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rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities *in vitro* and *in vivo*

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rho family GTPases link extracellular signals to changes in the organization of cytoskeletal actin. Serum stimulation of quiescent Swiss 3T3 fibroblasts leads to rho-dependent actin stress fibre formation and focal adhesions, whilst several growth factors initiate signalling pathways leading to rac-dependent actin polymerization at the plasma membrane, and membrane ruffling. The product of the breakpoint cluster region gene bcr, rho GTPase accelerating protein (rhoGAP) and rasGAP-associated p190 share structurally related rho GAP domains, and possess GAP activity for rho family members *in vitro*. We have directly compared the activities of the isolated GAP domains of these three proteins in regulating different rho family GTPases, both by *in vitro* assays and by microinjection, to address their possible physiologic functions. We show that bcr accelerates the GTPase activity of rac, but not rho *in vitro*, and inhibits rac-mediated membrane ruffling, but not rho-mediated stress fibre formation, after microinjection into Swiss 3T3 fibroblasts. *In vitro*, rhoGAP has a striking preference for G25K as a substrate, whilst p190GAP has marked preferential activity for rho. Furthermore, p190 preferentially inhibits rho-mediated stress fibre formation *in vivo*. Our data suggest that p190, rhoGAP and bcr play distinct roles in signalling pathways mediated through different rho family GTPases.

Key words: GAP/GTPases/rho/signalling

Introduction

Approximately 50 small GTP-binding proteins of the ras superfamily have been identified and these can be divided into four main subclasses, ras, rab, rho and ARF (Hall, 1990). These low molecular weight GTPases are thought to act as molecular switches, which are active when bound to GTP. Down regulation is controlled by GTPase activating proteins, or GAPs, which greatly stimulate the slow intrinsic rate of GTP hydrolysis, and are also implicated in mediating downstream signalling (Duchesne *et al.*, 1993).

The ras subfamily are critical components of signal transduction pathways emanating from protein tyrosine kinases which control cellular proliferation or differentiation. Two $p21^{ras}$-specific GAPs have been identified: p120-GAP (Trahey and McCormick, 1987) and neurofibromin, the product of the NF-1 gene (Xu *et al.*, 1990). The p120-GAP protein comprises an N-terminal region with two SH2 domains separated by an SH3 domain and a C-terminal domain which interacts with ras and stimulates its intrinsic GTPase activity (Trahey and McCormick, 1987; Vogel *et al.*, 1988; Marshall *et al.*, 1989; Koch *et al.*, 1991). Fibroblasts transformed by overexpression of c-*ras*, or the v-*src* oncoprotein, which requires ras for its mitogenic activity (Smith *et al.*, 1986), can be reverted by over-expression of full-length p120-GAP or the isolated GAP domain, or by low levels of GAP targetted to the plasma membrane (DeClue *et al.*, 1991; Nori *et al.*, 1991; Huang *et al.*, 1993), which down regulate ras and reduce cellular levels of rasGTP. Both p120-GAP and neurofibromin interact with ras at the effector domain, a region essential for downstream signalling (Adari *et al.*, 1988; Cales *et al.*, 1988), suggesting that they may also possess effector activity. Furthermore, ras and p120-GAP have been shown to synergize in blocking the opening of muscarinic potassium channels of atrial membranes and if the isolated SH2-SH3 regions of GAP are substituted, then ras is no longer required (Martin *et al.*, 1992). These data have led to the proposal that the interaction of activated ras with the p120-GAP C-terminus induces a conformational change to make the SH2/SH3 region accessible for binding to tyrosine phosphorylated effector proteins.

The mammalian rho subfamily comprises rho A, B and C, rac 1 and 2, TC10 and rho G, CDC42Hs, and G25K (Madaule and Axel, 1985; Didsbury *et al.*, 1989; Drivas *et al.*, 1990; Shinjo *et al.*, 1990; Vincent *et al.*, 1992). Stimulation of quiescent Swiss 3T3 fibroblasts with serum, or a constituent of serum, lysophosphatidic acid (LPA), results in the formation of actin stress fibres and focal adhesions, effects which are mediated by rho proteins (Paterson *et al.*, 1990; Ridley and Hall, 1992). In contrast, when quiescent fibroblasts are stimulated with a variety of growth factors, including platelet derived growth factor (PDGF), a signalling pathway mediated through rac proteins is initiated, leading to the accumulation of polymerized actin and formation of membrane ruffles at the plasma membrane (Ridley *et al.*, 1992). Hence, rac and rho proteins are critical components of signalling pathways which link extracellular stimuli with reorganization of polymerized actin.

Recently, a prototype GAP protein specific for rho family GTPases, termed rhoGAP, was purified. A partial cDNA, encompassing the complete C-terminal rhoGAP domain was cloned (Hall, 1992) and was found to be unrelated in sequence to rasGAP. rhoGAP was active on rho, with a 10-fold reduced activity towards rac *in vitro* (Diekmann *et al.*, 1991). Interestingly, several other signalling proteins also contain structurally related rhoGAP domains, and are therefore also putative rhoGAPs. Included in this family is the product of the breakpoint cluster region gene, bcr, a large protein which comprises several different functional regions,

including a C-terminal rhoGAP domain. At its N-terminus, bcr contains a region with intrinsic serine kinase activity, despite bearing none of the hallmark features of a serine kinase, and the central region shows homology to dbl, a guanine nucleotide exchange factor for CDC42. bcr has been demonstrated to possess GAP activity for rac, but not rho *in vitro* (Diekmann *et al.*, 1991). Another multifunctional domain protein p190, contains a C-terminal rhoGAP domain and has been shown to possess GAP activity for several rho family GTPases in *in vitro* assays (Settleman *et al.*, 1992b). At its N-terminus, p190 contains a region with homology to ras superfamily GTPases and the central region is identical to a putative transcriptional repressor (Settleman *et al.*, 1992a). p190 was originally identified as a tyrosine phosphorylated protein which associates with p120−rasGAP in cells stimulated with a number of growth factors or transformed with $p60^{v\text{-}src}$ (Ellis *et al.*, 1990), and which can bind *in vitro* to the isolated, bacterially expressed SH2−SH3−SH2 domain of GAP (Ellis *et al.*, 1991). Hence, p190 is a candidate p120-GAP target, and may provide a link between pathways involved in mediating cellular proliferation/differentiation and organization of the actin cytoskeleton.

