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Phosphorylation of MAP kinase and p90^{rsk} and its regulation during *in vitro* maturation of cumulus-enclosed rabbit oocytes

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Summary

Numerous studies have demonstrated that activation of the mitogen-activated protein (MAP) kinase is involved in the maturation of oocytes. In this study, the expression and phosphorylation of MAP kinase and p90^{rsk}, one of the substrates of MAP kinase, during rabbit oocyte maturation were studied. The results showed that MAP kinase phosphorylation began to occur after germinal vesicle breakdown (GVBD) and the active form was maintained until metaphase II. p90^{rsk} was also activated after GVBD following MAP kinase activation. Immunofluorescent analysis showed that p90^{rsk} was enriched in the nuclear area after GVBD and was gradually localised to the spindle. When GVBD was inhibited by increased cAMP or decreased protein kinase C activity, the phosphorylation of both MAP kinase and p90^{rsk} was blocked. Our data suggest that (1) MAP kinase/p90^{rsk} activation is not necessary for GVBD, but plays an important role in the post-GVBD events including spindle assembly in rabbit oocytes; and (2) MAP kinase/p90^{rsk} activation is down-regulated by cAMP and up-regulated by protein kinase C in cumulus-enclosed rabbit oocytes.

Keywords: MAP kinase, Microtubule, Oocyte, p90^{rsk}, Rabbit

Introduction

The meiotic division of oocytes is a protracted process that is naturally arrested at the diplotene stage of the first meiotic prophase, which corresponds to the G_2 phase of the cell cycle. This arrest can be released by liberation of oocytes from their follicular environment. The resumption of meiotic maturation is manifested by the germinal vesicle breakdown (GVBD), followed by chromatin condensation and microtubule reorganisation.

Several protein kinases have been shown to play a role in meiotic resumption. The importance of the role of maturation-promoting factor (MPF) in mammalian oocyte maturation is well defined, while the mitogen-activated protein (MAP) kinase cascade has not been fully studied. MAP kinase, which is also known as extracellular regulated kinase (ERK), belongs to a group of serine/threonine protein kinases that require dual phosphorylation on threonine and tyrosine residues to become fully activated (Nishida & Gotoh, 1993). MAP kinase activation occurs via a complex cascade of kinases including the immediately upstream activator, MAP kinase kinase (MAPKK, MEK), which in turn is phosphorylated by one of the MAP kinase kinase kinase (MAPKKK) (Gotoh & Nishida, 1995; Kosako *et al*., 1994). The MAPKKK/MEK/MAPK cascase is now believed to transmit various extracellular signals to their intracellular targets in eukaryotic cells.

Recent studies showed that MAP kinase activation plays a role in the meiotic maturation of oocytes in mammals. MAP kinase is involved in the induction of GVBD in bovine oocytes (Fissore *et al*., 1996), while other lines of evidence suggest that MAP kinase activation is not required for initiating oocyte maturation, but rather implicated in the post-GVBD events during oocyte maturation in mouse, rat and goat (Verlhac *et al*., 1993, 1994; Araki *et al*., 1996; Dedieu *et al*., 1996;

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Zernika-Goetz *et al*., 1997; Sun *et al*., 1999; Lu *et al*., 2001). MAP kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of MPF activity in bovine oocytes (Gordo *et al*., 2001). The role of MPF in rabbit oocyte maturation has been clarified (Jelinkova *et al*., 1994), but there is still no report about the function of MAP kinase in this species.

In this study, (1) the expression and phosphorylation of MAP kinase and $p90^{rsk}$ during rabbit oocyte maturation were studied; and (2) the cross-talk between cAMP or PKC and MAP kinase was also investigated in cumulus-enclosed rabbit oocytes.

Materials and methods

Oocyte collection and culture

Fully grown GV oocytes were isolated from ovaries of sexually mature female Japanese white rabbits after ovulation induction. After rupturing follicles of 1–3 mm in diameter, cumulus–oocyte complexes (COCs) with intact unexpanded cumulus cells were released in phosphate-buffered saline (PBS) solution containing 3 mg/ml bovine serum albumin (BSA) with hypoxanthine. After rinsing three times, the COCs were kept in the medium under paraffin oil pre-equilibrated at 38 °C in an incubator with 100% humidity and 5 % CO_2 in air. The basic maturation culture medium was TCM199 (Gibco, Grand Island, NY, USA) containing 100 IU/ml penicillin, 100 mg/ml streptomycin and 3 mg/ml BSA.

