The EMBO Journal vol.13 no.13 pp.3003-3010, 1994

Constitutive mutant and putative regulatory serine phosphorylation site of mammalian MAP kinase kinase (MEK1)

Gilles Pagès[1], Anne Brunet[1], Gilles L'Allemain and Jacques Pouysségur

Centre de Biochimie-CNRS, Université de Nice, 06108 Nice, France

[1]These authors contributed equally to this work

Communicated by J.Pouysségur

In response to various external stimuli, MAP kinases are activated by phosphorylation on tyrosine and threonine by MAP kinase kinase (MAPKK), a dual specificity kinase. This kinase is in turn activated via Raf-1 and MAPKK kinase (MAPKKK). To determine regulatory phosphorylation sites of MAPKK, we isolated a Chinese hamster cDNA, that we epitope-tagged and expressed in fibroblasts. This hamster MAPKK (MEK1 isoform) can reactivate recombinant p44^{mapk} when immunoprecipitated from growth factor-stimulated cells or when incubated with an active form of MAPKKK. Mutations at either of two residues that are conserved among kinases, D208N or S222A, abolished MAPKK activity. However, only S222A/MAPKK showed a reduction in phosphorylation in response to active MAPKKK and exerted a dominant negative effect on the serum-stimulated endogenous MAPKK. Finally, replacing Ser222 with Asp, a negatively charged residue, restored MAPKK activity independently of the upstream kinase. These results strongly suggest that Ser222 represents one key MAPKKK-dependent phosphorylation site switching on and off the activity of MAPKK, an event crucial for growth control.

Key words: constitutive mutations/fibroblasts/growth factors/kinase cascade/MAP kinase kinase

Introduction

Mitogen activated protein kinases, p42^{mapk} and p44^{mapk}, are two members of a family of serine/threonine kinases that relay an immense variety of extracellular signals to the nucleus (Cobb *et al.*, 1991; Sturgill and Wu, 1991; Pelech and Sanghera, 1992; Ruderman, 1993). Their activation proceeds via a kinase cascade, referred to as the MAP kinase module, that is conserved from yeast to mammals (Errede and Levin, 1993; Neiman, 1993). This module includes at least two upstream kinases: MAP kinase kinase kinase (MAPKKK), a mammalian homologue of the fission yeast byr2 (Lange-Carter *et al.*, 1993), and Raf-1 (Dent *et al.*, 1992; Howe, 1992; Kyriakis *et al.*, 1992). Both activate MAP kinase kinase (MAPKK), a dual specificity kinase. MAPKK, also referred to as MEK (MAPK/ERK kinase), (Ashworth *et al.*, 1992; Crews and Erikson, 1992a,b; Kosako *et al.*, 1992; Seger, 1992; Seger *et al.*, 1992) is unique in that it concomitantly phosphorylates two clustered tyrosine and threonine residues (TEY motif) of MAP kinases. Phosphorylation of the TEY motif, located in kinase subdomain VII just downstream of the well conserved S/APE sequence, is essential for activity (Anderson *et al.*, 1990; Payne *et al.*, 1991). MAPKK which possesses all the primary structural characteristics of protein serine/threonine kinases (Hanks *et al.*, 1988), first phosphorylates the tyrosine residue of the TEY motif of MAPKs (Haystead *et al.*, 1992). Replacing Thr with Ala does not impair tyrosine phosphorylation of the TEY motif, suggesting that this MAPK activator possesses a primary specificity for tyrosine. A second characteristic of MAPKK is its specificity for substrate. Other than MAPK, no additional substrates have been identified for MAPKK (Seger *et al.*, 1992). This extraordinary specificity among protein kinases seems to have been particularly well designed to 'forge' fidelity in signal transduction and cellular communication. The recent resolution of the unphosphorylated p42^{mapk} tertiary structure (Zhang *et al.*, 1994) reveals that Tyr185 of the TEY motif is buried in a largely hydrophobic pocket. A MAPKK/MAPK-mediated interaction is therefore required to induce a conformational change exposing Tyr185. This unique feature might be the basis for the restriction of MAPK phosphorylation to one specific group of protein kinases, MAPKKs. Approximately 20 MAPKK homologues have been cloned from a wide range of organisms [see Mordret (1993) for review]. While there is no doubt that MAPKK is itself dependent on Ser/Thr phosphorylation for activity (Kosako *et al.*, 1992, 1993; Ahn *et al.*, 1993), the mechanism of its activation has not yet been elucidated.

In our previous work with cells of mesenchymal origin (fibroblasts, vascular smooth muscle and mesangial cells), we showed that: (i) only potent growth factors induce a biphasic and sustained activation of both p42^{mapk} and p44^{mapk} (Kahan *et al.*, 1992; Meloche *et al.*, 1992a,b; Vouret-Craviari *et al.*, 1993; Wang *et al.*, 1992), (ii) the sustained phase of activation, lasting up to 6 h, parallels growth factor-induced MAP kinase nuclear translocation (Lenormand *et al.*, 1993a), and (iii) MAP kinase activation is a prerequisite for G_0-arrested fibroblasts to pass the 'restriction point' (Lenormand *et al.*, 1993b; Pagès *et al.*, 1993).

To determine the mechanism of MAPKK activation and to investigate further the time course of MAP kinase activation, in particular the basis of the biphasic activation, we cloned, epitope-tagged and expressed wild type and mutated forms of a Chinese hamster MAPKK. Here we report: (i) the functional expression of a cloned hamster MAPKK that, in contrast to MAPKs, responds to growth factors with a long lasting and monotonic phase of activation, (ii) the identification of a MAPKK Ser phosphorylation site critical for its activation by the upstream kinase MAPKKK and (iii) a mutation that constitutively activates MAPKK.

Results

Molecular cloning of the Chinese hamster MAPKK

A PCR product of 500 bp was amplified from hamster fibroblast mRNA using degenerated primers from a peptide sequence of *Xenopus* MAPKK (Matsuda *et al.*, 1992). A λgt10 cDNA library from Chinese hamster lung fibroblasts was screened at high stringency using the PCR product as a probe (see Materials and methods). The longest of the 30 positive clones analysed was subcloned in the PTZ18 vector and sequenced. The longest open reading frame (Figure 1) encodes a protein of 45 kDa that exhibits respectively 80 and 95% identity with the *Xenopus* (Kosako *et al.*, 1993) and murine amino acid sequences (Crews and Erikson, 1992a). This high level of identity indicates that we have cloned the Chinese hamster homologue of MAPKK corresponding to the human isoform MKK1 or MEK1 (Seger, 1992; Mordret, 1993).

