

Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2

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Mitogen-activated protein (MAP) kinases bind tightly to many of their physiologically relevant substrates. We have identified a new subfamily of murine serine/threonine kinases, whose members, MAP kinase-interacting kinase 1 (Mnk1) and Mnk2, bind tightly to the growth factor-regulated MAP kinases, Erk1 and Erk2. Mnk1, but not Mnk2, also binds strongly to the stress-activated kinase, p38. Mnk1 complexes more strongly with inactive than active Erk, implying that Mnk and Erk may dissociate after mitogen stimulation. Erk and p38 phosphorylate Mnk1 and Mnk2, which stimulates their *in vitro* kinase activity toward a substrate, eukaryotic initiation factor-4E (eIF-4E). Initiation factor eIF-4E is a regulatory phosphoprotein whose phosphorylation is increased by insulin in an Erk-dependent manner. *In vitro*, Mnk1 rapidly phosphorylates eIF-4E at the physiologically relevant site, Ser209. In cells, Mnk1 is post-translationally modified and enzymatically activated in response to treatment with either peptide growth factors, phorbol esters, anisomycin or UV. Mitogen- and stress-mediated Mnk1 activation is blocked by inhibitors of MAP kinase kinase 1 (Mkk1) and p38, demonstrating that Mnk1 is downstream of multiple MAP kinases. Mnk1 may define a convergence point between the growth factor-activated and one of the stress-activated protein kinase cascades and is a candidate to phosphorylate eIF-4E in cells.

Keywords: eIF-4E/Erk/Mapkap kinase-2/MAP kinase/stress-activated protein kinase

Introduction

Mammalian cells respond to a variety of extracellular stimuli via activation of specific mitogen-activated protein (MAP) kinase cascades (Davis, 1993; Marshall, 1994; Waskiewicz and Cooper, 1995). Utilization of a cascade of protein kinases provides for integration of different inputs, allows a sharp switch in output for a subtle change in stimulus (Huang and Ferrell, 1996) and allows the signal to reach different cellular compartments. Three MAP kinase cascades have been characterized in mammals. Treatment with peptide growth factors, phorbol

esters, Ca²⁺ and some G-protein-linked agonists activates the Erk cascade. This cascade starts with one or more Raf family kinases, which phosphorylate and activate MAP kinase kinase 1 (Mkk1) and Mkk2, permitting the Mkks to phosphorylate and activate the MAP kinases extracellular signal-regulated kinase 1 (Erk1) and Erk2 (Davis, 1993; Marshall, 1995). Erks then phosphorylate both cytoplasmic and nuclear substrates, including the epidermal growth factor receptor, cytosolic phospholipase A2, ribosomal S6 kinase II (Rsk), Elk-1 (a ternary complex factor), c-Jun and c-Myc (Sturgill *et al.*, 1988; Pulverer *et al.*, 1991; Takashima *et al.*, 1991; Davis, 1993; Lin *et al.*, 1993; Marais *et al.*, 1993; Gille *et al.*, 1995). One net result of activating the Erk cascade is induction of immediate early gene transcription leading to cell proliferation or differentiation.

UV radiation, submaximal concentrations of protein synthesis inhibitors and increased extracellular osmolarity activate two additional types of MAP kinase cascades. One type of stress-activated MAP kinase, known as the Jun N-terminal kinases (Jnks) or stress-activated protein (SAP) kinases, was characterized initially as a family of proline-directed kinases which phosphorylate the c-Jun transactivation domain (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Galcheva-Gargova *et al.*, 1994; Kyriakis *et al.*, 1994). The other class is comprised of p38/RK/MPK2 and p38 β , and is similarly activated by a variety of environmental stresses (Han *et al.*, 1994; Rouse *et al.*, 1994; Jiang *et al.*, 1996). Although they appear to be coordinately regulated, Jnks and p38s are activated by two distinct kinase cascades. The Mkk1 homolog, Mkk4 (also known as Sek1 and Jnkk), phosphorylates and activates Jnk1 (Sanchez *et al.*, 1994; Derijard *et al.*, 1995). Two other Mkks, Mkk3 and Mkk6, activate p38 (Derijard *et al.*, 1995; Raingeaud *et al.*, 1996). Active Jnk1, its relative Jnk2, and p38 phosphorylate numerous transcription factors including Jun, ATF2 and Elk-1 *in vitro* (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Gupta *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995). Collectively, the Erks, Jnks and p38 are known as MAP kinases, since they are related in sequence and have a common mode of activation, dual phosphorylation of a regulatory tyrosine and threonine residue.

A subset of MAP kinase targets are themselves kinases. For example, Erk1 and Erk2 phosphorylate and activate the serine/threonine kinases Rsk1 [also known as MAP kinase-activated protein (Mapkap) kinase-1], Rsk2 and Rsk3 (Sturgill *et al.*, 1988; Zhao *et al.*, 1995). Similarly, p38 phosphorylates and activates the serine/threonine kinase Mapkap kinase-2 (Stokoe *et al.*, 1992a; Rouse *et al.*, 1994) and the related kinase 3pk/Mapkap kinase-3 (McLaughlin *et al.*, 1996; Sithanandam *et al.*, 1996). Upon activation, Rsk translocates to the nucleus (Chen *et al.*, 1992), where it is thought to phosphorylate numerous

proteins, including serum response factor (SRF), Fos and the cAMP-response element-binding protein (CREB) (Chen *et al.*, 1993; Rivera *et al.*, 1993; Ginty *et al.*, 1994; Bohm *et al.*, 1995; Xing *et al.*, 1996). Although subcellular localization of Mapkap kinase-2 is undetermined, it has been shown to phosphorylate Hsp27 (heat shock protein of 27 kDa), ATF2 and CREB (Stokoe *et al.*, 1992b; Freshney *et al.*, 1994; Tan *et al.*, 1996). Since the substrate specificities of Rsk and Mapkap kinase-2 are somewhat different, their substrates combined with those of their activating MAP kinases generate a unique pool of targets for each cascade.

The MAP kinases—Jnks, p38s and Erks—possess overlapping substrate specificities, each phosphorylating the minimum sequence ψ X[S/T]P (where ψ is proline or aliphatic). In the cell, however, protein–protein interactions between MAP kinases and their substrates may increase specificity. Erk binds its substrate, Elk-1 (Rao and Reddy, 1994), and co-fractionates with Rsk (Scimeca *et al.*, 1992; Hsiao *et al.*, 1994). Jnk1 binds ATF-2 (Gupta *et al.*, 1995), while Jnk2 binds tightly to c-Jun (Kallunki *et al.*, 1994). p38 has been shown to partially co-purify with Mapkap kinase-2 (Freshney *et al.*, 1994).

To isolate novel Erk substrates, we performed a two-hybrid screen using rat Erk2 as bait. We have identified two serine/threonine kinases, MAP kinase-interacting serine/threonine kinase 1 (Mnk1) and Mnk2, which have the unique ability to interact with both p38 and Erk2 in two-hybrid tests. Mnk1 phosphorylates eukaryotic initiation factor-4E (eIF-4E, also known as eIF-4 α), a translation initiation factor which binds the 7-methyl-guanosine cap on all eukaryotic mRNAs.

The translation initiation factor eIF-4E plays a key role in both the mechanism and regulation of translation in mammalian cells (reviewed in Sonenberg, 1994; Flynn and Proud, 1996a). eIF-4E is a phosphoprotein, whose phosphorylation, at Ser209 near its C-terminus, is enhanced by insulin, mitogens and other agents which stimulate translation (Flynn and Proud, 1995; Joshi *et al.*, 1995). Phosphorylation of eIF-4E is believed to enhance its affinity for the 5'-cap (Minich *et al.*, 1994) and to facilitate its incorporation into complexes containing eIF-4G and eIF-4A (reviewed in Sonenberg, 1994; Flynn and Proud, 1996a).

We provide evidence that Mnk1 is activated *in vitro* by Erk and p38. In cells, Mnk1 activity is stimulated by agonists which are known to stimulate either Erk or p38 MAP kinase pathways. The Mnk proteins may provide a convergence between the mitogen-activated and one of the stress-activated MAP kinase cascades.

Results

Isolation of Mnk1 and Mnk2, kinases which bind Erk

To isolate novel Erk2-binding proteins, a two-hybrid screen was utilized to identify Erk2-interacting domains (Chien *et al.*, 1991; Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995; A.J.Waskiewicz and J.A.Cooper, unpublished data). Nine clones were isolated which possessed homology with the C-terminus of the known Erk substrate Rsk. Using the most novel Erk2-interacting clone as a probe, we screened a mouse embryo library and identified two related full-

length cDNA clones, Mnk1 and Mnk2. The cDNA sequence of Mnk1 predicts an ORF of 415 amino acids (Figure 1). The other, related cDNA, Mnk2, encodes a protein of 412 amino acids, 72% of which are identical with Mnk1 (Figure 1). The region found in the two-hybrid screen was identical to the C-terminal 194 amino acids of Mnk2 and is partially conserved in Mnk1, suggesting that this domain mediates interaction with Erk2.

Each cDNA clone possesses a putative initiator ATG which is preceded by an in-frame termination codon. Based on alignments with known proteins, Mnk1 and Mnk2 are predicted to encode serine/threonine kinases (Hanks *et al.*, 1988). The Mnk catalytic domains are most similar to the group of serine/threonine kinases comprised of Rsk (C-terminal domain), Ca²⁺/calmodulin (CaM)-dependent kinases, Mapkap kinase-2 and 3pk/Mapkap kinase-3 (McLaughlin *et al.*, 1996; Sithanandam *et al.*, 1996). A region rich in basic amino acids (9/11 lysine or arginine residues), located near the N-terminus of both kinases, may mediate localization of these proteins.

The expression of Mnk1 and Mnk2 was examined by Northern analysis. A single 3.1 kb Mnk1 message was detected in all adult mouse tissue samples studied (Figure 2A). A Mnk2 mRNA of 3.7 kb is also present in all tissues, with the exception of brain, where expression levels are greatly reduced (Figure 2B). Both messages are especially abundant in skeletal muscle.

