A phosphatase activity in *Xenopus* oocyte extracts preferentially dephosphorylates the MPM-2 epitope

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Abstract MPM-2 antigens are a large family of mitotic phosphoproteins that contain similar phosphoepitopes recognized by the anti-phosphoepitope antibody MPM-2 (MPM-2 epitopes). These proteins are phosphorylated during M phase induction and dephosphorylated from the onset of anaphase through interphase. Since biochemical characterization of the MPM-2 epitope phosphatase requires a specific assay for its activity, we tested different methods for measurement of the MPM-2 epitope phosphatase activity in crude cell lysates. First, an ELISA-based assay was designed that measured the phosphatase-induced reduction of the MPM-2 reactivity in crude M phase cell lysates. Using this assay to follow the phosphatase activity during sequential chromatography of *Xenopus* oocyte extracts, one predominant peak of phosphatase activity was detected which was separated from the majority of PP1 and PP2A activities. This phosphatase activity dephosphorylated the MPM-2 epitope on multiple MPM-2 antigens. The second method measured dephosphorylation of cdc25, a known MPM-2 antigen. Two major peaks of cdc25 dephosphorylating activities were detected during the sequential chromatography, one that copurified with the major peak of MPM-2 epitope phosphatase activity, and the other with the major peak of PP2A activity. Finally, we examined whether GST-MPM2, a fusion protein between glutathione *S*-transferase and a 19-residue peptide that contained two representative MPM-2 epitope sequences, could be dephosphorylated efficiently and specifically by the major MPM-2 epitope phosphatase activity in *Xenopus* oocyte extracts. Neither the crude extract nor the partially purified MPM-2 epitope phosphatase activity efficiently dephosphorylated the MPM-2 epitope on GST-MPM2. These results demonstrate that the ELISA-based assay preferentially detects the MPM-2 epitope phosphatase activity in crude cell lysates which may represent a physiological MPM-2 epitope phosphatase.

Key words: Phosphatase activity; MPM-2 antigen; Dephosphorylation; *Xenopus* oocyte extract

1. Introduction

Protein phosphorylation and dephosphorylation play important roles in controlling the entry into and exit from mitosis in the eukaryotic cell cycle. MPM-2 antigens, proteins that are recognized by the anti-phosphoepitope antibody MPM-2, are a large family of mitotic phosphoproteins that are phosphorylated during M phase induction and dephosphorylated from the onset of anaphase through interphase [1]. Because MPM-2 is a monoclonal antibody, the MPM-2 antigens that it recognizes are expected to contain similar phosphoepitopes which are dephosphorylated by one or few phosphatases. The phosphoepitopes recognized by MPM-2 (MPM-2 epitopes) contain phosphoserine or phosphothreonine followed by proline [2,3]. Genetic studies in *Aspergillus* suggest that PP1 activity is required for the dephosphorylation of MPM-2 antigens at the exit from mitosis [4]. However, there has been no biochemical characterization of the MPM-2 epitope phosphatase in any system.

Since the MPM-2 epitope phosphatase is likely to be a crucial regulator in the cell cycle, it is important to define its molecular identity and investigate its cell cycle regulation. One crucial step towards achieving these goals is the development of a specific assay that measures its activity in crude cell lysates. Such an assay would allow biochemical identification and purification of candidate phosphatases from crude cell lysates. It would also allow measurement of the MPM-2 epitope phosphatase activity in the cell cycle.

In developing a specific assay that measures the MPM-2 epitope phosphatase activity in crude cell lysates, three complications were considered. First, it is currently unknown whether one or several similar phosphatases dephosphorylate the MPM-2 epitope on MPM-2 antigens. Second, MPM-2 antigens may also contain phosphorylation sites unrelated to the MPM-2 epitope and thus be dephosphorylated by multiple phosphatases. Third, serine/threonine protein phosphatases often have broad substrate specificities when assayed in vitro using purified substrates. Because of these considerations, a phosphatase assay involving the use of a single purified MPM-2 antigen may not be selective enough to preferentially detect the MPM-2 epitope phosphatase activity in the presence of other phosphatase activities.

In this study, we tried three different methods for measurement of the MPM-2 epitope phosphatase activity. The first was an ELISA-based method that measures the reduction of the MPM-2 reactivity in crude M phase lysates. The second measures dephosphorylation of a single purified MPM-2 antigen, cdc25. The third measures dephosphorylation of the MPM-2 epitope consensus sequence in a fusion protein. Our results demonstrate that only the ELISA-based assay is both efficient and selective for detection of the MPM-2 epitope phosphatase activity.

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2. Materials and methods

2.1. Buffers and reagents

M phase egg extraction buffer (EB) contained 80 mM sodium β-glycerophosphate, 20 mM EGTA, and 15 mM $MgCl_2$, pH 7.3 [5]. Interphase egg extraction buffer (XB) contained 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, and 10 mM K-HEPES pH 7.4 [6]. The extract dilution buffer for the ELISA-based assay (DB) contained 100 mM KCl, 15 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 mM DTT, 50 mM sucrose and 10 mM K-HEPES, pH 7.4. Tris-based saline-Tween 20 (TBST) contained 10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20. The beads washing buffer (WB) was EB containing 0.5 M NaCl, 1 mM ATP, 5 mM NaF, 2 mM dithiothreitol (DTT), and 0.5% NP-40. Okadaic acid (OA) was purchased from LC Services Corporation (Woburn, MA) dissolved into DMSO to make a 1 mM stock solution, and stored at −20°C.

2.2. Preparation of Xenopus oocyte and egg extracts

Interphase-arrested *Xenopus* oocyte extract (IOE, 10 mg/ml) was prepared as previously described [7], except that the oocytes were homogenized in an equal volume of XB containing 250 mM NaCl, 5 mM DTT, and 1 μg/ml each of the protease inhibitors including benzamidine, aprotinin, and soybean trypsin inhibitor [8]. M phase-arrested *Xenopus* egg extract (MEE, 10 mg/ml) was prepared as previously described [9], except that the eggs were homogenized in an equal volume of EB containing 1 mM ATP, 1 μM OA, 5 mM DTT, and the same protease inhibitors. Interphase-arrested *Xenopus* egg cytosol (60 mg/ml) was prepared from unfertilized *Xenopus* eggs that had been activated by incubation with calcium ionophore A231587 in the presence of protein synthesis inhibitor, cycloheximide [10]. All extracts were immediately stored at −70°C.

