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Negative Regulation of MAPKK by Phosphorylation of a Conserved Serine Residue Equivalent to Ser^{212} of MEK1*

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Kailesh Gopalbhai‡, Gregor Jansen§¶, Geneviève Beauregard‡, Malcolm Whiteway§‖, France Dumas§, Cunle Wu§, and Sylvain Meloche‡**

From the ‡Institut de Recherches Cliniques de Montréal and the Department of Pharmacology, Université de Montréal, Montreal, Quebec H2W 1R7, Canada, the §Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec H4P 2R2, Canada, and the ‖Biology Department, McGill University, Montreal, Quebec H3A 1B1, Canada

The MAPKKs MEK1 and MEK2 are activated by phosphorylation, but little is known about how these enzymes are inactivated. Here, we show that MEK1 is phosphorylated *in vivo* at Ser^{212}, a residue conserved among all MAPKK family members. Mutation of Ser^{212} to alanine enhanced the basal activity of MEK1, whereas the phosphomimetic aspartate mutation completely suppressed the activation of both wild-type MEK1 and the constitutively activated MEK1(S218D/S222D) mutant. Phosphorylation of Ser^{212} did not interfere with activating phosphorylation of MEK1 at Ser^{218}/Ser^{222} or with binding to ERK2 substrate. Importantly, mimicking phosphorylation of the equivalent Ser^{212} residue of the yeast MAPKKs Pbs2p and Ste7p similarly abrogated their biological function. Our findings suggest that Ser^{212} phosphorylation represents an evolutionarily conserved mechanism involved in the negative regulation of MAPKKs.

Mitogen-activated protein kinase (MAPK)[1] pathways are evolutionarily conserved signaling modules by which cells transduce extracellular chemical and physical signals into intracellular responses (reviewed in Refs. 1–3). These modules are organized into an architecture of three sequentially acting protein kinases comprising a MAPK kinase kinase (MAPKKK or MEK kinase), a MAPK kinase (MAPKK or MEK), and the MAPK itself. The propagation of the signal through MAPK pathways is facilitated by specific protein-protein interactions between individual components of the pathway and scaffolding proteins (3, 4).

The prototypical and most studied MAPK pathway is the ERK1/2 pathway, which controls cell proliferation, differentiation, and development (1). Stimulation of cells with growth and differentiation factors leads to the activation of the MAPKKK Raf by a complicated mechanism involving cellular relocalization and multiple phosphorylation events (5, 6). Activated Raf isoforms bind to and activate the MAPKKs MEK1 and MEK2 by phosphorylation of two serine residues (corresponding to Ser^{218} and Ser^{222} in MEK1) in their activation loop (7, 8). Substitution of the two regulatory serines with acidic residues is sufficient to enhance the basal activity of MEK1/2 (7–12). The dual-specificity kinases MEK1 and MEK2 then catalyze the phosphorylation of the MAPKs ERK1 and ERK2 at threonine and tyrosine residues within the activation loop motif Thr-Glu-Tyr (13), causing a reorientation of the loop and activation of the enzyme (14). Both MEK1 and MEK2 stably associate with ERK1/2, and this association is required for efficient activation of the latter in cells (15, 16). The binding site for ERK1/2 is located at the N terminus of MEK1/2 and consists of a short basic region known as the D domain (16). MEK1 and MEK2 also contain a unique proline-rich insert between subdomains IX and X, which is required for full activation of ERK1/2 in intact cells (17, 18).

The magnitude and duration of MAPK activation are important determinants of the cellular response to extracellular signals (19, 20). Therefore, a tightly regulated balance between activation and inactivation mechanisms must exist to control the cellular activity of ERK1/2. Inactivation of the ERK1/2 enzymes is mainly achieved by dephosphorylation of the activating threonine and tyrosine residues. Biochemical and genetic studies have implicated both tyrosine-specific phosphatases and dual-specificity MAPK phosphatases in the negative regulation of ERK1/2 and other MAPKs (21, 22). Much less is known about the mechanisms that negatively regulate the pathway at the MAPKK level. The serine/threonine phosphatase protein phosphatase 2A was identified as the major phosphatase inactivating MEK1 in lysates of PC12 cells (23). Furthermore, overexpression of SV40 small t antigen, which binds to the A subunit of protein phosphatase 2A and inactivates the enzyme, was found to stimulate MEK and ERK activity in CV-1 cells (24). It is not known whether protein phosphatase 2A activity for MEK1/2 is regulated. Feedback inhibition of MEK1/2 activity may also occur by direct phosphorylation. Several protein kinases, including Cdc2 (25), ERK1/2 (9, 26–29), and Pak1 (30), have been shown to phosphorylate MEK1 at sites that are phosphorylated in intact cells. However, the impact of these phosphorylation events on the regulation of the

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** Investigator of the Canadian Institutes of Health Research. To whom correspondence should be addressed: Inst. de Recherches Cliniques de Montréal, 110 Pine Ave. West, Montreal, Quebec H2W 1R7, Canada. Tel.: 514-987-5783; Fax: 514-987-5536; E-mail: melochs@ircm.qc.ca.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK/MKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HEK, human embryonic kidney; HA, hemagglutinin; HPLC, high performance liquid chromatography; GST, glutathione *S*-transferase; HOG, high osmolarity glycerol.

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ERK1/2 pathway remains uncertain. Here, we show that MEK1 is phosphorylated at Ser[212] in intact cells. Substitution of Ser[212] with Ala enhanced the basal activity of MEK1 and MEK2, whereas phosphomimetic mutants completely inactivated the enzymes *in vivo*. We further show that mutations of the analogous Ser[212] residue in the yeast MAPKKs Pbs2p and Ste7p similarly regulate their biological activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Rat1 fibroblasts were cultured and synchronized by serum starvation as previously described (31). Rat1 cells were transfected with MEK1 expression plasmids using Lipofectin (Invitrogen). After 48 h, populations of stably transfected cells were selected by their ability to grow in complete minimum Eagle's medium containing 0.5 mg/ml Geneticin (Invitrogen). Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and were growth-arrested by serum starvation for 24 h. The cells were transiently transfected by the calcium phosphate precipitation method.

Plasmid Constructs and Mutagenesis—The sources of the plasmids used in this study were as follows: pGEX-2T/MEK2 (K.-L. Guan, University of Michigan, Ann Arbor, MI), pMT3-HA-SEK1 (J. Woodgett, Ontario Cancer Institute, Toronto, Canada), and pEF-Myc-MKK6 (A. Nebreda, European Molecular Biology Laboratory, Heidelberg, Germany). The plasmid pFA-Elk-1, which encodes a Gal4-Elk-1 fusion protein, and the Gal4-dependent luciferase reporter plasmid pFR-Luc were obtained from Stratagene.

