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Xp38γ/SAPK3 promotes meiotic G_2/M transition in *Xenopus* oocytes and activates Cdc25C

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We have studied the role of p38 mitogen-activated protein kinases (MAPKs) in the meiotic maturation of *Xenopus* oocytes. Overexpression of a constitutively active mutant of the p38 activator MKK6 accelerates progesterone-induced maturation. Immunoprecipitation experiments indicate that p38γ/SAPK3 is the major p38 activated by MKK6 in the oocytes. We have cloned *Xenopus* p38γ (Xp38γ) and show that co-expression of active MKK6 with Xp38γ induces oocyte maturation in the absence of progesterone. The maturation induced by Xp38γ requires neither protein synthesis nor activation of the p42 MAPK–p90Rsk pathway, but it is blocked by cAMP-dependent protein kinase. A role for the endogenous Xp38γ in progesterone-induced maturation is supported by the inhibitory effect of kinase-dead mutants of MKK6 and Xp38γ. Furthermore, MKK6 can rescue the inhibition of oocyte maturation by anthrax lethal factor, a protease that inactivates MAPK kinases. We also show that Xp38γ can activate the phosphatase XCdc25C, and we identified Ser205 of XCdc25C as a major phosphorylation site for Xp38γ. Our results indicate that phosphorylation of XCdc25C by Xp38γ/SAPK3 is important for the meiotic G_2/M progression of *Xenopus* oocytes.

Keywords: Cdc25/meiotic maturation/p38/SAPK3/*Xenopus* oocyte

Introduction

Fully grown *Xenopus* oocytes are arrested in G_2/prophase of meiosis I and are induced to proceed through meiosis by progesterone stimulation in a process called meiotic maturation (Ferrell, 1999; Nebreda and Ferby, 2000). A key enzymatic activity that regulates the G_2/M transition is the maturation-promoting factor (MPF), a protein complex formed by a B-type cyclin and the serine/threonine protein kinase Cdc2 (Cdk1) (Kobayashi *et al*., 1991; Doree and Hunt, 2002). In G_2 oocytes, this complex is maintained inactive by phosphorylation of the Cdc2 on a threonine and a tyrosine residues, a reaction which is probably catalysed by the Myt1 protein kinase (Mueller *et al*., 1995; Palmer *et al*., 1998). The dephosphorylation that is required to activate Cdc2 and make the oocyte progress into meiosis is catalysed by the phosphatase Cdc25C (Kumagai and Dunphy, 1992; Qian *et al*., 2001).

Several pathways have been described which regulate the phosphorylation–dephosphorylation of Cdc2 and hence MPF activity. The mitogen-activated protein kinase (MAPK) pathway formed by the protein kinase Mos, the MAPK kinase MEK1, the ERK-like MAPK Xp42Mpk1 and the protein kinase p90Rsk can lead to the inhibition of Myt1 and thus may contribute to MPF activation (reviewed by Nebreda and Ferby, 2000). Inhibition of the MAPK cascade delays Cdc2 activation and therefore oocyte maturation (Kosako *et al*., 1994; Gotoh *et al*., 1995; Fisher *et al*., 2000; Gross *et al*., 2000; Dupre *et al*., 2002). Conversely, activation of the Xp42Mpk1 MAPK pathway can induce Cdc2 activation and oocyte maturation in the absence of progesterone (Gotoh *et al*., 1995; Haccard *et al*., 1995; Huang *et al*., 1995).

Cdc25C can be phosphorylated and activated by the polo-like kinase Plx1 (Kumagai and Dunphy, 1996; Abrieu *et al*., 1998; Qian *et al*., 2001) and by MPF itself, forming a positive feedback loop that contributes to G_2/M transition (Kumagai and Dunphy, 1992; Hoffmann *et al*., 1993; Izumi and Maller, 1993). It has been proposed that Cdc25C is maintained inactive in G_2-arrested oocytes through binding to 14-3-3 proteins, which can be promoted by phosphorylation of Ser287 in *Xenopus* Cdc25C (XCdc25C) (Yang *et al*., 1999). This phosphorylation can be mediated by the protein kinase Chk1 (Oe *et al*., 2001) and by the cAMP-dependent protein kinase (PKA) (Duckworth *et al*., 2002).

Very little is known about the possible function of other MAPKs in *Xenopus* oocyte maturation. The stress-activated JNK pathway is activated during oocyte maturation and it may play a role in the regulation of oocyte apoptosis (Bagowski *et al*., 2001). The p38 subfamily of MAPKs traditionally has been implicated in environmental stress responses as well as in the regulation of inflammation and apoptosis (reviewed by Cohen, 1997; Kyriakis and Avruch, 2001). More recently, p38 MAPKs have been shown to participate in other cellular processes, including proliferation and differentiation of several cell types (reviewed by Nebreda and Porras, 2000). One of the best studied cases of p38 MAPK involvement in cell cycle regulation is probably the positive role of Sty1/Spc1 in mitotic initiation in *Schizosaccharomyces pombe* (Shiozaki and Russell, 1995). However, in higher eukaryotes, activation of p38 MAPKs usually correlates with cell cycle arrest. For example, the sea star p38 homologue Mipk is inactivated during G_2/M progression

and has been proposed to participate in G_2 arrest of oocytes (Morrison *et al*., 2000). p38 MAPKs can also induce both G_1 arrest (Bulavin *et al*., 1999; Kishi *et al*., 2001) and G_2/M arrest (Wang *et al*., 2000; Bulavin *et al*., 2001) in mammalian cells, as well as M phase arrest in *Xenopus* cleaving embryos (Takenaka *et al*., 1998).

In this report, we have investigated the role of p38 MAPKs during the meiotic G_2/M transition of *Xenopus* oocytes. We found that the activation of *Xenopus* p38γ (Xp38γ)/SAPK3 can trigger oocyte maturation, whereas p38α or p38β have no effect. We also show that the maturation induced by Xp38γ is independent of protein synthesis and that Xp38γ can phosphorylate and activate XCdc25C.

Results

Activation of endogenous p38 MAPKs accelerates G_2/M progression in Xenopus oocytes

To investigate the possible contribution of p38 MAPKs to the G_2/M transition of *Xenopus* oocytes, a constitutively active form of the p38 activator MKK6 (MKK6-DD) (Alonso *et al*., 2000) was injected into oocytes. Overexpression of MKK6-DD alone did not induce oocyte maturation. However, upon progesterone stimulation, MKK6-DD-injected oocytes reached germinal vesicle breakdown (GVBD)-50 (time when 50% of the oocytes show a white spot) ~3–4 h faster than water-injected oocytes (Figure 1A). Typically, >80% GVBD was observed in oocytes injected with MKK6-DD at the time when water-injected oocytes were still <10% GVBD. The same acceleration was observed in the activation kinetics of the biochemical markers Xp42Mpk1 MAPK and Cdc2–cyclin B (Figure 1B). Injection of MKK6-DD activated endogenous p38 MAPKs in the oocyte, as determined by the appearance of three bands in immunoblots with a phosphospecific p38 antibody (Figure 1B). The p38 inhibitor SB203580 did not affect the ability of MKK6-DD to accelerate progesterone-induced maturation (Figure 1A), indicating that p38α and p38β are unlikely to be implicated (Davies *et al*., 2000). Immunoblots using p38 phosphospecific antibodies also revealed the activation of endogenous p38 MAPKs in progesterone-treated oocytes, at around the time of white spot formation (Figure 1C).

