

A p90^{rsk} Mutant Constitutively Interacting with MAP Kinase Uncouples MAP Kinase from p34^{cdc2}/Cyclin B Activation in *Xenopus* Oocytes

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The efficient activation of p90^{rsk} by MAP kinase requires their interaction through a docking site located at the C-terminal end of p90^{rsk}. The MAP kinase p42^{mpk1} can associate with p90^{rsk} in G₂-arrested but not in mature *Xenopus* oocytes. In contrast, an N-terminally truncated p90^{rsk} mutant named D2 constitutively interacts with p42^{mpk1}. In this report we show that expression of D2 inhibits *Xenopus* oocyte maturation. The inhibition requires the p42^{mpk1} docking site. D2 expression uncouples the activation of p42^{mpk1} and p34^{cdc2}/cyclin B in response to progesterone but does not prevent signaling through p90^{rsk}. Instead, D2 interferes with a p42^{mpk1}-triggered pathway, which regulates the phosphorylation and activation of Plx1, a potential activator of the Cdc25 phosphatase. This new pathway that links the activation of p42^{mpk1} and Plx1 during oocyte maturation is independent of p34^{cdc2}/cyclin B activity but requires protein synthesis. Using D2, we also provide evidence that the sustained activation of p42^{mpk1} can trigger nuclear migration in oocytes. Our results indicate that D2 is a useful tool to study MAP kinase function(s) during oocyte maturation. Truncated substrates such as D2, which constitutively interact with MAP kinases, may also be helpful to study signal transduction by MAP kinases in other cellular processes.

INTRODUCTION

Xenopus oocytes are naturally arrested in prophase (late G₂) of the first meiotic division and are induced to reinitiate meiosis and enter M-phase by progesterone stimulation. This process is called meiotic maturation and is associated with dramatic structural changes in the oocyte. During maturation the nucleus migrates to the cortex of the oocyte, and the nuclear membrane disappears (this is known as germinal vesicle breakdown [GVBD]) concomitant with the appearance of a white spot at the animal pole of the oocyte. The chromosomes also condense and a metaphase spindle assembles. The meiotic maturation is associated with the activation of the ubiquitous M-phase- or maturation-promoting factor (MPF) (Masui and Markert, 1971; Masui and Clarke, 1979), which is required for the initiation of mitosis and is also likely to be responsible for most of the structural changes associated with the progression through M-phase.

MPF is a heterodimer of a B-type cyclin and the p34^{cdc2} protein kinase (reviewed by Nurse, 1990).

An essential requirement for progesterone-induced meiotic reinitiation is the translation of dormant maternal mRNAs stored in the oocyte. De novo synthesis of several proteins takes place during oocyte maturation (Sagata *et al.*, 1988; Nebreda *et al.*, 1995; Barkoff *et al.*, 1998), one of them being the Mos protein kinase (Sagata *et al.*, 1988, 1989; Freeman *et al.*, 1990; Sheets *et al.*, 1995). Mos is an efficient activator of the MAP kinase pathway (Nebreda *et al.*, 1993a; Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993), and several lines of evidence indicate that the activation of the *Xenopus* MAP kinase p42^{mpk1} (which is most similar to the mammalian ERK1/ERK2 MAP kinases) is required for meiotic reinitiation and p34^{cdc2}/cyclin B activation (reviewed by Kosako *et al.*, 1994a; Gotoh and Nishida, 1995). For example, injection of inhibitory anti-MAP kinase antibodies (Kosako *et al.*, 1994b, 1996) or a specific MAP kinase phosphatase (Gotoh *et al.*, 1995) inhibits meiotic reinitiation. On the other hand, injection of constitutively active MAP kinase kinase or thio-phosphorylated p42^{mpk1} induces meiotic reinitiation in the absence of progesterone (Matsuda *et al.*, 1992; Haccard *et al.*, 1995; Huang *et al.*, 1995). It has also been reported that MPF can

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Abbreviations used: GVBD, germinal vesicle breakdown; H1K, histone H1 kinase; IgG, immunoglobulin G; MPF, M-phase- or maturation-promoting factor.

trigger p42^{mpk1} activation, suggesting the existence of a positive feedback loop that links the activation of p42^{mpk1} and MPF (Gotoh *et al.*, 1991; Matsuda *et al.*, 1992). This may explain why the activation of p42^{mpk1} and MPF is coupled in progesterone-treated oocytes (Ferrell and Machleder, 1998). A positive feedback loop has also been described between p42^{mpk1} and the synthesis and/or stability of Mos (Matten *et al.*, 1996; Roy *et al.*, 1996; Howard *et al.*, 1999).

G₂-arrested oocytes contain a stockpile of p34^{cdc2}/cyclin B complexes, which are inactivated by the phosphorylation of p34^{cdc2} on Thr-14 and Tyr-15 (Cyert and Kirschner, 1988; Gautier and Maller, 1991; Kobayashi *et al.*, 1991). The kinase responsible for these inhibitory phosphorylations in oocytes is most likely to be Myt1, a membrane-associated member of the Wee1 protein kinase family (Atherton-Fessler *et al.*, 1994; Kornbluth *et al.*, 1994; Mueller *et al.*, 1995; Palmer *et al.*, 1998). The dephosphorylation of p34^{cdc2} at the onset of M-phase is triggered by the activation of the phosphatase Cdc25 (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). In G₂-arrested oocytes, Cdc25 is maintained inactive by Chk1 phosphorylation and association with 14-3-3 proteins (Nakajo *et al.*, 1999). The activation of the p34^{cdc2}/cyclin B complexes in progesterone-treated oocytes may be brought about by Myt1 inhibition and/or Cdc25 activation. Myt1 and Cdc25 are both hyperphosphorylated in mature oocytes as in M-phase-arrested extracts, and this correlates with the inhibition of Myt1 and the activation of Cdc25 (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Mueller *et al.*, 1995; Booher *et al.*, 1997; Palmer *et al.*, 1998). Both proteins can be phosphorylated by p34^{cdc2}/cyclin B complexes *in vitro* (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994; Booher *et al.*, 1997; Palmer *et al.*, 1998), and in the case of Cdc25 this phosphorylation up-regulates its phosphatase activity (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994). The ability of MPF to regulate Cdc25 (and potentially Myt1) constitutes a positive feedback loop, the so-called MPF autoamplification process, by which a small amount of active p34^{cdc2}/cyclin B would be able to trigger the activation of more p34^{cdc2}/cyclin B from the stored pre-MPF complexes (Masui and Markert, 1971). However, p34^{cdc2}/cyclin B is unlikely to be the triggering kinase that phosphorylates Myt1 and Cdc25 during oocyte maturation. We have recently found that the C-terminal regulatory domain of Myt1 can be efficiently phosphorylated by the p42^{mpk1}-activated protein kinase p90^{rsk}. Moreover, phosphorylation by p90^{rsk} decreases the inhibitory activity of Myt1 on p34^{cdc2}/cyclin B complexes *in vitro*, thereby providing a potential link between p42^{mpk1} and MPF activation at the onset of meiotic maturation (Palmer *et al.*, 1998). On the other hand, p90^{rsk} does not phosphorylate the N-terminal regulatory domain of Cdc25 (Palmer *et al.*, 1998), but this is efficiently phosphorylated by the Polo-like kinase of *Xenopus* Plx1 (Kumagai and Dunphy, 1996). Plx1 phosphorylation can activate Cdc25 *in vitro*, suggesting that Plx1 may be one of the triggering kinases responsible for the phosphorylation and activation of Cdc25 at the onset of M-phase (Kumagai and Dunphy, 1996; Qian *et al.*, 1998a). A protein kinase that can phosphorylate and activate Plx1 has been recently purified and cloned from *Xenopus* eggs and named xPlkk1 (Qian *et al.*, 1998b).

p90^{rsk} contains two protein kinase domains (Jones *et al.*, 1988): the N-terminal domain (D1) is required for the phosphorylation of exogenous substrates, and the C-terminal domain (D2) is involved in p90^{rsk} autophosphorylation (Bjørbaek *et al.*, 1995; Leighton *et al.*, 1995; Fischer and Blenis, 1996). Association between p90^{rsk} family members and ERK MAP kinases has been detected in various cell types, including *Xenopus* oocytes, mammalian fibroblasts, and PC12 cells (Scimeca *et al.*, 1992; Hsiao *et al.*, 1994; Zhao *et al.*, 1996). In G₂-arrested *Xenopus* oocytes the unphosphorylated and inactive p90^{rsk} is complexed with inactive p42^{mpk1}, but the complex dissociates during oocyte maturation concomitant with the phosphorylation and activation of both protein kinases (Hsiao *et al.*, 1994).

There is growing evidence that the efficient phosphorylation of some substrates requires specific sequences, which facilitate the kinase-substrate interaction and are distinct from the phosphoacceptor sites. These sequences, usually referred to as docking sites, were first described to be important for the phosphorylation of the transcription factor c-jun by JNK/MAP kinases (Kallunki *et al.*, 1994). Docking sites have been later identified on Smads phosphorylated by transforming growth factor- β receptors (Chen *et al.*, 1998; Lo *et al.*, 1998), on some cdk2/cyclin A substrates (Adams *et al.*, 1996; Schulman *et al.*, 1998), and on the transcription factor Elk-1 (Yang *et al.*, 1998a,b; Jacobs *et al.*, 1999). The interaction between Elk-1 and activating ERK, JNK, and p38 MAP kinases appears to involve several distinct and overlapping interaction motifs, which have been suggested to work either synergistically or competitively (Jacobs *et al.*, 1999). We and others have also recently characterized a novel MAP kinase docking site located at the C terminus of p90^{rsk}, which is required for the efficient phosphorylation and activation of p90^{rsk} both *in vitro* and *in vivo* (Gavin and Nebreda, 1999; Smith *et al.*, 1999). This docking site is specific for ERK MAP kinases and shows no amino acid sequence similarity with other MAP kinase docking sites, suggesting that it may represent the prototype of a novel ERK MAP kinase interaction motif.

In this report we use an N-terminally truncated form of p90^{rsk} named D2, which constitutively interacts with p42^{mpk1}, to investigate the function(s) of p42^{mpk1} during oocyte maturation. Our results indicate that activation of p42^{mpk1} normally precedes the activation of pre-MPF in progesterone-treated oocytes. We also found that p42^{mpk1} can trigger an MPF-independent pathway that leads to the activation of Plx1 and that the activation of p42^{mpk1} in the absence of pre-MPF activation can trigger nuclear migration in *Xenopus* oocytes.

