

# Casein Kinase 2 interacts with human mitogen- and stress-activated protein kinase MSK1 and phosphorylates it at Multiple sites

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**Mitogen- and stress-activated protein kinase (MSK1) plays a crucial role in the regulation of transcription downstream of extracellular-signal-regulated kinase1/2 (ERK1/2) and mitogen-activated protein kinase p38. MSK1 can be phosphorylated and activated in cells by both ERK1/2 and p38 $\alpha$ . In this study, Casein Kinase 2 (CK2) was identified as a binding and regulatory partner for MSK1. Using the yeast two-hybrid system, MSK1 was found to interact with the CK2 $\beta$  regulatory subunit of CK2. Interactions between MSK1 and the CK2 $\alpha$  catalytic subunit and CK2 $\beta$  subunit were demonstrated *in vitro* and *in vivo*. We further found that CK2 $\alpha$  can only interact with the C-terminal kinase domain of MSK1. Using site-directed mutagenesis assay and mass spectrometry, we identified five sites in the MSK1 C-terminus that could be phosphorylated by CK2 *in vitro*: Ser757, Ser758, Ser759, Ser760 and Thr793. Of these, Ser757, Ser759, Ser760 and Thr793 were previously unknown. [BMB reports 2009; 42(12): 840-845]**

## INTRODUCTION

Mitogen- and stress-activated protein kinase (MSK1) is a downstream kinase of mitogen-activated protein kinases (MAPKs). MAPK pathways converge on extracellular signal-regulated kinases (ERK, including ERK1 and ERK2 isoforms), c-Jun NH2-terminal kinases (JNK, including JNK1, JNK2 and JNK3 isoforms), and p38 MAPKs (including p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  isoforms) induce a variety of cellular functions such as

gene expression, mitosis, and apoptosis through the phosphorylation of specific serine and/or threonine residues of target proteins (1). MSK1 is most closely related to the p90 ribosomal S6 kinase (RSK) family kinases, and like RSK, MSK1 contains two distinct kinase domains within a single polypeptide (2-4). The N-terminal domain is a member of the protein kinase A/protein kinase G/protein kinase C/family kinases (AGC-type kinases), while the C-terminal kinase domain is related to the calmodulin-dependent protein kinase family (5). The molecular mechanism of MSK1 activation is complex. It requires the phosphorylation of MSK1 at Ser360 and Thr581 by either ERK1/2 or p38 $\alpha$  (2-4, 6). These events activate the C-terminal kinase domain of MSK1, which then autophosphorylates at Ser212, Ser376, and Ser381, resulting in the activation of the N-terminal kinase domain. Then Ser750, Ser752 and Ser758 were phosphorylated by the N-terminal kinase domain (7). In addition to these eight phosphorylation sites, five novel sites, Thr630, Ser647, Ser657, Ser695 and Thr700 have been recently identified (6). Among these five sites, Thr700 was found to be the third site phosphorylated by ERK1/2 or p38 $\alpha$ , perhaps playing a key role in the activation of MSK1. Another four sites can be phosphorylated by unknown kinases. Although thirteen phosphorylation sites have been identified in MSK1, the activation mechanism of it is unclear.

Activated MSK1 is required for the phosphorylation of several factors in response to mitogenic and cellular stress, including cAMP-response-element-binding protein (CREB), activating transcription factor 1 (ATF1) (8, 9), the chromatin protein histone H3, nuclear factor kappa B (NF- $\kappa$ B) (10) and high-mobility group protein 14 (HMG-14) (11, 12).

To further understand the phosphorylation regulation of MSK1, it will be important to identify and characterize its interacting kinases. We performed a yeast two-hybrid screen in our current study to detect. These experiments identified multiple isoforms of CK2 $\beta$  as direct MSK1 interacting partners. Casein Kinase 2 (CK2) is one of the most conserved Ser/Thr kinases, playing a key role in controlling gene expression, cell growth and proliferation. It is a tetrameric enzyme composed

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Received 19 May 2009, Accepted 26 August 2009

**Keywords:** CK2, Mass spectrometry, MSK1, Phosphorylation, Yeast two-hybrid

of two catalytic (CK2 $\alpha$  and/or CK2 $\alpha'$ ) subunits and two regulatory (CK2 $\beta$ ) subunits (13-16). CK2 can phosphorylate more than 300 proteins, including a striking number of signaling proteins. Here, we identified another five sites in the MSK1 C-terminus that could be phosphorylated by CK2 *in vitro*: Ser757, Ser758, Ser759, Ser760 and Thr793. Of these, Ser757, Ser759, Ser760 and Thr793 were previously unknown.

