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Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues

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MEK is a family of dual specific protein kinases which activate the extracellular signal-regulated kinases by phosphorylation of threonine and tyrosine residues. MEK itself is activated via serine phosphorylation by upstream activator kinases, including c-raf, mos and MEK kinase. Here, we report the activation phosphorylation sites of human MEK1 and yeast *STE7* kinase as determined by a combination of biochemical and genetic approaches. In human MEK1, substitution of either serine residue 218 or 222 with alanine completely abolished its activation by epidermal growth factor-stimulated Swiss 3T3 cell lysates or immunoprecipitated c-raf, suggesting that both serine residues are required for MEK1 activation. Phosphopeptide analysis demonstrated that serine residues 218 and 222 of human MEK1 are the primary sites for phosphorylation by c-raf. These two serine residues are highly conserved in all members of the MEK family, including the yeast *STE7* gene product, a MEK homolog in the yeast mating pheromone response pathway. Mutation of the corresponding residues in *STE7* completely abolished the biological functions of this gene. These data demonstrate that MEK is activated by phosphorylation of two adjacent serine/threonine residues and this activation mechanism is conserved in the MEK family kinases.

Key words: ERK/kinase/MEK activation/c-raf/STE7

Introduction

The mitogen activated protein kinase (MAPK) is acutely activated by mitogenic growth factors, including insulin, epidermal growth factor (EGF), platelet derived growth factor (PDGF) and nerve growth factor (NGF) (for review see Cobb *et al.*, 1991; Ahn *et al.*, 1992; Blenis, 1993; Crews and Erikson, 1993; Davis, 1993). Since numerous extracellular signals such as cytokines, T-cell antigens, phorbol esters and hormones binding to trimeric G-protein coupled receptors can induce a rapid activation of MAPK, it is also known as extracellular-signal regulated kinase (ERK) (for review see Cobb *et al.*, 1991). Accumulating evidence indicates that ERK activation is an essential event in mitogenic growth factor signal transduction. ERK has been indicated to phosphorylate and regulate transcription factors, protein kinases, EGF receptors, phospholipase A_2 and cytoskeletal proteins (for review see Blenis, 1993; Davis, 1993).

ERK is directly activated by an upstream activator kinase, MEK (also known as MAPK kinase, MAPKK), via phosphorylation on threonine and tyrosine. Threonine 183 and tyrosine 185 in the kinase subdomain VII and VIII of ERK2 are the activation phosphorylation sites (Payne *et al.*, 1991). *In vitro* phosphorylation of recombinant ERK by MEK can stimulate ERK activity as much as 1000-fold (Robbins *et al.*, 1993). The *in vitro* activated ERK has a specific activity comparable with that of ERK purified from mitogen-stimulated cells, suggesting that phosphorylation by MEK alone can fully activate ERK. Dephosphorylation of the threonine residue by phosphoprotein phosphatase 2A or the tyrosine residue by CD45 completely inactivates ERK, indicating that phosphorylation of both threonine and tyrosine are required for ERK activation (Anderson *et al.*, 1990; Gomez and Cohen, 1991). Recently, a mitogen-induced dual specific phosphatase has been shown to inactivate ERK by specifically dephosphorylating threonine and tyrosine residues (Alessi *et al.*, 1993; Charles *et al.*, 1993; Zheng and Guan, 1993a).

The proto-oncogene c-raf is a serine/threonine kinase whose activity is stimulated by growth factors. Cells transformed with v-raf, or the oncogenic mutants of c-raf, display elevated MEK activity, suggesting that MEK is downstream of raf (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). A MEK gene has been isolated in *Drosophila* as a downstream suppressor of raf, providing genetic evidence that raf functions upstream of MEK (Tsuda *et al.*, 1993). Furthermore, immunoprecipitated raf could phosphorylate and activate MEK *in vitro* (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). MEK kinase (MEKK) has been isolated based on sequence homology to the yeast *STE11* gene, and has been demonstrated to phosphorylate and activate MEK (Lange-Carter *et al.*, 1993). It has been suggested that MEKK may be responsible for trimeric G-protein mediated MEK activation. The proto-oncogene c-mos, a germ cell specific serine/threonine kinase, has been shown to activate MEK directly (Posada *et al.*, 1993).

Three distinct signal transduction pathways have been identified in the budding yeast *Saccharomyces cerevisiae* using a kinase cascade similar to the mammalian ERK/MEK pathway (for review see Errede and Levin, 1993). These include the mating pheromone response, protein kinase C mediated cell wall constructions and osmolarity regulation (Gartner *et al.*, 1992; Lee and Levin, 1992; Brewster *et al.*, 1993; Irie *et al.*, 1993; Lee *et al.*, 1993). The best studied system is the mating pheromone response signal transduction. A pair of functionally redundant kinases, *FUS3* and *KSS1* in the mating pathway share 55% amino acid sequence identity to the mammalian ERK (Courchesne *et al.*, 1989; Elion *et al.*, 1990; Boulton *et al.*, 1991). The upstream activator of *FUS3/KSS1* is the *STE7* gene product which shares high sequence identity (~50%) with MEK (Teague *et al.*, 1986; Crews *et al.*, 1992), and can activate the *FUS3* gene product *in vitro* (Errede *et al.*, 1993). Furthermore,

the *STE11* gene, which has been demonstrated to function upstream of *STE7* gene, is closely related to MEKK (Rhodes *et al.*, 1990; Cairns *et al.*, 1992; Lange-Carter *et al.*, 1993; Stevenson *et al.*, 1992), suggesting that the ERK/MEK kinase cascade is highly conserved through evolution.

Because MEK can be activated by several different kinases involved in different signaling pathways, it may serve as an integration point for different signals. The molecular mechanism of MEK activation is not known although evidence indicates that MEK activation is accompanied with serine and possibly threonine phosphorylation (Gomez *et al.*, 1992; Ahn *et al.*, 1993; Matsuda *et al.*, 1993). In this report, we demonstrate that c-raf activates human MEK1 by phosphorylation of both serine residues 218 and 222. Substitution of either residue by an alanine completely abolished MEK1 activation *in vitro*. Moreover, the biological significance of these activation phosphorylation sites was confirmed in yeast by using the *STE7* gene of the mating pheromone response pathway as a model.

Results

Serine 218 and 222 of human MEK1 are essential for activation

MEK activation has been found to correlate with serine and possibly threonine phosphorylation (Gomez *et al.*, 1992; Ahn *et al.*, 1993; Matsuda *et al.*, 1993). Selective dephosphorylation of serine residues completely inactivates MEK, indicating the importance of serine phosphorylation (Gomez *et al.*, 1992; Ahn *et al.*, 1993). Sequence alignment reveals that five serine/threonine residues are conserved in all members of the MEK family including mammalian, *Xenopus*, *Drosophila* MEKs, *STE7, MKK1, MKK2, PBS2* of *S.cerevisiae*, and byr1, wis1 of *Schizosaccharomyces pombe* (Ashworth *et al.*, 1992; Boguslawski and Polazzi, 1987; Crews *et al.*, 1992; Irie *et al.*, 1993; Kosako *et al.*, 1993; Nadin-Davis and Nasim, 1988; Seger *et al.*, 1992; Teague *et al.*, 1986; Warbrick and Fantes, 1991; Wu *et al.*, 1993; Yashar *et al.*, 1993; Zheng and Guan, 1993b), which correspond to serine residues 150, 212, 218, 222 and 248 of human MEK1. The schematic locations of these serine residues are shown in Figure 1. We envision that regulatory phosphorylation residues should be unique to MEK. Therefore, serine residue 248 is unlikely to serve as the regulatory phosphorylation site unique for MEK since it is also highly conserved in other serine/threonine kinases (Hanks and Quinn, 1991). To test the importance of these conserved serine residues in MEK activation, serines 150, 212, 218 and 222 were independently mutated to alanine by oligonucleotide directed mutagenesis. The MEK1 mutants were expressed as GST fusions and purified by glutathione−agarose affinity chromatography (Figure 2A).