Stable expression of epitope-tagged HA-MAPKK in hamster fibroblasts

To facilitate the studies of the hamster MAPKK, we introduced at the N-terminus the nine-residue epitope of the HA1 influenza virus haemagglutinin, which is recognized by the 12CA5 monoclonal antibody (Wilson *et al.*, 1984). Polyclonal antibodies raised against the MAPKK *Xenopus* N-terminal peptide were also used to compare the level of expression of the ectopically expressed kinase with that of the endogenous one. The epitope-tagged MAPKK construction together with the pECE expression vector used are depicted in Figure 2. PS 120, a Na^+/H^+ exchanger (NHE1)-deficient derivative of CCL39 cell line (Pouysségur *et al.*, 1984), was stably transfected with HA-MAPKK, and protein levels from control and transfected cell extracts were analysed by immunoblotting. Figure 3 (left) shows that the 12CA5 monoclonal antibody specifically recognizes a 47 kDa protein in the transfected cells. In contrast, the polyclonal anti MAPKK-derived peptide recognizes a single protein of 45 kDa in control cells and a doublet of 45 and 47 kDa in transfected cells (Figure 3, right). This doublet corresponds to the endogenous MAPKK (45 kDa) and to the more slowly migrating epitope-tagged MAPKK (47 kDa). The average level of expression of the recombinant HA-MAPKK is at least 4- to 5-fold higher than the endogenous kinase taking into account that about half of the transfected population has received the transgene.

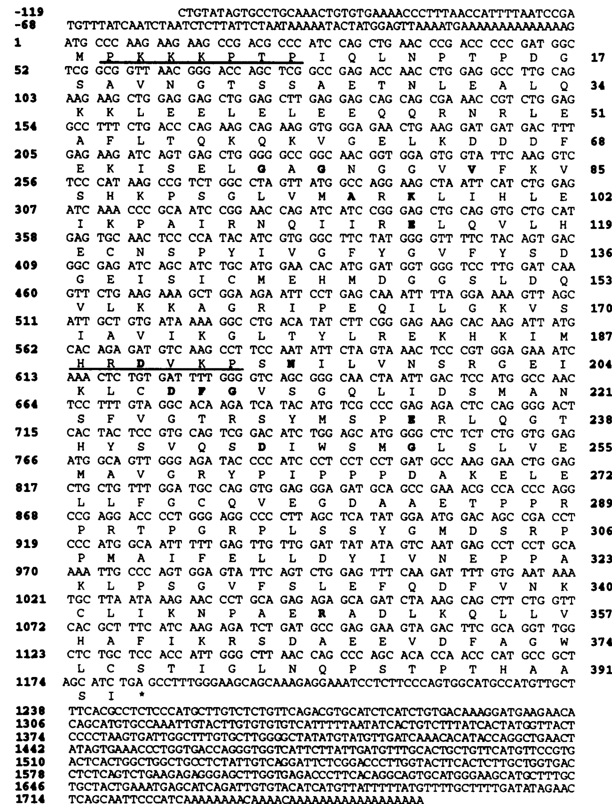

Fig. 1. Nucleotide and predicted amino acid sequence of Chinese hamster MAPKK. The sequence is derived from the full-length clone identified as described in Materials and methods. Underlined protein sequences correspond to the region used for the design of PCR oligonucleotides. Bold amino acids correspond to amino acids conserved among all the kinases.

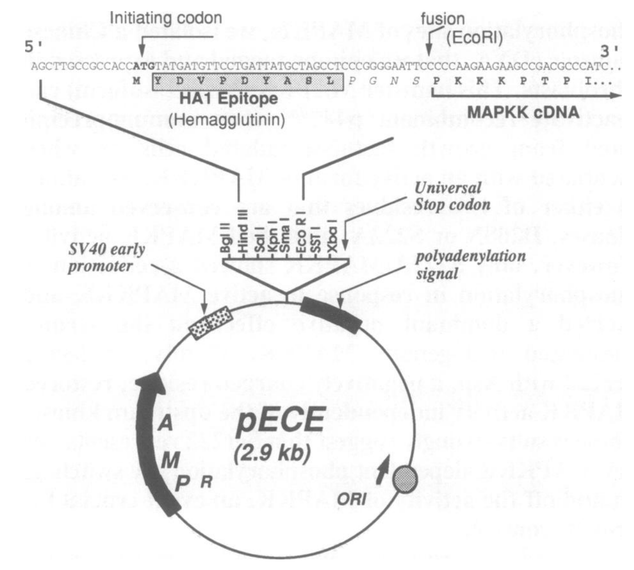

Fig. 2. Expression vector of the hamster MAPKK tagged with an HA epitope (HA-MAPKK). The construction of the vector is detailed in Materials and methods. The boxed amino acids correspond to the HA1 epitope and the underlined ones to the MAPKK protein. The italicized amino acids were created during the construction of the fusion protein.

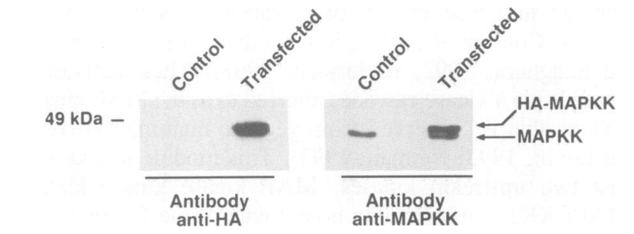

Fig. 3. Level of expression of the transfected HA-MAPKK protein. PS 120 cells transfected with vector alone or HA-MAPKK cDNA (see Materials and methods) were analysed for the endogenous and epitope-tagged MAPKK expression. 25 μg of Triton X-100 soluble proteins were resolved by SDS–PAGE (10% acrylamide gel) and immunoblotted with the 12CA5 monoclonal antibody specific for the HA epitope (left) or with the polyclonal anti-N-terminal peptide of *Xenopus* MAPKK (right). The position of the 49 kDa molecular weight standard is shown on the left.

Recombinant HA-MAPKK can be activated by growth factors

An *in vitro* assay was developed to test the activity of the endogenous and recombinant HA-MAPKK. As a substrate for the kinase reaction, we used the epitope-tagged hamster recombinant $p44^{mapk}$ (HA-MAPK) immunoprecipitated from resting fibroblasts (Meloche *et al.*, 1992a). PS 120 cells transfected with HA-MAPKK were arrested in G_0 and stimulated by 20% fetal calf serum for 5 min. Figure 4A shows that HA-MAPKK immunoprecipitated with the anti-HA epitope was able to phosphorylate HA-MAPK in a serum-dependent manner (compare lanes 3 and 4 of Figure 4A). A similar result was obtained using the deficient kinase HA-T192A/MAPK as a substrate (compare lanes 5 and 6 of Figure 4A). Note that in this case the extent of phosphorylation of HA-MAPK was reduced due to the elimination of one of the two phosphorylation sites (T192A). This serum-induced phosphorylation was specific to HA-MAPKK activity since it did not occur in cells transfected with the vector alone (see lanes 1 and 2).

