At Least Two Kinases Phosphorylate the MPM-2 Epitope during *Xenopus* Oocyte Maturation

Jian Kuang and Cheryl L. Ashorn

Department of Clinical Investigations, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract. MPM-2 antigens, a discrete set of phosphoproteins that contain similar phosphopeptides (the MPM-2 epitope), are associated with various mitotically important structures. The central mitotic regulator cdc2 kinase has been proposed to induce M-phase by phosphorylating many proteins which might include the MPM-2 antigens. To clarify the relationship of cdc2 kinase and the MPM-2 antigens, we developed an in vitro assay that enabled us to specifically detect the kinases that phosphorylate the MPM-2 epitope (ME kinases) in crude cell extracts. Two different ME kinase activities were identified in unfertilized *Xenopus* eggs, neither of which was cdc2 kinase, but both appeared to be activated by the introduction of cdc2 kinase into oocytes or oocyte extract. The two ME kinases differed in molecular size, substrate specificity, peptide components, and MPM-2 reactivity. The larger one, ME kinase-H, phosphorylated several MPM-2 antigens, while the smaller one, ME kinase-L, phosphorylated mainly one. We purified ME kinase-L to near homogeneity by sequential chromatography and showed that it has the characteristics of the 42-kD microtubule-associated protein (MAP) kinase. Our results support the previous finding that MAP kinase is activated during *Xenopus* oocyte maturation and suggest that MAP kinase may contribute to oocyte maturation induction by phosphorylating one subtype of MPM-2 epitope.

The entry of eukaryotic cells into mitosis is accompanied by a dramatic increase in the level of protein phosphorylation (Capony et al., 1986; Karsenti et al., 1987; Lohka et al., 1987; Nishimoto et al., 1987). This burst of protein phosphorylation results from the activation of multiple protein kinases (Cicirelli et al., 1988; Halleck et al., 1987) that phosphorylate a battery of proteins involved in both the regulatory and structural aspects of mitosis. It has been shown that a key mitotic kinase is cdc2 kinase, a protein complex composed of p34<sup>cdc2</sup> and a mitotic cyclin (Nurse, 1990). There is substantial evidence that phosphorylation by cdc2 kinase of certain structural proteins such as lamins, vimentin, caldesmon, nucleolin, and histone H1 plays an important role in the induction of mitosis (Nigg, 1991). However, since many mitotic phosphoproteins as well as kinases that phosphorylate them have not been identified or characterized, it is still not known how the different mitotic kinases orchestrate the multiple phosphorylation cascades which culminate in mitosis.

Using an immunological approach, mAbs such as MPM-2, MPM-12, and CHO3 (Davis et al., 1983; Ganju et al., 1992; and Kuriyama, 1989) were produced that recognized individual families of mitotic phosphoproteins. We are characterizing a large family of the phosphoproteins recognized by MPM-2 (Davis et al., 1989; Kuang et al., 1989; 1991a,b). MPM-2 is a mAb raised against mitotic HeLa cells and selected by preferential staining of mitotic versus interphase cells using indirect immunofluorescence (Davis et al., 1983). While MPM-2 staining can be detected on certain structures in some interphase cells (Vandre et al., 1984, 1986; Keryer et al., 1987), a dramatic increase in MPM-2 reactivity is observed during the G2/M transition in all species tested, both plant and animal (Davis et al., 1983; Vandre et al., 1986; Millar et al., 1987; Engle et al., 1988; Kuang et al., 1989; Kuriyama et al., 1990). On immunoblots of mitotic HeLa cell extract, MPM-2 recognizes many polypeptides that have been shown to be synthesized during interphase and phosphorylated during mitotic induction (Davis et al., 1983). Phosphatase treatment of MPM-2 antigens eliminates MPM-2 immunoreactivity, indicating that the mitosis-associated phosphorylation is required for MPM-2 reactivity (Davis et al., 1983). The MPM-2 antibody protects the antigens from being dephosphorylated, suggesting that the phosphorylation site resides in the epitope recognized by MPM-2 (Davis and Rao, 1987). Based on preliminary studies, the major phosphoamino acid in the MPM-2 epitope seems to be threonine (Zhao et al., 1989). One possible explanation for these observations is that the mitotic MPM-2 antigens contain similar or identical epitopes that are phosphorylated by a common kinase or kinases preferentially active during the G2/M transition. In this study, we...
collectively refer to the epitopes recognized by MPM-2 as the MPM-2 epitope and the kinases that phosphorylate them as MPM-2 epitope kinases (ME kinases).1

Several lines of evidence suggest that phosphorylation of the MPM-2 epitope during the G2/M transition plays a crucial role in the induction of mitosis. First, proteins containing the MPM-2 epitope are localized on structures important in mitosis, such as chromosomes (Hirano and Mitchison, 1991), spindle poles (Engle et al., 1988; Kuriyama et al., 1990; Vandre et al., 1991; Masuda et al., 1992), kinetochores (Vandre et al., 1984), the midbody (Vandre et al., 1984), and the nuclear envelope (Harper et al., 1990). Second, chromatography of Xenopus egg extract resulted in three fractions of M-phase promoting factor (MPF) activity, one of which was due to cdc2 kinase and the other two appeared to be due to MPM-2 antigens, suggesting that some MPM-2 antigens might be mitotic regulators (Kuang et al., 1991b). Third, microinjection of the MPM-2 antibody into Xenopus oocytes or fertilized eggs or Hela cells interferes with either the induction or the completion of M-phase (Davis et al., 1989; Kuang et al., 1989). Finally, phosphorylation of the MPM-2 epitope during M-phase induction is a highly conserved phenomenon, as described above. These observations suggest that phosphorylation and dephosphorylation of the MPM-2 epitope could be one mechanism by which several mitotic events are coordinate regulated.

