MPM-2 epitope sequence is not sufficient for recognition and phosphorylation by ME kinase-H

Shaoli Che[a], Michael M. Weil[b], Mayra Nelman-Gonzalez[a], Cheryl L. Ashorn[a], Jian Kuang[a,*]

[a]*Department of Clinical Investigation, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA*
[b]*Department of Experimental Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA*

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Abstract Monoclonal antibody MPM-2 recognizes a large family of mitotic phosphoproteins in a phosphorylation-dependent manner. The antigenic phosphoepitope, designated the MPM-2 epitope, putatively consists of hydrophobic residue-Thr/Ser-Pro-hydrophobic residue-uncharged/basic residue. In this study, we addressed whether this sequence motif contains all the information necessary for recognition and phosphorylation by the kinase that phosphorylates most MPM-2 antigens. A fusion protein between glutathione S-transferase and a 19-residue peptide that contained two representative MPM-2 epitope sequences overlapping with two potential MAP kinase phosphorylation sites was constructed. Both the MPM-2 epitope sequences in the fusion protein (GST-MPM2) were phosphorylated by *Xenopus* egg extract, making the fusion protein MPM-2 reactive. However, while MAP kinase phosphorylated both the MPM-2 epitope sequences, neither ME kinase-H, a good candidate for a major MPM-2 epitope kinase, nor mitotic cdc2 kinase, which is known to phosphorylate certain MPM-2 antigens in vitro, phosphorylated GST-MPM2 to any significant extent. Furthermore, depletion of MAP kinase activity removed most, if not all, of the GST-MPM2 phosphorylating activity from crude *Xenopus* egg extracts. These results suggest that additional or different structural information than that provided by the deduced MPM-2 epitope sequence is required for recognition and phosphorylation by ME kinase-H or other major MPM-2 epitope kinases. They also offer a valid explanation for selective phosphorylation of certain MPM-2 antigens by MAP kinase as well as selective recognition of certain phosphorylated MAP kinase substrates by MPM-2.

Key words: MPM-2 epitope; ME kinase-H; MAP kinase; Mitotic phosphorylation

1. Introduction

The entry of eukaryotic cells into M phase is accompanied by a dramatic increase in the level of protein phosphorylation [1–3], which plays a dominant role in the induction of various mitotic events. For mitotic phosphorylation to occur, multiple mitotic kinases must be activated in a coordinated manner at entry into mitosis. Each kinase then phosphorylates a specific family of mitotic phosphoproteins, which in turn participates in the induction of a specific set of mitotic events.

While many mitotic kinases have been identified, characterization of their in vivo substrates has been difficult owing to the lack of specific probes or assays. One exception is the MPM-2 antigens, the family of mitotic phosphoproteins recognized by the phosphoepitope monoclonal antibody MPM-2 [4]. MPM-2 antigens include regulators of mitotic cdc2 kinase, cdc25 phosphatase, wee1 kinase, and myt1 kinase [5–7]; M phase-promoting kinase nimA [8]; casein kinase II [9], which is also implicated in the G2/M transition [10]; structural proteins of the mitotic apparatus, MAP4 [11], topoisomerase II α [12], and INCEMP [13]; ubiquitin-conjugating enzyme APC, which is required for anaphase onset [14]; and several proteins associated with transcription or translation [9,13]. However, the identity of the physiological kinase that phosphorylates most MPM-2 antigens remains to be established.

Since MPM-2 is a monoclonal antibody, MPM-2 antigens are likely to contain a common phosphorylation site that is phosphorylated by common or similar kinases. By screening of a phage peptide display library, it was deduced that the MPM-2 epitope sequence consists of a string of five amino acids, with LTPLK being the most frequent amino acids at each position [15]. When the cloned MPM-2 antigens MPP1 and MPP2 were examined, multiple LTPLK-related sequences were found, among which at least FTPLQ was sufficient to confer MPM-2 reactivity. Finally, while both FTPLQ and LTPLK peptides were readily phosphorylated by mitotic kinases, FTPLQ exhibited a much higher binding affinity for MPM-2 than LTPLK. This suggests that a basic amino acid at the fifth position is tolerated but not preferred by the MPM-2 epitope sequence. It was also found that KTPVK exhibited no MPM-2 binding affinity upon phosphorylation, suggesting that a basic amino acid at the first position is not tolerated by the MPM-2 epitope sequence. In summary, it appears that a preferred MPM-2 epitope sequence contains hydrophobic residue-Thr/Ser-Pro-hydrophobic residue-uncharged residue.

Based on the deduced MPM-2 epitope sequence, several predictions can be made about the kinase that phosphorylates MPM-2 antigens, designated MPM-2 epitope kinase or ME kinase. First, the ME kinase should be a proline-directed serine/threonine protein kinase and thus most of the identified M phase-promoting protein kinases are not likely candidates. Second, mitotic cdc2 kinase, which is proline-directed, should be able to phosphorylate some but not all MPM-2 antigens since LTPLK but not FTPLQ satisfies S/T-P-X-(K/R), the phosphorylation consensus for mitotic cdc2 kinase [16]. This prediction is consistent with the results from several in vitro studies [7,8,13,15,17,18]. Third, the most preferred substrates of miotic cdc2 kinase are probably not MPM-2 antigens since the strongest phosphorylation sites for cdc2 kinase often contain two or three basic amino acids [19,20]. This has been confirmed by the finding that the most commonly used in vitro substrate of cdc2 kinase, histone H1, is not recognized by MPM-2 upon mitotic phosphorylation [4]. Based on these

*Corresponding author. Fax: (1) (713) 792-3754.
E-mail: Jian_kuang@isqm.mda.uth.tmc.edu

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analyses, it appears that the MPM-2 epitope does not represent the phosphorylation consensus sequence for mitotic cdc2 kinase or any other characterized mitotic kinase.