In Figure 4B we analysed the intrinsic activity of HA-MAPK by monitoring the phosphorylation of the substrate myelin basic protein (MBP). As expected, phosphorylation of HA-MAPK coincided with activation of the kinase (Figure 4B, compare lanes 3 and 4). Similar results were obtained by replacing HA-$p44^{mapk}$ with purified recombinant $p42^{mapk}$ (data not shown). However, this activation did not take place when the deficient kinase HA-T192A/MAPK was used (compare lanes 2 and 6 of Figure 4B) or when HA-MAPK was omitted (not shown). Altogether these results indicate that the recombinant HA-MAPKK expressed in fibroblasts is functional since its activation by serum growth factors specifically induces the phosphorylation and reactivation of MAPKs.

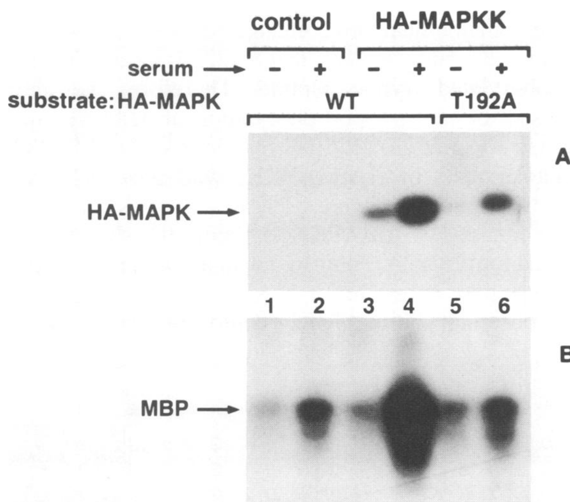

Fig. 4. HA-MAPKK phosphorylates and reactivates $p44^{mapk}$ in response to serum growth factors. PS 120 cells, transfected with the vector alone (control) or the vector expressing HA-MAPKK, were arrested in G_0 (−) or restimulated for 5 min with 20% fetal calf serum (+). (**A**) Phosphorylation of the MAPK substrates: HA-MAPK or HA-T192A/MAPK. The activities of HA-MAPKK immunoprecipitates from control (vector alone) and HA-MAPKK transfected cells were tested by measuring incorporation of ^{32}P on two substrates derived from the hamster $p44^{mapk}$: HA-MAPK (WT) and HA-T192A/MAPK (T192A). Both substrates were immunoprecipitated from non-stimulated transfected PS 120 cells (Pagès *et al.*, 1993). Equal protein samples were separated by SDS−PAGE (10% acrylamide), and the gel was dried and autoradiographed. (**B**) MBP kinase activity of the phosphorylated HA-MAPK. Autoradiogram of a 13% acrylamide gel showing the corresponding phosphorylated MBP as a reflection of MAPK activity in the experiment depicted in panel A (see Materials and methods).

Using this assay it was then possible to analyse the time course of MAPKK activation during the period that precedes S-phase entry. Resting fibroblasts expressing HA-MAPKK were stimulated with fetal calf serum. Figure 5A shows that the HA-MAPKK band resolved by SDS−PAGE rapidly acquires a lower mobility in response to serum growth factors that persists for at least 8 h. The band-shift experiment somewhat parallels activation of MAPKK (Figure 5B). Maximum activity is reached at 10−15 min, followed by a very slow decline, persistent activation still being detectable after 8 h of stimulation. A similar time course was observed with the endogenous MAPKK or when cells were stimulated with α-thrombin (data not shown). This kinetic of activation markedly contrasts with the biphasic activation of MAPKs that we observed under the same conditions of stimulation (Meloche *et al.*, 1992a,b).

In vitro reconstitution of the MAPK module

At least two different kinases appear to regulate MAPKK activity: the product of the *raf-1* proto-oncogene and a recently cloned MAPKKK, a mammalian homologue of byr2 (Lange-Carter *et al.*, 1993). Deletion of the N-terminal domain of Raf-1 is sufficient to generate an auto-active kinase (Beck *et al.*, 1987; Stanton *et al.*, 1989). Interestingly, the same type of truncation, eliminating the first 352 amino acids of MAPKKK, constitutively activated the kinase (our unpublished data; Gary Johnson, personal communication). We added the HA epitope at the new 5′-terminus and the HA-ΔMAPKKK, inserted in the pECE vector, was expressed transiently in 293 cells. When a kinase assay is performed with this immunoprecipitated HA-ΔMAPKKK, together with HA-MAPK as a substrate, no phosphorylation

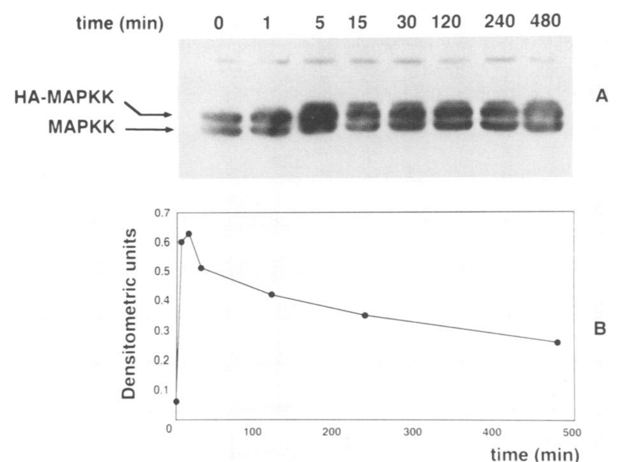

Fig. 5. Time-course of HA-MAPKK activation in response to serum. Cells expressing HA-MAPKK were arrested in G_0 and stimulated with 20% fetal calf serum for the times indicated. (**A**) Serum-induced band-shift of MAPKK. Triton X-100 lysates were run on an SDS−polyacrylamide gel (10% acrylamide/0.09% *bis*-acrylamide), then analysed by immunoblotting with the anti-MAPKK antibody. (**B**) Immunoprecipitates with the anti-HA epitope were assayed for MAPKK activity as described in the legend to Figure 4 and quantified by laser densitometry of the phosphorylated HA-MAPK.

is detected except the autophosphorylation of the truncated MAPKKK (Figure 6, lane 2). However, introduction of a non-activated HA-MAPKK in the kinase assay immediately restores the kinase cascade (Figure 6, lane 3). HA-MAPK is now phosphorylated as a consequence of phosphorylation and reactivation of HA-MAPKK since it does not occur in the absence of HA-ΔMAPKKK (Figure 6, compare lanes 1 and 3). If MBP is added in the assay, it becomes phosphorylated. This phosphorylation is, however, strictly dependent upon the addition of the truncated HA-ΔMAPKKK (data not shown). This result indicates that the HA-MAPKK is directly activatable by MAPKKK since the entire cascade can be reconstituted *in vitro*.

