

## Zinc ion acts as a cofactor for serine/threonine kinase MST3 and has a distinct role in autophosphorylation of MST3

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### Abstract

We examined the metal ion cofactor preference for MST3 (mammalian Ste20-like kinase 3) of the Ste20 serine/threonine kinase family. Four metal ions ( $\text{Mg}^{+2}$ ,  $\text{Mn}^{+2}$ ,  $\text{Zn}^{2+}$ , and  $\text{Co}^{2+}$ ) activate endogenous, exogenous, and baculovirus-expressed recombinant MST3 within the physiological concentration range. In contrast,  $\text{Fe}^{+2}$  and  $\text{Ca}^{+2}$  do not function as MST3 cofactors.  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Mg}^{2+}$ -dependent autophosphorylation of MST3 is mainly on threonine residue while  $\text{Zn}^{2+}$ -stimulated MST3 autophosphorylation is on both serine and threonine residues. The distinct autophosphorylation pattern on MST3 suggests that MST3 may exert various types of kinase reactions depending on the type of metal ion cofactor used. To our knowledge, this is the first report showing  $\text{Zn}^{2+}$  as the metal ion cofactor of a recombinant serine/threonine kinase.

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### 1. Introduction

MST3 (mammalian Ste20-like kinase 3) was originally identified by Schinkmann and Blenis [1] and belongs to the serine threonine Ste20 kinase family [2–4]. MST3 has an N-terminal kinase domain and a C-terminal regulatory region lacking the Cdc42/Rac1 interac-

tive motif [1,5]. Overexpression of either wild-type MST3 or dominant negative MST3 does not alter any known MAPK pathway [1]. We have previously reported that nuclear translocation and activation of MST3 occurred during apoptosis [6]. One unique feature for MST3 is the preference for  $\text{Mn}^{2+}$  as the cofactor in kinase reaction, which is unusual for a serine/threonine kinase [1].

The study of metal ion cofactor preference provides information on the electronic, substrate structural, and catalytic requirements for the enzymatic process [7]. In

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general, tyrosine kinases favor MnATP as the specific cofactor-substrate, while serine/threonine (Ser/Thr) kinases are MgATP dependent [8–10]. Receptor tyrosine kinases are additionally regulated by  $Zn^{2+}$  [11].  $Zn^{2+}$ -dependent tyrosine phosphorylation has been demonstrated in human platelet membrane, sheep platelet, and monkey brain [12–14].  $Zn^{2+}$  stimulates tyrosine phosphorylation on epidermal growth factor receptor and initiates insulin-like effects on insulin receptor [15,16]. Insulin receptor autophosphorylation in the presence of  $Mn^{2+}$  was mainly on tyrosine residues, whereas approximately equal serine and tyrosine residues were autophosphorylated in the presence of  $Zn^{2+}$  [17]. In addition to regulation of receptor functions, zinc ions bind to nerve growth factor and induce conformational changes within domains that participate in the recognition of TrkA and p75<sup>NTR</sup> [18]. The presence of zinc ions can alter cellular pathways mediated by nerve growth factor and other neurotrophins [18,19].

Since zinc ions play a key role in a lot of biological responses, we wish to study whether  $Zn^{2+}$  can act as a cofactor for MST3. In the present work, we compare the effect of common divalent metal ions  $Zn^{2+}$ ,  $Mn^{2+}$ ,  $Co^{2+}$ ,  $Mg^{2+}$ ,  $Ni^{2+}$ , and  $Cu^{2+}$  on the native endogenous, HA-tagged exogenous, and recombinant MST3. Our data shows that  $Zn^{2+}$  as well as  $Mn^{2+}$ ,  $Co^{2+}$ , and  $Mg^{2+}$ , can serve as the metal ion for the MST3 catalytic activity. The autophosphorylation pattern with  $Zn^{2+}$  is distinct from that with  $Mn^{2+}$ ,  $Co^{2+}$ , or  $Mg^{2+}$ . In addition,  $Zn^{2+}$  can serve as a cofactor at lower concentration and tends to inhibit MST3 activity at concentrations higher than 640  $\mu$ M. There may be a different role for MST3 under the regulation of  $Zn^{2+}$ . Choice of metal ion cofactors should be considered when investigating MST3 substrates and unique MST3 function in vitro or in vivo.

## 2. Experimental

### 2.1. DNA constructs

The DNA fragment encoding full length MST3 was generated by polymerase chain reaction and cloned into the Not I and Hind III sites of pMH vector (Roche). pMH vector is a mammalian expression vector containing a cytomegalovirus promoter and a C-terminal influenza hemagglutinin (HA) epitope tag.

### 2.2. Cell culture and transfection

TCCSUP bladder cancer cells and COS1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The day before transfection,  $4 \times 10^5$  COS1 cells were plated on 60 mm dishes. Transfections were performed using Lipofect-

AMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Cell extracts were harvested 48 h later for immunoprecipitation assays, kinase assays, and western analysis.

