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MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42

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MEK kinases (MEKKs) 1, 2, 3 and 4 are members of sequential kinase pathways that regulate MAP kinases including c-Jun NH_2-terminal kinases (JNKs) and extracellular regulated kinases (ERKs). Confocal immunofluorescence microscopy of COS cells demonstrated differential MEKK subcellular localization: MEKK1 was nuclear and in post-Golgi vesicular-like structures; MEKK2 and 4 were localized to distinct Golgi-associated vesicles that were dispersed by brefeldin A. MEKK1 and 2 were activated by EGF, and kinase-inactive mutants of each MEKK partially inhibited EGF-stimulated JNK activity. Kinase-inactive MEKK1, but not MEKK2, 3 or 4, strongly inhibited EGF-stimulated ERK activity. In contrast to MEKK2 and 3, MEKK1 and 4 specifically associated with Rac and Cdc42 and kinase-inactive mutants blocked Rac/Cdc42 stimulation of JNK activity. Inhibitory mutants of MEKK1–4 did not affect p21-activated kinase (PAK) activation of JNK, indicating that the PAK-regulated JNK pathway is independent of MEKKs. Thus, in different cellular locations, specific MEKKs are required for the regulation of MAPK family members, and MEKK1 and 4 are involved in the regulation of JNK activation by Rac/Cdc42 independent of PAK. Differential MEKK subcellular distribution and interaction with small GTP-binding proteins provides a mechanism to regulate MAP kinase responses in localized regions of the cell and to different upstream stimuli.

Keywords: Cdc42/epidermal growth factor/MEK kinases/Rac/subcellular distribution

Introduction

The mitogen-activated protein kinase (MAPK) family of serine/threonine kinases is composed of the extracellular regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs) and p38 kinases (for review, see Kyriakis and Avruch, 1996). The MAPKs are components of sequential kinase cascades which are phosphorylated and activated by an intermediate MAPK kinase in the pathway; MAPK kinases are themselves phosphorylated and activated by MAPK kinase kinases (for reviews, see Seger and Krebs, 1995; Denhardt, 1996). The MAPK kinase kinases include Raf which regulates the ERK pathway (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992) and a group of MEK (MAP/ERK kinase) kinases (MEKKs) which, although they have the ability to activate ERK (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996), appear to preferentially regulate the JNK pathway (Kyriakis *et al.*, 1994; Minden *et al.*, 1994; Yan *et al.*, 1994). In addition to Raf and MEKKs, other kinases have been proposed to function as MAPK kinase kinases including germinal center kinase (GCK), p21-activated kinase (PAK), TGF-β-activated kinase (TAK), tumor progression locus-2 (Tpl-2), mixed lineage kinase-3 (MLK-3), mitogen-activated protein kinase kinase kinase 5 (MAPKKK5) and apoptosis signal regulating kinase 1 (ASK1) (Bagrodia *et al.*, 1995; Pombo *et al.*, 1995; Yamaguchi *et al.*, 1995; Brown *et al.*, 1996; Rana *et al.*, 1996; Salmeron *et al.*, 1996; Teramoto *et al.*, 1996a; Wang *et al.*, 1996; Ichijo *et al.*, 1997). Thus, there are potentially many different kinases where biochemical and limited genetic evidence suggests these kinases could function as MAPK kinase kinases. Why so many kinases might function as MAPK kinase kinases is unclear; an obvious explanation would be that these kinases allow input into the MAPK pathways from many diverse stimuli (for review, see Fanger *et al.*, 1997).

Possibly the best example of multiple cellular stimuli feeding into the MAPK pathways is illustrated by the JNK pathway. In many cell types, growth factors such as EGF and cytokines including IL-1 and TNFα activate the JNK pathway (Coso *et al.*, 1995). Also, stress stimuli including heat shock, DNA damaging agents like cisplatin and irradiation can activate the JNK pathway (Xu *et al.*, 1996; for review, see Kyriakis and Avruch, 1996). Certainly, agents like cisplatin and growth factors such as EGF have dramatically different mechanisms of action and different initial cellular signaling responses leading to JNK activation.

We have cloned four MEKKs, referred to as MEKK1, 2, 3 and 4 based on the order in which they were cloned (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Gerwins *et al.*, 1997). Each functions as a MAPK kinase kinase capable of activating the JNK pathway. MEKK1, 2 and 3 are also capable of activating the ERK pathway to varying degrees in transfection experiments, whereas MEKK4 has little ability to activate the ERKs. MEKK1–4 do not appear to be involved in the regulation of the p38 pathway.

The deduced primary sequence of MEKK1, 2, 3 and 4 predicts specific properties for each (for review, see Fanger *et al.*, 1997). The kinase domain of each MEKK is encoded at the COOH-terminal end, with unique features in the NH_2-terminal regulatory moiety. MEKK1 is a 196 kDa protein having a proline-rich motif near its NH_2-terminus, one or two predicted pleckstrin homology domains and a modest cysteine-rich domain. The MEKK1 kinase domain region and a small sequence just upstream of the kinase

domain have been shown to bind Ras in a GTP-dependent manner (Russell *et al.*, 1995). MEKK2 and 3 are smaller kinases being ~70 kDa with no demonstrable regulatory domains except possible bipartite nuclear localization signals in their NH_2-terminus (Blank *et al.*, 1996). MEKK4 is a 180 kDa protein which is similar to MEKK1 and has a proline-rich motif near its NH_2-terminus, a predicted pleckstrin homology domain and a modified Cdc42/Rac interactive binding (CRIB) domain adjacent to its kinase domain. The kinase domains of MEKK2 and 3 are 96% conserved at the amino acid level indicating they are extremely homologous in their catalytic regions, whereas their NH_2-terminal moieties are quite different. Relative to MEKK2 and 3 the kinase domains of MEKK1 and 4 are ~55% conserved in amino acid sequence and similarly conserved towards each other. As with MEKK2 and 3, the NH_2-terminal domains of MEKK1 and 4 are also dramatically different. Therefore the sequences of MEKK1, 2, 3 and 4 indicate their regulation may be quite different even though they all appear capable of activating the JNK pathway.

In this report, we demonstrate that the MEKK family members have distinct intracellular distributions. In addition, we show activation of MEKK family members by epidermal growth factor and utilizing kinase-inactive mutants more clearly define the role of each MEKK, and the specificity inherent to each kinase, in mediating the effects of this growth factor on MAPK family members. Furthermore, evidence that the MEKK family members differentially interact with GTP-binding proteins, and are not required for JNK activation by PAK, provide a better mechanistic understanding of the sequence of events involved in regulation of not only the MEKKs, but also the MAPK family members. These findings define the MEKK proteins as localized sensors in the cell for regulation of MAPK pathways.

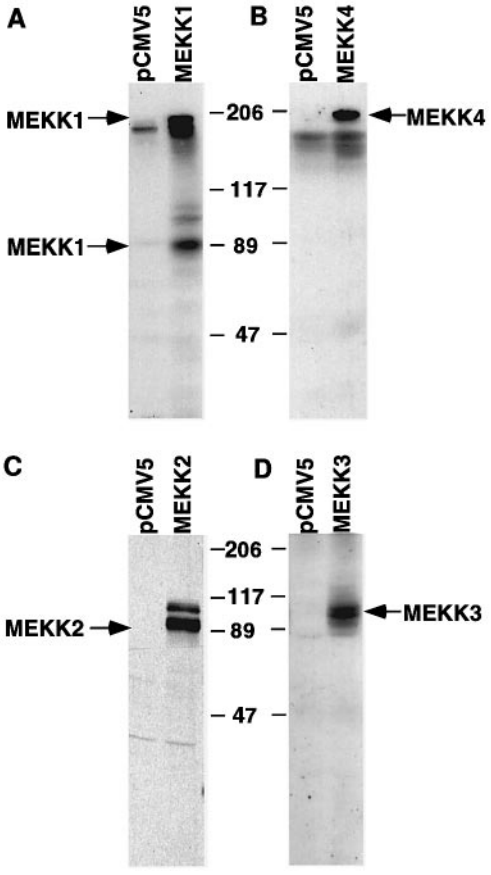

Fig. 1. Characterization of type-specific MEKK antibodies used for immunofluorescence. Peptides corresponding to unique sequence within each MEKK family member were generated and used to derive MEKK type-specific antibodies (see Materials and methods). Western blot analysis was performed on lysates from COS cells transfected with either empty mammalian expression vector (pCMV5) or pCMV5 vector expressing the full-length forms of (**A**) MEKK1, (**B**) MEKK4, (**C**) MEKK2 or (**D**) MEKK3. Relative sizes are indicated by molecular weight markers.

