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Activation of Myelin Basic Protein Kinases during Echinoderm Oocyte Maturation and Egg Fertilization

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At least five activated protein kinases were detectable in soluble extracts from maturing as compared to immature sea star oocytes. These kinases could be distinguished on the basis of the time courses of their activation following exposure of the oocytes to 1-methyladenine, their substrate specificities, and their chromatographic properties on DEAE-Sephacel and Sephacryl S-200. A histone H1 kinase (HH1K) (M_r 110,000) underwent maximal activation near the time of 1-methyladenine-induced germinal vesicle breakdown (GVBD). When myelin basic protein (MBP) was used as a substrate, HH1K and two additional kinases (MBPK-I and MBPK-II) were detectable. MBPK-II (M_r 110,000) was fully activated at the time of GVBD, whereas peak activation of MBPK-I (M_r 45,000) occurred after this event. Two "ribosomal protein S6 kinases" (S6K-I and S6K-II) could be detected with a synthetic peptide (RRLSSLRA), which was patterned after a major phosphorylation site in S6. The two S6 kinases (M_r 110,000 for both) underwent activation post-GVBD. HH1K and S6K-I coeluted from DEAE-Sephacel at a conductivity of 5.5-6.0 mmho, whereas MBPK-I, MBPK-II, and S6K-II coeluted from this resin in a second peak at a conductivity = 10-11 mmho. The HH1K and MBPK-II activities both declined prior to the emission of the first polar body (i.e., meiotic cell division), but the MBPK-I, S6K-I, and S6K-II activities remained elevated during this time. The activities of these kinases were also examined during the early cell divisions in sea urchin embryos. Within 5 min after fertilization, the high level of MBPK-I activity in sea urchin eggs rapidly declined. However, along with the HH1K and MBPK-II activities, the MBPK-I activity was transiently increased prior to each cell division. No appreciable postfertilization changes in the S6K-I and S6K-II activities were apparent during the first three cycles of cell division.

INTRODUCTION

Protein phosphorylation appears to play a pivotal role in the regulation of cell cycle progression during meiosis and mitosis. During meiotic maturation of oocytes from *Xenopus laevis* (Maller and Smith, 1985) and sea stars (Guerrier *et al.*, 1977), prior to germinal vesicle breakdown (GVBD),[5] there is a burst in net protein phosphorylation. Analysis of phosphoprotein from ^{32}P-labeled *Xenopus* oocytes by two-dimensional polyacrylamide gel electrophoresis has revealed that more than 150 proteins undergo enhanced phosphorylation near the time of GVBD.[6] Of these, only the 40 S ribosomal protein S6 (Hanocq-Quertier and Baltus, 1981; Nielsen *et al.*, 1982), nucleoplasmin (Cotton *et al.*, 1986), and nuclear lamins (Miake-Lye and Kirschner, 1985) have been identified.

The results of oocyte microinjection experiments indicate that the state of phosphorylation of a subset of proteins may be critical for meiotic maturation. Injection of alkaline phosphatase blocks progesterone-induced maturation of *Xenopus* oocytes, whereas the introduction of phosphoester inhibitors of protein phosphatases (e.g., β-glycerophosphate) accelerates the maturation process (Hermann *et al.*, 1984). With sea star oocytes, microinjection of alkaline phosphatase and purified preparations of the catalytic subunits of protein phosphatases 1 and 2A prevented 1-methyladenine (1-MeAde)-induced maturation (Meijer *et al.*, 1986), whereas the phosphatase inhibitor α-naphthylphosphate triggered maturation in the absence of 1-MeAde (Pondaven and Meijer, 1986). On the other hand, injection of the natural inhibitors of protein phosphatase 1, i.e., inhibitors 1 and 2, into *Xenopus* oocytes abolishes or retards progesterone-induced maturation (Huchon *et al.*, 1981; Foulkes and Maller, 1982; Boyer *et al.*, 1986). This implies further complexity in the amphibian meiotic maturation process, in which an early step may require the dephosphorylation of certain phosphoproteins. Several phosphoproteins become dephosphorylated during *Xenopus* oocyte maturation in addition to

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[5] Abbreviations: GVBD, germinal vesicle breakdown; HH1K, histone H1 kinase; MAK, M-phase-activated kinase; 1-MeAde, 1-methyladenine; MBP, myelin basic protein; MBPK-I, myelin basic protein kinase-I; MBPK-II, myelin basic protein kinase-II; MFSW, Millipore-filtered seawater; RRLSSLRA, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala; S6K-I, ribosomal protein S6 kinase-I; S6K-II, ribosomal protein S6 kinase-II.

[6] M. Cicirelli and E. Krebs, unpublished observations.

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those that undergo enhanced phosphorylation (Maller and Smith, 1985).

The dephosphorylation of a subset of the *Xenopus* oocyte proteins shortly after progesterone treatment may arise from the deactivation of cAMP-dependent protein kinase, since there is a rapid decline in the intracellular level of cAMP that accompanies meiotic maturation of *Xenopus* oocytes (Speaker and Butcher, 1977). A similar phenomenon is observed with sea star oocytes (Meijer and Zarutskie, 1987). Microinjection of *Xenopus* oocytes with the isolated subunits or an inhibitor of cAMP-dependent protein kinase has shown that a decrease in the amount of active cAMP-dependent protein kinase is sufficient to trigger maturation (Maller and Krebs, 1977; Huchon *et al.*, 1981). Since the cAMP level is still low at the time of GVBD, the burst in net protein phosphorylation that slightly precedes GVBD in maturing oocytes apparently reflects the activation of cAMP-independent protein kinases and not the inactivation of protein phosphatases 1 and 2A, which do not seem to fluctuate during sea star oocyte maturation (Pondaven *et al.*, 1987).

The increased phosphorylation of ribosomal protein S6 during oocyte maturation may be due to one or both of two cAMP- and Ca^{2+}-independent protein kinases, S6K-I and S6K-II, that are present in soluble extracts from *Xenopus* eggs (Erikson and Maller, 1985; 1986). The activation of S6 kinases has been detected in maturing *Xenopus* (Martin-Perez *et al.*, 1986; Cicirelli *et al.*, 1988) and sea star (Pelech *et al.*, 1987; Meijer *et al.*, 1987) oocytes. S6K-II has been purified to homogeneity from *Xenopus* eggs, and it is highly specific for S6 (Erikson and Maller, 1985; 1986). However, a synthetic peptide, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA), patterned after a major phosphorylation site in the C-terminal region of S6 (Wettenhall and Morgan, 1984), has been demonstrated to be a convenient probe for stimulated S6 kinases in extracts from maturing oocytes (Pelech *et al.*, 1987; Meijer *et al.*, 1987; Cicirelli *et al.*, 1988) and mitogen-treated Swiss mouse 3T3 cells (Pelech *et al.*, 1986; Pelech and Krebs, 1987). The oocyte S6K-II may be closely related to the mammalian mitogen-activated S6 kinase (Novak-Hofer and Thomas, 1984; Tabarini *et al.*, 1985; Erikson and Maller, 1985; 1986; Martin-Perez *et al.*, 1986; Pelech *et al.*, 1986; Nemenoff *et al.*, 1986; Hecht and Straus, 1986; Cobb, 1986; Pelech and Krebs, 1987; Cicirelli *et al.*, 1988).

