The EMBO Journal Vol.16 No.8 pp.1921–1933, 1997

MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates

Rikiro Fukunaga[1] and Tony Hunter[2]

Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

[1]Present address: Department of Genetics, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan

[2]Corresponding author
e-mail: hunter@salk.edu

We have developed a novel expression screening method for identifying protein kinase substrates. In this method, a λ phage cDNA expression library is screened by *in situ*, solid-phase phosphorylation using purified protein kinase and [γ-^{32}P]ATP. Screening a HeLa cDNA library with ERK1 MAP kinase yielded cDNAs of previously characterized ERK substrates, c-Myc and p90^{RSK}, demonstrating the utility of this method for identifying physiological protein kinase substrates. A novel clone isolated in this screen, designated MNK1, encodes a protein-serine/threonine kinase, which is most similar to MAP kinase-activated protein kinase 2 (MAPKAP-K2), 3pK/MAPKAP-K3 and p90^{RSK}. Bacterially expressed MNK1 was phosphorylated and activated *in vitro* by ERK1 and p38 MAP kinases but not by JNK/SAPK. Further, MNK1 was activated upon stimulation of HeLa cells with 12-*O*-tetradecanoylphorbol-13-acetate, fetal calf serum, anisomycin, UV irradiation, tumor necrosis factor-α, interleukin-1β or osmotic shock, and the activation by these stimuli was differentially inhibited by the MEK inhibitor PD098059 or the p38 MAP kinase inhibitor SB202190. Together, these results indicate that MNK1 is a novel class of protein kinase that is activated through both the ERK and p38 MAP kinase signaling pathways.

Keywords: expression cloning/MAP kinase/ phosphorylation/protein kinase/signal transduction

Introduction

A huge variety of protein kinases are involved in intracellular signal transduction leading to cell growth, differentiation and oncogenesis (Hunter, 1994; Hanks and Hunter, 1995). To elucidate these signal transduction pathways and how they are regulated, it is necessary to know the physiological substrates for individual protein kinases. Although the identification of physiological targets has been a high priority ever since the first protein kinase was purified, the conventional approach of purifying substrate proteins by biochemical techniques is a laborious and time-consuming task and is difficult in the case of scarce proteins. Recently, several new methods have been developed to identify protein kinase substrates. Interaction screens using phosphotyrosine-containing protein/peptide probes have identified novel proteins bearing phosphotyrosine-binding domains, some of which proved to be substrates for protein-tyrosine kinases (Skolnik *et al.*, 1991). Songyang and co-workers developed a technique for determining the preferred primary consensus sequence for a protein kinase by using a degenerate, oriented peptide library (Songyang *et al.*, 1994). Model experiments using several protein kinases revealed that optimal substrate sequences deduced by this method are consistent with the known substrate specificity (Songyang *et al.*, 1994). Although this method does not directly identify substrate proteins, potential substrates can be predicted by searching protein sequence data banks with the consensus sequence. There are also several reports of immunoscreening of phage expression libraries with antibodies that specifically recognize a group of phosphoproteins such as proteins phosphorylated upon epidermal growth factor (EGF) stimulation (Fazioli *et al.*, 1993) and M-phase proteins containing a phosphoamino acid-containing epitope (Westendorf *et al.*, 1994; Matsumoto-Taniura *et al.*, 1996). In addition, interaction cloning by the yeast two-hybrid system is a useful method for identifying potential protein kinase substrates (Yang *et al.*, 1992). In this study, we tested a more direct strategy, named phosphorylation screening, for the identification of protein kinase substrates. This method uses a protein-expressing phage cDNA library (Young and Davis, 1983) and is based on the fact that cellular proteins immobilized on a membrane filter can be phosphorylated by a soluble protein kinase with specificity similar to that obtained in conventional liquid-phase phosphorylation (Valtorta *et al.*, 1986).

To evaluate the efficacy of this new approach, we have applied it to the ERK1 MAP kinase. Mitogen-activated protein kinases (MAP kinases) are activated by a broad spectrum of extracellular signals, and play important roles in intracellular signal transduction pathways leading to gene induction (reviewed in Davis, 1993; Cooper, 1994; Cano and Mahadevan, 1995; Karin and Hunter, 1995). In mammals, three main classes of MAP kinase are recognized. In addition to the 'classic' MAP kinase, now known as the ERK (extracellular signal-regulated kinase) MAP kinases, two other distinct classes of MAP kinase, the JNK/SAPK (c-Jun N-terminal kinase or stress-activated protein kinase) (Cooper, 1994; Sanchez *et al.*, 1994; Cano and Mahadevan, 1995; Dérijard *et al.*, 1995; Lin *et al.*, 1995; Mohit *et al.*, 1995; Gupta *et al.*, 1996) and the p38/RK/p40/CSBP/Hog1/Mxi2 (Freshney *et al.*, 1994; Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994; Zervos *et al.*, 1995; Jiang *et al.*, 1996) MAP kinases have been identified. All these MAP kinases are activated by dual threonine/tyrosine phosphorylation of residues in the activation loop catalyzed by a specific MAP kinase kinase (MEK1, MEK2, SEK1/MKK4/JNKK, MKK3, MKK6/

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MEK6) (Cooper, 1994; Sanchez *et al.*, 1994; Cano and Mahadevan, 1995; Dérijard *et al.*, 1995; Lin *et al.*, 1995; Han *et al.*, 1996; Moriguchi *et al.*, 1996; Stein *et al.*, 1996), and the activated MAP kinases in turn phosphorylate various target proteins at Ser/Thr–Pro sequence. Although these MAP kinases have similar primary structures, activation mechanisms and the same minimal consensus sequence for substrate recognition, recent studies have indicated that the distinct classes of MAP kinase are activated differentially depending on extracellular stimulation, and in turn activate shared or specific downstream targets. ERK1 and ERK2 are the best characterized MAP kinases so far in terms of the signaling pathway involved in their activation and the identification of physiological targets. ERK MAP kinases are activated upon stimulation of cells by phorbol esters, which activate protein kinase C, and by growth factors such as EGF and platelet-derived growth factor, which activate receptor protein-tyrosine kinases. In the case of the growth factor receptor protein-tyrosine kinases, this leads to the sequential activation of Ras, Raf, MEK and the ERK1 and ERK2 MAP kinases (Davis, 1993). Several substrate proteins have been identified as physiological targets for ERK MAP kinases, including the 90 kDa ribosomal S6 protein kinase called RSK, cytosolic phospholipase A_2 and several transcription factors, including c-Myc, Elk-1, NF-IL6/C/EBPβ/NF-M, Tal-1, Ets-2 and possibly STAT proteins (Davis, 1993; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Kowenz-Leutz *et al.*, 1994; Wadman *et al.*, 1994; Gille *et al.*, 1995a; Wen *et al.*, 1995; Bunone *et al.*, 1996; Yang *et al.*, 1996). Based on peptide phosphorylation studies, Pro–X–Ser/Thr–Pro has been proposed as a consensus sequence for sites phosphorylated by ERK, with the proline at the +1 position being absolutely required and the proline at –2 being preferred but not essential (Davis, 1993). A similar consensus, Gly–Pro–X–Ser/Thr–Pro, was obtained for ERK1 using the oriented peptide library approach (Songyang *et al.*, 1996).

Here, we report a novel protein kinase substrate identification strategy that utilizes *in situ* phosphorylation screening, which we show is practically useful for the identification of substrates for a purified protein kinase. In a screen using ERK1 MAP kinase, we identified a cDNA clone which encodes a new class of MAP kinase-activated protein kinase. This protein kinase was phosphorylated and activated *in vivo* and *in vitro* by two distinct MAP kinases, ERK and p38, and is therefore designated as MNK1 (MAP kinase signal-integrating kinase).

