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Degradation of pig cyclin B1 molecules precedes MAP kinase dephosphorylation during fertilisation of the oocytes

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Summary

Pig oocytes at metaphase II were activated by penetration of spermatozoa in cycloheximide-free and cycloheximide-containing fertilisation media. The precise nuclear stage, and the kinetics of degradation of cyclin B1 and dephosphorylation of MAP kinase were assessed after insemination. After maturation culture, 96% of oocytes reached metaphase II. At 6 h after insemination in cycloheximide-free medium, 68% of the oocytes were activated and had progressed to anaphase II or beyond. After 8 h, 89% of the oocytes were activated: a female pronucleus had formed and the heads of penetrating spermatozoa had enlarged and changed to male pronuclei. In the cycloheximide-containing medium, activation of oocytes started earlier than in cycloheximide-free medium. After 4 h, 43% of the oocytes were activated, and the percentage increased to 97% after 6 h. Pig cyclin B1 disappeared in the oocytes at 6 h after insemination in both cycloheximide-containing and cycloheximide-free media. Pig oocytes at metaphase II contained two types of MAP kinase – ERK 1 and ERK 2 – in their active phosphorylated forms. At 8 h after insemination ERK 2 changed to the fast-migrating inactive form in the oocytes cultured in both cycloheximide-containing and cycloheximide-free media, although the shift-down was not complete. The change was delayed by 2 h after the degradation of cyclin B1 molecules. These results demonstrate that degradation of pig cyclin B1 molecules corresponds to the transition of the oocytes from metaphase II arrest to anaphase II/telophase II and was followed by MAP kinase dephosphorylation.

Keywords: Cyclin B1, Fertilisation, MAP kinase, Oocyte, Pig

Introduction

Fully grown mammalian oocytes are arrested at G2 phase in the ovarian follicles. The arrest is maintained until immediately before ovulation, when the oocytes resumes meiosis to enter M phase. The oocytes are arrested again at metaphase II in the meiotic cell cycle until they are released from arrest by sperm penetration or artificial activating stimuli. This second arrest is due to at least two factors (Masui & Markert, 1971):

metaphase-promoting factor (MPF), which is a complex of cyclin B1/B2 and the p34^{cdc2} protein kinase, and cytostatic factor (CSF), which is a product of *c-mos* proto-oncogene. Cyclins were originally identified in embryos of marine invertebrates as proteins displaying a striking periodicity in synthesis and degradation during the cell cycle (Evans *et al.*, 1983; Swenson *et al.*, 1986). B type cyclins are components of the universal mitotic inducer MPF (Draetta *et al.*, 1989; Labbe *et al.*, 1989; Meijer *et al.*, 1989; Gautier *et al.*, 1990), and they act through association with the highly conserved protein kinase p34^{cdc2}, the product of the cell division cycle gene *cdc2* in the fission yeast *Schizosaccharomyces pombe* (reviewed in Nurse, 1990).

MPF-associated protein kinase activity is also referred to as histone H1 kinase, since the enzyme exhibits a pronounced activity to this substrate. During mammalian oocyte maturation, H1 kinase activity increases around germinal vesicle breakdown. The increased activity is maintained during metaphase II and declines sharply after fertilisation or artificial

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activation of oocytes (mouse: Choi *et al.*, 1991; cattle: Collas *et al.*, 1993; pig: Kikuchi *et al.*, 1995a, b). The disappearance of the kinase activity is thought, based on the results in *S. pombe* (Glutzer *et al.*, 1991), to be triggered by associated cyclin B degradation via the ubiquitin pathway. It has been suggested that cyclin degradation by proteolysis is a key step governing exit from metaphase into the next cell cycle in both mitosis and meiosis. However, there are few available data showing the kinetics of degradation of cyclin B during fertilisation, especially in mammalian oocytes.

There is abundant evidence for the participation of mitogen-activated protein kinase (MAP kinase) in meiotic maturation of mammalian oocytes. During maturation, MAP kinase is reported to be activated in mouse (Sobajima *et al.*, 1993; Verlhac *et al.*, 1993; Choi *et al.*, 1996), pig (Inoue *et al.*, 1995), goat (Dedieu *et al.*, 1996), bovine (Fissore *et al.*, 1996) and rat oocytes (Zernicka-Goetz *et al.*, 1997). MAP kinase activity increases around germinal vesicle breakdown and remains high throughout the metaphase I and metaphase II transition. MAP kinase is inactivated after artificial activation (Verlhac *et al.*, 1994) or fertilisation (Moos *et al.*, 1995) of mouse oocytes. The change in MAP kinase activity has been shown by the phosphorylation of its substrate, myelin basic protein, as well as by the mobility change of the MAP kinase molecules in immunoblots.