The increase in the S6K-I and S6K-II activities is largely a post-GVBD event in maturing oocytes of the sea star *Evasterias troschelii* and does not appear to be critical for meiotic maturation (Meijer *et al.*, 1987). The activation of these S6 kinases can be prevented by forskolin treatment of the oocytes prior to exposure of the cells to 1-MeAde, even though treated oocytes can still undergo two cycles of meiotic division in response to 1-MeAde albeit with a delayed time course (Meijer *et al.*, 1987). By contrast, the commitment of sea star oocytes to meiotic maturation correlates very well with the marked stimulation of a histone H1 kinase (HH1K) activity that plateaus just prior to GVBD and subsequently declines prior to the first meiotic division (Sano, 1985; Picard *et al.*, 1985, 1987; Pelech *et al.*, 1987; Meijer *et al.*, 1987). Fertilization of sea star oocytes in the time frame between GVBD and the emission of the first polar body, as occurs during normal embryogenesis, leads to a transient increase in the HH1K activity with each cycle of mitotic division (Picard *et al.*, 1987). The HH1K has also been detected in extracts from maturing *Xenopus* oocytes (Cicirelli *et al.*, 1988). It elutes from DEAE-Sephacel with a conductivity of approximately 6 mmho (Pelech *et al.*, 1987; Cicirelli *et al.*, 1988). The oocyte HH1K exhibits many characteristics that are reminiscent of the "growth-associated histone kinase" described in various cultured mammalian cell lines (Lake and Salzman, 1972; Langan, 1978; Quirin-Stricker, 1984; Pelech *et al.*, 1987).

Recently, we found that myelin basic protein (MBP) is a useful probe for the detection of additional protein kinases that are activated in progesterone-treated *Xenopus* oocytes near the time of GVBD (Cicirelli *et al.*, 1988). DEAE-Sephacel chromatography of cytosol from mature *Xenopus* oocytes afforded the resolution of at least three activated MBP kinases. In the present study, these MBP kinases were further examined during the maturation of sea star oocytes and the early development of sea urchins. Our findings demonstrate that these enzymes are regulated in a cell cycle-dependent fashion during meiosis and mitosis.

EXPERIMENTAL PROCEDURES

Materials

The sea stars *Evasterias troschelii* and *Pisaster ochraceus*, from the Puget Sound area, were the generous gift of Mr. Roland Anderson of the Seattle Public Aquarium. The spawning seasons of these sea star species complemented each other and extended the period of availability of oocytes by 2 months. The sea urchin *Strongylocentrotus purpuratus* was also collected locally. Purified bovine brain myelin basic protein was provided by Dr. Nicholas Tonks (Department of Biochemistry, University of Washington, Seattle, WA). The peptides RRLSSLRA and TTYADFIASGRTGRRNAIHD (cAMP-dependent protein kinase inhibitor peptide [5-24]; K_i = 2–8 nM (Scott *et al.*, 1986; Cheng *et al.*, 1986)) were synthesized in this laboratory by Dr. Patrick Chou and Mr. Henry Zebroski. Histone Type-

IIIS, 1-MeAde, ATP, GTP, and most other reagents were purchased from Sigma. [γ-^{32}P]ATP and [γ-^{32}P]GTP were bought from New England Nuclear. Gel filtration standards were from Bio-Rad.

Preparation of Oocyte and Egg Extracts

The oocytes were released into ice-cold calcium-free artificial seawater when the sea star gonads were gently teased open with forceps (Meijer *et al.*, 1984). To remove contaminating follicle cells, the oocytes were washed three times in calcium-free artificial seawater, and then resuspended to 2% (v/v) in Millipore-filtered natural seawater (MFSW). The oocytes were treated with 2 μM 1-MeAde for 0–200 min at 15°C prior to harvesting.

The gametes from the sea urchins were released following injection of 5 ml of 0.5 *M* KCl intracoelomically through the peribuccal membrane. The sperm was collected "dry" and kept undiluted at 4°C, whereas the eggs were released into MFSW. A 2% (v/v) solution of eggs was fertilized in MFSW in the presence of 4 m*M* aminotriazole to prevent hardening of the fertilization membrane (Showman and Foerder, 1979), and at sperm:egg ratios of approximately 100 sperm:1 egg ($\sim 10^6$ settled eggs/ml; $\sim 5 \times 10^{10}$ packed "dry" sperm/ml). Ten min after sperm addition, the eggs were examined for successful fertilization (i.e., fertilization membrane elevation), the excess sperm was removed by washing the eggs once with MFSW, and the eggs were resuspended to 2% (v/v) in the presence of 2 m*M* aminotriazole. The incubations at 15°C were continued for up to 300 min prior to harvesting.

The maturing oocytes and fertilized eggs were harvested by subjecting 50 ml aliquots of the 2% cell suspensions to centrifugation (1500 rpm, 5 min) at 4°C. Three milliliters of homogenizing buffer (60 m*M* sodium β-glycerophosphate, 20 m*M* sodium Mops, pH 7.2, 15 m*M* disodium EGTA, 1 m*M* dithiothreitol, and 0.5 m*M* sodium vanadate) were added to the 1-ml packed cell pellets, and the cells were ruptured with 25 strokes of a glass Dounce homogenizer. The homogenates were immediately centrifuged for 15–20 min in a Beckman Airfuge, and the supernatants were quickly frozen and stored at −70°C in aliquots.

The details relevant to ion-exchange and gel permeation chromatography are provided in the figure legends.

Kinase Assays

Unless stated otherwise, all kinase assays contained in a final volume of 25 μl: 500 n*M* TTYADFIASGRTGRRNAIHD; 1 mg MBP/ml, 1 mg histone H1/ml (Sigma-Type III-S) or 0.25 mM RRLSSLRA; 50 μM [γ-^{32}P]ATP or [γ-^{32}P]GTP; and Buffer A (30 m*M* sodium β-glycerophosphate; 20 m*M* sodium Mops, pH 7.2, 20 m*M* $MgCl_2$, 5 m*M* disodium EGTA, 1 m*M* dithiothreitol, and 0.5 m*M* sodium vanadate). All reaction preincubations were performed at 0°C. Kinase reactions commenced upon addition of radioactive nucleotide, and were of 7.5 min (cytosolic assays) or 15 min (column fraction assays) duration at 30°C. With cytosolic sea star kinase assays, the phosphorylation of MBP, histone H1, and RRLSSLRA became nonlinear when the assay duration exceeded 10 min or when the final protein concentration was outside the range of 0.02–0.3 mg protein/ml. The cytosolic sea urchin kinase assays were linear with up to 0.9 mg protein/ml. Assays were terminated when 20-μl aliquots were spotted on to 1.5-cm^2 pieces of Whatman P81 phosphocellulose paper, and after 30 sec, the filters were washed five times (for at least 2 min each time) in a solution of 10 ml phosphoric acid/liter H_2O. The wet filters were transferred into 6-ml plastic scintillation vials containing 3 ml of Ecolite scintillation fluid (Westchem.) and counted for radioactivity in a Packard Counter.