To identify the MPM-2 epitope kinase, we developed an in situ ME kinase assay, with which we found two kinase activities in *Xenopus* egg extract that phosphorylate the MPM-2 epitope: the 42-kDa MAP kinase that phosphorylated a single polypeptide of approximately 150 kDa, and the ≥670-kDa ME kinase-H that generated MPM-2 reactivity on multiple polypeptides [18]. Mitotic cdc2 kinase did not exhibit ME kinase activity with this assay. Since ME kinase-H was the only prominent activity detected that phosphorylated the MPM-2 epitope on multiple polypeptides and did not seem to be related to any of the identified mitotic kinases, we considered it a good candidate for a major MPM-2 epitope kinase.

The objective of the present study was to test whether the deduced MPM-2 epitope sequence motif contains all the information necessary for recognition and phosphorylation by ME kinase-H or another major ME kinase. If so, it could be used as an in vitro substrate for purification and functional studies of ME kinase-H or to find other potential ME kinases. To achieve our goal, we fused two representative MPM-2 epitope sequences with glutathione S-transferase via gene fusion and determined whether the MPM-2 epitope sequences in the fusion protein could be phosphorylated by ME kinase-H or another mitotic kinase.

2. Materials and methods

2.1. Construction of MPM-2 epitope tagged glutathione S-transferase

To construct an MPM-2 epitope tagged glutathione S-transferase (GST-MPM2), an oligonucleotide encoding DPLTPLQMQKGPLSPMKEF was inserted into the *Bam*HI to *Eco*RI site of pRP259, which is a pGEX-1 vector for expression of GST. After transfection into DH 5α host cell, the expression of GST-MPM2 was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG, Boehringer Mannheim, Indianapolis, IN) at 37°C for 4 h. GST-MPM2 was purified from the cell lysates by affinity chromatography on glutathione agarose (Sigma, St. Louis, MO) as previously described [21], except that 0.1 mM PMSF and 0.1 mg/ml of each leupeptin, pepstatin A, and chymostatin (Boehringer Mannheim) were used as protease inhibitors. The washed beads, which contained ~0.2 μg/μl GST-MPM2, were either stored at −20°C directly or eluted with 10 mM glutathione containing 0.1 mg/ml of the protease inhibitors described above. The eluates, which contained ~1 mg/ml GST-MPM2, were stored at −20°C until use.

2.2. Preparation of Xenopus oocyte and egg extracts

Interphase-arrested *Xenopus* oocytes extracts were prepared as previously described [5] except that the oocytes were homogenized in extraction buffer XB [22] containing 250 mM NaCl, 5 mM DTT, and the protease inhibitors already mentioned. The M-phase arrested *Xenopus* egg extracts were prepared as previously described [23] except that the eggs were homogenized in extraction buffer EB [24] containing 1 mM ATP, 1 μM okadaic acid (OA, LC Services Corporation, Woburn, MA), 5 mM DTT, and the same protease inhibitors as described above.

2.3. GST-MPM2 phosphorylation

To detect GST-MPM2 phosphorylation by MPM-2 immunoblotting, 10 μl of the immobilized GST-MPM2 was mixed with 10 μl of kinase sample and 1 μl of a phosphorylation cocktail that gave a final concentration of 2 mM ATP, 10 mM NaF, and 0.5 μM OA. After the reaction mixture was incubated at 22°C for 30 min, the immobilized GST-MPM2 was washed three times with EB containing 0.5 M NaCl, 1 mM ATP, 5 mM NaF, 2 mM DTT, and 0.5% NP-40, and then rinsed once with EB. The eluted proteins were separated by SDS-PAGE and immunoblotted with MPM-2. For ^{32}P incorporation, the phosphorylation reaction mixture contained 10 μl of the immobilized GST-MPM2, 10 μl of kinase sample, and 5 μl of labeling cocktail that gave a final concentration of 0.5 μCi/μl [γ-^{32}P]ATP, 0.5 mM ATP, 10 mM NaF, and 0.5 μM OA. After incubation of the reaction mixtures at 22°C for 30 min, the beads were washed, and the eluted proteins were separated by SDS-PAGE. ^{32}P incorporation into GST-MPM2 was detected by autoradiography.