The *Xba*I/*Hin*dIII fragment of pGEX-MEK1, containing the entire human MEK1 coding sequence (32), and the *Eco*RI/*Pvu*II fragment of pGEX-MEK2 (33), containing the human MEK2 coding sequence, were subcloned into pALTER-1 (Promega). To generate HA-tagged constructs of MEK1 and MEK2, a synthetic oligonucleotide encoding the amino acid sequence YDVPDYASL was inserted at the N terminus of the respective cDNAs (after the initiator methionine) using the Altered Sites *in vitro* mutagenesis system (Promega). HA-MEK1 and HA-MEK2 cDNA constructs were then used as templates for *in vitro* mutagenesis to generate the various mutants described in this study. All mutations were confirmed by DNA sequencing. The HA-MEK1 and HA-MEK2 constructs were subcloned into the expression vector pRc/CMV (Invitrogen).

Immunoblot Analysis and Protein Kinase Assays—Cell lysis, immunoprecipitation, and immunoblot analysis were performed as described previously (34). Commercial antibodies were obtained from the following suppliers: anti-phospho-Ser[218]/Ser[222] MEK1/2 (Cell Signaling Technology) and anti-MEK1 (Transduction Laboratories). Monoclonal antibody 12CA5 raised against influenza was a gift from M. Dennis (SignalGene). Immunoblot analysis of MEK1/2 activating loop phosphorylation was carried out according to the manufacturer's specifications. The phosphotransferase activities of endogenous or ectopically expressed MEK1 and MEK2 were assayed by measuring their ability to increase the myelin basic protein kinase activity of recombinant ERK2 *in vitro* as previously described (35).

Luciferase Reporter Gene Assays—For reporter gene assays, 293 cells seeded in 24-well plates were cotransfected with 1 μg of pFR-Luc reporter construct, 50 ng of pFA-Elk-1, 300 ng of pCMV-β-gal, and 1 μg of MEK1 expression plasmids. The total DNA amount was kept constant at 3 μg with the pRc/CMV vector. After 48 h, the cells were harvested, and the activity of luciferase was assayed using a luciferase reporter assay kit (Promega). Transfection efficiency was normalized by measuring β-galactosidase activity.

HPLC Purification and N-terminal Sequencing of Phosphopeptides—For analysis of phosphorylated peptides, 10 Petri dishes (100 mm) of HEK 293 cells were transfected with HA-MEK1, and two of the dishes were metabolically labeled for 6 h with 2 mCi/ml [^{32}P]phosphoric acid. Cell lysates were prepared, and HA-MEK1 was immunoprecipitated as described above. The immunoprecipitated proteins were resolved by SDS-gel electrophoresis, and the gel was stained with Coomassie Brilliant Blue R-250 and exposed to x-ray film. The protein band corresponding to ^{32}P-labeled HA-MEK1 was excised from the gel, subjected to dithiothreitol reduction and iodoacetamide alkylation, and then digested overnight at 37 °C with 0.2 μg of sequencing-grade trypsin (Promega) (36). The tryptic peptides were extracted with 1% trifluoroacetic acid and 60% acetonitrile at 60 °C and separated by reverse-phase HPLC on a Vydac microbore C_{18} column using an Applied Biosystems 130A separation system. The column was developed at a flow rate of 150 μl/min using the following gradient program: 3 min in solvent A (0.1% trifluoroacetic acid in water), 0–50% solvent B (0.08% trifluoroacetic acid in 70% acetonitrile) during the next 60 min, and 50–100% solvent B during the remaining 7 min. The peptides were detected by absorbance at 220 nm, and the peaks were collected manually and subjected to Cerenkov counting to identify the radioactive phosphopeptides. Where necessary, HPLC-purified tryptic peptides were subjected to a second digestion with sequencing-grade endoproteinase Asp-N (Roche Molecular Biochemicals). The HPLC fractions were incubated for a total time of 5 h at 37 °C with two additions of 0.1 μg of Asp-N protease. The labeled peptides were applied to a Prosorb disc (Applied Biosystems) and subjected to automatic Edman degradation on a Procise Model 494 cLC sequencer using the general protocol of Hewick *et al.* (37). The phenylthiohydantoin-derivatives were analyzed on-line using an Applied Biosystems Model 140D capillary separation system and ultraviolet detection.

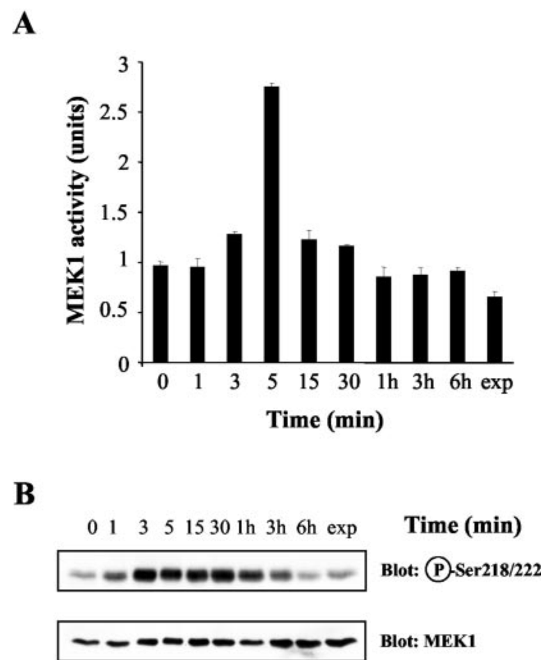

FIG. 1. **Time course of MEK1 activation and regulatory loop phosphorylation in response to serum.** *A*, quiescent Rat1 fibroblasts were stimulated with 10% serum for the times indicated. *exp*, exponentially proliferating cells. Cell lysates were prepared, and the activity of endogenous MEK1 was measured using an ERK2 reactivation assay. *B*, cell lysates were analyzed by sequential immunoblotting with a phospho-specific antibody to MEK1 activation loop residues Ser[218] and Ser[222] and with anti-MEK1 antibody. The results are representative of four different experiments.

