Journal of Cellular Biochemistry 75:272–287 (1999)

Characterization of Fertilization-Modulated Myelin Basic Protein Kinases From Sea Star: Regulation of Mapk

Diana L. Lefebvre,[1] **David L. Charest,**[1] **Arthur Yee,**[3] **Bruce J. Crawford,**[2] **and Steven L. Pelech**[1]*

[1]Department of Medicine, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada
[2]Department of Anatomy, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada
[3]Kinetek Pharmaceuticals, Inc., Vancouver, British Columbia V6P 6P2, Canada

Abstract The myelin basic protein (MBP)-phosphorylating enzymes present during maturation and early embryogenesis of the sea star (*Pisaster ochraceus*) were investigated. The major maturation-activated MBP kinase (p45 Mapk) was molecularly cloned based on tryptic sequence information obtained with the purified enzyme and shown to be highly related to human Erk1 with 76% amino acid identity. Kinase assays and immunoblotting studies revealed that Mapk remained highly active until 12 h post-fertilization (PF), after which it declined. By 4 days PF, Mapk protein was no longer detectable. At 3 h PF, about half of the detectable MBP phosphotransferase activity could be attributed to a 75 kDa protein kinase that was distinct from Mapk. Like Mapk, this protein phosphorylated MBP mostly on threonine residues, but it failed to phosphorylate a peptide (APRTPGGRR) based upon the Thr-97 MAP kinase phosphorylation site in MBP. Rather, it phosphorylated a peptide (AAQKRPSQRTKYLA) patterned after the N-terminus of MBP. Our studies also showed a dramatic increase in MBP phosphotransferase activity occurred by 4 days PF that arose from a third kinase that phosphorylated MBP solely on serine residues. This kinase exhibited the following substrate substrate preference: AAQKRPSQRTKYLA, peptide substrate for S6 kinases (AKRRRLSSLRASTSKSESSQK) > MBP > histone H1 > protamine > casein > APRTPGGRR. This kinase was not appreciably affected by addition of phosphatidylserine/diacylglycerol, or the staurosporine analogue Roche Compound 3, but it was partly inhibited by a protein kinase C pseudosubstrate peptide. Gel filtration analysis revealed an apparent molecular mass of 41 kDa for the enzyme. Therefore, at least two novel MBP-phosphorylating enzymes distinct from Mapk are preferentially activated following fertilization and early embryogenesis of the sea star. J. Cell. Biochem. 75:272–287, 1999.

Key words: MAP kinase; sea star; oocyte maturation; fertilization

Cell cycle progression during both meiosis and mitosis is orchestrated by an elaborate program of reversible protein phosphorylation events. Most immature oocytes, including those from sea star, are arrested at the G_2/M border of first meiotic prophase of oogenesis [Davidson, 1986]. The first morphological change that is evident during the induction of sea star oocyte maturation by the hormone 1-methyladenine (1-MeAde) is nuclear envelope disassembly, also referred to as germinal vesicle breakdown (GVBD) [Doree, 1983]. Subsequently, meiosis I is completed and the cell progresses to metaphase of meiosis II, where the mature oocytes are arrested until fertilization. A burst in net protein phosphorylation is triggered near the onset of GVBD [Maller and Krebs, 1977; Pelech et al., 1990]. Many of the protein kinases that are activated at meiotic maturation have been shown to be capable of phosphorylating myelin basic protein (MBP). These kinases can be distinguished from each other on the basis of their time courses of activation, their substrate specificities, and their chromatographic properties [Pelech et al., 1988].

The best characterized oocyte model for the investigation of the dynamics of protein kinase

Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; GVBD, germinal vesicle breakdown; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; 1-MeAde, 1-methyladenine; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; phenylmethylsulphonyl fluoride, PMSF; MOPS, 4-morpholine propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Grant sponsor: Medical Research Council of Canada (MRCC).

*Correspondence to: Dr. Steven Pelech, Department of Medicine, 2nd Floor, Koerner Pavilion, 2211 Wesbrook Mall, University of British Columbia, Vancouver, B.C., Canada, V6T 1Z3. E-mail: spelech@home.com

Received 26 February 1999; Accepted 7 May 1999

activation and their involvement in cell proliferation and differentiation has been *Xenopus laevis*. In this system, oocytes are matured by stimulation with agents including progesterone and insulin [reviewed in Pelech and Charest, 1995]. Early research demonstrated that the activity of the proline-directed, mitogen-activated protein (MAP) kinase known as ERK2 (extracellularly-regulated protein kinase) is tightly regulated during oogenesis and early development [Posada et al., 1991]. Near the time of GVBD, MAPK is activated and remains in this state in unfertilized eggs during meiotic arrest. Following fertilization, MAPK phosphotransferase activity rapidly declines and remains low until the onset of mesoderm induction where the activity of the kinase increases rapidly in response to fibroblast growth factor [Hartley et al., 1994].

The activation of Erk2 during meiotic maturation of echinoderm oocytes is distinct from the frog system, since the major MBP kinase that is stimulated during meiosis I near the onset of GVBD in sea star oocytes was found to be a 45-kDa kinase, p45 Mapk [Pelech et al., 1988; Sanghera et al., 1991]. Although this sea star Mapk possesses the regulatory tyrosine phosphorylation site before the subdomain VIII region of the catalytic domain, it appeared to lack the threonine phosphorylation site that is required for activation of other MAP kinase isoforms [Posada et al., 1991; Charest et al., 1993].

In the sea star system, the vast majority of information concerning the time course of activation (tyrosine phosphorylation) is limited to events surrounding the meiotic maturation and to only a few hours following fertilization. In this study, we employed the sea star oocyte system to investigate the changes in Mapk and other MBP kinases at various time points following fertilization, early development, and gastrulation. We have detected two novel MBP kinases that are activated during early embryogenesis.

MATERIALS AND METHODS

Materials

The *Pisaster ochraceus* sea stars were collected between May and July, the annual reproductive cycle of these sea stars, by scuba diving in the local waters surrounding Vancouver, British Columbia, Canada. Sea stars were individually sexed by sampling of gonadal tissue with a 20 ml syringe and 18-gauge needle. The 4G10 and anti-Erk1-CT antibodies were available from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-protein kinase C (cPKCα) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgGs and horseradish peroxidase-conjugated goat anti-mouse IgG were obtained from Bio-Rad. A staurosporine analogue, Roche Compound 3 (Cmpd3), was a gift from Dr. Michael Venuti (Department of Bio-Organic Chemistry, Genentech, San Francisco, CA). The pseudosubstrate inhibitor peptide of PKC (PKC-I; RFARKGALRQKNV) was produced by solid phase synthesis. Myelin basic protein (MBP) was purified from bovine brain [Deibler et al., 1984]. MAPK substrate peptide MBP-T97 (APRTPGGRR) was synthesized according to the Thr-97 phosphorylation site of MBP [Clark-Lewis et al., 1991]. The MBP-NT peptide (AAQKRPSQRTKYLA) was based on the amino-terminal portion of MBP, which is not phosphorylated by MAPK. The S6 peptide (AKRRRLSSLRASTSKSESSQK) was synthesized after a carboxyl terminal region of the S6 protein of the 40S ribosome complex that is phosphorylated by S6 kinase. The ECL detection reagent kit for Western blotting was obtained from Amersham (Canada). Protein A-Sepharose and HR5/5 Mono Q and Superose-12 were purchased from Pharmacia. Other reagents were purchased from Sigma.

Culture of Sea Star Oocytes and Preparation of Cellular Extracts

Immature oocytes arrested in the G_2/M border of meiosis were isolated from sea stars as previously described [Meijer et al., 1984]. Sea stars were induced to spawn by injection into the body cavity of a minimum of 1 ml per arm of 0.14 mM 1-methyladenine (1-MeAde) in Millipore-filtered seawater [Fraser et al., 1981]. Shedding of mature oocytes (characterized by GVBD as the disappearance of the nuclear and nucleolar membranes) typically commenced between 60 and 90 min following primary injection. During spawning, sea stars were inverted over 400 ml beakers that contained Millipore-filtered seawater. After approximately 60 min of shedding, the oocytes were allowed to settle and were washed with three changes of filtered seawater. At this time, an aliquot of mature oocytes was collected for an experimental time point and stored on ice prior to processing of

cellular extract. The remaining oocytes were suspended at a concentration of 1% (v/v) in filtered seawater. The container was placed in a refrigerator equilibrated to 12 ± 0.5°C and the seawater was gently aerated and oscillated at 40 rpm. The oocyte suspension was allowed to equilibrate at this temperature for at least 1 h prior to addition of sperm. Sperm was collected from male sea stars and diluted to 1:200 (v/v) with filtered seawater. Sperm viability and motility were verified by phase microscopy. Equilibrated mature oocytes were fertilized by addition of 1:100 (v/v) of the sperm dilution (effective dilution of 1:20,000 (v/v)). Fertilization of oocytes was confirmed in an aliquot of oocytes at approximately 1 h following sperm addition by observing the elevation of fertilization membranes. Embryo cultures with less than 70% fertilization of oocytes were discarded.

