

Phosphorylation of p90^{rsk} during meiotic maturation and parthenogenetic activation of rat oocytes: correlation with MAP kinases

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Summary

This paper reports on the activation of p90^{rsk} during meiotic maturation and the inactivation of p90^{rsk} after electrical parthenogenetic activation of rat oocytes. In addition, the correlation between p90^{rsk} and MAP kinases after different treatments was studied. We assessed p90^{rsk} activity by examining its electrophoretic mobility shift on SDS-PAGE and evaluated ERK1+2 activity by both mobility shift and a specific antibody against phospho-MAP kinase. The phosphorylation of p90^{rsk} during rat oocyte maturation was a sequential process that may be divided into two stages: the first stage was partial phosphorylation, which was irrelevant with MAP kinases because p90^{rsk} phosphorylation took place prior to activation of MAP kinases. The second stage inferred full activation occurred at the time when MAP kinases began to be activated (3 h after germinal vesicle breakdown). Evidence for the involvement of MAP kinases in the p90^{rsk} phosphorylation was further obtained by the following approaches: (1) okadaic acid (OA) accelerated the phosphorylation of both MAP kinases and p90^{rsk}; (2) OA induced phosphorylation of both MAP kinases and p90^{rsk} in the presence of IBMX; (3) when activation of MAP kinases was inhibited by cycloheximide, p90^{rsk} phosphorylation was also abolished; (4) dephosphorylation of p90^{rsk} began to take place at 3 h post-activation, temporally correlated with the completion of MAP kinase inactivation; (5) phosphorylation of both kinases was maintained in oocytes that failed to form pronuclei after stimulation; (6) OA abolished the dephosphorylation of both kinases after parthenogenetic activation. Our data suggest that MAP kinases are not required for early partial activation of p90^{rsk} but are required for full activation of p90^{rsk} during rat oocyte maturation, and that p90^{rsk} dephosphorylation occurs following MAP kinase inactivation after parthenogenetic activation of rat oocytes.

Keywords: Cell cycle, MAP kinase, Oocyte, p90^{rsk}, Rat

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Introduction

Protein phosphorylation/dephosphorylation regulated by protein kinase(s) and protein phosphatase(s) is thought to play a crucial role in regulating oocyte meiotic progression and the meiosis–interphase–mitosis transition (Bornslaeger *et al.*, 1986). Apart from the well-known maturation-promoting factor (MPF), recent studies have shown that MAP kinases are activated during meiotic progression of oocytes in many mammalian species (for review see Sun *et al.*, 1999a). Previous studies have shown that MAP kinase phosphorylation does not occur in oocytes that are incompetent to resume meiosis (Sun *et al.*, 1999b, 2001a) and

in germinal vesicle breakdown (GVBD)-inhibited, fully grown oocytes (Sun *et al.*, 1999c; Lu *et al.*, 2001), but premature activation of MAP kinases resulted in GVBD (de Vanterry Arrigh *et al.*, 2000). In general, functions of p42 and p44 MAP kinases include the participation in the reinitiation of meiosis I (cattle: Fissore *et al.*, 1996; pig: Inoue *et al.*, 1998), arrest of metaphase II (Dedieu *et al.*, 1996), control of microtubule dynamics (Verlhac *et al.*, 1993, 1994; Zernicka-Goetz *et al.*, 1997; Sun *et al.*, 2001b), regulation of pronuclear envelope assembly/disassembly (Moos *et al.*, 1995, 1996; Sun *et al.*, 1998, 1999d, e) and regulation of the APC for supporting cyclin B accumulation (Gross *et al.*, 2000).

