Activation of p90\textsuperscript{rsk} during meiotic maturation and first mitosis in mouse oocytes and eggs: MAP kinase-independent and -dependent activation

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SUMMARY

Mitogen-activated protein kinases (MAPK) become activated during the meiotic maturation of oocytes from many species; however, their molecular targets remain unknown. This led us to characterize the activation of the ribosomal subunit S6 kinase of M, \(82\times 10^3\)-\(92\times 10^3\) (p90\textsuperscript{rsk}; a major substrate of MAPK in somatic cells) in maturing mouse oocytes and during the first cell cycle of the mouse embryo. We assessed the phosphorylation state of p90\textsuperscript{rsk} by examining the electrophoretic mobility shifts on immunoblots and measured the kinase activity of immunoprecipitated p90\textsuperscript{rsk} on a S6-derived peptide. Germinal vesicle stage (GV) oocytes contained a doublet of M, \(82\times 10^3\) and \(84\times 10^3\) with a low S6 peptide kinase activity (12% of the maximum level found in metaphase II oocytes). A band of M, \(86\times 10^3\) was first observed 30 minutes after GV breakdown (GVBD) and became prominent within 2 to 3 hours. MAPK was not phosphorylated 1 hour after GVBD, when the p90\textsuperscript{rsk}-specific S6 kinase activity reached 37% of the M II level. 2 hours after GVBD, MAPK became phosphorylated and p90\textsuperscript{rsk} kinase activity reached 86% of the maximum level. The p90\textsuperscript{rsk} band of M, \(88\times 10^3\), present in mature M II oocytes when S6 peptide kinase activity is maximum, appeared when MAPK phosphorylation was nearly complete (2.5 hours after GVBD). In activated eggs, the dephosphorylation of p90\textsuperscript{rsk} to M, \(86\times 10^3\) starts about 1 hour after the onset of pronuclei formation and continues very slowly until the beginning of mitosis, when the doublet of M, \(82\times 10^3\) and \(84\times 10^3\) reappears. A role for a M-phase activated kinase (like p34\textsuperscript{cdc2}) in p90\textsuperscript{rsk} activation was suggested by the reappearance of the M, \(86\times 10^3\) band during first mitosis and in 1-cell embryos arrested in M phase by nocodazole. The requirement of MAPK for the full activation of p90\textsuperscript{rsk} during meiosis was demonstrated by the absence of the fully active M, \(88\times 10^3\) band in maturing c-mos\textsuperscript{-/-} oocytes, where MAPK is not activated. The inhibition of kinase activity in activated eggs by 6-DMAP after second polar body extrusion provided evidence that both MAPK- and p90\textsuperscript{rsk}-specific phosphatases are activated at approximately the same time prior to pronuclei formation.

Key words: rsk, mitogen activated protein kinase, MAP kinase, meiosis, activation, mouse, oocyte

INTRODUCTION

The functional significance of the activation of mitogen-activated protein kinases (MAPK) during mouse oocyte maturation (Verlhac et al., 1993, 1994) has not yet been determined precisely. The best evidences to date comes from the analysis of oocytes from the c-mos knock-out strains (Colledge et al., 1994; Hashimoto et al., 1994), which do not possess a functional Mos component (the physiological MAPK kinase kinase) and the MAPK is not activated (Verlhac et al., 1996). These oocytes also lack cytoplastic activity (CSF) activity (Colledge et al., 1994; Hashimoto et al., 1994) and show alterations in the organization of the microtubules as well as the chromatin during meiosis (Verlhac et al., 1996), suggesting a physiological role for the Mos.../MAPK cascade in the mouse oocyte. The best known physiological substrates of MAPK are the ribosomal subunit S6 kinases (RSK), a family of kinases of relative molecular mass (M,\textsubscript{r}) of about \(90\times10^3\) (p90\textsuperscript{rsk}) that were cloned originally on the basis of their ability to phosphorylate the S6 protein of the 40S ribosomal subunit in maturing Xenopus oocytes (Jones et al., 1988; Alcorta et al., 1989; Erikson, 1991; Blenis, 1993). It is believed, however, that in vivo most of the S6 phosphorylation is catalyzed by homologues of another S6 kinase family called p70\textsuperscript{s6k}/p85\textsuperscript{s6k} (review: Erikson, 1991; Ferrari and Thomas, 1994; Stewart and Thomas, 1994). There is only a partial homology between the p70\textsuperscript{s6k}/p85\textsuperscript{s6k} and p90\textsuperscript{rsk} kinases (Erikson, 1991) and they differ remarkably in their regulation, substrate specificity and physiological targets. MAPK (or ERK for extracellular regulated kinases) activate p90\textsuperscript{rsk} by phosphorylation in vitro...
and in vivo and they are not involved in the activation of p70^S6K/p85^S6K (Blenis, 1993; Sutherland et al., 1993; Stewart and Thomas, 1994).