Yeast Strains and Standard Methods—The yeast strains used in this study were W303-1AΔ*ste7* (*MAT***a** *ade2 leu2 trp1 his3 ura3* Δ*ste7*::*LEU2*) (B. Errede, University of North Carolina, Chapel Hill, NC), TM260 (*MAT***a** *ura3 leu2 trp1* Δ*pbs2*::*LEU2*) (H. Saito, Harvard Medical School, Boston, MA), YCW340 (*MAT***a** *ura3 leu2 his3 trp1 ssk2*::*LEU2 ssk22*::*LEU2 ste11*::*KanR*), YCW365 (*MAT***a** *ura3 leu2 his3 trp1 ssk2*::*LEU2 ssk22*::*LEU2 ste50*::*TRP1*) (38), and YGJ208 (*MAT***a** *ssk2*::*LEU2 ssk22*::*LEU2 sho1*::*TRP1*) (this study). Yeast cells were transformed by the method described (39), and the plasmid-containing cells were identified on selective plates. Mating of Δ*ste7* strains carrying the different *STE7* mutant alleles with the wild-type tester strain DC17 (MATα *his1*) (laboratory collection) was performed for 7 h before replicating the cells onto plates selecting for diploids. Cells with different *PBS2* mutant alleles were analyzed for osmosensitivity by transferring to rich medium containing 0.9 M NaCl and scoring growth after 3 days.

In Vivo Recombination and Construction of Mutant Plasmids—The construction of both *PBS2* and *STE7* plasmids and their mutant alleles was performed using the *in vivo* recombination procedure in the yeast *Saccharomyces cerevisiae* according to Jansen *et al.*[2] Two backbone plasmids (low copy number) with the promoter region and the N-terminal part of either *PBS2* or *STE7* were first constructed: 1) pGREG506-PBS2-N, containing 701 bp of the *PBS2* promoter region and the first 507 amino acids of *PBS2* coding sequence followed by an

[2] G. Jansen, C. Wu, B. Schade, D. Y. Thomas, and M. Whitney, submitted for publication.

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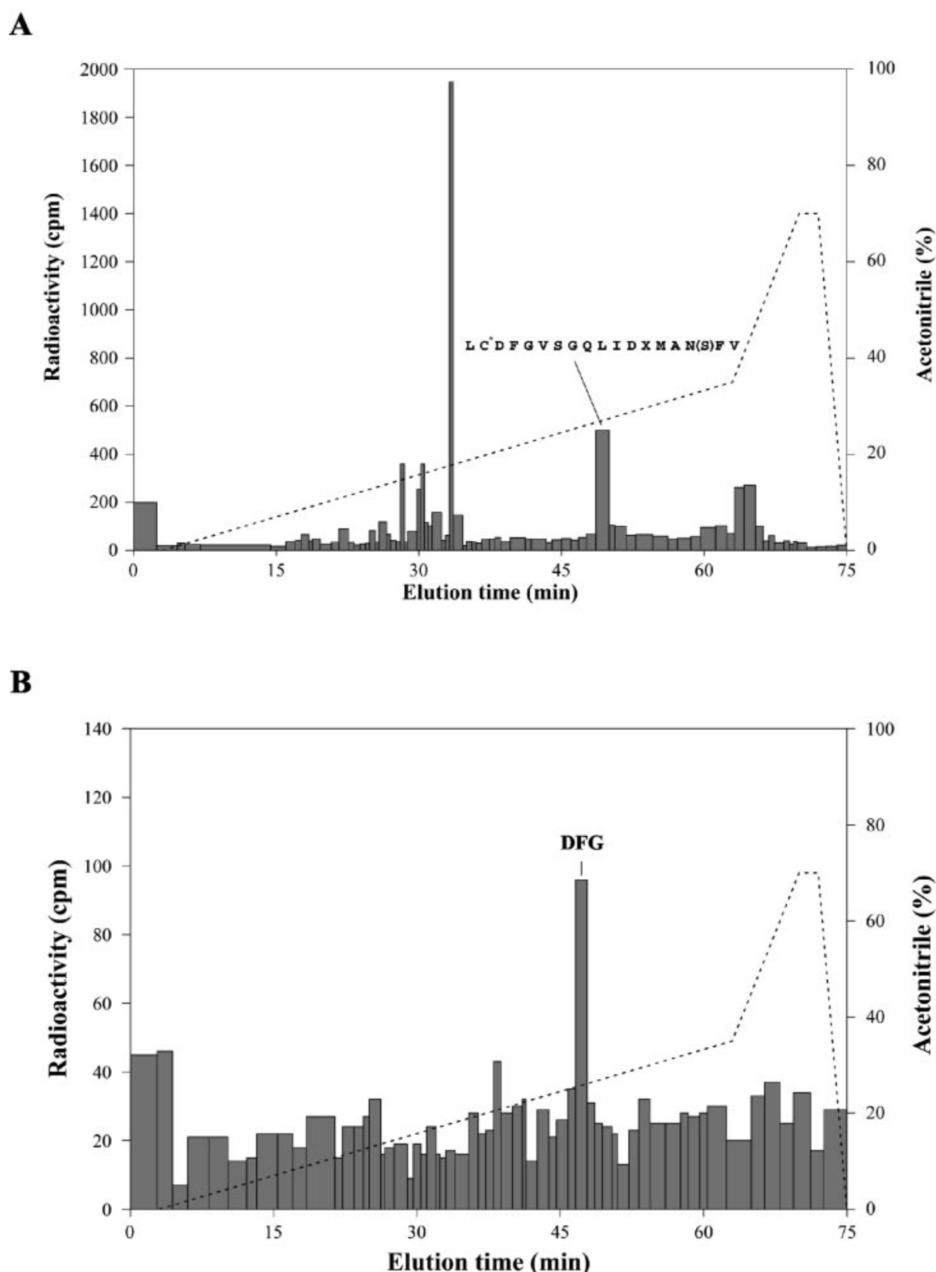

FIG. 2. **MEK1 is phosphorylated at Ser²¹² in intact cells.** *A*, HEK 293 cells transfected with HA-MEK1 were serum-starved for 24 h and metabolically labeled with [^{32}P]phosphoric acid. The ^{32}P-labeled HA-MEK1 protein was immunoprecipitated, resolved by SDS-gel electrophoresis, and digested in-gel with trypsin. The resulting peptides were then purified by reverse-phase HPLC. The radioactivity of each fraction was determined by Cerenkov counting. The sequence of the radioactive peptide eluting at 49 min was identified by automated Edman degradation and analysis of phenylthiohydantoin-derivatives. *B*, the purified tryptic peptide identified in *A* was further digested with endoproteinase Asp-N, and the digestion products were purified by HPLC. The sequence of the major ^{32}P-containing peptide was determined by automated amino acid sequencing.

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added unique *Not*I site and 2) pGREG506-STE7-N, containing 550 bp of the *STE7* promoter region and the first 352 amino acids of *STE7* coding sequence followed by a *Not*I site. To generate the mutant plasmid constructs by the *in vivo* recombination procedure, the backbone plasmids were first digested with *Not*I and *Xho*I and co-transformed into the appropriate yeast strain with the respective C-terminal parts of the genes carrying the desired mutations generated by PCR with mutant primers. The resulting mutants were sequenced to confirm the desired mutation and subcloned into the Gal1-GST yeast expression vector pGREG546 to verify the expression of the mutant proteins by anti-GST immunoblot analysis.