To determine the kinetics of meiotic progression and the activation of MAP kinase and $p90^{rsk}$ during maturation of rabbit oocytes cultured *in vitro*, oocytes were collected at different times from the start of culture. The surrounding cumulus cells were removed and the nuclear status of oocytes evaluated by acetoorcein staining (Yoshimura *et al*., 1990). In the meantime different groups of samples were collected for immunoblotting. Uncultured GV oocytes were used as controls.

Oocyte treatments

To investigate the regulation of MAP kinase and $p90^{rsk}$ by cAMP or protein kinase C (PKC), oocytes were treated with cAMP or PKC modulators. Stock solutions for IBMX, calphostin C and PMA were prepared with dimethyl sulfoxide (DMSO) and stored frozen at –20 °C. GV-stage oocytes were randomly allocated to culture medium containing IBMX (100 μM), or calphostin C (100 μM, light treatment for 15 min), or IBMX plus PMA (16.2 nM). GVBD was observed and samples for immunoblotting were collected.

Immunoblotting

Immunoblotting was performed as described by Sun *et al.* (1999), with some modifications. To determine the kinetics of MAP kinase activation, 30 cumulus-free oocytes at the appropriate stage of maturation were collected and extracted with double-strength sample buffer (100 mM Tris, 20% glycerol, 200 mM DTT, 4% SDS, 1% β-mercaptoethanol and 0.2% bromophenol blue) and frozen at –20 °C until use. Prior to electrophoresis, the samples were denatured at 100 °C for 4 min, cooled on ice for 4 min and centrifuged at 14000 rpm for 5 min. The proteins were separated by SDS-PAGE with a 4% stacking gel and a 12% separating gel for 3 h at 150 volts, and were electrophoretically transferred onto nitrocellulose membrane for 2.5 h at 200 mA, at 4 °C.

After blocking overnight at 4 °C in 5% skimmed milk in 10 mM Tris, 150 mM NaCl (TBS, pH 7.4) containing 0.1% Tween-20 (TBS-T), the membrane was cut into two for staining with different antibodies (MAP kinase and $p90^{rsk}$). The membrane was incubated for 2 h at 37 °C with anti-active MAPK mouse monoclonal antibody (Promega, Madison, WI, USA) diluted 1:1000 or anti-$p90^{rsk}$ goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:300 in 0.5% skimmed milk in TBS-T. After three washes of 10 min each in TBS-T, the membrane was incubated for 1 h at 37 °C with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000 in TBS-T containing 1% skimmed milk. The membrane was washed three times with TBS-T and then processed using an enhanced chemiluminescence (ECL) detection system.

For reprobing, the blots were stripped of the bound antibodies by washing in a stripping buffer (100 mM Tris, pH 6.7, 12.5 mM β-mercaptoethanol, 2% SDS) for 30 min at 50 °C with occasional agitation. The membrane was then reprobed with anti-ERK2 goat polyclonal antibody by the same procedure described above. Complete stripping of the blots was verified by exposing the membrane to the ECL detection system before immunoblotting with anti-ERK2 antibody. Image processing was conducted by using Photoshop 5.0 software. All the experiments were repeated at least three times.

Immunofluorescent localisation of $p90^{rsk}$

To monitor the dynamics of the subcellular distribution of $p90^{rsk}$, oocytes were collected at different times (GV stage, GVBD, MI) and processed by a method modified from Verlhac *et al.* (1993). Briefly, oocytes were stripped of the zona pellucida using 0.5% pronase, and allowed to recover for 30 min in TCM199 with 3 mg/ml BSA at 37 °C, then fixed in 2% fresh

formaldehyde, 0.1% Triton-X in PBS for 15 min at room temperature, and washed in 0.1% Tween-20 in PBS (PBS-T). After blocking with blocking solution (described above), the oocytes were incubated with anti-p90rsk monoclonal antibody (Santa Cruz, Biotechnology, Santa Cruz, CA) diluted 1:100 in blocking solution for 2 h at 37 °C, washed in PBS-T and incubated with FITC-conjugated sheep anti-mouse IgG (Sigma, St Louis, MO) diluted 1:1000 in blocking solution for 2 h at 37 °C. Chromatin was stained by 5 µg/ml propidium iodide. Stained oocytes were then mounted under a coverslip with anti-fade mounting medium to retard photobleaching, and sealed to glass slides with nail polish, and examined using laser scanning confocal microscopy (Leica TCS-4D, Leica, Heidelberg, Germany).