Results

Subcellular localization of MEKKs

Figure 1 shows immunoblots for type-specific MEKK antibodies using lysates from control and MEKK1, 2, 3 and 4 transfected COS cells. Endogenous MEKK1 is 196 kDa and, due to different phosphorylation states, the recombinantly expressed MEKK1 migrates as a triplet at about this size. MEKK1 also has a 90 kDa proteolytic cleavage fragment (C.Widmann and G.Johnson, submitted for publication) which due to low abundance is difficult to observe, but is present in untransfected lysates and readily apparent when expressed by transfection. Recombinant MEKK4 migrates as a slightly larger protein than the endogenous 180 kDa MEKK4 protein because of its hyperphosphorylated state and its epitope-tag. Endogenous MEKK2 and 3 are difficult to observe in immunoblots because of their low abundance. Transfected MEKK2 and 3 migrate as bands larger than their calculated size due in part to their phosphorylation, which also generates multiple gel-shifted bands. The antibodies for MEKK1 and 2 were immunoprecipitating, whereas those for MEKK3 and 4 were not. The antibody for MEKK3 did not stain cells using indirect immunofluorescence procedures that were successful with the antibodies raised against epitopes for MEKK1, 2 and 4.

Figure 2 shows the subcellular localization of MEKK1, 2 and 4 that was determined using confocal immunofluorescence microscopy. Analysis was performed by co-staining with either wheat germ agglutinin (WGA) to identify plasma membrane and Golgi or anti-adaptin (AP-1) antibody, a marker specific for the Golgi adaptor complex AP-1 (Ahle *et al.*, 1988). For reference, J, K and L show the profiles of Golgi, mitochondria and the endoplasmic reticulum, respectively. Figure 2A and B demonstrate that MEKK1 is found in the nucleus and dispersed in punctate vesicular-like structures in the cytoplasm. Figure 2D and E show that MEKK2 localization is different from that of MEKK1 in that it is found predominantly in the Golgi, as well as a distinct cytoplasmic punctate pattern. Similar to MEKK2, but in contrast to MEKK1, MEKK4 appears strictly Golgi-associated (Figure 2G and H). Using AP-1 as a marker for clathrin-coated membranes of the *trans*-Golgi network and brefeldin A (BFA) to induce rapid redistribution of coat proteins associated with clathrin-coated vesicles from the *trans*-Golgi (Orci *et al.*, 1991), Figure 2B/C, E/F and H/I demonstrate that MEKK2 and 4, but not MEKK1, are associated with structures that lead to their redistribution in response to BFA. These findings demonstrate that

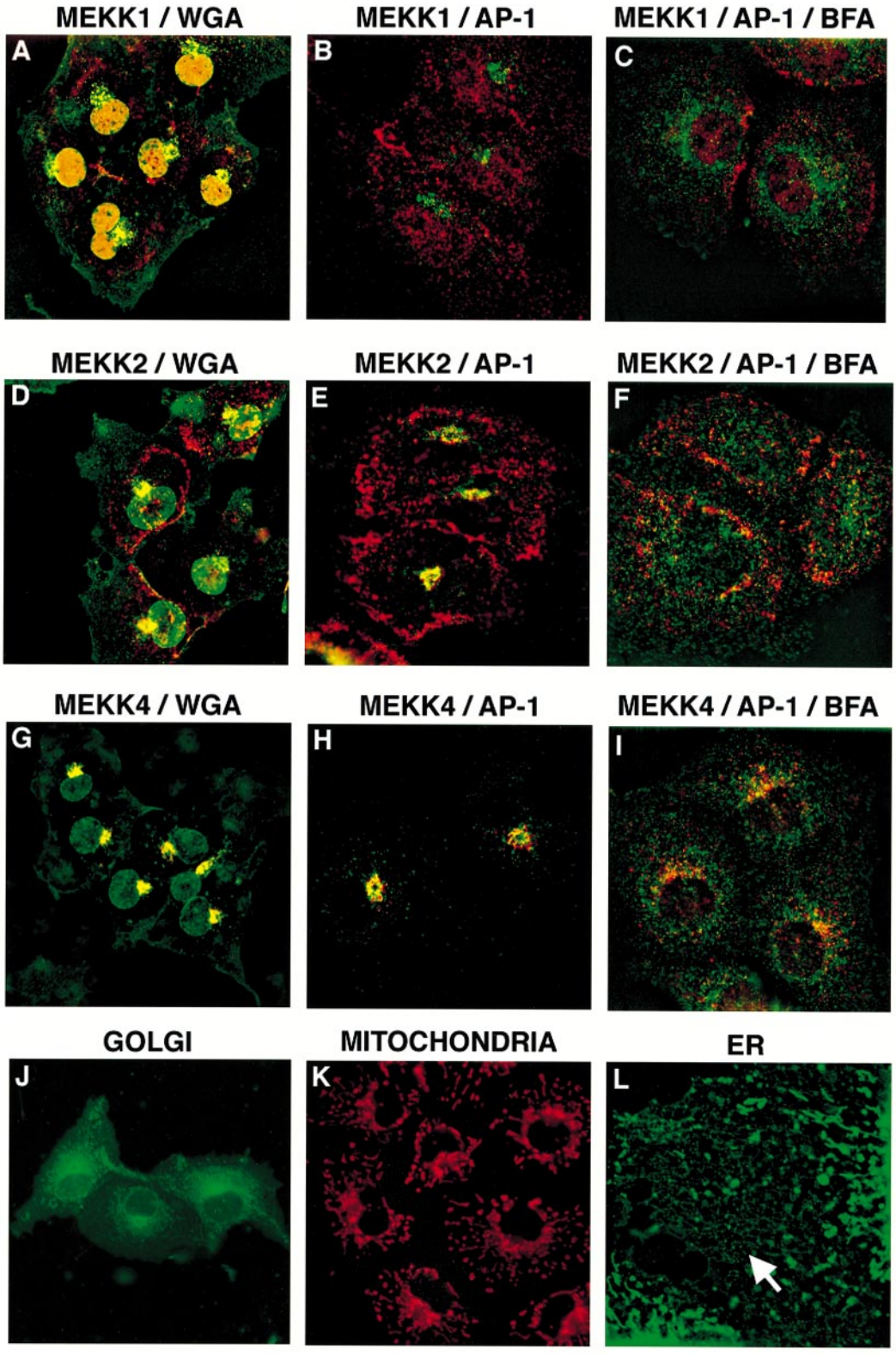

Fig. 2. Subcellular localization of MEKK family members. COS cells were plated onto glass coverslips and, using type-specific MEKK antibodies, digital confocal immunofluorescence was utilized to determine the subcellular localization of the MEKK family members. In (A), (D) and (G), cells were stained with a combination of fluorescein conjugated WGA (green) to better visualize cell membrane (WGA also stains the Golgi complex in COS cells), as well as with type-specific antibodies directed against either (**A**) MEKK1, (**D**) MEKK2 or (**G**) MEKK4 (red). In (B), (E) and (H), cells were stained with a combination of an anti-adaptin (AP-1) antibody that identifies the Golgi complex by recognizing the Golgi adaptor complex AP-1 (green), as well as with antibodies specific for (**B**) MEKK1, (**E**) MEKK2 and (**H**) MEKK4 (red). Cells were treated with brefeldin A (BFA) to induce redistribution of coat proteins associated with clathrin-coated vesicles from the *trans*-Golgi and stained with anti-AP-1 (green) in combination with antibodies specific for (**C**) MEKK1, (**F**) MEKK2 and (**I**) MEKK4. In order to better identify subcellular structures (**J**) the Golgi complex was identified with BODIPY-ceramide, (**K**) the mitochondria was identified with rhodamine 123 and (**L**) the endoplasmic reticulum (ER) (denoted by arrow) was identified with $DIOC_6(3)$.

