Biochem. J. (1997) **322**, 185–192 (Printed in Great Britain)

Regulation of the activity of MEK kinase 1 (MEKK1) by autophosphorylation within the kinase activation domain

Joseph C. DEAK* and Dennis J. TEMPLETON*†‡

*Institute of Pathology and †CWRU Cancer Center and Program in Cell Biology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland OH 44106, U.S.A.

MEK kinase 1 (MEKK1) shares sequence identity with the yeast kinases Ste11 and Byr2, and is capable of phosphorylation and activation of both mitogen-activated protein/extracellular signal-related protein kinase (MAP/ERK) kinase (MEK) and stress-activated protein kinase (SAPK)/ERK kinase (SEK) *in vitro*. *In vivo*, however, MEKK1 predominantly activates the SEK/SAPK kinase cascade. Mechanisms of activation of MEKK1 are unclear. We have identified a major site of autophosphorylation (Thr-575) within the 'activation loop' of MEKK1 between the kinase subdomains VII and VIII. Phosphatase treatment of a constitutively active MEKK1 decreased kinase activity by 59%. Dephosphorylated T575 was rapidly re-(auto)phosphorylated by MEKK1. Mutation of T575 to alanine decreased MEKK1 transphosphorylation activity with a SEK substrate to approx. 30% of wild-type. Mutation of a second threonine residue (Thr-587) to alanine eliminated the phosphorylation of MEK or SEK substrate but not autophosphorylation. MEKK1 autophosphorylation is an intramolecular reaction because active MEKK1 cannot transphosphorylate a kinase-inactive MEKK1. Inactive MEKK1 was not phosphorylated on Thr-575 within cells, suggesting that the phosphorylation of Thr-575 *in vivo* results from autophosphorylation rather than phosphorylation by an upstream kinase. Autoactivation of MEKK1 via autophosphorylation of Thr-575 might be an immediate response to initial kinase activation through non-phosphorylation mechanisms.

INTRODUCTION

MEK kinase 1 (MEKK1) is a proximate activator of a cascade of kinases leading to activation of the stress-activated protein kinase (SAPK). MEKK1 directly phosphorylates SAPK kinase 1 (SEK1), which in turn phosphorylates SAPK [1]. This cascade is entirely analogous to the mitogen-activated kinase cascade that leads from Raf phosphorylation of mitogen-activated/extracellular response kinase (MEK), and continues to MEK phosphorylation of mitogen-activated protein kinase (MAPK).

Although the overall schemes of the Raf–MEK–SAPK and MEKK–SEK–SAPK cascades are similar, the physiological and pharmacological activators of each are largely exclusive. Thus phorbol 12-myristate 13-acetate is a potent activator of MAPK but is unable to activate SAPK in NIH3T3 cells [1,2]. Other mitogens that strongly activate MAPK are in general weak activators of SAPK. Conversely, diverse cell stress agents such as ultraviolet, heat shock, anisomycin and tumour necrosis factor α (TNFα) treatments activate SAPK [2–4] far more than they activate MAPK. Thus the two activation cascades seem largely insulated from each other.

Although diverse agents as described above can activate SAPK, some of these agents apparently function through alternative SAPK activators [5]. Of these stimulators, only TNFα [6] and osmotic shock [7] have been shown to effect a rapid and transient increase in MEKK1 activity.

Stress signalling mechanisms proximal to MEKK1 are unclear. By analogy with Raf, the Ras oncoprotein or other small G-proteins are likely to be regulators of MEKK1 activity. Supporting this possibility, dominant inhibitory alleles of Ras have been reported to decrease MEKK1 activity in PC12 cells [8]. Recently, direct binding of Ras to the kinase domain of MEKK1 has been observed [7], a surprising contrast with the binding of Ras to the N-terminal regulatory domain of Raf. In contrast, TNFα-induced activation of SAPK/JNK in PC12 cells could not be blocked by transfection of a dominant negative form of Ras [9], suggesting either the presence of MEKK1-independent pathways for SAPK activation, or Ras-independent pathways of MEKK1 activation. The Ras-related G-proteins have been implicated in SAPK/JNK activation [10–13] but it is unclear whether these mediate the SAPK effect through MEKK1.

Like other signalling kinases, a likely means of MEKK1 activation is by phosphorylation. We sought to determine whether site-specific phosphorylation played a role in MEKK1 activity. Many kinases retain conserved sequences within their catalytic cores [14]. A stretch of residues between the conserved DFG motif of subdomain VII and the PE motif of subdomain VIII are often referred to as the 'activation loop' because many kinases contain demonstrable or putative phosphorylation sites within this loop (see Figure 1). The crystal structures of cAMP-dependent protein kinase (PKA) [15], extracellular signal-related protein kinase 2 (ERK2) [16] and cyclin-dependent kinase 2 [17] have been elucidated. Each of these contains phosphorylated residues within the activation loop, which lies at the threshold of the substrate-binding pocket of the kinases. Phosphorylation of the activation loop might therefore regulate substrate binding. PKA autophosphorylation of Thr-197 might also be important for protein folding and interaction with the regulatory subunit (reviewed in [15]). In contrast with the phosphorylation of PKA, MAPK residues Thr-183 and Tyr-185 within this activation loop

Abbreviations used: β-GP, β-glycerol phosphate; CIP, calf intestinal alkaline phosphatase; DTT, dithiothreitol; ERK, extracellular signal-related protein kinase; GST, glutathione S-transferase; MAP, mitogen-activated protein; MAPK/ERK, mitogen-activated protein kinase; MEK, mitogen-activated/extracellular response protein kinase kinase; MEKK1, MEK kinase 1; ΔMEKK1, 367-residue N-terminal truncation of MEKK1; PAA, phosphoamino acid analysis; PKA, cAMP-dependent protein kinase; SAPK, stress-activated protein kinase; SEK, SAPK kinase; TNFα, tumour necrosis factor α.

‡ To whom correspondence should be addressed.

are transphosphorylated by the upstream activator MEK [18], although autophosphorylation of MAPK on Tyr-185 also occurs [19].

These structures underscore the importance of phosphorylation events within the activation loop. Similarly to other kinases that are activated by phosphorylation, MEKK1 contains potential phosphorylation sites within this domain. Using an active truncated version of MEKK1 (Δ367 MEKK1, or ΔMEKK1), we examined the phosphorylation of MEKK1. We have identified a major site of phosphorylation on residue Thr-575 within the MEKK1 activation loop that results from the autophosphorylation of MEKK1 by an intramolecular reaction. Mutation of this threonine residue to alanine decreases MEKK1 kinase activity. The activation of MEKK1 by phosphorylation thus parallels that of several kinase families that are activated by phosphorylation within the activation domain.