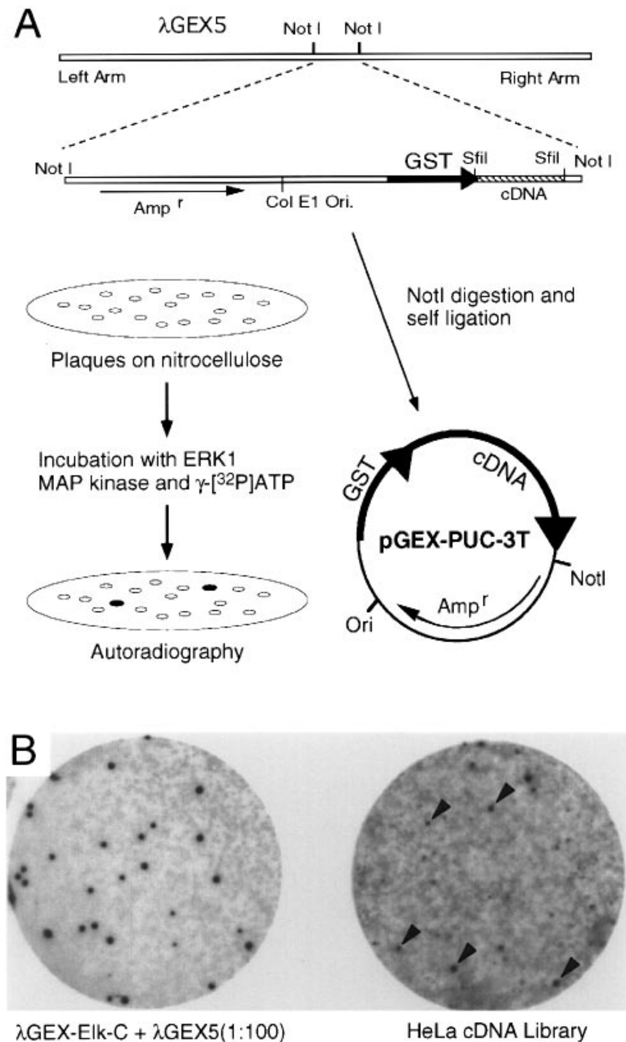

Fig. 1. Phosphorylation screening for cloning of protein kinase substrates. (**A**) Outline of the screening method. A HeLa cDNA library was constructed with a λ phage vector λGEX5 which contains plasmid sequences of a ColE1 origin, an ampicillin resistance gene and a GST gene between the two *Not*I sites. Recombinant proteins expressed in phage plaques were immobilized on nitrocellulose filters, which were then incubated with purified ERK1 MAP kinase in the presence of [γ-^{32}P]ATP as described in Materials and methods. Phosphorylated plaques were visualized by autoradiography. After plaque purification, cDNAs of interest were rescued as GST fusion protein-expressing plasmids (pGEX-PUC-3T) by *Not*I digestion of the phage DNA followed by self-ligation. (**B**) Autoradiograms of phosphorylation screening assay. Left panel: a mixture (1:100) of a positive control phage (λGEX-Elk-C) and a negative control (λGEX5) were plated at a density of 4000 plaques per 100 mm plate. Right panel: primary screen of the HeLa cDNA library at the same density. The arrowheads indicate positive clones that were confirmed after the second screening.

Results

Phosphorylation screening of a phage expression library

As the initial step in developing the new screening method, we constructed a phage expression vector, λGEX5, which has a PUC-derived plasmid sequence and a GST expression cassette (Figure 1A). Inserted cDNAs can be expressed as GST fusion proteins and immobilized on nitrocellulose filters. To test the feasibility of phosphorylation screening, a λGEX5 recombinant, which encodes the C-terminal region of the Elk-1 transcription factor (GST–Elk-C) containing multiple ERK phosphorylation sites (Marais *et al.*, 1993), was plated, and plaque proteins were transferred to nitrocellulose filters. The filters were blocked by treatment with bovine serum albumin (BSA) and then incubated with purified ERK1 MAP kinase in the presence of [γ-^{32}P]ATP to allow solid-phase phosphorylation. As shown in Figure 1B, the λGEX-Elk-C phage plaques gave a strong phosphorylation signal, whereas the background derived from phosphorylation of λ phage or *Escherichia coli* proteins was low.

Using the conditions developed with λGEX-Elk-C, we

Table I. Summary of expression screening for ERK1 substrates

Clone	Size of cDNA (kb)	Size of protein (kDa)[a]	Identity/homology
S3	1.1	30	$p90^{RSK2}$
S9	2.0	80	ZNF7
S16	2.0	4	$p90^{RSK2}$
S18	1.0	20	HSF-1
S20	1.7	59	c-Myc
S64	1.2	19	topoisomerase II-β
S115	1.8	40	RalGDS
S4	1.8	42	GTP-binding protein?
S101	1.6	15	protein kinase?
23 clones			no homology

[a]Sizes of recombinant proteins indicate their apparent molecular weights excluding the GST region.

screened a HeLa cDNA library of 3×10^5 independent clones with ERK1 (Figure 1B), and isolated 120 positive clones. Those cDNAs were rescued as expression plasmids, and their cognate proteins were expressed in *E.coli*, purified by glutathione–agarose and tested for phosphorylation by ERK1. Although almost all of the recombinant products were phosphorylated by ERK1 *in vitro* (data not shown), more than half of them produced GST proteins with a very short tail, which seemed to be artificial products derived from out-of-frame ligations. Therefore, we selected 32 clones that produced GST proteins with a fusion partner of >5 kDa, and determined their N-terminal amino acid sequences by DNA sequencing. The DNA and amino acid sequences were tested for identity/homology by searching with BLAST on the National Center for Biotechnology Information file server. Seven clones out of the 32 corresponded to fragments of structurally known proteins, including two already characterized ERK substrates, $p90^{RSK2}$ and c-Myc (Table I). Two independent clones of $p90^{RSK2}$ were obtained. Other clones were heat shock transcription factor-1 (HSF1) (Rabindran *et al.*, 1991), topoisomerase II-β (Jenkins *et al.*, 1992), the guanine nucleotide dissociation stimulator for the small G protein Ral (RalGDS) (Albright *et al.*, 1993) and a zinc finger protein called ZNF7 (Lania *et al.*, 1990). The amino acid sequences of all of these proteins contained possible MAP kinase recognition sequences, Ser–Pro or Thr–Pro. Two cDNAs were novel, but were related to known proteins, one of which (clone S4) showed sequence homology to GTP-binding proteins. The second clone, S101, showed a significant similarity to the C-terminal region of some protein kinases, and therefore we isolated and characterized full-length S101 cDNAs.

Structure of the S101 protein kinase, MNK1

Two independent clones (S101-17 and -19) isolated by hybridization with the original S101 cDNA (S101-0) probe were both 2.6 kb in length, and had identical sequences except for their 5′ ends. They encode an open reading frame of 424 amino acids with a calculated M_r of 47 372 (Figure 2A). Although there is no in-frame termination codon in the 5′ sequence upstream of the AUG codon at nucleotide 188, transient expression of the S101-17 cDNA in HeLa cells revealed that it encodes a protein whose size is identical to that of the endogenous gene product (see below and Figure 5), indicating that the S101-17 cDNA contains the entire coding region of the gene. A homology search using BLAST revealed that the S101 product, designated MNK1, contains a sequence typical of the catalytic domain of protein-Ser/Thr kinases (Figure 2A). The highest homology scores were obtained with the *Caenorhabditis elegans* putative protein kinase R166.5 (accession No. Z50795), human $p90^{RSK}$ (Moller *et al.*, 1994; Zhao *et al.*, 1995) and MAP kinase-activated protein kinase 2 (MAPKAP-K2) (Stokoe *et al.*, 1992, 1993; Engel *et al.*, 1993), whose kinase domains have 52, 36 and 34% amino acid identity to that of the MNK1 protein respectively (Figure 2B). The recently identified 3pK protein kinase/MAPKAP kinase-3, which is highly homologous to MAPKAP-K2 (McLaughlin *et al.*, 1996; Sithanandam *et al.*, 1996), also showed a similar identity score (33%) to MNK1. No obvious similarity was found in the N-terminal region beyond the catalytic domain, whereas the C-terminal region showed significant homology among these proteins. MAPKAP-K2 has a putative bipartite nuclear localization signal sequence in the C-terminal region (Engel *et al.*, 1993; Stokoe *et al.*, 1993), whereas MNK1 contains a putative nuclear localization signal sequence (RRRKKKRR) in its N-terminal region (Figure 2A). Recently, Ben-Levy and co-workers identified phosphorylation sites required for MAPKAP-K2 activation (Ben-Levy *et al.*, 1995). Those sites are Thr222, Ser272 and Thr334, of which the two Thr residues are conserved in MNK1 as Thr214 and Thr344 respectively, suggesting that similar phosphorylation events might be involved in the activation mechanism of the two protein kinases. The original clone (S101-0) obtained by phosphorylation screening contained the C-terminal region of MNK1 starting at Phe284 (Figure 2A). Determination of the ERK1 phosphorylation sites in the S101-0 product [GST–MNK1(284–424), see Figure 3C], showed that one of the major sites phosphorylated by ERK1 *in vitro* was Thr344 (data not shown), which was likely to have been phosphorylated in the expression screening. Northern blot analysis of RNAs from human and mouse cell lines such as fibroblast, T- or B-lymphoid and myeloid cell lines showed that the MNK1 mRNA of ~3.0 kb is expressed in all cell lines examined (data not shown), suggesting that the MNK1 protein is ubiquitously expressed.

MNK1 is phosphorylated and activated in vitro by ERK MAP kinase

We tested whether MNK1 could be phosphorylated and activated by ERK MAP kinase. For this purpose, the MNK1 coding region (2–424) was expressed as a GST fusion protein in *E.coli* and purified by glutathione–agarose chromatography. The GST–MNK1 fusion protein was phosphorylated efficiently by ERK1 MAP kinase *in vitro* and, upon phosphorylation, showed an electrophoretic mobility shift on SDS–PAGE (Figure 3A). We measured the protein kinase activity of GST–MNK1 by using a peptide substrate (MK-1 peptide; KKLNRTLSVA), which is derived from the N-terminus of glycogen synthase and is known to be a good substrate for $p90^{RSK}$ and MAPKAP-K2 (Stokoe *et al.*, 1993). Although the untreated GST–MNK1 protein did not have any detectable protein kinase activity towards exogenous substrates nor any autophosphorylating activity, after phosphorylation by ERK1 protein phosphotransferase activity was increased