The above results suggested that p38γ/SAPK3 and/or p38δ/SAPK4 might play a role in oocyte meiotic maturation. To determine which p38 MAPKs were phosphorylated in response to MKK6-DD, we performed immunoprecipitations with antibodies that specifically recognize different p38 family members (Supplementary figure 1 available at *The EMBO Journal* Online). Oocytes injected with MKK6-DD contained mostly phosphorylated p38γ, although lower levels of p38α and p38β were also detected (Figure 2A). We could also detect activation of p38γ in progesterone-treated oocytes by performing kinase assays on the p38γ immunoprecipitates (Figure 2B). These results indicate that p38γ is likely to be responsible for the MKK6-DD-induced acceleration of oocyte maturation.

To characterize further the role of p38γ in oocyte maturation, we cloned the *Xenopus* homologue. Xp38γ was ~75 and 65% identical to mammalian p38γ/SAPK3 and p38δ/SAPK4, respectively (Figure 3A and supplementary figure 2).

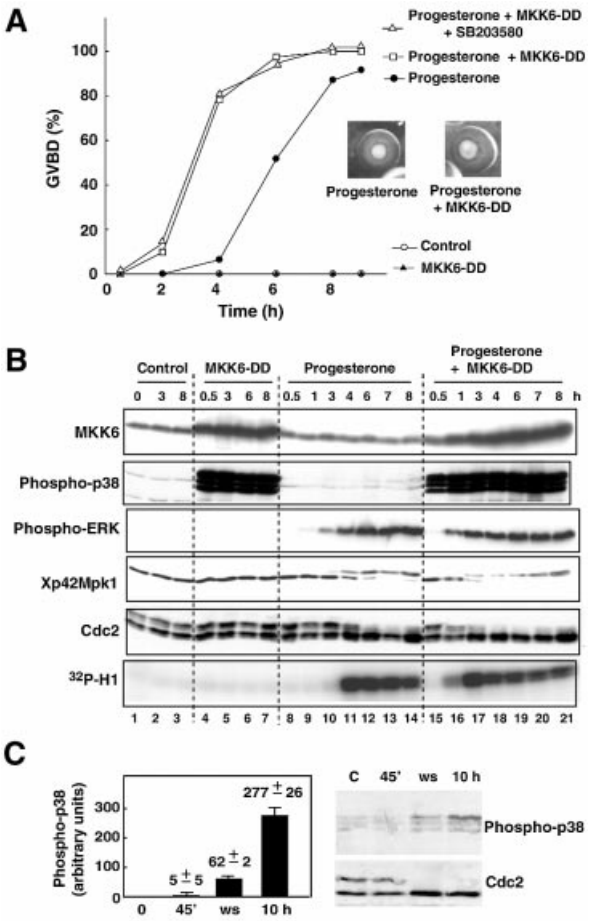

Fig. 1. Acceleration of *Xenopus* oocyte maturation by MKK6-DD. (**A**) Oocytes were incubated with progesterone 14 h after injection with MKK6-DD mRNA. Some oocytes were also injected with the p38α and p38β inhibitor SB203580 (10 μM). (**B**) Oocyte lysates were analysed by immunoblotting using the indicated antibodies. H1K assays were also performed. (**C**) Oocyte lysates were prepared 45 min and 10 h after progesterone stimulation and at the time of white spot appearance (ws). The experiment was performed with oocytes from three different frogs (GVBD-50 at 4.5, 5 and 8 h). Quantitation of the intensity of the upper band in the phospho-38 immunoblots of the three experiments is shown in the left panel. A representative example of the immunoblots (oocytes with GVBD-50 at 5 h) is in the right panel.

Overexpression of active Xp38γ/SAPK3 induces oocyte maturation in the absence of progesterone

Our results indicated that activation of endogenous p38 MAPKs, most probably p38γ, have a positive effect in progesterone-induced oocyte maturation. We tested whether overexpression of active Xp38γ would be sufficient to induce maturation in the absence of progesterone. Ooocytes were injected with MKK6-DD together with mRNAs encoding Myc-tagged forms of either *Xenopus* Xp38α/Mpk2 and Xp38γ/SAPK3 or mammalian p38β2, p38γ/SAPK3 and p38δ/SAPK4. The expression levels and activities of all p38 family members were comparable (Figure 3B). Nevertheless, maturation was only induced in MKK6-DD-injected oocytes that overexpressed Xp38γ/SAPK3 or mammalian p38δ/SAPK4. As expected from

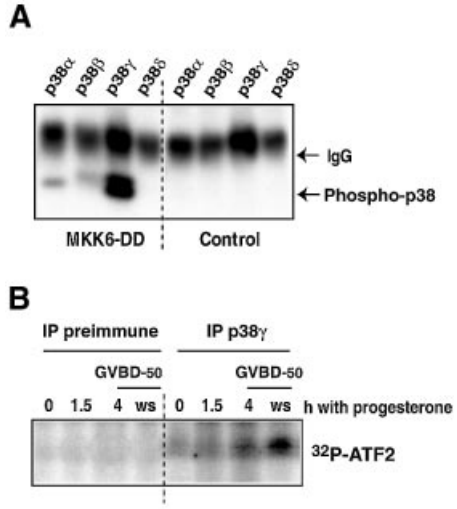

Fig. 2. Activation of endogenous p38s in oocytes by MKK6-DD. (**A**) Groups of 10 oocytes untreated or injected with MKK6-DD mRNA and incubated for 14 h were lysed, immunoprecipitated with p38α, p38β, p38γ and p38δ antibodies and analysed by immunoblotting using a phospho-p38 antibody. (**B**) Oocytes were untreated or treated with progesterone and collected 1.5 h later. At the time of GVBD-50 (4 h), both oocytes with and without white spot were collected. Immunoprecipitates were prepared with pre-immune and anti-p38γ antiserum and used for *in vitro* kinase assay with ATF2 as a substrate.

the results using SB203580 (Figure 1A), overexpression of active p38α or p38β2 did not induce maturation. Curiously, overexpression of murine p38γ plus MKK6-DD also did not induce oocyte maturation (Figure 3B).

The maturation induced by Xp38γ/SAPK3 was faster than with progesterone and usually resulted in a slightly different morphological appearance after GVBD. These oocytes produced a white spot similar to the one in progesterone-treated oocytes, but then underwent changes in the pigmentation of the animal pole (bigger white spot with a general pale-shadow appearance) (Figure 4A). The biochemical markers of maturation, including the phosphorylation of Xp42Mpk1 and p90Rsk and the tyrosine dephosphorylation of Cdc2, were also the same in Xp38γ/SAPK3- or progesterone-matured oocytes (Figure 4B). Moreover, the oocytes injected with active Xp38γ/SAPK3 appeared to undergo the meiosis I–meiosis II transition, based on the kinetics of MPF activation and transient inactivation in synchronized oocytes (Figure 4B). However, the levels of H1K were sometimes lower in these oocytes, especially at meiosis I (Figure 4B; see also Figures 7 and 9). Histological analysis of oocyte sections confirmed that most of the Xp38γ-injected oocytes formed a meiosis I spindle. However, only ~50% of these spindles were located at the membrane (the normal position of the spindle in progesterone-mature oocytes); the other 50% were located internally in the cytoplasm (see Supplementary table 1). At later times after GVBD, when 90% of the progesterone-treated oocytes contain meiosis II spindle and polar body, we found these structures in only 50% of the oocytes induced to mature by Xp38γ, suggesting that a fraction of the Xp38γ-injected oocytes that entered meiosis I were unable to progress to meiosis II.

