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# RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation

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**The RhoA GTPase regulates diverse cellular processes including cytoskeletal reorganization, transcription and transformation. Although many different potential RhoA effectors have been identified, including two families of protein kinases, their roles in RhoA-regulated events remain unclear. We used a genetic screen to identify mutations at positions 37–42 in the RhoA effector loop that selectively disrupt effector binding, and used these to investigate the role of RhoA effectors in the formation of actin stress fibres, activation of transcription by serum response factor (SRF) and transformation. Interaction with the ROCK kinase and at least one other unidentified effector is required for stress fibre formation. Signalling to SRF by RhoA can occur in the absence of RhoA-induced cytoskeletal changes, and did not correlate with binding to any of the effectors tested, indicating that it may be mediated by an unknown effector. Binding to ROCK-I, but not activation of SRF, correlated with the activity of RhoA in transformation. The effector mutants should provide novel approaches for the functional study of RhoA and isolation of effector molecules involved in specific signalling processes.**

*Keywords*: cytoskeleton/PKN/RhoA/ROCK/serum response element/SRF/transformation

## Introduction

The Rho subfamily of Ras-like GTPases transduce signals regulating many cellular processes, including cell morphology, cell motility, cell proliferation, gene expression and cytokinesis. In the case of mammalian RhoA, a number of potential effector molecules have been identified which interact preferentially with the GTP-bound form of RhoA (for review see Lim *et al*., 1996). By analogy with Ras, it is presumed that these interactions alter the subcellular localization or enzymatic activity of effector molecules and thereby transmit downstream signals (for review see Marshall, 1996). RhoA effectors include two families of protein kinases, the ROCKs (also known as ROKs; Leung *et al*., 1995, 1996; Nakagawa *et al*., 1996; Ishizaki *et al*., 1997) and PKN-related kinases (the PRK family; Palmer *et al*., 1994; Amano *et al*., 1996; Watanabe *et al*., 1996; Vincent and Settleman, 1997). In addition, RhoA can interact with the myosin binding subunit of MLC phosphatase (Kimura *et al*., 1996); putative adaptor proteins such as Rhotekin, Rhophilin and Citron (Madaule *et al*., 1995; Reid *et al*., 1996; Watanabe *et al*., 1996), two mouse Diaphanous homologues, p140mDia and mDia2 (Alberts *et al*., 1998b; Watanabe *et al*., 1997), and Kinectin (Hotta *et al*., 1996; Alberts *et al*., 1998b). Biochemical studies have provided strong evidence that the ROCKs, MLC phosphatase and possibly mDia participate in RhoA-induced cytoskeletal reorganization (Kimura *et al*., 1996; Leung *et al*., 1996; Matsui *et al*., 1996; Amano *et al*., 1997a,b; Ishizaki *et al*., 1997; Watanabe *et al*., 1997; reviewed by Lim *et al*., 1996; Narumiya *et al*., 1997).

In addition to its roles in cytoskeletal reorganization, RhoA-induced signalling is implicated in gene transcription, cell-cycle progression, and cell transformation. Functional RhoA is required for serum- and LPA-induced activation of the transcription factor SRF, and activated forms of the protein can activate SRF in the absence of external stimuli (Hill *et al*., 1995). In addition, RhoA can modulate TNFα-induced activity of the NF-κB transcription factor (Perona *et al*., 1997). Functional RhoA is required for the $G_1$–S transition in Swiss 3T3 cells, and microinjection of activated RhoA protein can induce quiescent Swiss 3T3 cells to enter the cell cycle (Yamamoto *et al*., 1993; Olson *et al*., 1995). RhoA is required for Ras-mediated transformation, and activated forms of the protein co-operate with Raf in focus formation assays in NIH 3T3 cells (Khosravi-Far *et al*., 1995; Olson *et al*., 1995; Qiu *et al*., 1995; Cerione and Zheng, 1996). Moreover, mutated derivatives of several Rho family guanine nucleotide exchange factors (GEFs) have been isolated in different screens for transforming genes (for review see Cerione and Zheng, 1996). Although it is tempting to speculate that the ability of RhoA to activate transcription is related to its role in the regulation of cell-cycle progression and transformation, there is no direct evidence to support this view since the signalling pathways involved remain unclear.

One way to investigate signalling pathways regulated by Ras-like GTPases is to examine the signalling properties of mutant proteins in which effector–protein binding is defective. Binding of Ras to its effectors is mediated by a sequence known as the effector loop, which changes conformation when GTP is bound (Willumsen *et al*., 1986; Pai *et al*., 1989; Milburn *et al*., 1990; reviewed by Marshall, 1996). Mutations in this region can selectively disrupt its interactions with different effectors, thereby allowing the functional consequences of the particular interactions to be distinguished (White *et al*., 1995; Joneson *et al*., 1996b; Khosravi-Far *et al*., 1996). Mutations of this type can also be generated in Rho-family proteins (Freeman *et al*., 1996; Joneson *et al*., 1996a; Lamarche *et al*., 1996; Westwick *et al*., 1997). In

this paper we have generated RhoA effector-loop mutants and investigated their abilty to induce cytoskeletal rearrangements, SRF activation and transformation. Our results show that RhoA-induced stress fibre formation requires interaction with ROCK-I and a distinct effector, and that RhoA-induced signalling to SRF does not correlate with its activity in transformation.

## Results

### *Generation of RhoA effector mutants*

We used the yeast two-hybrid system to identify RhoA effector mutants that selectively impair interaction of RhoA with its protein kinase effectors PKN and ROCK-I. For use in the assay, RhoA.V14/S190, an activated RhoA derivative lacking the C-terminal CAAX motif, was fused C-terminally to the Gal4 DNA-binding domain (Alberts *et al.*, 1998b). The yeast reporter strain HF7C was used to score interactions of Gal4-RhoA.V14/S190 with PKN and ROCK-I, each tagged with the Gal4 activation domain to allow detection in the assay. Interactions were monitored both by expression of the *His3* gene, which allows growth on medium lacking histidine, and by expression of the β-galactosidase gene, using a colony colour assay.

We first used the two-hybrid system to identify mutations that selectively impair interactions with PKN or ROCK-I (see Materials and methods). Cassette mutagenesis was used to generate a library of RhoA mutants in which codons 37, 38, 39 and 40 were individually randomized. In one screen, growth on plates lacking histidine was used to identify mutants able to interact with PKN, which were then rescreened for those unable to interact with mouse ROCK-I(348–1018). Four mutants, C20R/T37Y, C20R/F39L, E40L and E40W were recovered (the C20R mutation was presumably generated by the PCR). In a second screen, cassette mutants able to interact with ROCK-I(348–1018) were rescreened to identify those incapable of interaction with PKN. This screen identified the mutants F39V, E40N and E40T. We also created RhoA mutations analogous to those known to impair other GTPase–effector interactions, an approach used successfully to generate Rac1 and Cdc42 mutants (Lamarche *et al.*, 1996; Westwick *et al.*, 1997). The mutants constructed were F39A and Y42C, which in Rac1 and Cdc42 disrupt interaction with ROCK-I and PAK respectively (Lamarche *et al.*, 1996).

### *Binding properties of RhoA effector mutants*

We used the two-hybrid assay to examine the binding of the effector-loop mutants to different RhoA effectors. In addition to PKN and ROCK-I, we examined interaction with Rhophilin (Watanabe *et al.*, 1996), Citron (Madaule *et al.*, 1995), mDia2, a p140mDia-related protein (Alberts *et al.*, 1998b; Watanabe *et al.*, 1997), Kinectin (Hotta *et al.*, 1996; Alberts *et al.*, 1998b) and mNET1, a RhoA GEF (Chan *et al.*, 1996; Alberts *et al.*, 1998b; A.S.Alberts and R.Treisman, submitted). Interaction in yeast was assessed by growth on medium lacking histidine containing increasing amounts of aminotriazole. Results are shown in Table I. RhoA.V14/S190 exhibited a wide range of activities with the different effectors, ranging from growth on $His^-$ medium but sensitivity to 1 mM aminotriazole (intact PKN) to growth on $His^-$ medium containing 32 mM aminotriazole [mDia2(47–800)]. Comparison with β-galactosidase assays performed in parallel indicate that this represents a range of at least 100-fold (see Table I legend).

Of the four mutants recovered in the screen for RhoA.V14 derivatives that can interact with PKN but not ROCK-I(348–1018), C20R/F39L also showed greatly impaired interaction with Rhophilin and Citron (Table I). The other three mutants, C20R/T37Y, E40L and E40W, exhibited an increased background activity in the assay in the absence of the activator-tagged effector, which did not increase in the presence of the intact PKN (Table I); nevertheless, mutants E40L and E40W interacted indistinguishably from intact RhoA with the N-terminal region of PKN (Table I; see Discussion). The E40L and E40W mutations reduced interaction with ROCK-I(348–1018) and Kinectin to background levels, but other interactions were essentially unimpaired (Table I). The fourth mutation recovered in this screen, C20R/T37Y, showed no interaction above background level with any of the proteins tested apart from mNET1 and Rhophilin, whose interaction was greatly reduced relative to wild-type (Table I; see Discussion).