Statistical analysis

All data on oocyte maturation were evaluated by chi-square analysis. Oocytes that had degenerated or those with degenerating signs as indicated by darkening cytoplasm were not included. Differences at $p < 0.05$ were considered significant.

Results

Meiotic kinetics and phosphorylation of MAP kinase and p90rsk of rabbit oocytes

Oocytes recovered from ovarian follicles resumed meiotic maturation almost synchronously. GVBD was first observed at 2 h (10.1%) after incubation; 65.3% of the oocytes entered the first metaphase at 6 h, and 85.7% extruded the first polar body and remained arrested in metaphase II at 8 h (Table 1).

The dynamic changes in MAP kinase phosphorylation are shown in Fig. 1. MAP kinase was fully expressed in oocytes from GV to MII stages, and there was no difference in its expression, while MAP kinase was slightly phosphorylated at 3 h of culture when half the oocytes underwent GVBD (50.0%). MAP kinase was fully activated at 4 h, when 93.7% of the oocytes underwent GVBD, and its activity remained high throughout MII stage (Fig. 1).

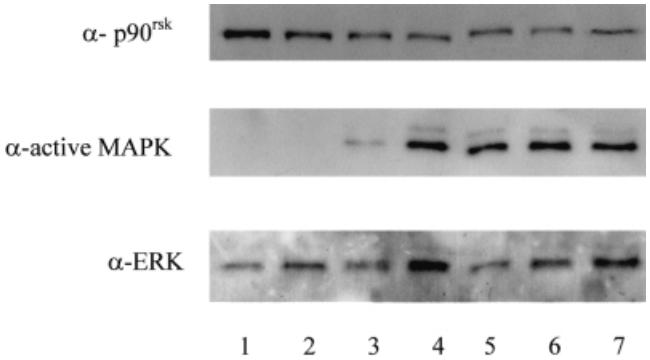

Figure 1 Expression (bottom panel) and phosphorylation (middle panel) of MAP kinase and p90rsk (top panel) during rabbit oocyte maturation *in vitro*. A total of 30 cumulus-free oocytes were loaded into each lane. Phosphorylation and expression of MAP kinase and p90rsk were detected on the same blot. Lane 1, GV oocytes collected before culturing; lanes 2–7, oocytes collected after 2, 3, 4, 8, 12 and 20 h of incubation. This blot is a representation of three similar experiments.

The phosphorylation of p90rsk was also detected. Two different mobility bands were observed. Oocytes at the GV stage had a fast shift band and p90rsk was not phosphoryated at 4 h after culture when MAP kinase was fully phosphorylated and the majority of oocytes had already been through GVBD. A slow shift band appeared after 8 h of culture (Fig. 1, lanes 5–7).

Activation of MAP kinase and p90rsk is regulated by cAMP or PKC

Our results showed that MAP kinase activation and meiosis resumption of rabbit oocytes were blocked by increased intra-oocyte cAMP caused by incubation with IBMX, or treatment with the PKC inhibitor calphostin C (Table 2; Fig. 2, lanes 1, 3). In the mean

Table 1 Kinetics of meiotic progression of rabbit oocytes matured *in vitro*

Time (h)	Total	GV (%)	GVBD (%)	MI (%)	MII (%)	Degeneration (%)
0	135	**131 (97.0)**				4 (3.0)
2	69	**62 (89.9)**	7 (10.1)			
3	52	25 (48.1)	**24 (46.2)**	2 (3.8)		1 (1.9)
4	95	4 (4.2)	**62 (65.3)**	**27 (28.4)**		2 (2.1)
6	63	1 (1.6)	20 (31.7)	**41 (65.3)**	1 (1.6)	
8	49	2 (4.1)		3 (6.1)	**42 (85.7)**	2 (4.1)
22	292	4 (1.4)		7 (2.4)	**266 (91.1)**	15 (5.1)

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Table 2 The effect of PKC or cAMP modulators on germinal vesicle breakdown

Treatment	4 h (GV%)	8 h (GV%)
100 μM IBMX	40/45 (88.9)[a]	28/35 (80.0)[a]
100 μM calphostin C	15/17 (88.2)[a]	10/21 (47.6)[b]
100 μM IBMX + 16.2 nM PMA	16/30 (53.3)[b]	6/31 (19.4)[c]
Control	5/42 (11.9)[c]	0/37 (0)[d]

Groups with different superscripts in the same column, $p < 0.05$.