Mnk1 and Mnk2 bind to Erk1, Erk2 and p38

The specificity of MAP kinase binding to Mnk1 and Mnk2 was studied by two-hybrid tests and *in vitro* association experiments. Yeast were transformed with LexA-Ras (Vojtek *et al.*, 1993), LexA-Mnk1 or LexA-Mnk2, together with VP16, VP16-Erk2, VP16-Jnk1 or VP16-p38. Two independent yeast transformants were assayed for trans-activation of the *HIS3* reporter gene, as indicated by growth on minimal medium lacking histidine (Figure 3A). LexA-Mnk1 and LexA-Mnk2, but not LexA-Ras, clearly interact with fusions from both Erk2 and p38, although the interaction between LexA-Mnk2 and p38 is noticeably weaker. Neither Mnk clone was capable of forming a complex with Jnk1, demonstrating the specificity of this interaction. Deletion of the C-terminal 83 amino acids of Mnk1 abrogates its interaction with Erk2 (data not shown), implying that this domain is required for MAP kinase interaction.

To confirm the results of the two-hybrid tests, GST fusion proteins of Mnk1 and Mnk2 were expressed and purified from 293T human embryonic kidney cells. These Mnk fusions were incubated with [³⁵S]methionine-labeled p38, Jnk1 or Erk2, washed to remove unbound proteins, and subsequently analyzed by SDS–PAGE and autoradiography (Figure 3B). Mnk1 bound both Erk2 and p38, while Mnk2 interacted strongly only with Erk2. Only low and non-specific binding of Jnk1 was detected.

To determine whether Mnks bind to MAP kinases in mammalian cells, we transiently expressed GST fusions of Mnk1 and Mnk2 in 293T cells. After purifying the proteins using glutathione–Sepharose, we analyzed the GST–Mnks for the presence of co-purifying MAP kinases (Figure 3C). GST–Mnk1 was expressed at ~10-fold higher levels than GST–Mnk2. Endogenous Erk1 and Erk2 co-purified with GST–Mnk1 and GST–Mnk2, but not with

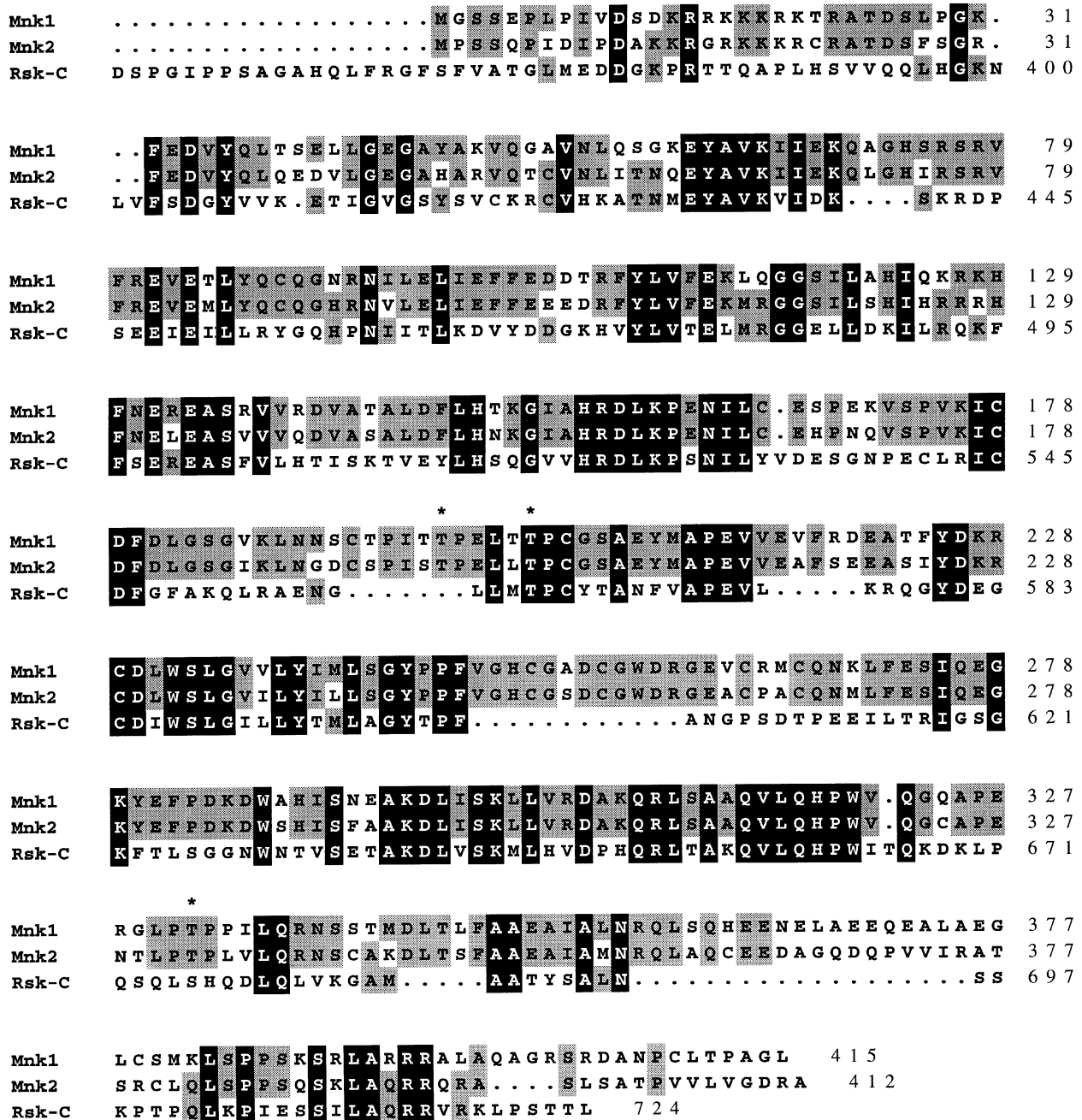


Fig. 1. The sequences of Mnk1, Mnk2 and Rsk1 were aligned. The amino acid sequence of Mnk1 and Mnk2 was generated by translating the cDNA into amino acids (shown here in single letter code). Each cDNA begins at an initiator ATG, which is preceded by an in-frame stop codon, and ends at a termination codon. An alignment with the C-terminal kinase domain of Rsk1 was generated using the PILEUP program. Identity among all three proteins is shown as a black box, while identity between two is shown as a gray box. Putative phosphorylation sites are labeled (*).

GST. p38 also co-purified with GST-Mnk1, but not with GST-Mnk2 or GST. The two-hybrid tests and binding studies suggest that Mnk2 prefers Erk over p38 as an *in vivo* binding partner, whereas Mnk1 binds both MAP kinases.

Phosphorylation of Erk2 reduces its affinity for Mnk1

To determine whether Erk-Mnk complexes were constitutive or regulated, we compared the ability of phosphorylated and dephosphorylated Erk to form complexes with Mnk. We incubated *in vitro* translated ³⁵S-labeled Erk2 with *in vitro* translated Mos. In the presence of wild-

type Mos, Erk2 becomes phosphorylated, presumably via activation of Mkk in the extract (Shibuya and Ruderman, 1993; Chen and Cooper, 1995). Incubation with either lower concentrations of Mos, or with a kinase-inactive Mos KM (K90R), generates a mixed population of dephosphorylated and phosphorylated Erk2. Using the electrophoretic mobility shift which results from phosphorylation of Erk2, we quantitated the ratio between dephospho and phospho forms. After incubating a mixed pool of Erk2 and phospho-Erk2 with GST-Mnk1 or GST-Mnk2, the population of Erk2 bound was depleted for phospho-Erk2 (Figure 4A, left). In a separate experiment, we compared

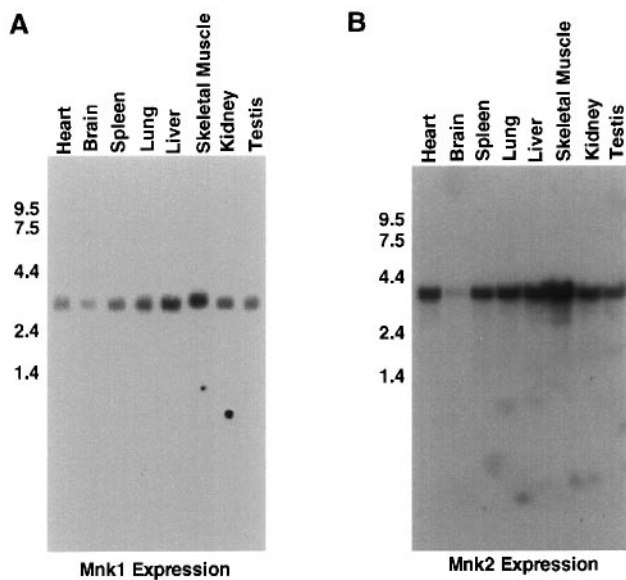


Fig. 2. Northern analysis of the expression patterns of Mnk1 and Mnk2 in adult mouse. **(A)** A multiple tissue Northern blot was probed with a probe from the 3'-untranslated region of Mnk1. The sizes of the molecular weight markers are shown on the left in kilobases. The approximate size of Mnk1 is 3.1 kb. **(B)** A multiple tissue Northern was probed with the 3'-untranslated region probe specific for Mnk2. The approximate size of the message is 3.7 kb. The amount of RNA was equal for all tissue samples.

the binding of populations of Erk2 that were either predominantly phosphorylated or dephosphorylated. GST-Mnk1 bound 44% of the dephosphorylated Erk2, versus 15% of the phosphorylated Erk2 (Figure 4A, right). These two independent measurements demonstrate that Mnks bind significantly more dephospho- than phospho-Erk2, *in vitro*.

To investigate whether Erk-Mnk1 complexes were constitutive or regulated *in vivo*, we expressed GST-Mnk1 in 293T cells, stimulated the cells with phorbol ester (TPA) and analyzed the phosphorylation state of Erks that co-purified with Mnk1. Treatment of 293T cells with two different doses of TPA induced phosphorylation of both Erk1 and Erk2, as shown by the mobility shift in the samples not bound to GST-Mnk1 (Figure 4B). However, Erk1 and Erk2 that co-purified with Mnk1 from TPA-stimulated cells were mostly dephosphorylated. These data are consistent with the release of phosphorylated Erk1 and Erk2 from Mnk1 following TPA stimulation of 293T cells.