Next we examined mutants recovered in the screen for interaction with ROCK-I(348–1018) but not PKN. Again mutation of different residues within the effector loop produced different effects on interaction. Mutant F39V exhibited profoundly impaired interaction with all effectors tested apart from ROCK-I and mNET1 (Table I, row 4). In contrast, mutants E40N and E40T exhibited substantially different behaviour. Although these mutants were selected by their inability to bind PKN, they interacted effectively with its N-terminal domain (Table I; see Discussion). Their interaction with other proteins was largely unimpaired, apart from Kinectin and mNET1 which were substantially reduced (Table I). Finally, we examined F39A and Y42C. These mutations block interaction with both PKN(1–511) and intact PKN. Mutant F39A blocked interaction with all other effectors tested apart from mDia2(47–800), while mutant Y42C affected no other interactions (Table I).

To corroborate the two-hybrid data we used an *in vitro* binding assay. GST fusion genes carrying PKN(1–511), ROCK-I(348–1018) and mDia2(47–257) were constructed and equal amounts of each fusion proteins bound to glutathione–Sepharose beads. Different 9E10 epitope-tagged RhoA.V14 mutants, each GTPγS-loaded, were allowed to interact with the GST fusion proteins, and the levels of bound protein measured by 9E10 immunoblot following extensive washing. The results are shown in Figure 1. The ability of the mutants to bind PKN(1–511) in general reflected the interactions seen in the two-hybrid assay, although weak interactions were detectable with mutants C20R/T37Y, F39V and Y42C upon prolonged exposure (see Figure 1B). Effector-loop mutants that interacted efficiently with ROCK-I(348–1018) in the two-hybrid assay also bound it strongly *in vitro*; however, in contrast to the two-hybrid data, mutants C20R/T37Y and E40W also bound weakly to ROCK-I(348–1018) *in vitro* (Figure 1C; see Discussion). All the RhoA mutants bound to mDia2(47–257) *in vitro* (Figure 1D). Taken together with the two-hybrid results, these data show that we have

**Table I.** Two-hybrid analysis of RhoA effector-loop mutants

| Gal4-DBD fusion[a] | His3 activity with Gal4-AD fusion[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | None | PKN 1–942 | PKN 1–511 | ROCK 348–1018 | Rhophilin 1–130 | Kinectin 1053–1327 | Citron 647–780 | mDia2 47–800 | mNET1 1–596 |
| RhoA.V14-WT | – | 1 | 2 | 4 | 6 | 3 | >4 | >4 | 3 |
| T37Y/C20R[c] | (1) | (1) | (1) | (1) | 2 | (1) | (1) | (1) | 3 |
| F39A[d] | – | – | – | – | – | – | – | >4 | – |
| F39V[e] | – | – | – | 4 | – | – | – | >4 | 4 |
| F39L/C20R[c] | – | 1 | 2 | 1 | 1 | – | – | >4 | 3 |
| E40L[c] | (1) | (1) | 2 | (1) | 4 | (1) | >4 | >4 | (1) |
| E40W[c] | (1) | (1) | 2 | (1) | 4 | (1) | 4 | >4 | (1) |
| E40N[e] | – | – | 2 | 4 | >4 | 1 | >4 | >4 | 3 |
| E40T[e] | – | – | 2 | 4 | >4 | – | >4 | >4 | 3 |
| Y42C[d] | – | – | – | 4 | 2 | 3 | >4 | >4 | 2 |

[a]pGBT9 fusion proteins comprise the Gal4 DNA binding domain fused N-terminally to each mutant RhoA, each containing the G14V and C190S mutations. All the mutants are expressed at comparable levels, as assessed by immunoblot (data not shown).
[b]Interaction was evaluated by a semi-quantitative plate assay for HIS3 activity by growth on plates containing increasing amounts of aminotriazole. Growth was scored as follows: (–) no growth on plates lacking histidine; (1 to >4) growth on plates containing 0 mM, 1 mM, 2 mM, 4 mM, ⩾8 mM aminotrizole, respectively. These scores correspond to LacZ activities as follows: 1, ⩽0.5 Miller units (⩽ background); 2, 0.5 units; 3, 16 units; 4, 52 units; >4, 67–153 units. With wild-type RhoA.V14, interactions corresponded to LacZ activities as follows: PKN(1–942), ⩽0.5 units; PKN(1–511), 0.5 units; ROCK-I(348–1018), 52 units; Rhophilin (1–130), 67 units; Kinectin (1053–1327), 16 units; Citron (674–780), 95 units; mDia2 (47–800), 153 units. Scores in parentheses emphasize that unlike wild-type RhoA, mutants C20R/T37Y, E40L and E40W promoted growth on plates lacking histidine in the absence of an effector plasmid.
[c]Mutants isolated from PKN+/ROCK– screen.
[d]Mutants analogous to previously characterized mutations in Rac1 and Cdc42.
[e]Mutants recovered from the ROCK+/PKN– screen.

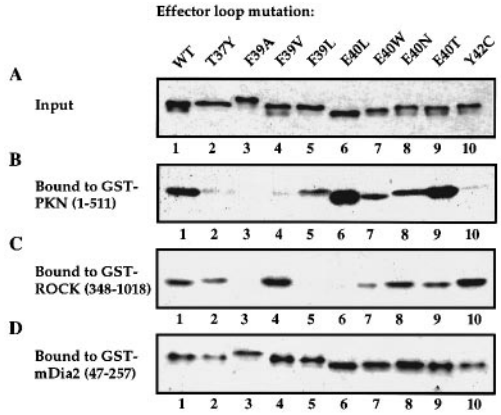


**Fig. 1.** *In vitro* interactions between Rho mutants and effectors. GTPγS-loaded bacterially produced 9E10 epitope-tagged RhoA mutants (100 ng, **A**) were incubated with the indicated bacterially produced GST-effector proteins (1000 ng) immobilized on glutathione–Sepharose beads. Mutant RhoA.V14 proteins bound to GST–PKN(1–511) (**B**), GST–ROCK-I(348–1018) (**C**) or GST–mDia2(47–257) (**D**) were detected with 9E10 antibody following elution, SDS–PAGE and immunoblotting.

identified RhoA effector-loop mutants that can discriminate between different RhoA effector proteins.

### *Regulation of cytoskeletal reorganization by RhoA effector mutants*

Studies with the ROCKs have implicated these kinases in cytoskeletal reorganization events including formation of stress fibres and focal adhesions (Kimura *et al.*, 1996; Leung *et al.*, 1996; Matsui *et al.*, 1996; Ishizaki *et al.*, 1997). We therefore investigated the effects of effector-loop mutations upon RhoA-induced cytoskeletal reorganization. Expression plasmids encoding 9E10 epitope-tagged RhoA.V14 or effector-loop mutants were microinjected into serum-deprived NIH 3T3 cells, and filamentous actin and RhoA visualized by staining with phalloidin and 9E10 antibody respectively. Serum-starved NIH 3T3 cells displayed low levels of filamentous actin, usually organized into a few short fibres with no particular organization with respect to one another. Expression of RhoA.V14 promoted the reorganization of filamentous actin, organized into parallel stress fibres 5 h following plasmid injection (Figure 2A, panels 1 and 2). We also examined focal adhesion formation by staining with vinculin antibodies; however, the relatively high basal level of focal adhesions in our serum-deprived NIH 3T3 cells precluded evaluation of the effects of the mutants, and we did not pursue these studies further (data not shown).