Among the many substrates for MAP kinases, including p82/CPEB, retinoblastoma protein (Rb), p90^{rsk} and SPIN (Oh *et al.*, 1998; Sturgill *et al.*, 1988; Taieb *et al.*, 1998; Katsu *et al.*, 1999), p90^{rsk} (90 kDa ribosomal S6 kinase, RSK, also known as MAP kinase-activated protein kinase-1, MAPKAP-K1) was the first to be discovered (Sturgill *et al.*, 1988) and it is a ubiquitous and versatile mediator of MAPK/ERK signal transduction involved in regulating cell survival and proliferation (Richards *et al.*, 1999; Frodin & Gammeltoft, 1999). Its functions include: (1) regulation of gene expression via association and phosphorylation of transcriptional regulators including c-Fos, estrogen receptor, NFkappaB/IkappaB alpha, cAMP-response element-binding protein (CREB) and CREB-binding protein; (2) a role in cell cycle regulation in *Xenopus laevis* oocytes by inactivation of the Myt1 protein kinase leading to activation of the cyclin-dependent kinase p34cdc2; (3) regulation of protein synthesis by phosphorylation of polyribosomal proteins and glycogen synthase kinase-3; and (4) phosphorylation of the Ras GTP/GDP-exchange factor, SOS, which leads to feedback inhibition of the Ras-ERK pathway (Palmer *et al.*, 1998; Xing *et al.*, 1998; Frodin & Gammeltoft, 1999; Palmer & Nebreda, 2000). Although p90^{rsk} is mainly phosphorylated and activated by MAP kinases, phosphoinositide-dependent protein kinase-1 (PDK1), a recently identified serine/threonine kinase, is also able to phosphorylate p90^{rsk} in the amino-terminal kinase-activation loop, leading to partial activation of the kinase in somatic cells (Richards *et al.*, 1999; Jensen *et al.*, 1999; Williams *et al.*, 2000).

Recently, it has been shown that p90^{rsk} plays crucial roles in oocyte meiotic progression. p90^{rsk} is known to be activated during *Xenopus* oocyte maturation (Gross *et al.*, 1999; Palmer & Nebreda, 2000). The effects of MAP kinases in suppressing entry into S phase, regulating the APC, arresting oocytes at metaphase II and supporting spindle formation are mediated by p90^{rsk} in *Xenopus* oocytes (Gross *et al.*, 1999, 2000). In mouse oocytes, p90^{rsk} is phosphorylated and activated in maturing oocytes (Gavin & Schorderet-Slatkine, 1997) and full activation of p90^{rsk} correlates with the activa-

tion of MAP kinases (Kalab *et al.*, 1996). However, the changes in p90^{rsk} phosphorylation/dephosphorylation and the correlation between p90^{rsk} and MAP kinases in oocytes of mammals other than mouse have never been reported.

The aim of the present study was to investigate, in rat oocytes, the changes in p90^{rsk} phosphorylation/dephosphorylation and its correlation with MAP kinase activity during meiotic maturation and after parthenogenetic activation as well as under various experimental conditions.

Materials and methods

Isolation and culture of oocytes

SD strain rats 25–30 days old were primed with 20 IU pregnant mare's serum gonadotropin (PMSG). Approximately 48 h later, cumulus-cell-enclosed oocytes (CEO) were collected from the ovaries in M2 medium containing 4 mg/ml bovine serum albumin (BSA, Sigma) and incubated in Dulbecco minimal essential medium (DMEM, Sigma) supplemented with 4 mg/ml BSA (DMEM/BSA) under paraffin oil at 37 °C, 5% CO₂ in air. After 1.5 h of culture, the cumulus cells surrounding the oocytes were removed by repeated gentle pipetting through a narrow-bore glass pipette and the denuded oocytes were further incubated until harvested for cell cycle evaluation and immunoblotting of MAP kinases and p90^{rsk}.

Collection and parthenogenetic activation of MII oocytes

To obtain ovulated oocytes, 25- to 30-day-old SD rats were superovulated by intraperitoneal injections of 20 IU PMSG and 20 IU human chorionic gonadotropin (hCG) 48 h apart. The animals were killed 16 h after hCG injection. Cumulus masses were dispersed by a brief exposure to 300 IU/ml hyaluronidase (Sigma). After washing four or five times in M2 medium, the oocytes were incubated in DMEM/BSA for 2 h at 37 °C, 5% CO₂ in air.

