

A link between MAP kinase and p34^{cdc2}/cyclin B during oocyte maturation: p90^{rsk} phosphorylates and inactivates the p34^{cdc2} inhibitory kinase Myt1

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M-phase entry in eukaryotic cells is driven by activation of MPF, a regulatory factor composed of cyclin B and the protein kinase p34^{cdc2}. In G₂-arrested *Xenopus* oocytes, there is a stock of p34^{cdc2}/cyclin B complexes (pre-MPF) which is maintained in an inactive state by p34^{cdc2} phosphorylation on Thr14 and Tyr15. This suggests an important role for the p34^{cdc2} inhibitory kinase(s) such as Wee1 and Myt1 in regulating the G₂→M transition during oocyte maturation. MAP kinase (MAPK) activation is required for M-phase entry in *Xenopus* oocytes, but its precise contribution to the activation of pre-MPF is unknown. Here we show that the C-terminal regulatory domain of Myt1 specifically binds to p90^{rsk}, a protein kinase that can be phosphorylated and activated by MAPK. p90^{rsk} in turn phosphorylates the C-terminus of Myt1 and down-regulates its inhibitory activity on p34^{cdc2}/cyclin B *in vitro*. Consistent with these results, Myt1 becomes phosphorylated during oocyte maturation, and activation of the MAPK–p90^{rsk} cascade can trigger some Myt1 phosphorylation prior to pre-MPF activation. We found that Myt1 preferentially associates with hyperphosphorylated p90^{rsk}, and complexes can be detected in immunoprecipitates from mature oocytes. Our results suggest that during oocyte maturation MAPK activates p90^{rsk} and that p90^{rsk} in turn down-regulates Myt1, leading to the activation of p34^{cdc2}/cyclin B.

Keywords: cell cycle/MAP kinase/MPF/oocyte maturation/p34^{cdc2}

Introduction

Xenopus laevis oocytes are naturally arrested in late G₂ of the first meiotic division and are induced to enter into M-phase of meiosis upon exposure to progesterone. The events of meiotic maturation, including germinal vesicle breakdown (GVBD), chromosome condensation and formation of the metaphase spindles are associated with the activation of maturation-promoting factor (MPF), a regulatory factor involved in mitosis initiation in eukaryotic cells (Masui and Markert, 1971; Masui and Clarke, 1979). MPF is composed of a regulatory subunit, cyclin B, and a catalytic subunit, the protein kinase p34^{cdc2}, whose phosphorylation state is very important for the activity

of the p34^{cdc2}/cyclin B complex. In particular, p34^{cdc2} phosphorylation on either Thr14 or Tyr15 mediated by Wee1 family kinases results in MPF inactivation (reviewed by Coleman and Dunphy, 1994; Morgan, 1995; Lew and Kornbluth, 1996).

Translation of mRNA for the protein kinase Mos is required for the progesterone-induced maturation of *Xenopus* oocytes (Sagata *et al.*, 1988, 1989; Kanki and Donoghue, 1991; Sheets *et al.*, 1995). Mos is an efficient activator of MAPK kinase (Nebreda and Hunt, 1993; Nebreda *et al.*, 1993b; Posada *et al.*, 1993; Shibuya and Ruderman, 1993), and injection of neutralizing antibodies against MAPK kinase inhibits Mos-induced oocyte maturation (Kosako *et al.*, 1994). This suggests that Mos activates p34^{cdc2}/cyclin B through the MAPK cascade. Consistent with this hypothesis, injection of either constitutively active MAPK kinase or thiophosphorylated MAPK can trigger the maturation of *Xenopus* oocytes independently of any progesterone stimulation (Gotōh *et al.*, 1995; Haccard *et al.*, 1995; Huang *et al.*, 1995). It should be noted, however, that although the Mos–MAPK pathway is likely to have an important role in oocyte maturation, there is also evidence that translation of mRNA(s) other than Mos is required to activate MPF during progesterone-induced maturation (Nebreda *et al.*, 1995; Barkoff *et al.*, 1998).

Little is known of how MAPK triggers MPF activation and the maturation of oocytes. A well characterized substrate for MAPK is the 90 kDa ribosomal S6 kinase (p90^{rsk} or S6KII), a serine/threonine protein kinase originally identified in *Xenopus* oocytes as a kinase that phosphorylates the S6 protein of the ribosomal 40S subunit *in vitro* (Erikson and Maller, 1986). *Xenopus* p90^{rsk} is most similar to the mammalian Rsk1/Rsk2 protein kinases (also referred to as MAPKAP kinase-1a and -1b) (Alcorta *et al.*, 1989), which can be phosphorylated and activated *in vitro* and *in vivo* by p42^{Erk2} and p44^{Erk1} MAPKs (Sturgill *et al.*, 1988; Chung *et al.*, 1991; Alessi *et al.*, 1995; Zhao *et al.*, 1996). In G₂-arrested *Xenopus* oocytes, inactive p90^{rsk} is associated with inactive MAPK in a heterodimer which dissociates upon phosphorylation and activation of the two kinases (Hsiao *et al.*, 1994), suggesting that p90^{rsk} has a role in oocyte maturation.

In *Xenopus* oocytes, there is a pre-formed stock of inactive p34^{cdc2}/cyclin B complexes (pre-MPF) in which p34^{cdc2} is phosphorylated on Thr14 and Tyr15 as well as Thr161 (Cyert and Kirschner, 1988; Gautier and Maller, 1991; Kobayashi *et al.*, 1991). Thus, the activation of pre-MPF during oocyte maturation requires the dephosphorylation of p34^{cdc2} on Thr14 and Tyr15, probably catalysed by the dual-specificity phosphatase Cdc25C (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). In addition to an increased activity of Cdc25C, inhibition of the kinases

that phosphorylate p34^{cdc2} on Thr14 and Tyr15 may also trigger the activation of p34^{cdc2}/cyclin B complexes (Atherton-Fessler *et al.*, 1994). The prototype of the p34^{cdc2} inhibitory kinases is Wee1, the negative regulator of mitosis in *Schizosaccharomyces pombe*. However, the first cloned human and *Xenopus* Wee1 homologues phosphorylate p34^{cdc2} only on Tyr15 but not Thr14, supporting the existence of a separate Thr14 kinase (Parker and Piwnicka-Worms, 1992; McGowan and Russell, 1993; Mueller *et al.*, 1995a). A kinase activity that can phosphorylate p34^{cdc2} on Thr14 was later detected in *Xenopus* extracts; this enzyme is associated with membranes and can be separated from a Tyr15-specific kinase activity in the soluble fraction (Atherton-Fessler *et al.*, 1994; Kornbluth *et al.*, 1994). A candidate cDNA for this activity, named Myt1, has been cloned from *Xenopus* (Mueller *et al.*, 1995b) and humans (Booher *et al.*, 1997; Liu *et al.*, 1997). Myt1 encodes a membrane-associated Wee1 homologue that can phosphorylate p34^{cdc2} on both Thr14 and Tyr15. As expected, and in contrast to Cdc25C whose activity increases during mitosis, the activity of both Wee1 and Myt1 declines during mitosis, therefore contributing to the fall in the inhibitory phosphorylation of p34^{cdc2} at this stage of the cell cycle. Cdc25C, Wee1 and Myt1 all become heavily phosphorylated during mitosis (M phase) and these phosphorylations correlate with the increased activity of Cdc25C (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992, 1996; Izumi and Maller, 1993; Hoffmann *et al.*, 1993) and the inhibition of Wee1 and Myt1 (Tang *et al.*, 1993; McGowan and Russell, 1995; Mueller *et al.*, 1995a,b; Booher *et al.*, 1997).

A possible connection between MAPK and the activation of pre-MPF in oocytes comes from the recent observation that MAPK down-regulates an activity which in turn inactivates p34^{cdc2}/cyclin B in G₂-arrested oocytes (Abrieu *et al.*, 1997). This inhibitory activity targeted by MAPK could correspond to Thr14–Tyr15 p34^{cdc2} kinases such as Wee1 and Myt1. Since Wee1 is not present in G₂-arrested oocytes and is synthesized only upon progesterone stimulation (Murakami and Vande Woude, 1998), it is not likely to keep pre-MPF in an inactive state. Thus, Myt1 may be the MPF inhibitory activity down-regulated by MAPK in *Xenopus* oocytes.

Here we report that Myt1 is a target of MAPK-activated p90^{rsk}. Thus, p90^{rsk} phosphorylates the C-terminal regulatory domain of Myt1 and down-regulates the inhibitory activity of Myt1 on p34^{cdc2}/cyclin B1 complexes *in vitro*. We also show that Myt1 becomes phosphorylated during oocyte maturation and that p90^{rsk} and Myt1 are associated in mature oocytes. Our results indicate that p90^{rsk}–Myt1 provides a link between the activation of MAPK and MPF during *Xenopus* oocyte maturation.