All the RhoA mutants were expressed at similar high levels and showed similar subcellular distribution (Figure 2A, insets). However, they varied in their ability to induce stress fibres. Expression of mutants E40N, E40T and Y42C triggered efficient stress fibre formation, although those formed by E40T appeared shorter and less numerous than those formed by RhoA.V14 (Figure 2A). Each of these mutants can interact with ROCK-I(348-1018). In contrast, mutants F39A, C20R/F39L, E40L and E40W, which do not interact with ROCK-I(348–1018), did not induce efficient stress fibre formation. Interestingly, F39V also did not promote stress fibre formation, even though its interaction with ROCK-I(348–1018) is unimpaired, suggesting additional effectors may be required for fibre formation. To test this we investigated whether the F39V mutant could co-operate functionally with F39A, F39L, E40L and E40W, none of which can bind ROCK-I, or induce stress fibre formation by itself. Simultaneous expression of F39V and E40W efficiently induced stress fibre formation, and the combination of F39V and E40L was partially active; F39A and F39L were ineffective (Figure 2). The inability of F39L, which binds PKN, to co-operate with F39V in this assay, together with the

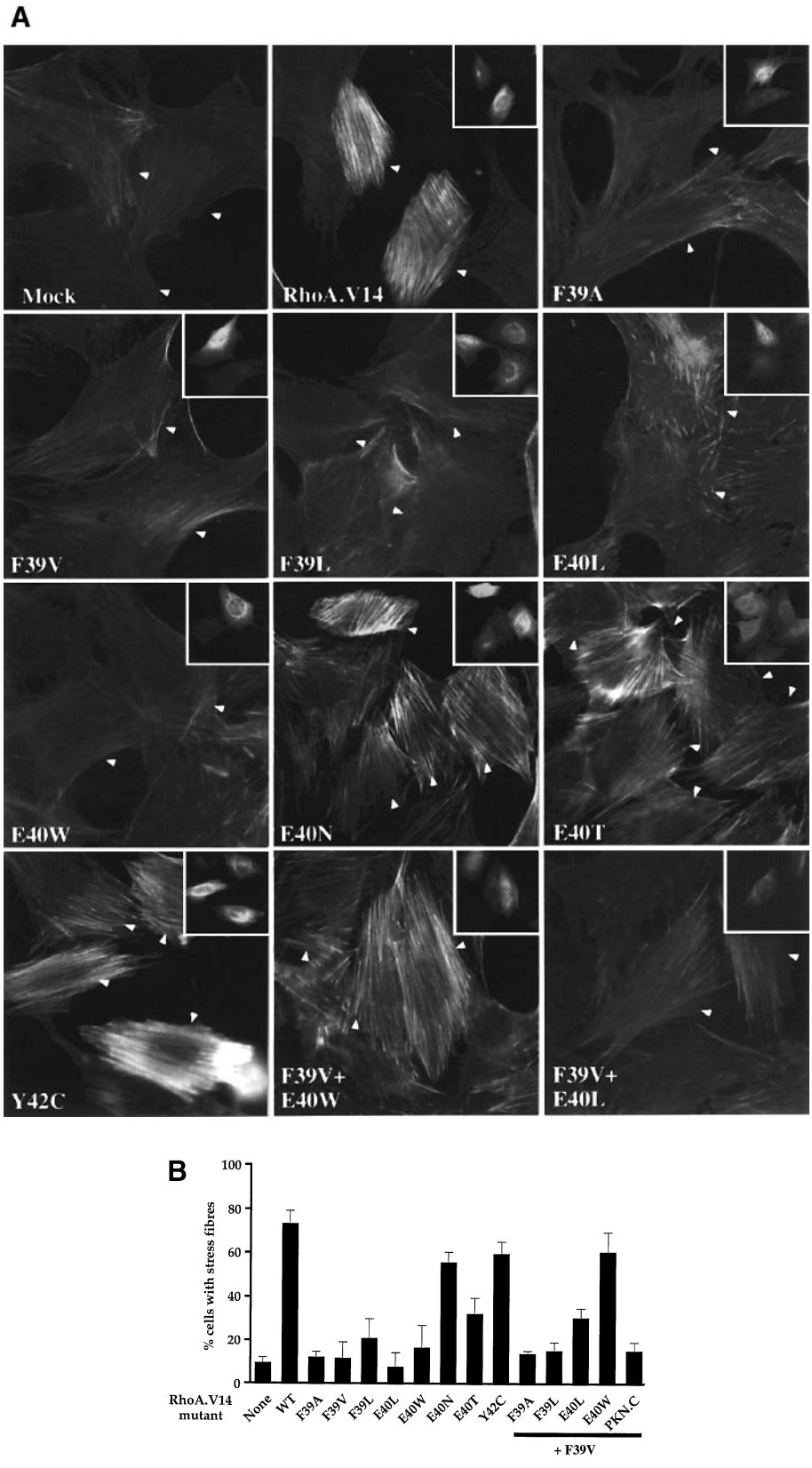


**Fig. 2.** Regulation of cytoskeleton by RhoA mutants. (**A**) NIH 3T3 cells were microinjected (arrowheads) with expression plasmids encoding 9E10 epitope-tagged RhoA.V14 mutants. Filamentous actin was visualized with TRITC-conjugated phalloidin (main panels) and expressed RhoA.V14 derivatives were visualized with 9E10 antibody (inset panels). (**B**) Summary of data. PKN.C expresses the catalytic domain of the RhoA effector kinase PKN (see Figure 4). Proportion of injected cells with large number of long stress fibres arranged in parallel sheets (average of three independent experiments, 20–30 cells were injected per coverslip; error bars indicate SEM). Note that mutant F39L also contains the C20R mutation.

finding that Y42C can induce stress fibres although it does not bind PKN, suggest that the additional effector cannot be PKN (see below).

### *Regulation of SRF activity by RhoA effector mutants*

Serum- or LPA-stimulated activity of SRF-controlled reporter genes in NIH 3T3 cells is dependent on RhoA, and SRF activity can be greatly increased by co-expression of activated RhoA (Hill *et al.*, 1995). To gain insight into which downstream effectors are involved in these phenomena, we examined the ability of effector-loop mutants to activate SRF. To facilitate comparison with other functional assays for RhoA.V14, we tested the mutants using both transfection and microinjection assays.

For the microinjection assay we used the reporter plasmid 3D.AFosHA, in which a minimal promoter containing three SRF binding sites controls expression of an HA epitope-tagged Fos protein which can be monitored by indirect immunofluorescence (Hill *et al*., 1995; Alberts *et al.*, 1998a). Microinjection of expression plasmids encoding RhoA.V14 and effector-loop mutants F39A, F39V, C20R/F39L, E40N, E40T and Y42C all activated the reporter efficiently. In contrast, mutant E40W triggered a partial response, while E40L did not lead to significant activation above background (Figure 3A).

To obtain a more quantitative measure of the relative efficacy of SRF activation by the different effector mutants we used a transfection assay. For this assay we used the reporter 3D.ACAT (Hill *et al.*, 1995), in which the bacterial chloramphenicol acetyltransferase (CAT) gene is controlled by exactly the same promoter used in microinjection assay. In each case varying amounts of RhoA-expression plasmids were co-transfected with the reporter, and upon harvesting the samples were split and processed to measure both CAT activity and RhoA expression level. As in the microinjection experiments, mutants F39V and Y42C activated the reporter as effectively as RhoA.V14 itself; in contrast activation by mutants E40L and E40W was at background levels (Figure 3B). The other mutants, F39A, C20R/F39L, E40N and E40T exhibited intermediate abilities to activate the reporter.

Taken together with the microinjection results, these data indicate that the identity of residue 40 within the effector loop is of particular importance for SRF activation. The data show that the ability of RhoA.V14 to potentiate SRF activity can be clearly dissociated from its ability to promote stress fibre formation (compare Figures 2A and 3A, mutants F39A, C20R/F39L and F39V), and that changes in cell morphology are not a prerequisite for potentiation of SRF activity. However, in neither assay does the ability of RhoA.V14 to potentiate SRF activity correlate with the binding of a single effector (see Discussion).

### *The ROCK-I and PKN kinase domains cannot activate SRF independently of RhoA function*

The data presented above suggest that either a novel RhoA effector is involved in signalling to SRF, or that multiple effectors are involved. In the light of the latter possibility, it was of interest to note that all mutants that bound to ROCK-I(348–1018) were able to signal to the SRE, consistent with the notion that although interaction with

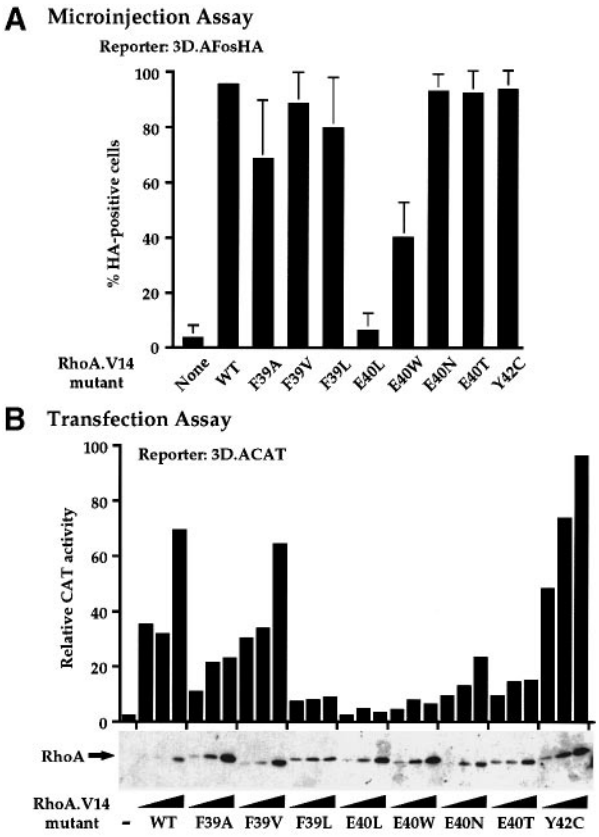


**Fig. 3.** Regulation of SRE by RhoA mutants. (**A**) Microinjection assay. NIH 3T3 cells were microinjected with 3D.AFosHA reporter plasmid and 9E10 epitope-tagged RhoA expression plasmids; RhoA and reporter expression was visualized 5 h later. The proportion of injected cells expressing reporter construct (average of three independent experiments; error bars indicate SEM). (**B**) Transfection assay. NIH 3T3 cells were co-transfected with the SRF-controlled reporter plasmid 3D.ACAT (1000 ng), together with increasing amounts of RhoA expression plasmids (200, 500 or 1000 ng) as indicated. Two days later, cell lysates were prepared and analysed for RhoA protein expression by immunoblot (top panel) CAT reporter gene activity (lower panel). Similar results were obtained in three independent experiments; a representative experiment is shown. Note that mutant F39L also contains the C20R mutation.

