

Mos Mediates the Mitotic Activation of p42 MAPK in *Xenopus* Egg Extracts

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Supplemental Results

We carried out several experiments aimed at determining whether the low levels of endogenous Mos protein are activated during mitosis in cleaving embryos. The main problem was that at best we saw ~10% of the p42 MAPK activated during mitosis (see the 75 and 80 min time points of Figure 3A), and sometimes we could not see the activation at all (see the 100–110 min time points of Figure 3A). We suspect that the amount of active Mos leading to this low level of p42 MAPK activation is quite small.

We therefore initially tried a technically easier experiment in which we drove cycloheximide-treated interphase extracts into a permanent mitosis with $\Delta 90$ -cyclin B and assessed the activity of the endogenous Mos protein. This experiment also proved to be difficult; the increase seen was sometimes small. However, in other experiments we got the expected result—the Mos from the $\Delta 90$ -cyclin B-treated extract had a higher activity than the Mos from the buffer-treated interphase extract (Figure S1A). We suspect that there might be a coprecipitating, constitutively active kinase activity (like B-Raf, which is two orders of magnitude more abundant than Mos) adding background to both the interphase and mitotic Mos assays.

We therefore tried another approach: adding recombinant FLAG-GST-Mos on FLAG-antibody beads and assessing its activity toward recombinant MEK with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The ability to fish the FLAG-tagged Mos out of the extract improved the background substantially. We found that $\Delta 90$ -cyclin B caused a substantial increase in the activity of the recombinant Mos. Treating the immobilized FLAG-GST-Mos with λ -phosphatase reduced the activity to near basal levels (Figure S1B, lanes 1–3). Addition of ethylenediaminetetraacetic acid (EDTA) to the λ -phosphatase blocked its ability to inactivate Mos (Figure S1B, lane 4), consistent with the phosphatase's require-

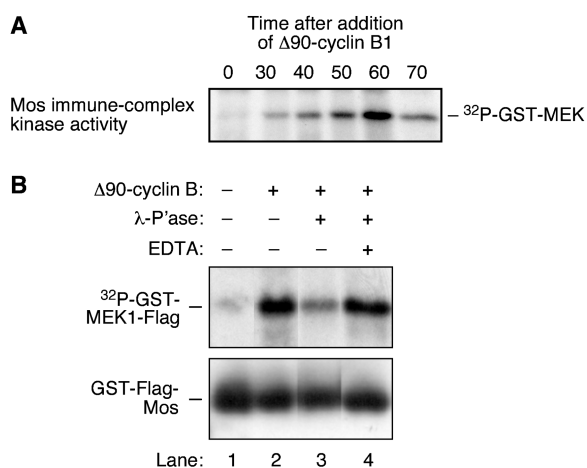


Figure S1. Cdc2-Cyclin B-Induced Mos Activation in *Xenopus* Egg Extracts

(A) Mos activation in $\Delta 90$ -cyclin-treated extracts. Recombinant cyclin was added to a cycloheximide-treated interphase extract at $t = 0$. Samples were collected, immunoprecipitated with a polyclonal Mos antibody (Santa Cruz Biotechnology, SC-86), and subjected to immune complex kinase assays with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and recombinant GST-MEK.

(B) Activation of GST-Flag-Mos by $\Delta 90$ -cyclin B in *Xenopus* egg extracts and inactivation by incubation with λ phosphatase. EDTA (lane 4) is an inhibitor of λ phosphatase.

ment for zinc for activity. Thus, $\Delta 90$ -cyclin B can bring about the activation of Mos, and Mos phosphorylation is required for the resulting activity. Further studies on the mechanism of Mos activation are in progress.

Supplemental Experimental Procedures

Preparation and Manipulation of Egg Extracts

Demembrated frog sperm nuclei, cycloheximide-treated interphase egg extracts, and cycling egg extracts were prepared as described [S1, S2]. To drive interphase extracts into a permanent mitotic state, we incubated extracts with 200 nM nondegradable sea urchin $\Delta 90$ -cyclin B for 60 min. The progress of cycling extracts was monitored by sperm morphology changes visualized by 4',6-diamidino-2-phenylindole (DAPI) staining and histone H1 kinase assay.

