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Characterization of Maturation-Activated Histone H1 and Ribosomal S6 Kinases in Sea Star Oocytes†

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ABSTRACT: DEAE-Sephacel chromatography of cytosolic extracts from sea star oocytes resolved at least two distinct peaks of maturation-activated protein kinase activity, each of which catalyzed the phosphorylation of histone H1, ribosomal protein S6, and Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA), a synthetic peptide based on the sequence of a phosphorylation site in the latter protein. The first peak (elution conductivity $\simeq$ 6 mmho) contained the major activated kinase with respect to the phosphorylation of histone H1, and the second peak (elution conductivity $\simeq$ 10.5 mmho) contained the major activated kinase with respect to the phosphorylation of S6 and RRLSSLRA. These kinase activities were barely detectable in extracts from immature oocytes. The major stimulated histone H1 kinase exhibited an apparent M_r of $\sim$90 000 on Sephacryl S-300 but eluted from TSK-400 with an apparent M_r of $\sim$10 000. After DEAE-Sephacel fractionation, this kinase was shown to utilize both ATP (apparent $K_m \simeq 45$ μM) and GTP (apparent $K_m \simeq 10$ μM), although the V_{max} was 8-fold higher with ATP than with GTP. The enzyme phosphorylated histone H1 with an apparent $K_m \simeq 50$ μg/mL. Its properties resembled those of the growth-associated histone kinase. The major stimulated RRLSSLRA kinase had an apparent M_r of $\sim$84 000 on Sephacryl S-300 and $\sim$40 000 on TSK-400. After DEAE-Sephacel chromatography, this kinase selectively utilized ATP (apparent $K_m \simeq 25$ μM). The kinase activities in both DEAE-Sephacel peaks were inhibited by NaF, $MnCl_2$, $CaCl_2$, zinc acetate, *N*-ethylmaleimide, trifluoperazine, and chlorpromazine, but the major stimulated RRLSSLRA kinase (peak at $\sim$10.5 mmho) was less sensitive than the major stimulated histone H1 kinase (peak at $\sim$6 mmho) to inhibition by β-glycerol phosphate, NaCl, pyrophosphate, quercetin, and heparin. The RRLSSLRA kinase (peak at $\sim$10.5 mmho) may be related to the mitogen-activated S6 kinase detected in mammalian and avian cells.

Protein phosphorylation may be instrumental in the orchestration of molecular events that facilitate progression through the cell cycle. In particular, chromosomal condensation and other nuclear changes during mitosis have been temporally linked with extensive phosphorylation of histone H1 subtypes (3–6 mol of phosphate/mol of H1) and histone H3. A chromatin-associated, cAMP-independent protein kinase (or kinases) detected in nuclear extracts of proliferating cells may be responsible for the cell cycle dependent phosphorylation of histone H1 (Lake & Salzman, 1972; Schlepper & Knippers, 1975; Langan, 1978b; Paulson & Taylor, 1982;

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Quirin-Stricker, 1984). This histone H1 kinase also appears to reside in the cytoplasmic compartment of cells (Lake & Salzman, 1972; Quirin-Stricker, 1984). Termed the growth-associated histone kinase, this enzyme catalyzes the phosphorylation of serine and threonine residues in the N- and C-terminal regions of histone H1 (Langan, 1978a) and probably corresponds to nuclear "kinase R" in mitotic *Physarum polycephalum* cells (Chambers et al., 1983). The mechanism of activation of the growth-associated histone kinase is unknown, but it is believed to be independent of alteration in the level of enzyme protein (Mitchelson et al., 1978; Zeilig & Langan, 1980).

Ribosomal subunit S6 is another protein that can undergo multisite phosphorylation (~6 mol of phosphate/mol of S6) when quiescent Swiss mouse 3T3 cells are prompted to reenter the cell cycle with mitogens (Wettenhall et al., 1983; Martin-Perez et al., 1984). The enhanced phosphorylation of S6 may promote the recruitment of mRNA for the formation of new polysomes and increased rates of protein synthesis initiation (Thomas et al., 1981; Glover, 1982; Keller et al., 1982; Burkhard & Traugh, 1983). Soluble extracts from mitogen-treated mammalian (Novak-Hofer & Thomas, 1984; Tabarini et al., 1985; Nemenoff et al., 1986; Cobb et al., 1986; Pelech et al., 1986; Pelech & Krebs, 1987) and avian (Blenis & Erikson, 1985; Lawen & Martini, 1985) cells exhibit an elevated S6 kinase activity that is also stimulated in response to 12-*O*-tetradecanoylphorbol acetate and Rous sarcoma virus infection (Tabarini et al., 1985; Blenis & Erikson, 1985; Cobb et al., 1986; Pelech et al., 1986; Pelech & Krebs, 1987). This cAMP-independent protein kinase has only been partially purified from mammalian and avian sources, and its mechanism of activation also remains to be established.

The process of oocyte maturation, which involves traverse through a meiotic cell cycle and has many parallels with somatic cell cycle progression, has been studied extensively in the frog. Ribosomal protein S6 is known to undergo enhanced phosphorylation during progesterone-induced maturation of *Xenopus laevis* oocytes (Hanocq-Quertier & Baltus, 1981; Nielsen et al., 1982). Martin-Perez et al. (1986) have shown that soluble extracts from unfertilized *Xenopus* eggs contain an S6 kinase activity that is not evident in immature oocytes. This may correspond to the S6 kinase that has been purified from *Xenopus* eggs by Erikson and Maller (1986). Recent studies in our laboratory (Cicirelli et al., 1987) have indicated that a family of protein kinases becomes activated during progesterone-induced *Xenopus* oocyte maturation. A stimulated *Xenopus* S6 kinase could be conveniently assayed with a synthetic peptide, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA),[1] patterned after a major phosphorylation site in S6 (Gabrielli et al., 1984).

Sea star oocyte maturation in response to 1-methyladenine (1-MeAde) is another model system that features a number of advantages for study over progesterone-induced maturation of *Xenopus* oocytes (Meijer & Guerrier; 1984). In particular, the germinal vesicle (nucleus) of sea star oocytes is readily observable under a light microscope, and the breakdown of the germinal vesicle (GVBD), one of the first morphological changes that is a characteristic of oocyte maturation, can occur within 20 min of addition of 1-MeAde to the oocytes. This is in sharp contrast with progesterone-induced GVBD in *Xenopus* oocytes, which occurs 4–9 h after initial exposure to the steroid. As has been observed with maturing *Xenopus* oocytes (Maller et al., 1977), there is a burst of protein phosphorylation that is triggered when sea star oocytes mature in response to 1-MeAde[1] treatment (Mazzei & Guerrier, 1982). We have observed a 2.5-fold increase in the phosphorylation of a protein (or proteins), which comigrates (comigrate) with the S6 protein on SDS–polyacrylamide gels, when *Evasterias troschelii* oocytes are exposed to 1-MeAde for 20 min, with early changes detectable within 2 min of addition of the hormone.[2] Soluble extracts from maturing sea star oocytes have not been examined for enhanced S6 kinase activity. However, Sano (1985) has reported 6–7-fold higher histone H1 phosphorylating activity in extracts from maturing oocytes from the sea star *Asterina pectinifera*. Work by Picard et al. (1985, 1987) with the sea star *Marthasterias glacialis* revealed that the enhanced cytosolic kinase activity (measured with mixed histones) from maturing oocytes subsequently declines just prior to first cell division.