## RESULTS

### Identification of MSK1/CK2 $\beta$ interaction in a yeast two-hybrid system

To identify proteins that interact with MSK1, we screened a human liver cDNA library in a yeast two-hybrid system using MSK1 as bait. Screening over  $2 \times 10^6$  clones yielded 25 candidates. Sequencing indicated that 12 of the 25 encoded the C-terminal portion of human CK2 $\beta$  (amino acids 74-216). To confirm the interaction between MSK1 and CK2 $\beta$ , full-length CK2 $\beta$  was cloned into the pPC86 vector, and co-transfected into Mav203 with pDBLeu-MSK1 or pDBLeu. Coexpression of MSK1 and CK2 $\beta$  showed activation of the reporter gene, but the negative control did not (Fig. 1A).

### Identification of MSK1/CK2 interactions *in vitro* and *in vivo*

To confirm the physical interaction between MSK1 and CK2, GST pull-down assays were employed. Bacterially expressed GST-MSK1 efficiently pulled down Myc-CK2 $\alpha$  and Myc-CK2 $\beta$ , but the control GST did not (Fig. 1B). This indicated that MSK1 could bind with the two subunits of CK2 *in vitro*.

To determine whether MSK1 interacted with CK2 in mammalian cells, *in vivo* binding assays were performed. Myc-CK2 $\alpha$  or Myc-CK2 $\beta$  was transiently co-transfected with Flag-MSK1 into HEK293T cells. As shown in Fig. 1C, Flag-MSK1 was detected in Myc-CK2 $\alpha$  and Myc-CK2 $\beta$  co-precipitated complex, but not in the control. This indicated that the two subunits of

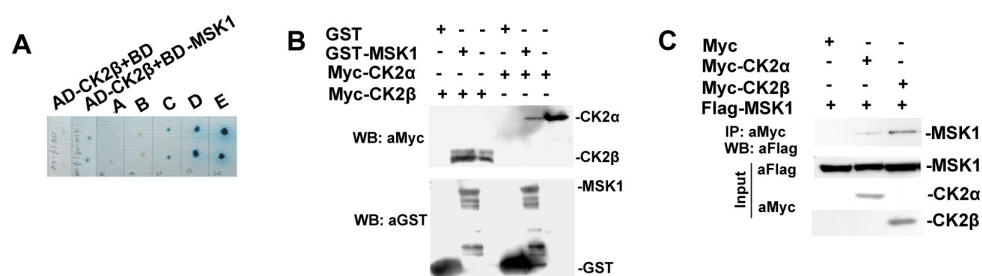
CK2 interacted with MSK1 directly *in vivo*.

