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# A new Rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-κB signalling pathways

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**The Rho, Rac and Cdc42 GTPases coordinately regulate the organization of the actin cytoskeleton and the JNK MAP kinase pathway. Mutational analysis of Rac has previously shown that these two activities are mediated by distinct cellular targets, though their identity is not known. Two Rac targets, p65$^{PAK}$ and MLK, are ser/thr kinases that have been reported to be capable of activating the JNK pathway. We present evidence that neither is the Rac target mediating JNK activation in Cos-1 cells. We have used yeast two-hybrid selection and identified a new target of Rac, POSH. This protein consists of four SH3 domains and ectopic expression leads to the activation of the JNK pathway and to nuclear translocation of NF-κB. When overexpressed in fibroblasts, POSH is a strong inducer of apoptosis. We propose that POSH acts as a scaffold protein and contributes to Rac-induced signal transduction pathways leading to diverse gene transcriptional changes.**

*Keywords*: apoptosis/JNK/NF-κB/POSH/Rac

## Introduction

Rho, Rac and Cdc42, three members of the Rho family of small GTPases, act as molecular switches cycling between an active GTP-bound and an inactive GDP-bound state. Activation, in response to extracellular agonists acting on membrane receptors, is mediated by the Dbl family of guanine nucleotide exchange factors (GEFs), while down-regulation involves a poorly characterized family of GTPase-activating proteins (GAPs) and RhoGDIs (Lamarche and Hall, 1994; Cerione and Zheng, 1996). In their active state, Rho, Rac and Cdc42 interact with a variety of target (effector) proteins to elicit cellular responses (Van Aelst and D'Souza-Schorey, 1997).

Activation of Rho, Rac and Cdc42 in quiescent Swiss-3T3 fibroblasts induces rearrangement of filamentous actin leading to the formation of actin stress fibres, lamellipodia and filopodia, respectively (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kozma *et al.*, 1995; Nobes and Hall, 1995). Growth factors such as platelet-derived growth factor (PDGF) and insulin, or constitutively activated (oncogenic) Ras protein stimulate Rac and in both cases this is mediated by PI 3-kinases (Kotani *et al.*, 1994; Wennstrom *et al.*, 1994; Hawkins *et al.*, 1995; Nobes *et al.*, 1995; Reif *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997). Once activated, Rac induces polymerization of monomeric actin at the cell periphery to produce a dense meshwork of actin filaments forming extending lamellipodia and membrane ruffles (Ridley *et al.*, 1992). There is growing evidence that this pathway plays a major role in directed cell migration and axonal guidance (Van Aelst and D'Souza-Schorey, 1997). In addition to inducing actin polymerization, Rac stimulates the formation of associated integrin-based adhesion complexes (Nobes and Hall, 1995). These structures contain many of the same constituents as classical focal adhesions, though morphologically they are distinct; they do not seem to be required for actin polymerization but instead may play a role in cell movement or signalling (Machesky and Hall, 1997).

Members of the Rho GTPase family also regulate gene transcription. All three GTPases have been reported to stimulate the JNK/SAPK and p38/HOG1 MAP kinase cascades, the transcription factor NF-κB and the transcription factor SRF (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Hill *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995; Sulciner *et al.*, 1996; Teramoto *et al.*, 1996b; Perona *et al.*, 1997). To date, most of these observations have been obtained by overexpressing GTPase constructs in transfected cells, but the genetic analysis of budding yeast and more recently *Drosophila* has confirmed that Rho GTPases coordinately regulate the organization of the actin cytoskeleton and the activity of MAP kinase pathways probably in all eukaryotic cells (Glise and Noselli, 1997; Leberer *et al.*, 1997). In addition, Rho, Rac and Cdc42 can trigger $G_1$ progression when introduced into quiescent fibroblasts and they are each required for serum-induced cell cycle progression and Ras-induced cell transformation (Olson *et al.*, 1995; Qiu *et al.*, 1995a,b, 1997). Moreover, many of the Dbl family of Rho GEFs are potent oncogenes and will transform NIH-3T3 cells to a malignant phenotype (Cerione and Zheng, 1996). How they do this is not known, though stimulation of $G_1$ progression and cell transformation correlate well with the ability of the GTPases to induce cytoskeletal changes and it is possible that signals are induced in response to actin polymerization or integrin complex assembly (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997).

To understand the biochemical mechanisms underlying the various activities of the Rho GTPases, there has been intense activity to identify target proteins (Van Aelst and D'Souza-Schorey, 1997). Mutational analysis of Rac has provided evidence for bifurcating pathways controlling cytoskeletal changes and MAP kinase activation; amino acid substitutions at codon 37, for example, block the induction of lamellipodia without affecting JNK activation, while changes at codon 40 have the opposite effect (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). It appears, therefore, that Rac interacts

with at least two distinct target proteins to trigger these pathways. It is not clear whether activation of NF-κB or other transcription factors such as SRF are a consequence of, or are independent of, Rac's effect on actin or the JNK pathway.

To date, around 10 targets for Rac have been identified using yeast two-hybrid and affinity chromatography techniques (Van Aelst and D'Souza-Schorey, 1997). The first Rac target to be identified was a serine/threonine kinase $p65^{PAK}$. It is closely related to a yeast kinase, Ste20p, which is known to regulate MAP kinase pathways in this organism and it has, therefore, been suggested that $p65^{PAK}$ probably mediates JNK activation by Rac in mammalian cells (Manser *et al.*, 1994). In agreement with this, the interaction of $p65^{PAK}$ is blocked by changes at codon 40, but not codon 37, of Rac (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). Furthermore, there have been a number of reports that a constitutively activated version of $p65^{PAK}$ leads to JNK activation and one report that a dominant-negative version inhibits Rac-mediated JNK activation (Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Brown *et al.*, 1996; Frost *et al.*, 1996). However, others have failed to find a role for $p65^{PAK}$ in JNK activation and its physiological role remains unclear (Teramoto *et al.*, 1996a,b; Westwick *et al.*, 1997). In addition to $p65^{PAK}$ another target of Rac, MLK, is also a potential regulator of JNK pathways, since it belongs to the family of MAP kinase kinase kinases (Burbelo *et al.*, 1995).

We report here that in Cos-1 cells, the interaction of Rac with $p65^{PAK}$ or MLK is not the trigger for JNK activation. Using a yeast two-hybrid screen, we have identified a new target for Rac, POSH, which contains four SH3 domains. When transfected into Cos-1 cells, POSH stimulates JNK activation. Expression of POSH in fibroblasts leads to nuclear translocation of NF-κB, independently of either actin reorganization or JNK activation. We conclude that POSH acts as a scaffold protein and participates in Rac-mediated signal transduction pathways leading to gene transcriptional changes.

