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Activation of ribosomal S6 kinase (RSK) during porcine oocyte maturation

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Summary

The normal kinetics of ribosomal S6 kinase (RSK) during the meiotic maturation of porcine oocytes were examined. The phosphorylation states of RSK and extracellular signal-regulated kinase (ERK), major mitogen-activated protein (MAP) kinases in maturating porcine oocytes, were detected by Western blotting analysis. The S6 protein kinase activity was assayed using a specific substrate peptide which contained the major phosphorylation sites of S6 kinase. Full phosphorylation of RSK was correlated with ERK phosphorylation and was observed before germinal vesicle breakdown. S6 kinase activity was low in both freshly isolated and 20 h cultured oocytes. S6 kinase activity was significantly elevated in matured oocytes to a level about 6 times higher than that in freshly isolated oocytes. Furthermore, full phosphorylation of RSK was inhibited when oocytes were treated with U0126, a specific MAP kinase kinase inhibitor, in dose-dependent manner, indicating that RSK is one of the substrates of MAP kinase. These results suggest that the activation of RSK is involved in the regulation of meiotic maturation of porcine oocytes.

Keywords: Oocyte maturation, Porcine oocyte, RSK, S6 kinase

Introduction

p90 ribosomal S6 kinase (RSK), one of the substrates of mitogen-activated protein (MAP) kinase, was first discovered in *Xenopus* oocytes as intracellular kinase activity that phosphorylated the 40 S ribosomal subunit protein S6 (Erikson & Maller, 1985; Erikson, 1991; Blenis, 1993). Homologues of RSK have been substantially cloned from many species including mouse, chicken and human (Alcorta *et al*., 1989; Moller *et al*., 1994). In mitotic cells, RSK acts as a regulator of gene expression via association and phosphorylation of various transcriptional regulators including c-Fos, estrogen receptor, IκBα/NFκB, cAMP-response element-binding protein (CREB), and CREB-binding protein (reviewed in Frodin & Gammeltoft, 1999).

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During the meiotic maturation of oocytes, the roles of RSK as a mediator of MAP kinase activity, such as inducing germinal vesicle breakdown (GVBD) (Palmer *et al*., 1998), mediating cytostatic factor (CSF) activity (Bhatt & Ferrell, 1999; Gross *et al*., 1999), suppressing entry into S phase after the first meiosis, and regulating the anaphase promoting complex (APC) (Gross *et al*., 2000), have been reported in *Xenopus laevis*. In *Xenopus* oocyte maturation, phosphorylation and activation of RSK have been shown in several studies (Erikson & Maller, 1989; Bhatt *et al*., 1999). In mammalian species, however, a study during meiotic reinitiation of mouse oocytes has been the only report to show phosphorylation and activation of RSK around GVBD (Gavin & Schorderet-Slatkine, 1997). No studies have attempted to elucidate in detail the phosphorylation and activation of RSK throughout the maturation period in mammalian species. Furthermore, whether MAP kinase participates in the activation of RSK during mammalian oocyte maturation has never been studied.

The present study, therefore, was designed to elucidate in detail the normal kinetics of RSK during the meiotic maturation of porcine oocytes. Our results

showed that the full phosphorylation of RSK correlated with MAP kinase phosphorylation and S6 protein kinase activity rose significantly during porcine oocyte maturation. Moreover, we showed that full phosphorylation of RSK was inhibited when the oocytes were treated with a specific inhibitor of MAP kinase activation.

Materials and methods

Collection and culture of porcine oocytes

Porcine ovaries were obtained from a local abattoir and transported to the laboratory in saline at 37–39 ºC. Follicles ranging in size from 2 to 5 mm in diameter were punctured and cumulus–oocyte complexes (COCs) with intact unexpanded cumulus cells were collected. Each of 20–25 COCs was cultured in 0.1 ml of culture medium consisting of modified Krebs-Ringer bicarbonate solution (Toyoda *et al*., 1971) containing 20% porcine follicular fluid, 1.0 IU/ml pregnant mare's serum gonadotropin (Pramex, Sankyo, Tokyo, Japan) and 3.2 mg/ml bovine serum albumin (Sigma, St Louis, MO) at 37 ºC in an incubator with 100% humidity and 5% CO_2 in air as previously reported (Sugiura *et al*., 1999; Naito *et al*., 1988). After culturing for up to 50 h, the COCs were treated with 150 IU/ml hyaluronidase (Sigma) in culture medium for a few minutes at room temperature, and the surrounding cumulus cells removed by pipetting gently with a fine-bore pipette.

