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Activation of p90^{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_r 82×10^3-92×10^3 (p90^{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^{rsk} by examining the electrophoretic mobility shifts on immunoblots and measured the kinase activity of immunoprecipitated p90^{rsk} on a S6-derived peptide. Germinal vesicle stage (GV) oocytes contained a doublet of M_r 82×10^3 and 84×10^3 with a low S6 peptide kinase activity (12% of the maximum level found in metaphase II oocytes). A band of M_r 86×10^3 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^{rsk}-specific S6 kinase activity reached 37% of the M II level. 2 hours after GVBD, MAPK became phosphorylated and p90^{rsk} kinase activity reached 86% of the maximum level. The p90^{rsk} band of M_r 88×10^3, 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^{rsk} to M_r 86×10^3 starts about 1 hour after the onset of pronuclei formation and continues very slowly until the beginning of mitosis, when the doublet of M_r 82×10^3 and 84×10^3 reappears. A role for a M-phase activated kinase (like p34^{cdc2}) in p90^{rsk} activation was suggested by the reappearance of the M_r 86×10^3 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^{rsk} during meiosis was demonstrated by the absence of the fully active M_r 88×10^3 band in maturing c-mos$^{-/-}$ 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^{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 cytostatic factor (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_r) of about 90×10^3 (p90^{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^{s6k}/p85^{s6k} (review: Erikson, 1991; Ferrari and Thomas, 1994; Stewart and Thomas, 1994). There is only a partial homology between the p70^{s6k}/p85^{s6k} and p90^{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^{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 phophorylated 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., 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 event required 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 p34cdc2/cyclin B kinase (or another kinase activated during M-phase) might be involved. Evidence for the involvement MAPK in the $p90^{rsk}$ phophorylation 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 rskmo-1 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% CO_2 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 (Laemmli, 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-

p90rsk and reprobed with the anti-ERK1+2. In the latter case, to remove the antibodies used for the p90rsk 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 p90rsk and 1/5000 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 p90rsk. 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 p90rsk

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_3VO_4, 20 mM NaF, 1 mM DTT, 5 mM EGTA, 0.1 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 Na3VO4, 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-p90rsk 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 Tris-HCl pH 7.2, 0.1% NP 40, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin), 2 times with 100 μl buffer D (100 mM NaCl, 10 mM Tris-HCl pH 7.2, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and 2 times with 100 μl of twice concentrated S6 kinase assay buffer (1×: 20 mM MOPS, pH 7.2, 10 mM $MgCl_2$, 1 μM PKA-I (UBI), 0.1 mg/ml BSA). After the last wash, the liquid phase was carefully removed and 15 μl of twice concentrated kinase assay buffer were added to each tube. The kinase reaction was started by the addition of 15 μl of freshly prepared twice concentrated ATP-[^{32}P]-substrate buffer (1×: 0.1 mM ATP, 0.25 mg/ml S6 peptide (UBI), 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 (2×2 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 ^{32}P incorporation was measured in a liquid scintillation counter.

RESULTS

p90rsk activation during maturation

Throughout the GV stage, p90rsk was observed as a doublet of M_r 82/84×10^3 (Fig. 1A). A diffuse staining above the M_r 84×10^3 band appeared 10 to 20 minutes after GVBD (Fig. 1A) and the M_r 86×10^3 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-