MATERIALS AND METHODS

cDNA Cloning and Site-directed Mutagenesis

Full-length *Xenopus* p90^{rsk} α and the N-terminal (D1, amino acids 1–308) and C-terminal (D2, amino acids 309–733) domains alone were cloned in the FTX5 expression vector as described (Palmer *et al.*, 1998). The mutants D2/KR (Lys-445 changed to Arg), D2 Δ 43 (stop codon following amino acid 690), and p90^{rsk} 6xAla (phosphorylation sites at positions Ser-221, Thr-359, Ser-363, Ser-380, Thr-571, and Ser-730 changed to Ala) were prepared using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. To prepare D1+43Ct, the C-terminal 43 amino acids of p90^{rsk} were amplified using a 5' oligonucle-

otide that introduced an *Nco*I site at position 2069 of p90^{rsk} (Lys-691 was changed to Met) and a 3' oligonucleotide that introduced a *Bam*HI site downstream of the stop codon. The PCR product was digested with *Nco*I and *Bam*HI and subcloned into the corresponding sites of the plasmid FTX5. The resulting plasmid FTX5-43Ct was linearized with *Nco*I and ligated to the *Nco*I-*Nco*I D1 fragment from FXT5-D1.

FTX5-p42^{mpk1} was prepared by subcloning a PCR fragment containing the p42^{mpk1} coding sequence from pMalcRI-p42^{mpk1} (Rouse *et al.*, 1994) into FTX5 previously digested with *Bam*HI and *Xho*I. The mutant p42^{mpk1} AEF (Thr-187 and Tyr-189 changed to Ala and Phe, respectively) was prepared using the QuikChange site directed mutagenesis kit. FTX5-Mos was prepared by subcloning a 1.2-kb *Nco*I-*Xho*I DNA fragment containing the full-length *Xenopus* c-mos protooncogene from MLV-Mos (Nebreda *et al.*, 1993a) into *Nco*I-*Xho*I digested FTX5.

Xp9 was amplified from a *Xenopus* oocyte cDNA library using oligonucleotides that created *Eco*RI sites both upstream of the first ATG and downstream of the stop codon. A PCR product with the same sequence as the cDNA described by Patra and Dunphy (1996) was cloned into *Eco*RI-digested FTX5.

Oocyte Maturation and Expression of myc-tagged Proteins

In vitro-transcribed mRNAs were obtained from FTX5 constructs linearized with either *Xba*I (p90^{rsk}, D1, D2, Mos, and Xp9) or *Xmn*I (p42^{mpk1}) using the MEGAscript kit (Ambion, Austin, TX) according to the manufacturer's instructions. The mRNAs were resuspended in 25 μ l of diethylpyrocarbonate-treated water and further diluted 1:5 in diethylpyrocarbonate-treated water before microinjection.

Fully grown oocytes were sorted either manually or after collagenase B treatment (Boehringer Mannheim, Indianapolis, IN; 0.5 mg/ml, 30–60 min) and left at 18°C in mBarth for at least 2 h before injection. Meiotic maturation was induced by incubation with 5 μ g/ml progesterone (Sigma, St. Louis, MO) or by injection with 50 nl of either malE-mos protein (40 ng, Nebreda and Hunt, 1993) or Mos mRNA. Maturation was scored by the appearance of a white spot at the animal pole of the oocyte, and GVBD was confirmed after either fixation in 5% trichloroacetic acid or by boiling for 90 s in PBS.

The oocytes were lysed in 10 μ l per oocyte histone H1 kinase (H1K) buffer (80 mM β -glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 2.5 mM benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 mM DTT, pH 7.4) and centrifuged at 14,000 rpm (Eppendorf centrifuge), and the supernatant (oocyte lysate) was stored at -70°C. To prepare concentrated extracts, oocytes were lysed in 1–2 μ l per oocyte H1K buffer or lysis buffer (80 mM β -glycerophosphate, pH 7.4, 10 mM EDTA, 1 mM sodium orthovanadate, 2 mM PMSF, 2 μ M microcystin, 10 mM *p*-nitrophenylphosphate, 100 μ g/ml leupeptin, and 100 μ g/ml aprotinin) and clarified by centrifugation at 14,000 rpm for 90 s. The clear cytoplasm was removed from between the overlying lipid layer and the yolk protein and was further clarified by a second centrifugation at 14,000 rpm for 10 min. Concentrated oocyte extracts were stored in aliquots at -70°C.

Immunoblotting and Immunoprecipitation

Immunoblotting was performed as previously described (Palmer *et al.*, 1998) using the following antibodies: the monoclonal antibody 3E1 (provided by J. Gannon and T. Hunt, Imperial Cancer Research Fund, South Mimms, United Kingdom) for p34^{cdc2} (Nebreda *et al.*, 1995); the rabbit antiserum 3297.1 for p42^{mpk1} (Palmer *et al.*, 1998); a mixture of purified anti-Rsk1 and anti-Rsk2 goat antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for p90^{rsk}; a rabbit anti-Plx1 antiserum (provided by A. Tavares, C. Avides, and D. Glover, University of Dundee, Dundee, United Kingdom) for Plx1; and the 9E10 monoclonal antibody for myc-tagged proteins.

For the immunoprecipitation of myc-tagged proteins, 10 μ l of anti-myc agarose conjugate (Santa Cruz; sc-40 AC) was incubated with 70 μ l of oocyte lysate for 2 h at 4°C. The beads were then washed three times in immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF, 100 μ M NaVO₄, 1 mM PMSF, 2.5 mM benzamidine, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 2 μ M microcystin) and once in S6 kinase buffer (see below) and immediately used for an in vitro kinase assay. Coimmunoprecipitation experiments were performed essentially as described above, except that 40–70 μ l of concentrated oocyte extracts were used.

For the immunoprecipitation of p42^{mpk1}, we used an anti-ERK2 antibody conjugated to agarose (Santa Cruz). As a control, we used purified rabbit immunoglobulin G (IgG; Sigma) covalently bound to protein A beads using dimethylpimelidate. Concentrated oocyte extracts (50–100 μ l) were precleared with 20 μ l of protein A beads for 30 min at 4°C and incubated for 2 h at 4°C with the bead-bound antibody. The beads were then washed three times in either H1K buffer supplemented with 2 μ g/ml microcystin, 4 mM *p*-nitrophenylphosphate, 1 mM PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin or lysis buffer and then used for immunoblotting.

For the immunoprecipitation of Plx1, 7 μ l of oocyte lysate were diluted to 200 μ l in buffer B (50 mM Tris, pH 8.0, 0.5% NP-40, 120 mM NaCl, 20 mM EDTA, and 1 mM DTT) and mixed with 5 μ l of anti-Plx1 antiserum and 20 μ l of protein A-Sepharose beads. After 2 h, the beads were washed twice in 10 mM Tris, pH 7.0, 0.1% NP-40, 1 M NaCl, and 1 mM DTT, twice in buffer B, and twice in kinase buffer (20 mM HEPES, pH 7.2, 2 mM DTT, 10 mM MgCl₂, 0.1 mM EGTA, and 0.01% Brij 35).

The immunoprecipitation of endogenous p90^{rsk} was performed as described by Palmer *et al.* (1998) but using a mixture of both anti-Rsk1 and anti-Rsk2 antibodies (Santa Cruz).

In Vitro Kinase Assays

Myc-tagged and endogenous p90^{rsk} immunoprecipitates were assayed for 40 min at room temperature in a final volume of 15 μ l of S6 kinase buffer (50 mM 3-morpholinopropanesulfonic acid, pH 7.2, 1 mM DTT, 10 mM MgCl₂, 20 mM *p*-nitrophenylphosphate, 0.1% Triton X-100, 1 mM NaVO₃, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 mM PMSF or 4-(2-aminoethyl)-benzenesulfonyl fluoride) containing 100 μ M ATP, 5 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and 0.4 μ g of GST-Myt1 Ct (Palmer *et al.*, 1998).

Plx1 immunoprecipitates were incubated for 30 min at 30°C in 15 μ l of kinase buffer containing 67 μ M ATP, 5 μ Ci [γ -³²P]ATP, and 1 μ g/ml dephosphorylated casein (Sigma).

Phosphorylation reactions were analyzed by SDS-PAGE followed by Coomassie staining and autoradiography.

Gel Filtration

Concentrated oocyte extracts were further clarified by centrifugation at 50,000 rpm in a Beckman Instruments (Palo Alto, CA) TLA 100 rotor at 4°C for 1 h. Samples (100 μ l) from 100 oocytes were chromatographed on a Superose 12 column (Pharmacia, Piscataway, NJ; flow rate 0.4 ml/min) equilibrated with lysis buffer containing 80 mM β -glycerophosphate, pH 7.4, 10 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM PMSF, 0.2 μ M microcystin, 0.5 mM *p*-nitrophenylphosphate, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin. Fractions of 100 μ l were collected and analyzed by immunoblotting. The molecular mass standards were aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa).

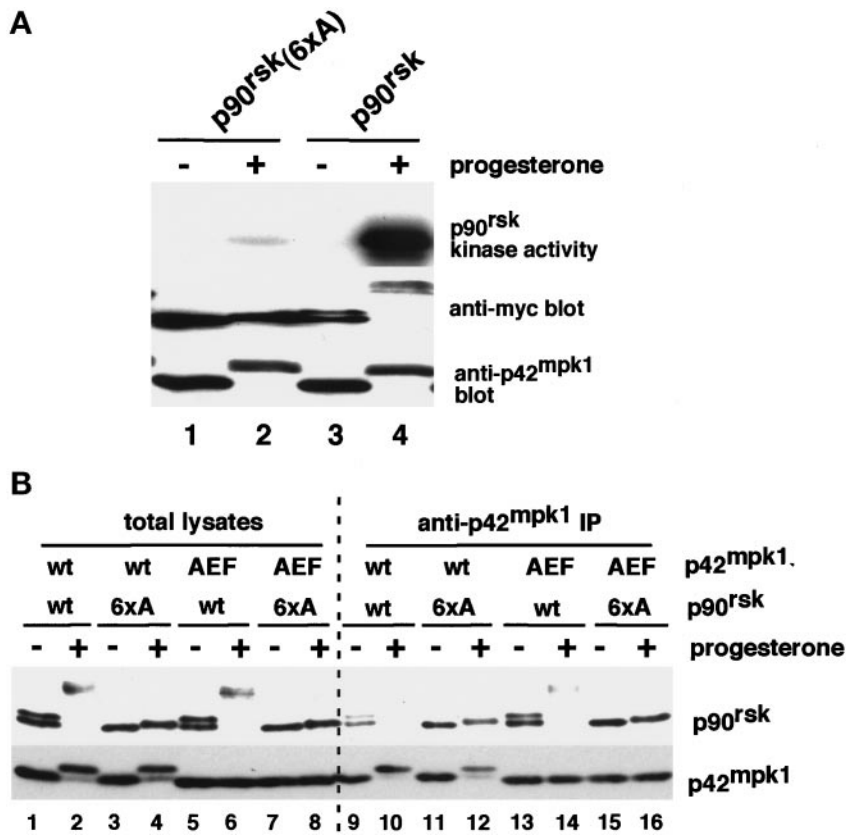


Figure 1. Mutation to alanine of six phosphorylatable residues in p90^{rsk} impairs its activation by progesterone and its ability to dissociate from p42^{mpk1}. (A) Lysates were prepared from untreated or progesterone-treated oocytes expressing either myc-tagged p90^{rsk} or p90^{rsk} (6xA) and were analyzed by immunoblotting using anti-myc and anti-p42^{mpk1}, as indicated. Anti-myc immunoprecipitates were prepared from the same oocyte lysates, and their associated kinase activity was assayed using GST-Myt1 as an *in vitro* substrate. (B) Lysates were prepared from either untreated or progesterone-treated oocytes expressing the indicated p90^{rsk} and p42^{mpk1} proteins and were immunoprecipitated with anti-p42^{mpk1} antibodies. The total lysates (lanes 1–8) and the anti-p42^{mpk1} immunoprecipitates (lanes 9–16) were analyzed by immunoblotting with anti-p90^{rsk} and anti-myc antibodies.