EXPERIMENTAL

Protein expression and purification

CV1 monkey kidney cells (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum. Mouse ΔMEKK1 was cloned by reverse transcriptase–PCR, with primers derived from the published sequence [20], and subcloned into a pTM1 plasmid vector downstream of an 'EE' epitope tag (EEEEYMPME) derived from the polyomavirus middle T antigen as described [21]. Cells were transfected with recombinant vTF7-3 vaccinia virus and co-transformed by lipofection with gene-encoding pTM1 vectors as described [21–23]. Cells were lysed in MLB buffer [50 mM Na Mops (pH 7.0)/250 mM NaCl/5 mM EDTA/1 mM dithiothreitol (DTT)/0.1% Nonidet P40 (NP40)/2.0 μg/ml aprotinin/2.0 μg/ml leupeptin/50 μg/ml PMSF/10 mM NaF/5 mM sodium pyrophosphate/1 mM sodium vanadate/10 mM β-glycerol phosphate (β-GP)] and lysates were cleared by centrifugation at 13000 ***g*** for 15 min. Proteins were immunoprecipitated from lysates by incubation with 20 μl of 'EE' monoclonal antibody-conjugated Affi-Gel 10 (Bio-Rad) beads (3 mg of antibody/ml of beads) for at least 1 h. After washing three times with 1 ml of MLB buffer and once with final wash buffer [50 mM Tris/HCl (pH 7.4)/0.5 mM DTT/1 mM Na_3VO_4], purified proteins were eluted overnight in 20 μl of elution buffer [final wash buffer containing 10 mM β-GP, 100 μg/ml 'EE' peptide, in 30% (v/v) glycerol]. Eluted samples were stored at −20 °C.

Expression and purification of bacterial protein

Glutathione S-transferase (GST)–SEK-KR and GST–ΔMEKK1 (a gift from Dr. John M. Kyriakis) fusion proteins were expressed in *Escherichia coli* BL21 cells by the induction of 600 ml Luria broth amp (80 μg/ml ampicillin) cultures (D_{600} approx. 0.1) with 50 μM isopropyl β-D-thiogalactoside followed by incubation overnight at room temperature. Cells were centrifuged at 5000 ***g*** for 15 min then resuspended in 10 ml of TNE5 [10 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA] supplemented with 500 μg/ml PMSF. Cell suspensions were sonicated and then incubated with 1% NP40 on ice for 5 min. Cell lysates were cleared by centrifugation at 12000 ***g*** for 15 min. Glutathione–agarose beads (200 μl; equilibrated with TNE5) were added to the supernatant. After 1 h of incubation at 4 °C the complexed beads were washed at least three times with TNE5 until no protein was detected in the wash (Bradford assay). Proteins were then eluted in 20 mM glutathione in 50 mM Tris/HCl, pH 9.0, and stored in 30% (v/v) glycerol. The concentration and purity of the eluted proteins were analysed by SDS/PAGE [10% (w/v) gel] followed by staining with Coomassie Blue, using a number of molecular mass standards in dilution series [β-galactosidase, BSA, egg albumin and carbonic anhydrase (Sigma)].

Site-directed mutagenesis

Mutagenesis was performed with a variation of the 'megaprimer' PCR method [24]. Diagnostic restriction sites were engineered into mutant sites. Mutant sequences were subcloned into unmutagenized plasmids and DNA-sequenced (Sequenase kit, USB) to ensure fidelity. A SEK1 cDNA clone was obtained from Dr. Leonard I. Zon.

Kinase assays *in vitro*

In vitro kinase assay reaction mixtures contained 20 μM ATP, 4 μCi of [γ-^{32}P]ATP, 10 mM $MgCl_2$, 5 mM $MnCl_2$ and 0.5 mM DTT in Tris/HCl, pH 7.4. Quantities of enzyme and substrate for particular experiments are given in the Figure legends. Time course reactions were initiated by adding ΔMEKK1 or ΔMEKK1 T575A to a pooled reaction mixture including 1.5 μg of bacterially expressed GST–SEK-KR per time point. At specified time points, 20 μl of the ongoing reaction was removed and stopped by addition to 20 μl of 2×SDS/DTT sample-loading buffer. Individual samples were immediately placed on ice. After SDS/PAGE, gels were stained with Coomassie Blue and dried. Quantification of incorporated [^{32}P]ATP was performed on an AMBIS β-detector. Kinase reactions *in vitro* not to be quantified were transferred to an Immobilon-P membrane (Millipore) after SDS/PAGE. Autoradiography was performed with Kodak X-OMAT AR-5 film. Immunoblotting was performed with an anti-(EE epitope) monoclonal antibody followed by an alkaline phosphatase-conjugated secondary antibody and a chromogenic substrate.

Treatments with calf intestinal alkaline phosphatase (CIP)

Immunopurified and eluted MEKKs were treated with 40 units of CIP in 50 mM Tris/HCl (pH 8.0)/100 mM NaCl/10 mM $MgCl_2$ for 30 min at room temperature followed by the addition of a cocktail of phosphatase inhibitors (10 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium vanadate and 10 mM β-GP) and placement on ice to stop the reaction. In parallel, equivalent aliquots of MEKK1 were incubated with CIP reaction buffer plus phosphatase inhibitors followed by either the addition of 40 units of CIP and placement on ice or incubation with 40 units of CIP in reaction buffer in the presence of the phosphatase inhibitors. MEKK1 activity was assayed by kinase reactions *in vitro* in the presence of phosphatase inhibitors and analysed as above.

CIP treatments that were followed by autokinase reactions were performed directly on MEKKs bound to EE-conjugated Affigel beads. After CIP treatment [in 50 mM Tris/HCl (pH 8.0)/100 mM NaCl/10 mM $MgCl_2$ for 30 min at 30 °C] using 0, 20 or 40 units, or 40 units in the presence of 10 mM NaF/5 mM sodium pyrophosphate/1 mM sodium vanadate/10 mM β-GP, the beads were washed three times with MLB buffer and once with final wash buffer. The bound MEKKs were then subjected to kinase reactions *in vitro* by addition of 20 μM ATP/4 μCi of [γ-^{32}P]ATP/10 mM $MgCl_2$/5 mM $MnCl_2$/0.5 mM DTT in Tris/HCl (pH 7.4). The reactions were analysed by SDS/PAGE followed by electroblotting and autoradiography as described. Quantification of phosphothreonine incorporated into CIP-treated and untreated controls was accomplished by partitioning each autokinase reaction. One-third of each reaction was sub-

jected to SDS/PAGE, electroblotting and phosphoamino acid analysis (PAA); the remaining two-thirds, along with a dilution series of mass standards, was subjected to SDS/PAGE followed by Coomassie Blue staining. Quantification of total phosphate and the measurement of the proportion of phosphothreonine incorporated into MEKK1 was performed on a Packard Instant Imager.