At specific time points during development, the oocytes or embryos were pelleted at 4°C in a Beckman J2-HS centrifuge (1,500 rpm for 5 min). A 33% oocyte suspension was prepared in chilled homogenization buffer (20 mM MOPS, pH 7.2, 50 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 1 mM sodium vanadate, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulphonyl fluoride [PMSF], and 1 mM benzamidine). Oocytes in suspension were disrupted with two 30 sec bursts at 19,000 rpm of a Polytron (PT3000, Brinkman, Westbury, NY) and cell breakage was verified by light microscopy. Homogenates were immediately centrifuged in a Sorval Combi Ultracentrifuge (Dupont, Canada) at 10,000*g* for 10 min. The supernatant was then decanted and centrifuged at 250,000*g* for 30 min. Supernatants were quickly aliquoted and stored at −70°C.

Mono Q Fractionation

Cellular homogenates (~5 mg protein, as determined by Bradford reagent assay with bovine serum albumin (BSA) as the protein standard) were fractionated on a HR5/5 Mono Q column (1 ml bed volume) equilibrated with buffer A (12.5 mM MOPS, pH 7.2, 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, and 1 mM DTT). The column was eluted with a 10 ml linear 0-0.8 M NaCl gradient in buffer A using the Pharmacia Fast Protein Liquid Chromatography system and 250 µl fractions were collected for enzyme assays and immunoblotting analysis.

Measurement of Phosphotransferase Activity

MBP, MBP-NT peptide, MBP-T97 peptide, S6 peptide, protamine, casein, and histone H1 phosphotransferase activities were measured by incubating in a final volume of 30 µl: 30 µg substrate, 10 µl Mono Q fraction, 0.5 mM cAMP dependent protein kinase inhibitor peptide (PKI), 50 µM [γ-^{32}P]-ATP (~2,000 cpm/pmol) and assay dilution buffer, pH 7.2 (20 mM MOPS, 25 mM β-glycerophosphate, 20 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 1 mM DTT, and 1 mM sodium vanadate). For assays that included a protein kinase C inhibitor or pseudosubstrate (final concentrations: 20 nM Cmpd3 or 1 µM of PKC-I) the total volume of the reaction was 35 µl. Reactions that required the presence of lipid, contained 5 µl of 30 µg/ml phosphatidylserine/diacylglycerol (PS/DAG) and the total volume of the reaction was 35 µl. Phosphotransferase activity assays were conducted for 10 min at 30°C and were terminated by spotting a 20 or 25 µl aliquot of the reaction mixture onto a 1.5-cm^2 piece of p81 phosphocellulose paper. The papers were washed 10 times in 1% phosphoric acid before their transfer into a 6-ml plastic scintillation vial that contained 0.5 ml scintillant and counting in a scintillation counter.

Electrophoresis, Immunoblotting, and Phosphotransferase Quantitation

SDS-polyacrylamide gel electrophoresis (PAGE) was performed essentially as described by Laemmli [1970]. Typically, a 10% separating gel and a 4% stacking gel were used. Samples, containing SDS-sample buffer (4× solution containing 125 mM Tris-HCl, pH 6.8, 4% SDS [w/v], 20% glycerol [v/v], 0.3 M β-mercaptoethanol, 0.01% bromophenol blue [w/v]) were boiled for 5 min and electrophoresed for 18 h at 10 mA per gel. Following SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes at 300 mA for 3 h. For immunoblotting, membranes were Ponceau-stained, to verify equivalent loading of proteins, and then blocked with TBS (0.5 M Tris, pH 7.5, and 1.5 M NaCl) containing 5% skim milk powder for 2 h at room temperature. Membranes were washed twice for 5 min with TBS containing 0.05% Tween-20 (TTBS) prior to incubation with primary antibody (in TTBS with 0.1% sodium azide) for 2–18 h at room temperature. Membranes were then washed twice with TTBS

before incubation with the secondary antibody (goat anti-rabbit IgG coupled to alkaline phosphatase in TTBS; 1:3,000 dilution) for 2 h at room temperature. The membranes were rinsed with two washes of TTBS for 5 min, one wash of TBS, and then incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) colour development solution (mixture of 3% NBT in 1 ml 70% dimethylformamide [DMF] and 1.5% BCIP in 1 ml 100% DMF before addition of 100 ml of 0.1 M Tris, 0.1 M NaCl and 5 mM $MgCl_2$, pH 9.5). Colour development typically occurred in 5–20 min and the reaction was stopped by rinsing membranes in a large volume of water. Essentially the same protocol was used for anti-phosphotyrosine blots, except that membranes were blocked with low-salt TBS (20 mM Tris, pH 7.5 and 50 mM NaCl) that contained 3% BSA and all washes were performed with low-salt TBS containing 0.05% Nonidet-40 (NP-40). If ECL was the development procedure of choice, the secondary antibody was either the goat anti-rabbit (for Erk1-CT and PKCα antibodies) or the goat anti-mouse (for 4G10 antibody) antibodies conjugated with horseradish peroxidase (1: 2,500 dilution) and the incubation time was 45 min.

Immunoprecipitation

MAPK was immunoprecipitated by incubation of 1 mg of cellular homogenate (containing 10 µg/ml aprotinin, 2 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, and 2 µg/ml PMSF) with 1% SDS in a microcentrifuge tube. An equal volume of 6% NETF (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 50 mM NaF, and 6% NP-40) and 10 µl of Erk1-CT antibody (1 mg/ml) were added to these tubes and the tubes were rotated at 4°C for 2 h. Protein A-Sepharose beads (20 µl) were added and the mixture rotated at 4°C for 2 h. The protein A-Sepharose beads were pelleted by centrifugation and washed successively with two washes each of 6% and 0% NETF (containing 6% and 0% NP-40, respectively). SDS-sample buffer (100 µl) was added to the beads and boiled for 5 min. Beads and sample buffer were loaded onto SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to nitrocellulose membranes and the blots were probed with Erk1-CT or 4G10 antibodies.

Phosphoamino Acid Analysis

MBP was phosphorylated by mature, 3 h PF and 5 day PF extracts using the same protocol as described for MBP phosphotransferase activity, except that the reaction was stopped by addition of 15 µl of SDS sample buffer and boiled for 5 min. Following electrophoresis on 12.5% SDS-polyacrylamide gels, the proteins were transferred on to PolyScreen Polyvinylidene fluoride (PVDF) membrane (Dupont, Canada) for 3 h at 300 mA. The membrane was exposed to Reflection autoradiography film (Dupont, Canada) for 2 h and the area on the membrane corresponding to phosphorylated MBP was excised, minced, and hydrolyzed in constant boiling HCl at 105°C for 1 h. The supernatants were transferred into 0.5-ml centrifuge tubes and evaporated in a Speed-Vac until dryness was achieved. The samples were washed consecutively with 300 µl, 100 µl, 50 µl, and 30 µl of distilled water. Dry samples were then resuspended in running buffer (0.5% Pyridine, 5% acetic acid) that contained 1 mg/ml standards (phospho-threonine, phospho-serine and phospho-tyrosine; Sigma Chemical Company). Samples (~2,000 cpm) were spotted on to a Kodak Thin-Layer Chromatography (TLC) cellulose plate (Dupont, Canada) and electrophoresed at 1,000 V for 1 h with cooling. The TLC plate was sprayed with a ninhydrin solution (0.2% in 100% ethanol) and heated at 90–95°C for 5 min to visualize the standards. The TLC plate was then exposed to Reflection film with an intensifying screen at −70°C for 3–7 days.

Superose-12 Gel Filtration Chromatography

A Superose 12 column (Pharmacia) was equilibrated with 0.25 M NaCl in KII buffer (containing 125 mM MOPS, pH 7.2; 125 mM β-glycerophosphate; 5 mM EGTA, 75 mM $MgCl_2$, 0.5 mM NaF) and developed at a rate of 0.2 ml/min. The elution positions of blue dextran (2,000,000 MW), BSA (67,000 MW), ovalbumin (43,000 MW), chymotrypsinogen A (25,000 MW), and ribonuclease A (13,700 MW) were determined prior to the sample run. The Mono Q fraction of 5 day PF extracts that possessed peak MBP phosphotransferase activity (fraction #30) was applied to the equilibrated column and 0.25 ml fractions were collected and assayed for MBP phosphotransferase activity (as described above).

Cloning and Sequencing of Mipk

RNA isolation from 300 µl of packed immature *P. ochraceus* oocytes was performed using the RNeasy isolation kit from Qiagen. The quantity of RNA was assessed following electrophoresis on a 1% agarose/5% formaldehyde gel in the presence of ethidium bromide. mRNA preparations were made using the Qiagen Oligotex mRNA kit. From approximately 440 µg of total sea star RNA, 4 to 22 µg of mRNA in 60 µl were isolated. The Perkin-Elmer GeneAmp RNA PCR kit was used in the synthesis of *P. ochraceus* cDNA from 10 ng of mRNA.