### 2.3. Western blot analysis

To detect endogenous MST3, cell extracts were resolved by 8–12% SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with rabbit anti-MST3 antiserum against the bacterially expressed His-tagged MST3 protein containing residues 310–432 as described previously [6]. To analyze the expression of exogenous HA-MST3, 4  $\mu$ g full-length pMH-HA-MST3 plasmid was transfected into COS1 cell on a 60-mm dish. Forty-eight hours after transfection, cells were washed three times with cold PBS and lysed with 300  $\mu$ l 2 $\times$  SDS lysis buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 200 mM dithiothreitol, 0.2% bromophenol blue and 20% glycerol. Whole cell extracts were resolved by SDS/PAGE, transferred to a nitrocellulose membrane, and blotted with anti-HA (1:3000) (Roche) antibody. The membranes were developed with the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

### 2.4. Construction and purification of baculovirus-expressed MST3

The cDNA for MST3 was PCR (polymerase chain reaction) amplified and cloned into BamHI site of pBacPAK-His1 (Clontech). MST3 cDNA was introduced into the baculovirus genome using the pBacPAK baculovirus expression system (PT1260-1, Clontech). Positive colonies from plaque assays were used in the production of virus stocks for the infection of Sf9 cell suspension cultures. Cells were infected at multiplicity of infection (MOI) 10 and grown for 72 h in an orbital shaker at 27 °C. Cultures were sedimented and lysed by French press in binding buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM imidazole) and protease inhibitors (Complete; Roche Molecular Biochemicals). His-MST3 was purified using Ni-NTA agarose (Qiagen), dialyzed in buffer containing 20 mM Tris-HCl, pH 8.0 and 20 mM NaCl, and stored at  $-70$  °C until used.

### 2.5. Immunoprecipitation and in vitro kinase assay

Cells on 60 mm dish were washed three times with cold PBS and then lysed using 300  $\mu$ l lysis buffer containing 150 mM NaCl, 50 mM  $NaH_2PO_4 \cdot 2H_2O$ , 6 mM deoxycholate, 1% IGEPAL CA-630, 1 mM sodium orthovanadate, 2 mM EGTA, 0.5% aprotinin (sigma A-6279), and 1 mM PMSF. After centrifugation at 8000 rpm for 15 min, supernatant containing 30  $\mu$ g total protein was incubated for 2 h at 4 °C with anti-MST3

antiserum or 1  $\mu\text{g}$  anti-HA (Roche) when cells were transfected with the HA-MST3 expression plasmid. The immuno-complex was harvested with 35  $\mu\text{l}$  protein A sepharose (Amersham Pharmacia Biotech) for 1.5 h at 4  $^{\circ}\text{C}$ . Immunoprecipitates were washed with lysis buffer three times, washed with 1 $\times$  TBS (Tris-buffered saline) three times, and suspended in 30  $\mu\text{l}$  1 $\times$  TBS buffer. The MST3-immunoprecipitates bound to protein A sepharose or recombinant MST3 was incubated with 30  $\mu\text{l}$  2 $\times$  kinase buffer containing 40 mM PIPES, 20  $\mu\text{Ci}$  [ $\gamma$ - $^{33}\text{P}$ ]-labeled ATP (2500Ci per mmol), 20  $\mu\text{M}$  unlabeled ATP, 4  $\mu\text{g}$  histone H3 or 60  $\mu\text{g}$  myelin basic protein, and various concentrations of each metal ion. Kinase reactions were performed at room temperature for 10–60 min and stopped by the addition of 6  $\mu\text{l}$  10 $\times$  SDS loading buffer containing 500 mM Tris-HCl (pH 6.8), 10% SDS, 50% sucrose, 0.2% bromophenol blue and 10% 2-mercaptoethanol. After boiling for 5 min, proteins in the kinase reactions were separated by SDS/PAGE. Gel was dried and substrate phosphorylation was visualized by autoradiography.

### 2.6. Evaluation of MST3 kinetic parameters

Recombinant MST3 enzyme activity was measured by phosphorylation of myelin basic protein (MBP). Thirty  $\mu\text{l}$  enzyme/substrate mixture containing increasing amounts of MBP and 0.5  $\mu\text{g}$  purified MST3 was added to 30  $\mu\text{l}$  2 $\times$  kinase buffer described as above. After different reaction time periods, the mixed samples were dropped onto circular Whatman P81 phosphocellulose papers. P81 paper was washed three times with 500 ml 0.5% phosphoric acid (for 3 min) and once with 200 ml acetone. The radioactivity of phosphorylated MBP on P81 paper was quantified by Cerenkov method in 3 ml scintillation cocktail. MBP phosphorylation was measured as incorporation of radioactivity from [ $\gamma$ - $^{33}\text{P}$ ]-labeled ATP into MBP substrate. Kinetic parameters were determined by varying the concentration of MBP from 1 to 24  $\mu\text{M}$  at a constant ATP concentration of 10  $\mu\text{M}$ . Metal ions of the following concentrations were used:  $\text{ZnCl}_2$  at 160  $\mu\text{M}$  and  $\text{MnCl}_2$ ,  $\text{CoCl}_2$ , and  $\text{MgCl}_2$  at 5 mM.  $K_m$  and  $V_{\text{max}}$  were determined from Lineweaver–Burk reciprocal plots of reaction rates and concentrations of substrates.