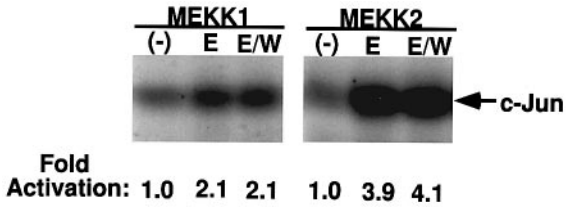

Fig. 3. Regulation of MEKK1 and 2 by EGF. Representative example of at least three complex kinase assays where either endogenous MEKK1 or 2 was immunoprecipitated following stimulation with EGF. Where indicated cells were pretreated with 100 nM wortmannin (W) prior to EGF (E) stimulation. c-Jun phosphorylation was measured following addition of recombinant wild type JNKK and JNK. Samples were separated by SDS–PAGE and visualized with autoradiography. PhosphorImager analysis was utilized to determine the relative changes in MEKK1 and 2 activity.

MEKK2 and 4 co-localize with Golgi-associated structures. However, a component of MEKK2 is also found localized in BFA resistant structures in the cytoplasm. In no instance did MEKK1, 2 or 4 appear associated with the mitochondria or endoplasmic reticulum. These findings were verified using additional antibodies recognizing independent epitopes of MEKK1 and 2 and a green fluorescent protein–MEKK4 fusion as described in the Materials and methods. Thus, MEKK1, 2 and 4 are found in unique subcellular locations in the cell with MEKK2 and 4 overlapping with BFA sensitive coat proteins in the *trans*-Golgi. The differential distribution of the MEKK proteins suggests selective roles for each in responses involving MAPK pathways.

MEKK1 and 2 are activated by EGF

It has been particularly difficult to demonstrate the regulation of MEKKs in response to extracellular stimuli primarily because transiently expressed MEKKs are constitutively active and immunoprecipitation of endogenous MEKKs results in high basal activity. The high basal activity of MEKKs when recombinantly expressed and/or immunoprecipitated is similar to STE11p in *Saccharomyces cerevisiae*, a MAPK kinase kinase component of the pheromone response pathway (Neiman and Herskowitz, 1994). To overcome this problem we used dilutions of MEKK1 and 2 immunoprecipitations from control and EGF stimulated COS cells in an *in vitro* sequential protein kinase assay (Gardner *et al.*, 1994; Blank *et al.*, 1996). In this assay, bacterially expressed JNK kinase and JNK are added to the kinase reaction and the MEKK activation of the pathway is assayed by monitoring JNK phosphorylation of GST–c-Jun (Figure 3). In three independent experiments imager analysis of the phosphorylated substrate demonstrated EGF stimulated the activity of MEKK1 an average of 2.1-fold and MEKK2 an average of 3.9-fold.

In mast cells we have also been able to demonstrate MEKK1 activation in response to crosslinking of the FcεRI receptor (Ishizuka *et al.*, 1996). MEKK1 and JNK activation in response to FcεRI ligation is inhibited by wortmannin, suggesting an involvement of phosphatidylinositol 3-kinase (PI3-K) in the regulation of mast cell MEKK1. Even though EGF elicited a 4.2-fold increase in PI3-K activity which was inhibited by wortmannin (data not shown), in contrast to the results with the FcεRI receptor, wortmannin did not inhibit either MEKK1 or 2 activation in response to EGF in COS cells (Figure 3). Thus, in COS cells, MEKK1 and 2 are activated by stimulation of the EGF receptor independent of PI3-K activation.

MEKK regulation of JNK and ERK activation in response to EGF

In order to define the role of MEKK1, 2, 3 and 4 in mediating activation of pathways associated with MAPK family members, JNK and ERK activation was measured in response to expression of activated MEKK family members or EGF stimulation in the presence of wortmannin or kinase-inactive mutants of each MEKK family member. Expression of MEKK1, 2, 3 and 4, where the NH_2-terminal regulatory domains have been deleted (ΔMEKK1–4), results in activation of JNK (Figure 4A) to similar (~5-fold) levels and ERK (Figure 4B) to varying degrees. Although sorbitol elicited a 3.5-fold activation, none of the ΔMEKKs were able to stimulate p38 activation (data not shown). In COS cells EGF is also capable of activating both JNK and ERK (Figure 4C and D, respectively) which, similar to the activation of MEKK1 and 2, is insensitive to the effects of wortmannin (compare Figure 3 with 4C and D) indicating PI3-K is not involved. As illustrated in Figure 4E, only expression of a kinase-inactive inhibitory mutant of MEKK1 and not the kinase-inactive inhibitory mutants of MEKK2, 3 or 4 inhibit ERK activation by EGF in COS cells. The inhibition of ERK activation by kinase-inactive MEKK1 was >80%. Immunoblotting of the epitope-tagged MEKKs indicated they were expressed at similar levels (not shown), demonstrating the selectivity of kinase-inactive MEKK1 in inhibiting ERK activation. The inhibitory effect of kinase-inactive MEKK1 on activation of the ERK pathway is most likely related to the interaction of MEKK1 with Ras (Russell *et al.*, 1995). Finally, kinase-inactive mutants of each MEKK expressed in COS cells only modestly inhibited EGF-mediated JNK activation, suggesting that multiple MEKKs and possibly additional kinases are involved in regulating the JNK pathways in response to EGF (data not shown).

Kinase-inactive MEKK1 and 4 block Rac and Cdc42 activation of the JNK pathway

Constitutively activated mutants of Cdc42 and Rac (Cdc42QL and RacQL) stimulate JNK activity, whereas inhibitory mutants of Cdc42 and Rac having high affinity for GDP (N17Cdc42 and N17Rac) inhibit JNK activation by EGF, indicating that these low molecular weight GTP-binding proteins are critical mediators of the effects of growth factors on JNK activation (Coso *et al.*, 1995; for review, see Vojtek and Cooper, 1995). Figure 5 demonstrates that kinase-inactive mutants of MEKK1 and 4 block Rac and Cdc42 stimulation of the JNK pathway. In contrast, kinase-inactive mutants of MEKK2 and 3 do not affect Rac and Cdc42 stimulation of the JNK pathway. Figure 5A shows a representative experiment, whereas Figure 5B demonstrates that equal amounts of HA-tagged JNK were immunoprecipitated from each sample. Figure 5C and D show the average of four to six experiments for Cdc42 and Rac, demonstrating, respectively, the regulation of the JNK pathway and selective inhibition by kinase-inactive MEKK1 and 4. These findings strongly

implicate both MEKK1 and 4 in Rac/Cdc42 regulated signal transduction pathways.

MEKK1 and 4 associate with Rac1 and Cdc42

Recombinant GST fusion proteins for Rac, Cdc42 and Rho were purified from bacteria and loaded with either GDP or GTP. The recombinant proteins were combined with COS cell lysates expressing truncated forms of the MEKK proteins that encode the COOH-terminal moiety including the kinase domain to determine if the MEKK proteins interacted with the low molecular weight GTP-binding proteins. MEKK1 associated with the GTP-bound Rac and Cdc42, but not when they were loaded with GDP (Figure 6A). MEKK1 did not associate with Rho in either a GTP or GDP bound state. MEKK2 and 3 did not associate with Cdc42, Rac or Rho in either nucleotide bound state (Figure 6B and C, respectively). The full-length forms of MEKK2 and 3 were also unable to associate with Rac, Cdc42 or Rho (data not shown) providing additional evidence that these two MEKKs do not bind to the Rho family of GTP-binding proteins. Although MEKK4 bound to all three GTP-binding proteins in both the GTP and GDP bound forms, MEKK4 did not bind to GST alone (Figure 6D) or to several other GST fusion proteins (data not shown).

To validate the findings with bacterially expressed GST fusions we prepared recombinant polyhistidine fusions of Cdc42 and Rho in *Sf*9 cells using baculovirus infection. Similar results were obtained where MEKK1 bound to Cdc42 in a GTP dependent manner and did not bind to Rho (Figure 6E). In contrast to MEKK1, MEKK4 bound to Cdc42 and Rho in both the GTP and GDP states (Figure 6F). These findings highlight the differential binding characteristics of MEKK1 and 4 to low molecular weight GTP-binding proteins and predict different potential regulatory functions for Cdc42/Rac in the control of MEKK1 and 4.