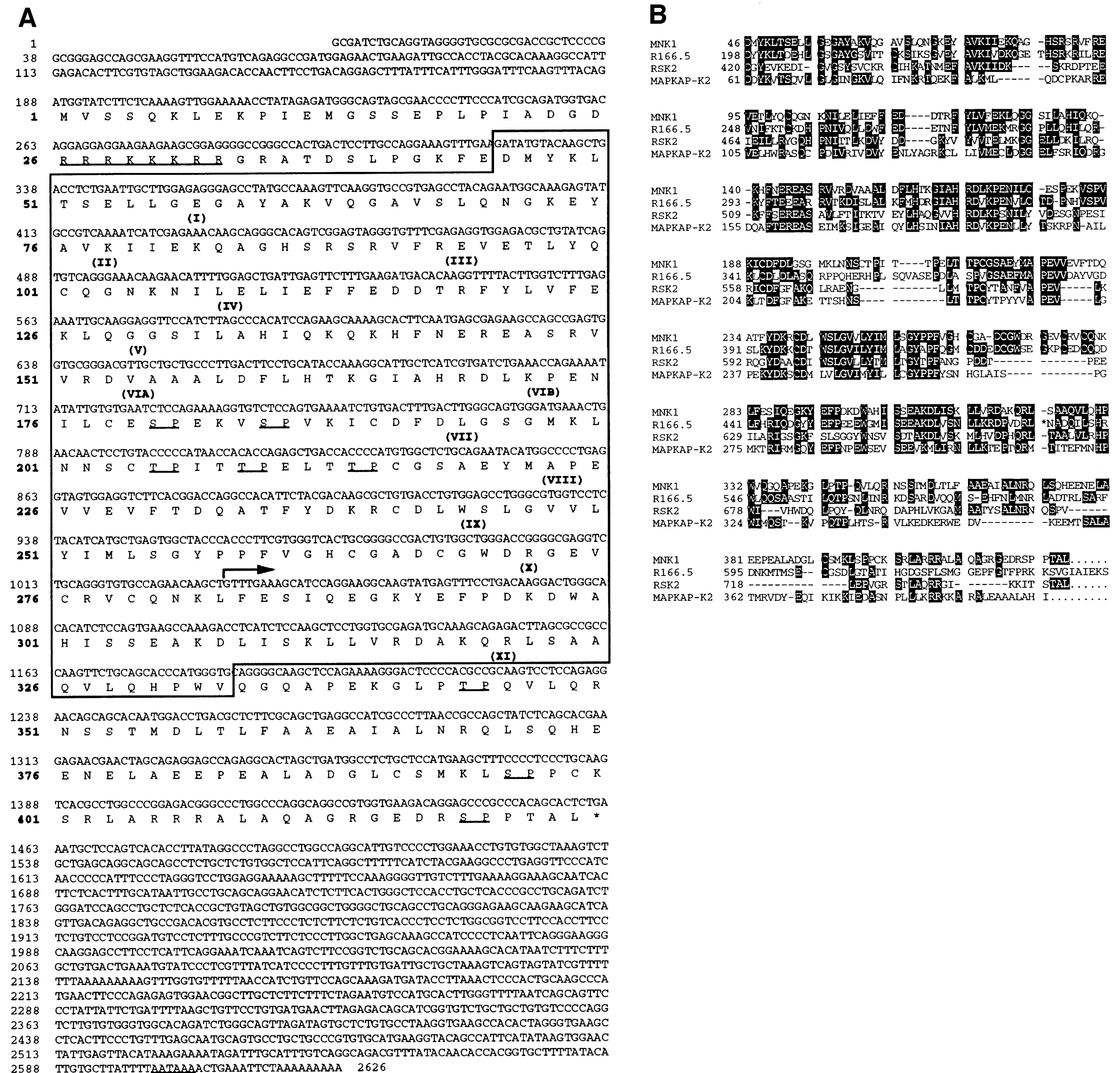

Fig. 2. cDNA and deduced amino acid sequences of human MNK1. (**A**) The S101-17 cDNA sequence is shown together with the predicted amino acid sequence of MNK1. The start point (nucleotide 1036) of the original S101-0 clone isolated by the phosphorylation screening is indicated by an arrow and a polyadenylation signal (AATAAA) is underlined. The protein kinase domain is boxed and its subdomains are shown in Roman numerals. A putative nuclear localization signal and potential MAP kinase phosphorylation sites are underlined. (**B**) A region containing the kinase and C-terminal domains of MNK1 is aligned with the *C.elegans* putative protein kinase R166.5, human p90^{RSK2} and human MAPKAP-K2. Residues conserved among more than two protein kinases are highlighted.

in a time-dependent manner (Figure 3B). On the other hand, a mutant protein (GST–MNK1-KN), which contains three point mutations (Lys78Arg, Gln100Arg and Ile107Thr), did not show detectable protein kinase activity even after extensive phosphorylation by ERK1 MAP kinase (Figure 3A and B). The wild-type GST–MNK1 gave many shifted bands upon phosphorylation by ERK1 but the mutant showed only one shifted band, suggesting that the activated GST–MNK1 has an autophosphorylating activity, which results in additional phosphorylations. We also found that myelin basic protein (MBP) can be phosphorylated on multiple serine residues by the activated MNK1.

To examine the roles of the N- and C-terminal regions lying outside the kinase domain, we constructed several MNK1 mutants and tested for phosphorylation by ERK1 and stimulation of protein kinase activity (Figure 3C). Deletion of the N-terminal 47 amino acid region did not affect phosphorylation or activation by ERK1, whereas deletion of the C-terminal 90 amino acids resulted in complete loss of ERK phosphorylation and kinase activation. GST–MNK1(284–424), which corresponds to the original clone isolated by phosphorylation screening, was phosphorylated very well by ERK. Tryptic phosphopeptide mapping showed that the phosphopeptides derived from GST–MNK1(284–424) were identical to the major

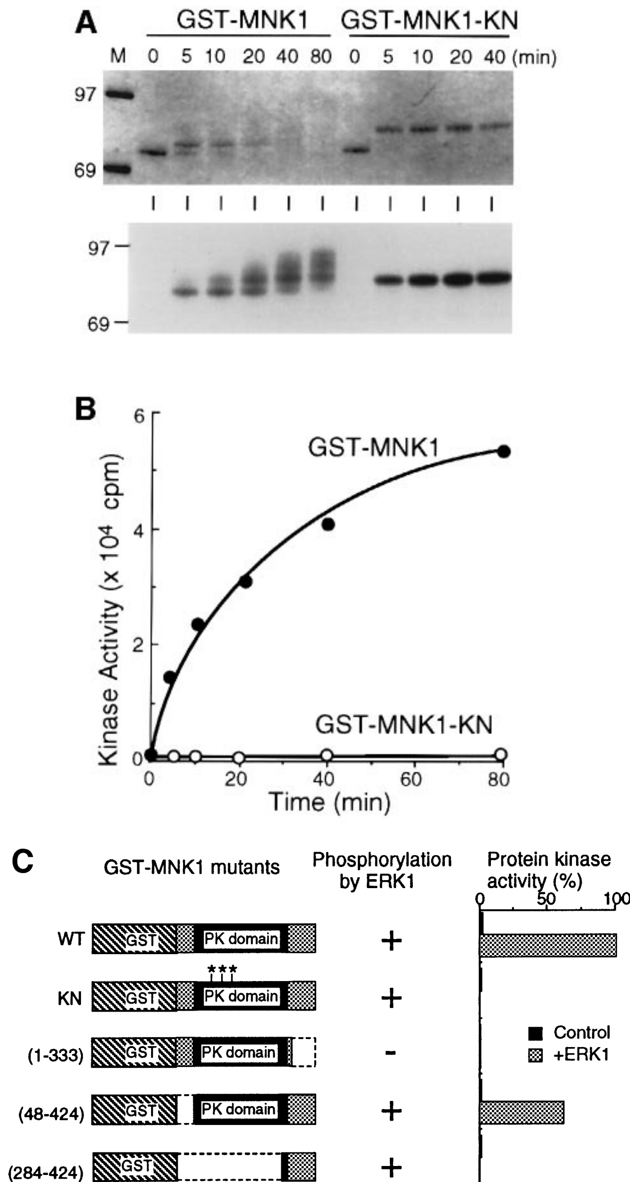

Fig. 3. Phosphorylation and activation of GST–MNK1 by ERK1 MAP kinase *in vitro*. (**A**) Time course of phosphorylation and mobility shift of GST–MNK1. Either GST–MNK1 or GST–MNK1-KN protein (~10 μg) on 70 μl of glutathione–agarose resin was incubated in 150 μl of the ERK kinase buffer with 0.6 μg of ERK1 in the presence of 0.4 mM [γ-^{32}P]ATP (0.5 Ci/mmol) at 30°C. At the indicated time, a portion (10 μl) was taken, resolved by SDS–PAGE and detected by Coomassie staining (top panel) or by autoradiography (bottom panel). The positions of protein size markers in kDa are shown on the left. (**B**) Time course of GST–MNK1 protein kinase activation. Either GST–MNK1 or GST–MNK1-KN protein bound to glutathione–agarose was phosphorylated by ERK1 as above in the presence of 0.4 mM unlabeled ATP. At the indicated time, 20 μl of suspension were taken and assayed for MNK1 kinase activity by using the MK-1 peptide as described in Materials and methods. (**C**) The wild-type GST–MNK1 (WT), the kinase-negative mutant (KN) and GST–MNK1 fusion proteins containing residues 1–333, 48–424 or 284–424 were produced in bacteria and purified by glutathione–agarose. Each protein was tested for phosphorylation by ERK1 and for protein kinase activity before (control) and after (+ERK1) phosphorylation by ERK1 as described in (A) and (B).

phosphopeptides from the full-length GST–MNK1 phosphorylated by ERK (data not shown), suggesting that the C-terminal region contains the primary ERK phosphorylation site(s) and is involved in the activation of the protein kinase.