Protein was estimated by the Method of Bradford (1976) using bovine serum albumin ($A^{1\%}_{280\,nm} = 6.5$) as a standard.

RESULTS

Elevation of Myelin Basic Protein Kinase Activity in Cytosol from Maturing Sea Star Oocytes

We have previously described a marked enhancement of histone H1 phosphorylating activity and a modest increase in ribosomal S6 peptide (i.e. RRLSSLRA) phosphorylating activity in soluble extracts of sea star oocytes induced to mature with 1-MeAde (Pelech *et al.*, 1987; Meijer *et al.*, 1987). We have now employed MBP as a probe for activated kinases in cytosol from maturing oocytes from the sea stars *E. troschelii* and *P. ochraceus*, and detected a dramatic (up to 15-fold) stimulation of MBP phosphorylating activity.

For a detailed examination of the time course of the 1-MeAde-induced increase in MBP phosphorylating activity, *P. ochraceus* oocytes were selected due to their slower rate of 1-MeAde-induced maturation as compared with *E. troschelii* oocytes. With *P. ochraceus* oocytes at 15°C, GVBD occurred 60–80 min after 1-MeAde addition (Fig. 1), and the first meiotic division (i.e., polar body emission) was evident about 2.5 hr later. For comparison, *E. troschelii* oocytes undergo GVBD after 20 min and meiosis I approximately 100 min after 1-MeAde treatment (Meijer *et al.*, 1987). The times after 1-MeAde exposure for half-maximal increases in the histone H1, MBP, and RRLSSLRA phosphorylating activities were 30, 50, and 60 min, respectively (Fig. 1).

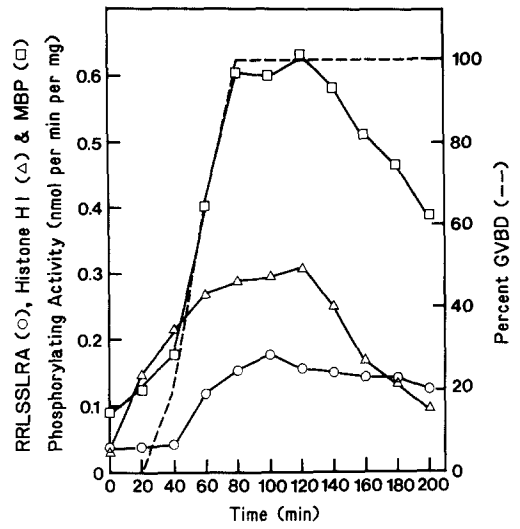

FIG. 1. Time course of the activation of protein kinases during sea star oocyte maturation. Oocytes from *P. ochraceus* were treated with 2 μM 1-MeAde for up to 4 hr prior to harvesting. MBP (1 mg/ml) (□), histone H1 (1 mg/ml) (△), and RRLSSLRA (0.25 mM) (○) phosphorylating activities in the presence of 25 μM [γ-^{32}P]ATP were determined with soluble extracts prepared from aliquots of oocytes removed at various times from a suspension at 15°C. Values are the means of duplicate determinations, and similar results were obtained in three independent experiments. The percentage of treated oocytes that underwent GVBD is indicated with the dashed line, and the emission of the first polar body occurred at 200–220 min.

Subsequent to GVBD and prior to the first meiotic division, the elevated MBP phosphorylating activity partially declined (Fig. 1). As previously described with *E. troschelii* oocytes (Pelech *et al.*, 1987), there was a more pronounced drop in the histone H1 phosphorylating activity and little change in the RRLSSLRA phosphorylating activity during this same period (Fig. 1).

Column Chromatography of Sea Star Oocyte MBP Kinases

To ascertain whether the enhanced MBP phosphorylating activity in maturing sea star oocytes stemmed from one or more kinases, soluble extracts from *E. troschelii* oocytes were subjected to DEAE-Sephacel chromatography (Fig. 2). A minor peak (conductivity $\approx$ 6 mmho) and a broad major peak (conductivity $\approx$ 10–12 mmho) of maturation-activated MBP phosphorylating activity were resolved (Fig. 2A). The elution conductivity and the time course of 1-MeAde-induced activation of the MBP kinase in the ~6 mmho peak indicated that it may correspond to the HH1K (Fig. 2B). This assignment was supported by the ability of the ~6 mmho MBP kinase to effectively utilize Mn^{2+} (1.5 mM free metal ion concentration) in place of Mg^{2+} (18 mM free metal ion concentration) in the presence of [γ-^{32}P]ATP (Fig. 3). Furthermore, the ~6 mmho MBP kinase exhibited substantial activity with [γ-^{32}P]GTP as the phosphate donor (Fig. 3). The use of Mn^{2+} and [γ-^{32}P]GTP by the sea star oocyte HH1K has been previously described (Pelech *et al.*, 1987).

Careful inspection of the 10- 12-mmho MBP phosphorylating peak in Fig. 2A revealed that it might contain two poorly separated MBP kinases, which will be referred to tentatively as MBPK-I and MBPK-II. (The 6 mmho peak will continue to be referred to as HH1K). Activation of MBPK-II (elution conductivity $\approx$ 11.5

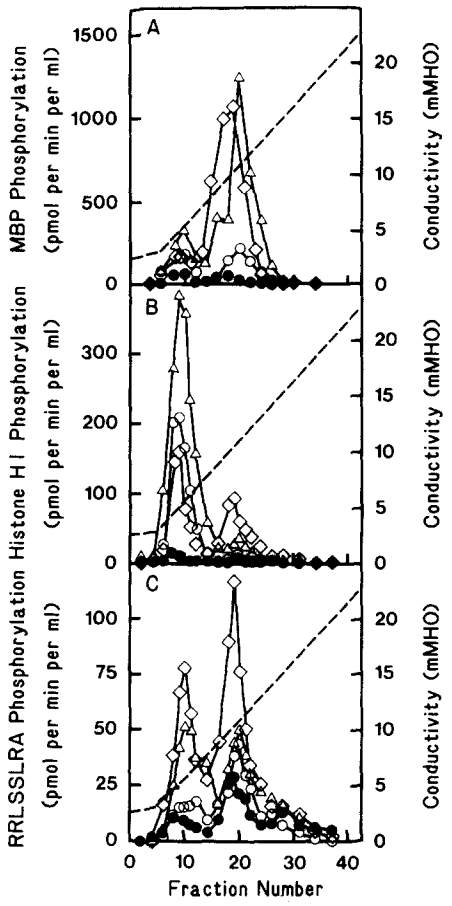

FIG. 2. DEAE-Sephacel chromatography of maturation-activated protein kinases in sea star oocyte cytosol. Cytosolic protein (2.5 mg) in Buffer B (see Experimental Procedures) from *E. troschelii* oocytes treated with 2 μM 1-MeAde for 0 (●), 15 (○), 45 (△), and 90 (◇) min was loaded on to a 2.5 ml DEAE-Sephacel column (Sigma) equilibrated in Buffer B. The column was washed with 5 ml of Buffer B, and developed with a 40-ml linear gradient of 0–450 mM NaCl in Buffer B with a flow rate of 0.4 ml/min. The collected fractions (~1.0 ml) were assayed with 1 mg MBP/ml (A), 1 mg histone H1/ml (B), and 0.25 mM RRLSSLRA (C) for kinase activity with 50 μM [γ-^{32}P]ATP. Almost no kinase activity with these substrates was detected in the wash through fractions. Typically 55–65% of the applied kinase activity toward each of these substrates was recovered in the gradient fractions. The conductivity gradient (---) is shown. A pure solution of 100 mM NaCl yielded a conductivity value of ~5 mmho. One hundred percent GVBD occurred 25–30 min after the addition of 1-MeAde to this population of oocytes.