In this study, we have further characterized the histone H1 and ribosomal protein S6 kinases that are stimulated during 1-MeAde-induced maturation of sea star oocytes. The results of this investigation support the notion that the major stimulated histone H1 kinase might be related to the "growth-associated histone kinase" and the major stimulated S6 kinase to the mitogen-activated S6 kinase. In addition, we provide suggestive evidence for a role for protein phosphorylation in the mechanism of activation of these kinases.

Experimental Procedures

Materials. *E. troschelii* and *Pisaster ochraceus* were collected in the Seattle area and were the generous gift of Roland Anderson of the Seattle Public Aquarium. Most of the experiments described were performed with *E. troschelii* oocytes, and very similar results were obtained with *P. ochraceus* oocytes. The peptides RRLSSLRA and TTYADFIASGRTGRRNAIHD [cAMP-dependent protein kinase inhibitor peptide (5–24); $K_i \simeq$ 2–8 nM (Scott et al., 1986; Cheng et al., 1986)] were synthesized in this laboratory. Rat liver 40S ribosomes were the generous gift of Dr. Robert Traut (Department of Biological Chemistry, University of California, Davis). Total calf thymus mixed histones were provided freely by Dr. Bassam Wakin (Department of Biochemistry, University of Washington). Histone type III-S, 1-MeAde, trifluoperazine, chlorpromazine, *N*-ethylmaleimide, sodium fluoride, spermine, heparin, hemin, ATP, and GTP were purchased from Sigma. [γ-^{32}P]ATP and [γ-^{32}P]GTP were bought from New England Nuclear. Electrophoresis and gel filtration standards were from Bio-Rad.

Preparation of Oocyte Extracts. The gonads were dissected out of each sea star arm, gently torn open in ice-cold calcium-free artificial sea water, and filtered through cheesecloth (Meijer et al., 1984). Oocytes were washed 3 or 4 times in calcium-free artificial sea water to remove contaminating follicle cells and subsequently resuspended in 9 volumes of filtered natural sea water. Oocytes were treated either with or without 2 μM 1-MeAde for 45–60 (*E. troschelii*) or 90 (*P. ochraceus*) min at 15 °C. Control (untreated) oocytes underwent a maximal spontaneous maturation rate of approximately 5–10%, whereas 100% GVBD was achieved within 20 (*E. troschelii*) and 45 (*P. ochraceus*) min in 1-MeAde-treated oocytes.

[1] Abbreviations: GVBD, germinal vesicle breakdown; I_{50}, dose producing a 50% inhibition; 1-MeAde, 1-methyladenine; RRLSSLRA, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

[2] S. Pelech, L. Meijer, and E. Krebs, unpublished observations.

Upon completion of the incubation period, 1-mL aliquots of the 10% oocyte suspension were rapidly centrifuged (5 s at full speed in Eppendorf microfuge). The supernatant was removed by aspiration, and the oocyte pellet was homogenized in 300 μL of buffer A [60 mM β-glycerol phosphate, 30 mM *p*-nitrophenyl phosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM sodium vanadate] with a glass Dounce homogenizer. The homogenate was immediately centrifuged for 15–20 min at 150000*g* at 5 °C in a Beckman airfuge, and the supernatant was quickly frozen and stored at −70 °C in aliquots.

For some experiments, after the oocytes were resuspended in buffer A, they were immediately frozen at −70 °C and processed further just prior to measurement of kinase activities. At this time, the samples were thawed and centrifuged for 15–20 min at 150000*g* at 5 °C, and the clear supernatant was used for the kinase assays. This approach was convenient for initial processing of a large number of samples in a short time period, but it resulted in lower recoveries of stimulated histone H1 and RRLSSLRA phosphorylating activities.

DEAE-Sephacel fractionation of sea star oocyte cytosol was generally performed as follows. One milliliter of the cytosol was diluted 7.5-fold in buffer B [25 mM β-glycerol phosphate, 5 mM EGTA, 0.8 mM EDTA, 4 mM Mops (pH 7.2), 0.3 mM dithiothreitol, and 50 μM vanadate] and applied to a 2-mL DEAE-Sephacel (Pharmacia) column equilibrated in buffer B. The column was sequentially washed with 12 mL each of 60, 120, 140, and 210 mM NaCl in buffer B, and 1-mL fractions were collected and stored at −70 °C. Other details relevant to ion-exchange and gel permeation chromatographies are provided in the figure legends.

Kinase Assays. Unless stated otherwise, all kinase assays contained the following in a final volume of 25 μL: 500 nM TTYADFIASGRTGRRNAIHD; 0.25 mM RRLSSLRA or 50 μM (1 mg/mL) histone H1 (Sigma type III-S); and 15 μM [γ-^{32}P]ATP or [γ-^{32}P]GTP (2 cpm/fmol for filter paper assays, 10 cpm/fmol for SDS–PAGE gels). Cytosolic assays contained 0.02–0.3 mg of cytosolic protein/mL and Buffer A, whereas column fractionated extracts were assayed in the presence of buffer B. All reaction preincubations were performed at 0 °C unless stated otherwise. Kinase reactions commenced upon addition of radioactive nucleotide and were usually of 5-min duration at 30 °C. Assays were terminated when 20-μL aliquots were spotted on to 1.5-cm^2 pieces of Whatman P81 phosphocellulose paper, and after 30 s, the filters were washed 5 times (for at least 2 min each time) in a solution of 10 mL of phosphoric acid/L of H_2O. The wet filters were transferred into 6-mL plastic scintillation vials containing 5 mL of Aquamix scintillation fluid and counted for radioactivity in a Packard counter.

Assays with 40S ribosomes and histones were stopped upon addition of 40 μL of SDS–PAGE sample buffer [5% β-mercaptoethanol, 0.5% SDS, 50 mM Tris-HCl (pH 6.8), 12.5% glycerol (v/v), and 0.04% bromophenol blue] and immediately boiled for 4 min. The samples were subjected to SDS–PAGE on 17.5% polyacrylamide gels as described by Laemmli (1970). Radiolabeled proteins were visualized upon exposure of Coomassie Blue stained and dried gels to Kodak X-Omat XRP-1 film with Du Pont Hi Plus Cronex intensifying screens.