Mnk1 and Mnk2 are *in vitro* substrates of Erk2 and p38

Inspection of the sequences of Mnk1 and Mnk2 revealed three consensus MAP kinase phosphorylation sites (ψ X[S/T]P, where ψ is aliphatic or proline) that are conserved between the two proteins (Alvarez *et al.*, 1991; Clark-Lewis *et al.*, 1991). Two sites, Thr197 and Thr202, are within the T-loop located between subdomains VII and VIII, and the third, Thr332, is in the C-terminus. To test the hypothesis that Mnk1 and Mnk2 are MAP kinase substrates, we synthesized GST-Mnk proteins in *Escherichia coli* and mixed them with active Erk2 or p38 in the presence of radiolabeled ATP. GST-Mnk1 does not incorporate ATP when incubated alone (Figure 5A, lane 2). When combined with active Erk2, Mnk1 became

phosphorylated (lane 3), although phosphorylation is low compared with a known Erk2 substrate, myelin basic protein (lane 1). In a similar assay, Mnk2 is better than Mnk1 as an Erk2 substrate (Figure 5B). Mnk1 and Mnk2 are phosphorylated equally well by p38 (Figure 5C and D). Further analysis will be needed to determine whether the detected phosphorylation is all catalyzed by the added MAP kinase, or whether phosphorylation by the MAP kinase is followed by autophosphorylation of Mnk.

Electrophoretic mobility shift of Mnk1 in NIH 3T3 cells treated with either growth factors or UV radiation

Gel mobility shifts have been utilized as indicators of protein phosphorylation for numerous phosphoproteins. GST-Mnk1 was transiently transfected into NIH 3T3 fibroblasts which were treated subsequently with a variety of stimuli. After gel electrophoresis, GST-Mnk1 from serum-starved, unstimulated cells migrated as one major and two minor slower mobility bands (Figure 6A). Upon stimulation with platelet-derived growth factor (PDGF), the proportion of one of the slower mobility species was increased. Treatment with UV also increased the proportion of this form. Prior treatment of the cells with SB203580, an inhibitor of p38 (Lee *et al.*, 1994), blocked the UV-induced shift in electrophoretic mobility. This inhibitor had no effect on the PDGF-induced Mnk1 bandshift. The shift in Mnk mobility is consistent with a rapid post-translational modification, possibly a phosphorylation event mediated by Erk and p38.

To determine whether activation of a MAP kinase cascade was necessary or sufficient for the modification in Mnk1, we expressed Mnk1 with a series of Mkk1a mutants (Seger *et al.*, 1994). In control cells expressing wild-type Mkk1a, the electrophoretic mobility of Mnk1 responded to PDGF (Figure 6B). Expression of a dominant-negative Mkk1a mutant (S222A) partially inhibited the PDGF-induced bandshift. The mobility of Mnk1 was constitutively altered in untreated cells expressing activated Mkk1a (S218E,S222E). These experiments indicate that the Mnk1 bandshift can be induced by activation of the Erk pathway, and that the PDGF-induced Mnk1 bandshift is dependent on the Erk cascade.

Mnk1 is a MAP kinase-activated eIF-4E kinase

To test whether Mnk1 is activated by MAP kinases, we incubated GST-Mnk1, purified from *E.coli*, with ATP and either active Erk1 or p38, and then measured its kinase activity against an added substrate. During the activation, the stoichiometry of Mnk1 phosphorylation by Erk exceeded 0.5 mol phosphate per mol of Mnk1 protein. Given that initiation factor eIF-4E phosphorylation is dependent on Erk activation (Flynn and Proud, 1996b), we tested eIF-4E as an *in vitro* substrate for Mnk1. Erk1-activated Mnk1 and p38-activated Mnk1 were potent eIF-4E kinases (Fig. 7). In addition, Erk and p38 stimulated Mnk1 autophosphorylation. Similar pre-incubation with Erk in the absence of ATP did not activate Mnk1 (data not shown), implying that phosphorylation of Mnk1 is required.

To determine whether Mnk's activity required phosphorylation of the T-loop of Mnk1, two potential MAP kinase phosphorylation sites within this loop (T197 and

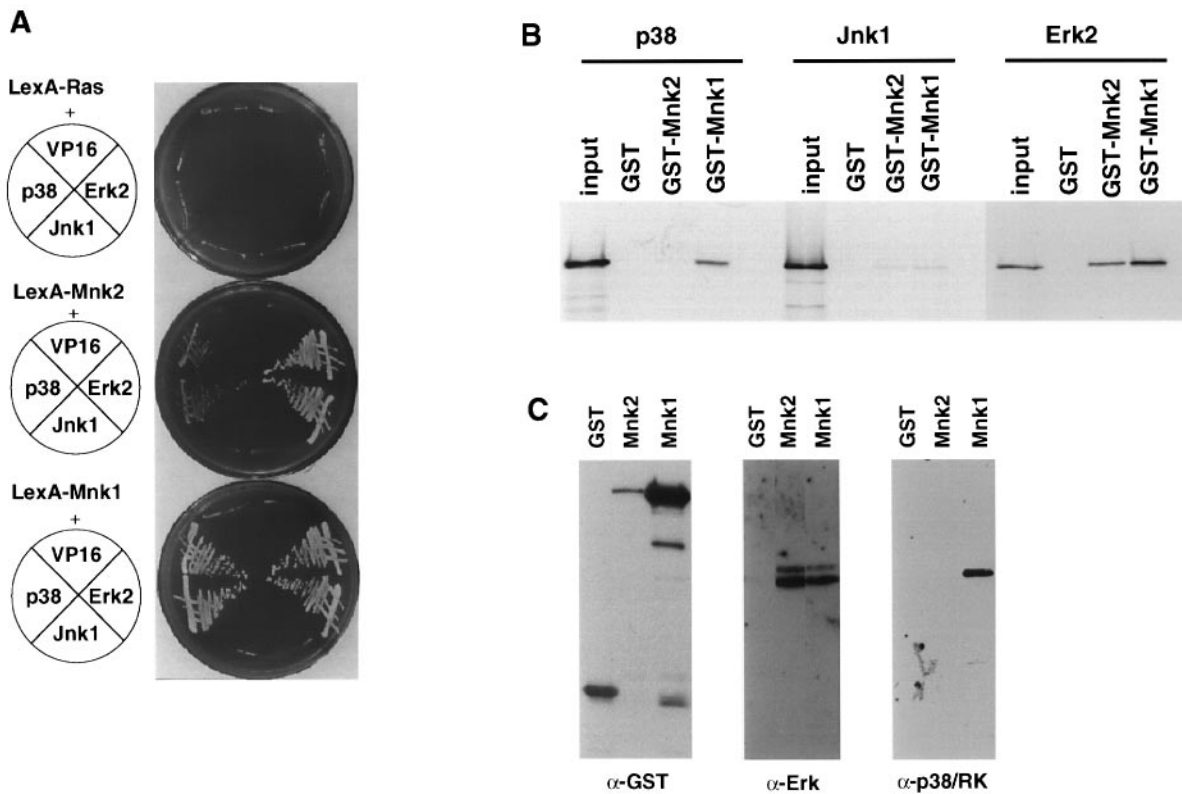


Fig. 3. Interaction between Mnks and MAP kinases. **(A)** Two-hybrid interaction between Mnks and the MAP kinases. *Saccharomyces cerevisiae* strain L40 was transformed with plasmids encoding LexA fusion proteins and VP16 fusion proteins. Two independent transformants were streaked from YC-leu-trp medium to YC-leu-trp-his medium and also to YC-leu-trp. All transformants regrew on the restreaked YC-leu-trp-his plates (data not shown). At the top, LexA-Ras was combined with VP16, VP16-Erk2 (Erk2), VP16-Jnk1 (Jnk1) and VP16-p38 (p38) and plated on YC-leu-trp-his. In the middle, LexA-Mnk2, which transactivates weakly on its own (data not shown), was combined with VP16 fusions containing either empty vector, Erk2, Jnk1 or p38. LexA-Mnk2 transformants were grown on YC-leu-trp-his medium containing 1 mM 3-amino-triazole. At the bottom, LexA-Mnk1 was combined with VP16 fusion proteins and plated to YC-leu-trp-his. All plates were grown for 48 h at 30°C. **(B)** MAP kinases bind to GST fusions of Mnk1 and Mnk2. GST fusion proteins were generated in 293T-HEK cells and purified with glutathione-Sepharose. *In vitro* translated ³⁵S-labeled p38, Jnk1 or Erk2 were mixed with each GST fusion, to assay for interaction. Bound proteins were separated by SDS-PAGE and visualized by autoradiography. One-sixth of the protein used in the binding reactions was loaded (input) as a molecular weight control. **(C)** MAP kinases co-purify with Mnk2 and Mnk1 from 293T cells. 293T cells were transiently transfected with vectors expressing GST alone, GST-Mnk2 or GST-Mnk1. Fusion proteins were purified and separated by SDS-PAGE. Immunoblotting with 38.4, an antiserum against GST, showed the expression levels of the three proteins. Fusion proteins were immunoblotted with 1913.3, to detect the presence of co-purifying Erk1 or Erk2. The upper band co-migrates with the 44 kDa Erk1 and the lower co-migrates with the 42 kDa Erk2 from lysates (data not shown). Fusion proteins were immunoblotted with αp38 to detect the presence of co-purifying p38. The band co-migrates with a single band in the lysates at ~43 kDa (data not shown).

T202) were mutated to alanine. Mnk1-T2A2 (T197A, T202A) is phosphorylated to a similar extent by Erk1 and p38 *in vitro* (data not shown), but does not become activated by either MAP kinase (Figure 7). This demonstrates that although Erk1 and p38 phosphorylate Mnk1 outside the T-loop, T197 and/or T202 are required for the MAP kinase-dependent activation of Mnk1.

eIF-4E is phosphorylated on Ser209 when cells are treated with insulin (Flynn and Proud, 1995; Joshi *et al.*, 1995). Mnk1 phosphorylates eIF-4E only on serine (Figure 8A), contained on a single tryptic phosphopeptide (Figure 8B). To determine whether Mnk1 phosphorylated the physiologically relevant site, the peptides phosphorylated by Mnk1 were compared with a synthetic phosphopeptide containing Ser209 (Figure 8B). The synthetic phosphopeptide and Mnk1-phosphorylated eIF-4E peptide had identical electrophoretic and chromatographic mobilities. Since this peptide contained four phosphorylatable amino acids, we utilized Edman degradation to distinguish which residue was phosphorylated. The third cycle of Edman degradation released free phosphate, implying that the

third residue of this tryptic peptide was phosphorylated (Figure 8C). Given the combination of phosphopeptide mapping and Edman degradation, eIF-4E Ser209 is the major site of phosphorylation by Mnk1.

