Active Maturation-promoting Factor Is Present in Mature Mouse Oocytes

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ABSTRACT Cytoplasmic extracts of meiotically mature mouse oocytes were injected into immature *Xenopus laevis* oocytes, which underwent germinal vesicle breakdown within 2 h. Germinal vesicle breakdown was not inhibited by incubaton of the *Xenopus* oocytes in cycloheximide (20 μg/ml). Identically prepared extracts of meiotically immature mouse oocytes, arrested at the germinal vesicle stage by dibutyryl cyclic AMP (100 μg/ml), did not induce germinal vesicle breakdown in *Xenopus* oocytes. The results show that maturation-promoting factor activity appears during the course of oocyte maturation in the mouse.

Oocytes arrest their meiotic progression during late G_2 phase of the first meiotic division. It is in this arrested stage that oocytes grow in preparation for fertilization and embryogenesis. Fully grown oocytes reinitiate meiosis in response to a hormonal signal at or near the time of ovulation. In amphibians, pituitary gonadotropin triggers the production of a maturation-inducing substance (presumably progesterone) by follicle cells encasing the oocyte. In turn, progesterone acts upon the surface of the oocyte to initiate meiotic maturation, which involves dissolution of the nuclear membrane (germinal vesicle breakdown [GVBD][1]), condensation of the interphase chromatin, spindle formation and completion of the first meiotic division, and arrest at metaphase of the second meiotic division (13, 20).

The action of progesterone in inducing GVBD is mediated by a cytoplasmic maturation-promoting factor (MPF). Cytoplasm from mature frog oocytes, injected into immature germinal-vesicle stage oocytes, will induce maturation in the absence of progesterone (15). MPF from *Xenopus laevis* oocytes has been purified >20-fold and has been characterized as a protein (or proteins) with a molecular weight of ~100,000 (28).

MPF appears to be ubiquitous. MPF activity has been demonstrated in the amphibians *Rana pipiens* (15), *X. laevis* (19), and *Ambystoma mexicanum* (17), and in the starfish *Asterina pectinifera* (6). MPF activity has also been demonstrated in amphibian blastomeres undergoing mitosis (15, 26), in HeLa cells and Chinese hamster ovary cells arrested in metaphase of mitosis (16, 23), and in yeast (27). Insofar as MPF controls the transition from G_2 to M phase in both meiosis and mitosis, Gerhart et al. (4) propose that the acronym MPF refers to "M-phase promoting factor."

MPF also appears to lack species specificity, which suggests that it has been highly conserved in evolution. MPF from each of three different species of starfish was effective in inducing maturation after injection into oocytes of the other two species (7). Similarly, MPF from *R. pipiens, X. laevis*, and *A. mexicanum* induced maturation in one another's oocytes (17). MPF from mammalian mitotic cells induced meiotic maturation in *X. laevis* (16, 23) and *A. pectinifera* oocytes (8), and MPF from *Bufo bufo japonicus* oocytes induced meiotic maturation in starfish and sea cucumber oocytes (8).

Recently, Kishimoto et al. (9) demonstrated maturation of starfish oocytes injected with cytoplasm from maturing mouse oocytes. It was the purpose of the study reported here to test for the appearance of MPF during mouse oocyte maturation by using *X. laevis* oocytes as the test system.

MATERIALS AND METHODS

For the collection of mature oocytes, 8–10-wk-old CF1 female mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were induced to superovulate by intraperitoneal injections of 5 IU of pregnant mare's serum gonadotropin (Teikoku Zoki Co., Tokyo, Japan) and human chorionic gonadotropin (Ayerst Laboratories, New York, NY) 48–52 h apart. For the collection of immature oocytes, nonsuperovulated 4–5-wk-old females were used. Mice were kept in the light for 14 h and in the dark for 10 h.

[1] *Abbreviations used in this paper:* FM I, flushing medium I; GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor.

14 h after injection of human chorionic gonadotropin, oviducts were dissected, swollen ampullae were nicked, and cumulus masses were expelled into modified Hanks' balanced salt solution (flushing medium I [FM I] [22]). Hyaluronidase (Type IV, Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.05%. Cumulus masses were incubated at room temperature until mature oocytes were freed (~15 min). Oocytes were washed in FM I several times to remove cumulus cells.