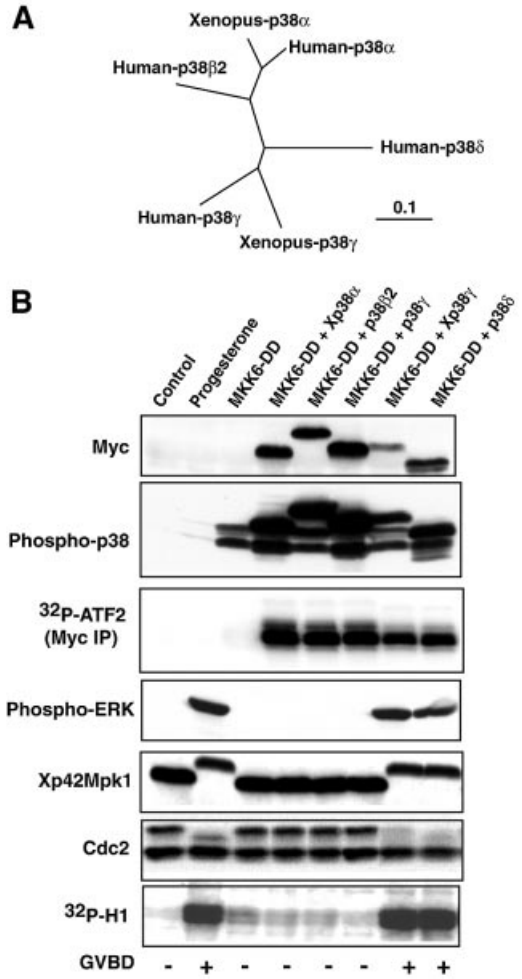

Fig. 3. Active Xp38γ/SAPK3 promotes oocyte maturation in the absence of progesterone stimulation. (**A**) Phylogram comparing the amino acid sequences of Xp38γ with other p38 family members generated using the ClustalX program (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/). (**B**) Oocytes were injected with mRNAs encoding Myc-tagged Xp38α/Mpk2, p38β2, p38γ/SAPK3, Xp38γ/SAPK3 and p38δ/SAPK4, and 14 h later were injected again with 50 ng of MKK6-DD protein. Oocytes were collected 7 h after the second injection and their lysates analysed by immunoblotting using the indicated antibodies. MPF activity was determined by H1K assay. The injected Myc-tagged p38s were assayed by anti-Myc immunoprecipitation followed by kinase assay using GST–ATF2 as substrate. Oocyte groups that underwent 100% GVBD or remained arrested in G_2 are indicated at the bottom of the panel by + and –, respectively.

MKK6 and Xp38γ/SAPK3 catalytically inactive mutants inhibit progesterone-induced maturation

To obtain evidence of a role for endogenous Xp38γ/SAPK3 in progesterone-induced maturation, we injected catalytically inactive mutants of either MKK6 (MKK6-DA) or Xp38γ/SAPK3 (Xp38γ-DA). These mutants have demonstrated a dominant-negative effect in some cases (Goedert *et al*., 1997; Ludwig *et al*., 1998; Wang *et al*., 2000). Expression of either of these two mutants significantly slowed down the kinetics of progesterone-induced maturation, delaying the time of GVBD-50 by at least 2 h (Figure 5A). We confirmed that both mutant proteins were overexpressed 10- to 20-fold (Figure 5B) and that they had no detectable kinase activity (Figure 5C). Importantly, co-expression of the wild-type (wt) MKK6 or Xp38γ proteins

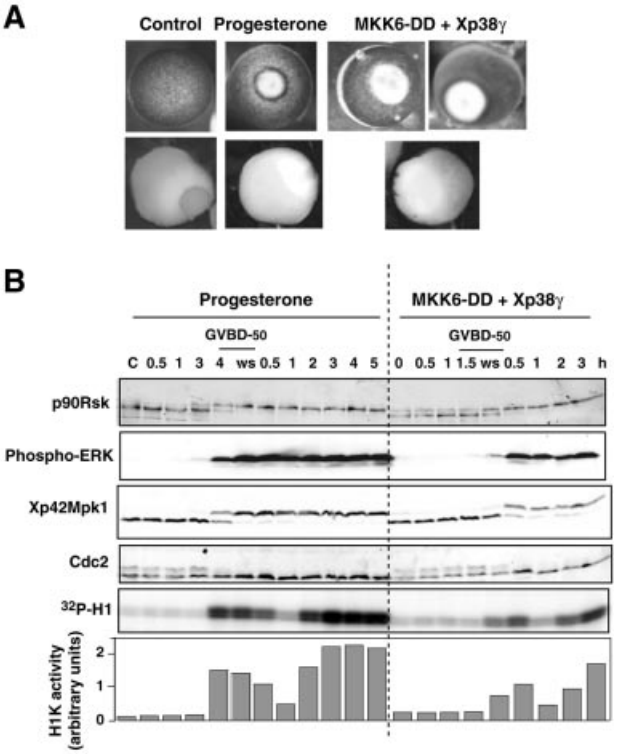

Fig. 4. Kinetics of Xp38γ/SAPK3-induced oocyte maturation. Oocytes were injected with Xp38γ/SAPK3 mRNA, and 14 h later were injected again with 50 ng of MKK6-DD protein. Water-injected oocytes were treated with progesterone and samples were taken at the indicated times. The oocytes were synchronized after white spot (ws) appearance (samples to the right of ws). (**A**) Morphological appearance of oocytes injected with Xp38γ/SAPK3 plus MKK6-DD and incubated for 4 h (left panel) and 7 h (right panel). Oocytes injected with water (control) and treated with progesterone for 8 h are also shown. The lower panels show the absence of a nucleus in fixed oocytes. (**B**) Oocyte lysates were analysed by immunoblotting using the indicated antibodies. H1K assays were also performed and were quantified using a PhosphorImager.

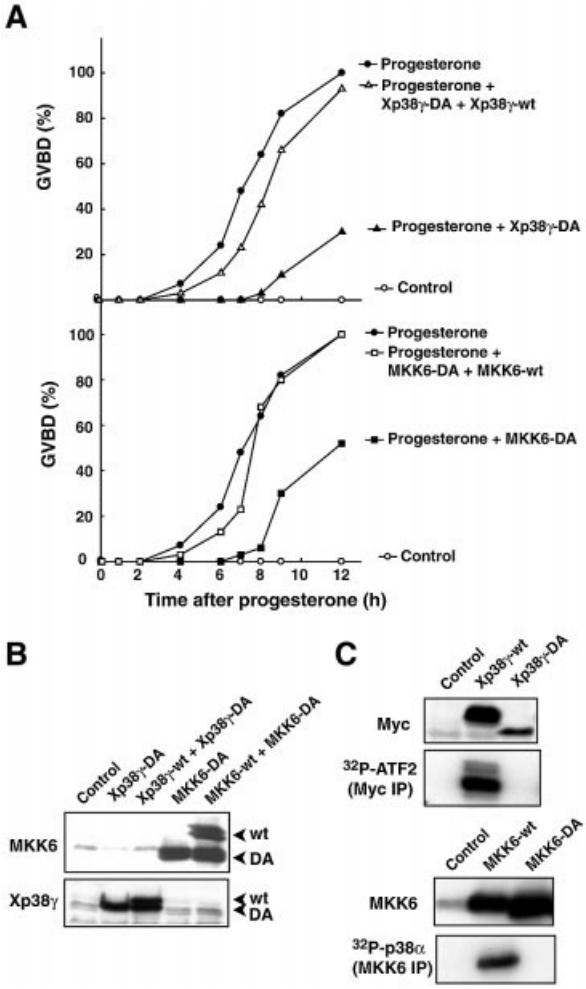

Fig. 5. Inhibition of oocyte maturation by MKK6 and Xp38γ/SAPK3 catalytically inactive mutants. (**A**) Oocytes were injected with MKK6-DA, MKK6-DA plus Myc-tagged MKK6-wt, Xp38γ-DA or Xp38γ-DA plus Xp38γ-wt mRNAs, and 14 h later were incubated with progesterone. (**B**) Lysates of oocytes collected at 12 h were analysed by immunoblotting using MKK6 and Xp38γ antibodies. (**C**) Kinase activities were assayed by immunoprecipitation with Myc or MKK6 antibodies followed by kinase assays using GST–ATF2 or GST–p38α as substrates for Xp38γ/SAPK3 (upper panel) or MKK6 (lower panel), respectively.

rescued the delay in maturation produced by the corresponding kinase-dead mutants (Figure 5A). As a further control, MKK6-DA did not affect maturation induced by Mos, a direct activator of the Xp42Mpk1 pathway (supplementary figure 3). These results support a role for the endogenous Xp38γ/SAPK3 in progesterone-induced oocyte maturation.