Western blotting

Twenty oocytes were put in 2 µl of saline supplemented with 0.1% polyvinylpyrrolidone (PVP), added to 0.5 µl of 5× Laemmli buffer (Laemmli, 1970) and denatured at 100 ºC for 5 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed using modified 10% polyacrylamide gel composed of 375 mM Tris-HCl (Sigma), 0.1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Wako, Japan), 10% acrylamide (Nacalai Tesque, Japan), 0.13% Bis (Wako), 0.025% ammonium persulphate (Wako) and 0.1% SDS (Wako). After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and detected with anti-RSK1 polyclonal antibody (C-21, Santa Cruz Biotechnology, Santa Cruz, CA) for detection of RSK, and with anti-MAP kinase polyclonal antibody (K-23, Santa Cruz Biotechnology) for detection of MAP kinase using a blotting detection kit in which the streptavidin–alkaline phosphatase conjugate was used as the signal-generating system (Amersham International).

Alkaline phosphatase treatment

Alkaline phosphatase treatment was performed as described previously (Naito *et al*., 1995). One hundred oocytes were put in 0.4 µl of saline supplemented with 0.1% PVP, added to 0.1 µl of 5× Laemmli buffer and denatured at 100 ºC for 5 min. The denatured extract was added to 4.5 µl of alkaline phosphatase buffer (Choi *et al*., 1991) and 0.5 µl of alkaline phosphatase (Sigma). The mixture was then incubated at 37 ºC for 16 h. After incubation, the extract was added to 1.25 µl of 5× Laemmli buffer and denatured at 100 ºC for 5 min. Western blotting was performed as described above.

Kinase assays

Ten oocytes were lysed in 2.5 µl assay buffer (pH 7.2) composed of 15 mM EGTA (Wako, Japan), 1% Nonidet p-40 (Nacalai Tesque), 60 mM sodium β-glycerophosphate (Sigma), 30 mM *p*-nitrophenylphosphate (Wako), 25 mM Mops (Wako), 15 mM $MgCl_2$ (Wako), 0.2 mM Na_3VO_4 (Wako), 1 mM dithiothreitol (Wako), 2 µg/ml leupeptin (Sigma), 2 µg/ml aprotinin (Sigma), 1 µg/ml pepstatin (Sigma), 1 mM phenylmethylsulphonylfluoride (Sigma) and 50 µM *p*-aminobenzoic acid (Sigma), and frozen at –80 ºC until used. Kinase assays were performed using an S6 kinase assay kit (Upstate Biotechnology, Lake Placid, NY). The lysate (2.5 µl) was added to 2.5 µl of assay dilution buffer (ADB) (composed of 20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM dithiothreitol), 2.5 µl of 250 µM substrate peptide (AKRRRLSSLRA) in ADB, 2.5 µl of inhibitor cocktail (composed of 20 µM PKC inhibitor peptide, 2 µM protein kinase A inhibitor peptide (PKI) and 20 µM Compound R24571 in ADB), 2.25 µl of magnesium/ATP cocktail (composed of 75 mM magnesium chloride and 500 µM ATP in ADB) and 0.25 µl of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham). The reaction was performed at 30 ºC for 1 h. The lysate (12.5 µl) was then transferred to P81 phosphocellulose paper and washed three times for 5 min with 0.75% phosphoric acid. After the paper had dried, the radioactivity was measured using a liquid scintillation counter.

U0126 treatment

Ten micromolar U0126 (Promega, Madison, WI) stock, dissolved in dimethylsulphoxide (DMSO), was added to culture medium to final concentrations of 10 or 20 µM. Oocytes were cultured in the U0126 supplemented culture medium for 50 h, and then subjected to Western blotting analysis. The control oocytes were treated with DMSO alone.

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Statistical analysis

Student's *t*-test was used for evaluation of the results. Probability of $p < 0.05$ was considered to be statistically significant.