### 2.7. In vitro labeling and phosphoamino acid analysis

Baculovirus-expressed MST3 was used to perform in vitro kinase reactions without adding any substrate in the reaction. All [ $\gamma$ - $^{33}\text{P}$ ]-labeled proteins in the reaction tube were directly hydrolyzed in 6 N HCl at 110  $^{\circ}\text{C}$  for 1 h and then lyophilized by speed-vacuum for 2 h. The hydrolyzed products were reconstituted in 15  $\mu\text{l}$  pH 1.9 first dimension buffer containing 1 mg/ml of unlabeled phosphoserine, phosphothreonine and phos-

photyrosine standards, respectively (Sigma). An aliquot sample with 3000 c.p.m. radioactivity was loaded on to a cellulose TLC (Thin Layer Chromatography) plate (HPTLC-platten 10 $\times$ 10 cm cellulose; Merck). Phosphorylated amino acids were separated in pH 1.9 first dimension buffer containing 2.2% formic acid, 7.8% glacial acetic acid at 1.5 kV for 18 min and then in pH 3.5 second dimension buffer containing 5% glacial acetic acid, 0.5% pyridine at 1.3 kV for 10 min. Comigrating standards were localized by spraying 0.25% ninhydrin in acetone (v/v) and the [ $\gamma$ - $^{33}\text{P}$ ]-labeled phosphoamino acids were detected by exposure the cellulose TLC plate to X-ray film.

## 3. Results

### 3.1. Metal ions other than manganese can serve as the cofactor of endogenous cellular MST3

MST3 prefers  $\text{Mn}^{2+}$  to  $\text{Mg}^{2+}$  as a divalent ion cofactor which is unusual for a serine/threonine kinase [5]. Despite the unusual manganese cofactor preference of MST3, systematic analysis of ion requirements of MST3 has not been undertaken previously. Endogenous MST3 activity in TCCSUP bladder cancer cells was immunoprecipitated by anti-MST3 antiserum [6] and assayed under standard conditions with ATP as the phosphate donor in the presence of different divalent ions. MST3 activity was observed when 0.16–10 mM  $\text{Mn}^{2+}$ , 0.16–10 mM  $\text{Co}^{2+}$ , or 10–160  $\mu\text{M}$   $\text{Zn}^{2+}$  was present; in addition, low MST3 kinase activity was detected when  $\text{Mg}^{2+}$  (0.16–10 mM) was used as the cofactor (Fig. 1A). Two other metal ions,  $\text{Ni}^{2+}$  and  $\text{Cu}^{2+}$ , showed no MST3 cofactor activity between 10  $\mu\text{M}$  and 10 mM. The highest MST3 activity in TCCSUP cells was obtained with  $\text{Mn}^{2+}$  as the cofactor, which was consistent with the previous finding in HeLa cells [5]. The MST3 activity measured with each metal ion as the cofactor was approximately in the order of  $\text{Mn}^{2+} > \text{Zn}^{2+} \approx \text{Co}^{2+} > \text{Mg}^{2+}$ .

### 3.2. $\text{Zn}^{2+}$ or $\text{Co}^{2+}$ can serve as the cofactor of exogenous HA-MST3

To exclude the possibility that the metal ion preference was due to the non-specific associated proteins immunoprecipitated by anti-MST3 antiserum, the metal ion preference for exogenous HA-MST3 kinase activity was examined. COS1 cells were transiently transfected with HA-tagged MST3, and the cell lysates were immunoprecipitated by anti-HA antibody. The immunoprecipitates were subjected to kinase assays with different metal ions as cofactors. The metal ion preference for exogenous HA-MST3 was overall similar to that observed with endogenous MST3 (Fig. 1B), with minor