For degenerate PCR, Mapk peptide sequences [Posada et al., 1991] were aligned with the protein sequence for human Erk1. From this information, degenerate oligonucleotides were designed in orientations that permitted amplifications of a ~576 bp fragment by PCR with Taq polymerase. The amplified fragments were cloned into the vector pBluescript SK and the cDNA sequence determined. Cloning into T-tailed pBluescript vector was based on the procedure of Holton and Graham [1991]. The Boehringer terminal deoxytransferase kit was used to T-tail the Bluescript vector. Transformations using the pBluescript vector into DH5a competent *E. coli* cells plated on LB/ampicillin/X-gal agar were performed as described [Sambrook et al., 1989]. Small quantities of plasmids were prepared using the Qiagen, QIAprep Plasmid Kit, whereas large quantity DNA preparations were performed using the Qiagen Plasmid Midi Kit.

The 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions were performed using the Clontech Marathon cDNA Amplification Kit [Chenchik et al., 1996]. PCR amplifications of the 3′ and 5′ ends of the Mapk cDNA were undertaken with the Clontech Advantage KlenTaq polymerase mix. To 5 µl of diluted Ad-cDNA were added 5 µl 10× KlenTaq buffer, 20 µM dNTPs, 0.2 µM gene specific primer, 0.2 µM adaptor primer. A touchdown PCR method of amplification was used. The reaction products were purified on a low melting point agarose gel and the appropriate product used for ligation using a T-tailed vector by the method of Kalvakolanu and Livingston [1991]. The cDNA sequence data from the RACE-derived fragments was used to design oligonucleotide primers for the PCR amplification of the full-length cDNA.

Clones were sequenced by automated fluorescent DNA sequencing at UCDNA (University of Calgary) and the NAPS Laboratory (University of British Columbia). Reactions were performed using the standard dideoxy chain termination method [Sanger et al., 1980] with custom primers using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Perkin Elmer). Reactions were performed using a DNA thermal cycler model 480 (Perkin Elmer) and products purified by ethanol precipitation.

RESULTS

Morphological Events During Oocyte Maturation and Following Fertilization

To investigate protein kinase regulation in fertilized sea star eggs, mature *Pisaster ochraceus* oocytes were treated with sea star sperm for 3 h to 5 days prior to harvest (as described in Experimental Procedures). Immature oocytes (Fig. 1A) collected from *Pisaster ochraceus* routinely underwent maturation after exposure to 1-MeAde for 60–80 min at 14°C, as determined by visual confirmation of GVBD (Fig. 1B; in the absence of fertilization, two polar body emissions occur by 5 h later). Typically, fertilization of the oocytes at 12°C following GVBD was first evident by the elevation of a fertilization membrane within 45 min after sperm addition (Fig. 1C). The first mitotic cell divisions occurred at 4 h PF and the second reductive cell cleavage took place by 6 h PF (Fig. 1D) and formation of a hollow blastula enveloped by a fertilization membrane could be observed at 24 h PF (Fig. 1G). The embryos hatch out of this membrane, become free swimming and a primitive gut endoderm begins forming shortly after 24 h PF. A primitive gut is actively forming by 72 h PF. By about 144 h PF (6 days), the embryo has developed into a free swimming, actively feeding organism.

Molecular Cloning of Mapk

We previously identified Mapk as the major MBP kinase that is activated during meiotic maturation of sea star oocytes [Pelech et al., 1988] and sequence analysis of tryptic peptides derived from purified Mapk indicated that it was related to the Erk1/Erk2 family of MAP kinases [Sanghera et al., 1990; Posada et al., 1991]. To confirm Mapk's identification as an echinoderm cognate of mammalian Erk1, we

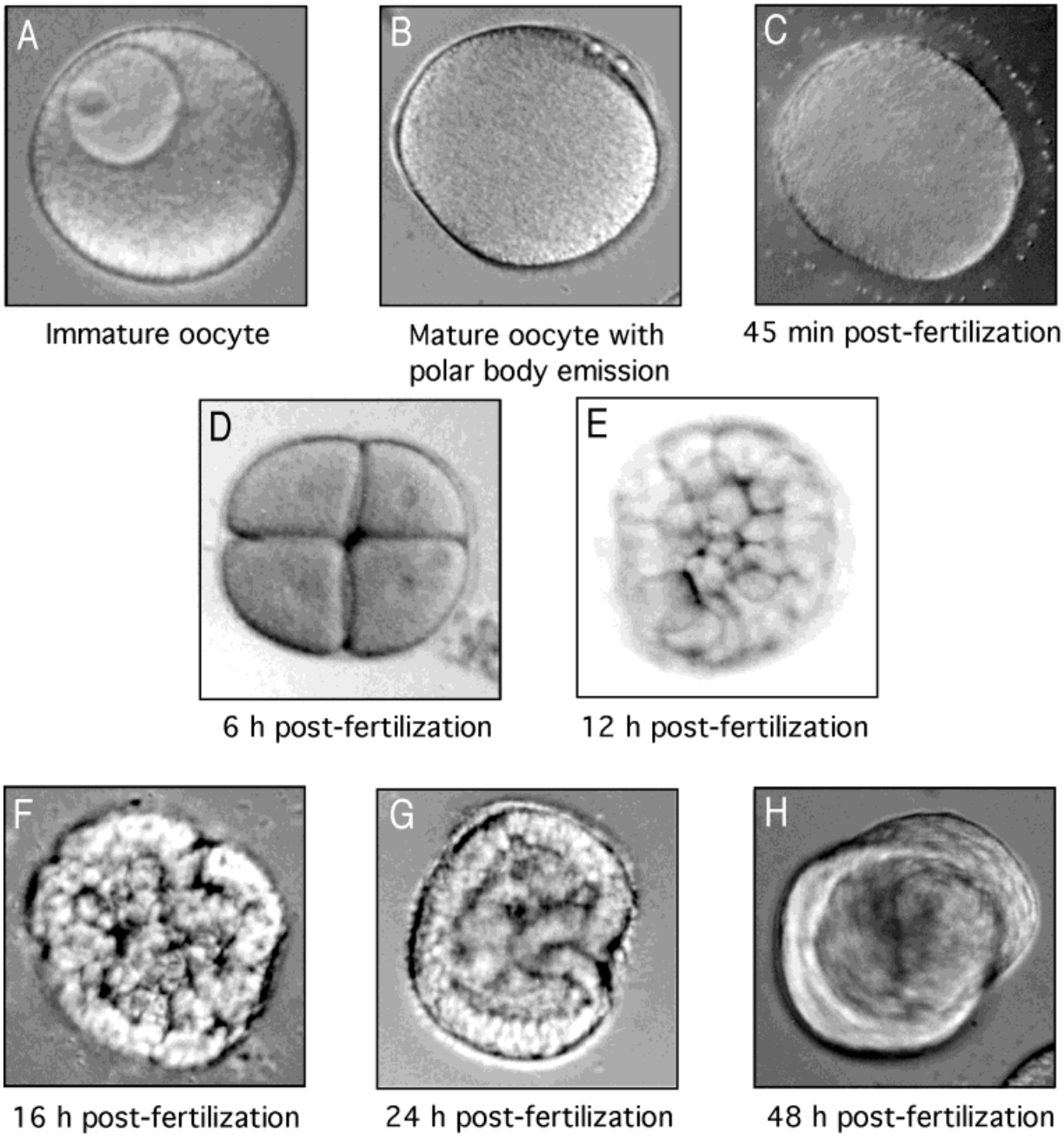

Fig. 1. Fertilization and early developmental stages of the *Pisaster ochraceus* eggs. **A:** An immature oocyte showing the germinal vesicle with its nucleolus and surrounded by a jelly coat. **B:** Mature oocyte following germinal vesicle breakdown and emission of two polar bodies. **C:** Fertilized egg 45 min after sperm addition showing raised fertilization membrane. **D:** Six h post-fertilization following first and second cell cleavages. **E:** Twelve h post-fertilization. **F:** Sixteen h post-fertilization. **G:** Twenty-four h post-fertilization showing an unhatched blastula with gastrulation. **H:** Forty-eight h post-fertilization with swimming blastula.