Immature (germinal-vesicle stage) oocytes were freed from their follicles by teasing apart ovaries in FM I. Cumulus cells were removed by repeated pipetting, and oocytes were washed in FM I several times. Oocytes were arrested at the germinal vesicle stage by the addition of 100 μg/ml dibutyryl cyclic AMP (Sigma Chemical Co.) to the FM I (3) from the moment the oocytes were freed until the final wash (~2 h).

Zonae pellucidae were digested by the incubation of oocytes in 0.5% pronase (Type B, Calibochem-Behring Corp., La Jolla, CA) at 37°C for 3 min. Oocytes were quickly washed in several changes of FM I. Just before extraction, oocytes were washed several times in calcium- and magnesium-free FM I and transferred to miniature centrifuge tubes constructed by flaming closed one end of a capillary tube (Kimax-51, 0.8-mm i.d., Kimble Products, Toledo, OH). Oocytes were washed through two changes of ice-cold extraction buffer (80 mM sodium β-glycerophosphate, 20 mM sodium EGTA, 15 mM $MgCl_2$, pH 7.3, plus 0.1 mM 8-thiolated ATP, 1 mM dithiothreitol, and the following protease inhibitors: 0.1 μg/ml pepstatin, 5 μM phenylmethylsulfonyl fluoride, 0.25 μg/ml leupeptin, 0.25 μg/ml aprotinin, 10 μM benzamidine HCl [28] by low-speed centrifugation. After removal of all but ~1 μl of extraction buffer, oocytes were disrupted by repeated pipetting through a mouth-controlled pipette with an inner diameter ~⅓ that of the oocyte. The homogenate was centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant (extract) was used to assay MPF activity (Fig. 1).

Adult *X. laevis* were obtained from Xenopus I (Ann Arbor, MI). Females were injected via the dorsal lymph sac with 20–50 IU of pregnant mare's serum gonadotropin 3 d before the surgical removal of ovaries. Stage 6 oocytes were dissected manually for use in the injection assay of MPF. Cycloheximide was obtained from the Sigma Chemical Co.

Mouse oocyte extracts were injected in 50-nl aliquots into the animal hemispheres of *Xenopus* oocytes. The total number of 50-nl aliquots recovered from the centrifuge tube was used to estimate the total volume of extract. *Xenopus* oocytes were incubated at room temperature after injection. 2 h after injection, GVBD was assessed by the appearance of a characteristic white spot in the pigmented animal hemisphere. Assessment was confirmed by fixing oocytes for 10 min in 10% trichloroacetic acid and dissecting them to observe the presence or absence of a germinal vesicle.

RESULTS

Extracts from mature ovulated mouse oocytes induced GVBD in immature *X. laevis* oocytes within 2 h after injection (Table I). Buffer-injected control *Xenopus* oocytes gave no response. The MPF activity was estimated by calculation of the number of mouse oocytes in the volume of extract injected. The minimum number of mouse oocytes that gave a positive response in the injected recipient oocytes was 23. Mature oocytes extracted by freezing and thawing were completely ineffective in inducing maturation. Two samples of *Xenopus* oocytes incubated in 20 μg/ml cycloheximide and injected with extracts from mature mouse oocytes also underwent GVBD (Table II). Treatment of *Xenopus* oocytes with this concentration of cycloheximide inhibits >95% of ^{35}S-[methionine] incorporation into protein (Gerhart, J., personal communication).

In contrast to the mature ovulated oocytes, extracts of immature germinal-vesicle stage ovarian oocytes arrested by incubation in dibutyryl cyclic AMP did not induce GVBD in *Xenopus* oocytes even though the number of mouse oocytes injected per recipient oocyte (34–64) was well above the number of mature oocytes demonstrated to cause GVBD (Table III). *Xenopus* oocytes co-injected with extract from immature mouse oocytes and with active *Xenopus* MPF did undergo GVBD (data not shown).