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 Protein Kinase Activity in Cytosol from Maturing Sea Star Oocytes. Histone H1 and RRLSSLRA were used as probes for the detection of M-phase-activated kinases in extracts from maturing oocytes from the sea stars *E. troschelii* and *P. ochraceus*. In *E. troschelii* oocytes, GVBD is usually completed within 20 min of 1-MeAde addition and emission of the first polar body takes another 100 min (Meijer et al., 1987). *E. troschelii* oocytes were harvested after 50–60-min incubation in the absence (control) or presence (mature) of 2 μM 1-MeAde, to ensure that stimulated kinase activities were examined (Picard et al., 1985, 1987). *P. ochraceus* oocytes required approximately 40–45-min exposure to 1-MeAde for 100% GVBD, and these cells were harvested after 90-min treatment with the hormone. When the oocytes were homogenized in the presence of β-glycerol phosphate and EGTA, soluble extracts from maturing oocytes exhibited a dramatic (>20-fold) stimulation of histone H1 phosphorylating activity and variable (1.5–5-fold) enhancement of RRLSSLRA phosphorylating activity relative to extracts from control oocytes (Figure 1). Phosphorylation of histone H1 and RRLSSLRA became nonlinear when the assay duration was extended beyond 10 min (Figure 1) or when the cytosolic protein concentration in the assay exceeded 0.5 mg/mL (not illustrated).

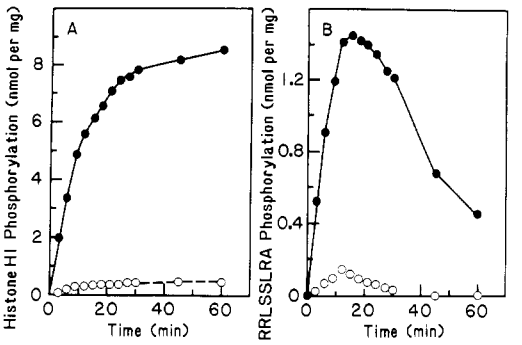

FIGURE 1: Dependence of histone H1 and RRLSSLRA phosphorylation on assay time. Protein kinase activity in cytosols (0.25 mg of protein/mL) from untreated (○) and 1-MeAde-treated (●) sea star oocytes was determined with 15 μM [γ-^{32}P]ATP in the presence of 1 mg of histone H1/mL (panel A) or 0.25 mM RRLSSLRA (panel B). Phosphate incorporation into the substrates was determined as described under Experimental Procedures. Similar results were obtained in three experiments.

To accentuate the maturation-associated increases in the histone H1 and RRLSSLRA phosphorylating activities in oocyte cytosol, suboptimal substrate concentrations were used (Pelech & Krebs, 1987). The apparent K_m's for ATP for phosphorylation of histone H1 and RRLSSLRA by cytosol were 100 and 40 μM, respectively. However, the use of ATP concentrations above 15 μM reduced the apparent fold stimulation of phosphorylating activity especially toward histone H1. While 1 mg of histone H1/mL was optimal for phosphorylation (apparent K_m was ~0.4 mg of histone H1/mL), concentrations greater than 3 mg/mL were inhibitory. The relative enhancement of the RRLSSLRA phosphorylating activity seen with maturation was most marked when the peptide concentration was 0.25 mM or less (apparent K_m was ~0.5 mM RRLSSLRA). Presumably, the use of higher substrate concentrations allowed the detection of additional kinases that were not activated during oocyte maturation.

DEAE-Sephacel Fractionation of Sea Star Oocyte Cytosol. Greater than 90% of the histone H1 and RRLSSLRA phosphorylating activities that were elevated during oocyte maturation were extracted into the cytosolic fraction of homogenized cells (data not shown). To ascertain whether the enhanced histone H1 and RRLSSLRA phosphorylating activities corresponded to one or more kinases, the soluble extracts from mature and control oocytes were subjected to

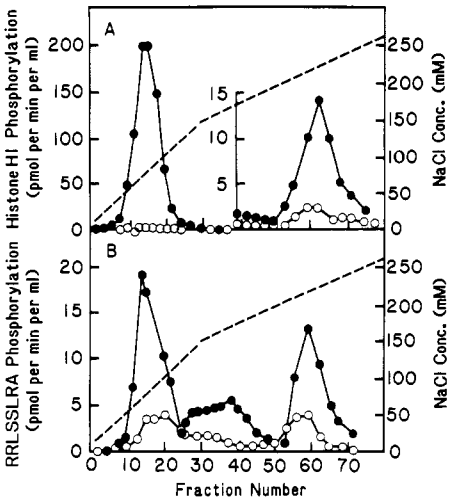

FIGURE 2: DEAE-Sephacel chromatography of histone H1 and RRLSSLRA phosphorylating activities in sea star oocyte cytosol. Cytosol (2 mg of protein) from untreated (○) and 1-MeAde-treated (●) *E. troschelii* oocytes was diluted 15-fold with buffer B and loaded onto a 4-mL DEAE-Sephacel column. The column was washed with 20 mL of buffer B and developed with a 100-mL linear gradient of 0–350 mM NaCl in buffer B with a flow rate of 0.5 mL/min. The collected fractions (~1.2 mL) were assayed in the presence of 15 μM [γ-^{32}P]ATP for kinase activity with 1 mg of histone H1/mL (panel A) and 0.25 mM RRLSSLRA (panel B). The scale is 10-fold greater for the insert to panel A. No histone H1 or RRLSSLRA kinase activity was detected in the wash-through fractions. The conductivity gradient (---) is shown. Fractions 13 and 60 corresponded to conductivities of 6 and 10.5 mmho, respectively.

