A MAP kinase docking site is required for phosphorylation and activation of p90^{rsk}/MAPKAP kinase-1

Anne-Claude Gavin and Angel R. Nebreda

Activation of the various mitogen-activated protein (MAP) kinase pathways converts many different extracellular stimuli into specific cellular responses by inducing the phosphorylation of particular groups of substrates. One important determinant for substrate specificity is likely to be the amino-acid sequence surrounding the phosphorylation site; however, these sites overlap significantly between different MAP kinase family members. The idea is now emerging that specific docking sites for protein kinases are involved in the efficient binding and phosphorylation of some substrates [1–4]. The MAP kinase-activated protein (MAPKAP) kinase p90^{rsk} contains two kinase domains [5]: the amino-terminal domain (D1) is required for the phosphorylation of exogenous substrates whereas the carboxy-terminal domain (D2) is involved in autophosphorylation. Association between the extracellular signal-regulated kinase (Erk) MAP kinases and p90^{rsk} family members has been detected in various cell types including *Xenopus* oocytes [6–8], where inactive p90^{rsk} is bound to the inactive form of the Erk2-like MAP kinase p42^{mpk1}. Here, we identify a new MAP kinase docking site located at the carboxyl terminus of p90^{rsk}. This docking site was required for the efficient phosphorylation and activation of p90^{rsk} *in vitro* and *in vivo* and was also both necessary and sufficient for the stable and specific association with p42^{mpk1}. The sequence of the docking site was conserved in other MAPKAP kinases, suggesting that it might represent a new class of interaction motif that facilitates efficient and specific signal transduction by MAP kinases.

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Results and discussion

Using a yeast two-hybrid assay, we confirmed that full-length p90^{rsk} associates with p42^{mpk1} (see Supplementary material published with this paper on the internet). Moreover, the D2 domain of p90^{rsk} (amino acids 309–733) alone, but not the D1 domain (amino acids 1–308) alone, could also associate with p42^{mpk1}. We tested the specificity of this interaction by using as a bait the related *Xenopus* MAP kinase p38^{mpk2}; this MAP kinase did not interact with either full-length p90^{rsk} or the D2 domain alone. As a further control, p38^{mpk2} but not p42^{mpk1} interacted with another MAPKAP kinase, MAPKAP kinase-2. Consistent with these results, we found that a fusion protein containing glutathione-S-transferase (GST) fused to the D2 domain (GST–D2), but not a similar fusion protein containing the D1 domain (GST–D1), was efficiently phosphorylated *in vitro* by purified active p42^{mpk1}. In contrast, p38^{mpk2} was much less efficient at phosphorylating GST–D2 (see Supplementary material).

The interaction between the D2 domain of p90^{rsk} and p42^{mpk1} was confirmed using bacterially produced GST fusion proteins bound to glutathione beads for 'pull-down' experiments. We found that Myc-tagged p42^{mpk1} expressed in *Xenopus* oocytes bound to GST–D2 but not to either GST alone or GST–D1 (see Supplementary material). The association was observed using either control oocytes or oocytes treated with progesterone to activate p42^{mpk1}, suggesting that GST–D2 can also bind active p42^{mpk1}. This was confirmed by using antibodies specific for the phosphorylated form of p42^{mpk1}, which could detect both Myc-tagged p42^{mpk1} and endogenous p42^{mpk1} in GST–D2 pull-down assays. The phosphorylated full-length form of p90^{rsk}, however, did not appear to bind efficiently to active p42^{mpk1} (data not shown).