RESULTS

Transient Activation of MEK1 Contrasts with Sustained Regulatory Loop Phosphorylation in Mitogen-stimulated Cells—MEK1 is activated by phosphorylation at Ser^{218} and Ser^{222} in the regulatory loop between kinase subdomains VII and VIII. To better understand the regulation of MEK1 activity, we monitored the enzymatic activation and Ser^{218}/Ser^{222} phosphorylation of MEK1 after serum stimulation of Rat1 fibroblasts. Detailed kinetic analysis revealed that MEK1 activation was very transient, reaching a peak at 5 min and returning to near basal levels by 15–30 min (Fig. 1*A*). A similar transient activation of endogenous MEK1/2 has been observed in other cell types (Ref. 23 and data not shown). In contrast, the phosphorylation of activating Ser^{218}/Ser^{222} residues, which was maximally induced at 3 min, was sustained for at least 3 h after serum addition (Fig. 1*B*). These results indicate that mechanisms other than dephosphorylation of regulatory Ser^{218}/Ser^{222} residues must contribute to inactivation of MEK1.

MEK1 Is Phosphorylated at Ser^{212} in Vivo—Phosphopeptide mapping analysis has revealed that MEK1 is phosphorylated on multiple peptides in both quiescent and serum-stimulated cells (Refs. 17 and 26 and data not shown), suggesting that phosphorylation of residues other than the Ser^{218}/Ser^{222} activation loop may also be involved in the regulation of the kinase. We initiated a series of experiments to identify new regulatory phosphorylation sites of MEK1. HEK 293 cells were transfected with HA-MEK1 and deprived of serum for 24 h. The cells were then metabolically labeled with [^{32}P]orthophosphate for 5 h, and ectopically expressed MEK1 was immunoprecipitated with anti-HA antibody. After resolution by SDS-gel electro-

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                          VII  212                                            VIII
Human MEK1           L C D F G V S G Q L I D - - S M A N S F - V G T R S Y M S P E
Human MEK2           L C D F G V S G Q L I D - - S M A N S F - V G T R S Y M A P E
Human MEK3           M C D F G I S G Y L V D - - S V A K T M D A G C K P Y M A P E
Human MEK4           L C D F G I S G Q L V D - - S I A K T R D A G C R P Y M A P E
Human MEK5           L C D F G V S T Q L V N - - S I A K T Y - V G T N A Y M A P E
Human MEK6           M C D F G I S G Y L V D - - S V A K T I D A G C K P Y M A P E
Human MEK7           L C D F G I S G R L V D - - S K A K T R S A G C A A Y M A P E
Mouse MEK1           L C D F G V S G Q L I D - - S M A N S F - V G T R S Y M S P E
Mouse MEK2           L C D F G V S G Q L I D - - S M A N S F - V G T R S Y M S P E
Drosophila DSOR1     I C D F G V S G Q L I D - - S M A N S F - V G T R S Y M S P E
C. elegans MEK-2     L C D F G V S G M L I D - - S M A N S F - V G T R S Y M A P E
X. laevis MEK        L C D F G V S G Q L I D - - S M A N S F - V G T R S Y M S P E
S. pombe PEK1        L C D F G V S G E L V N - - S L A G T F - T G T S Y Y M A P E
S. cerevisiae PBS2   L C D F G V S G N L V A - - S L A K T N - I G C Q S Y M A P E
S. cerevisiae STE7   L C D F G V S K K L I N - - S I A D T F - V G T S T Y M S P E
A. thaliana MEK1     I T D F G V S K I L T S T S S L A N S F - V G T Y P Y M S P E

Human ERK2           I C D F G L A R V A D P - - T E Y - - - - V A T R W Y R A P E
Human c-Raf-1        I G D F G L A T V K S R - - S G S Q Q V - T G S V L W M A P E
Human PKA            V T D F G F A K R V K G R - T W T L - - - C G T P E Y L A P E
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FIG. 3. **Conservation of Ser[212] in the MAPKK family.** Shown is an alignment of amino acid sequences between subdomains VII and VIII from MAPKK family members and related protein kinases. Residues equivalent to Ser[212] of MEK1 are indicated. *PKA*, cAMP-dependent protein kinase.

phoresis, the ^{32}P-labeled MEK1 protein band was cut from the gel, alkylated, and subjected to complete in-gel trypsin digestion. The resulting tryptic peptides were separated by reverse-phase HPLC, and the fractions recovered were counted for radioactivity (Fig. 2*A*). The radioactive fractions were subjected to automated Edman degradation, and the phenylthiohydantoin-derivatives were analyzed using a sensitive capillary separation system. The fraction eluting at 49 min was found to contain the peptide LCDFGVSGQLIDXMAN(S)FV, which corresponds to the tryptic fragment Leu^{206}–Arg^{227} of the human MEK1 sequence (Fig. 2*A*). This peptide contains four potential phosphorylation sites: Ser^{212}, Ser^{218}, Ser^{222}, and Thr^{226}. To refine our analysis, the HPLC fractions containing the Leu^{206}–Arg^{227} fragment were pooled and subjected to a second digestion with endoproteinase Asp-N, which cleaves before aspartate residues. Analysis of Asp-N digestion product by HPLC revealed the presence of a major radioactive peak (Fig. 2*B*). N-terminal sequencing of this peak yielded the sequence DFG, which corresponds to the double-digested peptide Asp^{208}–Ile^{216}. The only phosphorylatable residue within this peptide is Ser^{212}. These results unambiguously demonstrate that MEK1 is phosphorylated at Ser^{212} *in vivo*.