In this study we have investigated the possibility that these three rhoGAP proteins are involved preferentially in accelerating the intrinsic GTPase rate of different rho GTPases, by directly comparing the abilities of the isolated GAP domains of bcr, rhoGAP and p190 (bcrGAP, rhoGAP-C and p190GAP, respectively), to regulate rho, rac and G25K GTPases by *in vitro* assays. In addition, we microinjected isolated GAP domains of these proteins into Swiss 3T3 fibroblasts to address their possible physiological roles in signalling pathways specific for rho and rac GTPases. We show that *in vitro*, p190GAP has a striking preference for rho as a substrate, and furthermore, preferentially inhibits rho-mediated stress fibres after microinjection into Swiss 3T3 fibroblasts. bcrGAP accelerates the GTPase activity of rac, but not rho *in vitro* and specifically inhibits rac-induced membrane ruffling *in vivo*. Lastly, rhoGAP-C shows a marked preference for G25K as a substrate *in vitro*, but also inhibits rho, *in vitro* and *in vivo*. Our data suggest that p190, bcr and rhoGAP may play restricted roles in signalling pathways mediated through different rho family GTPases.

Results

Purification of p190, rhoGAP and bcrGAP domains

Expression of GST fusion proteins encompassing the complete GAP domains of p190GAP, bcrGAP or rhoGAP-C was induced with IPTG. Proteins were purified from bacterial cell lysates with glutathione−agarose beads, cleaved from the GST carrier with thrombin and dialysed into low salt buffer. Figure 1 shows aliquots of each purified protein on a 12% SDS gel stained with Coomassie blue. The same preparations of proteins were used for *in vitro* assays at various dilutions. For microinjection, bcrGAP was used at 0.2 or 0.1 mg/ml, p190GAP either at 0.5 or 0.3 mg/ml and rhoGAP-C at 2.0 or 0.8 mg/ml.

bcrGAP, p190GAP and rhoGAP-C show preferential activities for different rho family GTPases in vitro

To investigate the possibility that bcr, p190 and rhoGAP are involved preferentially in down regulating specific members

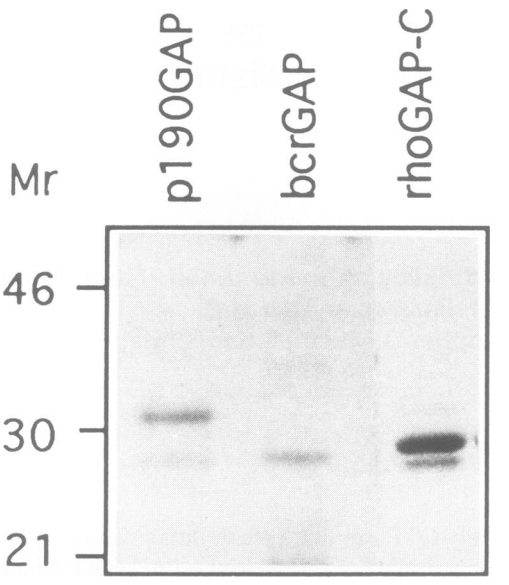

Fig. 1. Purification of p190GAP, bcrGAP, and rhoGAP-C. Recombinant bcrGAP, p190GAP and rhoGAP-C were purified from bacterial cell lysates using glutathione−agarose beads and cleaved from the GST carrier with thrombin. Aliquots were resolved by SDS−PAGE and stained with Coomassie blue.

Table I. *In vitro* GAP activities of p190GAP, rhoGAP-C and bcrGAP

	GAP activity (nM)		
	p190GAP	rhoGAP-C	bcrGAP
G25K	21	1	28
rac	18	8	20
rho	2	10	–

GAP activity represents the concentration of GAP protein required to stimulate the intrinsic GTPase rate by 50% in 5 min under assay conditions. Results are an average of three independent experiments. Recombinant GTPases were preloaded with [γ^{32}P]GTP for 10 min then incubated with GAP proteins. After 5 min the amount of radioactivity remaining bound to the GTPase was determined by liquid scintillation counting. The nM concentration of each GAP protein required to stimulate the intrinsic GTPase rate by 50% in 5 min is shown.

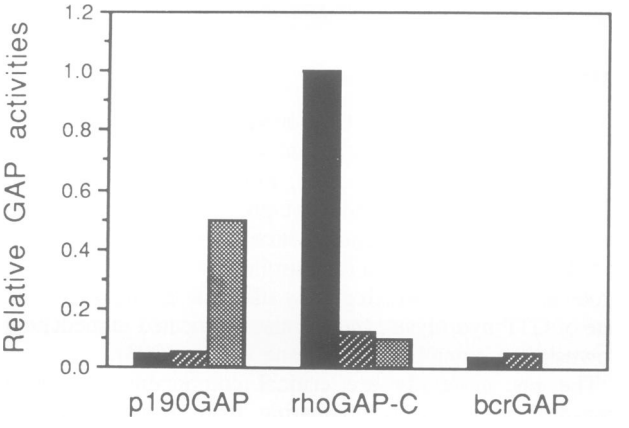

Fig. 2. Relative GAP activities of bcrGAP, rhoGAP-C and p190GAP for rho, rac and G25K proteins. The activity of G25K with rhoGAP-C is taken as 1. For further details see footnote to Table I. ■, G25K; ▨, rac; ▩, rho.