Labelling *in vivo*

Monkey kidney CV1 cells were transfected with pTM1 expression plasmids and infected with recombinant vTF7-3 vaccinia virus as described [21]. Cells were washed then incubated in phosphate-free Dulbecco's modified Eagle's medium supplemented with 2 % (v/v) dialysed calf serum for 4 h. Cells were then labelled for 4 h in fresh medium containing 250 μCi/ml [^{32}P]P$_i$. Labelled proteins were immunopurified as described above.

PAA

Phosphoproteins transferred to an Immobilon support were hydrolysed in 6 M HCl at 110 °C for 1 h by the method of Kamps and Sefton [25]. Samples were analysed by TLC at pH 3.5.

RESULTS

Characterization of the phosphorylation state of MEKK1 *in vivo*

MEKK1 is a phosphoprotein [20]. Our preliminary results demonstrated that a C-terminal fragment of MEKK1 (ΔMEKK1, encompassing the catalytic core of the enzyme, but lacking 367 amino acids of the putative regulatory domain at the N-terminus) is also phosphorylated *in vivo*. Residues between the conserved DFG motif of subdomain VII and the PE motif of subdomain VIII within the catalytic cores of many kinases [14] are referred to as the 'activation loop' because many kinases contain demonstrable or putative phosphorylation sites within this region (Figure 1).

Two threonine residues (Thr-575 and Thr-587) and one serine residue (Ser-572) reside within the putative activation loop of MEKK1. We targeted each of these residues for mutational analysis by site-directed mutagenesis. To assess the phosphorylation state of sites Thr-575, Thr-587 and Ser-572 *in vivo*, we labelled mutant MEKK1 proteins *in vivo* with ^{32}P and performed PAA (see the Experimental section).

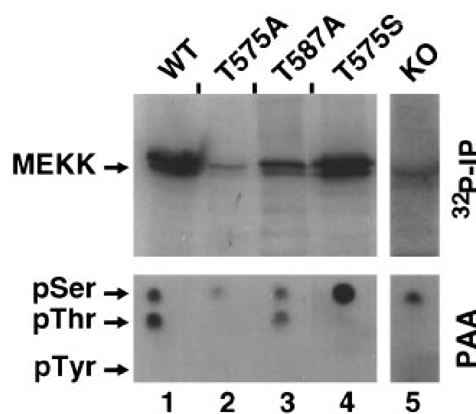

Figure 2 Labelling of ΔMEKKs *in vivo*

ΔMEKKs, expressed in vaccinia virus-transfected CV1 cells, were labelled *in vivo* with [^{32}P]P$_i$ for 4 h and then immunopurified by precipitation with 'EE' monoclonal antibody. Upper panel, autoradiograph of electroblotted wild-type (WT) ΔMEKK1 (lane 1), ΔMEKK1-T575A (lane 2), ΔMEKK1-T587A (lane 3), ΔMEKK1-T575S (lane 4) and the kinase-inactive ΔMEKK1-KO (lane 5); lower panel, PAA of the Western-blotted proteins showing no detectable phosphothreonine incorporated by either the T575A or T575S mutant MEKK1, or the inactive ΔMEKK1-KO mutant.

The threonine residue at position 587 was eliminated as a potential site of phosphorylation because mutation of Thr-587 to either alanine or serine did not change the relative amounts of phosphothreonine and phosphoserine from that of the wild-type ΔMEKK1 (Figure 2, lanes 1 and 3; T587S not shown). PAA of position 587 mutants has not shown Thr-587 to be phosphorylated under any condition tested. While Thr-587 is apparently not a site of phosphorylation, this residue is important in that its mutation to alanine abolishes phosphorylation activity with either MEK or SEK substrates, yet retains the ability to autophosphorylate Thr-575 (see below).

In contrast with Thr-587, mutation of Thr-575 to either alanine or serine completely abolished the incorporation of phosphothreonine in ΔMEKK1 *in vivo* (Figure 2, lanes 2 and 4). Mutant T575A also showed a large decrease in the total amount of incorporated 32p, relative to the wild-type ΔMEKK1, during labelling *in vivo*. The lack of phosphothreonine on the T575S

PKC-alpha	IKIADFGMCK	EHMMDG----	VTTRTFCGTP	DYIAPEII
cAPKa	IQVTDFGFAK	RVKGR-----	--TWTLCGTP	EYLAPEII
MAPK	LKICDFGLAR	VADPDHD-HT	GFLTEYVATR	WYRAPEIM
SAPK	LKILDFGLAR	TAG-----TS	FMMTPYVVTR	YYRAPEVI
CDC2hs	IKLADFGLAR	AFG----IPI	RVYTHEVVTL	WYRSPEVL
MEK1	IKLCDFGVSG	QLID------	SMANSFVGTR	SYMSPERL
c-Raf	VKIGDFGLAT	VKSRWSG--S	QQVEQPTGSV	LWMAPEVI
S6K	VKLTDFGLCK	ESIHDG----	TVTHTFCGTI	EYMAPEIL
MEKK1	LRIADFGAAA	RLASKGTGAG	EFQGGLLGTI	AFMAPEVL
		^ T575	^ T587	

Figure 1 Comparison of the activation loops of several kinases for which activating phosphorylation events have been characterized

Shaded amino acids represent conserved residues in domain 7 ('DFG') and domain 8 ('PE') [14]. Phosphorylation sites are shown in boxes for PKC-α [39], cAPKa [40], MAPK [41], SAPK, [2], CDC2 (cell division cycle 2) [42,43], MEK1 [21,30], Raf [44] and S6K [45] are shown. The locations of two threonine residues mutated in the current study are indicated. The conserved threonine residue corresponding to MEKK1 Thr-587 has not been identified as a phosphorylation site in any kinase, but might serve a structural role.