Results

In our maturation culture of porcine oocytes *in vitro*, GVBD took place from 25 to 30 h of culture (Fig. 1*A*), most of the oocytes were in the first meiotic metaphase after 30 h of culture, and then reached the second meiotic metaphase by 45 h of culture (data not shown). As shown in Fig. 1*B* (upper panel), four bands of RSK, which showed different mobility in electrophoresis, were detected with Western blotting analysis. In the present report, these bands are referred to (from upper to lower) as *a*, *b*, *c* and *d*, respectively, as indicated in the upper panel of Fig. 1*B*. When normal goat serum was used instead of anti-RSK antibody none of the bands were detected (data not shown). Up to 10 h of culture, only the two RSK bands *c* and *d* were detected, and another low-mobility band *b* was added at 15–20 h of culture. The lowest-mobility band *a* was detected from 25 h to 50 h of culture and the two high-mobility bands *c* and *d* had disappeared by this time (Fig. 1*B*, upper panel). Western blotting analysis for extracellular signal-regulated kinases (ERKs) (Fig. 1*B*, lower panel), major MAP kinases in maturing mammalian oocytes, showed that lower-mobility bands (arrowheads *e* and *g*), which indicate activation, can be detected from 25 h of culture as reported previously (Sobajima *et al.*, 1993; Inoue *et al.*, 1995). When the extracts were treated with alkaline phosphatase, band *c* in freshly isolated oocytes became weak and band *a* in the oocytes cultured for 50 h had disappeared, whereas a strong band *d* was detected (Fig. 1*C*, upper panel). These findings indicate that RSK is phosphorylated

