

Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins

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Mitogens promote cell growth through integrated signal transduction networks that alter cellular metabolism, gene expression and cytoskeletal organization. Many such signals are propagated through activation of MAP kinase cascades partly regulated by upstream small GTP-binding proteins. Interactions among cascades are suspected but not defined. Here we show that Rho family small G proteins such as Rac1 and Cdc42hs, which activate the JNK/SAPK pathway, cooperate with Raf-1 to activate the ERK pathway. This causes activation of ternary complex factors (TCFs), which regulate *c-fos* gene expression through the serum response element. Examination of ERK pathway kinases shows that neither MEK1 nor Ras will synergize with Rho-type proteins, and that only MEK1 is fully activated, indicating that MEKs are a focal point for cross-cascade regulation. Rho family proteins utilize PAKs for this effect, as expression of an active PAK1 mutant can substitute for Rho family small G proteins, and expression of an interfering PAK1 mutant blocks Rho-type protein stimulation of ERKs. PAK1 phosphorylates MEK1 on Ser298, a site important for binding of Raf-1 to MEK1 *in vivo*. Expression of interfering PAK1 also reduces stimulation of TCF function by serum growth factors, while expression of active PAK1 enhances EGF-stimulated MEK1 activity. This demonstrates interaction among MAP kinase pathway elements not previously recognized and suggests an explanation for the cooperative effect of Raf-1 and Rho family proteins on cellular transformation.

Keywords: cross-cascade activation/ERK/Rho family proteins/SRE/TCF function

Introduction

Many of the initial cellular responses to stimuli that promote proliferation or differentiation, as well as others that cause cellular stress, are mediated through the activ-

ation of distinct MAP kinase cascades (Hunter, 1995; Robinson and Cobb, 1997). The best characterized of these are the extracellular signal regulated kinase (ERK), c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAP kinase pathways. Most proliferative stimuli activate the ERK pathway, primarily through the small GTP binding protein Ras. Active Ras binds the MAP kinase kinase kinase Raf-1, thereby translocating it to the plasma membrane and promoting its activation. Active Raf-1 then phosphorylates and activates the MAP/ERK kinases (MEKs) 1 and 2, which in turn phosphorylate and activate ERK1 and ERK2. In a similar fashion, exposure of cells to cytokines and cellular stresses primarily activates the JNK/SAPK and p38 MAP kinase cascades. Through the activation of intermediary kinases, the Rho family small G proteins Rac and Cdc42hs can regulate the activation of JNK/SAPK, and to a much lesser extent the p38 MAP kinase pathways (Coso *et al.*, 1995; Minden *et al.*, 1995). In addition, all Rho family proteins have been shown to control aspects of cytoskeletal organization (Ridley and Hall, 1992; Ridley *et al.*, 1992; Coso *et al.*, 1995; Minden *et al.*, 1995; Nobes and Hall, 1995).

Among the consequences of MAP kinase activation are changes in the transcription of key growth-regulated genes. Such changes offer a sensitive readout of the cellular activity of these kinase cascades and have been monitored to determine if biochemical measurements of MAP kinase activation reflect cellular responses. One of the best characterized ERK-responsive promoters is that of the *c-fos* gene. ERK-stimulated transcription of *c-fos* requires an enhancer sequence within its promoter known as the serum response element (SRE), and is mediated by a complex of the serum response factor (SRF) and one of several ternary complex factors (TCFs). Phosphorylation of the TCFs Elk-1 and Sap-1a by ERKs leads to their high-affinity interaction in the ternary complex and a concomitant increase in transcriptional activation. While Sap-1a is activated only by ERK and p38 phosphorylation, Elk-1 is activated through phosphorylation by ERKs, JNK/SAPK and p38 MAP kinase (Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995; Price *et al.*, 1996; Janknecht and Hunter, 1997). Thus, these TCFs are differentially responsive to activation by different MAP kinase pathways.

Recently it has been reported that members of the Rho subfamily of small GTP binding proteins cooperate with Raf-1 to induce cellular transformation (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995a,b). Despite such observations, events downstream from the Rho family small G proteins that play significant roles in cooperating with Raf-1 to transform cells have not been described. It has been suggested that downstream targets, such as MAPKAP kinases and TCFs, integrate signals from the Ras-ERK and the Rac-JNK/SAPK cascades. Here we show that

Rho family small G proteins stimulate the ERK cascade, as well as TCF-dependent transcription. In this case, signal integration occurs further upstream, because these two pathways converge on MEK. The effects on MEK activity and TCF-mediated transcription occur in the absence of measurable effects on the JNK/SAPK and p38 MAP kinase pathways, and are insensitive to inhibition by dominant-negative mutants of Ras or SAPK. Rho family members require the activity of the p21-activated kinases (PAKs) to stimulate ERKs, and PAK activation leads to the phosphorylation of MEK1 on two sites within a domain required for interaction with Raf-1. Mutation of these sites to non-phosphorylatable residues impairs the cooperative activation of MEK1, as well as the interaction of Raf-1 and MEK1 *in vivo*. Furthermore, expression of interfering PAK1 partially blocks ligand stimulation of ERK-dependent transcription, and expression of active Rac2 or PAK1 potentiates the stimulation of the ERK pathway by EGF. Taken together, these findings identify MEKs as a target for convergent regulation by Raf-1 and Rho family small G proteins not previously appreciated.

Results

Rho family small G proteins cooperate with Raf-1 to activate ERK2

Though the ERK and JNK/SAPK pathways are activated by distinct cellular stimuli, regulation of these pathways is interconnected. It has been shown, for example, that expression of an interfering Rac mutant decreases the activation of ERKs by activated Ras (Frost *et al.*, 1996). Conversely, expression of activated Raf-1 (Raf BXB) and Rac results in the synergistic activation of ERK2 (Frost *et al.*, 1996). Because these results influence our view of MAPK pathway regulation and will have an impact on our understanding of cellular transformation caused by Raf-1 and Rho family small G proteins, we have studied the mechanism by which this cross-cascade cooperation occurs.

We first tested the ability of small G proteins to cooperate with wild-type Raf-1 to stimulate ERK2 activity. Co-transfected ERK2 was isolated from 293 cells with an antibody to an N-terminal hemagglutinin (HA) epitope tag and its activity was measured using myelin basic protein (MBP) as a substrate (Figure 1A). Expression of wild-type Raf-1 or any of the activated Rho family small G proteins alone did not significantly increase ERK2 activity (1- to 3-fold). However, co-expression of Raf-1 with activated forms of the small G proteins Rac2 (V^{12} Rac2), Cdc42hs (V^{12} Cdc42hs) or RhoA (L^{63} RhoA) caused a synergistic increase in ERK2 activity greatly in excess of the sum of their effects alone (25-, 16- and 25-fold, respectively; Figure 1A). Immunoblotting showed that this effect was not due to an increase in the amount of expressed Raf-1 in the soluble (Figure 1A) or particulate (data not shown) fractions. Similar results were found when two different activated forms of Raf-1 (Raf BXB and D^{259} Raf-1) were substituted for wt Raf-1 (Frost *et al.*, 1996; also data not shown). Raf-1 protein kinase activity was necessary for the effect on ERK2 activity because a kinase-deficient mutant of Raf-1 (W^{375} Raf-1) did not cooperate with V^{12} Rac2 (data not shown). Similar results were also found in NIH 3T3 cells (data not shown).

