Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocytes

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Summary. Porcine oocytes cultured in follicular fluid for various periods of up to 48 h were stained with Hoechst-33342 and classified according to maturation before assaying. Histone H1 kinase activity at metaphase I was ~10 times that at the germinal vesicle stage. An abrupt reduction in activity was observed in oocytes emitting the first polar body; then the activity increased again to the same level as at metaphase I. This pattern is similar to those reported in non-mammalian species and supports the concepts that histone H1 kinase is ubiquitous in eukaryotes and controls the meiotic cell cycle in mammals.

Keywords: histone H1 kinase; oocyte; fluorochrome; pig

Introduction

Histone H1 kinase was first identified in Chinese hamster cells as an enzyme whose activity increases during metaphase and that phosphorylates histone H1 in a cAMP-independent manner (Lake & Salzman, 1972). Recently, histone H1 kinase has been purified and suggested to be homologous with maturation-promoting factor (MPF) (Arion *et al.*, 1988; Labbe *et al.*, 1988a), which promotes meiosis in immature oocytes arrested at the germinal vesicle stage (Masui & Markert, 1971; Kishimoto & Kanatani, 1976). Furthermore, p34^{cdc2}, first identified in *Schizosaccharomyces pombe* as a gene product controlling the cell cycle (Nurse & Bissett, 1981), and cyclin, first identified in marine organisms as a protein that varies in concentration during the cell cycle (Evans *et al.*, 1983), have been reported as components of histone H1 kinase/MPF (Gautier *et al.*, 1988, 1990).

The high activity of histone H1 kinase during metaphase has been suggested to cause nuclear lamina disassembly (Peter *et al.*, 1990b), nucleolar disassembly (Peter *et al.*, 1990a), chromosome condensation (Moreno & Nurse, 1990), transcription regulation (Cisek & Corden, 1989), translation regulation (Bellé *et al.*, 1989) microfilament rearrangement (Morgan *et al.*, 1989), and reorganization of the intermediate filament network (Chou *et al.*, 1990).

Numerous studies have been reported on the changes of histone H1 kinase activity during oocyte meiotic maturation (Maller *et al.*, 1977; Doree *et al.*, 1983; Picard *et al.*, 1985; Karsenti *et al.*, 1987; Labbe *et al.*, 1988a, b) and its characteristics (Sano, 1985; Pelech *et al.*, 1987; Erikson & Maller, 1989) in *Xenopus* and marine organism oocytes. In mammalian oocytes, however, changes of histone H1 kinase activity during meiotic maturation have not been reported, although there are a few reports on the change in activity of MPF and H1 kinase at different times during maturation in mouse (Hashimoto & Kishimoto, 1988; Rime & Ozon, 1990) and porcine oocytes (Prochazka *et al.*, 1989).

In this study, we established an assay system for histone H1 kinase activity in porcine oocytes that matured *in vitro*, and investigated changes in activity during meiotic maturation.

Materials and Methods

Oocyte preparation. Gilt ovaries were obtained from a slaughterhouse and carried to the laboratory within 40 min in saline solution at 37–39°C. Follicular oocytes were collected from medium-sized follicles (2–5 mm in diameter) as described by Naito *et al.* (1988). Each of the 10 to 15 oocyte–cumulus complexes was cultured in 0.1 ml porcine follicular fluid (pFF) supplemented with 1.0 iu pregnant mares' serum gonadotrophin/ml (Peamex, Sankyo Co., Japan) for various periods of up to 48 h at 37°C in 5% CO₂ in air; pFF was collected from gilt ovaries and stored as described by Naito *et al.* (1988). After culturing, the oocytes were treated with 150 U hyaluronidase/ml (Sigma, Type IV-S) in a modified Krebs–Ringer bicarbonate solution (TYH; Toyoda *et al.*, 1971) for a few minutes at room temperature. Thereafter, the surrounding cumulus cells were removed by repeated pipetting with a fine-bore pipette. The denuded oocytes were then centrifuged at 15 000 *g* for 5 min, to localize the lipid granules, then stained in TYH containing 10 µg Hoechst-33342 (H-33342; Calbiochem Co., German Federal Republic)/ml for 15–60 min at 37°C in 5% CO₂ in air. H-33342 was dissolved in TYH at 1 mg/ml and stored in the dark at –70°C until use. The stained oocytes were examined under a fluorescent microscope (Nikon DIAPHOT-TMD microscope equipped with a 100-W mercury lamp and Nikon DM400 filter) and classified according to meiotic maturation stages (Fig. 1). Photographs were taken of the oocytes (Nikon FE2 camera with Fujichrom DX 400D film) through the fluorescent microscope at different times during culture.