To further characterise the interaction between *Xenopus* p90^{rsk} and p42^{mpk1}, we prepared truncated forms of p90^{rsk} and the D2 domain that lacked the last 43 amino acids (p90$^{rsk}\Delta$43 and D2Δ43, respectively). Deletion of this carboxy-terminal region has been shown to impair association between human Rsk3 and the MAP kinase Erk2 [8]. Using the yeast two-hybrid system we found that the removal of only the last 43 amino acids of p90^{rsk} completely abrogated the ability of both p90^{rsk} and the D2 domain to interact with p42^{mpk1} (Figure 1a). We then investigated whether these carboxy-terminal 43 amino acids were also required for the association between D2 and p42^{mpk1} in *Xenopus* oocytes. For this experiment, Myc-tagged D2 and D2Δ43 were expressed in *Xenopus* oocytes, immunoprecipitated with anti-Myc antibodies and analysed by immunoblotting using either anti-Myc or anti-p42^{mpk1} antibodies. Anti-Myc antibodies immunoprecipitated D2 and

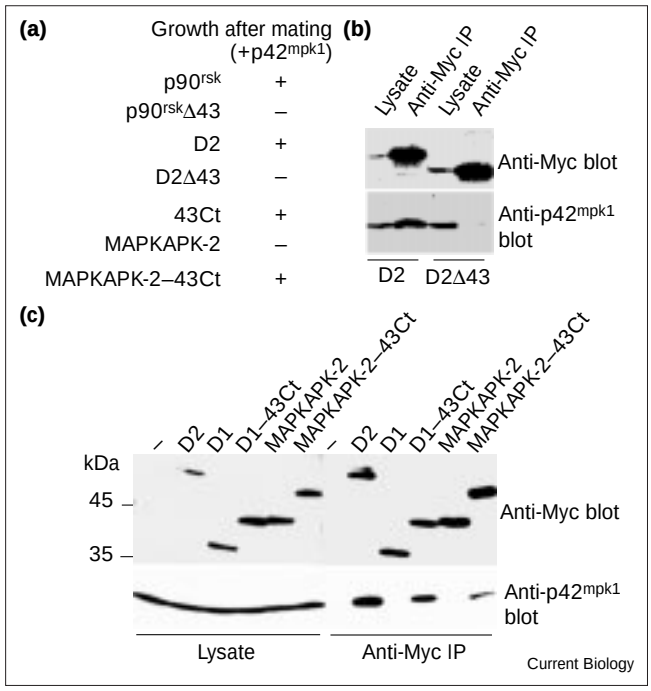
D2Δ43 with the same efficiency (Figure 1b, upper panel), but the endogenous p42^{mpk1} co-immunoprecipitated only with D2 (Figure 1b, lower panel), confirming that the removal of the last 43 amino acids completely abrogates the ability of D2 to interact with p42^{mpk1}.

We then wanted to determine whether the carboxy-terminal 43 amino acids of p90^{rsk} alone (43Ct) were sufficient for interaction with p42^{mpk1}. We found that 43Ct either alone or fused to the carboxyl terminus of MAPKAP kinase-2 (MAPKAPK-2–43Ct) was able to interact with p42^{mpk1} in a yeast two-hybrid assay (Figure 1a). To confirm these results, Myc-tagged forms of D2, D1, D1–43Ct, MAPKAP kinase-2 and MAPKAPK-2–43Ct, were expressed in *Xenopus* oocytes and analysed for binding to p42^{mpk1} as described above. All proteins were recovered in anti-Myc immunoprecipitates with the same efficiency (Figure 1c, upper panel). Although p42^{mpk1} was detected neither in D1 nor in MAPKAP kinase-2 immunoprecipitates, it did co-immunoprecipitate with both D1–43Ct and MAPKAPK-2–43Ct, albeit to a lesser extent than with D2 (Figure 1c, lower panel). These results demonstrate that the fusion of the 43Ct region to proteins that normally do not associate with p42^{mpk1} can target these proteins to this MAP kinase.