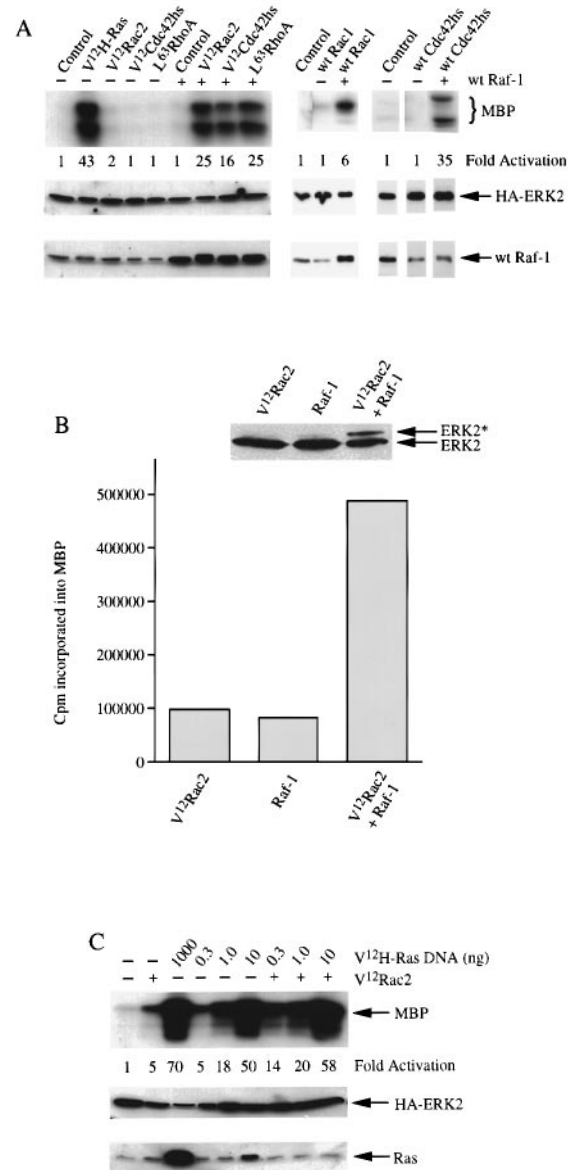


Fig. 1. Raf-1 synergizes with Rho family small G proteins to stimulate ERK activity. 293 cells were transfected with HA-ERK2 (5 μ g), the small G proteins shown (1 or 2 μ g each), plus or minus Raf-1 (1 μ g). Twenty hours after transfection, the cells were placed in medium without serum for an additional 20 h. (A) Activation of transfected HA-ERK2. The kinase activity of HA-ERK2 immunoprecipitated from cell lysates, normalized by immunoblotting for ERK2 expression, was assayed using MBP as a substrate. Shown in the top panel is the autoradiograph from a representative assay. Western blots for expressed HA-ERK2 and Raf-1 proteins are shown in the panels below. Fold activation refers to the fold increase in MBP phosphorylation over the control lane, as determined by scintillation counting. (B) Activation of endogenous ERK2. NIH 3T3 cells were transfected with the small G proteins shown plus or minus Raf-1. Twenty-four hours before harvest, cells were placed in medium lacking serum, and then incubated for another 24 h. The top panel shows a Western blot for ERK2. The slower-migrating band in lane 4 corresponds to active ERK2 (ERK2*). The graph shows total ERK activity immunoprecipitated from transfected lysates. (C) V^{12} H-Ras and V^{12} Rac2 do not cooperate to activate ERK2. 293 cells were transfected as described with V^{12} Rac2, HA-ERK2 and increasing amounts of V^{12} H-Ras. Immunoprecipitated HA-ERK2 activity was determined using MBP as a substrate (top panels). Shown below is a Western blot for V^{12} H-Ras. Fold activation refers to the fold increase in MBP phosphorylation over the control lane, as determined by scintillation counting.

Synergistic activation of ERKs was also observed with Raf-1 and activated RhoB (V^{14} RhoB, data not shown). Activated RhoG (V^{12} RhoG) was less effective than other Rho family proteins at synergizing with Raf-1 to activate ERK and activated RalA (V^{23} RalA) did not cooperate with Raf-1 to activate ERK2 (data not shown). Interestingly, co-expression of either wild-type Rac1 or wild-type Cdc42hs with Raf-1 also led to a synergistic activation of ERK2 (Figure 1A). Similar results were found when HA-ERK-ERK1 was substituted for HA-ERK2 (data not shown). Co-expression of Raf-1 with V^{12} Rac2 also increased the activity of endogenous ERK2 in NIH 3T3 cells, as measured both by a decreased mobility of ERK2 (Figure 1B, top panel) and an increase in immunoprecipitated ERK2 activity (Figure 1B, graph).

We also determined if other components of the ERK pathway could cooperate with V^{12} Rac2 to activate ERK2. Both V^{12} H-Ras and MEK1 were tested. Because V^{12} H-Ras is such a potent activator of ERK2, quantities of DNA ranging from pg to μ g were transfected to examine the potential for synergy with V^{12} Rac2. Even with as little as 0.3 or 1 ng of V^{12} H-Ras DNA transfected, little or no synergy with V^{12} Rac2 was exhibited (Figure 1C). Similarly, co-expression of MEK1 with V^{12} Rac2 did not lead to elevated ERK2 activity (data not shown). Thus, these experiments imply that activation of ERK2 by Rho family G proteins is contingent upon the elevation of Raf-1 kinase activity and also that synergy is more easily detected under conditions of submaximal Raf-1 activation.

TCF-dependent gene expression is activated by Raf-1 and Rho family small G proteins

To emphasize the biological consequences of ERK activation by Raf-1 and Rho family proteins, we examined transcriptional effects mediated by TCFs, which respond directly to proliferative stimuli. Growth responsive genes such as *c-fos* are controlled through a serum response element (SRE), which binds a complex of the serum response factor (SRF) and a member of the TCF family (Gille *et al.*, 1992; Marais *et al.*, 1993). This family includes Elk-1 (Hipskind *et al.*, 1991), Sap-1 (Dalton and Treisman, 1992) and Sap-2 (Net/Erp) (Giovane *et al.*, 1994; Lopez *et al.*, 1994; Price *et al.*, 1995). Activation via Elk-1 was examined because its phosphorylation by ERK1 and ERK2 correlates well with increased transcription of the *c-fos* gene (Janknecht *et al.*, 1993, 1995; Gille *et al.*, 1995a; Price *et al.*, 1995; Strahl *et al.*, 1996). Thus, a Gal-Elk chimera and a Gal-Elk-sensitive Gal4 reporter were used to measure the extent to which Raf-1 and Rho family members influenced transcriptional events in NIH 3T3 cells.

Initially, co-expression of activated Rac1 (L^{61} Rac1) and Raf BXB was tested. Expression of Raf BXB alone led to a 40-fold increase in Gal-Elk-dependent luciferase expression, which doubled when L^{61} Rac1 was also expressed (Figure 2A). Subsequently, wild-type Raf-1 was tested. In these experiments Gal-Elk-dependent reporter activity was only marginally increased by expression of either Raf-1, L^{61} Rac1, L^{61} Cdc42hs or L^{63} RhoA alone. However, in the presence of expressed Raf-1, L^{61} Rac1 L^{61} Cdc42hs expression markedly increased transcription from the Gal4 reporter (Figure 2B), consistent with their abilities to potentiate ERK activation. These results

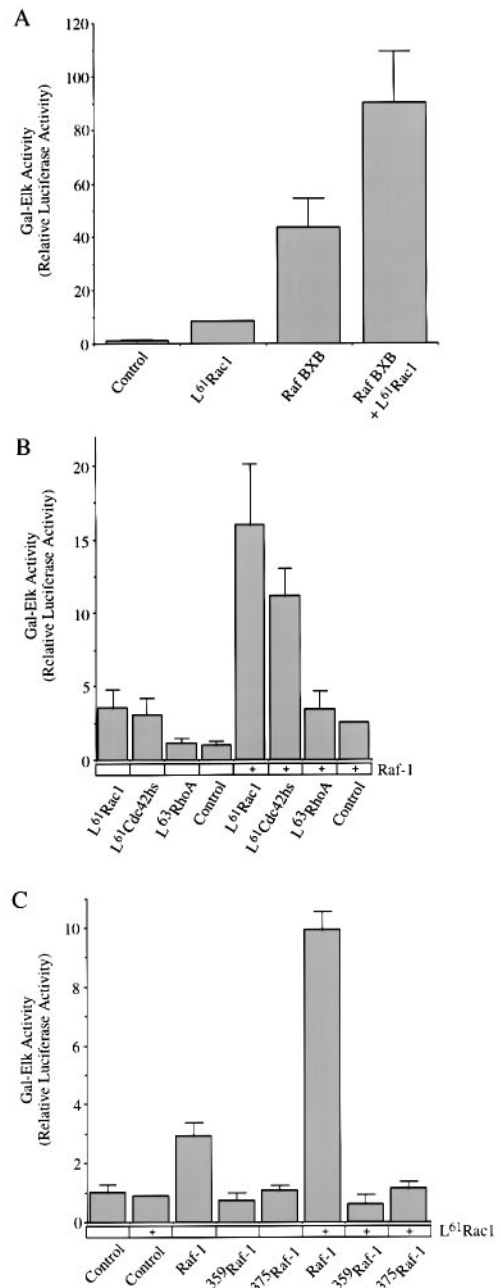


Fig. 2. Raf-1 and Rho family small G proteins synergize to activate TCF-dependent gene expression. NIH 3T3 cells were transfected with the Gal-Lux reporter, an expression vector for the chimeric Gal-Elk protein and a control plasmid or vectors for Raf-1 and small G proteins in various combinations. (A) Synergistic activation by Raf BXB and L^{61} Rac1. (B) Synergistic activation by wild-type Raf-1 and L^{61} Rac1, L^{61} Cdc42 or L^{63} RhoA. (C) Synergistic activation by L^{61} Rac1 and wild-type Raf-1, A³⁵⁹Raf-1 or W³⁷⁵Raf-1. Values are given as luciferase activity relative to control transfections and represent averages from at least three independent experiments.

depended on Elk-1 phosphorylation because mutation of a critical phosphorylation site in Elk1 (Ser383; Janknecht *et al.*, 1993; Gille *et al.*, 1995a) ablated reporter expression in response to Raf-1 and Rho family proteins (data not shown). In contrast to the synergistic activation of ERKs seen in 293 cells, Raf-1 and L^{63} RhoA had only an additive effect on Gal-Elk-dependent reporter expression in NIH 3T3 cells. This may reflect differences in signaling between