### Domains involved in the MSK1/CK2 interaction

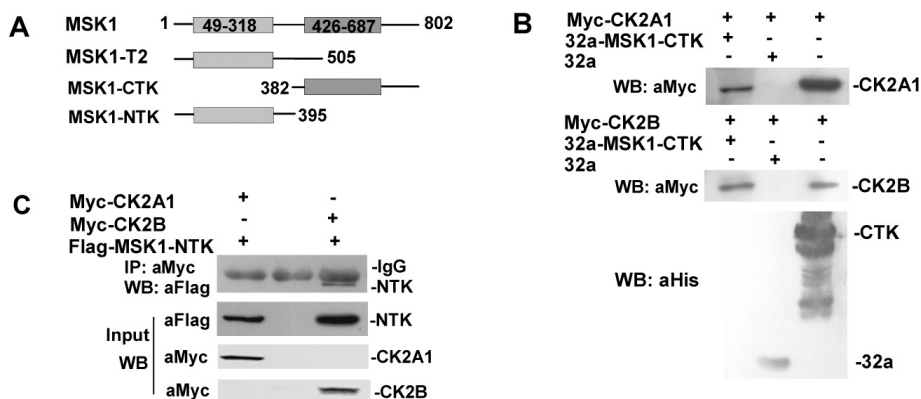
To further understand the association between MSK1 and CK2, we constructed two truncated mutants of MSK1, MSK1-NTK (amino acids 1-395) and MSK1-CTK (amino acids 382-802) according to its kinase domain (Fig. 2A). Co-immunoprecipitation assay was used to validate the interaction between MSK1-NTK and CK2. Flag-MSK1-NTK could only be detected in the co-precipitated complex co-transfected with Myc-CK2 $\beta$ , but not with Myc-CK2 $\alpha$  (Fig. 2B). This indicates that CK2 $\beta$  could interact with the N-terminal of MSK1 directly. Because Flag-MSK1-CTK can not expressed in HEK293T cells, GST pull-down assay was used to validate the interaction between MSK1-CTK and the two subunits. pET32a-MSK1-CTK expressed in bacterial could easily pull Myc-CK2 $\alpha$  and Myc-CK2 $\beta$  down. The pET32a itself could not (Fig. 2C). This result indicates that the two subunits of CK2 both can interact with the C-terminal of MSK1. Taken together, CK2 $\alpha$  only interact with the C-terminal kinase domain of MSK1, but CK2 $\beta$  can interact both with the N-terminal and C-terminal kinase domain of MSK1.

### CK2 specifically phosphorylates MSK1-CTK *in vitro*

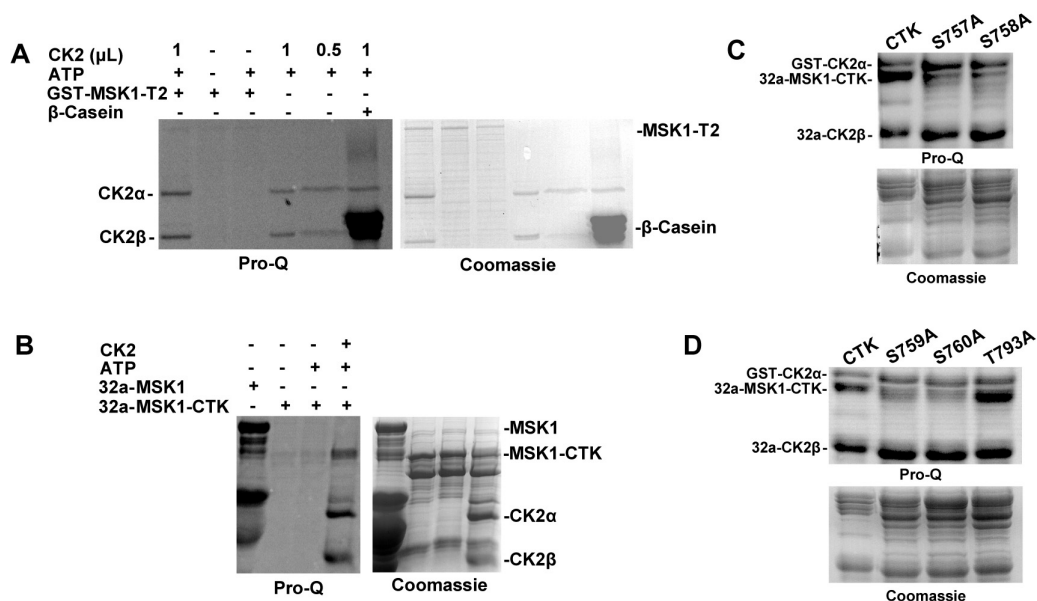
CK2 has been reported to phosphorylate a large variety of substrates, so we investigated whether MSK1 could be a substrate. MSK1 purified from bacterial systems autophosphorylates (6), so it can not be used as to test phosphorylation by CK2. GST-MSK1-T2 and pET32a-MSK1-CTK fusion proteins were tested as CK2 substrates. GST-MSK1-T2 was unphosphorylated, had no autophosphorylation ability and was not phosphorylated in the presence of CK2 (Fig. 3A), while  $\beta$ -casein, the positive control, was distinctly phosphorylated. In contrast, MSK1-CTK was readily phosphorylated in the presence of CK2 (Fig. 3B), while the control showed little phosphorylation sig-