Identification of a critical serine phosphorylation site of HA-MAPKK

HA-MAPKK is heavily phosphorylated in resting cells and its activation by growth factors is associated with increased Ser/Thr phosphorylation (Kosako *et al.*, 1992; Ahn *et al.*, 1993). Comparison of phosphorylated tryptic peptides derived from resting and activated MAPKK *in vivo*, highlights mainly two new phosphopeptides that correspond to retro-phosphorylation by MAPK. However, phosphorylation at these two Thr residues is not critical for MAPKK activity (Brunet *et al.*, 1994). To identify putative key Ser/Thr phosphorylation sites we aligned MAPKK sequences of various species together with those of MAPK, cdk1 and cAPK. Figure 7 shows conservation of Ser residues among these kinases between conserved domains VII and VIII of the protein kinases. This region is of particular interest because it contains the so-called 'autophosphorylation' domain of serine/threonine kinases (Hanks *et al.*, 1988) shown to be critical for MAPK, cdk1 and cAPK activity. We specifically mutated Ser222, the key phosphorylation site equivalent to Thr161, Thr192 and Thr197 of respectively cdk1, MAPK and cAPK. Figure 8 shows that replacing this Ser with a non-phosphorylatable residue, Ala, totally abolishes MAPKK activity. Indeed, HA-S222A/MAPKK can no longer phosphorylate and activate HA-MAPK in response to growth factors. In contrast, S228A, a similar mutation created at a Ser that is well conserved among the MAPKK family, did not affect the kinase activity (data not shown).

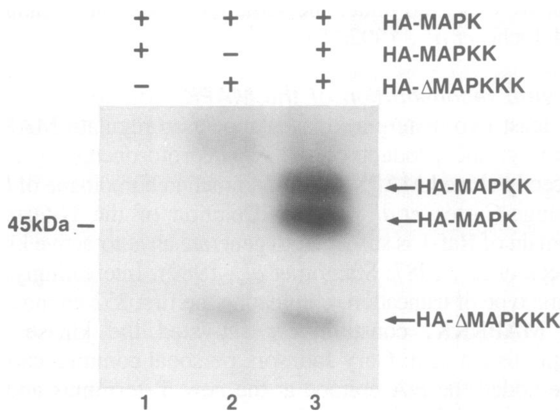

Fig. 6. *In vitro* reconstitution of the MAP kinase cascade. The truncated and constitutively active epitope-tagged MAP kinase kinase kinase (HA-ΔMAPKKK) was transiently expressed in 293 cells and immunoprecipitated with the 12CA5 antibody. Non-activated HA-MAPKK and HA-MAPK were immunoprecipitated as described in the legend to Figure 4. Lane 1: HA-ΔMAPKKK was omitted; lane 2: HA-MAPKK was omitted; lane 3, the three components were present. The kinase reaction was performed as described for the MAPKK assay (see Materials and methods). Note that the autoradiogram reveals the phosphorylation of the three members of the cascade in lane 3.

Because protein kinases display an extraordinarily well conserved tertiary structure (Taylor *et al.*, 1993; Zhang *et al.*, 1994), it is tempting to speculate that Ser222 also represents a key phosphorylation site of MAPKK. Unfortunately, comparison of phosphopeptide maps from *in vivo* ^{32}P-labelling of MAPKK and S222A/MAPKK did not reveal a major qualitative change (data not shown). This result might simply indicate that Ser222 is located in a multi-phosphorylated tryptic peptide. Therefore, we directly analysed the *in vitro* phosphorylation of MAPKK and its mutated form S222A by the upstream kinase MAPKKK. Equal amounts of HA-MAPKK, wild type, S222A and D208N (Figure 9B), were used as substrates for the auto-active kinase HA-ΔMAPKKK. Figure 9A shows that the mutated form S222A, compared with the wild type MAPKK, is a very poor substrate for MAPKKK. Alternatively, this low phosphorylation level could reflect a lack of

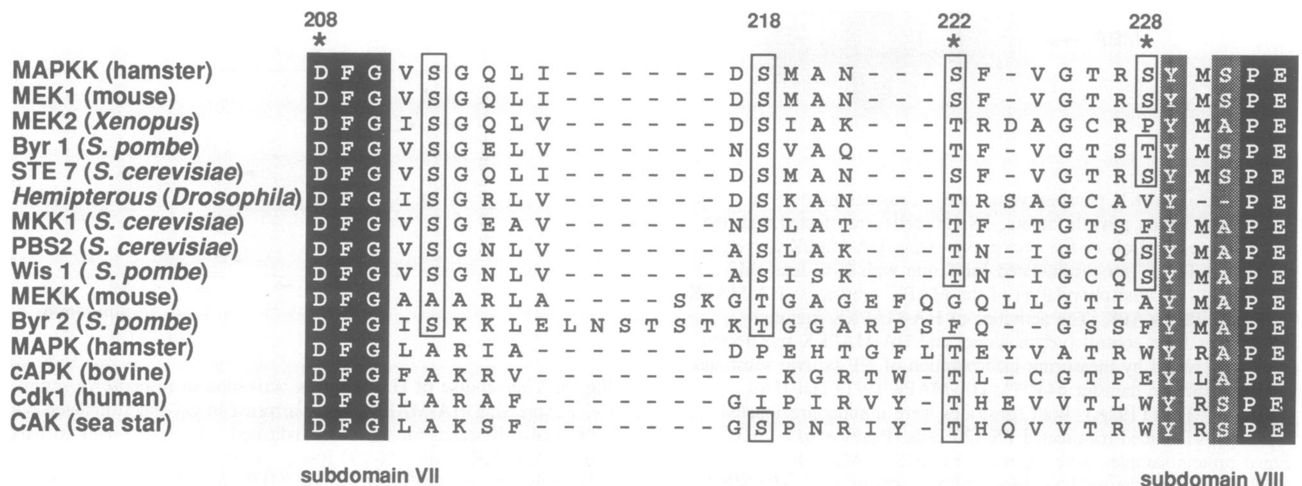

Fig. 7. Comparison of MAPKK sequences from different species with related kinases. Alignment of MAPKK and related kinases between the 'DFG' and 'A/SPE' motifs of the VII and VIII conserved subdomains of protein kinases. Conserved serine or threonine residues are boxed. Amino acids mutated in this study are shown by an asterisk (D208N; S222A; S228A) and positions correspond to the hamster MAPKK.

autophosphorylation since S222A/MAPKK is a 'dead kinase'. For this reason we compared the phosphorylation of another 'dead kinase', D208N/MAPKK, defective in the essential Asp residue of the well conserved DFG motif. This mutated form is in fact an excellent substrate for MAPKKK (Figure 9A). We therefore conclude that Ser222 represents a phosphorylation site essential for the activation of MAPKK by MAPKKK.

S222A behaves in vitro as a dominant-negative mutant

S222A/MAPKK stably expressed in PS 120 cells was tested for its capacity to interfere with the endogenous MAPKK. G_0-arrested cells were stimulated for 5 min with 20% fetal calf serum. Cell lysates were immunoprecipitated with a 10-fold excess of the antiserum directed against the N-terminal region of *Xenopus* MAPKK (see Materials and methods). Under these conditions, both the ectopically expressed and the endogenous forms of MAPKK are immunoprecipitated. The capacity to phosphorylate HA-MAPK was used as the MAPKK assay. Figure 10 shows that S222A/MAPKK significantly inhibits the endogenous MAPKK activity (compare lanes 2 and 6). Activity from cells transfected with WT HA-MAPKK is higher (compare lanes 2 and 4) because of the combined activities of endogenous and ectopically expressed MAPKK. The degree of inhibition that we observed in this assay varied among the clones tested and could be as high as 90% (data not shown). This interference appears specific of S222A mutant since it was not observed with the other inactive kinase, D208N/MAPKK, even when expressed at higher levels (data not shown).