ROCK-I might not be required for RhoA to signal to SRF, it may nevertheless be able to activate transcription at the SRE. Moreover, it has been reported that the PKN family kinase PRK2 kinase domain can potentiate SRF activity (Quilliam *et al.*, 1996). We therefore investigated whether the isolated kinase domains of ROCK-I and PKN were capable of potentiating SRF activity, using kinase-inactive derivatives to test whether any effects required kinase activity. To verify that any observed effects on SRF result from activation of pathways downstream of RhoA, we used co-expression of the specific Rho inhibitor C3 transferase to inactivate cellular RhoA (Hill *et al.*, 1995).

Microinjection of NIH 3T3 cells with an expression plasmid encoding intact ROCK-I had no effect on SRF reporter expression (Figure 4A), but promoted formation of thick actin filaments distinct from those induced by RhoA.V14 (Figure 4B). Next we investigated ROCKΔ3 (ROCK-I, codons 1–727), a truncated ROCK-I derivative which contains the N-terminal kinase domain and part of the coiled region, but lacks C-terminal sequences including the RhoA interaction domain (Ishizaki *et al.*, 1997).

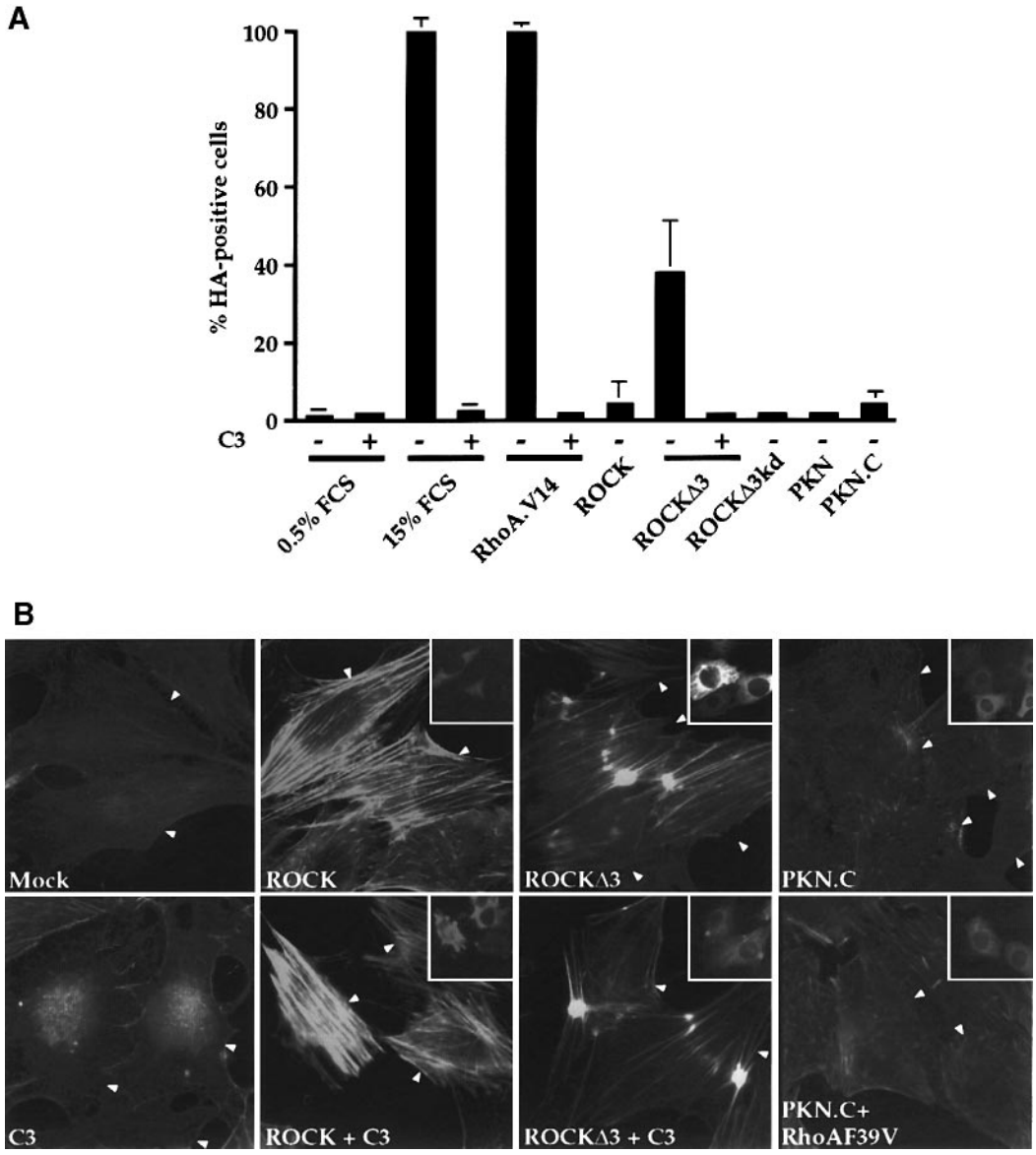


**Fig. 4.** Regulation of actin cytoskeleton and SRE by ROCK-I. (**A**) SRE activation by ROCK-I and PKN derivatives. Serum-deprived NIH 3T3 cells were microinjected with 3D.AFosHA reporter plasmid and either vector alone or the indicated combinations of 9E10 epitope-tagged ROCK-I and C3 expression plasmids. ROCK, codons 1–1354; ROCKΔ3, codons 1–727; ROCKΔ3kd, codons 1–727/K105A (kinase inactive); PKN, codons 1–942; PKN.C, codons 539–942. Five hours following injection, injected cells and FosHA protein were visualized by indirect immunofluorescence using anti-guinea-pig IgG or 9E10 and Y11 antibodies, respectively. Serum stimulation was for 1 h where indicated. Results are from three independent experiments, 20–50 cells injected per coverslip. Error bar indicates SEM. (**B**) Cytoskeletal rearrangements induced by ROCK-I derivatives. Serum-deprived NIH 3T3 cells were microinjected with the indicated combinations of ROCK-I, PKN, RhoA effector mutant and C3 expression plasmids as in (A). After 5 h, filamentous actin was visualized using TRITC–phalloidin. Between 20 and 30 cells were injected per coverslip; representative cells from one of three experiments are shown. For data summary see Figure 2B.

Immune complex kinase assays using extracts from transfected NIH 3T3 cells expressing ROCKΔ3 indicated that this protein possesses substantially increased histone kinase activity compared with over-expressed wild-type ROCK-I (data not shown). Upon microinjection of the ROCKΔ3 expression plasmid together with the 3D.AFosHA reporter, approximately one-third of injected cells showed reporter gene activation; surprisingly, however, this was dependent on endogenous Rho function, since it was blocked by C3 transferase expression (Figure 4A). All cells injected with ROCKΔ3 exhibited thick actin bundles radiating from hub-like actin structures very different from the stress fibres produced by RhoA.V14; as with intact ROCK-I, this was unaffected by C3 transferase (Figure 4B). Similar results have been reported for Swiss 3T3 cells (Amano *et al.*, 1997a), but not HeLa cells (Ishizaki *et al.*, 1997). Both reporter gene activity and cytoskeletal reorganization were not observed in cells injected with a kinase-inactive ROCKΔ3 derivative (data not shown).