cloned a cDNA of seastar Mapk by PCR using degenerate oligonucleotides based on the sequences of the tryptic peptides of Mapk. As shown in Figure 2, the primary structure of Mapk was 76% identical in amino acid sequence to human Erk1. In particular, the Thr-Glu-Tyr dual phosphorylation site motif located upstream of kinase catalytic subdomain VIII was completely conserved. Between subdomains V to X, the sequences were almost identical, and the greatest differences occurred in the N- and C-termini of these MAP kinases. This

```
                                                                    I
Seastar Mapk  MAA......FGSSASDKHPASSASDNMPEVVRGQIFDVAPRYTGLAYIGEGAYGMVCSAT
Human Erk1    MAAAAAQGGGGGEPRRTEGVGPGVPGEVEMVKGQPFDVGPRYTQLQYIGEGAYGMVSSAY
consensus     MAA       G                 E V GQ FDV PRYT L YIGEGAYGMV SA
                       II                III           IV
Seastar Mapk  DAKYLSKVAIKKISPFEHQTYCQRTLREIKILTRFRHENIINIQDIIHANSIDEMKDVYI
Human Erk1    DHVRKTRVAIKKISPFEHQTYCQRTLREIQILLRFRHENVIGIRDILRASTLEAMRDVYI
consensus     D      VAIKKISPFEHQTYCQRTLREI ILTRFRHEN I I DI  A   D M DVYI
               V                                              VI
Seastar Mapk  VQSLMETDLYKLLKTQKLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCDL
Human Erk1    VQDLMETDLYKLLKSQQLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLSNTTCDL
consensus     VQSLMETDLYKLLKTQKLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLL NTTCDL
                  VII                  * *      VIII               IX
Seastar Mapk  KICDFGLARIADPVHDHTGFLTEYVATRWYRAPEIMLNSKGYNKSIDIWSVGCILAEMLN
Human Erk1    KICDFGLARIADPEHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLS
consensus     KICDFGLARIADP HDHTGFLTEYVATRWYRAPEIMLNSKGY KSIDIWSVGCILAEML
                                     X
Seastar Mapk  GKPIFPGKHYLDQLNHILNILGSPSCEDLGCIHNDKARGYMQSLPNKPTVPWKRLYGAAD
Human Erk1    NRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKSD
consensus       PIFPGKHYLDQLNHIL ILGSPS EDL CI NDKAR Y QSLP K  V W  L    D
                            XI
Seastar Mapk  PKSLSLLDRMLTFNPDKRITVEDALKHDYLEQYHDPTDEPVAEEPFKFETELDDLPKEEL
Human Erk1    SKALDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDEPVAEEPFTFAMELDDLPKERL
consensus      K L LLDRMLTFNPDKRITVE AL H YLEQYHDPTDEPVAEEPF F  ELDDLPKE L

Seastar Mapk  KELIFEEAKKFALLPRNEMDGGEMS  379
Human Erk1    KELIFQETARFQPGVLEAP      379
consensus     KELIF E   F
```

Fig. 2. Primary structures of seastar Mapk and human Erk1. The locations of the kinase catalytic subdomains are indicated by Roman numerals and the highly conserved residues in these domains are bold-faced. The activating phosphorylation sites in the Thr-Glu-Tyr motif are asterisked.

validated the use of antibodies developed against human Erk1 for the analysis of sea star Mapk.

Activation of MBP Kinases After Fertilization

We next wanted to determine whether Mapk was responsible for the major MBP phosphotransferase activity shortly after oocyte fertilization [Pelech et al., 1988]. To assess MBP phosphorylating activity in oocytes following fertilization, cytosolic extracts were fractionated by fast protein liquid chromatography on a MonoQ column and phosphotransferase activities towards MBP were quantitated (Fig. 3). As previously demonstrated [Pelech et al., 1988], a dramatic increase in MBP phosphotransferase activity (approximately seven-fold) was detected following following GVBD in the oocyte (Fig. 3A). The major activity coeluted with immunoreactivity with Erk1-CT antibody with a 45-kDa protein that corresponded to phosphorylated Mapk (Fig. 3A, inset). There was also lesser amounts of a 42-kDa immunoreactive protein that eluted slightly later in the shoulder of the MBP kinase activity peak. This probably corresponded to a sea star Erk2 cognate. At 3 h PF, MBP phosphotransferase activity was 10-fold greater than that measured in immature oocytes, and about 30% more than the activity found in mature oocytes (Fig. 3B). However, by this time, approximately half of the Mapk was in the dephosphorylated state as shown by the appearance of the doublet of Mapk visualized by Western blotting analysis (Fig. 3B, inset). MBP phosphorylating activity detected in extracts from 6 h, 12 h, 24 h, and 2 days PF displayed a general decrease in activity to levels 30–50% of the level observed in mature oocytes. However, extracts from 4 day and 5 day PF (Fig. 3C) demonstrated a dramatic increase in MBP phosphorylating activity that amounted to a 3.5-fold stimulation over the activity observed in mature, unfertilized oocytes.

To determine the extent of the MBP phosphorylating activity that could be attributed to Mapk, we repeated these activity measurements in MonoQ fractions using the MBP-T97 peptide (APRTPGGRR) patterned after the Thr-97 site of MBP that is phosphorylated by sea star Mapk [Clark-Lewis et al., 1991] (Fig. 4A). After GVBD in maturing oocytes, the in-

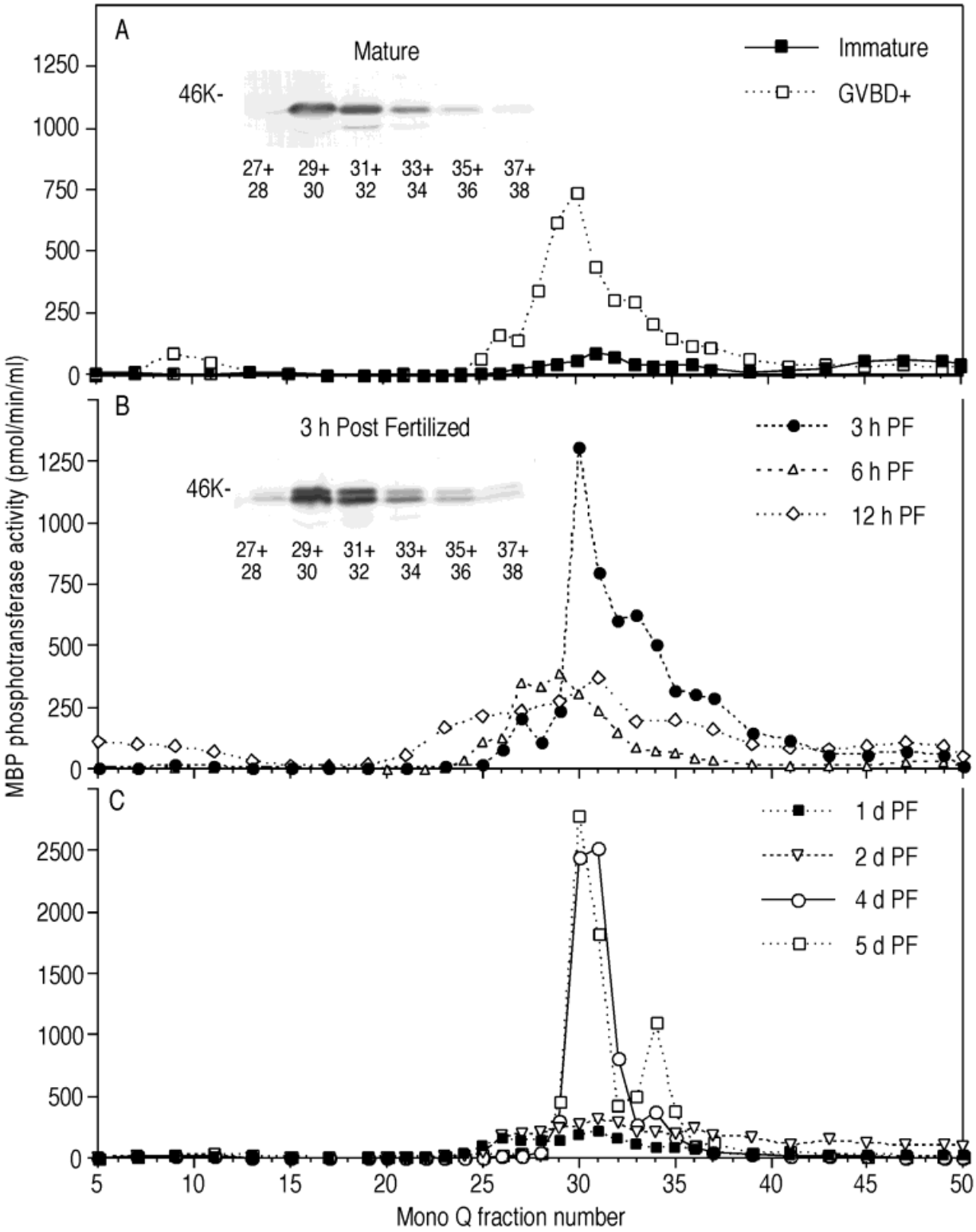

Fig. 3. MonoQ fractionation of MBP phosphotransferase activity at various developmental stages. Cytosolic homogenates (~5 mg) were fractionated separately on a Mono Q column (1 ml bed volume). The column was developed with a 10 ml linear 0–0.8 M NaCl gradient at a flow rate of 1 ml/min. The column fractions (0.25 ml) were assayed for phosphorylating activity towards 1 mg MBP/ml. Phosphotransferase activities are expressed in pmol/min/ml. Extract time points are given in hours post-fertilization (PF). This figure shows data from a representative experiment that was repeated at least three times for each extract. The insets in **A** and **B** show immunoblots probed with Erk1-CT antibody of MonoQ fractions from maturing, GVBD+ oocytes (A) and 3 h PF cells (B). **C:** Extracts from 1 to 5 days PF.