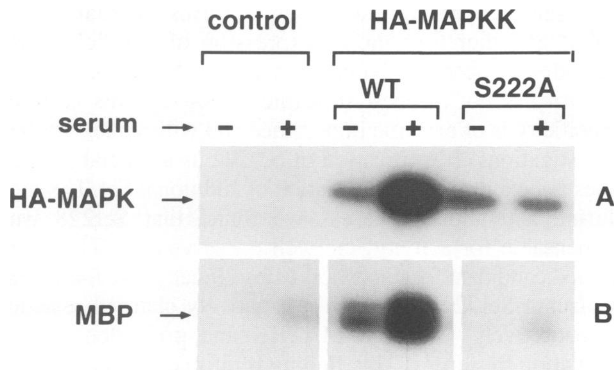

Fig. 8. The S222A/MAPKK mutant is totally deficient in kinase activity. This experiment was conducted exactly as described in the legend to Figure 4. (**A**) Phosphorylation of the substrate HA-MAPK from immunoprecipitates derived from cells transfected with either the vector alone (control), wild type HA-MAPKK (WT) or HA-S222A/MAPKK (S222A). (**B**) Corresponding activity of the HA-MAPK, using MBP as a substrate.

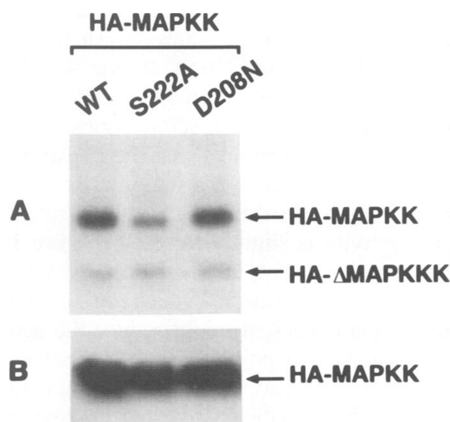

Fig. 9. The S222A/MAPKK mutant is a poor substrate for MAPKKK. Constitutively active HA-ΔMAPKKK was prepared as described in the legend to Figure 6 and used to *in vitro* phosphorylate wild type (WT) and S222A and D208N mutated forms of HA-MAPKK (**A**). The HA-MAPKK isoforms were immunoprecipitated from non-stimulated cells and used as a substrate for HA-ΔMAPKKK phosphorylation. (**B**) Shows immunoblotting with anti-HA monoclonal antibody from aliquots of extracts used in panel A.

Replacing Ser222 with Asp 'constitutively' activates HA-MAPKK

To reinforce the notion that Ser222 is a key residue in the conformation of the MAPKK catalytic site and that phosphorylation at this site 'switches on' MAPKK, we mutated this residue into Asp to mimic the negative phosphate charge. Interestingly, this mutant HA-S222D/MAPKK expressed in PS 120 cells displays constitutive activity as measured by phosphorylation and activation of HA-MAPK (Figure 11). Indeed, in unstimulated cells, S222D/MAPKK stimulates the phosphorylation of MAPK (Figure 11A, compare lanes 3 and 5). This phosphorylation involves tyrosine residues as it resists alkali treatment (Figure 11B, lane 5); this HA-MAPK-induced phosphorylation parallels MBP kinase activity (Figure 11C, compare lanes 3 and 5).

Discussion

Here, we report the cloning of a Chinese hamster MAPKK cDNA based on partial sequences of *Xenopus* MAPKK (Matsuda *et al.*, 1992). Three genes encoding MAPKK, and displaying distinct pattern of expression during development, have been identified in *Xenopus laevis* (Yashar *et al.*, 1993). Based on amino acid identity, the cDNA sequence we report here is the hamster homologue of the MEK1 isoform of *Xenopus* or murine MAPKK (Crews and Erikson, 1992a; Yashar *et al.*, 1993; Mordret, 1993). Using either polyclonal antibodies against the N-terminal peptide of *Xenopus* MEK1

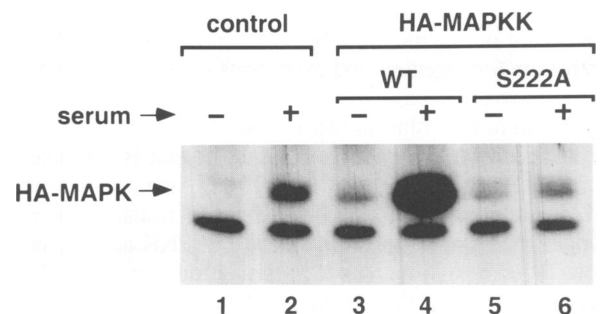

Fig. 10. S222A/MAPKK exerts a dominant-negative effect on endogenous MAPKK. G_0-arrested cells expressing either the vector alone (lanes 1 and 2), wild type HA-MAPKK (lanes 3 and 4) or HA-MAPKK mutated on serine 222 (S222A) were stimulated (+) or not (−) with 20% serum for 5 min. Cell lysates were immunoprecipitated with the N-terminal MAPKK antibody that recognizes both endogenous and transfected MAPKK. Activity of these different immunocomplexes was tested by incorporation of ^{32}P on HA-MAPK immunoprecipitated from unstimulated cells.

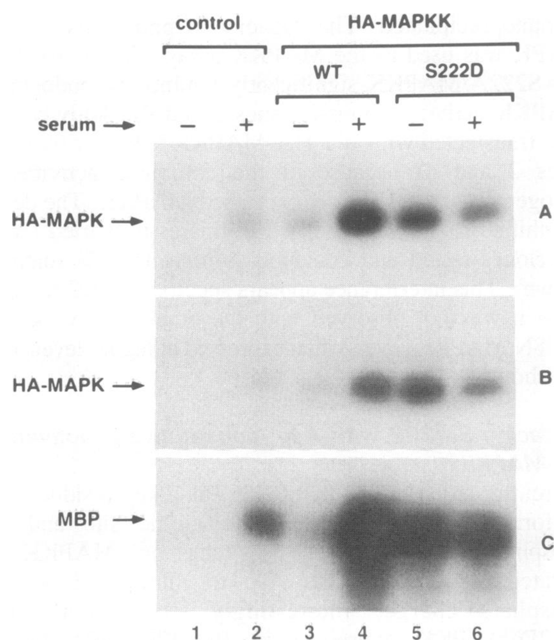

Fig. 11. Constitutive activity of the S222D/HA-MAPKK mutant. PS 120 cells were transfected either with the vector alone (control) or the vector expressing the wild type (WT) or the S222D/HA-MAPKK. HA-MAPKK was immunoprecipitated from resting cells stimulated (+) or not (−) by 20% serum. (**A**) Phosphorylation of non-active HA-MAPK; (**B**) same gel as in (A) treated for 1 h with KOH and (**C**) corresponding MBP kinase activity. A Western blot attested that similar amounts of HA-MAPKK were immunoprecipitated.