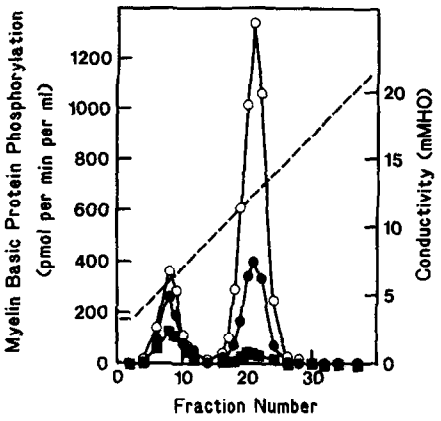

FIG. 3. Manganese and GTP utilization by the maturation-activated MBP kinases. Cytosolic protein (2.5 mg) in Buffer B from *P. ochraceus* oocytes treated for 100 min with 2 μM 1-MeAde was fractionated by DEAE-Sephacel chromatography as described in the legend to Fig. 2. The collected fractions (~1.0 ml) were assayed for kinase activity with 1 mg MBP/ml in the presence of 50 μM [γ-^{32}P]ATP and 18 mM free Mg^{2+} (○); 50 μM [γ-^{32}P]ATP and 1.5 mM free Mn^{2+} (●); and 50 μM [γ-^{32}P]GTP and 18 mM free Mg^{2+} (■). One hundred percent GVBD occurred 70–80 min after the addition of 1-MeAde to the oocytes.

mmho) peaked near the time of GVBD and subsequently declined prior to the first meiotic division (Fig. 2A). By contrast, maximal activation of MBPK-I (elution conductivity ≈ 10.5 mmho) occurred post-GVBD and the activity remained elevated during the time of polar body emission (Fig. 2A).

We have previously shown that S6K-I and S6K-II, as detected with both protein S6 and RRLSSLRA, elute from DEAE-Sephacel with conductivities of approximately 6 and 10.5 mmho, respectively (Pelech *et al.*, 1987; Meijer *et al.*, 1987). Although MBPK-I resembled S6K-II in terms of its DEAE-Sephacel elution profile and time course of 1-MeAde-induced activation of MBPK-I (Fig. 2C), gel filtration studies indicated that these were distinct kinases. When cytosol from maturing sea star oocytes was fractionated on Sephacryl S-200, MBPK-II, HH1K, S6K-I, and S6K-II all coeluted with an apparent M_r ~110,000 (Fig. 4). MBPK-I exhibited an apparent M_r ~45,000 on this gel filtration resin (Fig. 4A).

Modulation of MBP Kinases following Fertilization of Sea Star Oocytes

In sea stars, embryogenesis normally occurs when oocytes are fertilized between GVBD and the first meiotic division (Meijer and Guerrier, 1984). Picard *et al.* (1987) have recently shown that fertilization of oocytes from the sea star *M. glacialis* after completion of GVBD leads to a burst in protein phosphorylation and the transient activation of a histone H1 kinase just prior to the first mitotic cell division. Although this histone H1 kinase was reported to elute from a DEAE-HPLC column with 0.25 M NaCl (Picard *et al.*, 1987), it most probably corresponds to the HH1K that eluted from DEAE-Sephacel with 0.12 M NaCl in the present study.

To examine protein kinases in fertilized sea star eggs, mature *E. troschelii* oocytes that had completed the first and second polar body emissions (5 hr after 1-MeAde exposure) were subsequently treated with sea star sperm for up to 3 hr prior to harvest (under the conditions described under Experimental Procedures for sea urchin egg fertilization). When the soluble extracts from the eggs were assayed for histone H1 kinase activity, a ~2.5-fold stimulation peaked around 2 hr postfertilization at a time when less than 10% of the

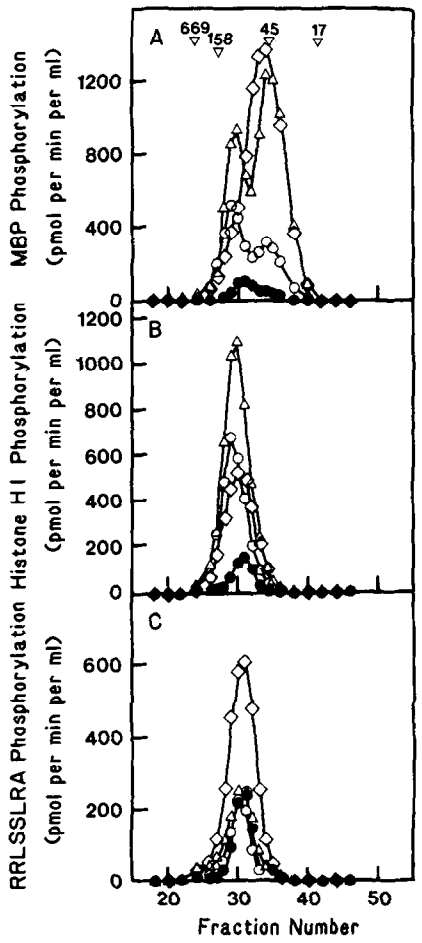

FIG. 4. Sephacryl S-200 chromatography of maturation-activated MBP kinases in sea star oocytes. Cytosolic protein (2.5 mg) from *E. troschelii* oocytes treated with 2 μM 1-MeAde for 0 (●), 22.5 (○), 45 (△), and 90 (◇) min was applied to a 1 × 55-cm Sephacryl S-200 (Sigma) column, equilibrated with Buffer B plus 100 mM NaCl and 0.1% Brij-35, and eluted at a flow rate of 0.15 ml/min. The collected fractions (~0.67 ml) were assayed with 1 mg MBP/ml (A), 1 mg histone H1/ml (B), and 0.25 mM RRLSSLRA (C) for kinase activity with 50 μM [γ-^{32}P]ATP. Typically 85–100% of the applied kinase activity toward each of these substrates was recovered in the column fractions. One hundred percent GVBD occurred 25–30 min after 1-MeAde addition. Elution positions of the marker proteins, thyroglobulin, γ-immunoglobulin, ovalbumin, myoglobin, and vitamin B_{12} are indicated in kilodaltons.

eggs had undergone the first mitotic cleavage. The enhanced histone H1 phosphorylating activity was due to activation of the HH1K as confirmed by DEAE-Sephacel chromatography (not illustrated). Following fertilization of the eggs, the MBP phosphorylating activity (shown to be due to MBPK-I by Sephacryl S-200 chromatography) in cytosol slowly declined by 85% over the period of 1 hr. During the second hour postfertilization, there was a modest increase (2-fold) in the MBPK-I and MBPK-II activities following resolution by DEAE-Sephacel chromatography (not illustrated). There did not appear to be any further increases in the S6 peptide phosphorylating activity after fertilization of the sea star oocytes. Due to the increasing asynchrony of the fertilized oocyte population, it was difficult to detect changes in protein kinase activities after the first mitotic cleavage.