MEKK1 and 4 bind directly to Cdc42 and Rac

To determine if the interaction of MEKK1 and 4 with Rac and Cdc42 was direct, MEKK family members were expressed as maltose binding protein (MBP) fusions in bacteria and purified. Recombinant Rac and Cdc42 were loaded with [γ-^{32}P]GTPγS and incubated with either MEKK1, 3 or 4 bound to amylose beads, washed, and the relative binding was determined for each MEKK–bead preparation. Rac and Cdc42 bound to MEKK1 and 4, but not to either MEKK3 or protein that encodes only MBP (Figure 7), confirming the findings in Figure 6 that MEKK1 and 4 interact with Rac and Cdc42. Interestingly, the ability of MEKK4 to bind Rho [γ-^{32}P]GTPγS was low relative to its association with Cdc42 and Rac (not shown). The reason for this is not apparent, because the specific activity of the Rho [γ-^{32}P]GTPγS was similar to that for Rac and Cdc42. This suggests that the interaction of Rho with MEKK4 (see Figure 6) may involve another protein and, although this binding event may be indirect, it

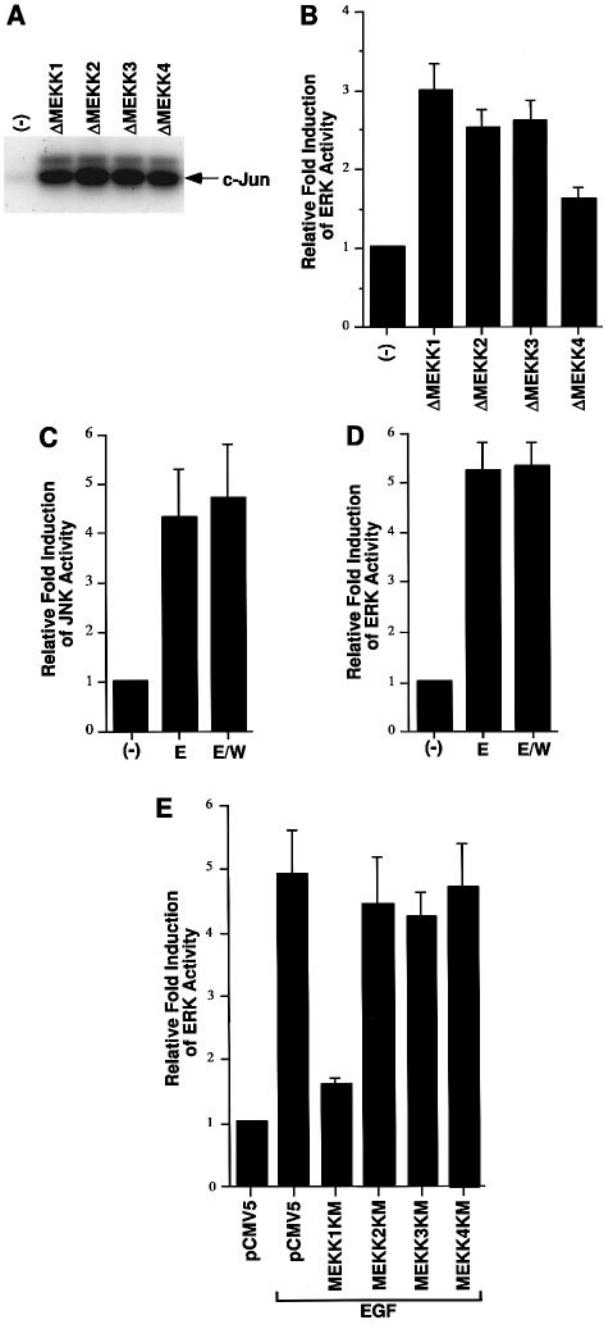

Fig. 4. Analysis of JNK and ERK pathways in COS cells. (**A**) Representative example of three experiments where endogenous JNK was precipitated from cell lysates with GST–c-Jun conjugated to Sepharose beads following transfection of either empty expression vector (–) or NH_2-terminally truncated forms of specific MEKK family members (ΔMEKK1–4). Activity was measured in a kinase reaction following the addition of [γ-^{32}P]ATP. Samples were separated by SDS–PAGE and the relative JNK activity was evaluated using PhosphorImager analysis. (**B**) Representative MAPK assay illustrating results from three separate experiments where either empty expression vector (–) or NH_2-terminally truncated MEKK family members (ΔMEKK1–4) were expressed and endogenous ERK activity was immunoprecipitated with an anti ERK2 antibody. Relative changes in ERK activity were assayed by [γ-^{32}P]ATP phosphorylation of substrate peptide (EGFR$^{662–681}$) and determined by scintillation counting. Average relative changes in JNK (**C**) and ERK2 (**D**) activity [measured as described in (A) and (B), respectively] following EGF (E) stimulation either with or without 100 nM wortmannin (W) pretreatment. Average of three separate experiments are shown. (**E**) Average EGF-mediated activation of MAPK activity following expression of kinase-inactive forms of the MEKK family members (MEKK1–4KM) and HA–ERK2. Empty expression vector (pCMV5) was included as necessary to maintain equal DNA concentrations during transfection. Averages represent four separate experiments. Error bars represent the standard deviation.

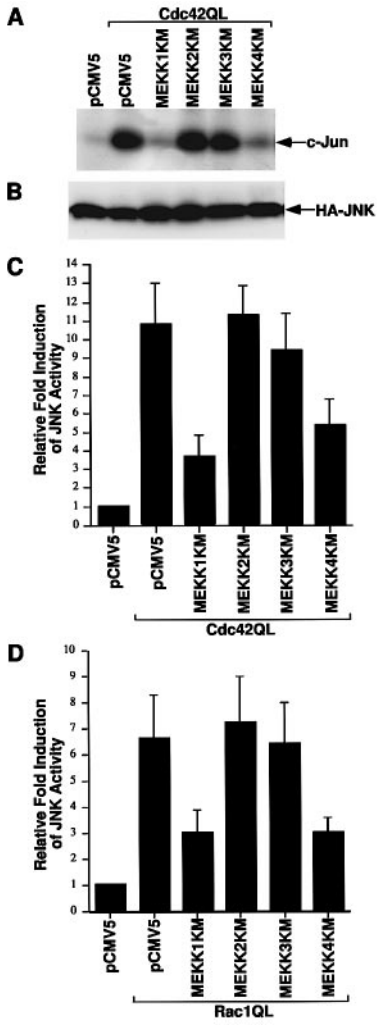

Fig. 5. MEKK1 and 4, but not MEKK2 and 3 contribute to JNK activation by Cdc42 and Rac. COS cells were transfected with activated forms of either Cdc42Q61L or RacQ61L along with kinase-inactive forms of each MEKK family member (MEKK1–4KM). Empty expression vector (pCMV5) was included as necessary to maintain equal DNA concentrations during transfection. JNK activity was measured following immunoprecipitation of HA-JNK. (**A**) Autoradiograph of a representative JNK assay. (**B**) Western blot of a portion of the HA–JNK immunoprecipitation demonstrating the equivalent levels of HA–JNK utilized in the assay. Average fold inductions of JNK activation from 4–6 separate experiments following stimulation with either (**C**) Cdc42 or (**D**) Rac. Error bars represent the standard deviation.

contrasts to the direct binding of MEKK1 and 4 to Cdc42 and Rac.