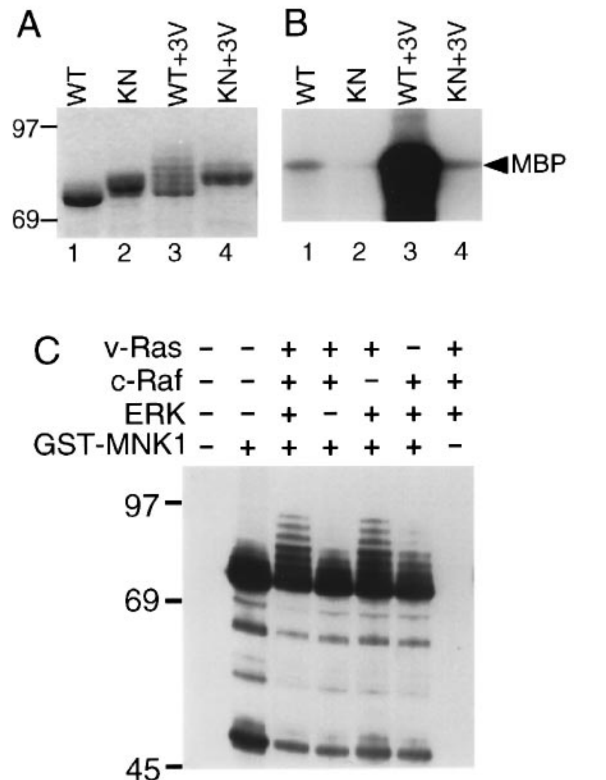

Fig. 4. Activation of GST–MNK1 in insect Sf9 cells by co-expression with ERK activators. (**A** and **B**) Sf9 cells were infected singly with a baculovirus encoding the wild-type GST–MNK1 (WT) or the kinase-negative mutant (KN), or co-infected with GST–MNK1 virus together with three baculoviruses encoding v-Ras, c-Raf and ERK1 (WT+3V or KN+3V). After incubation for 60 h, cells were lysed and GST–MNK1 proteins were purified using glutathione–agarose. GST–MNK1 proteins (4 μg) were analyzed on SDS–PAGE with Coomassie staining (A) and tested for protein kinase activity using MBP substrate as described in Materials and methods (B). (**C**) Sf9 cells were infected with the indicated combinations of baculoviruses of GST–MNK1, v-Ras, c-Raf and ERK1 and cultured for 60 h. Mobility shift of GST–MNK1 protein was analyzed by immunoblotting of the cell lysates using anti-MNK1 antiserum.

MNK1 is activated in insect cells by co-expression with active ERK MAP kinase

To assess the effect of the activation of the ERK signaling pathway on MNK1 activity, a GST–MNK1 protein was expressed in insect Sf9 cells using the baculovirus system (Williams *et al.*, 1993). GST–MNK1 expressed in Sf9 cells by single infection with the GST–MNK1 baculovirus was the same size as the bacterially produced GST–MNK1 and showed very weak kinase activity (Figure 4A and B). In contrast, co-infection with GST–MNK1, ERK1, c-Raf-1 and v-Ras baculoviruses resulted in a strong mobility shift and a dramatic increase in the kinase activity of GST–MNK1. The kinase-negative mutant (GST–MNK1-KN), which showed a small band shift even when infected singly, was not activated by co-infection with the upstream activators (Figure 4A and B). To test which component is required for the activation of GST–MNK1, Sf9 cells were infected with different baculovirus combinations and the expressed GST–MNK1 was analyzed by immunoblotting.

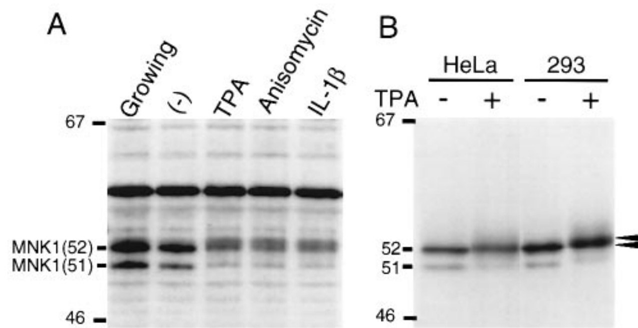

Fig. 5. Mobility shift of transiently expressed or endogenous MNK1 upon stimulation. (**A**) HeLa cells were transiently transfected with the unmodified S101-17 cDNA in pCMX vector and, after serum starvation, cells were stimulated with TPA, anisomycin or IL-1β for 15 min. Cell lysates (40 μg of protein) from growing cells, serum-starved but unstimulated cells (–), or stimulated cells were analyzed by immunoblotting using anti-MNK1 antiserum. (**B**) Detection of endogenous MNK1 in human cell lines. HeLa and 293 cells were serum starved for 16 h and left untreated (–) or stimulated with TPA for 15 min. MNK1 protein was precipitated with anti-MNK1 antibodies which had been affinity purified and covalently coupled to protein A–agarose. The immunoprecipitated MNK1 was detected by immunoblotting using anti-MNK1 antiserum.

As shown in Figure 4C, both ERK and v-Ras baculoviruses were necessary for the GST–MNK1 mobility shift, indicating that activated ERK is essential for the activation of GST–MNK1 in Sf9 cells. The fact that the GST–MNK1 mobility shift was observed in the absence of exogenous c-Raf expression implies that endogenous Raf and MEK are not rate-limiting for activation of exogenous ERK in this system (Williams *et al.*, 1993).

Multiple extracellular stimuli activate MNK1 through distinct MAP kinase pathways

Activation of MNK1 by phosphorylation was correlated with the electrophoretic mobility shift. To test whether MNK1 showed a mobility shift upon extracellular stimulation, a full-length MNK1 cDNA was transiently expressed by transfection in HeLa cells. Immunoblot analysis of cell lysates with an anti-MNK1 serum revealed MNK1 proteins of 52 and 51 kDa in both growing and serum-starved transfected HeLa cells (Figure 5A). Endogenous MNK1, which could be detected only by immunoblotting after immunoprecipitation, also showed two bands of the same sizes (Figure 5B). The 51 kDa band, which was less prominent in both cases, is likely to be a minor molecular species translated from an internal AUG codon 13 amino acids downstream of the major AUG codon (Figure 2A). Following stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), anisomycin or interleukin-1β (IL-1β) for 15 min, both MNK1 bands showed a mobility shift, suggesting that these stimuli induced its phosphorylation (Figure 5A). Endogenous MNK1 in HeLa and 293 cells showed a similar mobility shift upon TPA stimulation (Figure 5B).

To determine what kind of extracellular signals activate MNK1, HeLa cells were transiently transfected with an expression vector for an N-terminally hemagglutinin (HA) epitope-tagged version of MNK1 and treated with various growth stimulators, pro-inflammatory cytokines or exposed to environmental stresses (Figure 6A). Expression of HA-MNK1 in serum-starved, unstimulated HeLa cells yielded a single 53 kDa band, whereas multiple lower mobility bands were observed in cells stimulated with TPA, fetal calf serum (FCS), anisomycin, UV-C irradiation, tumor necrosis factor-α (TNF-α) or IL-1β. Osmotic shock with 0.7 M NaCl caused a very weak mobility shift. Treatment of cells with 0.1% dimethyl sulfoxide (DMSO), the vehicle for TPA and anisomycin, had no effect. When immunoprecipitated HA-MNK1 was treated with bacterial alkaline phosphatase (BAP), the slower mobility bands were mostly converted into a band at the position of HA-MNK1 from unstimulated cells, indicating that the mobility shift is a result of phosphorylation (Figure 6B).

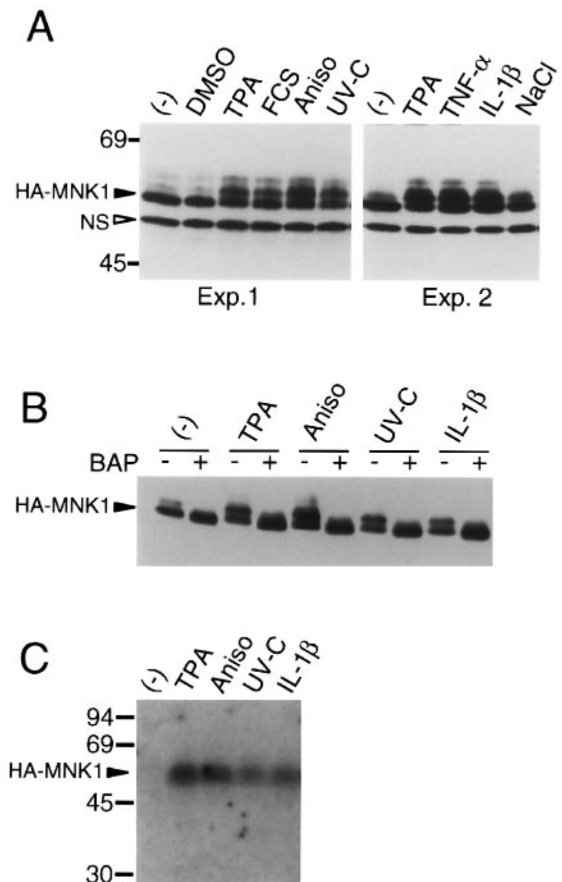

Fig. 6. Phosphorylation and activation of HA-MNK1 induced by various extracellular signals. (**A**) Mobility shift of HA epitope-tagged MNK1. HeLa cells were transfected with HA-MNK1 cDNA, serum starved, and left unstimulated (–) or stimulated with TPA (100 nM, 15 min), FCS (20%, 15 min), anisomycin (Aniso) (10 μg/ml, 15 min), UV-C irradiation (40 J/m^2, then incubated for 20 min), TNF-α (10 ng/ml, 30 min), IL-1β (10 ng/ml, 15 min) or osmotic shock (0.7 M NaCl, 30 min). Cell lysates (40 μg protein) were analyzed by immunoblotting with anti-HA epitope monoclonal antibody (12CA5). The positions of HA-MNK1 and protein size markers are shown on the left. NS indicates a non-specific band observed even in the untransfected cells (data not shown). (**B**) HA-MNK1 proteins were immunoprecipitated from the lysate of the transfected HeLa cells with affinity-purified anti-MNK1 antibodies. The precipitated HA-MNK1 was left untreated (–) or treated with bacterial alkaline phosphatase (+) as described in Materials and methods and analyzed by immunoblotting using 12CA5 antibody. (**C**) In-gel kinase assay for protein kinase activity of transiently expressed HA-MNK1. HeLa cells were transfected with the HA-MNK1 plasmid, serum starved and then stimulated as described in (A). HA-MNK1 protein was immunoprecipitated and assayed for protein kinase activity by the in-gel kinase assay using MBP as described in Materials and methods.

