Molecular Biology of the Cell
Vol. 5, 193–201, February 1994

MEK-1 Phosphorylation by MEK Kinase, Raf, and Mitogen-activated Protein Kinase: Analysis of Phosphopeptides and Regulation of Activity

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Submitted July 29, 1993; Accepted December 23, 1993
Monitoring Editor: Joseph Schlessinger

MEK-1 is a dual threonine and tyrosine recognition kinase that phosphorylates and activates mitogen-activated protein kinase (MAPK). MEK-1 is in turn activated by phosphorylation. Raf and MAPK/extracellular signal-regulated kinase kinase (MEKK) independently phosphorylate and activate MEK-1. Recombinant MEK-1 is also capable of autoactivation. Purified recombinant wild type MEK-1 and a mutant kinase inactive MEK-1 were used as substrates for MEKK, Raf, and autophosphorylation. MEK-1 phosphorylation catalyzed by Raf, MEKK, or autophosphorylation resulted in activation of MEK-1 kinase activity measured by phosphorylation of a mutant kinase inactive MAPK. Phosphoamino acid analysis and peptide mapping identified similar MEK-1 tryptic phosphopeptides after phosphorylation by MEK kinase, Raf, or MEK-1 autophosphorylation. MEK-1 is phosphorylated by MAPK at sites different from that for Raf and MEKK. Phosphorylation of MEK-1 by MAPK does not affect MEK-1 kinase activity. Several phosphorylation sites present in MEK-1 immunoprecipitated from ^{32}P-labeled cells after stimulation with epidermal growth factor were common to the in vitro phosphorylated enzyme. The major site of MAPK phosphorylation in MEK-1 is threonine 292. Mutation of threonine 292 to alanine eliminates 90% of MAPK catalyzed phosphorylation of MEK-1 but does not influence MEK-1 activity. The results demonstrate that MEKK and Raf regulate MEK-1 activity by phosphorylation of common residues and thus, two independent protein kinases converge at MEK-1 to regulate the activity of MAPK.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs), encoded by the extracellular signal-regulated kinase (ERK) genes (Boulton *et al.*, 1991), are serine-threonine protein kinases that are rapidly phosphorylated and activated in response to a variety of extracellular stimuli in many different cell types. Several substrates have been identified for MAPKs including MAP II (Ray and Sturgill, 1987), the 90-kDa ribosomal S6 protein kinase (Wood *et al.*, 1992), $cPLA_2$ (Lin *et al.*, 1993; Nemenoff *et al.*, 1993), c-Myc (Seth *et al.*, 1991), and the epidermal growth factor (EGF) receptor (Northwood *et al.*, 1991). Many of these proteins are involved in regulating mitogenesis, and the activation of MAPK appears pivotal in initiating DNA synthesis (Davis, 1993).

Several laboratories have elegantly demonstrated that MAPKs are themselves activated by phosphorylation (Ahn *et al.*, 1990; Gomez and Cohen, 1991; L'Allemain *et al.*, 1992). Activation of MAPK requires phosphorylation of the MAPK protein on both tyrosine and threonine (Ray and Sturgill, 1988; Anderson *et al.*, 1990; Boulton *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991), a reaction catalyzed by the protein kinase referred to as MAPK kinase (MAPKK) or MEK (MAPK/ERK kinase) (Ahn *et al.*, 1991; Gomez and Cohen, 1991; Crews and Erikson, 1992; Nakielny *et al.*, 1992; Seger *et al.*, 1992). MEKs have been described in EGF-stimulated Swiss 3T3

cells (Ahn *et al.*, 1991), nerve growth factor-stimulated PC12 cells (Gomez and Cohen, 1991), and phorbol ester-stimulated T cells (Crews and Erikson, 1992). MEK-1 is a dual recognition kinase capable of phosphorylating a tyrosine and threonine on MAPK both of which are required for activation (Crews and Erikson, 1992; Nakielny *et al.*, 1992; Seger *et al.*, 1992). MEK-1 is itself activated by phosphorylation on serine and threonine residues (Crews *et al.*, 1992; Crews and Erikson, 1992; Wu *et al.*, 1993b), effectively establishing a protein kinase cascade whose activity is controlled by tyrosine kinases and G protein-linked effectors.

MEK-1 is phosphorylated by Raf (Kyriakis *et al.*, 1992), MEK kinase (MEKK) (Lange-Carter *et al.*, 1993), Mos (Posado *et al.*, 1993), and by MAPK (Matsuda *et al.*, 1993). In this report, we compare the sites of phosphorylation on MEK-1 that are recognized by MEKK, Raf, and MAPK. Similar tryptic phosphopeptide maps are generated from MEK-1 phosphorylated by MEKK, or Raf, whereas MAPK phosphorylates distinct peptides. Several major in vitro phosphorylation sites are present on MEK-1 immunoprecipitated from ^{32}P-labeled cells after stimulation with EGF. Site-directed mutagenesis of the consensus MAPK phosphorylation sites in MEK-1 identified threonine 292 as the major site of phosphorylation. However, mutation of threonine 292 to alanine did not inhibit MEK-1 autoactivation. Thus, phosphorylation of recombinant MEK-1 by Raf and MEKK results in activation of the kinase, whereas phosphorylation of MEK-1 by MAPK does not directly affect its activity.

