascade-independent pathways play a role in the maturation process. However, many agents that can induce MAPK activation (such as insulin) can induce oocyte maturation (26, 27). Active forms of proto-oncogene products Ras and Raf are also capable of inducing both MAPK activation and GVBD (28–30). The fact that insulin-induced oocyte maturation requires MAPKK activity further suggests the involvement of the MAPK cascade in oocyte maturation (52). Ras is required for insulin-stimulated GVBD but not for progesterone-induced GVBD (53). Thus, several pathways including the Mos-induced pathway may converge at the point upstream of MAPK to initiate GVBD.

It is not impossible that STE11 is acting through a JNK/SAPK pathway, since MEKK, mammalian homolog of STE11, can activate a JNK/SAPK pathway in mammalian cells. However, only MAPK, and not JNK/SAPK-like kinase, was activated in STE11-incubated oocyte extracts. Therefore, STE11 may act through activating the MAPKK/MAPK pathway in Xenopus oocytes.

Besides the Mos-MAPK pathway, there may exist another important pathway for activating MPF, since progesterone can initiate oocyte maturation without protein synthesis in the presence of such a low dose of injected Mos protein that by itself cannot induce oocyte maturation (17). Fabian et al. (29) reported that induction of GVBD was observed with little or no activation of MAPK when dominant negative Raf was expressed in the presence of progesterone. A likely candidate for the alternate pathway for activating MPF is lowering of cAMP concentration, which results in decrease in the activity of cAMP-dependent protein kinase that is suggested to function as a negative regulator in oocyte maturation (Ref. 54; see also Ref. 55 and references therein). The observed synergistic effect of increasing amounts of Mos protein and the protein kinase A regulatory subunit on the induction of GVBD suggests counteracting cooperation between Mos and cAMP-dependent pro-

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4 H. Kawasaki, unpublished observations.
5 K. Takenaka, unpublished observations.
tein kinase for activation of MPF (55). Indeed, cAMP-dependent protein kinase is suggested to act at multiple points to inhibit oocyte maturation such as progesterone-stimulated Mos synthesis, Mos-induced activation of MPF, and MPF-induced activation of Cdc25 (56).

We could assume the existence of a positive feedback loop consisting of MPF, Mos, and the MAPK cascade as one of possible hypotheses that can account for the following results. 1) Injection of anti-MAPKK neutralizing antibody inhibited (or delayed) both MAPK activation and MPF activation induced by Mos injection (34). This suggests that Mos activates MPF through the MAPK cascade. 2) Injection of the active MAPKK mRNA or the active STE11 protein resulted in induction and/or accumulation of Mos protein as well as MPF activation (this study). 3) Injection of purified MPF into Xenopus oocytes induced activation of the MAPK cascade (21, 40). Injection of δ 90 cyclin A into oocytes induced induction of Mos and activation of MAPK. Activation of MAPK by cyclin was blocked in the presence of cycloheximide (this study) or c-mos antisense oligonucleotide (data not shown). These results are consistent with the idea that MPF activates the MAPK cascade through induction and accumulation of Mos. 4) In the presence of cycloheximide, injection of STE11 protein did not induce activation of MPF or increase in the protein level of Mos or full activation of MAPK (this study). 5) Mos is a MAPKK kinase (23, 25). On the other hand, the MAPK cascade may be important for induction and/or accumulation of Mos protein since ectopic expression of CL100 reduced the protein level of Mos (this study), and the activation of the MAPK cascade can induce Mos protein as described above. We cannot rule out completely the possibility, however, that CL100 abolished Mos synthesis through inactivating protein synthesis generally, although total protein synthesis was not markedly affected by injecting CL100 (data not shown). Anyway, these results taken together may be consist-

6 A. Nebreda and T. Hunt, personal communication.
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I. Introduction

II. Methods

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A. Analysis of Individual Oocytes Injected with STE11

B. Analysis of Individual Oocytes Injected with Cyclin A

IV. Discussion

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References

Figure 4. Analysis of individual oocytes injected with STE11.

Figure 5. Analysis of individual oocytes injected with cyclin A.

