Expression, purification and characterization of recombinant mitogen-activated protein kinase kinases

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Mitogen-activated protein (MAP) kinase kinases (MKKs) are dual-specificity protein kinases which activate $p42^{mapk}$ and $p44^{mapk}$ by phosphorylation of regulatory tyrosine and threonine residues. cDNAs for two isotypes of MKK, MKK1 and MKK2, have been isolated from several species. Here we describe construction of recombinant baculoviruses for high-level expression of histidine-tagged rat MKK1 and MKK2, and procedures for production of nearly homogeneous MKK1 and MKK2 fusion proteins, in both inactive and active forms. Co-infection of Sf9 cells with either MKK1 or MKK2 virus together with recombinant viruses for Raf-1, $pp60^{src}$ (Y527F) and c-Ha-Ras resulted in activations of 250-fold and 150-fold for MKK1 and MKK2 respectively. Specific activities towards kinase-defective $p42^{mapk}$ were of the order of several hundred nanomoles of phosphate transferred/min per mg of MKK protein. The Michaelis constants for both enzymes were approx. 1 μM. Preparations of activated MKK were apparently free of Raf-1 as assessed by Western blotting. Raf-1 phosphorylated MKK1 on one major tryptic phosphopeptide, the phosphorylation of which increased with time. This phosphopeptide contained only phosphoserine and possessed neutral overall charge at pH 1.9 on two-dimensional peptide mapping. Phosphorylation of MKK1 by Raf-1 correlated with activation and reached a plateau of $\sim$ 2 mol/mol.

INTRODUCTION

Mitogen-activated protein (MAP) kinases $p42^{mapk}$ (ERK2) and $p44^{mapk}$ (ERK1) (for reviews, see [1–3]) are important effectors for the transmission of signals to the nucleus to control gene transcription [2]. MAPK function is required for cell cycle progression in fibroblasts [4,5] and both $p42^{mapk}$ and $p44^{mapk}$ are translocated to the nucleus on prolonged stimulation [6–9]. These MAPKs phosphorylate ternary complex factor $p62^{TCF}$ and promote formation of a ternary complex with the serum response factor and the serum response element to transactivate the *fos* gene [10]. Myc also appears to be a physiological substrate for MAPK in the nucleus [11]. Reports demonstrating activation of *fos* by many stimuli have a parellel in current research aimed at defining the many upstream pathways for activation of MAPKs.

MAPKs are activated by MAP kinase kinases (MKKs or MEKs), possessing dual specificity for the regulatory tyrosine and threonine sites [1–3]. MKKs are an important site of convergence of pathways for activation of MAPK [1–3,12]. The protein kinases Raf-1, MEK kinase and Mos can activate MKK *in vitro* [1–3]. Two isozymes of MKK, MKK1 [13] and MKK2 [14], have been cloned from several species and shown to activate both $p42^{mapk}$ and $p44^{mapk}$ *in vitro* [13]. However, important differences in regulation and function of MKK1 and MKK2 may exist [15].

A necessary step in further biochemical studies of regulation and function of MKKs is development of reliable procedures for expression and purification of native proteins in significant quantities. MKKs expressed by pET vectors in *Escherichia coli* are mostly insoluble; MKKs expressed as fusion proteins with glutathione S-transferase are partially soluble [16] but are less than ideal because of the presence of many partially translated proteins and proteolytic fragments (P. Dent, Y.-H. Chow, J. Wu, D. K. Morrison, R. Jove and T. W. Sturgill, unpublished work). Expression in Sf9 insect cells using recombinant baculoviruses is an established technology for production of properly folded soluble proteins. Here, we describe recombinant MKK1 and MKK2 expressed in Sf9 cells and their biochemical properties. MKKs can be activated significantly by coexpression with c-Ha-Ras, $pp60^{Src}$ (Y527F) and Raf-1. These preparations are suitable for biochemical dissection of the MAPK pathways. Preparations of activated Raf-1 phosphorylated MKK1 to $\sim$ 2 mol/mol on serine residues, generating a single major phosphopeptide which correlated with activation of MKK catalytic activity.

MATERIALS AND METHODS

Sources of materials

Kinase-defective $p42^{mapk}$ (K52R) was prepared as described [14]. Protein determination was by the method of Bradford (kit available from BioRad, Richmond, CA, U.S.A.) with BSA as standard. Nickel–chelate nitrilotriacetate (NTA)–agarose was purchased from Qiagen (Chatsworth, CA, U.S.A.). Other chromatography reagents were obtained from Pharmacia–LKB biotechnology (Piscataway, NJ, U.S.A.). Rabbit polyclonal antibodies raised against residues 277–300 of murine MKK1 [15] were a gift from Dr. T. Rossomando (Miles Laboratories, New

Abbreviations used: MAP kinase, mitogen-activated protein kinase (ERK, extracellular signal-regulated protein kinase); MKK, MAP kinase kinase (MEK, MAPK/ERK kinase); K52R, kinase-defective $p42^{mapk}$ mutant; MOI, multiplicity of infection; Sf9 cells, *Spodoptera frugiperda* cells; NTA, nitrilotriacetate; FSBA, 5′-*p*-fluorosulphonylbenzoyladenosine.

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Haven, CT, U.S.A.). Raf-1 was detected by chemiluminescence (Amersham) in Western blots using a rabbit polyclonal antibody to the 13 C-terminal amino acids of Raf-1 [17]. The sources of other reagents and descriptions of standard methodologies have been given previously [12,13,18]. Recombinant baculoviruses expressing Raf-1 (Y340D), Raf-1 (K375M), pp60^{Src} (Y527F), and c-Ha-Ras have been described [17]. Baculoviruses expressing an activated mutant Δ7-Raf-1, containing a deletion in the N-terminal regulatory domain, and an activated N-terminally truncated $(His)_6$-tagged Raf-1 (22W [19]) were used in some experiments (Y.-H. Chow and R. Jove, unpublished work).