Basal activity of mutant GST-MEK1 was determined by *in vitro* ERK activation assay and results are shown in Figure 2B. Mutation of serine 150 of MEK1 had no effect on its ability to activate ERK. In contrast, substitution of serine 212 by an alanine enhanced the basal activity ~3- to 4-fold, while substitution of serine 218 or 222 by alanine decreased the basal activity (Figure 2B). The fact that all four mutants retain basal MEK activity and can be autophosphorylated (data not shown) indicates that alanine substitution of these serine residues does not inactivate the enzyme.

Mutants of GST-MEK1 were subjected to *in vitro* activation by EGF stimulated-Swiss 3T3 cell lysates. MEK1S150A and MEK1S212A were activated to a level comparable with the wild type (Figure 3A), suggesting that serines 150 and 212 are not essential for MEK activation. Interestingly, mutation of either serine 218 or 222 abolished MEK activation (Figure 3A).

The proto-oncogene c-raf has been demonstrated to be a MEK activator in response to growth factor (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). To determine whether serines 218 and 222 are essential for MEK activation by c-raf, *in vitro* activation of mutant MEK1 by immunoprecipitated c-raf was performed. Since the S150A mutant behaves similarly to wild type, only the MEK1 mutants S212A, S218A and S222A were used in this experiment. The immunoprecipitated c-raf increased the activity of the S212A mutant as well as the wild type, but not the S218A or S222A mutant (Figure 3B).

Serine residues 218 and 222 of MEK1 are phosphorylated by c-raf

To determine whether serines 218 and 222 are the activation phosphorylation sites, *in vitro* phosphorylation of MEK1 and mutants by immunoprecipitated c-raf was performed. In order to distinguish the raf-dependent phosphorylation from MEK-1 autophosphorylation, the catalytically essential lysine

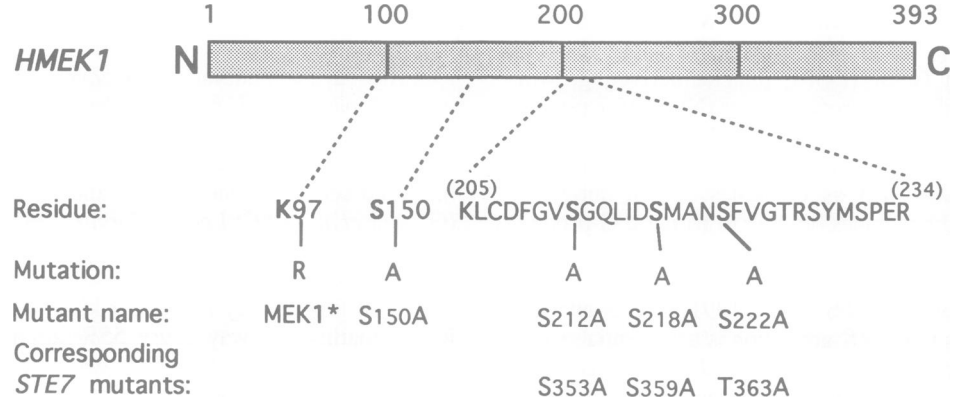

Fig. 1. Schematic representation of mutations of conserved serine/threonine residues in human MEK1 and yeast STE7. Numbers denote the positions of amino acid residues in human MEK1 or *S.cerevisiae STE7*. The amino acid sequence of the tryptic peptide (residues 206−234) containing the activation phosphorylation sites is shown. MEK1* denotes the kinase deficient mutant which contains the Lys97 substituted by an arginine.

residue 97 of MEK1 was mutated to an arginine to produce a kinase deficient MEK1* (see Figure 1). The kinase deficient mutants of GST-MEK1*, GST-MEK1*S150A, GST-MEK1*S212A, GST-MEK1*S218A and GST-MEK1*S222A were expressed and purified as in Figure 2. In addition, GST-MEK1*S218/222A, in which both serines 218 and 222 were substituted by alanine, was constructed. All of these mutants showed no apparent autophosphorylation (data not shown). The c-raf could phosphorylate GST-MEK1*, GST-MEK1*S150A, GST-MEK1*S212A, GST-MEK1*S218A and GST-MEK1*S222A. Phosphorylation of GST-MEK1*S218/222A by c-raf was significantly reduced (~8-fold lower than GST-MEK1*, data not shown). These results indicate that the double mutations of serines 218 and 222 eliminated the majority of c-raf-dependent phosphorylation of MEK1.

The raf-phosphorylated GST-MEK1* mutants were analyzed by two-dimensional tryptic phosphopeptide mapping. GST-MEK1*S150A and S212A displayed phosphopeptide patterns similar to that of the wild type (Figure 4, panels 1, 3 and 4), consistent with the activation results in Figures 2 and 3. In contrast, GST-MEK1*S218A did not contain phosphopeptides 2, 3, 4, 5, 6 and 7 (Figure 4, panels 1 and 5) while GST-MEK1*S222A mutant missed

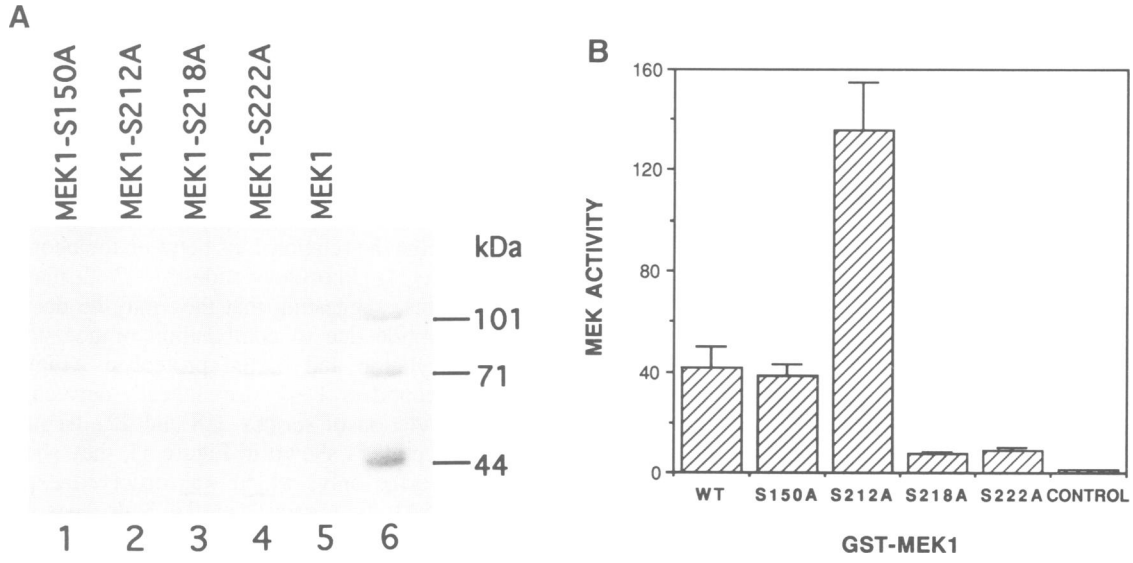

Fig. 2. (**A**) SDS-PAGE of recombinant GST-MEK1 and mutants. Molecular weight standards (kDa) are shown in lane 6. Purified recombinant proteins were resolved by a 10% SDS-PAGE followed by protein staining. (**B**) Basal activity of GST-MEK1, GST-MEK1S150A, GST-MEK1S212A, GST-MEK1S218A and GST-MEK1S222A. MEK activity was determined by the activation assay of recombinant human ERK1. An equal amount of recombinant protein was used in ERK activation assay for each GST-MEK mutant. The control denotes ERK activity in the absence of MEK and is given a value of one.