## Results

### *$p65^{PAK}$ and MLK do not mediate Rac-induced JNK activation*

To examine whether $p65^{PAK}$ could be a mediator of Rac-induced JNK activation, Cos-1 cells were co-transfected with a constitutively activated version of $p65^{PAK}$ (L107F, PAK1/PAKα isoform) and a JNK1 expression plasmid. As seen in Figure 1A, co-transfection with a constitutively activated Rac (V12) leads to a 21-fold stimulation of JNK activity, whereas activated $p65^{PAK}$ induced no significant activation. As a control, L107F-$p65^{PAK}$ was immunoprecipitated from the transfected cells and shown to be active using MBP as a substrate. It appears, therefore, that $p65^{PAK}$ does not activate the JNK kinase cascade in these cells.

It has been reported that another Rac target, MLK, is a potent activator of the JNK pathway and we have confirmed this in Cos-1 cells (Nagata *et al.*, 1998). It is possible, therefore, that the interaction of Rac with endogenous MLK proteins is the trigger for JNK activation. As shown previously, Rac containing a Y40C substitution can no longer activate JNK and as expected $p65^{PAK}$

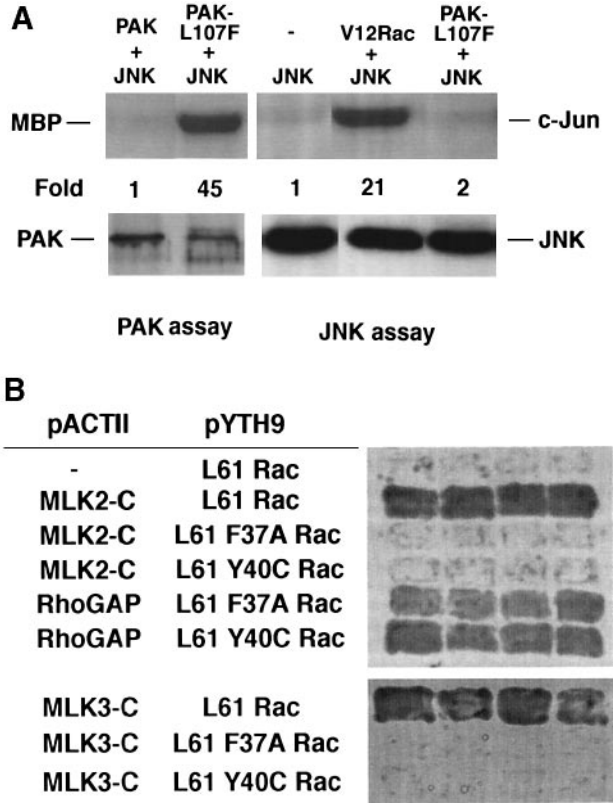


**Fig. 1.** $p65^{PAK}$ and MLK2/3 do not mediate Rac-induced JNK activation in Cos-1 cells. (**A**) Activated $p65^{PAK}$ does not cause JNK1 activation when overexpressed in Cos-1 cells. pCMV5FLAG-JNK1 was co-transfected with myc-tagged activated (L107F) $p65^{PAK}$, wild-type $p65^{PAK}$, V12 Rac or empty pRK5Myc vector (–), as described in Materials and methods. Kinase activities of $p65^{PAK}$ and JNK1 were measured on immune complexes (top panel) using Myelin Basic Protein (5 μg per reaction) or c-jun (2 μg per reaction). Expression levels were visualized with anti-myc (for $p65^{PAK}$) and anti-JNK1 antibodies (bottom panel). (**B**) MLKs do not interact with F37A or Y40C effector mutants of Rac. Yeast strains containing Rac mutants in the integrated bait vector pYTH9 were transformed with the pACTII prey vector containing MST1/MLK2 C-Terminus (aa 338–953), MLK3 C-Terminus (aa 348–847), RhoGAP as a positive control, or empty vector as a negative control. Colonies of equal size were replated in the presence of 25 mM 3-aminotriazole and allowed to grow for 3 days at 30°C.

(Lamarche *et al.*, 1996); MST1/MLK2 and MLK3 (Figure 1B) are no longer able to interact with Y40CRac in yeast two-hybrid or dot-blot assays. However, we had shown previously that Rac containing an F37A substitution was still able to activate JNK in Cos-1 cells (Lamarche *et al.*, 1996). As shown in Figure 1B, F37ARac does not interact with MST1/MLK2 or MLK3. We conclude, therefore, that the interaction of Rac with MLKs cannot be the trigger for JNK activation in this assay.

### *Isolation and characterization of a novel Rac-interacting protein*

In order to isolate potential downstream targets of Rac that might account for its ability to activate JNK, we have screened a yeast two-hybrid mouse cDNA library using L61 Rac as bait. Approximately $10^7$ clones were screened as described in Materials and methods and of the 30 fastest-growing clones picked from the selection plates, one (clone 4) tested positive upon rescreening and was

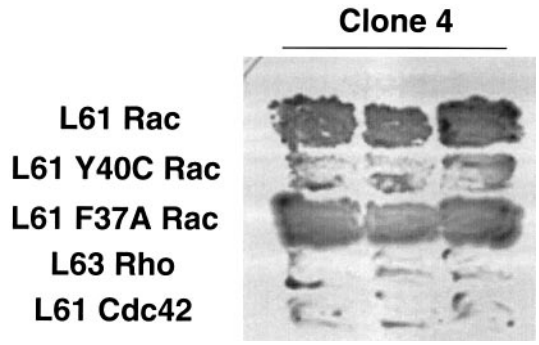


**Fig. 2.** Interaction of clone 4 with Rac. Clone 4 in the pGAD-10 prey vector was introduced into yeast strains containing integrated L61 Rac, L63 Rho, L61 Cdc42, F37A L61 Rac and Y40C L61 Rac. Colonies of equal size were replated in the presence of 25 mM 3-aminotriazole and allowed to grow for 3 days.

negative in the absence of the Rac bait. Figure 2 shows that clone 4 does not interact with Rho or Cdc42 in yeast, and in addition it still interacts with F37ARac, but not with Y40CRac. These observations are consistent with clone 4 encoding a mediator of Rac-induced JNK activation.

Sequence analysis of clone 4 (2.4 kb insert) revealed an open reading frame (ORF) for a protein of 67 kDa, but since no stop codon was present 5′ of the first methionine, it was possible that clone 4 represented an incomplete ORF. Using a primer derived from a region close to the 5′ end of clone 4 we looked for potential additional upstream sequences using a commercial 5′ RACE PCR library. One major RACE product was obtained (1.4 kbp in length) and sequence analysis of this confirmed that the original clone 4 represented an incomplete ORF. The full ORF encodes a protein of 892 amino acids (predicted mol. wt 93 kDa) (see Figure 3A); the first methionine is surrounded by a consensus Kozak sequence and is preceded by an in-frame stop codon.

Analysis of the protein sequence reveals a potential zinc finger structure (aa 18–82), but more interestingly four SH3 domains (underlined in Figure 3A). Accordingly, we have called the protein POSH (Plenty Of SH3s). Using a commercial mouse tissue Northern blot, a single mRNA species for POSH (at ~5 kb) can be seen in all tissues (Figure 3B), though skeletal muscle and spleen appear to have relatively low levels. We conclude that POSH is ubiquitously expressed.