Recombinant baculoviruses encoding MKK1 and MKK2

MKK1 cDNA sequence [12] was altered with two point mutations, GGCTGC to GGATCC, by PCR to generate a *Bam*HI-recognition site at position −9 to −4 upstream of the translational start site. The PCR-amplified region was sequenced by the dideoxy method to confirm the desired point mutations and the absence of other nucleotide changes. A *Bam*HI to *Hin*dIII fragment (2126 bp) containing the whole coding sequence of MKK1 was then isolated and subcloned between the *Bam*HI and *Hin*dIII sites of pBlueBacHisA (Invitrogen, San Diego, CA, U.S.A.), a baculovirus transfer vector, so that MKK1 could be translated in frame with an N-terminal $(His)_6$ tag. Full-length coding sequence of MKK2 cDNA [14] was isolated in a 1261 bp *Eag*I to *Eag*I fragment, followed by T4 DNA polymerase treatment to fill in the 3′ recessed ends generated by *Eag*I digestion. This blunt-ended fragment was subsequently subcloned into another baculovirus transfer vector, pBlueBacHisC, for expression of a $(His)_6$ fusion protein of MKK2. Structures of recombinants were confirmed by restriction mapping.

Recombinant baculoviruses were obtained by co-transfecting wild-type baculoviral DNA and the transfer vector containing either MKK1 or MKK2 sequences into Sf9 insect cells as described [18]. Viral supernatants were harvested and individual recombinant clones were isolated by serial soft agar plaque purification. Expression of the MKK proteins was screened by Western blotting using a STE7/byr1 anti-peptide antibody (Kintek Biotechnology, Vancouver, BC, Canada). Clones that expressed the highest levels of either MKK1 or MKK2 fusion proteins were amplified for protein purification.

Expression and purification of MKK1 and MKK2

Sf9 insect cells were grown at 27 °C in 50% oxygen/50% nitrogen in 75 ml spinner flasks using Grace's modified insect cell medium in 10% fetal calf serum. The Δ7-Raf-1 was not histidine tagged and was only used in *in vivo* activation experiments. Sf9 cells (2×10^6 per ml) were infected with MKK baculoviruses at an estimated multiplicity of infection (MOI) of 10. To prepare partially activated MKKs, Sf9 cells were also infected with MKK (as above) together with recombinant baculoviruses (MOI 3, each) expressing Δ7-Raf-1, pp60^{Src} (Y527F) and c-Ha-Ras. After 48 h of infection, cells were harvested by centrifugation (500 ***g***; 4 min), washed once with PBS, and repelleted. All subsequent steps were conducted at 4 °C. The cells were immediately placed on ice and lysed in 35 ml of ice-cold homogenization buffer [25 mM Tris/HCl (pH 7.9 at 4 °C), 2 mM imidazole/HCl (pH 7.9 at 4 °C), 500 mM NaCl, 25 mM NaF, 2 mM Na_3VO_4, 2 mM $Na_2P_2O_7$, 5 mM benzamidine, 40 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 40 μg/ml tosyl-lysine chloromethylketone, 40 μg/ml tosylphenyalanine chloromethylketone, 1 mM phenylmethanesulphonyl fluoride, 10% (v/v) glycerol, 1.0% (v/v) Nonidet P40, 0.1% (v/v) 2-mercaptoethanol]. The lysate was homogenized with ten passes of a tight fitting Teflon/glass homogenizer. The homogenate was spun at 10000 ***g*** for 10 min and batch adsorbed at 4 °C (45 min) on an end-over-end mixer to 2 ml of Ni^{2+}–NTA–agarose, which had been pre-equilibrated in homogenization buffer. The Ni–chelate beads were pelleted by centrifugation and washed twice with 50 ml of homogenization buffer and 50 ml of homogenization buffer containing 20 mM imidazole/HCl.

The Ni–chelate beads were resuspended in a small volume of homogenization buffer containing 20 mM imidazole/HCl and packed into a column (bed 1.5 cm × 1.0 cm) for chromatography using a Pharmacia f.p.l.c. system [buffer A, the same as the homogenization buffer but omitting NaCl and with 0.03% (v/v) Nonidet P40; buffer B, buffer A containing 250 mM imidazole/HCl]. The column was developed (0.25 ml/min; 1 ml fractions) with a 40 ml linear gradient of increasing buffer B concentration. Fractions containing recombinant MKK proteins (eluted between 50 and 125 mM imidazole) were identified by SDS/PAGE, pooled, diluted 10-fold with water, and applied to a Mono Q (HR 5/5) column equilibrated in buffer C (buffer A containing 0.1 mM EDTA and 0.1 mM EGTA). The column was eluted (0.25 ml/min; 1 ml fractions) with a 40 ml linear gradient of increasing concentration of buffer D (buffer C containing 500 mM NaCl). MKK1 and MKK2 were eluted between 100 and 150 mM NaCl as distinct protein peaks. Fractions to be pooled were selected after SDS/PAGE to achieve more than 90% purity and dialysed against buffer C containing 50% (v/v) glycerol and stored at −20 °C (see Figure 3a). Partially activated recombinant MKKs (from coexpression with oncogenes) remained active under these conditions for at least 2 months (Table 1).

Treatment and inactivation of recombinant MKK1 by 5′-*p*-fluorosulphonylbenzoyladenosine (FSBA)

Recombinant MKK1 (0.1–0.2 mg/ml) was incubated in the dark for 20 min at 30 °C with 100 mM $MgCl_2$ and 1 mM FSBA [20], followed by overnight incubation at 4 °C. The reaction mixture was diluted 20-fold with water (4 °C) and MKK repurified by Mono Q chromatography as above. Inactivated MKK was dialysed against buffer C containing 50% (v/v) glycerol and stored at −20 °C. Between 30 and 50% of recombinant MKK1 protein was recovered.