or the 12CA5 monoclonal antibody directed against the HA1 epitope 'grafted' onto the hamster MAPKK (HA-MAPKK), we demonstrated that the hamster cDNA encodes a functional MAPKK. Functionality of HA-MAPKK was assessed both by its capacity to phosphorylate T192A/MAPK [mutant of the TEY motif (Pagès *et al.*, 1993)] on tyrosine and by its capacity to activate MAPK measured by its MBP kinase activity. We showed that HA-MAPKK can be activated by serum, thrombin or FGF. As for MAPK activation (Kahan *et al.*, 1992; L'Allemain *et al.*, 1992; Meloche *et al.*, 1992a) and mitogenicity in CCL39 cells (Chambard *et al.*, 1987), thrombin-induced MAPKK activation is sensitive to pertussis toxin (data not shown). These results strongly support the notion that this MAPKK isoform is fully competent to transduce mitogenic signals. The time course of MAPKK activation (endogenous and exogenous expressed forms) does not, however, parallel that of MAPK. Activation of MAPK by serum or thrombin shows a typical biphasic mode (Kahan *et al.*, 1992; Meloche *et al.*, 1992a,b) that is not detected for MAPKK (Brunet *et al.*, 1994). Also, activation of MAPKK during the G_0/G_1 to S-phase transition persists longer than that of MAPK. Indeed MAPKK activity is still detectable 8 h after serum stimulation whereas MAPK activity returns to the resting value between 4 and 6 h after stimulation. This finding suggests that MAPK is specifically subjected to inactivation by specific phosphatases. The extinction of the second phase could very well be attributed to MAPK phosphatase (MKP1) that is maximally induced during this period (Noguchi *et al.*, 1993; Nebreda, 1994), whereas another phosphatase yet to be identified must be responsible for the early phase of inactivation.

In the second part of this work, we identified a key serine residue, Ser222, that switches on and off MAPKK activity upon phosphorylation by the upstream kinase, MAPKKK. This residue was chosen for site-directed mutagenesis because of its remarkable conservation among several protein kinases (Figure 7) and more importantly because it corresponds to the key phosphorylatable residue activating MAPKs (Thr of the TEY motif), cdk1 (Thr161) and cAPK (Thr197). Firstly we showed that Ser222 is indeed essential for kinase activity as S222A/MAPKK is not activatable. Secondly, we showed that, in contrast to wild type MAPKK or another dead kinase (D208N/MAPKK), S222A/MAPKK is a poor substrate for *in vitro* phosphorylation by active MAPKKK. Comparison of wild type and S222A/MAPKK expressed in CCL39 cells revealed a 4- to 5-fold decrease in ^{32}P incorporated into a cyanogen bromide-derived phosphopeptide obtained from the mutated MAPKK (data not shown). These results strongly suggest that Ser222 is indeed phosphorylated and represents a key residue for controlling MAPKK activity at least in response to the active MAPKKK isolated by Lange-Carter *et al.* (1993). With the emergence of multiple MAPKK isoforms (Yashar *et al.*, 1993) and therefore the co-expression of parallel kinase cascades, a possible explanation is that MAPKK (MEK1 isoform) is not the best substrate of Raf-1. This issue of specificity is of extreme importance and will require further investigations. Identification of Ser222 as a crucial residue does not exclude phosphorylation of additional Ser/Thr sites during activation. Whereas we found that Ser228 was dispensable for activity, the well conserved Ser/Thr218 is a good candidate for phosphorylation. Finally, we found that replacing Ser222 with Asp, a negatively charged residue, 'constitutively' activated MAPKK and prevented further activation by serum. This finding reinforces the notion that Ser222 represents a real switch for controlling MAPKK activity, and indicates that phosphorylation of this residue is sufficient to confer an active conformational change of the kinase.

Direct phosphorylation of Ser222 has not been directly demonstrated here, although all our results are consistent with that conclusion. Interestingly, after completion of this work, a paper by Zheng and Guan (1994) demonstrated that MAPKK is phosphorylated at Ser218 and Ser222 upon activation by Raf-1. Both residues appear to be critical for activation, and phosphorylation at one site by Raf-1 is independent of phosphorylation at the other, shown by the separate substitution of both Ser residues by Thr. In this regard it is interesting to note that the constitutive activity of S222D/MAPKK that is not maximal in the absence of growth factors is not further enhanced by serum stimulation. In fact the activity is slightly reduced (Figure 11, lane 6). One explanation is that in the S222D/MAPKK mutant, Ser218 cannot be phosphorylated or alternatively, if phosphorylation occurs, it might reduce the activity of the mutated enzyme. This point deserves further examination. Another point that is also worth considering is the substrate specificity of either MAPKKK or Raf-1 in regard to MAPKK (MEK1 isoform). Although Zheng and Guan (1994) reported activation of MAPKK (MEK1) in response to activated Raf, in our hands, activated Raf-1 or v-Raf are much weaker activators than activated MAPKKK (data not shown).

In addition, this report, together with those of Zheng and Guan (1994), provides another example in favour of the emerging picture that linker L12 or the 'lip' between domains

VII and VIII of protein kinases (Taylor *et al.*, 1993; Zhang *et al.*, 1994) contains the phosphoregulatory switch for enzyme activity. We anticipate that engineering a chimera within this 'lip' should break fidelity within the signalling kinase cascades. This approach will also indicate whether the variable region of the 'lip' is in itself sufficient to specify kinase recognition.

We previously demonstrated that interfering with the MAP kinase cascade in fibroblasts by expressing either a p44*mapk* antisense or a dominant-negative mutant prevented entry into the cell cycle (Pagès *et al.*, 1993). With the availability of a MAPKK constitutive allele, it will now be possible to analyse the incidence of this unregulated member of the cascade on growth factor relaxation and oncogenicity.

Materials and methods

Materials

[γ-^{32}P]ATP and the enhanced chemoluminesence (ECL) immunodetection system were obtained from Amersham; bovine myelin basic protein (MBP) and bovine serum albumin were purchased from Sigma; Triton X-100 was from Pierce. Polyclonal antibodies used for detection of endogenous MAPKK were directed against the N-terminal peptide of *Xenopus* MAPKK (PKK-KPTPIQLNPNPEG) synthesized by Neosystem SA (Strasbourg) [see Lenormand *et al.* (1993b) for details]. Monoclonal antibody 12CA5, raised to a peptide from influenza HA1 protein (Wilson *et al.*, 1984), was purchased from Babco (Emeryville, CA).