In Vitro Fertilization of *Xenopus* Eggs

Eggs were fertilized as described [S3]. Fertilized eggs were dejellied in 2% cysteine (pH 7.8), and embryos were collected at various times, frozen, and lysed as described [S4].

Immunodepletion

Depletion of Mos, Raf-1, B-Raf, or MEK1 from extracts was accomplished by two sequential rounds of immunodepletion, carried out at 4°C with the relevant antibody (Mos, SC-86, Santa Cruz Biotechnology; Raf-1, SC-133, Santa Cruz Biotechnology; B-Raf, SC-9002, Santa Cruz Biotechnology; and MEK1, antibody 662, raised in our laboratory [S5]) prebound to protein A-Sepharose beads (Sigma). Mock depletions were carried out with rabbit immunoglobulin G (IgG).

MEKK Assays

Crude extract samples (0.5–2 μl) diluted to 10 μl with EB buffer (80 mM β -glycerophosphate, 20 mM ethylene glycol-bis(aminoethyl ether)-*N,N,N,N*-tetraacetic acid [EGTA], and 15 mM MgCl_2 [pH 7.3]) or aliquots of 10 μl column fractions were added to 10 μl of reaction mixture consisting of 100 ng GST-MEK1-Flag, 10 μM cAMP-dependent protein kinase inhibitor, 0.75 mM Na_3VO_4 , 5 mM EGTA, 0.2 mM ATP, 13 mM HEPES-NaOH (pH 7.3), 3.2 mM EGTA, and 64 mM MgCl_2 and incubated at 30°C for 10 min. The reactions were stopped by the addition of SDS sample buffer. Proteins were subjected to electrophoresis through 10% sodium dodecyl sulfate (SDS) polyacrylamide gels (acrylamide: bisacrylamide, 29:1) and transferred to a Hybond-P (Amersham) blotting membrane. The active GST-MEK1-Flag was detected by Western blotting with anti-phospho-MEK antibody (9121, Cell Signaling Technology, Beverly, MA).

Dephosphorylation of ^{32}P -GST-MEK1-Flag in Mitotic and Interphase Extracts

B-Raf was immunoprecipitated from interphase extracts. For the preparation of radiolabeled, phosphorylated MEK1 protein, purified recombinant GST-MEK1-Flag protein (100 ng) was then incubated with aliquots of the B-Raf immunoprecipitate in kinase buffer (50 mM Tris [pH 7.0], 100 mM NaCl, 0.1 mg/ml BSA, and 10 mM MgCl_2) plus ATP (67 μM ; 0.33 $\mu\text{Ci}/\mu\text{l}$ carrier-free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$). The reaction was incubated for 2 hr at room temperature, and excess salt and ATP were removed by centrifugation through 100 μl of Sephadex G-25 resin (Sigma, St. Louis, MO) preequilibrated with kinase buffer. ^{32}P -GST-MEK1-Flag was added to interphase or M phase extracts, samples were taken at various times, and the samples were subsequently subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Table S1. Partial Purification of the Peak I MEK Activator

	Protein (mg)	Activity (Arbitrary Units)	Apparent Yield	Specific Activity (U/mg)	Purification
Interphase extract	310	342,000	-	-	-
M -phase extract	310	504,000	-	-	-
Δ	310	162,000	\approx 100%	523	\approx 1
Q -Sephrose	31	131,600	81%	4,245	8
40% AmSO ₄	4	90,750	56%	22,688	43
Superose 6	1.4	135,600	84%	96,857	185