2.3. ELISA-based assay for measurement of the MPM-2 epitope phosphatase activity

Wells of 96-well microtiter plates (Costar, Cambridge, MA) were individually coated with 200 μl of MEE that was diluted 1:80 with EB containing 1 mM ATP, 1 mM DTT, and 20 mM NaF either at 4°C overnight or at 37°C for 2 h. After the coated wells were rinsed four times with TBST, 200 μl of each phosphatase sample in DB or DB alone were added to two or three coated wells. The plate was then incubated at either room temperature (RT) or 37°C for the length of time indicated in the text and the reaction was stopped by rinsing the wells three times with TBST. For antibody binding, each well was first incubated with TBST plus 10% fetal calf serum (blocking solution) at RT for 1 h, then with 200 μl of 1:500 diluted MPM-2 or control ascites in the blocking solution at RT for 2 h, and finally with 200 μl of 1:5000 diluted goat anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) in the blocking solution at RT for 1 h. After each step, the wells were rinsed four times with TBST. For development, each well was incubated with 125 μl of 1 mg/ml *p*-nitrophenol phosphate (Bio-Rad Laboratories) in 1 M diethanolamine, pH 9.3–9.5, and 5 mM $MgCl_2$ at RT for 30 min which was followed by addition of 75 μl of 1 N NaOH to stop the development. Individual wells were then measured for absorbance at 405 nm (OD_{405}) using a Microtiter EIA reader (Bio-Rad Laboratories). The readings from the duplicate or triplicate wells were averaged and subtracted from the averaged value obtained from the control antibody-incubated wells, which were representative of the background. The percent reduction in the MPM-2 reactivity was calculated as (OD_{405} of the buffer treated−OD_{405} of the phosphatase treated):OD_{405} of the buffer treated×100%.

2.4. Measurement of ^{32}P release from the substrates

Interphase-arrested *Xenopus* egg cytosol which contained hypophosphorylated MPM-2 antigens [7] was mixed with an equal volume of EB containing 2 μM OA and 2 μCi/μl [γ-^{32}P]ATP and incubated at RT for 1 h. Incubation of the interphase egg cytosol with both EB and OA induced phosphorylation of most if not all MPM-2 antigens (unpublished results). The labeled cytosol was then diluted 1:250 with EB containing 1 mM ATP, 1 mM DTT, and 2 μM OA and coated onto microtiter plates at RT for 3 h. After the coated plate was washed four times with TBST, 200 μl of IOE of different dilutions were added to three coated wells, which were then incubated at RT for 1 h. The IOE samples were recovered and the wells washed four times with TBST. The IOE samples and the first wash were combined and subjected to scintillation counting. The cpms from the triplicate wells were averaged and subtracted from the value obtained from the control buffer-incubated wells, which were representative of the background. The washed wells were measured for the MPM-2 reactivity as described above.

2.5. Immunoblotting

Proteins were separated by 12.5% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. Immunoblotting was performed as previously described [9]. MPM-2 ascites was produced as previously described [1] and used upon 1:500 dilution. The anti-human PP1 or PP2A antibody was purchased from Upstate Biotechnology, Inc. (UBI, Lake Placid, NY) and used at 1 μg/ml. The alkaline phosphatase-conjugated goat anti-mouse or rabbit IgG was purchased from Promega Corporation (Madison, WI) and used at 1:15 000 dilution.

2.6. Chromatography of Xenopus oocyte extracts

Ammonium sulfate precipitation was done by incubation of the samples at 4°C for 30 min followed by centrifugation at 35 000×*g* at 4°C for 30 min. The ammonium sulfate-precipitated proteins were desalted through gel filtration with an Econo Pac 10 DG desalting column (Bio-Rad Laboratories). All chromatographies were performed at 4°C in DB unless otherwise indicated. For each sequential chromatography, 5 ml of IOE was precipitated with 45% ammonium sulfate and then desalted into DB. The desalted sample was applied to a 5 ml HiTrap Q-Sepharose column (Pharmacia Biotech Inc., Piscataway, NJ), which was then washed with 15 ml DB and sequentially eluted with 15 ml DB containing 0.2 M NaCl and 1.0 M NaCl. The 0.2 M NaCl eluate was concentrated by ammonium sulfate precipitation (45%) and desalted into 0.25×DB. The desalted sample was applied to a 5 ml HiTrap heparin-Sepharose column (Pharmacia Biotech Inc.), which was then washed with 15 ml of 0.25×DB and sequentially eluted with 15 ml of 0.25×DB containing 0.09 M NaCl and 1.0 M NaCl. The 0.09 M NaCl eluate was concentrated by ammonium sulfate precipitation (60%) and was further fractionated by FPLC on a Superose 6 gel filtration column in DB (Pharmacia Biotech Inc.).

2.7. Phosphorylase a phosphatase assay

The phosphorylase a phosphatase assay was performed as previously described [11], except that gel filtration was used to remove free [γ-^{32}P]ATP from the labeled phosphorylase a. Protamine (Sigma, St. Louis, MO) was added at a final concentration of 10 μg/ml to increase PP2A activity. Bovine serum albumin was added at a final concentration of 10 μg/ml to facilitate protein precipitation after the dephosphorylation reaction. The percentage dephosphorylation was calculated as (cpm from sample incubation−cpm from control buffer incubation):cpm from the added phosphorylase a×100%. Samples were diluted until they dephosphorylated ≤40% of the substrates.

2.8. Assay of cdc25 dephosphorylating activity

Recombinant *Xenopus* cdc25, fused as its N-terminus to glutathione *S*-transferase (GST-cdc25), was produced from *Escherichia coli* TG-1 cells harboring the expressing vector GEX-KG-cdc25 (a gift of A.R. Nebreda and T. Hunt). The expression of GST-cdc25 was induced with 0.08 mM isopropyl β-D-thiogalactopyranoside (IPTG, Boehringer Mannheim, Indianapolis, IN) overnight at 22°C when the bacteria grew to OD_{600} 0.8–1.0. Isolation of GST-cdc25 was done by affinity absorption of the induced cell lysates with glutathione agarose (Sigma) in the presence of 10 mM DTT, 0.1 mM PMSF and 0.1 mg/ml of each protease inhibitor (leupeptin, pepstatin A, and chymostatin, Boehringer Mannheim). The washed beads were stored at −70°C for future use as immobilized GST-cdc25.

For measurement of dephosphorylation of cdc25 by MPM-2 immunoblotting, GST-cdc25 was phosphorylated by incubation of the immobilized GST-cdc25 (0.2 μg/μl) with 1/3 volume MEE diluted 1:1 with EB containing 20 mM NaF, 1 mM DTT, 5 mM ATP and 0.5 μM OA at RT for 1 h. After the beads were washed three times with WB and rinsed two times with XB containing 1 mM DTT, 10 μl of the phosphorylated GST-cdc25 were incubated with 30 μl sample or XB at RT for 30 min. The reaction was stopped by addition of SDS-PAGE sample buffer. The proteins were then separated by 12.5% SDS-PAGE, transblotted onto nitrocellulose and immunoblotted with MPM-2.