MKK6 overexpression rescues the anthrax lethal factor inhibition of progesterone-induced maturation

Injection of anthrax lethal factor (LF), a metalloprotease that cleaves and inactivates MAPK kinases, into oocytes has a stronger inhibitory effect on progesterone-induced maturation (Duesbery *et al*., 1998) than specific inhibitors of the Xp42Mpk1 pathway such as U0126 (Gross *et al*., 2000). This suggests that LF may target other MAPK kinases (in addition to MEK1) that are required for oocyte maturation.

LF has been shown to inactivate the p38 activators MKK3 and MKK6 (Duesbery *et al*., 1998; Vitale *et al*., 2000). To test the possibility that cleavage of p38 MAPK kinases could contribute to the inhibition of oocyte maturation, we investigated the ability of wt MKK6 to rescue maturation in LF-injected oocytes. As expected, injection of LF led to the cleavage of the endogenous MEK1 and MKK6 proteins as well as the overexpressed wt MKK6, but to a lesser extent (Figure 6A). Interestingly, the overexpression of MKK6 restored the ability of progesterone to induce maturation, as determined by both morphological and biochemical markers (Figure 6A and B). The results indicate that activation of p38 MAPKs is necessary for progesterone-induced oocyte maturation.

Xp38γ/SAPK3-induced maturation is independent of the Xp42Mpk1 MAPK pathway

Previous reports have described cross-talk between p38 and ERK MAPK pathways (Ludwig *et al*., 1998; Chen *et al*., 2000; Westermarck *et al*., 2001). First, we verified that MKK6-DD did not activate the ERK Xp42Mpk1 pathway, both in oocytes and *in vitro* (Supplementary figure 4). Furthermore, our results with LF indicated that in

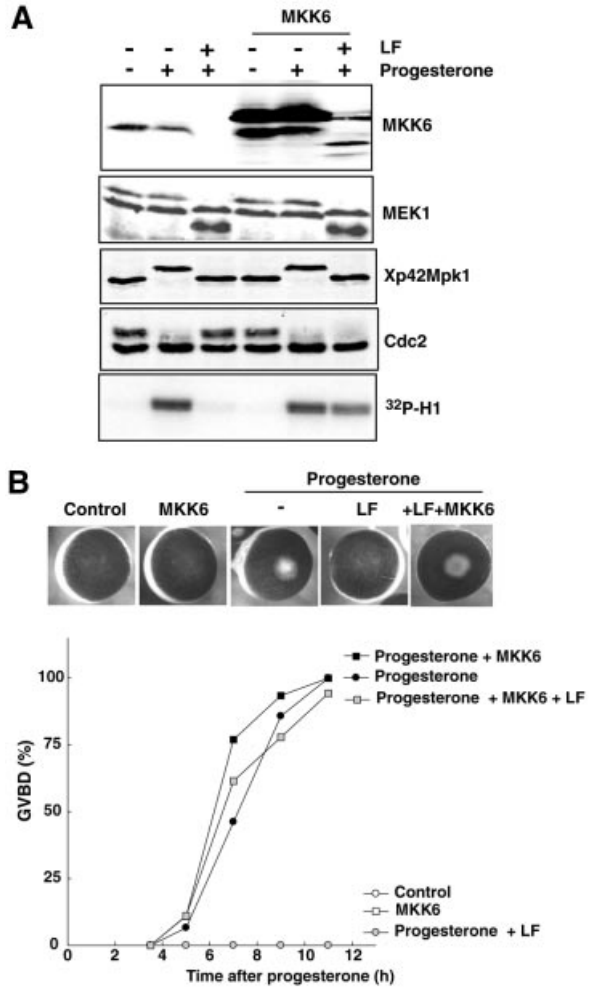

Fig. 6. MKK6 can rescue the inhibition of progesterone-induced maturation by LF. Oocytes were injected with MKK6 mRNA, incubated overnight and were then injected with 60 ng of purified LF followed by progesterone addition 1 h later. (**A**) Oocytes were collected 10 h after LF injection and the lysates were analysed by immunoblotting using the indicated antibodies and by H1K assay. (**B**) Maturation was scored by the appearance of a white spot and confirmed by manual dissection.

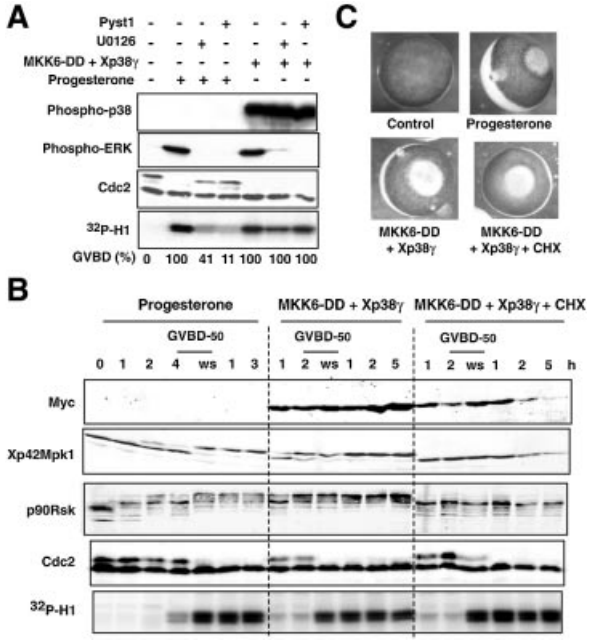

Fig. 7. Oocyte maturation induced by Xp38γ/SAPK3 is independent of Xp42Mpk1 MAPK activation and does not require protein synthesis. (**A**) Oocytes were injected with Xp38γ/SAPK3 mRNA, incubated overnight and then injected again with 50 ng of MalE-MKK6-DD protein. Some oocytes were either pre-incubated with 50 μM U0126 for 1 h or injected with the MAPK phosphatase Pyst1 and incubated overnight before MKK6-DD injection or progesterone treatment. Oocytes were collected after 12 h and their lysates analysed by immunoblotting using the indicated antibodies, and by H1K assays. The occurrence of GVBD in the different oocyte groups is indicated at the bottom of the panel. (**B**) Oocytes were injected with Xp38γ/SAPK3 mRNA, and 14 h later were injected with 50 ng of MKK6-DD protein. Some oocytes were incubated with 50 μg/ml CHX for 1 h before MKK6-DD injection. The oocytes were synchronized after white spot (ws) appearance (samples to the right of ws). Oocyte lysates were analysed by immunoblotting using the indicated antibodies. (**C**) Morphological appearance of the oocytes. Untreated (control) and progesterone-treated oocytes were collected at 7 h; MKK6-DD + Xp38γ-injected oocytes with or without CHX were collected at 4 h.