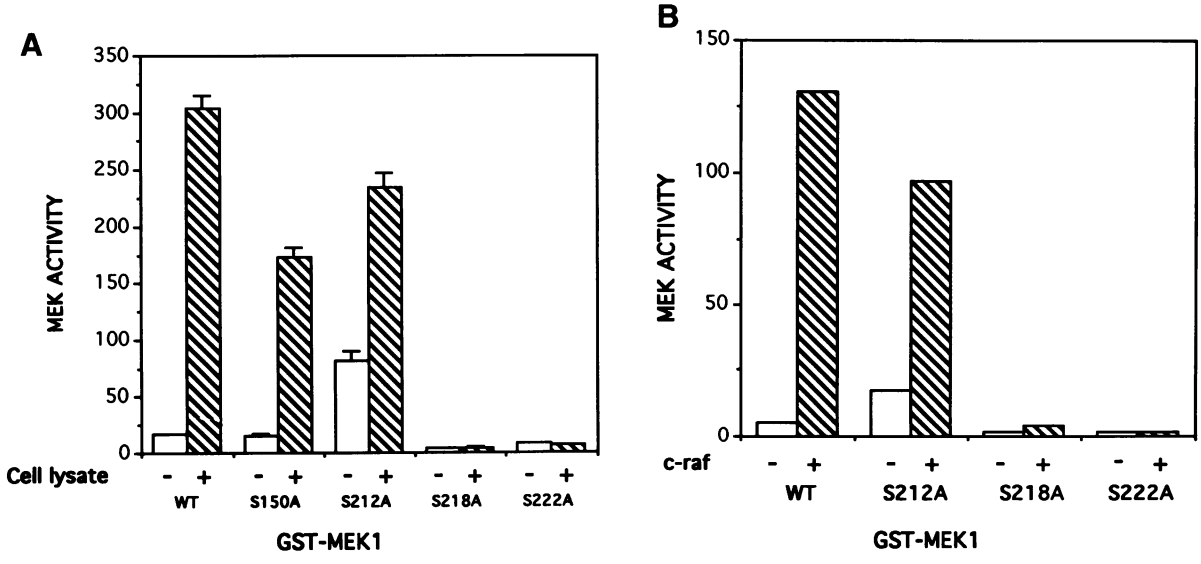

Fig. 3. (**A**) Activation of GST-MEK1 and mutants by EGF stimulated Swiss 3T3 cell lysates. GST-MEK1 and mutants were activated by cell lysates (+, hatched bars) or buffer (−, open bars), and purified from the reactions by glutathione−agarose affinity chromatography. MEK activity was determined by the ERK1 activation assay. Recombinant ERK1 has basal myelin basic protein (MBP) kinase activity. This MBP kinase activity can be activated by MEK. Data were from three independent assays. (**B**) Activation of GST-MEK1 and mutants by immunoprecipitated c-raf. GST-MEK1 and mutants were activated by immunoprecipitated c-raf (+, hatched bars) or buffer (−, open bars). MEK activity was measured by ERK activation assay.

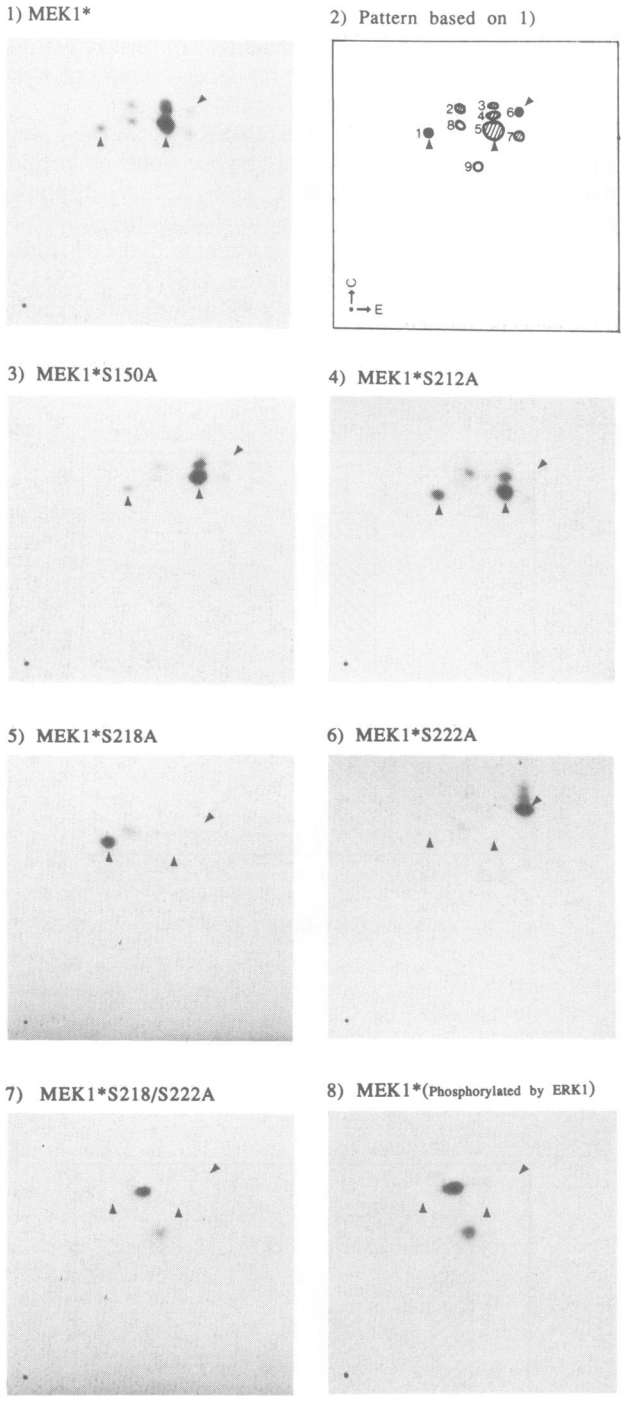

Fig. 4. Tryptic phosphopeptide mapping of MEK1* and mutants phosphorylated by c-raf or ERK1. GST-MEK1* and mutants were phosphorylated by immunoprecipitated c-raf and analyzed by two-dimensional tryptic phosphopeptide mapping (panels 1 and 3–7). Approximately 5-fold more sample was used in panel 7 than in panels 1 and 3–6. Phosphorylation of MEK1* by recombinant ERK1 is shown in panel 8. Schematic representation of phosphopeptides observed in wild type (panel 1) is shown in panel 2. The filled spots denote phosphopeptides containing single phosphorylation of serine 218 (spot 6) and serine 222 (spot 1). Hatched spots denote phosphopeptides likely to contain double phosphorylation of residues 218 and 222 (spots 2, 3, 4, 5 and 7). Open spots represent peptides phosphorylated by ERK1 (spots 8 and 9). The directions for electrophoresis (E, from cathode to anode) and TLC (C) are indicated in the lower left corner of panel 2. A dot in the lower left corner of each panel denotes the origin. Arrows (▲) indicate positions of phosphopeptides 1, 5 and 6 for references between different panels.