Mnk1 is activated *in vivo* by mitogens and stresses

To assay Mnk1 activation *in vivo*, we transfected 293 cells with vectors expressing GST-Mnk1 or GST-Mnk1-T2A2. Upon stimulation of the cells with phorbol ester, wild-type Mnk1 is activated ~3-fold in comparison with unstimulated cells, when assayed against the protein substrate, eIF-4E (Figure 9). Prior incubation with either PD098059, an inhibitor of Mnk1 (Dudley *et al.*, 1995), or SB203580 prevented this activation, indicating that TPA may activate both Erk- and p38-dependent mechanisms of Mnk1 phosphorylation. In cells treated with environmental stresses, NaCl or anisomycin, Mnk1 eIF-4E kinase activity increased 4- or 10-fold, respectively (Figure 9). The Mnk1 inhibitor, PD098059, had little effect on this stimulation, indicating that Erk is not responsible for the majority of

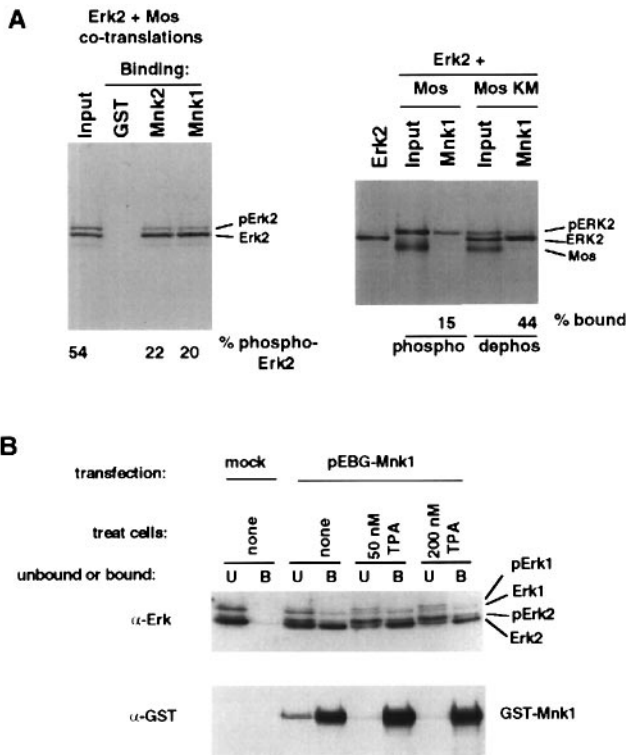


Fig. 4. Mnks associate preferentially with dephosphorylated Erk2. (A) Left panel: GST, Mnk1 and Mnk2 were purified from transiently transfected 293T cells. Erk2 was co-translated with a small amount of Mos DNA. The gel mobility of this translated Erk2 is a mixture of dephosphorylated and phosphorylated species, as shown in the Input lane. Quantitation by PhosphorImager demonstrated that the pool was 54% phosphorylated. Quantitation of the Erk which bound demonstrated that it was 22 (Mnk2) and 20% (Mnk1) phosphorylated. The approximate positions of phospho- and dephospho- Erk2 are shown on the right. Right panel: Erk2 was co-translated either with large amounts of wild-type Mos DNA or kinase-inactive Mos (KM). Using the predominantly phosphorylated (phos) Erk2 from the Mos co-translations, the percentage of binding to Erk2 was quantitated at 15%. Using the predominantly dephosphorylated (dephos) Erk2 from Mos KM translations, the binding was 44%. The positions of phospho-Erk2, dephospho-Erk2 and Mos are shown on the right. (B) 293T cells were transiently transfected with no DNA (mock) or a vector expressing GST-Mnk1 (pEBG-Mnk1). Two days after addition of the DNA, cells were serum starved for 16 h, and subsequently stimulated with either 50 or 200 nM phorbol ester (TPA). GST fusion proteins were purified and separated alongside supernatants. The purified proteins (B) and the supernatants (U) were assayed for the presence of Erk and GST immunoreactivity. The positions of phosphorylated and dephosphorylated Erks are shown.

stress-mediated Mnk1 activation. In contrast, the p38 inhibitor SB203580 was a potent inhibitor of both stress stimuli, indicating that p38 is required for stress-dependent Mnk1 stimulation. The eIF-4E phosphorylating activity is most likely due to Mnk1, not an associated kinase, because Mnk1-T2A2, which is not activated *in vitro* by either Erk or p38 (Figure 7), was not activated by TPA, NaCl or anisomycin (Figure 9).

Discussion

We have identified two new serine/threonine kinases which bind to MAP kinases. Together with Rsk1, Rsk2 and Rsk3, the new kinases, Mnk1 and Mnk2, share three features: (i) a conserved C-terminal Erk-interacting

domain; (ii) a catalytic domain with homology to the Ca²⁺/calmodulin-dependent family of kinases; and (iii) putative MAP kinase phosphorylation sites (ψ X[S/T]P) located within the T-loop of the kinase domain. Mnk1 and 2 differ from the Rsk's, however, in lacking a second, more N-terminal kinase domain. The Mnks are also homologous to Mapkap kinase-2 and 3pk, two kinases which are activated in cells by a p38-dependent stress-activated pathway.

Mnk1 and Mnk2 are distinct from the Rsk's because of their unique ability to interact with p38 as well as with Erk1 and Erk2. They do not interact with Jnk1, suggesting that they may not interact with other Jnk/SAPK-related MAP kinases. Mnk1 interacts with Erk or p38 equally well, based on two-hybrid tests, *in vitro* binding assays and detectable co-purification with both MAP kinases from tissue culture cells. Based on similar experiments, Mnk2 prefers to bind Erk. Mnk1 and Mnk2 can both be phosphorylated *in vitro* by either Erk or p38, but Mnk1 appears to be a relatively poor substrate for Erk and a good substrate for p38, whereas Mnk2 is a good substrate for both kinases. While the relative preference for *in vitro* phosphorylation parallels the binding preference, we had expected that both Mnks would be phosphorylated equally by Erk and unequally by p38. This anomaly could be due to misfolding of bacterial Mnk1, but other explanations are possible. Since activated Erk binds less well to Mnk than inactive Erk, the relative phosphorylation *in vitro* by pre-activated MAP kinases may not be a valid measure of *in vivo* activation. Indeed, preliminary phosphopeptide mapping studies indicate that one of the major sites phosphorylated by p38 in Mnk1 *in vitro* is not phosphorylated when Mnk1 is expressed in fibroblasts that are hypertonically stressed, even though Mnk1 is activated under these conditions and that activation is inhibited by a specific p38 inhibitor. This suggests that active p38 added to Mnk1 phosphorylates at least one site that is not accessible when the p38-Mnk1 complex is activated *in vivo*.

The importance of the Mnk-Erk interaction is underscored by the finding that it is regulated under conditions which are known to regulate Erk function. Following Erk activation in TPA-stimulated 293T cells, Mnk1 remains almost exclusively bound to dephosphorylated Erk1 and Erk2. This suggests that Erk-Mnk1 complexes may dissociate after activation, as suggested for Erk-Rsk complexes in *Xenopus* oocytes (Hsiao *et al.*, 1994). Dissociation of Rsk-Erk and Mnk-Erk complexes *in vivo* may be driven by phosphorylation and activation of Erk, since Mnk1 binds dephosphorylated Erk2 3-fold better than phosphorylated Erk2. We have not tested whether activation of Mnk1 may also affect its affinity for Erk.

Phosphorylation of residues within the T-loop (between subdomains VII and VIII) often leads to the stimulation of kinase activity (Johnson *et al.*, 1996). The Mnks both possess putative MAP kinase phosphorylation sites within their phosphoacceptor loops (IST¹⁹⁷P and LLT²⁰²P of Mnk2; ITT¹⁹⁷P and LTT²⁰²P of Mnk1). In addition, a more C-terminal site, LPT³³²P, is conserved in both Mnks, and phosphopeptide analysis of Mapkap kinase-2 has shown that the homologous residue is phosphorylated by p38 (Ben-Levy *et al.*, 1995). We have shown that Mnks are substrates for Erk and p38 *in vitro*. Phosphorylation

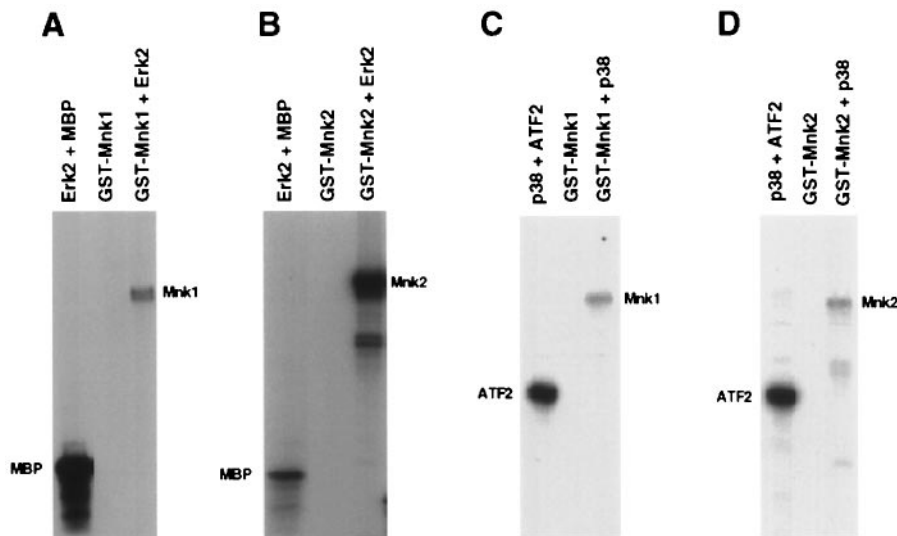


Fig. 5. GST-Mnk1 and GST-Mnk2 incorporate radiolabeled ATP when incubated with active MAP kinases. (A) GST-Mnk1 was synthesized in *E. coli*. Approximately 1 μ g of protein was incubated with or without active Erk2. One hundred ng of myelin basic protein (MBP) were mixed with active Erk2 to compare the relative rates of phosphorylation between Mnk1 and Mnk2. (B) GST-Mnk2 was synthesized in *E. coli* and mixed with or without active Erk2 and radiolabeled ATP. MBP phosphorylation was used to compare the phosphorylation of Mnk2 and Mnk1. (C) GST-Mnk1 was synthesized in *E. coli* and purified using glutathione-Sepharose. p38 was synthesized in NaCl-treated 293T cells and incubated with either ATF-2 or Mnk2 and radiolabeled ATP. (D) GST-Mnk2 and p38 were incubated in the presence of radiolabeled ATP.

of Mnk1 and Mnk2 *in vitro* stimulates intrinsic Mnk kinase activity against a protein substrate, eIF-4E. Either T197, T202 or both is required for activation by Erk or p38, suggesting that phosphorylation in the T-loop is important for activation.