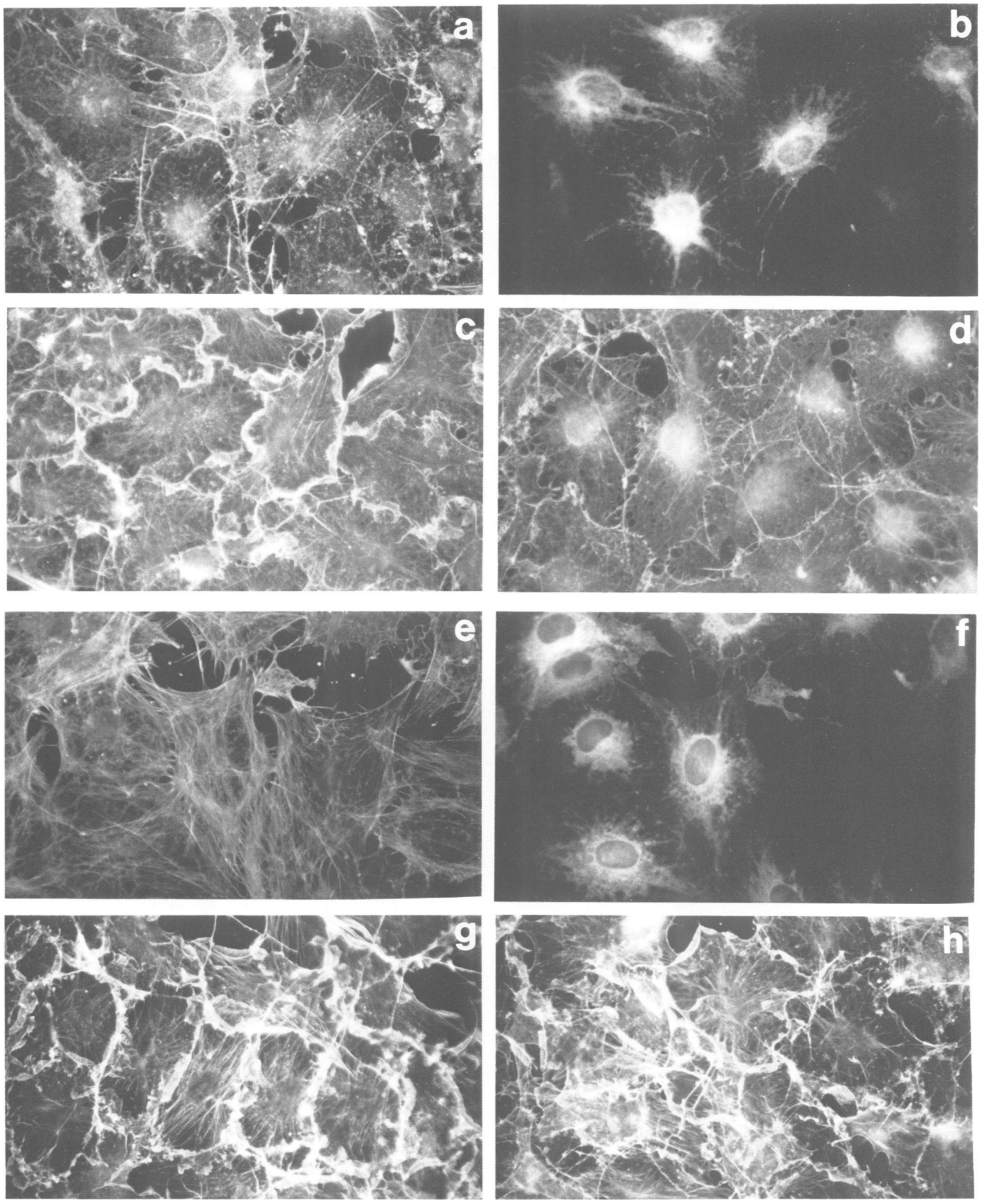

Fig. 3. Inhibition of PDGF-induced membrane ruffling, but not LPA-induced stress fibres by bcrGAP. Actin filaments are shown in serum starved Swiss 3T3 fibroblasts 25 min after microinjection with bcrGAP with no addition (**a**), microinjected with bcrGAP and after 15 min stimulated with 3 ng/ml PDGF for 10 min (**d**), or microinjected with bcrGAP and after 15 min stimulated with 20 ng/ml LPA for 10 min (**e**). Panel (**c**) shows membrane ruffles in control non-injected Swiss 3T3 cells stimulated with PDGF for 10 min. Panels (**g**) and (**h**) show membrane ruffles in serum starved Swiss 3T3 fibroblasts 20 min after microinjection with either 0.2 mg/ml V12rac1 alone (**g**), or co-injected with V12rac1 and 0.2 mg/ml bcrGAP (**h**). In panels (a), (c), (d), (e), (g) and (h), cells were stained for actin filaments with TRITC-labelled phalloidin. In panels (b) and (f), microinjected cells from (a) and (e) respectively, were identified by immunofluorescence with a rat immunoglobulin marker protein, microinjected at 0.5 mg/ml, followed by staining with FITC-labelled goat anti-rat IgG.

of the rho family of GTPases, we first compared the GTPase activating activity of p190GAP, bcrGAP and rhoGAP-C with rho, rac and G25K proteins in *in vitro* assays. To enable comparisons with microinjection studies, *in vitro* assays were carried out at physiological ionic strength.

These experiments revealed marked preferential activities

of the three GAP proteins for different GTPases (Table I and Figure 2). bcrGAP was completely inactive towards rho, but was active on rac, as previously reported (Diekmann *et al.*, 1991), and in addition we found that it accelerated the GTPase activity of G25K (Table I and Figure 2). In contrast, whilst p190GAP showed some capacity to down regulate all three rho family proteins, it was 9-fold more effective in accelerating the GTPase activity of rho, than either rac or G25K, and was the most effective GAP for rho. rhoGAP-C showed a striking preference for G25K as a substrate and had >20-fold more activity for this GTPase than either p190GAP or bcrGAP. In addition, rhoGAP-C had considerably reduced, but approximately equal activity for rac and rho at physiologic ionic strength. Interestingly, its activity for rho, but not rac, varied according to the salt conditions used, and at low ionic strength we found that rhoGAP-C was 10-fold more active on rho than on rac, as previously shown (data not shown; Diekmann *et al.*, 1991). However, under both salt conditions rhoGAP-C retained its strong preferential activity for G25K.

bcrGAP inhibits PDGF-induced membrane ruffling, but not LPA-induced stress fibres

Rac proteins are critical components of a signalling pathway that links extracellular growth factors with induction of membrane ruffling. Microinjection of an activated rac protein into confluent Swiss 3T3 fibroblasts induces membrane ruffling, whilst stimulation of membrane ruffles with growth factors such as PDGF, is blocked by microinjection of a dominant inhibitory N17rac protein (Ridley *et al.*, 1992).

To assess the role of bcr in down regulation of rho and rac proteins *in vivo*, we microinjected purified bcrGAP protein into the cytoplasm of confluent Swiss 3T3 fibroblasts. Microinjection at 0.2 mg/ml into serum starved cells reduced basal levels of membrane ruffling, but had no effect on the number or appearance of stress fibres (Figure 3a and b). To determine whether bcrGAP could inhibit growth factor-stimulated changes in the actin cytoskeleton, microinjected fibroblasts were treated with PDGF for 10 min. Normal PDGF-induced membrane ruffling was completely abolished in the microinjected cells (Figure 3c and d). In contrast, no inhibition of actin stress fibre formation was observed in cells microinjected with bcrGAP and stimulated with LPA (Figure 3e, and f) or fetal calf-serum (FCS) (data not shown). When microinjected at 0.1 mg/ml, bcrGAP was much less effective in inhibiting membrane ruffling, suggesting that this represents a threshold concentration of active protein.