phosphopeptides 1, 2, 3,4, 5 and 7 (Figure 4, panels 1 and 6). Double mutant GST-MEK1*S218/222A eliminated phosphopeptides 1–7 (Figure 4, panel 7, ~5-fold more sample was used in this panel than the others). These results indicated that phosphopeptide 1 was likely derived from serine 222 phosphorylation while phosphopeptide 6 was derived from serine 218 phosphorylation. Phosphopeptides 2, 3, 4, 5 and 7 may be derived from partial proteolytic cleavage of the double phosphorylated peptide (see below). Phosphopeptides 8 and 9 in GST-MEK1*S218/222A mutant showed identical mobility to the phosphopeptides of GST-MEK1* phosphorylated by ERK1 (Figure 4, panels 7 and 8), indicating that they were derived from ERK-dependent phosphorylation sites. Two-dimensional phosphopeptide mapping of raf phosphorylated MEK has been recently reported (Kyriakis *et al.*, 1993). However, it is not feasible to compare the phosphopeptide map in Figure 4 with that described by Kyriakis *et al.* (1993) because different conditions were used for separation.

Partial acid hydrolysis of phosphopeptides recovered from the two-dimensional phosphopeptide mapping was performed to determine the relationships between the phosphopeptides in Figure 4. Phosphopeptides 1–7 displayed similar fingerprints, suggesting that they may be derived from a single peptide due to combination of non-stoichiometric phosphorylation and partial proteolysis (Figure 5A). If phosphopeptides 1–7 are indeed derived from the phosphorylation of serines 218 and 222 (sequence of the tryptic peptide is shown in Figure 1), they should contain phosphoserine only, which was observed experimentally (Figure 5A). In contrast, partial hydrolysis of phosphopeptides 8 and 9 displayed fingerprints distinct from phosphopeptides 1–7, and contained phosphothreonine. Furthermore, partial hydrolysis of phosphopeptides 8 and 9 from the ERK1 phosphorylated MEK1 (panel 8 of Figure 4) showed fingerprints identical to those in lanes 8 and 9 of Figure 5A (data not shown). We conclude that phosphopeptides 8 and 9 were derived from ERK-dependent phosphorylation. These results are consistent with the fact that two putative ERK recognition consensus sites (threonine residues 292 and 386) are present in MEK1 (Seger *et al.*, 1992; Zheng and Guan, 1993b) and that residual ERK activity was detected in the raf immunoprecipitants (unpublished observation).

To provide further evidence that serine residues 218 and 222 of MEK1 are the raf-phosphorylation sites, serines 218 and 222 were independently mutated to threonine residues. Interestingly, both GST-MEK1*S218T and GST-MEK1*S222T could be phosphorylated by immunoprecipitated raf and showed tryptic phosphopeptide patterns similar to the wild type (data not shown). If phosphopeptide 1 is derived from phosphorylation of residue 222, phosphopeptide 1 in GST-MEK1*S222T should contain phosphothreonine rather than the phosphoserine found in GST-MEK1*. Similarly, phosphopeptide 6 in GST-MEK1*S218T should contain phosphothreonine. If a phosphopeptide contains phosphorylation of both residue 218 and 222, it should contain both phosphoserine and phosphothreonine in either GST-MEK1*S218T or GST-MEK1*S222T. Phosphoamino acid analysis of corresponding phosphopeptides from the raf-phosphorylated GST-MEK1*S218T and GST-MEK1*S222T showed results precisely as predicted (Figure 5B). Phosphopeptide 1 contained phosphothreonine in GST-MEK1*S222T (Figure

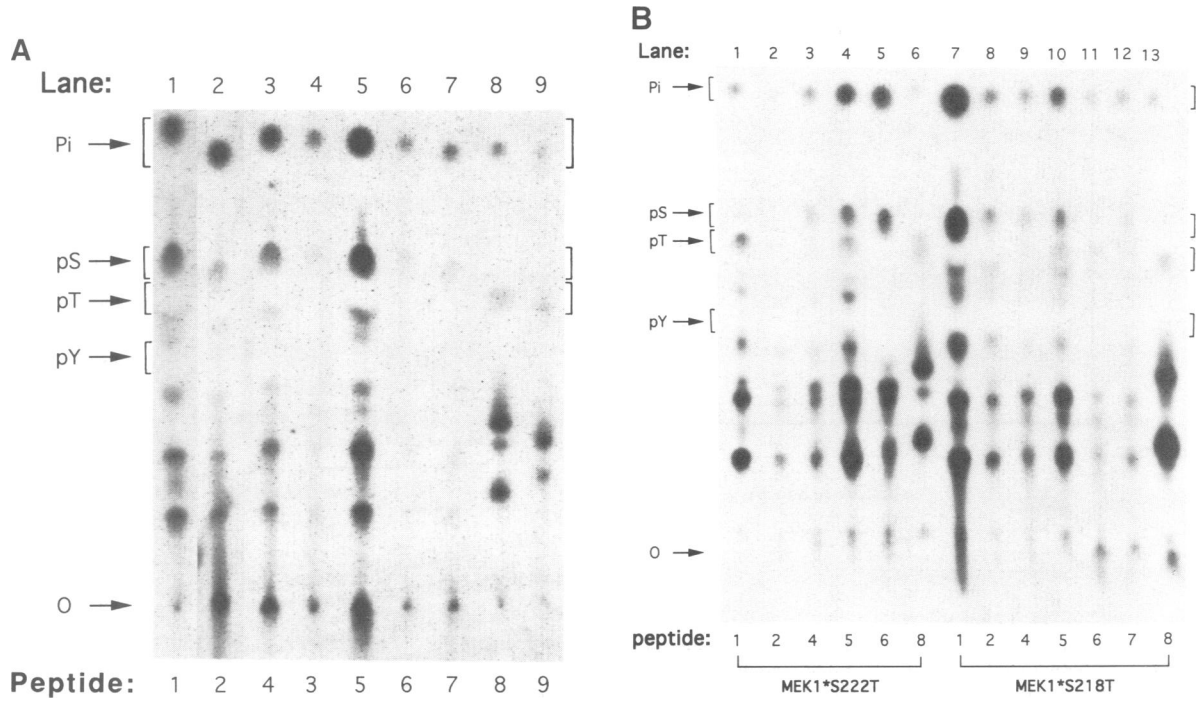

Fig. 5. Partial acid hydrolysis of phosphopeptides recovered from two-dimensional mapping. (**A**) The identity of phosphopeptide in each lane is indicated at the bottom of the figure. Phosphopeptides 1–7 (lanes 1–7) were recovered from panel 1 of Figure 4 while phosphopeptides 8 and 9 (lanes 8 and 9) were from panel 7 of Figure 4. The recovered phosphopeptides were hydrolyzed in 6 N HCl at 105°C for 1 h and resolved by one-dimensional electrophoresis. Pi, pS, pT, pY, and O denote positions of free phosphate, phosphoserine, phosphothreonine, phosphotyrosine and origin, respectively. (**B**) Partial acid hydrolysis of phosphopeptides from MEK1*S218T and MEK1*S222T mutants. GST-MEK1*S218T and GST-MEK1*S222T were phosphorylated by c-raf and subjected to two-dimensional phosphopeptide mapping. The resolved phosphopeptides were recovered and analyzed by phosphoamino acid analysis. Corresponding phosphopeptides are labeled at the bottom of the figure. Phosphopeptides from MEK1*S218T (lanes 7–13) and MEK1*S222T (lanes 1–6) are indicated.