Results

Identification of protein kinases in *Xenopus* cell-free extracts that phosphorylate Myt1 *in vitro*

In addition to the catalytic domain, Myt1 contains a region which includes many potential phosphorylation sites and is therefore likely to have a regulatory role. To identify protein kinases that can phosphorylate and potentially regulate *Xenopus* Myt1, we fused the non-catalytic regions of Myt1 (C-terminal half) to glutathione *S*-transferase

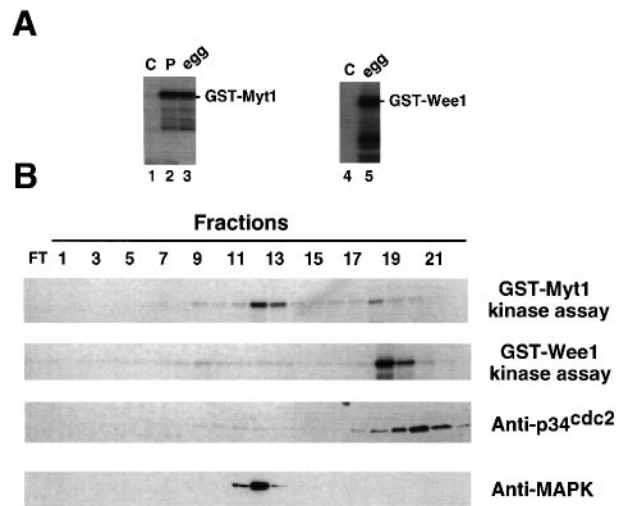


Fig. 1. Identification of protein kinase activities in *Xenopus* cell-free extracts that phosphorylate GST–Myt1 and GST–Wee1 *in vitro*. (A) Cell-free extracts prepared from either eggs (*in vivo* mature oocytes, lanes 3 and 5), oocytes matured *in vitro* by progesterone (lane 2) or control, G₂-arrested oocytes (lanes 1 and 4) were used to phosphorylate GST–Myt1 (lanes 1–3) or GST–Wee1 (lanes 4 and 5). (B) Egg extracts were ultracentrifuged and then fractionated by Mono Q chromatography. The fractions were either used in kinase assays with GST–Myt1 or GST–Wee1 as the substrate, or analysed by immunoblotting using anti-p34^{cdc2} or anti-Mpk1 MAPK antibodies, as indicated.

(GST) and used the bacterially produced fusion protein as a substrate in kinase assays with *Xenopus* cell-free extracts. As shown in Figure 1A, GST–Myt1 can be phosphorylated efficiently *in vitro* by cell-free extracts prepared from either eggs (lane 3) or progesterone-matured oocytes (lane 2), but not from control, G₂-arrested oocytes (lane 1). We then used GST–Myt1 to screen *Xenopus* egg extracts fractionated by Mono Q chromatography and we detected two peaks of kinase activity that phosphorylate GST–Myt1 *in vitro* (Figure 1B, GST–Myt1). As a control we also fused GST to the non-catalytic region of Wee1 (N-terminal half) and found that GST–Wee1 can also be phosphorylated *in vitro* by cell-free extracts prepared from unfertilized eggs (Figure 1A, lane 5) but not from control, G₂-arrested oocytes (Figure 1A, lane 4). When GST–Wee1 was used as a substrate to analyse the Mono Q-fractionated egg extracts, we found that most of the activity phosphorylating GST–Wee1 eluted in different fractions from those containing the major peak of activity that phosphorylates GST–Myt1 (Figure 1B). The same Mono Q fractions were also analysed by immunoblot with anti-p34^{cdc2} and anti-MAPK antibodies to identify the positions where these two protein kinases elute. We found that the major peak of kinase activity phosphorylating GST–Myt1 overlaps with the fractions where the p42^{Mpk1} MAPK is detected (Figure 1B, anti-MAPK), whereas the major kinase activity phosphorylating GST–Wee1 overlaps with some of the fractions where p34^{cdc2} eluted (Figure 1B, anti-p34^{cdc2}).

To gain further information on the protein kinase(s) that phosphorylates GST–Myt1, we used the fusion protein for pull-down experiments followed by a kinase assay. For these experiments, GST–Myt1 was first coupled to glutathione–Sepharose beads and then incubated with *Xenopus* egg extracts. The beads were recovered from the

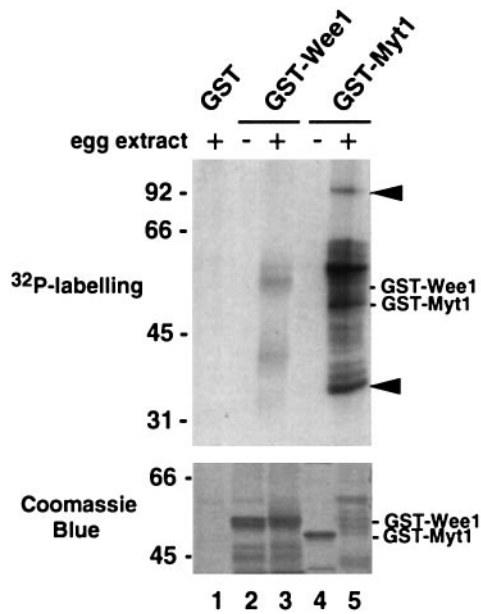


Fig. 2. Identification of protein kinase activities in egg extracts that bind to and phosphorylate GST-Myt1 and GST-Wee1 in pull-downs. Bacterially produced GST (lane 1), GST-Wee1 (lane 3) and GST-Myt1 (lane 5) proteins were incubated with egg extracts, recovered by centrifugation, incubated with [γ -³²P]ATP and analysed by polyacrylamide gel electrophoresis and autoradiography. Purified GST-Wee1 (lane 2) and GST-Myt1 (lane 4) were also incubated with [γ -³²P]ATP and analysed by polyacrylamide gel electrophoresis in parallel. The positions where the two purified recombinant proteins run are indicated. Arrowheads indicate two additional bands of ~34 and 90 kDa which are phosphorylated in GST-Myt1 pull-downs (lane 5). The upper panel shows an autoradiograph and the lower panel the same gel stained with Coomassie Blue.

extracts, washed and then incubated in a kinase reaction with [γ -³²P]ATP and Mg²⁺. The phosphorylated proteins were finally analysed by polyacrylamide gel electrophoresis and autoradiography. We found that protein kinases from the extracts were able to bind to and efficiently phosphorylate the GST-Myt1 fusion protein (Figure 2, lane 5 in upper panel). Moreover, the electrophoretic mobility of the GST-Myt1 recombinant protein shifted upwards upon phosphorylation in the pull-downs (Figure 2, lanes 4 and 5 in lower panel). We also detected in the GST-Myt1 pull-downs additional phosphorylated bands of ~34 and 90 kDa (Figure 2, arrowheads in upper panel). In the same experiments, GST-Wee1 was also phosphorylated but more weakly than GST-Myt1 (Figure 2, lane 3 in upper panel), although some variability was observed depending on the extracts. Moreover, we could not detect any significant size shift of the GST-Wee1 recombinant protein upon phosphorylation in pull-downs (Figure 2, lanes 2 and 3 in lower panel).

p34^{cdc2}-dependent and independent kinase activities can phosphorylate Myt1

Many regulatory and structural proteins become phosphorylated or hyperphosphorylated during M-phase, and p34^{cdc2}/cyclin complexes are known to play an important role in these phosphorylations. To investigate whether p34^{cdc2} could be detected in GST-Myt1 pull-downs, we analysed, by immunoblot with anti-p34^{cdc2} antibodies, GST and GST-Myt1 pull-downs prepared from *Xenopus* egg extracts. We found that in GST-Myt1 pull-downs

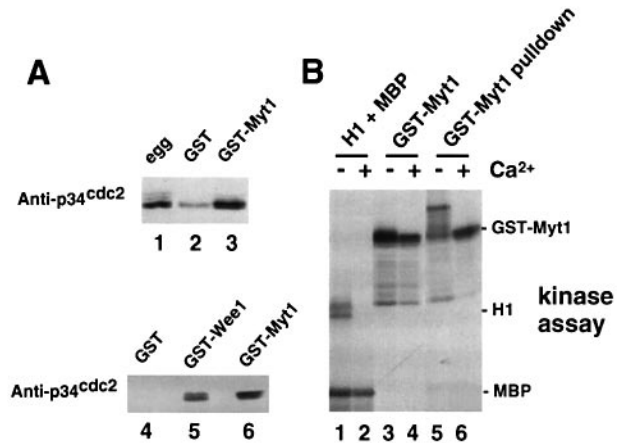


Fig. 3. p34^{cdc2} binds to Myt1. (A) Egg extracts (lane 1) and either GST (lanes 2 and 4), GST-Myt1 (lanes 3 and 6) or GST-Wee1 (lane 5) pull-downs of the extracts were analysed by immunoblotting with anti-p34^{cdc2} antibodies. (B) CSF-arrested egg extracts were incubated in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of Ca²⁺ for 30 min and then used for *in vitro* kinase assays using as substrates either histone H1 + MBP (lanes 1 and 2) or GST-Myt1 pull-downs (lanes 3 and 4). Aliquots of the extracts were also used for GST-Myt1 pull-downs (lanes 5 and 6) and then incubated with [γ -³²P]ATP and analysed by polyacrylamide gel electrophoresis and autoradiography.

there was significantly more p34^{cdc2} than in those with GST alone (Figure 3A, compare lanes 2 and 4 with lanes 3 and 6, respectively), indicating that p34^{cdc2} can associate specifically either directly or indirectly with the C-terminal regulatory region of Myt1. We also found p34^{cdc2} associated with GST-Wee1 pull-downs (Figure 3A, lane 5).