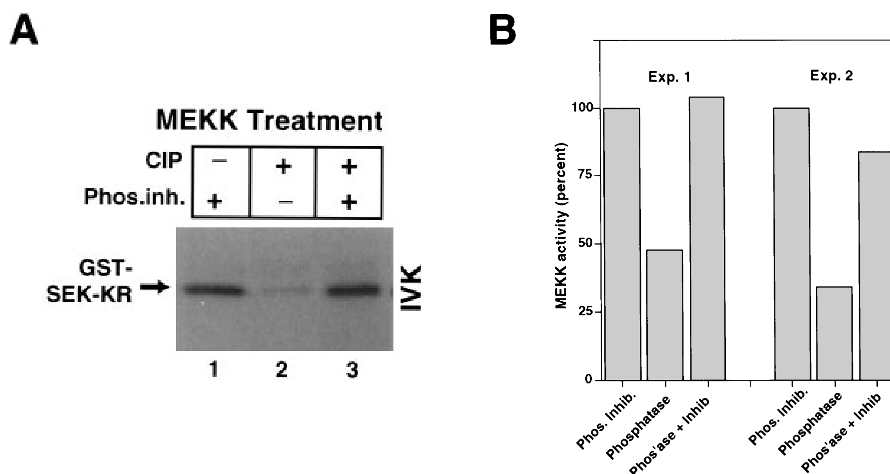

Figure 3 MEKK1 activity after CIP treatment

N-terminal 'EE' epitope-tagged ΔMEKK1 was expressed in monkey kidney CV1 cells and immunopurified with an 'EE' tag-specific monoclonal antibody. Equivalent amounts of eluted MEKKs (2 μl of eluate per reaction from a total of 22 μl derived from one 9 cm dish of cells) were preincubated for 30 min at 30 °C followed by kinase reactions *in vitro* (50 s) with a kinase-inactive GST–SEK (KR) substrate (2 μg per reaction). Treatments: a cocktail of phosphatase inhibitors (mock, lane 1); 40 units of CIP (lane 2); 40 units of CIP in the presence of phosphatase inhibitors (lane 3). At termination, preincubations were supplemented by the addition of CIP or phosphatase inhibitors to identical compositions before analysis by SDS/PAGE. (**A**) Representative autoradiograph. (**B**) Graphic representation of duplicate experiments showing percentage wild-type ΔMEKK1 activity (mock + phosphatase inhibitors as 100%) remaining after pretreatment with CIP or CIP + phosphatase inhibitors. Quantification of incorporated ^{32}P was performed with an AMBIS β-detector.

mutant strongly suggests that the source of phosphothreonine in the wild-type MEKK1 is predominantly from the phosphorylation of residue Thr-575.

Mutation of Ser-572 to alanine in combination with T575A (ΔMEKK1-S572A, T575A) did not abolish or decrease the relative amount of phosphoserine incorporated *in vivo* compared with the single-mutant protein MEKK1-T575A (results not shown). Kinase assays *in vitro* showed that both immunopurified ΔMEKK1-(S572A, T575A) and ΔMEKK1-T575A were able to phosphorylate SEK or MEK substrates to a similar degree (results not shown). Although our results do not exclude the possibility of a low level of phosphorylation of Ser-572, the lack of biological effect of the serine–alanine mutations at this site caused us to eliminate Ser-572 from further study.

The phosphorylation state *in vivo* was also determined for a catalytically inactive mutant of ΔMEKK1 containing a mutation of four residues encompassing Lys-447 within the ATP-binding pocket (AVK^{447}Q to EFL^{447}E). This mutant, termed ΔMEKK1-KO, was phosphorylated within CV1 cells (Figure 2, lane 5), but the phosphorylation was entirely in phosphoserine rather than phosphothreonine. This demonstrates that the ATP-binding domain is required for phosphorylation of Thr-575 and suggests that Thr-575 is phosphorylated via autophosphorylation rather than through the action of an upstream kinase. This is addressed further below.

Dephosphorylation decreases the kinase activity of ΔMEKK1

We sought to determine the role that phosphorylation of Thr-575 plays in the phosphorylation activity of MEKK1, using as substrate a bacterially expressed GST–SEK1 fusion protein containing an inactivating K123R mutation. We first tested whether pretreatment of MEKK1 with CIP influenced activity.

ΔMEKK1 is constitutively active and phosphorylates both MEK and SEK *in vitro* [1,21]. ΔMEKK1 was immunoprecipitated from vaccinia virus-transfected CV1 cell lysates, then incubated with 40 units of CIP. The phosphatase reaction was stopped by the addition of a cocktail of phosphatase inhibitors, and residual kinase activity was tested by phosphorylation of a kinase-inactive GST–SEK-KR fusion protein.

Treatment of ΔMEKK1 with CIP decreased kinase activity to an average of 41% (in replicate experiments) of the activity of ΔMEKK1 preincubated with phosphatase inhibitors only (Figure 3A, lanes 2 and 1 respectively). To control for possible contaminants within the CIP preparation that might affect MEKK1 activity independently of dephosphorylation, we incubated an equivalent amount of immunopurified ΔMEKK1 with CIP in the presence of phosphatase inhibitors (Figure 3A, lane 3). Treatment with CIP plus phosphatase inhibitors decreased MEKK1 activity only slightly compared with controls without CIP, as shown in Figure 3(B). These results indicate that phosphorylation of MEKK1 is important for its activity.

ΔMEKK1 is relatively resistant to CIP treatment, as pilot experiments with lower concentrations of CIP (1 unit in 25 μl of incubation buffer), showed a less dramatic effect (results not shown). Under no CIP concentration tested could ΔMEKK1 be completely and irreversibly inactivated. The residual MEKK1 activity observed after phosphatase treatment could be due in part to MEKK's ability to regain critical phosphates through autophosphorylation (see below).

Characterization of MEKK1 autophosphorylation

We sought to establish whether the phosphorylation of Thr-575 was an autocatalytic event or resulted from the activity of a second kinase. To test whether MEKK1 was able to autophosphorylate Thr-575, we dephosphorylated this residue on purified MEKK1 by CIP treatment *in vitro*.

ΔMEKK1, ΔMEKK1-T575S and ΔMEKK1-T575A were immunopurified from transfected cells as above. The bead-bound proteins were washed extensively and equivalent amounts of the bead-bound MEKKs were then treated for 30 min at 30 °C with CIP in the presence or absence of phosphatase inhibitors.

