Amphibian Oocyte Maturation Induced by Extracts of *Physarum polycephalum* in Mitosis

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Abstract. The orderly progression of eukaryotic cells from interphase to mitosis requires the close coordination of various nuclear and cytoplasmic events. Studies from our laboratory and others on animal cells indicate that two activities, one present mainly in mitotic cells and the other exclusively in G_1-phase cells, play a pivotal role in the regulation of initiation and completion of mitosis, respectively. The purpose of this study was to investigate whether these activities are expressed in the slime mold *Physarum polycephalum* in which all the nuclei traverse the cell cycle in natural synchrony. Extracts were prepared from plasmodia in various phases of the cell cycle and tested for their ability to induce germinal vesicle breakdown and chromosome condensation after microinjection into *Xenopus laevis* oocytes. We found that extract of cells at 10–20 min before metaphase consistently induced germinal vesicle breakdown in oocytes. Preliminary characterization, including purification on a DNA–cellulose affinity column, indicated that the mitotic factors from *Physarum* were functionally very similar to HeLa mitotic factors. We also identified a number of mitosis-specific antigens in extracts from *Physarum* plasmodia, similar to those of HeLa cells, using the mitosis-specific monoclonal antibodies MPM-2 and MPM-7. Interestingly, we also observed an activity in *Physarum* at 45 min after metaphase (i.e., in early S phase since it has no G_1) that is usually present in HeLa cells only during the G_1 phase of the cell cycle. These are the first studies to show that maturation-promoting factor activity is present in *Physarum* during mitosis and is replaced by the G_1 factor (or anti–maturation-promoting factor) activity in a postmitotic stage. A comparative study of these factors in this slime mold and in mammalian cells would be extremely valuable in further understanding their function in the regulation of eukaryotic cell cycle and their evolutionary relationship to one another.

DURING the interphase-to-mitosis transition, various dramatic changes occur in the eukaryotic cell. The major changes include the condensation of chromatin into discrete chromosomes, breakdown of the nuclear membrane, disassembly of the cytoskeletal arrays, and formation of the mitotic spindle (Adlakha et al., 1985*a*; Baserga, 1976, 1981; Karsenti et al., 1984; Mazia, 1974; Pardee et al., 1978; Prescott, 1976). Beginning with telophase, these processes are driven in the opposite direction until the initiation of DNA synthesis. The nuclear envelope re-forms and chromosomes begin to decondense. The chromatin reaches its most decondensed state by the end of the G_1 period when it becomes accessible for DNA replication. After replication of chromosomes, the whole process of major macromolecular reorganization of the nucleus associated with mitosis restarts. Although the mechanisms for the control of these events are not well understood, several lines of evidence suggest that there are factors (proteins) present in cells during mitosis and meiosis that are absent during interphase (Adlakha et al., 1982*a*, *b*; Al-Bader et al., 1978; Johnson and Rao, 1970; Sunkara et al., 1979*a*, *b*, 1982; Wasserman and Smith, 1978). Identification of these proteins would improve our understanding of how mitosis and meiosis are regulated.

Two lines of evidence suggest the existence of inducers for both mitosis and meiosis. The first comes from cell fusion experiments in which fusion of mitotic HeLa cells with interphase cells results in the breakdown of the nuclear envelope and the transformation of the interphase chromatin into prematurely condensed chromosomes (Johnson and Rao, 1970). The second line of evidence is that cytoplasmic extracts of mature *Xenopus laevis* frog oocytes (arrested in meiotic metaphase can induce germinal vesicle breakdown (GVBD)[1] and chromosome condensation when microinjected into immature oocytes (stage VI) (Masui and Markert, 1971; Smith and Ecker, 1971; Wasserman and Masui, 1976; Wasserman and Smith, 1978; Drury, 1978; Wu and Gerhart, 1980). These two approaches converged when it was shown that extracts

1. *Abbreviations used in this paper*: GVBD, germinal vesicle breakdown; IMFs, inhibitors of the mitotic factors; MPA, maturation-promoting activity; MPF, maturation-promoting factor.

of mitotic cells of different phylogenetic origins caused GVBD when microinjected into immature *Xenopus* oocytes (Wasserman and Smith, 1978; Sunkara et al., 1979*a*; Nelkin et al., 1980; Weintraub et al., 1982; Kishimoto et al., 1982; Halleck et al., 1984*a*). This suggested that some factor in both mitotic and meiotic cells is shared by both mitotic and meiotic induction pathways. It was called maturation-promoting factor (MPF) because it caused the maturation of immature oocytes; it was also called mitotic factor, because it was found in the extracts of all mitotic cells. Recently, it has been referred to as M-phase factor to represent both mitotic and meiotic sources (Gerhart et al., 1984).

Despite intense investigations, progress in the molecular characterization of M-phase factor has been limited (Adlakha et al., 1985*c*; for reviews see Adlakha and Rao, 1986, 1987*a*, *b*). Recently, several in vitro systems have been developed in which addition of crude or partially purified M-phase factor induces mitosis-like events. However, it is not clear how many intermediate steps occur between the addition of M-phase factor and the observed changes (Miake-Lye et al., 1983; Miake-Lye and Kirschner, 1985; Lohka and Masui, 1983; Lohka and Maller, 1985; Burke and Gerace, 1986; Newport and Spann, 1987; Murray, 1987*a*).