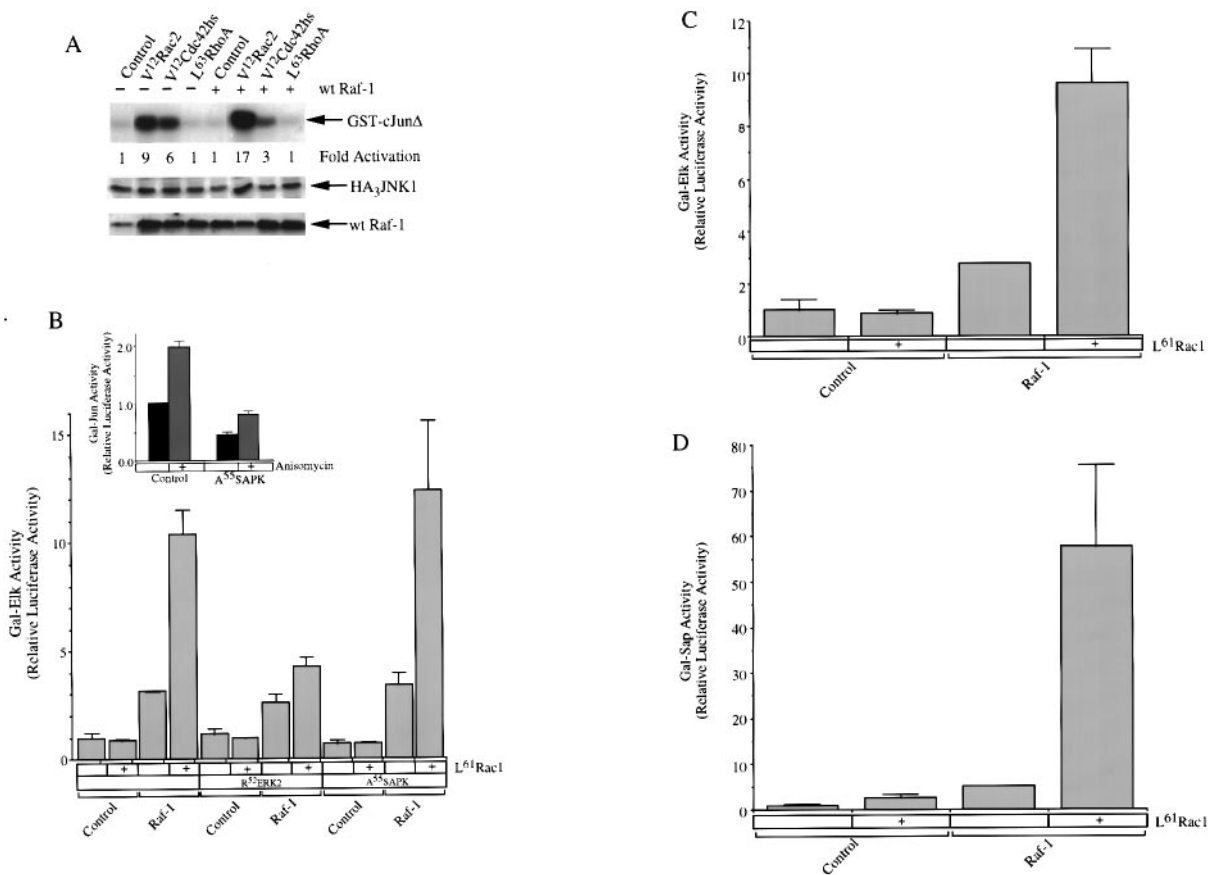


Fig. 3. Synergy between Raf-1 and Rho family small G proteins does not require JNK/SAPK activity. (A) 293 cells were co-transfected with HA₃JNK1 (5 μg), the small G proteins shown, and Raf-1, as described in Figure 1A. The top panel shows the activity of immunoprecipitated HA₃JNK1 using GST-cJunΔ (c-Jun 1-221) as a substrate. Shown in the panels below are Western blots for expressed HA₃JNK1 and Raf-1. Fold activation refers to the fold increase in activity over control, as determined by scintillation counting. (B) NIH 3T3 cells were transfected with a Gal-Lux reporter, an expression vector for the chimeric Gal-Elk protein, and either a control plasmid or vectors expressing wild-type Raf-1, L⁶¹Rac1, dominant-negative ERK2 (R⁵²ERK2) or dominant-negative SAPKβ (A⁵⁵SAPK), as indicated. Inset: NIH 3T3 cells were transfected with the Gal-Lux reporter and the Gal-Jun chimera, and a control plasmid or a vector encoding kinase defective A⁵⁵SAPK. Before harvest, cells were stimulated for 6 h with anisomycin (100 ng/ml) to activate the Gal-Jun chimera. (C and D) NIH 3T3 cells were transfected with the Gal-Lux reporter, an expression vector for either Gal-Elk (C) or Gal-Sap (D), as well as a control plasmid (pCMV5) or vectors for Raf-1 or L⁶¹Rac1. Values are given as luciferase activity relative to control transfections and are averaged from at least three independent experiments.

NIH 3T3 and 293 cells. The effect of co-expressing Raf-1 and V¹²Rac2 was also tested, but repression of the internal control (βGAL expression) by V¹²Rac2 prevented quantification of its ability to synergize with Raf-1 (data not shown).

The cooperation between Raf-1 and Rho family proteins was further examined by comparing wild-type Raf-1 with the kinase-deficient Raf-1 mutants W³⁷⁵Raf-1 and A³⁵⁹Raf-1. While Raf-1 cooperated with V¹²Rac1 to activate transcription, neither W³⁷⁵Raf-1 nor A³⁵⁹Raf-1 was able to do so, showing that the kinase activity of Raf-1 is required for cooperation (Figure 2C). Thus, activation of the ERK pathway by Raf-1 and Rho family small G proteins is also manifested in TCF-dependent gene expression, underscoring the functional significance of ERK activation by Rho family members.

JNK/SAPK activity is not affected by Raf-1 expression, nor is it required for the synergistic activation of transcription

Expression of active Rac or Cdc42hs stimulates the JNK/SAPK pathway (Coso *et al.*, 1995; Minden *et al.*, 1995). Therefore, we examined the effect of co-expressing Raf-1

and Rho family small G proteins on JNK/SAPK activity. Shown in Figure 3A is a representative experiment. Expression of either V¹²Rac2 or V¹²Cdc42hs alone elicited 9-fold and 6-fold increases in JNK/SAPK activity, respectively (Figure 3A). L⁶³RhoA was ineffective at stimulating JNK/SAPK activity (Figure 3A), consistent with previous reports (Coso *et al.*, 1995; Minden *et al.*, 1995). In the presence of Raf-1, the V¹²Rac2- and V¹²Cdc42hs-induced increases were 17- and 3-fold, little different from responses in its absence. Raf BXB co-expression with activated Rac2 or Cdc42hs also caused no further increase in JNK/SAPK activity (data not shown), indicating that JNK/SAPK is unaffected by overexpression of Raf BXB. Similar experiments indicated no effect of Raf-1 on the regulation of the p38 MAP kinase pathway (data not shown).

To determine whether JNK/SAPK activity was required for the synergistic activation of Gal-Elk, catalytically defective SAPKβ (A⁵⁵SAPK) was expressed in combination with Raf-1 and L⁶¹Rac1, and Gal-Elk-dependent reporter activity was measured. This SAPK mutant blocks stimulation of tumor necrosis factor-α translation by lipopolysaccharide, which is dependent upon SAPK activity

(Swantek *et al.*, 1997), and also inhibits anisomycin-stimulated Gal-Jun activity (Figure 3B, inset). Its co-expression, however, did not reduce Gal-Elk activity (Figure 3B). On the other hand, expression of kinase-defective ERK2 (R⁵²ERK2) inhibited Gal-Elk activity stimulated by L⁶¹Rac1 and Raf-1 by >50% (Figure 3B). In further experiments, expression of Raf-1 failed to influence the activation of Gal-Jun by either L⁶¹Rac1 or MEKK1, an activator of the JNK/SAPK pathway (data not shown). Together, these results indicate that Raf-1 and Rho family proteins stimulate Gal-Elk reporter activity through the activation of ERKs but not JNK/SAPKs, and that input from the Rac pathway occurs upstream of JNK/SAPK.

To provide additional evidence that the TCF-dependent transcriptional activity detected was a consequence of ERK2 activation, the activities of the Gal4 reporter mediated by Gal-Elk and Gal-Sap chimeras were compared. Whereas the Gal-Elk chimera is activated by the ERK pathway and, to a lesser extent, by the JNK/SAPK and p38 MAP kinase pathways, the Gal-Sap chimera is activated only by ERKs and p38, and does not respond to JNK/SAPK (Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995; Price *et al.*, 1996; Janknecht and Hunter, 1997). As shown in Figure 3C and D, Gal-Sap was 6-fold more effective than Gal-Elk at mediating Gal4 reporter activation in response to L⁶¹Rac1 and Raf-1 co-expression. This also suggests that TCF activation reflects the activity of the ERK pathway. Furthermore, because Rho family proteins and Raf-1 do not significantly activate p38 MAP kinase (see above; see also Coso *et al.*, 1995; Minden *et al.*, 1995), it is unlikely that the Gal-Sap activation we observe results from the activation of p38 MAP kinase. Thus, TCF activation most likely reflects the activity of the ERK pathway.