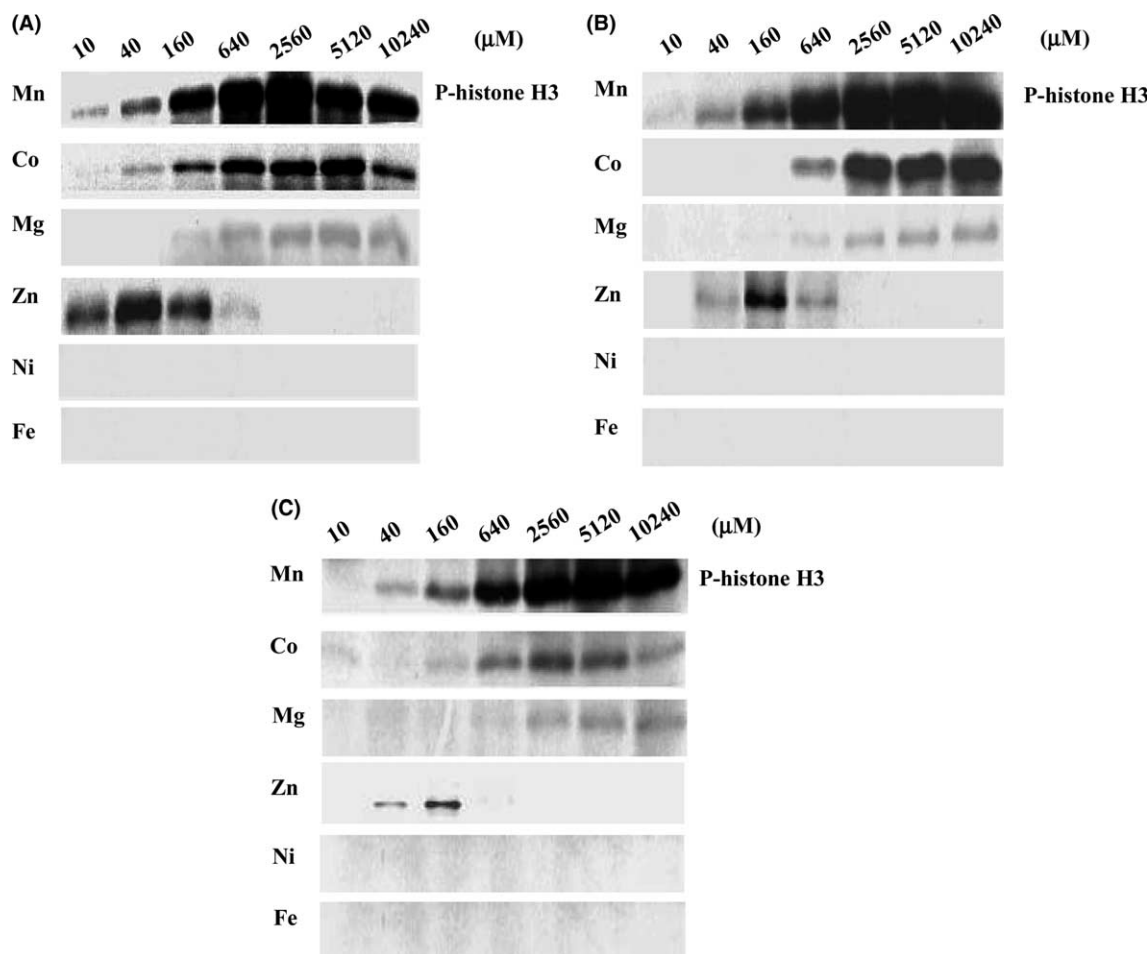


Fig. 1.  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Mg}^{2+}$  metal ions serve as cofactors of endogenous MST3, exogenous HA-MST3, and recombinant MST3. (A) Endogenous MST3 was immunoprecipitated from TCCSUP cells using anti-MST3 antiserum. (B) COS1 cells were transfected with full length HA-MST3, and exogenous HA-MST3 was immunoprecipitated with anti-HA antibody 48 hours after transfection. (C) Baculovirus-expressed MST3 was purified from  $\text{Ni}^{2+}$ -affinity column. The immunoprecipitated or recombinant MST3 was subjected to kinase assay with increasing concentrations of various metal ion cofactors. The MST3 activity was indicated by the phosphorylation level of substrate histone H3 as determined by autoradiography. The results were similar in two repetitions of each experiment.

differences in the optimal concentration for each divalent cation. Therefore, the unique preference for  $\text{Zn}^{2+}$  or  $\text{Co}^{2+}$  as a cofactor of MST3 is unlikely due to non-specific proteins precipitated by antibodies.

### 3.3. $\text{Zn}^{2+}$ or $\text{Co}^{2+}$ can serve as the cofactor of recombinant MST3

To further demonstrate the unique metal ions involved for MST3 activities, cofactor preference was examined with recombinant MST3 purified from baculovirus-infected insect cells. In agreement with the result of endogenous MST3 and HA-tagged MST3, similar profiles of ion preference were observed in recombinant MST3 (Fig. 1C). The optimal concentration of each ion cofactor for recombinant MST3 was similar to exogenous HA-MST3 kinase. The purity of baculovirus-expressed MST3 was examined on 12% SDS/PAGE with

coomassie blue staining. Only a few very weak protein bands besides MST3 were observed (Fig. 2A). These results indicated that MST3 kinase could use  $\text{Zn}^{2+}$  or  $\text{Co}^{2+}$  as the sole cofactor in kinase reactions.