For parthenogenetic activation metaphase II eggs that had extruded the first polar body were stimulated by an electrical stimulus (160 V, 80 μs) in electrofusion solution (0.3 M mannitol, 0.1 mM MgSO₄·7H₂O, 0.05 mM CaCl₂). The oocytes were washed four or five times in calcium-free M2 medium that contained 4 mg/ml BSA and then incubated in DMEM/BSA. Typically, pronuclei were observed after 4–7 h of activation. Oocytes were collected after different times of activation for cell cycle evaluation and MAP kinases and p90^{rsk} immunoblotting.

Drug treatments of oocytes

Okadaic acid (OA, Sigma), an inhibitor of protein phosphatase 1 and 2A, cycloheximide (CHX, Sigma), a protein synthesis inhibitor, and 3-isobutyl-1-methylxanthine (IBMX, Sigma), a phosphodiesterase inhibitor, were used to assess the correlation between MAP kinase activity and p90^{rsk} activity. OA and IBMX were dissolved in dimethylsulphoxide (DMSO) as stock solutions at concentrations of 100 μ M and 500 mM, respectively. When used, they were diluted in DMEM/BSA. The same concentration of DMSO as in the experimental group was added to the medium of the control group. CHX was dissolved directly in the culture medium.

GV-stage oocytes were incubated in DMEM/BSA containing 500 μ M IBMX to maintain meiotic arrest (Schultz *et al.*, 1983). OA was added to the culture medium at a final concentration of 2 μ M and was present throughout the incubation period. In the other experiments, GV-stage oocytes were incubated in IBMX-free DMEM/BSA containing OA at 2 μ M, or CHX at 35.5 μ M or 355 μ M, until harvested. Oocytes activated by electrical stimulation were incubated in DMEM/BSA containing OA at 2 μ M for the whole period of incubation.

Western immunoblotting

A total of 40 oocytes for each treatment were lysed in 15 μ l SDS sample buffer (Laemmli, 1970) and stored at -20°C . Before electrophoresis, they were heated to 100°C for 4 min and cooled on ice immediately after boiling. Electrophoresis was conducted on 10% SDS-PAGE gels and the proteins were electrically transferred onto nitrocellulose membranes. Following blocking with 5% skim milk in 10 mM Tris (pH 7.4), 150 mM NaCl containing 0.1% Tween-20 (TBST) at 4°C overnight, the membranes were cut into two parts, containing p90^{rsk} and ERK1+2, respectively, and were incubated separately with anti-ERK1+2 (1:300), anti-p90^{rsk} (1:50) antibodies (Santa Cruz Biotechnology) in TBST at 37°C for 2 h. After three washes of 10 min each in TBST, the membranes were incubated with secondary anti-rabbit antibody conjugated to peroxidase (Santa Cruz Biotechnology) diluted 1:5000 in TBST for 1 h at 37°C . After washing three times in TBST, the membranes were processed using the ECL detection system. The membrane containing ERK1+2 was then incubated in stripping buffer (2% SDS, 62.5 mM Tris, pH 6.7, 0.7% β -mercaptoethanol) at 50°C for 30 min to remove bound antibodies. After two washes of 15 min each in TBS (10 mM Tris, pH 7.4, 150 mM NaCl), the membrane was re-blocked with 5% skim milk in TBST at 4°C overnight, then anti-phospho-MAP kinase monoclonal antibody (Sigma) diluted 1:1000 in TBST

was used to further determine the phosphorylation of ERK1+2 using the same processes as above except that anti-mouse antibody conjugated to peroxidase (Santa Cruz Biotechnology) diluted 1:5000 in TBST was used. Two to six repeated experiments were conducted for different groups.

Statistical analysis

Data were processed by chi-square analysis. Differences at $p < 0.05$ were considered to be statistically significant.