To evaluate the importance of the docking site in the 43Ct region for efficient phosphorylation by p42^{mpk1}, we incubated different amounts of recombinant GST–D2 and GST–D2Δ43 with active p42^{mpk1} and [γ-^{32}P]ATP in an *in vitro* kinase assay (Figure 2a and Supplementary material). The deletion of the carboxy-terminal 43 amino acids reduced the efficiency of D2 phosphorylation by p42^{mpk1} *in vitro* by more than 10-fold (Figure 2a, left panel). This effect was not due to the removal of p42^{mpk1} phosphorylation sites on D2, because GST–43Ct alone was very poorly phosphorylated by p42^{mpk1} *in vitro* (Figure 2a, right panel), which is consistent with the absence of MAP kinase phosphorylation sites in 43Ct [9]. We also investigated the importance of 43Ct for the *in vivo* phosphory-lation and activation of p90^{rsk} by p42^{mpk1}. For this experiment we expressed both p90^{rsk} and p90^{rsk}Δ43 in *Xenopus* oocytes and then triggered the activation of p42^{mpk1} by progesterone stimulation (Figure 2b). We found that the deletion of the 43 carboxy-terminal amino acids in p90^{rsk}Δ43 severely impaired its ability to be phosphorylated during oocyte maturation, although both endogenous p90^{rsk} and endogenous p42^{mpk1} were phosphorylated to the same extent in all progesterone-treated samples (Figure 2b, left panels). Furthermore, when p90^{rsk}Δ43 was immunoprecipitated from oocyte lysates, it showed a significant inhibition of both its autophosphorylation activity (Figure 2c, upper panel) and its ability to phosphorylate exogenous substrates (Figure 2c, middle and lower panels) when compared with full-length p90^{rsk}. These results suggest that the 43 carboxy-terminal amino acids are required for the efficient phosphorylation and activation of p90^{rsk} by p42^{mpk1} in *Xenopus* oocytes. Moreover, GST–43Ct inhibited the phosphorylation of p90^{rsk} by p42^{mpk1} *in vitro* (Figure 2d), further suggesting an important role for the interaction between the 43Ct docking site and p42^{mpk1}.

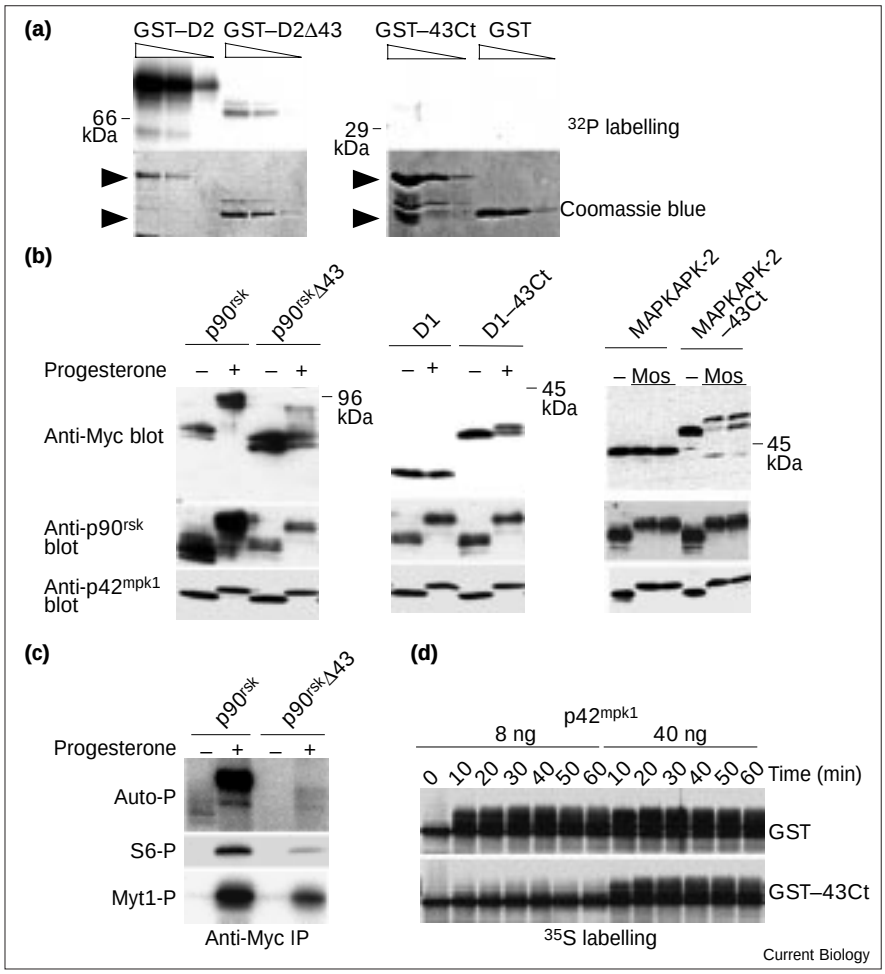
Interestingly, the D1 domain of p90^{rsk}, which is not phosphorylated by p42^{mpk1} either *in vitro* (see Supplementary material) or during oocyte maturation (Figure 2b and data not shown), might become phosphorylated by p42^{mpk1} when fused to 43Ct as suggested by the upward mobility shift of the D1–43Ct fusion protein following electrophoresis of progesterone-treated oocyte samples (Figure 2b, middle panels). Similarly, MAPKAP kinase-2 fused to 43Ct but not MAPKAP kinase-2 alone showed reduced electrophoretic mobility upon expression in oocytes injected with recombinant Mos protein to activate p42^{mpk1} (Figure 2b, right panels), suggesting that MAPKAPK-2–43Ct was more heavily phosphorylated by p42^{mpk1}. Furthermore, although MAPKAP kinase-2 is efficiently