We next examined if MNK1 was activated in response to extracellular stimuli. For this purpose, we measured the protein kinase activity of immunoprecipitated MNK1 by an in-gel protein kinase assay (Kameshita and Fujisawa, 1989) using MBP as substrate, since immune complex kinase assays using the MK-1 peptide or MBP gave a rather high background. As shown in Figure 6C, HA-MNK1 immunoprecipitated from unstimulated cells was inactive for MBP phosphorylation. Treatment of cells with TPA, anisomycin, UV-C, IL-1β (Figure 6C), FCS, TNF-α or NaCl (Figure 7C) caused increased MNK1 kinase activity. TPA and anisomycin were relatively strong activators of MNK1, whereas the activation by osmotic shock was very weak.

Recently, it has been established that various extracellular stimuli differentially activate distinct MAP kinase pathways. For example, growth factors and phorbol esters strongly activate the ERK MAP kinase through MEK activation but do not cause a marked increase in JNK/SAPK or p38 activity (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Minden *et al.*, 1994; Raingeaud *et al.*, 1995). In contrast, pro-inflammatory cytokines and various environmental stresses specifically activate JNK/SAPK and p38 MAP kinases through activation of MKK3 or SEK1/MKK4/JNKK (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Sanchez *et al.*, 1994; Sluss *et al.*, 1994; Yan *et al.*, 1994; Dérijard *et al.*, 1995; Lin *et al.*, 1995; Raingeaud *et al.*, 1995). To clarify which of these MAP kinase pathways is involved in the activation of MNK1 by individual stimuli, we tested the effect of the specific MAP kinase pathway inhibitors, PD098059 and SB202190, on MNK1 activation. PD098059, which was identified originally as an inhibitor of the unphosphorylated form of MEK1 and a constitutively active mutant of MEK1 (Dudley *et al.*, 1995), and shown subsequently to inhibit the activation of MEK by c-Raf or MEKK-1 but not the activation of MKK4 by MEKK-1 (Alessi *et al.*, 1995), can be utilized as a specific *in vivo* inhibitor of MEK activation (Alessi *et al.*, 1995). SB202190 is one of a series of pyridinyl-imidazoles, which specifically inhibit p38 MAP kinase both *in vitro* and *in vivo* (Lee *et al.*, 1994; Cuenda *et al.*, 1995). As shown in Figure 7A, pre-treatment of cells with PD098059 decreased the extent of the TPA- or FCS-induced mobility shift of transiently expressed HA-MNK1, but had no effect on the band shift caused by anisomycin, UV-C, TNF-α or IL-1β. In contrast, SB202190 inhibited the mobility shift caused by various stimuli other than TPA. The FCS-induced mobility shift, which was partially inhibited by either PD098059 or SB202190, was strongly inhibited when both inhibitors were combined.

To confirm these results in a physiological situation, we also examined mobility shift of the endogenous MNK1 (Figure 7B). Both 52 and 51 kDa forms of endogenous MNK1 showed mobility shifts upon stimulation of HeLa cells with TPA, FCS, anisomycin, UV-C, TNF-α or IL-1β. The effects of the inhibitors on each stimulation were consistent with those observed in the transient expression experiment, although PD098059 slightly inhibited the TNF-α- or IL-1β-induced band shift of the endogenous MNK1. Combined addition of both inhibitors inhibited the MNK1 mobility shift caused by TPA, FCS, TNF-α or IL-1β almost completely (Figure 7B), suggesting that both ERK and p38 MAP kinase cascades are involved in the activation of MNK1 by these stimuli, although which MAP kinase pathway predominates depends on the stimulus. Activation of the transiently expressed HA-MNK1, as measured by the in-gel kinase assay, was also differentially inhibited by these drugs in a manner well correlated with the band shift (Figure 7C). Thus, activation of HA-MNK1 by TPA was inhibited by PD098059 but not by SB202190, whereas the FCS-induced activation was inhibited equally by either inhibitor. On the other hand, SB202190 strongly

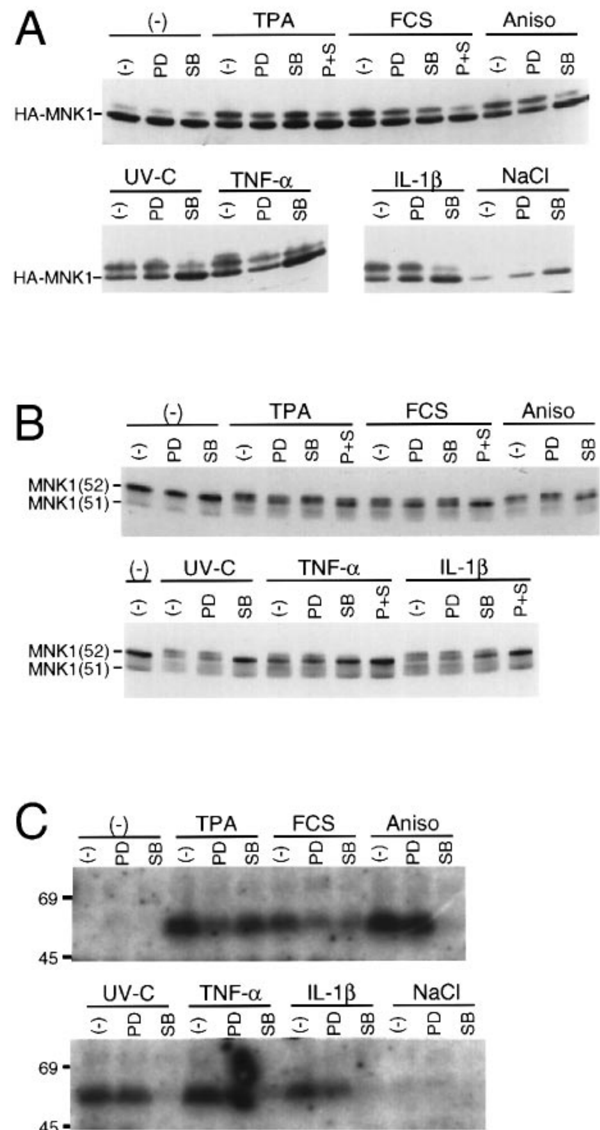

Fig. 7. Effect of protein kinase inhibitors on MNK1 activation. (**A**) Effect on the mobility shift of HA-MNK1 protein. HeLa cells were transfected with the HA-MNK1 plasmid, serum starved and treated with 30 μM PD098059 (PD) for 30 min or with 2 μM SB202190 (SB) for 60 min or with both inhibitors (P+S). As a control (–), cells were incubated with 0.1% DMSO, the solvent for the inhibitors. Then, the pre-treated cells were left unstimulated (–) or stimulated as indicated, and analyzed for the HA-MNK1 mobility shift by immunoblotting as described in Figure 6A. (**B**) Mobility shift of endogenous MNK1. Serum-starved HeLa cells were pre-incubated with the indicated inhibitor, and then stimulated as described in (A). Endogenous MNK1 was immunoprecipitated and analyzed by immunoblotting as described in Figure 5B. (**C**) In-gel kinase assay for MNK1 activity. HA-MNK1 protein was immunoprecipitated from the cell lysates prepared in (A) and tested for MBP-phosphorylating activity as described in Materials and methods.

inhibited activation of MNK1 by anisomycin, UV-C, TNF-α, IL-1β or osmotic shock, whereas PD098059 did not. These results indicate that anisomycin, UV-C, TNF-α, IL-1β and osmotic shock activate MNK1 mainly through the p38 MAP kinase pathway, whereas the activation of MNK1 by TPA is mediated mainly by the MEK–ERK pathway. The FCS-induced activation is likely to be mediated by both pathways.

MNK1 is phosphorylated and activated by ERK and p38 MAP kinases but not by JNK/SAPK

These results suggest that not only ERK but also p38 can directly phosphorylate and activate MNK1. To confirm this and to test whether JNK/SAPK can also phosphorylate and activate MNK1, we examined *in vitro* phosphorylation and activation of GST–MNK1 by purified ERK, JNK/SAPK and p38 MAP kinases. As shown in Figure 8A, p38 MAP kinase phosphorylated GST–MNK1 *in vitro* with efficiency similar to ATF2 (activating transcription factor 2), one of the best substrates for p38 so far known (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1995). In contrast, GST–MNK1 was not phosphorylated by JNK2 under the conditions where c-Jun, Elk-1 and ATF2 were phosphorylated very well. GST–MNK1 was also not phosphorylated by JNK1 (data not shown). Moreover, JNK2 did not phosphorylate the MNK1 protein which had been cleaved off from the GST portion by thrombin digestion (data not shown). In accordance with the extent of phosphorylation, GST–MNK1 protein kinase activity was increased upon phosphorylation by p38 MAP kinase as well as by ERK1 (Figure 8B). SB202190 strongly inhibited phosphorylation and activation of GST–MNK1 by p38, but not by ERK1, which confirmed the specificity of the inhibitor and excluded the possibility of contamination of ERKs in the p38 preparation.