The current model of M-phase induction is that the mitotic cdc2 kinase complex, which is composed of p34cdc2 and a mitotic cyclin, is a central regulator that induces individual mitotic events directly and indirectly (Lewin, 1990; Nigg, 1991). It has been suggested that the mitotic MPM-2 antigens may be substrates of cdc2 kinase (Tombs et al., 1991). However, none of the tested substrates of cdc2 kinase, including histone H1, lamin, and mitotic cyclin B, are reactive to MPM-2 after mitotic phosphorylation, suggesting that the ME kinases may be different from cdc2 kinase (Davis et al., 1983; Ottaviano and Gerace, 1985; Kuang et al., 1991a). Determining whether cdc2 kinase is an ME kinase is essential to understanding the role of phosphorylation of the MPM-2 epitope in M-phase induction.

The objective of this study was to identify and characterize the ME kinases from unfertilized Xenopus eggs and examine their relationship to cdc2 kinase. Unfertilized Xenopus eggs are naturally arrested at the second meiotic metaphase and exhibit a high level of MPM-2 epitope phosphorylation (Kuang et al., 1991b). The protein concentration of the oocyte and egg extracts was determined to be 8-10 mg/ml by Bradford protein assay (Bio Rad Laboratories, Richmond, CA).

### In Vitro Assay for ME Kinase Activity

To prepare substrate blots, 0.18-0.2 mg of immature oocyte extract in Laemmli sample buffer was loaded per lane for separation by 12.5% SDS-PAGE. Prestained molecular weight standards (Sigma SDS-7B) were loaded in alternate lanes. The separated proteins were transblotted onto a nitrocellulose membrane using a buffer of 25 mM Tris, 190 mM Glycine, and 20% methanol before blocking for 1 h at 22°C in Blotto (10 mM Tris-HCl, pH 7.6, with 150 mM NaCl, 3% powdered milk, 0.05% Tween-20, and 0.01% sodium azide). The substrate blots were then dried and stored at −20°C before use.

To detect phosphorylation by ME kinase, the substrate blots were cut into individual lanes and incubated with samples diluted with 0.25 × EB with 4 mM ATP, 20 mM NaF, 10 mM DTT, and 1 μM okadaic acid. Dilution of the samples varied according to their estimated protein concentration. After gentle agitation for 1 h at 22°C, the blots were rinsed in TBS-Tween 20 (10 mM Tris, 150 mM NaCl, and 0.05% Tween-20) to remove residual extract before washing. To remove any nonspecifically bound proteins, the blots were washed twice in a pH 3.0 buffer (50 mM Tris, 1 M NaCl, and 1% NP-40), twice in the same buffer at pH 10.0, and five times in Blotto for 10 min each time. The blots were immunostained 2 h with the primary antibody (MPM-2) diluted 1:500 in TBS-Tween 20 plus 10% calf serum before being washed four times (10 min each) with TBS-Tween 20. The secondary antibody, an alkaline phosphatase conjugate (Promega, Madison, WI), was diluted 1:15,000 in the same buffer as the primary antibody and added to the blots for 1 h. The washing procedure was repeated before development by 0.5 mg/ml nitro blue tetrazolium (NBT) and 0.25 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Bio Rad Laboratories) in 0.1 mM Tris-base, pH 9.5, plus 0.05 mM MgCl2, 0.1 mM NaCl. The development was stopped by washes with deionized water.

### Assay for Histone H1 Kinase Activity and Myelin Basic Protein Phosphorylation Activity

A 7.5-μl aliquot of each sample was mixed with 2.5 μl of reaction mixture. For the H1 kinase assay, the reaction mixture contained 0.1 mg/ml histone H1, 100 μM ATP, 4 μM cAMP-dependent protein kinase inhibitor (Sigma P-3294), and 0.5 μCi/μl [γ-32P]ATP in EB. For the myelin basic protein (MBP) kinase assay, it contained 0.5 mg/ml MBP, 500 μM ATP, 4 μM

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1. Abbreviations used in this paper: MAP, microtubule-associated protein; MBP, myelin basic protein; ME kinases, MPM-2 epitope kinases; MPF, M-phase promoting factor.
cAMP-dependent protein kinase inhibitor, and 0.5 μCi/μl [γ-32P]ATP in EB. The assay mixtures were incubated for 20 min at 22°C, and then Laemmli sample buffer was added to stop the reaction. The proteins were separated by SDS-PAGE and autoradiographed to reveal phosphorylation of histone H1 or MBP. To quantitate the phosphorylation, each histone H1 or MBP band was excised from the gel and the radioactivity in it was determined using a scintillation counter.