Phosphorylation of K52R p42^{mapk} by recombinant MKK1 and MKK2

Purified partially activated recombinant MKK proteins (MKK1 and MKK2) were used to phosphorylate K52R to determine kinetic parameters and the specific activity of each preparation. Assay mixtures (100 μl or a multiple thereof) contained 25 mM Tris/HCl (pH 7.5 at 4 °C), 15 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (10000 c.p.m./pmol), 50 nM recombinant MKK protein and various concentrations (1000, 600, 300, 100, 50, 25 and 10 nM) of K52R. Reactions were initiated by the addition of MgATP, incubated at 30 °C for 1 min and then terminated by using boiling SDS/PAGE sample buffer, followed by immediate heating (100 °C, 5 min). The incubation time was increased 20-fold for preparations of MKK1 and MKK2 from single infections to permit phosphorylation of K52R to be accurately measured. K52R was resolved by SDS/PAGE (10% gel). Coomassie-stained K52R protein bands were excised and ^{32}P incorporation

was determined by liquid-scintillation spectrometry in scintillation cocktail. Incorporation of phosphate into K52R was always less than 0.5 mol/mol of enzyme (25% maximal).

Phosphorylation and activation of recombinant MKK1 by recombinant Raf-1

A recombinant $(His)_6$-tagged partially active form of Raf-1 (Y340D mutant) was further activated during expression in Sf9 cells by coexpression with $pp60^{src}$ (Y527F) and c-Ha-Ras [17]. Sf9 cells were infected with Raf-1 (Y340D) virus and viruses encoding for activating oncogenes at approximate MOIs of 20 and 3 (each) respectively. Cells were harvested after 48 h and Raf-1 was partially purified by Ni–chelate and Mono Q chromatography exactly as described above. Active Raf-1, assayed by MKK phosphorylation and blotting, was eluted from the Mono Q column between 200 and 300 mM NaCl. Unlike the recombinant MKK proteins, the Raf-1 preparation was impure (estimated 5% purity) for unidentified reasons. Active Raf-1 (Y340D) preparations typically had specific activities towards recombinant MKK1 protein of 0.5–1.0 nmol of phosphate/min per mg of Raf-1 preparation. As a control, kinase-defective $(His)_6$Raf-1 (K375M) was coexpressed with the activating oncogenes and purified in exactly the same manner.

The assay for phosphorylation of MKK (or FSBA-treated MKK) by Raf-1 preparations (5 μg of total protein) was the same as that described above with the following exceptions: 1 mM $MnCl_2$ was present and the substrate was 0.5 μg of MKK1 (11 pmol) unless otherwise stated.

Tryptic digestion of recombinant MKK1 and phosphopeptide mapping

FSBA-treated MKK1 phosphorylated by activated Raf-1 (Y340D) was extracted from the acrylamide gel and processed for phosphopeptide mapping as described [21]. The recombinant MKK was digested for 18 h at 37 °C using 0.5 μg of methylated trypsin (Promega, Madison, WI, U.S.A.). Phosphoamino acid analysis was performed as described [21]. Peptides and phosphoamino acids were detected using Kodak X-Omat AR film with intensifying screens.

RESULTS AND DISCUSSION

Construction and characterization of recombinant MKK baculoviruses

For high-level expression of MKK proteins, we chose the insect cell–baculovirus expression system. cDNAs of MKK1 and MKK2 were cloned into similar baculovirus transfer vectors so that they would be expressed as fusion proteins with N-terminal $(His)_6$ tags to facilitate purification by metal–chelate chromatography. The structures of the resulting fusion proteins are shown in Figure 1. $(His)_6$-MKK1 is a 430-amino acid fusion protein, containing 36 additional residues from the poly-(His) tag and one residue from the 5′ untranslated sequence of MKK1 cDNA. $(His)_6$-MKK2, a 440-amino acid fusion protein, has 35 additional residues from the poly-(His) tag and five residues from the 5′ untranslated sequence. Both $(His)_6$-MKK1 and $(His)_6$-MKK2 contain an enterokinase-cleavage site that could be used to remove the poly(His) tag, leaving the authentic MKK proteins with six or nine extra amino acids respectively. The $(His)_6$-MKK recombinants were constructed into baculoviruses by homologous recombination on co-transfection of the transfer vectors with wild-type baculoviral DNA.

Infection of Sf9 cells with these recombinant baculoviruses resulted in high-level expression of MKK proteins (Figure 2). $(His)_6$-MKK1 protein has a molecular mass of 48 kDa, whereas $(His)_6$-MKK2 protein migrates at 50 kDa, both as predicted. Cells infected with $(His)_6$-MKK2 virus also contained a faster-migrating species reactive to anti-STE7 antibody (lane 4). This species was frequently present in preparations purified by