**Fig. 1.** MSK1 interacts with CK2. (A) Yeast two-hybrid interaction between MSK1 and CK2 $\beta$ . Yeast cells were co-transformed with pPC86-CK2 $\beta$ /pDBLeu and pPC86-CK2 $\beta$ /pDBLeu-MSK1.  $\beta$ -galactosidase assays were used to detect interaction. pPC86-CK2 $\beta$ /pDBLeu showed no evident interaction, pPC86-CK2 $\beta$ /pDBLeu-MSK1 indicated positive interaction. A, B, C, D, E are yeast controls with varying degrees of protein-protein interaction, E indicates the strongest interaction. (B) *In vitro* GST pull-down assay. GST and GST-MSK1 were expressed in bacteria and immunoblotted with anti-GST antibody. Cell lysate from mammalian cells expressing Myc-CK2 $\alpha$  or Myc-CK2 $\beta$  was incubated with glutathione beads purified with GST or GST-MSK1 fusion proteins. The bound proteins were eluted, subjected to SDS-PAGE analysis and immunoblotted with anti-Myc antibody. (C) *In vivo* co-immunoprecipitation assay. Myc-CK2 $\alpha$  or Myc-CK2 $\beta$  was co-transfected with Flag-MSK1 into HEK293T cells. Expression of proteins in co-transfected cells was analyzed by Western blotting with the relevant antibodies. The lysate was immunoprecipitated with anti-Myc antibody and analyzed by immunoblotting with anti-Flag antibody.



**Fig. 2.** Identification of domains of MSK1 required for the interaction with CK2. (A) Schematic diagram of the structure of the truncated mutant forms of MSK1. MSK1-T2, MSK1-NTK and MSK1-CTK are indicated. (B) CK2 $\alpha$  and CK2 $\beta$  both can interact with MSK1-CTK. pET32a and pET32a-MSK1-CTK recombinant proteins were expressed in *E. coli* BL21(DE)3 and purified with Ni<sup>2+</sup>-NTA agarose. Cell lysate expressed Myc-CK2 $\alpha$  or myc-CK2 $\beta$  in mammalian cells were incubated with the agarose. The agarose were eluted and detected with anti-myc antibody. (C) CK2 $\beta$  can interact with MSK1-NTK *in vivo*, but CK2 $\alpha$  can not. Myc-CK2 $\alpha$  and Myc-CK2 $\beta$  were co-transfected with Flag-MSK1-NTK into HEK293T cells respectively. Lysates were immunoprecipitated with anti-myc antibody and analyzed by immunoblotting with anti-flag antibody.



**Fig. 3.** MSK1 can be phosphorylated by CK2 *in vitro*, and the sites identified on MSK1 can be phosphorylated by CK2. (A) GST-MSK1-T2 recombinant protein was incubated with or without commercial CK2. Reactions were run on SDS-PAGE and visualized with Pro-Q Diamond Phosphoprotein Gel Stain to see phosphorylated protein, and with coomassie blue stain to see total protein. CK2 holoenzyme can be autophosphorylated (lanes 4, 5) and phosphorylates  $\beta$ -casein (2 mg/ml, last lane). But CK2 could not phosphorylate GST-MSK1-T2. (B) Recombinant protein pET32a-MSK1-CTK was used as the substrate of CK2 purified as described in Materials and Methods for *in vitro* kinase assays. (C) *In vitro* kinase assays were carried out in which recombinant protein pET32a-MSK1-Ser-757A and -Ser758A were used as substrates for CK2 purified as described in Materials and Methods. (D) pET32a-Ser759A, -Ser760A and -Thr793A recombinant proteins were used as substrates. Equal quantities of the five proteins and MSK1-CTK were used in the assays.

nal in the absence of CK2. GST-MSK1 which had been autophosphorylated was used as a positive control. These results indicated that MSK1 could be phosphorylated by CK2 *in vitro*,

with the potential phosphorylation site in the MSK1 C-terminus.