### *Interaction of POSH with Rac*

A bona fide target protein would be expected to interact preferentially with the GTP-bound state of Rac. To test whether the interaction of POSH is GTP-dependent, we have used a modified dot-blot assay using [α-$^{32}$P]GTP-loaded Rac (see Materials and methods) and [α-$^{32}$P]GDP-loaded Rac (obtained by pre-incubating [α-$^{32}$P]GTP-loaded Rac with a small amount of Rho GAP). As seen in Figure 4A, POSH interacts with the GTP form of Rac but not the GDP form. Using a dot-blot assay, we also confirmed that POSH interacts with the F37A mutant of Rac, but not with the Y40C mutant (Figure 4B).

Many, though not all, previously identified Rac targets contain a distinctive binding site, the CRIB site. Sequence analysis (Figure 3A) revealed that POSH does not contain a CRIB site. To identify the region of the protein that contains the Rac interaction site, a series of truncations were expressed as GST fusion proteins and tested for binding to L61 Rac in dot-blot assays. As shown in Figure 4C, Rac interacts with POSH in a region encompassing 70 residues (aa 292–362, dotted underline in Figure 3A). Extensive searching of EST and non-redundant databases, which includes all other known GTPase targets showed no significant matches to sequences within this region. The Rac interaction site in POSH is, therefore, so far unique.

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1   mdesalldll ecpvclerld asakvlpcqh tfckrcllgi vgsrnelrcp
51  ecrtlvgsgv delpsnillv rlldgikqrp wkpgpggggg ttctntlraq
101 gstvvncgsk dlqssqcgqq prvqawsppv rgipqlpcak alynyeqkep
151 qdlkfskqdt iilrrqvden wyhgevsqvh gffptnfvqi ikplpqpppq
201 ckalydfevk dkeadkdclp fakddvltvi rrvdenwaeq mladkiqifp
251 isyvefnsaa kqliewdkpp vpgvdtaecp sataqstsas khpdtkkntr
301 krhsftsltm ankssqgsqn rhsmeisppv lisssnptaa ariselsgls
351 csapsqvhis ttglivtppp sspvttgpaf tfpsdvpyqa algsmnpplp
401 pppllaatvl astpsgataa vaaaaaaaaa agmgprpvmg sseqiahlrp
451 qtrpsvyvai ypytprkede lelrkqemfl vfercqdqwy kqtsmhtski
501 qvfpqnyvap vtravtnasq akvsmstagq asrgvtmvsp staggptqkp
551 qgngvagnps vvptavvsaa hiqtspqakv llhmsgqmtv nqarnavrtv
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651 aavsisrtna pmacaagasl aspnmtsaml etepsgrtvt ilpglptspe
701 saasacgnss agkpdkdskk ekkgllklls gastkrkprv sppasptldv
751 elgageaplq gavgpelplg gshgrvgscp tdgdgpvaag taalaqdafh
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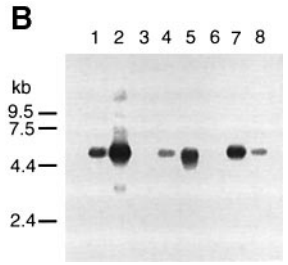


**Fig. 3.** Sequence and expression pattern of POSH. (**A**) Protein sequence of full-length POSH. SH3 domains are underlined. The minimal Rac-binding site is indicated with a dotted line. DDBJ/EMBL/GenBank accession number of POSH cDNA: AF030131. (**B**) Tissue expression of POSH. A mouse multiple tissue Northern blot was probed as described in the methods. Lane 1, testis; lane 2, kidney; lane 3, skeletal muscle; lane 4, liver; lane 5, lung; lane 6, spleen; lane 7, brain; lane 8, heart.

### *POSH triggers programmed cell death*

To test whether POSH can induce changes to the actin cytoskeleton, full-length POSH cDNA was first subcloned into the mammalian expression vector, pRK5, so as to introduce a myc epitope tag at its N-terminus. Plasmid DNA was microinjected into the nuclei of serum-starved, confluent Swiss-3T3 fibroblasts and any effects on the actin cytoskeleton observed 2–8 h later using fluorescently labelled phalloidin. Under conditions where Rac induced strong actin polymerization and lamellipodia, POSH induced no detectable assembly of actin filaments (data not shown). It was noticed, however that POSH did induce

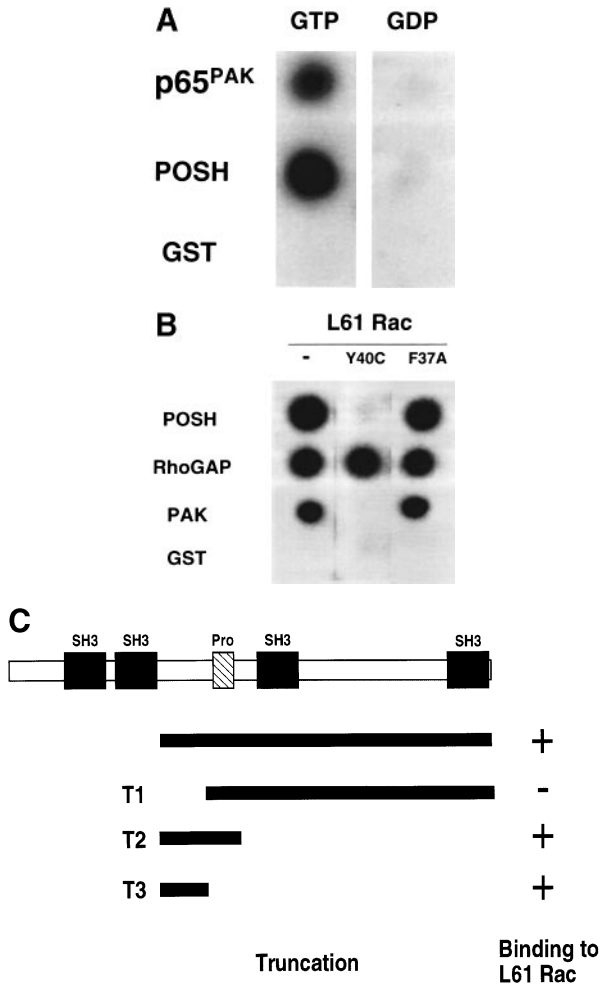


**Fig. 4.** Interaction of POSH with Rac *in vitro*. (**A**) POSH binds to Rac in a GTP-dependent manner. GST-POSH (10 μg, aa 292–892), GST-p65$^{PAK}$ (8 μg) and GST (10 μg) were spotted on strips of nitrocellulose which were probed with [α-$^{32}$P]GTP- or [α-$^{32}$P]GDP-bound wild-type Rac. (**B**) POSH binds to F37A but not Y40C Rac *in vitro*. GST-POSH (10 μg, aa 292–892), GST-p65$^{PAK}$ (8 μg), GST-RhoGAP (10 μg, aa 198–439), and GST (10 μg) were spotted on nitrocellulose and probed with [γ-$^{32}$P]GTP-bound Rac mutants. (**C**) Identification of a 70 amino acid fragment of POSH that is sufficient for binding to L61 Rac. Ten μg of GST-fusion proteins of POSH truncations were spotted on nitrocellulose and tested for interaction with L61 Rac as in (B). From top to bottom: original two-hybrid fragment (aa 292–892); Truncation 1 (aa 352–892); Truncation 2 (aa 292–398); Truncation 3 (aa 292–362).