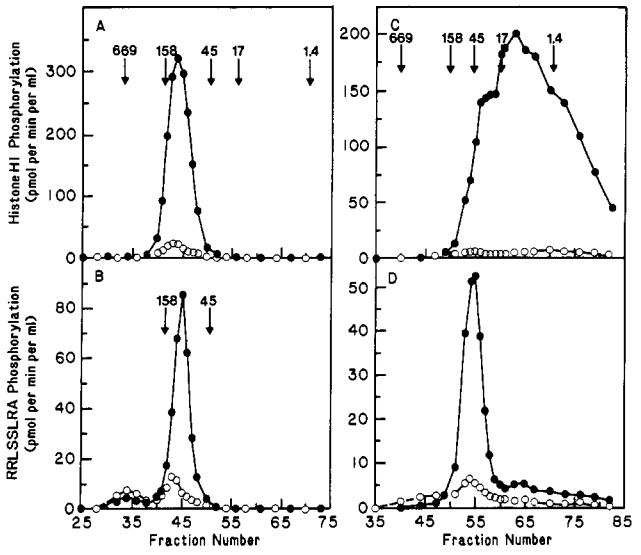

FIGURE 3: Gel filtration chromatography of histone H1 and RRLSSLRA phosphorylating activities in sea star oocyte extracts. Panels A and B: Cytosolic protein (1 mg) from untreated (○) and 1-MeAde-treated (●) *E troschelii* oocytes was applied to a 1 × 55 cm Sephacryl S-300 column (Sigma) equilibrated with buffer B plus 100 mM NaCl and 0.05% Brij-35 and eluted at a flow rate of 0.15 mL/min into ≃0.5-mL fractions. Panels C and D: Cytosolic protein (0.6 mg) from untreated (○) and 1-MeAde-treated (●) *E. troschelii* oocytes was applied to a 0.75 × 30 cm TSK-400 GSWP HPLC column (Bio-Rad) and eluted with buffer B plus 100 mM NaCl and 0.05% Brij-35 at a flow rate of 0.5 mL/min into 0.25-mL fractions. Histone H1 (panels A and C) and RRLSSLRA (panels B and D) phosphorylating activities were determined in the presence of 15 μM [γ-^{32}P]ATP. Elution positions of the marker proteins, thyroglobulin, γ-immunoglobulin, ovalbumin, myoglobin, and vitamin B_{12}, are indicated in kilodaltons.

DEAE-Sephacel chromatography (Figure 2). When the column fractions were assayed for histone H1 phosphorylating activity with [γ-^{32}P]ATP, one major peak (elution conductivity ≃ 6 mmho) and one minor peak (elution conductivity ≃ 10.5 mmho) of increased kinase activity were observed (Figure 2A). The ~6-mmho peak was always the predominant peak of stimulated histone H1 phosphorylating activity, but the height of the ≃6 mmho peak as detected with histone H1 relative to that of the ~10.5-mmho peak varied from 3- to 14-fold between experiments. When the DEAE-Sephacel column fractions were assayed with RRLSSLRA in the presence of [γ-^{32}P]ATP, two major peaks of stimulated phosphorylating activity were again released from the anion-exchange resin at conductivities of ~6 and ~10.5 mmho [Figure 2B and Meijer et al. (1987), cf. Figure 2B). With extracts from maturing *P. ochraceus* oocytes, the height of the ~10.5-mmho peak was usually greater than 10-fold that of the ~6-mmho peak as assessed with the S6 peptide (not shown). The ~6-mmho peak and the more prominent ~10.5-mmho peak of enhanced phosphorylating activity seen with RRLSSLRA were also detectable with rat liver 40S ribosomes as substrate (data not illustrated).

The ~10.5-mmho peak exhibited very little activity toward histone H1 and RRLSSLRA when GTP was substituted for ATP (data not shown). However, the ~6-mmho peak from DEAE-Sephacel could utilize GTP for phosphorylation of histone H1 and RRLSSLRA, although with 8- and 24-fold less activity than with ATP, respectively. It might be inferred from this difference in the ATP/GTP activity ratios for histone H1 and RRLSSLRA that more than one activated kinase was responsible for the phosphorylation of these substrates in the ~6-mmho peak.

Gel Filtration Chromatography of Sea Star Oocyte Cytosol. The increased histone H1 and RRLSSLRA phosphorylating activity in cytosol from mature oocytes migrated similarily, but not identically, on Sephacryl S-300 (Figure 3A,B). The histone H1 phosphorylating activity, which probably reflects the histone H1 kinase in the ~6-mmho peak from DEAE-Sephacel, eluted from this gel filtration column with an apparent M_r of ~90000 (Figure 3A). The stimulated RRLSSLRA phosphorylating activity eluted in a position corresponding to an apparent M_r of ~84000 (Figure 3B). When the ~6- and ~10.5-mmho peaks were first resolved by DEAE-Sephacel chromatography and then subjected separately to further fractionation on Sephacryl S-300, the histone H1 and RRLSSLRA kinases from both peaks exhibited apparent M_r's of 85000–90000 (not illustrated). Without these sequential fractionation experiments, assessment of the apparent M_r values for the stimulated RRLSSLRA kinase in the ~6-mmho peak and the histone H1 kinase in the ~10.5-mmho peak would have been equivocal.

The cytosols from control and mature sea star oocytes were also chromatographed on a TSK-400 HPLC column (Figure 3C,D). The major peak of histone H1 phosphorylating activity was greatly retarded by the TSK-400 resin and eluted over a broad range with a mean apparent M_r of 10000 (Figure 3C). The leading shoulder of this peak appeared to correspond in position to the increased RRLSSLRA phosphorylating activity, which emerged from this column as a sharp peak in the expected position of a protein with a M_r of ~40000 (Figure 3D). The results of this experiment, together with the anion-exchange chromatography studies, suggest that the enhanced phosphorylating activity in cytosol from maturing oocyte arises from several enzymes, at least two of which can catalyze the phosphorylation of RRLSSLRA. One of the latter is apparently found in the ~6-mmho peak from DEAE-Sephacel and one in the ~10.5-mmho peak. The major stimulated histone H1 kinase, which is present in the ~6-mmho peak and can utilize GTP as well as ATP, probably phosphorylates

Table I: Kinetic Constants for Substrate Phosphorylation by M-Phase-Activated Kinases after Resolution on DEAE-Sephacel

parameter	~6-mmho peak	~10.5-mmho peak
apparent K_m for histone H1[a]	2.3 μM	3.7 μM
apparent K_m for RRLSSLRA[a]	100 μM	25 μM
$V_{max,histone\ H1}/V_{max,RRLSSLRA}$[a,b,c]	3.0	1.0
apparent K_m for ATP	45 μM[b]	25 μM[c]
apparent K_m for GTP	10 μM	
$V_{max,ATP}/V_{max,GTP}$	8.0[b]	>100[c]

[a] Measured in the presence of 200 μM [γ-^{32}P]ATP. [b] Measured in the presence of 50 μM histone H1. [c] Measured in the presence of 0.5 mM RRLSSLRA.