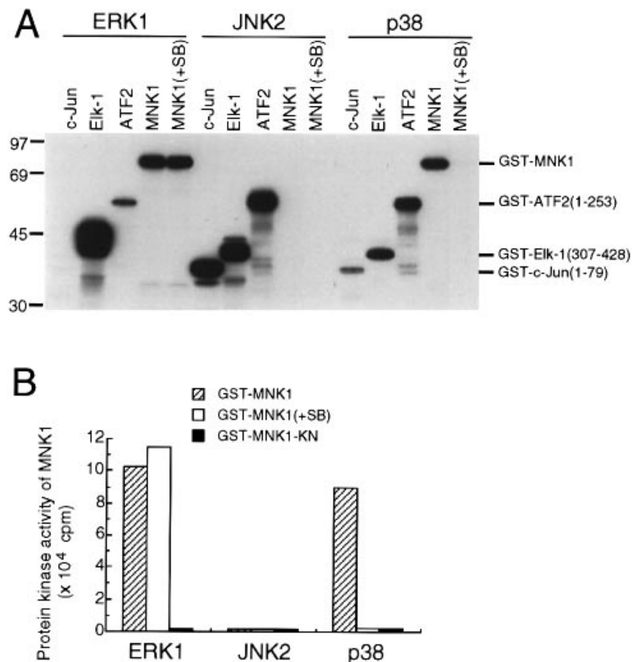

Fig. 8. Specificity of phosphorylation and activation of GST–MNK1 by MAP kinases. (**A**) Substrate specificity of the three MAP kinases against GST–MNK1 in comparison with known substrate proteins. Soluble GST fusion proteins (5 μg) of c-Jun (residues 1–79), Elk-1 (307–428), ATF2 (1–253) and MNK1 were incubated with purified ERK1 (2 ng) or JNK2 (20 ng) or FLAG-tagged p38 immunoprecipitated from transfected HeLa cells (from 0.2 ml of lysate; see Materials and methods) in 25 μl of p38 kinase buffer [25 mM HEPES–NaOH (pH 7.4), 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4] containing 25 μM [γ-^{32}P]ATP (10 Ci/mmol) for 60 min at 30°C. Incubations of GST–MNK1 with each MAP kinase were performed in the presence (+SB) or absence of 5 μM SB202190. After phosphorylation, GST fusion proteins (2 μg) were resolved by SDS–PAGE and exposed to X-ray film for 2 h (ERK1 and JNK2) or 10 h (p38) at –70°C with an intensifying screen. (**B**) Activation of GST–MNK1 by MAP kinases *in vitro*. Soluble GST–MNK1 and GST–MNK1-KN proteins (2.5 μg) were incubated with purified ERK1 (2 ng), JNK2 (20 ng) or immunoprecipitated FLAG-p38 (from 0.5 ml of cell lysate) in 50 μl of p38 kinase buffer containing 100 μM unlabeled ATP at 30°C in the presence (+SB) or absence of 5 μM SB202190. After 3 h, samples were diluted with 1 ml of the kinase wash buffer and centrifuged to remove the protein G–agarose resin used for the immunoprecipitation of FLAG-tagged p38. Then, GST–MNK1 in the supernatant was recovered by adding glutathione–agarose (20 μl resin) and washed three times with kinase wash buffer. GST–MNK1 protein kinase activity was assayed using MK-1 peptide as described in Materials and methods.

Discussion

Phosphorylation screening is practically useful for identifying physiological substrates for protein kinases

We have developed a novel method for identifying protein kinase substrates. This technique, termed phosphorylation screening, requires a purified protein kinase and a phage cDNA expression library. For this purpose, we constructed the λGEX5 vector, which has several advantages; identified cDNA clones can be subcloned rapidly as plasmids, and then used to provide predicted N-terminal amino acid sequences of the cDNA inserts, and can also be used for expression and easy purification of GST fusion proteins for further characterization and antibody preparation. The other reason for using the GST expression vector was to reduce background phosphorylation noise which might hamper identification of positive clones. Protein-based screening of a phage library, such as by South-western, West-western or immunoscreening, sometimes gives high backgrounds derived from endogenous phage or *E.coli* proteins. We designed the λGEX5 system to use together with glutathione-derivatized cellulose filters to immobilize recombinant proteins selectively onto the membrane, which should, in principle, reduce background noise derived from the phosphorylation of *E.coli* and/or phage proteins. Although the λGEX5–glutathione–cellulose filter system worked well in preliminary experiments, we did not need to use this system for ERK, since ERK did not give a high background with plaque proteins immobilized on nitrocellulose filters. Another modification of this method is likely to be effective for some protein kinases. In this protocol, plaque filters are incubated with a protein kinase to allow it to bind recombinant substrates in the absence of ATP, and, after washing, the filters are incubated in the presence of [γ-^{32}P]ATP to allow phosphotransfer to occur. We have not tested this option yet, but this may reduce background signal derived from non-specific phosphorylation, and would be a way of identifying substrates that have a high affinity binding site, as is the case for JNK and c-Jun (Hibi *et al.*, 1993; Kallunki *et al.*, 1994).

In a screen for ERK1 MAP kinase substrates, we isolated dozens of clones from a HeLa cDNA library, of which seven clones turned out to encode structurally known proteins including two already characterized ERK substrates, $p90^{RSK2}$ and c-Myc (Blenis, 1993; Davis, 1993). This result clearly indicates that the phosphorylation screening method is practically useful for identification of physiological substrates for protein kinases. We have not characterized other clones in detail, but some of them are attractive candidates as physiological ERK substrates. *In vivo* phosphorylation analysis will be required to elucidate whether these *in vitro* substrates are actually physiological ERK MAP kinase targets.

Phosphorylation screening has several advantages over other methods for substrate identification. The method of determining a consensus sequence using a peptide library is a powerful technique for identifying optimal substrate peptides for a protein kinase (Songyang *et al.*, 1994) and potential substrate proteins can be predicted by searching gene data banks. However, this method does not identify substrate proteins directly, and proteins containing a minimal consensus sequence for a certain protein kinase may not necessarily be good substrates because of tertiary structure constraints (Kemp and Pearson, 1990). The two-hybrid screening method overcomes this limitation and can identify substrate proteins directly (Yang *et al.*, 1992). However, this system is prone to detecting artifactual interactions, and one may also identify recombinants that bind to the bait protein kinase by interactions other than via an enzyme–substrate relationship (e.g. regulatory proteins or subunits, anchoring proteins and modification enzymes). Moreover, the affinity of the protein–protein interaction between a protein kinase and its substrates may not necessarily be great enough to be detected by the two-hybrid system, since in principle a protein kinase can interact with a substrate in a 'hit-and-run' manner via a short-lived intermediate complex. Phosphorylation screening may circumvent these problems and identify only substrate proteins for the protein kinase. Obviously, however, proteins identified by this technique are only '*in vitro*' substrates until proved to be a physiological target by *in vivo* experiments. In conclusion, utilizing these methods in combination would help to identify physiological targets for the variety of protein kinases.

MNK1 is a novel class of MAP kinase-activated protein kinase regulated by multiple MAP kinase pathways

We identified a novel protein kinase, MNK1, as a substrate for ERK1. Human MNK1 is most similar to mammalian MAP kinase-activated protein kinases, $p90^{RSK}$, MAPKAP-K2 and 3pK/MAPKAP-K3 (Figure 2B). The *C.elegans* R166.5 protein kinase has the highest similarity to MNK1 in the kinase domain but much less similarity in other regions, suggesting they are not true homologs. In contrast, the mammalian protein kinases also show significant similarity in their C-terminal regions, which seem to play an important role in phosphorylation and activation by MAP kinase (Figure 3C and Ben-Levy *et al.*, 1995). We have not determined all the sites phosphorylated in MNK1 by MAP kinases *in vitro* or *in vivo*, but preliminary results showed that Thr344 at least was one of the major sites phosphorylated by ERK1 *in vitro* and was the site phosphorylated inducibly *in vivo* upon TPA stimulation (R.Fukunaga and T.Hunter, unpublished observations). Thr344 in MNK1 corresponds to Thr334 of MAPKAP-K2, which is one of the sites phosphorylated by p38 MAP kinase and involved in its activation (Ben-Levy *et al.*, 1995). These results suggest that the enzymatic activity of these protein kinases is regulated by similar phosphorylation mechanisms. However, the existence of multiple mobility-shifted bands of MNK1 upon activation suggests that many phosphorylation sites may be involved in the regulation of MNK1. With regard to the mechanism of activation, it should be noted that MNK1 lacks an A-helix motif to the C-terminal side of the catalytic domain, and was not activated by deletion of the C-terminal region (Figure 3C), unlike MAPKAP-K2 (Engel *et al.*, 1995b).