significant cell death at the later time points in these conditions. Further analysis in NIH-3T3 cells revealed that 14 h after injection, full-length POSH induced cell death in ~90% of injected cells, even in the presence of serum (Figure 5, top) and that the few remaining cells had shrunken and condensed (pyknotic) nuclei typical of apoptosis (Figure 5, bottom). In contrast, a POSH Truncation (aa 352–892) lacking the N-terminal two SH3 domains and the Rac-binding domain, or activated L61Rac, did not induce significant cell death under these conditions (Figure 5). Time-lapse video microscopy revealed that cell contraction and intense surface blebbing could be seen in the majority of cells 5 h after injection (data not shown). Similar effects were observed in primary rat embryo fibroblasts (data not shown). We conclude that overexpression of POSH induces apoptotic cell death in primary and immortalized fibroblasts (Jacobson *et al.*, 1997).

### *POSH stimulates the JNK pathway*

To examine the effects of POSH on JNK activation, Cos-1 cells were co-transfected with POSH and JNK. As shown by Western blot analysis the toxic effects of full-length POSH, particularly when co-transfected with JNK (Figure 6A), were also apparent in this cell line [compare expression levels of full-length POSH (lane 2) with truncated POSH (lane 3)]. The toxic effects could be largely overcome by including the caspase inhibitor BocD-fmk (Deshmukh *et al.*, 1996; Weil *et al.*, 1997) in the transfection assays (Figure 6A, compare lane 1, + inhibitor with lane 2, – inhibitor). Under these conditions, it can be seen in Figure 6B that full-length POSH induces a 5.6-fold stimulation of JNK activity, compared with 11.0-fold by L61Rac. The truncated version of POSH was unable to stimulate JNK (Figure 6B), nor did it interfere with Rac-induced JNK activation (data not shown).

In order to test whether POSH-induced cell death is dependent on JNK activation, we co-injected POSH with a 5-fold molar excess of dominant-negative SEK1 (SAPK/ERK kinase 1) (S220A + T224L version or K129R version) into NIH-3T3 fibroblasts. Although both dominant-negative SEK1 constructs were expressed at relatively high levels, neither was able to prevent POSH-induced cell death (data not shown).

### *POSH stimulates nuclear translocation of NF-κB*

To examine whether POSH might contribute to Rac-induced NF-κB activation, expression constructs were microinjected into quiescent Swiss-3T3 cells. Translocation of NF-κB to the nucleus was visualized by immunofluorescence 5 h later. Full-length POSH, truncated POSH containing the two C-terminal SH3 domains (aa 362–892), activated L61 Rac and L61 F37A Rac were potent inducers of NF-κB translocation (Figure 7A). However, L61Y40CRac—which does not interact with POSH—was severely impaired in its ability to cause translocation. Interestingly, translocation of NF-κB in injected cells was accompanied by NF-κB translocation in neighbouring non-injected cells (Figure 7B, see arrowhead). We conclude that Rac and POSH can induce nuclear translocation of NF-κB and that this may involve the induction of autocrine/paracrine factors.

## Discussion

Rho, Rac and Cdc42 control the assembly and organization of the actin cytoskeleton in eukaryotic cells. In response to extracellular signals, the active conformation of the three GTPases leads to the assembly of actin–myosin contractile filaments, lamellipodia and filopodia, respectively, and it is likely that these proteins play important regulatory roles in cell movement (Murphy and Montell, 1996; Van Aelst and D'Souza-Schorey, 1997; Zipkin *et al.*, 1997). In addition to their effects on actin, members of the Rho GTPase family regulate changes in gene transcription. Rho, Rac and Cdc42 have each been reported to activate the JNK and p38 MAP kinase pathways, to activate the transcription factors NF-κB and SRF and to stimulate $G_1$ progression in quiescent Swiss cells (Bagrodia

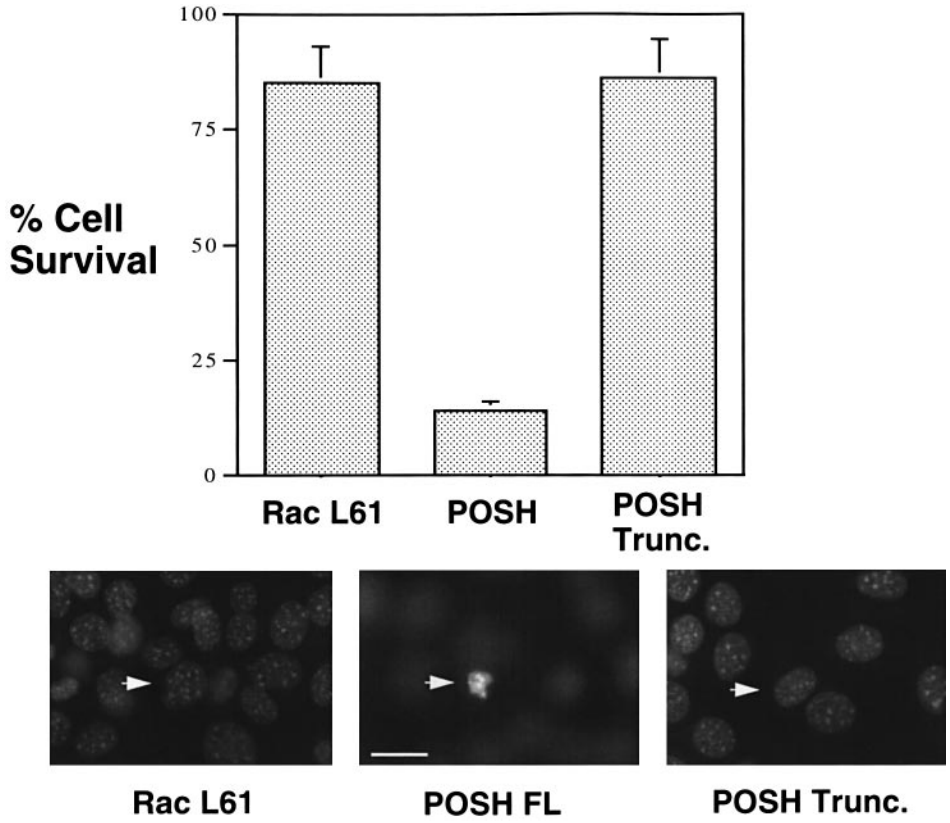


**Fig. 5.** Full-length POSH induces apoptosis in NIH-3T3 cells. NIH-3T3 cells were replated on glass coverslips and left for 24 h in DMEM containing 10% DCS and the nuclei of 50 cells were injected with an expression vector containing full-length POSH, POSH Truncation 1 (aa 352–892) or L61 Rac at a concentration of 0.04 mg/ml. The cells were fixed and stained as described after a 12 h incubation in the presence of serum. Bottom line shows typical nuclear morphology of injected cells (arrows) using Hoechst dye staining. In the middle panel, non-injected cell nuclei are in a different plane of focus from the rounded up POSH injected cells. Results were averaged over three independent experiments; error bars represent standard deviation. Scale bar = 20 μm.