RRLSSLRA poorly, if at all.[3]

Characterization of Sea Star Maturation-Activated Kinases after DEAE-Sephacel Chromatography. The aforementioned chromatography experiments indicated that several kinases were responsible for the stimulated phosphorylation of histone H1 and RRLSSLRA by cytosols from maturing sea stars. Since the activated kinases in the ~6-mmho peak could be step-eluted from DEAE-Sephacel in the 60–120 mM NaCl fraction of oocyte cytosol, this procedure was used to separate them from stimulated kinases in the ~10.5-mmho peak, which were recovered in the 140–210 mM NaCl fraction. Although not pure, these DEAE fractions afforded the opportunity carry out preliminary kinetics studies of the kinases in these fractions (Table I). The apparent K_m's of the kinases in the ~6- and ~10.5-mmho peaks for histone H1 were comparable (Table I), and in both instances, concentrations exceeding 3 mg of histone H1/mL were inhibitory. These apparent K_m values were 6–9-fold lower than that measured in crude cytosol (apparent $K_m \simeq 21$ μM). The apparent K_m's of the kinases in the two DEAE-Sephacel peaks for RRLSSLRA were also 5–20-fold lower than that observed in crude cytosol (apparent $K_m \simeq 0.5$ mM). S6 peptide concentrations above 0.3 mM were inhibitory to the ~10.5-mmho kinase, whereas the ~6-mmho kinase was not sensitive to inhibition by up to 2 mM RRLSSLRA (not shown). Although the histone H1 kinase in the ~6-mmho peak had a 4-fold lower apparent K_m for GTP than ATP, with saturating concentrations of these nucleotides, its activity was 8-fold greater with ATP than with GTP (Table I). The kinases in the ~10.5-mmho peak exhibited negligible phosphorylating activity in the presence of GTP.

With respect to the metal requirements for the major stimulated histone H1 kinase in the ~6-mmho peak and the major stimulated RRLSSLRA kinase in the ~10.5-mmho peak, it was found that both kinases required approximately 20 mM $MgCl_2$ in the presence of 5 mM EGTA (calculated $[Mg^{2+}]_{free} \simeq 18$ mM) for optimal activity (Figure 4A). A lower concentration of $MnCl_2$ (calculated $[Mn^{2+}]_{free} \simeq 1.5$ mM) could effectively substitute for $MgCl_2$ in the case of the histone H1 kinase, but $MnCl_2$ concentrations exceeding 8 mM in the presence of 5 mM EGTA were inhibitory (Figure 4B). The RRLSSLRA kinase with optimal concentrations of $MnCl_2$ showed only one-fifth the activity that could be achieved with 20 mM $MgCl_2$. As illustrated in Figure 4C, the activities of both kinases in the presence of 15 mM $MgCl_2$ and 5 mM EGTA were similarly reduced by $MnCl_2$. $CaCl_2$ and $CoCl_2$ were likewise inhibitory for these kinases, but the RRLSSLRA kinase appeared to be 2–3 times more sensitive to these metal cations (Table II). Zinc acetate was the most potent inhibitor of both kinases (Table II). It is possible that some of the effects of these metal cations were mediated indirectly, for

[3] As shall become evident in the following paper (Meijer et al., 1987), the RRLSSLRA phosphorylating activity in the ~6-mmho peak following DEAE-Sephacel chromatography probably arose from stimulated protein kinase that was distinct from the major one that phosphorylated histone H1.

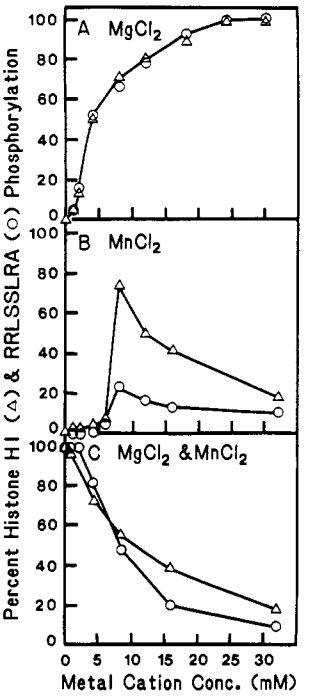

FIGURE 4: $MgCl_2$ and $MnCl_2$ concentration dependence of the major histone H1 and RRLSSLRA phosphorylating activities. The 60–120 mM NaCl DEAE-Sephacel fraction (△) of cytosol from 1-MeAde-treated *E. troschelii* oocytes was assayed for histone H1 phosphorylating activity with either 1–30 mM $MgCl_2$ (panel A), 1–32 mM $MnCl_2$ (panel B), or 15 mM $MgCl_2$ and 0–32 mM $MnCl_2$ (panel C). The 140–210 mM NaCl DEAE-Sephacel fraction (○) of cytosol from 1-MeAde-treated oocytes was assayed for RRLSSLRA phosphorylating activity with either 1–30 mM $MgCl_2$ (panel A), 1–32 mM $MnCl_2$ (panel B), or 15 mM $MgCl_2$ and 0–32 mM $MnCl_2$ (panel C). All incubations were performed with 15 μM [γ-^{32}P]ATP in the presence of 5 mM EGTA and 1 mM EDTA. Kinase activities with histone H1 or RRLSSLRA are expressed relative to the values determined in the presence of 30 mM $MgCl_2$ (panels A and C) or 15 mM $MgCl_2$ (panel C). Similar results were obtained in two experiments.

Table II: Summary of Various Inhibitors of the Major Maturation-Activated Histone H1 and RRLSSLRA Kinase Activities[a]

compound	I_{50} (mM) histone H1 kinase (~6 mmho)	I_{50} (mM) RRLSSLRA kinase (~10.5 mmho)
β-glycerolphosphate	70	100
EGTA	40	>100
NaCl	280	>600
NaF	25	30
pyrophosphate	2	8
$MnCl_2$	13	8
$CaCl_2$	22	11
$CoCl_2$	22	8
$Zn(CH_3COO)_2$	6	6
quercetin	0.025	3
N-ethylmaleimide	2.5	5
trifluoperazine	0.2	0.1
chlorpromazine	0.9	0.5

[a] The 60–120 mM NaCl (~6-mmho peak) and 140–210 mM NaCl (~10.5-mmho peak) DEAE-Sephacel fractions of cytosol from 1-MeAde-treated *E. troschelii* oocytes were assayed with 50 μM histone H1 and 250 μM RRLSSLRA, respectively, in the presence of 15 μM [γ-^{32}P]ATP and varying concentrations of several compounds. I_{50} refers to the concentration of substance that reduced the phosphorylating activity by 50%.

