Negative Regulation of MAPKKK by Phosphorylation of a Conserved Serine Residue Equivalent to Ser212 of MEK1*

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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 Ser212, a residue conserved among all MAPKK family members. Mutation of Ser212 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 Ser212 did not interfere with activating phosphorylation of MEK1 at Ser218/Ser222 or with binding to ERK2 substrate. Importantly, mimicking phosphorylation of the equivalent Ser215 residue of the yeast MAPKKs Pbs2p and Ste7p similarly abrogated their biological function. Our findings suggest that Ser212 phosphorylation represents an evolutionarily conserved mechanism involved in the negative regulation of MAPKKs.

Mitogen-activated protein kinase (MAPK) 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 MEKK), 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 Ser215 and Ser222 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
ERK1/2 pathway remains uncertain. Here, we show that MEK1 is phosphorylated at Ser212 in intact cells. Substitution of Ser212 with Ala enhanced the basal activity of MEK1 and MEK2, whereas phosphomimetic mutants completely inactivated the enzymes in vitro. We further show that mutations of the analogous Ser212 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 minimal 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-HEK-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 XbaI/HindIII fragment of pGEX-MEK1, containing the entire human MEK1 coding sequence (32), and the EcoRI/PvuII 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 YDYPSDAS 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-Ser218/Ser222 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 enzyme 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 μCi/ml [32P]phosphoric acid. Cell lysates were prepared, and HA-MEK1 was immunoprecipitated as described above. The immunoprecipitated proteins were resolved by SDS-PAGE, electroblotted, and the gel was stained with Coomassie Brilliant Blue R-250 and exposed to x-ray film. The protein band corresponding to Ser212-phosphorylated 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 in 70% acetonitrile at 60 °C and separated by reversed-phase HPLC on a Vydac micro bore C18 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 Proisorb 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, exposed 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 Ser212 and Ser222 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 W303-1AΔade2Δleu2Δtrp1Δhis3Δατa1ΔLEU2 (B. Errede, University of North Carolina, Chapel Hill, NC), TM260 (MATaΔura3Δleu2Δtrp1Δβα2ΔLEU2) (H. Saito, Harvard Medical School, Boston, MA), YCW340 (MATaΔura3Δleu2Δhis2Δtrp1Δskk2ΔLEU2Δαα22ΔLEU2ΔstI1ΔKanR), YCW305 (MATaΔura3Δleu2Δhis3Δtrp1Δskk2ΔLEU2Δskk22ΔLEU2Δst60ΔTRP1) (38), and YGJ208 (MATaΔskk2ΔLEU2Δskk22ΔLEU2Δshol1ΔTRP1) (this study). Yeast cells were transformed by the method described (39), and the plasmid-containing cells were identified on selective plates. Mating of Δατa1 strains carrying the different STE7 mutant alleles with the wild-type tester strain DC17 (MATaΔhis1Δ) (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. (4). Two backbone plasmids (low copy number) with the promoter region and the N-terminal part of either PBS2 or STE7 were first constructed: 1) pREG506-PBS2-N, containing 701 bp of the PBS2 promoter region and the first 507 amino acids of PBS2 coding sequence followed by an

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

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added unique NotI 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 NotI site. To generate the mutant plasmid constructs by the in vivo recombination procedure, the backbone plasmids were first digested with NotI and XhoI 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 Ser218 and Ser222 in the regulatory loop between kinase subdomains VII and VIII. To better understand the regulation of MEK1 activity, we monitored the enzymatic activation and Ser218/Ser222 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. 1A). 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 Ser218/Ser222 residues, which was maximally induced at 3 min, was sustained for at least 3 h after serum addition (Fig. 1B). These results indicate that mechanisms other than dephosphorylation of regulatory Ser218/Ser222 residues must contribute to inactivation of MEK1.

MEK1 Is Phosphorylated at Ser212 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 Ser218/Ser222 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 [32P]orthophosphate for 5 h, and ectopically expressed MEK1 was immunoprecipitated with anti-HA antibody.
phosphorylation, 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. 2A). The radioactive fractions were subjected to automated Edman degradation, and the phenylthiohydantoindervatatives were analyzed using a sensitive capillary separation system. The fraction eluting at 49 min was found to contain the peptide LCDFGVSQGLIDXMAN(S)FV, which corresponds to the tryptic fragment Leu-206–Arg-227 of the human MEK1 sequence (Fig. 2A). 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. 2B).