5B, lane 1) while phosphopeptide 6 contained phosphothreonine in GST-MEK1*S218T mutant (Figure 5B, lane 11). Furthermore, phosphopeptides 2, 4, 5 and 7 contained both phosphoserine and phosphothreonine, suggesting that these peptides were derived from the double phosphorylation of residues 218 and 222. Phosphopeptide 3 contained too little radioactivity to be analyzed. We do not know why double phosphorylated peptides showed multiple spots, although one possibility is due to partial proteolysis. The multiple spots may also be derived from partial oxidation of the cysteine and methionine residues in this peptide because the trypsin digested samples have not been oxidized by a reagent such as performic acid. Taken together, our data unambiguously demonstrated that serines 218 and 222 are the major raf phosphorylation sites.

The biochemical mechanism of MEK activation is conserved in yeast STE7

To study MEK activation *in vivo*, the yeast *STE7* gene was chosen as our model. The *STE7* gene product of *S.cerevisiae* shares ~50% amino acid sequence identity with human MEK1 (Teague *et al.*, 1986; Crews *et al.*, 1992). Serine residues 212 and 218 of MEK1 correspond to serines 353 and 359 in *STE7* while serine 222 is substituted by a threonine residue 363 (Figure 1). Mutations of the corresponding residues in *STE7* were made by site-directed mutagenesis. The mutated *STE7* genes were subcloned into pRS315 (Sikorski and Hieter, 1989), a low copy yeast shuttle vector, and the function of these mutant *STE7* genes was tested in yeast strain E929-6C-18 (MAT**a** *ste7-Δ3 FUS1-lacZ::URA3 can1 leu2-3,112 trp1-Δ1 ura3-52*). This strain has no functional *STE7* gene and contains a LacZ gene under the control of the *FUS1* promoter. Therefore, pheromone-dependent LacZ induction in this strain is impaired because it requires a functional *STE7* gene in the pheromone response pathway. Transformation of the wild type *STE7* gene into E929-6C-18 could confer LacZ induction by α-factor while vector alone could not (Figure 6A). Mutation of serine 353 (the MEK1 serine 212 equivalent) of *STE7* had no significant effect on α-factor-dependent LacZ induction, consistent with the observation that MEK1S212A can still be activated (Figure 3). It is worth noting that substitution of serine 353 by alanine in *STE7* did not elevate the basal LacZ expression (Figure 6A), although the corresponding mutation of MEK1 significantly elevated the basal activity (Figure 2B). Mutation of either serine 359 (the serine 218 equivalent in MEK1) or threonine 363 (the MEK1 serine 222 equivalent) completely eliminated the α-factor-induced LacZ expression (Figure 6A). The above observations can be best explained that mutation of either serine 359 or threonine 363 abolishes the pheromone induced *STE7* activation although it is possible that mutation of either residue completely inactivates the kinase.

Mating pheromone α-factor can induce a G_1 specific cell cycle arrest of mating type **a** cells. The pheromone induced cell cycle arrest cannot occur in strain E929-6C-18 due to the lack of functional *STE7* in this strain. The *STE7* wild type gene, S353A, S359A and T363A mutants were tested for α-factor induced G_1 cell cycle arrest in E929-6C-18. Both wild type *STE7* and *STE7*S353A mutant could complement the *STE7* deletion phenotype and cells arrested at G_1 upon pheromone stimulation, while cells containing