Although the effector mutant studies strongly suggest that interaction of RhoA.V14 with PKN is not a prerequisite for SRF activation, we also investigated whether expression of a constitutively active form of PKN is sufficient for SRE activation or stress fibre formation. Immune complex kinase assays using extracts from NIH 3T3 cells transfected with an expression plasmid encoding the PKN kinase domain (PKN.C; amino acids 539–942)

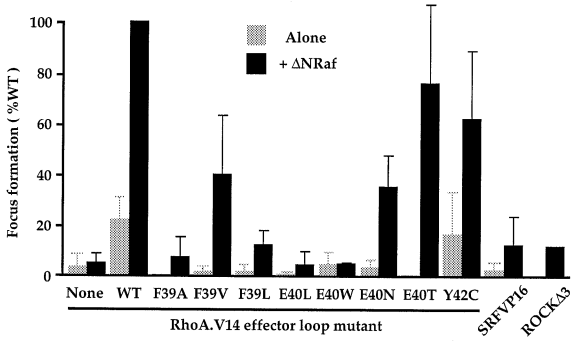


**Fig. 5.** Loss of contact inhibition caused by RhoA mutants and Raf. NIH 3T3 cells were transfected with expression plasmids encoding RhoA derivatives (500 ng), SRFVP16 (500 ng) or ROCK mutants (500 ng) either with (black bars) or without (grey bars) a plasmid encoding activated Raf (EXVΔNRaf; 500 ng). At 15 days post-transfection the number of foci of size >1.5 mm was counted. The efficiency of focus formation relative to RhoA.V14 + ΔNRaf is shown (average of three independent experiments, error bar indicates SEM). The combination of RhoA.V14 and ΔNRaf produced on average 38 foci per plate transfected. Note that mutant F39L also contains the C20R mutation.

indicated that this protein possesses substantial constitutive histone kinase activity (data not shown). However, expression of either intact PKN or PKN.C neither activated the SRF reporter gene (Figure 4A) nor induced actin stress fibres (Figure 4B; data not shown). Moreover, consistent with the effector mutant data which suggests that interaction between RhoA and PKN is not required for stress fibre formation, we found that the active PKN.C mutant did not co-operate with F39V to induce stress fibre formation (Figure 4B).

Taken together with the effector mutant data, these results are consistent with the view that neither ROCK-I nor PKN constitute single RhoA effectors that mediate SRF activation, and support the hypothesis that RhoA.V14-induced SRF activation is mediated either by a novel effector, or that multiple RhoA effectors are involved in the response (see Discussion).

### *Focus formation by RhoA effector mutants in co-operation with Raf*

Expression of activated derivatives of RhoA such as RhoA.V14 results in reduced serum- and anchorage-dependent growth (Perona *et al*., 1993), and RhoA.V14 can co-operate with activated Raf derivatives to induce transformation (Khosravi-Far *et al.*, 1995, 1996; Qiu *et al.*, 1995). We therefore used the effector mutants to investigate whether the ability of RhoA.V14 to co-operate with activated Raf in NIH 3T3 transformation correlates with its ability to potentiate SRF activity. NIH 3T3 cells were transfected with expression plasmids encoding either RhoA.V14 or its effector-loop mutants, with or without an expression plasmid encoding ΔNRaf (Leevers and Marshall, 1992), and the foci stained and counted 2 weeks later. In this assay, RhoA.V14 and its mutant derivatives F39V, E40N, E40T and Y42C, each of which can interact with ROCK-I(348–1018), promoted focus formation in co-operation with ΔNRaf (Figure 5). In contrast, the efficiency of transformation by the other effector mutants was not significantly greater than that observed with ΔNRaf alone; in particular, mutant F39A, which activates SRF with efficiency comparable to mutants E40N and E40T, was inactive in the transformation assay. Finally, SRF-VP16, an activated SRF derivative, did not co-operate with ΔNRaf in focus formation (Figure 5). Thus, co-operation between RhoA.V14 and ΔNRaf in transformation does not appear to reflect the ability of RhoA.V14 to activate SRF.

## Discussion

### *RhoA effector-loop mutants*

In this study we generated mutations in the effector-loop region of RhoA that selectively disrupted its interaction with downstream effector proteins. Such mutations have been generated previously in Ras, Rac and Cdc42 (White *et al*., 1995; Freeman *et al*., 1996; Joneson *et al.*, 1996a; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). We identified mutations at RhoA codons 37, 39, 40 and 42 that prevent interaction with PKN, ROCK-I, or both. Binding studies demonstrated that these mutations also selectively disrupt interactions with other RhoA effectors, indicating that the effector loop makes subtly different contacts with the different proteins. We used the mutants to investigate the role of RhoA effectors in cytoskeletal rearrangement, SRF activation and focus formation in NIH 3T3 cells. Although activated RhoA can potentiate the kinase activity of both ROCK-I and PKN (Kimura *et al.*, 1996; Watanabe *et al.*, 1996; Amano *et al.*, 1997b; Ishizaki *et al.*, 1997; Vincent and Settleman, 1997), the modest degree of activation precluded its investigation using the mutants. Stress fibre formation likely involves interaction both with ROCK and a second effector. SRF activation correlates with binding to neither PKN, ROCK-I nor any other single effector tested, suggesting a novel effector may be responsible. Focus formation by activated RhoA in combination with activated Raf correlates with ROCK-I binding rather than SRF activation.

### *Binding of RhoA to its effectors*

Interaction between PKN and RhoA.V14 could only be scored in the two-hybrid assay by use of the sensitive HIS3 reporter gene, although it was readily detectable *in vitro*. Mutations F39V and Y42C both abolish interaction with PKN, but maintain normal interaction with ROCK-I; in contrast, mutation C20R/F39L maintains interactions with PKN but abolishes interactions with ROCK-I. Mutant F39A abolishes interaction with both kinases. Several mutants at codon 40 isolated in the two-hybrid screen as defective for interaction with intact PKN retained the ability to interact with its RhoA-binding N-terminal region: the isolation of these mutants presumably reflects the extreme sensitivity of the HIS3 reporter assay to small changes in interaction strength, and it is possible that these mutations have no substantial effect on RhoA–PKN interaction. PKN and Rhophilin interact with RhoA via a common sequence motif (Watanabe *et al.*, 1996), and their binding is in general affected similarly by effector-loop mutations, although C20R/F39L specifically reduces Rhophilin binding. Effector-loop residues F39 and Y42 are conserved in Rac1 and in both proteins mutations F39A, F39L and Y42C have similar effects on ROCK-I binding, suggesting that the molecular contacts are similar (Joneson *et al.*, 1996a; Lamarche

*et al.*, 1996). Finally, ROCK-I and Kinectin interact with RhoA via related sequences (Alberts *et al.*, 1998b), but the interaction with Kinectin appears weaker, since it is abolished by all mutations save Y42C.

In the two-hybrid assay, mutant C20R/T37Y interacted with only the putative Rho-family GEF mNET1 (Chan *et al*., 1996), even though interactions with other effector proteins were readily detectable *in vitro*. Residue T37 or its equivalent co-ordinates a magnesium ion required for nucleotide binding, and is universally conserved in Ras-like GTPases (Pai *et al*., 1989; Hirshberg *et al.*, 1997; Rittinger *et al*., 1997). The failure of C20R/T37Y to manifest two-hybrid interactions may therefore reflect inefficient nucleotide binding, in contrast to the *in vitro* assays in which the GTPase is biochemically loaded with GTPγS. However, the C20R–T37Y mutant is impaired in *in vitro* interaction with GST–PKN(1–511), so this residue probably also makes specific effector interactions, as observed previously with Ras (White *et al.*, 1995).

The consequences of effector-loop mutations on interactions with Citron, mNET1, and mDia2, were distinct from those on PKN and ROCK-I. Mutations at position 39, but not those at 40 and 42, abolished interaction with Citron. The F39A, E40N and E40T mutants all exhibited defective binding to mNET1, and it is intriguing to note that corresponding residues in Ras may mediate its interaction with the *Saccharomyces cerevisiae* Ras GEFs Cdc25 and Sdc25 (Mistou *et al*., 1992; White *et al*., 1995). Interaction with mDia2, which is closely related in its RhoA-binding region to p140mDia (Watanabe *et al*., 1997), was unaffected by any of the mutations, including F39A, which abolished interactions with all other proteins tested. The primary site of mDia2 (and probably mDia) interaction with RhoA may therefore be distinct from the effector loop. In Rac proteins, the 'Rho family insert region' at codons 125–137, which constitutes an extra surface-exposed helix (Hirshberg *et al.*, 1997; Rittinger *et al*., 1997), is required for efficient and productive interaction with the NADPH oxidase complex (Freeman *et al*., 1996; Nisimoto *et al*., 1997). Perhaps the RhoA insert region, rather than the effector loop, interacts with mDia and mDia2.

### *RhoA requires multiple effectors to produce stress fibres*

Several previous studies in various cell types have suggested that the ROCKs are involved in RhoA-induced stress fibre formation (Leung *et al.*, 1996; Amano *et al.*, 1997a; Ishizaki *et al.*, 1997). However, both our data and previous studies of ROCK-I mutants (Ishizaki *et al.*, 1997) suggest that while ROCK-I is involved in the process it is not sufficient. Mutants F39A, C20R–F39L, E40L and E40W neither bind ROCK-I nor trigger stress fibre formation; in contrast, mutant F39V retains the ability to bind ROCK-I, but promotes stress fibre formation only in combination with mutant E40W (Table II). The data therefore show that formation of stress fibres requires the interaction of RhoA with both ROCK-I and another effector, which cannot bind F39V. Several observations suggest that this effector is unlikely to be PKN. First Y42C, which cannot bind PKN, nevertheless induces stress fibres; second, F39L, which can bind PKN, does not co-operate with F39V to induce stress fibre formation; third, an activated derivative of PKN co-operates with neither F39V nor the activated ROCK mutant ROCKΔ3 for stress fibre formation (see Figure 6). It has been proposed that p140mDia plays such a role (Watanabe *et al*., 1997), but all the mutants, including F39V, bind its relative mDia2, suggesting that proteins other than mDia–mDia2 may be involved. Our data do not rule out a role for Citron, and future experiments will address this possibility. Over-expression of the putative RhoA effector PI-4–P 5-kinase (Chong *et al.*, 1994; Ren *et al*., 1996) can also induce cytoskeletal changes independently of RhoA (Shibasaki *et al.*, 1997) and it will be interesting to examine the interaction of the mutants with this enzyme.

