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MEKK1 phosphorylates MEK1 and MEK2 but does not cause activation of mitogen-activated protein kinase

(extracellular signal-related protein kinase/protein kinase cascades/Jun N-terminal kinase/stress-activated protein kinase)

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ABSTRACT **A constitutively active fragment of rat MEK kinase 1 (MEKK1) consisting of only its catalytic domain (MEKK-C) expressed in bacteria quantitatively activates recombinant mitogen-activated protein (MAP) kinase/extracellular signal-regulated protein kinase (ERK) kinases 1 and 2 (MEK1 and MEK2) *in vitro*. Activation of MEK1 by MEKK-C is accompanied by phosphorylation of S218 and S222, which are also phosphorylated by the protein kinases c-Mos and Raf-1. MEKK1 has been implicated in regulation of a parallel but distinct cascade that leads to phosphorylation of N-terminal sites on c-Jun; thus, its role in the MAP kinase pathway has been questioned. However, in addition to its capacity to phosphorylate MEK1 *in vitro*, MEKK-C interacts with MEK1 in the two-hybrid system, and expression of mouse MEKK1 or MEKK-C in mammalian cells causes constitutive activation of both MEK1 and MEK2. Neither cotransfected nor endogenous ERK2 is highly activated by MEKK1 compared to its stimulation by epidermal growth factor in spite of significant activation of endogenous MEK. Thus, other as yet undefined mechanisms may be involved in determining information flow through the MAP kinase and related pathways.**

The mitogen-activated protein (MAP) kinase pathway has been implicated in the actions of numerous hormones, growth factors, and oncogene products including Ras (1, 2). The importance of intracellular processes thought to be regulated by the MAP kinases has focused attention on understanding the mechanism of regulation of this pathway. MAP kinase kinases or MAP kinase/extracellular signal-regulated protein kinase (ERK) kinases (MEKs) phosphorylate and activate the MAP kinases ERK1 and ERK2 (3). Extracellular signals that control the MAP kinase cascade through tyrosine kinase-coupled receptors, G-protein-coupled receptors, and protein kinase C all are thought to work through two closely related MEKs—MEK1 and MEK2. Thus, identifying the mechanisms of regulation of MEKs will be important for understanding the complexity and diversity of intracellular signaling mechanisms that impinge on this pathway.

In yeast, several distinct MAP kinase pathways called modules (4–6) have been identified that regulate disparate physiological responses to separate extracellular challenges. MAP kinase modules consist of a MAP kinase, an activating enzyme (or MEK), and an enzyme that activates MEK (a MAP kinase kinase kinase or MEKK). In the yeast pheromone signaling cascade, STE11 is the MEKK that activates the MEK homolog STE7, which then phosphorylates and activates the MAP kinase homolog FUS3. Lange-Carter *et al.* (7) have cloned a mammalian relative of STE11, MEKK1,§ based on its similarity to STE11, and demonstrated that MEKK1, when overexpressed in COS cells, increases MEK activity.

A second MAP kinase module has been identified in mammalian cells, which apparently does not involve the activation of ERK1 or -2. The terminal enzymes of this module are the Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) (8, 9). MEKK1 has recently been shown to cause greater activation of JNK/SAPK than MAP kinase in transfected cells, leading to the hypothesis that MEKK1 is not involved in the MAP kinase pathway (10).

In this study, we used a constitutively active catalytic domain fragment of MEKK1 (MEKK-C) expressed in bacteria to demonstrate the capacity of MEKK1 to activate MEKs *in vitro*. Using the two-hybrid system, we found that MEKK-C interacts with MEK1. Furthermore, both MEK1 and MEK2 are highly activated upon cotransfection with MEKK1, suggesting a strong relationship between MEKK1 and MEK1 and -2. On the other hand, neither cotransfected nor endogenous ERK2 is highly activated by MEKK1 in spite of activation of endogenous MEK.