Partial Purification of Cdc2-Cyclin B-Induced MEKK Activity

All chromatographic steps were carried out with a fast protein liquid chromatography (FPLC) station (Pharmacia) at 4°C. Interphase and M phase *Xenopus* egg extracts were prepared as described above. Egg extracts (8 ml) were applied to a 20 ml Q Sepharose column (Pharmacia) equilibrated with buffer A (20 mM Tris [pH 8.0], 1.5 mM MgCl₂, 2 mM sodium EDTA, 2 mM sodium EGTA, 1 mM dithiothreitol [DTT], 0.1 mM PMSF, 12.5 mM β -glycerophosphate, 1 mM Na₃VO₄, 2 mM NaF, and 10% glycerol). The column was washed with ten column volumes of buffer A and eluted with a linear gradient of 100 ml buffer A to buffer B (buffer A + 1 M NaCl). Fractions (1 ml) were collected and assayed for MEKK activity as described above. The active fractions were pooled, mixed with 40% ammonium sulfate for 1 hr, and centrifuged at 20,000 \times g for 15 min at 4°C. The resulting pellet was resuspended in 1 ml buffer A + 0.1 M NaCl and loaded to 25 ml Superose 6 column in five rounds (250 μ l each). Fractions of 1 ml were collected and assayed for MEKK activity.

Western Blot Analysis

Aliquots of egg extracts or column fractions were diluted in SDS sample buffer and submitted to electrophoresis on 10% SDS polyacrylamide gels (acrylamide: bisacrylamide, 100:1). Proteins were transferred to a Hybond-P (Amersham) blotting membrane, which was then blocked with 3% milk in Tris-buffered saline (20 mM Tris and 150 mM NaCl [pH 7.6]) and incubated with primary antibody (Mos, SC-86, Santa Cruz Biotechnology, 1:500 dilution; phospho-MAPK, #9106, Cell Signaling Technology, Beverly, MA, 1:1000 dilution; phospho-MEK, #9121, Cell Signaling Technology, Beverly, MA, 1:1000 dilution; B-Raf, SC-9002, Santa Cruz Biotechnology, 1:500 dilution; and Raf-1, SC-133, Santa Cruz Biotechnology, 1:500 dilution) for 1 hr. After being washed, the blots were probed with a secondary antibody for detection by chemiluminescence. ECL Advanced Detection System (Amersham) was used for detection of Mos.

Purification of Recombinant GST-Flag-Mos and GST-MEK1-Flag

The coding regions of *Xenopus* Mos and human MEK1 were obtained by polymerase chain reaction (PCR) and subcloned into pGEX4T-1 (Amersham), which added an N-terminal glutathione S-transferase (GST) tag and a Flag epitope tag. GST-MEK1-Flag and GST-Flag-Mos were expressed in bacteria and lysed essentially as described [S6]. The clarified cell extracts were loaded to a 5 ml column of glutathione agarose (Pharmacia). The column was washed with 10 vol of PBS, and fusion proteins were eluted with 10 mM HEPES-NaOH (pH 8.0) containing 15 mM glutathione.

Mos in vitro Kinase Assay

50 nM GST-Flag-Mos was added to 100 μ l cycloheximide-treated interphase extracts with or without cyclin B addition for 60 min. Extracts were then diluted with 100 μ l EB buffer and incubated with anti-Flag beads (Sigma) with rocking for 1 hr at 4°C. After three washes in EB containing 0.1% Nonidet P-40 and one wash in detergent-free EB, 25 μ l of reaction mixture, which contained 10 μ M cAMP-dependent protein kinase inhibitor, 0.75 mM Na₃VO₄, 5 mM EGTA, 100 ng GST-MEK1-Flag, 0.08 μ Ci/ μ l [γ -³²P]ATP, 0.2 mM ATP, 13 mM HEPES-NaOH (pH 7.3), 3.2 mM EGTA, and 64 mM MgCl₂, was added. After incubation at 30°C for 10 min, the reaction was stopped with SDS sample buffer, and the proteins were separated

on 10% SDS polyacrylamide gels (acrylamide: bisacrylamide, 29:1) and transferred to a Hybond-P (Amersham) blotting membrane.

Supplemental References

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