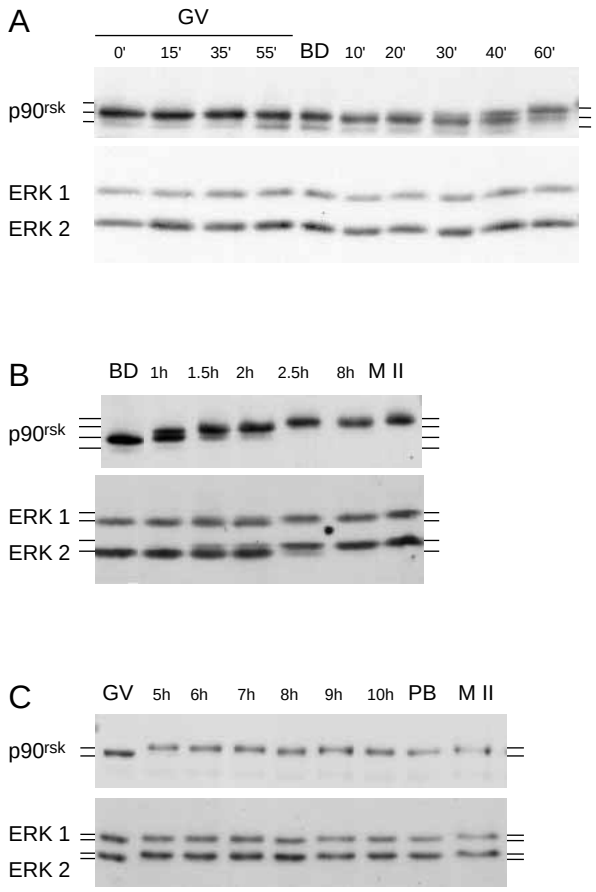

Fig. 1. p90rsk and MAPK activation during maturation. In this and all the following figures, p90rsk and MAPK were detected on the same blot with anti-p90rsk (upper part) and anti-ERK (lower part) antibodies. The position of individual p90rsk and ERK1+2 bands is marked with horizontal bars on both sides of the picture. The experiments A and B were performed 5 times and the experiment C three times and gave consistent results. (A) The first mobility shift of p90rsk (M_r 82/84×10^3 to 86×10^3) occurs in the absence of ERK1+2 phosphorylation. GV oocytes were cultured in vitro and extracted immediately after isolation in the absence of dbcAMP (GV 0') and 15, 35 and 55 minutes after removal of dbcAMP (GV 15'-55'). In the same experiment, oocytes having just completed GVBD (BD) were collected 55-60 minutes after removal from dbcAMP and other samples were taken 10-60 minutes after GVBD. The p90rsk forms of M_r 82×10^3, 84×10^3 and 86×10^3 (first shift) together with dephosphorylated ERK1 (M_r 42×10^3) and ERK2 (M_r 44×10^3) were detected. (B) The second mobility shift of p90rsk (M_r 86×10^3 to 88×10^3) is correlated with the phosphorylation of ERK1+2. GV oocytes were cultured in vitro and extracted immediately after GVBD (BD) and 1-8 hours after GVBD. Ovulated oocytes (MII) were obtained 14 hours after hCG. All the phosphorylation forms of p90rsk detectable under physiological conditions (bands of M_r 82, 84, 86 and 88 ×10^3) and both non phosphorylated and phosphorylated forms of ERK1+2 are visible. (C) The high level of p90rsk phosphorylation (M_r 88×10^3, second shift) does not change between metaphases I and II. GV oocytes (GV), oocytes cultured for 5 to 10 hours after GVBD (5-10h), oocytes collected at the time of first polar body extrusion (PB, 10 to 11 hours after GVBD) and ovulated oocytes (MII) were analyzed. The p90rsk bands of M_r 84×10^3 (GV) and 88×10^3 (5h-MII) and non-phosphorylated (GV) and phosphorylated forms (5h-MII) of ERK1+2 are visible.

phorylated and the M_r 82×10^3, 84×10^3 and 86×10^3 bands of p90^{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^{rsk} M_r 88×10^3 band was present (Fig. 1B). During maturation we did not detect the M_r 86×10^3 and 88×10^3 bands simultaneously, suggesting that the second shift of p90^{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^{rsk} (single band of M_r 88×10^3; 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^{rsk} in GV oocytes and after GVBD (data not shown).