Rac cooperation with Raf-1 does not involve an autocrine loop

Before we addressed the biochemical mechanism of this cross-cascade effect, we wanted to confirm that production of autocrine factors was not involved. We showed previously that medium from 293 cells co-transfected with V¹²Rac2 and Raf BXB did not activate ERK2 expressed in a second group of cells (Frost *et al.*, 1996). Similarly, expression of dominant interfering H-Ras (A¹⁵H-Ras) did not block the Rac/Raf BXB synergy. These findings suggested that an autocrine mechanism was unlikely to account for the enhanced ERK activity we observed. However, previous work in other cell lines has shown that autocrine factors may be released, some of which are known to signal through Ras and could elevate kinase activities in that manner (McCarthy *et al.*, 1995). Therefore, we determined if this was the case for the enhanced Gal-Elk activity observed in NIH 3T3 cells. First, L⁶¹Rac1 was co-expressed with Raf-1 in the presence or absence of the dominant-negative H-Ras mutant N¹⁷H-Ras. In either case, an equivalent increase in Gal-Elk reporter activity was observed (Figure 4A). Because N¹⁷H-Ras and A¹⁵H-Ras both interfere with endogenous Ras activation, these results parallel the lack of effect of A¹⁵H-Ras on ERK2 activation noted earlier (Frost *et al.*, 1996). Second, cells stably expressing V¹²Rac1 were co-cultured with another population of cells transiently transfected directly with Raf-1 and the Gal-Sap reporter system. After 20 h

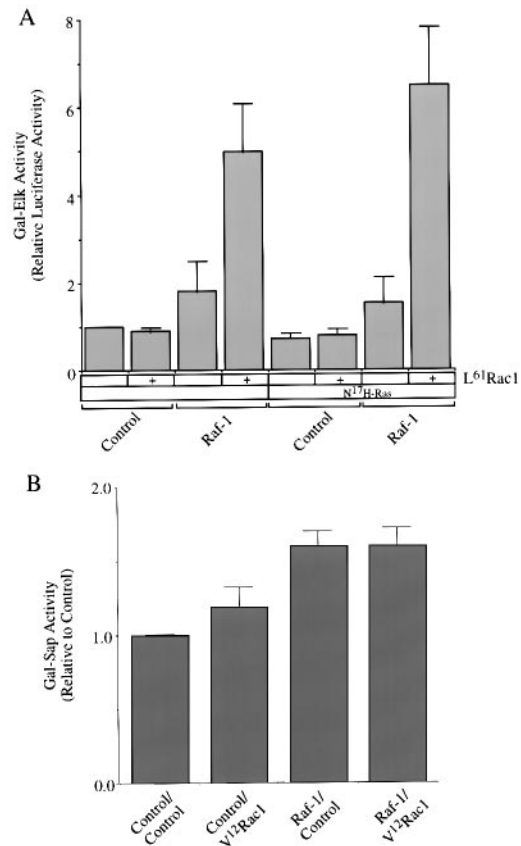


Fig. 4. Synergistic activation of TCF-dependent gene expression does not involve an autocrine loop. **(A)** Interfering H-Ras expression does not affect the synergistic activation of Gal-Elk. NIH 3T3 cells were transfected with the Gal-Lux reporter, an expression vector for the chimeric Gal-Elk protein and either a control plasmid (pCMV5) or vectors expressing wild-type Raf-1, L⁶¹Rac1 and a dominant interfering mutant of H-Ras (N¹⁷H-Ras), as indicated. **(B)** Media from active Rac1 expressing cells does not stimulate Gal-Sap activity in separately transfected cells. NIH 3T3 cells were transfected with the Gal-Lux reporter and the Gal-Sap expression vector with (Raf-1) or without (Control) an expression vector for Raf-1. After 20 h the cells were mixed with either control cells, or cells stably transfected with V¹²Rac1, replated and starved for a further 20 h before harvest. Values are given as luciferase units relative to control transfections (control/control) and represent the average of at least three independent experiments.

of co-culture, no increase in Gal-Sap activity was detected (Figure 4B). However, when the V¹²Rac1 cells were transfected with Raf-1 and the Gal-Sap reporter system, strong activation of the reporter by Gal-Sap was observed (data not shown). Based on these results, it is unlikely that the synergistic increase in TCF-dependent transcription due to co-expression of Rac1 and Raf-1 results from the release of autocrine factors.

Co-expression of Raf-1 and Rho family small G proteins increases MEK activity

To define the biochemical mechanisms underlying the cross-cascade activation of ERKs more precisely, we determined whether MEK1 or MEK2 activity was affected (Figure 5A and B). As expected, expression of V¹²H-Ras alone increased the activity of co-transfected MEK1 and MEK2 (29- and 9.9-fold, respectively). Expression of either V¹²Rac2 or Raf-1 alone caused only modest effects on MEK activity. On the other hand, expression of Raf-1

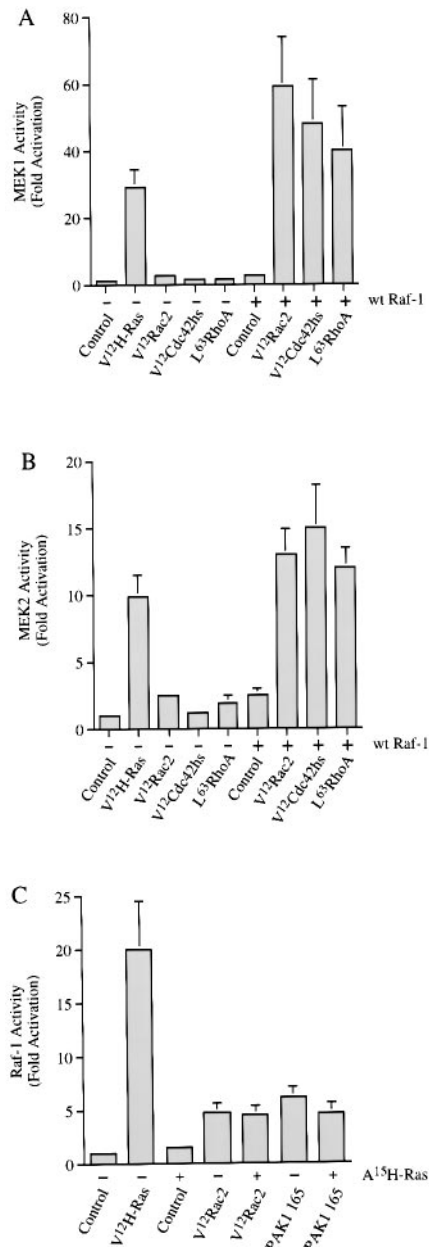


Fig. 5. Raf-1 and Rho family proteins synergize to activate MEKs and, to a lesser extent, Raf-1. 293 cells were transfected with HA-MEK1 (A), HA-MEK2 (B) or HA-Raf-1 (C), and the expression vectors shown. At 20 h after transfection, cells were placed in medium lacking serum, and then incubated for a further 20 h. R⁵²ERK2 was used as a substrate for immune complex kinase assays with HA-MEK1 and HA-MEK2. Wild-type MEK1 was used as a substrate for HA-Raf-1. In the panels below are Western blots for HA-MEK1, HA-MEK2 and Raf-1. Results in (A), (B) and (C) are averaged from at least three independent experiments. Error bars represent the standard error of the mean. Fold activation refers to the fold increase in substrate phosphorylation over control.

combined with V¹²Rac2, V¹²Cdc42hs or L⁶³RhoA led to a synergistic increase in the activity of HA-MEK1 and HA-MEK2 (40- to 59-fold for MEK1, and 12- to 15-fold for MEK2). MEK1 was consistently activated to a greater degree than MEK2, both in absolute activity and fold activation, suggesting that Rho family GTPases target MEK1 for activation more efficiently than MEK2. For both MEKs, the degree of activation resulting from co-

expression of Raf-1 and Rho family small G proteins was reproducibly equal to or greater than that stimulated by V¹²H-Ras. Comparable effects on endogenous MEK activity were obtained in NIH 3T3 cells (data not shown).

The impact on the activity of Raf-1 was also assessed. As anticipated, V¹²H-Ras led to a robust activation of HA-tagged Raf-1 (20-fold, Figure 5C). Surprisingly, co-expression with V¹²Rac2 also caused a small but reproducible increase in Raf-1 activity (5-fold, Figure 5C). This increase occurred even in the presence of interfering Ras (A¹⁵H-Ras), suggesting that the increase in Raf-1 activity did not require Ras activation. Ras-independent mechanisms of Raf-1 activation have been reported (Alessandrini *et al.*, 1996). Co-expression of a constitutively active, N-terminal truncation mutant of the Rac effector PAK1 (PAK1 165) also activated HA-Raf-1 (5-fold, Figure 5C). Similar results were found with endogenous Raf-1 in NIH 3T3 cells (data not shown). These results indicate that at least part of the mechanism for up-regulation of the ERK pathway by Rho family proteins can be accounted for by a modest, Ras-independent stimulation of Raf-1 activity. However, since the level of Raf-1 activation resulting from active Rac2 or PAK1 expression is much less than that stimulated by active Ras, and cross-cascade MEK activation is reproducibly greater than that caused by Ras expression, this suggests that additional mechanisms for MEK regulation by Rho family proteins exist.

PAK is required for the effect of Rho family proteins on the ERK cascade

The finding that constitutively active PAK1 expression stimulates Raf-1 activity suggests that Rho family proteins may use PAKs as effectors for synergy with Raf-1. However, PAKs are activated only by binding to active Rac or Cdc42hs, not active RhoA (Manser *et al.*, 1994, 1995; Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Martin *et al.*, 1995; Polverino *et al.*, 1995; Teo *et al.*, 1995). To test whether RhoA is signaling through Rac or Cdc42hs to synergize with Raf-1, we co-expressed interfering mutants of Rac, Cdc42hs or RhoA (N¹⁷Rac2, N¹⁷Cdc42hs and N¹⁹RhoA, respectively) with active Rho family members and Raf-1, and examined effects on ERK2 activation. As shown in Figure 6A, co-expression of N¹⁷Cdc42hs or N¹⁹RhoA did not block V¹²Rac/Raf-1 synergy. Similarly, co-expression of N¹⁷Rac2 or N¹⁹RhoA did not inhibit V¹²Cdc42hs/Raf-1 synergy. However, expression of interfering forms of Rac or Cdc42hs both inhibited the ability of active RhoA to cooperate with Raf-1 to activate ERK2. These data indicate that RhoA utilizes endogenous Rac or Cdc42hs to stimulate ERK2 activity. It is unclear whether this occurs through a direct stimulation of Rac or Cdc42hs activity, or by a RhoA-stimulated autocrine effect that activates endogenous Rac or Cdc42hs.