Recently, we described factors in G_1-phase HeLa cells that destroy the activity of mitotic factors. The G_1 factors seem to be activated, rather than newly synthesized, as the cell enters telophase and the chromosomes begin to decondense. Moreover, the G_1 factors can be activated in G_0 cells or Colcemid-arrested mitotic cells by UV irradiation. The G_1 cell extracts, upon incubation with mitotic cell extracts, inactivate maturation-promoting activity (MPA; Adlakha et al., 1983, 1984*a*), induce dephosphorylation of mitotic nonhistone proteins (Adlakha et al., 1984*b*) and mitosis-specific phosphoprotein antigens (Adlakha et al., 1985*a*), and specifically decrease the activity of the mitosis-specific kinases (Halleck et al., 1984*b*). Several other groups have recently described similar factors at the end of meiosis that inactivate MPF (Gerhart et al., 1984; Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Newport, 1987). These factors are called inhibitors of the mitotic factors (IMFs) or anti-MPF or G_1-phase factors (Adlakha and Rao, 1986, 1987*a*, *b*). These results suggest that the G_1 factors may play an important role in the transition of cells from mitosis to G_1 phase and in the process of nuclear envelope re-formation and chromosome decondensation.

In this report, we demonstrate the presence of the M-phase and the G_1-phase factors during the mitotic and postmitotic stages of the slime mold, *Physarum polycephalum*, in which mitosis is characterized by chromosome condensation and distribution in the absence of nuclear membrane breakdown. The preliminary biochemical characterization of these factors from *Physarum polycephalum* show them to be very similar, if not identical, to those from HeLa cells. Moreover, we have identified a number of mitosis-specific antigens in extracts from *Physarum* plasmodia, similar to HeLa mitotic cells and mature *Xenopus* oocytes, using mitosis-specific monoclonal antibodies.

Materials and Methods

Cells and Cell Synchrony

HeLa Cells. HeLa cells were routinely grown as suspension cultures in spinner flasks at 37°C in MEM (Gibco, Grand Island, NY) supplemented with 5% heat-inactivated FCS (KC Biological, Inc., Lenexa, KS), sodium pyruvate, nonessential amino acids, and a penicillin–streptomycin mixture (Gibco) in a humidified 5% CO_2 and air atmosphere, as described earlier (Rao and Engelberg, 1965). These cells have a cell cycle time of 22.0 h, consisting of a 10.5-h G_1 period, a 7.0-h S period, a 3.5-h G_2 period, and a 1.0-h mitosis (Rao and Engelberg, 1966).

To obtain mitotic cells, HeLa cells in exponential growth were plated either in 1,585-cm^2 borosilicate roller bottles (Bellco Glass Inc., Vineland, NJ) or in 150-mm Lux culture dishes as recently described (Adlakha et al., 1982*b*). Briefly, cells were partially synchronized into S phase by a single excess thymidine block (2.5 mM) of 20 h. After reversal of the block by washing with medium, cells were incubated in fresh medium containing Colcemid (0.05 μg/ml; Ciba Pharmaceutical Co., Summit, NJ) for another 20 h at 37°C or arrested in mitosis using N_2O blockade as described before (Rao, 1968). Mitotic cells were then harvested by selective detachment. These procedures routinely yielded a population with a mitotic index of 90–98%.

HeLa cells synchronized in G_1 phase were obtained by reversing the N_2O blockade of mitotic cells as described previously (Rao, 1968). Within 1.5 h after reversal of N_2O block, >90% of the cells had completed mitosis and entered G_1.

Physarum polycephalum Plasmodia. *Physarum polycephalum* strain TU29I was grown as an agitated suspension of microplasmodia for stock cultures, and macroplasmodia were prepared by fusion of microplasmodia in exponential growth phase on filter paper as described previously (Daniel and Baldwin, 1964; Mittermayer et al., 1965). Experiments were done during the second and third postfusion mitosis (MII and MIII). The cell cycle lasts 9.5 ± 0.5 h at 26°C, and metaphase of mitosis occurs with natural synchrony within 5 min throughout a whole macroplasmodium of an ~5-cm diam. The time of mitosis and the mitotic stages were determined by observation under a phase-contrast microscope. S phase begins in telophase and lasts 3 h.

Preparation of Extracts from Mitotic and Interphase HeLa Cells, Xenopus Oocytes, and Physarum Cultures. HeLa cells synchronized in various phases were collected by centrifugation at 600 *g* for 5 min at 4°C. After three to five washings with MEM without serum at 4°C, the extracts were prepared as described earlier (Adlakha et al., 1982*a*, 1983). Briefly, mitotic cells (4×10^7 cells/ml) were suspended in a buffer containing 10 mM Na_2HPO_4/NaH_2PO_4, 200 mM NaCl, 2 mM EGTA, 10 mM $MgSO_4$, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 5 mM sodium β-glycerolphosphate, and 10% glycerol at pH 6.5. Cells were disrupted by sonication with an ultrasonic cell disruptor (Heat Systems Ultrasonics, Inc., Farmingdale, NY) at a setting of six at 4°C. Three 10-s pulses were used for sonication, each separated by a 30-s interval. Phase-contrast microscopy indicated that sonication invariably disrupted >98% of the cells. The sonicate was then centrifuged at 100,000 *g* for 60 min at 4°C in an ultracentrifuge (L5-50; Beckman Instruments, Palo Alto, CA). The supernatants were stored at −70°C. For *Physarum*, plasmodia grown on filter papers were harvested at various stages during the cell cycle and immediately frozen in liquid nitrogen and lyophilized. Dried powdered plasmodia (1 gm lyophilized cells/ml) were extracted essentially as described above for HeLa cells except that the extraction buffer contained several additional protease inhibitors: 0.1 mM sodium *p*-tosyl-L-lysine chloromethyl ketone (TLCK), 0.5 mM *N*-ethyl maleimide (NEM), 5 μg/ml leupeptine, 2 μg/ml α-2 macroglobulin, and 0.1 mM *p*-chloro-mercurisulfonic acid, all of which were purchased from Sigma Chemical Co., St. Louis, MO. Extracts from immature and mature oocyte of *Xenopus laevis* stage VI were prepared exactly according to procedures described (Drury, 1978; Gerhart et al., 1984; Nguyen-Gia et al., 1986). The protein content of the soluble extracts was monitored by using the Coomassie Brilliant Blue G-250 binding assay according to Bradford (1976); BSA was used as the standard.