Preparation of oocyte extracts. Oocytes at different stages were picked up under the fluorescent microscope and collected in ice-cooled TYH for assaying the histone H1 kinase. Oocytes in which the maturation stage could not be determined under the fluorescent microscope were not used for assaying, but were mounted on a glass slide for determination of the stage after fixing and staining with 0.75% acetic orcein. The fresh oocytes of determined stages were washed 3 times in buffer A (60 mmol β-glycerol phosphate/l, 30 mmol *p*-nitro-phenyl phosphate/l, 25 mmol Mops/l (pH 7.2), 15 mmol EGTA/l, 15 mmol MgCl₂/l and 0.1 mmol sodium vanadate/l) and put into plastic tubes at 2 oocytes to 1 µl buffer A; they were then frozen to –70°C to break the oocyte membrane. Repeated freezing and thawing treatment in a preliminary experiment had been shown to reduce the enzyme activity and so all oocytes were subjected to frozen–thaw treatment only once. After thawing, the oocyte suspension was centrifuged at 15 000 *g* for 5 min at 4°C and mixed well; it was then centrifuged again for 20 min under the same conditions. The supernatants were used immediately for the kinase assay.

Kinase assay. Histone H1 kinase was assayed according to the method of Pelech *et al.* (1987), but with several modifications. Unless stated otherwise, all kinase assays contained the following in a final volume of 25 µl buffer: 500 nmol protein kinase inhibitor (TTYADFIASGRTGRRNAIHD; Sigma)/l, 50 µmol histone H1/l (Sigma type III-S), 1 mmol dithiothreitol/l (Wako Pure Chemical Ind., Japan), 20 µmol [γ -³²P]ATP/l (3–10 c.p.m./fmol; Amersham) and 5 µl oocyte extract. Kinase reactions commenced upon the addition of [γ -³²P]ATP and were usually completed within 60 min at 36°C. Assays were terminated by 0.4 ml of 20% trichloroacetic acid solution (TCA); 0.1 ml of 1% bovine serum albumin (BSA-FR.V.; Wako Pure Chemical Ind.) was added as a carrier protein for precipitation. The assay suspensions were centrifuged at 15 000 *g* for 5 min and supernatants were discarded. The precipitates were washed once with 0.4 ml of 20% TCA and dissolved in 0.4 ml of 1M-NaOH. The solution was then transferred into vials containing 5 ml of scintillation fluid (ACS II; Amersham) and radioactivity was measured using a liquid scintillation counter (Aloka, LSC-1000). The value for blank tubes which contained all materials except for a cytosol preparation was subtracted from each experimental value to obtain the kinase activity.

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

The maturation stages of most oocytes were distinguishable by fluorescent microscopy after staining with H-33342 (Fig. 1: b–h). The germinal vesicles were also visible by light microscopy after centrifugation (Fig. 1a). The maturation stages of some oocytes whose chromosomes were within the located lipid granules could not be determined without fixing and staining.

All oocytes remained at the germinal vesicle (GV) stage after 4 h in culture (Table 1); 24 h after the start of culturing, 39 and 29% of oocytes were at first prometaphase (PMI) and first metaphase (MI), respectively. The proportion of MI oocytes increased until 33 h after the start of culturing. Oocytes emitting the first polar body appeared after 24 h of culturing, but the incidence of these oocytes at first anaphase and telophase (ATI) remained <20% even at 33–37 h in culture. Most of the others were either at MI or at second metaphase (MII). Matured MII oocytes were observed mainly after 35 h in culture and increased gradually to reach 85% of the total at 45–48 h in culture.

Fig. 1. Porcine follicular oocytes at different stages of maturation stained with Hoechst-33342. The photographs were taken with a light microscope (a) or a fluorescent microscope (b–h), $\times 200$; (a) and (b) germinal vesicle after 4 h in culture, (c) prometaphase I at 31 h, (d) metaphase I at 31 h, (e) anaphase I (early stage) at 31 h, (f) anaphase I (late stage) at 33 h, (g) telophase I at 33 h and (h) metaphase II at 45 h.