Based on the findings of our *in vitro* phosphorylation and activation experiments, Mnk1 is activated by Erk or p38. *In vivo*, bandshift results demonstrate a change in Mnk1's electrophoretic mobility when NIH 3T3 cells are treated with either PDGF or UV-C irradiation. Since the p38 inhibitor, SB203580, blocks UV-dependent Mnk1 bandshifts, p38 is directly or indirectly responsible for the stress-induced bandshift. Kinase assays with Mnk1 indicate that these bandshifts correlate with an increase in the intrinsic activity of Mnk1 to phosphorylate exogenous substrates. Both phorbol esters and protein synthesis inhibitors reproducibly activated Mnk1 activity. Phorbol ester-dependent Mnk activation is inhibited by both Mnk1 inhibitors and p38 inhibitors, implying that mitogen activation of Mnk may depend on activation of multiple pathways. In contrast, stress-induced Mnk activation is only inhibited by the p38 inhibitor, indicating that p38 mediates stress-dependent activation of Mnk1.

The data presented here identify Mnk1 as a candidate for the kinase responsible for the phosphorylation of eIF-4E *in vivo*. Several lines of evidence are consistent with the idea that this kinase might be the enzyme which acts on eIF-4E in response to insulin and mitogens: (i) Mnk1 phosphorylates the same site in eIF-4E as that phosphorylated in intact cells in response to insulin (Flynn and Proud, 1995); (ii) the insulin-mediated phosphorylation of eIF-4E has been shown to require Erk (Flynn and Proud, 1995); (iii) activated Ras, which lies upstream of the MAP kinase cascade and thus of Mnk1, leads to enhanced phosphorylation of eIF-4E (Rinker-Schaeffer *et al.*, 1992); and (iv) the nerve growth factor-induced phosphorylation of eIF-4E is blocked by dominant-negative Ras (Frederickson *et al.*, 1992). Other MAP kinase-regulated

protein kinases, Rsk and Mapkinase-2, appear not to phosphorylate eIF-4E *in vitro* (A. Flynn and C.G. Proud, unpublished results).

Other workers have suggested previously that protein kinase C (PKC) might be directly responsible for the phosphorylation of eIF-4E (see, for example, Whalen *et al.*, 1996). However, this is not consistent with a requirement for the Erk cascade in the insulin-induced phosphorylation of eIF-4E (Flynn and Proud, 1996b) or the inability of inhibitors of PKC to block this effect (Flynn and Proud, 1996c). Makinje *et al.* (1995) have shown that a protamine kinase can phosphorylate Ser209 in eIF-4E although this enzyme also phosphorylates eIF-4E on threonine (a phenomenon not normally seen *in vivo* in response to stimuli which activate translation). The identity and upstream control of the protamine kinase are currently unknown.

Mnk1 is also activated *in vitro* by p38 MAP kinase and in intact cells by agents which stimulate this kinase cascade. However, studies on the relationship between activation of the p38 MAP kinase pathway [which occurs in response to cell stresses and to certain cytokines (Kyriakis and Avruch, 1996)] and the phosphorylation state of eIF-4E have not been reported so far.

Fukunaga and Hunter (1997) have identified a cDNA clone for a human protein closely related to Mnk1 (94% identity) in a screen for Erk1 substrates. They have also shown that Mnk1 is activated *in vitro* when phosphorylated by Erk1 or p38, and that Mnk1 is activated in cells by mitogenic and stress stimuli. The use of dominant-negative phosphorylation site mutant Mnks and constitutively activated mutants will be useful in determining their potential biological role in regulating eIF-4E phosphorylation and mRNA translation.

Materials and methods

Plasmids and nucleic acid manipulations

The plasmids pBTM116 (Bartel *et al.*, 1993a), pVP16 (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995), pLexA-lamin (Bartel *et al.*, 1993b),

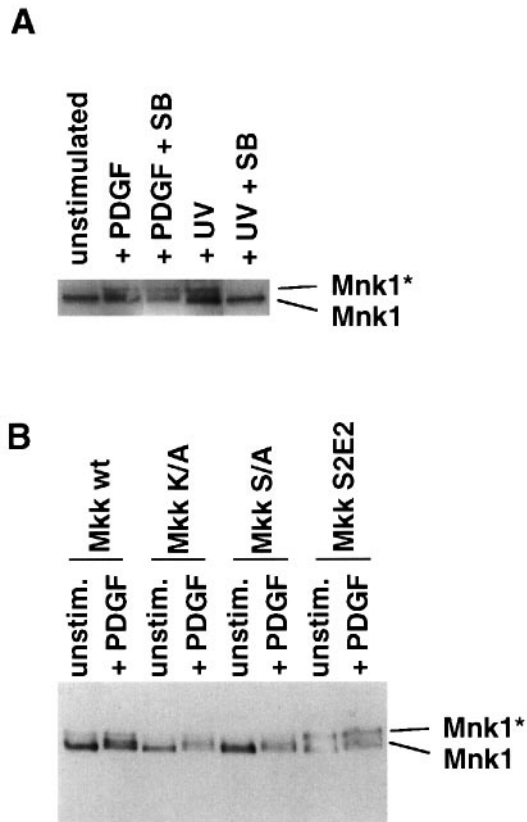


Fig. 6. Mnk1 undergoes a shift in gel mobility which correlates with the activation of MAP kinases. (A) NIH 3T3 cells were transiently transfected with vectors expressing GST-Mnk1. Cells were serum starved, and subsequently treated with either 10 ng/ml of PDGF or 75 J/m² UV-C radiation. Two dishes of cells underwent prior treatment for 120 min with the 30 μ M p38 inhibitor (SB203580). Mnk1 was purified, separated by SDS-PAGE and immunoblotted with GST antiserum. The positions of Mnk1 and the slower migrating Mnk1* are indicated. (B) NIH 3T3 cells were transiently transfected with vectors expressing GST-Mnk1 and vectors expressing wild-type Mkk1a (WT), kinase-inactive Mkk1a K/A (K97A), phosphorylation site mutant Mkk1a S/A (S222A) and activated mutant Mkk1a S2E2 (S218E, S222E). One out of two dishes was serum starved, while the other was starved and subsequently stimulated with 10 ng/ml of PDGF. The pattern of Mnk1 was visualized using an antiserum against GST.

pGEX3X, pSP64 (Promega), pSP64A-MT-Xp42 (wt, K57R and TY/VF) (Posada and Cooper, 1992), T7-Erk2 and pEBG-2T (Sanchez *et al.*, 1994) have been described previously. To complete the work in this study, the following modifications of the above vectors were made.

The ORF of rat Erk2 (gift of M.Cobb) was amplified by PCR using the primers 5'-TGACTAGGATCCGATGGCGGCGGGCG-3' and 5'-TATAAATGCATATTAAGATCTGTATCCTGG-3'. The resultant PCR product was digested with *Bam*HI and *Nsi*I and subcloned into *Bam*HI/*Pst*I-digested pBTM116 to create pLexA-Erk2. Mutant versions, pLexA-Erk2 (Lys52 replaced with arginine or K52R) and pLexA-Erk2 (T183E, Y185E) were constructed by amplifying the appropriate mutant template (gift of M.Cobb) with the above primers. Using the former primer listed above paired with 5'-CCCCGGATCCTTAAGATCTGTA-TCCTG-3', Erk2 was amplified by PCR. The product was digested with *Bam*HI and subcloned into pVP16, creating pVP16-Erk2.

The ORF from p38 (gift of R.J.Davis) was amplified by PCR using the following primers: 5'-CCCCGGATCCTCGGAAAATGTCTCAGGA-GA-3' and 5'-CCCCGGATCCTCAGGACTCCATCTCTT-3'. The product was digested with *Bam*HI and ligated into *Bam*HI-cut pVP16 to create pVP16-p38. The same digested product was also ligated into pCS2+ (Turner and Weintraub, 1995) to generate pCS2-p38. The product was also ligated into pEBG-3X to generate pEBG-p38.