Preparation of Xenopus laevis Oocytes and Assay for MPA. Stage VI oocytes were obtained by surgically removing a portion of an ovary from a *Xenopus* female. All operations on oocytes were conducted using Barth's modified medium (Merriam, 1972) supplemented with 0.6 mM $MgCl_2$. Cell extracts or protein fractions were assayed for MPA by injection of 60–70 nl into each oocyte. Injected oocytes were inspected for GVBD after 2–3 h. GVBD was detected as a depigmentation of an area of the animal hemisphere (Woodland, 1974). The presence or absence of the germinal vesicle was also determined by fixation in 7.5% TCA and dissection of the oocyte. For every set of oocytes removed from *Xenopus*, a batch was always tested for normal maturation by stimulation with progesterone (10 μg/ml) for 15 min and incubation for 8 h before scoring for GVBD induction. A batch of 10 oocytes injected with the extraction buffer served as control for every series of injections with extracts.

Assay for the Activity of IMFs or Anti-M Factors. HeLa G_1 cell ex-

tracts or extracts from postmitotic *Physarum polycephalum* cells with a protein concentration equal to that of the mitotic HeLa cell extracts to start with were mixed with HeLa mitotic cell extracts in various proportions to obtain mitotic HeLa extract concentrations of 100, 75, 66.6, 50, 33.3, 25, 20, 10, and 0% in the injection mixtures. Mixtures of mitotic extracts with extraction buffer in similar proportions served as controls. After incubation at 4°C for 1 h the mixtures were injected into *Xenopus* oocytes to determine their MPA. If an extract was able to inactivate the mitotic factors completely at a 50% dilution (i.e., mixtures containing mitotic and G_1 proteins in a ratio of 1:1), it was considered to have an anti-M-phase factor activity of 100%.

Purification of Mitotic Factors. M-phase factors from mitotic *Physarum polycephalum* cultures were purified on DNA-cellulose affinity chromatography and on a TSK-250 column by HPLC exactly as recently described for HeLa cell mitotic factors (Adlakha et al., 1985*c*; Adlakha and Rao, 1986, 1987*a*, *b*).

PAGE. Cell extracts or protein fractions with MPA were separated electrophoretically on either an 8 or a 6–20% acrylamide gradient gel containing 0.1% SDS according to Laemmli (1970). The gels were subsequently stained with Coomassie Brilliant Blue. Prestained high molecular mass standards from Sigma Chemical Co. were used as markers.

Antibodies. Isolation of two hybridoma clones designated, MPM-2 and MPM-7, that react specifically with mitotic cells have been described earlier (Davis et al., 1983, 1986). Monoclonal antibody MPM-2 is an IgG_1 antibody with kappa light chains and reacts with a family of phosphoprotein antigens found only during mitosis (Davis, 1983). Antibody MPM-7 is also an IgG with kappa light chains and recognizes a major phosphoprotein antigen with relative molecular mass of 100 kD in the extracts of mitotic HeLa cells (Rao et al., 1987).

Identification of Antibody-reactive Polypeptides and Immunoblotting. Polypeptides from crude extracts or partially purified mitotic fractions from HeLa, *Physarum polycephalum*, and *Xenopus laevis* oocytes were separated by 8% SDS-PAGE, electrophoretically transferred onto nitrocellulose sheets (Towbin et al., 1979), and stained with either MPM-2 or MPM-7 antibody. In some cases whole cells were washed with medium lacking serum and then solubilized in sample buffer containing 3% SDS; the proteins were separated by SDS-PAGE. The second antibody was goat anti-mouse IgG conjugated with alkaline phosphatase.

Immunofluorescence Staining. Indirect immunofluorescence was performed as described (Davis et al., 1983). Cells deposited on slides by using a cytocentrifuge were air dried, fixed in methanol for 10 min at 22°C, and air dried. The cells were overlaid with 75 μl of appropriate dilution of ascites fluid as primary antibody. FITC-conjugated rabbit anti-mouse IgG (heavy and light chains; Miles Scientific Div., Naperville, IL) was used as the indicator antibody. Cell surface and intracellular antigens were stained by this method. Fluorescence intensity of individual cells was measured at 550–600 nm on a Leitz Orthoplan microscope equipped with epifluorescence, an "I2" filter cube, and a MPV microscope photometer. Blank readings were obtained from areas on the slide adjacent to the cells measured. Cells on slides stained with antisera obtained before immunization (preimmune) yielded readings equivalent to that of the blanks.