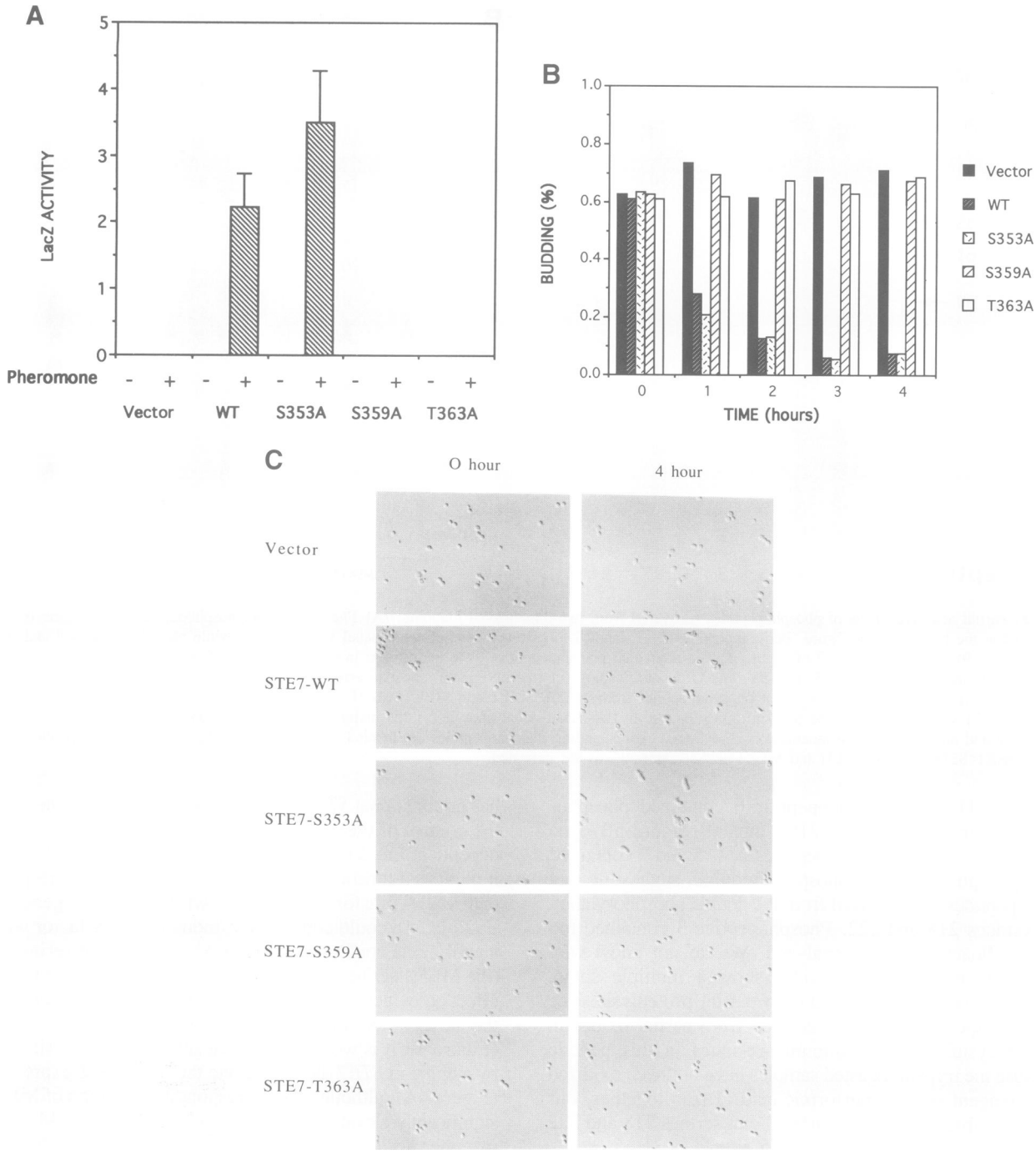

Fig. 6. Mating pheromone responses require serine 359 and threonine 363 of *STE7*. (**A**) Pheromone-dependent gene induction. The yeast strain E929-6C-18 contains a deletion of *STE7* and a *lacZ* gene under the control of the yeast *FUS1* promoter. Yeast cells were transformed with PRS315 (Vector), wild type *STE7* (WT), *STE7* Ser353Ala mutant (S353A), *STE7* Ser359Ala mutant (S359A), or *STE7* Thr363Ala mutant (T363A). *lacZ* expression (arbitrary unit) before (−) or 2.5 h after (+) pheromone (2.5 mM) induction. Data were from three independent yeast transformants for each plasmid. (**B**) Pheromone induced G_1 cell cycle arrest. Exponentially growing yeast cells were treated with α-factor (3 mM) for various times (0−4 h, *x*-axis). The budding percentage was determined microscopically. Yeast E929-6C-18 containing various plasmids are indicated. (**C**) Pheromone induced morphological change. Exponentially growing yeast cells were treated with α-factor for 4 h. Morphology of cells was examined microscopically. Yeast E929-6C-18 containing various plasmids are indicated on the left of the figure.

the *STE7*S359A or T363A mutant were unable to arrest at G_1 (Figure 6B). The mating type **a** cells undergo a dramatic morphological change when exposed to α-factor. No obvious morphological alteration was observed in E929-6C-18 cells harboring the vector, *STE7*S359A or *STE7*T363A mutant while cells containing the wild type *STE7* or *STE7S353A* mutant displayed a typical morphological change induced by α-factor (Figure 6C).

Mating pheromone treatment of the opposite mating type cells causes a sequence of events including induction of gene transcription, G_1 cell cycle arrest and mating. All these events depend on the integrity of the mating pheromone signal transduction pathway, and hence a functional *STE7* gene. The wild type *STE7* and mutant S353A, S359A or T363A were used to complement the mating defect in strain E929-16C-18 (Table I). Normal mating efficiency was

Table I. Effect of STE7 mutations on mating efficiency

Plasmid	Mating efficiency[a]
Vector	$<10^{-6}$
STE7	0.52
STE7S353A	0.29
STE7S359A	$<10^{-6}$
STE6T363A	$<10^{-6}$

[a]Yeast E929-6C-18 cells (10^6) containing various plasmids was mated with 5×10^6 test cells, MATα *thr4*. Mating efficincy was determined by counting the diploid colonies on synthetic minimal medium plates.

observed in cells containing wild type *STE7* or S353A mutant. In contrast, cells containing *STE7*S358A and S363A mutants were mating incompetent.

Substitution of serine 359 or threonine 363 by alanine in *STE7* completely eliminated the *STE7* gene function in pheromone-induced gene transcription, G_1 cell cycle arrest, morphological alteration and mating. These data support the hypothesis that serine 359 and threonine 363 are the activation phosphorylation sites although direct biochemical evidence has yet to be obtained. The genetic experiments in yeast are consistent with the *in vitro* biochemical data of human MEK1, and suggest that the molecular mechanism for MEK activation is conserved through evolution.

Discussion

We have shown that phosphorylation of both serine residues 218 and 222 is required for human MEK1 activation. Mutation of either one of these two residues completely abolished the *in vitro* activation of recombinant MEK1 by c-raf (Figure 3). Two-dimensional phosphopeptide mapping followed by phosphoamino acid analysis of GST-MEK1*, GST-MEK1*S150A, GST-MEK1*S212A, GST-MEK1*S218A, GST-MEK1*S222A, GST-MEK1*S218/222A, GST-MEK1*S218T and GST-MEK1*S222T unequivocally demonstrated that serine residues 218 and 222 are the raf phosphorylation sites in MEK1 (Figures 4 and 5). Phosphorylations of residues 218 and 222 can occur independently because mutation of one residue did not eliminate the phosphorylation of the other (Figure 4). However, double phosphorylation of serines 218 and 222 in wild type MEK1 appears to be a co-operative event because the intensity of double phosphorylated peptides was higher than expected for an independent event (Figure 4). Results from *in vivo* experiments with the *STE7* gene in yeast are consistent with the data obtained from human MEK1 and further demonstrate that activation of MEK is essential for its biological functions. Our data are supported by previous observations from other investigators that serine phosphorylation is associated with MEK activation (Gomez *et al.*, 1992; Ahn *et al.*, 1993; Matsuda *et al.*, 1993). The fact that the MEK1*S218/222A double mutant virtually eliminated all the raf-dependent phosphorylation suggests that phosphorylation of these two residues is sufficient for MEK activation.

Serine residues 218 and 222 are localized between the conserved 'DFG' and 'A/SPE' motifs of the kinase subdomains VII and VIII (Hanks and Quinn, 1991). Phosphorylation of residues in these subdomains is responsible for activation of several kinases, including ERK, $p90^{rsk}$, PKA, CDC2, CDK2 and the tyrosine kinase, src. The crystal structure of PKA demonstrates that phosphorylation of residues in this region can stabilize the kinase in an active conformation (Knighton *et al.*, 1991; De Bondt *et al.*, 1993). The same mechanism may also be employed in MEK activation. Activation of MEK and ERK shares another common feature that phosphorylation of two adjacent residues is required for activation. The requirement for double phosphorylation may be important to secure specificity. However, the regulatory phosphorylation sites of MEK appear to be more flexible than those of ERK. Threonine residue substitution at position 218 or 222 of MEK1 could still be phosphorylated by c-raf. In fact, the residue corresponding to serine 222 of MEK1 is naturally substituted by threonine in several members of the MEK family, consistent with our mutation data. In contrast, mutation of threonine 183 to serine, or tyrosine 185 to serine/threonine in ERK2 abolished their phosphorylation by MEK (E.Butch and K.-L.Guan, unpublished data), suggesting the extremely high specificity of ERK phosphorylation by MEK. This observation is consistent with the fact that MEK can be activated by several different activators including c-raf, mos and MEKK while ERK can only be activated by MEK.

Several lines of evidence support the hypothesis that phosphorylation of residues corresponding to serines 218 and 222 of MEK1 is a general molecular mechanism for activation of the MEK family kinases. First, these two serine resides are required for MEK activation by different upstream activators, including c-raf and a raf-independent activator (C.-F.Zheng and K.-L.Guan, unpublished data). Second, the corresponding residues are essential for STE7 function, suggesting that the activation mechanism is conserved through evolution. This observation also indicates that MEKK is likely to activate MEK by a similar mechanism because MEKK shares high sequence identity with STE11 (Lange-Carter *et al.*, 1993), the STE7 activator. Third, these two residues are highly conserved in all members of the MEK family including mammalian, *Xenopus*, *Drosophila* MEKs, *STE7, MKK1, MKK2, PBS2* of *S.cerevisiae* and byr1, wis1 of *S.pombe* (Teague *et al.*, 1986; Boguslawski and Polazzi, 1987; Nadin-Davis and Nasim, 1988; Warbrick and Fantes, 1991; Ashworth *et al.*, 1992; Crews *et al.*, 1992; Seger *et al.*, 1992; Irie *et al.*, 1993; Kosako *et al.*, 1993; Wu *et al.*, 1993; Yashar *et al.*, 1993; Zheng and Guan, 1993b) though serine 222 is substituted by a threonine in MEK homologs in yeast. The above observations also suggest that the MEK activation mechanism is conserved in distinct biological processes such as mating, cell wall construction and osmolarity regulation. Experiments to test the mechanism of MEK activation by MEKK or mos, and MKK1/MKK2 by BCK1 will provide further evidence for this hypothesis.