Results

Phosphorylation of p90^{rsk} during oocyte maturation

The phosphorylation of p90^{rsk} during oocyte maturation was judged by mobility shift of immunoblotting bands. GV oocytes collected immediately after removal from the follicles and GVBD oocytes collected at different times of *in vitro* culture were used. Oocytes still arrested at the GV stage 12 h after culture were also collected. As shown in the top panel of Fig. 1, p90^{rsk} was observed as double bands from the GV stage to GVBD (2 h after culture). The lower band may represent a totally non-phosphorylated form of p90^{rsk}, but the upper band was diffuse, indicating a low level of phosphorylation of this kinase during this period. Two hours after GVBD, a slight change in mobility shift of the p90^{rsk} upper band was detected. At this time the MAP kinase was still not phosphorylated. An evident shift of the upper band and disappearance of the lower band were observed 3 h after GVBD. A single sharp, narrow and retarded band was obvious during later stages of oocyte maturation, indicating the full activation of p90^{rsk}. MAP kinase phosphorylation was observed 3 h after GVBD and culminated at 6 h post-GVBD as detected both by mobility shift of ERK1 and ERK2 (Fig. 1, middle panel) and by immunoblots using anti-phospho-MAP kinase antibody (Fig. 1, bottom panel).

Correlation between MAP kinases and p90^{rsk} activation during meiotic maturation

To verify the correlation of p90^{rsk} activation with MAP kinase activation, OA and CHX were used to regulate MAP kinases. When GV-stage oocytes were treated with OA in the absence of IBMX, GVBD occurred in the same percentage as the controls (Table 1), while the phosphorylation of both MAP kinases and p90^{rsk} occurred earlier than in the controls (Fig. 2, lanes 1–3). IBMX inhibited spontaneous GVBD, while OA overcame this inhibitory effect (Table 1). Correspondingly,

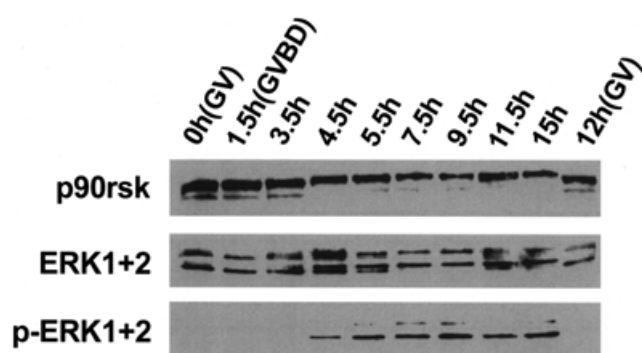


Figure 1 Phosphorylation of p90^{rsk} and ERK1+2 during rat oocyte maturation. Two bands of p90^{rsk} were observed and the upper band was diffuse in oocytes at GV stage and in oocytes that just went through GVBD (1.5 h of culture), implying a slight phosphorylation. By this time, the activation of ERK1+2 did not occur. At 2 h post-GVBD (3.5 h of culture), a slightly retarded mobility shift of p90^{rsk} could be detected in the absence of ERK1+2 activation. ERK1+2 activation occurred 3 h after GVBD (4.5 h of culture), correlated with full phosphorylation of p90^{rsk}. A single narrow retarded band of p90^{rsk} was observed at all following stages of oocyte maturation. Both p90^{rsk} and ERK1+2 were still in a dephosphorylated form (the first lane from the right) in oocytes that failed to undergo GVBD after 12 h of culture.

IBMX inhibited phosphorylation of both MAP kinases and p90^{rsk}, while both were phosphorylated after 6 h of treatment of oocytes with both OA and IBMX (Fig. 2, lanes 4–5). When oocytes were treated with CHX, GVBD occurred at the same time as the controls (Table 1), while the two kinases were not phosphorylated 4 h after GVBD when treated with a high concentration of CHX (Fig. 2, lanes 6–9). These results strongly suggest that p90^{rsk} activation is associated with MAP kinase activation.