RESULTS

A N-Terminally Truncated Form of p90^{rsk} Constitutively Interacts with p42^{mpk1}

In G₂-arrested *Xenopus* oocytes, p90^{rsk} and p42^{mpk1} are associated in a complex that dissociates upon activation of both kinases (Hsiao *et al.*, 1994), suggesting that the interaction may be regulated by the phosphorylation state of either p90^{rsk} or p42^{mpk1} or both. To investigate this possibility, we prepared a p90^{rsk} mutant with the amino acids that are known to be phosphorylated upon activation (Dalby *et al.*, 1998), being replaced by nonphosphorylatable Ala residues (p90^{rsk}/6xA). As expected, this mutated p90^{rsk} was neither hyperphosphorylated nor activated when expressed in progesterone-treated oocytes (Figure 1A, compare lanes 2 and 4 in the upper and middle panels). We also prepared a p42^{mpk1} mutant in which the sequence TEY in the activation loop was changed to AEF to prevent phosphorylation. The wild-type and mutant forms of both p90^{rsk} and p42^{mpk1} were coexpressed in oocytes, which were either left untreated or treated with progesterone to induce the activation of the p42^{mpk1} pathway and meiotic reinitiation (Figure 1B). The association between p90^{rsk} and p42^{mpk1} was determined by anti-p42^{mpk1} immunoprecipitation followed by immunoblotting using an anti-p90^{rsk} antibody (Figure 1B). The wild-type p42^{mpk1} associated with the wild-type p90^{rsk} only in G₂-arrested oocytes (Figure 1B, lanes 9 and 10). Similarly, p42^{mpk1}/AEF interacted much more efficiently with the unphosphorylated than with the phosphorylated p90^{rsk} (Fig-

ure 1B, lanes 13 and 14), suggesting that the interaction between p42^{mpk1} and p90^{rsk} might be regulated by the phosphorylation of p90^{rsk}. Consistent with this possibility, the nonphosphorylatable mutant p90^{rsk}/6xA was able to interact with p42^{mpk1} both in prophase- and progesterone-treated oocytes (Figure 1B, lanes 11, 12, 15, and 16), indicating that the phosphorylation of p90^{rsk} is responsible for the dissociation of the p42^{mpk1}/p90^{rsk} complex.

We have previously shown that a truncated form of p90^{rsk} named D2, which lacks the N-terminal kinase domain (Figure 2A), was able to bind to p42^{mpk1}. This interaction was mediated by a p42^{mpk1} docking site, which was located at the C-terminal 25 amino acids of p90^{rsk} (Gavin and Nebreda, 1999). Moreover, both nonphosphorylated and phosphorylated GST-D2 were able to bind p42^{mpk1} with similar affinity in pull-down experiments (Gavin and Nebreda, 1999). To further characterize the ability of this truncated p90^{rsk} to interact with p42^{mpk1}, myc-tagged D2 was expressed in *Xenopus* oocytes, which were then either treated with progesterone or left untreated. The association between p42^{mpk1} and either p90^{rsk} or D2 was determined by anti-p42^{mpk1} immunoprecipitation followed by immunoblotting using either anti-p90^{rsk} or anti-myc antibodies (Figure 2B). As expected, p90^{rsk} was found to coimmunoprecipitate with p42^{mpk1} in control oocytes (Figure 2B, lanes 1 and 3) but not in progesterone-treated oocytes, in which p90^{rsk} is phosphorylated (Figure 2B, lanes 2 and 4). In contrast, D2 associated with p42^{mpk1} both in control and in progesterone-treated oocytes (Figure 2B, lanes 3 and 4, anti-myc blot).

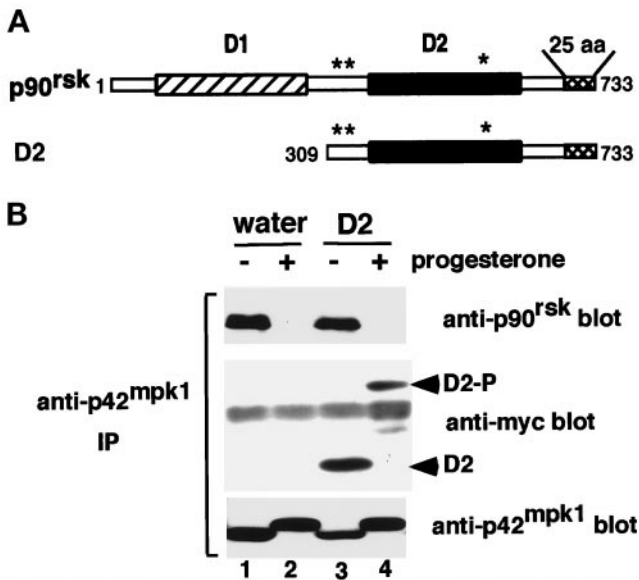


Figure 2. The C-terminal D2 domain of p90^{rsk} constitutively interacts with endogenous p42^{mpk1} in oocytes. (A) Schematic representation of full-length p90^{rsk} indicating the two kinase domains, D1 and D2, the p42^{mpk1} docking site located in the last 25 amino acids (25 aa) and the three p42^{mpk1} phosphorylation sites (asterisks). The N-terminally truncated p90^{rsk} protein D2 is also shown. (B) Oocytes injected with water (lanes 1 and 2) or expressing myc-tagged D2 (lanes 3 and 4) were induced to mature by progesterone or not as indicated. Anti-p42^{mpk1} immunoprecipitates prepared from the oocyte lysates were analyzed by immunoblotting with anti-p90^{rsk}, anti-myc and anti-p42^{mpk1} antibodies. The unphosphorylated and hyperphosphorylated D2 are indicated as D2 and D2-P, respectively.

Moreover, the electrophoretic mobility of D2 was significantly reduced upon progesterone treatment (Figure 2B, lanes 3 and 4), suggesting that it became hyperphosphorylated. This is consistent with previous reports showing that the major phosphorylation sites for p42^{mpk1} map in the C-terminal half of p90^{rsk} (Dalby *et al.*, 1998).

We further tested the ability of D2 to interact with p42^{mpk1} by gel filtration chromatography. Oocyte lysates were chromatographed on a Superose 12 column, and the fractions were analyzed by immunoblotting (Figure 3). In lysates prepared from G₂-arrested oocytes, p42^{mpk1} exhibited a broad size distribution, which, as described by others (Hsiao *et al.*, 1994), may correspond to two overlapping peaks of ~40 and 110 kDa (Figure 3, control/water). After progesterone treatment, the high-molecular-weight peak of p42^{mpk1} disappeared (Figure 3, progesterone/water), consistent with p42^{mpk1} being mainly present in its monomeric form (Hsiao *et al.*, 1994). Interestingly, in lysates prepared from D2-expressing oocytes, a significant amount of p42^{mpk1} was shifted toward a higher-molecular-weight form, which co-eluted with D2 (Figure 3, control/D2). Moreover, the pattern of elution of p42^{mpk1} (and D2) was not changed by progesterone treatment of the oocytes, despite the hyperphosphorylation of D2 (Figure 3, progesterone/D2). These results confirm that D2 interacts with p42^{mpk1} in the oocytes and that, in contrast to full-length p90^{rsk}, the association between D2 and p42^{mpk1} does not appear to be regulated by the phosphorylation state of D2.

Overexpression of the p42^{mpk1} Docking Site Negatively Regulates Oocyte Maturation

The overexpression of the p42^{mpk1} docking site located at the C terminus of p90^{rsk} interferes with the phosphorylation

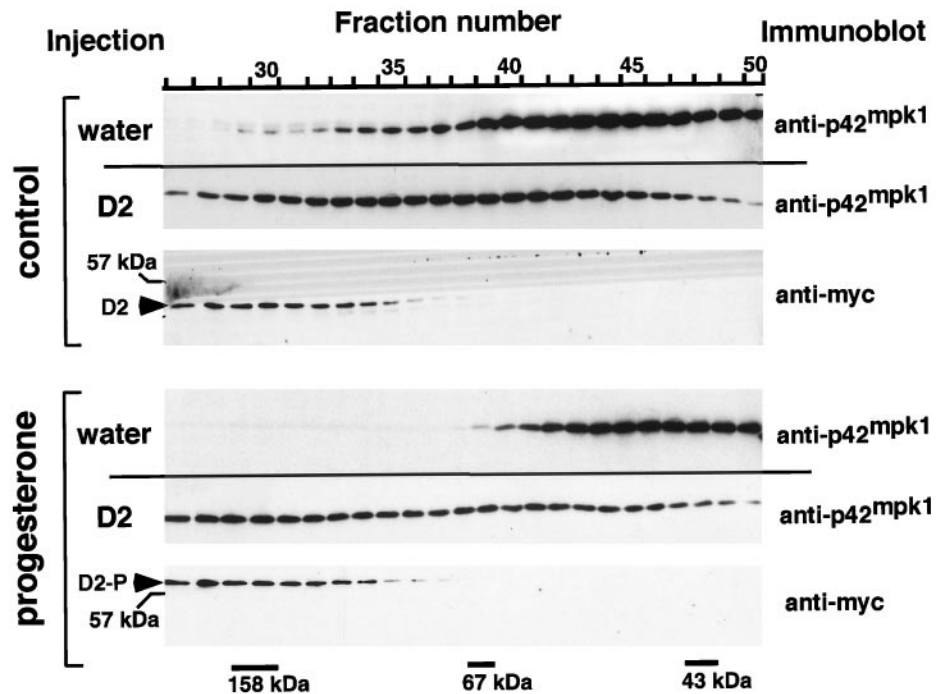


Figure 3. D2 expressed in oocytes comigrates with endogenous p42^{mpk1} in a high-molecular-weight complex upon gel filtration chromatography. Oocytes injected with water or expressing myc-tagged D2 were induced to mature by progesterone or not as indicated. The oocyte lysates were separated by gel filtration chromatography on Superose 12, and the fractions were analyzed by immunoblot using anti-myc and anti-p42^{mpk1} antibodies. The elution of the molecular weight markers is indicated at the bottom. The unphosphorylated and hyperphosphorylated D2 are indicated as D2 and D2-P, respectively.

