PROTEIN FACTOR(S) FROM MITOTIC CHO CELLS INDUCE MEIOTIC MATURATION IN *XENOPUS LAEVIS* OOCYTES

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1. Introduction

Although the morphologic aspects of mitosis have been studied for over a hundred years [1], the molecular mechanisms underlying the progression of mitotic events are virtually unknown. Considerable insight into the process of mitotic induction has been gained by the observation that when a mitotic cell is fused with an interphase cell (mediated via Sendai virus), the interphase cell nuclear membrane breaks down and the chromosomes condense [2]. These classic experiments imply that factors from the mitotic cell are able to induce mitosis in the non-mitotic cell.

Obvious questions arising from these experiments concern the nature and mode of action of the mitotic inducing factors. To answer such questions, a method for assaying the activity of factors from mitotic cells was needed. The immature frog oocyte provides such an assay [3]; extracts from early cleavage frog embryos during mitosis stimulate the meiotic maturation of the immature frog oocyte. Extracts of HeLa cells in mitosis also induce meiotic maturation in the frog oocyte [4]. We too, have used cytoplasmic extracts of mammalian cells to induce meiotic maturation in *X. laevis* oocytes. We show here that the active component(s) is probably a protein(s); the protein(s) is mol. wt >10 000, is calcium sensitive and is quite heat labile. This protein(s) will induce maturation in the absence of protein or RNA synthesis in the oocyte. Finally, we show that the protein(s) is dramatically stabilized by agents that inhibit phosphatases, suggesting that phosphorylation–dephosphorylation events may be important in the control of mitotic induction.

2. Materials and methods

2.1. *Materials*

Dulbecco's modified Eagle's medium was purchased from Microbiological Associates (Rockville, MD) and fetal calf serum from Sterile Systems (Logan, UT). Papain, antipain, actinomycin D, cycloheximide, progesterone and RNase were from Sigma Chemical Co. (St Louis, MO). DNase was from Worthington Biochemicals (Freehold, NJ).

2.2. *Preparation of mitotic and interphase cells*

The CHO-K1 line of Chinese hamster ovary cells was obtained from the American-Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium supplemented with proline (34 mg/l), HEPES (25 mM), penicillin (100 μg/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (DME-10). Cells were synchronized by a slight modification of a procedure developed for synchronization of CHO cells [5,6]. Briefly, cells at $1.5 \times 10^5/cm^2$ were blocked in G1 by isoleucine starvation for 44 h, then released into DME-10 containing hydroxyurea (2 mM) for 9 h to synchronize them at the G1/S boundary. The media was then replaced with fresh DME-10, and Colcemid was added 12 h later. After 3 h more, metaphase cells were collected by gently shaking the loosely adherent mitotic cells from the culture dish. The mitotic index of these cells was >98%. Interphase cells were prepared by trypsinization of monolayer cultures.

2.3. *Preparation of cell extracts*

All steps were performed at 0°C. Cells were

washed once in Hank's balanced salt solution with 10 mM $MgCl_2$ without calcium. Cells were then suspended (10^8 cells/ml) in extraction buffer containing 200 mM NaCl, 200 mM sucrose, 2 mM EGTA, 10 mM $MgCl_2$, 10 mM sodium phosphate (pH 6.7). This buffer had previously been shown to be effective for preparing meiotic induction factors from unfertilized frog eggs [7]. The suspension was sonicated for 4 s at 0°C with a Heat Systems sonicator (Plainview, NY), using a microprobe setting of 70 W output. The mixture was then centrifuged for 20 min at 27 000 × *g* in a Sorvall SS-34 rotor. The supernatant was filtered through a GF/C filter (Whatman) to remove any particulate matter, and was then used for injection.