MATERIALS AND METHODS

Plasmids. To express the catalytic domain of MEKK1, an *Nco* I/*Bam*HI fragment from a rat MEKK1 cDNA (S.X., D.R., and M.H.C., unpublished data) was subcloned into the vector pETHis$_6$TEV (GIBCO/BRL), which contains six histidine residues downstream of the start codon. The cDNA clones of mouse MEK1 (from Anne Gardner and Gary Johnson, National Jewish Center, Denver) and human MEK2 (from Kun-Liang Guan, University of Michigan, Ann Arbor) were introduced into the *Bam*HI site of pRSETA. Mutants of human MEK1 (MKK1) were prepared and expressed as described (11) and were the kind gifts of Sam Mansour and Natalie Ahn (University of Colorado, Boulder). To express hemagglutinin (HA) epitope-tagged MEKK1 (HA-MEKK), a fragment of mouse MEKK was created by PCR with the primers (5 → 3) GGACTAGTCTGGTTGGCAAGCTCTCT and CGGATCCCTACCACGTGGTACG with the codon for the first methionine of the MEKK cDNA and including the stop codon. After digestion with *Spe* I and *Bam*HI, the fragment was inserted into a mammalian expression vector that contained the HA epitope digested with *Nhe* I and *Bam*HI (pCEP4HA,

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Abbreviations: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAP kinase/ERK kinase; MEKK1, MEK kinase 1; MEKK-C, catalytic domain of MEKK1; JNK/SAPK, Jun N-terminal kinase/stress-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor.

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§Because there appear to be other related kinases, the MEKK identified by Lange-Carter *et al.* (7) is referred to throughout as MEKK1.

kindly provided by T. Geppert, University of Texas Southwestern Medical Center). The PCR product was sequenced and no mutations were found. Using similar strategies, ERK2 (12), a truncated form of MEKK1 containing the C-terminal 281 amino acids (MEKK-C), and the cDNAs of mouse MEK1 and human MEK2 were also subcloned into pCEP4HA. To express nontagged MEKK1, the rat MEKK1 cDNA was inserted into the *Eco*RI site of pCMV5 (from David Russell, University of Texas Southwestern Medical Center). For two-hybrid interaction analysis MEKK-C and wild-type ERK2 were subcloned into the GAL4 DNA-binding domain vector pAS1cyh and MEK1 and Elk1 were subcloned into the GAL4 activation domain vector pACT2 (from Steve Elledge, Baylor University, Houston).

Cell Culture, Transfection, and ^{32}P Labeling. 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% L-glutamine. Cells (≈80% confluent) were transfected with DNA by calcium phosphate coprecipitation (13) and maintained for 24–48 h after transfection. Transfection efficiency, judged by percentage of cells expressing β-galactosidase from pCMV5, was consistently 20–30%. To label cells with ^{32}P, cells were incubated in serum-free Krebs–Ringer bicarbonate solution containing 2% bovine serum albumin for 60 min, and then ^{32}P (1 mCi/ml; 1 Ci = 37 GBq) was added to the medium for an additional 60 min. Cells were washed with chilled phosphate-buffered saline and lysed on ice for 10 min in 0.5 ml of lysis buffer (7) per 60-mm dish. Lysates were collected and sedimented at 14,000 × *g* for 10–15 min at 4°C. Supernatants were stored at −80°C or used immediately as described.

Protein Quantitation. The concentrations of recombinant proteins were estimated by the Lowry method and by comparison of serial dilutions to dilutions of bovine serum albumin (quantified by measuring the OD_{280}) on Coomassie blue-stained SDS/polyacrylamide gels.

Kinase Assays. MEKK-C, expressed and purified as described (14), was assayed in 30 μl of 10 mM Hepes, pH 8.0/10 mM $MgCl_2$/1 mM benzamidine/1 mM dithiothreitol/100 μM ATP (1000 cpm/pmol) with the indicated concentration of MEK1 or MEK2 at 30°C for 60 min unless otherwise indicated. A 5-μl aliquot of the reaction mixture was removed to determine the activities of activated MEKs using a kinase-deficient ERK2 mutant (K52R ERK2) purified from bacteria as described (14) as a substrate at 50 μg/ml.