2.4. Immunoblotting and immunodepletion

Immunoblotting was performed as previously described [18]. MPM-2 ascites for immunoblotting was produced as previously described [4], and the anti-rat MAP kinase antibody for immunoblotting was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). For MAP kinase immunodepletion, IgG from 30 μl of anti-*Xenopus* MAP kinase immune serum and preimmune serum, which were kindly provided by Dr. J. Ferrell [25], was immobilized to 50 μl of protein A beads, which were then mixed at 4°C for 1.5–3 h with 45–50 μl of the samples. After the beads were spun down, the supernatants were recovered, and the beads washed three times with EB containing 0.5 M NaCl, 1 mM ATP, 5 mM NaF, 2 mM DTT, and 0.5% NP-40, and once with EB.

2.5. Chromatography

All chromatography was performed at 4°C in column buffer (CB), which was EB containing 20 mM NaF, 5 mM DTT, 10 μM ATPγS, and 0.5 μM OA except when noted otherwise. All ammonium sulfate precipitations (40%) were carried out by incubation at 4°C for 30 min followed by centrifugation at 35000×*g* at 4°C for 30 min. For sequential chromatography of *Xenopus* egg extracts, 40 ml of the extract was precipitated with 40% ammonium sulfate and then desalted into CB containing 1 mM ATPγS. After the desalted sample was incubated at 22°C for 2 h for thiophosphorylation to stabilize the mitotic kinases [18,23], it was applied to two 5-ml HiTrap Q-Sepharose columns (Pharmacia Biotech Inc., Piscataway, NJ), which were then washed and step eluted with CB containing first 0.2 M NaCl and then 0.4 M NaCl. The flowthrough was precipitated with 40% ammonium sulfate and further fractionated on two 40-ml Ultrogel AcA34 gel filtration columns as described previously [18]. The 0.2 M NaCl eluate was precipitated, thiophosphorylated, and applied to a 5-ml Affi-Gel blue column (Bio-Rad Laboratories, Hercules, CA), which was then washed and eluted with CB containing 0.7 M NaCl. The eluate was precipitated and further fractionated by FPLC on a Superose 6 gel filtration column.

2.6. Kinase assays, one-dimensional phosphopeptide mapping, and phosphoamino acid analysis

The in situ ME kinase assay and the assays of histone H1 kinase activity and MBP phosphorylating activity were performed as previously described [18]. Phosphoamino acid analysis was performed according to Boyle et al [26]. For one-dimensional phosphopeptide mapping, 50 μl of immobilized GST-MPM2 was phosphorylated by 100 μl of undiluted egg extract in the presence of 1.0 μCi/μl [γ-^{32}P]ATP. After the beads were washed as described above, the proteins on the beads were eluted, separated by 15% SDS-PAGE, and transblotted onto Immobilon-P. This was followed by Coomassie blue staining and autoradiography of the proteins on the blot. The top and bottom bands of the labeled GST-MPM2 were then sliced off and incubated with 1 μg of Glu-C (Boehringer Mannheim) in 50 μl of 50 mM NH_4HCO_3, pH 7.8, at 37°C for 3 h. After the peptides were eluted from the membrane by SDS-PAGE sample buffer, they were separated by tricine SDS-PAGE [27] followed by autoradiography.

3. Results

3.1. Construction of MPM-2 epitope-tagged glutathione S-transferase

We designed a 19-residue peptide DPLTPLQMQKGPLSPMKEF containing two representative MPM-2 epitope sequences (underlined). LTPLQ is similar to FTPLQ, which exhibits a high MPM-2 binding affinity upon phosphorylation, and LSPMK is similar to LTPLK, which was the MPM-2 epitope consensus from screening a phage peptide display library [15]. A proline was placed at the N-terminus of both LTPLQ and LSPMK for two reasons. First, 7 of the 19 MPM-2-reactive phage peptides contained a proline at or