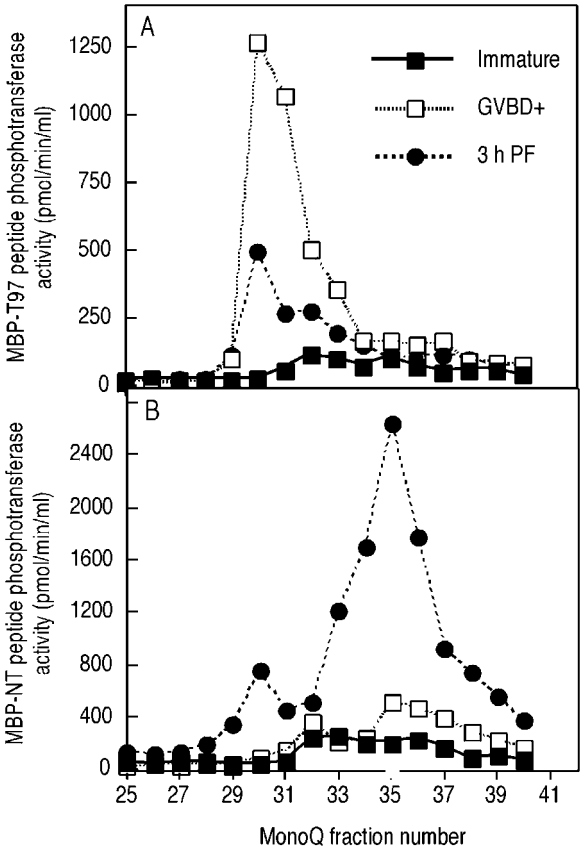

Fig. 4. MonoQ fractionation of MBP-T97 and MBP-NT peptide phosphotransferase activities at various developmental stages. The MonoQ column fractions described in Figure 3 from immature (filled squares), GVBD+ (open squares), and 3 h PF cells (filled circles) were assayed for phosphotransferase activity towards 1 mg/ml of peptides patterned after the Thr-97 phosphorylation site in MBP (**A**) or the N-terminus of MBP (**B**). This figure shows data from a representative experiment that was repeated at least three times for each extract.

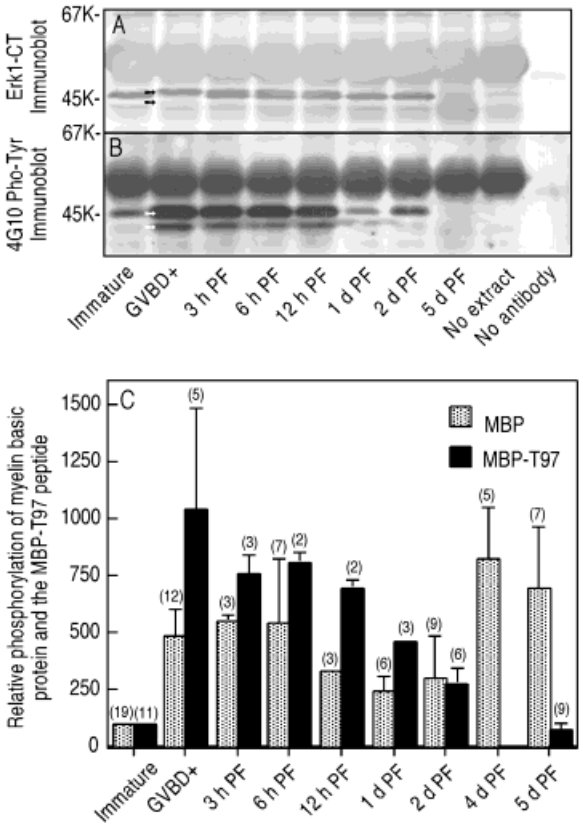

Fig. 5. Regulation of Mapk during embryonic development. Approximately 1 mg of cytosolic homogenate from different extracts was immunoprecipitated with the Erk1-CT antibody in the presence of 1% SDS. Following electrophoresis on a 10% SDS polyacrylamide gel, the proteins were transferred to nitrocellulose membranes and then probed with antibodies. **A:** This figure shows a representative Western blot of the immunoprecipitated Mapk probed with the Erk1-CT antibody. The antibody control contains all the components of reaction except extract; the extract/protein A-Sepharose control contains all components of reaction except antibody. The dark arrows point towards the putative phosphorylated forms of Mapk and Erk2. **B:** The same Western blot of immunoprecipitations was stripped and reprobed with the anti-phosphotyrosine (4G10) antibody. The tyrosine phosphorylated p45 Mapk and p43 Erk2 bands are indicated by white arrowheads. Migration positions of prestained molecular weight standards are indicated at the left. Similar results were obtained in four independent experiments. **C:** The same MonoQ fractions (#28–33) from various extracts were assayed for phosphorylating activity toward 1 mg/ml MBP (stippled bars) and toward 1 mg/ml of a peptide patterned after the Thr-97 phosphorylation site of MBP (black bars). Phosphotransferase activities are expressed relative to the activity with each substrate detected in MonoQ fractionated extracts from immature oocytes. Error bars depict the SD (or range if n = 2) of separate experiments, the number of which for each time point are indicated within the brackets on the figure.

crease in MBP phosphorylating activity was paralleled closely by the stimulation of MBP-T97 peptide phosphotransferase activity in the same MonoQ fractions. Thus, the majority of the MBP phosphotransferase activity in maturing oocytes appeared to be due to Mapk.

The level of MBP-T97 peptide phosphorylating activity declined rapidly following fertilization, in keeping with the dephosphorylation of Mapk by immunoblotting analysis. The pooled data from over 25 separate MonoQ fractionations of extracts from fertilized oocytes and embryos revealed that the MBP-T97 peptide phosphotransferase activity steadily declined after fertilization (Fig. 5C). As shown below, this generally correlated with the reappearance of the dephosphorylated form of Mapk and then the complete disappearance of the kinase (Fig. 5A).

To specifically investigate the regulation of Mapk during the various stages of oocyte maturation and fertilization, the Mapk was immunoprecipitated with the Erk1-CT antibody. The immunoprecipitates were further analyzed by

Western blotting with Erk1-CT antibody. Upon activation, the members of the MAPK family undergo reduced mobility in SDS-PAGE gels corresponding to an increase in tyrosine phosphorylation. A noticeable and quantitative shift in the mobility of the Mapk to a slower migrating form was apparent in the extracts from maturing GVBD positive oocytes as compared to that detected in immature oocytes (Fig. 5A). However, the Erk1-CT antibody detected a doublet of Mapk in the 3, 6, and 12 h PF extracts, with the upper band corresponding to the slower mobility Mapk seen in mature extracts and the lower band migrating at the same position as Mapk in immature extracts. By 24 h following fertilization, the slower mobility form of Mapk disappeared and the lower form persisted until 2 days PF. No Mapk protein was detected in the extracts from 4 day PF (data not shown) or 5 day PF embryos (Fig. 5A). It should be noted that a greater degree of proteolytic activity was observed in both the 4 day PF and 5 day PF extracts. Despite addition of 10-fold greater quantities of protease inhibitors, no Mapk was immunoprecipitated with this antibody for either of these time points.

Probing the Erk1-CT immunoprecipitates with the anti-phosphotyrosine-specific 4G10 antibody revealed that tyrosine residues were phosphorylated on Mapk in extracts from maturing oocytes and 3 h to 12 h PF cells (Fig. 5B). As expected, no tyrosine phosphorylation of Mapk was detected in the immunoprecipitates of immature extracts, indicating that Mapk was inactive in immature oocytes. This result, along with the visible shift in the electrophoretic mobility of Mapk, is in keeping with previous observations that a heavily phosphorylated, active form of MAP kinase has a slightly retarded migration pattern on SDS-PAGE [Sanghera et al., 1991]. Little or no tyrosine phosphorylation of the 45-kDa Mapk or the 42-kDa Erk2-like protein was detected in extracts from 1 to 5 day PF embryos. Therefore, Mapk was only significantly active during oocyte maturation and early mitosis and did not seem to be important after 24 h PF.

Relationship of Protein Kinase C to MBP Phosphotransferase Activity After Fertilization

Based on our data with the MBP-T97 peptide phosphotransferase assays and the Erk1-CT immunoprecipitations, the MBP phosphorylating activities detected in fertilized cells and embryos were largely due to protein kinases that were distinct from Mapk and the Erk2-like protein. Since the N-terminal portion of MBP features phosphorylation sites for other protein kinases such as protein kinase A (PKA), protein kinase C (PKC), and S6 kinase, we synthesized a peptide (AAQKRPSQRTKYLA) based on the first 14 residues of MBP. Analysis of the MonoQ fractions from immature, maturing and 3 h PF cells with this MBP-NT peptide as a substrate reveal the marked fertilization-associated activation of a protein kinase that eluted later (peak in fractions 34–36) from the column than the MBP-T97 kinase identified as Mapk (peak in fractions 30–31) (Fig. 4). Only a two-fold stimulation of this MBP-NT peptide kinase was evident at the time of GVBD, when Mapk was fully activated. The MBP-NT peptide was also an excellent substrate for the highly active protein kinase that eluted in MonoQ fractions 29 to 31 following fractionation of extracts from 4 and 5 day embryos (Fig. 5B).