Results

MPA during the Cell Cycle of Physarum polycephalum

Plasmodia of the slime mold *Physarum polycephalum* provides an important model system for the study of the cell cycle. These giant cells can contain 10^8 nuclei (5–7 cm diam) that divide every 8–10 h in virtually perfect natural synchrony. One can monitor the molecular details of the progress of a single cell throughout the mitotic cycle by repeated sampling from a single plasmodium. Observation of the synchronous mitosis defines the chronology of the cell cycle. Mitosis is followed by a 3-h S phase and a 6–7-h G_2 phase which leads to the next mitosis. The absence of a G_1 phase is a remarkable feature of the mitotic cycle of *Physarum* (Sauer, 1986). In *Physarum* the nuclear membrane does not breakdown during mitosis as in yeast cells (Lafontaine and Cadrin, 1982; Laffler and Carrino, 1986), yet the mitotic extracts of yeast can induce both GVBD and chromosome condensation in *Xenopus* oocytes (Weintraub et al., 1982). We, therefore, sought to determine if the M-phase factor activity could also be observed in *Physarum* cells.

Initially, we prepared extracts from synchronized plasmodia of *P. polycephalum* at various stages during the cell cycle using buffers identical to the ones we used for HeLa cells, and injected these into *Xenopus* oocytes to assay for MPA as described earlier (Adlakha et al., 1982*a*). However, we observed that extracts from every stage of the cell cycle were extremely toxic to the oocytes at dilutions up to 33%. At lower dilutions, these extracts did not cause any toxic effects when injected into oocytes, but when injected into progesterone-stimulated oocytes their maturation was completely inhibited. Increasing the concentration of EGTA or dialyzing the extract did not reduce the inhibitory effect. However, incubation of these extracts at 60°C for 15 min destroyed this inhibitory activity and toxicity (data not shown).

These results suggested that the extracts might contain a large pool of proteases that inactivate MPF in oocytes. To test this possibility, we included in our extraction buffer a number of other protease inhibitors (see Materials and Methods) and extracted *Physarum* plasmodia collected at different stages of the cell cycle. As shown in Table I, extracts prepared at either 10 or 20 min before mitosis consistently induced GVBD and chromosome condensation in oocytes within 2–3 h as was the case for HeLa mitotic cell extracts.

Table I. MPA of the Extract of Physarum polycephalum Cells from Different Stages of the Cell Cycle

Oocytes injected with extracts of cells from	Dilution extract/buffer	Proteins injected*	Oocytes showing GVBD/oocytes injected‡§	Induction of GVBD
	vol/vol	*ng*		%
20 min before mitosis‖	1:0	296	30/30	100
	1:1	148	36/36	100
	1:2	99	8/32	25
10 min before mitosis‖	1:0	308	40/40	100
	1:1	154	30/30	100
	1:2	103	20/25	80
	1:3	77	10/30	33
45 min after mitosis¶	1:0	328	0/25	0
2 h after mitosis	1:0	318	0/30	0
6 h after mitosis	1:0	288	0/20	0
Extraction buffer	–	–	0/40	0
HeLa mitotic cell extract	1:1	186	30/30	100

* A total volume of 65 nl of the extracts was injected into each oocyte, and oocytes were scored for GVBD 2–3 h after injection. Typically oocytes from the same female were used for a given experiment.

‡ Data presented is representative of several different injection experiments involving different batches of extracts with extremely identical results and differences in GVBD induction between experiments did not exceed 10%.

§ The percentage of GVBD in the injected oocytes was determined by scoring the oocytes for the appearance of a white spot in the animal hemisphere. These data were confirmed by fixing oocytes in 7.5% TCA and dissecting to check for the breakdown of the germinal vesicle.

‖ Extracts of *Physarum* in mitosis were injected into oocytes with or without pretreatment (incubation for 60 min at 19°C) with cycloheximide (20 μg/ml). After injection, incubation was continued in presence of cycloheximide. The cycloheximide treatment had no effect on the maturation of oocytes.

¶ For *Physarum* cells, extracts were prepared using ~1 g of dried cells/ml of extraction buffer containing various protease inhibitors as indicated in Materials and Methods.