Modulation of MBP Kinases following Sea Urchin Egg Fertilization

To investigate the possibility that the MBP kinases undergo cyclic activation during mitosis, it was necessary to employ a model system that featured a high degree of synchrony with respect to the cell cycle. Sea urchin eggs obtained upon spawning are naturally arrested in the female pronucleus stage after completion of the meiotic divisions. Fertilization of sea urchin eggs induces a series of rapid and synchronous mitotic divisions; in *S. purpuratus*, at 15°C, the first cleavage occurred at ~120 min, the second at ~180 min, and the third at ~260 min following fertilization (Fig. 5). Within the first 5 min of addition of the spermatozoa to a suspension of eggs, there was a marked (70%) drop in the MBP phosphorylating activity (Fig. 5) and a slight (20%) decrease in the histone H1 phosphorylating activity in the cytosolic fraction of these cells (latter not shown). However, both the MBP and histone H1 phosphorylating activities later peaked at ~90 min after fertilization and subsequently declined prior to cell division.[7] This cyclical activation of MBP and histone H1 kinases was detectable with each cell cleavage, but the amplitude of the phosphorylating activity peaks was highly dependent upon the synchrony of the egg population; a slight loss of synchrony resulted in wider peaks of activity of lower amplitude. Similar findings with respect to the cycling histone H1 phosphorylating activity have also been obtained with eggs from the sea urchin *Sphaerechinus granularis* (Pondaven *et al.*, 1987). The level of RRLSSLRA phosphorylating activity in the cytosol was essentially unchanged following egg fertilization (Figs. 5, 6C, 7C, and 7F).

Column Chromatography of Sea Urchin Egg MBP Kinases

DEAE-Sephacel chromatography of cytosol from untreated and fertilized sea urchin eggs indicated that MBPK-I (elution conductivity ≈ 10.5 mmho) was the predominant MBP kinase in unfertilized eggs (Fig. 6A). Within 5-min exposure of the eggs to sperm, the activity of MBPK-I was markedly reduced. However, by approximately 2 hr postfertilization, the activity of MBPK-I was again elevated, in concert with the stimulations of MBPK-II and HH1K activities (Figs. 6A and 6B). The reactivation of the 45-kDa MBPK-I was most clearly shown by Sephacryl S-200 chromatography of cytosol prepared from eggs 2 hr after fertilization (Fig. 7A). The properties of the sea urchin histone H1 kinase on DEAE-Sephacel (Fig. 6B) and on Sephacryl S-200 (Fig. 7B) were indistinguishable from the sea star oocyte HH1K.

We have previously described the anomalous behavior of the sea star HH1K on TSK-400 HPLC columns, from which it elutes with an apparent M_r ~10,000 (Pelech *et al.*, 1987). Likewise, the sea urchin HH1K exhibited an apparent M_r ~9000 on the TSK-400 gel filtration resin (Fig. 7E). MBPK-I had an apparent M_r ~30,000 on the TSK-400 column, whereas MBPK-II was greatly re-

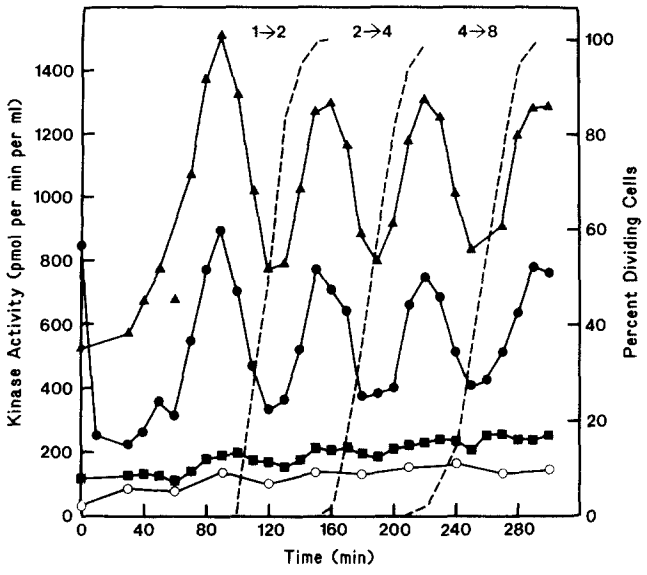

FIG. 5. Time course of the activation of protein kinases following fertilization of sea urchin eggs. *S. purpuratus* eggs were incubated with sperm from the same species starting at Time 0. The kinase activity in cytosolic extracts (0.8 mg protein/ml) was assayed with 25 μM [γ-^{32}P]ATP in the absence of exogenous substrates (○) or in the presence of MBP (1 mg/ml) (●), histone H1 (1 mg/ml) (▲), or 0.25 mM RRLSSLRA (■). Specific enzyme activity values (pmoles/min/mg protein) may be estimated by dividing the shown values by 4. The time course of mitotic cell divisions is indicated with the dashed line.

[7] There was some variation between experiments for the time at which peak activations of the MBP and histone H1 kinases were observed (i.e., from 90–120 min after fertilization). However, the peak kinase activities with MBP and histone H1 always coincided, and the first cell cleavage occurred only after these activities began to decline.

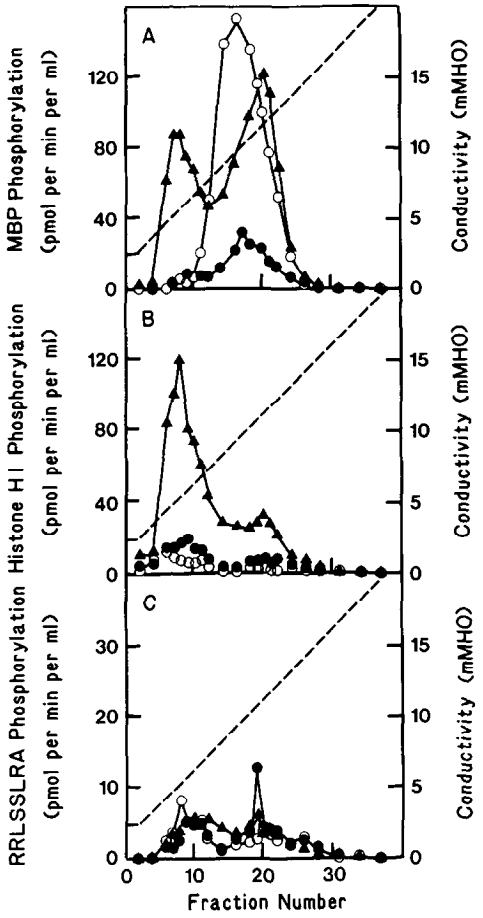

FIG. 6. DEAE-Sephacel chromatography of protein kinases in cytosol from fertilized sea urchin eggs. Cytosolic protein (2.5 mg) from eggs exposed to sperm for 0 (○), 5 (●), and 120 (▲) min was loaded on to a 2.5-ml DEAE-Sephacel column and eluted with a 40-ml linear gradient of 0–450 m*M* NaCl in Buffer B. The collected fractions (~1.0 ml) were assayed with 1 mg MBP/ml (A), 1 mg histone H1/ml (B), and 0.25 m*M* RRLSSLRA (C) for kinase activity with 50 μM [γ-^{32}P]ATP. Typically 55–65% of the applied kinase activity toward each of these substrates was recovered in the gradient fractions. Similar results were obtained in four experiments.

tarded by this resin and eluted after the included volume (Fig. 7D).