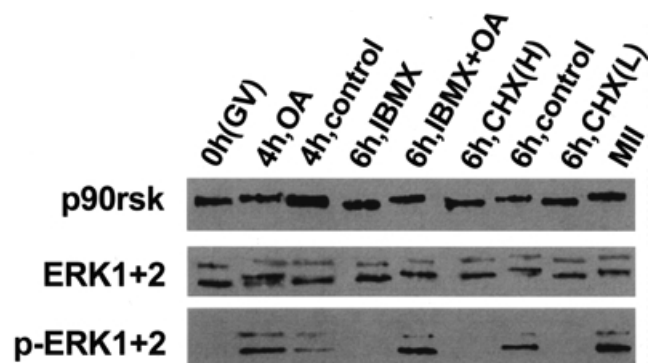


Figure 2 The effects of OA and CHX on the phosphorylation of ERK1+2 and p90^{rsk}. OA stimulated phosphorylation of both ERK1+2 and p90^{rsk}, and overcame the inhibitory effects of IBMX. CHX inhibited the phosphorylation of both ERK1+2 and p90^{rsk}. OA, okadaic acid (2 μ M); IBMX, 3-isobutyl-1-methylxanthine (500 μ M); CHX (H), cycloheximide (355 μ M); CHX (L), cycloheximide (35.5 μ M).

p90^{rsk} and MAP kinase dephosphorylation after parthenogenetic activation of metaphase II oocytes

An electrical stimulus (160 V, 80 μ S) resulted in second polar body emission after 1–2 h and pronucleus (PN) formation after 4–7 h. Oocytes with both PNs and the second polar body and those that underwent cleavage were regarded as activated. The ratio of parthenogenetic activation was 84%. Oocytes with extruded second polar bodies were used for analysis from 2 h of culture. Fig. 3 showed that active MAP kinases began to be dephosphorylated after 2 h and significantly dephosphorylated after 3 h as detected both by mobility shift of ERK1 and ERK2 and by immunoblotting using anti-phospho-MAP kinase antibody. Correspondingly, dephosphorylation of p90^{rsk} began 3 h after stimulation and further dephosphorylation continued for another 3 h.

Table 1 Meiotic resumption of rat oocytes after various treatments

Treatments	GVBD% (GVBD oocytes/total oocytes)			
	2h	4h	6h	24h
Control	76.2 (48/63) ^a	83.6 (51/61) ^a	–	95.6 (43/45) ^a
2 μ M OA	80.5 (70/87) ^a	84.3 (70/83) ^a	–	92.3 (36/39) ^a
500 μ M IBMX	3.6 (3/84) ^b	–	11.1 (8/72) ^b	46.7 (35/75) ^b
500 μ M IBMX + 2 μ M OA	75.3 (70/93) ^a	–	76.7 (66/86) ^a	96.2 (77/80) ^a
Control	72.3 (94/130) ^a	–	87.1 (81/93) ^a	96.5 (111/115) ^a
35.5 μ M CHX	70.0 (56/80) ^a	–	82.1(46/56) ^a	92.0 (46/50) ^a

The GV-stage rat oocytes were cultured in DMEM/BSA with drugs as described in the Table and in Materials and Methods. The percentages of oocytes that went through GVBD were calculated at the indicated times. GVBD, germinal vesicle breakdown; OA, okadaic acid; IBMX, 3-isobutyl-1-methylxanthine; CHX, cycloheximide. Different superscripts in the same column indicate $p < 0.01$.

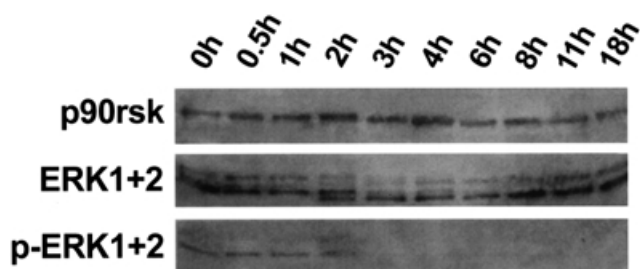


Figure 3 Dephosphorylation of MAP kinase and p90^{rsk} after parthenogenetic activation of rat oocytes by an electrical stimulus. Dephosphorylation of ERK1+2 began to occur at 2 h and became significant at 3 h post-activation. Dephosphorylation of p90^{rsk} began after 3 h, but the dephosphorylation period of p90^{rsk} was longer than that of ERK1+2.