Inhibitory mutant MEKKs do not block PAK stimulation of JNK activity

The PAKs bind Cdc42 and Rac in a GTP-dependent manner (Manser *et al.*, 1994) and mediate specific responses involving Cdc42/Rac (Zhang *et al.*, 1995; Westwick *et al.*, 1997). The homology between the PAKs and STE20p (Manser *et al.*, 1994), which mediates specific responses involving Cdc42p in *S.cerevisiae* (Leberer *et al.*, 1997), has prompted the hypothesis that PAKs are intermediate in the regulatory pathway Cdc42/Rac→PAK→MEKK (Minden *et al.*, 1995; for review, see Vojtek and Cooper, 1995). However, the association of MEKK1 and

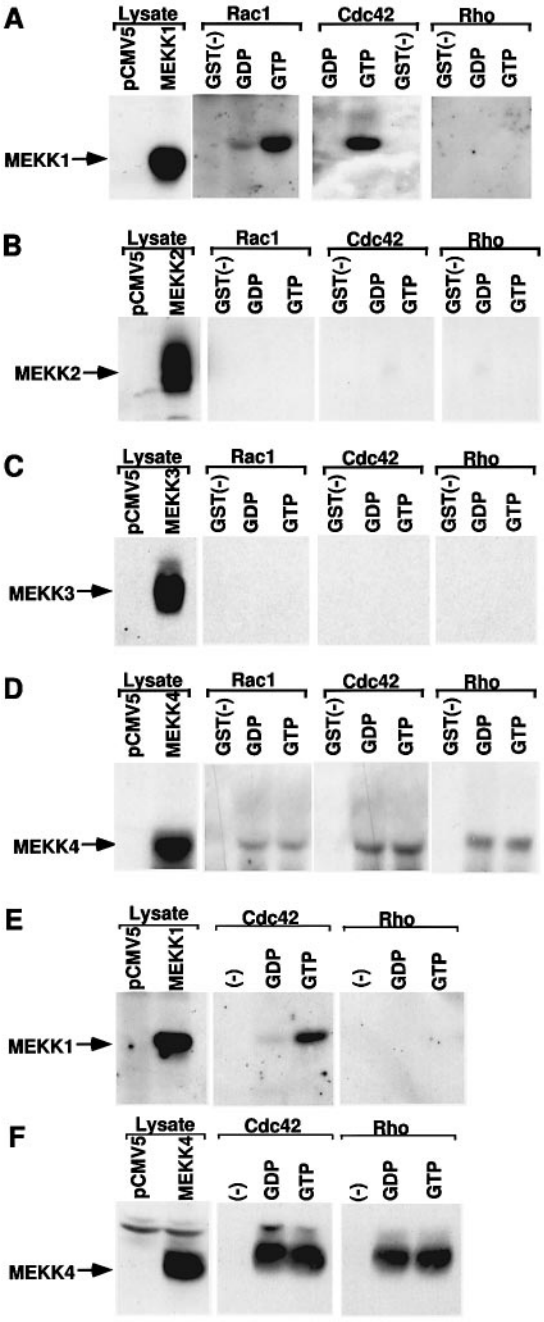

Fig. 6. MEKK1 and 4, but not MEKK2 or 3 associate with Rac and Cdc42. COS cells were transfected with mammalian expression plasmids expressing COOH-terminal kinase domains encoding either MEKK1, 2, 3 or 4. In (**A**), (**B**), (**C**) and (**D**) lysates were incubated with 10 μg of bacterially expressed GST fusions of either Cdc42, Rac, or Rho loaded with either GDP or GTPγS and conjugated to Sepharose beads. GST alone [GST(–)] was also included as a negative control for nonspecific binding. In (**E**) and (**F**), COS cell lysates recombinantly expressing either MEKK1 or 4 were incubated with 10 μg of *Sf*9 expressed polyhistidine (pHIS) tagged Cdc42 or Rho loaded with either GDP or GTPγS and conjugated to Sepharose beads. NTA-Ni Sepharose beads preincubated with *Sf*9 lysate (–) were included as a negative control for nonspecific binding. After washing, precipitates were separated by SDS–PAGE, transferred to nitrocellulose and Western blotted with type-specific antibodies for (A) and (E) MEKK1, (B) MEKK2, (C) MEKK3, and (D) and (F) MEKK4.

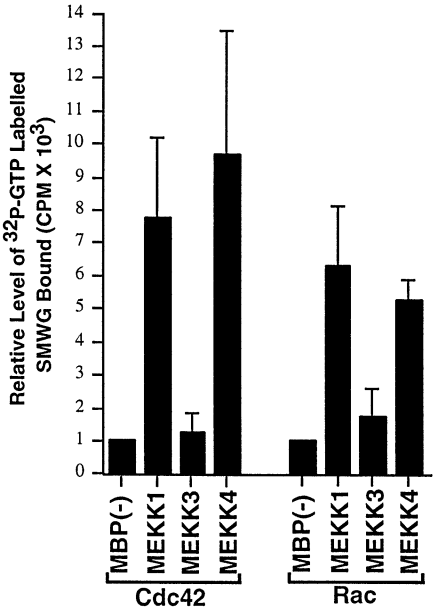

Fig. 7. MEKK1 and 4 bind directly to Rac and Cdc42. 1 μg of GST–Cdc42 or GST–Rac was loaded with [γ-^{32}P]GTPγS and incubated with 10 μg of bacterially expressed MBP fusions of MEKK1, 3 and 4 conjugated to amylose resin. MBP alone [MBP(–)] was used as a negative control for nonspecific binding. After washing, reactions were counted with a scintillation counter and plotted relative to control levels. Values represent at least three separate experiments. Error bars represent the standard deviation.

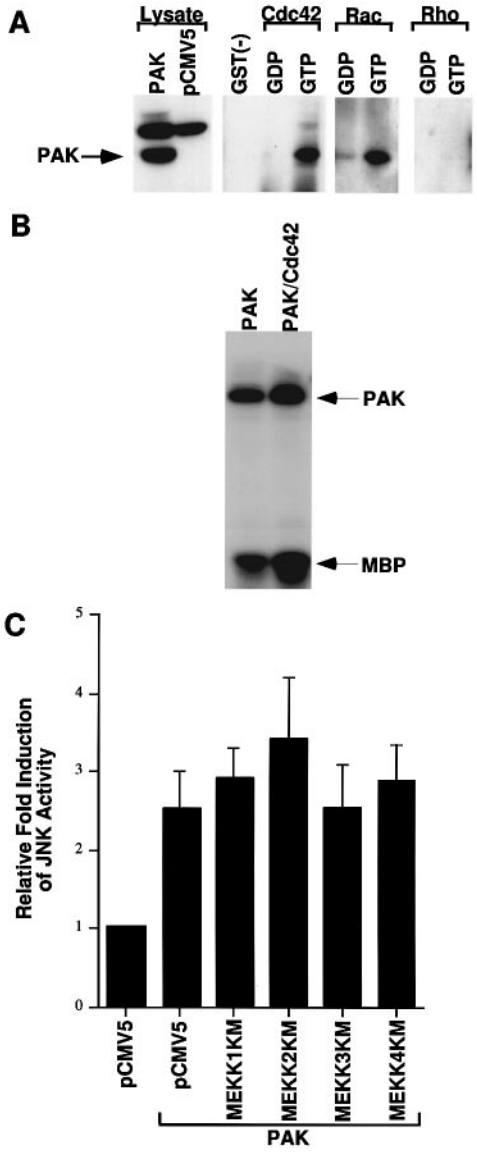

Fig. 8. MEKKs do not lie downstream and mediate the effects of PAK. **(A)** Western blot of haemagglutinin tagged PAK. COS cells were transfected with a plasmid expressing the full-length rat βPAK gene and lysates were incubated with 20 μg of GST fusions of either Cdc42, Rac, or Rho loaded with either GDP or GTPγS and conjugated to Sepharose beads. GST alone [GST(–)] was also included as a negative control for nonspecific binding. After washing, precipitates were separated by SDS–PAGE, transferred to nitrocellulose and Western blot analysis was performed with an anti-HA antibody. (**B**) Kinase assay of PAK incubated in either the presence or absence of GTPγS-loaded Cdc42Q61L. Myelin basic protein was included in the reaction as a substrate. (**C**) Relative levels of JNK activation following transfection of the wild type PAK with kinase-inactive forms of the MEKK family members (MEKK1–4KM). pCMV5 was included to maintain equal total levels of transfected plasmid. Western blot analysis of a portion of the immunoprecipitation indicated that equivalent levels of HA–JNK were immunoprecipitated in each sample. Averages represent five separate experiments. Error bars indicate the standard deviation.

4 with Rac and Cdc42 argues that a more likely scenario is a pathway where PAK and MEKK1/4 associate independently with Cdc42 and Rac and are parallel to one another in component signaling pathways. Consistent with previously published results (Manser *et al.*, 1994), Figure 8A shows that recombinant PAK associates with Cdc42 and Rac in the GTP-bound form and this interaction stimulates the kinase activity of PAK (Figure 8B). Whereas kinase-inactive inhibitory mutants of MEKK1 and 4 inhibit Cdc42 and Rac activation of the JNK pathway (Figure 5), these inhibitory mutants do not block PAK stimulation of the JNK pathway. Kinase-inactive mutants of MEKK2 and 3 also did not inhibit PAK stimulation of the JNK activity (Figure 8C). In addition, we have been unable to measure any detectable phosphorylation of catalytically inactive mutants of MEKK1–4 by PAK under conditions where PAK efficiently autophosphorylated and phosphorylated specific substrates (data not shown). Therefore, these results indicate that PAK does not lie directly upstream of MEKK1, 2, 3 or 4 in a pathway leading to JNK activation. Rather, the data supports the prediction of two independent pathways involving PAKs and MEKK1/4 that regulate JNK activity.