Table 1. Temporal relationship of the maturation stages in porcine follicular oocytes cultured *in vitro*

Culture period (h)	Total no. of ova examined	No. (%) of oocytes at the stage of				
		Germinal vesicle	Prometaphase I	Metaphase I	Anaphase and telophase I	Metaphase II
2–4	119	119 (100)	0	0	0	0
24–28	148	42 (28)	58 (39)	43 (29)	3 (2)	2 (1)
31	157	3 (2)	58 (37)	80 (51)	11 (7)	5 (3)
33	101	4 (4)	13 (13)	60 (60)	16 (16)	8 (8)
35	156	6 (4)	2 (1)	73 (47)	30 (19)	45 (29)
37	152	5 (3)	8 (5)	51 (34)	22 (14)	66 (43)
45–48	91	2 (2)	0	12 (13)	0	77 (85)

Data are means from 3 experiments.

In experiments using the cytosol of porcine oocytes cultured for 48 h at 36°C, the phosphorylation rate of histone H1 was almost constant for the first 2 h and then decreased (Fig. 2a). Therefore, an assay period of 1 h was chosen. Figure 2(c) shows the Lineweaver–Burk plot calculated from the relationship between histone H1 concentration and phosphorylation rate (Fig. 2b); it was an almost straight line (Fig. 2c) and the coefficient of correlation was 0.987. The Michaelis constant (K_m) for histone H1 calculated from the line was 9.9 $\mu\text{mol/l}$.

The activity of histone H1 kinase was low in oocytes at the germinal vesicle stage after 2–4 and 24–28 h in culture (Table 2, Fig. 3). At PMI, the activity had increased significantly and was greater at 31 than at 24–28 h in culture. Activity in MI oocytes was ~ 10 times greater than at the germinal vesicle stage and was similar in oocytes which reached MI after 31 or 33–37 h in culture. The activity then decreased markedly in oocytes emitting the first polar body (ATI) to about one-third of that in MI oocytes and was not statistically different from that at the germinal vesicle stage. At MII, the activity increased again to the same level as in the MI oocytes and the value did not change between 31–37 and 45–48 h in culture.

Fig. 2. Characteristics of histone H1 kinase in porcine oocytes. Cytosol preparations used were obtained from porcine follicular oocytes cultured for 48 h in porcine follicular fluid. (a) Time course of histone H1 phosphorylation at 36°C; (b) relationship between histone H1 concentration and phosphorylation rate; (c) a Lineweaver-Burk plot calculated from Fig. 2b.

Table 2. Activity of histone H1 kinase in porcine oocytes at various stages of meiosis

Stage of meiosis	Culture period (h)	No. of experiments	Total no. of ova used for assay	Histone H1 kinase activity* (fmol/h/oocyte)
Germinal vesicle	2-4	4	171	16.7 $\pm$ 5.8
	24-28	3	70	19.1 $\pm$ 10.9
Prometaphase I	24-28	3	51	69.0 $\pm$ 16.3 ^c
	31	3	51	95.1 $\pm$ 11.7 ^c
Metaphase I	31	4	106	190.1 $\pm$ 31.3 ^{cd}
	33	4	98	179.4 $\pm$ 28.8 ^{cd}
	35	4	86	180.9 $\pm$ 31.9 ^{cd}
	37	4	70	189.2 $\pm$ 38.0 ^{cd}
Anaphase and telophase I	31-37	3	68	62.1 $\pm$ 19.9
Metaphase II	31-37	3	114	184.7 $\pm$ 55.6 ^c
	45-48	3	70	162.4 $\pm$ 27.0 ^{cd}

*Mean $\pm$ s.e.

^cValues significantly higher than at 2-4 h in culture.

^dValues significantly higher than at prometaphase I after 24-28 h in culture and at anaphase and telophase I.

Fig. 3. Changes in histone H1 kinase activity during meiotic maturation in porcine oocytes cultured in porcine follicular fluid and used for cytosol preparation at each maturation stage. The activity was represented as the number of molecules of $^{32}\text{PO}_4$ incorporated into histone H1/h/oocyte. Columns and vertical bars represent the means $\pm$ s.e.m. of 3 or 4 experiments; data shown in Table 2.

Discussion

In this study, we used porcine oocytes maturing in pFF *in vitro* to assay histone H1 kinase activity during meiotic maturation. We reported previously that porcine oocytes which matured in pFF *in vitro* had normal ability to form male pronuclei (Naito *et al.*, 1988); after fertilization, their developmental ability was almost the same as those which matured *in vivo* (Naito *et al.*, 1989). The present results thus reflect physiological maturation processes.