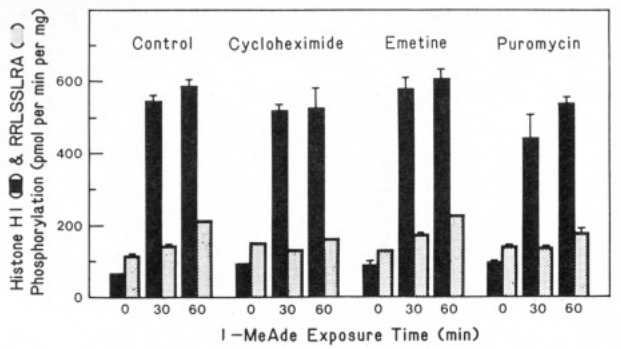

FIGURE 5: Effects of various protein synthesis inhibitors on 1-MeAde-induced stimulation of cytosolic histone H1 and RRLSSLRA phosphorylating activities. *E. troschelii* oocytes were treated for 10 min with cycloheximide (100 μg/mL), emetine (20 μg/mL), or puromycin (100 μg/mL) or left untreated (control). 1-MeAde was subsequently added at a final concentration of 1 μM. Before (time = 0) and 30 and 60 min after 1-MeAde addition, three aliquots of the oocyte suspension were removed and the oocytes were resuspended in buffer A and immediately frozen at −70 °C. Homogenization and preparation of cytosol were performed with thawed samples. Values are the means from three sets of extracts, and the standard deviation is shown. One hundred percent GVBD occurred in all batches.

example, through their action on contaminating protein phosphatases in the DEAE-Sephacel fractions.

Table II summarizes the I_{50} values for several substances that were found to be inhibitory for the major stimulated histone H1 (~6 mmho) and RRLSSLRA (~10.5 mmho) kinases. Both kinase activities were similarly decreased by β-glycerol phosphate, sodium fluoride, *N*-ethylmaleimide, trifluoperazine, and chlorpromazine. The histone H1 kinase was much more sensitive than the RRLSSLRA kinase to inhibition by EGTA, sodium chloride, sodium pyrophosphate, and quercetin. The inhibition of the histone H1 kinase by low concentrations of quercetin was consistent with the tendency of this compound to inhibit kinases that utilize both ATP and GTP (Srivastava, 1985). The histone H1 kinase activity was decreased 50% by 130 μg/mL heparin, whereas the RRLSSLRA kinase activity was unaffected by up to 180 μg/mL heparin (not illustrated). Triton X-100 at 0.1% reduced the RRLSSLRA kinase activity by 50% but doubled the histone H1 kinase activity ($A_{0.5} \simeq 0.0005\%$ Triton X-100) (not illustrated). Both kinase activities were insensitive to cAMP (50 μM), cGMP (50 μM), spermine (0.1–5 mM), and hemin (5–500 μM) (not shown).

Mechanisms of Activation of Maturation-Activated Kinases. The early steps of sea star oocyte maturation, including GVBD, have been demonstrated to be independent of the synthesis of protein, RNA, and DNA (Zampetti-Bosseler et al., 1973; Guerrier & Doree, 1975; Doree, 1982). Figure 5 shows that the enhancement of the histone H1 and RRLSSLRA phosphorylating activities in cytosols from 1-MeAde-treated oocytes was not prevented by the presence of protein synthesis inhibitors (cycloheximide, emetine, and puromycin). Therefore, the maturation-associated elevation of the histone H1 and RRLSSLRA kinase activities appeared to be due to activation of preexisting enzymes. The protein synthesis inhibitors caused small increases in the cytosolic RRLSSLRA phosphorylating activity of oocytes that were not exposed to 1-MeAde (Figure 5). Cycloheximide treatment of chicken embryo fibroblasts has been previously shown to activate a cytosolic S6 kinase in these cells (Blenis & Erikson, 1986).

Reversible protein phosphorylation has been implicated in the regulation of a wide variety of protein kinases. The possibility that the histone H1 and RRLSSLRA kinases in

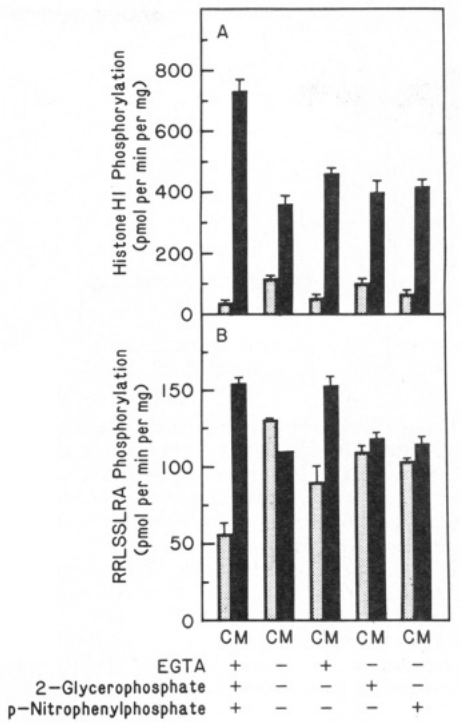

FIGURE 6: Phosphatase inhibitor requirement for preservation of maturation-associated activation of histone H1 and RRLSSLRA phosphorylating activities. Cytosols from untreated (C) and 1-MeAde-treated (M) *E. troschelii* oocytes, homogenized in buffer C [50 mM NaCl, 25 mM Mops (pH 7.2), 15 mM $MgCl_2$, and 2 mM dithiothreitol] ± the phosphatase inhibitors indicated (60 mM β-glycerol phosphate, 30 mM *p*-nitrophenyl phosphate, and 15 mM EGTA), were assayed under the same conditions for phosphorylating activity with 50 μM histone H1 (panel A) and 250 μM RRLSSLRA (panel B) in the presence of 15 μM [γ-^{32}P]ATP and 500 nM TTY-ADFIASGRTGRRNAIHD. Values are the mean ± standard deviation of three separate samples.

sea star oocytes exposed to 1-MeAde were activated by this mechanism warranted investigation. Earlier studies (Novak-Hofer & Thomas, 1984; Martin-Perez et al., 1986) have emphasized the importance of phosphatase inhibitors for the preservation of stimulated S6 kinase activity. In the present study, it was found that inclusion of EGTA in combination with β-glycerol phosphate or *p*-nitrophenyl phosphate in the homogenization buffer was important for maximal detection of the stimulated histone H1 phosphorylating activity; the presence of EGTA, β-glycerol phosphate, or *p*-nitrophenyl phosphate alone was insufficient (Figure 6A). EGTA not only aided in the preservation of the stimulated histone H1 phosphorylating activity but also depressed the basal phosphorylating activity toward this substrate, thereby accentuating the stimulation associated with oocyte maturation (Figure 6A). This ability of EGTA to stabilize stimulated kinase activity and decrease the level of basal kinase activity was also evident with RRLSSLRA as substrate (Figure 6B).