Microinjection of NIH 3T3 cells with an expression plasmid encoding intact ROCK-I induced formation of actin fibres independently of RhoA, but these were fewer in number and thicker than those induced by RhoA.V14. In contrast, expression of the truncated ROCKΔ3 mutant, which lacks the majority of ROCK-I C-terminal sequences, induced formation of thick actin spindles radiating from hub-like structures independent of functional RhoA. The observation that over-expression of ROCK-I induces structures distinct from those induced by RhoA.V14 is consistent with the idea that stress fibre formation requires both ROCK-I and additional effectors. Previous studies have disagreed as to the effects of over-expression of intact ROCK proteins upon the actin cytoskeleton, although they concur in the demonstration of efficient but aberrant actin reorganization by ROCK truncation mutants (Leung *et al*., 1996; Amano *et al.*, 1997a; Ishizaki *et al.*, 1997). In HeLa cells, over-expression of intact ROKα (ROCK-II) by microinjection was reported to induce identical structures to RhoA.V14 (Leung *et al.*, 1996) while transfection experiments with ROCK-I demonstrated only limited fibre formation by the intact protein (Ishizaki *et al.*, 1997). The reason for the discrepancy between these studies and the one reported here is unclear. A significant difference between studies performed with ROCK derivatives and those performed with RhoA effector mutants may be that in the latter studies stress fibre formation is dependent on activation of endogenous effectors. However, we cannot rule out effects due to different cell types, experimental conditions, or the use of different ROCK proteins.

### *Regulation of SRF by RhoA*

Since microinjection assays allow direct comparison of the effects of RhoA on cytoskeletal reorganization and SRF activity, we used both microinjection and transfection assays to investigate SRF activation. Only mutants F39V and Y42C showed wild-type activity in both assays. Signalling to SRF by activated RhoA did not correlate directly with any of the effectors tested, suggesting that it requires an as yet uncharacterized effector(s), and can occur independently of effector kinases such as ROCK-I or PKN (Figure 6; Table II). For example, mutant F39A, which is defective for all effectors tested apart from mDia2, was still functional in both assays, albeit with reduced efficiency. The identity of RhoA codon 40 is critically important for RhoA-mediated SRF activation: mutant E40L was inactive in both assays, while E40W was inactive in transfection assays but weakly active in the microinjection assay. (The failure of some effector-loop mutants to score as defective in the microinjection

**Table II.** Summary of results

| RhoA protein | Interaction[a] | | Activity | | |
|---|---|---|---|---|---|
| | PKN 1–511 | ROCK 348–1018 | Actin[b] | SRF[c] | Focus[d] formation |
| RhoA.V14-WT | ++ | ++ | ++ | ++ | ++ |
| F39A | – | – | – | + | – |
| F39V | – | ++ | – | ++ | + |
| F39L/C20R | ++ | –/+ | – | + | – |
| E40L[e] | ++ | – | – | – | – |
| E40W[e] | ++ | –/+ | – | – | – |
| E40N[e] | ++ | ++ | ++ | + | + |
| E40T[e] | ++ | ++ | + | + | ++ |
| Y42C | – | ++ | ++ | ++ | ++ |

[a]Interactions in the two-hybrid assay. ++, wild-type level of interaction or activity; –/+, weak interaction detectable only in one assay; –, no significant interaction or activity.
[b]++, 50–100% wild-type activity, + 20–50% wild-type activity, –, ⩽20% wild-type activity.
[c]++, wild-type activity in both transfection and microinjection assay; +, 50–100% wild-type activity in microinjection assay, 10–30% wild-type in transfection assay; –, <50% wild-type activity in microinjection assay, ⩽10% wild-type in transfection assay.
[d]++, 50–100% wild-type activity; +, 20–50% wild-type activity; –, ⩽20% wild-type activity.
[e]These mutants did not interact with intact PKN in the two-hybrid assay.

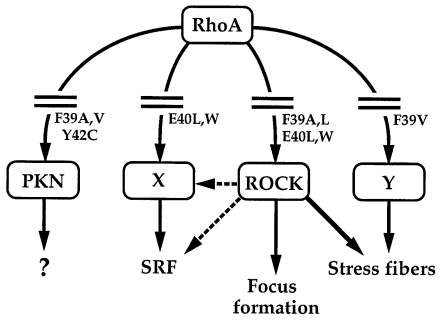


**Fig. 6.** RhoA effector pathways. Cross hatches indicate effector-loop mutations that block the indicated interactions. Mutations at position 40 impair interaction with intact PKN but not its N-terminal domain and are therefore not indicated. Dotted lines, potential signals between ROCK and SRF. For discussion see text.

assay probably reflects both saturating levels of RhoA produced in the assay and the use of a simple positive/negative system for the scoring of gene expression.) Cytoskeletal reorganization does not appear to be a prerequisite for SRF activation, since several mutants could activate SRF-controlled reporter genes without inducing stress fibre formation (Table II). It remains possible that SRF activation does require a functional cytoskeleton, but this is difficult to assess because agents that disrupt microtubules or actin filaments themselves trigger SRF activation (R.Treisman and C.S.Hill, unpublished observations).

Over-expression of the PKN kinase domain did not potentiate SRF activity in our assays, although a modest activation of SRF by PRK2 has been reported by others (Quilliam *et al.*, 1996). However, even though activation of SRF in our cells does not correlate directly with ROCK-I binding, we found that ROCKΔ3, a truncated ROCK-I derivative, weakly activated SRF reporter genes in Rho-dependent manner. Although a similar finding was reported by others while this paper was under review, that study did not investigate its dependence on RhoA (Chihara *et al.*, 1997). A trivial explanation for this is that activation of RhoA-mediated signalling pathways is a consequence of deregulated ROCK-I function; this is consistent with the observation that a specific ROCK inhibitor (Uehata *et al.*, 1997) does not block serum-induced SRF activation a (E.Sahai, unpublished data).

An alternative possibility, which we cannot exclude, is that ROCK-I either co-operates with or potentiates the activity of another RhoA effector to regulate SRF (Figure 6, dotted arrows). The lack of correlation between effector binding and function might either reflect the different conditions used for the functional and binding assays, or indirect effects of RhoA on effector function. The potential for such indirect effects is illustrated by the observations that GTP-bound RhoA binds both the myosin binding subunit (MBS), the regulatory subunit of myosin light-chain phosphatase, and ROCK-II, the kinase that phosphorylates MBS (Kimura *et al.*, 1996); and that the association of RhoA with phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Ren *et al.*, 1996) may also indirectly regulate PKN by altering PI-4,5–P2 concentrations (Palmer *et al.*, 1995). However, in microinjection experiments we have been unable to block F39V- or Y42C-induced SRF activation by expression of RhoA.N19, a dominant interfering mutant (E.Sahai, unpublished data). Further studies will be necessary to resolve this issue.

### *Transforming ability of RhoA correlates with binding to ROCK-I*

Activated RhoA is able to promote loss of contact inhibition and subsequent formation of foci in NIH 3T3 cells in co-operation with activated Raf (Khosravi-Far *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995), and a number of Rho-family GEFs have been isolated as oncogenes (for review see Cerione and Zheng, 1996). Since focus formation assays involve the generation of stable cell clones, comparison with the short-term microinjection and transfection assays is difficult: RhoA.V14 transformed cells do not contain a higher level of stress fibres than their non-transformed counterparts (E.Sahai, unpublished data). We found the ability of effector mutants to induce focus formation did not correlate with SRF activation, as previously observed with Rac1 effector mutants (Westwick *et al.*, 1997). Several other observations are consistent

with this. First, we found that the constitutively active SRF derivative SRF–VP16 did not co-operate with activated Raf in transformation assays; second, an intact lipid modification site in RhoB is required for transformation but not signalling to the SRE (Lebowitz *et al.*, 1997); finally, while activated RhoA is sufficient to activate transfected or microinjected immediate–early gene promoters, in microinjection experiments it requires additional signals to activate chromosomal genes (Alberts *et al.*, 1998a).