To investigate the possible connection between p34^{cdc2}/cyclin B and the kinase activity responsible for GST-Myt1 phosphorylation in egg extracts, we used Ca²⁺-treated egg extracts. Treatment of cytostatic factor (CSF)-arrested egg extracts with Ca²⁺ triggers cyclin B degradation, leading to MPF inactivation after 20–30 min as indicated by the absence of H1 kinase activity in the extracts (Figure 3B, lane 2). These H1 kinase-negative egg extracts can still phosphorylate GST-Myt1, albeit less efficiently than the untreated CSF-arrested egg extracts (Figure 3B, lanes 3 and 4). Moreover, in the Ca²⁺-treated extracts, there is still a kinase activity that can bind to and phosphorylate GST-Myt1 in pull-down experiments, but interestingly in this case the electrophoretic mobility of the GST-Myt1 protein does not shift as it does in the pull-downs from CSF-arrested egg extracts (Figure 3B, lanes 5 and 6). We conclude from this experiment that there are at least two protein kinase activities that can bind to and phosphorylate the C-terminal regulatory region of Myt1 *in vitro*. One of the kinase activities is dependent on MPF and may be either p34^{cdc2}/cyclin B or another p34^{cdc2}/cyclin complex. The other protein kinase activity can bind to and phosphorylate Myt1 independently of MPF activity.

To gain more information on this MPF-independent kinase activity that phosphorylates Myt1, we carried out in-gel kinase assays using GST-Myt1 as a substrate. As shown in Figure 4A, both in egg extracts (lane 3) and in lysates prepared from progesterone-matured oocytes (lane 2), we detected a protein kinase activity of ~90 kDa which can phosphorylate GST-Myt1 upon renaturation in the gel. This activity, however, was not detected in lysates

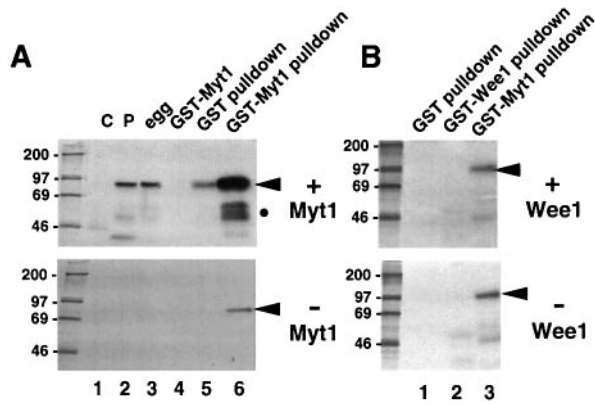


Fig. 4. A 90 kDa protein kinase binds to and phosphorylates Myt1. In-gel kinase assays using as substrates GST-Myt1 (A, upper), GST-Wee1 (B, upper) or GST alone (A and B, lower). (A) Control, G_2 -arrested oocyte lysates (lane 1), progesterone-matured oocyte lysates (lane 2), egg extracts (lane 3), purified GST-Myt1 (lane 4), GST (lane 5) and GST-Myt1 (lane 6) pull-downs from egg extracts. (B) GST (lane 1), GST-Wee1 (lane 2) and GST-Myt1 (lane 3) pull-downs from egg extracts. The arrowheads and the dot indicate kinase activities of 90 and 50–60 kDa, respectively.

prepared from unstimulated oocytes arrested in G_2 (lane 1). A protein kinase activity of 90 kDa was also strongly enriched in GST-Myt1 pull-downs prepared from egg extracts when compared with the same pull-downs using GST alone (~12-fold increase by PhosphorImager quantification; Figure 4A, compare lanes 5 and 6). In addition, we detected in GST-Myt1 pull-downs another kinase activity of 50–60 kDa (Figure 4A, dot), but we do not know at present whether this activity is related to the 90 kDa activity or corresponds to a totally different protein kinase. When a duplicate of these samples was analysed in gels cast with GST alone, we could detect a kinase of ~90 kDa which was able to renature, and presumably autophosphorylate, only in the GST-Myt1 pull-downs, but not in any of the other samples (Figure 4A, arrowhead in lower panel). This observation is consistent with the idea that the 90 kDa kinase that phosphorylates Myt1 is enriched in GST-Myt1 pull-downs, and confirms the previous detection of a phosphorylated band of 90 kDa in kinase assays using GST-Myt1 pull-downs (Figure 2, lane 5 in upper panel).

As a control, we performed in-gel kinase assays using GST-Wee1 polymerized in the gel. We observed that the 90 kDa protein kinase activity that binds to GST-Myt1 pull-downs did not phosphorylate GST-Wee1 (Figure 4B, lane 3 in upper panel). Moreover, using a gel with GST alone, we confirmed that the GST-Myt1-associated 90 kDa protein kinase is able to renature and autophosphorylate in the gel (Figure 4B, arrowhead in lower panel) but does not bind to GST-Wee1 (Figure 4B, lane 2), nor did we detect any protein kinase activity that is able to renature and phosphorylate GST-Wee1 (Figure 4B, lane 2 in upper panel).

p90^{rsk} strongly associates with Myt1

The protein kinase p90^{rsk} is activated during *Xenopus* oocyte maturation at the same time as MAPK (Nebreda *et al.*, 1993a; Hsiao *et al.*, 1994). Thus, we decided to investigate the connection between p90^{rsk} and the 90 kDa

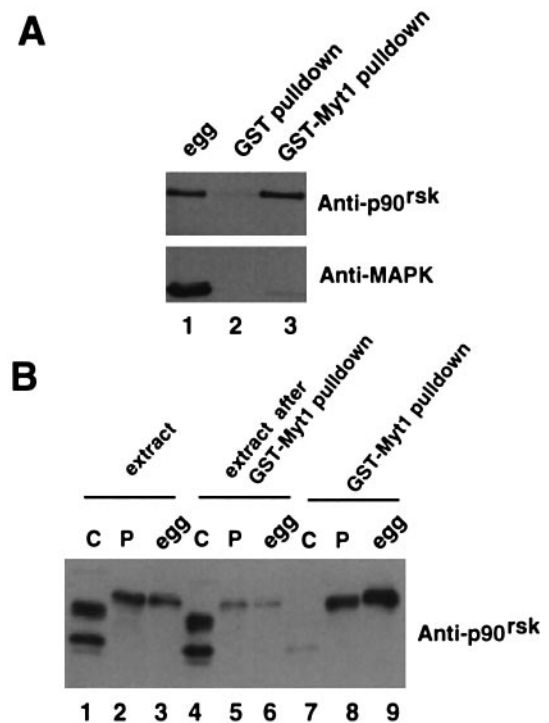


Fig. 5. p90^{rsk} binds to Myt1. (A) Egg extracts (lane 1) and either GST (lane 2) or GST-Myt1 (lane 3) pull-downs of the extracts were analysed by immunoblotting with both anti-p90^{rsk} and anti-Mpk1 MAPK antibodies. (B) GST-Myt1 pull-downs prepared from G_2 -arrested oocyte lysates (lane 7), progesterone-matured oocyte lysates (lane 8) or egg extracts (lane 9) were analysed by immunoblotting with anti-p90^{rsk} antibodies. The extracts before (lanes 1–3) and after the GST pull-downs (lanes 4–6) were also analysed in the same immunoblot.

protein kinase that associates with and phosphorylates the C-terminus of Myt1.