MAPK have been shown to be the major, if not exclusive, kinases able to phosphorylate p90^rsk on multiple serine and threonine sites in mitotic cells and maturing Xenopus oocytes, bringing about its activation (Chung et al., 1992; Grove et al., 1993; Sutherland et al., 1993). A sequence in the C-terminal domain containing a threonine that is phosphorylated exclusively by MAPK on rabbit p90^rsk (Sutherland et al., 1993) is completely conserved in all the known p90^rsk molecules (Alcorta et al., 1989; Moller et al., 1994). Phosphorylation by MAPK also increases the autophosphorylation activity of p90^rsk (Grove et al., 1993).

The activation of MAPK and p90^rsk are coordinated during the early response of quiescent somatic cells to extracellular signals (Hei et al., 1993; Yamazaki et al., 1993; Huang et al., 1994; Papkoff et al., 1994; Tordai et al., 1994). Moreover, a translocation of some activated MAPK and p90^rsk to the nucleus was observed in serum-activated HeLa cells (Chen et al., 1992). Finally, the phosphorylation by p90^rsk of histone H3, the transcription factors c-Fos and c-Jun (Chen et al., 1992, 1993) and the DNA-binding domain of Nur-77 (Davis et al., 1992) and the transcription factors c-Fos and c-Jun (Chen et al., 1992, 1993) suggests that it is an activator of specific transcription at the G0/G1 transition.

In this work, we describe the activation of p90^rsk in maturing mouse oocytes and during the first mitosis following parthenogenetic activation. We observed that p90^rsk is activated shortly after GVBD by a mechanism independent of MAPK, followed by a MAPK-dependent requirement for full activation of p90^rsk. During the first mitosis, only a low level of p90^rsk activation takes place, in the absence of MAPK activation, indicating that the p34^cdc2/cyclin B kinase (or another kinase activated during M-phase) might be involved. Evidence for the involvement of MAPK in the p90^rsk phosphorylation was obtained using oocytes from c-mos-deficient mice.

**MATERIALS AND METHODS**

**Antibodies**

We used polyclonal anti-mouse rsk antibodies (UBI, Lake Placid, #06-185) raised in rabbits immunized with a 44 amino acid peptide from the C terminus of mouse rsk-I S6 kinase (residues 681-724; Alcorta et al., 1989). The anti-ERK1+2 antibody (sc94, Santa-Cruz Laboratories) was characterized previously in mouse oocytes (Verlhac et al., 1993, 1994).

**Isolation and culture of oocytes**

GV stage oocytes were collected from ovaries of 6- to 8-week-old Swiss female mice in medium 2 containing 4 mg/ml PVP (M2/PVP) with 50 μg/ml dbcAMP and then removed from the drug and cultured in M2/PVP under paraffin oil at 37°C with 5% CO₂ in air. Oocytes undergoing GVBD were first observed about 45 minutes after removal of the dbcAMP. The culture was then checked every 3 to 5 minutes and newly formed GVBD oocytes were isolated into separate drops of medium. The collection lasted for 30-35 minutes after the onset of GVBD and typically about 80% of oocytes underwent GVBD within this time. Samples of oocytes were collected at various times before GVBD (‘GV’), within 5 minutes following GVBD (‘GVBD’) and at various times after GVBD.

To obtain metaphase II arrested oocytes (MII), mice were superovulated by intraperitoneal injections of 5 i.u. of pregnant mare’s serum gonadotrophin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet) 46 hours apart. Eggs were retrieved from the ampullae at 15 to 16 hours post-hCG except for activation experiments (see below) into medium 2 containing 4 mg/ml bovine serum albumin (M2/BSA; Fulton and Whittingham, 1978). The cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma).