The properties of the effector mutants show that transformation by RhoA correlates with the ability to bind ROCK rather than activate SRF. Mutants F39V, E40N, E40T and Y42C, all of which bind ROCK-I and mDia2 and activate the SRE, promote focus formation, but mutant F39A, which can also activate the SRE, does not. Transformation correlates with ROCK binding rather than stress fibre formation, since F39V transforms but fails to induce stress fibres. It is likely that activation of ROCK may not be sufficient for transformation, however, since the activated ROCK-I derivative ROCKΔ3 does not co-operate with ΔNRaf in the focus formation assay. In contrast, it appears that transformation by Rac1 correlates neither with ROCK binding nor cytoskeletal reorganization (Joneson *et al.*, 1996a; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). Our data suggest that transformation by RhoA and the ability to remodel the cytoskeleton must be, at least to some extent, interdependent. It will be interesting to test whether the recently developed ROCK inhibitors (Uehata *et al.*, 1997) can affect cellular transformation by derivatives of RhoA or its activators.

## Materials and methods

### Plasmids

All DNA manipulations were carried out using standard protocols. pGBT9RhoA.V14/S190 was made by subcloning RhoA.V14 from EF.RhoA.V14 (Hill *et al.*, 1995) into pGBT9 (*Eco*RI[blunt]–*Sal*I into *Nco*I[blunt]–*Xho*I); in addition codon 190 was changed from C to S. PCR across codons 1–45 of RhoAV14 was used to randomize codons 37–40 of EFRhoAV14 (forward primer: TGCTTACATTTGCTTCTG, reverse primers: CTCGATATCTGCCACATAGTTCTCAAACACNNNGGGCAC, CTCGATATCTGCCACATAGTTCTCAAANNNTGTGGGCAC, CTCGATATCTGCCACATAGTTCTCNNNCACTGTGGG, CTCGATATCTGCCACATAGTTNNNAAACACTGTGGG). PCR products were cut using *Nco*I and *Eco*RV, subcloned into EFRhoA, and subsequently recloned into pGBT9RhoA.V14/S190 to generate pGBT9RhoA$^{library}$. PGAD–PKN, pGAD–PKN.N, pGAD–ROCK, pGAD–mDia2, pGAD–Kinectin and pGAD–NET are pGAD424 derivatives containing PKN codons 1–942 and 1–511, ROCK-I codons 348–1018, mDia2 codons 47–800, Kinectin codons 1053–1327, and mNET codons 1–596; pVP16Citron and pVP16Rhophilin contain Citron codons 674–870 and Rhophilin codons 1–130, both with N-terminal HSV VP16 activation domains (Madaule *et al.*, 1995; Watanabe *et al.*, 1996). GST fusion proteins are described elsewhere (Alberts *et al.*, 1998b).

### Yeast manipulations and effector mutant screen

All yeast work was carried out using standard techniques. Transformation of Hf7C cells (Clontech) was done according to the supplier's protocol. For plasmid rescue, 2 ml of exponential liquid cultures were pooled, pelleted, resuspended in 3 ml of lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 2% Triton X-100, 1% SDS), mixed with 3 ml of phenol:chloroform (1:1) and 4 g of acid-washed glass beads (Sigma, 425–600 μm), and vortexed for 2 min. After centrifugation, DNA was recovered from the aqueous phase by ethanol precipitation.

*PKN+/ROCK– mutant screen.* Four hundred independent transformants carrying pGAD–PKN and pGBT9RhoA$^{library}$ were screened for HIS3 expression, yielding 100 positive colonies (the RhoA–PKN interaction, while specific, does not activate lacZ expression sufficiently to use the colony colour assay). Thirty-one colonies were grown, pooled, and the pGBTRhoA plasmids rescued by retransformation into *Escherichia coli* DH5α following digestion with *Cla*I to linearize the pGAD–PKN plasmid. Bacterial transformants were pooled and retransformed into yeast carrying pGAD–ROCK-I(348–1018); 107 transformants were screened for lack of growth on plates lacking histidine and containing 1 mM aminotriazole. pGBT9RhoA$^{library}$ plasmids were rescued from 36 positive colonies.

*ROCK+/PKN– mutant screen.* Four hundred and two independent transformants carrying pGAD–ROCK-I(348–1018) and pGBT9-RhoA$^{library}$ were screened for lacZ expression (strong blue colour within 3 h), and pGBT9RhoA plasmids were rescued from 68 positive clones. These were subsequently transformed into yeast carrying pGAD–PKN and rescreened for mutants unable to grow on plates lacking histidine. From 84 colonies screened, 16 positives were recovered and confirmed by retransformation into yeast before sequence analaysis to ascertain the mutant RhoA sequence.

### In vitro protein interaction assay

Purification of GST fusion proteins and 9E10 epitope-tagged RhoA.V14 proteins and their loading with GTPγS was as described elsewhere (Alberts *et al.*, 1998b). Following loading with GTPγS, 100 ng of RhoA protein were incubated with gentle agitation for 2 h at 10°C with ~1 μg of either GST–PKN(1–511), GST–mDia2(47–257), or GST–ROCK-I (831–1011), immobilized on gluthione–Sepharose (Pharmacia), in 200 μl of 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 125 μg/ml BSA, containing protease inhibitors. Beads were then washed once in PBS, 5 mM $MgCl_2$, 0.1% Triton X-100, once in 50 mM Tris–HCl pH 7.5, 200 mM NaCl (or 100 mM NaCl, 70 mM LiCl for ROCK-I binding assays), 5 mM $MgCl_2$, and finally in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$. RhoA proteins that remained bound to the beads were eluted by boiling in SDS–PAGE loading buffer, fractionated on 15% gels and detected by immunoblot with 9E10 antibody.

### Microinjection and immunofluorescence

NIH-3T3 cells were seeded onto glass coverslips 48 h before injection and maintained in E4–0.5% FCS for 24 h prior to injection. Injections were done on a Zeiss 5171 semi-automated machine using pulled glass capillaries. All DNAs were injected into the cell nucleus at 50 ng/μl, except for EFC3 (25 ng/μl) and the RhoA expression plasmids (100 ng/μl), together with guinea-pig marker IgG at 5 μg/μl. Cells were harvested 5 h after injection. Serum stimulation was with 15% FCS 1 h before harvesting. Cells were fixed in fresh 4% HCHO in PBS for 15 min followed by extraction in PBS, 0.3% Triton X-100. Antibodies were diluted 1:100 in PBS + 5% donkey serum and the cells were washed four times in PBS after antibody incubations and prior to mounting. Antibodies used were as follows: 9E10 (gift G.Evan), Y-11 (Santa Cruz), anti-rabbit-Texas Red or -FITC, anti-mouse-FITC or -AMCA, anti-guinea-pig-AMCA (all Jackson/Stratech, UK), and also TRITC-phalloidin (Sigma). Images were obtained using a Zeiss Axiovert microscope and Smart Capture system (Vysis, UK) and processed as PICT files using Adobe Photoshop.

### Transformation and reporter gene assays

For transformation assays, NIH 3T3 cells (a gift from Chris Marshall) were seeded at $2\times10^5$ per 35 mm well in E4 with 10% donor calf serum (DCS). For transfection, 1 μg of plasmid DNA in 100 μl of Optimem (GibcoBRL) was added to 108 μl Lipofectamine mix (100:8 Optimem: Lipofectamine; GibcoBRL) and incubated for 30 min at room temperature. For transfection cells were washed once with Optimem and the medium replaced with 800 μl of Optimem; the DNA mix was then added and the cells incubated at 37°C, 10% $CO_2$ for 5 h, following which the DNA mix was replaced with E4 + 10% DCS. After 24 h, the cells were trypsinized, transferred to 100-mm dishes, and maintained in E4 + 10% DCS for 3 days, following which they were maintained in E4 + 5% DCS for a further 12 days before staining for foci with 0.5% Crystal Violet in PBS–10% v/v methanol. For reporter gene assays NIH 3T3 cells grown on 60-mm dishes were transfected as above except that all quantities were doubled, and cells were maintained in E4 + 0.5% DCS for 48 h. Harvesting, CAT assays and immunoblots were carried out as described previously (Hill *et al.*, 1995).

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## References

Alberts,A., Geneste,O. and Treisman,R. (1998a) Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation. *Cell*, **92**, in press.

Alberts,A.S., Bouquin,N., Johnston,L.H. and Treisman,R.H. (1998b) Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein beta subunits and the yeast response regulator protein Skn7. *J. Biol. Chem.*, **273**, in press.

Amano,M., Mukai,H., Ono,Y., Chihara,K., Matsui,T., Hamajima,Y., Okawa,K., Iwamatsu,A. and Kaibuchi,K. (1996) Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. *Science*, **271**, 648–650.

Amano,M., Chihara,K., Kimura,K., Fukata,Y., Nakamura,N., Matsuura,Y. and Kaibuchi,K. (1997a) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science*, **275**, 1308–1311.

Amano,M., Ito,M., Kimura,K., Fukata,Y., Chihara,K., Nakano,T., Matsuura,Y. and Kaibuchi,K. (1997b) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.*, **271**, 20246–20249.

Cerione,R.A. and Zheng,Y. (1996) The Dbl family of oncogenes. *Curr. Opin. Cell Biol.*, **8**, 216–222.