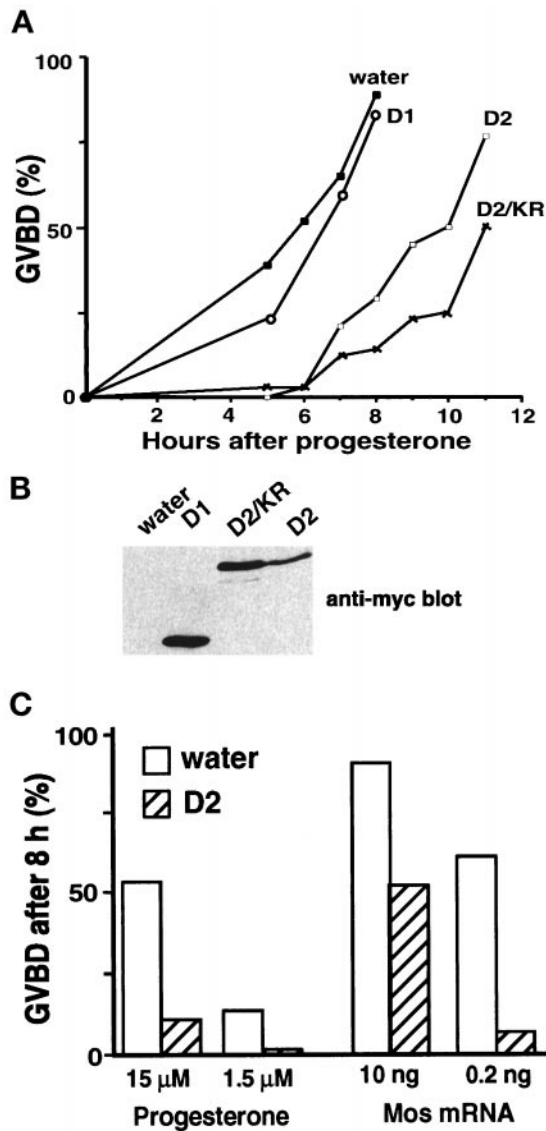


Figure 4. Expression of D2 inhibits oocyte maturation. (A) Groups of 25–30 oocytes were injected with water or mRNAs encoding the indicated proteins and after overnight incubation were treated with progesterone and scored for GVBD. D2 was found to inhibit or delay progesterone-induced maturation in 27 of 34 experiments. (B) Lysates were prepared from the same oocytes as in A after overnight incubation and analyzed by immunoblotting using anti-myc antibodies. (C) Groups of 25–30 oocytes were injected with water or D2 mRNA, incubated overnight, and then either injected with Mos mRNA or incubated with progesterone. GVBD was scored 8 h later.

of p90^{rsk} by p42^{mpk1} in reticulocyte lysates (Gavin and Nebreda, 1999). We wanted to investigate whether D2, which contains the p42^{mpk1} docking site and constitutively interacted with p42^{mpk1}, could also inhibit signaling by p42^{mpk1} in oocytes. We observed that expression of D2 in oocytes significantly delayed the kinetics of progesterone-induced maturation (Figure 4A). As a control, expression of similar levels of the D1 catalytic domain alone (Figure 4B) did not

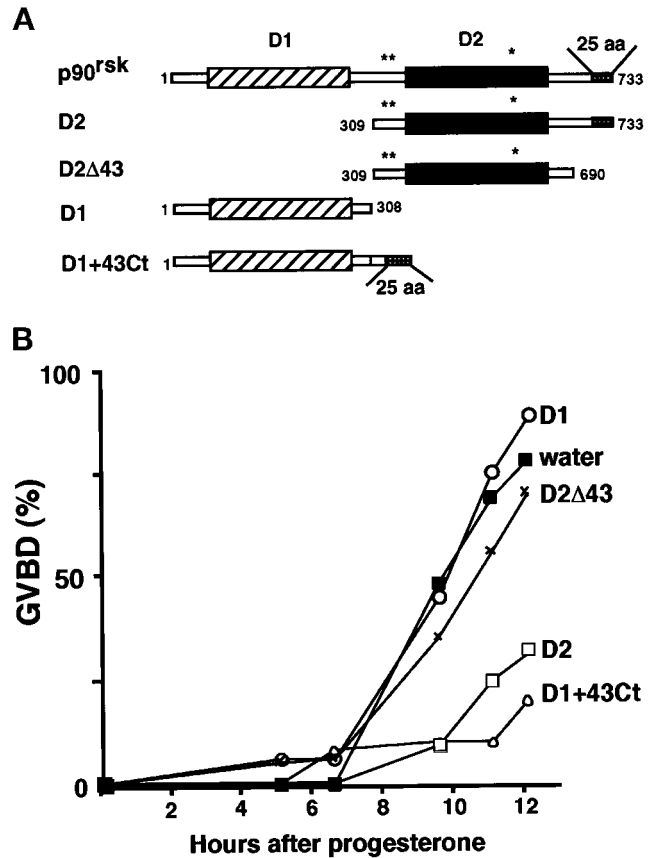


Figure 5. Expression of the p42^{mpk1} docking site is necessary and sufficient to inhibit oocyte maturation. (A) Schematic representation of the recombinant proteins expressed in oocytes. (B) Groups of 25–30 oocytes were injected with water or mRNAs encoding the indicated proteins and after overnight incubation were treated with progesterone and scored for GVBD.

affect meiotic reinitiation (Figure 4A). Moreover, a catalytically inactive mutant of D2 (D2/KR) was as efficient as D2 in delaying oocyte maturation (Figure 4A), indicating that the autophosphorylation activity of D2 (Fischer and Blenis, 1996; our unpublished data) was not required for the inhibition of oocyte maturation. The inhibitory effect of D2 was also observed when oocyte maturation was induced by the injection of either Mos mRNA or bacterially produced Mos protein (Figure 4C; also see Figure 7A).

To investigate whether the inhibitory effect of D2 required the p42^{mpk1} docking site, we used a truncated form of D2, which lacks the last 43 amino acids (Figure 5A, D2 Δ 43) and does not interact with p42^{mpk1} (Gavin and Nebreda, 1999). We also used a chimera in which the last 43 amino acids of p90^{rsk} have been added to the C terminus of D1 (Figure 5A, D1+43Ct) resulting in a fusion protein that can interact with p42^{mpk1} in oocytes (Gavin and Nebreda, 1999). Oocytes were injected with either water or the mRNAs encoding these proteins and then were treated with progesterone. We found that truncation of the C-terminal 43 amino acids abrogated the inhibitory effect of D2 (Figure 5B, D2 Δ 43). Furthermore, in contrast to D1 alone, which had no effect on

progesterone-induced maturation, D1+43Ct also delayed the kinetics of maturation to the same extent as D2 (Figure 5B). These results indicate that overexpression of the p42^{mpk1} docking site is able to inhibit the meiotic reinitiation in oocytes.

D2 Uncouples the Activation of p42^{mpk1} and MPF in Response to Progesterone

The observation that D2 can also interfere with oocyte maturation induced by Mos (Figures 4C) suggested that it might inhibit downstream of p42^{mpk1}. To test this possibility, oocytes were injected with either water or the D1 or D2 mRNAs and then treated with progesterone. When the oocytes started to mature (as determined by the appearance of a white spot), single oocytes were taken and analyzed by immunoblotting with anti-p42^{mpk1} and anti-p34^{cdc2} antibodies. The activation of p42^{mpk1} was scored based on the upward mobility shift of the p42^{mpk1} protein in SDS-PAGE, which correlates with its phosphorylation and the activation of pre-MPF for the disappearance of the slow-migrating p34^{cdc2} band, which corresponds to tyrosine-phosphorylated and cyclin B-bound p34^{cdc2} (Figure 6). We found that in every progesterone-treated oocyte that had a white spot there was activation of both p42^{mpk1} and pre-MPF, independently of whether they had been injected with water or the D1 or the D2 mRNAs (Figure 6, A, white spot, and B, ws, show a representative example). When progesterone-treated oocytes without a white spot were analyzed, we found two categories of oocytes from each injection with either water or the D1 mRNA (Figure 6, A, No white spot, and B, No ws): oocytes with neither p42^{mpk1} nor pre-MPF activation, which looked like G2-arrested oocytes (Figure 6, A, control, and B and C) or oocytes with both kinases in an active state as in oocytes with a white spot (these oocytes were probably about to undergo maturation when they were collected). This confirmed previous work showing that the activation of p42^{mpk1} is always coupled to the activation of preformed p34^{cdc2}/cyclin B complexes (pre-MPF) in response to progesterone stimulation (Ferrell and Machleder, 1998). Interestingly, when we analyzed single progesterone-treated oocytes expressing D2, we found that about half of the oocytes without a white spot fell into a third category in which p42^{mpk1} was partially activated (>40% of the p42^{mpk1} protein showed reduced electrophoretic mobility), but p34^{cdc2} was tyrosine phosphorylated, and thus preMPF was still inactive (Figure 6, A and B, D2).

The uncoupling between the activation of p42^{mpk1} and MPF in response to progesterone suggested that D2 interferes with p42^{mpk1} signaling in oocytes. These results also indicate that p42^{mpk1} activation normally precedes the activation of pre-MPF during progesterone-induced oocyte maturation. The partial activation of p42^{mpk1} is probably a consequence of the lack of an MPF-activated positive feedback loop, which has been proposed to be required for full p42^{mpk1} activation in response to progesterone (Gotoh *et al.*, 1991; Matsuda *et al.*, 1992). Consistent with this possibility, we observed that D2 expression in Mos-injected oocytes delayed GVBD (Figure 7A) and the activation of pre-MPF (Figure 7B, lower panel) but did not interfere with the activation of p42^{mpk1} (Figure 7B, middle panel). This suggested that D2 does not directly prevent p42^{mpk1} activation and is

more likely to act downstream of p42^{mpk1}, uncoupling the activation of p34^{cdc2}/cyclin B from the activation of p42^{mpk1}.

D2 Does Not Prevent p90^{rsk} Activation

We have recently shown that after activation by p42^{mpk1}, p90^{rsk} associates with and phosphorylates the p34^{cdc2} inhibitory kinase Myt1 (Palmer *et al.*, 1998). Thus, one possibility is that D2 uncouples p42^{mpk1} from p34^{cdc2}/cyclin B activation by interfering with p42^{mpk1} signaling through p90^{rsk}.

We observed, however, that overexpression of D2 inhibits neither the phosphorylation nor the activation of p90^{rsk} when p42^{mpk1} was activated by Mos injection (Figure 7B, upper panel; see Figure 9D). Furthermore, we could detect approximately the same amount of p90^{rsk} in the anti-p42^{mpk1} immunoprecipitates prepared from either control or D2-expressing oocytes (Figure 2B), suggesting that D2 did not disrupt the endogenous p42^{mpk1}/p90^{rsk} complexes in G₂-arrested oocytes. These results were confirmed when two consecutive rounds of anti-p42^{mpk1} immunoprecipitation were performed (Figure 7C).