As a control, confluent Swiss 3T3 fibroblasts were coinjected with bcrGAP and an activated rac protein, V12rac1 (equivalent to the V12 oncogenic mutation in ras), in which amino acid 12 (glycine) is mutated to valine. This mutant rac protein has a decreased intrinsic GTPase activity, is unresponsive to GAP proteins and induces the accumulation of membrane ruffles when microinjected into confluent, serum starved Swiss 3T3 fibroblasts (Ridley *et al.*, 1992; Figure 3g). Co-injection of bcrGAP had no effect on V12rac1-induced membrane ruffling (Figure 3h), indicating that bcrGAP specifically inhibits growth factor-induced rac activation *in vivo*, by increasing the rate of hydrolysis of racGTP to racGDP. The strict specificity of bcrGAP for rac GTPases relative to rho, observed *in vitro*, is thus reproduced *in vivo* after microinjection into Swiss 3T3 fibroblasts.

Table II. Percentage of fibroblasts showing inhibition of LPA-induced stress fibres, or PDGF-induced membrane ruffles, after microinjection of p190GAP

p190GAP	Total no. of analysed cells	−SF (%)	+SF (%)	−MR (%)	+MR (%)
A					
0.5 mg/ml	48	92	8	–	–
0.3 mg/ml	123	27	73	–	–
B					
0.5 mg/ml	76	–	–	8	92
0.3 mg/ml	106	–	–	2	98

p190GAP was microinjected into serum starved Swiss 3T3 cells at the indicated concentrations, together with 0.5 mg/ml rat IgG. After 10 min either 0.2% FCS (**A**) or 3 ng/ml PDGF (**B**) were added. Ten minutes later cells were fixed, actin filaments localised with TRITC-phalloidin and microinjected cells identified with FITC-labelled goat anti-rat IgG. Microinjected cells were scored for the presence of stress fibres significantly above background levels, or for membrane ruffles. SF = stress fibres; MR = membrane ruffles.

p190GAP and rhoGAP-C inhibit FCS-induced stress fibres, but not PDGF-induced membrane ruffles

Our *in vitro* assays suggested that p190GAP may possess preferential activity towards rho. To test its specificity *in vivo*, confluent Swiss 3T3 fibroblasts were microinjected with p190GAP. Cells became more rounded and refractile, and lost all actin stress fibres (Figure 4a and b). Stimulation of fibroblasts with FCS or LPA leads to the rapid formation of stress fibres, a process dependent on rho proteins (Ridley and Hall, 1992). When Swiss 3T3 fibroblasts microinjected with p190GAP were stimulated with serum, stress fibre formation was abolished (compare injected cell, indicated by an arrow, with a neighbouring uninjected cell, in Figure 4c). In contrast, when microinjected cells were stimulated for 10 min with PDGF, we observed no inhibition of growth factor-induced membrane ruffling (Figure 4d, e and f).

In a control experiment, we co-injected Swiss 3T3 fibroblasts with p190GAP and a constitutively activated rho mutant, V14rhoA. This mutant rho protein has a decreased intrinsic GTPase activity, is unresponsive to GAP proteins, and induces stress fibre formation when microinjected into confluent, serum starved Swiss 3T3 fibroblasts (Paterson *et al.*, 1990; Ridley and Hall, 1992: Figure 4g). Stress fibre formation induced by the mutant rho protein was not inhibited by the presence of p190GAP (Figure 4h). These results demonstrate that p190GAP directly inhibits LPA-stimulated stress fibre formation, by down regulating endogenous rho protein.

To confirm the significance of these observations, we recorded the percentages of microinjected fibroblasts which were inhibited in FCS or PDGF-induced changes in the actin cytoskeleton after microinjection of p190GAP at two different concentrations. Results from a typical experiment, in which 171 microinjected cells were analysed for the presence of FCS-induced stress fibres, and 182 microinjected cells were analysed for the presence of PDGF-induced membrane ruffles, are shown in Table II; these data are representative of three similar, independent experiments. At a concentration of 0.5 mg/ml, 92% of cells failed to produce