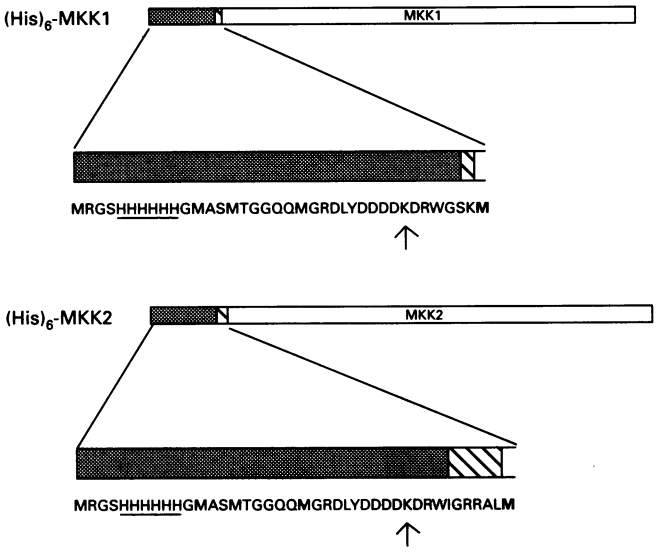

Figure 1 Structure of recombinant $(His)_6$-MKK proteins

MKK1 and MKK2 proteins (open boxes) were expressed as fusion proteins with 36 or 35 residues respectively of the $(His)_6$-tag (shaded boxes) and one or five residues respectively from the 5′ untranslated sequences of the cDNAs (hatched boxes). Amino acid sequences of the added residues are shown; histidine (H) residues are underlined; translational start methionine (M) of the MKKs are bold type. Arrows depict the positions of the lysine (K) residue after which enterokinase cleaves.

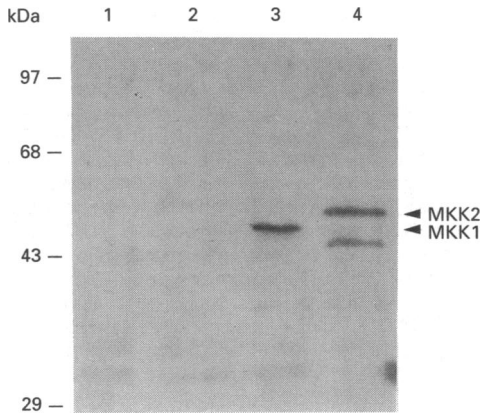

Figure 2 Expression of recombinant MKK1 and MKK2 proteins in infected insect cells

Immunoblot of whole-cell lysates prepared from uninfected Sf9 cells (lane 1), wild-type baculovirus-infected Sf9 cells (lane 2), MKK1 (lane 3) or MKK2 (lane 4) recombinant baculovirus-infected Sf9 cells. The blot was probed with anti-STE7/byr1 antibodies and detected as in the Materials and methods section. Recombinant $(His)_6$-MKK1 and $(His)_6$-MKK2 proteins are indicated. At this level of sensitivity, endogenous insect cell MKK proteins are not detected.

Table 1 Purification of recombinant MKK proteins from Sf9 cells

Each preparation used a single 75 ml spinner flask of Sf9 cells (2×10^6 per ml) infected as described in the Materials and methods section, and harvested 48 h after infection. The specific activity of each preparation is defined as nmol incorporated into K52R by the recombinant MKK protein/min per mg of MKK protein. MKK activity was assayed as described in the Materials and methods section using 1 μg of initial homogenate and 0.1 μg at each stage thereafter. The values shown are of single preparations of partially active MKK1 and MKK2, but are representative of a number of preparations.

Step	Volume (ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
MKK1						
Homogenate	35	1330	66.5	0.050	–	100
Nickel–agarose	6	0.78	59.3	76	1520	89
Mono Q	1.8	0.18	35.6	198	3960	54
MKK2						
Homogenate	35	1580	63.2	0.040	–	100
Nickel–agarose	6	0.75	57.0	68	1700	90
Mono Q	1.7	0.17	38.8	228	5700	61

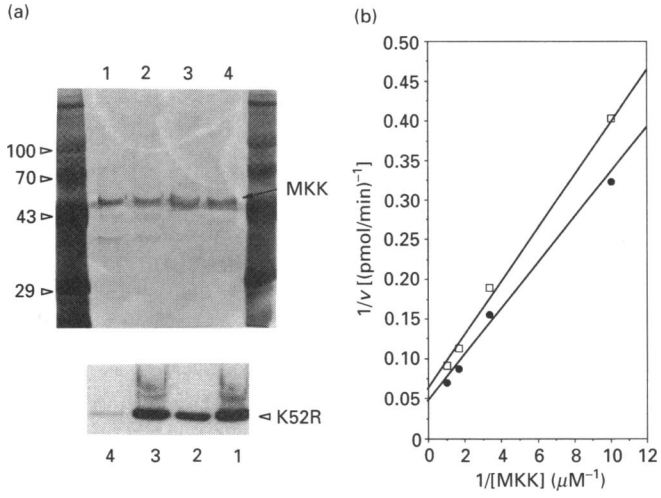

Figure 3 Characterization of recombinant MKK proteins

(**a**) Top, Silver-stained acrylamide gel (10% gel, 0.5 μg of protein/lane) or recombinant MKK2 expressed in the presence or absence of co-infected activating upstream oncogenes for Δ7-Raf-1, pp60^{Src} (Y527F) and c-Ha-Ras (lanes 1 and 2 respectively) and recombinant MKK1 expressed in the presence or absence of co-infected activating oncogenes for Δ7-Raf-1, pp60^{Src} (Y527F) and c-Ha-Ras (lanes 3 and 4 respectively) (Table 1). Staining was fully developed to reveal all minor contaminating proteins. This is a typical result from many experiments. Bottom, autoradiograph showing the phosphorylation of 2 μg (1.2 μM) of K52R by 0.5 μg (50 nM) of each MKK preparation [except 1.0 μg of MKK2 from lane 2 (top)] for 1 min at 30 °C. Lane allocation of MKK preparation is the same as the top. (**b**) Kinetic analysis of phosphorylation of K52R by recombinant partially active MKK1 (□) and MKK2 (●). Recombinant MKK1 and MKK2, purified from Sf9 cells coexpressing activating oncogenes (50 nM), were incubated with various concentrations of K52R under phosphorylating conditions to determine the rate of phosphate incorporation of K52R (see the Materials and methods section). This experiment was performed three times with duplicate values for each point; mean values are plotted. Incorporation (range 3000–120000 c.p.m.) was less than 25% of maximal. In order to show the ordinate intercept in detail, the 50–10 nM K52R concentrations are not shown. Incorporation into K52R by autophosphorylation was negligible.