Figure 1



Identification of a docking site in p90^{rsk} that is necessary and sufficient for p42^{mpk1} binding. (a) Interaction in the yeast two-hybrid assay between p42^{mpk1} and the indicated proteins. Growth on selective media after mating is indicated by +. All mating mixtures had the same rate of growth on non-selective media. (b,c) Interaction in co-immunoprecipitation (IP) experiments between the Myc-tagged versions of the indicated proteins expressed in *Xenopus* oocytes and endogenous p42^{mpk1}. Lysates prepared from 30 oocytes were immunoprecipitated with anti-Myc antibody and both the total lysates and the anti-Myc immunoprecipitates were analysed by immunoblotting with anti-Myc antibodies (upper panel) and anti-p42^{mpk1} antibodies (lower panel), as previously described [17].

Figure 2



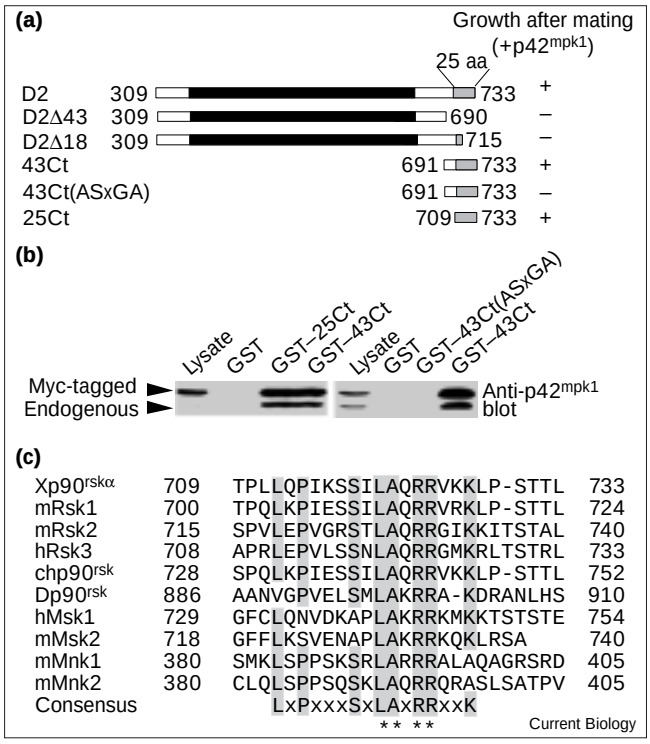
The MAP kinase docking site is required for efficient phosphorylation and activation of p90^{rsk}. (a) Active p42^{mpk1} (200 ng) was examined for its ability to phosphorylate decreasing amounts (1 μg, 500 ng, 100 ng) of either GST–D2, GST–D2Δ43, GST–43Ct or GST alone in an *in vitro* kinase assay. The arrowheads indicate the positions of the recombinant proteins. (b) Myc-tagged versions of the indicated proteins were expressed in *Xenopus* oocytes which then were either left untreated (–), treated with progesterone for 12 h (+) or injected with recombinant Mos protein (Mos) and incubated for 5 h. The phosphorylation states of the Myc-tagged proteins (upper panel), the endogenous p90^{rsk} (middle panel) and the endogenous p42^{mpk1} (lower panel) were analysed by mobility shifts on immunoblots. The anti-p90^{rsk} antibody does not recognise p90^{rsk}Δ43. (c) Lysates prepared from eight oocytes expressing either p90^{rsk} or p90^{rsk}Δ43 and either left untreated or treated with progesterone were immunoprecipitated with anti-Myc antibody and the activity of the Myc-tagged p90^{rsk} proteins was measured in an *in vitro* kinase assay as described [17]. The upper panel shows the autophosphorylation activity of p90^{rsk} (Auto-P) and the middle and lower panels the activity of the Myc-tagged p90^{rsk} proteins on the exogenous substrates S6 (S6-P; 2 μg) and GST–Myt1 (Myt1-P; 0.5 μg), respectively. (d) GST–43Ct inhibits phosphorylation of p90^{rsk} by p42^{mpk1} *in vitro*. Active p42^{mpk1} was preincubated with 4 μg of either GST–43Ct or GST alone and then incubated with ^{35}S-labelled p90^{rsk} prepared in rabbit reticulocyte lysate.