**Purification of ME Kinase-L**

All chromatography was performed at 4°C in column buffer (CB) (EB with 20 mM NaF, 50 mM ATP·S, and 1 mM DTT). For Ultrogel AcA 34 (LKB, Houston, TX) chromatography, 1 ml of egg extract was fractionated on a 40-ml (0.9 × 59 cm) column at a flow rate of 0.3 ml/min. 1-ml fractions were collected and measured for protein absorbance at 280 nm. Bio Rad gel filtration standards were used to calibrate the column. For Q-Sepharose Fast Flow chromatography, fractions 26–32 containing ME kinase-L activity from the Ultrogel AcA 34 chromatography were pooled and applied directly to a 4-ml Q-Sepharose Fast Flow (Pharmacia Fine Chemicals, Piscataway, NJ) column pre-equilibrated in CB. The column was then washed with 10 ml of CB with 50 mM NaCl and eluted with 10 ml of 0.2 M NaCl in CB and 10 ml of 0.4 M NaCl in CB sequentially. 1-ml fractions were collected and the peaks of flow through (QB), first eluate (QE1), and second eluate (QE2) could be easily identified by their protein absorbance at 280 nm. For Affi-Gel blue chromatography, QE1 from the Q-Sepharose chromatography was concentrated with a Centricon-30 microconcentrator (Amicon, Danvers, MA) and diluted with CB to reduce the concentration of NaCl to less than 0.02 M. Diluted QE1 from ten runs was pooled and applied to a 5-ml Affi-Gel blue (Bio Rad Laboratories) column pre-equilibrated in CB. The column was washed with 30 ml of CB and then eluted with 30 ml of 0.6 M NaCl in CB. 2-ml fractions were collected and the peaks of flow through and eluate could be easily identified by their protein absorbance at 280. For FPLC (Waters 650 purification system; Waters, Milford MA) on Protein Pak 300 SW column, the eluate from Affi-Gel blue was precipitated sequentially with 33 and 40% saturated ammonium sulfate. The 33–40% ammonium sulfate precipitate, which contained most of the ME kinase-L activity, was resuspended in 300 μl of CB and applied to a 15-ml Protein Pak 300 SW column (Waters) at a flow rate of 0.8 ml/min while collected 0.4-ml fractions. Bio Rad gel filtration standards were used to calibrate the column.

**Affinity Absorption**

MPM-2 or RDA-1 mouse ascites were produced as previously described (Davis et al., 1983). RDA-1 is a nucleolar antibody used as the control IgG for MPM-2. IgG from the ascites was bound to Affi-Prep protein A matrix with the binding buffer from a MAPs II kit (Bio Rad Laboratories). The anti-Xenopus MAP kinase (COOH terminus) antibody and the control pre-immune serum, kindly provided by Drs. J. Posada and J. Cooper, were bound to Affi-Gel blue protein A matrix. For immunodepletion, each sample was added to beads pre-equilibrated in EB and rotated at 4°C for at least 3 h. The beads were then pelleted and the supernatants recovered.

**Immunoblotting**

For Western blot analysis, proteins were separated by 7.5, 12.5, or 15% SDS-PAGE and transferred onto nitrocellulose. Immunostaining of the transblots was done as described for the in vitro ME kinase assay. The antibody against rat MAP kinase NH2 terminus was obtained from Upstate Biotechnology Inc. (USB) and used at a 1:1,250 dilution. The antibody against Xenopus MAP kinase COOH-terminus was used at a 1:2,000 dilution. The mAb against MAP2 protein was purchased from Boehringer Mannheim Corp. (Indianapolis, IN) and used at a 1:15,000 dilution. The anti-mouse and anti-rabbit secondary AP-conjugated antibodies were obtained from Promega and used at a 1:15,000 dilution.

**Phosphorylation of MAP2**

MAP2 protein was partially purified from rat brain tissue according to Kim et al. (1979). Briefly, microtubules were isolated by two cycles of assembly and disassembly. The total microtubule protein was heated at 100°C for 10 min to precipitate tubulin and MAP1, leaving MAP2 solubilized. After the precipitate was removed by centrifugation, supernatant containing approximately 30 μg of total protein was immunoprecipitated with 30 μl of anti-MAP2 antibody and 100 μl of protein A beads. For dephosphorylation, the immobilized MAP2 immunocomplex was incubated with 10 U of alkaline phosphatase (Boehringer Mannheim Corp.) in 50 mM Tris-HCl, pH 8.0, for 1 h at 22°C with protease inhibitors. The beads were washed thoroughly with EB at 1.0 M NaCl and 0.5% NP-40 and rinsed in plain EB. For phosphorylation, 20 μl of either purified ME kinase-L or EB buffer was added to the beads with 5 μl of 10 μCi/μl [γ-32P]ATP. After incubation at 22°C for 10 min, 2 mM cold ATP was added for 1 h to enhance phosphorylation. The reaction was stopped by addition of Laemmli sample buffer. The proteins were then separated by SDS-PAGE, transferred to nitrocellulose, probed with MPM-2, and autoradiographed to detect 32P incorporation by MAP2.