Antibodies. Antisera raised against a C-terminal peptide of MEKK1 (7) have been described. A monoclonal antibody to the HA epitope (12CA5) was purchased from Babco (Richmond, CA). Isoform-specific antisera to MEK1 (A2227) and MEK2 (A2228) were produced in rabbits (Peptide Express, Fort Collins, CO) with peptides from MEK1 (CQVEGDAAETPPR) and MEK2 (AIFGRPVVDGEEGEPHSIS).

Immunoprecipitation. Soluble extracts from cells were incubated with the indicated antibodies and protein A-Sepharose at 4°C with rotation for 2 h. The beads were washed with cold lysis buffer followed by cold 0.25 M Tris·HCl (pH 7.6) plus 0.1 M NaCl. MEK activity in immunoprecipitates was measured in 50 μl reaction mixtures with contents as described above plus 50 μg of recombinant K52R ERK2 per ml at 30°C for 60 min. Slurries of beads (10 μl) were assayed and the reactions were stopped by sedimenting the beads and adding an equal volume of 2× electrophoresis sample buffer to the supernatant.

Yeast Two-Hybrid Analysis. The yeast two-hybrid system was used as described (15) with the following modifications. For all clones, the yeast strain Y190 was used. Cells were transformed by the LiOAc method (16). Clones containing both the GAL4 DNA-binding domain and activation domain plasmids were grown on synthetic complete medium lacking tryptophan, leucine, and histidine. For interaction with MEKK-C, clones were grown on medium supplemented with 25 mM 3-aminotriazole (15). ERK2-interacting clones were grown in the presence of 50 mM 3-aminotriazole. Growth of clones was determined 5–7 days after transformation with the appropriate activation domain constructs.

RESULTS

Expression and Activity of a Fragment Containing the Catalytic Domain of Rat MEKK1. A rat MEKK1 cDNA clone encoding an enzyme ≈95% identical within the catalytic domain to mouse MEKK1 was isolated as described (S.X., D.R., and M.H.C., unpublished data). A catalytic domain fragment (MEKK-C), lacking the majority of N-terminal sequence, was constructed with an N-terminal hexahistidine tag, expressed in bacteria, and purified on Ni^{2+}-NTA agarose. It was recognized by antibodies raised against a C-terminal MEKK1 peptide (7) (Fig. 1*A*) and it phosphorylated MEK1 (Fig. 1*B Upper left*). This phosphorylation activated MEK1, as measured by the enhanced ability of MEK1 to phosphorylate catalytically defective (K52R) ERK2 (Fig. 1*B Lower left*). Under these conditions, MEKK-C does not significantly phosphorylate ERK2 (see Fig. 3). The phosphorylation of MEK1 (Fig. 2 *Upper*) was associated with a large increase in its protein kinase activity using wild-type ERK1 (data not shown), ERK2, or catalytically defective K52R ERK2 (Fig. 2 *Lower*) as substrates. Under the conditions of our assay, MEKK-C