Legend:

CHX - - - - - + + +
GVBD - - + + + + + +
MAPK - - - - - - - -
Mos - - - - - - - -
H1 - - - - - - - -

+ + + + + + + +
- - - - - - - -

Discussion

1. Increase in the protein level of Mos appeared to be divided into two phases: the induction of the protein synthesis in early phase before MPF activation and the dramatic increase of the protein level in late phase accompanying the activation of MAPK and MPF. Ectopically expressed CL100 might inhibit the late phase, as well as the activation of MAPK and MPF. This may also suggest a close relationship among Mos, MAPK, and MPF. Mos protein in the early phase may be insufficient to activate the positive feedback loop, as Mos might exist as an inactive form, or the amount of Mos protein might be less than a threshold to activate the feedback loop.

2. The mechanism by which MPF regulates the induction and accumulation of Mos protein remains to be determined, although MPF is known to up-regulate translation of protein synthesis and polyadenylation of maternal mRNAs (48), which increases translation efficiency. In fact, polyadenylation-mediated increase in Mos translation is recently reported to be required for Xenopus oocyte maturation (47). It is also possible that MPF is involved in the stabilization of Mos protein during oocyte maturation. On the other hand, the possibility cannot be ruled out that MAPK could be involved independent of MPF, in the stimulation of translation efficiency generally, since MAPK-induced phosphorylation and activation of PHAS-I, which is eIF-4E binding protein (4E-BP1), has been reported (57). How MAPK could activate MPF is also unknown. Wee1 and protein phosphatase 1 have the phosphorylation consensus sequence(s) for MAPK, and Cdc25 can be phosphorylated by MAPK in vitro, but no evidence for regulation of their activity by MAPK was reported.

3. There may exist a Mos-independent pathway in activation of the MAPK cascade by MPF, since the addition of nondegradable cyclin to oocyte extracts activates the MAPK cascade in the presence of cycloheximide (49). However, the contribution of this pathway in vivo may be minor, since no activation of MAPK was observed by injecting immature oocytes with cyclin A in the presence of cycloheximide.

4. It is known that MPF is capable of undergoing autoamplification even in the presence of cycloheximide (14), that is, in the absence of Mos. There are a number of observations that might be related to this autoamplification. Cdc25C has been shown to be phosphorylated and activated by p34cdc2/cyclin B directly (58). Both the reduction of the phosphatase activity acting on Cdc25 and the increase in the kinase activity acting on Cdc25 were observed when oocytes entered M phase (59). Both protein phosphatase 1 and protein phosphatase 2A are candidates of the phosphatase acting on Cdc25, protein phosphatase 1 activity is decreased transiently during M phase, and MPF inhibits protein phosphatase 1 activity by phosphorylating its near C-terminal site in Schizosaccharomyces pombe (60). Wee1 is also a candidate of the target for MPF. Wee1 was recently shown to be down-regulated by MPF-catalyzed phosphorylation (61).

5. After completion of this study, two reports (62, 63) appeared demonstrating that injection of an active MAPKK mutant (62) or thio phosphorylated MAPK (63) is capable of inducing GVBD in Xenopus oocytes in the absence of progesterone. Moreover, it has been demonstrated that injection of thio phosphorylated MAPK induces activation of endogenous MAPK and that this activation is inhibited by cycloheximide treatment (63). This is consistent with our results using STE11 protein. Although they did not show the data on the protein level of Mos, their data can be interpreted as suggesting that inhibition of Mos synthesis may cause the inhibition of activation of endogenous MAPK in the cycloheximide-treated oocytes. Thus, their results may be consistent with our tentative hypothesis of a positive feedback loop. In contrast, in the experiments of Huang et al. (62), cycloheximide treatment did not affect markedly the active MAPKK-induced activation of MAPK. Although the apparent difference between these observations cannot be easily explained, the possible difference in the strength of upstream activating enzymes for MAPK (e.g. STE11 and various mutants of MAPKK) might affect the results. In any case, if the upstream activating enzyme is strong enough, almost full activation of MAPK could be achieved even in the presence of cycloheximide. In addition, it might be possible that higher level activation of MAPK could induce MPF activation even in the presence of cycloheximide, since a large amount of Mos or Ras could induce GVBD with cycloheximide (17, 64, 65).

In summary, we may propose a working hypothesis, assuming the existence of a positive feedback loop consisting of Mos, the MAPKK/MAPK cascade, and MPF. That is, progesterone...
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triggers synthesis of Mos protein, and the positive feedback loop among Mos, the MAPK cascade, and MPF turns on. Then, when the level of MPF activity exceeds some threshold, MPF will undergo amplification without protein synthesis. Injection of a large amount of cyclin can bypass the first positive feedback loop and cause the promotion of MPF amplification independent of protein synthesis. Further studies will be necessary to elaborate or correct this tentative model.

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