**Partial Purification of cdc2 Kinase**

Unfertilized Xenopus egg extract, prepared as described (Kuang et al., 1991b), was fractionated on Ultrogel AcA 34 in CB. The MPF-positive fractions, which did not include the ME kinase-H fractions, were pooled and subjected to Q-Sepharose chromatography (Kuang et al., 1991b). While cdc2 kinase went into the flow through, the bound proteins contained ME kinase-L and the two non-cdc2 MPFs. To further purify cdc2 kinase, the flow through was diluted with an equal volume of water to reduce the ionic strength and reapplied to a Q-Sepharose column pre-equilibrated in 0.5 × CB. After washing the column with 0.75 × CB which eliminated ~90% of the total protein, the bound cdc2 kinase was eluted with CB plus 50 mM NaCl. Hi kinase activity and MPF activity copurified indicating that cdc2 kinase was responsible for the MPF activity recovered. The cdc2 kinase eluate was concentrated with Centricon 30 microconcentrator and depleted of MPM-2 antigens by immunosorbption with MPM-2 affinity beads which immobilized neither Hi kinase nor MPF activity in the partially purified cdc2 kinase. On the other hand, p34cdc2 depletion (Kuang et al., 1991b) was able to cause the loss of MPF activity from the sample and its ability to induce the phosphorylation of a broad spectrum of MPM-2 antigens upon microinjection into Xenopus oocytes.

**Results**

**Development of an In Vitro Assay for ME Kinases**

To identify ME kinase, we developed an in vitro assay that detects its activity. The unique feature of this assay is that the substrates are immobilized on nitrocellulose and MPM-2 immunostaining is used to detect the phosphorylation. In our previous studies, we showed that immature Xenopus oocytes contain unphosphorylated MPM-2 antigens (Kuang et al., 1989). We therefore used interphase extract prepared from immature Xenopus oocytes as a source of unphosphorylated substrates for ME kinase. To prevent potential inhibitors of ME kinase or activation of latent interphase ME kinase from interfering with the assay, the crude interphase substrates were separated by SDS-PAGE and transblotted onto nitrocellulose under conditions that allowed their partial renaturation (see Materials and Methods). To perform the assay, the substrate blots were incubated with candidate kinase samples in the presence of ATP and the phosphatase inhibitors, sodium fluoride and okadaic acid. The blots were then rigorously washed under harsh pH conditions known to break even antibody-antigen binding to eliminate a false positive result due to residual MPM-2 antigens from the kinase sample binding to the polypeptides on the blot. To specifically detect phosphorylation of the MPM-2 epitope in the immobilized substrates, the blots were immunostained with the MPM-2 antibody, which recognizes only the phosphorylated version of the substrates of ME kinase.

We tested the specificity of this assay by incubating substrate blots with either interphase extract from immature Xenopus oocytes or M-phase extract from unfertilized Xenopus eggs in the presence of [γ-32P]ATP. After incubation, the blots were processed for both autoradiography and
MPM-2 immunostaining. Fig. 1 shows that interphase extract did not phosphorylate any of the polypeptides on the blot to a significant extent (lanes 1 and 2). On the other hand, M-phase extract catalyzed the phosphorylation of a large number of polypeptides (lane 4) of which three major high-molecular weight bands reacted with the MPM-2 antibody (lane 3) under these conditions. M-phase extract contains a large number of MPM-2 antigens, which could produce a false positive signal if any of the antigens bound to the polypeptides on the substrate blots. To address this concern, two experiments were performed. First, 60 mM EDTA was included in the M-phase extract to chelate ions required by kinases for phosphorylation. The EDTA prevented the generation of MPM-2 reactive bands on the substrate blot (data not shown) suggesting that de novo phosphorylation is the cause of the MPM-2 reactivity. Second, we included 0.1% SDS in the wash buffers which could effectively break protein-protein binding without eluting the majority of the transferred polypeptides from the nitrocellulose. We found that the MPM-2 reactive bands still appeared on the substrate blot. Together, these results indicate that 1) phosphorylation of the MPM-2 epitope could occur on some of the denatured substrates; 2) MPM-2 staining distinguished phosphorylation of the MPM-2 epitope from other kinds of phosphorylation; and 3) the ME kinase activity detected by this assay was M-phase-specific, as anticipated. It should be noted that in extracts of unfertilized Xenopus eggs, MPM-2 ordinarily recognizes many polypeptides ranging from 40 to 400 kD (Kuang et al., 1989). Thus, many of the substrates on the blots were not sufficiently renatured to be phosphorylated by ME kinase and/or recognized by MPM-2 in this in vitro assay. It has been our experience that in immunoblot analysis, partial renaturation of the MPM-2 antigens from M-phase extract is required for MPM-2 recognition, suggesting that a secondary or tertiary structure is essential in the formation of the MPM-2 epitope.