Discussion

In this study, we have better defined the mechanisms controlling regulation of the MEKK family members (MEKK1–4), as well as their role as growth factor-mediated MAPK kinase kinases that regulate the activity of JNK and ERK. From these findings we propose a model shown in Figure 9, wherein the MEKK family members potentially play a role in JNK activation by EGF, but only MEKK1 and 4 regulate the JNK pathway in a Cdc42/Rac dependent manner. MEKK-mediated regulation of JNK activity by these low molecular weight GTP-binding proteins is parallel to PAK. MEKK1 is also capable of binding to Ras (Russell *et al.*, 1995) and kinase-inactive MEKK1 inhibits ERK activation in response to EGF. The mechanisms that may allow for MEKK2 and 3

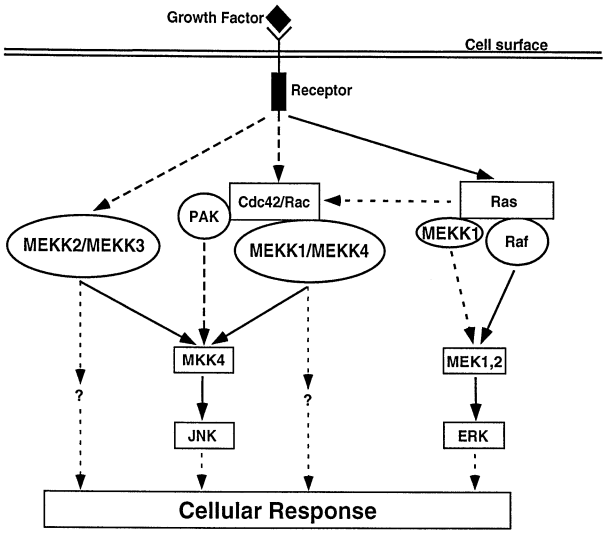

Fig. 9. Summary of the Cdc42/Rac-mediated regulation of the MEKK family members by tyrosine kinase receptors. The binding of growth factors to their respective receptors activates the receptor's intrinsic tyrosine kinase activity which leads to activation (GTP loading) of low molecular weight GTP-binding proteins and sequential protein kinase pathways controlling JNK and ERK. The changes in activity of these, as well as other less well characterized response pathways, can control critical cellular responses. With respect to the epidermal growth factor (EGF) receptor, the MEKK family of kinases are critical in mediating the effects of this receptor and all of the MEKKs play a role in JNK activation, whereas only MEKK1 appears to be involved in ERK activation by this growth factor receptor. MEKK1 and 4 associate with and mediate the effects of Cdc42 and Rac on activation of the JNK pathway. Furthermore, MEKK1 has been demonstrated to associate with Ras in a GTP-dependent manner, providing additional evidence that MEKK1 is an important component of ERK activation (Russell *et al.*, 1995). PAK mediates its effects on the JNK pathway independent of any of the MEKK family members suggesting that the MEKK1 and 4 coordinate with other kinases such as PAK and Raf to mediate the effects of specific low molecular weight GTP-binding proteins. Although MEKK2 and 3 play a role in JNK activation by EGF the proteins that control their activity and the mechanisms by which these kinases are regulated is not well defined at this time. Dashed arrows indicate unresolved mechanisms of signaling regulation, whereas solid arrows indicate established and relatively well-defined aspects of signaling regulation.

regulation of the JNK pathway independent of the low molecular weight GTP-binding proteins is at present undefined. However, EGF stimulates MEKK2 and its activation is involved in JNK regulation by this growth factor. Thus, there appears to be considerable specificity inherent to the regulation of the MEKK family members, and in lieu of the relatively conserved kinase domains this suggests that the highly divergent NH_2-terminal regions of these kinases encode domains or motifs responsible for their selective regulation.

Although EGF stimulates MEKK1 activation and transfection of activated forms of Cdc42 and Rac yield a modest, yet reproducible activation of MEKK1 (G.Fanger and G.Johnson, unpublished observations), the addition of Cdc42Q61L *in vitro* does not directly stimulate MEKK1 activation as it does for PAK. This is consistent with other kinases regulated by low molecular weight GTP-binding proteins, where it has been problematic to show that recombinant Ras–GTP stimulates Raf activation (Zhang *et al.*, 1993), suggesting that another input possibly from another kinase may be required to mediate activation of Raf (Marais *et al.*, 1995; Jelinek *et al.*, 1996), as well as MEKK1. Unlike PAK and Raf, the MEKKs also display high constitutive activity when expressed recombinantly or when the endogenous kinases are immunoprecipitated from cells which contributes to obscuring activation incurred by addition of recombinant Cdc42 or Rac. High constitutive activity is also observed for STE11, a MEKK1 homologue from *S.cerevisiae* (Neiman and Herskowitz, 1994), and is a feature of this family of kinases. It appears that when the MEKK proteins are isolated or overexpressed in cells that, like STE11, they become activated. This activation most likely involves an autophosphorylation and/or loss of interaction with a regulatory protein.

The differential subcellular localization of the MEKKs indicates that they function to regulate localized signaling in response to different stimuli. The finding that MEKK2 and 4 are redistributed from a Golgi localization to cytoplasm by BFA argues strongly that they are associated with vesicles budding from the Golgi complex. Recently, Cdc42 was shown to be localized to the Golgi allowing for it to be co-localized with MEKK4 (Erickson *et al.*, 1996). MEKK2 also has a punctate cytoplasmic distribution that is not affected by BFA; similarly, MEKK1 has a cytoplasmic distribution that is distinct from that determined for MEKK2. In addition, a component of the MEKK1 in cells has a nuclear localization. We have shown that MEKK1 is activated by agents such as cisplatin and mitomycin C (C.Widmann and G.L.Johnson, submitted for publication), suggesting it could function in part in the nucleus as a stress sensor to DNA damaging agents. The differential localization of specific MEKKs in cytoplasmic and Golgi vesicular-like structures, as well as the nucleus indicates that each could function as a sensor to specific stimuli in localized regions of the cell. The implication is that the different MEKKs would regulate signaling pathways in defined cellular locations. This model is consistent with the growing number of MAPK kinase kinases that appear capable of activating the JNK pathway (Bagrodia *et al.*, 1995; Pombo *et al.*, 1995; Yamaguchi *et al.*, 1995; Brown *et al.*, 1996; Rana *et al.*, 1996; Salmeron *et al.*, 1996; Teramoto *et al.*, 1996a; Wang *et al.*, 1996; Ichijo *et al.*, 1997) and to interact with Cdc42 or Rac (Manser *et al.*, 1994; Teramoto *et al.*, 1996a). The low molecular weight GTP-binding proteins Rac/Cdc42/Rho also appear to be found in several different subcellular locations (Adamson *et al.*, 1992; Erickson *et al.*, 1996) and to regulate a diverse set of cytoskeletal and morphological functions in addition to multiple kinase pathways (for reviews, see Chant and Stowers, 1995; Symons, 1996). Thus, MEKK1 and 4 are members of a family of diverse effectors for the Rho family GTP-binding proteins.

There are distinct differences in the abilities of MEKK1 and 4 to interact with the Rho family of low molecular weight GTP-binding proteins. Although MEKK1 did not associate with Rho, it clearly associated with Rac and Cdc42 in a strict GTP-dependent fashion. In contrast, MEKK4 associated with either the GDP or GTP bound Rac and Cdc42. The binding of MEKK4 to both the GDP and GTP bound Rac and Cdc42 is similar to other proteins such as PIP5-kinase (Ren *et al.*, 1996) that have been shown to bind to Rac in both GDP and GTP states. MEKK4 expressed in COS cells also associated with Rho,

but this interaction could not be seen with a MBP–MEKK4 fusion protein purified from bacteria. The reason for failure to see MEKK4 interaction directly with Rho is unclear, but it could suggest that when MEKK4 is expressed in COS cells it binds a protein that allows interaction with Rho. It has been proposed that Rho can regulate the JNK pathway in a cell type specific manner (Teramoto *et al.*, 1996b), but this suggestion and our observations with Rho require further investigation to determine their significance.