For measurement of dephosphorylation of cdc25 by ^{32}P removal, the immobilized GST-cdc25 was ^{32}P-labeled by incubation with 1/3 volume MEE containing 2 μCi/μl [^{32}P]ATP and 2 mM ATP. After the beads were washed as described above, the labeled GST-cdc25 was eluted with 10 mM glutathione in XB. The free GST-cdc25 (2 μg/10 μl) was then incubated with 30 μl sample from Superose 6 fractions at RT for 30 min. The proteins were separated by 12.5% SDS-PAGE and the gel was subjected to autoradiography.

2.9. Assay of dephosphorylation of GST-MPM2

GST-MPM2 immobilized onto glutathione agarose was prepared as previously described [12]. The beads, which contained ~0.2 μg/μl GST-MPM2, were phosphorylated by MEE as described for phosphorylation of GST-cdc25. After the beads were washed three times with WB and rinsed twice with XB, 10 μl beads were incubated with 30 μl of 1:20 diluted IOE at RT for 0–60 min. The proteins were then eluted, separated by SDS-PAGE and immunoblotted with MPM-2.

3. Results

3.1. Development of an ELISA-based assay that measures MPM-2 epitope phosphatase activity

The design of the ELISA-based assay to measure the MPM-2 epitope phosphatase was based on the principle that the recognition of MPM-2 antigens by the antibody MPM-2 is phosphorylation-dependent [1]. Thus dephosphorylation of the MPM-2 epitope on MPM-2 antigens by a phosphatase activity should result in a reduction of their reactivity to the MPM-2 antibody (MPM-2 reactivity). The substrates used for the assay were total proteins extracted from M phase-arrested *Xenopus* eggs (MEE), which presumably contained most, if not all, of the MPM-2 antigens in *Xenopus laevis* [13]. MEE was coated on 96-well microtiter plates and candidate phosphatase samples were added for the dephosphorylation reaction. After the plates were washed, ELISA was used to quantitate the MPM-2 reactivity of the substrates. Presumably, immobilization of MEE had disabled proteins in MEE which might interfere with dephosphorylation of the MPM-2 epitope. The buffer used in the assay contained 15 mM Mg^{2+} and 0.3 mM Ca^{2+}, allowing detection of all four major types of serine/threonine protein phosphatases [14].

To establish the validity of the assay, we first confirmed the specificity of the detection of the phosphorylated MPM-2 epitope by ELISA. It was previously shown that proteins extracted from interphase-arrested *Xenopus* oocytes (IOE) and MEE contained unphosphorylated and phosphorylated MPM-2 antigens respectively [13]. Thus, IOE and MEE were immobilized on the same microtiter plate and their MPM-2 reactivities were measured by ELISA. As shown in Fig. 1A, the ELISA detected a 20-fold higher MPM-2 reactivity in MEE relative to IOE; both extracts showed little reactivity to the control antibody, RDA-1, which recognizes a nuclear protein throughout the cell cycle. These results demonstrated that the ELISA specifically detected the phosphorylated MPM-2 epitope.

We next determined the ability of IOE to decrease the MPM-2 reactivity in the substrates. IOE was chosen as a possible source of MPM-2 epitope phosphatase activity because extracts made from interphase-arrested cells that lack significant levels of phosphorylated MPM-2 antigens should contain high levels of MPM-2 epitope phosphatase activity. The substrates were first incubated with 1:20 diluted IOE at either RT or 37°C for 30 min. The 30 min incubation with 1:20 diluted IOE reduced the substrate MPM-2 reactivity by 30–40% at RT and by 40–50% at 37°C (data not shown).

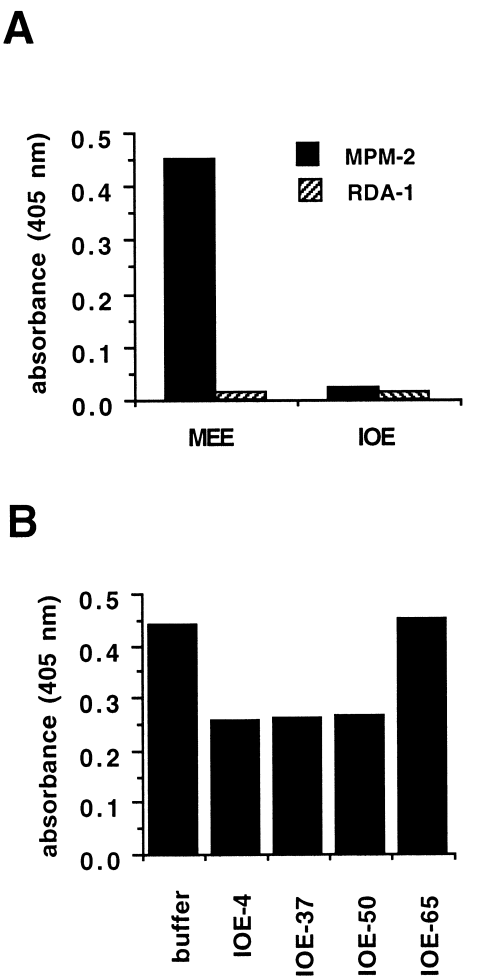

Fig. 1. Measurement of MPM-2 reactivity by ELISA. A: Individual wells of a microtiter plate were coated with either MEE or IOE. The immobilized proteins were then measured for their reactivity to either MPM-2 or control antibody RDA-1 by ELISA, which was indicated by absorbance at 405 nm. B: MEE immobilized on a microtiter plate was incubated for 30 min at 37°C with either control buffer or 1:20 diluted IOE that had been preincubated at 4°C, 37°C, 50°C, or 65°C for 30 min. The MEE on the plate was then measured for MPM-2 reactivity by ELISA.

Substrates were then incubated at 37°C for 30 min with 1:20 diluted IOE that had been preincubated at 4°C, 37°C, 50°C or 65°C for 30 min. Data presented in Fig. 1B show that while the incubation with IOE that had been preincubated at ≤50°C significantly reduced the substrate MPM-2 reactivity as compared to the buffer control, the incubation with IOE that had been preincubated at 65°C did not decrease the substrate MPM-2 reactivity. To determine the kinetics of the IOE-induced reduction in the MPM-2 reactivity, the substrates were incubated with 1:20 diluted IOE at 37°C for 0–4.5 h. The data in Fig. 2A show that the rate of the reduction in the substrate MPM-2 reactivity was almost linear for the first 30 min, and progressively decreased thereafter. To determine the concentration dependency of the reaction, the substrates were incubated with serially diluted IOE (1:2.5–1:1280) at 37°C for 30 min. The reduction in MPM-2 reactivity was plotted as a function of concentration of IOE (Fig. 2B). For convenience, the concentration of undiluted IOE which contained 10 mg/ml of protein was defined as 100%.

Only at IOE concentrations of ≤2.5% of the original concentration (or ≥1:40 dilution) was the concentration of IOE proportional to the reduction in the substrate MPM-2 reactivity. Taken together, these results demonstrated that this assay detected an enzymatic activity in IOE that decreased the MPM-2 reactivity of the substrates.