MATERIALS AND METHODS

Expression and Purification of Recombinant MEK-1

Wild-type and kinase inactive murine MEK-1 (Lys 97 to Met) and kinase inactive *Xenopus* MAPK (Lys 57 to Met) were cloned into the pRSET expression vector (Invitrogen, San Diego, CA). These constructs were used for expression in the LYSS strain of *Escherichia coli* BL21(DE3) of fusion proteins containing polyhistidine sequences at their NH_2 termini. The recombinant proteins were purified on Ni^{2+}-NTA-agarose (Qiagen, Chatsworth, CA) columns (0.5 ml) (Gardner *et al.*, 1993).

Isolation of Activated MEKK, Raf-1, and MAPK

COS-1 cells were transfected with pCMV5-MEKK (Lange-Carter *et al.*, 1993). After 48 h, the cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in 70 mM β-glycerophosphate pH 7.2, 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM dithiothreitol (DTT). After centrifugation for 10 min at maximum speed in a microcentrifuge, cell lysates containing 1–2 mg soluble protein were applied to a Mono Q HR 5/5 FPLC column (Pharmacia, Piscataway, NJ) and equilibrated in loading buffer (70 mM β-glycerophosphate pH 7.2, 100 μM sodium vanadate, 1 mM EGTA, and 1 mM DTT). The column was washed with 2 ml of buffer, and the bound proteins eluted with a 30 ml linear 0–350 mM NaCl gradient in loading buffer. The peak of MEKK activity eluted at 250 mM NaCl (fraction 22). MAPK was purified by Mono Q-FPLC from EGF-stimulated COS cells (30 ng/ml, 5 min) as for MEKK, except the peak of MAPK activity elutes at 150 mM NaCl (fraction 14). Raf-1 was immunoprecipitated from EGF-stimulated COS cells (30 ng/ml, 5 min). Cells were washed twice in cold PBS, lysed by scraping in RIPA buffer (50 mM tris(hydroxymethyl)aminomethane[RIPA Tris SDS Tris] pH 7.2, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1.0% Triton X-100, 10 mM sodium pyrophosphate, 25 mM sodium glycerophosphate, 2 mM sodium vanadate, 2.1 μg/ml aprotinin) and were microfuged for 10 min to remove nuclei. The supernatants were normalized for protein content and precleared with protein A Sepharose before immunoprecipitation with rabbit antiserum to the C terminus of Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A Sepharose for 2–3 h at 4°C. The beads were washed twice with ice cold RIPA and twice with PAN (10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid [PIPES] pH 7.0, 100 mM NaCl, 20 μg/ml aprotinin).

In Vitro Kinase Reactions

Wild-type recombinant MEK-1 was autophosphorylated by incubation in kinase buffer (20 mM PIPES pH 7.0, 10 mM $MnCl_2$, 0.1–2 μg wild-type recombinant MEK-1, 20–100 μCi [γ^{32}P]ATP, and 20 μg/ml aprotinin) in a final volume of 80 μl for 2–3 h at 30°C. The Raf immune complexes were resuspended in kinase buffer with 100–300 ng kinase-inactive MEK-1, 50 μCi [γ^{32}P]ATP in a final volume of 80 μl for 30 min at 30°C. Kinase-inactive MEK-1 was phosphorylated by MEKK and MAPK under similar conditions, except 25 μl of a Mono Q FPLC fraction containing activated MEKK or MAPK was added and the reactions were incubated for 30–45 min. All reactions were terminated by the addition of 20 μl of 5× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled and resolved by SDS-PAGE.

In Vivo Labeling of MEK-1 with ^{32}P

Rat 1a fibroblasts, which were 80% confluent (4 to 24, 10-cm dishes), were washed once with Tris-buffered saline (25 mM Tris pH 7.5, 150 mM NaCl) and were serum-starved to induce quiescence in 10 ml phosphate-free Dulbecco's minimal essential medium (GIBCO BRL, Grand Island, NY) supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% bovine serum albumin. After 12–18 h, 30 μl of $^{32}PO_4$ (10 mCi/ml H_2O, New England Nuclear, Boston, MA) was added to the media and the cells were incubated for ≤6 h. The cells were stimulated with EGF (30 ng/ml, 5 min), washed twice with ice-cold PBS, and lysed by scraping in RIPA buffer. The nuclei were removed by centrifugation at 14 000 × *g* for 10 min, and 3 μl of the supernatant was quantitated by liquid scintillation counting. An equal amount of radioactivity from stimulated and nonstimulated cell lysates was immunoprecipitated with rabbit antiserum to the C terminus of MEK-1 and protein A Sepharose at 4°C. The immune complexes were washed three times with ice cold 1% Triton X-100, 0.5% NP-40, 150 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 2.1 μg/ml aprotinin; eluted in 1× SDS-PAGE sample buffer, boiled for 5 min; resolved by SDS-PAGE; and analyzed by tryptic phosphopeptide mapping.

Phosphoamino Acid Analysis

Recombinant MEK-1 phosphorylated by in vitro kinase reactions was resolved by SDS-PAGE and detected by autoradiography. The labeled MEK-1 bands were cut out of the wet gel and eluted in 50 mM NH_4HCO_3 pH 7.4–7.6, 0.1% SDS, and 0.5% β-mercaptoethanol. The protein was hydrolyzed with 6 N HCl and analyzed by two-dimensional thin layer electrophoresis (20 min, 2.0 kV at pH 1.9, followed by 16 min, 1.9 kV at pH 3.5) (Boyle *et al.*, 1991). Phosphoamino acid standards were included in each sample and were detected with 0.2% (wt/vol) ninhydrin in acetone.