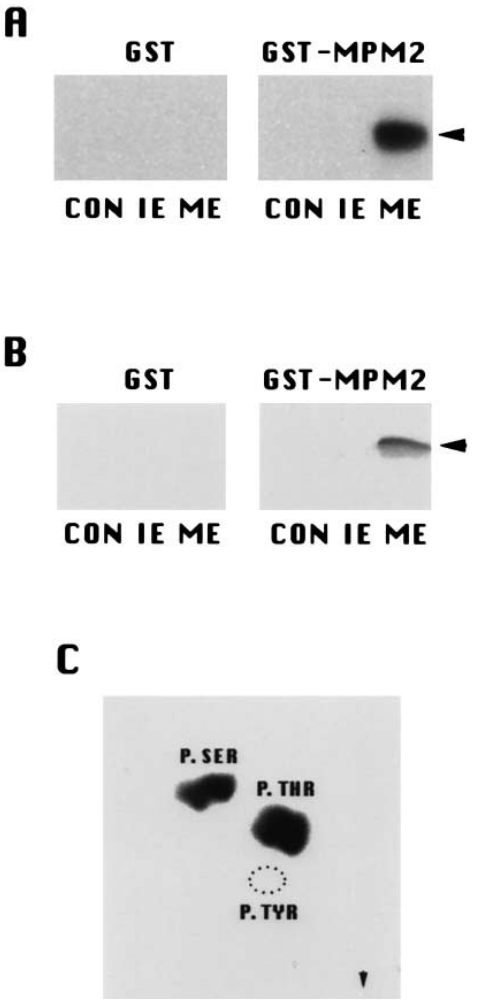

Fig. 1. Phosphorylation of GST-MPM2 in vitro. (A) Immobilized GST or GST-MPM2 was incubated with either control buffer (CON), 1:10 diluted IE, or ME at 22°C for 30 min in the presence of [γ-^{32}P]ATP. Autoradiography of the SDS-PAGE separated proteins is shown. The arrow indicates the position of the labeled GST-MPM2. (B) Immobilized GST or GST-MPM2 was phosphorylated as described for (A) except in the absence of the radiolabeled ATP. MPM-2 immunoblotting of the SDS-PAGE separated proteins is shown. The arrow indicates the position of the MPM-2-reactive GST-MPM2. (C) The ^{32}P-labeled GST-MPM2 was hydrolyzed with HCl and subjected to phosphoamino acid analysis. The positions of phosphorylated serine (P. SER), threonine (P. THR), and tyrosine (P. TYR) are indicated.

near the amino-terminus of the MPM-2 epitope sequence, suggesting that a proline in this position might favor phosphorylation or MPM-2 recognition. Second, adding proline at this position would generate an overlapping MAP kinase phosphorylation site, PX(S/T)P, whose phosphorylation by MAP kinase would indicate the availability of the MPM-2 epitope sequence for phosphorylation. The spacing sequence between the two MPM-2 epitope sequences, MQKG, was copied from the sequence following LTPLK in the cognate MPM-2-reactive peptide selected from the phage peptide library. Finally, two MPM-2 epitope sequences were placed in proximity because 5 of the 19 MPM-2 reactive phage peptides contained two MPM-2 epitope sequences and increasing the number of the MPM-2 epitope sequences would favor MPM-2 recognition [15].

For production and purification of the designed peptide (MPM-2 peptide), the peptide sequence was attached to the carboxy-terminus of recombinant glutathione S-transferase (GST) via gene fusion (see Materials and Methods) so that it could be easily purified by affinity chromatography on glutathione agarose. This approach had been used successfully by Holmes and Solomon to characterize the fine-structure specificity of cyclin-dependent protein kinases [19].

To determine whether the MPM-2 peptide in the fusion protein, designated GST-MPM2, could be phosphorylated specifically by M phase-arrested *Xenopus* egg extract (ME), the immobilized GST or GST-MPM2 was incubated with control buffer, 1:10 diluted interphase-arrested *Xenopus* oocyte extract (IE), or ME in the presence of [γ-^{32}P]ATP. Autoradiography of the gel-separated proteins showed that while GST was not ^{32}P-labeled under any conditions, GST-MPM2 became ^{32}P-labeled upon incubation with ME but not the control buffer or IE (Fig. 1A). This result indicated that at least one MPM-2 epitope sequence in the MPM-2 peptide was phosphorylated by a kinase that is active only in ME. To confirm that phosphorylation of the MPM-2 epitope in GST-MPM2 generated MPM-2 reactivity, the phosphorylated GST-MPM2 was immunoblotted with MPM-2. Consistent with the result from the ^{32}P incorporation experiment, only the GST-MPM2 that had been incubated with ME was recognized by MPM-2 (Fig. 1B). This result confirmed that phosphorylation of the MPM-2 epitope sequence in GST-MPM2 is sufficient for generating MPM-2 immunoreactivity.

To determine whether one or both of the MPM-2 epitope sequences in GST-MPM2 was phosphorylated, GST-MPM2 that had been ^{32}P-labeled by 1:10 diluted ME was hydrolyzed with 6 N HCl at 110°C for 2 h and subjected to phosphoamino acid analysis. As shown in Fig. 1C, the recovered phosphoamino acids contained both phosphoserine and phosphothreonine. Scintillation counting of each of the phosphoamino acids showed that the phosphothreonine was twice as abundant as phosphoserine. However, we later found that during phosphoamino acid analysis, phosphothreonine was recovered twice as efficiently as phosphoserine. Thus, both MPM-2 epitope sequences were phosphorylated with similar efficiencies.

If GST-MPM2 was phosphorylated with undiluted ME as described above, an additional slower migrating band appeared that was both ^{32}P-labeled and MPM-2 reactive (Fig. 2A). This suggested that at higher kinase concentration, some GST-MPM2 was phosphorylated on both LTPLQ and LSPMK, and some on only one of the sequences. To confirm this idea, we first treated the phosphorylated GST-MPM2 with potato acid phosphatase and then separated it by SDS-PAGE. Phosphatase treatment eliminated the slower migrating species of GST-MPM2 (Fig. 2B), indicating that the appearance of the slower migrating molecular species was not due to a modification of GST-MPM2 in addition to phosphorylation. We next performed a one-dimensional phosphopeptide mapping of the two bands. A partial digestion of each band with Glu-C resulted in a similar collection of phosphopeptides except that each phosphopeptide from the slower migrating band exhibited a slightly higher apparent molecular weight than its counterpart from the faster migrating band (Fig. 2C). This result indicated that the slower migrating molecular species was not due to a forced phosphorylation of the GST portion of the molecule or a contamination of a labeled MPM-2 antigen from the kinase source. We thus concluded