2.4. *Oocyte assay*

Oocytes were obtained from *X. laevis* females from South Africa supplied by a local distributor. Individual oocytes of >1.2 mm diam. were dissected out manually. In some experiments, the ovary was treated for 3–4 h with collagenase (Calbiochem B grade) in amphibian Ringer's solution without calcium before dissection. Oocytes were kept in amphibian Ringer's solution (with calcium) and injected with 70 nl fluid. After 3 h, meiotic maturation was assessed in the injected oocytes. The assay consisted of observation for the appearance of a white 'maturation spot' centered at the animal pole and the disappearance of the germinal vesicle (nucleus). The white maturation spot represents pigment displaced by the movement of the germinal vesicle towards the animal pole [8]. The presence or absence of the germinal vesicle was determined after fixation of the oocytes in 5% trichloroacetic acid. Some oocytes were fixed in Smith's fluid, embedded in paraffin, sectioned, and stained with Feulgen or Unna to examine the state of the chromosomes.

3. Results

When a cytoplasmic extract prepared from mitotic cells is injected into immature oocytes, 100% of the oocytes undergo meiotic maturation (table 1). The signs of maturation always appeared between 1.75–2.25 h after injection of mitotic cell extract. These signs included a white spot centered at the animal pole and germinal vesicle breakdown (see section 2). Histological studies showed that the chromosomes had condensed on a spindle near the oocyte cortex in the animal hemisphere. Hence, true maturation, rather than pseudomaturation [9], was induced.

Control experiments showed that extracts identically prepared from interphase cells did not induce maturation. This was true whether the interphase cells were in logarithmic growth or had been pre-

Table 1
Meiotic maturation in oocytes treated with extracts of CHO cells

Material injected[a]	No. of oocytes injected	Meiotic maturation (%)
Mitotic cell extract	50	100
Mitotic cell extract diluted 1:1	16	100
Mitotic cell extract diluted 1:2	16	100
Mitotic cell extract diluted 1:4	16	50
Mitotic cell extract diluted 1:10	16	6
Interphase cell extract[b]	30	0
S phase cell extract[c]	16	0
Interphase cell extract (Colcemid treated)[d]	16	0
Extraction buffer only	40	5
Cytoplasm from mature oocytes[e]	16	100

[a] All extracts prepared with 10^8 cells/ml. Where indicated, extract was diluted with extraction buffer
[b] Obtained from an exponentially growing cell population
[c] Cells were synchronized in S phase by isoleucine deprivation and hydroxyurea treatment followed by a 6 h growth period in DME-10
[d] Exponentially growing cells were treated with Colcemid for 3 h. The cells remaining after detaching the mitotic cells were used to prepare the extract
[e] Oocytes were matured in vitro by exposure to progesterone, 10 μg/ml, for 15 min

treated with isoleucine deficient medium and hydroxyurea, or Colcemid (table 1). Cytoplasm from progesterone matured oocytes served as a positive control in induction of meiotic maturation. Extraction buffer alone never induced a full meiotic maturation (i.e., complete with white spot and condensed chromosomes adjacent to cortex), but sometimes induced a small percentage of injected oocytes ($\leqslant$5%) to undergo germinal vesicle breakdown, perhaps due to damage by the injection needle.

We next inquired as to whether the mitotic factors were active if RNA or protein synthesis was inhibited in the injected oocyte. It is known that cytoplasm from mature oocytes or cleaving embryos will induce meiotic maturation in the absence of protein or RNA synthesis [3,10,11], but protein synthesis inhibitors block progesterone maturation [9,11,12]. The data in table 2 show that CHO mitotic cell extract is active in the presence of cycloheximide or actinomycin D. Therefore, the active components in CHO mitotic cell extract must bypass some step involving protein synthesis required for progesterone-induced maturation. This is consistent with the fact that maturation induced by CHO mitotic cell extract occurs 1.75–2.25 h after injection, while progesterone-induced maturation takes 4–12 h.