To test whether PAK expression can influence ERK activation, we co-expressed either wild-type or constitutively active PAK1 (PAK1 165) with Raf-1 and measured ERK2 and Gal-Elk activity. As shown in Figure 6B, co-expression of PAK1 165 with Raf-1 led to a synergistic activation of ERK2 (207-fold), whereas expression of either molecule alone only weakly stimulated ERK2 activity. The activation of ERK2 by PAK1 165 and Raf-1 was reproducibly greater than that observed with active Rac and Raf-1. Co-expression of PAK1 165 with Raf-1

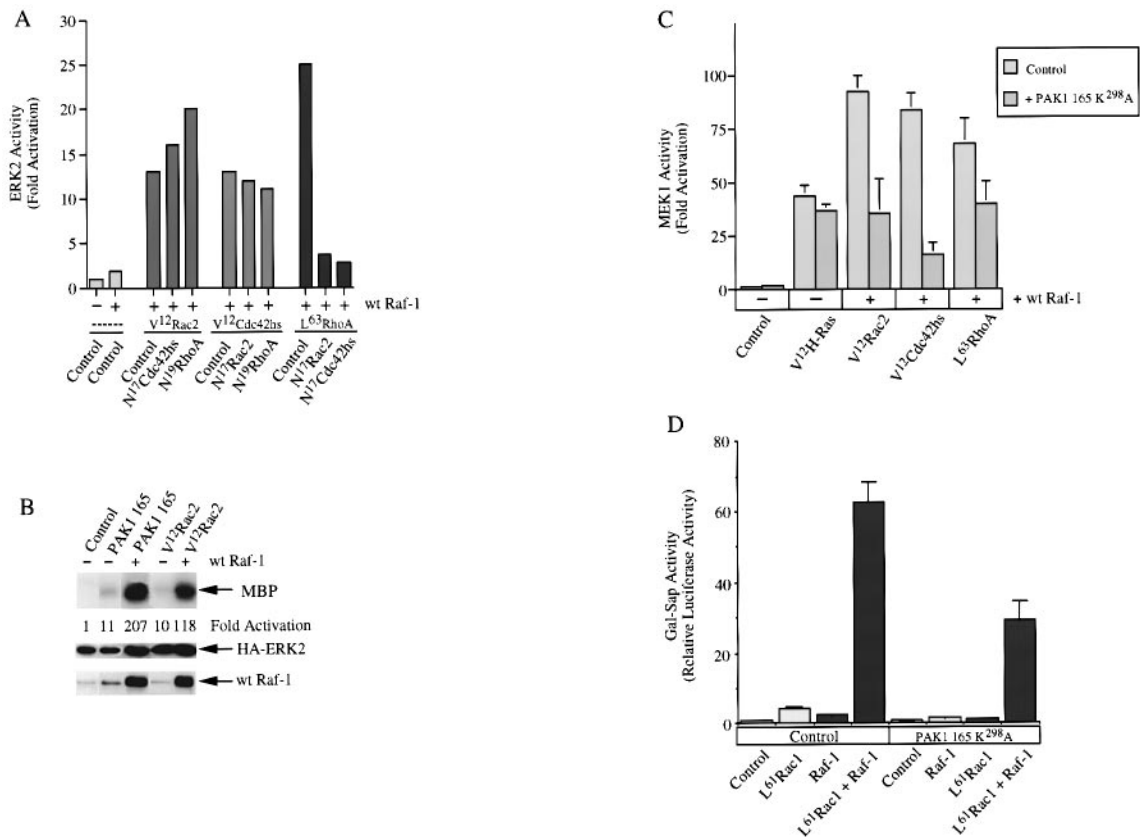


Fig. 6. Co-expression of active PAK1 and wild-type Raf-1 leads to activation of HA-ERK2, HA-MEK1 and the Gal-Elk chimera. **(A)** Interfering Rac and Cdc42hs block RhoA-mediated synergy with Raf-1. 293 cells were transfected as described with HA-ERK2, wild-type Raf-1 and the cDNAs shown. Fold activation refers to the fold increase in MBP phosphorylation over control. Shown is a representative experiment. **(B)** Active PAK1 cooperates with Raf-1 to activate ERK2. 293 cells were transfected as described with HA-ERK2, wild-type Raf-1 and the cDNAs shown (5 μ g transfected for the PAK plasmids). PAK1 165 refers to the constitutively active, N-terminal truncation mutant PAK1 165-544. Shown is a representative experiment. In the top panel is MBP phosphorylation by immunoprecipitated HA-ERK2. Below are Western blots for expressed HA-ERK2 and Raf-1. Fold activation refers to the fold increase in MBP phosphorylation over control. **(C)** Kinase-defective PAK1 165 expression blocks Rho family synergy with Raf-1. 293 cells were transfected with HA-MEK1 and the plasmids shown. The fold increase in MEK1 activity is depicted. Results are the average of three independent experiments. Light bars refer to cells transfected with the plasmids shown and the control vector pCMV5. Dark bars refer to cells transfected with the plasmids shown and kinase-deficient PAK1 165 K²⁹⁸A. **(D)** Interfering PAK1 165 blocks the cooperative activation of Gal-Elk. NIH 3T3 cells were transfected as described in Figure 2 with the Gal-Elk chimera/Gal4-luciferase reporter system, Raf-1, and L⁶¹Rac1 plus or minus PAK1 165 K²⁹⁸A, as indicated. Control refers to cells transfected with pCMV5 instead of interfering PAK1 165.

also led to a synergistic activation of both HA-MEK1 and HA-MEK2 (data not shown). Similarly, co-expression of wild-type PAK1 and Raf-1 increased MEK and ERK activity, though to a lesser degree than that caused by expression of constitutively active PAK1 (data not shown). When Gal-Elk reporter activity was examined, the results mirrored those found for ERKs and MEKs (data not shown). These findings demonstrate that active PAK1 can substitute for Rho family small G proteins in activating ERKs.

To determine if PAKs mediate the effects of Rho family small G proteins on MEK1, we co-transfected catalytically defective PAK1 165 (PAK1 165 K²⁹⁸A) with Raf-1 and Rho family small G proteins, and then measured the activity of immunoprecipitated, HA-tagged MEK1. Because PAK1 165 K²⁹⁸A lacks a small G protein binding domain, any interference observed must occur downstream of the small G proteins. Co-expression of interfering PAK1 had little effect on MEK1 activation by V¹²H-Ras (Figure 6C). This is consistent with the finding that V¹²Rac2 did not significantly increase V¹²H-Ras-stimulated ERK2 activity at any level of V¹²H-Ras transfected (Figure 1D).

On the other hand, co-expression of interfering PAK1 reproducibly decreased MEK1 activation by Rho family small G proteins and Raf-1 (Figure 6C, 50–80% inhibition). Similar results were found when HA-ERK2 was transfected in place of HA-MEK1 (data not shown). Similarly, interfering PAK1 inhibited the activation of Gal-Sap by Raf-1 and L⁶¹Rac1 (Figure 6D). Thus, these findings indicate that Rho family proteins utilize PAKs to activate MEK1, ERK2 and TCF-dependent transcription.

PAK1 phosphorylates MEK1 on Ser298, a site important for MEK1-Raf-1 interaction

Expression of active Rac causes the phosphorylation of MEK1 in transfected cells (data not shown). To determine which residues become phosphorylated, either V¹²Rac2 or PAK1 165 were co-transfected into NIH 3T3 cells with human MEK1 or MEK1 mutants lacking one of several potential phosphorylation sites. Phosphorylation of MEK1 and the mutants was deduced by a shift in electrophoretic mobility of MEK1. Expression of either V¹²Rac2 or PAK1 165 resulted in the phosphorylation of co-expressed MEK1

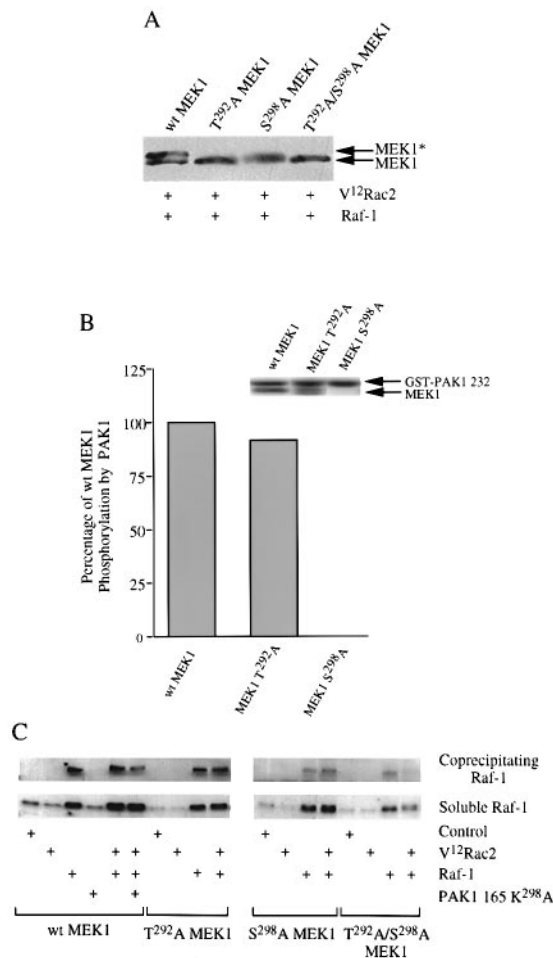


Fig. 7. PAK1 phosphorylates human MEK1 on Ser298, one of two sites important for synergistic activation by Raf-1 and Rho family proteins and MEK1–Raf-1 Binding. (A) *In vivo* phosphorylation of MEK1. HA-tagged wild-type T²⁹²A, S²⁹⁸A or T²⁹²A/S²⁹⁸A MEK1 was co-transfected with V¹²Rac2 and Raf-1. MEK1 expression was detected by Western blotting using the mouse anti-HA antibody. Phosphorylation of MEK1 on Thr292 and Ser298 results in a reduced mobility of MEK1 (MEK1*). (B) Phosphorylation of bacterially expressed, human MEK1 proteins by GST–PAK1 232–544. The graph corresponds to the percentage of counts incorporated into MEK1. Inset is a representative autoradiograph. (C) Raf-1 co-precipitates more efficiently with wild-type MEK1 than mutant MEK1. 293 cells were transfected with the MEK1 constructs and the plasmids shown. HA-MEK1 proteins were immunoprecipitated from soluble lysates as described in Materials and methods. Co-precipitated Raf-1 protein (top panel), as well as Raf-1 present in soluble lysates before immunoprecipitation (bottom panel) was detected by Western blotting. Shown is a representative experiment.

on Thr292 and Ser298, based on a loss of the induced shift in electrophoretic mobility of those mutants (Figure 7A).