Chan,A.M., Takai,S., Yamada,K. and Miki,T. (1996) Isolation of a novel oncogene, *NET1*, from neuroepithelioma cells by expression cDNA cloning. *Oncogene*, **12**, 1259–1126.

Chihara,K. *et al*. (1997) Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J. Biol. Chem.*, **272**, 25121–25127.

Chong,L.D., Traynor,K.A., Bokoch,G.M. and Schwartz,M.A. (1994) The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell*, **79**, 507–513.

Freeman,J.L., Abo,A. and Lambeth,J.D. (1996) Rac 'insert region' is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. *J. Biol. Chem.*, **271**, 19794–19801.

Hill,C.S., Wynne,J. and Treisman,R. (1995) The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell*, **81**, 1159–1170.

Hirshberg,M., Stockley,R.W., Dodson,G. and Webb,M.R. (1997) The crystal structure of human rac1, a member of the rho-family complexed with a GTP analogue. *Nat. Struct. Biol.*, **4**, 147–152.

Hotta,K., Tanaka,K., Mino,A., Kohno,H. and Takai,Y. (1996) Interaction of the Rho family small G proteins with kinectin, an anchoring protein of kinesin motor. *Biochem. Biophys. Res. Commun.*, **225**, 69–74.

Ishizaki,T., Naito,M., Fujisawa,K., Maekawa,M., Watanabe,N., Saito,Y. and Narumiya,S. (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.*, **404**, 118–124.

Joneson,T., McDonough,M., Bar Sagi,D. and Van Aelst,L. (1996a) RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. *Science*, **274**, 1374–1376.

Joneson,T., White,M.A., Wigler,M.H. and Bar Sagi,D. (1996b) Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science*, **271**, 810–812.

Khosravi-Far,R., Solski,P.A., Clark,G.J., Kinch,M.S. and Der,C.J. (1995) Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. *Mol. Cell. Biol.*, **15**, 6443–6453.

Khosravi-Far,R., White,M.A., Westwick,J.K., Solski,P.A., Chrzanowska-Wodnicka,M., Van Aelst,L., Wigler,M.H. and Der,C.J. (1996) Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Mol. Cell. Biol.*, **16**, 3923–3933.

Kimura,K. *et al.* (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*, **273**, 245–248.

Lamarche,N., Tapon,N., Stowers,L., Burbelo,P.D., Aspenstrom,P., Bridges,T., Chant,J. and Hall,A. (1996) Rac and Cdc42 induce actin polymerization and $G_1$ cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell*, **87**, 519–529.

Lebowitz,P.F., Du,W. and Prendergast,G.C. (1997) Prenylation of RhoB is required for its cell transforming function but not its ability to activate serum response element-dependent transcription. *J. Biol. Chem.*, **272**, 16093–16095.

Leevers,S.J. and Marshall, C.J. (1992) Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *EMBO J.*, **11**, 569–574.

Leung,T., Manser,E., Tan,L. and Lim,L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.*, **270**, 29051–29054.

Leung,T., Chen,X.Q., Manser,E. and Lim,L. (1996) The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell. Biol.*, **16**, 5313–5327.

Lim,L., Manser,E., Leung,T. and Hall,C. (1996) Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur. J. Biochem.*, **242**, 171–185.

Madaule,P., Furuyashiki,T., Reid,T., Ishizaki,T., Watanabe,G., Morii,N. and Narumiya,S. (1995) A novel partner for the GTP-bound forms of rho and rac. *FEBS Lett.*, **377**, 243–248.

Marshall,C.J. (1996) Ras effectors. *Curr. Opin. Cell Biol.*, **8**, 197–204.

Matsui,T. *et al.* (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.*, **15**, 2208–2216.

Milburn,M.V., Tong,L., deVos,A.M., Brunger,A., Yamaizumi,Z., Nishimura,S. and Kim,S.H. (1990) Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. *Science*, **247**, 939–945.

Mistou,M.Y., Jacquet,E., Poullet,P., Rensland,H., Gideon,P., Schlichting,I., Wittinghofer,A. and Parmeggiani,A. (1992) Mutations of Ha-ras p21 that define important regions for the molecular mechanism of the SDC25 C-domain, a guanine nucleotide dissociation stimulator. *EMBO J.*, **11**, 2391–2397.

Nakagawa,O., Fujisawa,K., Ishizaki,T., Saito,Y., Nakao,K. and Narumiya,S. (1996) ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.*, **392**, 189–193.

Narumiya,S., Ishizaki,T. and Watanabe,N. (1997) Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett.*, **410**, 68–72.

Nisimoto,Y., Freeman,J.L.R., Motalebi,S.A., Hirshberg,M. and Lambeth,J.D. (1997) Rac binding to p67(phox). Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase. *J. Biol. Chem.*, **272**, 18834–18841.

Olson,M.F., Ashworth,A. and Hall,A. (1995) An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through $G_1$. *Science*, **269**, 1270–1272.

Pai,E.F., Kabsch,W., Krengel,U., Holmes,K.C., John,J. and Wittinghofer,A. (1989) Structure of the guanine-nucleotide-binding domain of the *Ha-ras* oncogene product p21 in the triphosphate conformation. *Nature*, **341**, 209–214.

Palmer,R.H., Ridden,J. and Parker,P.J. (1994) Identification of multiple, novel, protein kinase C-related gene products. *FEBS Lett.*, **356**, 5–8.

Palmer,R.H., Dekker,L.V., Woscholski,R., Le Good,J.A., Gigg,R. and Parker,P.J. (1995) Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. A comparison with protein kinase C isotypes. *J. Biol. Chem.*, **270**, 22412–22416.

Perona,R., Esteve,P., Jimenez,B., Ballestero,R.P., Ramon y Cajal,S. and Lacal,J.C. (1993) Tumorigenic activity of *rho* genes from *Aplysia californica*. *Oncogene*, **8**, 1285–1292.

Perona,R., Montaner,S., Saniger,L., Sanchez Perez,I., Bravo,R. and Lacal,J.C. (1997) Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac–1 proteins. *Genes Dev.*, **11**, 463–475.

Prendergast,G.C., Khosravi-Far,R., Solski,P.A., Kurzawa,H., Lebowitz,P.F. and Der,C.J. (1995) Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene*, **10**, 2289–2296.

Qiu,R.G., Chen,J., McCormick,F. and Symons,M. (1995) A role for Rho in Ras transformation. *Proc. Natl Acad. Sci. USA*, **92**, 11781–11785.

Quilliam,L.A. *et al*. (1996) Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *J. Biol. Chem.*, **271**, 28772–28776.

Reid,T., Furuyashiki,T., Ishizaki,T., Watanabe,G., Watanabe,N., Fujisawa,K., Morii,N., Madaule,P. and Narumiya,S. (1996) Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the Rho-binding domain. *J. Biol. Chem.*, **271**, 13556–13560.

Ren,X.D., Bokoch,G.M., Traynor-Kaplan,A., Jenkins,G.H., Anderson,R.A. and Schwartz,M.A. (1996) Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Mol. Biol. Cell*, **7**, 435–442.

Rittinger,K., Walker,P.A., Eccleston,J.F., Nurmahomed,K., Owen,D., Laue,E., Gamblin,S.J. and Smerdon,S.J. (1997) Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. *Nature*, **388**, 693–697.

Shibasaki,Y., Ishihara,H., Kizuki,N., Asano,T., Oka,Y. and Yazaki,Y. (1997) Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase *in vivo*. *J. Biol. Chem.*, **272**, 7578–7581.

Uehata,M. *et al.* (1997) A key role for p160ROCK-mediated Ca++ sensitization of smooth muscle in hypertension. *Nature*, **389**, 990–994.

Vincent,S. and Settleman,J. (1997) The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. *Mol. Cell. Biol.*, **17**, 2247–2256.

Watanabe,G. *et al.* (1996) Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. *Science*, **271**, 645–648.

Watanabe,N. *et al.* (1997) p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.*, **16**, 3044–3056.

Westwick,J.K., Lambert,Q.T., Clark,G.J., Symons,M., Van Aelst,L., Pestell,R.G. and Der,C.J. (1997) Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. *Mol. Cell. Biol.*, **17**, 1324–1335.

White,M.A., Nicolette,C., Minden,A., Polverino,A., Van Aelst,L., Karin,M. and Wigler,M.H. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell*, **80**, 533–541.

Willumsen,B.M., Papageorge,A.G., Kung,H.F., Bekesi,E., Robins,T., Johnsen,M., Vass,W.C. and Lowy,D.R. (1986) Mutational analysis of a ras catalytic domain. *Mol. Cell. Biol.*, **6**, 2646–2654.

Yamamoto,M., Marui,N., Sakai,T., Morii,N., Kozaki,S., Ikai,K., Imamura,S. and Narumiya,S. (1993) ADP-ribosylation of the rhoA gene product by botulinum C3 exoenzyme causes Swiss 3T3 cells to accumulate in the G1 phase of the cell cycle. *Oncogene*, **8**, 1449–1455.

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