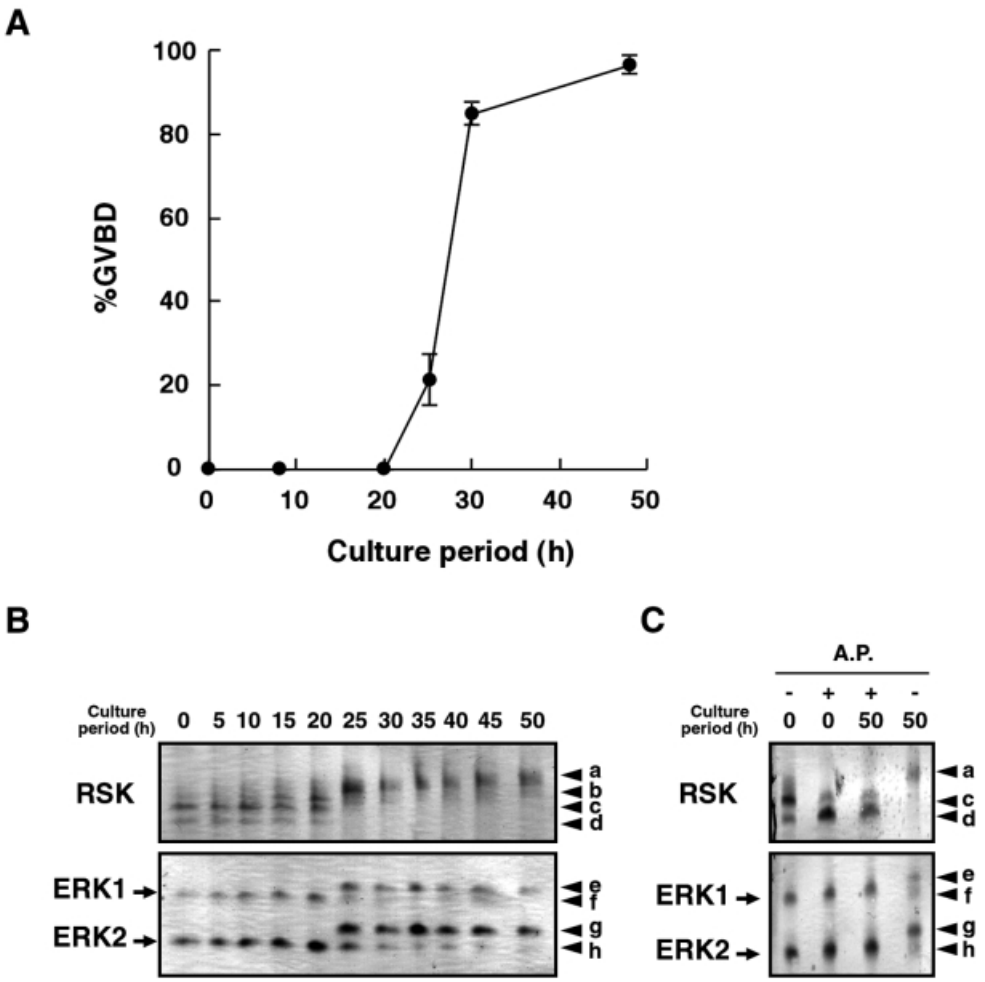

Figure 1 Relation of GVBD and phosphorylation states of RSK and ERK during porcine oocyte maturation. (*A*) The ratio of GVBD (mean ± SEM) during maturation culture of porcine oocyte *in vitro*. (*B*) Detection of RSK and ERK by Western blotting analysis. The oocytes were collected at each culture period, and subjected to Western blotting analysis with either anti-RSK antibody (upper panel) or anti-MAP kinase antibody (lower panel). Some of the oocytes were treated with alkaline phosphatase (A.P.) before being subjected to Western blotting analysis (*C*).

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during porcine oocyte maturation. The lower-mobility bands of ERKs were also shifted down by the treatment with alkaline phosphatase (Fig. 1*C*, lower panel), as reported previously (Inoue *et al.*, 1998).

To elucidate whether these phosphorylations accompanied activation of RSK, S6 protein kinase assays were performed using a specific substrate peptide which contained the major phosphorylation sites of S6 kinase. In experiments using 50 h cultured porcine oocytes, the phosphorylation rate of the substrate peptide was almost constant up to at least 120 min (Fig. 2*A*). Therefore, an assay period of 60 min was chosen. S6 kinase activity was low in oocytes which had bands *c* and *d* (freshly isolated), and activity was not significantly elevated in oocytes which had bands *b*, *c* and *d* (20 h cultured) (Fig. 2*B*). In contrast, S6 kinase activity was significantly elevated in those oocytes which had only band *a* (50 h cultured). The activity in the oocytes with only band *a* was about 6 times higher than that of the oocytes with bands *c* and *d* ($p < 0.005$) (Fig. 2*B*). These data indicate that RSK is activated only when it is fully phosphorylated.

To elucidate whether MAP kinase participates also in the activation of RSK in porcine oocyte maturation, as reported in *Xenopus* oocyte maturation, we used U0126, a specific inhibitor of MAP kinase kinase, to inhibit MAP kinase activation. When oocytes were treated with U0126, bands *e* and *g* in the oocytes cultured for 50 h became weak, and bands *f* and *h* became strong in a dose-dependent manner (Fig. 3, lower panel, lanes 4 and 5). This indicates that MAP kinase activation in porcine oocyte maturation is inhibited incompletely by U0126 treatment as reported previously (Kagii *et al.*, 2000). In these oocytes, the decrease in the fully phosphorylated band *a* of RSK, which represents the active form of RSK, and the increase in bands *c* and *d*, which represent the inactive forms of RSK, were observed in a dose-dependent manner (Fig. 3, upper panel, lanes 4 and 5), indicating that RSK is phosphorylated by MAP kinase during porcine oocyte maturation.

Discussion

The objective of the present study was to elucidate the normal kinetics of RSK during meiotic maturation of porcine oocytes. Our results show that phosphorylation of RSK increases before GVBD, that full phosphorylation correlates with activation of MAP kinase, and that S6 protein kinase activity is significantly higher in mature oocytes than in freshly isolated oocytes. Furthermore, activation RSK was inhibited when the oocytes were treated with a specific inhibitor of MAP kinase activation.