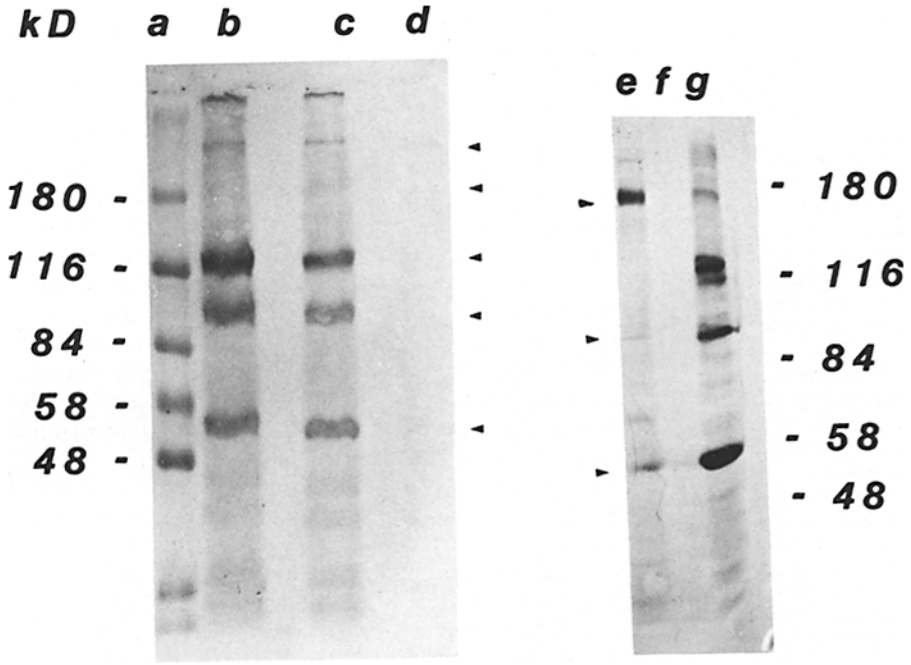

Figure 1. Electroimmunoblots of extracts of HeLa, *Xenopus* oocytes, and *Physarum polycephalum* cells in mitosis and interphase. Polypeptides were separated by 8% SDS-PAGE, electrophoretically transferred to nitrocellulose sheets, and stained with MPM-2 antibody. The second antibody was goat anti-mouse IgG conjugated with alkaline phosphatase. Approximately 40 μg of protein per slot was loaded. Lane *a*, molecular mass markers; lane *b*, synchronized mitotic HeLa cells; lane *c*, *Physarum* cells 10 min before mitosis; lane *d*, *Physarum* cells 45 min after mitosis; lane *e*, mature oocytes of *Xenopus*; lane *f*, immature oocytes of *Xenopus*; lane *g* mitotic HeLa cells. Arrows point to the major bands recognized by MPM-2. It is worth noting that the antigens stained by MPM-2 in HeLa (lane *b*) and *Physarum* (lane *c*) appear to be identical.

This activity could be detected in these extracts even when they were diluted to 25 or 33%, respectively. The extracts from cells collected 10 min before mitosis were slightly more active than those collected 20 min before mitosis. Injection of HeLa mitotic extract and extraction buffer into oocytes served as the positive and negative controls, respectively. Cytological examination of the injected oocytes followed by dissection revealed the breakdown of the germinal vesicle in oocytes that matured; the oocytes that did not mature had normal germinal vesicles. Extracts of plasmodia collected at 45 min, 2 h, or 6 h after mitosis, representing early S, late S, and G_2 phases, did not induce any GVBD in *Xenopus* oocytes at all the dilutions tested, or even when two- to threefold concentrated extracts were prepared. These results show for the first time that the MPA is present in *Physarum polycephalum*, initially appearing in late G_2 phase, reaching a peak in mitosis, and becoming undetectable in postmitotic cells.

Biochemical Characteristics of the M-Phase Factor from Physarum polycephalum

Using the GVBD-inducing system in oocytes, we studied certain characteristics of the M-phase factor activity in *Physarum*. These studies indicated that this activity was Ca^{2+} sensitive, Mg^{2+} dependent, and resided in a heat-labile protein with a molecular mass of 100 kD as determined by chromatography on Sephacryl S-200 (data not shown). We have also attempted to purify this activity using affinity chromatography on DNA-cellulose followed by HPLC on TSK-250 column as previously reported for HeLa mitotic factors (Adlakha et al., 1985*c*; Adlakha and Rao, 1986, 1987*a*, *b*). The activity from the *Physarum* extracts eluted exactly in the same fractions as HeLa mitotic factor activity did. These procedures usually result in a purification of ~1,600-fold (Adlakha and Rao, 1987*b*). Furthermore, we observed that the activity from *Physarum* cells was also stabilized by the presence of phosphatase inhibitors (ATP, NaF, and sodium β-glycerol phosphate) and induced maturation even in *Xenopus* oocytes pretreated with the protein synthesis inhibitor cycloheximide (Table I). These results further support the notion that the M-phase factors involved in the initiation of mitosis, meiosis, or prematurely condensed chromosomes are very similar, if not identical.

Identification of Mitosis-specific Antigens in Physarum

Davis et al. (1983, 1986), in our laboratory, have recently reported the isolation of two monoclonal antibodies, designated MPM-2 and MPM-7, that specifically react only with mitotic cells of all species tested and not with interphase cells. In mitotic HeLa cells, the major antigens recognized by MPM-2 are of 70, 118, and 182 kD (Fig. 1), and by MPM-7 is a protein of ~100 kD (Fig. 2). We determined whether the antigens recognized by MPM-2 and MPM-7 antibodies in *Physarum* were similar to those recognized in HeLa cells. The proteins from metaphase HeLa cells, immature and mature *Xenopus* oocytes, and interphase and mitotic *Physarum* cells were electrophoretically separated by 8% SDS-PAGE, electrophoretically transferred to nitrocellulose paper, and incubated with MPM-2 (Fig. 1) and MPM-7 (Fig. 2). We observed that both these antibodies recognized antigens very strongly in mitotic *Physarum* cells with an insignificant staining in interphase. Furthermore, the molecular masses of the antigens recognized in *Physarum* were very similar to the ones recognized by MPM-2 and MPM-7 in HeLa cells. Indirect immunofluorescence staining with MPM-2 antibodies located the antigens into the *Physarum* nuclei, while antibody MPM-7 stained the cytoplasm as well as nuclei in metaphase plasmodia as is the case in mammalian cells (Davis et al., 1983, 1986). Preimmune serum did not show any staining (data not shown). These results suggest that these mitotic-specific protein antigens are well conserved during evolution.