Ser^{212} Regulates the Activity of MEK1 and MEK2—Alignment of MAPKK sequences from different species revealed that Ser^{212}, which lies in the activation loop between kinase subdomains VII and VIII, is conserved in all members of the MAPKK family from yeast to mammals (Fig. 3). However, this residue is not found in Raf MAPKKKs, MAPKs, cyclin-dependent kinases, or cAMP-dependent protein kinase. Notably, replacement of Ser^{212} with aspartic acid was shown to completely abolish the basal kinase activity of MEK1 *in vitro* (40). To evaluate the impact of Ser^{212} on the regulation of MEK1 activity in intact cells, we generated a series of MEK1 mutants by site-directed mutagenesis. The various HA-MEK1 constructs were transiently expressed in HEK 293 cells, and their phosphotransferase activity was measured using a specific ERK2 reactivation assay. Immunoblotting of total cell extracts with anti-HA antibody confirmed that all mutants were expressed to similar levels (Fig. 4*A*). Replacement of Ser^{212} with alanine significantly enhanced the enzymatic activity of MEK1 (from 3- to 5-fold) in exponentially growing HEK 293 cells, whereas mutation to the phosphomimetic acidic residue aspartate completely abolished it (Fig. 4*A*). As previously reported, substitution of the activating phosphorylation sites Ser^{218} and Ser^{222} with acidic residues (S218D/S222D) strongly potentiated the activity of MEK1, whereas substitution with alanine residues (S218A/S222A) impaired activation. Replacement of Ser^{212} with alanine did not further enhance the activity of the MEK1(S218D/S222D) mutant, nor did it rescue the compromised activation of the S218A/S222A mutant. However, substitution of Ser^{212} with aspartate completely abrogated the constitutive activation of the MEK1(S218D/S222D) mutant. We also tested whether the equivalent Ser^{216} residue of the related MAPKK MEK2 had similar regulatory effects. As shown in Fig. 4*B*, replacement of Ser^{216} with alanine increased the basal activity of MEK2, whereas the aspartate mutation completely suppressed the activation of wild-type MEK2 and the constitutively activated MEK2(S222D/S226D) mutant.

To examine the functional consequences of MEK1 regulation by Ser^{212}, we tested the ability of MEK1 mutants to potentiate the transcriptional activation of the ERK1/2 target Elk-1 in exponentially growing HEK 293 cells. Under these experimental conditions, Elk-1-dependent reporter activity was not significantly enhanced by expression of the wild-type MEK1 protein (Fig. 4*C*). However, expression of the MEK1(S212A) mutant caused a small but reproducible 2-fold stimulation of Elk-1 transcriptional activity. In agreement with the results of enzymatic assays, transfection of activated MEK1(S218D/S222D) strongly potentiated Elk-1-dependent transcription, and this effect was completely prevented by substitution of Ser^{212} with a phosphomimetic Asp residue.

To further investigate the role of Ser^{212} in the regulation of MEK1 activity, we generated populations of Rat1 fibroblasts stably expressing HA-MEK1 Ser^{212} mutants. The cells were made quiescent by serum starvation and restimulated for different period of times with serum, and the activity of ectopically expressed MEK1 was measured. Similar to the endogenous protein, activation of ectopic MEK1 was transient, reaching a peak at 5 min and returning to basal levels by 30 min (Fig. 5*A*). The MEK1(S212A) mutant displayed constitutive activity in serum-deprived cells. Stimulation with serum induced a further increase in MEK1(S212A) activity at 5 min, which declined thereafter, but remained elevated for at least 24 h. Mutation of Ser^{212} to Asp lowered the basal activity of MEK1 and abrogated activation of the enzyme by serum growth factors. Immunoblot analysis confirmed that the mutants were expressed at levels comparable to the wild-type protein (Fig. 5*B*). These results are consistent with the idea that phosphorylation of Ser^{212} plays a role in the regulation of MEK1/2 activity and of downstream signaling events.

Mutations of Ser^{212} Do Not Modulate Activating Loop Phosphorylation of MEK1 or Binding to ERK2—MEK1 is activated by phosphorylation of Ser^{218}/Ser^{222} in the activation loop. We therefore tested whether the effects of Ser^{212} mutations on MEK1 activity could be related to differences in activating phosphorylation of the kinase. For these studies, HA-MEK1 constructs were transiently expressed in HEK 293 cells. The cells were serum-starved and restimulated with serum for 5

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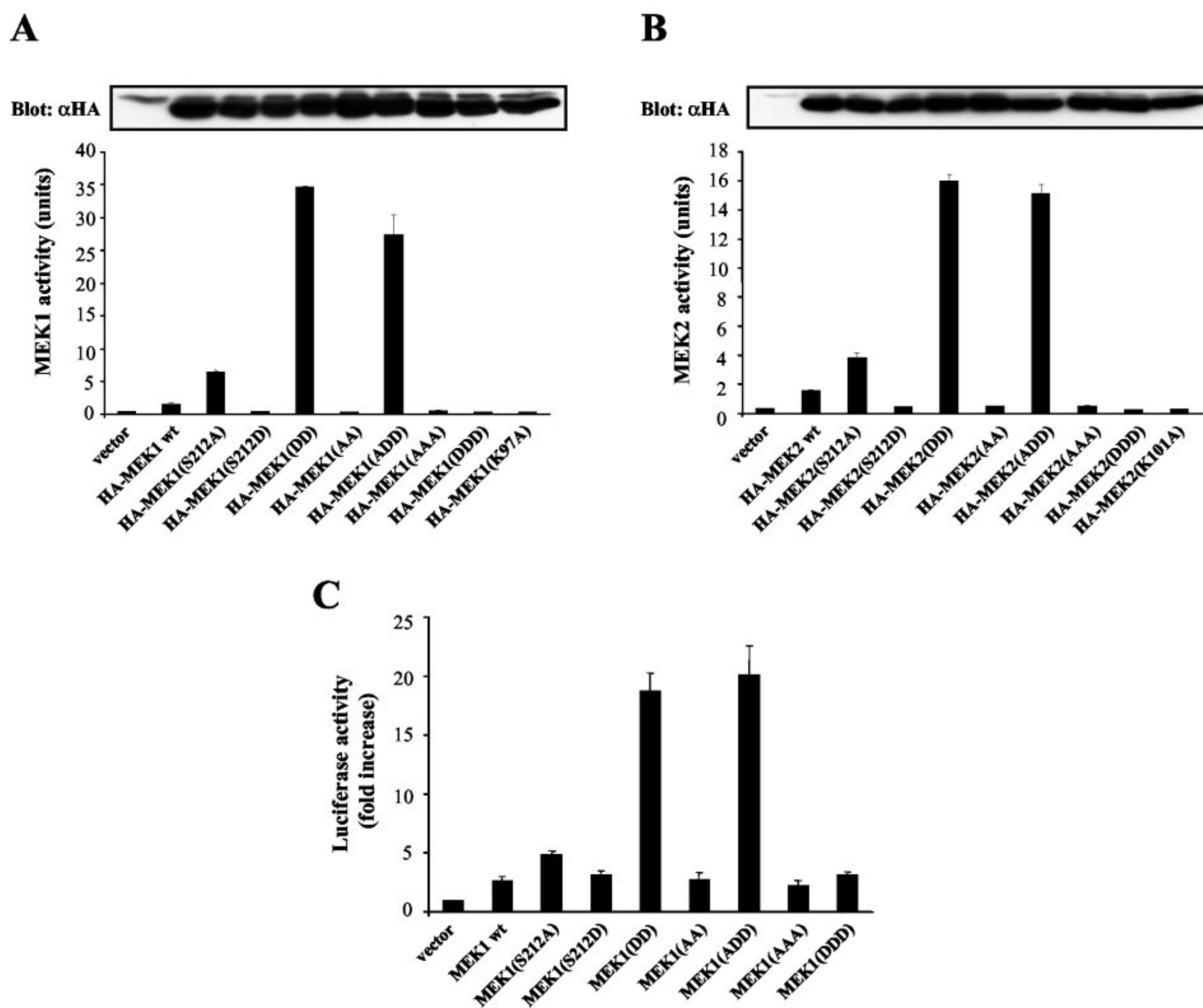