With Western blotting analysis of RSK, two high-mobility bands were detected prior to GVBD. The lowest-mobility bands were then detected around GVBD and remained present until the end of maturation culture. Alkaline phosphatase treatment of lysates

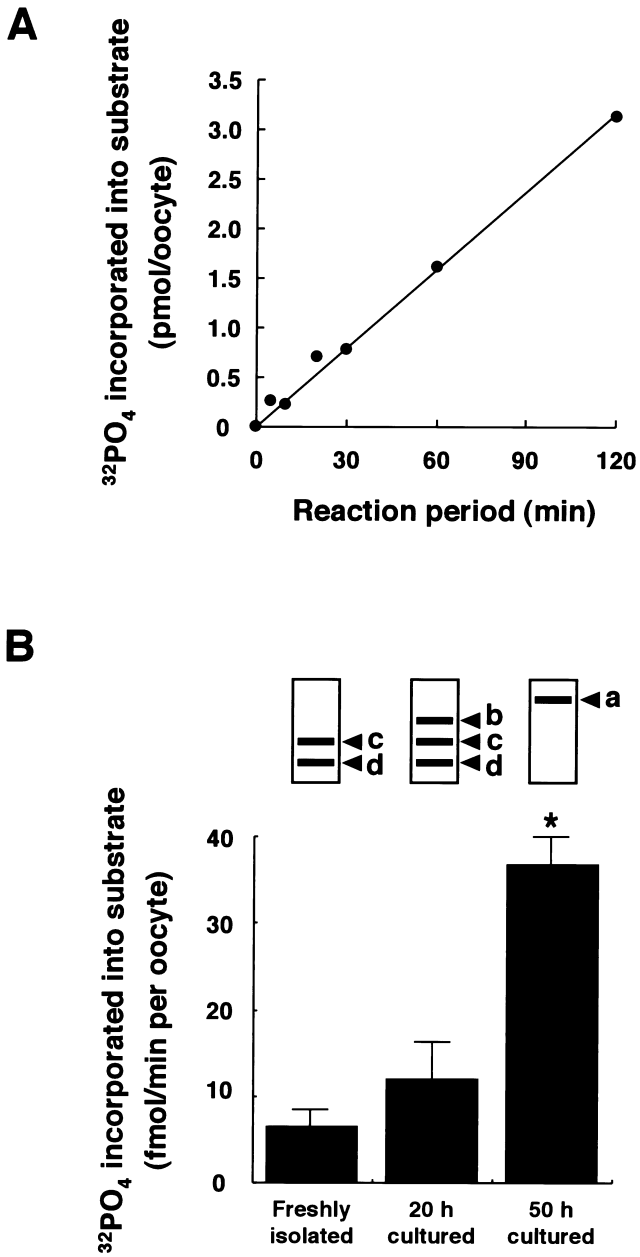

Figure 2 Activation of S6 protein kinase during porcine oocyte maturation. (*A*) Time course of S6 substrate phosphorylation at 30 ºC. Cytosol preparations used were obtained from porcine oocytes cultured for 50 h *in vitro*. (*B*) S6 protein kinase activity in the porcine oocytes. The oocytes with bands *c* and *d* (freshly isolated), bands *b*, *c* and *d* (20 h cultured) and band *a* (50 h cultured) were used for the kinase assay. The RSK band patterns in Western blotting are shown schematically at the top. The value (mean ± SEM) indicated by the asterisk is significantly different from that of freshly isolated oocytes ($p < 0.005$). Experiments were repeated three times.

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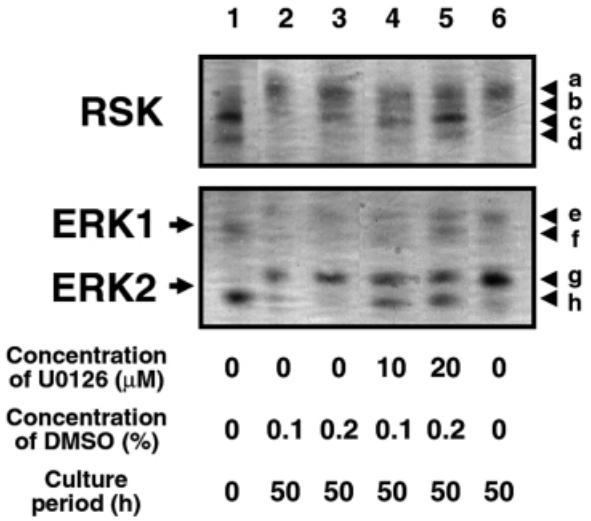