**Parthenogenetic activation of MII eggs**

Eggs for activation were collected at 17 to 18 hours post hCG and the granulosa cells were removed by hyaluronidase treatment. At 19 hours post hCG, oocytes were exposed to 8% ethanol in M2/BSA for 6.5 minutes (Cuthbertson, 1983). The eggs were then washed carefully in a large volume of M2/BSA without calcium and then rinsed two times and placed into drops of T6 media equilibrated in the incubator. Only oocytes that extruded the second polar body within 45 to 90 minutes after activation were used for the experiment. Typically, pronuclei were formed between 2 and 3.5 hours postactivation and nuclear envelope breakdown (NEBD) was initiated 11.5 to 13.5 hours postactivation. To assess the involvement of the p34^cdc2/cyclin B kinase in p90^rsk activation, following nuclear envelope breakdown (NEBD), 1-cell embryos were incubated for 2-4 hours in 10 μM nocodazole (NZ) in M2/BSA (Kubiak et al., 1993).

**c-mos-deficient mice**

GV oocytes were isolated from the ovaries of mice homozygous (+/−) or heterozygous (+/−; controls) for the c-mos disrupted gene (Colledge et al., 1994) and cultured in vitro for 3 hours after GVBD, when MAPK is fully activated in the controls (Verlhac et al., 1994, 1996). All manipulations and oocyte culture were performed in conditions identical to those used for oocytes from Swiss mice.

**Bisection experiments**

GV oocytes and activated eggs were bisected manually (Czolowska et al., 1986). Briefly, zonae pellucidae were removed by treatment with acid Tyrode’s and then the oocytes or activated eggs were bisected manually in M2/BSA medium containing 10 μg/ml cytochalasin D and 10 μM nocodazole. In the case of GV oocytes, the media was supplemented with dbcAMP. The bisected karyoplasts and cytoplasts were washed in M2/BSA and incubated as described in the Results section until collection for analysis. Measurement of the diameter of these halves on photographs showed that the karyoplasts and cytoplasts were of similar sizes.

**6-DMAP treatment of activated eggs**

MII eggs were activated as described and transferred 1 hour later (when 95% had extruded the second polar body) to M2/BSA medium with or without 2.5 mM 6-dimethylaminopurine (6-DMAP). Their development was scored at half hourly intervals until the formation of pronuclei had occurred. Samples were then collected hourly.

**Electrophoresis and immunoblotting**

Groups of oocytes were washed in M2/PVP, extracted in SDS electrophoresis sample buffer, boiled for 3 minutes and either electrophoresed immediately or frozen at −80°C. Electrophoresis was carried on 7.5% SDS-PAGE gels (Laemmlli, 1970) prior to the transfer of proteins onto Immobilon (Millipore) membrane (150 mA for 2 hours). In all experiments, 20 oocytes per sample were used except for the experiment using oocytes of the c-mos-deficient mice where groups of 12 oocytes were used. The blots were blocked with 10% Teleostei gelatin (Sigma) in 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 (TTBS) for 1 to 2 hours. To detect both p90^rsk and ERK1+2, blots were cut in two parts containing the proteins above and below the 68 kDa molecular weight marker and incubated separately with 1.3 μg/ml of anti-p90^rsk antibodies for the upper part of the membrane and with 0.2 μg/ml of anti-ERK1+2 for the lower part of the membrane. Alternatively, the same blot was first probed with the anti-
p90<sup>sk</sup> and reprobed with the anti-ERK1+2. In the latter case, to remove the antibodies used for the p90<sup>sk</sup> detection, membranes were incubated twice for 20 minutes in 50 mM glycine pH 2.3, reblocked with gelatin and probed with the anti-ERK1+2 antibodies. The secondary anti-rabbit antibody conjugated to peroxidase (Amersham) was diluted 1/2000 for the detection of p90<sup>sk</sup> and 1/50000 for the detection of ERK1+2. In some cases, we used biotinylated secondary antibodies and peroxidase-labelled avidin-biotin complexes (ABC Elite, Vector laboratories) to enhance the detection of p90<sup>sk</sup>. Enhanced chemiluminiscence (ECL, Amersham) was used to visualize the specific staining. The specificity of the immunoblotting detection was verified using an unrelated rabbit antiserum as a control (data not shown).