### 3.4. Effect of divalent metal ions on the kinetic parameters of the MST3 kinase activity

The kinetic parameters were determined with each metal ion by increasing concentrations of MBP substrate in the presence of 10  $\mu\text{Ci}$  [ $\gamma$ - $^{33}\text{P}$ ]-labeled ATP and 10  $\mu\text{M}$  unlabeled ATP. The following optimal concentrations of metal ions were used: 160  $\mu\text{M}$  of  $\text{Zn}^{2+}$  and 5 mM of  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ , or  $\text{Mg}^{2+}$ . The apparent  $K_m$ ,  $V_{\text{max}}$ , and  $V_{\text{max}}/K_m$  values were summarized in Table 1. The lowest  $K_m$  value was observed when  $\text{Zn}^{2+}$  was used as cofactor. The  $K_m$  value in the presence of  $\text{Zn}^{2+}$  for purified MST3 was approximately 4.1  $\mu\text{M}$  and the  $V_{\text{max}}$  is

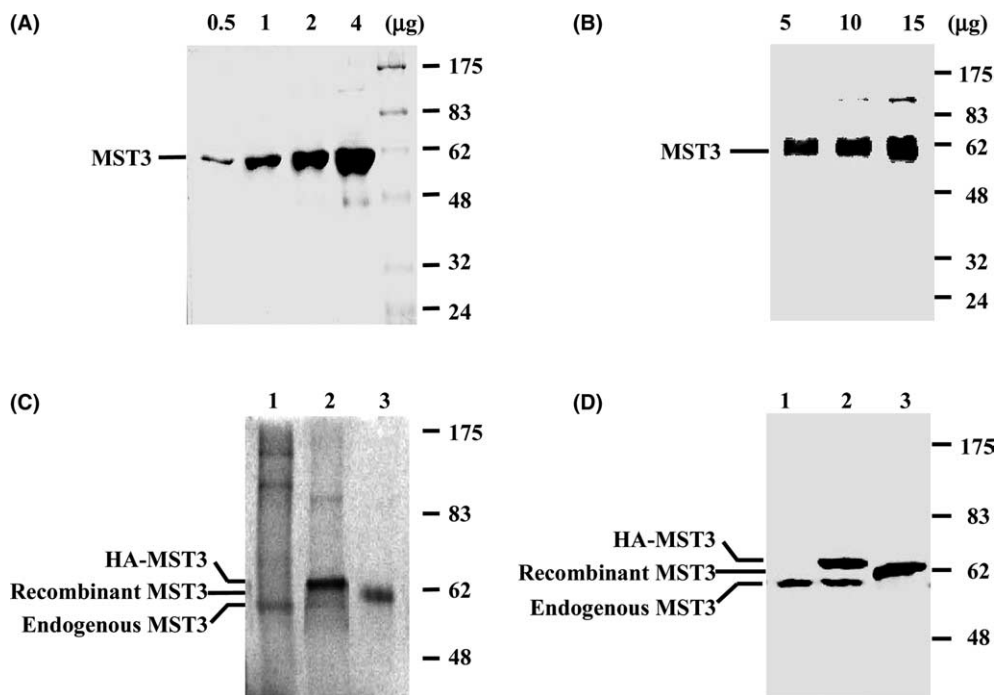


Fig. 2. Characterization and autophosphorylation of MST3 with  $Zn^{2+}$ . (A) The purity of baculovirus-expressed MST3 was examined on 12% SDS/PAGE with coomassie blue staining. The molecular weights of the protein standards are indicated. (B)  $Zn^{2+}$ -dependent autophosphorylation of recombinant MST3. Five  $\mu$ g, 10  $\mu$ g, or 15  $\mu$ g purified baculovirus-expressed MST3 was subjected to kinase reaction in the presence of 160  $\mu$ M  $Zn^{2+}$  and [ $\gamma$ - $^{33}P$ ]-labeled ATP for 90 min. The reaction mixture was analyzed on 12% SDS/PAGE followed by autoradiography. (C) Autophosphorylation patterns of the endogenous MST3 (lane 1), exogenous HA-MST3 (lane 2), and one  $\mu$ g of baculovirus-expressed recombinant MST3 (lane 3). Immunoprecipitated or recombinant MST3 was subjected to kinase reaction with 160  $\mu$ M  $Zn^{2+}$ . The reaction mixture was analyzed on 8% SDS/PAGE followed by autoradiography. (D) Western blotting of MST3. Thirty  $\mu$ g of TCCSUP cell extract (lane 1), 30  $\mu$ g of HA-MST3-transfected COS1 cell extract (lane 2), and 0.25  $\mu$ g baculovirus-expressed MST3 (lane 3), were analyzed on 8% SDS/PAGE and probed with anti-MST3 antiserum.