**Identification of Two ME Kinases in Unfertilized Xenopus Eggs**

Using the assay described above, we analyzed the ME kinase activity observed in unfertilized Xenopus eggs by chromatography. Egg extract was fractionated by gel filtration on Ultrogel AcA 34 (LKB) and individual fractions were assayed for ME kinase activity. As shown in Fig. 2 B, the assay detected two major peaks of ME kinase activity. One activity eluted slightly earlier than a 670-kD marker protein (Fig. 2 A, arrow 1) and phosphorylated mainly three major bands of 210, 150, and 116 kD plus several minor bands. The second peak of ME kinase activity eluted at 17 kD (Fig. 2 A, arrow 2) and phosphorylated mainly one band of 150 kD. The high-molecular weight ME kinase was designated ME kinase-H and the low-molecular weight one ME kinase-L. To determine whether either ME kinase contained an MPM-2 epitope, the peak fractions of either kinase activity were pooled and incubated with MPM-2 affinity beads, and the supernatants were assayed for ME kinase activity. We found that ME kinase-H activity could be depleted by incubation with MPM-2 affinity beads, while ME kinase-L activity could not (data not shown), demonstrating that ME kinase-H contains an MPM-2 epitope. The findings that ME kinase-H and ME kinase-L differ in both substrate specificity and MPM-2 reactivity suggest that ME kinase-H is not an aggregate of ME kinase-L and that the ME kinase activity in the M-phase extract is due to at least two different kinases that phosphorylate different subsets of MPM-2 antigens in vitro.

To determine whethercdc2 kinase exhibits ME kinase activity, the same fractions were also assayed for H1 kinase activity, a commonly used indicator of cdc2 kinase (Arion et al., 1988; Draetta et al., 1989). As shown in Fig. 2 C, two peaks of H1 kinase activity were observed, one eluting at 670 kD and the other at 150 kD. The larger H1 kinase was a novel M-phase-specific H1 kinase recognized by MPM-2, while the 150-kD H1 kinase was the mitotic cdc2 kinase, as shown in our previous studies (Kuang et al., 1991a). Therefore, ME kinase-H was completely separated from cdc2 kinase but copurified with the larger H1 kinase while ME kinase-L overlapped but did not copurify with cdc2 kinase. To examine the relationship between ME kinase-H and the larger H1 kinase, fractions with ME kinase-H and the larger H1 kinase activity were pooled and fractionated on an Affi-Gel blue column. While ME kinase-H activity was recovered in the flow-through, all the activity due to the larger H1 kinase remained bound to the column (data not shown), indicating that ME kinase-H and the larger H1 kinase are different enzymes. To determine whether ME kinase-L could be separated from cdc2 kinase, the fractions with cdc2 kinase and ME kinase-L activity were pooled and fractionated on a Q-Sepharose column. As shown in Fig. 3, most of the H1 kinase activity due to cdc2 kinase was in the flow-through (QF) which agrees with our previous observation that p34cdc2 could only be detected in QF (Kuang et al., 1991b). However, ME kinase-L was recovered in the first eluate (QE1). To eliminate the possibility that the inability of cdc2 kinase to phosphorylate the MPM-2 epitope was due to the presence of some inhibitors in the QF fraction, purified cdc2 kinase (Solomon et al., 1990), that was highly active in phosphorylating histone H1, was assayed for ME kinase activity. We found that the purified cdc2 kinase could not phosphorylate any MPM-2 epitope on the substrate blots (data not shown) confirming that cdc2 kinase is not responsible for the ME kinase activity detected in this in vitro assay.

**Purification of ME Kinase-L**

To better characterize the ME kinases, we used sequential chromatography in an attempt to purify them to near homogeneity. Thus far we succeeded in purifying ME kinase-L from M-phase egg extract with a purification strategy consisting of five steps: (a) gel filtration on Ultrogel AcA 34; (b)
ion exchange chromatography on Q-Sepharose (Fast Flow); (c) affinity chromatography on Affi-Gel blue; (d) 33–40% ammonium sulfate precipitation; and (e) FPLC on Protein Pak 300 SW (see Materials and Methods). To optimize the recovery and stability of the kinase, we included adenosine-5'-o-(3-thiotriphosphate) (ATPγS) in both the crude extract and the column running buffer. In each purification step, ME kinase-L activity eluted as a single peak with >50% recovery. At the final step of purification, only one major polypeptide of 42 kD was present in the fractions containing ME kinase-L as determined by SDS-PAGE and silver staining (Fig. 4). The intensity of this band correlated well with the kinase activity (which was quantitated by the maximum fold of diluted that still allowed the detection of ME kinase activity), suggesting that the ME kinase activity is associated with the 42-kD protein.

**Identification of ME Kinase-L as the 42-kD MAP Kinase**

Since the 42-kD MAP kinase has been shown to be active in M-phase Xenopus egg extract (Ferrell et al., 1991; Gotoh et al., 1991a,b; Posada et al., 1991), it was of interest to determine whether the purified 42-kD ME kinase-L was the 42-kD MAP kinase. We immunoblotted the fractions from the final step of purification (FPLC on Protein Pak 300 SW) with an antibody against the highly conserved NH2-terminal domain of rat MAP kinase (Boulton et al., 1990). In addition, each fraction was assayed for its ability to phosphorylate myelin basic protein (MBP), a preferred substrate of MAP kinase (Gotoh et al., 1991a). As seen in Fig. 5, fractions 28-31 that contained ME kinase-L activity also contained both a 42-kD immunoreactive band and high MBP-
phosphorylating activity, indicating copurification of ME kinase-L and MAP kinase. The two polypeptides in fractions 16–21, which cross-reacted with the antibody but did not have a corresponding MBP-phosphorylating activity, could not be active MAP kinase and therefore were not considered in this study. If MAP kinase and ME kinase-L were identical, an antibody to the 42-kD *Xenopus* MAP kinase should immunodeplete ME kinase-L activity. Using a polyclonal antibody against the COOH-terminal sequence of the 42-kD *Xenopus* MAP kinase (Posada and Cooper, 1992), we found