**Immunocomplex assay of S6 peptide kinase activity of p90<sup>sk</sup>**

100 oocytes were collected in approximately 1 μl of M2/PVP and lysed in 10 μl of buffer A (20 mM MOPS, pH 7.2, 10 mM p-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF, 1 mM DTT, 1 mM EDTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM benzamidine), frozen immediately on dry ice and kept subsequently at −80°C. Before the assay, 30 μl of buffer B (150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 0.5% NP 40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF, 1 mM EDTA, 2 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin) were added. The lysates were incubated on ice for 5 minutes and the zonae pellucidae were removed by centrifugation at 6000 g for 3 minutes at 4°C. An equal amount of either anti-p90<sup>sk</sup> antibody (UBI # 06185, in individual experiments 2.0 to 3.5 μg/sample) or control affinity-purified rabbit IgG specific to human IgG was added to each sample and the mixture was incubated under agitation (rotator) for 2 hours at 4°C. Subsequently, 40 μl of 30% Protein A Sepharose in buffer B were added and followed by a 1 hour incubation at 4°C under agitation (rotator). The Protein A Sepharose beads were washed 3 times with 100 μl of buffer C (1 M NaCl, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM DTT, 10 mM Tris-HCl pH 7.2, 0.1% NP 40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Bovine serum albumin (1 μg/ml), 500 μCi/ml [32 P]-g ATP) and carried out at 30°C for 30 minutes with a brief vortexing of the samples every 5 minutes. The reaction was stopped by chilling on ice and centrifugation. The whole reaction mixture was spotted onto two pieces (2x2 cm) of phosphocellulose paper (Whatman P81) that were transferred immediately to 0.85% orthophosphoric acid and washed extensively with at least five changes of 0.85% orthophosphoric acid for a minimum of 2 to 3 hours. Dried papers were placed into scintillation vials and the 32P incorporation was measured in a liquid scintillation counter.

**RESULTS**

**p90<sup>sk</sup> activation during maturation**

Throughout the GV stage, p90<sup>sk</sup> was observed as a doublet of M<sub>r</sub> 82/84×10<sup>3</sup> (Fig. 1A). A diffuse staining above the M<sub>r</sub> 84×10<sup>3</sup> band appeared 10 to 20 minutes after GVBD (Fig. 1A) and the M<sub>r</sub> 86×10<sup>3</sup> band was clearly observed 30 to 40 minutes after GVBD (Fig. 1A). 1 hour after GVBD, when ERK1 and ERK2 (ERK1+2), the two major species of MAPK present in mouse oocytes (Verlhac et al., 1993) were not yet phos-
phosphorylated and the $M_r 82\times 10^3$, $84\times 10^3$ and $86\times 10^3$ bands of p90$\text{rsk}$ were observed. The phosphorylation of ERK1+2 usually began 1.5 hours after GVBD and was completed 2 to 3.5 hours after GVBD, when the p90$\text{rsk}$ $M_r 88\times 10^3$ band was present (Fig. 1B). During maturation we did not detect the $M_r 86\times 10^3$ and $88\times 10^3$ bands simultaneously, suggesting that the second shift of p90$\text{rsk}$ is rapid, taking place in less then 0.5 hour. During the transition between metaphase I and metaphase II, the phosphorylated forms of ERK1+2 were observed and there was no change in the mobility of p90$\text{rsk}$ (single band of $M_r 88\times 10^3$; Fig. 1C).

Of note, neither the use of dbcAMP in the medium at the beginning of the culture, nor the priming of the female mice with PMSG had any effect on the phosphorylation patterns of p90$\text{rsk}$ in GV oocytes and after GVBD (data not shown).