Four experiments were performed to confirm that the enzyme in IOE that decreased the MPM-2 reactivity in the substrates was a phosphatase. We first examined whether the ability of IOE to reduce the substrate MPM-2 reactivity was sensitive to phosphatase inhibitors. The substrates were incubated with 1:20 diluted IOE in the presence of either 2 μM okadaic acid, which inhibits both PP1 and PP2A activities [14], or 20 mM NaF, which inhibits a broad spectrum of serine/threonine protein phosphatases. Both phosphatase inhibitors dramatically blocked the IOE-induced reduction in the MPM-2 reactivity (Fig. 3A). Second, we examined

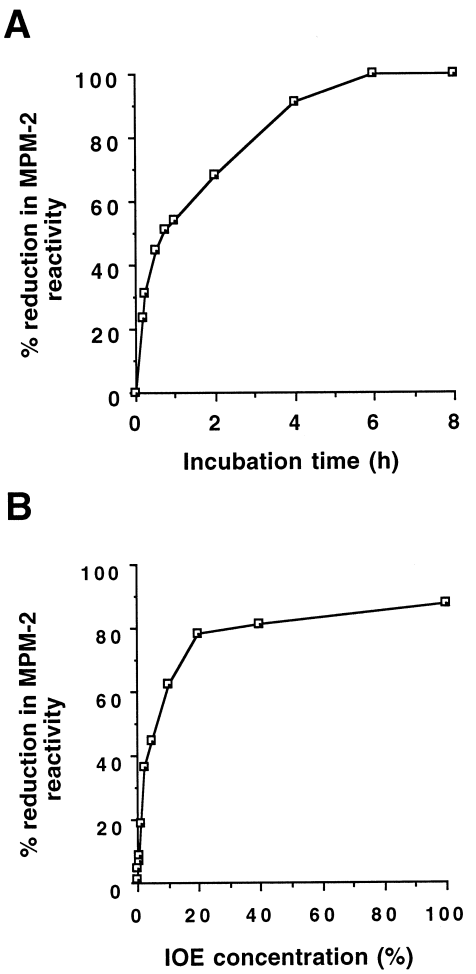

Fig. 2. Time and concentration dependencies of the IOE-induced reduction in the MPM-2 reactivity. A: MEE immobilized on a microtiter plate was incubated with 1:20 diluted IOE at 37°C for 0–8 h. The MPM-2 reactivity of the MEE at each time point was measured by ELISA. The IOE-induced % reduction in the MPM-2 reactivity was calculated as described in Section 2 and plotted as a function of the incubation time (h). B: MEE immobilized on a microtiter plate was incubated at 37°C for 30 min with IOE that had been serially diluted 1–1280-fold. After the MPM-2 reactivity of the MEE was measured by ELISA, the IOE-induced % reduction in the MPM-2 reactivity was plotted as a function of the IOE concentration.

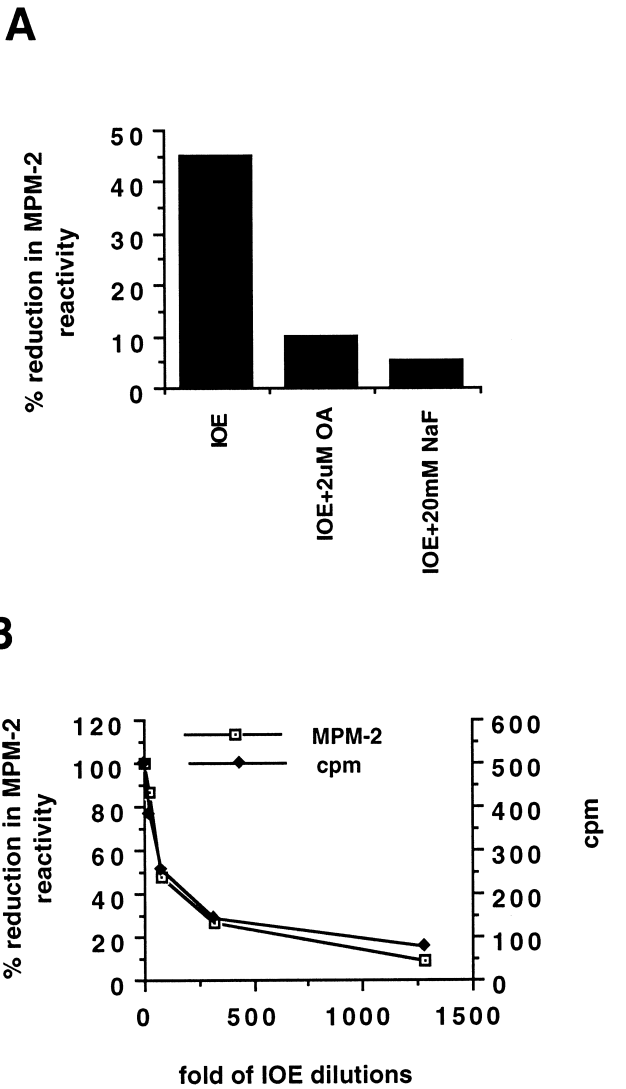

Fig. 3. The reduction of the MPM-2 reactivity was due to dephosphorylation. A: MEE immobilized on a microtiter plate was incubated at RT for 30 min with 1:20 diluted IOE alone, 1:20 diluted IOE containing 2 μM OA, or 1:20 diluted IOE containing 20 mM NaF. After the MPM-2 reactivity of the MEE was measured by ELISA, the % reduction in the MPM-2 reactivity due to incubation with each sample was calculated. B: ^{32}P-labeled MPM-2 antigens on a microtiter plate were incubated with IOE of indicated dilutions at RT for 1 h. After the IOE sample and the first wash from each well were recovered and combined, the relative amount of ^{32}P in each sample was measured in a scintillation counter. The proteins remaining on the plate were measured for MPM-2 reactivity by ELISA and the IOE-induced % reduction in the MPM-2 reactivity was calculated.

whether the reduction in the substrate MPM-2 reactivity was accompanied by release of free phosphate. For this, ^{32}P-labeled MPM-2 antigens were prepared (see Section 2), coated onto microtiter plates and incubated with either control buffer or IOE of different dilutions at RT for 1 h. This allowed measurement of loss of both the MPM-2 reactivity and phosphate from the substrates. Data in Fig. 3B showed that ^{32}P was released from the substrates during incubation with IOE which correlated with the reduction in the substrate MPM-2 reactivity. Third, we examined whether there was significant proteolysis during the incubation of the substrates

with IOE. For this, MEE proteins were immobilized on nylon membrane and then incubated with IOE. The choice of nylon membrane disks instead of the 96-well microtiter plates to immobilize the substrates was simply due to the membrane's much higher protein binding capacity which was required for this experiment. The immobilized proteins were then eluted, separated by SDS-PAGE, and silver stained. As shown in Fig. 4A, similar profiles of polypeptides were recovered irrespective of whether the substrates had been incubated with control buffer or IOE. This indicated that there was no significant proteolysis during the incubation. Finally, we addressed the concern that the reduction in the substrate MPM-2 reactivity observed by ELISA might be caused by masking of the MPM-2 epitope by a binding protein in IOE such as Pin 1 [3]. For this, the substrates eluted from the membrane after the incubation were separated by SDS-PAGE and immunoblotted with MPM-2, a procedure that should break any non-covalent protein-protein interactions. As shown in Fig. 4B, the IOE-treated substrates exhibited a significant decrease in the entire spectrum of the MPM-2 antigens on the immunoblot. Collectively, these results indicated that this ELISA-based assay measured a phosphatase activity in IOE that dephosphorylated the majority of the MPM-2 antigens.