The Jnk1 ORF (gift of R.J.Davis) was amplified using the primers 5'-CCCCGGATCCTCATGAGCAGAAGC-3' and 5'-CCCCGATCTTCACTGCTGCACCTGTG-3'. The resultant product was digested

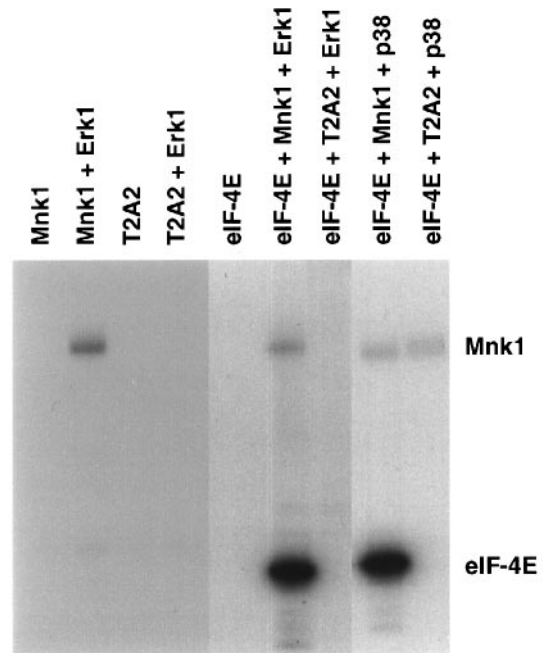


Fig. 7. Incubation with MAP kinases and ATP activates the kinase activity of Mnk1. GST fusion proteins from *E.coli* were incubated with either active Erk1 or active p38 in the presence of 400 μ M ATP. Following a 2 h incubation at 30°C, the GST fusions were washed to remove excess ATP and bound Erk. Samples were assayed for autophosphorylation and substrate phosphorylation activity by mixing the activated fusion proteins with radiolabeled ATP with or without 1 μ g of eIF-4E protein. The Mnk1 T2A2 mutant (T197A, T202A) was also assayed using this protocol. Both autophosphorylation and eIF-4E phosphorylation were detected with wild-type but not T2A2 mutant that had been incubated with Erk1 or p38. Phosphorylation of the T2A2 mutant in the sample pre-incubated with GST-p38 is probably due to phosphorylation by residual GST-p38, not to autophosphorylation.

with *Bam*HI and *Bgl*II and ligated into *Bam*HI-linearized pVP16 to generate pVP16-Jnk1. The same product was also ligated into pCS2+, generating pCS2-Jnk1.

The ORF of Mnk2 was PCR amplified using the following primer pair: 5'-CCCTGACTGACTGACAATGCCTTCCAGT-3' and 5'-CCCTG-ATGATCATCATGCGCGATCCCCCA-3'. The base pair substitution at the 3' end of the first oligo was unintended, but does not cause a change in the encoded amino acid residue. The product was digested with *Bcl*II and subcloned into (i) *Bam*HI-cut pBTM116, generating pLexA-Mnk2, (ii) *Bam*HI-cut pEBG3X, generating pEBG-Mnk2, and (iii) *Bam*HI-cut pGEX3X, generating pGEX-Mnk2.

The ORF of Mnk1 was amplified using 5'-CCCCGGATCCTGGAGA-TGGGAGCAGT-3' and 5'-CCCCGGATCCTGAGGTTTCAGAGCCCTG-3'. The PCR product was digested with *Bam*HI and ligated into: (i) *Bam*HI-cut pBTM116, generating pLexA-Mnk1, (ii) *Bam*HI-cut pEBG3X, generating pEBG-Mnk1, and (iii) *Bam*HI-cut pGEX3X, generating pGEX-Mnk1. To generate Mnk1 [Thr193Ala, Thr197Ala(T2A2)], we created two additional primers spanning the sites of mutations. Using the former Mnk1 oligo and 5'-GGAGCAGTGAGCTCTGGCGCGGTT-ATGGGAG-3', we amplified the N-terminal Mnk1 (T2A2) fragment with Vent polymerase (New England Biolabs). Using the latter Mnk1 oligo and 5'-AACCAGCCAGAGCTCACTGCTCCATGCGGC-3', we similarly amplified the C-terminal Mnk1 (T2A2) portion. The two fragments were mixed and used as a template for a second round of *Taq*-based PCR, using only the above non-mutagenic flanking oligos. The resultant product was digested with *Bam*HI and subcloned into the same series of vectors as wild-type Mnk1.

Isolation of cDNAs and sequence analysis

A probe from clone 209 (A.J.Waskiewicz and J.A.Cooper, unpublished data) was generated using *Taq* (Perkin-Elmer) amplification with a single primer in the presence of [α -³²P]dCTP. Unincorporated nucleotides were removed by ethanol precipitating the single-stranded probe. The

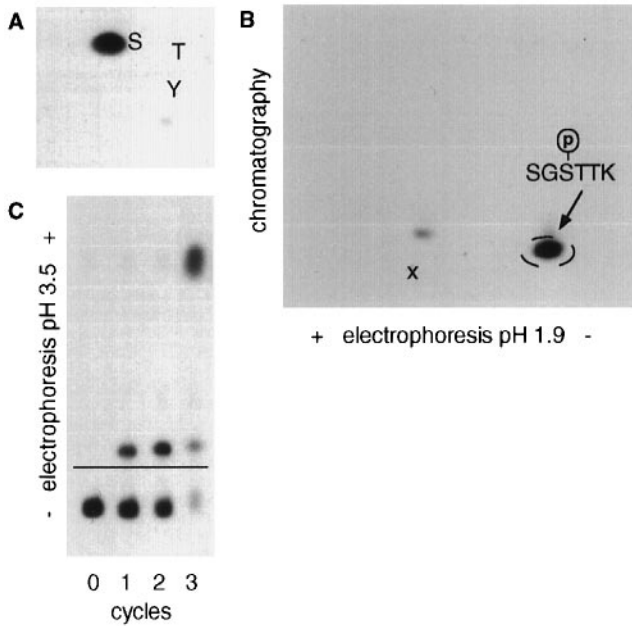


Fig. 8. Mnk1 phosphorylates eIF-4E at the physiologically relevant site, Ser209. (A) Mnk1-phosphorylated eIF-4E was acid hydrolyzed and subsequently examined to determine its phosphoamino acid content. The positions of phosphoserine, phosphothreonine and phosphotyrosine standards were visualized with ninhydrin staining. (B) Mnk1-phosphorylated eIF-4E was digested with trypsin. Resultant phosphopeptides were separated by electrophoresis at pH 1.9 and chromatography. The synthetic phosphopeptide, SGSpTTK, was spotted and separated simultaneously to determine the expected migration of the Ser209 tryptic peptide. The location of this peptide was determined with ninhydrin staining and is indicated by the dotted ellipse. (C) The major phosphopeptide was subjected to Edman degradation. The products were then separated by thin layer electrophoresis at pH 3.5 and visualized by autoradiography. Free phosphate is released upon the third cycle of degradation, indicating that the third residue of this phosphopeptide is phosphorylated.

denatured probe was added to filters which were pre-hybridized with Church-Gilbert buffer and hybridized at 65°C. The filters displayed a λ phage E16 stage mouse embryo poly(A)-primed cDNA library (Novagen). After incubating overnight, filters were washed and analyzed by autoradiography. Positive plaques were purified through two additional rounds of hybridization using the 209 probe. DNA was isolated and subcloned into pBS KS II+ (Stratagene). The ORFs of the clones 209.11 (Mnk2) and 209.5 (Mnk1) were sequenced on both strands using an ABI automated fluorescent dye terminator protocol. The sequences were analyzed using GAP, PILEUP, TRANSLATE and MAILFASTA programs of GCG. The sequences of Mnk2 and Mnk1 have been deposited in the EMBL database and are accessible under the accession numbers Y11091 (Mnk1) and Y11092 (Mnk2)

Northern hybridizations

An adult mouse multiple tissue Northern (Clontech) was probed according to the manufacturer’s recommended protocol. Briefly, a 730 bp *NheI* fragment from nucleotides 1294 to 2024 of the Mnk2 cDNA was subcloned into pBS KSII+ (Stratagene). Using a vector oligo and [α -³²P]dCTP (New England Nuclear), a probe was generated by *Taq* synthesis of the antisense strand. The blot was pre-hybridized and hybridized in 6–10 ml of Hyb2 [0.75 M NaCl, 0.05 M NaH₂PO₄, 0.005 M Na₂EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin (BSA), 100 μ g/ml sheared salmon sperm DNA, 2% SDS, pH 7.4] at 65°C. Following 18 h of hybridization, the blot was washed four times with Wash1 (0.3 M NaCl, 0.03 M Na citrate, 0.05% SDS, pH 7.0) at room temperature and twice with Wash2 (0.015 M NaCl, 0.0015 M Na citrate, 0.1% SDS, pH 7.0) at 50°C. Blots were exposed to X-ray film (Kodak) and developed after 24–48 h. The same protocol was used to determine the expression of Mnk1, with the exception that a 3’-untranslated region *NcoI*–*EcoRV* fragment was used to generate the probe.

GST purifications

The *E. coli* strain TG1 was transformed with the appropriate plasmids, either pGEX3X or pGEX3X encoding a fusion of GST with the polypeptide of interest. An appropriate culture volume (100 ml to 1 l) was grown in the presence of 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cultures were resuspended in 8 ml of lysis buffer (0.14 M NaCl, 0.0027 M KCl, 0.0015 M KH₂PO₄, 0.0065 M Na₂HPO₄, 0.0025 M EDTA, 1% Triton X-100, 1 μ g/ml lysozyme) and sonicated. Particulate matter was removed by centrifugation. To the supernatant, we added an appropriate volume of glutathione–Sepharose (Pharmacia), typically 0.5 ml bead volume. After the beads were allowed to bind, they were washed four times with lysis buffer.