The two mobility shifts of p90$\text{rsk}$ during maturation were correlated with increases in the S6 kinase activity of the immunoprecipitated p90$\text{rsk}$ (Fig. 2A, compare with Fig. 1). The very low p90$\text{rsk}$-specific S6 peptide kinase activity in GV oocytes (12±6% of MII levels) is due to the protein of $M_r 84\times 10^3$, since the isoform of $M_r 82\times 10^3$ is unstable under the assay conditions (Fig. 2B, IP control). Further, it was not possible to prevent the disappearance of the $M_r 82\times 10^3$ band by extraction in buffer A supplemented with PKA inhibitor, PKC inhibitor or the kinase inhibitor 6-DMAP (data not shown). 1 hour after GVBD, when the $M_r 84\times 10^3$ and $86\times 10^3$ forms are present, the p90$\text{rsk}$-specific activity had reached 37±4% of the MII level. 2 hours after GVBD, when oocytes containing the $M_r 88\times 10^3$ and $86\times 10^3$ forms existed, the S6 kinase activity reached 86±4% of the level found in MII oocytes. Fig. 2B demonstrates the high efficiency of the p90$\text{rsk}$ immunoprecipitation used in the S6 kinase assay and confirms that the early increase of p90$\text{rsk}$ activity, observed 1 hour after GVBD, took place when ERK1+2 were still not phosphorylated. 2 hours after GVBD, the major increase of p90$\text{rsk}$ activity occurred when the phosphorylated forms of ERK1+2 were prominent. In conclusion, the p90$\text{rsk}$ isoforms of $M_r 86\times 10^3$ and $88\times 10^3$ are both active kinases, although only the $M_r 88\times 10^3$ isomorf appears to be fully active.

Behavior of p90$\text{rsk}$ during the first mitotic cycle
In parthenogenetically activated eggs, MAPK dephosphorylation started 0.5 to 1 hour before the formation of the pronuclei and was completed approximately 1 hour later (3 to 3.5 hours after activation), when the dephosphorylation of p90$\text{rsk}$ began, as shown by a downward shift in $M_r$ from $88\times 10^3$ to $86\times 10^3$ (Figs 3, 7). The shift of p90$\text{rsk}$ from $M_r 86\times 10^3$ to $84\times 10^3$ occurred slowly until the onset of first mitosis, when the $84\times 10^3$ band was prominent (the $86\times 10^3$ and $82\times 10^3$ bands were also present; Fig. 3). In metaphase of first mitosis, p90$\text{rsk}$ was partially respophorylated shifting to $M_r 86\times 10^3$ and the doublet of $M_r 84/86\times 10^3$ was observed in 2-cell embryos for up to 2 hours after cleavage. Furthermore, since MAPK is not reactivated during first mitosis (Figs 3, 4; Verlhac et al., 1994), this suggests that MAPK is not required for the first mobility shift of p90$\text{rsk}$. 2-cell embryos collected later than 7 hours after cleavage contained only the $M_r 84\times 10^3$ p90$\text{rsk}$ band (Fig. 3). ERK1+2 were not respophorylated during and after first mitosis (Figs 3, 7).

The treatment of metaphase 1-cell embryos with 10 $\mu$M NZ (Kubiak et al., 1993), induced a permanent and reversible block to mitosis (data not shown). Subsequently, we have observed that the level of the $M_r 86\times 10^3$ p90$\text{rsk}$ band was increased markedly in these embryos in the absence of ERK1+2 phosphorylation (Fig. 4). The treatment of 2-cell embryos with NZ had no effect on the phosphorylation state of either ERK1+2 or p90$\text{rsk}$ when compared to controls (data not shown). This experiment suggests that the upregulation of the p34$\text{cdc2}$ kinase that is observed following the NZ treatment (Kubiak et al., 1993) cannot induce the phosphorylation of p90$\text{rsk}$ beyond the level of the first mobility shift.

p90$\text{rsk}$ is not fully phosphorylated after GVBD in c-mos-deficient mice
MAPK is not activated and ERK1+2 are not phosphorylated in maturing oocytes of c-mos-deficient mice (Verlhac et al., 1996). In addition, p90$\text{rsk}$ is only detected as a $M_r 86\times 10^3$ band in the mos$^{-/-}$ oocytes 3 hours after GVBD while the $M_r 88\times 10^3$ band is observed in mos$^{-/-}$ oocytes (Fig. 5). This provides evidence that active MAPK is required for the second mobility shift of p90$\text{rsk}$, corresponding to the fully active form of p90$\text{rsk}$.
The involvement of the nucleus in the phosphorylation and dephosphorylation of MAPK and p90\textsuperscript{rsk}

The nuclear and cytoplasmic halves of bisected GV oocytes contained an approximately equal amount of p90\textsuperscript{rsk}. In addition, there was no visible effect of the absence or presence of the nuclei on the onset of p90\textsuperscript{rsk} and ERK1+2 phosphorylation at GVBD and 1 hour later (data not shown).