By immunoblotting using anti-p90^{rsk}-specific antibodies, we found that p90^{rsk} can be detected specifically in GST-Myt1 pull-downs prepared from *Xenopus* egg extracts but not in the pull-downs prepared with GST alone (Figure 5A, p90^{rsk}). In contrast, the p42^{Mpk1} MAPK is not present at detectable levels in the same GST-Myt1 pull-downs (Figure 5A, MAPK). Moreover, the C-terminus of Myt1 associates more strongly with the active and hyperphosphorylated p90^{rsk} present either in egg extracts (Figure 5B, lane 9) or in lysates prepared from progesterone-matured oocytes (Figure 5B, lane 8) than with the inactive and hypophosphorylated p90^{rsk} present in lysates from G_2 -arrested oocytes (Figure 5B, lane 7).

p90^{rsk} is an unusual protein kinase in that it contains two catalytic (kinase) domains: the N-terminal D1 domain is related to protein kinase A (PKA) and p70 S6K, while the C-terminal D2 domain is related to the γ -subunit of phosphorylase kinase (Jones *et al.*, 1988). To characterize further the association between p90^{rsk} and the C-terminus of Myt1, we expressed both proteins in a yeast two-hybrid system (Figure 6A). We found that Myt1 and p90^{rsk} can also associate in this assay. Moreover, the association between p90^{rsk} and Myt1 was only detected with the full-length p90^{rsk}, neither the D1 nor the D2 kinase domain alone were able to bind to the C-terminus of Myt1. MAPKAP K-2, a kinase that also can be phosphorylated and activated by MAPK *in vitro* (Stokoe *et al.*, 1992), did

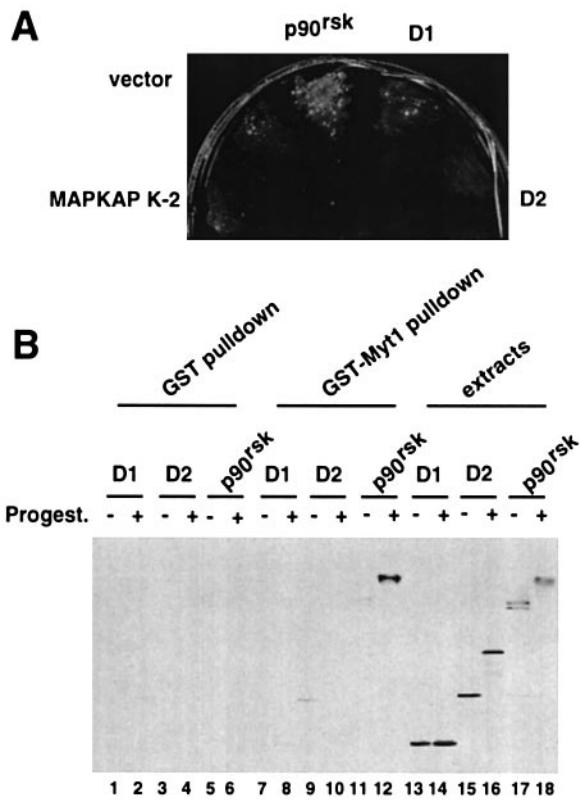


Fig. 6. Myt1 interacts in yeast and in *Xenopus* oocytes with full-length p90^{rsk} but not the D1 or D2 kinase domains independently. (A) *Saccharomyces cerevisiae* α cells expressing the Gal4 DNA-binding domain fused in-frame to the C-terminal half of Myt1 were mated with *S.cerevisiae* a cells expressing the Gal4 activation domain either alone (vector) or fused in-frame to MAPKAP K-2, full-length p90^{rsk}, D1 kinase domain alone or D2 kinase domain alone, as indicated. Positive interactions were selected by plating the cells on medium lacking histidine. The five mating cultures grew to the same extent on medium supplemented with histidine. (B) *In vitro* transcribed mRNAs encoding myc-tagged full-length p90^{rsk} or either myc-tagged D1 or D2 kinase domains alone were injected into *Xenopus* oocytes which, after overnight incubation, were either left untreated (lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17) or matured by progesterone stimulation (lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18). Total lysates prepared from these oocytes (lanes 13–18) together with GST pull-downs (lanes 1–6) and GST-Myt1 pull-downs (lanes 7–12) obtained from the same oocyte lysates were analysed by immunoblot with anti-myc antibodies.

not associate with Myt1. These results strongly suggest a direct interaction between p90^{rsk} and the C-terminus of Myt1 in yeast. Interestingly, other p90^{rsk}-interacting proteins such as the p42^{Mapk1} MAPK can interact in yeast both with the full-length p90^{rsk} and with the D2 kinase domain alone (A.-C.Gavin and A.R.Nebreda, unpublished results). Association between the C-terminus of Myt1 and full-length p90^{rsk}, but not the D1 or the D2 kinase domain, was also detected by expressing these proteins in *Xenopus* oocytes. *In vitro* transcribed mRNAs encoding either full-length p90^{rsk} or the D1 and D2 kinase domains were microinjected into oocytes and, after overnight incubation to allow for expression of the proteins, the corresponding oocyte lysates were used for GST-Myt1 pull-downs. The results of this experiment shown in Figure 6B confirm that Myt1 associates more strongly with the active, hyperphosphorylated p90^{rsk} than with the hypophosphorylated form.

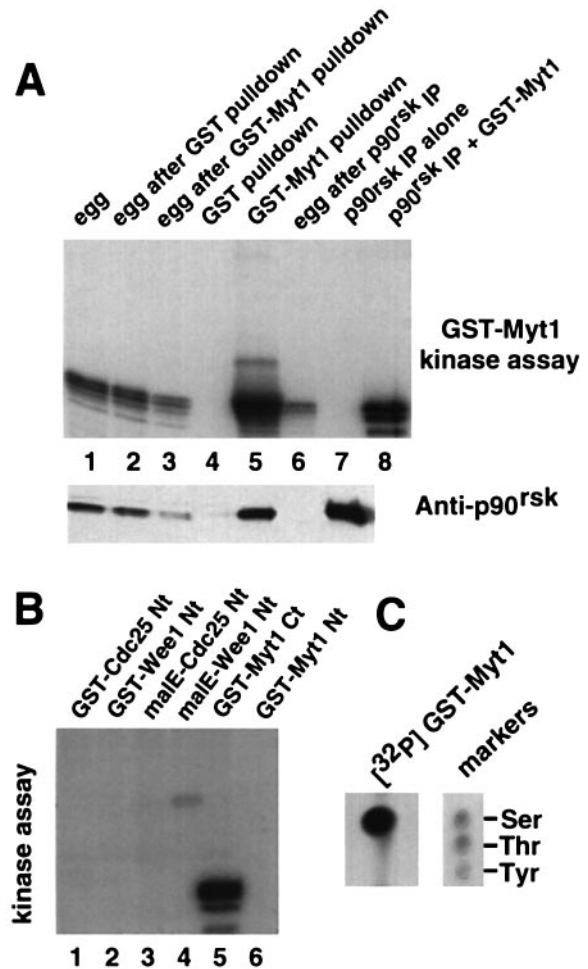


Fig. 7. p90^{rsk} phosphorylates Myt1 on serine residues. (A) Upper panel: *in vitro* kinase assay using GST-Myt1 as a substrate (except for lane 7 where no substrate was added). Lane 1, egg extracts; lane 2, egg extracts after GST pull-down; lane 3, egg extracts after GST-Myt1 pull-down; lane 4, GST pull-down from egg extracts; lane 5, GST-Myt1 pull-down from egg extracts; lane 6, egg extracts after p90^{rsk} immunoprecipitation; lanes 7 and 8, p90^{rsk} immunoprecipitates from egg extracts incubated alone or in the presence of GST-Myt1, respectively. Lower panel: immunoblot with anti-p90^{rsk} antibodies of the same samples. (B) *In vitro* kinase assay using p90^{rsk} immunoprecipitates from egg extracts and as a substrate either GST-Cdc25 Nt (lane 1), GST-Wee1 Nt (lane 2), malE-Cdc25 Nt (lane 3), malE-Wee1 Nt (lane 4), GST-Myt1 Ct (lane 5) or GST-Myt1 Nt (lane 6), as indicated. (C) GST-Myt1 was *in vitro* phosphorylated by p90^{rsk} immunoprecipitates in the presence of [γ -³²P]ATP and then subjected to phosphoamino acid analysis.

p90^{rsk} phosphorylates Myt1

We next investigated whether p90^{rsk} can phosphorylate the C-terminus of Myt1. Immunoprecipitates prepared from egg extracts with anti-p90^{rsk}-specific antibodies can phosphorylate exogenously added GST-Myt1 efficiently *in vitro* (Figure 7A, lane 8). Moreover, the Myt1-phosphorylating kinase activity in the egg extracts can be immunodepleted using p90^{rsk} antibodies (Figure 7A, compare lane 6 with lanes 1 and 2). As expected, GST-Myt1 pull-downs can also phosphorylate exogenously added GST-Myt1 efficiently (Figure 7A, lane 5). In addition, a significant reduction in Myt1-phosphorylating activity in the extract was observed following GST-Myt1 pull-downs (Figure 7A, lane 3). When the same set of samples was analysed by immunoblot with anti-p90^{rsk}

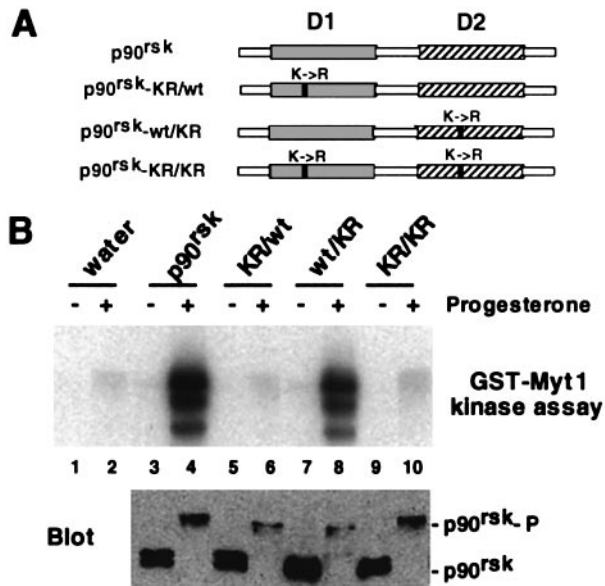


Fig. 8. The D1 but not the D2 kinase domain of p90^{rsk} is required for phosphorylation of Myt1. (A) Schematic representation of the lysine to arginine mutations introduced in p90^{rsk}. (B) *In vitro* transcribed mRNAs encoding the myc-tagged p90^{rsk} mutants were injected into *Xenopus* oocytes which then were either left untreated (lanes 1, 3, 5, 7 and 9) or matured by progesterone stimulation (lanes 2, 4, 6, 8 and 10). The p90^{rsk} proteins expressed in the oocytes were recovered by immunoprecipitation with anti-myc antibodies and the immunoprecipitates were then used in kinase assays with GST-Myt1 (upper panel). An immunoblot with anti-myc antibodies of the same samples is shown in the lower panel and the positions where p90^{rsk} and the hyperphosphorylated p90^{rsk} (p90^{rsk}-P) run are indicated.