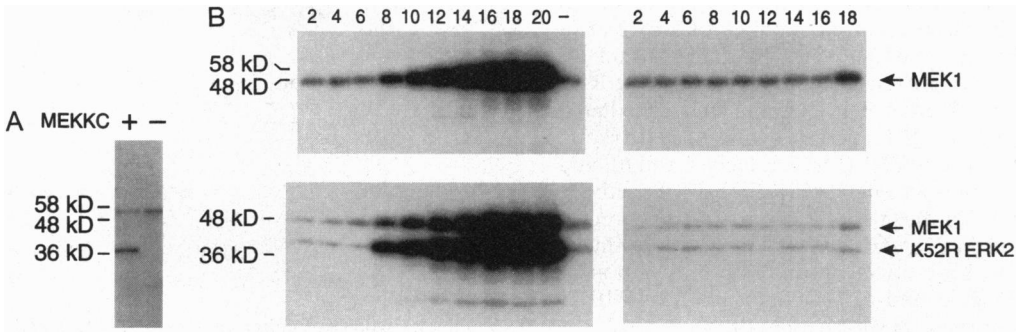

FIG. 1. Bacterial expression of His-MEKK-C. Bacterial strains that did and did not express His_6-MEKK-C were cultured at room temperature and induced with 10 mM isopropyl β-D-thiogalactopyranoside for 15 h. After induction, the bacteria were lysed and histidine-tagged protein was purified on Ni^{2+}-NTA agarose. (*A*) Expression of MEKK-C protein in lysates was detected by using anti-MEKK-C antibody. (*B*) (*Upper*) MEK kinase activity in the Ni^{2+}-NTA agarose fractions from lysed bacteria with (*Left*) and without (*Right*) His_6-MEKK-C in the eluted fraction was analyzed by incubation with MEK1 (50 μg/ml) in the presence of [γ-^{32}P]ATP and Mg^{2+}. Numbers of fractions assayed are indicated above lanes. Lane −, control containing substrate but no fraction. (*Lower*) Activation of MEK1 was measured by using bacterially expressed K52R ERK2 as a substrate.

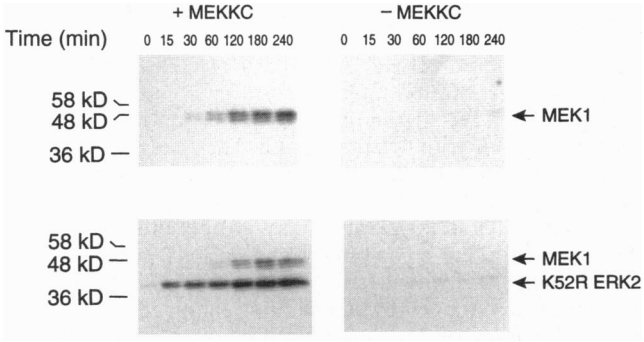

FIG. 2. Time course of MEK1 phosphorylation by MEKK-C. MEK1 (10 μg/ml) was incubated with or without MEKK-C in the presence of [γ-^{32}P]ATP and Mg^{2+} at 30°C. At the indicated times, 20-μl aliquots of the reaction mixtures were removed and placed on ice. Half of each sample was transferred to a tube containing an equal volume of 2× electrophoresis sample buffer (*Upper*), and the rest was used to measure phosphorylation of K52R ERK2 at 30°C for 5 min (*Lower*).

phosphorylated MEK1 and MEK2 with specific activities of 46.8 nmol·min^{-1}·mg^{-1} and 20.5 nmol·min^{-1}·mg^{-1}, respectively. MEKK-C catalyzed the incorporation of ≈2 mol of phosphate per mol of MEK1 and 2.7 mol per mol of MEK2. Purified MEKK-C used either magnesium or manganese as divalent cation to phosphorylate MEKs, with optima of 5 and 3 mM, respectively.

Properties of Activated MEK1 and MEK2. Phosphorylation resulted in the appearance of two labeled bands in the MEK1 and MEK2 preparations, one with nearly the same mobility as unphosphorylated protein and one with a clearly decreased mobility. The most highly phosphorylated proteins were largely shifted in electrophoretic mobility and had specific activities of 105 and 113 nmol·min^{-1}·mg^{-1}, in reasonable agreement with the specific activity reported for enzyme purified from A431 cells (3). In contrast, after autophosphorylation for 8 h the specific activities of MEK1 and MEK2 were only 0.6 and 1.6 nmol·min^{-1}·mg^{-1}, indicating that autophosphorylation does not result in substantial activation.

Phosphorylation of MEK1 Mutants by MEKK-C. The sites of phosphorylation on MEK1 by Raf-1 (17) and c-Mos (11)—S218 and S222—have been determined. We tested MEK1 mutants with substitutions at these sites as well as S212, another potential phosphorylation site (11), to ascertain whether MEKK-C phosphorylates the same sites *in vitro* as do c-Mos and Raf-1. MEKK-C phosphorylated S212A MEK1 as well as wild-type MEK1 (Fig. 3*A*, lanes 2 and 8). Activation of S212A MEK1 was comparable to wild-type MEK1, as shown by a reduction in the electrophoretic mobility of ERK2 after phosphorylation by these MEKs (Fig. 3*B*, lanes 2 and 8). Retardation of mobility correlated with activation of ERK2 confirmed in a coupled assay (Fig. 3*C*). On the other hand, neither S218A nor S222A MEK1 was as highly phosphorylated by MEKK-C as wild-type MEK1 (Fig. 3*A*, lanes 4 and 6), and their ability either to phosphorylate K52R ERK2 or to activate wild-type ERK2 was significantly reduced when compared to wild-type protein (Fig. 3 *B* and *C*, lanes 4 and 6). Interestingly, these mutations had little effect on autophosphorylation of MEK1 [Fig. 3*A*, lanes 3 and 5, although in S212A MEK1 autophosphorylation (lane 1) was increased].