Xenopus oocyte maturation, these two pathways could act independently. We used specific ERK inhibitors to confirm this possibility. As previously described, the MEK1 inhibitor U0126 (Gross *et al*., 2000) or the ERK MAPK-specific phosphatase Pyst1/MKP-3 (Groom *et al*., 1996; Fisher *et al*., 1999) delayed maturation induced by progesterone. However, in both cases, Xp38γ/SAPK3-induced maturation was not affected (Figure 7A). Observation of the biochemical markers showed that pre-MPF was active in Xp38γ/SAPK3-injected oocytes, whereas Xp42Mpk1 was either only slightly phosphorylated (U0126) or quantitatively dephosphorylated (Pyst1) (Figure 7A). Taken together, the results confirm that Xp38γ/SAPK3 can induce G_2/M progression in oocytes in the absence of Xp42Mpk1 MAPK activation.

Maturation induced by Xp38γ/SAPK3 does not require protein synthesis

Pre-incubation of oocytes with cycloheximide (CHX) is known to block progesterone-induced maturation, indicating that the process has an absolute requirement for protein synthesis. We found that incubation of the oocytes with CHX did not inhibit maturation induced by Xp38γ/SAPK3, indicating that translation of maternal mRNAs and synthesis of new proteins were not required (Figure 7B). The appearance of these oocytes at GVBD was also the same with and without CHX (Figure 7C). Xp42Mpk1 and p90Rsk, however, were not phosphorylated in the CHX-treated oocytes, suggesting that activation of the ERK Xp42Mpk1 pathway during Xp38γ/SAPK3-induced maturation is probably the consequence of positive feedback loops.

Activation of XCdc25C by Xp38γ/SAPK3

The lack of inhibitory effect of CHX on Xp38γ/SAPK3-induced pre-MPF activation suggests that Xp38γ/SAPK3 could be directly targeting the cell cycle regulators Myt1 or Cdc25C. In mammalian cells, p38 MAPKs can directly or indirectly lead to the phosphorylation of Cdc25 family members (Wang *et al*., 2000; Bulavin *et al*., 2001). We therefore investigated if Xp38γ/SAPK3 could also phosphorylate XCdc25C and modulate its phosphatase activity.

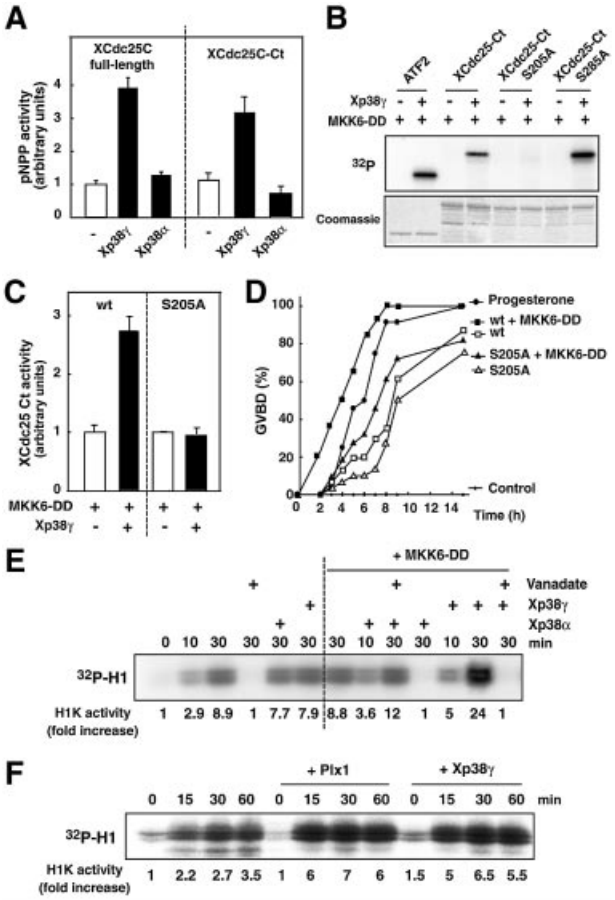

Fig. 8. Xp38γ/SAPK3 activates Cdc25C. (**A**) The phosphatase activities of full-length GST–XCdc25C and the XCdc25C-Ct mutant (lacking the first 199 amino acids) were assayed using pNPP as substrate after *in vitro* phosphorylation with activated Xp38α or Xp38γ recombinant proteins. The experiment was performed in triplicate and standard error was calculated with the Sigmaplot 8.0 software. The data are compiled from 12 different experiments. (**B**) Phosphorylation of bacterially produced wt XCdc25-Ct and the indicated mutant proteins by either MKK6-DD alone or MKK6-DD plus Xp38γ/SAPK3. The experiment was repeated five times. (**C**) The *in vitro* phosphatase activity on pNPP of the XCdc25-Ct wt and S205A proteins was determined as in (A). (**D**) Oocytes were injected with MKK6-DD mRNA and incubated overnight before being injected again with mRNAs encoding either wt or S205A XCdc25C. Some oocytes were treated with progesterone. (**E**) Full-length GST–XCdc25C (0.5 μg) was pre-incubated in kinase assays for 1 h with MalE-MKK6-DD (0.2 μg) or MalE (1 μg) in the presence or absence of 0.2 μg of recombinant Xp38α or Xp38γ and then added to high-speed oocyte extracts (25 μl). Some aliquots of extracts were pre-incubated with 5 mM vanadate for 5 min. At the times indicated after GST–XCdc25C addition, Cdc2–cyclin B activity in the extracts was determined by H1K assays and quantified using a PhosphorImager. (**F**) Myc-Xp38γ and Flag-Plx1 were immunoprecipitated from oocytes injected with Xp38γ and MKK6-DD mRNAs or injected with Plx1 mRNA and treated with progesterone, respectively, extensively washed and then incubated in kinase assays for 30 min with GST–XCdc25C. The phosphatase activity in the supernatant was analysed in oocyte extracts as in (E).