Table 1

Effect of divalent metal ions on the kinetic parameters of the MST3 kinase activity

Me <sup>2+</sup>	Apparent $K_m$ ( $\mu$ M)	Apparent $V_{max}$ ( $\text{pmol min}^{-1} \text{mg}^{-1}$ )	$V_{max}/K_m$
Mn <sup>2+</sup>	12.8	2623.1	204.9
Co <sup>2+</sup>	34.9	1746.1	50.0
Mg <sup>2+</sup>	22.7	129.1	5.7
Zn <sup>2+</sup>	4.1	22.3	5.4

*Note.* The initial velocity of MST3 kinase activity was determined by the addition of 6–8 various concentrations of myelin basic protein and increasing reaction time from 0 to 240 min in the presence of fixed concentrations of 5 mM Mn<sup>2+</sup>, 5 mM Co<sup>2+</sup>, 5 mM Mg<sup>2+</sup>, or 160  $\mu$ M Zn<sup>2+</sup>.  $K_m$  and  $V_{max}$  were determined from Lineweaver–Burk reciprocal plots of rates and concentrations of substrates. Data are the average of duplicate data points.

22.3  $\text{pmol min}^{-1} \text{mg}^{-1}$ . The highest  $V_{max}/K_m$  was observed in the presence of Mn<sup>2+</sup>. The catalytic efficiencies ( $V_{max}/K_m$ ) in the presence of Mn<sup>2+</sup> were 4-fold faster than those of Co<sup>2+</sup> and 40-fold faster than those of Mg<sup>2+</sup> and Zn<sup>2+</sup> (Table 1). The divalent metal ion cofactor was important in determining the affinity and turn-over efficiency between MST3 and the phospho-accepting substrate.

### 3.5. Autophosphorylation of MST3 in the presence of zinc ion

As MST3 can phosphorylate itself [1], we examine whether MST3 can utilize  $Zn^{2+}$  to catalyze autophosphorylation. Five to 15  $\mu$ g baculovirus-expressed MST3 was used for autophosphorylation reactions (without adding any substrate) in the presence of  $Zn^{2+}$ . As shown in Fig. 2B, the major phosphorylated band was MST3 itself. Autophosphorylation of different preparations of MST3 were further examined in the presence of  $Zn^{2+}$ . Exogenous HA-MST3 showed a similar autophosphorylation pattern (Fig. 2C). When immunoprecipitated endogenous MST3 was used in autophosphorylation reactions, a few additional phosphorylated proteins were observed. However, the molecular weights of these proteins were different from those observed when HA-MST3 or recombinant MST3 was used in autophosphorylation reactions (Fig. 2C). We show here that the  $Zn^{2+}$ -dependent autophosphorylation can occur in three different preparations of MST3. The minor differences in the mobility of these three preparations of MST3 were indicated by western blotting (Fig. 2D).



### 3.6. Selective inhibitor CaEDTA abolishes $Zn^{2+}$ -dependent MST3 kinase activity

The requirement of  $Zn^{2+}$  for MST3 activity was further demonstrated by the selective inhibitor CaEDTA [18,19]. Addition of equimolar of CaEDTA (160  $\mu$ M) to the kinase reaction completely abolished the endogenous MST3, exogenous MST3, and recombinant MST kinase activity (Fig. 3). Autophosphorylation of MST3 with  $Zn^{2+}$  was also inhibited by CaEDTA (data not shown).

### 3.7. $Zn^{2+}$ -dependent autophosphorylation of MST3 is on both serine and threonine residues

$Zn^{2+}$  and  $Mn^{2+}$  are important cofactors for several tyrosine kinases and can catalyze autophosphorylation on tyrosine residues. Phosphoamino acid analysis was performed with baculovirus-expressed recombinant MST3. Different concentrations of purified MST3 for each metal ion were used to achieve similar levels of autophosphorylation (Fig. 4). None of divalent metal ions ( $Mn^{2+}$ ,  $Co^{2+}$ ,  $Mg^{2+}$ , or  $Zn^{2+}$ ) supported phosphorylation of tyrosine residues on MST3. Major autophosphorylation on threonine residues was observed with  $Mn^{2+}$ ,  $Co^{2+}$ , and  $Mg^{2+}$  while autophosphorylation on both serine and threonine residues were observed with  $Zn^{2+}$  (Fig. 4). Unique phosphorylation pattern of