In the present study, pig oocytes from ovarian follicles were cultured to induce meiotic maturation to metaphase II, and were then activated by penetration of ejaculated boar spermatozoa. The precise nuclear stage, and the kinetics of degradation of cyclin B1 and dephosphorylation of MAP kinase were assessed after insemination. The results demonstrate that degradation of pig cyclin B1 corresponds to the activation of pig oocytes and was followed by MAP kinase dephosphorylation.

Materials and methods

Maturation culture

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. Following three washes in Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA), antral follicles of 4–6 mm diameter were dissected in PBS-PVA. After the follicles were opened in 25 mM Hepes-buffered medium 199 (Earle's salt, Nissui Pharmaceutical, Tokyo, Japan) containing 0.08 mg/ml kanamycin sulphate (Sigma, St Louis, MO), cumulus-oocyte complexes with pieces of parietal granulosa (cumulus-oocyte-granulosa cell complexes) were isolated from the follicles (Mattioli *et al.*, 1988). Following two washes in culture medium,

cumulus-oocyte-granulosa cell complexes were cultured in 2 ml of culture medium, which was bicarbonate-buffered medium 199 supplemented with 10% fetal calf serum (Biocell, CA), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulphate, 0.1 IU/ml hMG (Pergonal, Teikoku Zoki, Tokyo, Japan) and two everted thecal shells. Maturation culture was carried out in an atmosphere of 5% CO₂ in humidified air at 38.5 °C with gentle agitation for 36–42 h.

In vitro fertilisation

After maturation culture, oocytes with an expanding cumulus were transferred into 2 ml of fertilisation medium, which was bicarbonate-buffered medium 199 supplemented with 10% fetal calf serum, 0.1 mg/ml sodium pyruvate, 0.05 mg/ml glucose, 0.9 mg/ml calcium lactate, 0.08 mg/ml kanamycin sulphate and 2 mM caffeine. The oocytes were inseminated with ejaculated boar spermatozoa based on the methods described by Kano *et al.* (1994). Briefly, spermatozoa were washed with centrifugation for 3 min at 500 g in PVS-BVA and were introduced into a fertilisation medium at a concentration of 2×10^6 cells/ml. Then a portion of the sperm suspension was introduced into the fertilisation medium containing *in vitro*-matured oocytes to give a final concentration of 1×10^5 cells/ml, and cultured at 38.5 °C in 5% CO₂ in air for up to 8 h. To block the presumptive continuous synthesis of cyclin B1 and to detect clearly the degradation of cyclin B1, cycloheximide at a concentration of 35 µM was added to the fertilisation medium in some experiments. This concentration of cycloheximide has been reported to reduce the protein synthesis of pig oocytes to 25% of its control value without any effects on sperm penetration or pronuclear development (Ding *et al.*, 1992). As the control, pig oocytes were transferred to the fertilisation medium containing cycloheximide at the same concentration and cultured for 8 h without spermatozoa.

Some oocytes before and 2, 4, 6 and 8 h after insemination were freed from cumulus cells by a narrow-bore pipette and fixed with acetic alcohol (1:3 v/v) to confirm the nuclear stage and sperm penetration. Other oocytes were used for immunoblotting as described below. The fixed oocytes were stained with 1% orcein to examine in whole mount. Oocytes having both unswollen or swollen sperm head(s) and/or male pronucleus(ei) with corresponding detached sperm tail(s), and a female pronucleus in the cytoplasm, were considered to be penetrated. Experiments were repeated three times. Percentages of activated and penetrated oocytes did not differ among replicates. The results were combined and the differences analysed using the chi-square test; *p* values less than 0.05 was considered statistically significant.

Immunoblotting

After insemination, groups of 50 oocytes were denuded, washed three times in PBS-PVA and transferred into Eppendorf tubes in a small volume (about 1 µl) of PBS-PVA. They were dissolved in 15 µl of SDS sample buffer (Laemmli, 1970) by boiling for 3 mins, and frozen at -20 °C before use. Oocytes before maturation culture and oocytes before insemination were also collected and treated in the same manner for the comparison.