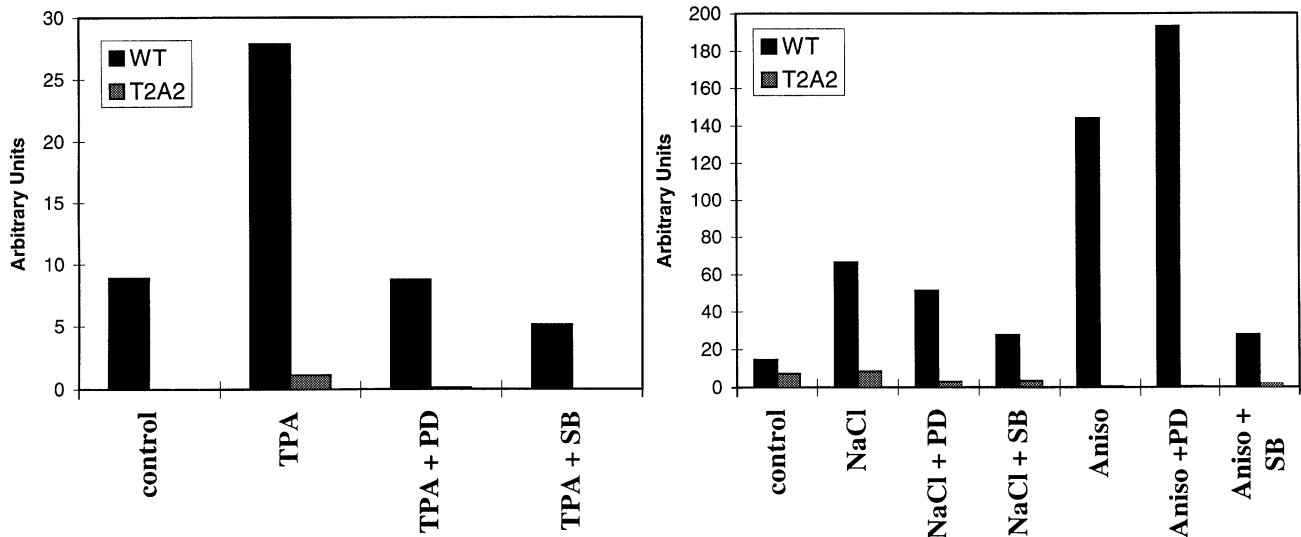


Fig. 9. Mnk1 kinase activity is activated in cells stimulated with either mitogens or stresses. Shown on the left is the activity of Mnk1 expressed in 293 cells stimulated with phorbol ester (TPA). The activity of wild-type (WT) and Mnk1-T197A,T202A (T2A2) were calculated using a phosphoimager. On the right, 293 cells expressing Mnk1 were treated with environmental stress, either 0.4 M NaCl or anisomycin. To dissect the requirements for mitogen- and stress-dependent activation of Mnk1, cells were also pre-treated for 60 min with 50 μ M PD098059 (PD) or 120 min with 30 μ M SB203580 (SB).

From mammalian cells, GST fusion proteins were generated subsequent to pEBG3X transient transfections. 293T and NIH 3T3 cells were lysed 48–72 h post-transfection in a Triton lysis buffer (1% Triton X-100, 50 mM NaF, 10 mM HEPES, 2 mM EDTA, 2 mM sodium orthovanadate, 0.1% β -mercaptoethanol, 0.002 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Cell membranes and unlysed cells were removed by centrifugation for 30 min at 14 000 r.p.m. in a Sorvall SS-34 rotor. A sample from the resulting clarified supernatant was used as cell lysate for immunoblotting. To the remainder, an appropriate volume of glutathione–Sepharose was added (0.02–0.5 ml) and allowed to mix at 4°C for 1 h. The bead pellets were washed with either lysis buffer (for co-purification experiments) or lysis buffer containing 0.5 M LiCl (when Mnk kinase activity was measured).

In vitro association

Using the plasmids pSP64A-MT-Erk2, pSP64A-MT-Erk2(K57R), pSP64A-MT-Erk2(TY/VF), pCS2-Jnk1, pCS2-p38 and pT7-Erk2, proteins were synthesized using a coupled *in vitro* transcription and translation reaction (TnT, Promega). Reactions were carried out according to the manufacturer's specifications.

Binding reactions were based on a published protocol (Keegan and Cooper, 1996). Briefly, ~1 μ g of GST fusion protein was mixed with 1/5 of a translation product reaction (3 μ l out of 14 μ l). Then 0.5 ml of Triton lysis buffer was added and the binding reaction was mixed at 4°C for 1 h. Subsequently, 20 ml of glutathione–Sepharose was added as carrier and the reactions were centrifuged and washed three times with lysis buffer. Electrophoretic sample buffer (2.5 mM EDTA, 2% SDS, 2.8 M β -mercaptoethanol, 10% glycerol, 100 mM Tris–HCl, 0.01% bromophenol blue, pH 6.0) was added. The reactions were boiled, separated by SDS–PAGE and visualized by autoradiography.

Cell culture and transient transfection

293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v/v*) fetal bovine serum (Gemini). NIH 3T3 cells (generous gift of C.Der) were grown in DMEM supplemented with 10% (*v/v*) calf serum (Hyclone).

For transient transfections, 4×10^5 293T cells were split onto a 60 mm dish 18–24 h prior to adding calcium phosphate precipitates. Alternatively, 5×10^5 NIH 3T3 cells were split prior to lipofectamine (Gibco-BRL) transfection. Five and 10 μ g of DNA were added to the cells for lipofection and CaPO₄ transfection, respectively. At 48 h after the addition of DNA, cells were quiesced by resuspension in serum-free medium; 72 h after the addition of DNA, cells were stimulated with the appropriate agonist, harvested and lysed as described elsewhere.

SDS–PAGE and immunoblotting

Glutathione–Sepharose-purified proteins were resuspended in electrophoretic sample buffer (2.5 mM EDTA, 2% SDS, 2.8 M β -mercaptoethanol, 10% glycerol, 100 mM Tris–HCl, 0.01% bromophenol blue, pH 6.0) and separated on 15% acrylamide, 0.87% bis-acrylamide SDS gels.

Gels were either dried or electroblotted onto Immobilon-P (Millipore). Filters were incubated for 1 h in block (150 mM NaCl, 10 mM Tris–HCl, 1% BSA, 1% ovalbumin, 0.005% Na₂S₂O₈, 0.05% polyoxyethylenesorbitan monolaureate, pH 7.5). Immunoblots were performed using a 1:10 000 dilution of 38.4 (a rabbit polyclonal antiserum which recognizes GST), 1913.3 (a rabbit polyclonal anti-C-terminal peptide Erk2 antiserum) or α p38 (a rabbit polyclonal antiserum against p38). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (BioRad) allowed the detection of proteins using enhanced chemiluminescence (Amersham).

In vitro activation of Mnk2 and Mnk1

Approximately 40 μ g of either Mnk1 or Mnk2 GST fusion proteins purified from *E.coli* were incubated for 2 h at 30°C in buffer (20 mM HEPES, 1 mM dithiothreitol, 10 mM MgCl₂, pH 7.4) containing 400 μ M ATP and 0.7 μ g of Erk1 or GST–p38.

Following incubation, GST–Mnk1 and GST–Mnk2 were washed twice with 0.5 M LiCl to remove excess ATP and bound Erk. The washed beads were resuspended in freezing buffer (100 mM NaCl, 1 mM PIPES, 1% aprotinin, 10% glycerol, pH 7.0) and stored at –70°C prior to assaying their activity.

Kinase assays

GST fusion proteins were assayed for activity by adding 1 μ g of purified eIF-4E to the appropriately purified GST–bead mixture. Recombinant eIF-4E was expressed in *E.coli* and purified by chromatography on

7-methyl-guanosine–Sepharose essentially as described previously (Stern *et al.*, 1993). Kinase–substrate reactions were carried out at 30°C in the presence of kinase buffer (20 mM HEPES, 10 mM MgCl₂, 10 mM β -glycerophosphate, 25 μ M ATP, 10 μ Ci [γ -³²P]ATP, pH 7.4). Reactions were separated by SDS–PAGE and visualized by autoradiography. eIF-4E phosphorylation was quantified with a Molecular Dynamics PhosphorImager.

Phosphorylation site analysis

Recombinant eIF-4E was labeled *in vitro* using Erk-activated Mnk1. Labeled 4E was then purified by SDS–PAGE. The labeled protein was analyzed as described (Boyle *et al.*, 1991; Flynn and Proud, 1995). Specifically, peptides were electrophoresed in pH 1.9 buffer and chromatographed in phospho chromatography buffer.

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References

- Alvarez,E., Northwood,I.C., Gonzalez,E.A., Latour,D.A., Seth,A., Abate,C. and Curran,T. (1991) Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate phosphorylation. Characterization of the phosphorylation of c-myc and c-jun proteins by an epidermal growth factor receptor threonine 669 kinase. *J. Biol. Chem.*, **266**, 15277–15285.
- Bartel,P., Chien,C., Sternglanz,R. and Fields,S. (1993a) In Hartley,D.A. (ed.), *Cellular Interactions in Development: A Practical Approach*. Oxford University Press, Oxford, UK, pp. 153–179.
- Bartel,P., Chien,C., Sternglanz,R. and Fields,S. (1993b) Elimination of false positives that arose in using the 2-hybrid system. *Biotechniques*, **14**, 920–924.
- Ben-Levy,R., Leighton,I.A., Doza,Y.N., Attwood,P., Morrice,N., Marshall,C.J. and Cohen,P. (1995) Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. *EMBO J.*, **14**, 5920–5930.
- Bohm,M., Moellmann,G., Cheng,E., Alvarez-Franco,M., Wagner,S., Sassone-Corsi,P. and Halaban,R. (1995) Identification of p90^{RSK} as the probable CREB-Ser¹³³ kinase in human melanocytes. *Cell Growth Differ.*, **6**, 291–302.
- Boyle,W.J., Van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phospho amino acid analysis by two-dimensional separation on thin layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.
- Chen,M. and Cooper,J.A. (1995) Ser-3 is important for regulating Mos interaction with and stimulation of mitogen-activated protein kinase kinase. *Mol. Cell. Biol.*, **15**, 4727–4734.
- Chen,R.-H., Sarnecki,C. and Blenis,J. (1992) Nuclear localization and regulation of erk and rsk-encoded protein kinases. *Mol. Cell. Biol.*, **12**, 915–927.
- Chen,R.H., Abate,C. and Blenis,J. (1993) Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc. Natl Acad. Sci. USA*, **90**, 10952–10956.
- Chien,C., Bartel,P.L., Sternglanz,R. and Fields,S. (1991) The two-hybrid system: a method to identify proteins encoded by a plasmid library that interact with a protein of interest. *Proc. Natl Acad. Sci. USA*, **88**, 9578–9582.
- Clark-Lewis,I., Sanghera,J.S. and Pelech,S.L. (1991) Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem.*, **266**, 15180–15184.
- Davis,R.J. (1993) The mitogen-activated protein kinase pathway. *J. Biol. Chem.*, **268**, 14553–14556.