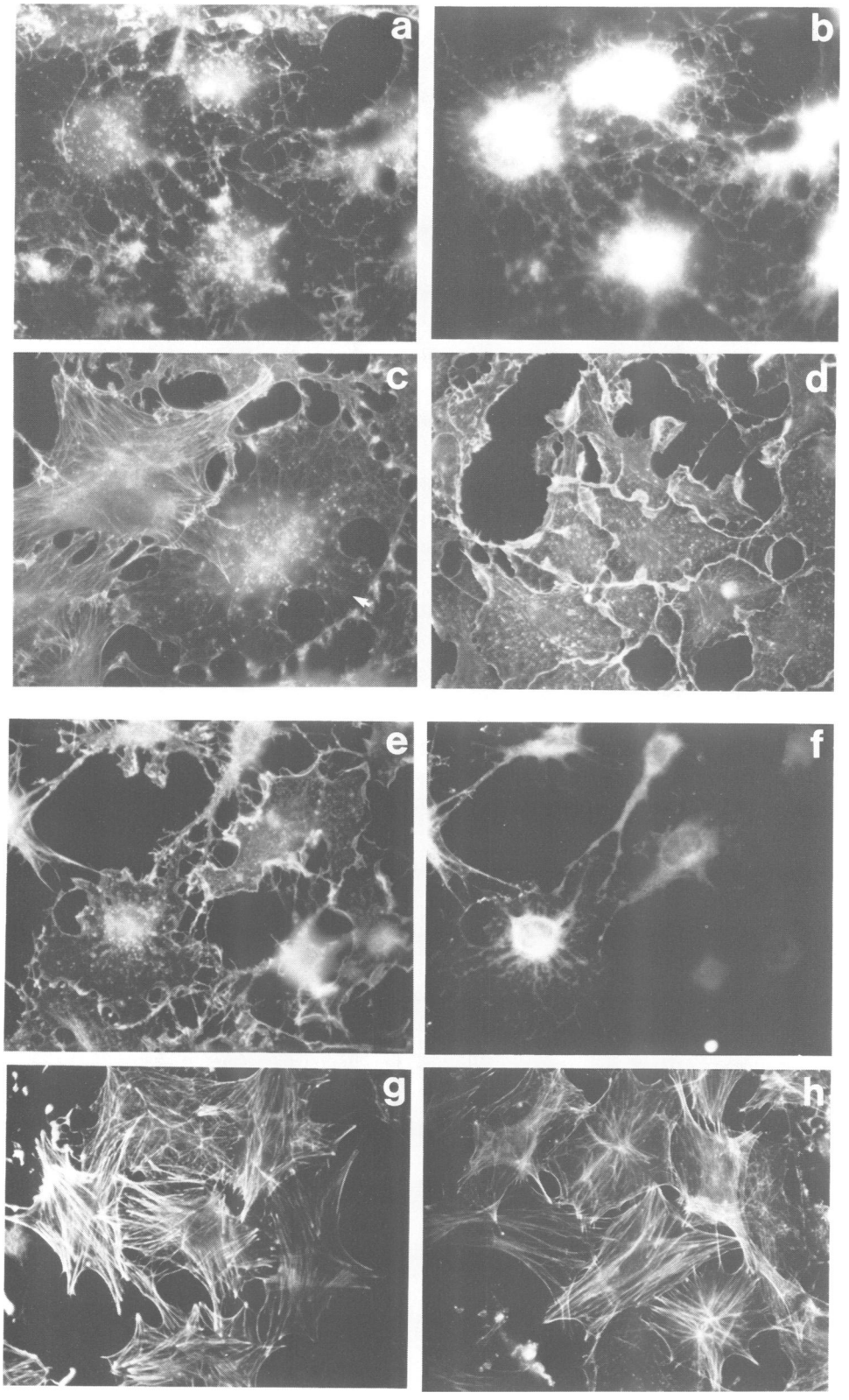

Fig. 4. Inhibition of FCS-induced stress fibres, but not PDGF-induced membrane ruffles by p190GAP. Actin filaments are shown in serum starved Swiss 3T3 fibroblasts 25 min after microinjection with p190GAP at 0.5 mg/ml with no addition (**a**), microinjected with p190GAP and after 15 min stimulated with 0.2% FCS for 10 min (**c**), or microinjected with p190GAP and after 15 min stimulated with PDGF for 10 min (**e**). Panel (**d**) shows membrane ruffles in control non-injected Swiss 3T3 cells stimulated with PDGF for 10 min. Panels (**g**), and (**h**), show stress fibres in serum starved Swiss 3T3 fibroblasts 30 min after stimulation with either 0.1 mg/ml V14rhoA alone (g), or coinjected with V14rhoA and 0.5 mg/ml p190GAP (h). In panels (a), (c), (d), (e), (g) and (h), cells were stained for actin filaments with TRITC-labelled phalloidin. In panels (b) and (f), the microinjected cells from panels (a) and (e) respectively, were identified as described in legend to Figure 2. In panel (c), the p190GAP-microinjected fibroblast is indicated by an arrow. Adjacent to the microinjected fibroblast is a non-injected fibroblast showing normal FCS-induced stress fibres.

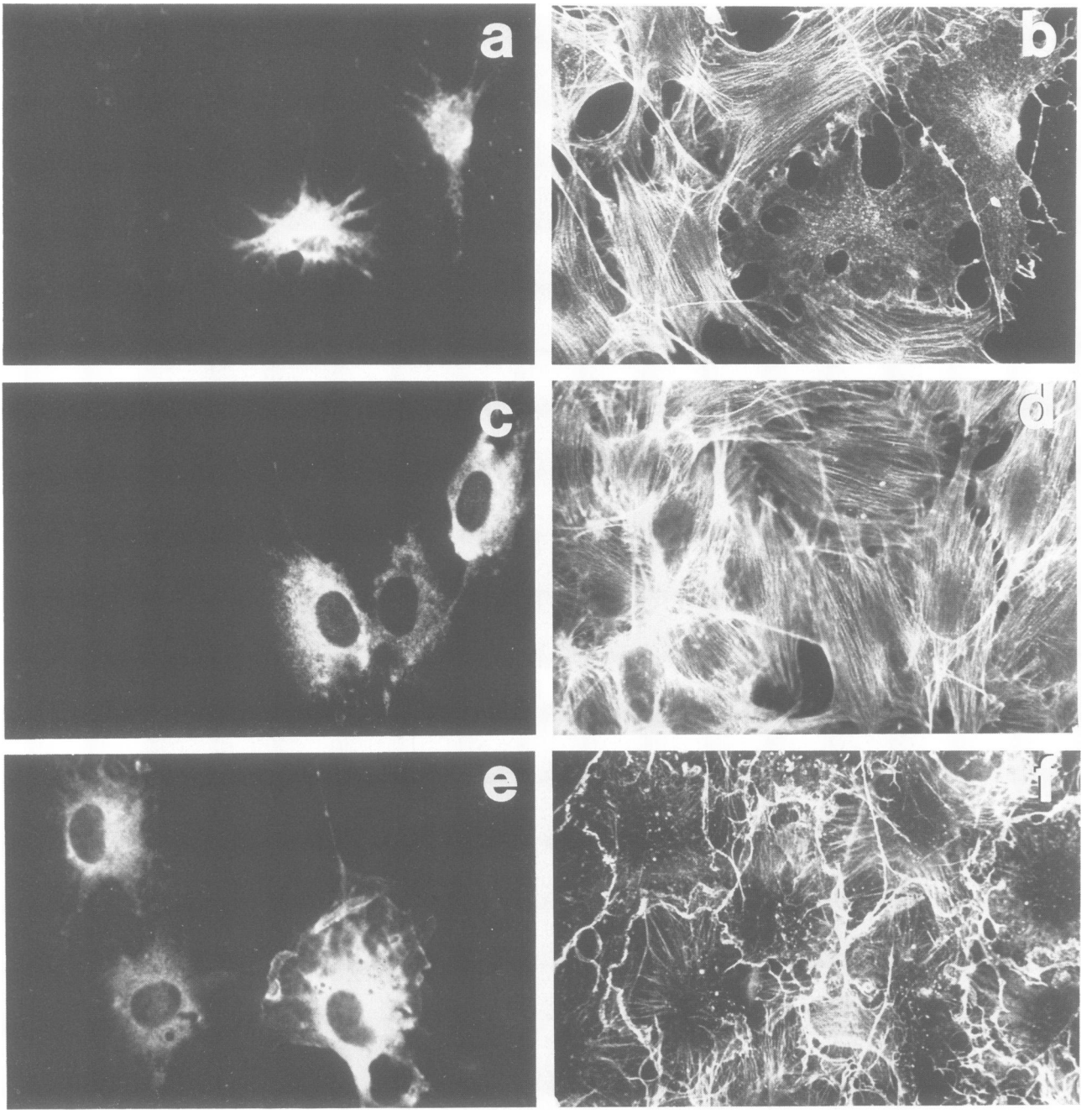

Fig. 5. Inhibition of serum-induced stress fibres, but not PDGF-induced membrane ruffles, by rhoGAP-C. Actin filaments are shown in serum starved Swiss 3T3 fibroblasts microinjected with rhoGAP-C either at 2.0 mg/ml (panels b and f), or 0.8 mg/ml (d). Panels (**a**), (**b**), (**c**) and (**d**) show cells microinjected with rhoGAP-C and after 15 min stimulated with 0.2% FCS for 10 min, and panels (**e**) and (**f**) show cells microinjected with rhoGAP-C and after 15 min stimulated with 3 ng/ml PDGF for 10 min. In panels (b), (d) and (f), cells were stained for actin filaments with TRITC-labelled phalloidin. In panels (a), (c) and (e), microinjected cells from (b), (d) and (f), respectively, were identified as described in legend to Figure 2.