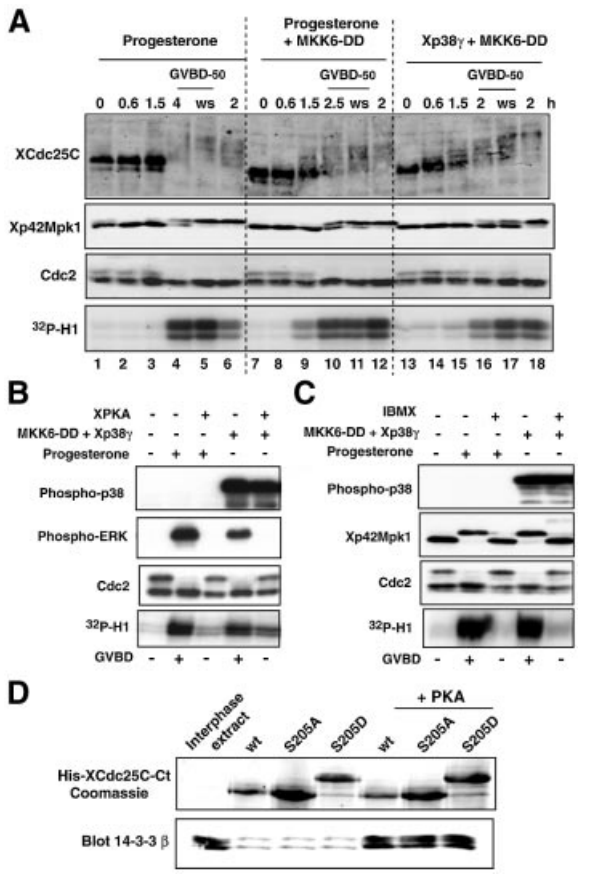

Fig. 9. Xp38γ/SAPK3-induced oocyte maturation correlates with earlier phosphorylation of XCdc25C and is inhibited by PKA. Oocytes were injected with Xp38γ/SAPK3 or MKK6-DD mRNAs. After 14 h, oocytes were treated with progesterone or injected again with 50 ng of MKK6-DD protein. At the time of GVBD-50 (2–4 h), both oocytes with and without white spot (ws) were collected. Oocytes were also synchronized at the time of ws appearance and collected 2 h later. Oocyte lysates were analysed by immunoblotting using the indicated antibodies and by H1K assays. (B and C) Oocytes were injected with Xp38γ/SAPK3 mRNA and then incubated overnight before being injected again with 50 ng of MKK6-DD protein. Some oocytes were injected with 50 ng of XPKA mRNA (**B**) or pre-incubated with 1 mM IBMX (**C**) 1 h before MKK6-DD injection or progesterone stimulation. Oocytes were collected after 12 h and their lysates analysed by immunoblotting with the indicated antibodies, and by H1K assays. Oocyte groups that underwent 100% GVBD or remained arrested in G_2 are indicated at the bottom of the panels by + and –, respectively. (**D**) Recombinant wt XCdc25-Ct and the indicated mutant proteins (2 μg) were purified on Talon beads (Clontech) and incubated in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 200 μM ATP in the presence or absence of 50 U of purified PKAc (Promega). After 30 min at 30°C, the beads were incubated with 100 μl of *Xenopus* interphase extract (diluted 1:10) for 1 h and then washed three times with IP buffer and once with H1K buffer. The proteins bound to beads were analysed by immunoblotting with anti-14-3-3 β antibody (Santa-Cruz).

We performed *in vitro* assays using bacterially produced XCdc25C and *p*-nitrophenyl phosphate (pNPP) as a substrate. The phosphatase activity of XCdc25C increased by >3-fold upon incubation with Xp38γ/SAPK3, but was barely affected by Xp38α (Figure 8A). Xp38γ/SAPK3 also stimulated the phosphatase activity of an N-terminally truncated XCdc25C mutant (XCdc25C-Ct, lacking the first 199 amino acids) to about the same extent (Figure 8A). These results indicate that the N-terminal non-catalytic domain, which contains most of the Cdc2 phosphorylation sites (Izumi and Maller, 1993), is not involved in the regulation of XCdc25C activity by Xp38γ/SAPK3.

We found two potential MAPK phosphorylation sites in XCdc25C-Ct with the consensus Ser–Pro at positions Ser205 and Ser285, respectively. Interestingly, the mutation S205A abolished *in vitro* phosphorylation by Xp38γ/SAPK3, whereas the mutation S285A had no effect

(Figure 8B). The lack of phosphorylation of XCdc25C-Ct Ser205Ala also correlated with the inability of Xp38γ/SAPK3 to stimulate the phosphatase activity of this mutant (Figure 8C). These results indicated an important role for Ser205 phosphorylation in the regulation of XCdc25C activity by Xp38γ/SAPK3. Further support for this idea came from the observation that MKK6-DD strongly accelerated oocyte maturation promoted by wt XCdc25C (GVBD-50 from 8.5 to 3.5 h), but had little effect on maturation induced by the S205A mutant (GVBD-50 from 8.5 to 7.5 h) (Figure 8D).

To confirm that Xp38γ/SAPK3 phosphorylation was able to activate XCdc25C activity on the physiological substrate Cdc2–cyclin B, we used oocyte extracts as a source of pre-MPF (Ferby *et al*., 1999). Addition of purified GST–XCdc25C to these extracts can trigger the activation of pre-MPF, which can be inhibited by the tyrosine phosphatase inhibitor sodium vanadate (Figure 8E). We found that addition of recombinant GST–XCdc25C plus activated Xp38γ/SAPK3 increased MPF activity by ~2- to 3-fold, as determined by H1K, and this activation was inhibited by vanadate, indicating that it was likely to come from XCdc25C. When compared with Plx1, Xp38γ/SAPK3 appeared to be somewhat less efficient at activating XCdc25C *in vitro* (Figure 8F).

Xp38γ/SAPK3-induced maturation correlates with earlier phosphorylation of XCdc25C and is inhibited by PKA

We investigated whether induction of oocyte maturation by Xp38γ/SAPK3 correlated with phosphorylation of endogenous XCdc25C. During the G_2/M transition, XCdc25C undergoes a shift in electrophoretic mobility due to extensive phosphorylation on its N-terminus, probably by both Cdc2–cyclin B and Plx1 (Kumagai and Dunphy, 1996; Qian *et al*., 2001). Consistent with previous reports, we observed the hyperphosphorylation and shift-up of XCdc25C around the time of GVBD in progesterone-treated oocytes (Figure 9A, lanes 4–6). However, in oocytes either injected with MKK6-DD before progesterone treatment or matured by co-injection of Xp38γ/SAPK3 and MKK6-DD, XCdc25C started to shift earlier (Figure 9A, compare lanes 2 and 3 with lanes 8 and 9, and 14 and 15). For example, XCdc25C was already partially shifted only 40 min after injection of active Xp38γ/SAPK3 (~1 h before GVBD-50; Figure 9, lane 14), whereas in progesterone-treated oocytes no shift of XCdc25C could be detected until the time of GVBD-50 (Figure 9A, lanes 1–3). These results support a connection between the activation of Xp38γ/SAPK3 and XCdc25C in oocyte maturation.

PKA is known to inhibit XCdc25C-induced oocyte maturation (Matten *et al*., 1994; Duckworth *et al*., 2002; Schmitt and Nebreda, 2002). We found that either overexpression of *Xenopus* PKA (XPKA) or incubation with the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) efficiently inhibited Xp38γ/SAPK3-induced maturation (Figure 9B and C). These results are consistent with the idea that Xp38γ/SAPK3 activates XCdc25C. Furthermore, in agreement with the dominant inhibitory effect of XPKA, mutation of Ser205 (the major Xp38γ/SAPK3 phosphorylation site) to either alanine or aspartate did not affect the ability of 14-3-3 proteins to bind PKA-phosphorylated XCdc25C (Figure 9D).

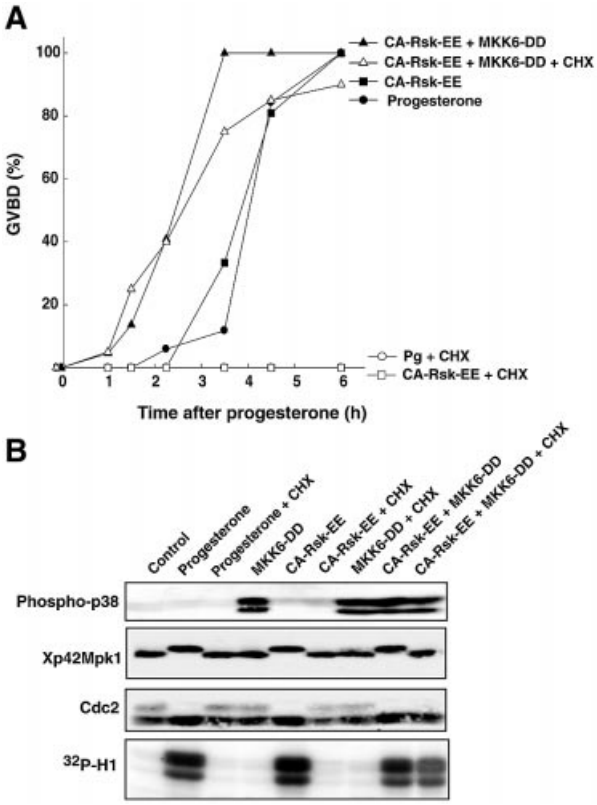

Fig. 10. Endogenous Xp38γ/SAPK3 synergizes with p90Rsk to induce oocyte maturation in the absence of progesterone. (**A**) Oocytes were untreated or treated with 50 μg/ml CHX for 30 min prior to injection with either 25 ng of CA-Rsk-EE, 25 ng of MKK6-DD or both recombinant proteins together. Some oocytes were treated with progesterone. (**B**) Lysates of ocytes collected at 6 h after injection or progesterone treatment were analysed by immunoblotting using the indicated antibodies and by H1K assays.