For the immunoblotting, rabbit anti-pig cyclin B1 antibody and rabbit anti-MAP kinase antibody were used. The anti-pig cyclin B1 antibody was produced in rabbits against the N-terminal of the molecule (Dai *et al.*, unpublished data). The anti-MAP kinase antibody was purchased from Santa Cruz (#sc-94CA, USA). Samples were run in 10% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes (Hybond-C super, Amersham Life Science, UK). Each membrane was cut into two pieces at the position of the 46 kDa molecular weight marker. Sets of membranes containing proteins with higher and lower molecular weights were blocked with 10% fetal calf serum in PBS containing 0.1% Tween 20 (PBS-Tween) for 2 h, then incubated with anti-cyclin B1 antibody (1:1000) and anti-MAP kinase antibody

(1:500) at room temperature for 4 and 2 h, respectively. After three washes in PBS-Tween, the membranes were treated with horseradish-peroxidase-labelled donkey anti-rabbit immunoglobulins (1:1000, Amersham Life Science) in the blocking buffer for 1 h at room temperature. After three washes with PBS-Tween, peroxidase activity was visualised using the ECL Western blotting detection system (Amersham Life Science).

Results

Sperm penetration and activation of pig oocytes

After maturation culture, 96% of oocytes reached metaphase II (Table 1). At 4 h after insemination one of the oocytes was activated in the cycloheximide-free medium, although no oocytes were penetrated by spermatozoa. After 6 h percentages of activated oocytes and penetrated oocytes increased to 68% and 54%, respectively. Most of the activated oocytes (23/28) were at anaphase II or telophase II, and sperm heads had not transformed to male pronuclei. After 8 h, 89% of the oocytes were activated: a female pronucleus had formed, and heads of penetrating spermatozoa had enlarged and changed to male pronuclei.

Table 1 Activation and sperm penetration of pig oocytes inseminated in cycloheximide-free and cycloheximide-containing media

Insemination ¹	Time after insemination (h)	Addition of cycloheximide ²	No. of oocytes examined ³	No. of matured oocytes	No. (%) of oocytes activated			No. (%) of oocytes penetrated			
					Total	AII-TII ⁴	FPN ⁵	Total	USH ⁶	SSH ⁷	MPN ⁸
	0		27	26 (96)	0 (0)	0 (0)	0 (0)	–	–	–	–
+	2	–	31	28 (90)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
+	4	–	36	34 (94)	1 (3) ^d	1 (3) ^c	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
+	6	–	41	39 (95)	28 (68) ^b	23 (56) ^a	5 (12) ^b	22 (54) ^b	8 (20) ^b	14 (31) ^b	0 (0)
+	8	–	35	34 (97)	31 (89) ^{ab}	5 (14) ^{bc}	26 (74) ^a	30 (86) ^a	5 (14) ^b	13 (32) ^b	12 (29) ^a
+	2	+	32	31 (97)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
+	4	+	37	34 (92)	16 (43) ^c	11 (30) ^b	5 (14) ^b	4 (11) ^c	4 (11) ^b	0 (0)	0 (0)
+	6	+	36	36 (100)	35 (97) ^a	5 (14) ^{bc}	30 (83) ^a	34 (94) ^a	16 (44) ^a	16 (44) ^b	2 (6) ^b
+	8	+	36	36 (100)	36 (100)	0 (0)	36 (100)	35 (97) ^a	2 (6) ^b	28 (78) ^a	5 (14) ^{ab}
–	2	+	31	29 (94)	0 (0)	0 (0)	0 (0)	–	–	–	–
–	4	+	25	23 (92)	2 (8) ^d	0 (0)	2 (8) ^b	–	–	–	–
–	6	+	28	27 (96)	1 (4) ^d	0 (0)	1 (4) ^b	–	–	–	–
–	8	+	23	22 (96)	2 (9) ^d	0 (0)	2 (9) ^b	–	–	–	–

¹ Oocyte–cumulus–granulosa cell complexes cultured for 36–42 h were inseminated with ejaculated boar spermatozoa.

² Cycloheximide was added at a concentration of 35 µM to the fertilisation medium.

³ Each value represents the total number of three replicated experiments.

^{4,5} Oocytes at anaphase II (AII) or telophase II (TII), and oocytes with a female pronucleus (FPN).

^{6–8} Oocytes with an unswollen sperm head(s) (USH), a swollen sperm head(s) (SSH) and a male pronucleus(ei) (MPN).

^{a–d} Values with different superscripts in the same column are significantly different ($p < 0.05$).

In the cycloheximide-containing medium, activation of oocytes started earlier than in cycloheximide-free medium. After 4 h, 43% (16/34) of the oocytes had been activated, of which 11 were at telophase II or anaphase II, and 4 were penetrated by spermatozoa. At 6 h after insemination the percentages of activated oocytes and penetrated oocytes had increased to 97% and 94% respectively. The percentages of activation and penetration were significantly higher in the oocytes in the cycloheximide-containing medium at 4 and 6 h than in the oocytes inseminated in the cycloheximide-free medium. Formation of a female pronucleus was also observed earlier in the oocytes in the cycloheximide-containing medium. After 6 h, 83% of the oocytes had formed a female pronucleus.