Activation of HA-Tagged MEK1 and MEK2 Cotransfected with MEKK-C. To determine whether MEK activities were increased in cells expressing MEKK-C, 293 cells were cotransfected with MEKK-C plus either MEK1 or MEK2 tagged with the HA epitope (HA-MEK1, HA-MEK2). HA-MEK1 and HA-MEK2 were immunoprecipitated with the HA antibody and aliquots of the immunoprecipitates were immunoblotted with the HA antibody (Fig. 4, *Lower right*) as well as with MEK

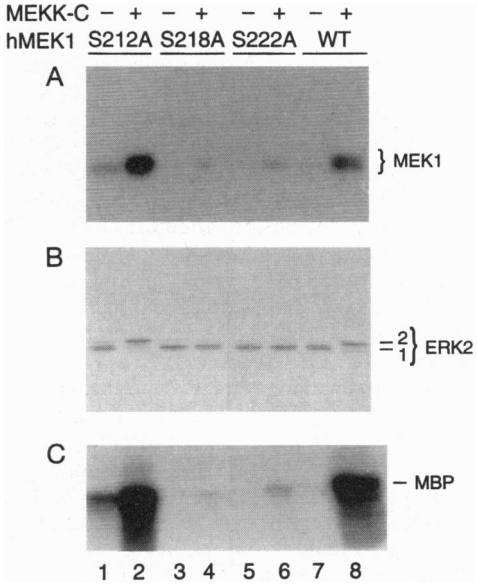

FIG. 3. Phosphorylation and activation of MEK1 mutants. (*A*) Wild-type (WT) and mutant human MEK1 (hMEK1) proteins (20 μg/ml) were incubated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) MEKK-C at 30°C for 4 h. cpm incorporated into MEK1 S218A and S222A were 23–25% of wild-type protein. (*B*) A 1-μl aliquot of each reaction mixture was used to phosphorylate ERK2, and the ERK2 mobility shift was detected by using 9% polyacrylamide gels with an acrylamide/bisacrylamide ratio of 29:1. ERK2 was stained with Coomassie blue. A reduced ERK2 mobility correlated with phosphorylation on the activating sites. (*C*) ERK2 kinase activity was also measured by phosphorylation of myelin basic protein (MBP) (0.3 mg/ml) (18).

isoform-specific antibodies (Fig. 4 *Lower left* and *center*) to confirm that nearly equal amounts of epitope-tagged protein were present in all immune complexes. The activities of both MEKs in the HA immune complexes as assessed by phosphorylation of K52R ERK2 as substrate (Fig. 4 *Upper right*) were increased by coexpression with MEKK-C. Coexpression with MEKK-C also increased the incorporation of phosphate into each MEK (Fig. 4 *Upper left*). These data indicate that