DISCUSSION

In a previous study (Cicirelli *et al.*, 1988), three peaks of activated MBP phosphorylating activity in soluble extracts from maturing *Xenopus* oocytes were sequentially eluted from DEAE-Sephacel with 0.12, 0.17, and 0.21 *M* NaCl. These were designated MAK-H, MAK-S, and MAK-M, respectively; "MAK" was an abbreviation of M-phase activated kinase, whereas "H," "S," and "M" referred to the major or distinguishing substrates, histone H1, ribosomal protein S6 and MBP, for detection of these peaks from DEAE-Sephacel (Cicirelli *et al.*, 1988). It was unresolved whether the *Xenopus* oocyte MBP kinases in the MAK-H and MAK-S peaks were distinct from HH1K, S6K-I, and S6K-II. A given chromatographic peak of kinase activity toward a protein substrate does not necessarily designate one enzyme. In the present study, M-phase-activated MBP kinases were further investigated in maturing and fertilized sea star oocytes and fertilized sea urchin eggs. From echinoderm sources, M-phase-activated MBP kinases eluted from DEAE-Sephacel with 0.12, 0.21, and 0.24 *M* NaCl, respectively. By several criteria, the MBP kinase that eluted from DEAE-Sephacel with 0.12 *M* NaCl appeared to correspond to the HH1K that has already been described (Sano, 1985; Picard *et al.*, 1985, 1987; Pondaven *et al.*, 1987; Pelech *et al.*, 1987; Meijer *et al.*, 1987; Cicirelli *et al.*, 1988). On the other hand, the MBP kinases, referred to as MBPK-I and MBPK-II, are novel cAMP- and Ca^{2+}-independent protein kinases. All three MBP kinases are distinct from the two S6 kinases that are stimulated during oocyte maturation with respect to the time course of activation and/or chromatographic

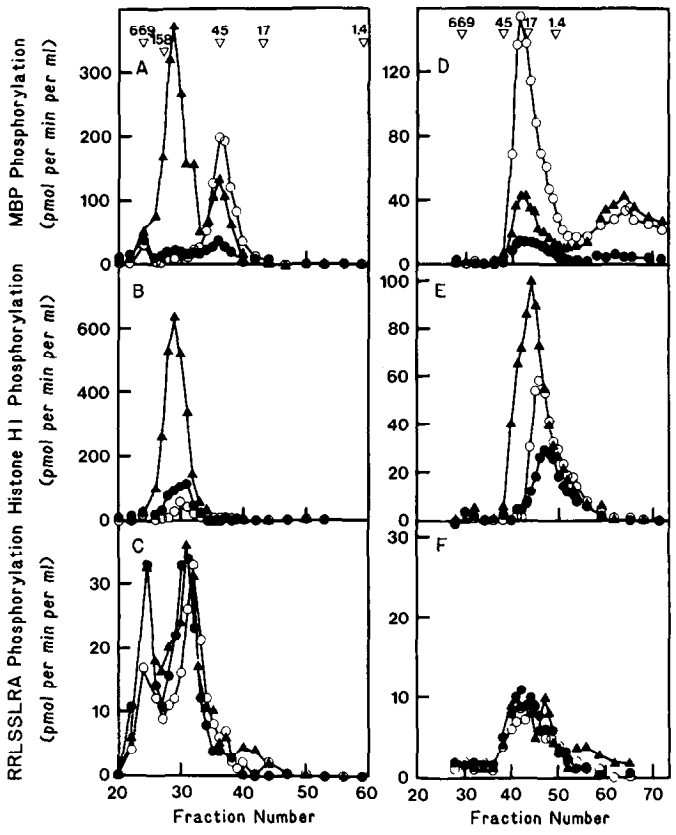

FIG. 7. Gel filtration chromatography of protein kinases in cytosol from fertilized sea urchin eggs. (A–C) Cytosolic protein (2.5 mg) from eggs exposed to sperm for 0 (○), 5 (●), and 120 (▲) min was fractionated on Sephacryl S-200 as described in the legend to Fig. 4. (D–F) Cytosolic protein (1.0 mg) from eggs exposed to sperm for 0 (○), 5 (●), and 120 (▲) min was applied to a 0.75 × 30-cm TSK-400 GSWP HPLC column (Bio-Rad) and eluted with Buffer B plus 100 m*M* NaCl and 0.1% Brij-35 at a flow rate of 0.33 ml/min into 0.33 ml-fractions. MBP (A, D), histone H1 (B, E), and RRLSSLRA (C, F) kinase activities were determined in the presence of 50 μM [γ-^{32}P]ATP. Elution positions of the marker proteins, thyroglobulin, γ-immunoglobulin, ovalbumin, myoglobin, and vitamin B_{12} are indicated in kilodaltons.

behavior on DEAE-Sephacel and Sephacryl S-200. In particular, the activity levels of HH1K, MBPK-I and MBPK-II, unlike that of the S6 kinases, fluctuate in a cell cycle-dependent fashion following egg fertilization.

The lack of detectable S6 kinase activation following sea urchin egg fertilization was somewhat surprising in view of a report that within 4 min of sperm addition to the sea urchin *Arbacia punctulata* there is enhanced labeling of S6 in eggs preloaded with $^{32}PO_4$ (Ballinger and Hunt, 1981). However, an S6-specific protein phosphatase appears to become inactivated at about the same time S6 phosphorylation is increased in fertilized sea urchin eggs (Ballinger and Hunt, 1981). Although S6 is the only protein that has been clearly shown to undergo increased phosphorylation postfertilization, there is a burst of total cellular protein phosphorylation following fertilization of sea star oocytes that correlates with the activation of the HH1K (Picard *et al.*, 1987). Presumably histone H1 is a physiological substrate for the HH1K, since elevated histone H1 phosphorylation is a characteristic feature of mitosis in mammalian cells (Wu *et al.*, 1986). The physiological substrates of MBPK-I and MBPK-II, which is unlikely to include MBP in meiotic cells, remain to be identified. Possible candidates include nucleoplasmin (Cotton *et al.*, 1986), nuclear lamins (Miake-Lye and Kirschner, 1985) and phosphoproteins associated with mitotic microtubule organizing centers (centrosomes, kinetochores, and midbodies) (Vandre *et al.*, 1984, 1986), but the occurrence of MBPK-I and MBPK-II in the nucleus remains to be investigated.