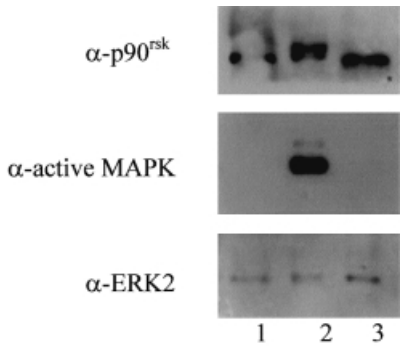

Figure 2 Regulation of MAP kinase and p90[rsk] phosphorylation by cAMP and PKC modulators. Lane 1, oocytes cultured with 100 μM IBMX for 8 h; lane 2, oocytes cultured with 100 μM IBMX + 16.2 μM PMA for 8 h; lane 3, oocytes cultured with 100 μM calphostin C for 8 h.

time, the activation of p90[rsk] was also inhibited. When oocytes were incubated with IBMX plus PMA, an activator of PKC, the inhibitory effect of increased intra-oocyte cAMP on GVBD, MAP kinase and p90[rsk] phosphorylation were partly overcome (Table 2; Fig. 2, lane 2).

Localisation of p90[rsk] during oocyte maturation

Immunofluorescent microscopy showed that p90[rsk] diffusely distributed in the cytoplasm at the GV stage, enriched in the nuclear area after GVBD and was localised to the spindle later on (Fig. 3).

Discussion

The meiotic cell cycle is mainly controlled by a protein phosphorylation/dephosphorylation regulatory cascade (Maller *et al*., 1977; Kastrop *et al*., 1990). Among the kinases involved in oocyte maturation, maturation (or M-phase) promoting factor (MPF) (Masui & Market, 1971) has been found to be a universal cell cycle regulator of both mitosis and meiosis. MPF activation is correlated with a G_2/M transition (review by Eppig *et al*., 1996). The activation of another protein kinase, MAP kinase, is also correlated with GVBD in bovine oocytes (Fissore *et al*., 1996). However, other lines of evidence suggest that MAP kinase activation is not required for initiating oocyte meiosis resumption, but rather implicated in the post-GVBD events during oocyte maturation in mouse, rat and goat (Verlhac *et al*., 1994; Araki *et al*., 1996; Dedieu *et al*., 1996; Zernika-Goetz *et al*., 1997; Sun *et al*., 1999; Lu *et al*., 2001). p90[rsk] phosphorylates the p34[cdc2] inhibitory kinase Myt1, decreasing its activity, resulting in progression of oocytes through the G_2/M phase of meiosis (Palmer *et al*., 1998). Our results indicate that MAP kinase and p90[rsk] phosphorylation occur after GVBD during rabbit oocyte meiotic maturation. Verlhac (1994) reported that microtubule and chromatin behaviour follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Our previous studies showed that spindle assembly was temporarily correlated with MAP kinase phosphorylation in pig oocytes (Sun *et al*., 2001). In the present study, we found that MAP kinase/p90[rsk] activation was temporally correlated with spindle assembly. Immunofluorescent analysis showed p90[rsk] was enriched in the nuclear area and was localised to the spindle, suggesting a role of the MAP kinase cascade in spindle assembly. In *Xenopus* oocyte maturation, kinetochore attachment/spindle assembly checkpoint protein is downstream of 90[rsk] (Frank-Vaillant *et al*., 2001). It could be concluded that MAP kinase/p90[rsk] activation is not required for GVBD, but participates in post-GVBD events, for example spindle assembly, in rabbit oocytes.