We next investigated the nature of the components responsible for the activity of mitotic cell extracts. Table 3 shows that the active factor(s) is quite heat labile, exhibiting no activity after a 2 h incubation at 37°C. The active factor(s) behaved as a molecule of mol. wt >10 000 upon dialysis. In addition, the factor was sensitive to calcium, losing all activity in the presence of 10 mM calcium.

One of the most interesting characteristics examined was the increased stability of the active factor(s) when the extract was maintained in a buffer designed to inhibit phosphatases (consisting of 150 mM NaCl, 25 mM NaF, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM ATP, pH 6.6). In phosphatase inhibiting buffer, the extract was active for $\geqslant$6 days (at 0°C), while mitotic extract in the original extraction buffer lost all of its activity after 6–12 h. On the basis of this increased stability, we now prepare extract by sonicating mitotic cells in phosphatase inhibiting buffer rather than extraction buffer. These experiments were suggested by the finding [13] that phosphatase inhibitors would prolong the stability of cytoplasmic factors from in vitro matured oocytes; such buffers also stabilize the activity of extracts prepared from cleaving frog embryos (Wasserman, personal communication).

We then tested whether the heat sensitivity of the mitotic inducing factor(s) was due to activation of phosphatases inherently present in the extract. As shown in table 4, the factor(s) was still heat sensitive (37°C, 6 h) in the presence of phosphatase inhibiting buffer. This suggests that activation of phosphatases is not the cause of the heat sensitivity; however, the possibility that phosphatase inhibiting buffer does not completely inhibit phosphatase activity at 37°C cannot be ruled out at present.

Finally, we showed that the active mitotic factor(s) was protein dependent as demonstrated by its sensitivity to papain. Papain was chosen because it is a protease that can be used at room temperature in the presence of EGTA and because it is readily inhibited by antipain; active proteases are toxic to frog oocytes

Table 2
Effect of protein and RNA synthesis inhibitors on activity of mitotic extract

Inducing agent	Treatment of oocytes	% Meiotic maturation[a]
Mitotic cell extract	None	100
Mitotic cell extract	Cycloheximide[b]	100
Mitotic cell extract	Actinomycin D[b]	100
Progesterone[c]	None	100
Progesterone	Cycloheximide[b]	12
Progesterone	Actinomycin D[b]	94

[a] 16 oocytes were used for each experiment
[b] Cycloheximide (40 μg/ml) or actinomycin D (50 μg/ml) were present in the amphibian Ringer's solution bathing the oocytes from 2 h prior to treatment with the inducing agent until fixation of the oocytes
[c] Oocytes were treated with progesterone (10 μg/ml) for 15 min and fixed 12 h later

Table 3
Effects of various treatments of mitotic cell extracts on the induction of meiotic maturation

Treatment of extract	% Meiotic maturation[a]
None	100
Heated to 37°C for 2 h	0
Dialysis[b]	100
Calcium added[c]	0
Papain treated[d]	10
Papain buffer only[e]	100
Ammonium sulfate, 0–20% precipitate[f]	0
Ammonium sulfate, 20–40% precipitate[f]	100
Ammonium sulfate, 40–60% precipitate[f]	10
Ammonium sulfate, 60–100% precipitate[f]	0

[a] 10 oocytes were injected in each experiment
[b] Mitotic cell extract was dialyzed in a Schleicher and Schuell (Keene, NH) collodion bag (exclusion limit of mol. wt 10 000) against extraction buffer to a total dilution of 10^{12}
[c] Mitotic cell extract was dialyzed as above in extraction buffer (without EGTA) containing 10 mM $CaCl_2$
[d] Mitotic cell extract was treated with papain (500 μg/ml) in phosphatase inhibiting buffer containing cysteine (5 mM) and β-mercaptoethanol (0.05 mM) for 1 h at 25°C. Antipain (80 μg/ml) was then added for 10 min at 25°C before injection
[e] Extract was incubated 1.25 h at 25°C in phosphatase inhibiting buffer containing cysteine (5 mM) and β-mercaptoethanol (0.06 mM) before injection
[f] Saturated ammonium sulfate (0.25 vol.) was added to 1 vol. extract at 0°C. The resulting precipitate was centrifuged, resuspended in phosphatase inhibiting buffer and dialyzed against phosphatase inhibiting buffer before injection. More ammonium sulfate was added to the supernatant, and the above process repeated to get the fractions indicated in the table