Cell culture

The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection), its derivative PS 120, which lacks NHE1 antiporter activity (Pouysségur *et al.*, 1984), and corresponding transfected cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, from Gibco/BRL) containing 7.5% fetal calf serum, penicillin (50 U/ml) and streptomycin sulfate (50 μg/ml). The human 293 cells were cultivated in DMEM containing 7.5% decomplemented fetal calf serum. Cells were growth-arrested by completely depriving them of serum for 16–24 h.

Stable transfection

We used NHE1 as a selective marker and the H^+-killing selection technique as previously described (Sardet *et al.*, 1989; Pagès *et al.*, 1993). PS 120 cells (10^6 cells per 10 cm plate) were co-transfected by the calcium phosphate technique with 2 μg of pEAP expression vector (NHE1 cDNA) (Wakabayashi *et al.*, 1992) and 20 μg of pECE expression vector (Ellis *et al.*, 1986) containing different constructs of MAPKK. Forty-eight hours after transfection, cells were subjected to an acid-load selection that killed non-transfected cells, usually 90–95% of the cell population. Two additional acid-load selections were applied usually on days 4 and 8 after transfection. Stable clones were either picked up for analysis or mixed to provide the transfected population. The population or individal clones were passaged with application of the acid-load selection once a week.

Isolation and characterization of MAPKK cDNA clones

First strand cDNA was synthesized from 1 μg of CCL39 poly(A)$^+$ RNA using avian myeloblastosis virus reverse transcriptase with an oligo(dT) primer. This material was used as template for polymerase chain reaction (PCR) amplification. The following degenerate oligonucleotides derived from *Xenopus* MAPKK sequence (Matsuda *et al.*, 1992) were synthesized and used as primers: 5'- CC(C/G)AA(G/A)AA(G/A)AA(G/A)CC(C/G)AC(C/G/T/A)CC-3' and 5'-CA(G/A)CT(C/T/GA)AA(C/T)CC(C/G)AA(C/T)CC-3'. An aliquot of cDNA was amplified in 50 μl reaction volume with 1 μg of each primer, 200 μM dNTPs and 2.5 U Taq DNA polymerase (Boehringer) in a buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin. The PCR amplification was performed in a DNA thermal cycler (Biotechnia) using the following parameters: 1 min at 95°C, 2 min at 55°C and 2 min at 72°C for 30 cycles followed by an extra cycle with a 10 min extension step at 72°C. A fragment of the expected size (~500 bp) was obtained. After purification on a 2% agarose gel (Bio-Rad), it was ^{32}P-labelled by random priming and used as a probe to screen an amplified Chinese hamster lung fibroblast cDNA library in λgt10 (1.4 × 10^6 recombinants). Phage plaques were screened according to Benton and Davis (1977) then pre-hybridized at 55°C for 1 h in 6× SSC, 2× Denhardt's solution and hybridized in the same solution at 55°C with 10^6 c.p.m./ml of ^{32}P-labelled probe and 500 μg/ml of denatured salmon sperm DNA for 16 h. Filters were washed in 2× SSC, 0.1% SDS at 55°C and exposed to film for 24 h. Of the 30 positive clones identified, 10 were isolated and plaque purified. The longest clone, which contained a 1.8 kb insert, was subcloned in PTZ 18 vector and subjected to sequence analysis. The cDNA clone was digested with different restriction enzymes and the suitable restriction fragments were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia). Sequences were analysed with the MacVector program for Macintosh (IBI, New Haven, CT).

DNA constructs and expression vector

Two oligonucleotides, JP39 and JP40, were designed to introduce the hamster MAPKK sequence in-frame in the pECE/HAP vector (Meloche *et al.*, 1992a). This plasmid already contains the Kozak consensus sequence for initiation of translation and a sequence coding for a nine-residue immunodominant peptide from influenza virus haemagglutinin HA1 inserted between a *Hin*dIII site (5') and an *Eco*RI (3') site. JP39 (5'-GTGAATTCG-CC(C/G)AA(G/A)AA(G/A)AA(G/A)CC(C/G)AC(C/G/T/A)CC-3') corresponds to the N-terminal coding region and possesses an *Eco*RI site allowing an in-frame insertion in the *Eco*RI site of the vector. JP40 (5'-GTAATTCGCAACATGGCATGCCACTGGG-3') also contains an *Eco*RI site and is located within the 3' untranslated region. The expression vector containing the tagged p44*mapk* (HA-MAPK) has already been described (Meloche *et al.*, 1992a; Pagès *et al.*, 1993). The vector coding for the constitutively active HA-ΔMAPKKK was constructed as follows. The full-length mouse MAPKKK cDNA (Lange-Carter *et al.*, 1993) subcloned in pBluescript vector was cut by *Nco*I (position 1541), blunted with T4 polymerase and then cut by *Xba*I (which cuts in the pBluescript plasmid's polylinker). This fragment was introduced into the pECE/HAP vector described above between the *Sma*I and *Xba*I sites.

Immune complex kinase assays

Quiescent PS 120 cells expressing different forms of HA-MAPKK were stimulated in HEPES-buffered DMEM with 20% serum for 5 min at 37°C. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in Triton X-100 lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethyl-sulfonyl fluoride, 10 mM *p*-nitrophenyl phosphate, 4 μg/ml aprotinin, 1% Triton X-100) for 15 min at 4°C. Insoluble material was removed by centrifugation at 12 000 *g* for 5 min at 4°C. Proteins from cell lysates were incubated with the 12CA5 antibody preadsorbed to protein A–Sepharose coated beads for 2 h at 4°C. The same procedure was followed for preparing the HA-MAPK substrate from non-stimulated CCL39 cells. Immune complexes were washed three times with Triton X-100 lysis buffer and the beads coming from HA-MAPKK and HA-MAPK immune complexes were mixed, then washed again with kinase buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 10 mM *p*-nitrophenylphosphate). MAPKK activity was assayed by resuspending the final pellet in 50 μl of kinase buffer containing 5 μM of [γ-^{32}P]ATP(5000 c.p.m./pmol). The reaction mixture was incubated for 30 min at 30°C. To test MAPK activity, reactivation by MAPKK was carried out as described above for 30 min at 30°C with non-radioactive ATP and the reaction was started by simultaneous addition of 50 μM [γ-^{32}P]ATP (5000 c.p.m./pmol) and MBP (0.25 mg/ml). The reactions were stopped by Laemmli sample buffer (Laemmli, 1970). The samples were heated at 95°C for 5 min and protein separated by SDS–PAGE (10% acrylamide gel for the resolution of MAPK and 13% acrylamide gels for the resolution of MBP). For detection of phosphotyrosine-containing proteins, the gels were incubated in 1 M KOH at 55°C for 1 h (Cooper *et al.*, 1983). The alkali-treated gels were neutralized in 10% acetic acid, 10% methanol and subjected to autoradiography.

Other methods

Protein concentration was measured using the bicinchonic acid protein assay kit (Pierce) with bovine serum albumin as standard. Site-directed mutagenesis was performed according to the Clontech double strand DNA strategy based on the Deng and Nickoloff method (1992).