When matured oocytes were transferred into cycloheximide-containing fertilisation medium and cultured for 8 h without spermatozoa as the control, a few oocytes (total 5/107) were activated and formed a female pronucleus, although the remainder were at metaphase II.

Degradation of cyclin B1 after insemination of pig oocytes

Anti-pig cyclin B1 antibody recognised three proteins of 66, 64 and 57 kDa in pig oocytes at metaphase II in the immunoblots (Fig. 1). The upper two bands showed no change during meiotic maturation and fertilisation. On the other hand, the lowest (57 kDa) band changed dramatically during meiosis of pig oocytes. According to the estimated molecular weight from the sequencing data of pig cyclin B1 (Dai *et al.*, unpublished data), we determined the 57 kDa band to be pig cyclin B1. Pig cyclin B1 was scarcely observed in the oocytes at germinal vesicle stage before maturation culture (GV in Fig. 1A), and it was accumulated in the

oocytes at metaphase II. The cyclin B1 band disappeared at 6 h after insemination in both cycloheximide-containing and cycloheximide-free media (Fig. 1B, C). This disappearance corresponded to the time when the most of the pig oocytes progressed from metaphase II to anaphase II/teleophase II. In the control oocytes which were cultured in the cycloheximide-containing medium without spermatozoa, cyclin B1 bands were observed throughout the culture period, although the intensity was gradually diminished (Fig. 1A). Such a decrease was also observed in the oocytes inseminated with spermatozoa in the cycloheximide-containing medium at 2 and 4 h after insemination.

Dephosphorylation of MAP kinase after insemination of pig oocytes

In the oocytes at germinal vesicle stage, two proteins of 42 and 44 kDa were detected by anti-MAP kinase antibody, while another set of bands with lower electrophoretic mobility was detected in the oocytes at metaphase II (Fig. 1A). The former 42 and 44 kDa bands are described as mammalian MAP kinase – ERK 1 and ERK 2 – in the dephosphorylated inactive forms, and the latter two bands are the active forms of ERK 1 and ERK 2 by phosphorylation with a modification of their electrophoretic mobilities (Verlhac *et al.*, 1994). In the oocytes inseminated in the cycloheximide-free medium, both ERK 1 and phosphorylated ERK 1 showed no significant change until 8 h (Fig. 1B). On the other hand, phosphorylated ERK 2, which was dominant in the oocytes at metaphase II, showed a shift-down at 8 h after insemination. However, phosphorylated ERK 2 was still observed in the oocytes. MAP kinase molecules in the oocytes inseminated in the cycloheximide-containing medium showed similar change (Fig. 1C). In the control oocytes no shift-down

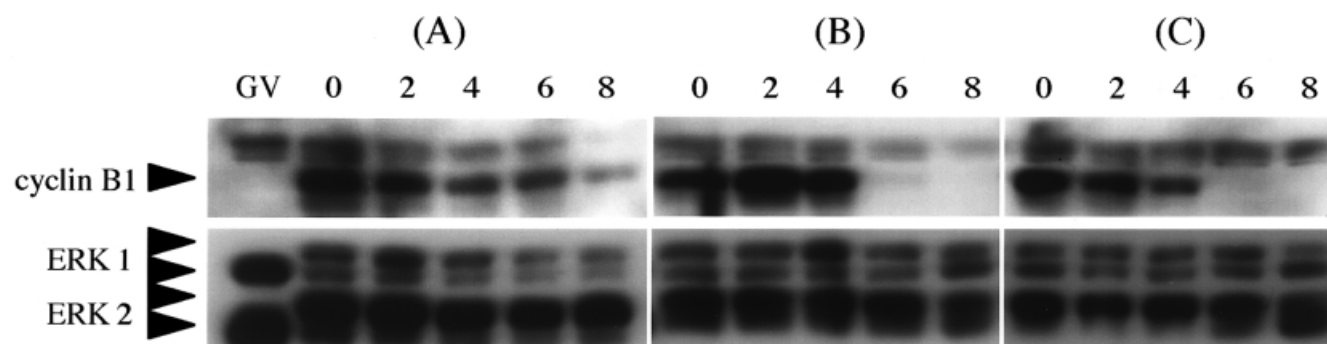


Figure 1 The changes in cyclin B1 and MAP kinase molecules (ERK 1, ERK 2) of pig oocytes inseminated with spermatozoa *in vitro*. *In vitro*-matured pig oocytes were inseminated at time 0, and groups of 50 oocytes were collected every 2 h after insemination. Whole oocyte lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and reacted with anti-pig cyclin B1 and anti-MAP kinase antibody. (A) Pig oocytes transferred into fertilisation medium without spermatozoa. (B) Pig oocytes inseminated with spermatozoa in cycloheximide-free fertilisation medium. (C) Pig oocytes inseminated with spermatozoa in cycloheximide-containing fertilisation medium. GV represents the oocytes at germinal vesicle stage before maturation culture.

of the molecules was observed during the culture period.