Tryptic Phosphopeptide Mapping

Labeled MEK-1 was resolved by SDS-PAGE, transferred to nitrocellulose, and identified by autoradiography. The MEK-1 bands were excised and digested with trypsin by the method of Luo *et al.* (1991). The tryptic phosphopeptides were analyzed by electrophoresis (1.2 kV, 20 min at pH 8.9) followed by chromatography in isobutyric acid: pyridine: acetic acid: n-butanol: H_2O 65:5:3:2:29 (Boyle *et al.*, 1991).

2-(2'-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-Skatole) (Pierce, Rockford, IL) Cleavage

Phosphorylated wild-type and kinase-inactive MEK-1 were resolved by SDS-PAGE (Fling and Gregerson, 1986) and transferred electrophoretically to polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) paper (Matsudaira, 1987) in transfer buffer (10 mM 3-(cycloexylamino)-1-propanesulfonic acid (CAPS (Sigma, St. Louis, MO) pH 11, 10% methanol) for 1 h at 1 amp. Radiolabeled MEK-1 proteins were identified by staining with Ponceau S and autoradiography. The Ponceau S-stained band was excised from the PVDF paper and cleaved with BNPS-skatole as described (Crimmins *et al.*, 1990). Briefly, the electroblotted sample was feathered into 1–2-mm^2 pieces and placed into a 1.5-ml screw-cap tube with 100 μl of 1 mg/ml BNPS-skatole in 75% aldehyde-free acetic acid. The tube was kept in the dark, and after 1 h at 47°C, 300 μl of H_2O was added to the reaction. The entire reaction was transferred to a 1.5-ml screw-cap tube, and 400 μl of ethyl acetate was added to extract the BNPS-skatole. After mixing the aqueous and organic phases, the tube was placed in a microcentrifuge and spun for 5 min at 15 000 rpm. The organic phase was removed and discarded, and the aqueous phase was concentrated by lyophilization.

The cleaved protein that remained bound to PVDF paper was eluted with 30 μl of 50 mM Tris pH 9.1, 2% SDS (wt/vol), and 1% Triton X-100 (vol/vol). After 2 h at 37°C, the detergent solution was removed from the PVDF paper and mixed with 30 μl 2× SDS-PAGE sample buffer. This solution was combined with the lyophilized aqueous layer, and the sample was heated at 100°C for 3 min.

Electrophoresis, Electrotransfer, and Protein Staining

Cleaved proteins were resolved by SDS-PAGE (Schägger and von Jagow, 1987). The acrylamide stock solution was stored over a mixed-bed ion-exchange resin (AG 510-X8, Bio-Rad, Richmond, CA), and 1 mM 3-mercaptopropionic acid was added to the electrophoresis buffer to prevent NH_2-terminal blocking of peptides (Ploug *et al.*, 1989). The gel was placed in transfer buffer (10 mM CAPS pH 11, 20% methanol) for 10 min while the PVDF paper was soaked for 5 min in 100% methanol and 5 min in transfer buffer. The gel was placed between three pieces of Whatman 3MM paper (Clifton, NJ) and transferred in a Transphor apparatus (Hoeffer Scientific Instruments, San Francisco, CA) for 45 min at 1 amp. The PVDF paper was rinsed in water and then stained in 0.1% (wt/vol) Coomassie (Sigma) blue R-250 in 50% methanol for 1 min. Nonspecifically bound Coomassie blue was removed with 40% (vol/vol) methanol and 10% (vol/vol) aldehyde-free acetic acid for 10 min. Amino acid sequence analysis was obtained by automated Edman degradation in an Applied Biosystem gas-phase sequencer 477A (Foster City, CA). Phenylthiohydantion amino acid derivatives were identified by high-performance liquid chromatography. This service was provided by Joe Leykam at the Macromolecular Structure Facility (Michigan State University) and the Molecular Resource Center at National Jewish Center for Immunology and Respiratory Medicine.

MEK-1 Activity Assays

Autoactivation of MEK-1 was measured by incubating purified recombinant wild-type MEK-1 (1.25 μg) in the presence or absence of recombinant kinase inactive MAPK (1.25 μg) in buffer (50 mM β-glycerophosphate, 100 μM sodium vanadate, 20 mM $MgCl_2$, 40 μM [γ^{32}P ATP] (100 cpm/fmol), 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] pH 7.2, 50 μg/ml IP_{20}, 1 mM EGTA) at 30°C. At various times, aliquots were removed and the reactions were terminated by the addition of 5× SDS PAGE sample buffer. The samples were boiled for 5 min and analyzed by SDS-PAGE on 10% acrylamide gels followed by autoradiography and quantitation using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Activation of recombinant wild-type MEK-1 by MEKK was performed using MEKK purified by Mono Q FPLC, as described above. MEKK was incubated in the presence or absence of purified recombinant wild-type MEK-1 (200 ng) and in the presence of purified kinase inactive MAPK (300 ng) in kinase buffer (20 mM PIPES pH 7.0, 10 mM $MnCl_2$, 20 μCi γ^{32}P-ATP, and 20 μg/ml aprotinin) for 30 min. Activated Raf was immunoprecipitated with anti-Raf serum from COS cells stimulated with EGF (30 ng/ml) for 5 min. Control basal Raf activity was immunoprecipitated from unstimulated COS cells. The beads were incubated in the presence or absence of purified recombinant wild-type MEK-1 (50 ng) and in the presence of purified kinase inactive MAPK (400 ng) for 15 min. For MAPK phosphorylation of MEK-1, recombinant wild-type MEK-1 (150 ng) and kinase inactive MAPK (250 ng) were preincubated for 20 min in the absence or presence of activated MAPK partially purified by Mono Q FPLC. After preincubation, [γ^{32}P]ATP was added (T = 0), and aliquots were removed at various times. All reactions were terminated and analyzed as for the autophosphorylation reactions.