As the MBP-NT peptide is strongly phosphorylated by PKC, we investigated whether the major MBP-NT kinases that were evident in the 3 h and 5 day PF extracts corresponded to PKC. Initially, we tested the ability of the PKC activators phosphatidylserine, diacylglycerol and calcium [reviewed by Hug and Sarre, 1993] to stimulate MBP-NT phosphotransferase activity in MonoQ fractions of extracts from maturing oocytes, since these extracts had a lower level of MBP-NT kinase activity. As shown in Figure 6A, these lipid activators and calcium stimulated by up to three-fold the measurable phosphotransferase activity towards MBP-NT in MonoQ fractions 29 to 36. However, there was little or no activation of the MBP-NT phosphotransferase activity with these lipids and calcium for the MBP-NT kinase peaks in 3 h PF (MonoQ fractions 33 to 37) and 5 day PF (MonoQ fractions 29–31) cell extracts (Fig. 6B,E). Immunoblotting with an antibody for PKC-alpha confirmed the presence of a broad band of an appropriately size (~85 kDa) for this kinase in MonoQ fractions of the maturing oocytes (Fig. 6C), but the immunoreactivity was not precisely coincident with the MonoQ peak of MBP-NT phosphotransferase activity in the 5 day PF sample (Fig. 6D). The MBP-NT kinases in the 3 h PF and 5 day PF extracts were also relatively insensitive to the potent PKC inhibitor Roche Compound 3, which is a staurosporine analogue (Fig. 6E). However, a pseudo-substrate inhibitor peptide for PKC produced a partial inhibi-

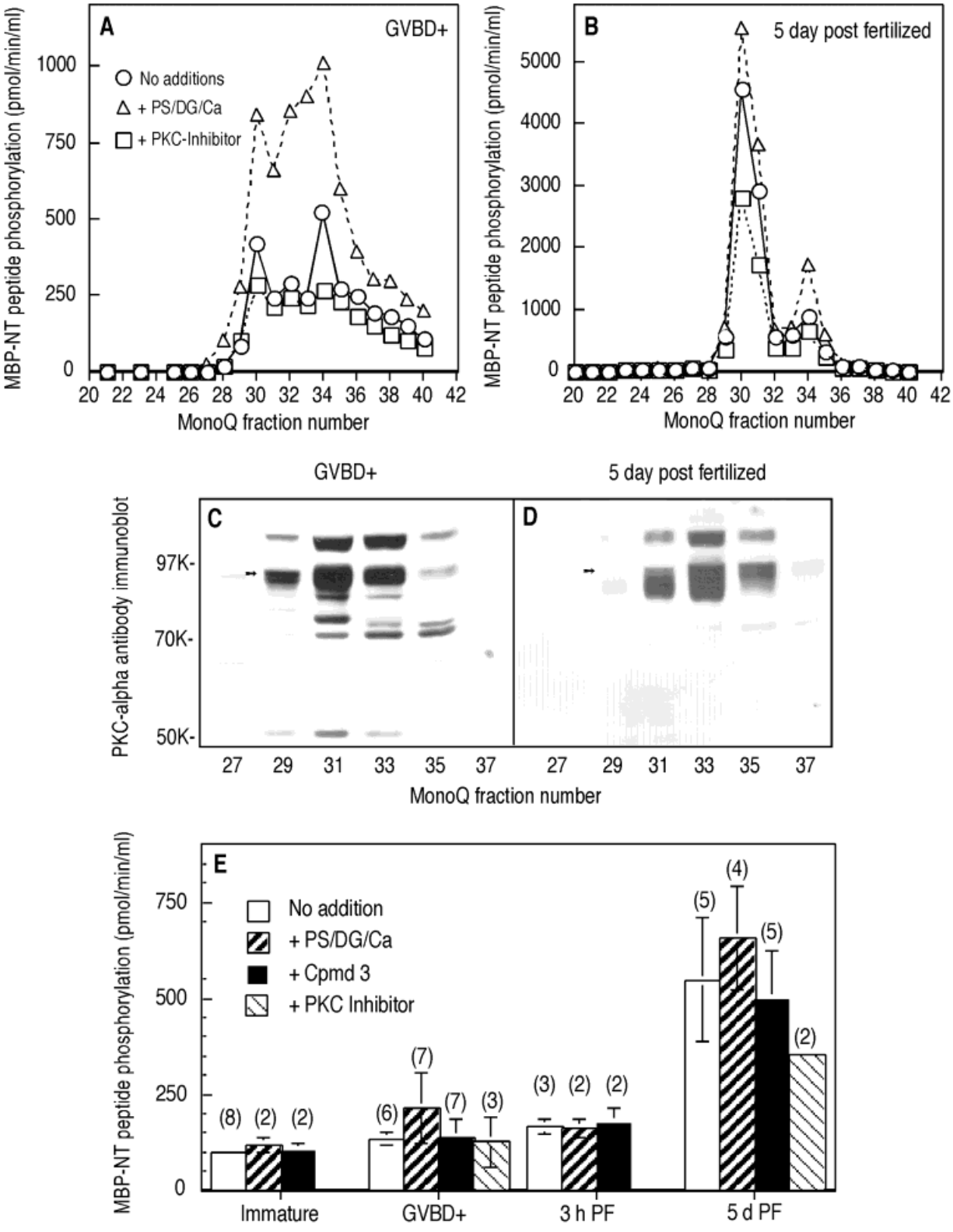
A
GVBD+
No additions
+ PS/DG/Ca
+ PKC-Inhibitor
MBP-NT peptide phosphorylation (pmol/min/ml)
MonoQ fraction number
B
5 day post fertilized
MBP-NT peptide phosphorylation (pmol/min/ml)
MonoQ fraction number
GVBD+
5 day post fertilized
C
D
PKC-alpha antibody immunoblot
97K-
70K-
50K-
MonoQ fraction number
E
No addition
+ PS/DG/Ca
+ Cpmd 3
+ PKC Inhibitor
MBP-NT peptide phosphorylation (pmol/min/ml)
Immature
GVBD+
3 h PF
5 d PF

Figure 6.

tion of the MBP-NT kinase in the 5 day PF samples (Fig. 6E). Our findings tend to rule out PKC as the kinase that is principally responsible for the bulk of the MBP-NT and MBP phosphotransferase activity in the developing embryos following fertilization of the mature oocytes. It is possible that the PKC inhibitor peptide was able to inhibit the MBP-NT kinase in 5 day PF cell extracts, because the substrate specificity of this sea star kinase was very similar to that of PKC.

In our assay system, a peptide inhibitor of PKA was included in all reactions, thereby eliminating the possibility that the MBP phosphorylating activity peak observed in 3 h and in 5 day PF extracts was due to PKA. Previous work in our laboratory has also shown that S6 kinases do not elute from MonoQ at the same position as the MBP phosphorylation peak in this system. Thus, S6 kinase was not a likely candidate for the MBP phosphotransferase activity peak at 3 h and 5 day PF samples.

Substrate Specificity of Fertilization-Activated MBP Kinases

To determine the amino acids on MBP that were phosphorylated by the MBP protein kinases in 3 h and 5 day PF extracts, phosphoamino acid analysis was performed (Fig. 7B). MBP appeared to be phosphorylated mainly on threonine residues by the major MBP kinase peaks in MonoQ fractions from the mature and 3 h PF extracts. By contrast, MBP was phosphorylated exclusively on serine residues by the MBP kinase in 5 day PF extracts and not on threonine or tyrosine residues.

In an attempt to determine the substrate specificity of the MBP kinases in the 3 h PF and 5 day extracts, we performed phosphotransferase assays using a variety of substrates. The substrate preference of the kinase in 5 day PF extract, in descending order, was as follows: S6 peptide, MBP-NT > MBP > histone H1 > protamine > casein (Fig. 7A). A similar substrate profile was also observed in the MonoQ fractions (34–36) containing the peak of MBP-NT phosphorylating activity in 3 h PF extracts. It is likely that some of the measured phosphotransferase activity towards different substrates was due to distinct kinases.

Further characterization of the protein kinases in Mono Q fraction 30 of 3 h PF and 5 day PF extracts was achieved by subjecting these fractions to chromatographic fractionation on a Superose 12 gel filtration column (Fig. 8). Size fractionation of the Mono Q fraction #30 of the 3 h PF extract revealed at least two major peaks of MBP phosphotransferase activity (Fig. 8A). One peak had an apparent molecular mass of 45,000 Daltons. This activity peak correlated well with our activity assays and immunoblotting data which demonstrated the presence of active sea star Mapk in this extract. The second peak of activity possessed an apparent molecular mass of 75,000 Daltons. The enzyme responsible for the major MBP phosphotransferase activity at 113 h PF exhibited an apparent molecular mass of 41,000 Daltons. These findings further confirmed that the MBP phosphotransferase peak measured in 3 h and 5 day PF extracts was either partially (3 h PF) or entirely (5 day PF) due to the activation of protein kinases distinct from Mapk.