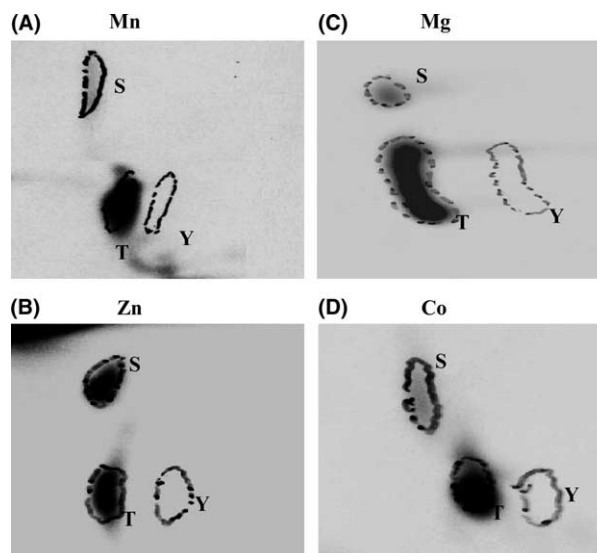


Fig. 4. Phosphoamino acid analysis on autophosphorylated recombinant MST3 in the presence of various metal ion cofactors: (A) 0.05  $\mu$ g MST3 and 5 mM  $Mn^{2+}$ , (B) 10  $\mu$ g MST3 and 160  $\mu$ M  $Zn^{2+}$  were autophosphorylated in the presence of 10  $\mu$ Ci [ $\gamma$ - $^{33}P$ ]-labeled ATP for 90 min, (C) 0.5  $\mu$ g MST3 and 5 mM  $Mg^{2+}$ , and (D) 0.5  $\mu$ g MST3 and 5 mM  $Co^{2+}$ . The reaction product was directly hydrolyzed, lyophilized and electrophoresed along with unlabeled standards. Positions of phosphoamino acids were identified by autoradiography and staining unlabeled standards with 0.25% ninhydrin. Only phospho-threonine and phospho-serine were noted. S, phospho-serine; T, phospho-threonine; Y, phospho-tyrosine. Three repetitions were performed.

$Zn^{2+}$  suggests a distinct role of  $Zn^{2+}$  in the regulation of MST3 activity.

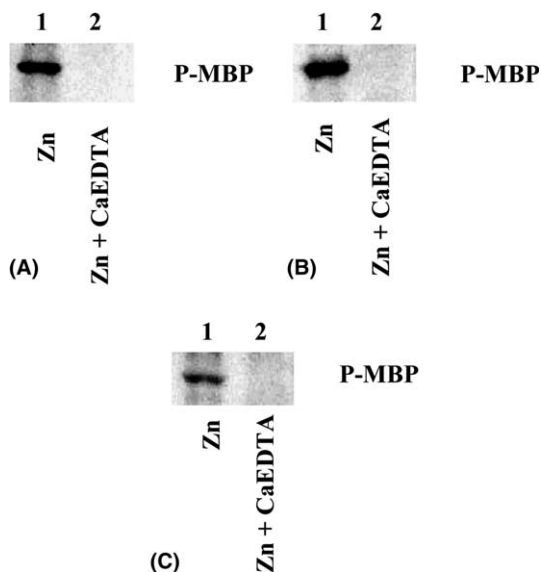


Fig. 3.  $Zn^{2+}$ -dependent MST3 activity was abolished by selective inhibitor CaEDTA. (A) Endogenous MST3, (B) exogenous transfected HA-MST3, and (C) baculovirus-expressed MST3 were incubated with myelin basic protein (MBP) and the 160  $\mu$ M  $Zn^{2+}$  in a kinase reaction in the absence of CaEDTA (lane 1) or in the presence of equimolar CaEDTA (lane 2). The phosphorylation level of substrate MBP represents the MST3 kinase activity. The data shown is a representative experiment of three independent experiments.

## 4. Discussion

In this report, we demonstrated that MST3 could use  $Zn^{2+}$  or  $Co^{2+}$  ion as a cofactor in kinase reactions. As for other cofactors, our finding was consistent with the previous report that MST3 could be activated by both  $Mn^{2+}$  and  $Mg^{2+}$  divalent cations [1]. Intracellular concentration of  $Mn^{2+}$  is 0.2–58  $\mu$ M [20,21] and  $Mg^{2+}$  0.3–2 mM [22,23]. The total intracellular  $Zn^{2+}$  is around 80–200  $\mu$ M [24,25].  $Zn^{2+}$  concentration at synapses of the hippocampus have been estimated to be as high as 300  $\mu$ M, although the free  $Zn^{2+}$  in other regions may be in the range of 1–20  $\mu$ M [18,19,26]. Intracellular free  $Zn^{2+}$  in isolated hepatocytes was estimated to be approximately 1  $\mu$ M [27]. Therefore, the MST3 may use Zinc ion as cofactor if the free  $Zn^{2+}$  concentration was raised by intracellular movement of  $Zn^{2+}$  or release of  $Zn^{2+}$  from metallothionein. cAMP dependent protein kinase [28] and c-terminal Src kinase (Csk) [29] are just a few examples of cobalt dependent kinases. Classification of human MST3 as a cobalt enzyme further expanded the array of cobalt dependent kinases. Only a few protein kinases have been identified to be stimulated by  $Mn^{2+}$  or  $Zn^{2+}$  *in vitro*. Protein kinase C, a Ser/

Thr kinase, is activated by cooperation of  $Zn^{2+}$  and  $Ca^{2+}$  [30]. Among these kinases, MST3 was the only Ser/Thr kinase activated by sole presence of  $Zn^{2+}$  in a pure recombinant form (Fig. 1C).