A Possible Role for p42^{mpk1} Upstream of Plx1

Because D2 did not interfere with p42^{mpk1} signaling through p90^{rsk}, we investigated alternative pathways that could potentially be regulated by p42^{mpk1}. One obvious candidate was the Plx1/Cdc25 pathway (Kumagai and Dunphy, 1996; Abrieu *et al.*, 1998; Qian *et al.*, 1998a). To investigate the possible role of p42^{mpk1} upstream of Plx1, oocytes were injected with Mos to activate p42^{mpk1}. We then analyzed by immunoblotting the phosphorylation state of p42^{mpk1}, p34^{cdc2}, and Plx1 in single oocytes. As expected, the appearance of the white spot always correlated with the activation of pre-MPF (tyrosine dephosphorylation of p34^{cdc2}) and with the phosphorylation of both p42^{mpk1} and Plx1 (Figure 8A, GVBD). When Mos-injected oocytes not showing a white spot were analyzed, we observed some Plx1 phosphorylation occurring before any detectable tyrosine dephosphorylation of p34^{cdc2} (Figure 8A, Mos). This result was observed in three experiments of five; in the other two experiments Plx1 phosphorylation was coupled to the activation of preMPF (see example in Figure 9A). To confirm that Plx1 could be phosphorylated independently of pre-MPF activation, we injected the small p34^{cdc2}-binding protein Xp9, which can interfere with p34^{cdc2}/cyclin B activation and delay M-phase entry in *Xenopus* egg extracts (Patra and Dunphy, 1996). We found that expression of Xp9 inhibited Mos-induced (Figure 8B) as well as progesterone-induced (our unpublished results) oocyte maturation, as measured by the absence of GVBD. These Xp9-injected oocytes, however, had a less pigmented area, which resembled an irregular white spot (see below and Figure 10A), although the nucleus was still detected upon dissection of fixed oocytes. The activation of p42^{mpk1} by Mos was not affected in Xp9-expressing oocytes, whereas p34^{cdc2}/cyclin B activation was either blocked or severely delayed depending on the batch of oocytes (Figure 8A). However, Plx1 phosphorylation was still observed in the absence of pre-MPF activation in oocytes coinjected with Mos and Xp9 (Figure 8A).

The early Plx1 phosphorylation in Mos-injected oocytes was associated with a partial increase in the kinase activity of Plx1 immunoprecipitates (usually 20–50% of the activity

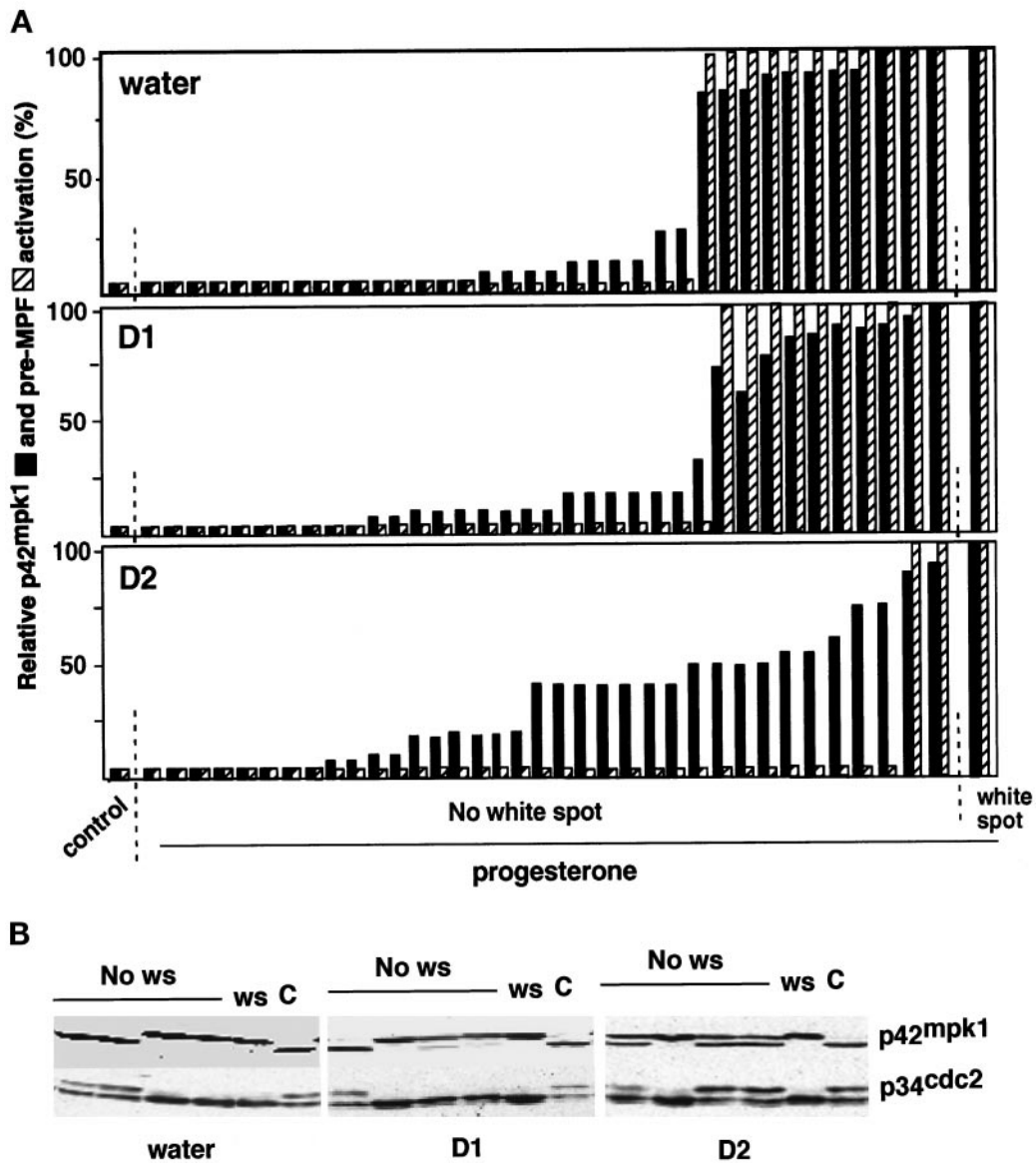


Figure 6. Expression of D2 uncouples the activation of p42^{mpk1} and pre-MPF in response to progesterone. (A) Groups of 30 oocytes were injected with water or mRNAs encoding either D1 or D2 and after overnight incubation were treated with progesterone. Every hour, starting 3 h after the addition of progesterone, two or three progesterone-treated oocytes without a white spot were transferred to dry ice. Untreated oocytes (control) and progesterone-treated oocytes that had a white spot were also taken for comparison. Oocyte lysates were prepared from single oocytes and analyzed by immunoblotting with anti-p42^{mpk1} and anti-p34^{cdc2} antibodies. Each bar represents a single oocyte. A total of 30 oocytes were analyzed in three independent experiments. The activation of p42^{mpk1} was scored by the phosphorylation of the protein, which causes its upward mobility shift in the blots. The activation of pre-MPF was scored for the disappearance in the blots of the slow migrating p34^{cdc2} form, which corresponds to Tyr-phosphorylated cyclin B-bound p34^{cdc2}. (B) Representative examples of the p42^{mpk1} and p34^{cdc2} immunoblots from single oocytes described in A.

measured in GVBD oocytes) (Figure 8C), in agreement with previous reports showing that the phosphorylation of Plx1 correlates with its activation (Qian *et al.*, 1998a; Karaiskou *et al.*, 1998). These results suggested that the activation of p42^{mpk1} could lead to partial Plx1 activation. However, full Plx1 activation apparently required the activation of MPF, which is consistent with the existence of a positive feedback loop linking p34^{cdc2}/cyclin B and Plx1 (Abrieu *et al.*, 1998;

Karaiskou *et al.*, 1998). Interestingly, although p42^{mpk1} can be activated as early as 1 h after Mos injection, the phosphorylation and activation of Plx1 was detected only 3–5 h later (Figure 8A). This suggested that the link between p42^{mpk1} and Plx1 was not direct. To determine whether Plx1 activation by p42^{mpk1} might require protein synthesis, oocytes were preincubated with cycloheximide before injection of the recombinant Mos protein. We observed that the phos-

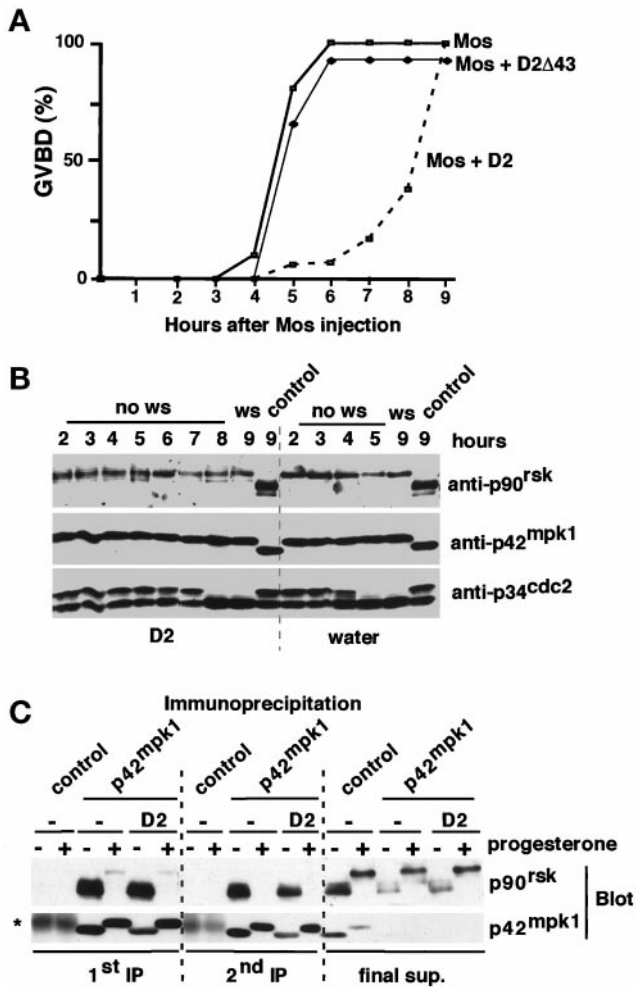


Figure 7. Expression of D2 does not affect the Mos-induced activation of p42^{mpk1} and p90^{rsk}. (A) Groups of 25–30 oocytes were injected with water or mRNAs encoding either D2 or D2Δ43 and after overnight incubation were injected with male-mos protein and scored for GVBD. (B) Lysates were prepared from the same oocytes as in A and analyzed by immunoblotting with anti-p90^{rsk}, anti-p42^{mpk1}, and anti-p34^{cdc2} antibodies. (C) Groups of 100 oocytes were injected with either water or D2 mRNA, incubated overnight, and then treated or not treated with progesterone for 12 h. Oocyte lysates were subjected to two sequential rounds of immunoprecipitation using either anti-p42^{mpk1} or control rabbit IgG. The immunoprecipitates and the final supernatant (after the second round of immunoprecipitation) were analyzed by immunoblotting with anti-p90^{rsk} and anti-p42^{mpk1} antibodies. The asterisk indicates a nonspecific band that probably corresponds to control IgG leaking from the beads.

phorylation of Plx1 induced by Mos injection was completely inhibited in the presence of cycloheximide (Figure 9A). The same results were obtained when Xp9 was overexpressed before Mos injection to uncouple Plx1 phosphorylation from the activation of pre-MPF. Altogether these results suggested that p42^{mpk1} can induce partial phosphorylation and activation of Plx1 via a pathway that requires protein synthesis but is independent of MPF activity. We

then investigated whether D2 expression could interfere with the p42^{mpk1}-dependent phosphorylation of Plx1. For this experiment, we coexpressed in oocytes Xp9 with either D2 or D2Δ43 and then injected Mos protein to activate p42^{mpk1}. We observed that expression of D2 but not D2Δ43 inhibited Plx1 phosphorylation in Mos/Xp9-injected oocytes, whereas p90^{rsk} activation was not significantly affected (Figure 9, B and D). This also correlated with a reduction in the extent of Plx1 activation in D2 versus D2Δ43-expressing oocytes (Figure 9C). The results suggested that D2 interfered with this pathway and further supports that the early Plx1 phosphorylation observed after Mos injection is mediated by p42^{mpk1}.