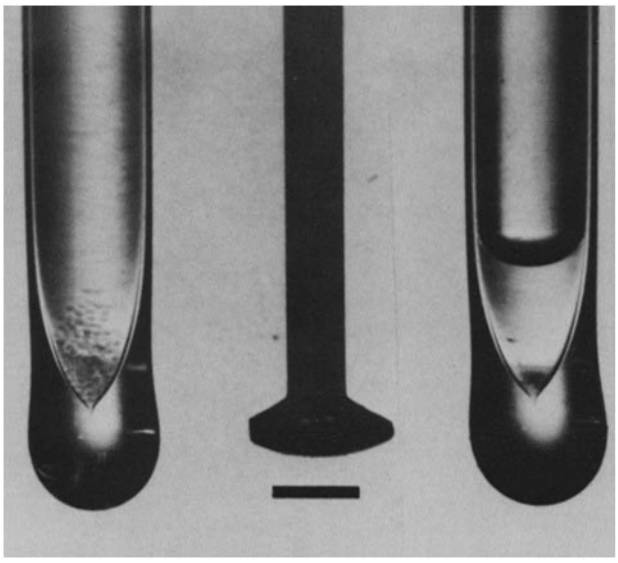

FIGURE 1 Mature oocytes before (*left*) and after (*right*) extraction. Whole oocytes were disrupted and centrifuged in capillary tubes. A common straight pin is included to demonstrate scale. Bar, 1 mm. × 11.4.

DISCUSSION

The results reported allow the mouse oocyte to be included among the known meiotic and mitotic cell types in which the transition from G_2 to M phase is regulated by a cytoplasmic factor. The appearance of a cytoplasmic MPF in maturing mouse oocytes was first suggested by Balakier and Czolowska (2), who observed that anucleate fragments of immature mouse oocytes, when fused to interphase blastomeres from two-celled mouse embryos, induced premature dissolution of the nuclear membrane and premature chromatin condensation in the blastomeres when the nucleate sister fragment underwent GVBD.

The precocious induction of GVBD (<2 h) compared with the time of progesterone-induced maturation (>3 h) supports the conclusion that the active agent in extracts of mature mouse oocytes is MPF. According to the evolving model of progesterone control of amphibian oocyte maturation (12, 14), it is the phosphorylation of preexisting MPF that triggers GVBD. Progesterone, the maturation-inducing substance in amphibians, is proposed to release calcium ions from the cell surface that activate a cytoplasmic phosphodiesterase, which in turn decreases the concentration of cyclic AMP. A lowered cyclic AMP concentration leads to inactivation of protein kinase, resulting in dephosphorylation of a hypothetical initiator protein, changing it from an inactive to an active state. The initiator protein is proposed to be a cyclic AMP–independent protein kinase responsible for the phosphorylation and activation of inactive MPF. MPF also may be a protein kinase capable of phosphorylating itself, thus explaining its observed autocatalytic behavior on serial transfer (15). The distal position of MPF in this elaborate protein phosphorylation cascade explains the more rapid response of oocytes to MPF injection compared with the time of oocyte response to progesterone treatment.

The induction of GVBD in the presence of cycloheximide, an inhibitor of protein synthesis, can be considered definitive evidence for MPF activity in mature mouse oocyte extract. Progesterone-treated *Xenopus* oocytes do not mature in cycloheximide, but become increasingly resistant to the inhibitor as the maturation process progresses (25). Cycloheximide does

TABLE I

Injection of Extract from Mature Mouse Oocytes into Immature Xenopus Oocytes

Experiment No.	No. of mouse oocytes extracted	Total volume of extract	Volume of extract injected	No. of mouse oocytes per injection	No. of *Xenopus* oocytes receiving injection	No. of *Xenopus* oocytes undergoing GVBD	*Xenopus* oocytes undergoing GVBD
		μl	nl				%
1	600	2.6	100	23	9	7	78
			150	35	6	2	33
			200	46	4	4	100
2	450	1.5	50	16	8	0	0
			100	32	5	4	80
3	567	0.6	25	24	3	3	100
			50	47	4	4	100
			100	95	3	3	100

TABLE II

Injection of Extract from Mature Mouse Oocytes into Cycloheximide-treated Immature Xenopus Oocytes