antibodies (Figure 7A, lower panel), we found that following GST-Myt1 pull-downs the total amount of p90^{rsk} in the extract was strongly reduced (Figure 7A, compare lanes 2 and 3 in the lower panel). This suggests a strong interaction between the C-terminus of Myt1 and p90^{rsk}, which would allow for the almost quantitative removal of p90^{rsk} from the egg extract. We then analysed *Xenopus* egg extracts, fractionated using Mono Q chromatography, by immunoblot with anti-p90^{rsk} antibodies, and found that the fractions where p90^{rsk} was detected overlapped with the major peak of kinase activity phosphorylating GST-Myt1 *in vitro* (data not shown).

To assess the specificity of p90^{rsk} for the C-terminus of Myt1, we investigated the ability of p90^{rsk} immunoprecipitates to phosphorylate GST fused to the N-terminus of Myt1 (Myt Nt). GST-Myt1 Nt was not phosphorylated by p90^{rsk} *in vitro* (Figure 7B, lane 6). We also used recombinant proteins containing the N-terminal regulatory regions of either Wee1 or Cdc25C, but neither was phosphorylated significantly by p90^{rsk} *in vitro* (Figure 7B, lanes 1-4). Phosphoamino acid analysis of *in vitro* phosphorylated GST-Myt1 showed that phosphorylation by p90^{rsk} only involves serine residues (Figure 7C), consistent with the known substrate specificity of p90^{rsk} (Leighton *et al.*, 1995).

To investigate which of the two p90^{rsk} kinase domains is responsible for Myt1 phosphorylation, mRNAs encoding myc-tagged p90^{rsk} proteins mutated in either one or both domains (Figure 8A) were microinjected into *Xenopus* oocytes. The expressed p90^{rsk} mutant proteins were then recovered by immunoprecipitation (Figure 8B, lower

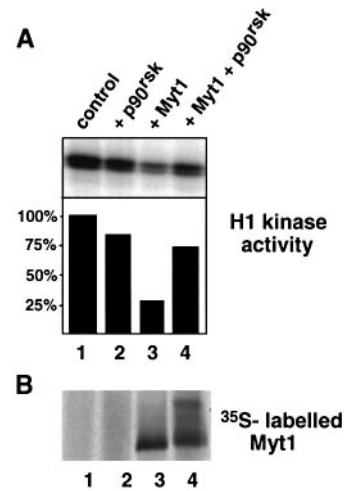


Fig. 9. p90^{rsk} down-regulates Myt1 activity. (A) H1 kinase activity of p34^{cdc2}/cyclin B1 complexes which were pre-incubated with control immunoprecipitates (lanes 1 and 2) or immunopurified Myt1 (lanes 3 and 4) either in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of active p90^{rsk}. The phosphorylation reactions were analysed by polyacrylamide gel electrophoresis and autoradiography, and the results were quantified using a PhosphorImager. We observed inhibition of Myt1 activity by p90^{rsk} in four independent experiments. (B) The ³⁵S-labelled Myt1 protein in the same samples: control (lane 1), p90^{rsk} (lane 2), Myt1 immunoprecipitates (lane 3), Myt1 immunoprecipitates + p90^{rsk} (lane 4).

panel) and tested for their ability to phosphorylate GST-Myt1 *in vitro* (Figure 8B, upper panel). As for the endogenous p90^{rsk} present in oocytes, we found that activation of the myc-tagged p90^{rsk} required that the oocytes are first stimulated by progesterone treatment (Figure 8B, lanes 3 and 4). Moreover, mutation of both p90^{rsk} kinase domains (D1 and D2) totally abolished the activity of p90^{rsk} in progesterone-treated oocytes (Figure 8B, lane 10). However, mutation of the D2 kinase domain alone only slightly reduced the activity of p90^{rsk} on GST-Myt1 (Figure 8B, lane 8), whereas mutation of only D1 totally impaired the ability of p90^{rsk} to phosphorylate GST-Myt1 *in vitro* (Figure 8B, lanes 6). This is consistent with results published for chicken Rsk2 and rat p90^{rsk1} which showed that the D1 kinase domain is required for the phosphorylation of exogenous substrates (Leighton *et al.*, 1995; Fisher and Blenis, 1996). These results together with the in-gel kinase assay strongly suggest that the activity phosphorylating Myt1 is p90^{rsk} itself rather than a p90^{rsk}-associated protein kinase.

p90^{rsk} inactivates Myt1

To test the effect of phosphorylation by p90^{rsk} on Myt1 activity, we expressed myc-tagged Myt1 in reticulocyte lysate in the presence of microsomal membranes and then immunoprecipitated the Myt1 protein with anti-myc antibodies. The immunopurified Myt1 was able to inhibit the histone H1 kinase activity of p34^{cdc2}/cyclin B1 complexes purified from baculovirus-infected insect cells (Figure 9A, lanes 1 and 3). The inhibition was between 45 and 75% using as a reference the activity of p34^{cdc2}/cyclin B1 complexes incubated with anti-myc immunoprecipitates prepared from reticulocyte lysates not expressing Myt1. Interestingly, pre-incubation of Myt1 immunocomplexes with active p90^{rsk} significantly reduced the

ability of Myt1 to inhibit the kinase activity of p34^{cdc2}/cyclin B1 complexes (Figure 9A, lanes 3 and 4). Active p90^{rsk} alone had essentially no effect on the kinase activity of p34^{cdc2}/cyclin B1 complexes (Figure 9A, lanes 1 and 2). We also found that the down-regulation of Myt1 inhibitory activity upon incubation with active p90^{rsk} correlated with an upward shift of the electrophoretic mobility of the Myt1 protein (Figure 9B, lanes 3 and 4). These results show that p90^{rsk}-induced phosphorylation of Myt1 inhibits its activity towards p34^{cdc2}/cyclin B complexes. In contrast, in parallel assays, we have not been able to detect down-regulation by p90^{rsk} of the inhibitory activity of myc-tagged Wee1 immunopurified from reticulocyte lysates (data not shown).

Endogenous Myt1 is phosphorylated during maturation and associates with p90^{rsk} in mature oocytes

Our results indicate that inhibition of Myt1 kinase activity may have an important role in oocyte maturation. To obtain further support for this hypothesis, we investigated the status of endogenous Myt1 in oocytes. We found that Myt1 protein is present in G₂-arrested oocytes (Figure 10A, lane 1) in contrast to Wee1 (Murakami and Vande Woude, 1998). Moreover, the electrophoretic mobility of the endogenous Myt1 protein is reduced in mature oocytes, consistent with the protein being hyperphosphorylated (and inactivated) during oocyte maturation (Figure 10A, lane 2). To investigate whether the MAPK-p90^{rsk} cascade has an effect on endogenous Myt1, oocytes were injected with Mos mRNA and then incubated for 4 h. Under these conditions where MAPK and p90^{rsk} are activated but MPF is not, the band corresponding to endogenous Myt1 protein also shifted upwards, although the shift was less pronounced than in mature oocytes (Figure 10A, lane 3). These results show that the MAPK-p90^{rsk} cascade can trigger some Myt1 phosphorylation in the absence of MPF activity and that in Mos-injected oocytes this phosphorylation precedes the activation of pre-MPF.

We next investigated whether a complex between the endogenous Myt1 and p90^{rsk} is present in mature oocytes. Using anti-Myt1 antibodies, we detected a band of ~80 kDa in immunoblots prepared with the membrane fraction isolated from egg extracts (Figure 10B, lane 1). A band of the same electrophoretic mobility was also detected by the anti-Myt1 antibodies in p90^{rsk} immunoprecipitates prepared from egg extracts (Figure 10B, lanes 3 arrow), but not in control immunoprecipitates prepared in parallel (Figure 10B, lanes 2). These results suggest that the hyperphosphorylated Myt1 protein forms a complex with p90^{rsk} in mature oocytes.