The two mobility shifts of p90^{rsk} during maturation were correlated with increases in the S6 kinase activity of the immunoprecipitated p90^{rsk} (Fig. 2A, compare with Fig. 1). The very low p90^{rsk}-specific S6 peptide kinase activity in GV oocytes (12±6% of MII levels) is due to the protein of M_r 84×10^3, since the isoform of M_r 82×10^3 is unstable under the assay conditions (Fig. 2B, IP control). Further, it was not possible to prevent the disappearance of the M_r 82×10^3 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 84×10^3 and 86×10^3 forms are present, the p90^{rsk}-specific activity had reached 37±4% of the MII level. 2 hours after GVBD, when oocytes containing the M_r 88×10^3 and 86×10^3 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^{rsk} immunoprecipitation used in the S6 kinase assay and confirms that the early increase of p90^{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^{rsk} activity occured when the phosphorylated forms of ERK1+2 were prominent. In conclusion, the p90^{rsk} isoforms of M_r 86×10^3 and 88×10^3 are both active kinases, although only the M_r 88×10^3 isoform appears to be fully active.

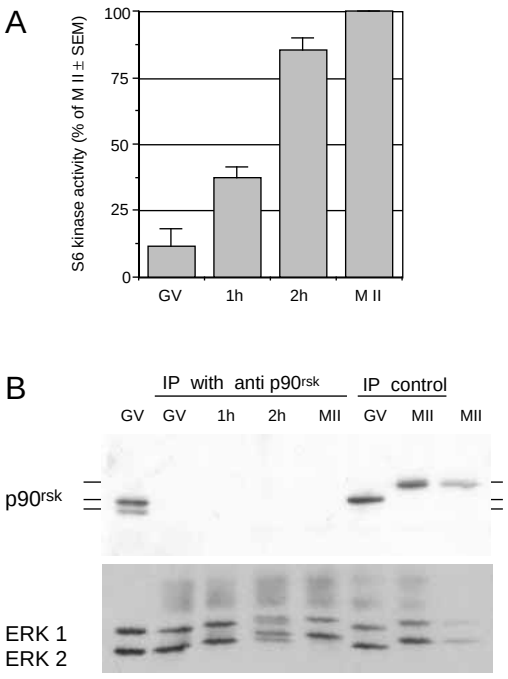

Fig. 2. p90^{rsk} is activate during meiotic maturation. (A) Relative S6 peptide kinase activity (±s.e.m.) of p90^{rsk} immunoprecipitated from MII oocytes, GV oocytes and oocytes taken 1 and 2 hours after GVBD. Background noise of the assay was determined using identical samples of GV and MII oocytes immunoprecipitated with an irrelevant antibody. Data are from four experiments, using 100 oocytes/sample in all cases. (B) Analysis of the supernatants left after p90^{rsk} immunoprecipitation used for one of the S6 kinase assay represented above. Following the S6 kinase assay, 30% of each lysate remaining after immunoprecipitation of p90^{rsk} was loaded per lane (IP with anti-p90^{rsk} and IP controls). 20 GV oocytes and 20 MII oocytes were loaded for comparison in the side lanes of the gel. In the IP controls, note that the p90^{rsk} band of M_r 82×10^3 is missing in the GV sample after immunoprecipitation. Supernatants of 3 (out of 4) S6 kinase assays were analyzed this way with identical results.

Behavior of p90^{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^{rsk} began, as shown by a downward shift in M_r from 88×10^3 to M_r 86×10^3 (Figs 3, 7). The shift of p90^{rsk} from M_r 86×10^3 to 84×10^3 occured slowly until the onset of first mitosis, when the 84×10^3 band was prominent (the 86×10^3 and 82×10^3 bands were also present; Fig. 3). In metaphase of first mitosis, p90^{rsk} was partially rephosphorylated shifting to M_r 86×10^3 and the doublet of M_r 84/86×10^3 was observed in 2-cell embryos for up to 2 to 4 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^{rsk}. 2-cell embryos collected later than 7 hours after cleavage contained only the M_r 84×10^3 p90^{rsk} band (Fig. 3). ERK1+2 were not rephosphorylated during and after first mitosis (Figs 3, 7).