activated by p42^{mpk1} when overexpressed in oocytes, we could still measure a small but reproducible increase (about twofold) in the *in vitro* kinase activity of MAPKAPK-2–43Ct upon p42^{mpk1} activation in oocytes. Addition of the 43Ct region to MAPKAP kinase-2, however, did not affect the activation of this MAPKAP kinase by p38^{mpk2} in oocytes (data not shown). These results indicate that 43Ct can direct p42^{mpk1}-mediated phosphorylation of proteins other than p90^{rsk} *in vivo*. To define a shorter sequence for the MAP kinase docking site in 43Ct, we prepared a truncated form of D2 lacking the last 18 amino acids (D2Δ18), which did not associate with p42^{mpk1} in the yeast two-hybrid assay (Figure 3a). We also found that the carboxy-terminal 25 amino acids of p90^{rsk} (25Ct) alone were able to interact with p42^{mpk1} in the yeast two-hybrid assay (Figure 3a). Furthermore, GST–25Ct was able to pull down p42^{mpk1} from oocyte lysates with the same efficiency as GST–43Ct (Figure 3b) and GST–D2 (data not shown).

In summary, we have identified an Erk MAP-kinase docking site at the carboxyl terminus of p90^{rsk} that is specific for p42^{mpk1} binding and is required for the efficient phosphorylation and activation of p90^{rsk} by p42^{mpk1} both *in vitro* and *in vivo*. This 25 amino acid sequence contains a motif that is highly conserved in all the Rsk isoforms identified so far [10–12], as well as in the Erk substrates Mnk and Msk [13–15] (Figure 3c). Interestingly, the carboxy-terminal half of both Mnk1 and Mnk2 is involved in Erk2 binding [14]. This suggests that the p42^{mpk1} interaction motif that we have identified in *Xenopus* p90^{rsk} may be a relevant docking site in other Erk MAPKAP kinases.