We also found that in oocytes coinjected with Mos and Xp9 the animal pole tends to become either mottled or opaque, suggesting that sustained p42^{mpk1} activation in the absence of MPF activity may have some unexpected effects. These oocytes also had a less pigmented area, which resembled a kind of irregular white spot (Figure 10A, Mos+Xp9). However when the oocytes were fixed and dissected, ~50% of them still contained a nucleus, which was usually located closer to the cortex than in uninjected oocytes and sometimes was also flatter in shape (we refer to these oocytes as pseudomatured; Figure 10B). In the same experiment, the majority of Mos-injected oocytes showed a normal white spot, and the nucleus was not detected inside (Figure 10, A and B, Mos). This suggested that some of the morphological changes associated with meiotic maturation, such as the germinal vesicle migration, can occur independently of pre-MPF activation. A role for p42^{mpk1} in this process was confirmed by the observation that when D2 was expressed together with Mos and Xp9, the pseudomatured was blocked, and both the oocytes and their nucleus had the appearance of normal uninjected oocytes (Figure 10, A and B, Mos+Xp9+D2). In contrast, the expression of D2Δ43 did not block oocyte pseudomatured (Figure 10, A and B, Mos+Xp9+D2Δ43), consistent with the inability of this truncated form to associate with p42^{mpk1}. These results suggest that nuclear migration may be regulated by the activation of p42^{mpk1}; however, GVBD appears to require MPF activation.

DISCUSSION

In this report we show that expression of D2, an N-terminally truncated p90^{rsk} that contains a MAP kinase docking site, interferes with *Xenopus* oocyte maturation. D2 inhibition is mediated by the p42^{mpk1} docking site and uncouples the activation of p42^{mpk1} and p34^{cdc2}/cyclin B in response to progesterone. The uncoupling does not correlate with the inhibition of p90^{rsk}, suggesting that p42^{mpk1} can target pre-MPF activation through alternative pathways. Consistent with this hypothesis, we found that in Mos-injected oocytes the activation of p42^{mpk1} is followed by the partial phosphorylation and activation of Plx1 via a pathway that requires protein synthesis. Plx1 activation triggered by p42^{mpk1} was independent of pre-MPF activation and was decreased by D2 expression in the oocytes. We also provide evidence that D2 interferes with nuclear migration triggered by the sustained activation of p42^{mpk1} in the absence of MPF activity (Figure 11).

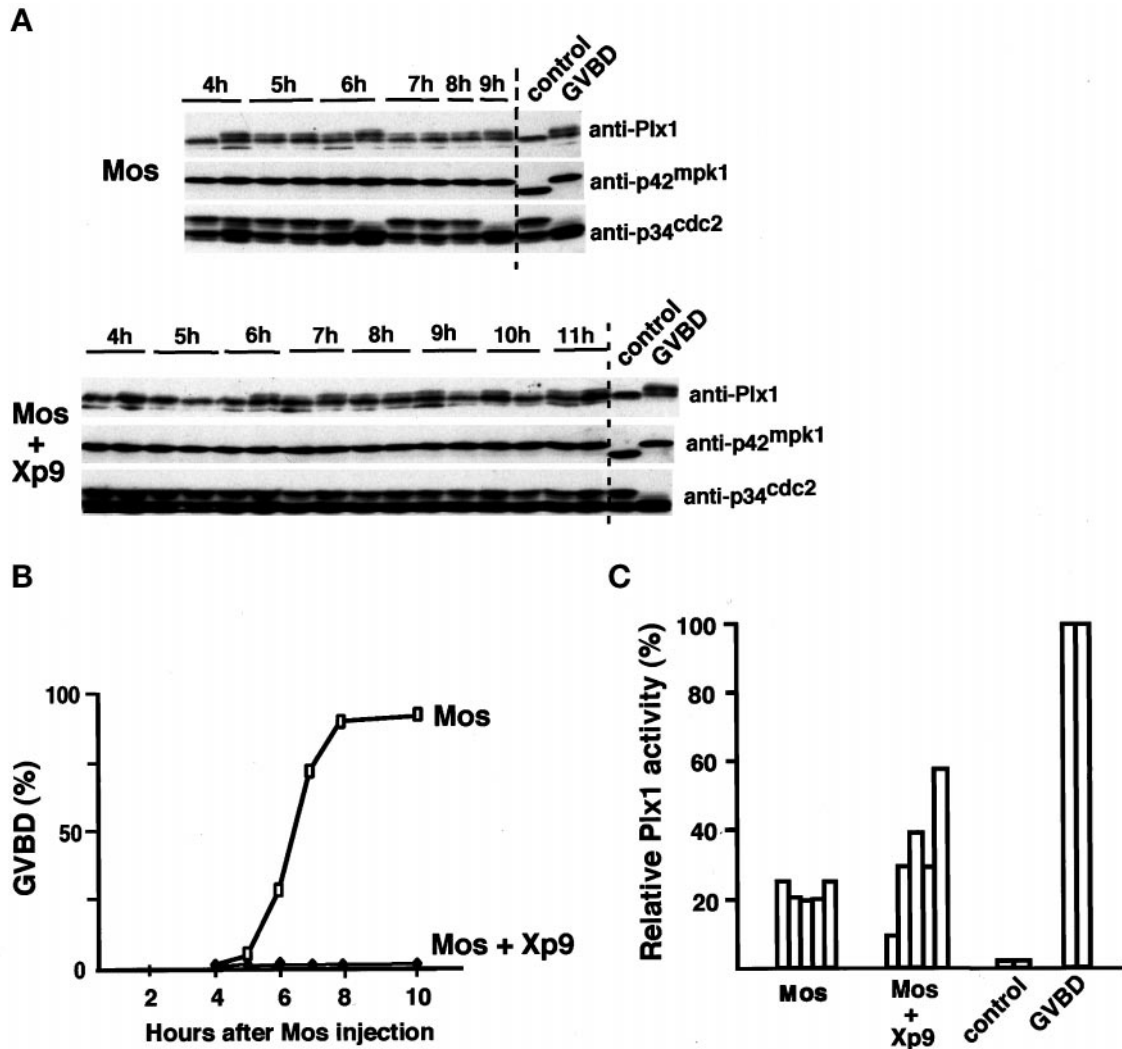


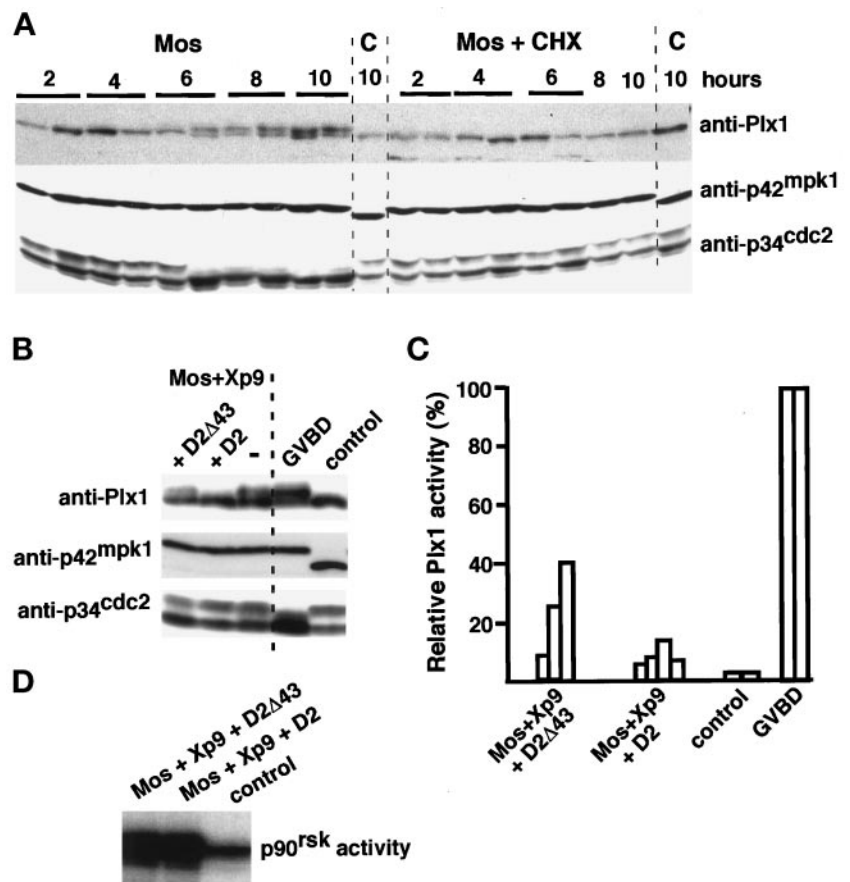
Figure 8. Mos induces partial phosphorylation and activation of Plx1 independently of pre-MPF activation. (A) Groups of 35 oocytes were injected with either Xp9 mRNA or water and after overnight incubation were injected again with malE-mos protein. At the indicated times injected oocytes that did not have a normal white spot were transferred to dry ice. Uninjected oocytes (control) and malE-mos matured oocytes (GVBD) were also taken for comparison. Lysates were prepared from single oocytes, and half of this lysate was analyzed by immunoblotting with anti-Plx1, anti-p42^{mpk1}, and anti-p34^{cdc2} antibodies. (B) Kinetics of maturation of the same oocytes as in A. (C) Lysates corresponding to half an oocyte (the second half of the lysates prepared from single oocytes that in A were used for immunoblotting) were immunoprecipitated with anti-Plx1 antibodies, and their associated kinase activity was assayed using casein as an *in vitro* substrate. Each bar represents single oocytes that were uninjected (control), malE-mos injected and matured (GVBD), or injected with malE-mos either alone or plus Xp9 but had no GVBD (these corresponded to the oocytes that in A showed partial Plx1 phosphorylation but had no preMPF activation). The Plx1 activity taken as 100% in mature oocytes (GVBD) was usually 30- to 40-fold higher than in control oocytes.