The treament of metaphase 1-cell embryos with 10 μM 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 86×10^3 p90^{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^{rsk} when compared to controls (data not shown). This experiment suggests that the upregulation of the p34^{cdc2} kinase that is observed following the NZ treatment (Kubiak et al., 1993) cannot induce the phosphorylation of p90^{rsk} beyond the level of the first mobility shift.

p90^{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^{rsk} is only detected as a M_r 86×10^3 band in the *mos*$^{-/-}$ oocytes 3 hours after GVBD while the M_r 88×10^3 band is observed in *mos*$^{+/-}$ oocytes (Fig. 5). This provides evidence that active MAPK is required for the second mobility shift of p90^{rsk}, corresponding to the fully active form of p90^{rsk}.

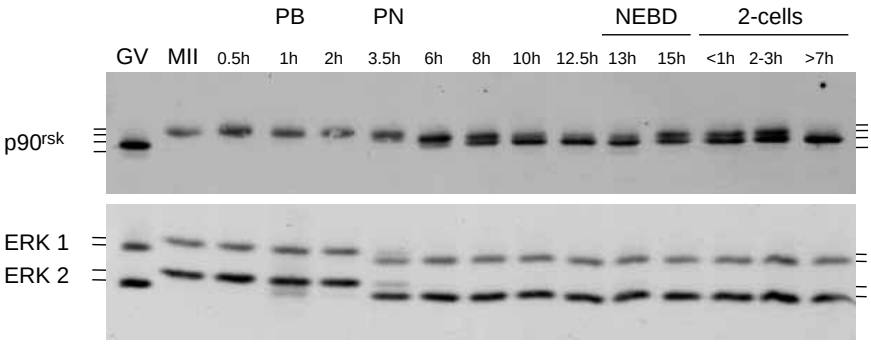

Fig. 3. Behaviour of p90rsk and MAPK during the first two mitotic cell cycles. MII oocytes were activated by ethanol 19 hours after hCG and samples were taken before activation (MII) and at various times after activation. All the eggs taken for analysis had extruded the second polar body 1 hour after activation and in all samples collected later than 3.5 hours after activation, pronuclei were formed. Samples of 1-cell embryos were collected just after NEBD (13 hours after activation) and when large metaphase spindles were present (15 hours after activation). 2-cell embryos were collected just after cleavage (<1 hour), 2 to 3 and 7 to 10 hours later. All the samples were obtained from the same batch of activated MII oocytes. The experiment was repeated 5 times with similar results.

The involvement of the nucleus in the phosphorylation and dephosphorylation of MAPK and p90rsk

The nuclear and cytoplasmic halves of bisected GV oocytes contained an approximately equal amount of p90rsk. In addition, there was no visible effect of the absence or presence of the nuclei on the onset of p90rsk and ERK1+2 phosphorylation at GVBD and 1 hour later (data not shown).

In activated eggs, p90rsk was also distributed equally between the karyoplasts and cytoplasts (Fig. 6). The M_r 88×10^3 p90rsk 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).

p90rsk dephosphorylation and pronuclear formation

To determine whether there was any relationship between pronuclear formation and the dephosphorylation of p90rsk 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 p90rsk dephosphorylation (that is the downward mobility shifts from M_r 88×10^3 to M_r 86×10^3 and from M_r 86×10^3 to 84×10^3) were also accelerated by about 1 hour. 4.5 hours after activation, the major p90rsk isoform observed was the M_r 86×10^3 band in 6-DMAP-treated eggs, while in controls a similar pattern was not observed until 1.5 hour later. The M_r 84×10^3 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 p90rsk dephosphorylation depend upon a drop in the activity of a kinase. In addition, the p90rsk-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.