We have also found that mutation of the four most conserved amino acids in 25Ct (LAQRR to ASQGA) impaired p42^{mpk1} binding (Figure 3). A sequence of 16 amino acids that is required for Erk1/2 binding and also contains the motif LxxRR has been identified recently in the protein tyrosine phosphatases PTP-SL and STEP [16]. In contrast, the 25Ct region shows no significant sequence similarity with the motif R/KxxxxLxL, which is involved in the binding of MAP kinases to nuclear substrates [4]. Thus, 25Ct may represent the first of a new class of MAP

Figure 3



The carboxy-terminal 25 amino acids of p90^{rsk} are sufficient for p42^{mpk1} binding and mutation of four amino acids in this sequence impairs p42^{mpk1} binding. (a) Interaction in the yeast two-hybrid system between p42^{mpk1} and the indicated proteins was determined as in Figure 1. GST–43Ct(ASxGA) represents GST–43Ct with the sequence LAQRR mutated to ASQGA (in the single-letter amino-acid code). (b) Lysates were prepared from oocytes expressing Myc-tagged p42^{mpk1} and used for pull-downs with either GST, GST–25Ct, GST–43Ct or GST–43Ct(ASxGA). The total lysate and the pull-down assays were analysed by immunoblotting with anti-p42^{mpk1} antibodies. (c) Comparison of the carboxy-terminal amino-acid sequences in protein kinases activated by Erk MAP kinases: *Xenopus* p90$^{rsk\alpha}$, mouse Rsk1and Rsk2, human Rsk3, chicken p90 S6 kinase II, *Drosophila* p90 S6 kinase II, human Msk1, mouse Msk2 and mouse Mnk1 and Mnk2. Amino-acid numbers of the amino-terminal and carboxy-terminal residues are indicated. The consensus shows the amino acids that are conserved in at least 8 out of the 10 sequences. The asterisks indicate the four amino acids that are conserved in all of the sequences and are required for p90^{rsk} binding to p42^{mpk1}.

kinase docking site, which is present on cytoplasmic targets of Erks and allows the preassembly of complexes between MAP kinases and substrates before MAP kinase activation. This association would ensure the preferential phosphorylation of the associated substrate, especially for those cases in which partial or transient levels of active MAP kinase are produced, and it may be of particular importance when the targets themselves are protein kinases with the potential for further signal amplification.

Supplementary material

Additional methodological details and three figures showing interaction between D2 and p42^{mpk1} but not p38^{mpk2}, specific phosphorylation of D2 by p42^{mpk1} and the requirement for 43Ct for efficient D2 phosphorylation *in vitro* are published with this paper on the internet.

Acknowledgements

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Supplementary material

A MAP kinase docking site is required for phosphorylation and activation of p90^{rsk}/MAPKAP kinase-1

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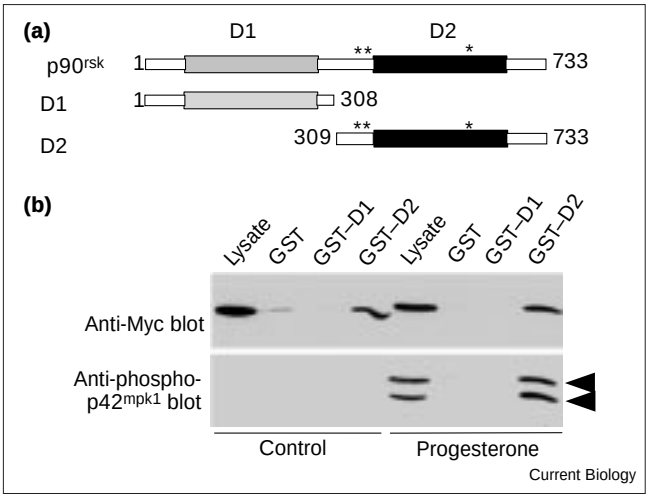
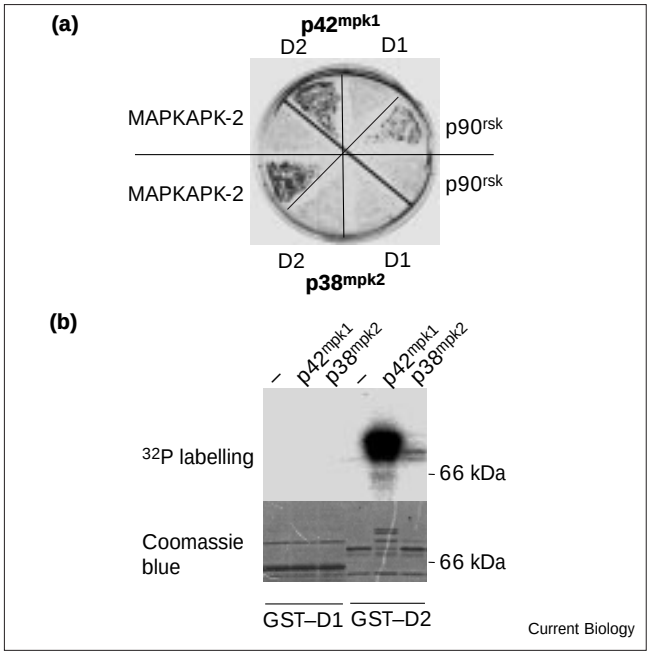
Figure S1



