

absence or presence of inhibitors, P6 + 5 DIV cultures were washed twice with medium (BME) containing 5 mM KCl and no serum (starvation medium) and then placed in this medium in the presence or absence of the survival factor (BDNF, insulin, or IGF-1) and in the presence of MAPK, PI-3K inhibitors, or their vehicle, dimethyl sulfoxide (DMSO). In experiments in which biochemical analyses were done, the medium [BME + calf serum (10%) + 25 mM KCl] was switched to starvation medium 1 hour before stimulation with the survival factor. In the last 30 min of the 1-hour starvation period, the inhibitor or its vehicle control (DMSO) was added.

11. Immunoblotting was carried out as described (31, 38). Briefly, proteins from lysates were separated by polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and immunoblotted with the appropriate primary antibody. Antibody binding was detected by enhanced chemiluminescence (ECL, Amersham) with a secondary antibody conjugated to horseradish peroxidase (dilution: 1:20,000).
12. Transfections of cerebellar granule cultures were done with a calcium phosphate transfection method as described [Z. Xia et al., *J. Neurosci.* **16**, 5425 (1995)]. In the experiments shown in Fig. 1C and in Fig. 4, B to D, cultures were transfected with test plasmid (2 µg per well) together with the β-galactosidase expression plasmid (0.5 µg per well) in a 24-well plate. Each well contained 6×10^5 cells. In the experiments shown in Fig. 3C, cultures were transfected with 0.5 µg of the test plasmid and 0.5 µg of the β-galactosidase expression plasmid per well of a 24-well plate. In Fig. 3A, each well was transfected with 1.5 µg of MEK-1ca, 0.05 µg of the BAD or BADS112A expression plasmid, and 0.5 µg of the β-galactosidase expression plasmid. In Fig. 3B, each well was transfected with 1.5 µg of MEK-1ca with 1 µg of Rsk2-WT or Rsk2-KN [Rsk2KR100 (32)], 0.125 µg of the BAD or BADS112A expression plasmid, and 0.5 µg of the β-galactosidase expression plasmid.
13. Indirect immunofluorescence was done as described [A. Bonni, D. A. Frank, C. Schindler, M. E. Greenberg, *Science* **262**, 1575 (1993)]. Cultures were fixed in 4% paraformaldehyde and subjected to indirect immunofluorescence with a mouse monoclonal antibody (mAb) to β-galactosidase (Promega, dilution 1:500) and the DNA dye bisbenzimidazole (Hoechst 33258). Determination of cell death in neurons was made on the basis of integrity of neurites and nuclear morphology. Cell counts were carried out in a blinded manner by two independent observers.
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24. We transfected 100-mm plates of 293T cells as described (20) with expression plasmids encoding hemagglutinin (HA) epitope-tagged constitutively active Akt [myrΔPH Akt (15)] (15 µg), Rsk2-WT (5 µg), or Rsk-KN (5 µg) together with MEK-1ca [MEK-S218D/S222D; A. Brunet et al., *Oncogene* **9**, 3379 (1994)] (10 µg). Two days after transfection, cells were lysed and the HA epitope-tagged proteins were immunoprecipitated as described (15). Immunoprecipitated proteins were subjected to an in vitro kinase assay as described (15, 30) using BAD peptide substrates (14 amino acids in length encompassing Ser¹¹² or Ser¹³⁶).
25. Six-well plates of 293T cells containing 7.5×10^5 cells per well were transfected by a calcium phosphate trans-

fection method as described (38). In Fig. 2B, cells were transfected with Rsk2-WT or Rsk2-KN (2 µg) together with MEK-1ca (1 µg) and BAD (1 µg). In Fig. 2C, cells were transfected with 5 µg of the MEK-1ca expression plasmid together with 0.75 µg of the BAD. One day after transfection, cultures were starved for 7 hours. Cell lysates were prepared and immunoblotted with the antibody to phospho-Ser¹¹² (11, 26).