- Derijard,B., Hibi,M., Wu,I.-H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1037.
- Derijard,B., Raingeaud,J., Barrett,T., Wu,I.-H., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, **267**, 682–685.
- Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J. and Saltiel,A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **92**, 7686–7689.
- Flynn,A. and Proud,C.G. (1995) Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells. *J. Biol. Chem.*, **270**, 21684–21688.
- Flynn,A. and Proud,C.G. (1996a) The role of eIF4 in cell proliferation. *Cancer Surv.*, **27**, 293–310.
- Flynn,A. and Proud,C.G. (1996b) Insulin stimulation of the phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway. *FEBS Lett.*, **389**, 162–166.
- Flynn,A. and Proud,C.G. (1996c) Insulin and phorbol ester stimulate eIF-4E phosphorylation by distinct pathways in Chinese hamster ovary cells overexpressing the insulin receptor. *Eur. J. Biochem.*, **236**, 40–47.
- Frederickson,R.M., Mushynski,W.E. and Sonenberg,N. (1992) Phosphorylation of translation initiation factor eIF-4E is induced in a ras-dependent manner during nerve growth factor-mediated PC12 cell differentiation. *Mol. Cell. Biol.*, **12**, 1239–1247.
- Freshney,N.W., Rawlinson,L., Guesdon,F. and Jones,E. (1994) Interleukin-1 activates a novel protein kinase cascade which results in the phosphorylation of hsp27. *Cell*, **78**, 1039–1049.
- Fukunaga,R. and Hunter,T. (1997) Mnk1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.*, **16**, 1921–1933.
- Galcheva-Gargova,Z., Derijard,B., Wu,I.-H. and Davis,R.J. (1994) An osmosensing signal transduction pathway in mammalian cells. *Science*, **265**, 806–808.
- Gille,H., Kortenjann,M., Thomae,O., Moomaw,C., Slaughter,C., Cobb,M.H. and Shaw,P.E. (1995) ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.*, **14**, 951–962.
- Ginty,D.D., Bonni,A. and Greenberg,M.E. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell*, **77**, 713–725.
- Gupta,S., Campbell,D., Derijard,B. and Davis,R.J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*, **267**, 389–393.
- Han,J., Lee,J.-D., Bibbs,L. and Ulevitch,R.J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, **265**, 808–811.
- Hanks,S., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.
- Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, **7**, 2135–2148.
- Hollenberg,S.M., Sternglanz,R., Cheng,P.F. and Weintraub,H. (1995) Identification of a new family of tissue-specific basic helix–loop–helix proteins with a two-hybrid system. *Mol. Cell. Biol.*, **15**, 3813–3822.
- Hsiao,K.M., Chou,S.Y., Shih,S.J. and Ferrell,J.E. (1994) Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer. *Proc. Natl Acad. Sci. USA*, **91**, 5480–5484.
- Huang,C.-Y.F. and Ferrell,J.E. (1996) Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **93**, 10078–10083.
- Jiang,Y., Chen,C., Li,Z., Guo,W., Gegner,J.A., Lin,S. and Han,J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J. Biol. Chem.*, **271**, 17290–17296.
- Johnson,L.N., Noble,M.E.M. and Owen,D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell*, **85**, 149–158.
- Joshi,B., Cai,A.L., Keiper,B.D., Minich,W.B., Mendez,R., Beach,C.M., Stolarski,R., Darzynkiewicz,E. and Rhoads,R.E. (1995) Phosphorylation of eukaryotic protein synthesis initiation factor 4E at Serine 209. *J. Biol. Chem.*, **270**, 14597–14603.
- Kallunki,T., Su,B., Tsigelny,I., Sluss,H.K., Derijard,B., Moore,G., Davis,R.J. and Karin,M. (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.*, **8**, 2996–3007.
- Keegan,K. and Cooper,J.A. (1996) Use of the two-hybrid system to detect the association of the protein-tyrosine phosphatase, SHPTP2, with another SH2-containing protein, Grb7. *Oncogene*, **12**, 1537–1544.
- Kyriakis,J.M. and Avruch,J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.*, **271**, 24313–24316.
- Kyriakis,J.M., Banerjee,P., Nikolaki,E., Dai,T., Rubie,E.A., Ahmad,M.F., Avruch,J. and Woodgett,J.R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156–160.
- Lee,J.C. et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.
- Lin,L.L., Wartmann,M., Lin,A.Y., Knopf,J.L., Seth,A. and Davis,R.J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell*, **72**, 269–278.
- Makkinje,A., Xiong,H., Li,M. and Damuni,Z. (1995) Phosphorylation of eukaryotic protein synthesis initiation factor by insulin stimulated protamine kinase. *J. Biol. Chem.*, **270**, 14824–14828.
- Marais,R., Wynne,J. and Treisman,R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell*, **73**, 381–393.
- Marshall,C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.
- Marshall,C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179–185.
- McLaughlin,M.M., Kumar,S., McDonnell,P.C., Van Horn,S., Lee,J.C., Livi,G.P. and Young,P.R. (1996) Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J. Biol. Chem.*, **271**, 8488–8492.
- Minich,W.B., Balasta,M.L., Goss,D.J. and Rhoads,R.E. (1994) Chromatographic resolution of *in vivo* phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form. *Proc. Natl Acad. Sci. USA*, **91**, 7668–7672.
- Posada,J. and Cooper,J.A. (1992) Requirements for phosphorylation of MAP kinase during meiosis in *Xenopus* oocytes. *Science*, **255**, 212–215.
- Pulverer,B.J., Kyriakis,J.M., Avruch,J., Nikolakaki,E. and Woodgett,J.R. (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature*, **353**, 670–674.
- Raingeaud,J., Whitmarsh,A.J., Barrett,T., Derijard,B. and Davis,R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.*, **16**, 1247–1255.
- Rao,V.N. and Reddy,E.S. (1994) Elk-1 proteins interact with MAP kinases. *Oncogene*, **9**, 1855–1860.
- Rinker-Schaeffer,C.W., Austin,V., Zimmer,S. and Rhoads,R.E. (1992) Ras transformation of cloned rat embryo fibroblasts results in increased rates of protein synthesis and phosphorylation of eukaryotic initiation factor 4E. *J. Biol. Chem.*, **267**, 10659–10664.
- Rivera,V.M., Miranti,C.K., Misra,R.P., Ginty,D.D., Chen,R.H., Blenis,J. and Greenberg,M.E. (1993) A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. *Mol. Cell. Biol.*, **13**, 6260–6273.
- Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) A novel kinase cascade triggered by chemical stress and heat shock which stimulates MAP kinase-activated protein kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, **78**, 1027–1037.
- Sanchez,I., Hughes,R.T., Mayer,B.J., Yee,K., Woodgett,J.R., Avruch,J., Kyriakis,J.M. and Zon,L.I. (1994) Role of SAPK/ERK kinase-1 in stress-activated pathway regulating transcription of c-Jun. *Nature*, **372**, 794–798.
- Scimeca,J.C., Nguyen,T.T., Filloux,C. and VanObberghen,E. (1992) Nerve growth factor-induced phosphorylation cascade in PC12 pheochromocytoma cells. Association of S6 kinase II with microtubule-associated protein kinase, ERK1. *J. Biol. Chem.*, **267**, 17369–17374.
- Seeger,R. et al. (1994) Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. *J. Biol. Chem.*, **269**, 25699–25709.
- Shibuya,E.K. and Ruderman,J.V. (1993) Mos induces the *in vitro* activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. *Mol. Biol. Cell*, **4**, 781–790.
- Sithanandam,G. et al. (1996) 3pK, a new mitogen-activated protein kinase-activated protein kinase located in the small cell lung cancer tumor suppressor gene region. *Mol. Cell. Biol.*, **16**, 868–876.

- Sonenberg,N. (1994) Regulation of translation and cell growth by eIF-4E. *Biochimie*, **76**, 839–846.
- Stern,B.D., Wilson,M. and Jagus,R. (1993) Use of nonreducing SDS–PAGE for monitoring renaturation of recombinant protein synthesis initiation factor, eIF4 alpha. *Protein Expr. Purif.*, **4**, 320–327.
- Stokoe,D., Campbell,D.G., Nakielny,S., Hidaka,H., Leever,S.J., Marshall,C.J. and Cohen,P. (1992a) MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.*, **11**, 3985–3994.
- Stokoe,D., Engel,K., Campbell,D.G., Cohen,P. and Gaestel,M. (1992b) Identification of MAPKAP kinase-2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett.*, **364**, 223–228.
- Sturgill,T.W., Ray,L.B., Erickson,E. and Maller,J.L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature*, **334**, 715–718.
- Takahima,K., Griswold-Prenner,I., Ingebritsen,T. and Rosner,M.R. (1991) Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated 'MAP' kinase. *Proc. Natl Acad. Sci. USA*, **88**, 2520–2524.
- Tan,Y., Rouse,J., Zhang,A., Cariati,S., Cohen,P. and Comb,M.J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.*, **15**, 4629–4642.
- Turner,D.L. and Weintraub,H. (1995) Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.*, **8**, 1434–1447.
- Vojtek,A.B., Hollenberg,S.M. and Cooper,J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell*, **74**, 205–214.
- Waskiewicz,A.J. and Cooper,J.A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeasts. *Curr. Opin. Cell Biol.*, **7**, 798–805.
- Whalen,S.G., Gingras,A.C., Amankwa,L., Mader,S., Branton,P.E., Aebersold,R. and Sonenberg,N. (1996) Phosphorylation of eIF-4E on Ser209 by protein kinase C is inhibited by the translation repressors, 4E-binding proteins. *J. Biol. Chem.*, **271**, 11831–11837.
- Whitmarsh,A.J., Shore,P., Sharrocks,A.D. and Davis,R.J. (1995) Integration of MAP kinase signal transduction at the serum response element. *Science*, **269**, 403–407.
- Xing,J., Ginty,D.D. and Greenberg,M.E. (1996) Coupling of the RAS–MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science*, **273**, 959–963.
- Zhao,Y., Bjorbaek,C., Weremowicz,S., Morton,C. and Moller,D.E. (1995) RSK3 encodes a novel pp90^{sk} isoform with a unique N-terminal sequence: growth factor stimulated kinase function and nuclear translocation. *Mol. Cell. Biol.*, **15**, 4353–4363.
- Zinck,R., Cahill,M.A., Kracht,M., Sachsenmaier,C., Hipskind,R.A. and Nordheim,A. (1995) Protein synthesis inhibitors reveal differential regulation of MAPK and SAPK pathways that converge on Elk-1. *Mol. Cell. Biol.*, **15**, 4930–4938.

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