*et al.*, 1995; Coso *et al.*, 1995; Hill *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995; Sulciner *et al.*, 1996; Teramoto *et al.*, 1996b; Perona *et al.*, 1997). To what extent these pathways are interdependent is not clear, although $G_1$ progression can be triggered by Rac mutants that can no longer activate the JNK pathway (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997).

To characterize the biochemical pathways mediating the various cellular responses induced by GTPases, many groups have used yeast two-hybrid and affinity chromatography techniques to identify target proteins and approximately ten candidate targets for Rac have been isolated so far (Van Aelst and D'Souza-Schorey, 1997). Little progress has yet been made in identifying which is responsible for triggering actin polymerization after interacting with Rac GTP, but several targets have been implicated in JNK activation. A number of groups have reported that the interaction of Rac with the ser/thr kinase, $p65^{PAK}$, might lead to activation of the JNK and p38 pathways and in some cell types overexpression of $p65^{PAK}$ does lead to JNK activation (Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Brown *et al.*, 1996; Frost *et al.*, 1996). The MLK kinases are also known to be potent activators of the JNK pathway and these proteins have binding sites for Rac (Rana *et al.*, 1996; Teramoto *et al.*, 1996b; Nagata *et al.*, 1998). We show here that constitutively activated $p65^{PAK}$ (PAK1/PAKα isoform) does not stimulate the JNK pathway in Cos-1 cells. Furthermore, a Rac mutant (L61F37ARac) that can no longer interact with MLK (MST1/MLK2 or MLK3 isoforms) is still able to activate JNK. We conclude, therefore, that neither target is likely to mediate JNK activation by Rac in Cos-1 cells. Although it remains a possibility that one of the other two PAK isoforms (PAK2/PAKγ or PAK3/PAKβ) or another member of the MLK family might trigger JNK activation in our Cos-1 cells, this seems unlikely given their high degree of sequence homology. Two new MAP kinase kinase kinases, MEKK4 and MEKK1, have recently been reported to interact directly with Rac in a GTP-dependent manner and to activate the JNK cascade (Fanger *et al.*, 1997). It is possible, therefore, that either of these could be the cellular target of Rac in Cos-1 cells responsible for activation of JNK.

We have searched for new Rac targets that might contribute to JNK activation by screening a yeast two-hybrid library with constitutively active L61Rac. We have identified a ubiquitously expressed protein, POSH, that interacts with Rac (but not Rho or Cdc42) in a GTP-dependent manner. This 93 kDa protein contains four SH3 domains (and a putative zinc finger) and it interacts with F37ARac, but not Y40CRac. It is possible, therefore, that this protein might play a role in Rac-mediated JNK activation. Consistent with this, expression of POSH in Cos-1 cells induces activation of JNK. Activation of JNK by inflammatory cytokines or by stress is often accompanied by nuclear translocation of the transcription

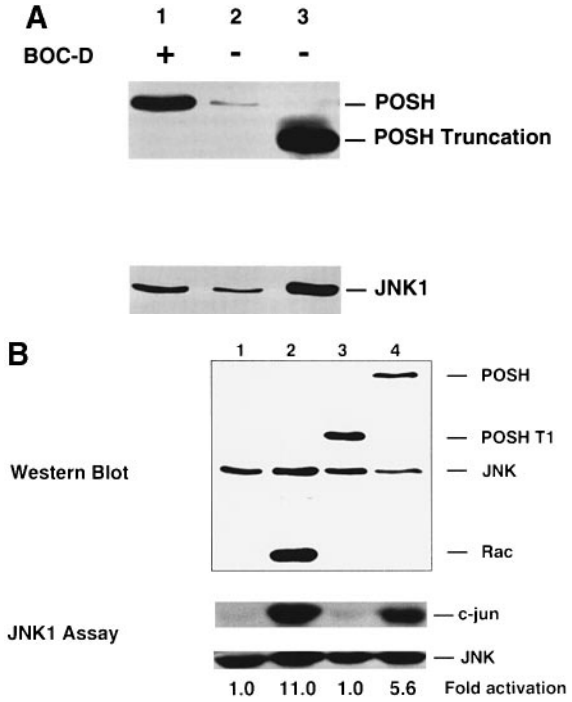


**Fig. 6.** POSH activates JNK1 in Cos-1 cells. (**A**) Cos-1 cells were co-transfected with pCMV5FLAG-JNK1 (5 μg) and pRK5myc-full-length POSH (lanes 1 and 2) or pRK5myc-POSH Truncation 1 (lane 3) (all 3 μg) in the presence (+) or absence (–) of BocD-fmk at 20 μM. Cells were harvested in 300 μl of 3× protein sample buffer and 30 μl of each lysate was analysed by Western blotting with anti-myc and anti-JNK1 antibodies. (**B**) pCMV5FLAG-JNK1 was co-transfected with empty pRK5myc vector (lane 1), pRK5myc-L61 Rac (lane 2), pRK5myc-POSH Truncation 1 (aa 352–892) (lane 3), and pRK5myc-full-length POSH (lane 4) all in the presence of 20 μM BocD-fmk (Enzymes Systems Products). Aliquots of each transfection were electrophoresed on an SDS–PAGE gel and expression levels of transfected constructs were visualized on Western blots using anti-myc (for POSH, Rac), and anti-FLAG (for JNK1) antibodies followed by $^{125}$I-labelled protein A (top panel). JNK1 activity was assayed on immune complexes using GST-c-jun as a substrate and quantified on a Bio-Rad Molecular Imager (middle panel). Levels of JNK1 in the immunoprecipitates were visualized using an anti JNK1 antibody (bottom panel).

factor NF-κB (Verma *et al.*, 1995). Furthermore, there have been reports that Rac can activate NF-κB when transfected into cells (Sulciner *et al.*, 1996; Perona *et al.*, 1997). Using an immunofluorescence assay, we have shown that expression of POSH in Swiss-3T3 cells leads to nuclear translocation of NF-κB. Interestingly, the two C-terminal SH3 domains are sufficient to induce NF-κB translocation, but not JNK activation. We conclude that POSH may play a role in both JNK and NF-κB activation mediated by Rac. In agreement with this, Y40CRac—which is inactive in both assays—no longer interacts with POSH, while F37ARac—which interacts with POSH—is active in both assays.