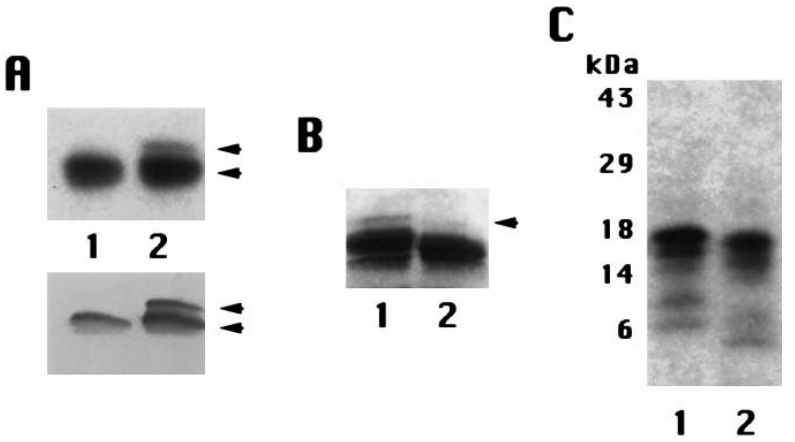

Fig. 2. Phosphorylation of GST-MPM2 with different concentrations of *Xenopus* egg extract. (A) Immobilized GST-MPM2 was phosphorylated with either 1:10 diluted ME (lane 1) or the same extract with no dilution (lane 2) at 22°C for 30 min. Top panel: The phosphorylation was done in the presence of [γ-^{32}P]ATP; autoradiography of the gel-separated proteins is shown. Bottom panel: The phosphorylation was done in the absence of radiolabeled ATP; MPM-2 immunoblot of the gel separated proteins is shown. The arrows indicate the positions of the phosphorylated GST-MPM2. (B) The immobilized GST-MPM2 that had been phosphorylated with undiluted ME and washed was incubated with either control buffer (lane 1) or potato acid phosphatase at 37°C for 2 h before the proteins were separated by SDS-PAGE. Coomassie blue staining of gel-separated proteins is shown. (C) After GST-MPM2 was phosphorylated with undiluted ME in the presence of [γ-^{32}P]ATP, the top (lane 1) and bottom (lane 2) bands of the labeled GST-MPM2 were each digested with Glu-C. The SDS-PAGE separated peptides from the digestion are shown.

that while the faster migrating species of phosphorylated GST-MPM2 was phosphorylated on either LTPLQ or LSPMK, the slower migrating species was phosphorylated on both MPM-2 epitopes.

Since the slower migrating species of the phosphorylated GST-MPM2 should contain equal amounts of phosphoserine and phosphothreonine, phosphoamino acid analysis of it would reveal the relative recovery efficiencies of phosphoserine and phosphothreonine from hydrolysis of the phosphorylated GST-MPM2, which would be useful for evaluating