stress fibres after LPA addition, whereas at 0.3 mg/ml, only 27% of fibroblasts did not produce stress fibres, suggesting that this represents a threshold concentration of active p190GAP protein. In comparison, in two separate experiments, only 8 and 4% respectively of a random background of non-injected cells failed to produce stress fibres in response to LPA. In contrast, we found that 92 and 98% of cells which had been microinjected with p190GAP at 0.5 or 0.3 mg/ml, respectively, showed normal membrane ruffling in response to PDGF stimulation (Table II), compared with 92 and 96% of a random background of non-injected fibroblasts. Thus, at both concentrations tested, p190GAP had no effect on PDGF-induced membrane ruffling relative to control cells. This inability to down regulate rac proteins *in vivo* correlates with the reduced activity of p190GAP towards rac GTPases that we have observed *in vitro*.

In vitro assays showed that whilst rhoGAP-C has a striking preferential activity for G25K, it can in addition accelerate the GTPase activity of both rac and rho at physiological ionic

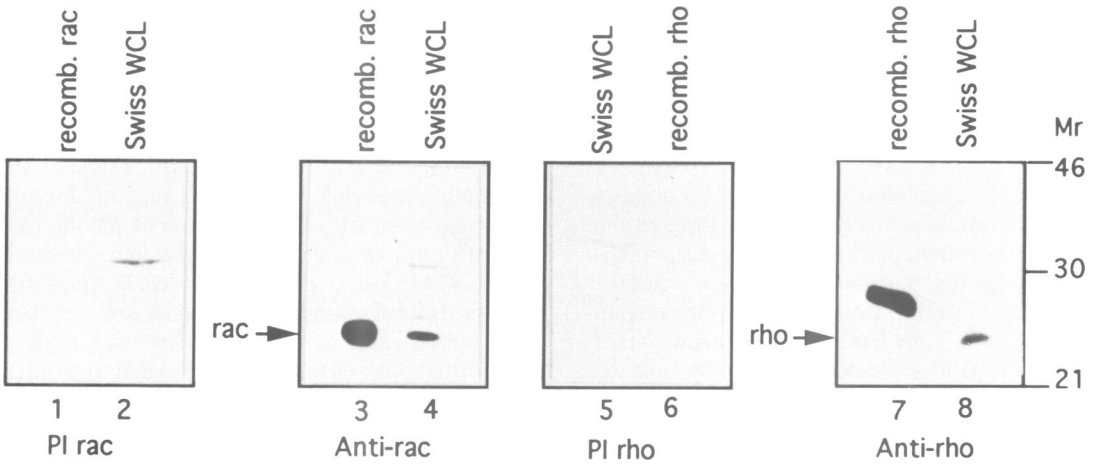

Fig. 6. Expression of rac and rho in rodent fibroblasts. Whole cell lysates from confluent Swiss 3T3 fibroblasts (equivalent amounts of protein) were resolved on SDS–polyacrylamide gels, adjacent to 10 ng samples of recombinant, bacterially expressed rho or rac proteins, and transferred to nitrocellulose. Membranes were blocked and probed with either pre-immune serum (lanes 1 and 2), or anti-rac antibodies (lanes 3 and 4), or pre-immune serum (lanes 5 and 6), or anti-rho antibodies (lanes 7 and 8), followed by detection with ECL reagents. The positions of rho and rac proteins are indicated by arrows. Bacterially expressed rho protein showed a decreased mobility relative to the mammalian protein (lane 7).

strength. We tested its activity on rac and rho proteins *in vivo*, but not G25K, since the physiological function of this GTPase is not known. When Swiss 3T3 fibroblasts were microinjected with rhoGAP-C at a concentration of 2.0 mg/ml and stimulated for 10 min with FCS, we observed a loss of normal stress fibre formation (Figure 5a and b). In contrast, microinjection of rhoGAP-C at 0.8 mg/ml had no effect on serum-stimulated stress fibre formation; thus, this concentration lies below the threshold for inhibition of rho proteins (Figure 5c and d). The ability to inhibit serum stimulated stress fibres is therefore a direct consequence of the concentration of rhoGAP-C microinjected into fibroblasts, and is not a non-specific effect of the injection procedure. In contrast, rhoGAP-C microinjected at 2.0 mg/ml had no effect on PDGF-induced membrane ruffling (Figure 5e and f).

Expression of rac and rho proteins in fibroblasts

The apparent preference of p190GAP and rhoGAP-C for down regulation of rho proteins that we observed *in vivo*, could be due to considerably lower levels of expression of endogenous rho proteins, relative to rac, in Swiss 3T3 fibroblasts. This would necessitate the introduction of much higher concentrations of p190GAP and rhoGAP-C to effectively inhibit rac-mediated changes in the actin cytoskeleton.

To estimate the amounts of endogenous rho and rac proteins present in Swiss 3T3 fibroblasts, whole cell lysates (equivalent amounts of protein) were run on SDS gels, adjacent to 10 ng samples of recombinant, bacterially expressed rho or rac proteins. After transfer to nitrocellulose, Western blots were blocked and probed with anti-rac or anti-rho antibodies, or pre-immune sera, followed by detection with ECL reagents (Figure 6). Anti-rac antibodies detected both recombinant, bacterially expressed rac (lane 3) and endogenous rac protein from the Swiss 3T3 fibroblast lysate (lane 4); these proteins were not detected by the pre-immune serum (lanes 1 and 2). In addition, anti-rac antibodies did not cross-react with other rho family GTPases (data not shown). Anti-rho antibodies detected specifically both recombinant, bacterially expressed rho protein (which had an anomalously high molecular weight of ~27 kDa; lane 7) and endogenous rho protein from the Swiss 3T3 fibroblast whole cell lysate (lane 8); these proteins were not detected by the pre-immune serum (lanes 5 and 6). The reason for the slower mobility of recombinant rho protein is not clear, but may be due to geranylgeranylation of the mammalian, but not the bacterially expressed protein, as has been previously suggested (Hori *et al.*, 1991). In addition, the anti-rho antibodies did not cross-react with other rho family GTPases (data not shown). Thus, anti-rho and anti-rac antibodies, but not their pre-immune sera, specifically recognize both bacterially expressed and endogenous, mammalian rho or rac proteins.