Finally, activation of JNK and NF-κB pathways is often seen in cells stimulated to undergo apoptosis though the relative contributions of the two pathways to cell death are highly cell type-dependent (Liu *et al.*, 1996; Baichwal and Baeuerle, 1997; Chuang *et al.*, 1997; Herdegen *et al.*, 1997). We have found that overexpression of POSH in Swiss or NIH-3T3 cell lines, in primary rat embryo fibroblasts or in Cos cells is highly toxic and leads to cell death via apoptosis. The two C-terminal SH3 domains of POSH, which can activate NF-κB but not JNK, do not induce cell death. Co-injection of POSH with dominant-negative SEK failed to block POSH-induced cell death, though this construct has been reported to block JNK activation induced by MEKK1 and to inhibit cell death induced by *cis*-platinum, UV irradiation or heat (Sanchez *et al.*, 1994; Zanke *et al.*, 1996). It is possible, therefore, that POSH induces cell death independently of JNK activation. Furthermore, even though POSH is a Rac target, overexpression of Rac does not lead to apoptosis. One possible explanation for this apparent discrepancy is that Rac, through its multiple downstream targets, might also activate survival signals. If this is the case, then the survival signal cannot be NF-κB, since this is also activated by POSH.

SH3 domain-containing proteins have previously been shown to play important roles in mediating Rho GTPase signals. In phagocytic cells, $p67_{phox}$, a component of the NADPH oxidase enzyme complex is a target of Rac (Diekmann *et al.*, 1994). $p67_{phox}$ has no catalytic activity, but consists of two SH3 domains—the second of which shows close similarity to the first and fourth SH3 domains of POSH (51.9% with POSH 1, 42.6% with POSH 4). The membrane-bound oxidase is responsible for the generation of superoxide, which forms a part of the pathogen-killing mechanism of professional phagocytes, and both Rac and $p67_{phox}$ are essential for its activity (Abo *et al.*, 1991; Segal and Abo, 1993; Diekmann *et al.*, 1994). Rac has also been reported to induce the formation of reactive oxygen species (ROS) in non-phagocytic cells and in HeLa cells, this appears to account for Rac-mediated NF-κB activation (Sulciner *et al.*, 1996). Interestingly, during the POSH and Rac injection experiments, we see translocation of NF-κB in neighbouring, non-injected cells and although we cannot rule out mechanisms involving cell–cell contact, it raises the possibility that a paracrine factor (perhaps ROS) is produced. We are currently looking at whether Rac can induce ROS in Swiss-3T3 cells and if so, whether this is mediated by $p67_{phox}$ or perhaps POSH.

In *Saccharomyces cerevisiae*, the SH3-containing protein Bem1p is a key component of Cdc42-mediated signals during cell division and in the pheromone mating response (Leberer *et al.*, 1997). In the mating response, Bem1p acts as a scaffold protein and interacts with multiple proteins including Cdc24p (an exchange factor for Cdc42p), with Ste20p (a target of Cdc42p and a relative of mammalian $p65^{PAK}$), with Ste5p (a scaffold protein required for MAP kinase activation), with actin and with Far1p (an inhibitor of the cell cycle) (Leberer *et al.*, 1997). It is thought that a major role of Cdc42p in the pheromone pathway is to localize this multi-molecular signalling complex to the mating projection. Interestingly, in fission yeast the homologue of Bem1p, scd2, is also required for the mating response. In this case, scd2 interacts directly with scd1 (the exchange factor for cdc42) and with cdc42 (Chang *et al.*, 1994). No Bem1p/scd2 homologues have been identified in higher eukaryotes and it is tempting to speculate that POSH fulfils an analogous role and acts as a scaffold protein in Rac-mediated signalling pathways in mammalian cells. If this is the case, POSH might be predicted to interact with other components of the Rac signal transduction pathway (e.g. an exchange factor or