Ni–chelate chromatography, implying that proteolysis had occurred at or near the C-terminus of the protein.

Isolation of MKKs by Ni–chelate and Mono Q chromatography

After batch adsorption, MKKs were eluted from Ni^{2+}–chelate–agarose using a gradient of increasing imidazole concentrations. Analysis of eluted proteins by Coomassie Blue staining demonstrated that this single step resulted in significant purification (results not shown). No significant differences were observed between MKK1 and MKK2 in copurifying proteins or degree of purification achieved (Table 1).

To purify the MKK proteins further, peak fractions containing MKK were pooled and subjected to Mono Q chromatography. Two major peaks of protein were eluted (results not shown); the first peak eluted corresponded to the recombinant MKK protein. The purity of the final preparation (see the Materials and methods section) was assessed by SDS/PAGE followed by silver staining (Figure 3a). By this criterion, the preparations are estimated to be more than 90% pure.

Raf has been identified as an upstream activator of MKK/MEK, implying that these proteins interact [1]. Association of some protein kinases with their protein substrates has been documented. In particular, both biochemical methods [22] and the two-hybrid system for detecting protein–protein interactions in yeast have demonstrated binding of Raf to MKK [23]. We have detected MKK immunoreactive protein in partially purified v-Raf preparations ([14], results not shown). Huang et al. [24] observed copurification of epitope-tagged MEK1 and Raf-1 from insect cells co-infected with recombinant baculoviruses, confirming interaction of Raf and MEK.

Western blotting with anti-Raf 1 was performed on the final preparations to determine whether any Δ7-Raf-1 had copurified with the activated MKK proteins. None was observed (results not shown). Δ7-Raf-1 is expressed at levels significantly higher than endogenous Raf proteins and should have outcompeted any endogenous Raf for binding to MKK. Incubation of purified MKK preparations, activated *in vivo* by coexpression with Raf-1, Src and Ras, with MgATP failed to increase MKK specific activity, consistent with the absence of active Raf-1. Furthermore, C-terminally tagged Raf-1, which was partially purified from Sf9 cells by a similar procedure to that described herein, did not contain detectable copurifying insect cell MKK (P. Dent, Y.-H. Chow, J. Wu, D. K. Morrison, R. Jove and T. W. Sturgill, unpublished work). Thus, Raf-1 appears not to copurify with MKK proteins under our conditions. The reasons for the differences between our observations and those of Huang et al. [24] are not clear. Our recombinant MKK proteins were tagged at the N-terminus, whereas recombinant MKK proteins that were shown to co-purify with Raf-1 were tagged at the C-terminus. It is thus possible that the N-terminal tag may decrease interaction between MKK and Raf-1. Alternatively, our con-

Table 2 Activation *in vivo* and *in vitro* of recombinant MKK proteins by Raf-1

MKK specific activity (A) from various *in vivo* and *in vitro* conditions (indicated in column headings) is expressed as pmol of phosphate incorporated into K52R protein/min per μg of MKK protein. The column headings for *in vivo* MKK activation summarize the baculoviruses co-infected; the column headings for *in vivo* MKK activation summarize the results obtained with a portion (see the Materials and methods section) of the partially purified $(His)_6$-tagged Raf protein [one of: none, 22WRaf-f, Δ7-Raf-1, Raf-1 (Y340D) or Raf-1 (K375M)] from such infections. The *in vivo* data are the average of three to five separate experiments, differing by less than 10%. MKK phosphorylation (B) is expressed as pmol of phosphate incorporated into recombinant MKK1 (1 μg) from a single infection during incubation *in vitro* for 20.5 min at 30 °C (see the Materials and methods section). MKK activity was assessed by the addition of 2 μg of K52R added during the last 30 s of the reaction. The incorporation of phosphate into K52R by Raf-1 preparations was less than 1% of the total counts. The *in vitro* results are an average of three to five separate experiments, differing by less than 20%. nd, not determined.

(A) MKK1 activity (pmol/min per μg of protein)

	MKK1	MKK1 + 22W-Raf-1	MKK1 + Ras + Src + Δ7-Raf-1	MKK1 + Ras + Src + Raf-1 (K375M)
In vivo	0.5	25	190	1.0
In vitro	0.5	3.0	25	0.75

(B) MKK1 phosphorylation (pmol of phosphate incorporated into MKK1)

	MKK1	MKK1 + 22W-Raf-1	MKK1 + Ras + Src + Raf-1 (Y340D)	MKK1 + Ras + Src + Raf-1 (K375M)
In vivo	nd	nd	nd	nd
In vitro	0.1	1.0	5.0	0.25

ditions of homogenization and purification are more likely to have disrupted Raf–MKK complexes at some stage, most likely during column chromatography. In our experiments, MKKs are immobilized on Ni–agarose and washed with a total of 75 bed volumes of buffer containing salt and detergent followed by an additional chromatography.

Activation of MKKs by oncogenes results in preparations with specific activities in the nmol/min per mg region

Studies of Raf-1 activation in the insect cell system by co-expression with combinations of proto-oncogenes or oncogenes suggested a strategy for activating MKK *in vivo* [25]. Fabian et al. [17] identified a partially activating point mutant of Raf-1, Y340D, and demonstrated that coexpression with activated $pp60^{Src}$ and c-Ha-Ras further enhanced activity of the Raf-1 mutant. $(His)_6$-tagged MKK proteins were expressed together with an untagged mutant of Raf-1, Δ7-Raf-1, bearing an activating deletion in the N-terminal regulatory domain (Y.-H. Chow and R. Jove, unpublished work) and with activated $pp60^{Src}$ and c-Ha-Ras to evaluate the activation of MKK.