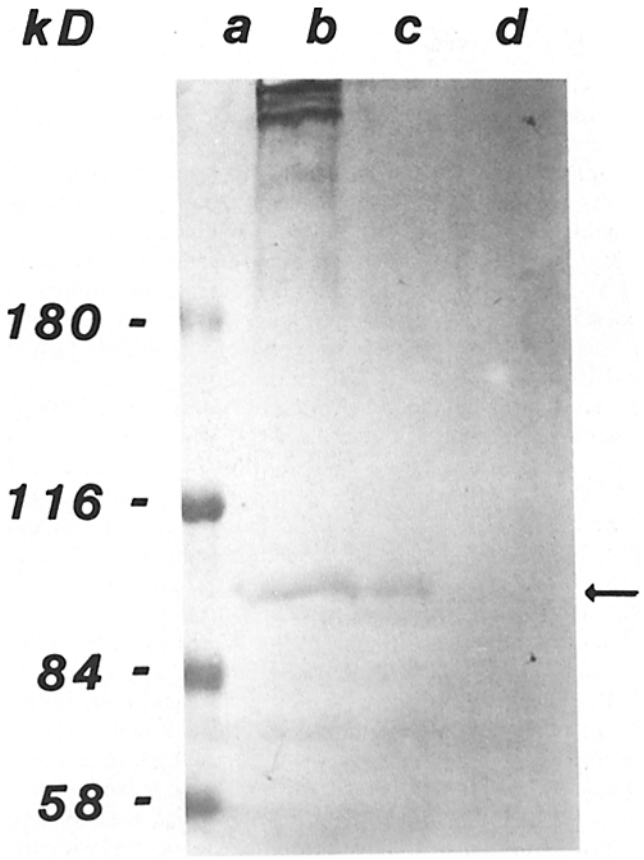

Figure 2. Electroimmunoblots of extracts of HeLa and *Physarum polycephalum* cells synchronized in mitosis and an interphase population. Polypeptides were separated by 8% SDS-PAGE, electrophoretically transferred to nitrocellulose sheets, and stained with MPM-7. The second antibody was goat anti-mouse IgG conjugated with alkaline phosphatase. Approximately 40 μg of protein per slot was loaded. Lane *a*, molecular mass markers; lane *b*, synchronized mitotic HeLa cells; lane *c*, *Physarum* cells 10 min before mitosis; lane *d*, *Physarum* cells 45 min after mitosis. Arrow points to a 100-kD band recognized by MPM-7.

The Inhibitors of the Mitotic Factors or Anti-M-phase Activity during the Cell Cycle of Physarum

We have previously demonstrated that when mitotic cell extracts were mixed with extracts of G_1-phase cells in various proportions and the mixtures injected into *Xenopus* oocytes, the MPA of the mitotic factors was inhibited in a dose-dependent manner. This inhibitory activity was present only in G_1-phase cells and not in S- or G_2-phase cells (Adlakha et al., 1983). However, in V79-8 cells, which lack G_1 and G_2 periods in their cell cycle, this activity was manifest in only early S-phase cells (Rao and Adlakha, 1985). These factors have been called IMFs or G_1-phase factors. We have, therefore, examined whether similar activity is present in postmitotic *Physarum* even though it lacks a G_1 phase in its cell cycle. Extracts were prepared from *Physarum* plasmodia at 10 min, 45 min, 2 h, or 6 h after mitosis (i.e., in anaphase or telophase, in early S, late S, and mid-G_2, respectively), mixed with HeLa mitotic extracts in various proportions, and injected into *Xenopus* oocytes. Dilutions of HeLa mitotic extract with extraction buffer in similar proportions served as control. As shown in Table II, extracts of *Physarum* at 45 min after mitosis completely neutralized the activity of HeLa M-phase factors when the *Physarum* extract concentration was 33% or higher. Extracts of *Physarum* plasmodia from 2 h after mitosis also exhibited this anti-M-phase activity but were slightly less active than extracts of *Physarum* at 45 min after mitosis. No inhibitory activity was observed in extracts of *Physarum* at 6 h after mitosis at every dilution tested (data not shown). These assays have been repeated several times with different batches of extracts with very similar results. We also observed that the extracts of *Physarum* at 45 min after mitosis completely inhibited the M-phase activity present in *Physarum* at 10 min before mitosis (data not shown). These data indicate that an anti-M-phase activity similar to the G_1-phase factor activity described earlier in HeLa cells (Adlakha et al., 1983) is present in postmitotic *Physarum* and that this activity fluctuates in a cyclical manner during the cell cycle.

Table II. Inactivation of the MPA of the HeLa Mitotic Cells by Extracts from Physarum in a Postmitotic Stage

Oocytes injected with HeLa mitotic cell extract diluted with	Dilution* HeLa mitotic extract/buffer or *Physarum* extract	Proteins injected	Oocytes showing GVBD/oocytes injected	Induction of GVBD
	vol/vol	*ng*		%
Extraction buffer (control)	1:0	408	25/25	100
	1:1	204	30/30	100
	1:2	136	36/40	90
	1:3	102	10/30	33
Extracts of *Physarum* at 10 min after mitosis	1:0	408	25/25	100
	2:1	398	0/10	0
	1:1	393	0/15	0
	1:2	388	0/15	0
	1:3	385	0/15	0
Extracts of *Physarum* at 45 min after mitosis	2:1	382	0/25	0
	1:1	368	0/30	0
	1:2	356	0/40	0
	1:3	382	0/30	0
Extracts of *Physarum* at 2 h after mitosis	2:1	382	12/30	40
	1:1	363	0/25	0
	1:2	348	0/36	0
	1:3	376	0/30	0

* HeLa mitotic cell extract was mixed with either the extraction buffer (control) or with *Physarum* extracts and incubated for 1 h at 4°C before injection into oocytes. All conditions for preparation of extracts, injection into oocytes, and scoring of GVBD were as described in footnote to Table I. The data presented here are an average of five different experiments.