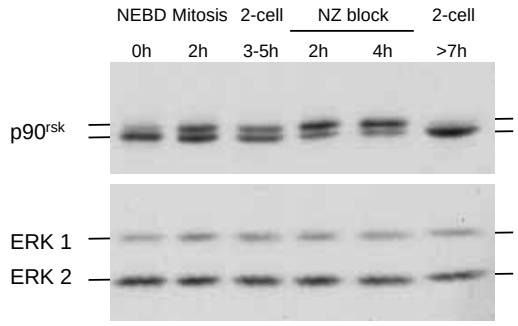

Fig. 4. The M_r 86×10^3 isoform of p90rsk accumulates in eggs blocked in first mitosis by nocodazole in the absence of MAPK activation. Activated eggs were incubated in vitro until NEBD (0 hours) when a subgroup of the embryos was transferred to 10 μM nocodazole (NZ) and cultived for further 2-4 hours (NZ block). This treatment causes up regulation of the $p34^{cdc2}$ kinase (Kubiak et al., 1993) and resulted in an increased phosphorylation of p90rsk to M_r 86×10^3 in comparison to untreated mitotic 1-cell embryos (mitosis 2 hours) and freshly cleaved 2-cell embryos (3-5 hours). The phosphorylation of p90rsk did not proceed to the level of M_r 88×10^3, even after 8 to 10 hours of exposure to NZ (data not shown). Note that MAPK is not phosphorylated during the NZ block.

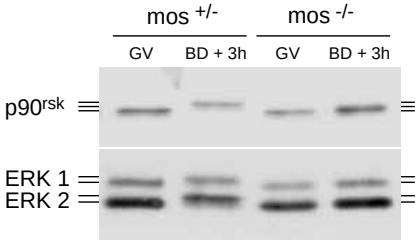

Fig. 5. MAPK is not activated and the second shift of p90rsk does not take place during maturation of *mos*$^{-/-}$ oocytes. GV oocytes were collected in parallel from homozygote mice carrying the c-mos disruption mutation (*mos*$^{-/-}$; Colledge et al., 1994; Verlhac et al., 1995) and from heterozygote mice (*mos*$^{-/+}$) and cultured until 3 hours after GVBD. At this time, the ERK1+2 had shifted and the M_r 88×10^3 p90rsk isoform (second shift) was present in oocytes from heterozygotes (*mos*$^{-/+}$), but not in those from homozygotes (*mos*$^{-/-}$), where the phosphorylation of p90rsk did not progress beyond the level of the first shift (M_r 86×10^3).

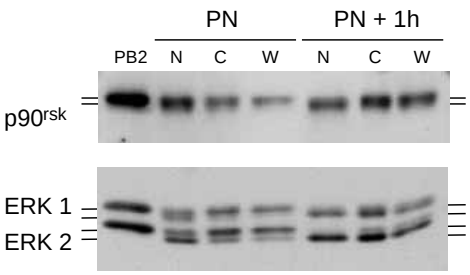

Fig. 6. p90^{rsk} and MAPK in activated eggs. Zona-free activated MII eggs were bisected in the presence of cytochalasin D to give halves with or without the second polar body and incubated until pronuclei were formed. All polar body containing halves formed pronuclei (N), while none of the halves without polar body formed pronuclei (C). 40 karyoplasts or cytoplasts (N and C) and 20 non-bisected controls (W) were collected at the time of pronuclear formation (PN) and 1 hour later (PN+1h). 20 untreated activated eggs with the second polar body were also analysed for comparison (PB2). Note that while the dephosphorylation of MAPK takes place more rapidly in karyoplasts (PN, compare ERK1+2 in N with C and W samples), there is only a subtle concurrent downward shift of p90^{rsk} as seen by a slight increase of the lower part of the band of M_r 88×10^3 (PN+1 hour, compare N with C and W). The experiment was repeated twice with similar results.