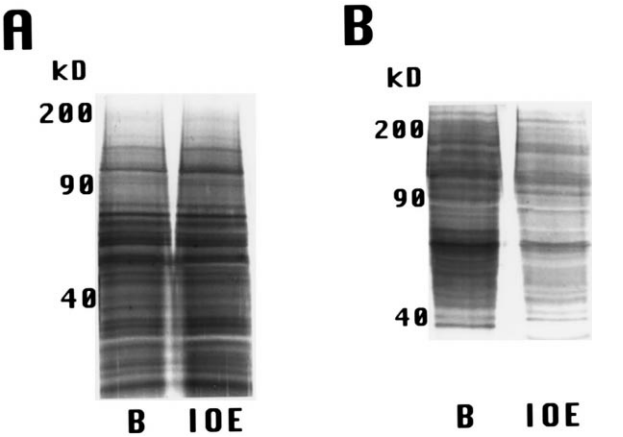

Fig. 4. Analysis of eluted proteins. Five nylon membrane disks (0.5 mm in diameter) were soaked in 1:80-diluted MEE at 4°C for 2 h and then rinsed four times with TBST. The coated disks were then incubated with buffer (lane B) or 1:20 diluted IOE (lane IOE) at 37°C for 30 min. After the discs were washed again four times with TBST, the proteins were eluted by incubation with 3% SDS at 22°C for 2 h and then at 95°C for 5 min. A: The eluted proteins were separated by 12.5% SDS-PAGE and silver stained. B: The eluted proteins were separated by 12.5% SDS-PAGE and immunoblotted with MPM-2.

3.2. Separation of the major MPM-2 epitope phosphatase activity from the majority of PP1 and PP2A activity

To examine whether the MPM-2 epitope phosphatase activity in IOE observed by the ELISA-based assay was due to a specific phosphatase, we fractionated IOE by sequential chromatography and followed both the MPM-2 epitope phosphatase activity and the phosphatase activity that dephosphorylates phosphorylase a which is a common substrate for PP1 and PP2A, the major serine/threonine protein phosphatases in most eukaryotic cells [11]. In step 1, IOE was precipitated with 45% ammonium sulfate. The precipitated proteins contained ≥90% of the MPM-2 epitope dephosphorylating activity and ≥80% of the phosphorylase a phosphatase activity (data not shown). In step 2, the 45% ammonium sulfate precipitate was loaded onto a Q-Sepharose column, which was sequentially eluted with 0.2 M NaCl and 1.0 M NaCl. The majority of the MPM-2 epitope and phosphorylase a phosphatase activities were recovered in the 0.2 M NaCl eluate (data not shown). In step 3, the 0.2 M eluate from the Q-Sepharose chromatography was loaded onto a heparin-Sepharose column which was sequentially eluted with 0.09 M NaCl and 1.0 M NaCl. The flowthrough (FT) contained high phosphorylase a phosphatase activity and low MPM-2 epitope phosphatase activity, whereas the 0.09 M eluate (E1) contained low phosphorylase a phosphatase activity and high MPM-2 epitope phosphatase activity. In contrast, the 1.0 M eluate (E2) contained low levels of both activities (Fig. 5A). In step 4, E1 from the heparin-Sepharose chromatography was fractionated by FPLC on a Superose 6 gel filtration column. The MPM-2 epitope phosphatase activity was eluted as a distinct peak at an apparent molecular mass of ~158 kDa, which correlated with the low-level phosphorylase a phosphatase activity (data not shown). The recovered MPM-2 epitope phosphatase activity was able to dephosphorylate the MPM-2 epitope on the majority of the MPM-2 antigens like crude IOE (data not shown). In contrast, little MPM-2 epitope phosphatase activity was recovered from fractionation of FT through the Superose 6 column although high levels of phosphorylase a phosphatase activity were detected (data not shown).

Since the results described above suggested that the major MPM-2 epitope phosphatase activity was separated from the majority of PP1 and PP2A activities, IOE and the three fractions from the heparin-Sepharose chromatography were immunoblotted with commercial anti-human PP1 and PP2A antibodies which work across species. Immunoblots with anti-PP1 antibodies showed that IOE contained a readily detectable polypeptide of the expected size whereas none of the three fractions contained significant levels of this polypeptide (Fig. 5B). This indicated that most if not all of PP1 protein had been removed prior to this step and thus could not account for the detected MPM-2 epitope and phosphorylase a phosphatase activities. Immunoblots with anti-PP2A antibodies showed that both IOE and FT contained a strong polypeptide of the expected size, whereas E1 and E2 contained much less and little of this polypeptide respectively (Fig. 5B), indicating that the recovered phosphorylase a phospha-

Table 1
Purification of MPM-2 epitope phsophatase in *Xenopus* oocytes

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Oocyte extract	48.15	820	17.03	100	1
Ammonium sulfate	20.05	735	36.66	89.6	2.15
Q-Sepharose	11.3	697.5	61.88	85.1	3.62
Heparin-Sepharose	1.78	612.5	344.1	74.7	20.2
Superose 6	0.113	110.4	977.0	13.5	57.4