Bacterially produced GST–MNK1 was phosphorylated and activated *in vitro* by ERK1 MAP kinase, and the phosphorylation resulted in multiple mobility-shifted MNK1 bands on SDS–PAGE (Figure 3). A similar band shift and enzymatic activation of GST–MNK1 was observed in insect Sf9 cells only when the GST–MNK1 baculovirus was co-infected together with baculoviruses encoding activators of the ERK pathway (Figure 4). Stimulation of HeLa cells with TPA or FCS resulted in phosphorylation and activation of MNK1, both of which were inhibited by the MEK inhibitor PD098059 (Figures 5–7). In addition, transient co-expression of MEKK-C, which is a constitutive activator of JNK/SAPK but can also activate ERK when overexpressed, or a constitutively active mutant of MEK1 [MEK1(DE)] with HA-MNK1 in HeLa cells both caused a strong HA-MNK1 mobility shift, whereas the wild-type MEK1 did not (R.Fukunaga and T.Hunter, unpublished observations). These results clearly establish that the protein kinase activity of MNK1 is regulated *in vivo* by ERK MAP kinase. In addition to growth stimulation, however, MNK1 could also be activated by various environmental stresses such as anisomycin, UV-C and osmotic shock, and by pro-inflammatory cytokines, such as TNF-α and IL-1β. Intriguingly, the activation of MNK1 by these stimuli was strongly inhibited by the p38 inhibitor SB202190, whereas no obvious effect was shown by PD098059 (Figures 5–7). Moreover, MNK1 was phosphorylated and activated *in vitro* by p38 MAP kinase, but not by JNK/SAPK (Figure 8). These results demonstrate that MNK1 is regulated not only by ERK but also by p38.

Activation of MNK1 through dual MAP kinase pathways is a unique property of this MAPKAP kinase. $p90^{RSK}$ was reported to be activated by growth factors and phorbol esters through the ERK pathway *in vivo* (Blenis, 1993), but is unlikely to be activated by the p38 pathway (Brunet and Pouysségur, 1996). MAPKAP-K2 was identified originally as a protein kinase that could be (re)activated by ERK MAP kinase *in vitro* (Stokoe *et al.*, 1992), but recent studies demonstrated that MAPKAP-K2 is activated through the p38/RK pathway rather than the ERK pathway *in vivo* (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Engel *et al.*, 1995a). The inability of ERK to activate MAPKAP-K2 *in vivo* is likely to be due to the N-terminal domain of MAPKAP-K2, which acts to suppress activating phosphorylation by ERK but not by p38/RK (Ben-Levy *et al.*, 1995). We did not observe any inhibitory effect of the N-terminal domain of MNK1 on its activity

(Figure 3C), which may explain the different behavior of the two protein kinases. Recently, 3pK/MAPKAP-K3 has been shown to be activated by all three MAP kinase pathways (Ludwig *et al.*, 1996). The physiological significance of the activation of MNK1 by two out of the three MAP kinase pathways is not clear. The transcription factor Elk-1, which binds in conjunction with serum response factor to the serum response element in the c-*fos* promoter to form a ternary transcriptional complex, can be phosphorylated and activated strongly *in vivo* by JNK/SAPK (Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995) as well as by ERK (Janknecht *et al.*, 1993; Marais *et al.*, 1993) but is only weakly activated by p38 (Janknecht and Hunter, 1997), providing an example of selective integration of MAP kinase pathways leading to transcriptional activation. Likewise, MNK1 may integrate multiple extracellular signals leading to a common target.

We have not identified the physiological target proteins for MNK1 or its precise function in signaling downstream of the ERK and p38 MAP kinases. It is reported that $p90^{RSK}$ undergoes nuclear translocation following serum stimulation, suggesting a potential function in phosphorylating transcription factors (Blenis, 1993; Zhao *et al.*, 1995). Although a putative nuclear localization signal sequence is found in MNK1 as well as in MAPKAP-K2 and 3pK/MAPKAP-K3 (Stokoe *et al.*, 1993; Sithanandam *et al.*, 1996), it remains to be determined whether these protein kinases translocate into the nucleus upon activating stimulation. As a substrate for MNK1, we used a peptide MK-1 which is known to be a good substrate for $p90^{RSK}$ and MAPKAP-K2 (Stokoe *et al.*, 1993). However, the K_m value of the MK-1 peptide for MNK1 (2.8 mM, data not shown) was much higher than that for MAPKAP-K2 (12.7 μM) (Stokoe *et al.*, 1993), suggesting that the substrate specificity of these two protein kinases may be quite different. Identification of substrate proteins and downstream pathways are necessary to elucidate the physiological function of MNK1 (but see below). We note that phosphorylation screening would be one means of doing this. Another way of examining the roles of MNK1 in signal transduction is to develop molecular probes such as chemical inhibitors and mutant forms of MNK1. Identification of sites phosphorylated in MNK1 by MAP kinases *in vivo* may allow construction of dominant-negative and/or constitutively active mutants of MNK1.

Independently, Waskiewicz and co-workers have identified two closely related mouse protein-Ser/Thr kinases by yeast two-hybrid screening using ERK2 as a bait, one of which has 93% amino acid identity to MNK1, and is presumably the mouse homolog of human MNK1 (Waskiewicz *et al.*, 1997). They have shown that mouse Mnk1 and Mnk2 interact with ERK1 and ERK2 and with p38, but not with JNK/SAPK, through a C-terminal domain. Both Mnk1 and Mnk2 are substrates for ERK2 and p38, and Mnk1 is activated upon MAP kinase phosphorylation *in vitro*; Mnk1 is also activated *in vivo* by stimuli that activate the ERK and p38 MAP kinases. These results are largely consistent with our conclusions about the *in vivo* and *in vitro* activation specificity of human MNK1 by MAP kinases. Waskiewicz *et al.* (1997) have also shown that Thr197 and/or Thr202, which lie in the activation loop, are required for activation of Mnk1. In addition, they have identified the translational initiation factor eIF-4E as a potential physiological substrate for Mnk1/2; Mnk1 phosphorylates eIF-4E at Ser209, an event that enhances its affinity for the 5′ cap structure of mRNAs.

In summary, we have developed a phosphorylation screening method to identify protein kinase substrates and have shown its utility in identifying substrates for the ERK MAP kinases. With further refinement, it should be possible to apply this technique to other protein kinases, which can be purified in an active form, including protein-tyrosine kinases and multisubunit protein kinases such as cyclin/Cdks and receptor-type protein-Ser/Thr kinases.

Materials and methods

Production and purification of ERK1 MAP kinase

Active human ERK1 was produced in insect Sf9 cells as described (Williams *et al.*, 1993). Briefly, Sf9 cells were infected with three recombinant baculoviruses encoding v-Ras, c-Raf-1 and ERK1 (Williams *et al.*, 1993) at multiplicities of infection of 3, 3 and 10, respectively, and cultured for 60 h at 27°C. Cells were disrupted by Dounce homogenization in a hypotonic buffer and the recombinant ERK1 was purified by DE-52, phenyl-Sepharose, Mono Q and Superose 12 column chromatographies.

Construction of the λGEX5 phage vector and preparation of a λGEX5 HeLa cDNA library

The GST expression plasmid, pGEX-KG (Guan and Dixon, 1991), was modified by inserting a 0.42 kb stuffer sequence flanked by *Sfi*I sites to produce pGEX-Stuffer. Then, a region containing the stuffer and GST gene was cloned into the 1.99 kb *Pvu*II–*Aat*II fragment of pUC119. The resulting plasmid, pGEX-PUC-3T, was linearized and inserted between the *Eco*RI and *Xba*I sites of λgt11 DNA (Young and Davis, 1983) using a *Not*I linker. The recombinant phage (λGEX5) was recovered by *in vitro* packaging reaction followed by plaque purification using *E.coli* BB4 as host strain. Double-stranded cDNA was synthesized from HeLa poly(A)$^+$ RNA with oligo(dT) primer using a cDNA synthesis kit (Amersham) or RNase H$^-$ reverse transcriptase (SuperScript II, Gibco BRL), and ligated to an adaptor which consisted of 5′-phosphorylated oligonucleotides, pCCAGCACCTGCA and pAGGTGCTGG. The size-fractionated cDNA (>0.8 kb) was ligated to the *Sfi*I-digested λGEX5 arms and packaged into bacteriophage λ particles. The cDNA library was amplified by growth in *E.coli* BB4 on agar plates. To construct λGEX-Elk-C, a 1 kb *Msc*I–*Xba*I fragment from pGEX-Elk-C (Marais *et al.*, 1993), containing the C-terminal part of GST and Elk-C, was cloned into *Msc*I- and *Xba*I-digested pGEX-PUC-3T. The resulting plasmid, which encoded the same fusion protein as pGEX-Elk-C, was digested with *Not*I and inserted between the *Eco*RI and *Xba*I sites of λgt11 DNA as described above.