26. A rabbit antiserum to BAD phosphorylated at Ser¹¹² was generated by injecting New Zealand rabbits with the phosphopeptide C-METRSRHpSSYPAG (20). The specificity of the antibody to BAD phosphorylated at Ser¹¹² was confirmed by its reactivity to recombinant BAD protein that was phosphorylated by PKA but not to unphosphorylated BAD, and by its recognition of Rsk2- or PKA-induced phosphorylated wild-type BAD but not S112A BAD that was expressed in 293T cells.
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The Protein Kinase p90 Rsk as an Essential Mediator of Cytostatic Factor Activity

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Persistent activation of p42 mitogen-activated protein kinase (p42 MAPK) during mitosis induces a "cytostatic factor" arrest, the arrest responsible for preventing the parthenogenetic activation of unfertilized eggs. The protein kinase p90 Rsk is a substrate of p42 MAPK; thus, the role of p90 Rsk in p42 MAPK-induced mitotic arrest was examined. *Xenopus laevis* egg extracts immunodepleted of Rsk lost their capacity to undergo mitotic arrest in response to activation of the Mos-MEK-1-p42 MAPK cascade of protein kinases. Replenishing Rsk-depleted extracts with catalytically competent Rsk protein restored the ability of the extracts to undergo mitotic arrest. Rsk appears to be essential for cytostatic factor arrest.

Masui identified two hypothetical M-phase regulators in his classic studies of *Rana pipiens* oocyte maturation. The first, maturation-promoting factor (MPF), was an activity present in mature oocytes that was able to induce immature oocytes to mature even in the absence of protein synthesis (1). MPF ultimately proved to be a complex of the universal M-phase regulators Cdc2 and cyclin B (2). Cytostatic factor (CSF) was de-

defined as an activity present in mature oocytes that induced mitotic arrest when injected into cleaving embryos (1). The underlying hypothesis was that CSF activity is responsible for the maintenance of mature oocytes in their normal metaphase arrest state.

Studies over the past decade have identified the proto-oncoprotein Mos as CSF and the protein kinases MEK and p42 MAPK as essential mediators of CSF activity (3). The introduction of Mos mRNA (4) or protein (5), constitutively active MEK (3), or thio-phosphorylated, active p42 MAPK (6) into *Xenopus laevis* embryos or cell-free cycling extracts (7, 8) causes a metaphase arrest. Depletion of Mos from extracts of mature

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oocytes depletes the extracts' CSF activity (4). In addition, injection of a neutralizing MEK antibody prevents Mos from causing a CSF arrest (9), as do a pharmacological inhibitor of MEK activation (10) and the CL100 MAPK phosphatase (7). Moreover, in Mos-deficient mice oocytes do not arrest properly in metaphase of meiosis II and therefore do not develop into normal fertilizable eggs (11). These findings demonstrate the importance of CSF and Mos in normal reproduction. The Mos-MEK-p42 MAPK cascade arrests embryos in mitosis at least in part by preventing the destruction of B-type cyclins (3), although the cascade also functions to maintain the mitotic state even after cyclins have been degraded (12, 13).

Members of the p90 Rsk family of protein kinases are activated by p42 MAPKs in diverse biological contexts and may be important MAPK effectors (14). Rsk-2 has been implicated in MAPK-induced transcriptional changes (15), and mutations in the human Rsk-2 gene are associated with Coffin-Lowry syndrome, an X-linked syndrome characterized by severe psychomotor retardation and facial and digital dysmorphisms (16).

Two closely related Rsk isoforms, Rsk-1 and Rsk-2, are present and active in CSF-arrested *Xenopus* eggs (17, 18). We initially focused on Rsk-2 as a possible mediator of CSF arrest because it is the more abundant isoform (18). We chose cycling *Xenopus* egg extracts (19) for our studies. Cycling extracts respond to the properly timed addition of recombinant Mos or activated MEK by undergoing a classical CSF arrest, with high Cdc2 activity, dissolved nuclear envelopes, and condensed chromatin aligned on metaphase spindles (7, 8, 20). Moreover, extracts offer the possibility of abolishing Rsk function by immunodepletion and restoring Rsk function by addition of recombinant Rsk protein.