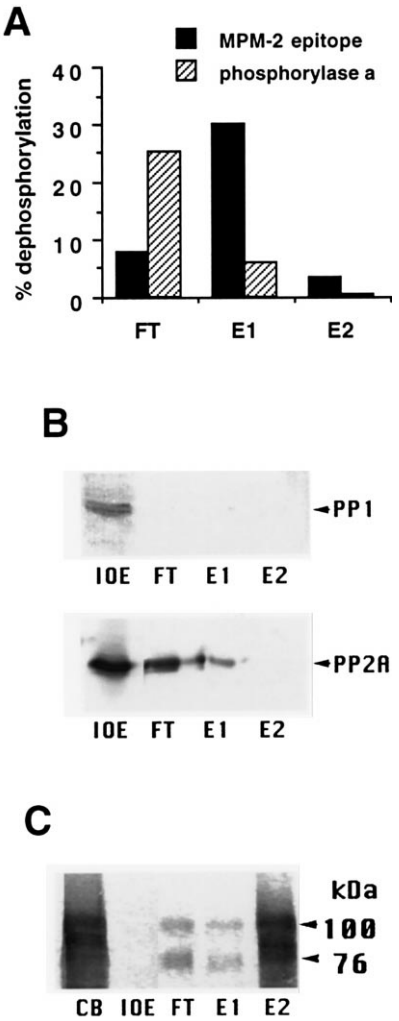

Fig. 5. Fractionation of *Xenopus* oocyte extracts. IOE was sequentially fractionated by 45% ammonium sulfate precipitation. Q-Sepharose chromatography and heparin-Sepharose chromatography as described in Section 2. The flowthrough (FT), the 0.09 M NaCl eluate (E1), and the 1.0 M NaCl eluate (E2) from the heparin-Sepharose chromatography were adjusted to same volume as the starting IOE. A: The three fractions were diluted 1:20 and measured for the MPM-2 epitope phosphatase activity by incubation for 30 min at 37°C. The samples were also measured for phosphorylase a phosphatase activity as described in Section 2. B: Aliquots of 20 μl from the starting IOE and each of the three fractions were separated by 12.5% SDS-PAGE and immunoblotted with anti-PP1 or anti-PP2A antibody. C: Phosphorylated GST-cdc25 (2 μg/10 μl) was incubated with 30 μl of either control buffer (CB) or 1:20 diluted IOE, FT, E1 and E2 at RT for 1 h. Proteins were separated by 12.5% SDS-PAGE and immunoblotted with MPM-2. The arrows indicated the positions of the unphosphorylated 100 kDa and 76 kDa GST-cdc25.

tase activity was mostly due to PP2A. Although further studies are required to determine the relationship between the major MPM-2 epitope phosphatase activity and the minor PP2A activity in E1, this result indicated that the major MPM-2 epitope phosphatase activity was separated at least from the majority of PP2A activity.

Coomassie blue staining of the SDS-PAGE separated proteins from the last step of the fractionation showed that further chromatography was required in order to identify the putative polypeptides that comprise this phosphatase (data not shown). Table 1 summarizes recoveries from each step of the fractionation in a typical purification. For quantitation, one unit of the MPM-2 epitope dephosphorylating activity was arbitrarily defined as the amount of enzyme that caused a 20% reduction in the MPM-2 reactivity. After the four-step fractionation procedure, approximately 13% of the starting MPM-2 epitope phosphatase activity was recovered and a 57-fold enrichment achieved. Recovery of the MPM-2 epitope phosphatase activity was nearly quantitative until the last step, FPLC on Superose 6. In this step, the overall recovery of protein was low as well, suggesting that the loss of MPM-2 epitope phosphatase activity was non-specific.

Overall, these results demonstrate that there is a specific phosphatase activity in IOE that preferentially dephosphorylates the MPM-2 epitope on multiple MPM-2 antigens. This phosphatase activity is separated from the majority of PP1 and PP2A activities.

3.3. The major MPM-2 epitope phosphatase activity copurified with one of the two major cdc25 dephosphorylating activities

Since a phosphatase assay which uses a defined substrate would be less complicated and more efficient than the ELISA-based assay, we next explored the possibility that the major MPM-2 epitope phosphatase activity in IOE could be assayed by using a single purified MPM-2 antigen as the substrate. For this, GST-cdc25, a fusion protein between GST and cdc25, a known MPM-2 antigen [7], was tested. Non-treated GST-cdc25 migrated on SDS-PAGE as two major bands of 100 kDa and 76 kDa which represented the full-length and a cleaved version of GST-cdc25. Both bands migrated more slowly and became MPM-2 reactive upon phosphorylation with MEE while GST alone did not acquired any MPM-2 reactivity (data not shown). The phosphorylated and repurified GST-cdc25 was incubated with various chromatographic fractions from the four-step purification scheme and then subjected to SDS-PAGE and MPM-2 immunoblotting. This method measured the specific dephosphorylation of the MPM-2 epitope on cdc25. Samples were also separated by SDS-PAGE and stained with Coomassie blue to check for any degradation of the GST-cdc25. No degradation of GST-cdc25 was observed (data not shown).

In the initial 45% ammonium sulfate precipitation, the cdc25 dephosphorylating activity was recovered mostly in the precipitated proteins like the recovered MPM-2 epitope phosphatase activity (data not shown). In step 2, the Q-Sepharose chromatography of the 45% ammonium sulfate precipitate, the cdc25 dephosphorylating activity was mostly detected in the 0.2 M NaCl eluate, again like the recovered MPM-2 epitope phosphatase activity (data not shown). However, in step 3, the heparin-Sepharose chromatography of the 0.2 M eluate from the Q-Sepharose chromatography, a high cdc25 dephosphorylating activity was recovered in both FT and E1 (Fig. 5C), whereas a high MPM-2 epitope phosphatase activity was detected mostly in E1 (Fig. 5A). In step 4, the Superose 6 chromatography of E1, there was a good correlation between the MPM-2 epitope phosphatase activity and the dephosphorylation of GST-cdc25. The fractions 31–33 which contained the highest MPM-2 epitope phosphatase activity removed most of the MPM-2 reactivity from GST-cdc25 (Fig. 6A,B). These results suggest that there are two major phosphatase activities in IOE that dephosphorylate the MPM-