Rsks, also referred to as MAPKAP kinases-1, are a family of 85–90 kDa proteins that are widely expressed in higher eukaryotes (Frodin *et al*., 1999). Mammals have at least three isoforms – Rsk-1, Rsk-2 and Risk-3 – that are all specifically activated through phosphorylation by MAP kinase. It has been proposed that Rsks are important intermediates that connect MAP kinase with the transcriptional activation of key regulatory genes (Nebreda & Gavin, 1999). Two additional reports demonstrate that Rsks are also involved in MAPK-mediated arrest in the metaphase II stage of meiosis (Gross *et al*., 1999; Bhatt & Ferrell, 1999). MAP kinase has been shown to be the major kinase that is able to phosphorylate p90[rsk] on multiple serine and threonine sites in maturing *Xenopus* oocytes (Grove *et al*., 1993). We and others previously reported that initial activation of p90[rsk] is independent of MAP kinase, but full activation of p90[rsk] needs MAP kinase phosphorylation in mouse and rat oocytes (Kalab *et al*., 1996; Tan *et al*., 2001). The present study showed that phosphorylation of MAP kinase is followed by p90[rsk] phosphorylation, and that inhibition of MAP kinase phosphorylation also blocked p90[rsk] phosphorylation. Thus, we suggest that p90[rsk] is downstream of MAP kinase in rabbit oocytes.

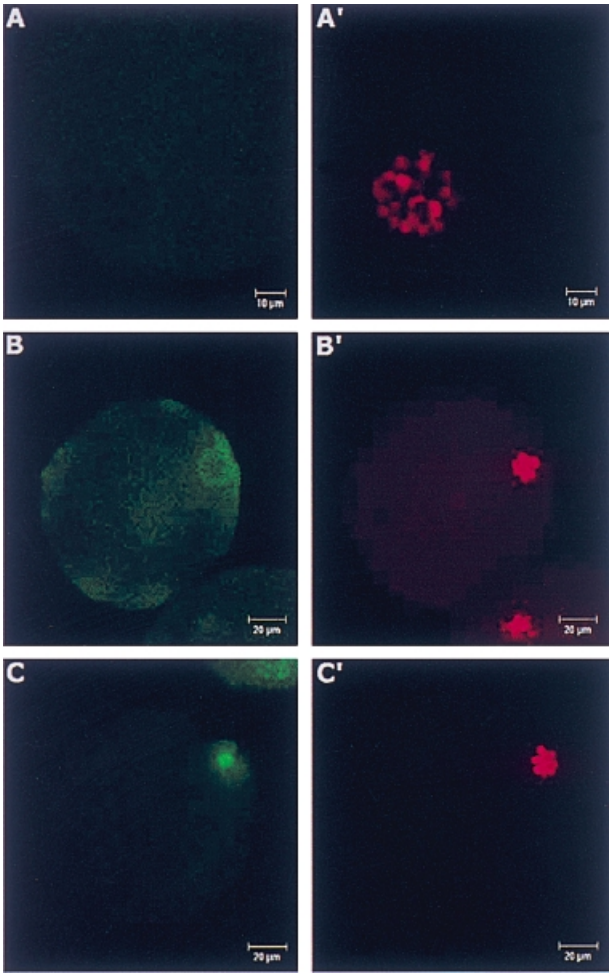

Figure 3 Localisation of p90^{rsk} in rabbit oocytes matured *in vitro*. Oocytes were double-stained for indirect immunofluorescent microscopy with anti-p90^{rsk} polyclonal antibody (*A*, *B*, *C*) and with PI (*A'*, *B'*, *C'*). Each pair of photographs (*A*–*A'*, *B*–*B'*, *C*–*C'*) was taken in the same oocyte. *A*–*A'*, oocyte at the GV stage; *B*–*B'*, oocyte cultured for 4 h, immediately after GVBD; *C*–*C'*, oocyte cultured for 8 h.

We previously reported that MAP kinase phosphorylation was down-regulated by cAMP or PKC during *in vitro* maturation of mouse and rat cumulus-free oocytes (Sun *et al*., 1999; Lu *et al*., 2001). The present study has shown that increased cAMP and inhibition of PKC blocked GVBD. In the meantime, the activation of MAP kinase and p90^{rsk} was also inhibited. When oocytes were incubated with both IBMX and PMA, an activator of PKC, the inhibitory effect of increased intra-oocyte cAMP on GVBD and MAP kinase/p90^{rsk} activation was partly overcome. These results suggest that MAP kinase/p90^{rsk} is down-regulated by cAMP but up-regulated by PKC during *in vitro* maturation of cumulus-enclosed rabbit oocytes.

In conclusion, MAP kinase/p90^{rsk} activation is not necessary for GVBD but is required for post-GVBD events including spindle assembly in rabbit oocytes. Activation of MAP kinase/p90^{rsk} is down-regulated by cAMP, but is up-regulated by PKC during *in vitro* maturation of cumulus-enclosed rabbit oocytes.

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