Stabilization of the stimulated kinase activities by EGTA and β-glycerol phosphate was time-dependent, and these components were most effective when added at the time of homogenization (Figure 7). This time dependence implied that the phosphatase inhibitors blocked an inactivating reaction, possibly catalyzed by protein phosphatases. After DEAE-Sephacel chromatography, the histone H1 kinase (~6 mmho) activity was stable to at least 30-min preincubation at 30 °C in the presence of EGTA and β-glycerol phosphate. In contrast, the RRLSSLRA kinase (~10.5 mmho) activity following anion-exchange chromatography was decreased 70% after 5-min preincubation at 30 °C, even in the presence of

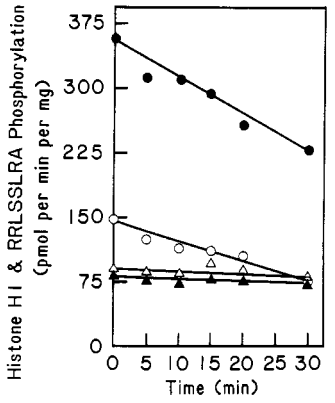

FIGURE 7: Time dependence of loss of maturation-associated activation of histone H1 and RRLSSLRA phosphorylating activities in the absence of phosphatase inhibitors. Untreated (△, ▲) and 1-MeAde-treated (○, ●) *E. troschelii* oocytes were homogenized in buffer C (see Figure 6 legend), and aliquots were incubated at 20 °C for 30 min prior to preparation of cytosols. β-glycerol phosphate (60 mM), EGTA (15 mM), and *p*-nitrophenyl phosphate (30 mM) were added to the various aliquots at 0–30 min during the 20 °C incubation. Histone H1 (●, ▲) and RRLSSLRA (○, △) phosphorylating activities were determined in the soluble extracts under the standard conditions in the presence of 15 μM [γ-^{32}P]ATP. Similar results were obtained in two separate experiments.

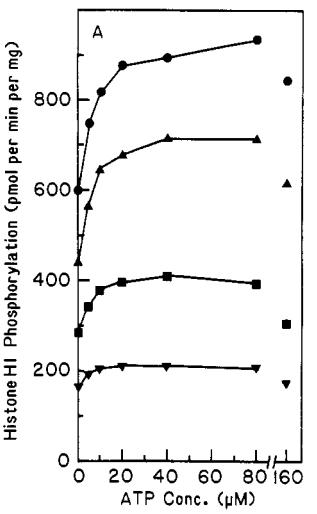

FIGURE 8: Influence of ATP on the ability of oocyte cytosolic histone H1 phosphorylating activity to utilize [γ-^{32}P]GTP. Cytosol from 1-MeAde-treated *E. troschelii* oocytes was assayed for histone H1 phosphorylating activity with 7.5 (▼), 15 (■), 30 (▲), and 45 μM (●) [γ-^{32}P]GTP in the presence of 0–160 μM ATP. Phosphorylating activity refers specifically to the transfer of radiolabel from [γ-^{32}P]GTP.

these components. However, the RRLSSLRA kinase activity could be partially stabilized by the addition of MgATP (this explained why the activity remained linear with time for up to 10 min during enzyme assays). For example, 5-min preincubation of the RRLSSLRA kinase at 30 °C in the presence of 50 μM ATP resulted in only a 15% loss of the kinase activity.

The possible role of a phosphorylation reaction in the stimulation of the histone H1 kinase activity was further implied by the ability of ATP to enhance histone H1 phosphorylation with [γ-^{32}P]GTP by mature oocyte cytosol (Figure 8). Alternately, it may be that ATP acts as a positive allosteric effector of this kinase. Figure 8 demonstrates that the ability of the histone H1 kinase to use [γ-^{32}P]GTP did not simply result from the production of [γ-^{32}P]ATP, since the specific radioactivity of such [γ-^{32}P]ATP would have been decreased, in some instances, 20-fold or more by the added unlabeled ATP. Otherwise, a substantially reduced incorporation of radiolabel into histone H1, rather than the observed activation, would have been found.

As the preceding experiments with DEAE-Sephacel fractionated material indicated that the presence of ATP might facilitate activation of the histone H1 and RRLSSLRA kinases, the effect of preincubation of cytosol with ATP on these kinase activities was investigated. The histone H1 phosphorylating activity in cytosol was not appreciably decreased by preincubation at 30 °C for 10 min in the absence or presence of 50 μM ATP. By contrast, this concentration of ATP prevented the loss of RRLSSLRA phosphorylating activity that was observed when cytosol was preincubated (data not shown).

The possibility that cytosol from mature oocytes contained a kinase (or kinases) that could stimulate the activities of latent histone H1 and RRLSSLRA kinases in cytosol from immature oocytes was tested by pooling the cytosols. However, there was no detectable stimulation of either the histone H1 or RRLSSLRA phosphorylating activity when the pooled cytosols were preincubated for 10 min at 30 °C in the absence or presence of ATP (not shown). These mixing studies also indicated that the mature oocyte cytosol did not specifically contain an activator that could stimulate the M-phase-activated kinases in the immature oocyte cytosol or, the converse, that the immature oocyte extract did not selectively contain an inhibitor of these kinases that was absent in the mature oocyte extract.

Discussion

Identification of Sea Star M-Phase-Activated Kinases. The present study has provided evidence for the existence of at least three cAMP-independent protein kinases that become stimulated during sea star oocyte maturation. Activation of the histone H1 kinase in the ~6-mmho peak from DEAE-Sephacel was the most striking, and the characteristics of this enzyme (or these enzymes) compare most favorably with those of the growth-associated histone kinase (Langan, 1978a).

The growth-associated histone kinase(s) from mammalian cells (Lake & Salzman, 1972; Schlepper & Knippers, 1975; Gurley et al., 1975; Langan, 1978a,b; Ajiro et al., 1981; Quirin-Stricker & Schmitt, 1981; Zampetti-Bosseler et al., 1973; Quirin-Stricker, 1984) and *P. polycephalum* (Chambers et al., 1983) shares (share) one or more of the following properties with the major stimulated histone H1 kinase in sea star oocytes: (i) activation in proliferating cells; (ii) relatively loose interaction with DEAE resins; (iii) an apparent M_r of 90 000 on gel permeation columns; (iv) no obvious autophosphorylation; (v) a strong substrate preference for histone H1; (vi) use of both ATP and GTP; (vii) effective use of manganese in place of magnesium; (viii) inhibition by NaCl and divalent metal cations; and (ix) distribution in both the cytoplasmic and nuclear compartments of cells. This latter point was of particular interest, since the nuclear envelope becomes ruptured during GVBD (as well as during oocyte homogenization), and it was possible that the nucleus was a major source of the M-phase-activated kinases. When enucleated oocytes and isolated intact nuclei were prepared from sea star oocytes prior to GVBD via cytochalasin B treatment, stimulated histone H1 and RRLSSLRA kinase activities were detectable in both the cytosolic and nuclear fractions, but the most dramatic activation of these kinases was evident with the nuclei preparations.[2]