To test whether PAK1 directly phosphorylates either of these residues, we assayed wild-type, T²⁹²A and S²⁹⁸A MEK1 as substrates for bacterially expressed PAK1 *in vitro*. We found that wild-type MEK1 was phosphorylated on serine with a stoichiometry of one mole of phosphate per mole of MEK1 (Figure 7B; also data not shown). T²⁹²A MEK1 is similarly phosphorylated by PAK1. However, S²⁹⁸A MEK1 is not phosphorylated by PAK1 (Figure 7B). Wild-type MEK2, MEK3, MEK4 and MEK6 also were not appreciably phosphorylated by PAK1 *in vitro* (data not shown). Given that expression of active PAK1 or Rac2 results in the phosphorylation of MEK1

on Ser298 *in vivo* and that this site is phosphorylated efficiently by PAK1 *in vitro*, MEK1 is likely to be a physiological substrate for PAK1. The kinase that phosphorylates Thr292 remains unknown, although ERK2 efficiently phosphorylates this site *in vitro*. When tested *in vitro*, phosphorylation of MEK1 on either Thr292 or Ser298 neither increased nor decreased its kinase activity towards R⁵²ERK2 (data not shown).

Both Thr292 and Ser298 are located within a poly-proline insert in the catalytic domain of MEK1 that has previously been shown to be important for MEK1–Raf-1 interaction (Jelinek *et al.*, 1994; Catling *et al.*, 1995). Thus, we tested whether mutation of these sites to alanine affected the association of MEK1 with Raf-1 in cells. When immunoprecipitated MEK1 was tested for co-precipitating Raf-1, less Raf-1 co-precipitated with S²⁹⁸A MEK1 than wild-type MEK1 (Figure 7C). In some experiments, T²⁹²A MEK1 also bound less Raf-1 than wild-type MEK1, but this effect was variable. The T²⁹²A/S²⁹⁸A double mutant consistently bound co-transfected Raf-1 less well than either single point mutant (Figure 7C). In the case of wild-type MEK1, expression of interfering PAK1 165 reproducibly decreased the amount of co-precipitating Raf-1 (Figure 7C). Expression of constitutively active Rac2 or PAK1, however, did not further increase the amount of co-precipitating Raf-1. Although it is not clear as to why this was the case, we can conclude that Thr292 and Ser298 are required for maximal MEK1–Raf-1 association. This suggests that the cross-cascade effect of Rho family proteins on MEK1 may arise, in part, from the phosphorylation of MEK1 on these sites, thus enhancing the association of MEK1 and Raf-1 and leading to an increase in MEK1 activation. This model fits with the finding that MEK2 is activated less well by Rho family proteins than MEK1, since MEK2 is not appreciably phosphorylated by PAK1.

Rac and PAK influence the activation of MEK1 and TCF-dependent transcription by extracellular ligands

Overexpression of Raf-1 is likely to result in an increase in total cellular Raf-1 activity. This increase in Raf-1 activity must be small, however, because only a modest stimulation of MEKs and ERKs occurs. The experiments described thus far, in which Rho family proteins increased MEK activation by expressed Raf-1, suggest that Rho-type proteins may potentiate MEK activation by other ERK pathway stimuli. To test this hypothesis we examined whether EGF-stimulated MEK1 activity was enhanced by expression of active Rac2 or PAK1. EGF activates the ERK and, to a lesser extent, the JNK/SAPK pathways (Kyriakis *et al.*, 1994). Stimulation of 293 cells with EGF (50 ng/ml) resulted in a 5-fold activation of MEK1 that peaked at 5 min and returned to near-basal levels after 30 min (Figure 8A). Expression of active Rac (V¹²Rac2), on the other hand, had little effect on basal MEK1 activity, but greatly increased the response of MEK1 to EGF from 5-fold up to 27-fold. MEK1 activation was slightly delayed, reaching a maximum 10 min after stimulation, but its activation was prolonged. MEK1 activity did not return to basal levels until nearly 2 h after addition of EGF (Figure 8A). The response to EGF in cells expressing active PAK1 was similar to that in cells expressing

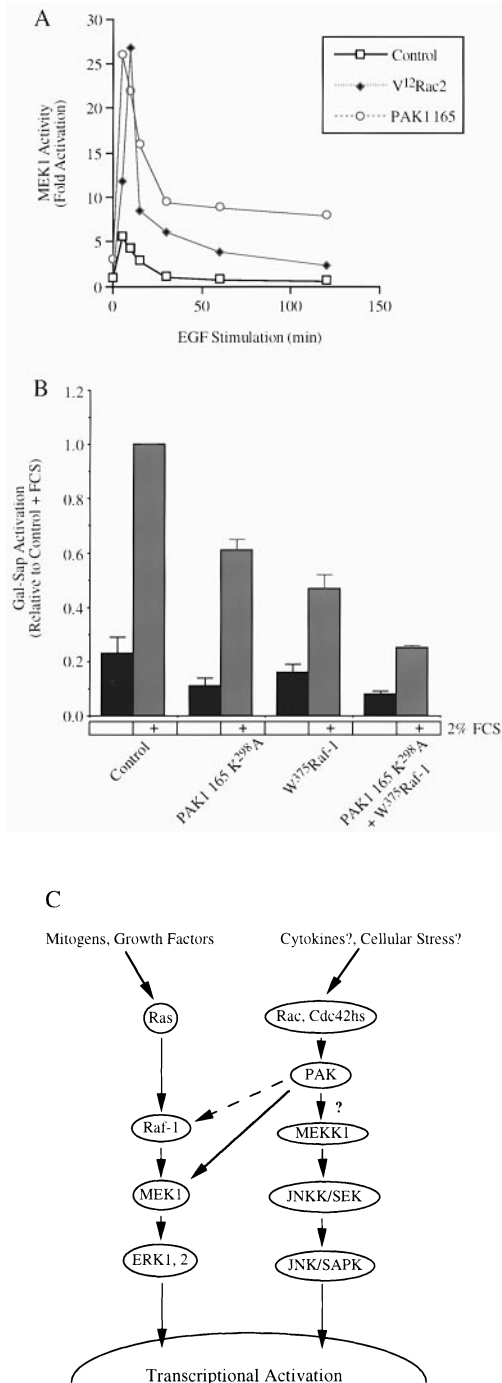


Fig. 8. Involvement of PAK1 in growth factor induced activation of Gal-Sap and MEK1. **(A)** Expression of active Rac2 or PAK1 potentiates EGF-mediated activation of MEK1. 293 cells were transfected as described previously with HA-MEK1 and either the control plasmid pCMV5 (□), V¹²Rac2 (◆) or PAK1 165 (○). Before harvest, cells were stimulated with EGF (50 ng/ml) for the time periods shown. Shown are the results from a representative experiment. Fold activation refers to the fold increase in MEK1 activity as compared with MEK1 activity recovered from unstimulated cells transfected with control plasmid. **(B)** Interfering PAK1 165 partially blocks activation of Gal-Sap stimulated by fetal calf serum addition. NIH 3T3 cells were transfected with the Gal-Lux reporter and the Gal-Sap chimera, as well as pCMV5 (control), PAK1 165 K²⁹⁸A, and/or W³⁷⁵Raf-1 as described previously. Before harvest, cells were stimulated with 2% fetal calf serum (FCS) for 8 h. Shown are the results of three independent experiments. Gal-Sap activity is shown relative to control cells stimulated with 2% FCS. **(C)** A model for the potentiation of MEK1 activation by PAKs.

V¹²Rac2, except that the basal activity of MEK1 was slightly increased (3-fold, Figure 8A), and the duration of MEK1 activation was even longer (still 8-fold above basal at 120 min). Similar results were observed when ERK2 activity was tested (data not shown). Thus, expression of active forms of either Rac2 or its effector PAK1 greatly potentiates both the amplitude and duration of the response of the ERK pathway to external ligands such as EGF.

To test whether EGF utilizes Rho family GTPases to activate the ERK pathway, we examined whether expression of interfering Rac2, Cdc42hs, RhoA or PAK1 would inhibit EGF-stimulated ERK2 activation. In these experiments, it was clear that only expression of interfering H-Ras blocked the stimulation of ERK2 activity by EGF. Expression of interfering Rho family proteins or interfering PAK1 was without effect (data not shown). Thus, EGF does not appear to use either Rho family proteins or PAKs to activate the ERK pathway, suggesting that the potentiating effect of active Rac2 or PAK1 expression on EGF-stimulated MEK1 activity represents a distinct regulatory input.