Coexpression with these upstream signalling proteins dramatically increased the specific activity of purified MKK proteins (Figure 3a (bottom) and Table 2], as measured by phosphorylation of K52R. The apparent kinetic properties of these preparations of partially activated MKK1 and MKK2 were determined (Figure 3b). The $V_{max.}$ of MKK1 and MKK2 in the standard assay were 15 and 20 pmol/min respectively. The specific activities of these preparations of MKK1 and MKK2 were 200 and 230 nmol/min per mg respectively, in a 1 min standard assay (50 nM MKK; 1.2 μM K52R). The estimated K_m values for MKK1 and MKK2 were 0.57 μM and 0.60 μM respectively (Figure 3b), in general agreement with the K_m determined for phosphorylation of dephospho-MAPK using partially purified skeletal-muscle MKK [26]. Active recombinant MKK1 and MKK2 were also shown to be capable of phosphorylation and activation of $p44^{mapk}$/ERK1 (not shown). Neither MKK1 nor MKK2 were capable of phosphorylating ERK3, a protein kinase with sequence similarities to ERK1/ERK2 [3] (not shown). Although MKK purified from Sf9 cells coexpressing oncogenes is very active, it can be further activated by Raf-1 *in vitro* and thus consists of a mixture of active and inactive forms (results not shown).

Purified recombinant MKK2 from a single viral infection appeared to be significantly more active (20-fold) than MKK1 from a single infection. This is a consistent but surprising finding. These data may suggest a difference in regulation of MKK1 and MKK2 *in vivo*. Indeed, MKK1, but not MKK2, can be phosphorylated and inactivated *in vitro* by $p34^{cdc2}$ kinase [15]. Infection of insect cells with wild-type baculovirus may activate a subset of signal-transduction pathways. Kozma et al. [27] demonstrated that viral infection activated both forms of $p70^{s6k}$, which are thought to lie on a distinct pathway from MAPK. By analogy, infection *per se* might account for the basal activities of recombinant MKK1 and MKK2 from single infections. MKK1 or MKK2 purified from single infections of Sf9 cells can be completely inactivated by incubation with protein phosphatase 2A catalytic subunit. Thus the basal observed activity of MKK1 and MKK2 is caused by phosphorylation. These inactivated preparations could be re-activated by incubation *in vitro* using partially purified activated Raf-1 (results not shown).

These active recombinant MKK proteins will greatly facilitate generation of homogenous active $p42^{mapk}$ (including thio-phosphorylation) to study downstream targets. Furthermore, these characterized preparations of MKK1 and MKK2 will facilitate efforts to identify novel MKK-activating protein kinases and their regulation.

In vivo and *in vitro* activation of recombinant MKK1

The effect of coexpression of activating upstream oncogenes on MKK1 activity was quantified, and the effects of substitutions of kinase-defective Raf-1 and N-terminally truncated Raf-1 were determined. Recombinant MKK1 purified from Sf9 cells and infected with baculoviruses expressing Δ7-Raf-1, $pp60^{Src}$ (Y527F) and c-Ha-Ras was 200-fold more active than MKK1 from Sf9 cells singly infected (Table 2). Recombinant MKK2 was activated 140-fold by coexpression (results not shown). Substitution of kinase-defective Raf-1 (K375M) for Δ7-Raf-1 resulted in MKK1 specific activities no different from single infections, consistent with studies supporting direct activation of MKK/MEK by Raf-1. Baculovirus expressing N-terminally truncated 22W-Raf-1 [19] (nearly equivalent to the activating truncation in v-Raf) was ~ 8-fold less effective than Δ7-Raf-1 in catalysing the activation of MKK1.

Recombinant MKK1, isolated from singly infected Sf9 cells, could be activated *in vitro* with partially purified activated Raf-1 (Y340D) (Table 2), in agreement with previous reports [17]. MKK1 was phosphorylated by activated Raf-1 (Y340D) but not by kinase-defective Raf-1 (K375M) isolated from cells under identical conditions, including co-infected viruses for c-Ha-Ras and activated $pp60^{Src}$. Phosphorylation of MKK1 by activated Raf-1 correlated with increases in enzyme activity as judged by K52R phosphorylation (Table 2).