**Figure 4.** Gel filtration on Protein Pak 300 SW. (A) Individual fractions were measured for protein absorbance at 280 nm (OD 280 nm). The hatched areas indicate the ME kinase-L positive fractions. The arrows indicate the protein standards: (1) thyroglobulin (670 kD), (2) IgG (158 kD); (3) ovalbumin (44 kD); (4) myoglobin (17 kD); and (5) vitamin B12 (1.35 kD). (B) 20 μl of the individual fractions were separated by 20% SDS-PAGE and silver stained. The maximum fold dilution (MFD) is the extent to which the fractions could be diluted and ME kinase-L activity still detected. The minus sign (−) indicates that no ME kinase-L was detected in that fraction.

**Figure 5.** Copurification of ME kinase-L activity and MAP kinase. (A) 50 μl of fractions 16–33 from the Protein Pak 300 SW gel filtration were separated by 12.5% SDS-PAGE, transblotted onto nitrocellulose, and immunostained with anti-rat MAP kinase (NH2 terminus) antibody. The arrow indicates the 42-kD immunoreactive band. (B) The same fractions were also assayed for ME kinase activity at a 1:10 dilution and for MBP phosphorylating activity, an indicator of MAP kinase activity. MFD is defined in the legend to Fig. 4. The arrow indicates the phosphorylated MBP.
that not only did it recognize the 42-kD band in the purified ME kinase-L fractions (data not shown) but it immunodepleted ME kinase-L-activity (Fig. 6). Finally, like ME kinase-L, purified sea star and \textit{Xenopus} MAP kinases were both able to phosphorylate a 150-kD polypeptide in the in vitro assay (Fig. 7). Taken together, these results indicate that MAP kinase is responsible for ME kinase-L activity.

To determine whether ME kinase-L could phosphorylate the MPM-2 epitope in immature oocyte extract which represents a more physiological condition, immature oocyte extract was incubated with or without ME kinase-L in the presence of ATP and sodium fluoride, a phosphatase inhibitor. Immunoblot analysis of the extracts indicated that addition of ME kinase-L could cause the appearance of a 165-kD MPM-2 reactive polypeptide (Fig. 8). The result indicates that the phosphorylation of at least one MPM-2 antigen can be induced by ME kinase-L under non-denaturing conditions. Whether this 165-kD MPM-2 antigen is the phosphorylated version of the p150 observed in the ME kinase assay remains to be established.

While ME kinase-H was not purified to the same extent as ME kinase-L, it was still of interest to determine whether ME kinase-H also contained the 42-kD MAP kinase as a subunit. Fractions from AcA 34 gel filtration of unfertilized egg extract, which separated the two ME kinases completely, were immunoblotted with the NH$_2$-terminal anti-MAP kinase antibody and also assayed for MBP-phosphorylating activity. While fractions with ME kinase-L activity contained both the 42-kD anti-MAP kinase reactive peptide and high MBP-phosphorylation activity, the fractions with ME kinase-H activity contained neither (data not shown). Therefore, it is unlikely that the 42-kD MAP kinase is a peptide component of ME kinase-H.

**Phosphorylation of the MPM-2 Epitope on MAP2 by ME Kinase-L**

Phosphorylation of the MPM-2 epitope by ME kinase-L/ MAP kinase raised the possibility that the MPM-2 epitope may be identical with the phosphorylation consensus sequence of MAP kinase. To explore this possibility, we tested whether MAP2 and MBP, two preferred in vitro substrates of MAP kinase (Gotoh et al., 1991a), became MPM-2 reactive after phosphorylation by ME kinase-L. We found that MAP2 protein isolated from rat brain tissue was reactive to MPM-2 on immunoblots (data not shown), indicating that MAP2 contained the phosphorylated MPM-2 epitope. To test whether ME kinase-L could phosphorylate the MPM-2 epitope on MAP2, we immunoprecipitated MAP2 onto anti-MAP2 affinity beads, dephosphorylated it with alkaline phosphatase, and then incubated it with purified ME kinase-L or control buffer in the presence of $[^{32}P]ATP$ before MPM-2 immunoblotting. As shown in Fig. 9A, after incubation with ME kinase-L, MAP2 became both $^{32}$P-labeled and reactive to MPM-2, demonstrating that the kinase phosphorylates MAP2 on the MPM-2 epitope. In contrast, when MBP was also incubated with ME kinase-L in the presence of $[^{32}P]ATP$ and immunoblotted with MPM-2, MBP became strongly labeled with $^{32}$P but did not become reactive to MPM-2 (data not shown). To rule out the possibility that phosphorylated MBP did contain the MPM-2 epitope but that it was undetectable because of denaturation, we incubated phosphorylated MBP with MPM-2 or control antibody affinity beads. Neither antibody immobilized any $^{32}$P-labeled MBP, confirming that phosphorylated MBP does not contain the MPM-2 epitope (Fig. 9B). These findings suggest that not all substrates of MAP kinase become MPM-2 antigens after phosphorylation. We do not think that these results indicate that MAP2 and MBP contain two different phosphorylation sequences for MAP kinase. Instead, it is more likely that while MAP2 and MBP both contain the MAP kinase phosphorylation consensus sequence, this consensus sequence plus the surrounding amino acid sequence on MAP2 comprises the MPM-2 epitope, whereas on MBP it does not.