In activated eggs, p90\textsuperscript{rsk} was also distributed equally between the karyoplasts and cytoplasts (Fig. 6). The $M_\text{r} 88\times10^3$ p90\textsuperscript{rsk} band was still present 1 hour after pronuclei formation both in cytoplasts and karyoplasts, while the dephosphorylation of ERK1+2 progressed more rapidly in karyoplasts (Fig. 6B).

p90\textsuperscript{rsk} dephosphorylation and pronuclear formation

To determine whether there was any relationship between pronuclear formation and the dephosphorylation of p90\textsuperscript{rsk} and ERK1+2, we used 6-DMAP, a kinase inhibitor able to speed up pronuclear formation (Szöllösi et al., 1993) and inhibit MAPK (Verlhac et al., 1994). When activated eggs were exposed to 2.5 mM 6-DMAP just after polar body extrusion, pronuclei formed 1 hour earlier than in controls (2 hours after activation versus 3 hours). Under these conditions, the time course of MAPK dephosphorylation was virtually unaffected (Fig. 7). However, both levels of p90\textsuperscript{rsk} dephosphorylation (that is the downward mobility shifts from $M_\text{r} 88\times10^3$ to $M_\text{r} 86\times10^3$ and from $M_\text{r} 86\times10^3$ to $84\times10^3$) were also accelerated by about 1 hour. 4.5 hours after activation, the major p90\textsuperscript{rsk} isofrom observed was the $M_\text{r} 86\times10^3$ band in 6-DMAP-treated eggs, while in controls a similar pattern was not observed until 1.5 hour later. The $M_\text{r} 84\times10^3$ band appeared between 4.5 and 5.5 hours after activation in 6-DMAP-treated eggs, while it only became apparent between 6 and 7 hours after activation in controls. These data suggest that, in activated eggs, both pronuclear formation and p90\textsuperscript{rsk} dephosphorylation depend upon a drop in the activity of a kinase. In addition, the p90\textsuperscript{rsk}-specific phosphatase(s) is(are) apparently activated at about the same time as the MAPK phosphatase, that is 2 to 3 hours after oocyte activation, just before pronuclear formation.

![Fig. 4. The $M_\text{r} 86\times10^3$ isoform of p90\textsuperscript{rsk} accumulates in eggs blocked in first mitosis by nocodazole in the absence of MAPK activation. Activated eggs were incubated in vitro until NEBD (0 hours) when a subgroup of the embryos was transferred to 10 \textmu M nocodazole (NZ) and cultivated for further 2-4 hours (NZ block). This treatment causes up regulation of the p34\textsuperscript{cdk2} kinase (Kubiak et al., 1993) and resulted in an increased phosphorylation of p90\textsuperscript{rsk} to $M_\text{r} 86\times10^3$ in comparison to untreated mitotic 1-cell embryos (mitosis 2 hours) and freshly cleaved 2-cell embryos (3-5 hours). The phosphorylation of p90\textsuperscript{rsk} did not proceed to the level of $M_\text{r} 88\times10^3$, even after 8 to 10 hours of exposure to NZ (data not shown). Note that MAPK is not phosphorylated during the NZ block.](image)

![Fig. 5. MAPK is not activated and the second shift of p90\textsuperscript{rsk} does not take place during maturation of mos\textsuperscript{-/-} oocytes. GV oocytes were collected in parallel from homozygote mice carrying the c-mos disruption mutation (mos\textsuperscript{+/+}; Colledge et al., 1994; Verlhac et al., 1995) and from heterozygote mice (mos\textsuperscript{+/}) and cultured until 3 hours after GVBD. At this time, the ERK1+2 had shifted and the $M_\text{r} 88\times10^3$ p90\textsuperscript{rsk} isoform (second shift) was present in oocytes from heterozygotes (mos\textsuperscript{-/+}), but not in those from homozygotes (mos\textsuperscript{-/-}), where the phosphorylation of p90\textsuperscript{rsk} did not progress beyond the level of the first shift ($M_\text{r} 86\times10^3$).](image)
DISCUSSION

Our studies show that two major electrophoretic mobility shifts of p90rsk take place during meiotic maturation of the mouse oocyte. These shifts are apparently produced by two major phosphorylation events and are correlated with changes in the S6 kinase activity of p90rsk. The first event induced a shift from a doublet of $M_t 82/84 \times 10^3$ to a single band of $M_t 86 \times 10^3$, while the second event induced a shift from $M_t 86 \times 10^3$ to $M_t 88 \times 10^3$.