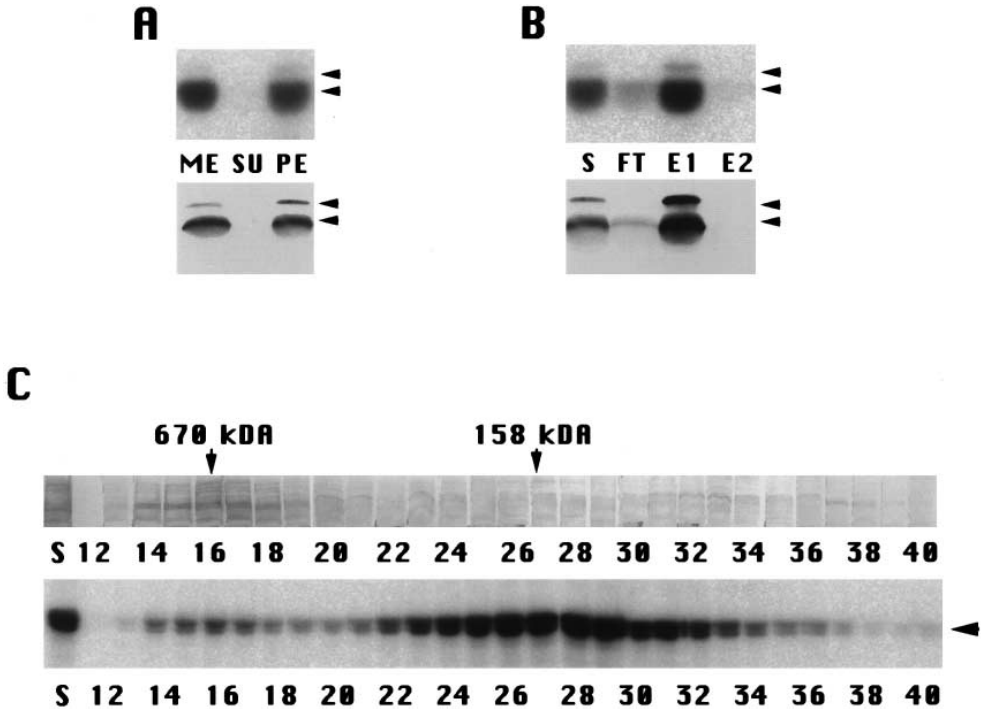

Fig. 3. Fractionation of *Xenopus* egg extracts. (A) ME was precipitated with 40% ammonium sulfate. The starting material (ME), the unprecipitated proteins (SU), and the precipitated proteins (PE) were assayed for their ability to phosphorylate GST-MPM2 by ^{32}P incorporation (top panel) or MPM-2 immunoblotting (bottom panel). (B) The thiophosphorylated 40% ammonium sulfate precipitate was fractionated by Q-Sepharose chromatography. The starting material (S), flowthrough (FT), 0.2 M NaCl eluate (E1), and 0.4 M NaCl eluate (E2) were each assayed for their ability to phosphorylate GST-MPM2 by ^{32}P incorporation (top panel) or by MPM-2 immunoblotting (bottom panel). (C) The flowthrough from Q-sepharose chromatography was precipitated by 40% ammonium sulfate and fractionated on a 40-ml UltroGel AcA 34 gel filtration column. The starting material (S) and fractions 12–40 were assayed for ME kinase-H activity by the in situ ME kinase assay (top panel) and for mitotic cdc2 kinase activity by histone H1 kinase assay (bottom panel). The positions of the standard proteins thyroglobulin (670 kDa) and bovine IgG (158 kDa) are indicated. The arrow indicates the phosphorylated histone H1.