Site-directed Mutagenesis

MEK-1 contains four consensus MAPK phosphorylation sites (PXTP or TP) (reviewed in Davis, 1993). The threonine residue in these sites at amino acids 7, 286, 292, and 386 were mutated to alanine using specific oligonucleotides and the Promega Altered Sites Mutagenesis kit (Madison, WI). All mutations were confirmed by DNA sequencing and were subcloned into the wild-type and kinase inactive pRsetA-MEK-1 expression vectors. Recombinant proteins containing these mutations were expressed and purified and were used as substrates for phosphorylation by MAPK and in MEK-1 activity assays.

RESULTS

Regulation of MEK-1 Activity

Phosphorylation of recombinant MEK-1 by MEKK or Raf increased MEK-1 kinase activity five- and twofold, respectively, as measured by the ability of MEK-1 to phosphorylate MAPK (Figure 1, A and B). This level of activation is significant but lower than has been reported for in vivo stimulation of MEK (Ahn *et al.*, 1991; Gomez and Cohen, 1991), probably because we are measuring the effect of individual purified enzymes on MEK-1 activation, whereas growth factor stimulation of MEK-1 is likely to involve multiple activation pathways. In addition, the purified recombinant MEK-1 used in these assays is capable of autoactivation (Figure 1C), and this level is subtracted from MEKK and Raf catalyzed activation. Interestingly, preincubation of recombinant MEK-1 with Mg^{+2}ATP does not increase the initial rate of MEK-1 phosphorylation of MAPK. Therefore, it appears that MEK-1 is partially activated as purified from *E. coli.* Incubation with either MEKK or Raf markedly accelerates the initial rate of MEK-1 phosphorylation of MAPK, such that the maximal rate of MEK-1 activity is achieved in minutes rather than hours. Thus, both MEKK and Raf are independent kinases that

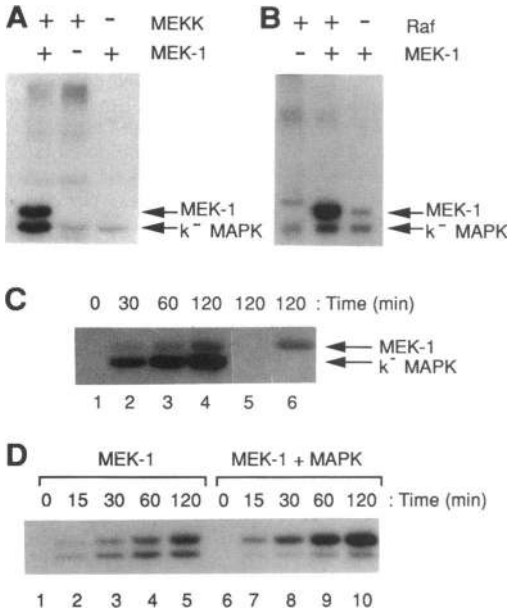

Figure 1. MEK-1 activity assays. (A) Activation of recombinant wild-type MEK-1 by MEKK. MEKK purified by Mono Q FPLC was incubated in the presence (+) or absence (−) of purified recombinant wild-type MEK-1 (200 ng) and in the presence of purified kinase inactive MAPK (300 ng) for 30 min. (B) Activation of MEK-1 by Raf. Raf was immunoprecipitated with anti-Raf serum (lanes 1 and 2) or with nonimmune serum (lane 3) from COS-cells stimulated with EGF (30 ng/ml) for 5 min and was incubated in the presence (+) or absence (−) of purified recombinant wild-type MEK-1 (50 ng) and in the presence of purified kinase inactive MAPK (400 ng) for 15 min. (C) Autoactivation of recombinant wild-type MEK-1. Recombinant wild-type MEK-1 (1.25 μg) was incubated in the presence (lanes 1–4) or absence (lane 6) of recombinant kinase inactive MAPK (1.25 μg). Lane 5, autophosphorylation of kinase inactive MAPK. At the indicated times, aliquots were removed and the reactions were terminated. (D) MAPK phosphorylation of MEK-1. Recombinant wild-type MEK-1 (150 ng) and kinase inactive MAPK (250 ng) were preincubated for 20 min in the absence (lanes 1–5) or presence (lanes 5–10) of activated MAPK partially purified by Mono Q FPLC. After preincubation, [γ^{32}P]ATP was added (T = 0), and aliquots were removed at the indicated times. The reactions were terminated by the addition of 5× SDS-PAGE sample buffer.

recognize and phosphorylate MEK-1, resulting in increased MEK-1 catalytic activity.

MAPK has also been shown to phosphorylate MEK-1 (Matsuda *et al.*, 1993). Activated MAPK was used to phosphorylate MEK-1 to determine the consequence of this phosphorylation on MEK-1 catalytic activity (Figure 1D). MAPK catalyzed phosphorylation of MEK-1 did not result in activation of MEK-1 kinase activity, even though MEK-1 is an excellent substrate for MAPK. The phosphorylation sites of MEK-1 were therefore analyzed to define the differential regulation of MEK-1 activity by Raf and MEKK versus MAPK.