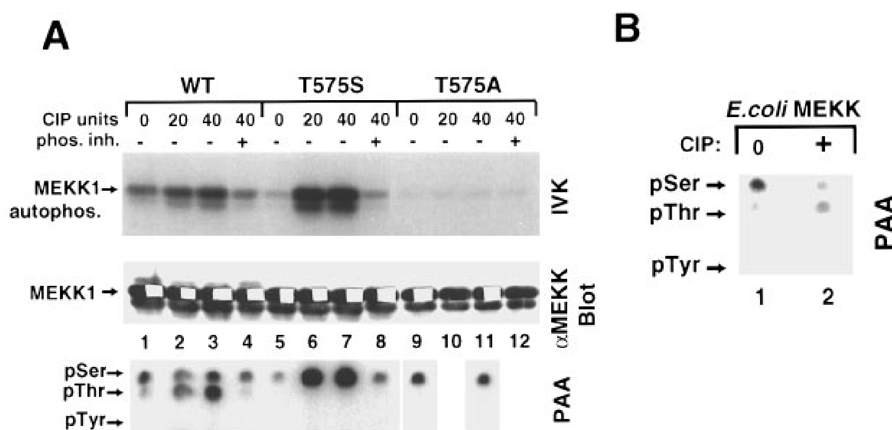

Figure 4 Autophosphorylation of MEKK1 on residue 575

(**A**) Characterization of MEKK1 autophosphorylation after phosphatase treatment. 'EE' epitope-tagged ΔMEKK1 (lanes 1–4), ΔMEKK1 T575S (lanes 5–8) and ΔMEKK1 T575A (lanes 9–12) were expressed in vaccinia virus-transfected CV1 cells. Immunoprecipitated MEKKs were preincubated for 30 min at 30 °C with: no CIP (lanes 1, 5 and 9); 20 units of CIP (lanes 2, 6 and 10); 40 units of CIP (lanes 3, 7 and 11); or 40 units of CIP in the presence of phosphatase inhibitors (lanes 4, 8 and 12). Autophosphorylation of washed MEKKs was assessed by kinase reactions *in vitro* in the presence of [γ-^{32}P]ATP followed by electroblotting, autoradiography and PAA. Top panel, autoradiograph showing increased incorporation of radiolabel on both the wild-type and the conservative T575S mutant after treatment with CIP. ΔMEKK1 T575A shows drastically decreased autophosphorylation that does not increase on treatment with CIP. Middle panel, immunoblot probed with a chicken anti-MEKK antibody, raised against bacterially expressed ΔMEKK1, to demonstrate approximately equivalent protein levels (excised areas were used for PAA). The MEKK antibody recognizes a more rapidly migrating untagged MEKK species, which probably represents an internal initiation product lacking the EE epitope tag. Bottom panel, PAA showing increasing phosphothreonine incorporation in the wild-type MEKK1 and increasing phosphoserine incorporation on the T575S mutant MEKK1 after treatment with phosphatase. ΔMEKK1 T575A does not incorporate phosphothreonine (lanes 9 and 11; exposed for 1 week as opposed to 1 day for the other lanes). No phosphotyrosine was detected in any sample. (**B**) Autophosphorylation of *E. coli*-expressed ΔMEKK1. Equivalent amounts of bacterially expressed GST–ΔMEKK1 fusion protein were subjected to autophosphorylation *in vitro* in the presence of [γ-^{32}P]ATP, followed by PAA. Lane 1, mock pretreatment; lane 2, pretreatment with 40 units of CIP. Without phosphatase treatment, primarily phosphoserine was detected. After CIP treatment an increased amount of phosphothreonine was detected. The experiment was performed four times with similar results.

After removal of the CIP by washing the beads, kinase reactions were performed *in vitro* in the presence of [γ^{32}P]ATP without added substrate. After SDS/PAGE and transfer to Immobilon, the autophosphorylated MEKKs were subjected to PAA.

Wild-type untreated ΔMEKK1 shows a low level of threonine autophosphorylation (Figure 4A, lane 1). After treatment with CIP, ΔMEKK1 showed increased incorporation of phosphothreonine (Figure 4A, lanes 2 and 3). The amount of phosphothreonine increased in proportion to the amount of CIP in the preceding reaction. This suggests that CIP dephosphorylation of Thr-575 allowed radioactive rephosphorylation of this site during the autophosphorylation reaction. The small amount of phosphothreonine recovered on MEKK1 without CIP treatment suggests that as recovered from cells, MEKK1 was nearly fully phosphorylated on Thr-575. As a control, one fraction of immunopurified MEKK1 was treated with CIP in the presence of phosphatase inhibitors. No difference from untreated controls in autophosphorylation pattern was detected (Figure 4; compare lanes 1 and 4). ΔMEKK1 T575S was phosphorylated only on serine in all reactions (Figure 4, lanes 5–8). Like wild-type ΔMEKK1, the amount of ^{32}P incorporated increased in response to phosphatase treatment. This demonstrates that the mutant ΔMEKK1 with a serine residue at position 575 is phosphorylated *in vivo* and can be dephosphorylated by CIP and rephosphorylated during the autophosphorylation reaction. ΔMEKK1-T575A also incorporated label only on a serine residue (Figure 4A, lanes 9 and 11; exposed seven times longer than the rest of the PAA samples). However, unlike MEKKs containing a serine or threonine residue at position 575, ΔMEKK1-T575A showed the same low level of phosphoserine labelling regardless of the amount of CIP used during the treatment.

To quantify the amount of phosphate that could be reincorporated on Thr-575, we repeated the above experiment with wild-type ΔMEKK1 and measured the total amount of incorporated ^{32}P. An aliquot of each reaction was subjected to PAA to determine the proportion of phosphothreonine incorporation relative to phosphoserine. The amount of phosphothreonine incorporated increased approx. 5-fold in samples pretreated with CIP, from 1.6 mmol of phosphothreonine per mol of MEKK to an average of 8.15 mmol of phosphothreonine per mol of MEKK in the CIP-treated samples. This indicates that slightly less than 1% of MEKK molecules incorporated phosphate on the threonine residue at position 575 after CIP treatment. This relatively small percentage might reflect incomplete efficiency of the phosphatase or instability of the MEKK during the prolonged incubation before the kinase activity assay. In other experiments performed to assess the effect of CIP treatment on the kinase activity of MEKK, preincubations in buffer alone decreased MEKK activity to approx. 10% of controls kept on ice and not subjected to pretreatment (results not shown).

Although these experiments with mammalian-cell-derived MEKK1 suggest that ΔMEKK undergoes autophosphorylation, an alternate possibility is that a cell-derived kinase binds to ΔMEKK through immunopurification. To guard against the presence of a confounding mammalian kinase contaminating the ΔMEKK preparation, a GST–ΔMEKK1 fusion protein was prepared from bacteria. Bacterial ΔMEKK1 phosphorylates both SEK-KR (results not shown) and MEK-KM [7], indicating at least partial independence from an activating kinase. PAA of bacterially expressed ΔMEKK1, allowed to autophosphorylate in a radioactive kinase reaction *in vitro*, again shows primarily phosphoserine (Figure 4B, lane 1). After treatment with 40 units of CIP a relative increase in phosphothreonine incorporation was observed (Figure 4B, lane 2). This result suggests that Thr-575 is phosphorylated within bacteria, as it is in mammalian

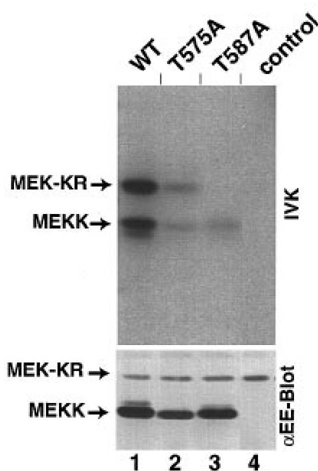

Figure 5 Kinase activities of ΔMEKK1 T575A and T587A relative to the wild-type

EE-tagged ΔMEKK species and kinase-inactive MEK (KR) substrate were immunopurified from vaccinia virus-transfected CV1 cells with equivalent amounts of 'EE' monoclonal antibody-bound Affi-Gel beads. The products of kinase reactions *in vitro* (30 min) were subjected to SDS/PAGE [10% (w/v) gel] and Western blotted. Upper panel, representative autoradiograph showing transphosphorylation and autophosphorylation activity of: wild-type ΔMEKK1 (lane 1), ΔMEKK1-T575A (lane 2), ΔMEKK1-T587A (lane 3), and MEK-KR alone (negative control) (lane 4). Lower panel, immunoblot of the same blot with an anti-(EE epitope) monoclonal antibody to detect the MEKK species and MEK-KR substrate. Abbreviation: αEE, anti-EE.