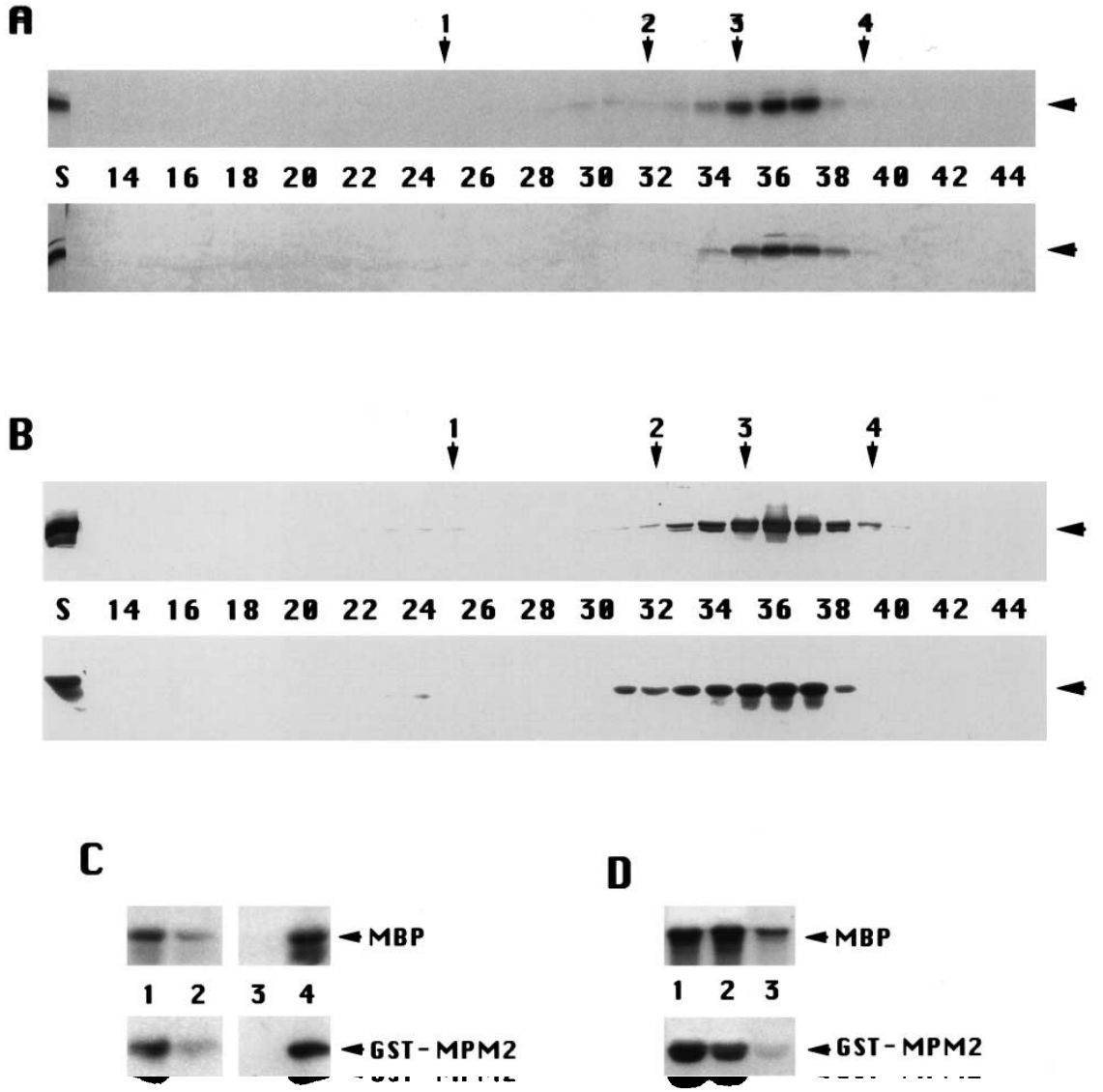

Fig. 4. MAP kinase accounted for the GST-MPM2 phosphorylating activity. After the 0.2 M NaCl eluate from the Q-sepharose chromatography was fractionated by Affi-Gel blue chromatography, the 0.7 M NaCl eluate from it was further fractionated by FPLC on Superose 6 column. The numbered vertical arrows indicate the positions of the standard proteins: 1, thyroglobulin (670 kDa); 2, bovine IgG (158 kDa); 3, chicken ovalbumin (44 kDa); and 4, equine myoglobin (17 kDa). (A) Starting material (S) and fractions 14–44 were assayed for their ability to phosphorylate GST-MPM2 by MPM-2 immunoblotting (top panel) or by ^{32}P incorporation (bottom panel). (B) The same samples were immunoblotted with anti-MAP kinase antibody (top panel) or assayed for their ability to phosphorylate MBP by ^{32}P incorporation (bottom panel). (C) Aliquots of fractions 35–37 from the Superose 6 gel filtration were pooled and immunodepleted by IgG from preimmune serum or from anti-*Xenopus* MAP kinase immune serum bound to protein A beads. After the beads were pelleted, both the supernatants were assayed for MBP phosphorylating activity (top panel) and GST-MPM2 phosphorylating activity (bottom panel) by ^{32}P incorporation. Lanes: 1, control antibody-absorbed supernatant; 2, anti-MAP kinase-absorbed supernatant; 3, control antibody beads; 4, anti-MAP kinase antibody beads. (D) ME was immunodepleted as in (C). The starting material (lane 1), the control antibody-absorbed supernatant (lane 2), and the anti-MAP kinase antibody-absorbed supernatant (lane 3) were assayed for their ability to phosphorylate MBP (top panel) and GST-MPM2 (bottom panel) by ^{32}P incorporation.

whether LTPLQ was more preferentially phosphorylated than LSPMK by 1:10 diluted ME. We found that the recovered phosphothreonine was approximately twice as abundant as the recovered phosphoserine (data not shown), which was consistent with the previous finding that phosphothreonine was generally more stable than phosphoserine during acid hydrolysis [28]. Since this result was similar to that obtained from phosphoamino acid analysis of the singly phosphorylated GST-MPM2, we concluded that LTPLQ and LSPMK were phosphorylated with similar frequencies.

3.2. The GST-MPM2 phosphorylating activity was not due to ME kinase-H or mitotic cdc2 kinase

To determine whether the MPM-2 epitope sequences in GST-MPM2 could be phosphorylated by ME kinase-H or other mitotic kinases, we fractionated ME by sequential chromatography to separate ME kinase-H, mitotic cdc2 kinase, and MAP kinase, any one or all of which might phosphorylate the MPM-2 epitope sequences in GST-MPM2. The GST-MPM2 phosphorylating activity was followed at each step by both ^{32}P incorporation and MPM-2 immunoblotting. ME was

first precipitated with 40% ammonium sulfate to enrich mitotic kinases and remove the inhibitor of M phase induction, INH, which otherwise might destabilize mitotic kinases [29–31]. Only the pelleted proteins contained a high GST-MPM2 phosphorylating activity (Fig. 3A). The thiophosphorylated ammonium sulfate precipitate was then fractionated by Q-sepharose chromatography. Most GST-MPM2 phosphorylating activity was recovered in the 0.2 M eluate, a little in the flowthrough, and none in the 0.4 M NaCl eluate (Fig. 3B). Since our previous studies showed that mitotic cdc2 kinase [32] and most ME kinase-H activity (unpublished data) were recovered in the flowthrough and MAP kinase was recovered in the 0.2 M NaCl eluate [18], this result suggested that while the major MPM2-GST phosphorylating activity in the egg extract was not due to ME kinase-H or cdc2 kinase, it might be due to MAP kinase.

To confirm that ME kinase-H activity and mitotic cdc2 kinase activity were actually recovered from the Q-sepharose chromatography, the flowthrough from Q-sepharose chromatography was fractionated by gel filtration on an UltroGel AcA 34 column, which is a diagnostic chromatography for both ME kinase-H and mitotic cdc2 kinase [18]. An in situ ME kinase assay of the collected fractions showed that a peak of ME kinase activity was recovered in the 670-kDa fractions and generated MPM-2 reactivity on multiple polypeptides (Fig. 3C top panel), which is characteristic of ME kinase-H activity [18]. A histone H1 kinase assay of the fractions showed that a strong peak of histone H1 kinase activity was recovered in the 158-kDa fractions (Fig. 3C bottom panel), which did not did not exhibit ME kinase activity in the in situ ME kinase assay. This was expected of mitotic cdc2 kinase [18]. Recovery of both ME kinase-H and mitotic cdc2 kinase activities from the chromatography confirmed that they did not contribute significantly to the GST-MPM2 phosphorylating activity in ME.