Fig. 6. Determination of PKC involvement in MBP phosphotransferase activity in maturing oocytes and embryos. The MonoQ column fractions described in Figure 3 from GVBD+ (**A**) and 5 days PF cells (**B**) were assayed for phosphotransferase activity towards 1 mg/ml of a peptide patterned after the N-terminus of MBP in the absence (open circles) and presence of phosphatidylserine, diacylglycerol and calcium (open triangles) or PKC pseudosubstrate inhibitor peptide (open squares). The MonoQ column fractions in the region of the peak MBP-NT phosphotransferase activities from GVBD+ (**C**) and 5 day PF (**D**) cells were subjected to immunoblotting with a PKC-α antibody. The dark arrow indicates the position of PKC. **E:** The results from multiple experiments where the peak MonoQ fractions from immature (#29–32), GVBD+ (#29–32), 3 h PF (#33–37), and 5 day PF (#29–32) with 1 mg/ml MBP-NT peptide as the substrate were assayed for phosphotransferase activity in the absence (white bars) and presence of phosphatidylserine, diacylglycerol and calcium (dark striped bars), Compound 3 (black bars) or PKC pseudosubstrate inhibitor peptide (light striped bars). All data are expressed as percent of the MBP-NT phosphotransferase activity in immature oocyte extracts without additions. Error bars depict the SD (or range if n = 2) of separate experiments, the number of which for each time point are indicated within the brackets on the figure.

DISCUSSION

Many studies have examined the regulation of MBP kinases during meiotic resumption of oocyte maturation in various model systems. However, while studies using the *Xenopus laevis* oocytes have been fairly abundant, very little information has been obtained from the sea star system following fertilization of mature oocytes. Previous studies using sea star and sea urchin oocytes have described at least three MBP kinases activated upon the onset of maturation [Pelech et al., 1988]. Two of these

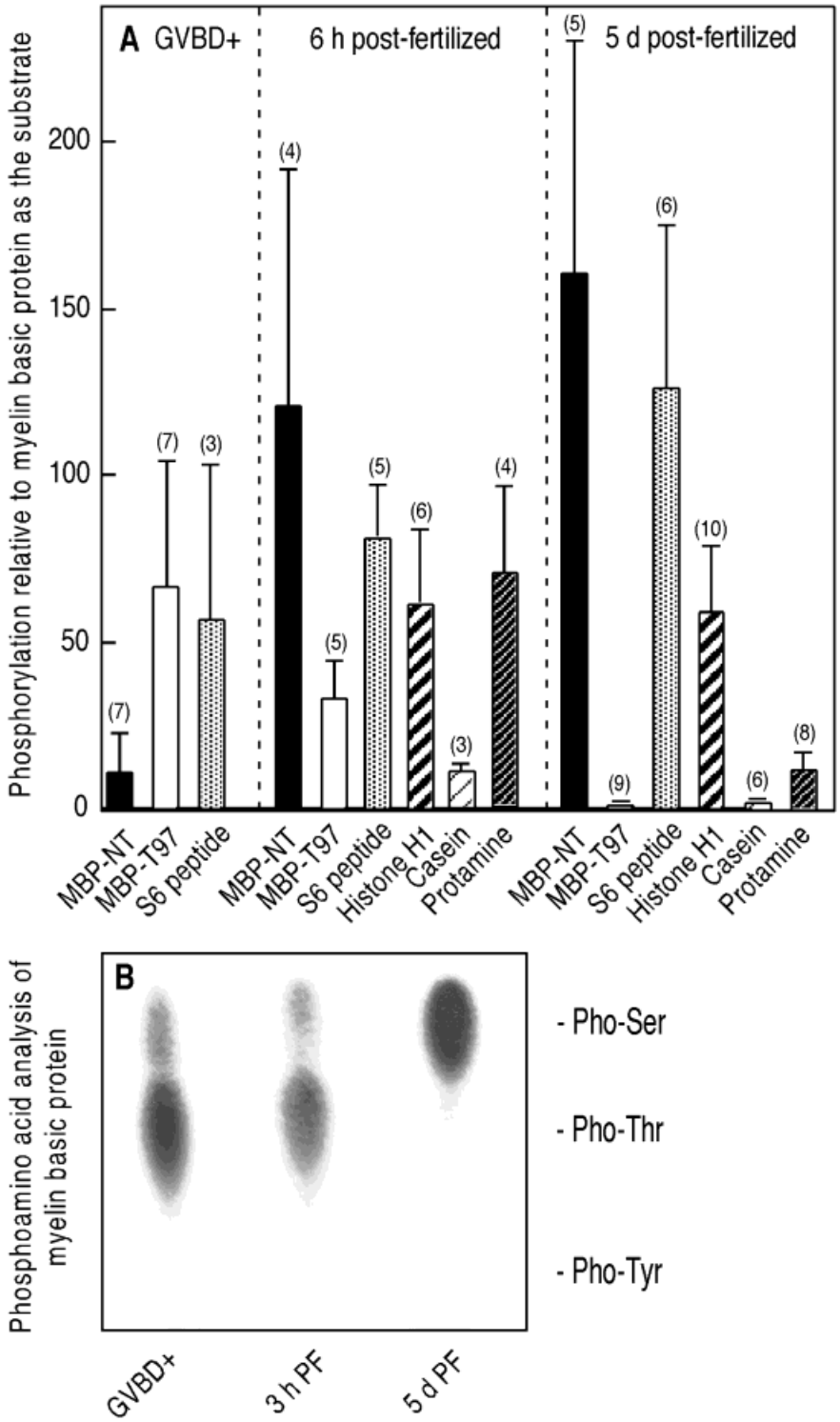

Fig. 7. Substrate specificity of MBP kinases. **A:** The MonoQ fractionated MBP kinases from GVBD+ (fractions 29–32), 6 h PF (fractions 33–37), and 5 day PF (fractions 29–32) cells were assayed for phosphotransferase activity towards 1 mg/ml of the following substrates: MBP-NT (~0.7 mM), MBP-T97 (~1 mM), S6 peptide (~0.4 mM), MBP, casein, histone H1, and protamine. Error bars depict the SD of separate experiments, the number of which for each time point are indicated within the brackets on the figure. **B:** MBP phosphorylated by the three MonoQ fractionated MBP kinases described above was subjected to phosphoamino acid analysis. The positions of the phosphoamino acid standards on the TLC plate are indicated.

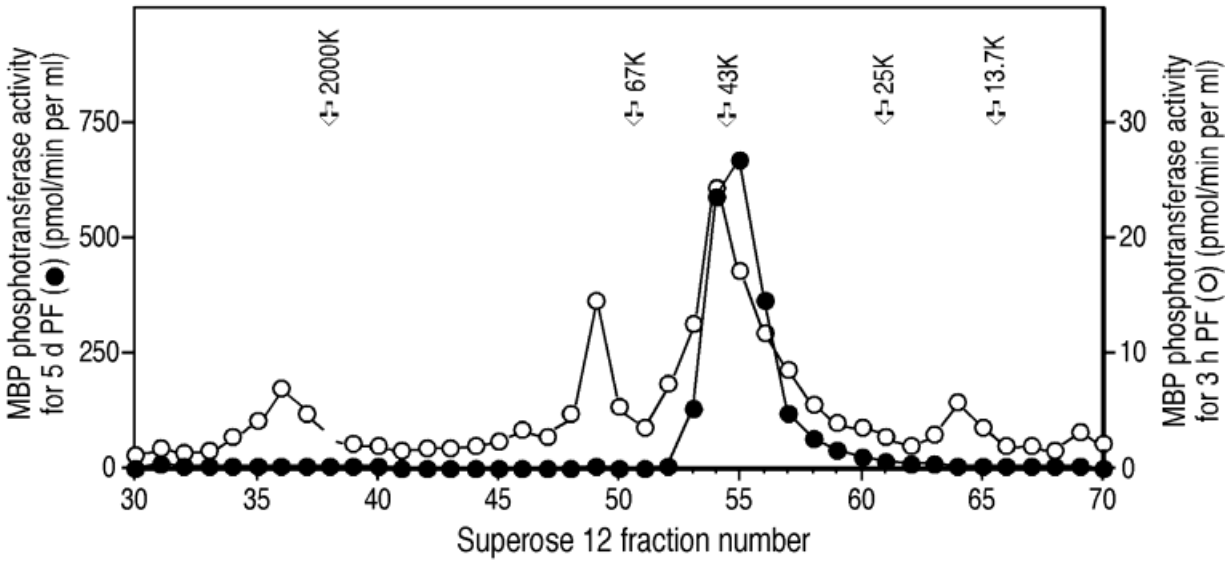

Fig. 8. Gel filtration chromatography of the 3 h and 5 day PF MonoQ fractions with peak MBP phosphotransferase activity. The MonoQ fractions (#30) that possessed peak MBP phosphotransferase activity from 3 h PF (open circles) and 5 day PF (filled circles) were applied to a Superose-12 column equilibrated in 0.25 M NaCl in KII buffer and 0.25 ml fractions were collected and assayed for MBP phosphotransferase activity. The elution positions of blue dextran (2,000,000 MW), BSA (67,000 MW), ovalbumin (43,000 MW), chymotrypsinogen A (25,000 MW), and ribonuclease A (13,700 MW) are indicated with arrowheads in kilodaltons.

enzymes underwent maximal activation near the time of GVBD, declining prior to the emission of the first polar body (i.e., meiotic cell division). However, a third MBP kinase (MBPK-I, M_r = 45,000) displayed peak activation after first meiotic cell division and appeared to be transiently activated prior to each cell division. This MBP kinase was purified from mature sea star oocytes and identified as a novel MAP kinase family member, Mapk [Posada et al., 1991]. Here we have provided the complete primary protein structure of sea star Mapk.