**Table 1.** MSK1 phosphorylation sites identified for CK2 by mass spectrometry. Three samples were prepared for MS/MS analysis. Sample 1 was the purified recombinant proteins pET-32a-MSK1-CTK. Sample 2 was autophosphorylation sample. Sample 3 was CK2 kinase assay. The right column of the table was the corresponding phosphorylation sites located by MS

MSK1	Sample 1				Sample 2				Sample 3			
	T	G	P	K	T	G	P	K	T	G	P	K
S436	✓		✓		✓		✓		✓		✓	
T581				✓				✓				✓
T585				✓				✓				✓
S628	✓			✓			✓	✓			✓	✓
T630	✓			✓	✓		✓	✓	✓		✓	
T632	✓			✓	✓				✓		✓	
S757									✓			
S758									✓			
S759									✓			
S760									✓			
T793									✓			
S798	✓								✓	✓	✓	
S800	✓	✓							✓	✓	✓	

T, digestion by trypsin; G, digestion by Glu-C; P, digestion by pepsin; K, digestion by proteinase K; ✓, indicates the phosphorylation site identified in this work.

### Analysis of MSK1 phosphorylation sites by mass spectrometry and *in vitro* kinase assays

To further analyze the CK2 phosphorylation sites in MSK1, three samples described in methods were analyzed by MS. The confidence of phosphopeptide identifications was set at a false discovery rate (FDR) <2%. The identified phosphopeptides and locations of MSK1 phosphorylation sites are in Table 1. These results showed five additional phosphorylation sites (Ser757, Ser758, Ser759, Ser760, Thr793) in sample 3, in addition to the sites in samples 1 and 2. These data suggested that the five additional sites in MSK1 were phosphorylated by CK2. The phosphorylated sites identified by MS were validated by *in vitro* kinase assays. Five constructs were generated, in which Ser757, Ser758, Ser759, Ser760 and Thr793 were mutated to alanine. *In vitro* kinase assays were carried out using these five mutant recombinant proteins, all adjusted to the same concentration, as CK2 substrates. Ser757A, Ser758A, Ser759A, and Ser760A all showed weak phosphorylation compared to the wild type protein (Fig. 3C, D), which suggested that these four sites were phosphorylated by CK2. The phosphorylation signal of Thr793A was unchanged, possibly because it is weakly phosphorylated, and could be detected by the highly sensitive MS, but was under the detection limit of the Pro-Q assay. Hence, use of both MS and *in vitro* kinase assays validated the identification of phosphorylation sites.

## DISCUSSION

In this work, specific *in vitro* and *in vivo* interactions between MSK1 and CK2 were identified. MSK1 is a downstream kinase

of p38 MAPK and can be activated by p38 $\alpha$  under cellular stress (2-4, 6). CK2 also contributes to p38 MAPK signaling pathway regulation. In a phosphorylation-dependent manner, p38 $\alpha$  can directly interact with the  $\alpha$  and  $\beta$  subunits of CK2 to activate the holoenzyme (17). p38 and CK2 both co-immunoprecipitate with p53 (18, 19). Anisomycin and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced phosphorylation of p53 at Ser392 requires p38 MAPK kinase and CK2 activities (17). In addition, CK2 can phosphorylate I $\kappa$ B $\alpha$  at a cluster of C-terminal sites through a mechanism that depends on the ultraviolet-induced activation of p38 MAPK kinase (20).

CK2 is one of the most highly conserved Ser/Thr kinases, and participates in many signal pathways, phosphorylating numerous substrates. To determine if MSK1 is a substrate for CK2, *in vitro* kinase assays were carried out using a series of recombinant MSK1 proteins. MSK1 could be phosphorylated by CK2 in its C-terminal kinase domain. CK2 $\alpha$  interacted only with the C-terminal kinase domain of MSK1, consistent with the location of the phosphorylated sequences.

Five sites (Ser757, Ser758, Ser759, Ser760 and Thr793) in the C-terminus of MSK1 were identified as CK2-phosphorylated sites by *in vitro* kinase assays and MS. Among these sites, Ser758 has been reported as an autophosphorylated site *in vitro* (7). Further research is necessary to determine if this site is phosphorylated by CK2 or is autophosphorylated *in vivo*. All five sites are located in the MSK1 C-terminus, close to Thr700, which is conserved in MAPK-integrating kinase 1 and 2 (MNK1 and MNK2) (6). According to a recent activation model of MSK1, phosphorylation of Thr700 relieves inhibition of MSK1 by a C-terminal autoinhibitory helix, and helps induce a conformational shift that protects Thr581 from dephosphorylation (6). The five sites located C-terminal to Thr700 have no equivalent sites in MSK2, RSK1, MAPKAPK2 and MNK, so they are specific to MSK1. We hypothesize that they play a similar role as Thr700. Under cellular stress, activated p38 $\alpha$  could phosphorylate MSK1 at Ser360, Thr581 and Thr700. Simultaneously, the CK2 holoenzyme could be activated and phosphorylates MSK1 at these five sites. Phosphorylation of Thr700 relieves the inhibition of MSK1. These five phosphorylated sites phosphorylated by CK2 may help Thr700 induce the shift of the C-terminal peptide of MSK1, contributing to activation by providing a more stable MSK1 activated structure.