In the present study, the time course of maturation and the incidence of oocytes at each stage were in good agreement with previous reports of the *in-vivo* and *in-vitro* maturation of porcine oocytes (Hunter & Polge, 1966; McGaughey & Polge, 1971). In those reports, the first polar body emitting oocytes appeared at 27–43 h after the start of meiotic maturation, and the incidence of those oocytes did not exceed 25% at any time, *in vivo* or *in vitro*. Considering these and the present results, which showed the asynchronous emission of the first polar body, it was suspected that histone H1 kinase activity would not have changed significantly, if the oocytes had been subjected to assay without confirming their meiotic stage. Therefore, we stained the oocytes with fluoro-chrome, Hoechst-33342, and separated the oocytes emitting the first polar body from those at metaphase before assaying the kinase activity.

The single straight line in the Lineweaver–Burk plot revealed that our assay system evaluated activity of a single enzyme. The apparent K_m for histone H1 of 9.9 $\mu\text{mol/l}$ was of the same order as, but a little higher than, others (2–3 $\mu\text{mol/l}$) reported in non-mammalian oocytes (Pelech *et al.*, 1987; Erikson & Maller, 1989) and cultured human cells (Brizuela *et al.*, 1989). Such a discrepancy might be due to species differences or the conditions used in the assays.

Histone H1 kinase was first identified in cultured mitotic hamster cells (Lake & Salzman, 1972) and thereafter, in *Xenopus* eggs (Karsenti *et al.*, 1987; Labbe *et al.*, 1988b), sea urchin eggs (Meijer & Pondaven, 1988) and cultured human cells (Brizuela *et al.*, 1989), with high and low activity in metaphase and interphase, respectively. During the meiotic maturation of oocytes, as in mitosis, low activity in prophase (germinal vesicle stage), high activity at metaphases I and II and reduced activity at anaphase I and telophase I have been reported in *Xenopus* (Labbe *et al.*, 1988b; Murray & Krischner, 1989) and starfish oocytes (Picard *et al.*, 1985; Labbe *et al.*, 1988a). The present results for porcine oocytes showed the same pattern of activity during meiotic maturation as in those reports. The activity in one oocyte at metaphase, which was ~ 200 fmol/h in pig (present study), 5–20 pmol/h in starfish (Labbe *et al.*, 1987) and 600–900 pmol/h in *Xenopus* (Labbe *et al.*, 1988b), correspond well to the oocyte volumes, which were ~ 1 nl, 100 nl and 2 μ l, respectively. These results support the concepts that histone H1 kinase is ubiquitous in eukaryotes and plays an important role in controlling the cell cycle.

Histone H1 kinase has been reported to have a component, p34^{cdc2} (Arion *et al.*, 1988; Labbe *et al.*, 1988a), and is suggested to be homologous with MPF. In mammalian oocytes, the fluctuation pattern of MPF activity during meiotic maturation has been reported only in mice (Hashimoto & Kishimoto, 1988) and is similar to that found in the present study. Prochazka *et al.* (1989) have shown low and high activity in porcine oocytes at the germinal vesicle stage and in matured oocytes, respectively, which agrees with our results. These results also support the concept of histone H1 kinase as being homologous with MPF in mammals.

Histone H1 kinase is responsible for phosphorylating many different substrates. Peter *et al.* (1990b) found that the highly purified p34^{cdc2} kinase phosphorylates B1 and B2 lamins, solubilizes lamin B from nuclei and causes nuclear lamina disassembly. RNA polymerase II (Cisek & Corden, 1989), elongation factors EF-1 β and EF-1 γ (Bellé *et al.*, 1989), NO38 and nucleolin (Peter *et al.*, 1990a), p60^{c-src} tyrosine kinase (Morgan *et al.*, 1989) and vimentin (Chou *et al.*, 1990) have also been reported to be phosphorylated by p34^{cdc2} kinase *in vivo* and *in vitro*, suggesting the possible roles of this kinase in metaphase on transcription regulation, translation regulation, nucleolar disassembly, microfilament rearrangement and reorganization of the intermediate filament network, respectively. It has been suggested that phosphorylation of histone H1 alters the nucleosome packing and contributes to chromosome condensation (Moreno & Nurse, 1990). Although further studies are required for molecular explanations of the events in metaphase, these substances might be phosphorylated by p34^{cdc2} kinase in mammalian oocytes also and responsible for the induction of germinal vesicle breakdown and meiotic maturation.

We assayed histone H1 kinase activity during meiotic maturation in porcine oocytes for the first time and showed a fluctuation pattern similar to those reported in non-mammalian species. Studying this activity in mammalian oocytes might promote the understanding of regulatory mechanisms of oocyte maturation and refinement of culture conditions for in-vitro maturation of mammalian oocytes.

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