In the present study, we performed detailed analyses of these MBP kinases throughout early sea star embryogenesis. Our data complements previous studies by demonstrating that Mapk protein levels and phosphotransferase activities decline 12 h after fertilization and remain low throughout the first 5 days of sea star embryogenesis. The role of Mapk in meiotic maturation is unclear. We observed that the Mek1 inhibitor PD98059 failed to block GVBD and oocyte maturation in response to 1-MeAde (unpublished data). Therefore, Mapk activation does not appear to be required for conversion into a fertilizable egg. However, microinjection into immature oocytes of a constitutively active [S202E, S204E] form of murine Mek1 was able to induce meiotic maturation in about 50% of injected oocytes (H. Paddon, D.L.C. and S.L.P., unpublished data). Since this activated form of Mek1 was also capable of phosphorylating and activated sea star Mapk expressed as a glutathione S-transferase fusion protein in bacteria, it would appear that activation of Mapk may be sufficient for meiotic maturation. It has been speculated that activation of MAP kinases during oocyte maturation may serve to prevent DNA replication during the meiotic cell divisions [reviewed in Murray, 1998]. However, Mapk activity was very high in the *Pisaster ochraceus* early embryos during the first 12 h PF when several rounds of mitotic cell division transpired with DNA replication. Further studies will be required to evaluate the critical functions of Mapk in sea star oocyte meiosis and early development.

Unlike previous studies, we have shown that although Mapk phosphotransferase activity towards MBP is evident at 3 h PF, less than half of this measured activity can be assigned to Mapk or Erk2. Phosphotransferase activity assays using various other peptide and protein substrates have not provided any clues to the identity of this novel MBP kinase. However, phosphoamino acid analysis of MBP that is phosphorylated by this novel kinase in 3 h PF

extracts indicates that it phosphorylates MBP on threonine residues, implying that it is a protein-serine/threonine kinase family member. Furthermore, gel filtration chromatography indicated that this MBP kinase had an apparent molecular mass of 69,000 Daltons. Extensive purification and sequencing of this enzyme will be required for its further characterization.

We have also described for the first time that a novel protein kinase is responsible for the majority of the MBP phosphotransferase activity in later sea star embryogenesis. Immunoblotting with a specific MAP kinase antibody and phosphotransferase activity assays using the MBP-T97 peptide ruled out the possibility that Mapk was responsible for the dramatic rise in MBP phosphotransferase activity in later embryogenesis. Likewise, this kinase appeared to be distinct from PKC, although there was some similarity in substrate specificity. It was not lipid inducible nor calcium dependent, therefore ruling out the conventional family of PKCs (i.e., PKC α, βI, βII, and γ) [reviewed by Hug and Sarre, 1993]. Our size determination experiments of the MBP kinase in 5 day PF extracts revealed a protein of apparent molecular mass of 41 kDa. No known PKC isoform to date has an apparent molecular mass of less than 50 kDa. When we performed Western blot analysis with PKC-α antibody of the 5 day PF MonoQ fractions that contained this MBP kinase, no immunoreactive bands were detected below the 75 kDa (Fig. 6D). PKC can be cleaved by calpains thus producing two distinct fragments, a protein comprising the regulatory domain and one containing the catalytic domain which is active in the absence of any activators [Kishimoto et al., 1989; Saido et al., 1992]. Thus, it is possible that the MBP kinase in 5 day PF extracts corresponded to a proteolytic cleavage fragment of a known PKC isoform. However, we have no evidence for this except for the partial inhibition of this kinase with a pseudosubstrate peptide for PKC.

In summary, we have described the activation of three distinct MBP phosphorylating enzymes at different stages of sea star oocyte maturation and embryonic development. We have confirmed that the principal MBP kinase activated during the maturation process of the oocyte corresponds to the sea star MAP kinase Mapk. The MBP kinase activated at 3 h postfertilization was distinct from Mapk. However, the identity of this MBP kinase is uncelar at present. A third MBP kinase was detected in 4 and 5 day post-fertilization cytosolic extracts, and our experimental data implies that this enzyme was also novel. To further characterize these unidentified MBP kinases, purification, and sequencing of these enzymes will be required.

ACKNOWLEDGMENTS

We thank Dr. J. Sanghera and members of the Pelech laboratory, especially Donna Morrison and Lorin Charlton for moral support and critical review of the manuscript. Also, we thank Dan Leung and Stephanie Buchanan for their expert technical assistance. This work was supported by grants from the Medical Research Council of Canada (MRCC) to S.L.P. D.L.L. was a recipient of a MRCC post-doctoral fellowship, D.L.C. was the holder of an MRCC studentship award, and S.L.P. was a MRCC Scientist.

REFERENCES

Charest DL, Mordret G, Harder KW, Jirik F, Pelech SL. 1993. Molecular cloning, expression, and characterization of the human mitogen-activated protein kinase $p44^{erk1}$. Mol Cell Biol 13:4679–4690.

Chenchik A, Diachenko L, Moqadam F, Tarabykin V, Lukyanov S, Siebert PD. 1996. Full-length cDNA cloning and determination of mRNA 5′ and 3′ ends by amplification of adaptor-ligated cDNA. BioTechniques 21:526–534.

Clark-Lewis I, Sanghera JS, Pelech SL. 1991. Definition of a consensus sequence for peptide substrate recognition by $p44^{mpk}$, the meiosis-activated myelin basic protein kinase. J Biol Chem 266:15180–15184.

Davidson EH. 1986. Gene activity in early development, Third edition. London: Academic Press.

Deibler GE, Boyd LF, Kies MW. 1994. Proteolytic activity associated with purified myelin basic protein. In: Progress in clinical and biological research. 146:249–256.

Doree M, Peaucellier G, Picard A. 1983. Activity of the maturation promoting factor and the extent of protein phosphorylation oscillate simultaneously during meiotic maturation of starfish oocytes. Dev Biol 99:489–501.

Fraser A, Gomez J, Hartwick EB, Smith MJ. 1981. Observations on the reproduction and development of *Pisaster ochraceus* (Brandt). Can J Zool 59:1700–1707.

Hartley RS, Lewellyn AL, Maller JL. 1994. MAP kinase is activated during mesoderm induction in *Xenopus laevis*. Dev Biol 163:521–524.

Holton TA, Graham MW. 1991. A simple and effective method for direct cloning of PCR products using ddT-tailed vectors. Nucleic Acids Res 19:1156.

Hug H, Sarre TF. 1993. Protein kinase C isoenzymes: Divergence in signal transduction? Biochem J 291:329–343.

Kalvakolanu DVR, Livingston WH. 1991. Rapid and inexpensive protocol for generating >95% recombinants in subcloning experiments. Biotechniques 10:176–177.

Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S-I, Tominaga M, Kuroda T, Nishizuka Y. 1989. Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem 264: 4088–4092.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Maller JL, Krebs EG. 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 252:1717–1718.

Meijer L, Pondaven P, Guerrier P, Moreau M. 1984. A starfish oocytes user's guide. Cah Biol Mar 25:457–480.

Murray AW. 1998. MAP kinases in meiosis. Cell 92:157–159.

Pelech SL, Charest DL. 1995. MAP kinase-dependent pathways in cell cycle control. In Meijer L, Guidet S, Tung HYL, editors. Progress in cell cycle research, vol 1. New York: Plenum Press. p 33–52.

Pelech SL, Tombes RM, Meijer L, Krebs EG. 1988. Activation of myelin basic protein kinases during echinoderm oocyte maturation and egg fertilization. Dev Biol 13: 28–36.

Pelech SL, Sanghera JS, Daya-Makin M. 1990. Protein kinase cascades in meiotic and mitotic cell cycle control. Biochem Cell Biol 68:1297–1330.

Posada J, Sanghera J, Pelech S, Aebersold R, Cooper JA. 1991. Tyrosine phosphorylation and activation of homologous protein kinases during oocyte maturation and mitogenic activation of fibroblasts. Mol Cell Biol 11:2517–2528.

Saido TC, Mizuno I, Konno Y, Osada S, Ohno S, Suzuki K. 1992. Purification and characterization of protein kinase C epsilon from rat brain. Biochemistry 31:482–490.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p 168.

Sanger F, Coulson AR, Barrell BG, Smith AJH, Roe BA. 1980. Cloning in single stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol 143:161–178.

Sanghera JS, Paddon HB, Bader SA, Pelech SL. 1990. Purification and characterization of a maturation-activated myelin basic protein kinase from sea star oocytes. J Biol Chem 265:52–57.

Sanghera JS, Paddon HB, Pelech SL. 1991. Role of protein phosphorylation in the maturation-induced activation of a myelin basic protein kinase from sea star oocytes. J Biol Chem 266:6700–6707.

Sanghera J, McNabb C, Tonks N, Pelech S. 1991. Tyrosyl phosphorylation and activation of the myelin basic protein kinase $p44^{mpk}$ during sea star oocyte maturation. Biochim Biophys Acta 1095:153–160.