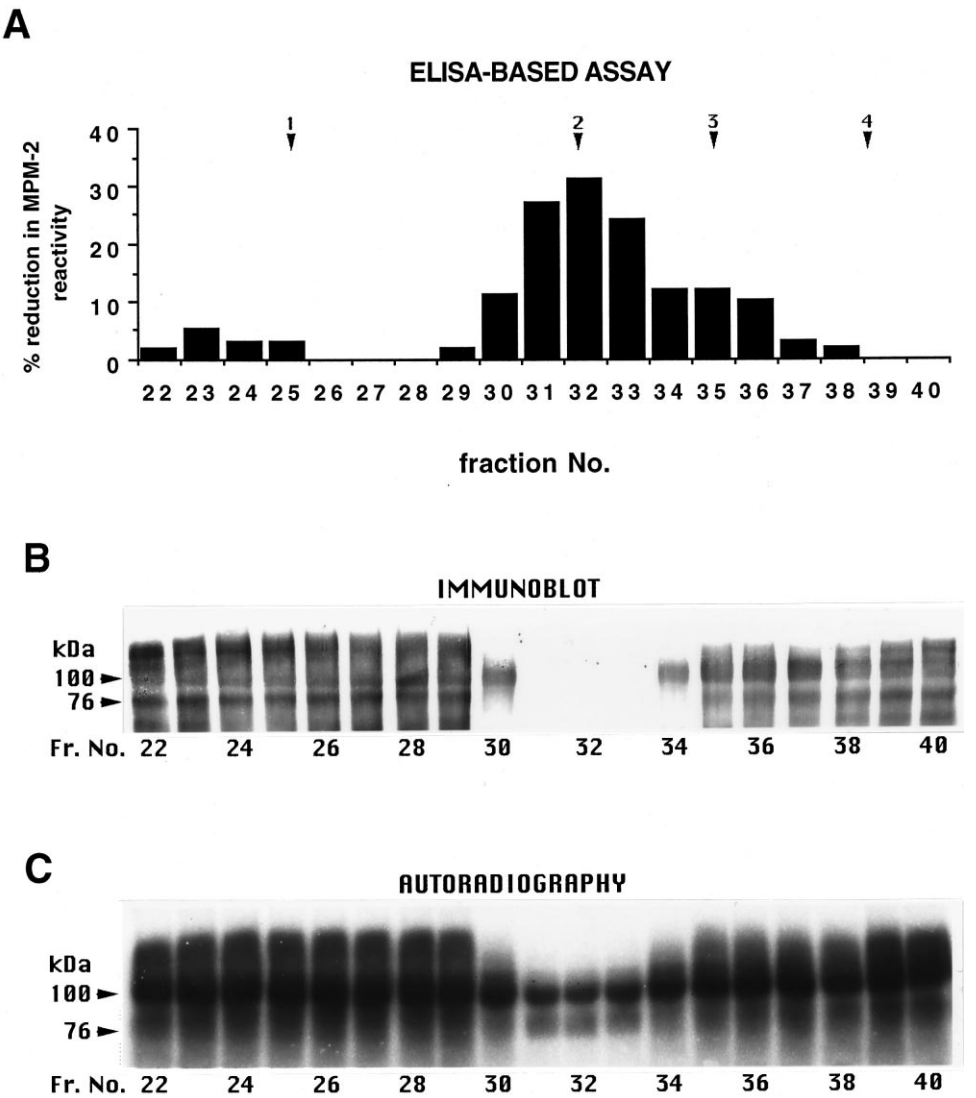

Fig. 6. Cdc25 dephosphorylating activity during Superose 6 chromatography. A: Fractions 22–40 from the Superose 6 gel filtration of the E1 from the heparin-Sepharose chromatography were 1:5 diluted and measured for MPM-2 epitope phosphatase activity by the ELISA-based assay for 30 min at RT. The numbered 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). B: Phosphorylated GST-cdc25 (2 μg/10 μl) was incubated with 30 μl aliquots from the same fractions at RT for 1 h. The samples were separated by SDS-PAGE and immunoblotted with MPM-2. C: ^{32}P-labeled GST-cdc25 (2 μg/10 μl) were incubated with 30 μl aliquots from the same fractions at RT for 30 min. The samples were separated by SDS-PAGE and subjected to autoradiography.

2 epitope on GST-cdc25, and that only one of them copurified with the major MPM-2 epitope phosphatase activity.

cdc25 is known to be phosphorylated on more than seven sites [15,16], however, the percentage of them that are MPM-2 epitopes has not been determined. To examine whether the major MPM-2 epitope phosphatase activity dephosphorylated cdc25 on most of the phosphorylation sites, the fractions from Superose 6 were incubated with ^{32}P-labeled GST-cdc25 and the total dephosphorylation of GST-cdc25 was measured by SDS-PAGE and autoradiography. The fractions 31–33 that contained the highest MPM-2 epitope phosphatase activity removed most of the ^{32}P from the labeled GST-cdc25 (Fig. 6C), indicating that most of the phosphorylation sites on GST-cdc25 could be efficiently dephosphorylated by the major MPM-2 epitope phosphatase activity.

Since FT from the heparin-Sepharose chromatography contained high levels of both PP2A protein and phosphorylase a phosphatase activity, it seemed likely that the cdc25 dephosphorylating activity in it was due to the major PP2A activity. To test this, FT was fractionated by FPLC on Superose 6 and the fractions were both assayed for cdc25 and phosphorylase a dephosphorylating activities and immunoblotted with anti-PP2A antibody. The cdc25 dephosphorylating activity copurified with both phosphorylase a phosphatase activity and PP2A protein (data not shown), supporting the possibility that the one of the cdc25 dephosphorylating activities was due to the major species of PP2A.

3.4. The major MPM-2 epitope dephosphorylating activity did not dephosphorylate the MPM-2 epitope in the GST-MPM2 fusion protein

Since the results described above indicated that cdc25 is not

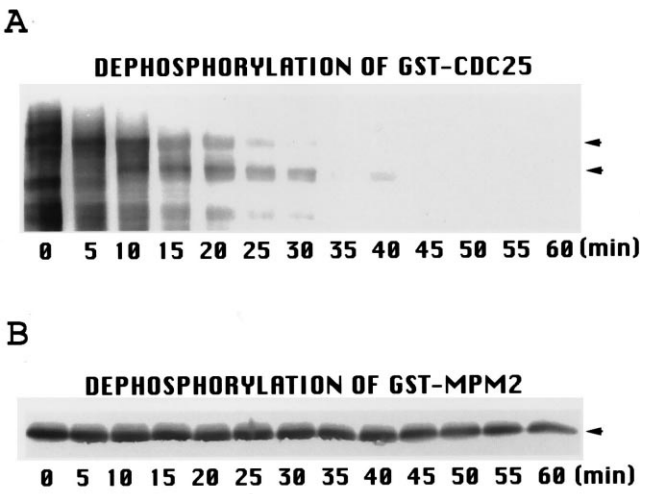

Fig. 7. Comparison of dephosphorylations of GST-cdc25 and GST-MPM2 by IOE. Phosphorylated GST-cdc25 (A) or GST-MPM2 (B) was incubated with 1:20 diluted IOE at RT for the indicated lengths of time. The proteins were separated by 12.5% SDS-PAGE and immunoblotted with MPM-2. The arrows in A indicate the positions of 100 kDa and 76 kDa unphosphorylated GST-cdc25. The arrow in B indicates the position of GST-MPM2.

a specific substrate for the major MPM-2 epitope phosphatase activity, we explored the possibility that the MPM-2 epitope consensus sequence could be used as the specific substrate to assay the major MPM-2 epitope phosphatase activity. For this, GST-MPM2, a fusion protein between GST and a 19-residue peptide that contained two representative MPM-2 epitope sequences [12], was used as the substrate for the phosphatase assay. It was reported that GST-MPM2 became MPM-2 reactive upon phosphorylation with MEE and all the phosphorylation was on the MPM-2 epitope. The phosphorylated GST-MPM2 could be both immunoprecipitated with MPM-2 and dephosphorylated by potato acid phosphatase (data not shown), indicating that the phosphorylation site was open for dephosphorylation.