Acknowledgements

We thank Dr G.Johnson for kindly providing the mouse MAPKKK cDNA, D.Grall and M.Valetti for skilled technical and secretarial assistance, and C.Cibre for computing artwork. We also thank Prof. R.Stanley for stimulating discussions and for reading the manuscript. This work was supported by the Centre National de la Recherche Scientifique (UMR 134),

the Institut National de la Santé et de la Recherche Médicale, and the Association pour la Recherche contre le Cancer.

References

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.
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.
Beck,T.W., Huleihel,M., Gunnell,M., Bonner,T.I. and Rapp,U.R. (1987) *Nucleic Acids Res.*, **15**, 595–609.
Benton,W.D. and Davis,R.W. (1977) *Science*, **196**, 180–182.
Brunet,A., Pagès,G. and Pouysségur,J. (1994) *FEBS Lett.*, in press.
Chambard,J.C., Paris,S. and Pouysségur,J. (1987) *Nature*, **326**, 800–803.
Cobb,M.H., Boulton,T.G. and Robbins,D.J. (1991) *Cell Regul.*, **2**, 965–978.
Cooper,J., Hunter,T. and Sefton,B. (1983) *Methods Enzymol.*, **99**, 387–402.
Crews,C.M. and Erikson,R.L. (1992a) *Science*, **258**, 478–480.
Crews,C.M. and Erikson,R.L. (1992b) *Proc. Natl Acad. Sci. USA*, **89**, 8205–8209.
Deng,W. and Nickoloff,J. (1992) *Anal. Biochem.*, **200**, 81.
Dent,P. *et al.* (1992) *Science*, **257**, 1404–1407.
Ellis,L. *et al.* (1986) *Cold Spring Harbor Symp. Quant. Biol.*, **51**, 773–784.
Errede,B. and Levin,D.E. (1993) *Curr. Opin. Cell Biol.*, **5**, 254–260.
Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42–52.
Haystead,T., Dent,P., Wu,J., Haystead,C. and Sturgill,T. (1992) *FEBS Lett.*, **306**, 17–22.
Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P. and Marshall,C.J. (1992) *Cell*, **71**, 335–342.
Kahan,C., Seuwen,K., Meloche,S. and Pouysségur,J. (1992) *J. Biol. Chem.*, **267**, 13369–13375.
Kosako,H., Gotoh,Y., Matsuda,S., Ishikawa,M. and Nishida,E. (1992) *EMBO J.*, **11**, 2903–2908.
Kosako,H., Gotoh,Y. and Nishida,E. (1993) *EMBO J.*, **12**, 787–794.
Kyriakis,J.M. *et al.* (1992) *Nature*, **358**, 417–421.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
L'Allemain,G., Pouysségur,J. and Weber,M. (1991) *Cell Regul.*, **2**, 675–684.
Lange-Carter,C.A., Pleinman,C.M., Gardner,A., Blumer,K.J. and Johnson,G.L. (1993) *Science*, **260**, 318–319.
Lenormand,P., Sardet,C., Pages,G., L'Allemain,G., Brunet,A. and Pouysségur,J. (1993a) *J. Cell Biol.*, **122**, 1079–1088.
Lenormand,P., Pagès,G., Sardet,C., L'Allemain,G., Meloche,S. and Pouysségur,J. (1993b) In Markovac,J. (ed.), *Advances in Second Messenger and Phosphoprotein Research*. Raven Press, New York, Vol. 28, pp. 237–244.
Matsuda,S., Kosako,H., Tanenaka,K., Moriyama,K., Sakai,H., Akiyama,T., Gotoh,Y. and Nishida,E. (1992) *EMBO J.*, **11**, 973–982.
Meloche,S., Pagès,G. and Pouysségur,J. (1992a) *Mol. Biol. Cell*, **3**, 63–71.
Meloche,S., Seuwen,K., Pagès,G. and Pouysségur,J. (1992b) *Mol. Endocrinol.*, **638**, 845–854.
Mordret,G. (1993) *Biol. Cell*, **79**, 193–207.
Nebreda,A. (1994) *Trends Biochem. Sci.*, **19**, 1–2.
Neiman,A.M. (1993) *Trends Genet.*, **9**, 390–394.
Noguchi,T., Metz,R., Chen,L., Mattéi,M.G., Carrasco,D. and Bravo,R. (1993) *Mol. Cell. Biol.*, **13**, 5195–5205.
Pagès,G., Lenormand,P., L'Allemain,G., Chambard,J.C., Meloche,S. and Pouysségur,J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8319–8323.
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.
Pelech,S. and Sanghera,J.S. (1992) *Science*, **257**, 1355–1356.
Pouysségur,J., Sardet,C., Franchi,A., L'Allemain,G. and Paris,S. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 4833–4837.
Ruderman,J.V. (1993) *Curr. Opin. Cell. Biol.*, **5**, 207–213.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Sardet,C., Franchi,A. and Pouysségur,J. (1989) *Cell*, **56**, 271–280.
Seger,R. (1992) *J. Biol. Chem.*, **267**, 25268–25631.
Seger,R., Ahn,N.G., Posada,J., Munar,E.S., Jensen,A.M., Cooper,J.A., Cobb,M.H. and Krebs,E.G. (1992) *J. Biol. Chem.*, **267**, 14373–14381.
Stanton,V.P., Nichols,D.W., Laudano,A.P. and Cooper,G.M. (1989) *Mol. Cell. Biol.*, **9**, 639–647.
Sturgill,T.W. and Wu,J. (1991) *Biochim. Biophys. Acta*, **1092**, 350–357.
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
Vouret-Craviari,V., Van Obberghen-Schilling,E., Scimeca,J.C., Van Obberghen,E. and Pouysségur,J. (1992) *Biochem. J.*, **289**, 209–214.
Wakabayashi,S., Fafournoux,P., Sardet,C. and Pouysségur,J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2424–2428.
Wang,Y., Simonson,M.S., Pouysségur,J. and Dunn,M.J. (1992) *Biochem. J.*, **287**, 589–594.
Wilson,I.A., Niman,H.L., Hougten,R.A., Cherenson,A.R., Connolly,M.L. and Lerner,R.A. (1984) *Cell*, **37**, 767–778.
Yashar,B., Kelley,C., Yee,K., Errede,B. and Zon,L. (1993) *Mol. Cell. Biol.*, **13**, 5738–5748
Zhang,F., Strand,A., Robbins,D., Cobb,M. and Goldsmith,E. (1994) *Nature*, **367**, 704–711.
Zheng,C.-F. and Guan,K.-L. (1994) *EMBO J.*, **13**, 1123–1131.

Received on March 8, 1994; revised on April 11, 1994

Note added in proof

During the submission of this report, another study appeared on the phosphorylation sites regulating MAPKK in response to activated Raf-1 [Alessi,D. *et al.* (1994) *EMBO J.*, **13**, 1610–1619].