Concurrent studies in this laboratory with *X. laevis* have revealed the presence of kinases analogous to the histone H1 (~6 mmho) and RRLSSLRA (~10.5 mmho) kinases in

maturing frog oocytes (Cicirelli et al., 1987). The latter enzyme may have already been purified from *Xenopus* eggs (Erikson & Maller, 1986).[4] The properties of this *Xenopus* S6 kinase include the following: (i) activation during progesterone-induced oocyte maturation; (ii) relatively tight interaction with DEAE resins; (iii) an apparent M_r of 85 000 on Sephacryl S-300; (iv) the ability to autophosphorylate; (v) a strong substrate preference for ribosomal subunit S6; (vi) use of ATP but not GTP; (vii) inability to effectively use manganese in place of magnesium; and (viii) inhibition by divalent metal cations, but reduced sensitivity to NaCl inhibition as compared with the histone H1 kinase[4] (Martin-Perez et al., 1986; Erikson & Maller, 1986; Cicirelli et al., 1987). A second *Xenopus* egg S6 kinase has also been detected (Erikson & Maller, 1986); this might correspond to the first stimulated RRLSSLRA kinase (~6 mmho) that eluted from DEAE-Sephacel in this study.

The major stimulated RRLSSLRA kinase (~10.5 mmho) features a number of similarities with the mitogen-activated S6 kinase described in various mouse 3T3 cell lines (Novak-Hofer & Thomas, 1984, 1985; Tabarini et al., 1985; Pelech et al., 1986; Pelech & Krebs, 1987). Among other things, both kinases require the presence of phosphatase inhibitors to stabilize their enzymatic activity (Novak-Hofer & Thomas, 1984, 1985), and they exhibit similar chromatographic properties on DEAE-Sephacel, Sephacryl S-300, and TSK-400 (Pelech et al., 1986; Pelech & Krebs, 1987). The insulin-activated S6 kinase from 3T3-L1 cells and the *Xenopus* equivalent of this RRLSSLRA kinase both yielded the same apparent M_r (~55 000) by gradient sedimentation analysis (Tabarini et al., 1985; Erikson & Maller, 1986). Moreover, an S6 kinase that resembles the major RRLSSLRA kinase is activated in extracts from insulin-treated *Xenopus* oocytes (Stefanovic et al., 1986).

Mechanism of Activation of Sea Star Oocyte Maturation-Activated Kinases. The mechanism by which 1-MeAde treatment of sea star oocytes culminates in the stimulation of M-phase-activated kinases remains open. However, a variety of observations suggest regulation of these kinase activities by reversible phosphorylation reactions. (i) The activation process is rapid in the case of the histone H1 kinase (~6 mmho) (Meijer et al., 1987) and, for both the histone H1 and RRLSSLRA kinases, could proceed in the presence of inhibitors of protein synthesis (Figure 5). (ii) The presence of phosphatase inhibitors is essential to preserve maximum activation of these kinases (Figure 6). (iii) The loss of activity in the absence of phosphatase inhibitors occurs in a time-dependent manner (Figure 7), consistent with an enzyme-catalyzed reaction. (iv) Preincubation of the RLSSLRA kinase (~10.5 mmho) in the presence of ATP stabilizes the kinase activity. (v) The activity of the histone H1 kinase with [γ-^{32}P]GTP can be stimulated in the presence of unlabeled ATP (Figure 8). (vi) The activated states of the maturation-activated kinases were stable to fractionation by three different types of chromatography systems (Figures 2 and 3). (vii) The *Xenopus* egg S6 kinase can apparently autophosphorylate in vitro[4] (Erikson & Maller, 1986).

If the histone H1 and RRLSSLRA kinase activities are indeed stimulated by phosphorylation of these kinases, identification of the putative kinase(s) that is (are) responsible will become paramount. An attractive candidate is MPF (maturation- or M-phase-promoting factor), a poorly understood activity that appears to control both meiotic and mitotic cell division [for review see Maller (1985)]. Elucidation of the phosphorylation cascade that is triggered by 1-MeAde binding to its receptor and leads to activation of M-phase-activated kinases may provide important insights into the regulation of cell cycle events in both sea and land animals.

[4] M. Cicirelli, S. Pelech, and E. Krebs, unpublished observations.

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Differential Regulation of Histone H1 and Ribosomal S6 Kinases during Sea Star Oocyte Maturation†

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ABSTRACT: In the preceding paper [Pelech, S. L., Meijer, L., & Krebs, E. G. (1987) *Biochemistry* (preceding paper in this issue)], at least three activated kinases were detected in soluble extracts from sea star oocytes induced to undergo maturation by 1-methyladenine (1-MeAde). Coincident with nuclear envelope breakdown (20 min after exposure to 1-MeAde), there was a rapid activation of a histone H1 kinase that eluted from DEAE-Sephacel with a conductivity of ~6 mmho. By contrast, 60-min treatment of the oocytes with 1-MeAde was required for maximal activation of two kinases, each of which phosphorylated a synthetic peptide, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA), patterned after a phosphorylation site sequence from ribosomal protein S6. These RRLSSLRA kinases were released from DEAE-Sephacel with elution conductivities of ~6 and ~10.5 mmho. The 1-MeAde dose–response curves for maturation induction and activation of the histone H1 and RRLSSLRA kinases were superimposable. Both oocyte maturation and the activation of the kinases required the presence of 1-MeAde during the hormone-dependent period. When 1-MeAde was removed after this period, full histone H1 kinase activation still occurred and maturation was induced. Forskolin pretreatment of the oocytes, by elevating the basal cAMP level more than 35-fold, doubled the hormone-dependent period and similarly delayed the onset of histone H1 kinase activation by 1-MeAde. However, postmaturation activation of the RRLSSLRA kinases was completely blocked by forskolin. The phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibited both 1-MeAde-induced oocyte maturation and activation of the histone H1 kinase at equivalent phorbol ester concentrations, but only when introduced prior to the completion of the hormone-dependent period. On the other hand, TPA elicited modest activations of these kinases in the absence of 1-MeAde.

A fundamental problem in developmental biology is the delineation of the chain of intracellular molecular events that are responsible for oocyte maturation, i.e., the resumption of meiotic divisions. In most animals, oocytes are arrested in the first prophase stage of meiosis and feature a prominent nucleus or germinal vesicle. The rupture of the nuclear envelope or germinal vesicle breakdown (GVBD)[1] serves as a useful marker for oocyte maturation in response to 1-methyladenine

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[1] Abbreviations: cAMP, adenosine 3′,5′-monophosphate; GVBD, germinal vesicle breakdown; I_{50}, dose producing a 50% inhibition; 1-MeAde, 1-methyladenine; MPF, maturation-promoting factor; PB1, emission of the first polar body; RRLSSLRA, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate).

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