Discussion

In this report, we have demonstrated the existence of two activities, one present mainly in mitotic cells and the other exclusively in postmitotic plasmodia of the slime mold, *Physarum polycephalum*. Using microinjection of extracts from plasmodia at various stages during the cell cycle into *Xenopus* oocytes we have shown the presence of an activity similar to the M-phase factor. This activity was first detectable in late G_2 cells, reached a peak in mitosis, and was undetectable

in postmitotic cells. Using two monoclonal antibodies directed against proteins present during mitosis of HeLa cells, MPM-2 and MPM-7 (Davis et al., 1983, 1986), we have identified *Physarum* proteins on Western blots with identical mobilities. We observed that the levels of the respective *Physarum* antigens fluctuate in the cell cycle and reach a maximum level around mitosis. These results correlate with the changes in *Physarum* M-phase factor activity. We have previously shown that MPM-2 reacts with a family of phosphoproteins in mitotic cells and that phosphatase treatment abolished their detection (Davis et al., 1983). MPM-7 also recognizes a phosphoprotein of ~100 kD in mitotic cells (Rao et al., 1987).

M-phase factor activity was initially identified as a natural inducer of meiosis in frog oocytes (Masui and Markert, 1971; Smith and Ecker, 1971) and has subsequently been found in mitosis in all eukaryotic cells from yeast to human cells in culture (Wasserman and Smith, 1978; Sunkara et al., 1979*a*; Nelkin et al., 1980; Weintraub et al., 1982; Kishimoto et al., 1982; Halleck et al., 1984*a*). More recently it has been shown that the addition of partially purified M-phase factor to cells or cell lysates containing interphase nuclei caused these nuclei to go into mitosis even in the presence of cycloheximide, a protein synthesis inhibitor (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985; Burke and Gerace, 1986; Newport and Spann, 1987). A gradual disappearance of M-phase activity in these cells or inactivation by calcium resulted in the exit of cells from M phase as evidenced by the decondensation of chromosomes, nuclear envelope re-formation, and resumption of DNA synthesis (Lohka and Masui, 1984). These results suggest that the addition and removal of M-phase factor activity is sufficient to induce multiple cycles of mitosis and interphase, respectively. The demonstration of this activity in *Physarum* cells is important because *Physarum* may provide an inexpensive and convenient source of these factors. Furthermore, the investigation of the biology of this activity in *Physarum* is even more significant since mitosis in this organism is characterized by chromosome condensation in the absence of nuclear envelope breakdown (Sauer, 1986).

What is M-phase factor? Is the M-phase activity present in *Physarum* similar to the one observed in HeLa mitotic cells and mature *Xenopus laevis* oocytes? What is its role in the induction of mitosis and meiosis? One approach to identifying and characterizing this activity has been to purify it by traditional biochemical methods. However, progress in the purification of M-phase factor from either mature oocytes or mitotic HeLa cells has been slow (Wu and Gerhart, 1980; Adlakha et al., 1985*c*; Nguyen-Gia et al., 1986). Using affinity chromatography on DNA-cellulose in combination with HPLC we can generally obtain a 1,600–2,000-fold, and sometimes even a 5,000-fold purification of M-phase factor from mitotic HeLa cells (Adlakha and Rao, 1986; 1987*a*, *b*). Although this is the purest preparation of M-phase factor yet obtained, it is still not homogeneous by SDS-PAGE analysis and its activity is very unstable. Due to its low abundance and instability, it is very difficult to purify M-phase factor to homogeneity using only standard biochemical methods.

Recently Nguyen-Gia et al. (1986) for the first time reported a 200-fold purification of MPF from mature oocytes of *Xenopus laevis*. Analysis by SDS-PAGE of the 200-fold purified MPF exhibited five proteins with molecular masses ranging from 37 to 62 kD. We have also observed that a protein kinase activity independent of cAMP, Ca^{2+}, or calmodulin, and neither inhibited by heparin nor stimulated by spermine appears to be associated with the 1,600-fold purified mitotic factors. This purified fraction, like the crude extracts, can induce maturation even in the presence of cycloheximide (Adlakha et al., 1985*b*; Adlakha and Rao, 1986, 1987*a*, *b*). Our present results indicate that the characteristics of M-phase factors from *Physarum* are extremely similar, if not identical, to those from HeLa cells.