MEKK4 contains a modified Cdc42/Rac interactive binding (CRIB) domain (Burbelo *et al.*, 1995), whereas MEKK1 does not. The modified CRIB domain in MEKK4 is CDTPKSYDNVMHVG corresponding to residues 1311–1324 in the MEKK4 sequence. The proposed CRIB motif is ISXP (χ_{2-4}) FXH (χ_2) HVG where the underlined sequences in the MEKK4 sequence are found in the consensus CRIB sequence. Preliminary studies indicate that deletion of the CRIB motif diminishes but does not eliminate binding of MEKK4 to Cdc42 suggesting more than one interaction site in the two proteins (P.Gerwins and G.L.Johnson, unpublished observations). Regions that mediate interaction of MEKK1 to Cdc42 and Rac are not yet defined but are being pursued in our laboratory.

There is a growing awareness that specific effectors for GDP/GTP-binding proteins can in fact bind to both the GDP and GTP states (Chi *et al.*, 1997). In fact, the crystal structures of heterotrimeric G proteins indicates that the major effector binding domain does not undergo conformational changes in the GTP state relative to GDP (Noel *et al.*, 1993; Coleman *et al.*, 1994; Mixon *et al.*, 1995; Wall *et al.*, 1995). The prediction is that a second region that interacts with the effector undergoes a conformational change in the GTP state relative to GDP, and it is this site that activates the effector enzyme (Mixon *et al.*, 1995; Wall *et al.*, 1995). A two-site interaction for Rac with specific effectors also has been proposed (Ren *et al.*, 1996). In yeast, Bud1 binds Bem1 only when Bud1 is in a GDP, but not a GTP bound state; Bud1 binds a second protein, Cdc24, when Bud1 is in the GTP bound state (Herskowitz *et al.*, 1995; Leberer *et al.*, 1996; Peter *et al.*, 1996). Thus, the binding of effector proteins to GDP/GTP-binding proteins can be GTP-dependent, GDP-dependent or bind in either GDP or GTP bound states. The relevance of these observations must be realized in defining the regulation of effector proteins for Rac/Cdc42/Rho in mammalian cells where regulation of effector pathways generally is conserved to that found in yeast. Our results would suggest Rac/Cdc42 would regulate MEKK1 only when GTP is bound; in contrast MEKK4 might be localized by binding to Rac/Cdc42 but that additional regulatory inputs including protein interactions would be required for its activation.

As indicated by our studies on the MEKKs, differential subcellular localization and/or interactions with specific low molecular weight GTP-binding proteins are key mechanisms that control the specificity and regulation of the MAPK kinase kinases. It is becoming apparent that to define the regulation of the MAPK kinase kinases and their relevant downstream substrates, their localization in the cell, as well as their specific interaction with and access to regulators and substrates, must be considered. We are currently defining the domains within each MEKK that are responsible for their unique subcellular locations and interactions with regulatory proteins.

Materials and methods

Plasmids, protein purification and antibodies

Activated forms of Rac (Q61L) and Cdc42 (Q61L) were expressed in pCMV5. NH_2-terminally truncated, activated forms of MEKK1, 2, 3 and 4 (ΔMEKK) were expressed in pCMV5, as were kinase-inactive, competitive inhibitory mutants (MEKKKM) which were created by substitution of methionine for lysine in the ATP binding domain. Since the full-length MEKK1 protein undergoes a proteolytic cleavage event which is critical for formation of the biologically active form of MEKK1 (see Figure 1; C.Widmann and G.Johnson, submitted for publication), the kinase-inactive forms of MEKK1 that act as competitive inhibitors and were used in this study corresponded to shorter naturally occurring, proteolytically cleaved MEKK1 products. Otherwise, for MEKK2, 3 and 4 which do not contain proteolytic cleavage sites, the full-length kinase-inactive forms functioned as competitive inhibitory molecules. Since the wild type and kinase-inactive MEKK constructs were epitope tagged, their relative expression was determined by Western blot and found to be equivalent (not differing by more than 10%) in all cases examined.

The β form of PAK was cloned from a rat brain cDNA using PCR and primers that flanked the 5′ ATG start and the 3′ TAG stop site, 5′GCTGGGATCCAAAATGTCTGACAGCTTGGATAACGAAG3′ and 3′GGCTGCGGCCGCTAACGGCTACTGTTCTTAATGGCTTC-C5′. The full-length rat βPAK was cloned into pCMV5 and used for subsequent transfections.

Glutathione *S*-transferase (GST) fusions of RacQ61L, Cdc42Q61L, and RhoQ63L were expressed in *Escherichia coli* with the pGEX-5X expression vector (Pharmacia, Uppsala, Sweden) and purified from bacterial cell lysates with glutathione–Sepharose beads (Pharmacia, Uppsala, Sweden). Polyhistidine (pHIS) fusions of Cdc42Q61L and RhoQ63L were constructed by subcloning into the expression vector pAcHLT-A (Pharmingen, San Diego, CA) which was expressed in *Sf*9 insect cells. Ni-NTA Sepharose beads (Quiagen, Santa Clarita, CA) were used to purify recombinant pHIS-tagged protein from cell lysates. In all preparations from either *E.coli* or *Sf*9 cells the protein was analyzed by gel electrophoresis followed by loading assays with [γ-^{32}P]GTPγS (ICN Biologicals, Costa Mesa, CA) in order to determine the quality and concentration of the recombinant protein prepared. The COOH-terminal, kinase domain portions of MEKK1 (nucleotides 3474–4542), MEKK3 (nucleotides 1324–2277), and MEKK4 (nucleotides 3909–4791) were subcloned into the bacterial expression vector pMAL-c2 (New England Biolabs, Beverly, MA) which fuses a small portion of the maltose binding protein (MBP) to MEKK. Recombinant MBP-tagged MEKK was expressed in *E.coli* and purified with maltose-amylose resin (New England Biolabs).

Type specific MEKK antibodies were generated in rabbits by injecting peptides corresponding to COOH-terminal regions of each MEKK with divergent amino acid sequence.

Cell culture and transfection

COS cells were maintained in a humidified CO_2 environment in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5% newborn calf serum and 5% calf serum (Gibco Laboratories, Grand Island, NY). For growth factor studies, cells were starved for either 4 h or overnight with DMEM containing 0.1% calf serum before being stimulated with 100 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA). Cells were transfected with DEAE dextran (Ausubel *et al.*, 1987).

Immunofluorescence

Cells were plated onto uncoated glass coverslips at least 1 day prior to being fixed for 10 min in a solution containing 3% paraformaldehyde and 3% sucrose in PBS (pH 7.4). Cells were then washed with PBS and permeabilized with 0.2% Triton X-100 for 10 min, washed again and allowed to incubate for 15 min in normal growth media. Coverslips were then incubated for 1 h with the indicated combinations of fluorescein-conjugated WGA (Molecular Probes, Eugene, OR) and/or affinity-purified rabbit anti-MEKK1, anti-MEKK2, anti-MEKK4 or mouse monoclonal anti-γ-adaptin (Sigma, St Louis, MO) antibody. Following washing with PBS, cells were incubated with 1.5 mg/ml of Cy^3-conjugated affinity purified donkey anti-rabbit Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) and/or fluorescein-conjugated donkey anti-mouse

Ig (Jackson ImmunoResearch Laboratories). Coverslips were washed with PBS and allowed to incubate for 12 h in PBS supplemented with 0.2% sodium azide before being mounted onto slides with 20 mg/ml *o*-phenylenediamine (OPDA) in 1 M Tris (pH 8.5). Cells were visualized by digital confocal immunofluorescence and images were captured with a cooled CCD camera mounted on a Leica DMR/XA microscope using a 40× Plan Neo objective. In order to remove out of focus immunofluorescence, images were deconvolved using Slidebook (Intelligent Imaging Innovations, Inc., Denver, CO) on a 9500 Power PC Macintosh computer.