Endogenous Xp38γ/SAPK3 synergizes with p90Rsk to induce oocyte maturation in the absence of progesterone

The experiments injecting MKK6-DD in oocytes suggested that activation of the endogenous Xp38γ/SAPK3 was necessary but not sufficient for G_2/M progression. Hence, the phosphorylation of Ser205 does not seem to be sufficient to activate XCdc25C fully and trigger oocyte maturation. We tested whether inhibition of Myt1, which maintains Cdc2–cyclin B inactive in G_2-arrested oocytes (Palmer *et al*., 1998), could complement Xp38γ/SAPK3 activity in the oocytes. For these experiments, we took advantage of the existence of constitutively active mutants of the protein kinase p90Rsk, such as CA-RSK2 (Frodin *et al*., 2000). Injection of the CA-RSK2 derivative CA-Rsk-EE can induce oocyte maturation in the absence of progesterone (data not shown), as has been described for other active p90Rsk mutants (Gross *et al*., 2001). However, addition of CHX to the medium inhibits oocyte maturation induced by low concentrations of CA-Rsk-EE (Figure 10). Interestingly, oocytes co-injected with MKK6-DD and CA-Rsk-EE underwent rapid maturation (faster than with progesterone) even in the presence of

CHX (Figure 10A), as determined by both cytological and biochemical markers (Figure 10B). These results are consistent with the ability of Xp38γ/SAPK3 to phosphorylate and activate XCdc25C which, together with the inhibition of Myt1 by p90Rsk phosphorylation (Palmer *et al*., 1998), will lead to meiotic G_2/M progression.

Discussion

We have shown that overexpression of a constitutively active form of the p38 MAPK activator MKK6 strongly accelerates progesterone-induced G_2/M transition in *Xenopus* oocytes. Our results indicate that this is probably due to the activation of the endogenous Xp38γ/SAPK3 in the oocyte.

In contrast to Xp38γ/SAPK3, expression of active mammalian p38γ/SAPK3 cannot induce oocyte maturation in the absence of progesterone, whereas p38δ/SAPK4 does. It should be noted that the homology between mammalian and *Xenopus* p38γ is not as high as between p38α of different species. In fact, some regions of Xp38γ, such as the common docking (CD) domain that has been implicated by mutagenesis analysis in MAPK substrate recognition (Tanoue *et al*., 2000), appear to be more related to mammalian p38δ (70% identical) than to p38γ (50% identical). Moreover, the residue Ile116 that has been found in p38α crystals to interact with peptide substrates (Chang *et al*., 2002) is conserved in both Xp38γ and mammalian p38δ, but is replaced by leucine in mammalian p38γ (Supplementary figure 2).

Two different approaches demonstrate that activation of p38 MAPKs is necessary for the meiotic maturation of oocytes. First, overexpression of either MKK6 or Xp38γ/SAPK3 catalytically inactive mutants significantly delayed progesterone-induced maturation, and the inhibitory effect could be rescued by co-expression of the corresponding wt proteins. Moreover, overexpression of wt MKK6 rescues the inhibition of oocyte maturation produced by injection of anthrax LF. The ability of LF to target both the p38 and ERK pathways may explain why it is a stronger inhibitor of progesterone-induced oocyte maturation (Duesbery *et al*., 1998) than the ERK MAPK-specific inhibitors (Gross *et al*., 2000).

Our analysis of the mechanism by which Xp38γ/SAPK3 can trigger meiosis I entry in oocytes strongly supports a link with XCdc25C. We found that Xp38γ-induced maturation is independent of the activation of the Mos–Xp42Mpk1 pathway and does not require protein synthesis. Thus, Xp38γ/SAPK3 may be acting close to the cell cycle machinery that regulates the activation of pre-MPF in the oocyte. We found that meiotic maturation induced by overactivation of Xp38γ/SAPK3 correlated with earlier phosphorylation of XCdc25C. Moreover, Xp38γ-induced maturation can be blocked by PKA. This is consistent with the ability of PKA to inhibit Cdc25C-induced oocyte maturation (Matten *et al*., 1994; Schmitt and Nebreda, 2002), probably via direct phosphorylation of Ser287 and generation of a 14-3-3-binding site on XCdc25C (Duckworth *et al*., 2002).

In agreement with these results, we showed that Xp38γ/SAPK3 is able to phosphorylate and increase the phosphatase activity of XCdc25C *in vitro*. Cdc25 phosphatases have also been shown to be phosphorylated by p38α in mammalian cells, but in connection with cell cycle arrest (Bulavin *et al*., 2001). In contrast, p38 MAPKs can stimulate G_2/M progression in yeast via both Cdc25-dependent and independent pathways (reviewed by Ambrosino and Nebreda, 2001). Our studies identify Ser205 phosphorylation as an important event for the activation of XCdc25C by Xp38γ. However, mutagenesis analysis suggest that this phosphorylation does not interfere with binding of 14-3-3 to XCdc25C. Then, how does overexpression of active Xp38γ induce oocyte maturation? One possibility is that the increase in XCdc25C phosphatase activity that we observe upon phosphorylation of Ser205 by Xp38γ *in vitro* suffices to trigger pre-MPF activation in the oocyte. In addition, phosphorylated Ser205 may function as a docking site for regulatory proteins that contribute to full activation of XCdc25C. The nature of these proteins remains to be elucidated, but phosphatases that dephosphorylate Ser287 (leading to the release of 14-3-3 proteins) and protein kinases that directly stimulate Cdc25C activity (by conformational changes induced upon phosphorylation) are good candidates (see below).

Taken together, our results implicate the phosphorylation of Cdc25C by Xp38γ/SAPK3 in *Xenopus* oocyte meiotic maturation. However, expression of constitutively active MKK6-DD is not sufficient to release the G_2 arrest of oocytes in the absence of progesterone stimulation, unless Xp38γ/SAPK3 is overexpressed. These results indicate that activation of endogenous Xp38γ/SAPK3 is not sufficient to trigger oocyte maturation, perhaps because other pathways activated by progesterone are normally required. Experimental support for this hypothesis was obtained by using a constitutively active mutant of p90Rsk. We found that in oocytes expressing CA-Rsk-EE, the activation of endogenous Xp38γ/SAPK3 is sufficient to induce MPF activation and GVBD independently of both protein synthesis and progesterone stimulation. Since p90Rsk can phosphorylate and inhibit Myt1 (Palmer *et al*., 1998), these results are consistent with the idea that Xp38γ/SAPK3 can activate XCdc25C in the oocyte. We cannot rule out the possibility that Xp38γ/SAPK3 might also be able to downregulate Myt1, but in this case activation of endogenous Xp38γ/SAPK3 would be expected to induce oocyte maturation. Our results also suggest that both activation of XCdc25C and inhibition of Myt1 are normally required for meiotic G_2/M progression in oocytes. It should be noted, however, that either inhibition of Myt1 by injection of specific antibodies (Nakajo *et al*., 2000) or overexpression of Cdc25C are sufficient to induce oocyte maturation (Yang *et al*., 1999; Oe *et al*., 2001; Schmitt and Nebreda, 2002). This suggests that the level of Cdc25C activity achieved upon phosphorylation of Ser205 by endogenous Xp38γ/SAPK3 may not be high enough to bypass the negative regulators that maintain pre-MPF inactive in the G_2-arrested oocyte. Thus, it is likely that further events, in addition to phosphorylation by Xp38γ/SAPK3, are required for full activation of XCdc25C in progesterone-treated oocytes.