Discussion

We have shown that the C-terminal, non-catalytic region of the p34^{cdc2} inhibitory kinase Myt1 can be phosphorylated by protein kinases which are present in M-phase egg extracts. In parallel experiments, we do not detect significant phosphorylation of the N-terminal half of Myt1 which includes the kinase domain (data not shown). Since Myt1 hyperphosphorylation during M-phase correlates with decreased activity of the enzyme (Mueller *et al.*, 1995b), our results suggest a correlation between phospho-

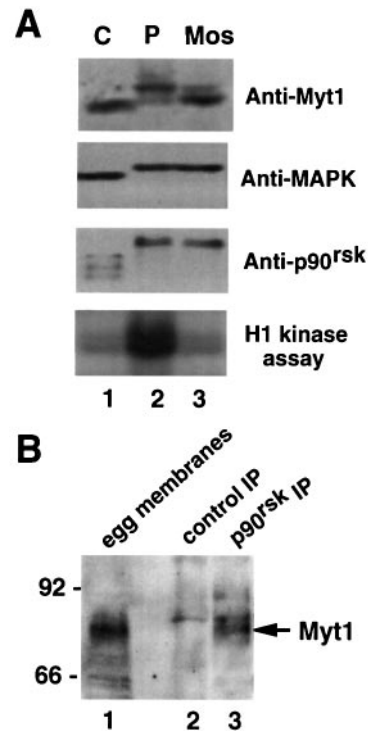


Fig. 10. The endogenous Myt1 is phosphorylated during oocyte maturation and associates with p90^{rsk} in mature oocytes. (A) Membrane fractions (equivalent to ~30 oocytes) were isolated from G₂-arrested oocytes (lane 1), oocytes matured by incubation with progesterone for 16 h (lane 2) or oocytes injected with *in vitro* transcribed Mos mRNA and incubated for 4 h (lane 3) and then analysed by immunoblot with anti-Myt1 antibodies. Aliquots of the same total lysates (equivalent to about one oocyte) before membrane purification were either used for H1 kinase assays or analysed by immunoblot with anti-MAPK and anti-p90^{rsk} antibodies, as indicated. (B) Egg extracts (300 μ l) were immunoprecipitated using either control (lane 2) or anti-p90^{rsk} (lane 3) antibodies and then analysed by immunoblot with anti-Myt1 antibodies. Membrane fractions isolated from 50 μ l of egg extracts were analysed in parallel (lane 1). The arrow indicates a band of ~80 kDa which is recognized by the affinity-purified anti-Myt1 antibodies and is present both in isolated membrane fractions and in p90^{rsk} immunoprecipitates.

rylation of the C-terminus of Myt1 and down-regulation of Myt1 kinase activity. We have identified at least two protein kinases that can bind to and phosphorylate the non-catalytic domain of Myt1. One of them is dependent on MPF activity and may be p34^{cdc2}/cyclin B or another p34^{cdc2}/cyclin complex since we detected p34^{cdc2} in GST-Myt1 pull-downs. This suggests that phosphorylation of Myt1 by p34^{cdc2}/cyclin B (or a downstream kinase) may be involved in a feedback loop of autoamplification, as has been proposed for Cdc25C and Wee1 (Hoffmann *et al.*, 1993; Mueller *et al.*, 1995a). The other kinase activity is p90^{rsk} which can bind to and phosphorylate the C-terminus of Myt1 independently of MPF activity.

p90^{rsk} associates with and phosphorylates Myt1

The interaction between p90^{rsk} and the C-terminus of Myt1 is strong enough to allow bacterially produced GST-Myt1 to almost deplete the endogenous p90^{rsk} from *Xenopus* egg extracts. In contrast, GST-Myt1 does not bind detectable amounts of MAPK from egg extracts. We also found association between p90^{rsk} and the C-terminus of Myt1 in a yeast two-hybrid system, which strongly

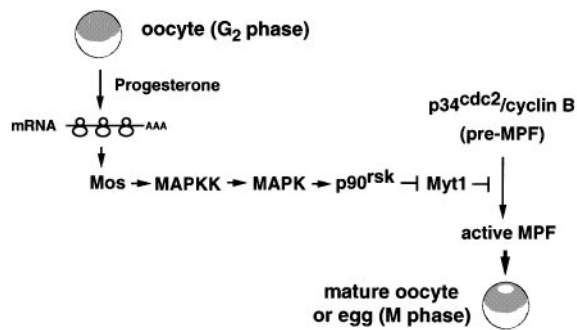


Fig. 11. A pathway to link the activation of MAPK and p34^{cdc2}/cyclin B during oocyte maturation. In G₂-arrested oocytes, progesterone stimulates unidentified signalling pathways that lead to the translation of maternal mRNAs including Mos. Accumulation of the protein kinase Mos induces the activation of MAPK, via MAPK kinase, and MAPK in turn activates p90^{rsk}. Our results suggest that p90^{rsk} down-regulates the p34^{cdc2} inhibitory kinase Myt1, thus leading to the activation of p34^{cdc2}/cyclin B (pre-MPF) and entry into M-phase of meiosis. For clarity, feedback loops and other proteins that may also have a role in triggering meiotic maturation are omitted from the scheme.

suggests a direct interaction between the two proteins. Moreover, the interaction of p90^{rsk} and Myt1 is only detected when the full-length p90^{rsk} is used; neither of the two p90^{rsk} kinase domains D1 or D2 alone interact with Myt1. This is in contrast to the ability of MAPK to interact with the D2 kinase domain of p90^{rsk} alone, and suggests that MAPK and Myt1 probably interact with different regions of p90^{rsk}. Finally, the association between p90^{rsk} and Myt1 is enhanced by the hyperphosphorylation that normally accompanies the activation of p90^{rsk}, which is consistent with the observation that inactive p90^{rsk} is associated mostly with MAPK and supports the idea that Myt1 may be a relevant substrate for p90^{rsk} (see below). Our results also indicate that the requirements for binding to p90^{rsk} are very different for MAPK (a p90^{rsk} activator) and Myt1 (a p90^{rsk} substrate).

The specificity of the phosphorylation of the C-terminal region of Myt1 by p90^{rsk} is supported by the observation that p90^{rsk} efficiently phosphorylates neither the N-terminus of Myt1 nor the regulatory regions of either Cdc25C or Wee1. Moreover, as reported for other p90^{rsk} substrates (Leighton *et al.*, 1995; Fisher and Blenis, 1996), the ability of p90^{rsk} to phosphorylate Myt1 is only slightly affected by mutation of the D2, C-terminal kinase domain of p90^{rsk}, but it is totally impaired by mutation of the D1 N-terminal kinase domain.

A link between MAPK and MPF during oocyte maturation

The strong and specific interaction between p90^{rsk} and Myt1 as well as the ability of p90^{rsk} to phosphorylate Myt1 suggest a functional connection between the two proteins. This is supported further by the observation that phosphorylation by p90^{rsk} interferes with the inhibitory activity of Myt1 on p34^{cdc2}/cyclin B1 complexes *in vitro*. This result suggests a direct link between the MAPK pathway and the activation of p34^{cdc2}/cyclin B (MPF) during oocyte maturation (Figure 11). At the same time, this is consistent with the work showing that MAPK down-regulates a factor which can inactivate MPF (Abrieu *et al.*, 1997). Our model implies that p90^{rsk} is an important

mediator of the triggering activity of MAPK in oocyte maturation. Several reasons, however, make it difficult to demonstrate directly a role for p90^{rsk} in oocyte maturation. First, both MAPK and p90^{rsk} are always activated at the same time in *Xenopus* oocytes, which is probably related to the fact that they are associated in their inactive forms. Secondly, so far it has not been possible to activate p90^{rsk} independently of MAPK and there are no constitutively active p90^{rsk} mutants available. Finally, inhibitors of p90^{rsk} activity *in vitro* (Alessi, 1997) do not appear to be effective when used in intact *Xenopus* oocytes (unpublished observations).

Another implication of our model is that Myt1 may be the activity responsible for maintaining the pre-formed p34^{cdc2}/cyclin B complexes in their inactive state (pre-MPF). Consistent with this possibility, we find that Myt1 protein is present in G₂-arrested oocytes, in contrast to Wee1 which is only detected upon progesterone stimulation (Murakami and Vande Woude, 1998). We also find that during oocyte maturation the electrophoretic mobility of the endogenous Myt1 protein shifts upwards, which is most likely due to hyperphosphorylation. As reduced electrophoretic mobility of Myt1 has been shown to correlate with down-regulation of Myt1 activity (Mueller *et al.*, 1995b; Booher *et al.*, 1997), our results suggest that Myt1 is active in G₂-arrested oocytes and later down-regulated during oocyte maturation. Moreover, we detected partial phosphorylation of the endogenous Myt1 protein under conditions where the MAPK–p90^{rsk} pathway is activated but MPF is not. This is the case, for example, in Mos-injected oocytes, where activation of MAPK–p90^{rsk} and phosphorylation of Myt1 precede the activation of pre-MPF. We have also found that endogenous Myt1 and p90^{rsk} form a complex in mature oocytes. These results, together with the experiments showing phosphorylation and inactivation of Myt1 by p90^{rsk} *in vitro*, indicate that p90^{rsk} is an excellent candidate for triggering the inhibition of Myt1 kinase activity during oocyte maturation. However, it should be noted that human Myt1 is a substrate for p34^{cdc2}/cyclin B1 complexes *in vitro* (Booher *et al.*, 1997), and we have also shown that the C-terminus of *Xenopus* Myt1 can interact either directly or indirectly with p34^{cdc2}. Thus Myt1 regulation might require a multistep mechanism which could ensure the proper timing for inactivation of Myt1. This would be consistent with our observation that in M-phase extracts, at least two protein kinases can phosphorylate the C-terminus of Myt1.