**Activation of ME Kinases by cdc2 Kinase**

During \textit{Xenopus} oocyte maturation, phosphorylation of the MPM-2 antigens appears to coincide with the activation of cdc2 kinase (Kuang et al., 1991b), suggesting that there exists a regulatory relationship between them. To determine whether cdc2 kinase can induce the activation of ME kinases, we added partially purified cdc2 kinase or buffer to immature oocyte extract for 30 min and then assayed the extract for ME kinase activity. Fig. 10A shows that while neither cdc2 kinase alone nor immature oocyte extract exhibited ME kinase activity, addition of the cdc2 kinase to the oocyte extract resulted in the activation of ME kinases.
extract produced an ME kinase activity comparable to that of unfertilized egg extract, suggesting that both ME kinase-H and ME kinase-L can be activated by cdc2 kinase. If the in vivo activation of ME kinases is an event downstream of cdc2 kinase, we would expect that cdc2 kinase would induce phosphorylation of MPM-2 antigens upon microinjection. To test this possibility, we first injected partially purified cdc2 kinase into Xenopus oocytes, made extracts of the injected oocytes, and assayed the extracts for the appearance of the MPM-2 antigens. As shown in Fig. 10 B, 30 min after the injection, a broad spectrum of MPM-2 antigens appeared and remained for at least 150 min. This result supports the possibility that cdc2 kinase can induce the activation of ME kinases. Finally, to determine when the ME kinases were activated in relation to cdc2 kinase in vivo, their activation was monitored during progesterone-stimulated oocyte maturation. Oocytes were treated with progesterone and harvested at 30-min intervals to produce extracts which were assayed for both HI kinase and ME kinase activity. We found that cdc2 kinase and the ME kinases were activated during the same interval (data not shown), demonstrating a close connection between activation of cdc2 kinase and the ME kinases under physiological conditions. Taken together, these results suggest that during Xenopus oocyte maturation, cdc2 kinase induces the phosphorylation of MPM-2 antigens indirectly via activation of the ME kinases.

Discussion

In this study, we used Xenopus oocyte maturation as a model system to identify kinases that phosphorylate the MPM-2 epitope (ME kinases) during M-phase induction and examine their relationship to the central M-phase regulator cdc2 kinase. To achieve this, we developed an in vitro assay that enabled us to detect ME kinase activity in crude M-phase egg extract. Our findings are that (a) there exist at least two ME kinases in unfertilized Xenopus eggs; (b) one of the ME kinases, ME kinase-L has the characteristics of the 42-kD MAP kinase, which in turn exhibits ME kinase-L activity; and (c) cdc2 kinase can activate the latent ME kinases in vivo in immature oocytes although it does not exhibit ME kinase activity in our in vitro assay.

The two ME kinases identified in unfertilized Xenopus eggs differ in molecular size, peptide components, substrate specificity, and MPM-2 reactivity. We do not exclude the possibility that there are more than two ME kinases in unfertilized Xenopus eggs since only a portion of the denatured substrates in the in vitro assay was sufficiently renatured for phosphorylation of their MPM-2 epitope. Regarding the two ME kinases detected, it is probable that the larger ME kinase-H is the major ME kinase responsible for phosphorylating the majority of the MPM-2 antigens while the smaller ME kinase-L is a minor ME kinase which phosphorylates only a few MPM-2 antigens. The basis of this speculation is two fold. First, we showed that in the in vitro assay, ME kinase-H phosphorylated multiple MPM-2 antigens while ME kinase-L phosphorylated mainly one. Next,
we showed that ME kinase-H is an MPM-2 antigen which can be bound by MPM-2. In our previous studies, we found that microinjection of MPM-2 into Xenopus oocytes before progesterone stimulation inhibits the phosphorylation of most of the MPM-2 antigens suggesting that a major ME kinase is an MPM-2 antigen (Kuang et al., 1989). Since ME kinase-H is able to both phosphorylate multiple MPM-2 antigens and react with MPM-2, it is likely to be the major ME kinase inhibited in the microinjection studies. We have not yet addressed the role of phosphorylation of the MPM-2 epitope on ME kinase-H in its activation. An intriguing possibility is that in Xenopus oocytes, ME kinase-H might be initially activated via phosphorylation of its MPM-2 epitope by ME kinase-L, or another unidentified ME kinase, and then further activated by autophosphorylation.