MEK activation by mitogenic growth factors is a rapid and transient event. Although the activation mechanism by kinases is emerging, little is known about the MEK inactivation process which occurs immediately after the activation. It is possible that a unique phosphatase specifically dephosphorylates serines 218 and 222 and inactivates MEK. Although phosphoprotein phosphatase 2A can inactivate MEK *in vitro*, there is no direct evidence to support that this phosphatase is a physiological MEK inactivator. Identification of a MEK phosphatase could be a necessary step toward the full understanding of MEK regulation.

Materials and methods

Site-directed mutagenesis

The *Bam*HI fragment from pGEX-2T/HMEK1 encoding the entire human MEK1 (Zheng and Guan, 1993b) and the *Hin*dIII fragment of pSTE7.4 (a generous gift of Dr B.Errede; Teague *et al.*, 1986) which contains the entire coding sequence for STE7 were subcloned into pALTER (Promega). All point mutations were made using the Altered Site Mutagenesis System (Promega) according to manufacturer's instructions, with synthetic oligonucleotides containing the mutated nucleotide. Mutations were confirmed by DNA sequencing.

Expression and purification of recombinant MEK

Human MEK1, as well as all the mutants used in this study, were subcloned into pGEX-KG, expressed as glutathione-*S*-transferase fusions and purified by glutathione−agarose (Sigma) affinity chromatography essentially as described (Guan and Dixon, 1991).

Cell culture

Swiss 3T3 cells were obtained from ATCC and maintained in DMEM supplemented with 10% calf serum (GIBCO-BRL). Cells cultured on 100 mm plates were starved with the same medium containing 0.1% serum for 24 h and then treated with EGF (100 ng/ml, Sigma) for 2.0 min. Cells were washed twice with ice cold PBS and scraped into 0.5 ml of RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.2 mM sodium vanadate, 25 mM NaF, 10 mM sodium pyrophosphate and 25 mM glycerophosphate).

Immunoprecipitation of c-raf

Cells in RIPA buffer (1 ml, 2.1 mg/ml protein) were sonicated briefly and centrifuged in a microfuge for 15 min. The supernatant was transferred to a new tube and mixed with 10 μl of pre-immune or anti-raf antiserum (a generous gift of Dr S.Decker) (Ohmichi *et al.*, 1992). After incubation on ice for 1 h, protein A−agarose (20 μl, Pierce) was added, and shaken for another 30 min on ice. The protein A−agarose was separated from the supernatant by centrifugation, washed three times with RIPA buffer and once with kinase buffer (18 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM DTT) without ATP and resuspended in the same buffer.

In vitro activation of GST-MEK and kinase assays

In vitro activation of GST-MEK was performed essentially as described (Zheng and Guan, 1993c). Briefly, GST-MEK1 or mutants (1 μg) were incubated with Swiss 3T3 cell lysates (300 μl, 2.0 mg/ml protein) or immunoprecipitated c-raf in kinase assay buffer for 45 min at 37°C. If cell lysates were used, GST-MEK1 was purified from cell lysates by glutathione−agarose resin. If immunoprecipitated raf was used, the raf protein was removed by centrifugation to remove protein A−agarose because the raf protein was bound to the protein A−agarose beads via antibodies. MEK activity was assayed by its ability to activate ERK whose activity was measured by phosphorylation of myelin basic protein as described (Sturgill *et al.*, 1991).

Phosphorylation of kinase deficient MEK1 by c-raf*

In order to study the activation phosphorylation, a kinase deficient mutant of MEK1, having lysine reside 97 substituted by arginine, was made and designated as MEK1* (Figure 1). GST-MEK1*, GST-MEK1*S150A, GST-MEK1*S212A, GST-MEK1*S218A, GST-MEK1*S222A, GST-MEK1*218/222A, GST-MEK1*S218T or GST-MEK1*S222T (2 μg) was incubated in 20 μl of kinase buffer containing 0.5 μCi [γ-^{32}P]ATP and 10 μl immunoprecipitated c-raf for 45 min at 37°C with shaking. The reaction mixture was then centrifuged to remove the protein A−agarose bound raf. The supernatant was mixed with SDS sample treatment buffer and resolved by 8% SDS−PAGE.

Phosphopeptide mapping and phosphoamino acid analysis

Phosphorylated GST-MEK1*s were resolved on SDS−PAGE and transferred onto Immobilon-P membrane (Amersham Corp.). For phosphopeptide mapping, GST-MEK1* band was excised and incubated in 0.5 ml of 0.5% PVP-40 dissolved in 100 mM acetic acid for 30 min at 37°C according to Aebersold (1989). The samples were then digested with 20 μg of TPCK-treated trypsin (Sigma) at 37°C in 75 mM sodium bicarbonate buffer, pH 8.0, containing 5% acetonitrile. After digestion for 8 h, an additional 20 μg of trypsin was added for an additional 4 h. The peptides released from the membrane after trypsin digestion were dried in speed vacuum. The dried peptides were dissolved in 1 ml of water and dried by speed vacuum (repeated three times). Finally, the peptides were dissolved in 10 μl of water and spotted on to a cellulose plate (20×20 cm, Kodak). Electrophoresis was run using 1% sodium bicarbonate buffer, pH 8.9; the second dimension was developed in water:1-butanol:pyridine:acetic acid (30:20:24:6) as described (Boyle *et al.*, 1991). The phosphopeptide pattern was then obtained by exposing the dried cellulose plates to X-ray film (Kodak). Phosphopeptides were recovered from cellulose plates and used for phosphoamino acid analysis (Boyle *et al.*, 1991).

Yeast strain and construction of STE7 mutants

The yeast strain used in this study, E929-6C-18 (MATa *ste7-Δ3 FUS1-lacZ::URA3 can1 leu2-3,112 trp1-Δ1 ura3-52*), was a generous gift from Dr B.Errede. The *Hin*dIII fragment from pSTE7.4 (Teague *et al.*, 1986) containing the entire *STE7* gene was subcloned into low copy yeast shuttle vector pRS315 (Sikorski and Hieter, 1989). The *Hin*dIII fragment of *STE7* gene was also subcloned into pALTER vector (Promega) for site-directed mutations of serine residues 353, 359 and threonine 363. The mutations were confirmed by DNA sequencing and subcloned into vector pRS315. Plasmids were introduced into yeast cells by electroporation (Becker and Guarente, 1991).

Assay of yeast pheromone response and mating reaction

Quantitative mating was performed according to Sprague (1991). β-galactosidase activity of yeast cells induced by 3 μM α-factor for 2.5 h was assayed following published procedures (Guarente and Mason, 1983). Percentage of unbudded cells was determined microscopically (Sprague, 1991).

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References

Aebersold,R. (1989) In Matsudaira,P.T. (ed.), *A Practical Guide to Protein and Peptide Purification for Microsequencing*. Academic Press, Inc., pp. 73-88.

Ahn,N.G., Seger,R. and Krebs,E.G. (1992) *Curr. Opin. Cell Biol.*, **4**, 992−999.

Ahn,N.G., Campbell,J.S., Seger,R., Jensen,A.L., Graves,L.M. and Krebs,E.G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5143−5147.