In conclusion, we have identified an interaction between MSK1 and CK2, and located five potential CK2 phosphorylation sites within the MSK1 C-terminal domain. Among these, Ser757, Ser759, Ser760 and Thr793 are novel. According to their spatial position, we hypothesize that these five phosphorylation sites may help MSK1 maintain a stable structure for subsequent activation. CK2 participates in many signal pathways, phosphorylating numerous substrates resulting in regulating of gene activity, localization and protein-protein interaction. Whether this phosphorylation of MSK1 by CK2 influence the processes such as MSK1 localization or activity need further research. Nevertheless, our results found a

new regulative kinase CK2 for MSK1. And we proposed an activation model for phosphorylation modification of MSK1.

## MATERIALS AND METHODS

### Plasmid constructs

For protein interaction assays in yeast, MSK1 was inserted into vector pDBLeu (GIBCO) and fused to Gal4 DNA binding domain (BD). CK2 $\beta$  was cloned into pPC86 (GIBCO) vector and fused to Gal4 activation domain (AD). For *in vitro* binding analysis, MSK1 was cloned into a GST-tag vector pGEX-6p-1 (Amersham Biosciences, Uppsala, Sweden). MSK1 deletion mutant MSK1-CTK (amino acids 382-802) was inserted into a 6  $\times$  His-tag vector pET32a (Novagen, Madison, WI, USA). For co-immunoprecipitation assays, MSK1 and deletion mutant MSK1-NTK (amino acids 1-395) were cloned into Flag-tag vectors. For binding assays, CK2 $\alpha$  and CK2 $\beta$  were cloned into Myc-tag vectors. For kinase assays, MSK1 transcript variant 2 (MSK1-T2, amino acids 1-505) was cloned into pGEX-6p-1. MSK-CTK and its mutagenesis were cloned into pET32a. Mutagenesis of MSK-CTK was performed using the Quik Change<sup>®</sup> Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

### Yeast two-hybrid assays

The yeast two-hybrid screen and co-transformation assay were performed by GIBCO yeast two-hybrid screen manufacturer's protocol. Briefly, the autoactivation of the *lacZ* reporter gene by pDBLeu-MSK1 was tested in Mav203 cells. Then the stable BD-MSK1-transformed yeast cells were transformed with a human liver cDNA library (Invitrogen, Grand Island, NY, USA) constructed in the pPC86 plasmid. More than 2  $\times$  10<sup>6</sup> cDNA colonies were screened on plates containing 25 mM 3AT (Sigma, St Louis, MO, USA) and lacking histidine, leucine and tryptophan. Positive clones were verified by  $\beta$ -galactosidase assay. Prey plasmids were isolated from His<sup>+</sup>/Leu<sup>+</sup>/LacZ<sup>+</sup> colonies and re-transformed into yeast along with either pDBLeu-MSK1 or pDBLeu to verify specific interactions.

### *In vitro* GST pull-down assays

GST fusion proteins were expressed in 0.1 mM IPTG-induced *E. coli* strain BL21 and purified with glutathione-Sepharose 4B beads (Invitrogen). The beads with bound proteins were incubated with lysate from HEK293T cells expressing Myc-CK2 $\alpha$  or Myc-CK2 $\beta$  at 4°C for 4 h with gentle rotation. Beads were washed three times with cell lysis buffer to disrupt non-specific interactions, and bound proteins were separated by SDS-PAGE. Mouse monoclonal antibody against GST was from Sigma. Rabbit anti-mouse horseradish peroxidase second antibody was from Rockland (Philadelphia PA, USA).