The first step of p90rsk activation does not depend upon MAPK activation

The absence of ERK1+2 phosphorylation until 1 to 1.5 hours after GVBD is in agreement with our previous studies where MAPK activation was observed a few hours after GVBD (Verlhac et al., 1993, 1994) and provides evidence that MAPK does not play a role in the first step of p90rsk activation (Figs 1, 2). The p90rsk of $M_t 84 \times 10^3$ in GV oocytes is not an active kinase (Fig. 2), thus autophosphorylation is not likely to be responsible for p90rsk activation following GVBD (see Grove et al., 1993).

It was shown previously that the p34cdc2 kinase is activated at GVBD (as measured by histone H1 kinase activity) and its activity rises approximately 3-fold within 1 hour after GVBD (Choi et al., 1991; Gavin et al., 1994; Verlhac et al., 1994). It is thus probable that p34cdc2 (or another kinase activated during M-phase) is involved in p90rsk activation at GVBD. This putative role of p34cdc2 was further supported by the observed phosphorylation of p90rsk to $M_t 86 \times 10^3$ during first mitosis, after oocyte activation, where we have previously observed that p34cdc2 is transiently activated in the absence of MAPK activation (Verlhac et al., 1994). Moreover, the permanent upregulation of the p34cdc2 kinase in mitotic eggs by nocodazole, a drug that inhibits the microtubule-dependent degradation of cyclin B (Kubiak et al., 1993), leads to the accumulation of the $M_t 86 \times 10^3$ isoform of p90rsk in the absence of phosphorylated forms of ERK1+2. This demonstrates clearly that the p34cdc2 kinase is unable to induce the second mobility shift of p90rsk.

To understand the potential involvement of p34cdc2 in the early activation of p90rsk, we may question whether there is some mechanism preventing p90rsk phosphorylation before GVBD in the presence of the already rising H1 kinase activity (Gavin et al., 1994; Verlhac et al., 1994). In response to this query, besides examining the level of p90rsk-specific phosphatase activity in GV oocytes, it would be interesting to determine whether dephosphorylated p90rsk is sequestered in
complexes with dephosphorylated MAPK, as was suggested by Hsiao et al. (1994) in the case of Xenopus oocytes.

To our knowledge, this article is the first report showing that p90rsk is phosphorylated during mitosis and that the p34cdc2 kinase may play a role in the low-level initial activation of p90rsk. However, the sequence of the mouse rsk (Alcorta et al., 1989) does not contain any phosphorylation sites for the p34cdc2 kinase. Thus it is possible that an as yet unidentified intermediary kinase controlled by p34cdc2 phosphorylates p90rsk after GVBD and during the first mitosis.

**Active MAPK is required for the complete phosphorylation and activation of p90rsk**

Consistent with MAPK being the dominant p90rsk kinase in mouse oocytes, the presence of the most active form of p90rsk (Mr 88×10^3) is strictly correlated with the presence of phosphorylated ERK1+2 in maturing oocytes and activated eggs (from 2.5 hours after GVBD until 1 hour after the beginning of pronuclear formation). The best evidence to date that active MAPK is required for the complete phosphorylation of p90rsk was provided by the absence of p90rsk phosphorylation to Mt 88×10^3 in the oocytes from the c-mos-deficient mouse (Fig. 4). Finally, the first phosphorylation event of p90rsk, leading to the Mt 86×10^3 isoform, was not inhibited in mos^-/- oocytes demonstrating further that this event is MAPK independent.