We determined whether Rsk-2, like p42 MAPK (12, 20), was activated during mitosis in cycling extracts. Rsk-2 was transiently activated (Fig. 1A) concomitantly with p42 MAPK (Fig. 1B) (12). Like p42 MAPK activation, Rsk-2 activation lagged behind that of Cdc2 and preceded mitotic exit (Fig. 1, A and B). Cycling extracts responded to an appropriately timed addition of recombinant Mos (21) by entering a CSF arrest state with active Cdc2, condensed chromatin, and disassembled nuclear envelopes. This CSF arrest was accompanied by increased phosphorylation of Rsk-2 as indicated by a decreased electrophoretic mobility (Fig. 1C), and activation of the enzyme as assessed by immune complex kinase assays (22). Similar results were found for Rsk-1 (22). Thus, Rsk-2 are potential mediators of CSF activity.

We were able to deplete essentially all of the Rsk-2 from *Xenopus* egg extracts with a

single round of immunodepletion, with no measurable effect on the levels of p42 MAPK and only partial depletion of Rsk-1 (Fig. 2A) (23). We then examined whether Rsk-2 depletion compromised the extract's ability to undergo a CSF arrest. We prepared mock-depleted (Fig. 2, B to D) and Rsk-2-depleted (Fig. 2, E to M) extracts, and supplemented the Rsk-2-depleted extracts with either no added p90 Rsk-2 (Fig. 2, E to G) or physiological concentrations of catalytically inactive KR Rsk-2 (Fig. 2, H to J) (23) or wild-type Rsk-2 (Fig. 2, K to M).

The mock-depleted extract (Fig. 2, B to D) responded to added Mos by undergoing a typical CSF arrest. The extract entered mitosis by 30 min, with increased Cdc2 activity (Fig. 2C), condensed chromatin and dissolved nuclear envelopes (Fig. 2D), increased Rsk-2 phosphorylation (Fig. 2B), and activated p42 MAPK (Fig. 2B). Cdc2 activity remained increased for the duration of the experiment (Fig. 1C) and as a consequence the extract remained in mitosis (Fig. 2, B and D).

In contrast, the Rsk-2-depleted extract (Fig. 2, E to G) did not undergo CSF arrest in response to Mos. Instead, the extract entered and exited mitosis normally, with the mitotic activation of Cdc2 followed by its rapid inactivation (Fig. 2F). The Rsk-2-depleted, Mos-treated extract reformed nuclei with decondensed chromatin and intact nuclear envelopes about 20 min after nuclear envelope breakdown (Fig. 2G). Mock-depleted and Rsk-2-depleted extracts incubated without added Mos cycled with the same 20-min duration of mitosis (22). Thus, Rsk-2-depleted extracts lost their ability to undergo a CSF

arrest in response to added Mos (24).

Adding back physiological concentrations of a catalytically inactive KR Rsk-2 (25) protein did not restore the ability of the extract to undergo a CSF arrest (Fig. 2, H to J). The extract still underwent a transient mitosis in the presence of Mos (Fig. 2I), with inactivation of Cdc2 (Fig. 2I) followed by reformation of interphase nuclei with intact nuclear envelopes and decondensed chromatin (Fig. 2, I and J). However, addition of purified recombinant wild-type Rsk-2 did restore the ability of the extract to undergo a CSF arrest in response to Mos (Fig. 2, K to M). When treated with Mos, the extract to which Rsk-2 had been restored underwent a sustained mitotic arrest (Fig. 2, L and M) with increased Cdc2 activity (Fig. 2L); it behaved indistinguishably from the mock-depleted extract (Fig. 2, K to M and B to D). Recombinant Rsk-2 had no effect on the duration of mitosis in the absence of added Mos (18). Moreover, neither the depletion of Rsk-2 nor the addition of KR Rsk-2 or wild-type Rsk-2 had any appreciable effect on Mos-induced p42 MAPK phosphorylation (Fig. 2, B, E, H, and K). Taken together, these data establish Rsk-2 as an essential mediator of the CSF activity of the Mos-MEK-1-p42 MAPK cascade (26).