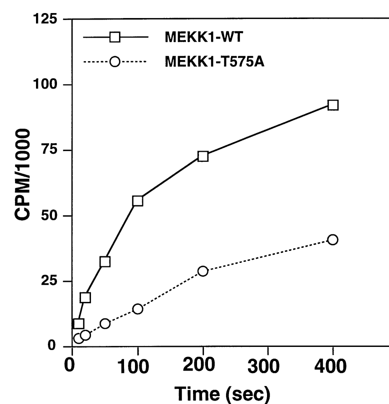

Figure 6 Relative kinase activity of ΔMEKK1 T575A *in vitro* compared with the wild-type (WT)

EE epitope-tagged ΔMEKK1 and ΔMEKK1-T575A were eluted from anti-EE immunoprecipitates of vaccinia virus-transfected CV1 cells. The activity of MEKK species was assessed by the reaction of approx. 1.5 μg of kinase-inactive, bacterially expressed, GST–SEK-KR for 10, 20, 50, 100, 200 and 400 s. The graph shows the amount of incorporated ^{32}P on the GST–SEK-KR substrate for wild-type and T575A ΔMEKK1. Quantification was performed on an Ambis β-detector. Abbreviations: CPM, c.p.m.; sec, seconds.

cells. Autophosphorylation within bacteria of PKA has similarly been observed [26].

Although Thr-575 phosphorylation is increased after CIP treatment, the total amount of phosphate incorporated into bacterially expressed ΔMEKK1 does not increase significantly. This is probably due to the instability of ΔMEKK1 during preincubation (see above). Alternatively, phosphatase treatment might reverse structurally important phosphorylation sites such as those identified in PKA (reviewed in [15]).

Both bacterially expressed ΔMEKK1 and ΔMEKK1 expressed in monkey kidney CV1 cells showed increased phosphothreonine incorporation on CIP treatment (Figures 4A and 4B). Neither ΔMEKK1 T575A nor T575S expressed in CV1 cells showed any detectable phosphothreonine under similar conditions. We conclude that Thr-575 is a major site of autophosphorylation.

Relative kinase activity of ΔMEKK1 site-directed mutants

Because the use of phosphatase in the previous experiments could have dephosphorylated residues other than Thr-575 that might themselves be responsible for the decreased kinase activity, we examined whether site-directed threonine-to-alanine mutations would affect MEKK1 activity. Mutation of Thr-575 to alanine resulted in decreased kinase activity of MEKK1 towards an immunopurified MEK-KR substrate *in vitro*, and also decreased autophosphorylation *in vitro* (Figure 5, lane 2) compared with the wild-type (Figure 5, lane 1). We also analysed a mutant of a nearby conserved threonine residue (see Figure 1 for orientation). Mutation of Thr-587 to alanine resulted in an enzyme incapable of phosphorylation of either SEK or MEK (Figure 5, lane 3, and results not shown). However, in contrast with its lack of transphosphorylation of substrate, ΔMEKK1-T587A retained a capacity for autophosphorylation (Figure 5, lane 3), although at a reduced level compared with wild-type ΔMEKK1.

Loss of kinase activity as a result of mutation at Thr-587, which by our earlier analysis is not a site of phosphorylation, suggests that structural changes were probably the cause of the loss of kinase activity. Thr-587 lies six residues N-terminal to the PE consensus of domain VIII. Threonine residues are found at equivalent positions in ERK2 (Thr-188) and PKA (Thr-201), and are not phosphorylation sites in these kinases. Instead they are probably important constituents of a hydrogen-bonding network within the catalytic domains of both enzymes [27].

To quantify the decreased transphosphorylation activity of ΔMEKK1-T575A more accurately, we performed kinetic experiments *in vitro* with immunopurified MEKK1 mutants. Eluted ΔMEKK1 and ΔMEKK1-T575A proteins were normalized for protein concentrations by comparison of immunoblot intensity, then used to phosphorylate purified SEK-KR substrate in phosphorylation reactions *in vitro* over a time course of 10–400 s. Radiolabelled proteins were quantified with an AMBIS β-detector. Figure 6 depicts such a time course experiment. Phosphorylation of SEK substrate proceeded linearly for both kinases until approx. 100 s, then declined for each kinase, possibly through instability of the kinase. The T575A mutant showed an initial rate of 28% of the wild-type at the 50 s time point, and 38% at 100 s. Kinase activity of MEKK1-T575A was thus decreased in transphosphorylation activity compared with the wild-type, but a significant residual activity was retained. An extrapolation of this result is that phosphorylation of Thr-575 might not be absolutely required for activity of MEKK1, but the phosphorylation of this site considerably increases kinase activity.

The reduction of the specific activity of the ΔMEKK1-T575A mutant is congruent with the level of decrease in MEKK1 activity on treatment with phosphatase. Nevertheless other 'silent' phosphorylation sites could contribute to MEKK1 stability and activity, such as those found in PKA (reviewed in [15]). However, treatment of ΔMEKK1 T575A with phosphatase did not alter the activity or increase the amount of phosphate incorporated during autophosphorylation compared with untreated controls (results not shown, and Figure 4, lanes 9 and 11). These results indicate that dephosphorylation of Thr-575 is