The c-mos-deficient mouse oocytes present a unique model with which to study the mechanism of p90rsk regulation by MAPK. For example, the injection of exogenous constitutively active MAPK (Haccard et al., 1993) into mos^-/- oocytes kept in the presence or absence of protein synthesis inhibitors could provide the evidence required to determine whether previous low level phosphorylation by a p34cdc2-dependent kinase is required for the maximal activation of p90rsk by MAPK.

**In activated eggs, p90rsk is slowly dephosphorylated by a phosphatase that is activated at the same time as the MAPK phosphatase**

The bisection experiments showed that the nucleus, or some nucleus-associated structures, are involved in the dephosphorylation of MAPK in activated eggs, either by activation of a specific MAPK phosphatase, or by accelerating the down-regulation of the MAPK kinase through Mos degradation (Weber et al., 1991; Verlhac et al., 1996). However, the sustained high level of p90rsk in karyoplasts indicates that the presence of a small amount of active MAPK can overcome the effect of the phosphatases involved in p90rsk dephosphorylation.

A closer insight into the control of MAPK and p90rsk specific phosphatases after activation was provided by inhibiting phosphorylation in activated eggs after polar body extrusion with 6-DMAP, a potent kinase inhibitor. In contrast to our previous study, where 6-DMAP was applied before oocyte activation (Szöllösi et al., 1993; Verlhac et al., 1993), the early events of oocyte activation took place in the presence of kinase activity. If 6-DMAP inhibits the MAPK kinase, as suggested by our previous work (Verlhac et al., 1994), the unaltered rate of MAPK dephosphorylation in the presence of 6-DMAP that we observe indicates that the MAPK phosphatase is not active until 2-3 hours after oocyte activation. Similarly, the simultaneous delayed onset of p90rsk dephosphorylation in the presence of 6-DMAP shows that the phosphatase(s) responsible for both MAPK and p90rsk dephosphorylation become active at about the same time, that is shortly before pronuclear formation. It should be emphasized that the delay in the dephosphorylation of both kinases after 6-DMAP treatment is not likely due to a delayed distribution of 6-DMAP into the eggs (leading to a residual kinase activity that could mask the onset of dephosphorylation), since the formation of pronuclei was dramatically accelerated (Fig. 7). Moreover, in vitro, the same concentration of 6-DMAP was able to inhibit completely the S6 kinase activity of the immunoprecipitated p90rsk (data not shown).

From the time of activation of the p90rsk phosphatase, the phosphorylation of p90rsk leading to the Mt 88×10^3 isoform must be maintained by a continuous kinase activity. A gradual decrease in MAPK activity is most likely to be responsible for the slow return of p90rsk to an Mt 86×10^3, as suggested by the simultaneous disappearance of the Mt 88×10^3 p90rsk isoform and of the phosphorylated forms of ERK1+2 about 1 hour after pronuclei formation. Our data on the timing of pronuclei formation and that of MAPK and p90rsk dephosphorylation are consistent with the suggestion that a decrease in MAPK activity is required for the process of pronuclei formation to take place in fertilized eggs (Szöllösi et al., 1993; Verlhac et al., 1994; Moos et al., 1995; Verlhac et al., 1996). The kinase activity of the Mt 86×10^3 p90rsk isoform may maintain its own phosphorylation state by autophosphorylation in the absence of both p34cdc2 and MAPK activities after pronuclear formation in activated eggs and after first mitosis in 2-cell embryos. However, it is possible that the activity of a yet unidentified kinase and/or the lack of phosphatase activity may be involved as well.

More information on the qualitative and quantitative changes of phosphorylation sites of p90rsk will be necessary to understand its precise regulation during meiosis and embryonic development. This will also permit the identification of some of the kinases involved in p90rsk activation. Phosphorylation ‘fingerprints’ on p90rsk could then potentially serve as a cellular reporter of the activity of upstream regulatory kinases, such as MAPK and p34cdc2.

We thank N. Winston for critical reading of the manuscript and R. Schwartzmann for his expert photographic work. This work was supported by grants to B. M. from La Ligue contre le Cancer, l’Association pour la Recherche contre le Cancer and le Centre National de la Recherche Scientifique. P. K. was sponsored by the EDB short term fellowship 94/027 and partly by grants of the Grant Agency of Czech Republic (301/94/0509) and of the Czech Academy of Sciences (A545401).

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(Accepted 5 March 1996)