The molecular mechanisms by which the mitotic factors induce meiotic maturation in *Xenopus* oocytes are not yet completely understood; however, recent studies from our laboratory and those of others strongly suggest that protein phosphorylation is the most likely possibility (Gerace and Blobel, 1980; Sahasrabuddhe et al., 1984; Karsenti et al., 1987; Lohka et al., 1987; and for reviews see Adlakha and Rao, 1987*a*, *b*; Halleck et al., 1987). These findings taken together support the notion that the mitotic factors or MPF either act as a kinase in vivo or as an activator of an in vivo protein kinase cascade responsible for the induction of GVBD and chromosome condensation (Miake-Lye and Kirschner, 1985; Newport and Spann, 1987; Adlakha and Rao, 1987*a*, *b*). John Newport's laboratory has recently shown, using an in vitro system, that MPF is a pleiotropic cytoplasmic factor that activates various other activities involved in lamin depolymerization, membrane vesicularization, and chromosome condensation. Several of these processes associated with nuclear breakdown during mitosis were biochemically independent of each other. They have shown that mitotic extracts can be depleted of activities needed to induce the breakdown of the nucleus without removing MPF itself. The depleted extracts, although no longer able to breakdown nuclei themselves, retain the ability to turn on mitotic activities in interphase eggs or extract (Newport, 1987; Newport and Spann, 1987). These results might explain why, though MPF activity in *Physarum* is present, the nuclear envelope does not break down; yet these plasmodia extracts can induce nuclear envelope breakdown in oocytes, which are known to contain stockpiles of other activities (Woodlands and Adamson, 1977; Newport and Kirschner, 1982, 1984; Masui and Clarke, 1979; Ford, 1985; Zierler et al., 1985). The *Physarum* system could therefore provide a less complicated source for the isolation of M-phase activity.

Additionally, *Physarum* may provide an alternative and suitable model for studying the molecular biology of the cell cycle–regulated gene expression. Recently, several genes related to the expression of M-phase factor have been cloned. One of them is defective in the $tsBN_2$ cell line (Kai et al., 1986). The mutant phenotype suggests that the messenger RNA for mitotic inducers is transcribed during S phase but only translated rapidly in G_2-M phase. The action of the gene defective in the mutant is hypothesized to be responsible for the posttranscriptional control of the synthesis of mitotic inducers. Therefore, the failure of it to appropriately function at the nonpermissive temperature causes premature induction of mitotic events.

Another gene which is related to M-phase factor and already cloned is cyclin A from surf clam (Swenson et al., 1986). Cyclin A rises and falls in parallel with the entry into and exit from meiosis and mitosis in several species other

than surf clam (Rosenthal et al., 1980; Evans et al., 1983). More interestingly, it can induce meiosis when it is synthesized in *Xenopus* oocytes after microinjection of its mRNA (Swenson et al., 1986). These results point to the possibilities that cyclin A could be either a component of active M-phase factor or the activator of M-phase factor (Murray, 1987*a*, *b*). Meiosis I occurs perfectly well in clam in the absence of detectable cyclin A, and immunoblots with anti-clam cyclin A antibody reveal no cyclin A-related proteins in clam, *Xenopus* oocytes, or in metaphase II-arrested *Xenopus* eggs. Furthermore, fertilized clam oocytes proceed perfectly well through meiosis I (but not meiosis II or mitosis) in the absence of any preexisting or newly synthesized cyclin A (Swenson et al., 1986). These data argue against cyclin A being the M-phase factor.

The laboratory of Paul Nurse has cloned three yeast genes involved in mitotic induction (cdc2, cdc25, and wee 1) using the fission yeast *Schizosaccharomyces pombe* (Russell and Nurse, 1986, 1987; Lee and Nurse, 1987). Of these, the cdc2 gene product is absolutely required for the entry into mitosis and S phase. However, a recent report from their laboratory showed that the human cdc2 gene, as well as yeast cdc2, encodes a protein kinase of 34 kD (Lee and Nurse, 1987), which is much smaller than MPF. Thus, though related, the cdc2 gene product and MPF are most likely different. At present there has been no successful attempt to clone the M-phase factor gene from vertebrate cells, and all the hypotheses about the relationship of the already cloned genes with M-phase factor remain to be tested directly after it has been identified and cloned.

After completion of mitosis, the cell must reassemble the nucleus and chromosomes should decondense. Like nuclear envelope breakdown and chromosome condensation, this reassembly process has also been shown by both in vivo and in vitro studies to require another protein factor that is antagonistic to the action on M-phase factor (Adlakha et al., 1983; Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985; Suprynowicz and Gerace, 1986; Gerace, 1986). Using inactivation of HeLa M-phase factors as the biological assay, we demonstrated in postmitotic plasmodia of *Physarum*, the presence of an anti-M-phase factor activity. This activity rises and falls in a cyclical manner. Since these cells do not have a G_1 period, this activity manifests mainly in early S phase. We could detect this anti-M-phase activity as early as 10 min after mitosis when the cells were in anaphase or telophase. We have previously shown that in V79-8 cells lacking G_1 and G_2 periods in their cell cycle, this inhibitory activity was observed also only in early S-phase cells (Rao and Adlakha, 1985). Although it has been shown that the progression of mitotic cells into anaphase can be triggered by an increased concentration of calcium (Lohka and Masui, 1984; Suprynowicz and Gerace, 1986), our results indicate that the inhibitory activity observed in postmitotic cells of *Physarum* is due to a protein, as shown previously for IMF in HeLa cells (Adlakha et al., 1983), and that the anti-M-phase factor may play an active role in triggering anaphase.

In conclusion, the results of the present study further support the notion that the initiation and completion of mitosis is regulated by positive and negative controlling elements and that these activities do not exhibit any species specificity. A comparative study of these activities and the antigens recognized by mitosis-specific monoclonal antibodies in *Physarum* and in mammalian cells should broaden our understanding of their function in the regulation of the eukaryotic cell cycle and their evolutionary relationship to one another.

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