Phosphoamino Acid Analysis of Recombinant MEK-1

Phosphoamino acid analysis of MEK purified from phorbol 12-myristate 13-acetate-stimulated T cells has demonstrated that MEK-1 is phosphorylated predominantly on threonine and serine but also on tyrosine (Crews and Erikson, 1992). To compare these sites of phosphorylation with those regulated by MEKK, Raf, and MAPK, purified recombinant wild-type MEK-1 was autophosphorylated in vitro and in parallel samples a recombinant kinase inactive MEK-1 was phosphorylated by MEKK, Raf, and MAPK. As shown in Figure 2A, recombinant MEK-1 autophosphorylated predominantly on threonine and serine, but also on tyrosine. In contrast, the phosphorylation by MEKK and Raf occurred predominantly on serine residues and to a much lesser extent on threonine and tyrosine (Figure 2, B and C). MAPK preferentially phosphorylated threonine residues (Figure 2D).

Tryptic Phosphopeptide Maps

Two-dimensional tryptic phosphopeptide maps of autophosphorylated wild-type MEK-1 or kinase inactive MEK-1 phosphorylated by MEKK, Raf, and MAPK are shown in Figure 3. The patterns of phosphopeptides of autophosphorylated wild-type MEK-1 and kinase inactive MEK-1 phosphorylated by MEKK and Raf were similar. One major and three minor phosphopeptides (labeled 1, 2, 3, and 4) were observed for MEK-1 au-

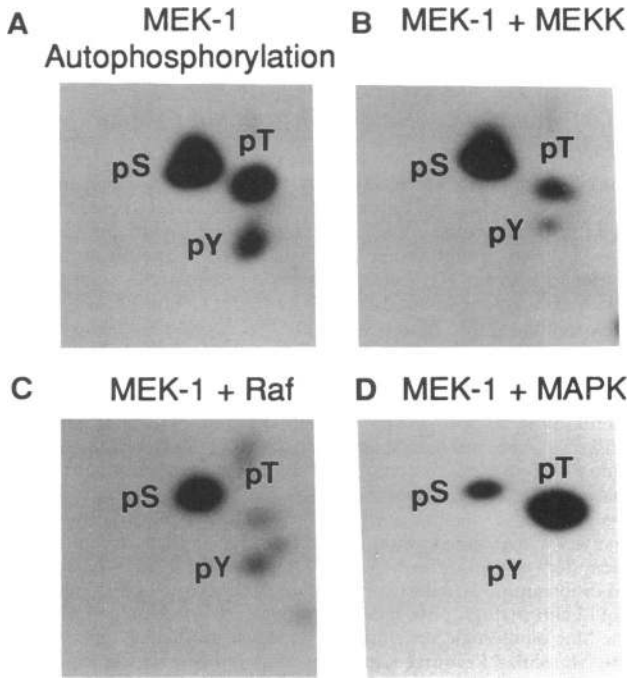

Figure 2. Phosphoamino acid analysis of recombinant MEK-1. ^{32}P-labeled amino acids were generated from recombinant MEK-1 phosphorylated by autophosphorylation (A), MEKK (B), Raf (C), or MAPK (D). The positions of the phosphoamino acid standards (pS, phosphoserine; pT, phosphothreonine; and pY, phosphotyrosine) are indicated.

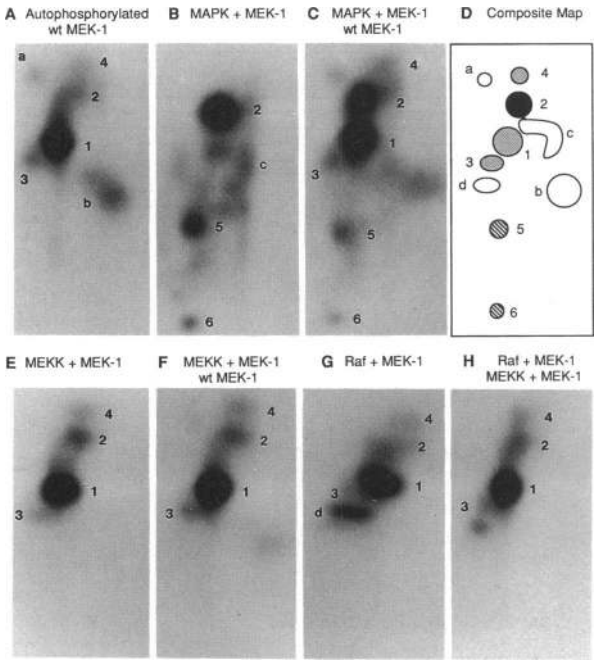

Figure 3. Tryptic phosphopeptide analysis of MEK-1. Autoradiograms of two-dimensional tryptic phosphopeptide maps of MEK-1. (A) Autophosphorylated wild-type MEK-1. Phosphorylation of kinase inactive MEK-1 by MAPK (B) and a mixture of A and B (C). (D) Schematic diagram of the migration of the phosphopeptides found in MEK-1. (E) MEKK. (F) Mixture of A and E. (G) Raf. (H) Mixture of E and G. Peptide 2 is common to all maps. Peptides 1, 3, and 4 are the major phosphopeptides resulting from autophosphorylation or phosphorylation by MEKK or RAF. Peptides 5 and 6 are unique to phosphorylation of MEK-1 by MAPK. Spots marked a–d are minor peptides that are unique to autophosphorylation (a and b), phosphorylation by MAPK (c), or Raf (d) and did not appear consistently on all maps.

tophosphorylation and phosphorylation by MEKK and Raf (Figure 3, A, E, and G). Additional minor phosphopeptides unique to autophosphorylated MEK-1 or MEK-1 phosphorylated by Raf were occasionally apparent (peptides a and b or d, respectively). The pattern of phosphopeptides observed with MAPK phosphorylation of MEK-1 was substantially different (Figure 3B); the intensity of peptide 1 was diminished, whereas that of peptide 2 was greatly increased. Furthermore, two unique phosphopeptides (5 and 6) were detected, as well as a minor peptide c with MAPK-catalyzed phosphorylation of MEK-1. Mixing experiments of the MEK-1 tryptic phosphopeptides generated from the different reactions confirmed the similarity of the phosphorylation sites on MEK-1 for Raf, MEKK, and MEK-1 autophosphorylation (Figure 3, F and H). Mixing experiments also clearly demonstrated that the major MAPK phosphorylation site on MEK-1 was unique (Figure 3C).