The mechanisms that regulate the activities of MBPK-I and MBPK-II during oocyte maturation and during the initial divisions of sea urchin embryos have also yet to be established. By analogy with the HH1K (Pondaven *et al.*, 1987; Picard *et al.*, 1985, 1987), the cyclic activation of MBPK-I and MBPK-II might involve three steps: protein synthesis of inactive kinase, activation via phosphorylation by another protein kinase, and proteolysis leading to kinase inactivation. We have provided suggestive evidence for stimulation of the sea star oocyte HH1K, S6K-I, and S6K-II activities by protein phosphorylation during maturation (Pelech *et al.*, 1987). The role of protein phosphorylation in the activation of MBPK-I and MBPK-II also merits careful scrutiny.

There is already a precedent for activation of another MBP kinase by protein phosphorylation. Recently, a 36-kDa MBP kinase from pig liver, which eluted from DEAE-cellulose with ~0.11 *M* NaCl, was shown to undergo Mg · ATP-mediated activation via intramolecular phosphorylation (Yang *et al.*, 1987). Although the chromatographic properties of the pig liver MBP kinase are not quite the same as those of MBPK-I in this study, it is intriguing that the pig liver enzyme and the *Xenopus* oocyte DEAE-Sephacel MAK-S peak, which appears to contain MBPK-I, both phosphorylate glycogen synthase (Yang *et al.*, 1987; Cicirelli *et al.*, 1988). Extensive purification of MBPK-I and MBPK-II will be required for proper characterization of the substrate specificity of these kinases. The sea star oocyte system should prove to be an excellent purification source for these novel cell-cycle regulated protein kinases.

Recent genetic studies (Simanis and Nurse, 1986; Russell and Nurse, 1987a,b; Mendenhall *et al.*, 1987) have implicated a regulatory network of at least four protein kinases (encoded by the *cdc2*$^+$, *CDC28*, *wee1*$^+$, and *nim1*$^+$ genes) in the regulation of yeast mitosis initiation. Their relationship to the MBP kinases described in the present study remains to be established. Future studies should facilitate delineation of the cascade of kinases that controls cell cycle progression through meiosis and mitosis.

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REFERENCES

BALLINGER, D. G., and HUNT, T. (1981). Fertilization of sea urchin eggs is accompanied by 40S ribosomal subunit phosphorylation. *Dev. Biol.* **87,** 277–285.

BOYER, J., ASSELIN, J., BELLE, R., and OZON, R. (1986). Progesterone and cAMP-dependent protein kinase regulate in vivo the level of phosphorylation of two proteins (M_r 20,000 and M_r 32,000) in *Xenopus* oocytes. *Dev. Biol.* **113,** 420–428.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

CHENG, H. C., KEMP, B. E., PEARSON, R. B., SMITH, A. J., MISCONI, L., VAN PATTEN, S. M., and WALSH, D. A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J. Biol. Chem.* **261,** 989–992.

CICIRELLI, M., PELECH, S. L., and KREBS, E. G. (1988). Activation of multiple protein kinases during the burst in protein phosphorylation that precedes the first meiotic cell division in *Xenopus* oocytes. *J. Biol. Chem.* **263,** 2009–2019.

COBB, M. H. (1986). An insulin-stimulated ribosomal protein S6 kinase in 3T3-L1 cells. *J. Biol. Chem.* **261,** 12,994–12,999.

COTTON, M., SEALY, L., and CHALKLEY, R. (1986). Massive phosphorylation distinguishes *Xenopus laevis* nucleoplasmin isolated from oocytes or unfertilized eggs. *Biochemistry* **25,** 5063–5069.

ERIKSON, E., and MALLER, J. L. (1985). A protein kinase from *Xenopus* eggs specific for ribosomal protein S6. *Proc. Natl. Acad. Sci. USA* **82,** 742–746.

ERIKSON, E., and MALLER, J. L. (1986). Purification and characterization of a protein kinase from *Xenopus* eggs highly specific for ribosomal protein S6. *J. Biol. Chem.* **261,** 350–355.

FOULKES, J. G., and MALLER, J. L. (1982). In vivo actions of protein phosphatase inhibitor-2 in *Xenopus* oocytes. *FEBS Lett.* **150,** 155–159.

GUERRIER, P., MOREAU, M., and DOREE, M. (1977). Hormonal control of meiosis in starfish: Stimulation of protein phosphorylation induced by 1-methyladenine. *Mol. Cell Endocrinol.* **7,** 137–150.

HANOCQ-QUERTIER, J. and BALTUS, E. (1981). Phosphorylation of ribosomal proteins during maturation of *Xenopus laevis* oocytes. *Eur. J. Biochem.* **120,** 351-355.

HECHT, L. B., and STRAUS, D. S. (1986). Insulin-sensitive serum-sensitive protein kinase activity that phosphorylates ribosomal protein S6 in cultured fibroblast-melanoma hybrid cells. *Endocrinology* **119,** 470-480.

HERMANN, J., MULNER, O., BELLE, R., MAROT, J., TSO, J., and OZON, R. (1984). In vivo effects of microinjected alkaline phosphatase and its low molecular weight substrates on the first meiotic cell division in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. USA* **81,** 5150-5154.

HUCHON, D., OZON, R., and DEMAILLE, J. G. (1981). Protein phosphatase-1 is involved in *Xenopus* oocyte maturation. *Nature (London)* **294,** 358-359.

HUCHON, D., OZON, R., FISCHER, E. H., and DEMAILLE, J. G. (1981). The pure inhibitor of cAMP-dependent protein kinase initiates *Xenopus laevis* meiotic maturation: A 4-step scheme for meiotic maturation. *Mol. Cell. Endocrinol.* **22,** 211-222.

LAKE, R. S., and SALZMAN, N. P. (1972). Occurrence and properties of a chromatin-associated F1-histone phosphokinase in mitotic Chinese hamster cells. *Biochemistry* **11,** 4817-4826.

LANGAN, T. A. (1978). Isolation of histone kinases. *Methods Cell Biol.* **19,** 143-152.

MALLER, J. L., and KREBS, E. G. (1977). Progesterone-stimulated meiotic cell division of *Xenopus* oocytes: Induction by regulatory subunit and inhibition of catalytic subunit of cAMP-dependent protein kinase. *J. Biol. Chem.* **252,** 1712-1718.

MALLER, J., and SMITH, D. S. (1985). Two-dimensional polyacrylamide gel analysis of changes in protein phosphorylation during maturation of *Xenopus* oocytes. *Dev. Biol.* **109,** 150-156.

MARTIN-PEREZ, J., RUDKIN, B. B., SIEGMANN, M., and THOMAS, G. (1986). Activation of ribosomal protein S6 phosphorylation during meiotic maturation of *Xenopus laevis* oocytes: in vitro ordered appearance of S6 phosphopeptides. *EMBO J.* **5,** 725-731.