The subcellular localization of MEKK1, 2 and 4 was verified by at least two antibodies to each kinase made against different epitopes. For MEKK1 and 2, antibodies made against fusion proteins recognizing the kinase domain or NH_2-terminal regulatory domain gave similar staining patterns (not shown) to the antibodies raised using peptides corresponding to divergent COOH-terminal regions of each protein (see Figure 2). For MEKK4, a green fluorescent protein–MEKK4 fusion gave a similar peri-nuclear Golgi localization as that observed with the anti-COOH-terminal antibody. It was observed that with transfection the expression of wild-type MEKK or green fluorescent protein constructs appeared to also give a diffuse cytoplasmic staining, presumably because 'docking sites' for the MEKKs were saturated (not shown). For this reason, antibody staining for the endogenous protein rather than transfected green fluorescent protein fusions were performed for analysis of MEKK protein distribution in cells.

In some cases cells were treated with 5 μg/ml BFA (Sigma) for up to 30 min at 37°C in a CO_2 supplemented environment before being fixed and stained with indicated antibody as described above. In order to visualize the Golgi complex, cells were washed on ice with cold HEPES-supplemented Minimum Essential Medium (HMEM) and incubated on ice for 30 min in HMEM containing 0.3 mg/ml defatted bovine serum albumin (BSA) and 5 μM BODIPY-ceramide (Molecular Probes). The BODIPY-ceramide was prepared as described in Ladinsky *et al.* (1994). Cells were washed with HMEM containing defatted BSA and incubated at 37°C in a CO_2 free environment for 30 min before being visualized live in the FITC channel. To visualize the endoplasmic reticulum, cells were fixed and permeabilized as described above. Cells were then incubated in 2.5 μg/ml of 3, 3′dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Molecular Probes) for 10 s before being washed with PBS, mounted as described above, and observed in the FITC channel. To visualize mitochondria, cells were incubated with 5 μg/ml of rhodamine 123 (Molecular Probes) for 25 min at 37°C in CO_2 supplemented environment. Cells were observed live in the rhodamine channel.

Jun kinase assay

Cells were lysed in buffer containing 20 mM Tris (pH 7.6), 0.5% NP-40, 250 mM sodium chloride, 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM ethylene glycolbis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium vanadate, 21 μg/ml aprotinin, 5 μg/ml leupeptin and 1 mM dithiothreitol (DTT). Protein concentration was determined using the method of Bradford (1976) and under conditions where HA–JNK was transfected, JNK activity was immunoprecipitated from 400 μg of cell lysate with an anti-haemagglutinin (HA) antibody (Berkeley Antibody Company, Richmond, CA). JNK immunoprecipitates were washed and activity was measured using an *in vitro* kinase assay which utilized 10 μCi [γ-^{32}P]ATP (ICN Biologicals) and an excess of recombinantly expressed glutathione *S*-transferase–c-Jun(1–79) (GST–c-Jun) as substrate. Alternatively, where indicated, JNK was precipitated from 400 μg of cell lysate with GST–c-Jun conjugated to Sepharose beads, washed and activity was measured in a kinase assay following addition of 10 μCi [γ-^{32}P]ATP (ICN Biologicals). Kinase reactions were performed for 20 min at 30°C in buffer containing 20 mM HEPES (pH 7.5), 10 mM β-glycerophosphate (βGP), 10 mM p-nitrophenyl phosphate (PNPP), 10 mM magnesium chloride, 1 mM DTT, and 50 μM sodium vanadate. The kinase reaction was terminated by the addition of SDS sample buffer (0.31 M Tris pH 6.8, 11.5% SDS, 50 mM DTT, 50% glycerol), samples were boiled, size fractionated by SDS–PAGE, and ^{32}P-labeled GST–c-Jun was visualized by autoradiography. PhosphorImager analysis was utilized to quantitate the relative differences in GST–c-Jun phosphorylation as a measure of JNK activity.

MAP kinase assay

Cells were lysed in buffer containing 20 mM HEPES (pH 7.5), 50 mM βGP, 100 μM sodium vanadate, 2 mM magnesium chloride, 1 mM EGTA, 0.5% Triton X-100, 5 μg/ml leupeptin, 21 μg/ml aprotinin and 1 mM DTT. Protein concentration was determined as described above and ERK activity was immunoprecipitated from 400 μg of cell lysate with either an anti-HA antibody (Berkeley Antibody Company) when HA–ERK2 was transfected or an anti-ERK2 antibody (Santa Cruz, Santa Cruz, CA) when endogenous ERK activity was assayed. Immunoprecipitates were washed and *in vitro* kinase assays were carried out at 30°C for 15 min in buffer containing 20 mM HEPES (pH 7.5), 50 mM βGP, 100 μM sodium vanadate, 20 mM magnesium chloride, 0.1 mM EGTA, 0.2 mM ATP, 10 μCi [γ-^{32}P]ATP (ICN Biologicals), 50 μg/ml IP-20 peptide and 80 μM of epidermal growth factor receptor 662–681 peptide ($EGFR^{662–681}$) as a selective substrate for MAPK activity. Reactions were terminated with the addition of 10 μl of 25% trichloroacetic acid, spotted onto P81 phosphocellulose ion exchange paper (Whatman, Maidstone, UK), washed three times with 75 mM phosphoric acid, and washed once with acetone. The level of EGFR peptide ^{32}P incorporation was determined by scintillation counting.

MEKK assay

Either endogenously expressed MEKK1 or 2 was immunoprecipitated with type-specific antibodies in lysis buffer (20 mM Tris pH 7.6, 0.5% NP-40, 250 mM sodium chloride, 3 mM EDTA, 3 mM EGTA, 1mM PMSF, 2 mM sodium vanadate, 21 μg/ml aprotinin, 5 μg/ml leupeptin and 1 mM DTT) from serum-starved cells that were either unstimulated or stimulated with 100 μg/ml EGF (Collaborative Biomedical Products) for 10 min. Where indicated, cells were preincubated for 10 min with 100 nM wortmannin (Sigma). Each immunoprecipitate was incubated at 30°C for 20 min in kinase buffer (20 mM HEPES pH 7.5, 10 mM βGP, 10 mM PNPP, 10 mM magnesium chloride, 1 mM DTT, and 50 μM sodium vanadate) containing wild type JNK, wild type JNKK, and 2 mM ATP. This reaction was incubated with GST–c-Jun substrate conjugated to Sepharose beads for 20 min at 4°C, before being washed and incubated for 20 min at 30°C in kinase buffer containing 10 μCi of [γ-^{32}P]ATP (ICN Biologicals). Kinase reactions were stopped by the addition of SDS sample buffer, separated by SDS–PAGE, and visualized with autoradiography. To quantify the relative changes in GST–c-Jun phosphorylation PhosphorImager analysis was utilized.

Affinity precipitation

GST or pHIS fusion proteins of either Rac, Cdc42 or Rho, which were still attached to the glutathione–(for GST) or Ni-NTA–(for pHIS) Sepharose beads, were loaded with either guanosine 5′-diphosphate (GDP; Sigma) or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS; Sigma) at 30°C for 30 min in loading buffer containing 20 mM Tris (pH 7.5), 1 mM dithiothreitol, and 0.5 mg/ml BSA. This reaction was stopped by the addition of 1 mM magnesium chloride. COS cells recombinantly expressing either rat βPAK, MEKK1, 2, 3 or 4 were lysed in buffer containing 1% Triton X-100, 10 mM Tris, 5 mM EDTA, 50 mM sodium fluoride, 50 mM sodium chloride, and 20 μg/ml aprotinin and 400 μg of cell lysate was incubated with 10 μg of the indicated fusion protein preparation of the low molecular weight GTP-binding protein attached to Glutathione beads for 1 h at 4°C. Each reaction was washed three times, run on SDS–PAGE and immunoblotted with the appropriate antibody. Alternatively, 10 μg of either Rac or Cdc42 protein dissociated from Sepharose beads and loaded with [γ-^{32}P]GTPγS (ICN Biologicals) was incubated with 1 μg of amylose resin conjugated MBP fusion proteins of either MEKK1, 3 or 4 for 1 h at 4°C in PAN buffer [100 mM sodium chloride, 10 mM piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid) (PIPES), and 38 μg/ml aprotinin]. Reactions were washed three times with PAN buffer and quantitated with a scintillation counter.

Western blot

Protein was separated by SDS–PAGE, transferred to nitrocellulose (Schleicher and Schuell, Keene, NH), and immunoblotted with the indicated antibody in 5% milk solution. Enhanced chemiluminescence (Dupont, Boston, MA) was used to detect the immune complex following incubation with protein A–horseradish peroxidase. Autoradiography was utilized to visualize immunoreactive bands.

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