Figure 3 Phosphorylation of RSK by MAP kinase in porcine oocyte maturation. Freshly isolated oocytes were cultured in culture medium supplemented with 10 or 20 μM U0126, a specific inhibitor of MAP kinase activation (lane 4 or 5). After 50 h of culture the oocytes were subjected to Western blotting analysis for RSK (upper panel) and ERK (lower panel). Control oocytes were treated with DMSO alone (lanes 2 and 3).

from either freshly isolated or mature oocytes revealed that these mobility shifts were due to the phosphorylation of RSK. The kinetics of these mobility shifts in Western blotting analysis agreed well with previous reports in *Xenopus* oocyte maturation (Palmer *et al*., 1998; Gavin *et al*., 1999). From 15 h to 20 h of culture another band, described as band *b* in the present report, also became detectable, although its characteristics are unknown at present. The meaning of the appearance of this band, including the phosphorylation states and its relation with MAP kinase activity, is currently under investigation.

The present results show that S6 protein kinase activity is significantly elevated during porcine oocyte maturation in the oocytes with fully phosphorylated RSK, compared with oocytes having non-phosphorylated and partially phosphorylated RSK. There are two major S6 protein kinases *in vivo*: RSK and $p70^{S6K}$ (Banerjee *et al*., 1990). Biochemical and molecular cloning studies have indicated that RSK is the protein kinase responsible for S6 phosphorylation during meiotic maturation of *Xenopus* oocytes (Erikson & Maller, 1985, 1986, 1989). Furthermore, $p70^{S6K}$ was inactive throughout the maturation period of *Xenopus* and mouse oocytes (Gavin & Schorderet-Slatkine, 1997; Schwab *et al*., 1999). These indicate that the present S6 protein kinase activity should be the activity of RSK. The role of the activated RSK during meiosis might be to phosphorylate various substrates, such as glycogen-synthase kinase-3, IκBα/NFκB, estrogen receptor and CREB, which results in changes in the regulation of transcriptional and translational activity as reported in mitotic cells (reviewed in Frodin & Gammeltoft, 1999). We are now attempting to identify the role of RSK in the regulation of mammalian oocyte maturation.

Phosphorylation and activation of RSK during the meiotic reinitiation of oocytes have been previously reported in *Xenopus laevis* (Erikson & Maller, 1989; Bhatt *et al*., 1999). According to these studies, phosphorylation and activation of RSK occurred shortly before GVBD. In the present results, RSK was fully phosphorylated after 25 h of culture, when most of the oocytes were at the GV stage. This indicates that activation of RSK occurred before GVBD in porcine oocytes, as has been reported in *Xenopus* oocytes (Erikson & Maller, 1989). In mouse oocytes, however, activation and phosphorylation of RSK were observed after GVBD (Gavin & Schorderet-Slatkine, 1997). The reason why the timing of RSK activation differs between mouse and porcine oocytes is not clear. RSK is one of the substrates of MAP kinase, and the increase in RSK phosphorylation in parallel with MAP kinase activation has been reported during *Xenopus* and mouse oocyte maturation (Gross *et al*., 2000; Gavin & Schorderet-Slatkine, 1997). The present results with the specific MAP kinase kinase inhibitor, U0126, suggested that this is the case also in porcine oocyte maturation. In mouse oocyte maturation the requirement of MAP kinase activity for GVBD was discounted on the basis of studies on Mos knockout mice, in which oocytes had no MAP kinase activity (Colledge *et al*., 1994; Hashimoto *et al*., 1994). In contrast, MAP kinase activation is a prerequisite for the initiation of *Xenopus* oocyte maturation (Kosako *et al*., 1994; Gotoh *et al*., 1995). In porcine oocytes a relationship between MAP kinase activation and the initiation of oocyte maturation was suggested (Inoue *et al*., 1998; Kagii *et al*., 2000). These differences in the requirements of MAP kinase activity might be one reason for the different timing of MAP kinase activation and the subsequent RSK phosphorylation in mouse, porcine and *Xenopus* oocytes.

In conclusion, the present results showed that the full phosphorylation of RSK correlated with MAP kinase phosphorylation, and that S6 protein kinase activity rose significantly during porcine oocyte maturation. We also showed that full phosphorylation of RSK was inhibited when the oocytes were treated with a specific inhibitor of MAP kinase activation. These results suggest that the activation of RSK is involved in the regulation of meiotic maturation of porcine oocytes.

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