MEIJER, L., and GUERRIER, P. (1984). Maturation and fertilization in starfish oocytes. *Int. Rev. Cytol.* **86,** 129-196.

MEIJER, L., PELECH, S. L., and KREBS, E. G. (1987). Differential regulation of histone H1 and ribosomal S6 kinases during sea star oocyte maturation. *Biochemistry* **26,** 7968-7974.

MEIJER, L., PONDAVEN, P., GUERRIER, P., and MOREAU, M. (1984). A starfish oocytes user's guide. *Cah. Biol. Mar.* **25,** 457-480.

MEIJER, L., PONDAVEN, P., TUNG, H. Y. L., COHEN, P., and WALLACE, R. W. (1986). Protein phosphorylation and oocyte maturation: Inhibition of starfish oocyte maturation by intracellular microinjection of protein phosphates 1 and 2A and alkaline phosphatase. *Exp. Cell Res.* **163,** 489-499.

MEIJER, L., and ZARUTSKIE, P. (1987). Starfish oocyte maturation: 1-Methyladenine triggers a drop of cAMP concentration related to the hormone-dependent period. *Dev. Biol.* **121,** 306-315.

MENDENHALL, M. D., JONES, C. A., and REED, S. I. (1987). Dual regulation of the yeast CDC28-p40 protein kinase complex: Cell cycle, pheromone, and nutrient limitation effects. *Cell* **50,** 927-935.

MIAKE-LYE, R., and KIRSCHNER, M. W. (1985). Induction of early mitotic events in a cell-free system. *Cell* **41:**165-175.

NEMENOFF, R. A. GUNSALAS, J. R., and AVRUCH, J. (1986). An insulin-stimulated (ribosomal S6) protein kinase from soluble extracts of H4 hepatoma cells. *Arch. Biochem. Biophys.* **245,** 196-203.

NIELSEN, P. J., THOMAS, G., and MALLER, J. L. (1982). Increased phosphorylation of ribosomal protein S6 during meiotic maturation of *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **79,** 2937-2941.

NOVAK-HOFER, I., and THOMAS, G. (1984). An activated S6 kinase in extracts from serum- and epidermal growth factor-stimulated Swiss 3T3 cells. *J. Biol. Chem.* **259,** 5995-6000.

PELECH, S. L., and KREBS, E. G. (1987). Mitogen-activated S6 kinase is stimulated via protein kinase C-dependent and independent pathways in Swiss 3T3 cells. *J. Biol. Chem.* **262,** 11,598-11,606.

PELECH, S. L., MEIJER, L., and KREBS, E. G. (1987). Characterization of maturation-activated histone H1 and ribosomal S6 kinases in sea star oocytes. *Biochemistry* **26,** 7960-7968.

PELECH, S. L., OLWIN, B. B., and KREBS, E. G. (1986). Fibroblast growth factor treatment of Swiss 3T3 cells activates a subunit S6 kinase that phosphorylates a synthetic peptide substrate. *Proc. Natl. Acad. Sci. USA* **83,** 5968-5972.

PICARD, A., LABBE, J. C., PEAUCELLIER, G., LE BOUFFANT, F., LE PEAUCH, C., and DOREE, M. (1987). Changes in the activity of the maturation promoting factor are correlated with those of major cyclic AMP and calcium-independent protein kinase during the first mitotic cell cycles in the early starfish embryo. *Dev. Growth Differ.* **29,** 93-103.

PICARD, A., PEAUCELLIER, G., LE BOUFFANT, F., LE PEAUCH, C., and DOREE, M. (1985). Role of protein synthesis and proteases in production and inactivation of maturation-promoting activity during meiotic maturation of starfish oocytes. *Dev. Biol.* **109,** 311-320.

PONDAVEN, P., and MEIJER, L. (1986). Protein phosphorylation and oocyte maturation: Induction of starfish oocyte maturation by intracellular microinjection of a phosphatase inhibitor, α-naphthylphosphate. *Exp. Cell Res.* **163,** 477-488.

PONDAVEN, P., MEIJER, L., and PELECH, S. L. (1987). Protein phosphorylation in starfish oocyte meiotic divisions and sea urchin egg mitotic divisions. *Adv. Protein Phosphatases* **4,** 229-251.

QUIRIN-STRICKER, C. (1984). Histone H1 kinase from mouse plasmacytoma: Further characterization and molecular structure. *Eur. J. Biochem.* **142,** 317-322.

RUSSELL, P., and NURSE, P. (1987a). Negative regulation of mitosis by wee1$^+$, a gene encoding a protein kinase homolog. *Cell* **49,** 559-567.

RUSSELL, P., and NURSE, P. (1987b). The mitotic inducer nim1$^+$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* **49,** 569-576.

SANO, K. (1985). Calcium- and cyclic AMP-independent labile protein kinase appearing during starfish oocyte maturation: Its extraction and partial characterization. *Dev. Growth Differ.* **27,** 263-275.

SCOTT, J. D., GLACCUM, M. B., FISCHER, E. H., and KREBS, E. G. (1986). Primary-structure requirements for inhibition by the heat-stable inhibitor of the cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **83,** 1613-1616.

SHOWMAN, R. M., and FOERDER, C. A. (1979). Removal of the fertilization membrane of sea urchin embryos employing aminotriazole. *Exp. Cell Res.* **120,** 253-255.

SIMANIS, V., and NURSE, P. (1986). The cell cycle control gene cdc2$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45,** 261-268.

SPEAKER, M. G., and BUTCHER, F. R. (1977). Cyclic nucleotide fluctuations during steroid induced meiotic maturation of frog oocytes. *Nature (London)* **267,** 848-849.

TABARINI, D., HEINRICH, J., and ROSEN, O. M. (1985). Purification of a bovine liver S6 kinase. *Proc. Natl. Acad. Sci. USA* **82,** 4369-4373.

VANDRE, D. D., DAVIS, F. M., RAO, P. N., and BORISY, G. G. (1984). Phosphoproteins are components of mitotic microtubule organizing centres. *Proc. Natl. Acad. Sci. USA* **81,** 4439-4443.

VANDRE, D. D., DAVIS, F. M., RAO, P. N., and BORISY, G. G. (1986). Distribution of cytoskeletal proteins sharing a conserved phosphorylated epitope. *Eur. J. Cell Biol.* **41,** 72-81.

WETTENHALL, R. E. H., and MORGAN, F. J. (1984). Phosphorylation of hepatic ribosomal protein S6 on 80 and 40S ribosomes: Primary structure of S6 in the region of the major phosphorylation sites for cAMP-dependent protein kinases. *J. Biol. Chem.* **259,** 2084-2091.

WU, R. S., PANUSZ, H. T., HATCH, C. L., and BONNER, W. H. (1986). Histones and their modifications. *CRC Crit. Rev. Biochem.* **20,** 201-263.

YANG, S.-D., CHANG, S.-Y., and SODERLING, T. R. (1987). Characterization of an autophosphorylation-dependent multifunctional protein kinase from liver. *J. Biol. Chem.* **262,** 9421-9427.