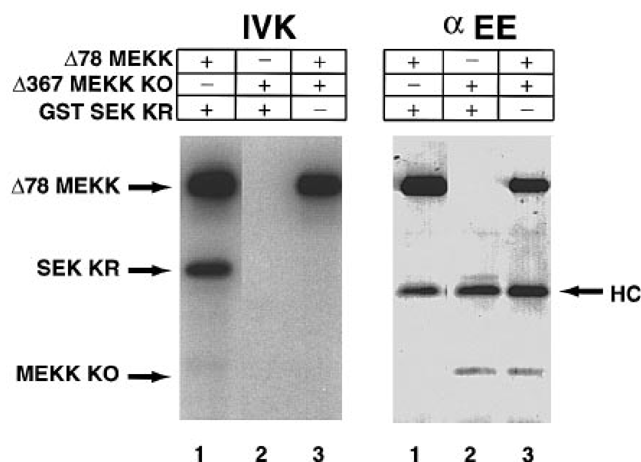

Figure 7 Intramolecular but not intermolecular autophosphorylation of MEKK1

The electrophoretically distinct Δ78 MEKK1 (lane 1) and Δ367 MEKK1-KO (lane 2) were expressed in CV1 cells, immunoprecipitated individually and tested for kinase activity with GST–SEK-KR substrate (lanes 1 and 2). To test whether Δ78 MEKK1 could transphosphorylate ΔMEKK1-KO, the same lysates were mixed and both kinases were co-immunoprecipitated and subjected to the same kinase reaction without GST–SEK-KR substrate (lane 3). The kinase reactions were subjected to SDS/PAGE, electroblotting and autoradiography (left panel). The same blot was probed with an anti-(EE epitope) antibody (αEE) (right panel). No transphosphorylation of the of the ΔMEKK1-KO protein was detected even at a much longer exposure. Abbreviation: HC, IgG heavy chain.

the mechanism by which wild-type MEKK1 activity is decreased by phosphatase.

Autophosphorylation of MEKK1 is intramolecular

'Autophosphorylation' of a kinase can represent either a true intramolecular autophosphorylation, or a transphosphorylation of one kinase molecule by another. To distinguish between the two processes we performed a kinase reaction using two electrophoretically distinct forms of MEKK1, one of which was catalytically competent and the other inactive. An epitope-tagged 78-residue deletion of MEKK1, like Δ367, was able to phosphorylate SEK-KR substrate (Figure 7, lane 1). The Δ367 MEKK1-KO mutant was absolutely inactive (lane 2). In a kinase reaction with mixed kinases, the active Δ78 kinase was unable to phosphorylate Δ367 MEKK-KO but was able to autophosphorylate strongly (Figure 7, lane 3). Thus MEKK1 autophosphorylation seems to be an intramolecular rather than an intermolecular reaction. Also, because the kinase-inactive ΔMEKK1 KO did not become labelled in this reaction *in vitro*, it is unlikely that autophosphorylation of other MEKK1s is a result of co-precipitation of an endogenous MEKK1 kinase.

DISCUSSION

Some essential features of the catalytic domain of protein kinases are highly conserved (reviewed in [14]). Amino acids between subdomains VII and VIII often contain demonstrable or potential phosphorylation sites. Activation of the mitogen-activated protein (MAP) kinase ERK2 requires both threonine (Thr-183) and tyrosine (Tyr-185) phosphorylation within this region [28]. In the same region, the MAP kinase activator MEK1 requires activating phosphorylation of two serine residues (Ser-218 and Ser-222) [21,29,30]. In addition to transphosphorylation by an activating kinase, autophosphorylation within this region is important for the regulation of some kinases. On ligand binding, the insulin receptor autophosphorylates three tyrosine residues (Tyr-1158, Tyr-1162 and Tyr-1163) within this region, two of which (Tyr-1162 and Tyr-1163) are required for activating the kinase function [31]. PKA autophosphorylates a threonine residue (Thr-197) within this loop, which interacts with the side chains of His-87, Arg-165 and Lys-189 [32]. Autophosphorylation of Thr-197 is also thought to be important for the binding of the PKA regulatory subunit [33,34]. As an alternative mechanism of kinase activation through the activation loop, the co-crystal structure of CDK2 with its activator cyclin A [35] has revealed that the cyclin serves to hold open the activation loop and presumably allows substrate binding. This activation by cyclin binding is apparently co-operative with phosphorylation of the loop at T160.

That MEKK1 is activated by phosphorylation of sequences in the activation loop places this kinase in the expanding group of kinases that are similarly activated. This activation mechanism is so broad that it is likely to have been a feature of the primordial kinase as well as the highly specialized kinases involved in cell signalling. The catalytic domain of MEKK1 is able to autophosphorylate these residues, enhancing its own activity. This is a feature shared with PKA [32] but is distinct from the cascade phosphorylation demonstrated by MEKK–SEK–SAPK and RAF–MEK–MAPK phosphorylation. Our results do not exclude the possibility that other cellular kinases serve to additionally phosphorylate this activation site of MEKK1, and thus pass activating signals along to MEKK1, but we have not identified such activity with stimulated cell extracts and bacterially expressed MEKK1-KO (results not shown). The ability of MEKK1 to auto-activate would apparently relieve MEKK1 from a requirement for phosphorylation by an upstream kinase. However, this result leaves open the question of how MEKK1 activity is controlled in signal transduction. We suggest that this mechanism will probably not involve regulated phosphorylation of this site by an upstream kinase, although phosphorylation within the regulatory domain remains a possibility.

The two mutants of the MEKK1 activation loop that we have described here are phenotypically distinct. Whereas ΔMEKK1-T757A displays reduced phosphorylation of substrate and autophosphorylation, the behaviour of the MEKK1-T587A mutant is unique in our experience. ΔMEKK1-T587A is incapable of phosphorylation of substrate but it is capable of autophosphorylation on Thr-575. This suggests that autophosphorylation of the activation loop is a 'simpler' reaction, without the necessity for substrate recognition. On the basis of the crystal structures available for model kinases, the activation loop apparently obstructs the substrate-binding pocket. In the unphosphorylated state, the activation loop bearing Thr-575 is positioned similarly to a genuine substrate in the binding pocket. MEKK1 activation and catalysis might thus be thought of in three steps: (1) activation through the regulatory domain (uncharacterized), (2) intramolecular autophosphorylation of Thr-575, resulting in an open substrate-binding pocket, and (3) substrate binding and phosphorylation. MEKK1 T575A is thus deficient in steps (2) and (3) of this model, whereas MEKK1-T587A is defective in step (3) only.

If we consider the entire MEKK1 polypeptide, the overall regulation of MEKK1 is probably complex. Bacterially expressed full-length MEKK1 is capable of both autophosphorylation as well as phosphorylation of GST–SEK (results not shown). Thus the N-terminal 'regulatory domain', retained in the full-length native protein, does not inhibit either autophosphorylation or substrate recognition.

As with Raf, MEKK1 activation might require Ras activity [8,9]. MEKK1 is similar to the α-factor signalling kinase Ste11 [20], which associates with the MAPK/SAPK homologue Fus3 and the SEK homologue Ste7 via a nucleating protein Ste5 [36]. By analogy, mammalian MAPK cascade intermediates might also be regulated, in part, by subcellular organization. This model would predict the existence of a Ste5-equivalent protein in mammalian cells that might couple MEKK1, SEK and SAPK, and regulate the cascade activity. Evidence for similar clustering of members of the MAPK cascade with Ras has already been found [37]. Such coupling could serve to modulate the apparently intrinsic ability of MEKK1 to auto-activate and could prove to be the proximal activation mechanism of MEKK1.