Discussion

Histone H1 kinase activity in mammalian oocytes at metaphase II shows a rapid decline after fertilisation or artificial activation. Kikuchi *et al.* (1995a) examined the nuclear stage of activated pig oocytes after insemination under a fluorescence microscope, and found that kinase activity was low in the oocytes shortly after sperm penetration and maintained at the basal level throughout female pronuclear formation. It has been speculated in mammalian oocytes that sperm penetration or artificial activation induces a rise in intracellular calcium which then leads to the degradation of cyclin by a ubiquitin-dependent proteolytic pathway, causing histone H1 kinase activity of MPF to be lost (Glotzer *et al.*, 1991). The cyclin B1 molecule is reported to be synthesised in the pig oocyte progressing to metaphase II (Naito *et al.*, 1995). The present experiments clearly showed that pig cyclin B1 degrades in oocytes during their transition from metaphase II to anaphase II/telophase II.

In both cycloheximide-free and cycloheximide-containing media, pig cyclin B1 disappeared at 6 h after insemination, although there was a time-dependent decrease in cyclin B1 protein in the oocytes inseminated in the cycloheximide-containing medium. Collas *et al.* (1993) investigated the influence of electrical stimulation on the histone H1 kinase activity in bovine oocytes at metaphase II. In their experiment, histone H1 kinase was rapidly inactivated after a single electrical stimulation, although the kinase reactivated at 2 h after the stimulation. We had presumed that the turnover of cyclin B molecules was rapid, and arranged *in vitro* fertilisation using cycloheximide-containing medium to make clear the pig cyclin degradation. Ding *et al.* (1992) reported that sperm penetration and pronuclear formation of pig oocytes were not dependent on new protein synthesis, because they occurred even in the presence of cycloheximide in the culture medium. In the present study, sperm penetration and pronuclear formation of the oocytes occurred in cycloheximide-containing medium similarly to cycloheximide-free medium, although the transition from metaphase II to anaphase II/telophase II of the oocytes progressed slightly earlier in the cycloheximide-containing medium than in cycloheximide-free medium. It is thought that cycloheximide inhibits the *de novo* synthesis of cyclin B1 molecules and blocks the recruitment of the newly synthesised molecules, in turn the disappearance of cyclin B1 molecules in the oocytes being promoted.

Pig oocytes contains two forms of MAP kinase:

p44^{ERK1} and p42^{ERK2} (Inoue *et al.*, 1995). The results in the present experiment are consistent with previous reports. The two forms of MAP kinase were already present in the oocytes at germinal vesicle stage, and they showed a shift-up of MAP kinase bands in the oocytes at metaphase II depending on their phosphorylation. During the transition from germinal vesicle stage to metaphase, MAP kinase has been reported to be activated after histone H1 kinase activation in mouse (Verlhac *et al.*, 1994), goat (Dedieu *et al.*, 1996) and rat oocytes (Zernicka-Goetz *et al.*, 1997). During the transition from metaphase II to interphase in mouse oocytes, MAP kinase is inactivated after activation (Verlhac *et al.*, 1993, 1994) or fertilisation (Moos *et al.*, 1995), and this inactivation follows the decrease in histone H1 kinase activity. Following activation of mouse oocytes, the slow-migrating phosphorylated MAP kinases were dephosphorylated and shifted down to the fast-migrating forms (Verlhac *et al.*, 1993). It has also been suggested by the mouse experiments that the decrease in MAP kinase activity regulates pronuclear formation, since no pronuclear envelope formed when MAP kinase remained active following fertilisation (Moos *et al.*, 1996). In the present experiment, pig ERK 2 changed to the fast-migrating form at 8 h after insemination, although the shift-down was not completed. Moreover, the change was delayed by 2 h compared with the degradation of cyclin B1 molecules. Therefore, the time courses of the inactivation of p34^{cdc2} protein kinase and MAP kinase in pig oocytes are thought to be similar to those in mouse oocytes. However, female pronuclear formation was almost completed at 8 h after insemination in pig oocytes. This result suggests that cyclin B1 degradation, not dephosphorylation of MAP kinase, leads pig oocytes from metaphase II arrest to interphase.

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