The observation that Raf-1 (Y340D or Δ7) was more

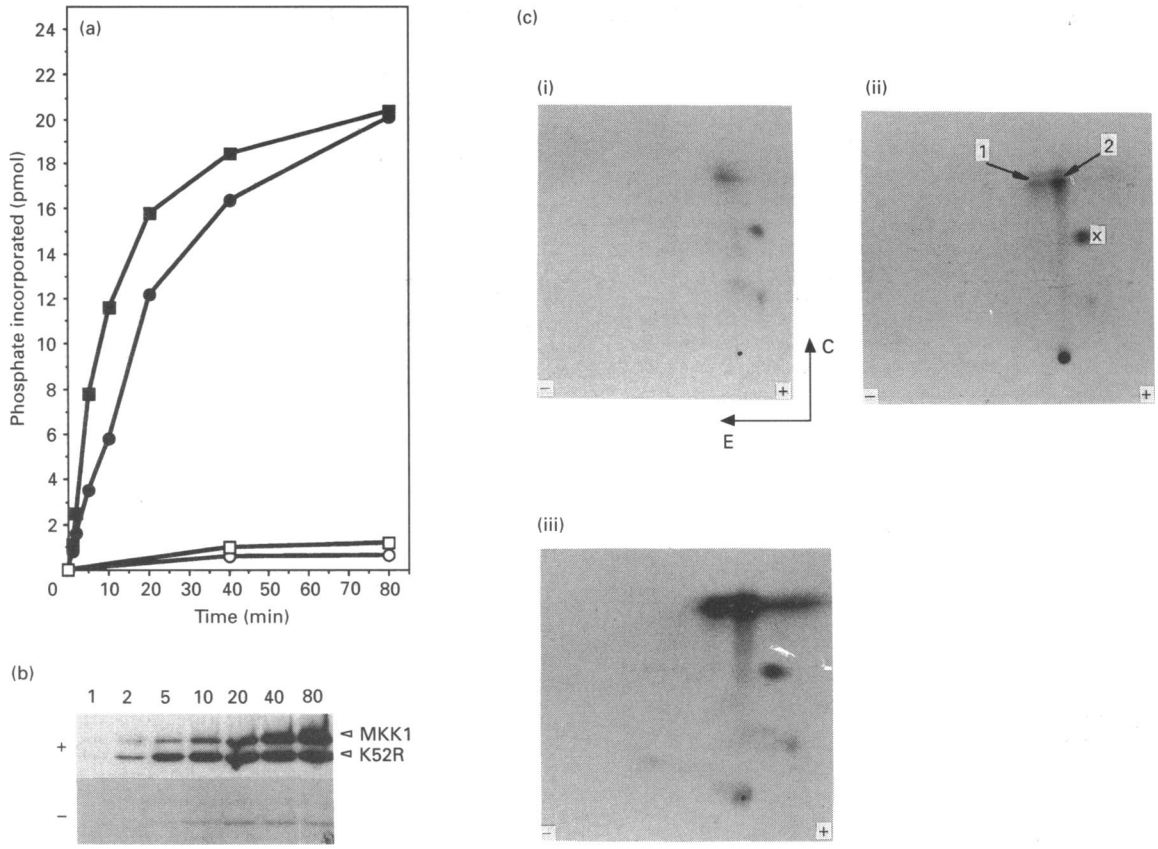

Figure 4 Correlation of MKK1 activation by Raf-1 with serine phosphorylation of a tryptic phosphopeptide

(**a**) Time course of phosphorylation of recombinant MKK1 by Raf-1 (Y340D) (●) and activation towards K52R (■). Activated Raf-1 (Y340D) (5 μg), MKK1 (0.5 μg) and K52R (2 μg) were incubated together as described in the Materials and methods section, and the incorporation of [^{32}P]phosphate into K52R was determined. A similar experiment was performed using FSBA-treated MKK1 0.5 μg and omitting K52R to measure the incorporation of [^{32}P]phosphate into MKK1. Control incubations were performed identically using Raf-1 (K375M) (○, □). (**b**) Autoradiograph directly showing that phosphorylation of MKK1 by Raf-1 (Y340D) correlates with activation of MKK1 versus K52R. The assays presented in the autoradiograph marked + contained activated Raf-1 (Y340D), whereas the autoradiograph marked — contained Raf-1 (K375M), as a control. The time (min) of MKK1 incubation with Raf-1 protein preparation is shown above each lane. This experiment is representative of several, each using different preparations of MKK1 and Raf-1 (Y340D). (**c**) Autoradiographs of tryptic [^{32}P]phosphopeptide maps (see the Materials and methods section) of recombinant ^{32}P-labelled FSBA-treated MKK1 phosphorylated by activated Raf-1 (Y340D) for 1, 10 and 40 min (i, ii and iii, respectively) performed in parallel with (**a**) and (**b**). The exposure time for all panels was 5 h at —70 °C (5–20000 c.p.m. loaded per plate). The electrophoretic (E) dimensions are indicated by + and —, and the ascending chromatography (C) dimension is indicated by the arrow to the right of (i). The origin is marked by a dot in (i). The tryptic phosphopeptides are marked 1 and 2 in (ii).

efficacious in activating MKKs *in vivo* than 22W-Raf-1 was surprising considering that the 22W protein possessed the most transforming potential of a series of N-terminal Raf-1 mutants, including v-Raf itself [19]. This difference in efficacy may result from differences in levels of expression of 22W-Raf-1 and Raf-1 and/or inherent differences in kinetics. Alternatively, the N-terminal histidine tag of 22W-Raf-1 might interfere with kinase activity or protein stability. 22W-Raf-1 appeared to be labile to incubation at 30 °C *in vitro*. 22W-Raf-1 phosphorylated MKK1 substoichiometrically to an apparent plateau of ~ 0.10 mol/mol of MKK1 *in vitro* (results not shown), in contrast with activated Raf-1 (Y340D) which could incorporate up to 2 mol/mol of MKK1 (see below). However, further addition of 22W-Raf-1 to phosphorylation reactions that appeared to have plateaued resulted in increased levels of MKK1 phosphorylation (results not shown), implying lability of 22W-Raf-1.

Activation of MKK1 by Raf-1 is correlated with serine phosphorylation of a tryptic phosphopeptide

MKK is regulated by serine/threonine phosphorylation as judged by inactivation with protein serine/threonine phosphatases but not protein tyrosine phosphatases [1]. To correlate activation of MKK1 by Raf-1 with phosphorylation of site(s) within MKK1, parallel temporal studies of MKK1 phosphorylation and activation were performed (Figure 4). Recombinant MKK1 undergoes endogenous autophosphorylation at unidentified sites. To prevent autophosphorylation from complicating the interpretation of the phosphate-incorporation of peptide-mapping experiments, MKK1 was catalytically inactivated by treatment with FSBA (see the Materials and methods section).