Experiment No.	No. of mouse oocytes extracted	Total volume of extract	Volume of extract injected	No. of mouse oocytes per injection	No. of *Xenopus* oocytes receiving injection	No. of *Xenopus* oocytes undergoing GVBD	*Xenopus* oocytes undergoing GVBD
		μl	nl				%
1	450	1.50	100	32	3	1	33
2	285	0.65	50	22	5	5	100
			100	44	4	4	100

Xenopus oocytes were incubated for 1 h in 20 μg/ml cycloheximide before injection and incubated continuously in 20 μg/ml cycloheximide after injection.

TABLE III

Injection of Extract from Immature Mouse Oocytes into Immature Xenopus Oocytes

Experiment No.	No. of mouse oocytes extracted	Total volume of extract	Volume of extract injected	No. of mouse oocytes per injection	No. of *Xenopus* oocytes receiving injection	No. of *Xenopus* oocytes undergoing GVBD	*Xenopus* oocytes undergoing GVBD
		μl	nl				%
1	385	0.60	100	64	5	0	0
2	309	0.90	100	34	6	0	0

Maturation of mouse oocytes was arrested by the addition of 100 μg/ml dibutyryl cyclic AMP to the collection medium.

not prevent maturation of oocytes injected with MPF (25, 28) and does not prevent the appearance of high concentrations of MPF when oocytes are injected with low concentrations of MPF (4). These data suggest that MPF is present in the oocyte before GVBD and is activated post-translationally, most likely by phosphorylation. The hypothesis that the phosphorylation cascade depends upon the synthesis of an initiator protein nominates it as a target of cycloheximide action. Since MPF acts "downstream" from the initiator protein, injected MPF would not be sensitive to the inhibition of protein synthesis. Other substances capable of inducing GVBD upon injection, such as the R and I proteins of cyclic AMP metabolism (11), are sensitive to cycloheximide owing to their "upstream" position in this hypothetical cascade.

Consistent with the cascade model for progesterone action, the maintenance of a high cyclic AMP concentration would prevent MPF activation. The failure of an extract of dibutyryl cyclic AMP–arrested mouse oocytes to induce GVBD after injection into *Xenopus* oocytes is therefore predicted. Dibutyryl cyclic AMP–arrested mouse oocytes, however, do undergo initial chromosome condensation and extensive convolution of the germinal vesicle membrane (24), two changes associated with incipient GVBD. These initial maturational events, therefore, do not appear to be regulated by MPF.

Sunkara et al. (23) calculated that a minimum of 1,000 mitotic HeLa cells (with a mean diameter of 17 μm) was capable of causing 100% maturation after injection into *Xenopus* oocytes. With a diameter of 72 μm (10), mouse oocytes have 75 times the volume of a HeLa cell. Accordingly, 13 mature mouse oocytes should be the minimum number capable of causing a response in *Xenopus* oocytes, assuming an equivalent MPF concentration in both HeLa cells and mouse oocytes. This value is close to the estimated value in this study.

The question of the relationship of MPF to the development of meiotic competence in mouse oocytes is intriguing. Sorensen and Wassarman (21) observed that small oocytes recovered from mice younger than 15 d *post partum* were incompetent to mature in culture. Balakier (1) reported that incompetent young oocytes that were fused with maturing oocytes or four-cell blastomeres were arrested in meiosis, underwent GVBD, and progressed to metaphase of the first meiotic division. The injection of MPF into small *Xenopus* oocytes incapable of responding to progesterone treatment

induced GVBD (5, 18). Our preliminary attempts to microinject either concentrated *Xenopus* MPF (28) or freshly prepared extract of mature mouse oocytes into incompetent mouse oocytes have failed to induce GVBD (Sorensen, R. A., and R. A. Pedersen, unpublished results). Insofar as the microinjected volume is considerably less than that introduced by fusion of a whole oocyte, either incompetent mouse oocytes are completely deficient in MPF, which can only be provided by the massive transfusion of cytoplasm afforded by cell fusion, or they do not possess sufficient MPF to be activated by the injection of a small volume of active MPF.

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