Activation of the p42 MAPK cascade can sustain the mitotic state even after Cdc2 activity has dropped to low interphase levels (12, 13). The suppression of mitotic exit by p42 MAPK may be important for establishing normal mitotic timing. Accordingly, we examined whether Rsk-2 was required for p42 MAPK-induced suppression of mitotic exit. Even when the addition of Mos was too late to prevent Cdc2 inac-

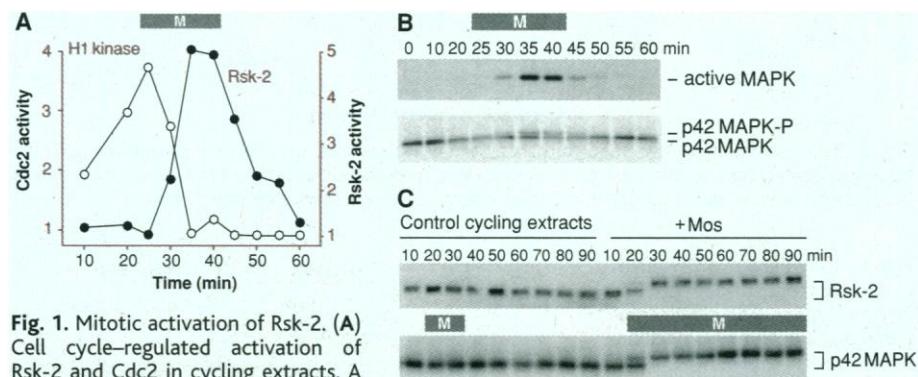
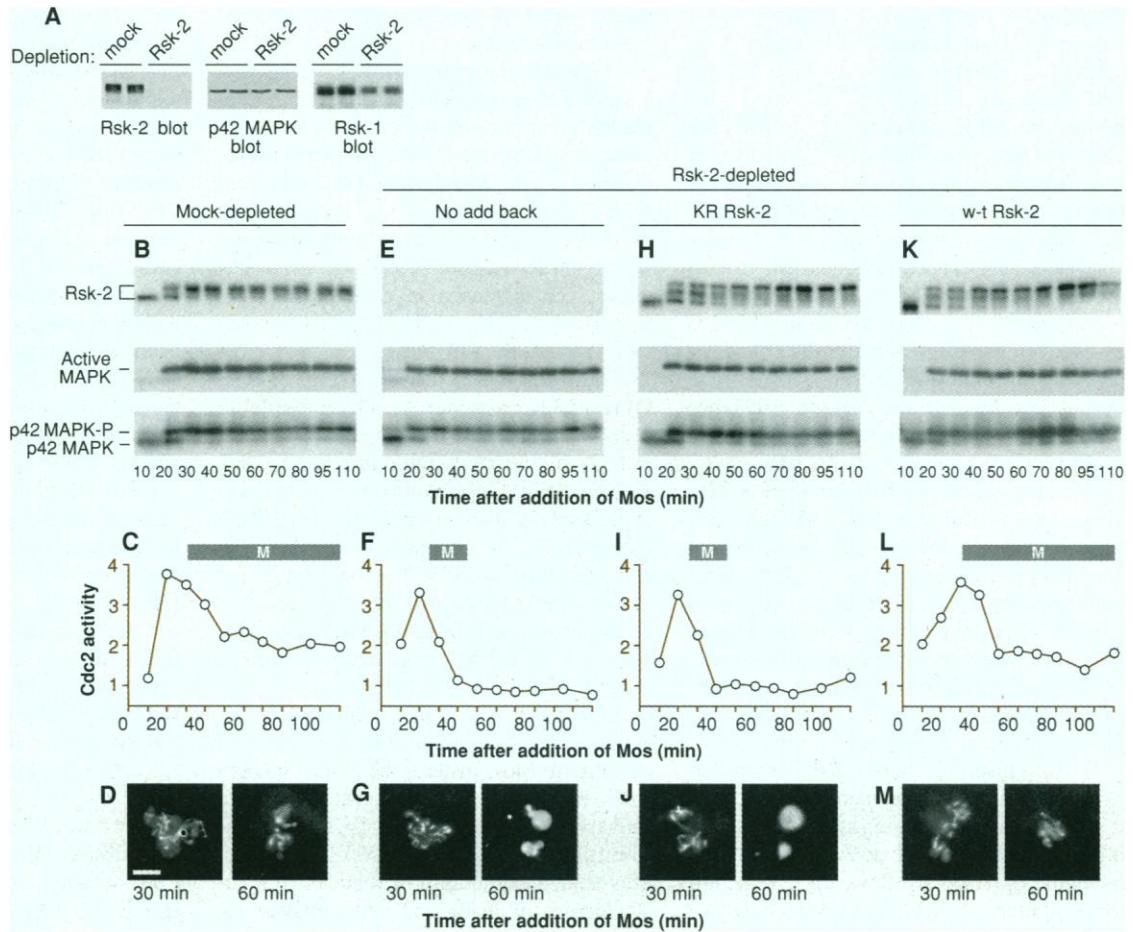