To further investigate the role of Ser-212 in the regulation of MEK1 activity, we generated populations of Rat1 fibroblasts stably expressing HA-MEK1 Ser212 mutants. The cells were made quiescent by serum starvation and restimulated for different periods 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. 5A). The MEK1(S212A) mutant displayed constitutive activation of the MEK1/2 activity and of downstream signaling events.

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**Fig. 3. Conservation of Ser212 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 Ser212 of MEK1 are indicated. PKA, cAMP-dependent protein kinase.
min, and the phosphorylation of MEK1 at Ser\textsuperscript{218}/Ser\textsuperscript{222} was analyzed by immunoblotting using a phospho-specific antibody. Mutation of Ser\textsuperscript{212} to Ala or Asp did not affect the phosphorylation of MEK1 at activating Ser\textsuperscript{218}/Ser\textsuperscript{222} residues in serum-stimulated cells (Fig. 6A).

We also investigated whether Ser\textsuperscript{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\textsubscript{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. 6B). Similar results were obtained in co-immunoprecipitation experiments (data not shown). These observations indicate that Ser\textsuperscript{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\textsuperscript{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\textsuperscript{212} in MEK1 corresponds to Ser\textsuperscript{353} in Ste7p and Ser\textsuperscript{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 W303-1A/*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 W303-1AΔste7 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 W303-1AΔste7 restores the mating ability of the cells, whereas the empty vector does not. Mutation of Ste7p Ser\textsuperscript{353} to alanine had no significant effect on mating efficiency (Fig. 7A). However, substitution of Ser\textsuperscript{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,
type (HA-MEK1 or HA-MEK1 Ser212 mutants) were made quiescent by serum. A by immunoblotting with anti-HA antibody (H9251) assayed as described above. Expression of MEK1 proteins was analyzed by immunoblotting with anti-HA antibody (aHA). Results are representative of three different experiments.

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 gene. Corresponding mutations were also made in the STE7 gene.

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**In a Dominant Manner**—It has been shown that substitution of SerT114 and ThrT118 with phosphomimetic amino acid residues (either Ghu or Asp) leads to constitutively activated forms of Pbs2p (42). We changed these two residues to aspartate residues of both wild-type and mutant Pbs2p-GST fusion proteins were expressed at comparable levels (data not shown).

Pbs2p Negative Regulatory Phosphorylation Site SerT508 Acts in a Dominant Manner—It has been shown that substitution of SerT514 and ThrT518 with phosphomimetic amino acid residues (either Ghu or Asp) leads to constitutively activated forms of Pbs2p (42). We changed these two residues to aspartate residues of both wild-type and mutant Pbs2p-GST fusion proteins were expressed at comparable levels (data not shown).

**FIG. 5.** Effect of Ser212 mutation on the kinetics of MEK1 activation by serum. A, populations of Ral1 cells stably expressing wild-type (wt) HA-MEK1 or HA-MEK1 Ser212 mutants were made quiescent and restimulated with 10% serum for the indicated times. The activated Pbs2p(S514D/T518D) mutant, substitution of Ser 508 with either Ala or Asp was made in combination with 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).

**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-SerT222 MEK1/2 and anti-HA antibodies. B, cell extracts from HEK 293 cells transfected with HA-MEK1 constructs were incubated with immobilized recombinant His6-ERK2 fusion protein. ERK2 complexes were pulled-down with covalt-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 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).

The Pbs2p signaling pathway is required for the hyperosmolarity stress response, and cells defective in Pbs2p function are unable to grow on hyperosmotic medium. Wild-type Pbs2p allowed the growth of TM260 cells on medium containing 0.9M NaCl, and the S508A mutant displayed a similar phenotype (Fig. 7A). In contrast, replacement of SerT508 with an aspartate residue blocked the growth of TM260 cells on hyperosmotic medium, suggesting that the S508D mutation, similar to the corresponding mutation in ST7, 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).

**FIG. 7.** A, 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).
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**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 MEKK1 and MEKK2 and in the yeast MAPKKS Ste7p and Pbs2p negatively regulates enzymatic activity in *vivo*. These findings suggest that both activation and inactivation of MAPK 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\(^{217}\)/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 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\(^{218}\) 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.

**Acknowledgments**—We thank J. Noel and M. Arcand for technical assistance; M. H. Cobb, K.-L. Guan, A. Nebreda, and J. Woodgett for reagents; and H. Saito and B. Errede for strains.

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J. Biol. Chem. 2003, 278:8118-8125.
doi: 10.1074/jbc.M211870200 originally published online December 27, 2002

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