The D2 domain of p90^{rsk} interacts with p42^{mpk1}. (a) Schematic representation of full-length p90^{rsk} and the two p90^{rsk} kinase domain constructs D1 and D2. The three phosphorylation sites at Thr360, Ser364 and Thr574 are indicated by asterisks. (b) Lysates prepared from 10 oocytes expressing Myc-tagged p42^{mpk1} and either left untreated (control) or treated with progesterone were used in pull-down assays with 4 μg of either GST, GST–D1 or GST–D2, as previously described [S1]; a 'lysate only' sample is also shown. The samples were analysed by immunoblotting with anti-Myc antibodies (upper panel) and anti-phospho-p42^{mpk1} antibodies (Promega; lower panel). The two arrowheads indicate the positions of the Myc-tagged (upper) and the endogenous (lower) forms of phosphorylated p42^{mpk1}.

Figure S2



The D2 domain of p90^{rsk} interacts with and is phosphorylated by p42^{mpk1} but not p38^{mpk2}. (a) Interaction in the yeast two-hybrid system of p42^{mpk1} and p38^{mpk2} with full-length p90^{rsk}, the D1 or D2 domains alone or MAPKAP kinase-2. Growth on selective media after mating is shown. All mating mixtures grew at the same rate on non-selective media. We observed the same results using as a bait either the wild-type p42^{mpk1}, a kinase-inactive mutant (K56R), mutants with changes in the phosphorylation sites (TEY to either AEF or EEY, in the single-letter amino-acid code), or a mutant with the activation loop sequence DHTGFLTEY replaced by GATL. (b) *In vitro* phosphorylation of GST–D1 and GST–D2 (1 μg) by the same activity (normalised using myelin basic protein (MBP) as a substrate) of p42^{mpk1} (100 ng) and p38^{mpk2} (200 ng). After electrophoresis and Coomassie blue staining, the radioactive bands were detected by autoradiography.

Supplementary materials and methods

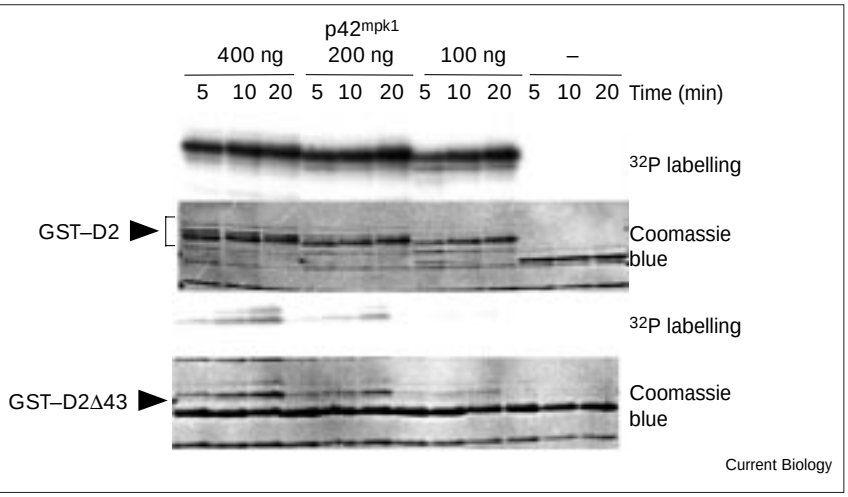
Cloning, mutagenesis and expression of Myc-tagged proteins in Xenopus *oocytes*