Fig. 1. Mitotic activation of Rsk-2. **(A)** Cell cycle-regulated activation of Rsk-2 and Cdc2 in cycling extracts. A cycling extract was prepared (19) and warmed to room temperature to initiate cycling. Demembrated sperm chromatin (500 sperm per microliter) was added to allow monitoring of progression into and out of mitosis. The times indicated are times after warming. Rsk-2 activity was assessed by immune complex kinase assay with S6 peptide as the phosphoacceptor. Cdc2 activity was assessed by histone H1 phosphorylation and quantified by PhosphorImager analysis (Molecular Dynamics). The duration of mitosis was determined by phase contrast microscopy. **(B)** Cell cycle-regulated activation of p42 MAPK. Portions of the same cycling extract were subjected to SDS-PAGE and immunoblotting with antibodies to active MAPK (New England Biolabs) or p42 MAPK antiserum X15. **(C)** Phosphorylation of p42 MAPK and Rsk-2 in Mos-treated cycling extracts. Purified Mos was added (final concentration, 1 μ M). Phosphorylation of Rsk-2 and p42 MAPK was detected by shifts in their electrophoretic mobilities on immunoblots. The antisera used were from Santa Cruz Biotechnology (Rsk-2) and our laboratory (X15).

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Fig. 2. Requirement of Rsk-2 for the Mos-induced mitotic arrest. (A) Specificity of Rsk-2 immunodepletion. Extracts were mock-depleted or immunodepleted of Rsk-2. The extracts were subjected to immunoblotting to detect Rsk-2 (left), p42 MAPK (middle), and Rsk-1 (right). (B to M) Effect of Rsk-2 immunodepletion on the response of cycling extracts to Mos. (B, E, H, and K) Rsk-2 and p42 MAPK phosphorylation in Mos treated extracts. Rsk-2 protein was depleted and replenished (final concentration 100 nM) as indicated. Mos (final concentration, 1 μ M) and sperm chromatin (500 per microliter) were added when cycling was initiated. Rsk-2 and p42 MAPK phosphorylation was assessed by immunoblotting with antibodies to Rsk-2 (top), active MAPK (middle), or total p42 MAPK (bottom). (C, F, I, and L) Cdc2 activity in Mos-treated extracts. Cdc2 activity was assessed with histone H1 as substrate and quantitated by PhosphorImager analysis (Molecular Dynamics). Mitotic progression was assessed morphologically by phase contrast microscopy and DAPI (4',6'-diamidino-2-phenylindole) staining. (D, G, J, and M) Nuclear morphol-



ogy of Mos-treated extracts. Nuclei were stained with DAPI and observed by fluorescence microscopy. Bar, 10 μ m.

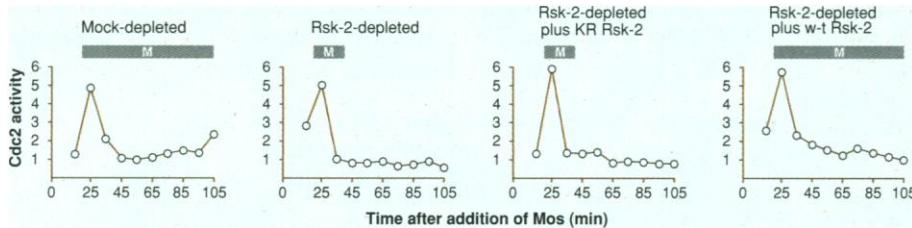
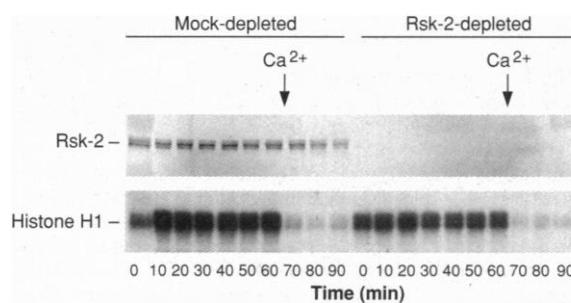


Fig. 3. Rsk-2-dependent sustained mitosis after cyclin destruction. The Mos protein (1 μ M) was added when cycling was initiated. Cdc2 activity was monitored by histone H1 kinase assay. Mitotic progression was assessed by phase contrast microscopy and DAPI staining.