To test whether other ligands require PAK1 activity to stimulate the ERK pathway, we examined whether expression of interfering PAK1 could block the transient activation of the ERK-responsive Gal-Sap reporter by factors present in fetal calf serum (FCS). Stimulation of NIH 3T3 cells with 2% FCS resulted in a 5-fold increase in Gal-Sap activity (Figure 8B). This activation was partially blocked by expression of interfering PAK1 (40% reduction, Figure 8B). Expression of catalytically inactive Raf-1 (W³⁷⁵Raf-1) reduced Gal-Sap activation by 50%. When expressed together, PAK1 165 K²⁹⁸A and W³⁷⁵Raf-1 reduced the overall stimulation of Gal-Sap to that observed in unstimulated control cells (Figure 8B). These data indicate that PAK activity is required for full activation of ERK-regulated transcriptional elements stimulated by ligands present in fetal calf serum.

Discussion

To define mechanisms of cross-cascade signaling between MAP kinase pathways, we examined how the Rho family small G proteins Rac and Cdc42, which activate the JNK/SAPK pathway, influence the regulation of ERK pathway elements. We found that Rho family proteins have little effect alone but potentiate the ability of wild-type Raf-1 or growth factors to stimulate recombinant or endogenous ERKs and MEKs in multiple cell types. Within the ERK pathway, MEK1 was highly activated by co-expression of Rho proteins with Raf-1, while Raf-1 activity was only marginally enhanced. The Rac effector PAK1 substituted for Rho family proteins and phosphorylated MEK1 directly on Ser298, one of two sites required for the maximal association of MEK1 with Raf-1. Thus, we identify MEK1 as a point of convergent regulation by distinct upstream signals (Figure 8C).

The biological importance of cross-cascade activation of MEK1 and ERK2 has been demonstrated by measuring the impact on TCF-dependent transcription. Rho family proteins strongly increase Raf-stimulated, TCF-dependent transcription. Compelling evidence, in addition to the selective increase in ERK pathway activity, indicates that the induced TCF activity requires ERKs. First, expression of

an interfering ERK2 mutant blocks TCF stimulation by Rho family members, but a dominant-interfering SAPK mutant does not. Second, the activity of a Gal-Sap chimera, which is regulated by ERK and p38, but not JNK/SAPK phosphorylation (Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995; Fan *et al.*, 1996; Price *et al.*, 1996; Janknecht and Hunter, 1997), is also greatly stimulated. This occurs in the absence of a measurable increase in p38 activity. Thus, it is unlikely that the JNK/SAPK or p38 MAP kinases mediate the increase in TCF activity. These effects do not appear to be mediated by an autocrine loop, because they are neither blocked by expression of dominant-interfering H-Ras, nor do they occur when cells stably expressing active Rac1 are co-cultured with others transiently transfected with Raf-1 and the Gal-Sap reporter system. The cross-cascade activation of TCFs reported here occurs independently of SRF and, as such, is distinct from the transcriptional activation by SRF reported previously (Hill *et al.*, 1995).

Rho family proteins, signaling through either Rac or Cdc42hs, most likely contribute to MEK activation through the stimulation of PAKs. This is based on three lines of evidence. First, interfering forms of Rac and Cdc42hs each inhibit ERK activation by RhoA and Raf-1, while interfering RhoA does not block Rac- or Cdc42hs-mediated cooperation with Raf-1. Second, constitutively active PAK1 effectively substitutes for Rho family GTPases in synergizing with Raf-1 to activate ERKs. Third, expression of a catalytically defective PAK1 truncation mutant, which cannot bind to Rac or Cdc42hs, interferes with the ability of Rho family proteins to cooperatively activate MEKs and ERKs. Based on these data, one or more members of the PAK family is likely to mediate potentiation of the ERK pathway by Rho-type proteins.

Consistent with this conclusion is the finding that expression of either active Rac2 or active PAK1 causes the phosphorylation of human MEK1 on Thr292 and Ser298 *in vivo*. Both of these sites are contained within a poly-proline insert between catalytic subdomains IX and X of MEK1, a region previously shown to be important for MEK1-Raf-1 interaction (Jelinek *et al.*, 1994; Catling *et al.*, 1995). PAK1 phosphorylates Ser298 to high stoichiometry *in vitro*. Mutation of Ser298 to alanine significantly reduces the interaction of MEK1 and Raf-1 *in vivo*, as determined by the co-immunoprecipitation of Raf-1 with MEK1. Thr292 may also contribute to Raf-1-MEK1 association, since mutation of both Thr292 and Ser298 to alanine impairs MEK1-Raf-1 binding to a greater extent than mutation of either site alone. In this light, it is important to note that MEK2 is not efficiently phosphorylated by PAK1 *in vitro*. Thus, if phosphorylation of Thr292 and Ser298 promotes interaction between MEK1 and Raf-1 *in vivo*, this might explain why MEK1 is consistently activated to a greater degree than MEK2 by Rho family proteins.

Thus, PAK affects ERK pathway activation through multiple mechanisms. One is through the phosphorylation of MEK1 on sites important for MEK1-Raf-1 interaction. This may favor the binding of MEK1 over MEK2 by Raf-1. Another contributing factor is a modest but reproducible activation of both endogenous and recombinant Raf-1. This apparently occurs in the absence of Ras activation, as expression of interfering H-Ras does not prevent this increase in Raf-1 activity. Since both endogenous and

recombinant Raf-1 are activated, it is unclear as to why MEK activity is more potently stimulated when Raf-1 is overexpressed. It is possible that Raf-1 overexpression alters its subcellular localization, thus allowing a more productive interaction with MEK in a quiescent cell than otherwise possible. In support of this notion is the finding that expression of active Rac2 or PAK1 greatly potentiates MEK1 activation by growth factors in the absence of Raf-1 overexpression. It is likely that endogenous Raf-1 and MEKs form complexes in growth factor-stimulated cells that do not occur in the resting cell. Others have, in fact, reported the formation of complexes between Ras, Raf-1 and MEK1 that are dependent upon the presence of active Ras (Moodie *et al.*, 1993; Jelinek *et al.*, 1994; Catling *et al.*, 1995). Thus, it is possible that the potentiating effect of PAK can occur only when such complexes form.

The increase in EGF-stimulated MEK1 activation caused by active Rac2 or PAK1 expression is striking. This effect, however, is not limited to EGF. We have also observed this potentiation with such diverse ligands as LPA, bradykinin and carbachol (J.A.Frost and M.H.Cobb, unpublished observations). Therefore, it appears that PAK can potentiate MEK activation by many ligands that can normally stimulate MEK1 activity. In the case of EGF, at least, it appears that the activation of Rho family proteins and PAKs are not required for ERK activation. This is based on the observation that expression of interfering Rac, Cdc42hs, RhoA or PAK1 does not inhibit EGF-mediated stimulation of ERKs. This suggests that Rho family regulation of the ERK pathway represents a separate input from agents which on their own do not strongly activate ERK activity. This idea is supported by the finding that stimulation of the Gal-Sap chimera by fetal calf serum is inhibited by expression of interfering PAK1. The identity of the ligand(s) which are able to potentiate MEK activation by Ras-linked growth factors are, however, unknown. We have tried unsuccessfully to potentiate EGF stimulation of MEK1 activity with agents that primarily activate the JNK/SAPK pathway. For example, the cytokine interleukin 1 β , which activates the JNK/SAPK pathway in 293 cells, does not synergize with EGF to activate ERKs. It is possible, however, that ligand stimulation of pathways that regulate Rho family GTPases cannot supplant active Rac or PAK1 expression as a positive regulatory factor for MEK1. This may be due to the transient nature of their activation, or the fact that they activate multiple cellular enzymes in addition to Rho GTPases. By selectively expressing individual activated small G proteins or PAK, one is stimulating individual pathways to a greater degree and more selectively than occurs following ligand stimulation. Future work will be required to determine if any ligand can replace active Rac or PAK expression in this context.

Increases in ERK activation as a result of cross-cascade signaling may have important consequences for the cell. For example, both EGF and NGF activate ERKs in PC12 cells, but the duration of ERK activation stimulated by EGF is much shorter than that by NGF. It has been speculated that it is this difference in the temporal activation of the ERK pathway that results in the stimulation of growth by EGF and the stimulation of neuronal differentiation by NGF in PC12 cells (Cowley *et al.*, 1994), and

distinguishes non-mitogenic from mitogenic stimuli in CCL39 cells (Lenormand *et al.*, 1993). Perhaps activation of Rho family proteins and the subsequent effects on MEK activity can account for the differences in the kinetics of ERK activation by these stimuli. An increase in ERK activation may also have relevance to cellular transformation (Lenormand *et al.*, 1993; Cowley *et al.*, 1994; Mansour *et al.*, 1994). For instance, such an increase may be important to the initial events in cellular transformation which would not necessarily be evident in the transformed cell. Further work will be necessary to determine the contributions of the regulatory mechanisms described here to cellular growth and transformation.

MAP kinase cascades were initially viewed as linear, independent signaling pathways controlled by ligands and often involving small G proteins. This view has been revised as a consequence of the discovery by many laboratories of interactions among distinct MAP kinase pathways (Xia *et al.*, 1995; Xu *et al.*, 1995), revealing added layers of regulatory complexity (Figure 8C). Here we have documented the cross-cascade effects of Rho-family small G proteins on the ERK pathway and its impact on TCF-mediated transcription, and we have elucidated underlying biochemical mechanisms. Significant regulatory interactions across protein kinase cascades may occur on many levels. These interactions are found not only at the level of activation of the upstream small G proteins (Ridley and Hall, 1992; Ridley *et al.*, 1992) and the downstream substrates such as TCFs and MINK (Fukunaga and Hunter, 1997), but also, as we have shown here, within the kinase cascades themselves.