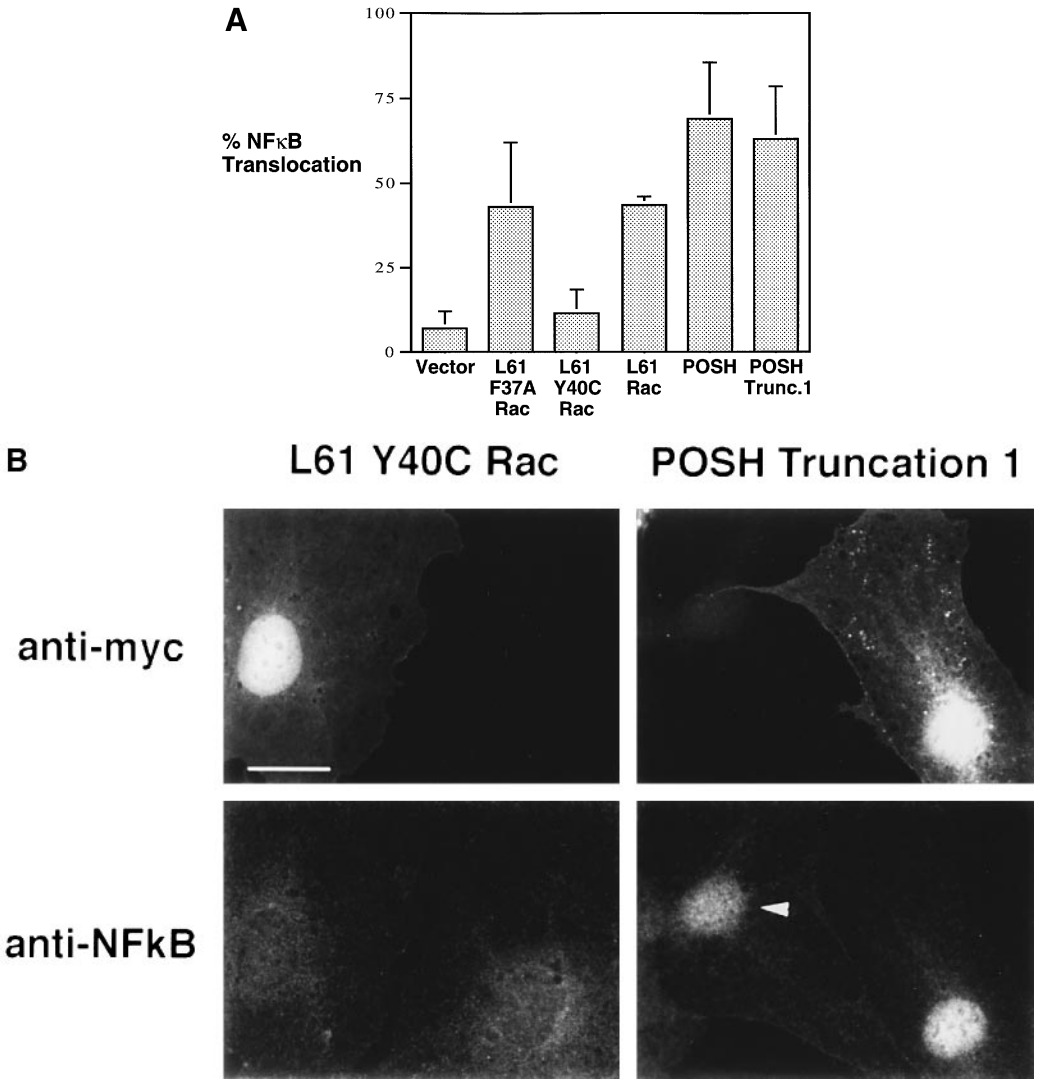


**Fig. 7.** POSH induces nuclear translocation of NF-κB in Swiss-3T3 cells. (**A**) Myc-tagged pRK5 plasmids (0.04 mg/ml) encoding POSH (full-length), POSH Truncation1 (aa 352–892), L61Y40CRac, L61F37ARac, L61Rac or empty vector (co-injected with FITC-dextran) were microinjected into serum-starved, confluent Swiss-3T3 cells and the cells fixed 5 h later. POSH/Rac expression was visualized using anti-myc antibody, while NF-κB localization was visualized using an anti-NF-κB antibody (Santa Cruz). The percentage of myc-positive cells in which clear nuclear fluorescence of NF-κB was seen were scored as positive. Results were averaged over four independent experiments; between 29 and 85 myc-expressing cells were analysed per experiment and the error bar represents standard deviation. (**B**) Right panel: a typical phenotype of a myc-positive, nuclear NF-κB-positive cell after injection with truncated POSH. The arrowhead points to a neighbouring, non-injected cell that is nuclear NF-κB positive. Left panel: a typical phenotype of a myc-positive, nuclear NF-κB-negative cell after injection with L61Y40CRac. Scale bar = 20 μm.

other targets). We have been unable to detect an interaction between $p65^{PAK}$ and POSH after Cos cell transfections (data not shown) and we are currently using yeast two-hybrid screens to identify binding partners of POSH.

The biochemical mechanisms through which overexpression of POSH leads to activation of JNK in fibroblasts is unclear. Expression of other adaptor-like molecules with SH3 domains can also have profound cellular effects; the SH2/SH3-containing protein v-Crk, for example, can induce malignant transformation (Mayer *et al.*, 1989). Furthermore, overexpression of Bem1p in yeast has been reported to activate the mating pheromone pathway independently of mating pheromone when expressed in a *STE11-4* mutant background (Lyons *et al.*, 1996). We propose that POSH either triggers the formation of a signalling complex leading to activation of JNK, NF-κB and apoptosis or acts by titrating out inhibitors of these processes.

In conclusion, we have shown that the two Rac targets, $p65^{PAK}$ and MLK are unlikely to account for Rac-induced JNK activation in Cos-1 cells. We have identified a new SH3-containing Rac target, POSH, which activates JNK when transfected into Cos-1 cells and induces nuclear translocation of NF-κB. We propose that POSH acts as scaffold protein required for the assembly of signalling complexes that control gene transcriptional events downstream of Rac.

## Materials and methods

### *Yeast two-hybrid screen*

A Ras-transformed NIH-3T3 cDNA library fused to the GAL-4 activation domain in the pGAD-10 vector (kind gift of Dr C.C.Kumar, USA) was

screened using L61 Rac in an integrated pYTH6 vector as a bait, as previously described (Aspenstrom *et al.*, 1996; Lamarche *et al.*, 1996). Approximately $10^7$ yeast colonies were screened for their ability to grow on selective medium containing 25 mM 3-aminotriazole. The 30 fastest-growing clones were replated and plasmids were rescued using the Wizard clean-up kit (Promega) and retransformed into the original yeast strain. One of these, clone 4 was strongly positive in the plate lift assay for expression of the LacZ reporter gene after the second round of transformation. The 2.4 kb insert of clone 4 was sequenced and found to be a novel cDNA potentially encoding a 67 kDa protein.

### Cloning of full-length clone 4

The full-length clone 4 sequence was isolated using the Clonetech Marathon RACE PCR kit (Mouse whole embryo) with Advantage Klentaq. A primer was generated corresponding to the minus strand of bp 156–178 of the original yeast clone (5′-GTCACCGGGCTGCTA-GGGGGTGGGG-3′). This primer was used with the Clonetech adaptor primer in the standard touchdown PCR protocol described in the Clonetech manual. Analysis of the RACE reaction by agarose gel electrophoresis revealed a major RACE product at 1.4 kb. The product was ligated into pYTH6 after *Nco*I–*Not*I digestion and sequenced. Two independent clones of the 1.4 kb fragment were analysed to eliminate any errors due to PCR amplification.

### Mammalian cell transfections

Cos-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10% FCS (Sigma). These were transfected using the DEAE–dextran method as previously described (Olson *et al.*, 1995). Plasmid amounts per 10 cm dish were as follows: pRK5myc-POSH, 5 μg; pRK5myc-POSH Truncation T1, 2 μg; pRK5myc-L107F$p65^{PAK}$ (PAK1/PAKα isoform), 5 μg; pRK5myc-V12/L61Rac1, 1.5 μg; pCMV-FLAG-JNK1, 5 μg. Empty vector was added where appropriate to ensure all transfections contained equal amounts of DNA. Cells were serum-starved 24 h after transfection and harvested 16 h later. After transfection, the cells were kept in serum-containing or serum-free media supplemented with the caspase inhibitor BocD-fmk (Boc-Aspartic acid-fluoromethylketone; Enzyme Systems Products) at 20 μM (from a 10 mM stock in DMSO).

### Kinase assays

Transfected Cos-1 cells were harvested in lysis buffer (20 mM Tris pH 8.0, 40 mM Na pyrophosphate, 50 mM NaF, 5 mM $MgCl_2$, 100 μM Na vanadate, 10 mM EGTA, 1% Triton X-100, 3 mM PMSF, 20 μg/ml leupeptin/aprotinin) using a cell scraper (Falcon). JNK1 or $p65^{PAK}$ were immunoprecipitated using anti-FLAG antibody (Scientific Imaging) for JNK1 or anti-myc antibody for $p65^{PAK}$ (Olson *et al.*, 1995). Kinase activity of immunocomplexes was measured using GST-c-jun for JNK1 or Myelin Basic Protein for $p65^{PAK}$ as substrates, as described (Olson *et al.*, 1995; Lamarche *et al.*, 1996). The relative levels of substrate phosphorylation were determined on a Bio-Rad PhosphorImager, following SDS–PAGE and transfer to nitrocellulose. Amounts of immunoprecipitated kinases were checked using anti-JNK1 antibody (Santa Cruz) or anti-myc antibody for $p65^{PAK}$.

### Co-precipitation of $p65^{PAK}$ and POSH

Cos-1 cells were transfected with pRK5myc-L107F$p^{65PAK}$ or pRK5FLAG-full-length POSH (3 μg each). Cell were harvested as above and the lysates were combined. Half of the lysate was used for immunoprecipitation using anti-myc antibody and the other half using anti-FLAG antibody (Scientific Imaging). Immunoprecipitations and washing were carried out in 40 mM Tris, pH 7.5, 50 mM NaCl, 0.2% NP-40, 50 mM NaF, 20 μg/ml leupeptin/aprotinin as for JNK assays (Olson *et al.*, 1995).