Fig. 4. Maintenance of CSF arrest in the absence of Rsk-2. CSF-arrested egg extracts were prepared and subjected to mock depletion or Rsk-2 depletion. Calcium (400 μ M) was added at 65 min to release the extracts from their arrest state. Amounts of Rsk-2 were assessed by immunoblotting. Cdc2 activity was monitored by H1 kinase assay.



it still suppressed mitotic exit (Fig. 3), in agreement with previous reports (12). Depleting Rsk-2 eliminated the ability of

Mos to suppress mitotic exit, and wild-type Rsk-2 but not KR Rsk-2 restored the sup-

pression of mitotic exit (Fig. 3). Therefore, Rsk-2 is essential for both suppression of Cdc2 inactivation and post-inactivation suppression of mitotic exit. Any direct effects p42 MAPK might have on mitotic substrates [such as CENP-E (27)] are evidently insufficient to maintain the mitotic state in the absence of Rsk function.

Finally, we asked whether Rsk-2 is required for the maintenance of mitotic arrest, or just for initiation of mitotic arrest. We prepared CSF-arrested extracts from *Xenopus* eggs, subjected them to mock depletion or Rsk-2 depletion, and examined whether the depletion caused the extracts to exit their CSF arrest. Both the mock-depleted and Rsk-2-depleted extracts maintained high mitotic levels of Cdc2 activity for at least 60 min, and responded normally to Ca^{2+} by rapidly inactivating their Cdc2 (Fig. 4). Thus, although Rsk-2 function is essential for the initiation of mitotic arrest, it appears to be dispensable for maintenance of the arrest. Alternatively, it is possible that eggs possess a back-up CSF-like activity that is absent from cycling egg extracts.

Our findings demonstrate that the Rsk protein kinase is essential for cyostatic factor ar-

rest. Rsk-2 is required for Mos-MEK-1-p42 MAPK-induced suppression of Cdc2 inactivation and also for suppression of mitotic exit subsequent to Cdc2 inactivation. As shown in the accompanying report (26), an activated form of Rsk-1 is capable of causing a CSF arrest; thus, Rsk activation appears to be both necessary and sufficient for CSF arrest. Rsk proteins are therefore critical targets of p42 MAPK in the regulation of cell cycle progression and the development of fertilizable eggs.

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18. *Xenopus* Rsk-2 is 92% identical to human Rsk-2 (ISPK-1) and 79% identical to *Xenopus* Rsk-1. From quantitative immunoblotting experiments with purified recombinant Rsk-1 and Rsk-2 proteins as standards, we have estimated the oocyte concentration of Rsk-1 to be 2 to 6 nM and Rsk-2 to be ~100 nM. The *Xenopus* Rsk-2 sequence has been submitted to the National Center for Biotechnology Information (accession number AF165162).
19. Cycling extracts were prepared as described (28) with one modification; we waited 30 min after electrically activating the eggs before crushing them, rather than the usual 15 min. This resulted in extracts that were usually too far progressed to undergo G₂ arrest in response to added Mos (7, 8), making the extracts better suited for examining Mos-induced mitotic arrests. CSF extracts were prepared as described (28).
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21. Mos was expressed in bacteria as a maltose-binding protein (malE) fusion (5). The malE-Mos fusion protein was purified as described (5).
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23. Rsk-2 immunodepletion was accomplished by incubating extracts for 75 min on ice with antibodies to Rsk-2 (Santa Cruz Biotechnology) that had been pre-

bound to protein G agarose beads (Gibco-BRL). Mock depletions were carried out similarly, with the antibody omitted.