Alessi,D.R., Smythe,C. and Keyse,S.M. (1993) *Oncogene*, **8**, 2015−2020.

Anderson,N.G., Maller,J.L., Tonks,N.K. and Sturgill,T.W. (1990) *Nature*, **343**, 651−653.

Ashworth,A., Nakielny,S., Cohen,P. and Marshall,C. (1992) *Oncogene*, **7**, 2555−2556.

Becker,D.M. and Guarente,L. (1991) *Methods Enzymol.*, **194**, 182−187.

Blenis,J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5889−5892.

Boguslawski,G. and Polazzi,J.O. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 5848−5852.

Boulton,T.G., Nye,S.H., Robbins,D.J., Ip,N.Y., Radziejewska,E., Morgenbesser,S.D., DePinho,R.A., Panayotatos,N., Cobb,M.H. and Yancopoulos,G.D. (1991) *Cell*, **65**, 663−675.

Boyle,W.J., van de Geer,P. and Hunter,T. (1991) *Methods Enzymol.*, **201**, 508−519.

Brewster,J.L., de Valoir,T., Dwyer,N.D., Winter,E. and Gustin,M.C. (1993) *Science*, **259**, 1760−1763.

Cairns,B.R., Ramer,S.W. and Kornberg,R.D. (1992) *Genes Dev.*, **6**, 1280−1292.

Charles,C.H., Sun,H., Lau,L.F. and Tonks,N.K. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5292−5296.

Cobb,M.H., Boulton,T.G. and Robbins,D.J. (1991) *Cell Regul.*, **2**, 965−978.

Courchesne,W.E., Kunisawa,R. and Thorner,J. (1989) *Cell*, **58**, 1107−1119.

Crews,C.M. and Erikson,R.L. (1993) *Cell*, **74**, 215−217.

Crews,C.M., Alessandrini,A. and Erikson,R.L. (1992) *Science*, **258**, 478−480.

Davis,R.J. (1993) *J. Biol. Chem.*, **268**, 14553−14556.

De Bondt,H.L., Rosenblatt,J., Jancarik,J., Jones,H.D., Morgan,D.O. and Kim,S.H. (1993) *Nature*, **363**, 595–602.
Dent,P., Haser,W., Haystead,T.A., Vincent,L.A., Roberts,T.M. and Sturgill,T.W. (1992) *Science*, **257**, 1404–1407.
Elion,E.A., Grisafi,P.L. and Fink,G.R. (1990) *Cell*, **60**, 649–664.
Errede,B. and Levin,D.E. (1993) *Curr. Opin. Cell Biol.*, **5**, 254–260.
Errede,B., Gartner,A., Zhou,Z., Nasmyth,K. and Ammerer,G. (1993) *Nature*, **362**, 261–264.
Gartner,A., Nasmyth,K. and Ammerer,G. (1992) *Genes Dev.*, **6**, 1280–1292.
Gomez,N. and Cohen,P. (1991) *Nature*, **353**, 170–173.
Gomez,N., Traverse,S. and Cohen,P. (1992) *FEBS Lett.*, **314**, 461–465.
Guan,K.L. and Dixon,J.E. (1991) *Anal. Biochem.*, **192**, 262–267.
Guarente,L. and Mason,T. (1983) *Cell*, **32**, 1279–1286.
Hanks,S.K. and Quinn,M. (1991) *Methods Enzymol.*, **200**, 38–62.
Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P. and Marshall,C.J. (1992) *Cell*, **71**, 335–342.
Irie,K., Takase,M., Lee,K.S., Levin,D.E., Araki,H., Matsumoto,K. and Oshima,Y. (1993) *Mol. Cell. Biol.*, **13**, 3076–3083.
Knighton,D.R., Zheng,J., Eyck,L.F.T., Xuong,N., Tylor,S.S. and Sowadski,J.M. (1991) *Science*, **253**, 414–420.
Kosako,H., Nishida,E. and Gotoh,Y. (1993) *EMBO J.*, **12**, 787–794.
Kyriakis,J.M., App,H., Zhang,X.F., Banerjee,P., Brautigan,D.L., Rapp,U.R. and Avruch,J. (1992) *Nature*, **358**, 417–421.
Kryiakis,J.M., Force,T.L., Rapp,U.R., Bonventre,J.V. and Avruch,J. (1993) *J. Biol. Chem.*, **268**, 16009–16019.
Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,K.J. and Johnoson,G.L. (1993) *Science*, **260**, 315–319.
Lee,K.S. and Levin,D.E. (1992) *Mol. Cell. Biol.*, **12**, 172–182.
Lee,K.S., Irie,K., Gotoh,Y., Watanabe,Y., Araki,H., Nishida,E., Matsumoto,K. and Levin,D.E. (1993) *Mol. Cell. Biol.*, **13**, 3067–3075.
Matsuda,S., Gotoh,Y. and Nishida,E. (1993) *J. Biol. Chem.*, **268**, 3277–3281.
Nadin-Davis,S. and Nasim,A. (1988) *EMBO J.*, **7**, 985–993.
Ohmichi,M., Pang,L., Decker,S.J. and Saltiel,A.R. (1992) *J. Biol. Chem.*, **267**, 14604–14610.
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.
Posada,J., Yew,N., Ahn,N.G., Vande Woude,G.F. and Cooper,J.A. (1993) *Mol. Cell. Biol.*, **13**, 2545–2553.
Rhodes,N., Connell,L. and Errede,B. (1990) *Genes Dev.*, **4**, 1862–1874.
Robbins,D.J., Zhen,E., Owaki,H., Vanderbilt,C.A., Ebert,D., Gepper,T.D. and Cobb,M.H. (1993) *J. Biol. Chem.*, **268**, 5097–5106.
Seger,R., Seger,D., Lozeman,F.J., Ahn,N.G., Graves,L.M., Campbell,J.S., Ericsson,L., Harrylock,M., Jensen,A.M. and Krebs,E.G. (1992) *J. Biol. Chem.*, **267**, 25628–25631.
Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
Sprague,G.F. (1991) *Methods Enzymol.*, **194**, 77–93.
Stevenson,B.J., Rhodes,N., Errede,B. and Sprague,G.F. (1992) *Genes Dev.*, **6**, 1293–1304.
Sturgill,T.G., Ray,L.B., Anderson,N.G. and Erickson,A.K. (1991) *Methods Enzymol.*, **200**, 342–351.
Teague,M.A., Chaleff,D.T. and Errede,B. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 7371–7375.
Tsuda,L., Inoue,Y.H., Yoo,M.A., Mizuno,M., Hata.M., Lim,Y.M., Masamune,Y. and Nishida,Y. (1993) *Cell*, **72**, 407–414.
Warbrick,E. and Fantes,P.A. (1991) *EMBO J.*, **10**, 4291–4299.
Wu,J., Harrison,J.K., Vincent,L.A., Haystead,C., Michel,H., Hunt,D.F., Lynch,K.R. and Sturgill,T.W. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 173–177.
Yashar,B.M., Kelley,C., Yee,K., Errede,B. and Zon,L.I. (1993) *Mol. Cell. Biol.*, **13**, 5738–5748.
Zheng,C.F and Guan,K.L. (1993a) *J. Biol. Chem.*, **268**, 16116–16119.
Zheng,C.F. and Guan,K.L. (1993b) *J. Biol. Chem.*, **268**, 11435–11439.
Zheng,C.F. and Guan,K.L. (1993c) *J. Biol. Chem.*, **268**, 23933–23939.

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