It has been proposed that the activation of XCdc25C in *Xenopus* egg extracts occurs in two steps, with the second one requiring protein phosphatase 2A downregulation for XCdc25C to become hyperphosphorylated and fully active (Karaiskou *et al*., 1999). The first step may require a

triggering protein kinase activated in response to progesterone, which may be Plx1 (Qian *et al*., 2001). Recently the binding of human Plk1 to Cdc25C has been shown to require the priming phosphorylation of Cdc25C by a proline-directed kinase, which was proposed to be Cdc2–cyclin B (Elia *et al*., 2003). Since the same phosphorylated motifs can be generated by the action of Cdks and MAPKs, Xp38γ might also function as a priming kinase that facilitates phosphorylation and full activation of XCdc25C by Plx1. Future experiments will investigate the regulatory effect of interplay between Xp38γ/SAPK3 and other protein kinases on XCdc25C activity.

Materials and methods

DNA cloning and mutagenesis

A *Xenopus* oocyte cDNA library (gift from J.Shuttleworth, University of Birmingham, UK) was screened with the full-length rat SAPK3 cDNA as a probe (provided by Michael Goedert, LMB, Cambridge, UK) (Mertens *et al*., 1996). We obtained several positives that corresponded to the same cDNA encoding a protein of 363 amino acids, which was most similar to human p38γ and murine SAPK3, and we named it Xp38γ/SAPK3 (accession No. AJ551272). The FTX5 constructs to express in *Xenopus* oocytes human MKK6 wt, MKK6-DD (S207D/T211D), MKK6-DA (D197A) and murine p38γ SAPK3 and the bacterial expression construct for MalE-MKK6-DD protein have been described previously (Alonso *et al*., 2000). Xp38αMpk2 (Rouse *et al*., 1994) was cloned in FTX5 as an *Nco*I–*Xho*I fragment. Human p38β2 and p38δ/SAPK4 (Goedert *et al*., 1997) were provided by Philip Cohen (MRC, Dundee, UK) and were cloned in FTX5 as *Eco*RI fragments. Pyst1/MKP-3 (Groom *et al*., 1996) in pSG5 was provided by Steve Keyse (NineWells Hospital, Dundee, UK). CA-Rsk-EE was a derivative of CA-RSK2 (Frodin *et al*., 2000) containing the mutations T365E and S369E and was subcloned into pGEX-KG as an *Xho*I–*Hin*dIII fragment. Flag-tagged Plx1 (Qian *et al*, 2001) was provided by James Maller (Howard Hughes Medical Institute, Denver, CO).

To express XCdc25C-Ct with an N-terminal His_6 tag in *Escherichia coli*, a DNA fragment encoding amino acids 200–550 of XCdc25C (Xcdc25a in Kumagai and Dunphy, 1992) was cloned into the *Nde*I–*Xho*I sites of the pET16b vector (Novagen). Full-length XCdc25C (lacking only the first eight amino acids) was prepared by fusing the N-terminal half of XCdc25C (amino acids 9–203), which was cloned in pGEX-KG (Palmer *et al*., 1998), in-frame to a *Bam*HI DNA fragment encoding amino acids 204–550 of XCdc25C (obtained from pET16b-XCdc25C-Ct).

The mutants Xp38γ-D171A, Cdc25C-Ct-S205A and Cdc25C-Ct-S285A were generated by using the QuikChange mutagenesis kit (Stratagene) and verified by DNA sequencing. Recombinant proteins were purified as described (Palmer *et al*., 1998).

Oocyte maturation and mRNA expression

Xenopus oocytes were obtained, microinjected, treated with progesterone and lysed as described (Ferby *et al*., 1999; Perdiguero and Nebreda, 2003). GVBD was scored by the appearance of a white spot at the animal pole of the oocyte and confirmed by manual dissection (Perdiguero and Nebreda, 2003). For synchronization experiments, oocytes that underwent GVBD within a time window of 10 min were pooled and used for biochemical analysis. Capped mRNAs were obtained as described (Palmer *et al*., 1998; Ferby *et al*., 1999).

Protein kinase inhibitors

U0126 (Promega) and IBMX (Sigma) were added to the oocyte medium to a final concentration of 50 μM and 1 mM, respectively. SB203580 (Promega) was dissolved in dimethylsulfoxide (DMSO) at 1 mM and the stock solution was diluted 1:10 in water for injection into oocytes of a final concentration of 10 μM. Control oocytes were injected with the same dilution of DMSO. LF protein was purified from culture supernatants of *Bacillus anthracis* BH445 as described (Duesbery *et al*., 1998).

Immunobloting, immunoprecipitation and kinase assays

Immunoblotting was performed as described (Palmer *et al*., 1998; Ferby *et al*., 1999). The following antibodies were used: Cdc2 3E1 monoclonal antibody (provided by J.Gannon and T.Hunt, Cancer Research UK, Clare Hall Laboratories, South Mimms, UK), Xp42Mpk1 rabbit antiserum (Palmer *et al*., 1998), MEK1 (Transduction Laboratories), Rsk1 and Rsk2 goat antisera (Santa Cruz), phospho-ERK (p44/p42) MAPK and phospho-p38 (Cell Signalling Technology), p38α/XMpk2 (Rouse *et al*., 1994), p38γ/SAPK3 and MKK6 (Alonso *et al*., 2000), XCdc25C (Schmitt and Nebreda, 2002), Xp38γ/SAPK3 (prepared by immunizing rabbits with purified GST–Xp38γ) and Myc 9E10 monoclonal antibody. For detection, we used Alexa Fluor 680- (Molecular Probes) or Li-Cor IRDye 800- (Rockland) labelled antibodies with the Odyssey Infrared Imaging System (Li-Cor). The intensity of the bands was quantified using the Odyssey software.

Immunoprecipitations were performed as described (Gavin *et al*., 1999; Perdiguero and Nebreda, 2003) using 3 μl of anti-Myc agarose conjugate (Santa Cruz) or the following rabbit antisera covalently coupled to protein A beads using dimethylpimelimidate (Palmer *et al*., 1998): 3 μl of anti-MKK6 or 30 μl of anti-p38γ (Alonso *et al*., 2000), 30 μl of anti-p38α (Rouse *et al*., 1994) and 30 μl of anti-p38β2 or anti-p38δ (obtained by immunizing rabbits with the purified GST fusion proteins).

H1K assays were performed as described (Palmer *et al*., 1998). Kinase assays using GST–ATF2 (amino acids 19–96) or recombinant p38α as substrates were carried out as described (Alonso *et al*., 2000).

Recombinant XCdc25C phosphatase assays

The catalytic activity of full-length XCdc25C and XCdc25C-Ct towards pNPP (Sigma) was measured in 100 mM imidazole pH 7.5, 10 mM dithiothreitol (DTT) and 20 mM pNPP using 1 μg of GST–Cdc25C or His-Cdc25C-Ct. Reactions were incubated at 30°C for 16 h and stopped by the addition of 0.2 M NaOH. The absorbance was measured at 410 nm. In some experiments, the XCdc25C and XCdc25C-Ct proteins were pre-incubated at 30°C for 1 h with 1 μg of activated recombinant p38 MAPKs in 20 μl of 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 200 μM ATP and 2 mM DTT and then used directly for the phosphatase assay as described above. Bacterially expressed p38 MAPKs were activated with MalE-MKK6-DD (5:1 ratio) by incubation at 30°C for 30 min in 50 μl of 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 200 μM ATP and 2 mM DTT.

The activity of GST–XCdc25C on inactive Cdc2–cyclin B complexes was assayed using the supernatant of high-speed oocyte extracts as described (Ferby *et al*., 1999).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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