Phosphorylation of FSBA-treated MKK1 by activated Raf-1 (Y340D) reached a plateau after 80 min at ~ 1.9 mol of phosphate/mol of enzyme (Figure 4a). In a separate assay, the extent of phosphorylation of MKK1 correlated well with the extent of activation of untreated MKK1, as judged by phosphorylation of K52R (Figure 4b). Time-course progression curves for MKK activity and MKK phosphorylation are similar in shape and approximately superimposable by normalization. These data are most consistent with a requirement for two sites for activation but do not rule out the possibility that only one is required. To determine which phosphopeptides correlate with

activation, we performed tryptic phosphopeptide mapping of ^{32}P-labelled FSBA-treated MKK1 at selected time points.

FSBA-treated MKK1 protein phosphorylated by activated Raf-1 (Y340D) was purified by SDS/PAGE, and the FSBA-treated MKK1 protein was extracted from the acrylamide gel, and subjected to two-dimensional mapping (Figure 4c). At 1 min, one major phosphopeptide was observed [Figure 4c (i)]. However, as phosphorylation and activation progressed, a more negatively charged phosphopeptide (with lower electrophoretic mobility) but with similar chromatographic mobility predominated [Figure 4c (ii) and (iii)]. Phosphoamino acid analysis of the peptides marked 1 and 2 [Figure 4c, (ii)] revealed that they both contained phosphoserine (P. Dent, Y.-H. Chow, J. Wu, D. K. Morrison, R. Jove and T. W. Sturgill, unpublished work).

An additional spot, labelled X, was detected and increased in apparent ^{32}P content from 1 min to 10 min but did not appear to contain a phosphoamino acid. Thus spot X may be a contaminant or a phosphopeptide, assuming technical difficulties. Regardless, spot X correlates poorly with activation in comparison with phosphopeptides 1 and 2.

We suspected that activating phosphorylation of MKK1 by Raf-1 might occur at Ser^{222}, nine amino acids N-terminal to the conserved (S)PE motif because the mutant produced by mutation of the corresponding Thr in STE7 [28] is non-functional (B. Errede, personal communication) and because activating phosphorylations of MAPK, pp90^{rsk}, cyclin-dependent protein kinase and glycogen synthase kinase 3 also occur in this region [29]. However, a synthetic tryptic peptide from MKK1 containing Ser^{222} [MKK1-(206–227) was not a substrate *in vitro* for our purified activated Raf-1 preparations. Furthermore, 20 μM MKK1-(206–227) failed to competitively inhibit the phosphorylation of MKK1 by activated Raf-1 (Y340D) or Δ7-Raf-1 (results not shown). These results precluded comparative peptide-mapping experiments.

While this manuscript was in preparation, Zheng and Guan [30] demonstrated that both Ser^{218} and Ser^{222} in bacterially expressed MEK1/MKK1 appeared to be critical for MKK1 activation *in vitro*, using Raf-1 immunoprecipitates from stimulated fibroblasts to phosphorylate MKK1. The replacement of either serine residue with threonine resulted in MKK1 phosphorylation on serine and threonine residues by Raf-1 immunoprecipitates, whereas only phosphoserine was detected without mutagenesis. The mutation of either residue to alanine resulted in an MKK1 enzyme that was incapable of activation. Mutation of both serine residues to alanine also generated an MKK1 protein completely incapable of phosphorylation. Thus by site-directed mutagenesis, these workers provide strong evidence that MKK1 is regulated by dual serine phosphorylation. Furthermore, mutagenesis of either of the two corresponding residues in STE7 [28] (Ser^{359} and Thr^{363}) to alanine (B. Errede, personal communication) also resulted in an enzyme that was completely incapable of activation *in vivo* and *in vitro*.

Tryptic digestion of activated MKK1 (phosphorylated *in vivo*) from Sf9 cells labelled with [^{32}P]orthophosphate revealed a similar pattern of phosphopeptides tentatively assumed to correspond to singly and doubly phosphorylated MKK-(206–227), which co-migrated in mixing experiments with phosphopeptides 1 and 2 generated *in vitro* (P. Dent, Y.-H. Chow, J. Wu, D. K. Morrison, R. Jove and T. W. Sturgill, unpublished work). In addition, three minor phosphopeptides were observed in MKK1 phosphorylated *in vivo* (unpublished work). These data indicate that Raf-1 phosphorylates the same sites *in vivo* and *in vivo*. The sequence surrounding Ser^{218} and Ser^{222} is conserved in MKK2. Tryptic phosphopeptide maps of MKK2 phosphorylated by Raf-1 are identical with those for MKK1 (unpublished work). Thus it appears reasonable to assume that activation of MKK2 by Raf-1 also occurs by phosphorylation of Ser^{218} and Ser^{222}.

The most likely explanation for our data is that we are observing an increase in phosphorylation of the two sites of serine phosphorylation by Raf-1 within MKK1 in our peptide maps [Figure 4c, compare (i) with (ii) and (iii)]. At the earliest time point examined (1 min), only one phosphopeptide was observed (peptide 1). However, by 10 min a major more acidic version of the first phosphopeptide had appeared (peptide 2). This implies that the purified Raf-1 preparation had incorporated more phosphate into the same peptide. Of note, the predicted charge of the doubly phosphorylated tryptic peptide containing Ser^{218} and Ser^{222} is neutral at pH 1.9, which agrees with our data [Figure 4c (i) and (ii), peptide 2]. Thus our biochemical results are compatible with the dual-site identification by Zheng and Guan [30]. The inability of Raf-1 to phosphorylate a synthetic tryptic peptide containing these two serines is not exceptional and is analogous to the inability of MKK to phosphorylate a peptide containing the regulatory phosphorylation sites in the MAPK protein. Further biochemical studies of phosphorylation of Ser^{218} and Ser^{222} are needed to test the proposed mechanism rigorously. It should be emphasized that novel activating kinases may utilize MKK 1 sites other than Ser^{218} and Ser^{222}.

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