FIG. 4. **Ser^{212} regulates the biological activity of MEK1 and MEK2.** *A* and *B*, HEK 293 cells were transiently transfected with HA-tagged MEK1 or MEK2 constructs, respectively. After 48 h, the ectopically expressed MEK protein was immunoprecipitated with anti-HA antibody (*αHA*), and phosphotransferase activity was measured using an ERK2 reactivation assay. Expression of HA-tagged MEK1 and MEK2 proteins was analyzed by immunoblotting with anti-HA antibody. *C*, HEK 293 cells were transfected with expression plasmids for wild-type (*wt*) MEK1 or the indicated mutants in combination with Gal4-Elk-1 and the Gal4-dependent luciferase reporter gene. After 48 h, the activity of luciferase was measured and normalized to that of β-galactosidase. Results are presented as -fold activation over vector-transfected cells. All results are representative of four different experiments. MEK1 mutants: *DD*, S218D/S222D; *AA*, S218A/S222A; *ADD*, S212A/S218D/S222D; *AAA*, S212A/S218A/S222A; and *DDD*, S212D/S218D/S222D.

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min, and the phosphorylation of MEK1 at Ser^{218}/Ser^{222} was analyzed by immunoblotting using a phospho-specific antibody. Mutation of Ser^{212} to Ala or Asp did not affect the phosphorylation of MEK1 at activating Ser^{218}/Ser^{222} residues in serum-stimulated cells (Fig. 6*A*).

We also investigated whether Ser^{212} mutations interfere with the ability of MEK1 to bind its substrates ERK1 and ERK2. Cell extracts prepared from HEK 293 cells transiently transfected with HA-MEK1 constructs were incubated with His_6-ERK2 beads, and the resulting complexes were analyzed by anti-HA immunoblotting. No differences were observed in the abilities of the various MEK1 mutants to bind ERK2 in this pull-down assay (Fig. 6*B*). Similar results were obtained in co-immunoprecipitation experiments (data not shown). These observations indicate that Ser^{212} mutations are unlikely to alter the global three-dimensional structure of the MEK1 enzyme. They also demonstrate that the inactivation of MEK1 observed upon mutation of Ser^{212} to a phosphomimetic residue cannot be explained by inhibition of activating loop phosphorylation or by interference with substrate binding.

Mechanism of MAPKK Inactivation by Phosphorylation Is Conserved in Yeast—To determine whether the inhibitory mechanism of MAPKK regulation by phosphorylation has been conserved during evolution, we extended our studies to the yeast *S. cerevisiae STE7* and *PBS2* MAPKK genes (41). The *STE7* and *PBS2* gene products, Ste7p and Pbs2p, display significant amino acid sequence identity to mammalian MEK1/2. Ser^{212} in MEK1 corresponds to Ser^{353} in Ste7p and Ser^{508} in Pbs2p (Fig. 3). Mutations of the corresponding serine residues in Ste7p and Pbs2p were made by site-directed mutagenesis, and the resulting mutants were subcloned into a low copy yeast shuttle plasmid vector by *in vivo* recombination in yeast. The function of these alleles was tested in yeast strain W303-1AΔ*ste7* for *STE7*-related functions and in yeast strain TM260 for *PBS2*-related functions.

The mating ability of the yeast *S. cerevisiae* requires the function of Ste7p. Strain W303-1AΔ*ste7* has no functional *STE7* and therefore is unable to mate with a partner of opposite mating type. Transformation of the wild-type *STE7* gene into strain W303-1AΔ*ste7* restores the mating ability of the cells, whereas the empty vector does not. Mutation of Ste7p Ser^{353} to alanine had no significant effect on mating efficiency (Fig. 7*A*). However, substitution of Ser^{353} with a phosphomimetic aspartate residue led to a sterile phenotype, suggesting that Ste7p(S353D) is nonfunctional. To rule out the possibility that Ste7p(S353D) is not expressed or has decreased stability,

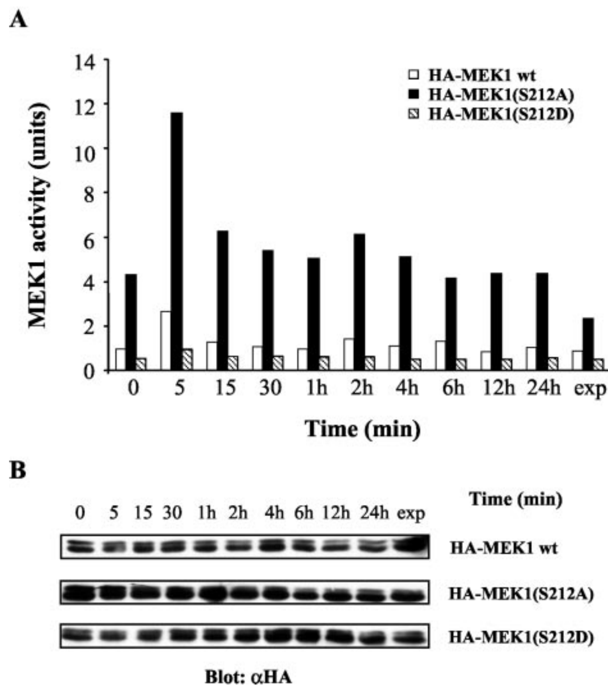

FIG. 5. **Effect of Ser²¹² mutation on the kinetics of MEK1 activation by serum.** *A*, populations of Rat1 cells stably expressing wild-type (*wt*) HA-MEK1 or HA-MEK1 Ser[212] mutants were made quiescent and restimulated with 10% serum for the indicated times. The enzymatic activity of the ectopically expressed MEK1 constructs was assayed as described above. *B*, expression of MEK1 proteins was analyzed by immunoblotting with anti-HA antibody (*αHA*). Results are representative of three different experiments.