Correlation of cell cycle progression with phosphorylation of p90^{rsk} and MAP kinases after parthenogenetic activation

Both ERK1+2 and p90^{rsk} were dephosphorylated in oocytes with second polar bodies and PNs after 14 h of activation. In contrast, the dephosphorylation of ERK1+2 and p90^{rsk} was not detected in oocytes where no PN formed after the extrusion of second polar bodies 14 or 26 h after activation. When OA was used, PN formation was completely inhibited, and ERK1+2 and p90^{rsk} remained in a phosphorylated form 8 h after parthenogenetic activation (Fig. 4). Phosphorylation of neither p90^{rsk} nor MAP kinases was detected in cleaved parthenogenetic embryos (Fig. 4).

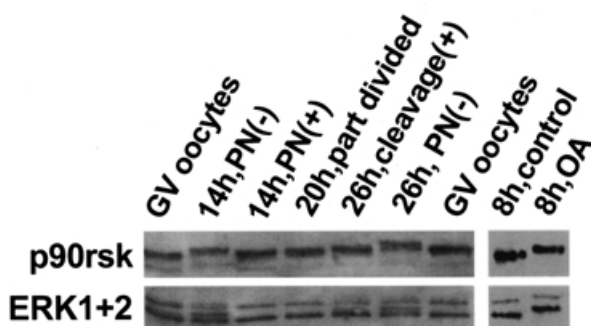


Figure 4 The relationship between pronucleus (PN) formation, cell division and the phosphorylation of MAP kinase and p90^{rsk}. GV oocytes were used as a non-phosphorylated control. In eggs where the PN formed and in cleaved embryos ERK1+2 and p90^{rsk} were dephosphorylated, while dephosphorylation was not detected in eggs where second polar bodies were extruded but PN formation failed. OA abolished both PN formation and protein kinase dephosphorylation in electrically stimulated eggs. OA, okadaic acid (2 μ M).

Discussion

In this study we examined the activity changes of ERK1+2 and p90^{rsk} during meiotic maturation and parthenogenetic activation of rat oocytes by evaluating phosphorylation/dephosphorylation. The phosphorylation of p90^{rsk} was determined by mobility shift on SDS-PAGE, and the phosphorylation of ERK1+2 was shown by both antibody specific to phosphorylated ERK1+2 and their mobility shift on SDS-PAGE. We showed two-phase activation of p90^{rsk} during oocyte maturation: MAP kinase-independent partial activation within 2 h post-GVBD and MAP kinase-correlated full activation at 3 h post-GVBD. A series of experiments provided further evidence for the involvement of MAP kinase in p90^{rsk} phosphorylation/dephosphorylation during rat oocyte maturation and activation.

p90^{rsk} is unusual in that its single polypeptide contains two distinct protein kinase domains (Jones *et al.*, 1988). The N-terminal kinase domain (NTD) is necessary for the phosphorylation of peptide substrates, whereas the C-terminal kinase domain (CTD) is required for activation of the N-terminal domain (Dalby *et al.*, 1998; Poteet-Smith *et al.*, 1999). Both domains contribute to autophosphorylation of the molecule (Bjorbaek *et al.*, 1995). Activation of p90^{rsk} occurs sequentially by a series of phosphorylations of serine or threonine sites along the molecule (Frodin & Gammeltoft, 1999), which may involve different kinases and autophosphorylation activity of p90^{rsk}. The diffuse upper bands of p90^{rsk} at GV stage and immediately following GVBD as shown in this study indicated that a slight phosphorylation took place at this stage. This is in agreement with the work conducted by Dalby *et al.* (1998), which identified two sites (Ser222 and Ser733) in the molecule that are partially phosphorylated in unstimulated cells when the NTD and CTD are inactivated (Dalby *et al.*, 1998). The kinase(s) responsible for this phosphorylation remains to be identified. Two hours after GVBD, the mobility shift of p90^{rsk} became slightly retarded compared with that at GV and immediate GVBD stages, indicating that new phosphorylation took place in addition to the existing phosphorylation. In contrast, ERK1+2 had not been activated by this time. These results were similar to those obtained in mice (Kalab *et al.*, 1996). Thus, the early partial activation of p90^{rsk} is independent of MAP kinases, but other kinase(s) may be involved. The unknown kinase may directly or indirectly be activated by p34cdc2/cyclin B kinase because this kinase is activated just at the time of GVBD (Zernicka-Goetz *et al.*, 1997). Some studies indicate that PDK1, a newly identified Ser/Thr kinase, can partially activate p90^{rsk} in somatic cells (Richards *et al.*, 1999; Jensen *et al.*, 1999; Williams *et al.*, 2000). Whether this kinase has any