### Cell culture and co-immunoprecipitation assays

HEK293T cells were maintained in DMEM supplemented with 10% bovine calf serum, grown on 60 mm dishes at a concentration of 6  $\times$  10<sup>5</sup> cell/dish before the day of transfection. The relevant

plasmids were transfected with Lipofectamine (Invitrogen) into HEK293T cells. After 48 h, cells were lysed in 400  $\mu$ l lysis buffer (Cell Signaling, Beverly, MA). Lysate was pretreated with protein A/G agarose (Santa Cruz, CA, USA) and immunoprecipitated with 1-2  $\mu$ g of relevant antibody and protein A/G agarose at 4°C overnight. After washing three times with lysis buffer, the precipitates were analyzed by Western blot. Mouse monoclonal antibodies against c-Myc, Flag M2, and 6  $\times$  His were from Sigma.

### *In vitro* kinase assays

pET32a-CK2 $\beta$ , pET32a-MSK1-CTK and their mutated variants were expressed in *E. coli* BL21 (DE3). Recombinant proteins were purified with Ni<sup>2+</sup>-NTA agarose and eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 8.0). GST-MSK1 and GST-CK2 $\alpha$  were expressed in *E. coli* BL21. Recombinant proteins were purified with Sepharose 4B beads and eluted with glutathione solution (50 mM Tris-HCl, 20 mM reduced glutathione, pH 8.0). Approximately 1-2  $\mu$ g recombinant protein was incubated at 30°C for 30 min in 20  $\mu$ l kinase buffer with 1 mM ATP and either 1  $\mu$ l CK2 holoenzyme (New England Biolabs, Herts, United Kingdom), or a mixture of 1  $\mu$ g GST-CK2 $\alpha$  and 2  $\mu$ g pET32a-CK2 $\beta$  purified as described above, that had CK2 holoenzyme activity (Supporting Information 1).  $\beta$ -casein (Sigma, St. Louis, MO, USA) was a special substrate for CK2, used as the positive control. The reaction was run on SDS-PAGE followed by PrO-Q Diamond Phosphoprotein Gel Stain (Invitrogen) detection. For mass spectrometry, three samples were prepared: 1) 1 mg purified recombinant protein MSK1-CTK; 2) 1 mg recombinant protein in 1 ml kinase buffer with 1 mM ATP; 3) 0.8 mg recombinant protein in 600  $\mu$ l kinase buffer with 1 mM ATP and 15  $\mu$ l CK2 holoenzyme (New England Biolabs). The second and third reactions were carried out at 30°C for 30 min and all samples were denatured with 8 M urea.

### Identification of phosphorylation sites in MSK1 by mass spectrometry

Mass spectrometry (MS) was used to localize phosphorylation sites, using an MS<sup>2</sup>/MS<sup>3</sup> target-decoy database search strategy with multi-protease digestion (21). Briefly, the six steps in this approach were: 1) separate digestions of the phosphoprotein sample with multiple proteases; 2) enrichment of phosphopeptides by Ti<sup>4+</sup>-IMAC (22, 23) from the individual peptide mixtures; 3) analysis of enriched phosphopeptides with LC-MS<sup>2</sup>-MS<sup>3</sup>; 4) submission of acquired MS<sup>2</sup> and MS<sup>3</sup> spectra to an MS<sup>2</sup>/MS<sup>3</sup> target-decoy search using a composite database that included MSK1 and two subunits of CK2 as target sequences, and a reversed yeast database with 1,000 entries as the decoy database; 5) filtering the candidate phosphopeptides with parameters (Rank'm,  $\Delta$ Cn'm and Xcorr's) designed to achieve phosphopeptide identification at a specific false discovery rate (FDR); 6) determination of phosphorylation sites by Tscore as described by Jiang et al. (24). Analysis was performed on a nano-RPLC-MS/MS system using a LTQ linear ion trap mass spectrometer (Thermo

Finnigan). MS data were searched against database using SEQUEST (version 0.27) in Bioworks (version 3.2).

### Acknowledgements

We thank Dr. Jiahuai Han for pcDNA3-HA-RLPK plasmids, and thank Dr. Jinsheng Dong and Dr. Guojun Hwang for proofreading the manuscript and active discussion. This work was supported by the China High Technology Research Program Grant (2006AA02A310, 2006AA02A309, 2004BA711A19); and the National Natural Sciences Foundation of China (20735004, 90713017).

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