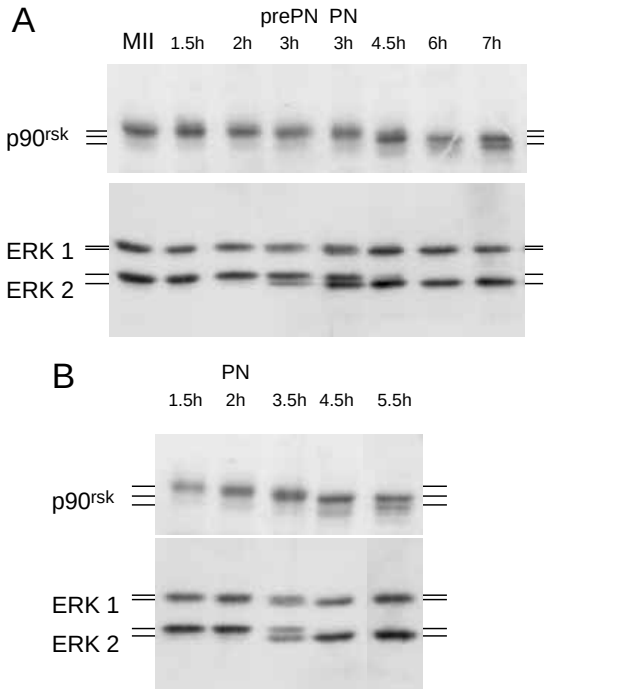

Fig. 7. The inhibition of kinase activity after polar body extrusion by 6-DMAP accelerates p90^{rsk} dephosphorylation and pronuclear formation in activated eggs. (A) MII oocytes before activation (MII) and activated eggs were collected after culture in control media for various period of times after activation. In all activated eggs, the second polar body formed about 1 hour after activation. At 3 hours postactivation, eggs containing recently formed pronuclei (PN, 3h) as well as eggs without pronuclei (pre PN, 3h) were collected. Samples of eggs that were taken 1, 2 and 3 hours after pronuclei formation were collected 4.5, 6 and 7 hours after activation, respectively. (B) In the same experiment, some activated eggs were transferred to medium containing 2.5 mM 6-DMAP immediately after second polar body extrusion (1 hour after activation). All activated eggs in the 2 hour sample contained recently formed pronuclei. Samples of eggs that were taken 1, 2 and 3 hours after pronuclei formation were collected 3.5, 4.5 and 5.5 h after activation, respectively. Note that in 6-DMAP treated eggs both the rate of pronuclear formation and the dephosphorylation of p90^{rsk} (from M_r 88×10^3 to 86×10^3 and from 86×10^3 to 84×10^3) was accelerated by approximately 1 hour. The experiment was repeated twice with similar results.

DISCUSSION

Our studies show that two major electrophoretic mobility shifts of p90^{rsk} 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 p90^{rsk}. The first event induced a shift from a doublet of M_r 82/84×10^3 to a single band of M_r 86×10^3, while the second event induced a shift from M_r 86×10^3 to M_r 88×10^3.

The first step of p90^{rsk} 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 p90^{rsk} activation (Figs 1, 2). The p90^{rsk} of M_r 84×10^3 in GV oocytes is not an active kinase (Fig. 2), thus autophosphorylation is not likely to be responsible for p90^{rsk} activation following GVBD (see Grove et al., 1993).

It was shown previously that the p34^{cdc2} 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 p34^{cdc2} (or another kinase activated during M-phase) is involved in p90^{rsk} activation at GVBD. This putative role of p34^{cdc2} was further supported by the observed phosphorylation of p90^{rsk} to M_r 86×10^3 during first mitosis, after oocyte activation, where we have previously observed that p34^{cdc2} is transiently activated in the absence of MAPK activation (Verlhac et al., 1994). Moreover, the permanent upregulation of the p34^{cdc2} 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_r 86×10^3 isoform of p90^{rsk}, in the absence of phosphorylated forms of ERK1+2. This demonstrates clearly that the p34^{cdc2} kinase is unable to induce the second mobility shift of p90^{rsk}.

To understand the potential involvement of p34^{cdc2} in the early activation of p90^{rsk}, we may question whether there is some mechanism preventing p90^{rsk} 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 p90^{rsk}-specific phosphatase activity in GV oocytes, it would be interesting to determine whether dephosphorylated p90^{rsk} 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 (M_r 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 M_r 88×10^3 in the oocytes from the c-mos-deficient mouse (Fig. 4). Finally, the first phosphorylation event of p90rsk, leading to the M_r 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 about 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 M_r 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 M_r 86×10^3, as suggested by the simultaneous disappearance of the M_r 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 M_r 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.

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