24. Immunodepletion of Rsk-1 yielded extracts that still underwent a CSF arrest in response to Mos. Thus, Rsk-1 appears not to be necessary for CSF arrest.
25. (His)₆-tagged Rsk-2 proteins (with His tag inserted directly after the putative translation initiation methionine codon) were produced in Sf9 cells and purified to homogeneity by nickel chelate chromatography. Catalytically inactive Rsk-2 (KR Rsk-2) was engineered by substituting arginine for lysine at residue 97.
26. We also titrated different concentrations of Rsk-1 or Rsk-2 into Rsk-2-depleted extracts. We found that 100 nM Rsk-1 or Rsk-2 was sufficient to restore the arrest, but 20 nM, 4 nM, and 1 nM were insufficient.

These findings indicate that the Rsk's are at least partially interchangeable, but by virtue of its higher normal concentration (18) Rsk-2 is the more important Rsk in this context. Our results fit well with the recent finding that a gain-of-function form of Rsk-1 can cause CSF arrest [S. G. Gross, M. S. Schwab, A. L. Lewellyn, J. L. Maller, *Science* **286**, 1365 (1999)].

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Induction of Metaphase Arrest in Cleaving *Xenopus* Embryos by the Protein Kinase p90^{Rsk}

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Before fertilization, vertebrate eggs are arrested in metaphase of meiosis II by cytostatic factor (CSF), an activity that requires activation of the mitogen-activated protein kinase (MAPK) pathway. To investigate whether CSF arrest is mediated by the protein kinase p90^{Rsk}, which is phosphorylated and activated by MAPK, a constitutively activated (CA) form of Rsk was expressed in *Xenopus* embryos. Expression of CA Rsk resulted in cleavage arrest, and cytological analysis showed that arrested blastomeres were in M phase with prominent spindles characteristic of meiotic metaphase. Thus, Rsk appears to be the mediator of MAPK-dependent CSF arrest in vertebrate unfertilized eggs.

The unfertilized eggs of most vertebrates are naturally arrested at metaphase of meiosis II. Upon fertilization, an increase in free calcium activates the anaphase-promoting complex, which drives exit from mitosis and entry into the first embryonic cell cycle. The enzymatic activity that causes metaphase arrest is CSF; it appears in meiosis II during oocyte maturation and disappears shortly after fertilization (1). Microinjection of CSF into blastomeres of cleaving embryos causes arrest in metaphase of the next cell cycle (CSF arrest).

CSF has not been purified, and therefore its molecular composition is unknown. However, the MAPK pathway is required for the generation of CSF activity. The *c-mos* proto-oncogene product (Mos), which is a MAPK kinase, is synthesized during meiosis in *Xenopus* and mice, and its expression is sufficient to produce CSF arrest in injected blastomeres (2, 3). CSF arrest can also be induced by a thiophosphorylated, phosphatase-resistant form of MAPK (4).

In *Xenopus* eggs and other systems, the 90-

kD ribosomal protein S6 kinase (p90^{Rsk}) is directly phosphorylated and activated by MAPK (5); during oocyte maturation, activation of p90^{Rsk} closely parallels that of MAPK, and both enzymes are dephosphorylated and deactivated after fertilization, when CSF activity also disappears (3, 6). Cloning of p90^{Rsk} in *Xenopus* and mammalian systems revealed a structure with two distinct kinase domains (7). Three different isoforms of p90^{Rsk}, termed Rsk1, Rsk2, and Rsk3, are present in mammalian cells, and all have the same basic two-domain structure (7, 8). Activation of p90^{Rsk} requires phosphorylation at two specific sites (Thr⁵⁷⁰ and Ser³⁶²) by MAPK and autophosphorylation at a specific site (Ser³⁷⁸) by the COOH-terminal kinase domain (Fig. 1A) (9). Activity also requires phosphorylation by an unidentified kinase of a site in the middle of the activation loop of the NH₂-terminal kinase domain (Ser²²⁰) (9).

To evaluate a possible role for p90^{Rsk} in mediating CSF arrest, we generated a constitutively active form of the enzyme. Inasmuch as the phosphorylation of exogenous substrates by p90^{Rsk} is mediated by the NH₂-terminal kinase domain (10), we generated a construct in which the COOH-terminal domain was deleted completely with or without a truncation of the NH₂-terminal domain similar to that which causes constitutive activation of MAPK kinase (11,

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