In vivo Phosphorylation of MEK-1

We analyzed the sites of in vivo phosphorylation of MEK-1 to determine which phosphopeptides detected in vitro are the same as those phosphorylated on MEK-1 in intact cells. Serum-starved Rat 1a cells were labeled with ^{32}P orthophosphate and MEK-1 was immunoprecipitated from lysates of unstimulated cells (basal) or cells stimulated with EGF for 5 min. The immunoprecipitated MEK-1 was fractionated by SDS-PAGE and identified by autoradiography and comigration with immunoblotted MEK-1. Representative tryptic phosphopeptide maps of in vivo–and in vitro–labeled MEK-1 are shown in Figure 4. EGF stimulation of cells greatly enhanced the phosphorylation of MEK-1 (compare Figure 4, A and B). Longer exposure of the basal maps indicated that similar spots were present, thus growth factor stimulation results in quantitative rather than qualitative differences in the phosphorylation of MEK-1. Several of the spots detected by in vitro labeling are also present in the in vivo–labeled MEK-1. Notably, the two major in vitro phosphopeptides 1 and 2 detected

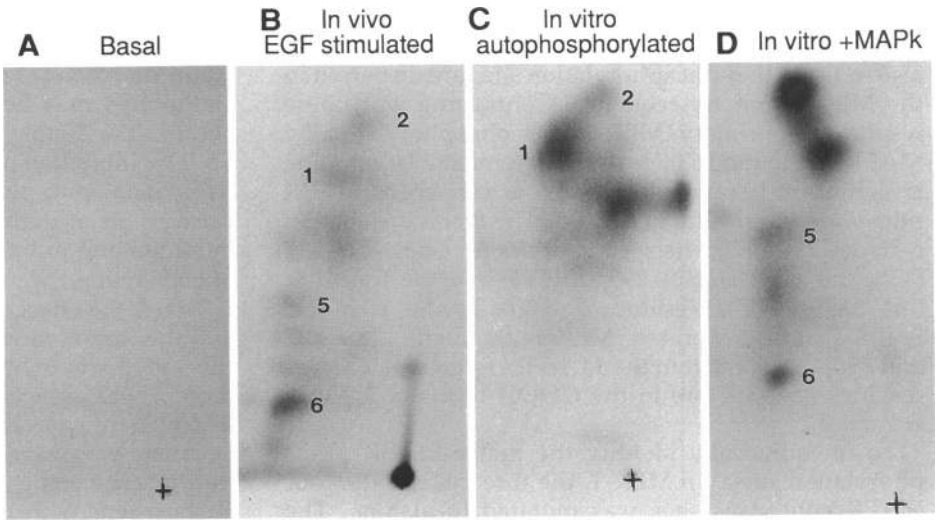

Figure 4. Comparison of in vivo and in vitro sites of phosphorylation on MEK-1. MEK-1 was immunoprecipitated from ^{32}P-labeled cells, purified by SDS-PAGE, and analyzed by tryptic phosphopeptide mapping as in Figure 3. (A) In vivo basal phosphorylation of MEK-1. (B) In vivo phosphorylation of MEK-1 from cells stimulated with EGF for 5 min. (C) In vitro autophosphorylation of MEK-1. (D) In vitro phosphorylation of kinase inactive MEK-1 by MAPK. Spots are identified by the same numbers as in Figure 3. + marks the origin.

on MEK-1 phosphorylated by MEKK and Raf are present in the in vivo–phosphorylated MEK-1 polypeptide (Figure 4, B and C). In addition, spots 5 and 6, which are unique to MEK-1 phosphorylated by MAPK (Figure 4D), are also present in the in vivo–phosphorylated enzyme. Because these phosphorylation sites were found with the in vivo–phosphorylated enzyme, they are likely to be physiologically relevant to the regulation of MEK-1, and this analysis corroborates the importance of the sites detected by in vitro phosphorylation.