### Dot-blot assay

Interaction of GTPases with their targets was determined by dot-blot assay (Diekmann *et al.*, 1994). 10 μg of GST-fusion proteins were spotted on strips of nitrocellulose. The strips were air-dried and incubated for 1 h in 1 M glycine, 5% milk powder, 1% ovalbumin, and 5% fetal calf serum. The strips were then washed in buffer A [50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT)] and incubated for 5 min at 4°C with the indicated GTPase radiolabelled with [γ-$^{32}$P]GTP in a total volume of 2.5 ml of buffer A. The strips were washed three times with 5 ml of cold buffer A containing 0.1% Tween. Remaining radioactivity was visualized by autoradiography and quantified by densitometry.

### Purification and expression of recombinant proteins

Rac GST fusion proteins were purified on glutathione–Sepharose beads (Sigma) and cleaved using human thrombin as previously described (Self and Hall, 1995). POSH, $p65^{PAK}$ and RhoGAP were produced using a modification of this protocol. A culture of bacteria bearing pGEX-4T3 containing the relevant insert was grown in 500 ml of L-Broth containing 100 μg/ml ampicillin overnight at 37°C with vigorous agitation. The cells were diluted with 500 ml of fresh L-Broth containing ampicillin and left to grow for 2 h. Fusion protein was induced for 2 h by addition of IPTG to 1 mM. The cells were harvested and lysed as previously described (Self and Hall, 1995). Following purification on glutathione–Sepharose beads, the proteins were eluted with three washes of 500 μl resuspension buffer (15 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT) containing 1.5 mM glutathione. Eluted proteins were concentrated on centricon-10 columns and stored in liquid nitrogen. Protein concentration was assayed using the Bradford method and the quality was checked on SDS–PAGE gels using Coomassie blue.

### GTP dependence

To determine the relative binding of RacGTP and RacGDP to target proteins a modified dot-blot protocol was used. GST-fusion proteins were spotted onto nitrocellulose strips. 200 ng of wild-type Rac were loaded with [α-$^{32}$P]GTP and the exchange reaction stopped by addition of $MgCl_2$ on ice as previously described (Diekmann *et al.*, 1994). The sample was split in two and 10 ng of RhoGAP were added to one of the tubes. This tube was incubated for 10 min at 30°C (to produce predominantly [α-$^{32}$P]GDP), while the other tube was left on ice (predominantly [α-$^{32}$P]GTP). The GTPase aliquots were used in a dot-blot assay as described above. Bound radioactivity was quantified by autoradiography and densitometry.

### DNA constructs

Standard DNA protocols were used (Sambrook *et al.*, 1989). pGEX-4T3-clone 4 was generated by digestion of pGAD10-clone 4 with *Bam*HI and *Hin*dIII followed by ligation of clone 4 cDNA into pGEX-4T3. Truncations of clone 4 in pGEX were made by PCR with Pfu polymerase (Stratagene). Clone 4 and the truncations were transferred from pGEX-4T3, using *Bam*HI and *Hin*dIII, into the eukaryotic expression vector, PRK5-myc (Lamarche *et al.*, 1996). Full-length POSH in PRK5-myc was generated by amplifying the RACE product with Pfu polymerase using the following oligonucleotides: 5′-GCCGGATCCGATGAGT-CTGCCTTGTTGGAC-3′, and 5′-GACTTGTTGGCCATGG TGAGGG-AGGTGAAGG-3′. This fragment was then combined with the remainder of the POSH coding sequence and inserted into PRK5-myc using *Bam*HI and *Nco*I. The amplified fragment was checked by sequencing. A pRK5FLAG vector was constructed by digesting pRK5myc with *Cla*I and *Hin*dIII and introducing the FLAG epitope using the following oligonucleotides: 5′-CGATAGCCACCATGGACTACAAGGACGATG-ACGATAAGGGATCCCGGGTCTAGAATTCGGGA-3′ and 5′-AGCT-TCCCGAATTCTAGACCCTGGATCCCTTATCGTCATCGTCCTTGT-AGTCCATGGTGGCTAT-3′. Full-length POSH was inserted into pRK5FLAG from pRK5myc using *Bam*HI and *Hin*dIII.

The following constructs were kind gifts from colleagues: human MST-1/MLK2 from Dr M.Terada, National Cancer Center Research Institute, Tokyo; PCMV-FLAG-JNK1 and pGEX-c-Jun from Dr M.Karin, UC San Diego and Dr J.Ham, Eisai London Research Laboratories; pRK5mycSEK-1 (K129R) from Dr M.Olson, Chester Beatty Laboratories, London; HA-tagged SEK-1 AL (S220A, T224L) from Dr L.Zon, Dana Farber Cancer Institute, Boston; and PAK (L107F) from Dr J.Chant, Harvard University.

### Microinjection

Swiss-3T3 cells were maintained in DMEM containing 10% FCS and antibiotics. NIH-3T3 cells were maintained in DMEM containing 10% donor calf serum (DCS) and antibiotics. Swiss-3T3 cells were plated on acid-washed, round 13 mm coverslips at $6\times10^4$ cells/coverslip. At 7–10 days after plating, the confluent quiescent cells were serum-starved for 16 h in DMEM containing 2 g/l $NaHCO_3$. NIH-3T3 cells were plated on acid-washed coverslips at $5\times10^4$ cells/coverslip and left for 24 h in DMEM with 10% DCS before injection. Cells were microinjected as described (Nobes and Hall, 1995).

### Immunofluorescence microscopy

After microinjection and incubation at 37°C for the indicated times, the coverslips were rinsed in PBS and fixed for 10 min with 4% (w/v) paraformaldehyde in PBS. Coverslips were rinsed in PBS between each step of the staining procedure. Following fixation, the cells were

permeabilized for 5 min in 0.2% Triton X-100 in PBS (10 min for NF-κB staining). Free aldehyde groups were reduced by treatment with 0.5 mg/ml sodium borohydride for 10 min. Labelling of the cells was as previously described (Nobes and Hall, 1995). Anti-myc (9E10) antibody (kind gift of D.Drechsel) was diluted 1/200 in PBS; anti-NF-kB antibody (Santa Cruz) was diluted 1/200 in PBS. Primary antibodies were left on the coverslips for 1 h. After washing, the coverslips were incubated for 30 min with secondary antibodies: goat anti-mouse FITC (Pierce), donkey anti-rabbit TRITC (Jackson), diluted 1/100 in PBS. Coverslips were mounted on moviol mountant containing *p*-phenylenediamine as an anti-bleaching agent. After 1 h at 37°C, the coverslips were examined and the cells counted on a Zeiss axiophot microscope using Zeiss 40×1.3 and 63×1.4 oil-immersion objectives. Pictures were taken with a Hamamatsu C5985-10 video camera, then transferred to Kodak T-MAX 400 ASA film.

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