The cloning of *Xenopus* p90^{rsk} and the truncated D1 and D2 domains [S1] and of *Xenopus* p42^{mpk1} and p38^{mpk2} [S2] has been described. A rabbit MAPKAP kinase-2 cDNA was provided by P. Cohen (MRC, Dundee). The p90^{rsk}Δ43 and D2Δ18 deletion mutants were prepared by the introduction of stop codons following amino acid 690 and 715, respectively, using the QuikChange mutagenesis kit (Stratagene). DNA fragments containing either the last 43 or the last 25 amino acids of p90^{rsk} were obtained by PCR. All constructs were verified by DNA sequencing. Complete details of the oligonucleotides and PCR conditions will be provided upon request. All mRNAs were prepared from linearized FTX5 expression constructs [S1] using the MEGAscript *in vitro* transcription kit (Ambion).

Immunoblotting, immunoprecipitation, GST pull-downs and in vitro *kinase assays*

Oocyte microinjection and lysis, SDS–PAGE, immunoblotting with anti-Myc, anti-p90^{rsk} and anti-p42^{mpk1} antibodies and the *in vitro* kinase assay with Myc-tagged p90^{rsk} were performed as described [S1]. For co-immunoprecipitation experiments, oocytes were lysed in 1 μl H1 kinase buffer per oocyte [S1] and clarified by two sequential centrifugation steps at 14,000 rpm (Eppendorf) for 90 sec and 10 min, respectively. GST pull-down assays were performed as described [S1] using 4 μg of GST fusion proteins pre-bound to glutathione beads (20 μl) which were mixed with lysates prepared from 10 oocytes. After 2 h at 4°C, the beads were washed three times and analysed by immunoblotting. For the *in vitro* phosphorylation, GST fusion proteins were incubated for 20 min at 30°C with active maltose-binding protein (MalE)–p42^{mpk1} or MalE–p38^{mpk1} in 50 mM Tris pH 7.5, 2 μM microcystin, 10 mM $MgCl_2$, 10 μM ATP, 2 mM DTT, 2 μCi [γ-^{32}P]ATP (3000 Ci/mmol). MalE–p42^{mpk1} and MalE–p38^{mpk2} were activated by *in vitro* phosphorylation with recombinant MKK1 and MKK6, respectively. The ^{35}S-labelled p90^{rsk} was prepared in rabbit reticulocyte lysate and then incubated with active MalE–p42^{mpk1} at 30°C in the above kinase buffer but without [γ-^{32}P]ATP.

Figure S3



The 43Ct docking site is required for phosphorylation of the D2 domain of p90^{rsk} by p42^{mpk1} *in vitro*. Decreasing amounts of active p42^{mpk1} (400 ng, 200 ng and 100 ng) were used to phosphorylate 1 μg of either GST–D2 or GST–D2Δ43 for the indicated times. After SDS–PAGE, the gels were stained with Coomassie blue and the radioactive bands detected by autoradiography.

Yeast transformation and mating

The GAL4 DNA-binding domain constructs were prepared in vector pAS2-1 and the GAL4 activation domain constructs in vector pACT2 (Clontech). Transformation, mating and plating on both selective (His$^-$) and non-selective media were performed as described [S1].

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S1. Palmer A, Gavin AC, Nebreda AR: A link between MAP kinase and p34cdc2/cyclin B during oocyte maturation: p90^{rsk} phosphorylates and inactivates the p34cdc2 inhibitory kinase Myt1. *EMBO J* 1998, 17:5037-5047.

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