GST-MPM2 was first incubated with 1:20 diluted IOE for 0–60 min. GST-cdc25 was incubated in parallel as a positive control. Proteins were then separated by SDS-PAGE and immunoblotted with MPM-2. As shown in Fig. 7, the MPM-2 epitope on GST-cdc25 was quantitatively dephosphorylated within 30 min whereas a significant portion of GST-MPM2 remained phosphorylated even after the 60 min incubation. Similar results were obtained when 1:1 diluted IOE or partially purified MPM-2 epitope phosphatase activity was used for dephosphorylation (data not shown). These results indicated that the MPM-2 epitope on GST-MPM2 could not be efficiently dephosphorylated by the major MPM-2 epitope phosphatase activity in IOE.

4. Discussion

Protein dephosphorylation is required for cells to exit from mitosis and remain in interphase during eukaryotic cell cycles [17,18]. MPM-2 antigens comprise one of the major families of mitotic phosphoproteins that are simultaneously dephosphorylated from the onset of anaphase and remain dephosphorylated throughout interphase [1]. In this first attempt to characterize the phosphatase that is responsible for the dephosphorylation of MPM-2 antigens (MPM-2 epitope phosphatase), we tried to develop a specific assay to measure its activity. To this end, three approaches were used. The first was an ELISA-based assay, which measured the phosphatase-induced reduction in the MPM-2 reactivity of crude M phase lysates. Using this method to follow the phosphatase activity during sequential chromatography of *Xenopus* oocyte extracts, one predominant phosphatase activity was detected which was separated from the majority of PP1 and PP2A activities. This phosphatase activity dephosphorylated the MPM-2 epitope on the majority of the MPM-2 antigens. The second method measured dephosphorylation of GST-cdc25, a single purified MPM-2 antigen. Using this assay, two major phosphatase activities were detected from the sequential chromatography, one that copurified with the major MPM-2 epitope phosphatase activity, and the other that copurified with the major PP2A activity. The third method measured dephosphorylation of the MPM-2 epitope consensus sequences in GST-MPM2, a fusion protein between GST and a 19-residue peptide that contained two representative MPM-2 epitope sequences. The MPM-2 epitopes in the fusion protein could not be efficiently dephosphorylated by the major MPM-2 epitope phosphatase activity in *Xenopus* oocyte extracts.

Since the ELISA-based assay efficiently detects the major phosphatase activity that dephosphorylates the MPM-2 epitope on multiple MPM-2 antigens but not the majority of PP1 and PP2A activities under the same conditions, this assay appears to be both efficient and selective for the MPM-2 epitope phosphatase activity. Most protein serine/threonine protein phosphatases have broad substrate specificities when assayed in vitro. Three unique features might account for the preferential detection of the MPM-2 epitope phosphatase activity by the ELISA-based assay. First, the M phase cell lysates contain not only high levels of the entire spectrum of phosphorylated MPM-2 antigens which are the substrates for the MPM-2 epitope phosphatase, but also substrates for other protein phosphatases. Thus, the non-specific dephosphorylation of the MPM-2 epitope by other protein phosphatases may be decreased. Second, since the substrates contain multiple MPM-2 antigens, even if a phosphatase is able to dephosphorylate certain MPM-2 antigens non-specifically, its activity should be much less than that of the phosphatase that dephosphorylates many or all MPM-2 antigens. Third, dephosphorylation of sites other than MPM-2 epitopes are not measured by the ELISA-based assay. Because of the specificity of the assay, there is a good possibility that the major phosphatase activity in *Xenopus* oocyte extracts detected by this assay represents a physiological MPM-2 epitope phosphatase. To confirm this possibility, the ability of this phosphatase activity to dephosphorylate multiple MPM-2 antigens will be examined in vivo in future studies.

The cdc25 dephosphorylation method efficiently detected both the major MPM-2 epitope phosphatase activity and the major PP2A activity. Dephosphorylation of cdc25 by PP2A was also suggested by others previously [19]. Although it is possible that both the major MPM-2 epitope phosphatase activity and the major PP2A activity are important for dephosphorylation of the MPM-2 epitope on cdc25 in vivo, it is unlikely that the major PP2A activity which did not dephosphorylate the MPM-2 epitope on multiple MPM-2 antigens efficiently in vitro is important for the dephosphorylation of many MPM-2 antigens in vivo. Thus, the use of the cdc25

dephosphorylation method would generate misleading information if used alone to assay the MPM-2 epitope phosphatase activity in crude samples, although it would be an excellent method for analysis of purified MPM-2 epitope phosphatase activity.

The finding that the MPM-2 epitope in GST-MPM2 could not be dephosphorylated by the major MPM-2 epitope phosphatase activity in IOE efficiently suggests that the MPM-2 epitope phosphatase requires more structural information than the deduced MPM-2 epitope sequence for the recognition and dephosphorylation by the MPM-2 epitope phosphatase. It was recently found that the MPM-2 epitope in GST-MPM2 was not sufficient for the recognition and the phosphorylation by the major kinase activity in MEE that phosphorylates the MPM-2 epitope on multiple MPM-2 antigens [12]. On the other hand, the MPM-2 epitope sequences were sufficient for the recognition by the prolyl isomerase Pin 1, a mitotic regulator, in a phosphorylation dependent manner [3]. This suggests that MPM-2 epitope may have two functions. First, it acts in concert with an additional domain(s) to direct the MPM-2 kinase and phosphatase. Second, once phosphorylated, it is an acceptor site for the regulatory protein Pin 1.

Genetic studies in *Aspergillus* showed that mutation of PP1 catalytic subunit inhibited both the completion of anaphase and dephosphorylation of the MPM-2 antigens [4]. This raises the possibility that PP1 is the phosphatase that dephosphorylates the MPM-2 antigens. However, our results show that the major MPM-2 epitope phosphatase activity in interphase-arrested *Xenopus* oocyte extracts is separated from most or all of PP1 activity. This suggests that either a specific and transient PP1 activity dephosphorylates the MPM-2 antigens at the exit from mitosis or PP1 is not directly involved in the dephosphorylation of MPM-2 antigens.

Previous studies showed that ABC, the predominant species of PP2A, was the major phosphatase activity that dephosphorylated mitotic cdc2 kinase substrates [20–22]. Since the major MPM-2 epitope dephosphorylating activity was separated from the major PP2A activity, it appears that the major MPM-2 epitope phosphatase activity detected by the ELISA-based method is different from the phosphatase activity that dephosphorylates the cdc2 kinase substrates. Supporting this possibility, while the MPM-2 epitope phosphatase activity was not suppressed in undiluted *Xenopus* oocyte extracts (Fig. 2B), the overall PP2A activity is strongly suppressed in concentrated cell extracts [23]. The apparent difference between the two phosphatase activities suggest that MPM-2 antigens and cdc2 kinase substrates are dephosphorylated either by different phosphatases or by different subtypes of PP2A.

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