In summary, our results indicate that down-regulation of Myt1 activity by p90^{rsk} phosphorylation is very likely to be of physiological relevance for the activation of MPF during progesterone-induced oocyte maturation (Figure 11). We cannot rule out, however, that alternative pathways, leading for example to the up-regulation of the Cdc25C phosphatase, may also be activated in parallel during the process. Further work is needed to elucidate fully the regulation of Myt1 function by phosphorylation and its contribution to the signalling pathways that trigger MPF activation and the meiotic maturation of *Xenopus* oocytes.

Materials and methods

cDNA cloning and manipulation

Xenopus Myt1 (Mueller *et al.*, 1995b), Wee1 (Mueller *et al.*, 1995a), Cdc25C (Kumagai and Dunphy, 1992) and p90^{rsk} (Jones *et al.*, 1988)

were cloned from *Xenopus* oocyte and egg cDNA libraries by PCR using *Pfu* polymerase (Stratagene) and oligonucleotides based on published sequences. Complete details of the oligonucleotides and PCR conditions will be provided upon request. All the constructs were confirmed by DNA sequencing.

The N-terminal half of Myt1 which includes the kinase domain (Myt1 Nt, amino acids 1–329) was cloned using a 5' oligonucleotide designed to create a *Sma*I site in front of the first ATG and a 3' oligonucleotide that introduced a stop codon at nucleotide 1011 followed by a *Sall*I site. The C-terminal half of Myt1 which includes the transmembrane domain (Myt1 Ct, amino acids 322–518) was isolated using oligonucleotides designed to create a *Bam*HI site at nucleotide 990 and a *Sall*I site downstream of the stop codon. Both PCR fragments were digested with the corresponding restriction endonucleases and cloned into the pGEX-KG vector. To prepare the GST–Myt1 Ct recombinant protein, we generated a modified Myt1 Ct which lacks the transmembrane domain (Myt1 Ct₂, amino acids 389–518) using an oligonucleotide that created a *Bam*HI site at nucleotide 1188. Myt1 Ct₂ was also subcloned into the *Nde*I–*Sall*I sites of the yeast vector pAS2-1 using an oligonucleotide designed to create a *Nde*I site at amino acid 389. Full-length Myt1 was cloned using a 5' oligonucleotide that introduced a *Nco*I site at the first ATG and a 3' oligonucleotide that created a *Xho*I site downstream of the stop codon. The PCR product was cloned between the *Nco*I and *Xho*I sites of the FTX5 vector (provided by C.Hill, ICRF, London).

The N-terminal, non-catalytic half of Wee1 (Wee1 Nt, amino acids 6–209) was cloned using a 5' oligonucleotide that created a *Bam*HI site at nucleotide 22 downstream of the first ATG and a 3' oligonucleotide that introduced an *Eco*RI site at nucleotide 625. The PCR product was digested with *Bam*HI and *Eco*RI and inserted between the *Bam*HI and *Eco*RI sites of pGEX-KG, in order to express the GST–Wee1 Nt protein. Wee1 Nt was also subcloned from pGEX–Wee1 Nt into pMalc2 as a *Bam*HI–*Sall*I fragment to produce the malE–Wee1 Nt fusion protein.

The N-terminal half of Cdc25C (Cdc25 Nt, amino acids 9–205) was cloned using a 5' oligonucleotide that created a *Bcl*I site at nucleotide 22 downstream of the first ATG and a 3' oligonucleotide based on the sequence of Cdc25 from nucleotides 672 to 645, downstream of the internal *Bam*HI site at position 609. The PCR product was digested with *Bcl*I and *Bam*HI and cloned into the *Bam*HI site of pGEX-KG. Cdc25 Nt was also subcloned into pMalc2 by PCR from pGEX–Cdc25 Nt using a 5' oligonucleotide that reconstituted the *Bam*HI site at the *Bcl*I–*Bam*HI site and a 3' oligonucleotide that eliminated the *Bam*HI site at position 609 and introduced immediately downstream a stop codon followed by a new *Bam*HI site. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pMalc2.

p90^{rsk} was cloned using a 5' oligonucleotide that introduced a *Nco*I site at the first ATG and a 3' oligonucleotide that created an *Xho*I site downstream of the stop codon. The PCR products were cloned into the PCRII vector (Invitrogen) and analysed by DNA sequencing. One of the clones was found to be identical in sequence to the p90^{rsk} α isoform described by Jones *et al.* (1988) and was chosen for further work. To subclone the N-terminal (D1) and C-terminal (D2) domains of p90^{rsk}, PCRII–p90^{rsk} was digested with *Nco*I and *Xho*I and the 923 bp *Nco*I–*Nco*I fragment (D1) and 1279 bp *Nco*I–*Xho*I fragment (D2) were ligated into both pACT2 (Clontech) and FTX5. The full-length p90^{rsk} was also subcloned into the pACT2 and FTX5 vectors by digestion of PCRII–p90^{rsk} with *Xho*I followed by partial digestion with *Nco*I. Site-directed mutagenesis to change the conserved lysine residues to arginine in the D1 (K94R) and D2 (K445R) catalytic domains was done by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). The mutations were confirmed by DNA sequencing. Capped mRNAs were obtained from *Xba*I-linearized FTX5 constructs using the MEGAscript *in vitro* transcription kit (Ambion) according to the manufacturer's instructions. After LiCl precipitation, mRNAs were resuspended in 25 μ l of diethylpyrocarbonate (DEPC)-treated water and stored at –70°C. All proteins expressed from FTX5 constructs contained the myc epitope at the N-terminus (Howell and Hill, 1997).

Bacterial expression and purification of GST and malE fusion proteins

Recombinant GST or malE fusion proteins were expressed in *Escherichia coli* TG-1 or in *E. coli* DE3. For the GST fusion proteins, fresh overnight LB-ampicillin (100 μ g/ml) cultures were diluted 10-fold with fresh medium and incubated at 37°C until the A₆₀₀ was 0.4–0.5. Induction was for 6 h at 23°C using 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The cells were harvested, washed with cold phosphate-buffered saline (PBS) and lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM

benzamidine, 0.05% NP-40 and 0.5 mg/ml lysozyme, for 30 min at 4°C. After sonication and centrifugation at 10 000 g for 15 min, the supernatant was mixed with glutathione–Sepharose (Pharmacia) for 30 min at 4°C. Beads were washed in lysis buffer containing neither NP-40 nor lysozyme, and the GST fusion proteins were eluted with 10 mM glutathione in 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (DTT). For the purification of malE fusion proteins, the *E. coli* cultures were obtained as above and induced with 0.1 mM IPTG. Bacterial pellets were lysed for 30 min at 4°C in 50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF and 1 mg/ml lysozyme. The lysate was sonicated, and 1 mM DTT was added before centrifugation. The supernatants were incubated with amylose beads (New England Biolabs) for 30 min at 4°C and the beads were washed twice with PBS and once with 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT. The malE-fused protein was eluted in the same buffer supplemented with 0.1 mM EDTA and 12 mM maltose. Fractions containing the purified GST or malE fusion proteins were dialysed overnight against 20–50 mM Tris, pH 7.5–8.0, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 5% glycerol and stored in aliquots at –70°C.

Preparation and fractionation of egg extracts

CSF-arrested egg extracts were prepared as described by Murray (1991). Ca²⁺ treatment was performed by the addition of 0.4 mM CaCl₂ to the egg extracts followed by incubation at 25°C. For fractionation, the egg extracts were first diluted 1:10 in H1K buffer (80 mM β -glycerophosphate, pH 7.5, 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 1 mM PMSF or AEBSEF, 2.5 mM benzamidine and 2 μ g/ml each of leupeptin and aprotinin) and ultracentrifuged at 100 000 g for 90 min. Ammonium sulfate was added to the supernatant to 35% saturation and, after incubation for 30 min at 4°C, the solution was centrifuged at 10 000 g for 30 min. The pellet was resuspended in 20 mM Tris, pH 7.4, 1 mM DTT, 0.1 mM EDTA, dialysed and applied to an anion exchange column of Mono Q HR 5/5 (Pharmacia Biotech) equilibrated in 20 mM Tris, pH 7.4. The column was developed with a 20 ml linear salt gradient up to 0.5 M NaCl in equilibration buffer. The flow rate was 1 ml/min and fractions of 1 ml were collected and assayed for kinase activity or immunoblotting.