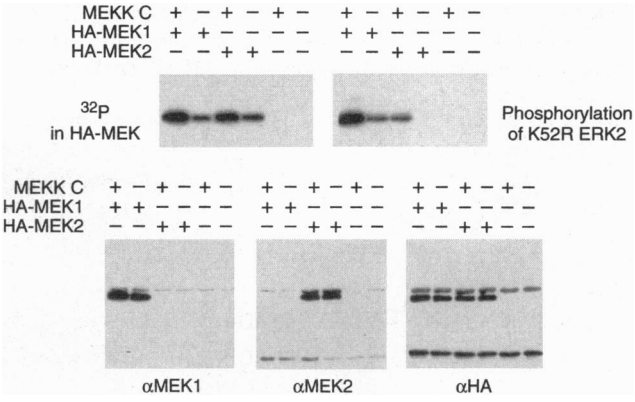

FIG. 4. MEKK-C enhances phosphorylation and activity of MEK1 and MEK2 in mammalian cells. MEKK-C was cotransfected with either HA-MEK1 or HA-MEK2 as indicated in 293 cells; 48 h later, these cells were labeled with $^{32}P_i$. HA-MEK1 and HA-MEK2 were immunoprecipitated with anti-HA antibodies. (*Upper left*) Autoradiograph of ^{32}P-labeled immunoprecipitates. (*Upper right*) Autoradiogram of immune complex kinase assay using K52R ERK2 as a substrate. Western blots of immunoprecipitated MEK1 and MEK2 with antibodies to MEK1 (*Lower left*), MEK2 (*Lower center*), and HA (*Lower right*).

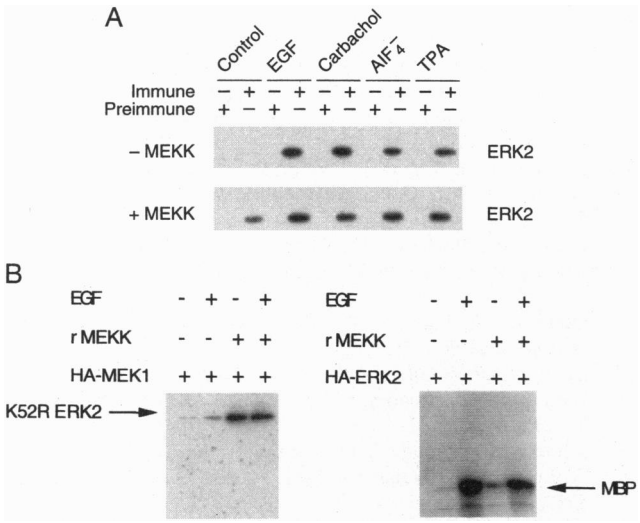

FIG. 5. Activation of MEK1 but not ERK2 in cells transfected with MEKK1. (*A*) Cells were transfected with or without MEKK1 and then treated with EGF, carbachol, AlF_4^-, or phorbol 12-myristate 13-acetate (TPA) for 5, 15, 15, and 15 min, respectively. Endogenous MEK1 was immunoprecipitated and assayed using K52R ERK2. (*B*) Cells were transfected with either HA-MEK1 or HA-ERK2 as indicated. Half of the dishes were also transfected with MEKK1. After 48 h, cells were not stimulated or stimulated with EGF for 5 min before lysis and immunoprecipitation. Activity of HA-MEK1 was assayed with K52R ERK2 and activity of HA-ERK2 was assayed with myelin basic protein (MBP).

MEKK-C activates both MEK1 and MEK2 when expressed in eukaryotic cells.

MEKK1 Activates MEK1 and MEK2 but Not ERK2. Endogenous and cotransfected MEK1 (Fig. 5) and MEK2 (data not shown) are activated in cells transfected with MEKK1 even in the absence of a growth factor signal. The fraction of endogenous MEK1 activated is the same as the proportion of cells expressing MEKK1. Transfected HA-MEK1 is more highly activated by cotransfection with MEKK1 (17.4-fold) than by epidermal growth factor (EGF) (2.9-fold; Fig. 5*B Left*). In contrast, activation of endogenous ERK2 by MEKK1, as assessed by mobility shift, is minimal (data not shown). Furthermore, cotransfection of HA-ERK2 with MEKK1 increases its myelin basic protein kinase activity only slightly (2.4-fold) compared to activation of HA-ERK2 achieved with EGF (12.7-fold; Fig. 5*B Right*). In fact, expression of MEKK1 reduces EGF-stimulated kinase activity of ERK2 by nearly one-third.