Zinc ion modulates protein module recognition, protein kinase activity, and protein phosphatase activity. Treatment with the chemical agent *o*-tetradecanoylphorbol-13-acetate (TPA) resulted in the distribution of zinc from nucleus and mitochondria to the cytosol and microsomes in T lymphocyte [31]. Activation of PKC by  $Zn^{2+}$  and  $Ca^{2+}$  contributes to the binding of PKC onto membranes in T lymphocyte [32]. Metallothionein and zinc are localized mainly in the cytoplasm in myoblasts but are translocated into the nucleus of newly formed myotubes during early stage of differentiation [33]. Garchon et al. [34] showed that  $Zn^{2+}$  supported a significant retention of XLR protein in the nucleus and that  $Co^{2+}$  caused moderate retention. In contrast,  $Mn^{2+}$  and  $Mg^{2+}$  did not cause a withholding protein in the nuclear fraction.

Phosphorylation of several MST members affects their location in the cells [35,36]. We have previously demonstrated that MST3 has a dual nuclear and cytoplasmic localization in response to stress [6]. Thus, changes in the intracellular zinc concentration might affect the localization and function of MST3. The regulation of autophosphorylation by different metal ions may have a role in keeping MST3 in an active or inactive form until it contacts naturally occurring substrates, leading to localized high catalytic specificity within the cell. Furthermore, phosphoamino acid analysis demonstrated unique phosphorylation patterns by  $Zn^{2+}$ , compared to those by  $Mn^{2+}$ ,  $Co^{2+}$ , or  $Mg^{2+}$ .  $Zn^{2+}$  may employ a mechanism distinguished from other metals in activating MST3. The phosphorylation of MST3 on serine residues may be regulated in  $Zn^{2+}$ -involved signal pathway in vivo.

The effect of metal ions on the enzyme activity depends on the binding affinity of enzyme and substrate, phosphotransfer, and product release steps [28]. High concentrations of  $Zn^{2+}$  inhibited the MST3 kinase activity (Fig. 1). It is possible that there was a second binding site for  $Zn^{2+}$  with relative low affinity. The second binding site may be occupied to inhibit MST3 kinase activity when the concentration of  $Zn^{2+}$  was above physiologic range. There were two  $Mg^{2+}$ -binding sites for cAMP dependent protein kinases. The first binding site was required for catalysis while the other binding site was for inhibition [8]. In Csk,  $Zn^{2+}$  showed better affinity at the second  $Mg^{2+}$  binding site than  $Mn^{2+}$ ,  $Co^{2+}$ , or  $Mg^{2+}$  although it did not support Csk kinase activity [29]. The question of whether only one or more divalent metal ion binding sites is present in MST3 requires further investigation.

$Zn^{2+}$  was as effective as  $Mn^{2+}$  on endogenous MST3 and exogenous HA-MST3 activity in the immunoprecip-

itation assay (Fig. 1). However, the activity of baculovirus-expressed recombinant MST3 in the presence of  $Zn^{2+}$  was not as potent as that of  $Zn^{2+}$ -stimulated endogenous MST3 and exogenous HA-MST3. The minor discrepancy might be due to two possible causes. First, the post-translational modification of baculovirus-expressed MST3 was not identical to that of endogenous and exogenous MST3 immunoprecipitated. Post-translational modification may affect MST3 activity. Secondly,  $Zn^{2+}$  is a potent inhibitor of Ser/Thr phosphatase, i.e., lambda phosphatase and calcineurin (PP2B), at micro molar concentrations [21,37,38]. It is possible the MST3 kinase activity was further enhanced by inhibition of phosphatase activity in immunoprecipitates. Therefore, in the presence of  $Zn^{2+}$ , the  $V_{max}/K_m$  of MST3 in vivo could be higher than that of recombinant MST3 in vitro.

The concentration of  $Zn^{2+}$  was relatively high in the brain of seizure-prone strain mice i.e., 304  $\mu M$ , and  $Zn^{2+}$  was involved in regulation of neuronal activity. The electrical stimulation of hippocampus slices was reported to facilitate the uptake of  $Zn^{2+}$  [39]. A human brain-specific isoform of MST3 has been identified [40], and the role of  $Zn^{2+}$  on activation or inhibition of MST3 might be important for regulation of neuronal activity. The role of MST3 in neuronal cell warrants future investigation.

## 5. Abbreviations

CaEDTA	calcium ethylene diamine tetraacetic acid
CSK	c-terminal Src kinase
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis( $\beta$ -aminoethyl ether) <i>N,N,N',N'</i> -tetraacetic acid
GCK	germinal center kinase
HPTLC	high pressure thin layer chromatography
MBP	myelin basic protein
MST3	mammalian Ste20-like kinase 3
MOI	multiplicity of infection
PAK	p21-activated kinase family
PDGF	platelet derived growth factor
PIPES	piperazine- <i>N,N'</i> -bis-[2-ethanesulfonic acid]
PMSF	phenylmethyl-sulfonyl fluoride
PP2B	lambda phosphatase and calcineurin
PTPase	phospho-tyrosine phosphatase
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
Ser/Thr	serine/threonine
TPA	<i>o</i> -tetradecanoylphorbol-13-acetate
Tris-HCl	Tris(hydroxymethyl)methylammonium chloride

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