The catalytic domain of MEKK1 is also phosphorylated on one or more unidentified serine residues in our experiments. The serine residue(s) do not lie within the activation loop because mutation of the only serine residue located in that kinase domain did not affect the kinase activity and did not alter the stoichiometry of serine phosphorylation. Serine phosphorylation of MEKK1 could represent alternative regulating functions such as feedback inhibition. Such feedback regulation of MEK by MAPK has been postulated [38]. Additional serine residues located at the N-terminus of MEKK1 are also phosphorylated, as shown by the high stoichiometry of serine phosphorylation seen in autophosphorylation reactions using larger MEKK1 polypeptides (J. C. Deak, unpublished work).

The functional importance of the autophosphorylation of Thr-575 of MEKK1 awaits further physical characterization of the enzyme. It is likely that this phosphorylation serves to move aside the activating loop that (in model kinase crystal structures) lies in front of the substrate-binding pocket. Additional roles such as recognition of a regulatory subunit (e.g. PKA) [33,34] or induction by a kinase effector (e.g. the insulin receptor) [31] are more speculative. Thr-575 phosphorylation of MEKK1 is likely to serve as one element of a multi-component regulatory path.

We thank Dr. Leonard I. Zon for the SEK cDNA and Dr. John M. Kyriakis for the GST ΔMEKK1 construct; Dr. James Woodgett, M. Yan, M. Mahalingam and L. Horton for helpful discussion; and Y. Qian and M. Lewis for technical assistance. This work was supported by NIH grant CA66134 to D. T. and by NRSA grant CA68738 to J. D.

REFERENCES

1 Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R. and Templeton, D. J. (1994) Nature (London) **372**, 798–800
2 Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) Nature (London) **369**, 156–160
3 Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R. J. (1994) Cell **76**, 1025–1037
4 Hibi, M., Lin, A., Smeal, T., Minden, A. and Karin, M. (1993) Genes Dev. **7**, 2135–2148
5 Pombo, C. M., Kehrl, J. H., Sanchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T. and Kyriakis, J. M. (1995) Nature (London) **377**, 750–754
6 Winston, B. W., Lange-Carter, C. A., Gardner, A. M., Johnson, G. L. and Riches, D. W. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 1614–1618
7 Russell, M., Lange-Carter, C. A. and Johnson, G. L. (1995) J. Biol. Chem. **270**, 11757–11760
8 Lange-Carter, C. A. and Johnson, G. L. (1994) Science **265**, 1458–1461
9 Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L. and Karin, M. (1994) Science **266**, 1719–1723
10 Bagrodia, S., Derijard, B., Davis, R. J. and Cerione, R. A. (1995) J. Biol. Chem. **270**, 27995–27998
11 Hill, C. S., Wynne, J. and Treisman, R. (1995) Cell **81**, 1159–1170
12 Minden, A., Lin, A., Claret, F. X., Abo, A. and Karin, M. (1995) Cell **81**, 1147–1157
13 Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S. (1995) Cell **81**, 1137–1146
14 Hanks, S. K. and Quinn, A. M. (1991) Methods Enzymol. **200**, 38–62
15 Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S. and Zoller, M. J. (1993) Trends Biochem. Sci. **18**, 84–89
16 Zhang, F., Robbins, D. J., Cobb, M. H. and Goldsmith, E. J. (1993) J. Mol. Biol. **233**, 550–552
17 De-Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. and Kim, S. H. (1993) Nature (London) **363**, 595–602
18 Crews, C. M., Alessandrini, A. and Erikson, R. L. (1992) Science **258**, 478–480
19 Wu, J., Rossomando, A. J., Her, J. H., Del-Vecchio, R., Weber, M. J. and Sturgill, T. W. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 9508–9512
20 Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. and Johnson, G. L. (1993) Science **260**, 315–319
21 Yan, M. and Templeton, D. J. (1994) J. Biol. Chem. **269**, 19067–19073
22 Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A. and Fuerst, T. R. (1990) Nature (London) **348**, 91–92
23 Elroy-Stein, O., Fuerst, T. and Moss, B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6126–6130
24 Sarkar, G. and Sommer, S. S. (1990) Biotechniques **8**, 404–407
25 Kamps, M. P. and Sefton, B. M. (1989) Anal. Biochem. **176**, 22–27
26 Yonemoto, W. M., McGlone, M. L., Slice, L. W. and Taylor, S. S. (1991) Methods Enzymol. **200**, 581–596
27 Zhang, F., Strand, A., Robbins, D., Cobb, M. H. and Goldsmith, E. J. (1994) Nature (London) **367**, 704–711
28 Ray, L. B. and Sturgill, T. W. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1502–1506
29 Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J. and Cowley, S. (1994) EMBO J. **13**, 1610–1619
30 Zheng, C.-F. and Guan, K.-L. (1994) EMBO J. **13**, 1123–1131
31 Zhang, B., Tavaré, J. M., Ellis, L. and Roth, R. A. (1991) J. Biol. Chem. **266**, 990–996
32 Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1991) Science **253**, 414–420
33 Gibbs, C. S., Knighton, D. R., Sowadski, J. M., Taylor, S. S. and Zoller, M. J. (1992) J. Biol. Chem. **267**, 4806–4814
34 Orellana, S. A. and McKnight, G. S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4726–4730
35 Jeffrey, P., Russo, A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. and Pavletich, N. (1995) Nature (London) **376**, 313–320
36 Choi, K. Y., Satterberg, B., Lyons, D. M. and Elion, E. A. (1994) Cell **78**, 499–512
37 Moodie, S. A., Willumsen, B. M., Weber, M. J. and Wolfman, A. (1993) Science **260**, 1658–1661
38 Brunet, A., Pages, G. and Pouysségur, J. (1994) FEBS Lett. **346**, 299–303
39 Cazaubon, S. M. and Parker, P. J. (1993) J. Biol. Chem. **268**, 17559–17563
40 Shoji, S., Titani, K., Demaille, J. G. and Fischer, E. H. (1979) J. Biol. Chem. **254**, 6211–6214
41 Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J. and Sturgill, T. W. (1991) EMBO J. **10**, 885–892
42 Gould, K. L., Moreno, S., Owen, D. J., Sazer, S. and Nurse, P. (1991) EMBO J. **10**, 3297–3309
43 Krek, W. and Nigg, E. A. (1992) Nature New Biol. **4**, 323–329
44 Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993) Nature (London) **364**, 249–252
45 Weng, Q. P., Andrabi, K., Klippel, A., Kozlowski, M. T., Williams, L. T. and Avruch, J. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 5744–5748

Received 10 June 1996/20 September 1996; accepted 3 October 1996