BNPS-Skatole Cleavage

BNPS-skatole was used to chemically cleave MEK-1 at tryptophans (Crimmins *et al.*, 1990). Complete cleavage of MEK-1 by BNPS-skatole generated four peptides of predicted molecular weight 3.4, 27.5, 14.1, and 1.9 kDa (Figure 5A). Partial digestion generated additional fragments as diagrammed in Figure 5A. Phosphorylated MEK-1 was digested by BNPS-skatole, resolved on Tricine-acrylamide gels, and transferred to PVDF paper. A Coomassie blue–stained lane of BNPS-skatole–digested MEK-1 is shown in Figure 5B. The major peptides are doublets of ~45.8–47.7, 41.8–43.7, 31.7, 27.5, 16, and 14.1 kDa. By molecular weight these peptides correspond to full length MEK-1 or MEK-1 lacking the NH_2 and/or COOH terminal peptides (41.8–47.7 kDa), the NH_2 terminal half of MEK-1 (27.5 and 31.7 kDa), and the COOH terminal region of MEK-1 (14.1 and 16 kDa), respectively. The identities of the 14.1 and 16 kDa peptides were confirmed by protein microsequencing. The NH_2-terminus of the larger peptides was blocked, but amino acid composition verified their assignment in the MEK-1 primary sequence. Major phosphorylation sites for MEK-1 autophosphorylation and MEK-1 phosphorylation by MEKK and Raf were found in both the 31.7/27.5 and 16/14.1 kDa BNPS-skatole–generated fragments (Figure 5B). This finding is consistent with the multiple tryptic MEK-1 phosphopeptides observed for MEK-1 autophosphorylation and MEK-1 phosphorylation by MEKK and Raf (Figure 3). The results also indicate that phosphorylation sites are dispersed in the MEK-1 primary sequence. Contrasting with these results, the primary MEK-1 site phosphorylated by MAPK was in the 16.0/14.1 kDa fragments. This finding is significant because there are four consensus MAPK phosphorylation sites in MEK-1. The first is encoded in residues 7–8 (TP), the second at amino acids 286–287 (TP), the third at 290–293 (PRTP), and the fourth at 384–387 (PSTP). Residues 7–8 are in the 27.5-kDa BNPS-skatole–generated MEK-1 fragment, 286–287 and 290–293 are within the 14.1-kDa fragment, whereas residues 384–387 are in the COOH-terminal 1.9-kDa fragment.

To unequivocally identify the major MAPK phosphorylation site(s) in MEK-1, the threonine in the four MAPK consensus sites was mutated to alanine. The

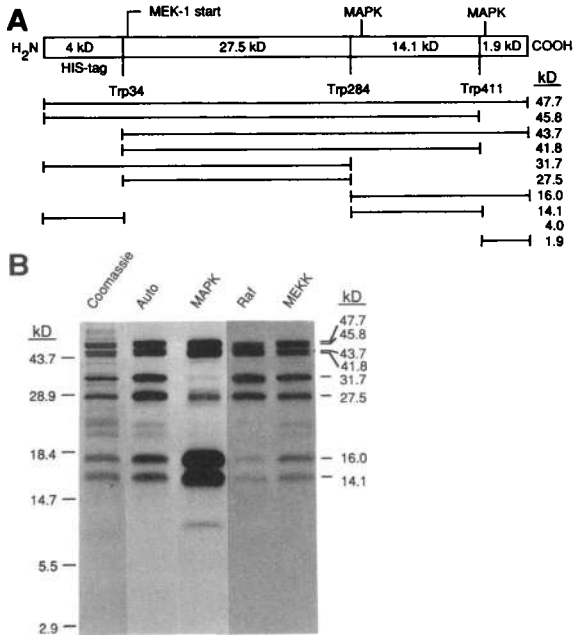

Figure 5. BNPS-skatole cleavage of phosphorylated MEK-1. (A) Schematic diagram of BNPS-skatole cleavage sites in recombinant HISTIDINE-tagged MEK-1. Predicted molecular masses of the peptides are indicated. (B) Cleavage of MEK-1 with BNPS-skatole. Recombinant MEK-1 was phosphorylated in vitro by the indicated kinase, purified by SDS-PAGE, digested with BNPS-skatole, resolved by SDS-PAGE, and blotted to PVDF membrane. Predicted molecular masses of the peptides are indicated to the right, and molecular weight markers are to the left. Lane 1, Coomasie-stained blot of MEK-1 cleaved with BNPS-skatole; lanes 2–5, autoradiographs of BNPS-skatole–cleaved MEK-1; lane 2, autophosphorylated MEK-1; lane 3, MEK-1 phosphorylated with MAPK; lane 4, MEK-1 phosphorylated with Raf; lane 5, MEK-1 phosphorylated with MEKK.

mutant kinase inactive MEK-1 proteins were recombinantly expressed, purified, and used as substrates for MAPK. Mutation of threonine 292 to alanine reduced MAPK catalyzed phosphorylation of MEK-1 by 80–90% (Figure 6A). None of the other mutations substantially affected phosphorylation. In some assays, phosphorylation of MEK-1 T386A was reduced ~5–10%. This amino acid may be a minor site of phosphorylation, because the double T292A/T386A routinely exhibited a >90% inhibition of phosphorylation (Figure 6A). The reduction in phosphorylation of MEK-1 T292A was not because of an inability to express the protein as immunoblotting indicated approximately equal amounts of each mutant were used in the assay (Figure 6B).

To test the effect of these mutations on MEK-1 activity, the triple mutation T286A/T292A/T386A was subcloned into wild-type MEK-1, and the recombinant protein was assayed for the ability to phosphorylate recombinant kinase inactive MAPK. As shown in Figure 6C, there was no reduction in MEK-1 activity with the triple mutant compared to wild-type MEK-1. This result is consistent with our observation in Figure 1C that