Materials and methods

Cell culture and transfections

HEK 293 and NIH 3T3 cells were grown in DMEM plus 10% FBS, 1% L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. 60 mm dishes (100 mm for transcription assays) of cells 60–80% confluent were transfected by calcium phosphate co-precipitation as previously described (Lange-Carter *et al.*, 1993). At 18–24 h after transfection, cells were placed in medium without serum and incubated for a further 18–24 h. Cells were then harvested for immunoprecipitation kinase or transcription assays as previously described (Frost *et al.*, 1996).

Immunoprecipitation and immunoblotting

HA-tagged kinases were immunoprecipitated and assayed for kinase activity as described previously (Xu *et al.*, 1995). For immunoprecipitation of endogenous ERK2, cells were lysed in 0.5% Triton X-100 lysis buffer and supernatants recovered as previously described (Frost *et al.*, 1996). Beta-galactosidase (β GAL) activity derived from a co-transfected β GAL expression plasmid was used to correct for transfection efficiencies. Lysates containing equal β GAL activities were then incubated with anti-ERK2 antibody A249 (Whitehurst *et al.*, 1992) for 1 h at 4°C with mixing. A 50% slurry of protein A-Sepharose (Pharmacia) in Triton X-100 lysis buffer (30 μ l) was then added and lysates were incubated for another 1 h at 4°C with mixing. Immune complexes were sedimented by centrifugation and washed three times with 1 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, then once with 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂. MBP kinase activity was determined as described previously for HA-tagged ERK2 (Frost *et al.*, 1996). For co-immunoprecipitation of Raf-1 with HA-MEK1, anti-HA-MEK1 immunoprecipitates were washed three times with 20 mM Tris-HCl, pH 8.0, 1 M NaCl, and once with kinase buffer. After kinase reactions for MEK1 activity were carried out, equal portions of the pellets were used for Western blotting with rabbit anti-Raf-1 antibody SC-133 (Santa Cruz Biotech). For HA-MEK1 and HA-MEK2, 1 μ M recombinant, kinase-defective ERK2 (R^{S2}ERK2) was used as a substrate. Reactions were carried out for 15 or 30 min at 30°C. For HA-Raf-1, wild-type recombinant MEK1 (500 nM) was used as a substrate. Reactions were carried out at 30°C

for 15 min. For the coupled assay, one-tenth of this reaction was used to assay for MEK1 activity using R^{S2}ERK2 as a substrate (30°C, 30 min).

HA-tagged proteins were detected by Western blotting using mouse anti-HA antibody 12CA5. Raf-1 proteins were detected using rabbit anti-Raf antibody SC-133 (Santa Cruz Biotech) at 1:400 dilution. Expression of V¹²H-Ras was detected with rabbit anti-panRas (Oncogene Science) diluted 1:200. Overexpression of wild-type MEK1 was monitored using anti-MEK1 antibody A2227 (Xu *et al.*, 1995) (1:500 dilution). Expression of myc epitope-tagged PAK1 mutants was detected using the mouse anti-myc antibody 9E10 (Santa Cruz Biotech). All antibodies were diluted in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.1% Tween 20 (TBST) plus 0.25% non-fat milk. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham) were used at 1:2500 or 1:3300 dilutions in TBST plus 0.25% non-fat milk. Before immunoblotting, all transfers were blocked for at least 1 h at room temperature in TBST plus 5% non-fat milk. All antibody incubations were for 1 h at room temperature. Blots were washed for at least 15 min in TBST before and after antibody incubations. Blots were developed with ECL (Amersham) according to the manufacturer's protocol.

Luciferase reporter assays

Unless indicated otherwise, NIH 3T3 cells were transfected with 1 μ g of reporter plasmid, 1 μ g of expression vector for Gal-Elk or Gal-Sap, 1 μ g of expression vector for Raf-1 derivatives and 4 μ g of expression vector for small G protein derivatives in a mixture of 10 μ g of DNA per 100 mm plate. Plasmids encoding dominant-negative alleles of ERK2, SAPK β and Ras were transfected at 4 μ g/plate.

Cells were harvested in cold PBS and lysed in 250 mM KCl, 50 mM HEPES, pH 7.5, 0.1% NP40, 10% glycerol. Transfection efficiencies were monitored by measuring the β GAL activity resulting from co-transfection of 1 μ g of pCH110 (Pharmacia) per plate. The results of the luciferase assays were normalized to the relative transfection efficiency of each experimental point.

Plasmids

V¹²H-Ras, V¹²Rac2, V¹²Cdc42hs and L⁶³RhoA were as described previously (Frost *et al.*, 1996). L⁶¹Rac1 and L⁶¹Cdc42hs were contained in pCDNA3. N¹⁷Rac2 and N¹⁹RhoA were contained in pCMV5. N¹⁷Cdc42hs and C3 transferase were contained in pEFmyc. V¹²RhoG was created from wild-type RhoG by PCR with Vent polymerase using primers that add 5' *Eco*RI and 3' *Xho*I restriction sites. Correct amplification was determined by sequencing. V¹²RhoG was then subcloned into pCMV5 with an N-terminal myc tag (pCMV5M). HA-ERK1, HA-ERK2, HA-MEK1 and HA-MEK2 were as described previously, as was pCMV5/MEKK1 (Frost *et al.*, 1996). pCMV5M/PAK1 165-544 and pCMV5M/PAK1 165-544 K²⁹⁸A were created by PCR using Vent, adding 5' *Eco*RI and 3' *Xho*I sites. Amplicons were subcloned into pGEX-KG first, and then pCMV5M. During subcloning procedures, these PAK molecules were found to be susceptible to inactivating mutations in the catalytic domain. To reduce the frequency of these mutations, the bacterial strain TG1 was used and all clones were grown at 30°C. Correct subcloning was determined by sequencing the entire catalytic domain for each construct upon their creation, as well as from later plasmid preparations.

Eukaryotic expression plasmids for Raf-1 (pCMV-craf) and K³⁷⁵WRaf-1 (pCMV-301-1) were produced by cloning the cDNAs from the corresponding pMNC constructs (Wagner *et al.*, 1992) into pCMV5 (Rubin, 1994). Raf BXB, S²⁵⁹D (D²⁵⁹Raf-1) and S³⁵⁹A (A³⁵⁹Raf-1) were also sub-cloned into pCMV5. The cDNA of A³⁵⁹Raf-1 originated from pBS/KS-S359A and the cDNA for the BXB domain of Raf-1 originated from MHS-BXB, both of which were provided by W.Kolch. The cDNA for the D²⁵⁹Raf-1 mutant originated from pUC-CMV-Raf259D (Häfner *et al.*, 1994). N-terminally HA-tagged Raf-1 and D²⁵⁹Raf-1 were contained in pCEP4. All Raf-1 point mutations were verified by sequencing.

The expression vector for N¹⁷H-Ras consisted of the cDNA insert from pcEXVN17 subcloned into pCMV5. Dominant-negative ERK2 K⁵²R and SAPK β K⁵⁵A were also contained in pCMV5. Human wild-type, T²⁹²A, S²⁹⁸A and T²⁹²A/S²⁹⁸A MEK1 were contained in pCEP4, as was human wild-type MEK2.

The Gal-Lux reporter (pG₅E₄D38-Lux) contained five copies of a Gal4 binding site upstream of the adenovirus E4 promoter (Carey *et al.*, 1990) and was a gift from K.-H.Klempnauer. The expression vectors for Gal-Elk and Gal-Sap, pSG.Gal-Elk (Kortenjann *et al.*, 1994) and pSG.Gal-Sap (Strahl *et al.*, 1996), have been described previously. pRSV-Gal-Jun was a gift from P.Angel.

Recombinant protein expression in *Escherichia coli* and corresponding *in vitro* kinase assays

Histidine-tagged, mouse and human MEK1 proteins were expressed and purified as previously described (Xu *et al.*, 1995). GST-PAK1 232-544 was expressed in JM109 cells. Cultures were grown at 30°C in M9 minimal medium (Sambrook *et al.*, 1989) with 10 g/l bactotryptone, 0.2% glucose, 100 µM CaCl₂, 1 mM MgSO₄ and 0.01% thiamine added. At OD₆₀₀ = 0.6, IPTG was added to 400 µM and the cells were incubated for 4 h more, after which they were pelleted by centrifugation and frozen. Bacteria were lysed by incubation for 30 min on ice in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 µM aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin) plus 1 mg/ml lysozyme, followed by extensive sonication on ice. Insoluble material was pelleted by centrifugation at 27 000 g for 15 min at 4°C. Soluble GST fusions were purified using glutathione-agarose beads by incubating for 1 h at 4°C with mixing, followed by extensive washing with lysis buffer. GST fusions were eluted with 5 mM glutathione in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 10% glycerol. Eluted proteins were dialyzed into elution buffer lacking glutathione, flash-frozen in liquid nitrogen, and stored at -80°C.

PAK1 phosphorylation of MEK1 was carried out for 1 h at 30°C in 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 100 µM ATP and [γ -³²P]ATP added to a specific activity of ~4 c.p.m./fmol. MEK1 and mutants were present at a concentration of 200 nM, while GST-PAK1 232-544 was present at 2 µM. Reactions were stopped by addition of 5× Laemmli sample buffer followed by boiling, and proteins were resolved by SDS-PAGE. Phosphoamino acid analysis was carried out on proteins transferred to PVDF membranes (Millipore) and then eluted by exhaustive TPCK-trypsin digestion, as described previously (Boyle *et al.*, 1991).

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