By comparing the density of the bands corresponding to fibroblast rho and rac proteins with the known quantities (10 ng) of recombinant rho and rac proteins run out on the same gel, we estimated that the 70 μg aliquots of Swiss 3T3 fibroblast whole cell lysates contained 1.5 ng rho protein and 2.4 ng rac. Similar estimates were obtained from a number of independent experiments (data not shown). Thus, these results demonstrate that rho and rac proteins are expressed at similar levels in Swiss 3T3 fibroblasts.

Discussion

Recently, considerable progress has been made in understanding the functions of rho and rac in cellular transduction pathways: these GTPases have been shown to be critical to pathways linking extracellular signals with reorganization of polymerized actin. The identification of a family of structurally related rho GTPase activating proteins raises the question of whether *in vivo*, different rhoGAPs share overlapping and broad specificities, or function to mediate signals through particular members of the rho subfamily.

In vitro assays have previously suggested that p190 has a broad activity and down regulates several members of the rho subfamily (Settleman *et al.*, 1992b). Similar studies have shown that rhoGAP has GTPase activating activity for rho

and 10-fold less activity for rac, whereas bcr is a GAP for rac, but not rho (Diekmann *et al.*, 1991). In this study, we have directly compared the ability of isolated, recombinant GAP domains of p190, bcr and rhoGAP to down regulate rho, rac and G25K GTPases in *in vitro* assays, and have microinjected purified p190GAP, bcrGAP and rhoGAP-C proteins into Swiss 3T3 fibroblasts to examine their abilities to inhibit directly signalling pathways mediated through rho and rac proteins *in vivo*. Both p190 and bcr are large multi-domain proteins, suggesting that they may possess a number of different biological functions. Hence, the use of recombinant GAP domains in this study has enabled investigation of GAP activities of bcr, p190 and rhoGAP *in vivo*, in isolation from possible additional activities of these proteins towards other low molecular weight GTPases.

Our *in vitro* assays revealed marked preferential activities of bcrGAP, p190GAP and rhoGAP-C for the three different rho family GTPases (Table I and Figure 2). For example, p190GAP showed preferential GTPase accelerating activity for rho and had 9-fold more activity for this GTPase than for rac or G25K. We confirmed that bcrGAP is inactive against rho *in vitro* and active against rac, and have further demonstrated that it accelerates the GTPase activity of G25K. rhoGAP-C showed >20-fold higher activity for G25K than either p190GAP or bcrGAP, implicating it as an important regulator of this GTPase.

Consistent with the marked preferential activity of p190GAP for rho *in vitro*, microinjection of p190GAP into Swiss 3T3 fibroblasts inhibited rho-mediated stress fibre formation, but had no effect on rac-mediated membrane ruffling. p190GAP had no effect on stress fibre formation induced by a mutant rho protein (V14rhoA) which is unresponsive to GAP proteins, indicating that p190GAP inhibits LPA stimulated stress fibre formation by direct down regulation of endogenous rho protein. Thus, results from *in vitro* assays and microinjection experiments show that p190GAP has a preferential activity for rho and are consistent with the idea that p190 plays an important role in signalling pathways linking extracellular stimuli to cytoskeletal changes controlled by rho proteins. rhoGAP-C was also active against rho *in vitro* and *in vivo*, although it appeared to be less effective than p190GAP. For example, *in vitro* at physiological ionic strength, 5-fold higher concentrations of rhoGAP-C than p190GAP were required to stimulate the intrinsic GTPase rate of rho by 50%, whilst *in vivo*, rhoGAP-C inhibited rho-induced actin stress fibres only when microinjected at ~4-fold higher concentration than p190GAP (2 mg/ml rhoGAP-C, compared with 0.5 mg/ml p190GAP). From these results it is possible that p190 may be the major rho GTPase activating protein *in vivo*.

Correlating with its *in vitro* activities, microinjection of bcrGAP specifically inhibited rac-mediated membrane ruffling, but not rho-mediated stress fibres. Its ability to inhibit membrane ruffling was a direct consequence of its ability to convert racGTP to racGDP, since bcrGAP showed no activity against a constitutively activated rac mutant (V12rac1) which is unresponsive to GAP proteins. Taken together, the activity of bcrGAP for rac that we have observed *in vitro* and *in vivo* suggests that bcr may play an important role in signal transduction pathways linking extracellular signals with rac-mediated reorganization of polymerized actin.

Thus, for p190 and bcr, the *in vivo* activity correlates with their *in vitro* preferences for substrates. However, for rhoGAP this appears not to be the case, since rhoGAP-C showed approximately equal activity for rac and rho *in vitro* at physiological ionic strength, yet was specifically active against rho *in vivo*. Interestingly, however, rhoGAP activity towards rho, but not rac, is increased 10-fold under low ionic strength conditions. The significance of this is not clear, although a similar effect is observed for the ras−rasGAP interaction. It does demonstrate that the susceptibility of rho to rhoGAP can be altered *in vitro*, presumably by a conformational change induced under low ionic strength, and it is possible that this *in vitro* effect may mimic an *in vivo* conformational change induced when rho interacts with another protein. This discrepancy therefore emphasizes the value of microinjection studies, in addition to *in vitro* assays, in attempts to dissect the physiologic functions of GAP proteins in rho family signalling pathways. It remains possible that p190GAP and rhoGAP-C have some reduced activity for rac proteins *in vivo*. However, since rac and rho proteins are expressed at similar levels in Swiss 3T3 fibroblasts (Figure 6), and the concentrations of p190GAP and rhoGAP-C used for microinjection were effective in inhibition of rho, our data clearly demonstrate that in these cells p190GAP and rhoGAP-C are considerably less active on rac than on rho.

In addition to their GAP activities for different rho subfamily members, structural features of both p190 and bcr suggest they may be involved in signalling through other low molecular weight GTPases. For example, the N-terminus of p190 contains structural features in common with other small GTP-binding proteins, suggesting that it may be able to bind and hydrolyse GTP (Settleman *et al.*, 1992a), whilst bcr shares a motif in common with the *dbl* proto-oncogene, which has been shown to act as an exchange factor for CDC42, suggesting that bcr may have exchange activity, or interact with other low molecular weight GTPases.