Screening of a cDNA library by solid-phase phosphorylation

The HeLa cDNA library was plated on *E.coli* BB4 strain at a density of 1.5×10^4 plaques per 150 mm agar plate. After incubation for 3.5 h at 37°C, the plates were overlaid with nitrocellulose membrane filters (BA85, Schleicher & Schuell) that had been impregnated with 10 mM isopropyl-β-thiogalactopyranoside (IPTG). After incubation of the plates at 37°C for another 6–10 h, the filters were peeled off and immersed in blocking solution [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 3% BSA] and gently agitated at room temperature for 60 min. The filters were washed three times for 20 min in TWB [Triton wash buffer; 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 1 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and rinsed for 10 min in MRB [MAPK reaction buffer; 20 mM HEPES–NaOH (pH 7.5), 10 mM $MgCl_2$, 50 μM Na_3VO_4, 5 mM β-glycerophosphate, 5 mM NaF, 2 mM DTT, 0.1% Triton X-100]. Then, the filters were incubated for 60 min at room temperature in MRB containing 25 μM unlabeled ATP to mask proteins which have autophosphorylating and/or ATP-binding activities. Following washing for 10 min in the MRB without ATP, filters were incubated for 60 min at room temperature with gentle shaking in MRB (2 ml of the solution per 137 mm filter) containing 25 μM unlabeled ATP, 5 μCi/ml [γ-^{32}P]ATP and 1 μg/ml purified human ERK1. The filters were washed six times for at least 5 min in MWB [MAPK wash buffer; 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 20 mM NaF] containing 0.1% Triton X-100, and then once for 10 min in MWB

without Triton X-100. The filters were air-dried and exposed to X-ray films for 12–48 h at –70°C with intensifying screens. Positive clones were plaque purified by secondary screening and the phage DNA was prepared by the plate lysate method. The phage DNA was digested with *Not*I and the cDNA-containing plasmid (pGEX-PUC-3T) was recovered by self-ligation followed by transformation of *E.coli* XL-1 Blue. Overnight cultures (1.5 ml) of the transformed bacteria were used for plasmid preparation and for purification of GST fusion protein after IPTG induction.

Expression of GST–MNK1 fusion proteins in bacteria and insect cells

To express a GST–MNK1 fusion protein, an *Sse*8387I site was introduced to the 5′ side of the initiation codon of MNK1 cDNA by PCR, which was inserted into the pGEX-Stuffer plasmid. A kinase-inactive mutant (GST–MNK1-KN) was constructed by mutating Lys78 (AAA) to Arg (AGA) using the recombinant PCR method. One clone thus obtained contained two additional mutations at Gln100 (CAG) and Ile107 (ATT) to Arg (CGG) and Thr (ACT), respectively. Since the GST–MNK1 mutant containing only the Lys78Arg single mutation was catalytically as active as the wild-type protein after phosphorylation by ERK1, the above mutant containing three mutations of Lys78Arg, Gln100Arg and Ile107Thr was used as a kinase-negative mutant (MNK1-KN). The N-terminal deletion mutant, GST–MNK1(48–424), was constructed by deleting the 0.16 kb *Sse*8387I–*Bsr*GI region of the GST–MNK1 construct. The C-terminal deletion mutant, GST–MNK1(1–333), was constructed by truncating the 3′ region from the unique *Nco*I site of the cDNA. The wild-type and mutant GST–MNK1 proteins were produced in *E.coli* BL21 cells and purified as described (Guan and Dixon, 1991). To express GST–MNK1 in insect cells, two baculovirus transfer plasmids were constructed by ligating the 1.6 kb *Bam*HI fragment of the pGEX-MNK1 or pGEX-MNK1-KN to the *Bam*HI-digested pAcG2T vector (Pharmingen). Recombinant baculoviruses were produced by using BaculoGold DNA (Pharmingen) according to the manufacturer's instructions.

Expression and activation of HA-tagged MNK1 in mammalian cells

To construct mammalian expression plasmids for HA epitope-tagged MNK1 protein, the MNK1 cDNA starting at the second codon (Val2) was ligated to a DNA fragment encoding an HA epitope sequence (MVYPYDVPDYASLVD) and inserted into the *Hin*dIII and *Bam*HI sites of the pCMX vector (Umesono *et al.*, 1991). For the expression of the native form of MNK1, the whole S101-17 cDNA was inserted into the *Eco*RV and *Bam*HI sites of the pCMX plasmid. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS. Cells grown on a 10 cm plate were transfected with 20 μg of plasmid DNA by the calcium phosphate co-precipitation method using Bes-buffered saline. At 32 h after transfection, the medium was changed to 10 ml of DMEM with 0.5% calf serum and the cells were serum starved for 16 h. Then the cells were stimulated with TPA (100 nM, 15 min), FCS (20%, 15 min), anisomycin (10 μg/ml, 15 min), UV-C irradiation (40 J/m^2, then incubated for 20 min), TNF-α (10 ng/ml, 30 min), IL-1β (10 ng/ml, 15 min) or osmotic shock (0.7 M NaCl, 30 min). For experiments with protein kinase inhibitors, 10 μl of 30 mM PD098059 or 2 mM SB202190 in DMSO were added to the medium (10 ml) of serum-starved cells and incubated for 30 or 60 min respectively, before stimulation. After stimulation, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of NLB [NP-40 lysis buffer; 50 mM HEPES–NaOH (pH 7.4), 150 mM NaCl, 1% NP-40, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM DTT, 1 mM PMSF, 10 U/ml aprotinin and 10 μg/ml leupeptin). The supernatant was recovered after centrifugation for 20 min at 4°C.

Immunological procedures and phosphatase treatment

Anti-MNK1 antiserum was prepared by immunizing rabbits with the C-terminal region of MNK1 protein (residues 284–424) which had been cleaved off with thrombin from GST–MNK1(284–424) produced in *E.coli*. Anti-MNK1 antibody was affinity purified on a glutathione–agarose resin which had been covalently coupled to GST–MNK1(284–424) protein. Immunoblotting was performed with anti-MNK1 antiserum (5000-fold dilution) or anti-HA epitope monoclonal antibody (12CA5), and horseradish peroxidase-conjugated protein A or anti-mouse immunoglobulin antibody, respectively, using the ECL detection system (Amersham or Pierce). For immunoprecipitation, cell lysates (1.2 mg of protein) were incubated for 2 h at 4°C with 20 μl of protein A–agarose beads, which had been bound to the affinity-purified anti-MNK1 antibody. Then, the resin was washed once with NLB (1 ml), twice with 0.5× NLB containing 1 M NaCl, once with NLB and once with 20 mM HEPES–NaOH (pH 7.4). For the phosphatase treatment, the immunoprecipitated HA-MNK1 from 50 μl of cell lysate was washed with 50 mM Tris–HCl (pH 7.5) containing 20 mM β-mercaptoethanol and incubated with bacterial alkaline phosphatase (3 μg, Pharmacia) for 1 h at 37°C. After the treatment, HA-MNK1 samples were resolved on 10% SDS–PAGE and detected by immunoblotting using 12CA5 antibody.

Protein kinase assays for MNK1

GST–MNK1 protein (10 μg) bound to glutathione–agarose was incubated with 0.6 μg of ERK1 in 150 μl of the ERK kinase buffer [20 mM HEPES–NaOH (pH 7.4), 10 mM $MgCl_2$ and 1 mM DTT] in the presence of 0.4 mM ATP at 30°C for the indicated time. After phosphorylation, the beads were washed three times with kinase wash buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA and 10% glycerol] and then once with the ERK kinase buffer supplemented with 20 mM β-glycerophosphate. The assays were initiated by adding kinase buffer containing 50 μM [γ-^{32}P]ATP (2 Ci/mmol) and 1.5 mM substrate peptide (MK-1 peptide; NH_2-KKLNRTLSVA-COOH; Stokoe *et al.*, 1993). For the assay using MBP as a substrate for GST–MNK1, 5 mg of MBP (Sigma) was first phosphorylated with 15 μg of ERK1 in the ERK kinase buffer containing 1 mM unlabeled ATP to obtain stoichiometric phosphorylation at the ERK phosphorylation site(s). The ERK-phosphorylated MBP was purified from ERK1 and ATP by DE-52 and Mono S column chromatography and used as a substrate for GST–MNK1 produced by Sf9 cells. For the in-gel kinase assay, HA-MNK1 protein was immunoprecipitated from transfected HeLa cell lysate (600 μg protein) with affinity-purified anti-MNK1 antibody and separated on SDS–PAGE whose resolving gel had been polymerized with 0.3 mg/ml of unmodified MBP. The in-gel kinase assays were carried out as described (Kameshita and Fujisawa, 1989).

Preparation of JNK and p38 MAP kinases

Purified human JNK1 and JNK2 (Dérijard *et al.*, 1994; Kallunki *et al.*, 1994) produced in Sf9 cells were provided by Dr Helen Brady (Signal Pharmaceuticals). Active p38 MAP kinase was produced by transient transfection of HeLa cells with FLAG-tagged p38 cDNA (Dérijard *et al.*, 1995) followed by osmotic shock with 0.7 M NaCl for 30 min. FLAG-tagged p38 MAP kinase was immunoprecipitated with M2 monoclonal antibody bound to protein G–agarose, washed extensively and used for *in vitro* phosphorylation of GST fusion proteins.

Acknowledgements

We are grateful to Richard Treisman (Imperial Cancer Research Fund) for pGEX-Elk-C; Tom Roberts (Dana-Farber Cancer Institute) for baculoviruses; Anning Lin and Michael Karin (University of California, San Diego) for pGEX-ATF2 (1–253) and SB202190; Helen Brady (Signal Pharmaceuticals Inc.) for pGEX-c-Jun (1–79) and purified JNK1 and JNK2; Roger Davis (University of Massachusetts Medical School) for the FLAG-tagged p38 plasmid; and David Dudley (Parke Davis Pharmaceutical, Warner-Lambert Co.) for PD098059. We thank Martin Broome for his kind help with baculoviruses and Sf9 cells. We are also grateful to Andrew Waskiewicz and Jon Cooper for sharing unpublished data. This work was supported by US Public Health Service grants CA14195 and CA39780. R.F. was supported by a fellowship from AMGEN. T.H. is an American Cancer Society Research Professor.

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Received on September 6, 1996; revised on January 9, 1997