MEKK-C and MEK1 Interact in the Two-Hybrid System. As a further test of the possible relationship of MEKK1 and MEK1, their interaction was tested in the yeast two-hybrid system (Table 1). In this system, yeast cells containing interacting proteins are able to grow on medium lacking histidine and also show a marked increase in the activity of a *lacZ* reporter gene. The strength of interaction is roughly proportional to both the rate of growth of these clones on selective medium and their respective β-galactosidase activities. As shown in Table 1, the interaction of MEKK-C and MEK1 in this system appears to be stronger than that of MEK1 and ERK2, two proteins that are known to interact *in vivo* (19). As expected, MEKK-C did not bind to the transcription factor Elk1, thus suggesting that the MEKK-C–MEK1 interaction is specific. In parallel experiments, ERK2 was shown to interact with its substrate Elk1.

Table 1. Interaction of MEKK-C and MEK1 in the yeast two-hybrid system

	GAL4 Act	
GAL4 DBD	MEK1	Elk1
MEKK-C	+++	–
ERK2	++	+

Yeast strain Y190 containing either MEKK-C or ERK2 fused to the GAL4 DNA-binding domain (GAL4 DBD) was transformed with GAL4 activation domain (GAL4 Act) fusions containing either MEK1 or Elk1. Interaction between fusion proteins promotes growth on medium lacking histidine. +, Rate of growth of clones; –, no growth.

DISCUSSION

MEKK-C, a constitutively active, catalytic domain fragment of rat MEKK1 expressed in bacteria, catalyzed the quantitative activation of MEK1 and MEK2. Recombinant MEKK-C from bacteria, unlike preparations of Raf-1 and c-Mos, is free from contaminating eukaryotic protein kinases that might otherwise contribute to the catalytic activity of the preparation. Not surprisingly, MEK1 is phosphorylated by MEKK-C on the same sites that are phosphorylated in intact cells (20, 21) and by c-Mos and Raf-1 (11, 20) *in vitro*.

In 293 cells, expression of either MEKK-C or the complete mouse MEKK1 sequence leads to phosphorylation and activation of both MEK1 and MEK2 in the absence of extracellular stimuli. Furthermore, MEK1 interacts with MEKK-C in the yeast two-hybrid system. Together, the results from *in vitro*, two-hybrid, and eukaryotic expression studies support a relationship between MEKK1 and MEK. However, in spite of the activation of MEK, activation of MAP kinase in cells expressing MEKK1 is minimal. This is reminiscent of effects of the inducible expression of an oncogenic form of Raf-1 (22) on activation of MAP kinase by EGF. Thus, our findings suggest that the ultimate functions of MEK may depend on the manner in which it is activated.

MEKK1 has been reported to activate JNK/SAPK more efficiently than ERKs (10). This conclusion was largely based on the respective activities of JNK/SAPK and ERKs after overexpression of MEKK1 and therefore does not preclude the possibility that MEKs, as well as the JNK/SAPK activator, may be MEKK1 substrates. If this is true, an interesting question is how are MEKs significantly activated within the cell without the concomitant activation of ERKs. It may be that overexpression of MEKK1 leads to expression of ERK-specific phosphatases such as MKP-1, which would rapidly inactivate MAP kinase. It is also possible that MEKK1 may form complexes with MEK that prevent interactions with ERKs, or that MEK activated by MEKK1 may be abnormally localized so that ERK1 and ERK2 are no longer accessible as substrates. A preliminary experiment with sodium vanadate suggests that inhibition of tyrosine phosphatase has no effect on MAP kinase activity in MEKK1-expressing cells. The detection of MEK1 but not MEK2 in a complex with Ras and Raf-1 in mammalian cells (19) suggests that such specificity in complex formation may exist. In *Saccharomyces cerevisiae*, the issue of complex formation among MAP kinase module components has been more thoroughly investigated. The STE5 protein promotes formation of a signaling complex among STE11, STE7, and FUS3/KSS1 and is absolutely required for efficient signaling through this module (23, 24). Given the known conservation between yeast and mammals of MAP kinase signaling cascades, it seems likely that nucleation factors like STE5 exist in metazoans and that proteins such as these may restrict the function of potentially promiscuous enzymes, such as MEKK1, to maintain the specificity of individual signaling pathways.

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