A Regulated Interaction between p42^{mpk1} and p90^{rsk} Is Essential for Signal Transduction by p42^{mpk1}

The efficient activation of p90^{rsk} requires binding to the *Xenopus* ERK MAPK p42^{mpk1} through a specific docking site located in the last 25 amino acids of p90^{rsk}. In G₂-arrested oocytes, this interaction is regulated, and the p90^{rsk}/p42^{mpk1} complex dissociates during maturation (Hsiao *et al.*, 1994). We found that in contrast to wild-type p90^{rsk}, a mutant with Ala substitutions in the six residues that are known to be phosphorylated upon p90^{rsk} activation (p90^{rsk}/6xA) was

able to interact with both the inactive and the active p42^{mpk1}. This suggests that the phosphorylation state of p90^{rsk} regulates the interaction between the full-length p90^{rsk} and p42^{mpk1}. However, D2, which lacks the N-terminal kinase domain but is efficiently phosphorylated by p42^{mpk1} (Fischer and Blenis, 1996; Dalby *et al.*, 1998; Gavin and Nebreda, 1999), can still constitutively interact with p42^{mpk1}. This suggests that upon p90^{rsk} phosphorylation, the D1 kinase domain is somehow required for the dissociation of the p90^{rsk}/p42^{mpk1} complex. D1 has been reported to phosphor-

Figure 9. Activation of Plx1 by Mos requires protein synthesis and is inhibited by expression of D2. (A) Groups of 30 oocytes that had been either preincubated for 45 min or not with cycloheximide (CHX, 50 μ g/ml) were injected with malE-mos protein. At the indicated times oocytes were individually transferred to dry ice. Uninjected oocytes (C) were also taken for comparison. Oocyte lysates were prepared from single oocytes and analyzed by immunoblotting with anti-Plx1, anti-p42^{mpk1}, and anti-p34^{cdc2} antibodies. (B) Groups of 25 oocytes were injected first with mRNAs encoding either D2 or D2 Δ 43 and 2 h later with Xp9 mRNA. After overnight incubation the oocytes were injected again with malE-mos protein and incubated for 12 h. Lysates prepared from three oocytes were analyzed by immunoblotting with anti-Plx1, anti-p42^{mpk1}, and anti-p34^{cdc2} antibodies. Uninjected oocytes (control) and Mos-matured oocytes (GVBD) were also analyzed in parallel. (C) Groups of 35 oocytes were injected first with mRNAs encoding either D2 or D2 Δ 43 and 2 h later with Xp9 mRNA. After overnight incubation the oocytes were injected again with malE-mos protein, and 4 h later oocytes that did not have a normal white spot were individually taken every hour and stored on dry ice. Lysates were prepared from single oocytes and immunoprecipitated with anti-Plx1 antibodies to assay their associated kinase activity using casein as an in vitro substrate. Each bar represents single oocytes that were uninjected (control), malE-mos injected and matured (GVBD), or injected with malE-mos plus Xp9 and either D2 or D2 Δ 43. The Plx1 activity taken as 100% in mature oocytes (GVBD) was usually 30- to 40-fold higher than in control oocytes. (D) Groups of 25 oocytes were injected first with mRNAs encoding Xp9 and either D2 or D2 Δ 43 and later with malE-mos protein as in A. After 12 h, lysates were prepared from three oocytes, immunoprecipitated with anti-p90^{rsk} antibodies, and assayed for kinase activity using GST-Myt1 Ct as an in vitro substrate. Uninjected oocytes (control) were also analyzed in parallel.



ylate Ser-730 of p90^{rsk}, which is located in the p42^{mpk1} docking site (Dalby *et al.*, 1998), but this is unlikely to prevent the binding to p42^{mpk1}, because Ser-730 is apparently phosphorylated both in active and inactive p90^{rsk} (Dalby *et al.*, 1998). An alternative possibility is that the phosphorylation of p90^{rsk} induces some conformational change, so that D1 will then sterically prevent p42^{mpk1} from accessing its docking site in p90^{rsk}. Finally, it is also possible that the D1 kinase domain is required for the interaction of phosphorylated p90^{rsk} with other proteins, which in turn might prevent p42^{mpk1} binding. Interestingly, the C-terminal regulatory domain of Myt1 can specifically bind to the phosphorylated form of p90^{rsk} and this association requires the full-length p90^{rsk} (Palmer *et al.*, 1998). It is thus possible that p90^{rsk} interaction with Myt1 prevents p42^{mpk1} binding.

The observation that a truncated form of p90^{rsk}, which constitutively binds to p42^{mpk1} can inhibit signaling by p42^{mpk1}, suggests an important role for the regulated interaction between p42^{mpk1} and p90^{rsk}. The association between these two kinases in G₂-arrested oocytes, in which p42^{mpk1} is inactive, would ensure the preferential phosphorylation of the bound p90^{rsk}. However, upon progesterone stimulation, dissociation of the complex is probably necessary for the accessibility of p42^{mpk1} and p90^{rsk} to other substrates. Ex-

pression of peptides based on the sequence of an ERK MAP kinase docking site from Elk-1 has been also reported to inhibit *Xenopus* oocyte maturation (Jacobs *et al.*, 1999). Thus, truncated substrates and docking site peptides may be helpful inhibitory tools to study signal transduction by MAP kinases. They might also allow the differential inhibition of specific MAP kinase pathways, because some docking sites appear to target specific MAP kinase family members. Furthermore, these tools might preferentially inhibit particular subsets of substrates, because their ability to compete with endogenous substrates may depend on the relative affinities of the different docking sites for the MAP kinases.

The Inhibitory Effect of D2 Requires Binding to p42^{mpk1}

The inhibitory effect of D2 on meiotic reinitiation does not depend on its kinase activity, suggesting that D2 inhibits by interaction with endogenous proteins and not catalytically. Fragments of monomeric enzymes can sometimes work in a dominant negative way by interfering with the folding of the wild-type endogenous protein (Michaels *et al.*, 1996). However, it appears unlikely that D2 works by targeting p90^{rsk} in a similar way. We have not detected interactions

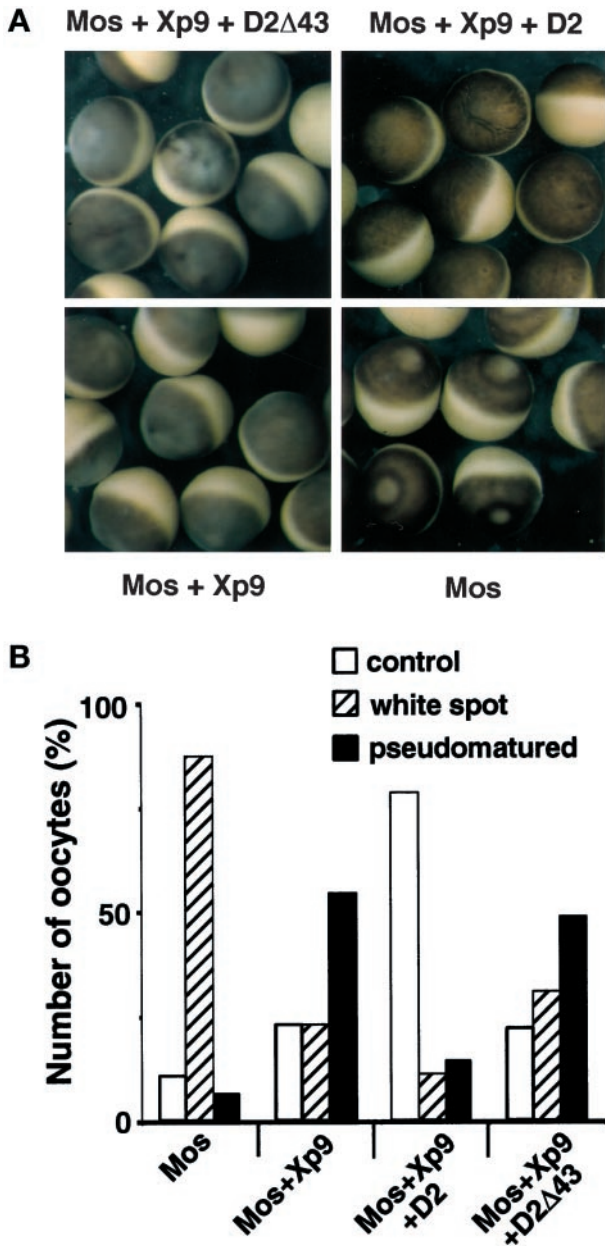


Figure 10. The sustained activation of p42^{mpk1} in the absence of pre-MPF activation produces morphological changes in the oocytes. (A) Oocytes were injected with Xp9 mRNA alone or together with either D2 or D2Δ43 mRNAs, incubated overnight, and then injected again with malE-mos protein. Pictures were taken 10 h after malE-mos injection. (B) Oocytes were injected as in A and 8–24 h later were fixed by boiling for 90 s in PBS. After dissection, the oocytes were monitored for the presence of the nucleus (control), GVBD (white spot), or nuclear migration (pseudomatured). The results represent four independent experiments with ~40 oocytes being dissected for each treatment.

between D2 and either p90^{rsk} or the D1 or D2 kinase domain alone in a yeast two-hybrid system (our unpublished results). Furthermore, we showed that D2 associates with

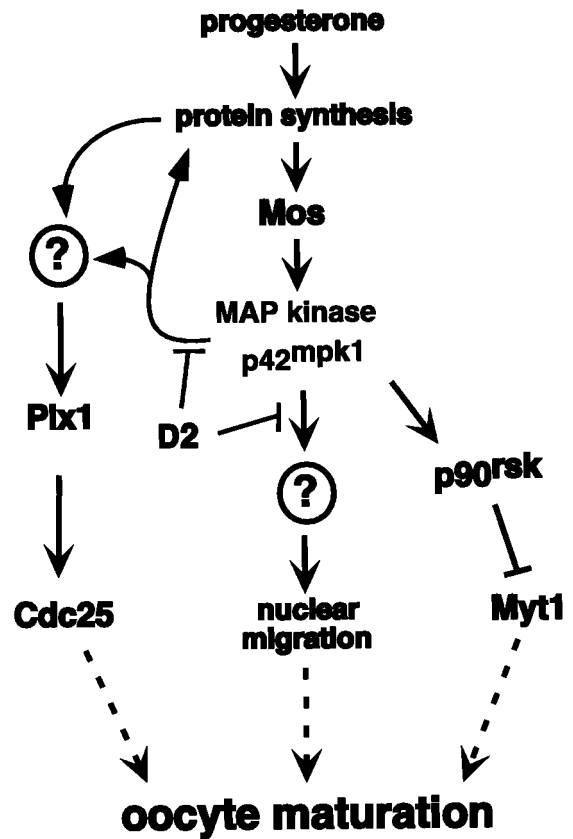


Figure 11. A summary of possible p42^{mpk1} functions during *Xenopus* oocyte maturation. Progesterone stimulates the translation of maternal mRNAs and triggers the synthesis of the protein kinase Mos, which activates p42^{mpk1} via MAP kinase kinase. p42^{mpk1} activates p90^{rsk}, which in turn down-regulates the p34^{cdc2} inhibitory kinase Myt1. Our results indicate that D2 interferes with p42^{mpk1} signaling in oocytes but does not affect the activation of p42^{mpk1} and p90^{rsk}. Instead, D2 interferes with the phosphorylation and activation of Plx1. This pathway, which may lead to Cdc25 up-regulation, is triggered by p42^{mpk1} independently of pre-MPF activation but requires protein synthesis. Expression of D2 also inhibits the nuclear migration and pseudomatured, which appears to be triggered by the sustained activation of p42^{mpk1} in the absence of MPF activation.

endogenous p42^{mpk1} in the oocytes (which is recruited to higher-molecular-weight complexes), whereas the elution pattern of p90^{rsk} in gel filtration was unchanged upon D2 expression (our unpublished results). These results together with the observation that D2 inhibition is mediated by the p42^{mpk1} docking site suggest that the inhibitory effect of D2 is brought about by binding to p42^{mpk1}.