relevance to the activation of p90^{rsk} during oocyte maturation needs further investigation.

ERK1+2 were not activated in oocytes in which GVBD had not occurred, either in the oocytes having no ability to enter meiotic division spontaneously *in vitro* or in the oocytes being incubated in the presence of IBMX, a drug that maintains an elevated level of cAMP by inhibiting phosphodiesterase of oocytes and cumulus cells (Mitra & Schultz, 1996). p90^{rsk} was also not phosphorylated and activated in oocytes in which GVBD did not occur. Significant phosphorylation of p90^{rsk} occurred 3 h after GVBD when ERK1+2 were activated. These results suggest that MAP kinases may be the p90^{rsk} kinase. Our experiment showed that the full phosphorylation of p90^{rsk} and activation of MAP kinase were inhibited by CHX, a protein synthesis inhibitor, after 6 h of culture. CHX may inhibit protein synthesis that is required for the activation of ERK1+2, while it has no influence on GVBD and less influence on the activation of MPF in mouse oocytes (Gavin & Schorderet-Slatkine, 1997). OA, an inhibitor of phosphatase 1 and 2A, was able to induce the activation of both ERK1+2 and p90^{rsk} in the presence of IBMX or accelerate the activation of the two kinases in IBMX-free medium, while p34cdc2/cyclin B kinase was not significantly influenced by the treatment (Gavin *et al.*, 1994; Oh *et al.*, 1998). These results imply that cAMP inhibits GVBD, activation of ERK1+2 and p90^{rsk}, but not activation of p34cdc2/cyclin B kinase, and this effect may be mediated by OA-sensitive phosphatases in rat oocyte maturation. Another possibility is that phosphorylation of ERK1+2 and p90^{rsk} reflects a balance between the kinases and phosphatases acting upon ERK1+2 and p90^{rsk}. The use of OA destroys the balance by inhibiting phosphatases, resulting in the activation of the two kinases. The activation of p90^{rsk} was either a function of active ERK1+2 activated by OA or the direct effect of OA on the phosphatase(s) specific to p90^{rsk}. Further, more detailed experiments are needed to distinguish the two possibilities. In any case, the time of full activation of p90^{rsk} is highly coincident with that of ERK1+2 activation.

ERK1+2 began to be dephosphorylated at about 2 h after parthenogenetic activation, and p90^{rsk} dephosphorylation began after the dephosphorylation of ERK1+2 was completed. This implies that the activity of p90^{rsk} was mainly maintained by phosphorylation of ERK1+2 on p90^{rsk} sites. A proportion of ERK1+2 molecules were still in active form 2 h after activation. This amount of active ERK1+2 was sufficient to maintain the phosphorylation of p90^{rsk}. The inactivation period of p90^{rsk}, which lasted to 6 h after activation, was longer than that of ERKs. The autophosphorylation activity (Dalby *et al.*, 1998) of p90^{rsk} may also take effect during this period. Pronuclei formed after the inactivation of ERKs; absence of ERK inactivation resulted in

failure to form pronuclei and to inactivate p90^{rsk} in these oocytes up to 26 h after parthenogenetic activation. These results also confirm that activity of p90^{rsk} is maintained by the phosphorylation effect of ERKs and is in agreement with the hypothesis that inactivation of ERKs is a prerequisite for the formation of pronuclei. When OA was used to depress the action of phosphatases 1 and 2A, the activity of both ERKs and p90^{rsk} were retained and the formation of pronuclei was inhibited.

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