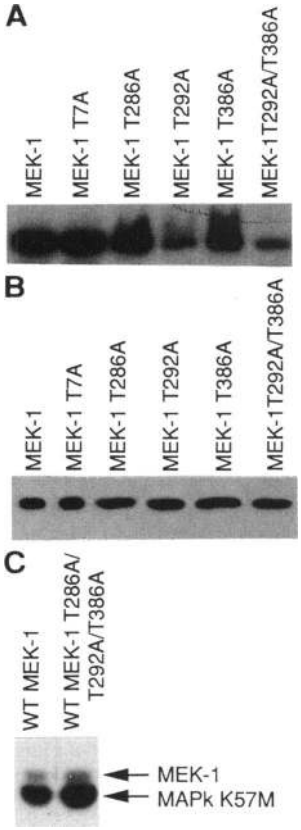

Figure 6. Analysis of mutant MEK-1 polypeptides. (A) Phosphorylation of recombinant mutant MEK-1 polypeptides by MAPK. The indicated kinase inactive mutant MEK-1 polypeptides were used as substrates for MAPK in in vitro kinase reactions. (B) Immunoblot of mutant MEK-1 polypeptides showing the relative amount of each purified recombinant MEK-1 polypeptide used in A. (C) MEK-1 activity assay. The indicated kinase active MEK-1 polypeptide (100 ng) was incubated with kinase inactive MAPK in kinase buffer at 30°C for 1 h. The reactions were terminated by the addition of SDS-PAGE sample buffer, fractionated by SDS-PAGE, and autoradiographed.

phosphorylation of wild-type recombinant MEK-1 with MAPK does not affect MEK-1 activity. In addition, the data indicate that these mutations do not influence the ability of MEK-1 to assume an active conformation.

The mutant MEK-1 polypeptides were analyzed by tryptic phosphopeptide mapping. Mutations at 286, or 386 did not significantly alter the maps (Figure 7, A–E). In contrast, all of the prominent spots (2, 2A, 5, 6) are missing from the MEK-1 T292A map (Figure 7C). Thus, tryptic phosphopeptide mapping of the mutant MEK-1 proteins confirmed that threonine 292 is the major site of MAPK phosphorylation in MEK-1.

DISCUSSION

Our findings demonstrate that MEKK and Raf regulate MEK-1 activity by phosphorylating common sites in the MEK-1 polypeptide. In contrast, the phosphorylation of MEK-1 by MAPK does not affect MEK-1 catalytic activity. In yeast, MAPK-catalyzed phosphorylation of MEK-1 has been proposed to be involved in a feedback regulatory control of MEK-1 activity (Errede *et al.*, 1993). It is possible that MAPK phosphorylation of MEK-1 may influence its interaction with other proteins in the MAPK regulatory network but that our assay does not detect these changes. The very rapid inactivation of MAPK in some cell types is consistent with a rapid feedback control of upstream regulators of the MAPK activation system. Demonstration of a feedback control mechanism will require in vitro reconstitution of all the components of the MAPK regulatory network.

The results indicate that MEK-1 acts as a convergence point for multiple protein kinases that are involved in MAPK activation. The placement of MEK-1 between MAPK and upstream regulators, such as MEKK and Raf provides integration but also independence of signal transduction networks initiated by growth factor receptors. MEKK and Raf phosphorylate MEK-1 but may phosphorylate other proteins as well. Thus, the differential regulation of MEKK and Raf could allow a common activation of MEK-1 and MAPK but independent regulation of other phosphorylation substrates. The dual threonine and tyrosine recognition properties of MEK-1 and its function as a convergence point for multiple serine/threonine protein kinases makes it unique among the

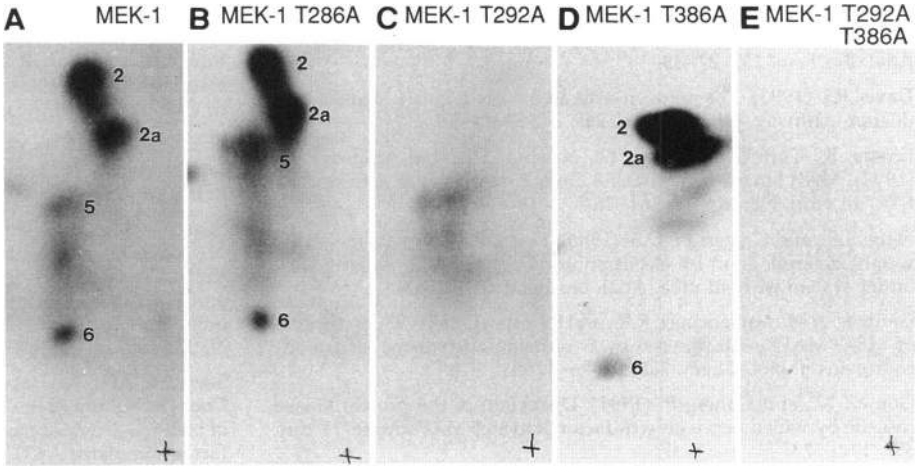

Figure 7. Tryptic phosphopeptide analysis of mutant MEK-1 polypeptides. The indicated mutant MEK-1 polypeptides were phosphorylated by MAPK as in Figure 6, purified by SDS-PAGE, and equal amounts of each protein were analyzed by two-dimensional tryptic phosphopeptide mapping. Spots are identified by the same numbers as in Figure 3. Spot 2A most likely represents a partial digestion product, as it was not consistently present. Spot 5 was present on other maps of MEK-1 T386A. + marks the origin.

members of the protein kinase family. The fact that multiple MEKs are being discovered (Wu *et al.*, 1993a; Zhang and Guan, 1993) suggests that the number of upstream regulators of MAPK may be much more diverse than presently appreciated.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health (NIH) grants DK-37871, GM-30324, and CA-58187 to G.L.J. A.M.G. is a fellow of the Cancer League of Colorado and was supported in part by a research grant from the American Cancer Society. C.L.-C. and R.R.V. are supported by NIH grants GM-15843 and DK-08897.

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