A New Pathway That Links the Activation of p42^{mpk1} and p34^{cdc2}/Cyclin B during Oocyte Maturation

We have taken advantage of the inhibitory effect of D2 to investigate the function of p42^{mpk1} during *Xenopus* oocyte maturation. We found that D2 expression uncouples the activation of p42^{mpk1} and p34^{cdc2}/cyclin B. This confirms

previous results showing that the interference with p42^{mpk1} activation prevents meiotic reinitiation and p34^{cdc2}/cyclin B activation (Kosako *et al.*, 1994b, 1996; Gotoh *et al.*, 1995). However, in contrast to previous studies in which the activation of p42^{mpk1} was also prevented, we use a tool that does not block p42^{mpk1} activation but inhibits downstream of p42^{mpk1} and prevents the activation of p34^{cdc2}/cyclin B. Thus, our work indicates that p42^{mpk1} activation normally precedes the activation of pre-MPF during progesterone-induced meiotic maturation.

We found that D2 did not disrupt the preformed p90^{rsk}/p42^{mpk1} complexes present in G₂-arrested oocytes. This observation is surprising because we have not detected significant differences in the affinities of either D2 or p90^{rsk} for p42^{mpk1}. One possible explanation is that in G₂-arrested oocytes the interaction between p42^{mpk1} and p90^{rsk} might not be dynamic. In support of this possibility, when either p42^{mpk1} or full-length p90^{rsk} is overexpressed, it does not bind to the endogenous partners (our unpublished results); however, the overexpressed p42^{mpk1} and p90^{rsk} are able to interact with each other (Figure 1B). These observations suggest that there may be mechanisms to stabilise the preformed p90^{rsk}/p42^{mpk1} complexes in oocytes. Whether this is due to the binding of additional proteins that stabilize the complex or to the localization of the complexes in particular subcellular compartments where they are not accessible to the exogenous, overexpressed proteins remains to be investigated. Consistent with the observation that D2 did not disrupt the preformed p90^{rsk}/p42^{mpk1} complex, D2 did not delay the kinetics of phosphorylation and activation of p90^{rsk} by p42^{mpk1}, suggesting that D2 does not interfere with the p90^{rsk}/Myt1 pathway. Thus, p42^{mpk1} might target pre-MPF activation through alternative pathways. In G₂-arrested oocytes only part of the p42^{mpk1} is found associated with p90^{rsk}, and our gel-filtration experiments suggest that D2 interacts with the p42^{mpk1} subpopulation, which is not associated with p90^{rsk}. This p42^{mpk1} subpopulation is likely to target substrates other than p90^{rsk}, which might also participate in the regulation of p34^{cdc2}/cyclin B activity (and hence the activation of pre-MPF) during oocyte maturation. We cannot rule out, however, that upon progesterone stimulation D2 might also interact with the active p42^{mpk1} released from the p42^{mpk1}/p90^{rsk} complex.

The Cdc25 activating kinase Plx1 can be phosphorylated and activated by the protein kinase xPlkk1, which itself is also regulated by phosphorylation by one or more protein kinases (Qian *et al.*, 1998b). Several reports indicate that Plx1 activation may be triggered by p34^{cdc2}/cyclin B as part of the MPF autoamplification loop (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1998). Here we provide evidence for a p42^{mpk1}-triggered pathway, which also regulates Plx1 phosphorylation and activation. We observed that in Mos-injected oocytes, the activation of p42^{mpk1} is followed by partial phosphorylation of Plx1 before any detectable pre-MPF activation. In some batches of oocytes, however, the phosphorylation of Plx1 induced by Mos injection coincides with pre-MPF activation. One interpretation is that Plx1 phosphorylation normally precedes the activation of pre-MPF, although in some oocytes the two events may take place too close in time to be separated experimentally. To rule out an implication of p34^{cdc2}/cyclin B in the phosphorylation of Plx1 induced by Mos, we coexpressed the *Xenopus* Suc1/Cks

protein Xp9 together with Mos. Xp9 is a p34^{cdc2}-binding protein that appears to be important for targeting p34^{cdc2}/cyclin complexes to some substrates (Kusubata *et al.*, 1992; Patra and Dunphy, 1998). For example, Xp9 can enhance the phosphorylation of the Cdc27 component of the anaphase-promoting complex by p34^{cdc2}/cyclin B and affect cyclin B stability (Patra and Dunphy, 1998). When overexpressed in *Xenopus* egg extracts, Xp9-like proteins can delay entry into mitosis by interfering with p34^{cdc2} tyrosine dephosphorylation (Dunphy and Newport, 1989; Patra and Dunphy, 1996). Consistent with this, we found that injection of Xp9 into oocytes prevented Mos-induced meiotic reinitiation by interfering with the activation of p34^{cdc2}/cyclin B. In these oocytes, however, ectopic Mos was still able to activate p42^{mpk1}, and we could confirm that Plx1 phosphorylation and activation occurred independently of pre-MPF activation. Importantly, the phosphorylation and partial activation of Plx1 induced by Mos was inhibited by the expression of D2, indicating that it involves p42^{mpk1}. A link between p42^{mpk1} and Plx1 has not been found in extracts prepared from G₂-arrested oocytes (Karaiskou *et al.*, 1998). In these extracts, however, the activation of p42^{mpk1} and p34^{cdc2}/cyclin B appear to be independent events (Huang and Ferrell, 1996) probably attributable to the impaired ability of the oocyte extracts to translate mRNAs (our unpublished results). In agreement with this, we show that the p42^{mpk1}-dependent phosphorylation of Plx1 is abolished by cycloheximide and thus requires protein synthesis. This observation together with the 3- to 5-h delay observed between the activation of p42^{mpk1} and Plx1 argue against a direct link between p42^{mpk1} and either Plx1 or xPlkk1 and rather suggest that p42^{mpk1} triggers the accumulation of a protein or proteins that are required for the activation of Plx1. Whether this link between p42^{mpk1} and Plx1 activation is related to the triggering pathway that leads to Plx1 activation at the onset of M-phase remains to be determined. In any case, the delayed kinetics observed between p42^{mpk1} and Plx1 activation suggest that Plx1 activation is probably not an early p42^{mpk1}-dependent step in maturation and may be the basis of a positive feedback loop ensuring the coordinated activation of both p42^{mpk1} and MPF.

During oocyte maturation p42^{mpk1} can participate in p34^{cdc2}/cyclin B activation via several pathways (Figure 11). One pathway may involve phosphorylation and inhibition of Myt1 by p90^{rsk} (Palmer *et al.*, 1998). Accumulation of Mos is also stimulated by p42^{mpk1} through a positive feedback loop (Matten *et al.*, 1996; Roy *et al.*, 1996), and it has recently been shown that p42^{mpk1} can up-regulate cytoplasmic polyadenylation of the Mos mRNA (Howard *et al.*, 1999). However, the initial progesterone-induced synthesis of Mos is probably independent of p42^{mpk1}, because it occurs before any detectable p42^{mpk1} activation. Our results suggest a new connection between p42^{mpk1} and a protein or proteins that are required for the phosphorylation and partial activation of Plx1. p42^{mpk1} could stimulate synthesis of these proteins and/or post-translationally regulate their activity, for example, at the level of protein stability. As in the case of Mos, synthesis of these proteins might be also initiated during progesterone-induced maturation before the activation of p42^{mpk1}. This suggests the possibility that the susceptibility of the oocytes to inhibition by D2 may depend on the initial rate of protein synthesis induced by progesterone.

A Possible Role for p42^{mpk1} in Nuclear Migration

MPF is a key regulator of M-phase progression during the cell cycle and is likely to be responsible for most of the structural changes associated with M-phase. ERK MAP kinases and p34^{cdc2}/cyclin B, however, have some overlapping substrate specificity. For example, both can phosphorylate nuclear lamins at the sites that are involved in lamin disassembly before nuclear envelope breakdown (Peter *et al.*, 1992). In mouse oocytes and embryos ERK MAP kinases have also been implicated in some M-phase events (Gavin *et al.*, 1994; Moos *et al.*, 1996; Verlhac *et al.*, 1996). However, a possible role for p42^{mpk1} in controlling M-phase structural changes in *Xenopus* oocytes has been difficult to address, because p42^{mpk1} is usually activated at the same time as MPF during meiotic maturation. Here we show that activation of p42^{mpk1} by Mos in the absence of p34^{cdc2}/cyclin B activation may induce some of the morphological changes associated with meiotic maturation such as nuclear migration to the cortex of the oocyte. This is likely to be triggered by p42^{mpk1} activation, because it can be inhibited by the overexpression of D2, but not of a truncated version of D2, which lacks the p42^{mpk1} docking site. However, the sustained activation of p42^{mpk1} in the absence of MPF activation appears to lead to an abnormal maturation as indicated by the morphological appearance of the oocytes. Similar effects (including germinal vesicle migration without breakdown) have also been described when maturation is induced by the injection of oncogenic Ras in the presence of either drugs that increase cAMP levels (Birchmeier *et al.*, 1985) or protein synthesis inhibitors (Nebreda *et al.*, 1993b). Our results also show that activation of p42^{mpk1} alone is not enough to trigger GVBD in *Xenopus* oocytes, indicating that nuclear envelope breakdown during M-phase is likely to require the activation of MPF.

In summary, we have found that overexpression of the p42^{mpk1} docking site located at the C-terminal end of p90^{rsk} can inhibit *Xenopus* oocyte maturation, and we have used this as a tool to elucidate the role(s) of p42^{mpk1} in the process. Our main conclusions are that activation of p42^{mpk1} normally precedes the activation of pre-MPF and that there is an MPF-independent pathway that links the activation of p42^{mpk1} and the Cdc25-activating kinase Plx1 during oocyte maturation. We also show that p42^{mpk1} may have a role in nuclear migration during *Xenopus* oocyte maturation. Finally, our results indicate that the regulated interaction with substrates is essential for signal transduction by MAP kinases.

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