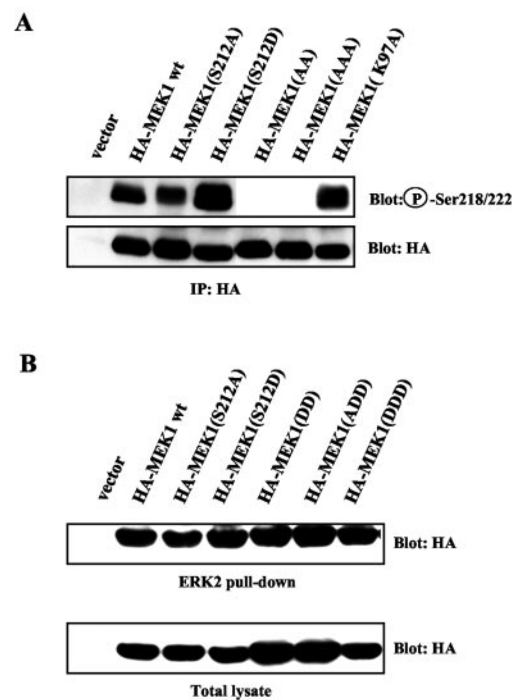

FIG. 6. **Effect of Ser²¹² mutation on activating phosphorylation of MEK1 and binding to ERK2 substrate.** *A*, HEK 293 cells were transiently transfected with HA-MEK1 constructs. The cells were serum-starved for 24 h and then restimulated with serum for 5 min. The ectopically expressed MEK1 protein was immunoprecipitated (*IP*) with anti-HA antibody and analyzed by immunoblotting with anti-phospho-Ser[218]/Ser[222] MEK1/2 and anti-HA antibodies. *B*, cell extracts from HEK 293 cells transfected with HA-MEK1 constructs were incubated with immobilized recombinant His_6-ERK2 fusion protein. ERK2 complexes were pulled-down with cobalt-agarose beads and analyzed by immunoblotting with anti-HA antibody. Expression of the various MEK1 mutants in total cell lysates was comparable. Results are representative of three different experiments. *wt*, wild-type; *AA*, S218A/S222A; *AAA*, S212A/S218A/S222A; *DD*, S218D/S222D; *ADD*, S212A/S218D/S222D; *DDD*, S212D/S218D/S222D.

the wild-type and mutant versions of Ste7p were expressed in yeast as GST fusion proteins and analyzed by immunoblotting. The results confirm that both the S353D and S353A mutants have steady-state levels of expression similar to those of wild-type Ste7p (data not shown).

Corresponding mutations were also made in the *PBS2* gene. The Pbs2p signaling pathway is required for the hyperosmolarity stress response, and cells defective in Pbs2p function are unable to grow on hyperosmotic medium. The sensitive yeast strain TM260 was transformed with different alleles of *PBS2*, and the transformants were tested for their ability to grow on hyperosmotic medium. Wild-type Pbs2p allowed the growth of the hyperosmolarity-sensitive cells on medium containing 0.9 M NaCl, and the S508A mutant displayed a similar phenotype (Fig. 7*A*). In contrast, replacement of Ser[508] with an aspartate residue blocked the growth of TM260 cells on hyperosmotic medium, suggesting that the S508D mutation, similar to the corresponding mutation in *STE7*, results in a nonfunctional allele of the MAPKK protein. This loss of function was not due to differences in expression levels, as both wild-type and mutant Pbs2p-GST fusion proteins were expressed at comparable levels (data not shown).

Pbs2p Negative Regulatory Phosphorylation Site Ser⁵⁰⁸ Acts in a Dominant Manner—It has been shown that substitution of Ser[514] and Thr[518] with phosphomimetic amino acid residues (either Glu or Asp) leads to constitutively activated forms of Pbs2p (42). We changed these two residues to aspartate residues to obtain a constitutively activated Pbs2p kinase (Pbs2p(S514D/T518D)). Unlike wild-type Pbs2p, whose activity requires at least one of the upstream activating kinases Ssk2p, Ssk22p, or Ste11p, Pbs2p(S514D/T518D) was able to activate the HOG pathway independent of these activating kinases. To assess the regulatory effect of Ser[508] on the constitutively activated Pbs2p(S514D/T518D) mutant, substitution of Ser[508] with either Ala or Asp was made in combination with the S514D/T518D mutation. The resulting constructs were transformed into strains YCW340 (Δ*ssk2* Δ*ssk22* Δ*ste11*), YCW365 (Δ*ssk2* Δ*ssk22* Δ*ste50*), and YGJ208 (Δ*ssk2* Δ*ssk22* Δ*sho1*) and tested for activation of the HOG pathway. As shown in Fig. 7*B*, the S508D mutation completely blocked the ability of Pbs2p(S514D/T518D) to activate the HOG pathway, whereas no significant effect of the S508A mutation was observed on Pbs2p(S514D/T518D) activity. These results indicate that the regulatory effect of Ser[508] is dominant over the effect of activating phosphorylation of Pbs2p at Ser[514] and Thr[518].

To test whether mutation of the dominant inhibitory phosphorylation site Ser[508] to alanine is sufficient to render Pbs2p constitutively activated, wild-type Pbs2p, Pbs2p(S508A), and Pbs2p(S508D) were transformed into strains YCW340, YCW365, and YGJ208 and assayed for activation of the HOG pathway. As expected, no HOG pathway activity was observed in any strain transformed with Pbs2p(S508D) under all conditions tested (Fig. 7*B*). This is consistent with previous observations that *SHO1-STE11/STE50* signaling is essential in the absence of the *SLN1* two-component osmosensor branch that activates the MAPKKKs Ssk2p and Ssk22p (38, 43). However, Pbs2p(S508A) displayed a significant increase in *SHO1*-independent HOG pathway activity, as judged by the ability of cells to grow in medium containing 0.9 M NaCl. However, this activity was not observed when either *STE11* or *STE50* was deleted in the absence of *SSK2* and *SSK22*. Thus, the HOG pathway activity observed was *SHO1*-independent, but *STE11*- and *STE50*-dependent.