We have purified ME kinase-L to near homogeneity and shown that it is a 42-kD protein. Since Xenopus MAP kinase is also a 42-kD protein (Ferrell et al., 1991; Gotoh et al., 1991a,b; Posada et al., 1991), we performed three independent experiments to address whether ME kinase-L is the MAP kinase previously identified in Xenopus oocytes. First, we found that a major 42-kD polypeptide in the purified ME kinase-L fractions immunoreacted with two different MAP kinase antibodies. Secondly, we showed that anti-Xenopus MAP kinase antibody immunodepleted ME kinase-L activity. Finally, we found that MAP kinase purified from Xenopus by Gotoh et al. (1991a,b) and from sea star (UBI) exhibited ME kinase-L activity. Taken together, these observations indicate that the ME kinase-L purified in this study is the 42-kD Xenopus MAP kinase. Two variants of Xenopus MAP kinase have been identified which are 98% homologous with each other (Gotoh et al., 1991b; Posada et al., 1991). Without knowing the amino acid sequence of ME kinase-L, we cannot yet determine whether it is one of these variants. However, since even the 44-kD sea star MAP kinase (UBI) exhibits ME kinase activity, it is likely that both variants of the 42-kD Xenopus MAP kinase can phosphorylate the MPM-2 epitope.

We showed that ME kinase-L not only phosphorylated the MPM-2 epitope on a denatured–partially renatured polypeptide on the substrate blot, it also phosphorylated the MPM-2 epitope on the native protein, MAP2. Furthermore, the purified ME kinase-L could induce phosphorylation of the MPM-2 epitope on at least one protein (p165) in immature oocyte extract in solution. We cannot eliminate the possibility that ME kinase-L did not phosphorylate the p165 protein directly but activated another kinase which did. This seems unlikely considering that ME kinase-L can directly phosphorylate the MPM-2 epitope in vitro on both the substrate blot and native MAP2. The most reasonable explanation is that ME kinase-L, which is MAP kinase, is one of the kinases that phosphorylates the MPM-2 epitope in vivo during Xenopus oocyte maturation.

MAP kinase was originally identified as a family of kinases activated during various signal transduction pathways (Thomas, 1992; Pelech and Sanghera, 1992). Recently, it was proposed that MAP kinase is also involved in M-phase induction since MAP kinase was demonstrated to be activated during Xenopus and sea star oocyte maturation (Ferrell et al., 1991; Gotoh et al., 1991a,b; Posada et al., 1991; Sanghera et al., 1991). It was argued that the role of MAP kinase during oocyte maturation may still be in a signal transduction pathway and not in M-phase induction (Ferrell et al., 1991; Shibuya et al., 1992). In this study, we showed that MAP kinase phosphorylates the MPM-2 epitope, which apparently supports the actual involvement of MAP kinase in M-phase induction. However, although the phosphorylation of the MPM-2 epitope is greatly elevated during the G2/M transition of various cells, it is not restricted to M-phase induction. For example, it has been reported that MPM-2 recognizes the microtubule structures of interphase neuronal cells, the basal bodies of flagellate cells, and certain areas of interphase nuclei in Pt KI cells (Vandere et al., 1986; Keryer et al., 1987). Since MAP-2 reactivity in those interphase cells is confined to a few specific structures whereas the entire M-phase cell reacts to MPM-2, it is probable that the kinase(s) responsible for the interphase phosphorylation of the MPM-2 epitope would be different from the one(s) responsible for phosphorylating mitotic MPM-2 antigens. Therefore, it is possible that MAP kinase may be an interphase ME kinase which is also expressed during oocyte maturation for a function not related to M-phase induction. To determine the role of MAP kinase during Xenopus oocyte maturation, we are presently purified the MPM-2 antigen phosphorylation by MAP kinase.

Finally, we showed that although cdc2 kinase did not exhibit ME kinase activity in our in vitro assay, it could activate ME kinases in immature oocyte extract and lead to the phosphorylation of a broad spectrum of MPM-2 antigens when microinjected into immature oocytes. The simplest interpretation of these results is that in vivo, cdc2 kinase induces phosphorylation of MPM-2 antigens indirectly via ME kinases. One concern we have about this explanation is that we cannot eliminate the possibility that in vivo, cdc2 kinase might be able to phosphorylate the MPM-2 epitope in some substrates which are not sufficiently renatured for phosphorylation in the in vitro assay. However, it is unlikely that cdc2 kinase is responsible for phosphorylating most MPM-2 antigens. Statistically, if cdc2 kinase was the major ME kinase, it would be highly improbable that none of its MPM-2 epitope-containing substrates were sufficiently renatured for phosphorylation while several substrates of the other ME kinases were. Therefore, even if cdc2 kinase is able to phosphorylate the MPM-2 epitope in some MPM-2 antigens, its major role in the phosphorylation of MPM-2 antigens during M-phase induction is probably to activate the other ME kinases. It has been hypothesized that cdc2 kinase induces mitosis mainly by activating an array of intermediary enzymes (Lewin, 1990; Nigg, 1991), although few of them have been identified. The present study raises the possibility that the ME kinases act as intermediary kinases by which cdc2 kinase can induce certain events of mitosis. One issue that we have not addressed is the mechanism by which cdc2 kinase activates the ME kinases. However, it has been shown that the 42-kD MAP kinase, which is ME kinase-L, is activated by cdc2 kinase indirectly via MAP kinase kinase (Matsuda et al., 1992). Whether ME kinase-H can also be activated indirectly by cdc2 kinase through ME kinase-L or an unidentified kinase will be addressed in our future studies.

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