Table 4
Effects of phosphatase inhibitors on stability of mitotic cell extracts

Treatment of mitotic cell extract[a]	% Meiotic maturation[b]
None	100
0°, 20 h in extraction buffer[c]	10
0°, 20 h in phosphatase inhibiting buffer[d]	100
0°, 144 h in phosphatase inhibiting buffer[d]	100
37°C, 2 h in phosphatase inhibiting buffer[d]	10
Phosphatase inhibiting buffer (no extract)	0

[a] Extracts were originally prepared in extraction buffer
[b] 10 oocytes were injected in each experiment
[c] Extract was dialyzed against extraction buffer (final dilution 10^6 over 6 h) before incubation at 0°C
[d] Extract was dialyzed against phosphatase inhibiting buffer (final dilution 10^6 over 6 h) before incubation at the indicated temperatures

(B. Vogelstein, unpublished data). When mitotic cell extract was treated with papain, and the papain subsequently inhibited with antipain, no meiotic maturation inducing activity remained (table 3). Control experiments showed the amount of antipain used was sufficient to inhibit all papain activity. Other control experiments showed that mitotic cell extract incubated at 25°C for 1.25 h in the buffer used for papain treatment retained its activity. Treatment with RNase and DNase (covalently attached to beads) did not inhibit the activity of the mitotic cell extract (data not shown).

It is also shown in table 4 that the active factor is precipitable with ammonium sulfate at 20–40% saturation. This provides a small (3-fold) purification, as measured by protein determination [14]. This recovery of activity after ammonium sulfate precipitation was not possible in the original extraction buffer which did not contain phosphatase inhibitors.

4. Discussion

The above results show that mitotic CHO cell extracts consistently induce meiotic maturation in immature *X. laevis* oocytes, while extracts identically prepared from interphase cells do not. The data in tables 3, 4 show that the active component in these mitotic extracts is papain sensitive, is probably of mol. wt >10 000, is heat sensitive and precipitable with ammonium sulfate, and stabilized with phosphatase inhibiting reagents. All these data are consistent with the interpretation that the active component is a protein [7]. We have tentatively termed this protein(s) from mitotic CHO cells MIP, mitotic inducing protein(s). Although the assay for its activity is induction of meiotic maturation, it seems reasonable to assume that its natural function in the CHO cell is to induce mitosis.

Biological evidence indicates that the initiating events in mitosis and meiosis must have some features in common; mitotic cell extracts induce meiosis in frog oocytes ([3,4] this work), and meiotic cell cytoplasm induces mitosis in interphase nuclei [15,16]. This work indicates that, similarly, the factor(s) initiating mitosis has physical properties in common with the factors characterized [7,13] initiating meiosis from frog oocytes. Both factors are Ca^{2+} sensitive, heat-labile proteins which are stabilized by phosphatase inhibitors. It is noteworthy that these similarities extend between species as evolutionarily diverse as frog and hamster.

The results in table 4 indicate that reagents which are known to inhibit phosphatases (NaF, β-glycerophosphate, ATP) stabilize the activity of MIP. However, these agents exert many diverse effects besides the inhibition of phosphatases and ATPase [13,17–21]. A variety of studies have suggested that phosphorylation may play a role in meiosis [13,22–24]. It is intriguing to speculate on the possiblity that phosphorylation–dephosphorylation reactions might be involved in the induction and cessation of mitotic events. Definitive evidence to support such speculation must await the further purification of MIP.

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