3.3. *The GST-MPM2 phosphorylating activity was due to MAP kinase*

To test whether the GST-MPM2 phosphorylating activity was due to MAP kinase, the 0.2 M eluate from the Q-sepharose chromatography was fractionated by chromatography on an Affi-Gel blue column followed by FPLC on a Superose 6 column. This procedure had been utilized before to purify MAP kinase in ME to near homogeneity [18]. In the Affi-Gel blue chromatography, the GST-MPM2 phosphorylating activity was eluted at 0.7 M NaCl (data not shown) like MAP kinase [18]. In the Superose 6 gel filtration, the GST-MPM2 phosphorylating activity peaked in fractions 35–37 (Fig. 4A), which correlated with both MAP kinase protein, as determined by immunoblot, and MAP kinase activity, as assayed by phosphorylation of myelin basic protein (MBP, Fig. 4B). Moreover, when fractions 35–37 were pooled and absorbed with either anti-MAP kinase antibody or control antibody affinity beads, the anti-MAP antibody absorption removed most of the MBP phosphorylating activity and GST-MPM2 phosphorylating activity as compared to the control (Fig. 4C). Similar results were also obtained if the immunodepletion was done with crude egg extracts (Fig. 4D). Taken together, these results clearly indicated that GST-MPM2 phosphorylating activity in ME was mainly due to MAP kinase.

4. Discussion

MPM-2 antigens are a distinct family of 50–100 mitotic phosphoproteins that contain similar phosphorylated epitopes recognized by the monoclonal antibody MPM-2. Although the MPM-2 epitope sequences in cellular MPM-2 antigens have not been determined, the five-residue peptides FTPLQ and LTPLK have been demonstrated to be sufficient for recognition by MPM-2 upon phosphorylation [15]. This suggests that both contain essential structural information for the MPM-2 epitope. In the present study, we made a fusion protein between GST and a 19-residue peptide containing LTPLQ, which is very similar to FTPLQ, and LSPMK, which is very similar to LTPLK. Using the fusion protein as the substrate, we tested whether either or both of the two MPM-2 epitope sequences were phosphorylated by ME kinase-H or other mitotic kinases in M phase-arrested *Xenopus* egg extract. We found that while both could be phosphorylated by MAP kinase, indicating that they were available for phosphorylation, neither was phosphorylated by ME kinase-H or other mitotic kinases, which suggests that more or different structural information than that provided by the minimal MPM-2 epitope sequence is required for recognition and phosphorylation by the kinase that phosphorylates most MPM-2 antigens.

One possibility to consider is that the MPM-2 epitope sequence is only part of the phosphorylation consensus sequence. This idea is supported by our experience that when we make the substrate blots for the in situ ME kinase assay [18], transferring the proteins slowly in the absence of SDS but in the presence of methanol is crucial for the MPM-2 epitope on the substrate blots to be phosphorylated by ME kinase-H activity. Since such conditions favor renaturation of the SDS-PAGE denatured proteins, this suggests that more than a short amino acid sequence is required for recognition and phosphorylation by ME kinase-H. An additional requirement may involve a distant sequence or a secondary structure.

It is also possible that the MPM-2 epitope sequences we tested were not the type phosphorylated by ME kinase-H. Although we assume that the phosphoepitopes recognized by the monoclonal antibody MPM-2 possess a common feature, we cannot exclude the possibility that subtypes of the MPM-2 epitope exist that are beyond resolution by MPM-2 recognition. If such is the case, it is possible that ME kinase-H phosphorylates only one subtype of the MPM-2 epitope and that his subtype significantly differs from the two MPM-2 epitope sequences tested in the present study.

While the inability of mitotic cdc2 kinase to phosphorylate LTPLQ, which does not match the phosphorylation consensus sequence for mitotic cdc2 kinase, S/TPXK/R, was expected, the inability of mitotic cdc2 kinase to phosphorylate LSPMK, which matches S/TPXK/R, was at first surprising. However, since screening of a synthetic peptide library showed that the optimal sequence for cdc2 kinase phosphorylation is RRRKSPRK [20], indicating that having basic residues both amino-terminal and carboxy-terminal of the cdc2 kinase phosphorylation site facilitates cdc2 kinase phosphorylation, the possibility should be considered that inclusion of an acidic residue carboxy-terminal of LSPMK in GST-MPM2 would have greatly hindered its phosphorylation by cdc2 kinase.

The consensus sequence for MAP kinase phosphorylation,

PXS/TP, overlaps with the MPM-2 epitope sequence LT/SPLQ/K. This predicts that if an MPM-2 epitope sequence is preceded by a proline, this MPM-2 epitope should be phosphorylatable by MAP kinase. We confirmed this prediction by showing that both PLTPLQ and PLSPMK in GST-MPM2 were phosphorylated by MAP kinase. It may thus offer a valid explanation on why only some of the MAP kinase substrates are MPM-2 antigens and why MAP kinase phosphorylates only a small subset of MPM-2 antigens [18]. For example, we showed that, while MAP kinase phosphorylates both MBP and MAP2 very well, only MAP2 became MPM-2 reactive upon phosphorylation [18]. Consistent with the prediction, only the latter protein sequence contains MPM-2 epitope sequences preceded by a proline. Therefore, an ideal in vitro substrate specific for MPM-2 epitope kinase should be one that only contains MPM-2 epitope sequences not proceded by a proline.

In summary, the findings in the present study suggest that while the deduced MPM-2 epitope sequence is sufficient for MPM-2 recognition, it is not equivalent to the phosphorylation consensus sequence for the MPM-2 epitope kinase. Further studies are required to define the relationship between them.

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