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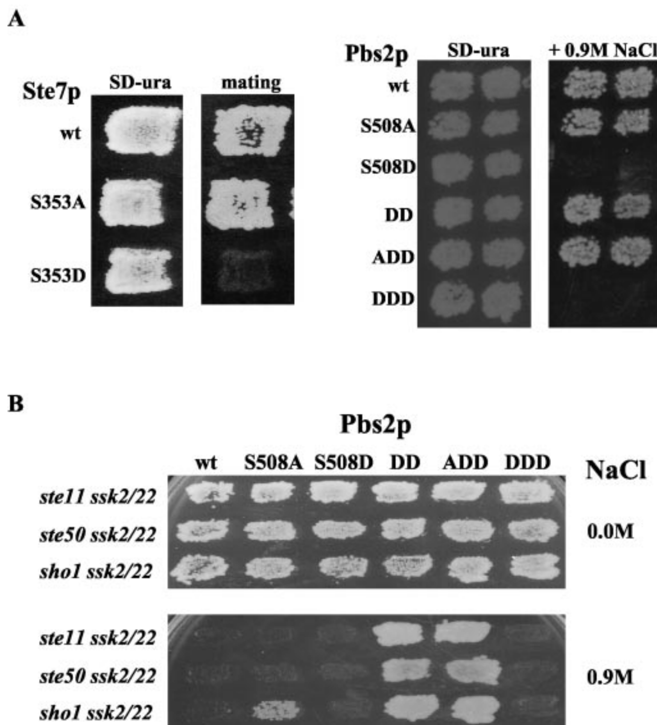

FIG. 7. **Regulation of Ste7p and Pbs2p function *in vivo*.** *A*, *left panels*, complementation assay of different alleles of Ste7p in the mating pheromone-response pathway. Δ*ste7* strains carrying different *STE7* mutant alleles were mated with the wild-type tester strain DC17, and diploids were selected. *Right panels*, complementation assay of different alleles of Pbs2p in the hyperosmolarity-response pathway. Δ*pbs2* cells bearing different *PBS2* alleles were analyzed for growth on medium containing 0.9 M NaCl. *B*, functional analysis of the regulatory effect of Ser[508] of Pbs2p and its relationship with the upstream activator kinase. Yeast cells of strains YCW340 (*ssk2*::*LEU2 ssk22*::*LEU2 ste11*::*KanR*; no MAPKKK for Pbs2p), YCW365 (*ssk2*::*LEU2 ssk22*::*LEU2 ste50*::*TRP1*; no activable MAPKKK for Pbs2p), and YGJ208 (*ssk2*::*LEU2 ssk22*::*LEU2 sho1*::*TRP1*; no membrane osmosensor) bearing different *PBS2* alleles were tested for osmosensitivity on medium containing the indicated concentrations of NaCl. All MAPKK constructs were expressed from their own promoters in low copy centromere plasmids. The results are representative of three to four different experiments. *SD-ura*, synthetic dextrose with uracil; *wt*, wild-type; *DD*, S514D/T518D; *ADD*, S508A/S514D/T518D; *DDD*, S508D/S514D/T518D.

DISCUSSION

Enzymatic activation of MEK1 requires phosphorylation of Ser[218] and Ser[222] in the activation loop (7, 8). However, the mechanisms responsible for MEK1/2 inactivation remain to be established. Our observation that sustained phosphorylation of MEK1 at regulatory Ser[218]/Ser[222] residues contrasts with the transient nature of MEK1 activation in Rat1 fibroblasts led us to believe that mechanisms other than the simple involvement of protein phosphatases are involved in MEK1 inactivation. MEK1 is phosphorylated on multiple peptides in cells, suggesting that phosphorylation of residues other than Ser[218] and Ser[222] might be involved in other aspects of MEK1 regulation (17, 26). Here, we have reported that MEK1 is phosphorylated at Ser[212] in intact cells. Importantly, we have provided biochemical and genetic evidence that phosphorylation of the equivalent Ser[212] residue in human MEK1 and MEK2 and in the yeast MAPKKs Ste7p and Pbs2p negatively regulates enzymatic activity *in vivo*. These findings suggest that both activation and inactivation of MAPKK family members are mediated by common evolutionarily conserved mechanisms.

Replacement of Ser[212] with acidic residues does not prevent activating phosphorylation of MEK1 at Ser[218]/Ser[222], nor does it affect binding to ERK2 substrate, thereby suggesting that Ser[212] phosphorylation may directly interfere with the catalytic reaction. Consistent with this hypothesis, a previous study has shown that substitution of Ser[212] with aspartate completely abolishes the basal kinase activity of MEK1 for exogenous substrates *in vitro* (40). Conversely, replacement of Ser[212] was alanine was shown to increase the rate of autophosphorylation of recombinant MEK1 (44) and to enhance the basal phosphotransferase activity of MEK1-GST by 3–4-fold (8) in *in vitro* kinase assays. We also observed that the equivalent S212A mutation significantly increases the enzymatic activity of MEK1 and MEK2 in intact cells (Fig. 4). It is noteworthy that Ser[212] is localized within the activation loop of MEK1, close to the activating phosphorylation sites. Although Ser[212] phosphorylation does not interfere with phosphorylation of Ser[218]/Ser[222], the presence of an additional phosphate group might compete for or establish undesirable electrostatic interactions with one or more basic residues in the catalytic domain. Thus, Ser[212] phosphorylation may hinder the correct positioning of the aspartate residue essential for catalysis or perturb the conformation of the activation loop, blocking access of the substrate to the active site. Given the evolutionarily conserved nature of the MAPKK family, elucidation of the crystal structure of MEK1 in the inactive and active conformations will add greatly to our understanding of the mechanisms controlling both activation and inactivation of this family of enzymes.

Studies by different groups have shown that MEK1 is also phosphorylated at Thr[292], Ser[298], and Thr[386] *in vivo* (9, 25–28, 30). However, the exact biological consequences of these phosphorylation events remain to be established. It has been suggested that the MAPKs ERK1 and ERK2 phosphorylate MEK1 at Thr[292]/Thr[386] and inhibit its activation by a negative feedback mechanism (26). In contrast, another study reported that the MEK1(T292A) mutant is inactivated more rapidly than wild-type MEK1 in serum-stimulated cells (17). We did not observe any effect of the T292A mutation on MEK1 activity in exponentially growing 293 cells (data not shown). In a more recent study, it was reported that Akt phosphorylates MKK4 at Ser[78] and negatively regulates its activity by interfering with substrate binding (45). MKK4 is the only member of the mammalian MAPKK family that has a consensus Akt phosphorylation motif. It is likely that MAPKKs are regulated by phosphorylation mechanisms common to all members as well as by more subtle mechanisms that allow differential regulation of individual isoforms. Identification of the physiological kinases and phosphatases that control the phosphorylation level of Ser[212] and other regulatory sites will be necessary for a complete understanding of MAPKK regulation.

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Negative Regulation of MAPKK by Phosphorylation of a Conserved Serine Residue Equivalent to Ser ^{212} of MEK1

Kailesh Gopalbhai, Gregor Jansen, Geneviève Beauregard, Malcolm Whiteway, France Dumas, Cunle Wu and Sylvain Meloche

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