To prepare membrane fractions from eggs, 400 μ l of concentrated CSF-arrested extracts were centrifuged at 260 000 g for 90 min. The pellet was resuspended in 60 μ l of 10 mM HEPES, pH 7.7, 2.5 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.25 M sucrose, 1 mM PMSF, 2.5 mM benzamidine, 1 μ M calyculin A and 2 μ g/ml each of leupeptin and aprotinin and stored at –70°C.

Oocyte maturation and mRNA expression

Meiotic maturation of *Xenopus* oocytes was induced by incubation of manually dissected oocytes with 5 μ g/ml of progesterone (Sigma) in modified Barth's medium. For expression of myc-tagged proteins, oocytes were microinjected with 50 nl of the *in vitro* transcribed mRNAs (diluted 1:5 in DEPC-treated water) and maintained overnight at 18°C before progesterone treatment. For the preparation of lysates, oocytes were homogenized in 10 μ l per oocyte of ice-cold H1K buffer. The lysates were centrifuged at 10 000 g for 10 min and the cleared supernatants stored at –70°C. To prepare membrane fractions from oocytes, the low speed supernatants corresponding to 100 oocytes were centrifuged further at 260 000 g for 90 min. The pellet was resuspended in 50 μ l of the same buffer as indicated above for egg extracts and stored at –70°C.

Kinase assays

Protein kinases normally were assayed in a final volume of 12 μ l of H1K buffer containing 4 μ l of egg extract (diluted 1:10) or oocyte lysate, 50 μ M cold ATP, 2 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and either 4 μ g of histone H1 (Sigma), 3 μ g of myelin basic protein (MBP, Sigma) or 1 μ g of the GST or malE bacterially expressed fusion proteins. After 15 min at room temperature, the phosphorylation reactions were terminated by addition of sample buffer and were analysed by polyacrylamide gel electrophoresis and autoradiography.

Myc-tagged 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 MOPS, pH 7.2, 1 mM DTT, 10 mM MgCl₂, 10 mM *p*-nitrophenylphosphate, 0.1% Triton X-100, 1 μ M NaVO₃, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 100 μ M PMSF or AEBSEF) containing 100 μ M ATP, 5 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and 0.4 μ g of GST–Myt1.

For in-gel kinase assays, proteins were separated by polyacrylamide gel electrophoresis on 10% Laemmli gels which were polymerized in the presence of 100 μ g/ml of either GST–Myt1, GST–Wee1 or GST

alone. After electrophoresis, the gels were processed following the protocol described by Peverali *et al.* (1996).

To assay Myt1 activity, myc-tagged Myt1 protein was expressed from FTX5-Myt1 using TNT reticulocyte lysate supplemented with canine pancreatic microsomal membranes as recommended by the supplier (Promega). After translation, the reticulocyte lysate (50 μ l) was diluted 1:1 in immunoprecipitation (IP) buffer (see below) and then incubated for 2 h at 4°C with 10 μ l of 9E10 anti-myc antibody conjugated to agarose (Santa Cruz Biotechnology). As a control, the same 9E10 antibody agarose conjugate was used to immunoprecipitate reticulocyte lysates which did not express Myt1. The Myt1 and the control immunocomplexes were washed three times in IP buffer, twice with kinase buffer (50 mM HEPES-KOH, pH 7.3, 10 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 2.5 mM benzamidine, 1 μ M calyculin A and 10 μ g/ml each of leupeptin and aprotinin) and then incubated in 12 μ l of kinase buffer containing 250 μ M ATP and 6 μ l of p34^{cdc2}/cyclin B1 complexes prepared from baculovirus-infected Sf9 cells as described by Kumagai and Dunphy (1997). After 30 min at room temperature, the activity of the p34^{cdc2}/cyclin B1 complexes was assayed by adding 12 μ l of kinase buffer containing 4 μ g of histone H1 and 2 μ Ci of [γ -³²P]ATP, and incubating for a further 15 min at room temperature. The samples finally were boiled in sample buffer and analysed by polyacrylamide gel electrophoresis and autoradiography. To determine the effect of p90^{rsk} phosphorylation on Myt1 activity, the Myt1 immunocomplexes were pre-incubated with active p90^{rsk} for 30 min at room temperature in 12 μ l of kinase buffer supplemented with 350 μ M ATP. We observed down-regulation of Myt1 activity using either active GST-p90^{rsk} fusion protein purified from mammalian cells (0.1 μ g, provided by D.Alessi, MRC, Dundee, Scotland) or *Xenopus* p90^{rsk} immunopurified from egg extracts as described below.

Immunoblotting, immunoprecipitation and GST pull-down experiments

For immunoblotting, protein samples normally were separated by polyacrylamide gel electrophoresis on 15% Anderson gels (Nebreda *et al.*, 1995) and then transferred to 0.2 μ m nitrocellulose membranes using a semi-dry blotting apparatus (Hofer). Blocking was done in TTBS (25 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween-20) containing 4% milk, and the antibodies were incubated in TTBS containing 1% milk. The monoclonal antibody 3E1 (provided by J.Gannon and T.Hunt, ICRF South Mimms) was used to detect p34^{cdc2} (Nebreda *et al.*, 1995), and the rabbit antiserum 3297.1 prepared against the same C-terminal peptide of the p42^{Erk2} MAPK as described by Leever and Marshall (1992) was used for the Xp42/Mpk1 MAPK. p90^{rsk} was detected using a purified anti-Rsk1 goat antibody (C-21-G, Santa Cruz Biotechnology). The 9E10 monoclonal antibody was used to detect myc-tagged proteins. The anti-Myt1 antibodies were prepared in rabbits against a keyhole limpet haemocyanin (KLH)-coupled peptide corresponding to the C-terminal end of Myt1 (CPRNLLGMFDDATEQ) and were affinity purified on GST-Myt1 Ct coupled to Affi-Gel 10 (Bio-Rad). In all cases, horseradish peroxidase (HRP)-coupled secondary antibodies (Dakko) were used and the binding was detected using ECL (Amersham).

The endogenous p90^{rsk} was immunoprecipitated from egg extracts using 5 μ g of the anti-Rsk1 antibody C-21-G pre-bound to 10 μ l of protein G-Sepharose 4B (Pharmacia). In a typical experiment, the antibody-bound beads were washed in IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 20 mM NaF, 0.5–1% NP-40, 100 μ M Na₃VO₄, 1 mM PMSF, 2.5 mM benzamidine, 10 μ g/ml each of leupeptin and aprotinin and either 2 μ M microcystin or 1 μ M calyculin A) and then incubated for 2 h at 4°C in 100 μ l of the 100 000 g supernatant from egg extracts (diluted 1:10). The immunocomplexes were washed twice with IP buffer and once with H1K buffer and then either used immediately for *in vitro* kinase assay as indicated above or analysed by immunoblot. To investigate the co-immunoprecipitation of Myt1 and p90^{rsk}, 300 μ l of concentrated egg extracts were mixed 1:1 with IP buffer, left on ice for 20 min and then centrifuged at 10 000 g for 10 min. p90^{rsk} immunocomplexes were prepared from the supernatant as described above and then analysed by immunoblot with affinity-purified anti-Myt1 antibodies.

For immunoprecipitation of myc-tagged p90^{rsk} expressed in oocytes, 3 μ g of the anti-myc antibody 9E10 were pre-bound to 20 μ l of protein G beads in IP buffer and then incubated for 2 h at 4°C in 70 μ l of lysate prepared from oocytes expressing either wild-type or mutant forms of p90^{rsk}. The immunocomplexes were washed three times in IP buffer and once in S6 kinase buffer (see above) and then were used in kinase assays or analysed by immunoblotting.

For GST pull-down experiments, 5–10 μ g of the bacterially produced

GST fusion proteins were pre-bound to 20 μ l of glutathione-Sepharose beads and then incubated in 80–100 μ l of either oocyte lysates or the 100 000 g supernatant from egg extracts (diluted 1:10). After 2 h rocking at 4°C, the beads were washed three times with H1K buffer and then either used immediately for kinase assay as indicated above or analysed by immunoblot.

Phosphoamino acid analysis

GST-Myt1 was phosphorylated with p90^{rsk} immunopurified from egg extracts as indicated above. After polyacrylamide gel electrophoresis, the proteins were transferred to a PVDF membrane (Immobilion, Millipore) and the band corresponding to GST-Myt1 was cut out. Phosphoamino acids were analysed by HCl hydrolysis of the protein followed by thin-layer chromatography using standard procedures (Kamps, 1991).

Yeast transformation and mating

For analysis of protein interactions, yeast transformations were performed using the lithium/acetate protocol as recommended by Clontech. The constructs pAS2-1-Myt1 Ct₂, pACT2-p90^{rsk}, pACT2-D1 and pACT2-D2 were prepared as indicated above. pACT2-MAPKAP K-2 was prepared by PCR using a rabbit MAPKAP K-2 cDNA provided by P.Cohen (MRC, University of Dundee, Scotland). For mating, pAS2-1 constructs were transformed into Y187 α cells (Clontech) and pACT2 constructs into Y190 α cells (Clontech). Both cell types were incubated together in 0.5 ml of YPD medium for ~8 h and then 10 μ l of the culture were plated on selective medium.

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