In addition, p190 associates with rasGAP in cells stimulated with growth factors, or transformed by $p60^{v\text{-}src}$ (Ellis *et al.*, 1990). The p190−rasGAP complex has a 4-fold reduced GTPase activating activity for p21ras (Moran *et al.*, 1991), suggesting that one function of this complex may be to increase the activity of ras in response to mitogenic or oncogenic stimulation. p120-GAP also associates with activated receptor tyrosine kinases via its SH2 domains, and may therefore function to couple tyrosine kinases and ras to control of the actin cytoskeleton, through its association with p190. bcr is also implicated in interactions with SH2-containing signalling proteins. For example, in the generation of the bcr−abl fusion protein in Philadelphia chromosome positive human leukaemias, a fusion protein is formed between N-terminal bcr sequences and the abl tyrosine kinase. Phosphoserine residues from bcr have been shown to interact with the SH2 domain of abl, resulting in activation of abl tyrosine kinase activity (Maru and Witte, 1991; Pendergast *et al.*, 1991). Moreover, a limited number of isolated SH2 domains bind *in vitro* with high affinity to serine/threonine phosphorylated bcr (Muller *et al.*, 1992), suggesting the potential for such interactions in normal cell signalling. Thus, the likely multiple functions of the rhoGAP family members p190 and bcr may enable them to coordinate a network of signalling pathways linking protein tyrosine kinases to different rho family proteins and other low molecular weight GTPases involved in mediating cellular

proliferation, differentiation and changes in the organization of the actin cytoskeleton, in response to extracellular signals.

Materials and methods

Materials

Bovine thrombin and LPA were obtained from Sigma Chemical Co., and PDGF was from Amersham International. Radiochemicals were obtained from NEN.

Expression and purification of recombinant proteins

The GAP domain of p190 was obtained by PCR amplification from a rat brain cDNA library, using oligonucleotide probes corresponding to sequences of rat brain p190 cDNA (Settleman *et al.*, 1992a). p190GAP contains the complete C-terminal GAP domain (aa 1260–1469), with additional N- and C-terminal flanking sequences. *Bam*HI and *Eco*RI sites incorporated into the oligonucleotides enabled PCR products to be subcloned into a derivative of the bacterial expression vector pGEX 2T (Smith and Johnson, 1988). A transformant which expressed a protein of the expected size upon induction with isopropyl-β-D-thiogalactosidase (IPTG) was sequenced to ensure that point mutations had not been introduced into the GAP domain by Taq polymerase. rhoGAP-C and bcrGAP were obtained by subcloning the complete C-terminal GAP domains (aa 720–1044 and aa 1050–1271, respectively) into pGEX2T (details will be published elsewhere).

Recombinant rho family GTPases, rho, rac and G25K, rho and rac mutants V14RhoA and V12Rac1, and GAPs, rhoGAP-C, p190GAP and bcrGAP, were expressed as GST fusion proteins, purified to homogeneity and cleaved from the GST carrier as previously described (Ridley and Hall, 1992).

GAP assay

Recombinant rho, rac and G25K (30 ng) were preloaded with [γ-^{32}P]GTP (10 μCi, 6000 Ci/mmol, NEN) in 20 μl of 20 mM Tris–HCl, pH 7.6, 0.1 mM DTT, 25 mM NaCl and 4 mM EDTA for 10 min at 30°C. $MgCl_2$ was then added to a final concentration of 17 mM. Three microlitres (3.5 ng) of the preloaded protein (to give a final concentration of 6 nM) were diluted with 20 mM Tris–HCl, pH 7.6 and 0.1 mM DTT, and an aliquot of GAP protein added to a final volume of 30 μl. NaCl was added to a final concentration of 150 mM and the mixture incubated at 22°C. Five microlitre samples were removed after 5 min and diluted to 1 ml with cold buffer A (50 mM Tris–HCl, 5 mM $MgCl_2$ and 50 mM NaCl) and filtered through nitrocellulose filters (prewetted with buffer A). Filters were washed with 10 ml of cold buffer A, dried and counted.

Cell culture and microinjection

Quiescent, serum starved Swiss 3T3 fibroblasts were obtained and microinjected as previously described (Ridley and Hall, 1992). Examination of membrane ruffles and actin stress fibres was precluded in a proportion of fibroblasts, due to a non-specific toxic effect. This effect increased with the concentration of protein preparations used for microinjection, therefore preventing introduction of higher concentrations of proteins.

Immunofluorescence

Fibroblasts were fixed and permeabilized, and actin filaments localized with TRITC-labelled phalloidin (from Sigma), as described (Ridley and Hall, 1992). Cells injected with rat Ig marker protein were identified by incubation with FITC-labelled goat anti-rat IgG (Sigma). Cells were viewed and photographed on a Zeiss Axiophot microscope for Figures 3 and 4, and a laser confocal microscope (MRC 600) for Figure 5.

Preparation of anti-rac and anti-rho antibodies

Recombinant full-length rho A and rac 1 were expressed as GST fusion proteins, purified to homogeneity and cleaved from the GST carrier. Purified proteins were mixed with an equal volume of Freund's complete adjuvant and injected into lymph nodes of rabbits. After 3 weeks rabbits were boosted subcutaneously with protein mixed with Freund's incomplete adjuvant, followed by a further intramuscular injection after 2 weeks. A first bleed of antisera was obtained 1 week later.

Preparation of cell lysates and Western blotting

A confluent 10 cm plate of Swiss 3T3 fibroblasts was washed twice in ice-cold TBS and cells were lysed on ice in 250 μl SDS sample buffer. The lysate was heated for 3 min at 70°C, then passed three times through a 25 gauge needle. After boiling at 100°C for 3 min, and preclearing by brief centrifugation, equal aliquots (~70 μg protein) were run out on a 13.5% SDS gel, adjacent to known quantities of recombinant, bacterially expressed rho or rac proteins. Proteins were then transferred to nitrocellulose.

Filters were blocked overnight in Blotto (1 M glycine, 5% w/v milk powder, 1% ovalbumin and 5% bovine calf-serum), then washed twice for 5 min each in TBS containing 0.1% Tween 20 (TBST). Filters were probed for 2 h with anti-rho or anti-rac antibodies, or pre-immune sera (all at 1:10 000 dilution), in TBST containing 2% milk powder, and were then washed six times for 10 min in TBST. After incubation for 1 h with anti-rabbit horseradish peroxidase (1:15 000 dilution), in TBST containing 2% milk powder, filters were washed and developed using ECL reagents (Amersham).

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