Regulation of p190 Rho-GAP by v-Src is linked to cytoskeletal disruption during transformation

V. J. Fincham, A. Chudleigh and M. C. Frame*

The Beatson Institute for Cancer Research, CRC Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK

*Author for correspondence (e-mail m.frame@beatson.gla.ac.uk)

Accepted 6 January; published on WWW 25 February 1999

SUMMARY

The v-Src oncoprotein perturbs the dynamic regulation of the cellular cytoskeletal and adhesion network by a mechanism that is poorly understood. Here, we have examined in detail the effects of a temperature-dependent v-Src protein on the regulation of p190 RhoGAP, a GTPase activating protein (GAP) that has been implicated in disruption of the organised actin cytoskeleton, and addressed the dependence of v-Src-induced stress fibre loss on inhibition of Rho activity. We found that activation of v-Src induced association of tyrosine phosphorylated p190 with p120RasGAP and stimulation of p120RasGAP-associated RhoGAP activity, although p120RasGAP itself was not a target for phosphorylation by v-Src in chicken embryo cells. These events required the catalytic activity of v-Src and were linked to loss of actin stress fibres during morphological transformation and not mitogenic signalling. Furthermore, these effects were rapidly reversible since switching off v-Src led to dissociation of the p190/p120RasGAP complex, inactivation of p120RasGAP-associated RhoGAP activity and re-induction of actin stress fibres. In addition, transient transfection of Val14RhoA, a constitutively active Rho protein that is insensitive to RhoGAPs, suppressed v-Src-induced stress fibre loss and cell transformation. Thus, we show here for the first time that an activated Src kinase requires the inactivation of Rho-mediated actin stress fibre assembly to induce its effects on actin disorganisation. Moreover, our work supports p190 as a strong candidate effector of v-Src-induced cytoskeletal disruption, most likely mediated by antagonism of the cellular function of Rho.

Key words: Rho, p190, Rho-GAP, v-Src, Cytoskeleton

INTRODUCTION

Morphological transformation by the v-Src oncoprotein is a consequence of disruption of the organised actin cytoskeleton and cellular focal adhesions. It occurs independently of Src-induced nuclear changes (Beug et al., 1978) and most likely involves tyrosine phosphorylation of targets that control assembly and disassembly of the cellular actin and adhesion network. Although many v-Src-interacting proteins and substrates have now been identified, there is still no clear understanding of how this tyrosine kinase initiates cytoskeletal disruption.

Previous studies have shown that v-Src, in its active state, resides in cellular focal adhesions, structures that link the actin filaments to the membrane points of attachment of integrins with the extracellular matrix (ECM) (reviewed by Turner and Burridge, 1991; Fincham and Frame, 1998). v-Src induces tyrosine phosphorylation and, in chicken embryo fibroblasts (CEF), subsequent degradation of several focal adhesion components; one of these, focal adhesion kinase (pp125FAK) (Fincham et al., 1995) has been implicated in the regulation of both focal adhesion assembly (Richardson and Parsons, 1996) and disassembly (Fincham et al., 1995; Ilic et al., 1995; Fincham and Frame, 1998). Tyrosine phosphorylation of pp125FAK in CEF temporally precedes, but is linked to, v-Src-induced focal adhesion turnover as cells round up during transformation (Fincham et al., 1995). Thus, tyrosine phosphorylation of pp125FAK is a likely component of the v-Src-induced signalling events that lead to focal adhesion disruption and the altered shape of transformed cells.

In addition to focal adhesion components, v-Src also induces tyrosine phosphorylation of proteins that more directly influence regulation of the actin cytoskeleton. In particular, p190 was first identified as a tyrosine phosphorylated species that associated with p120RasGAP in Src transformed cells (Ellis et al., 1990) and was subsequently shown to possess GTPase-activating protein (GAP) activity towards the small GTP binding protein Rho A (Settleman et al., 1992a; Ridley et al., 1993). Since microinjection of the p190 GAP domain into Swiss 3T3 cells inhibits serum-induced, Rho-mediated stress fibre formation in vivo (Ridley et al., 1993), one possibility is that altered regulation of p190 may contribute to v-Src-induced cytoskeletal disruption by antagonising the stress fibre assembly function of Rho A. However, to date there is no information on the regulation of p190 RhoGAP activity by tyrosine phosphorylation and it has not been demonstrated that v-Src induced inactivation of cellular Rho function is required for cytoskeletal disruption and transformation. Thus, we
examined the effects of the v-Src tyrosine kinase on p190, its association with \( p120^{RasGAP} \) and \( p120^{RasGAP} \)-associated RhoGAP activity. Activation of temperature-sensitive (ts) v-Src induced the binding of tyrosine phosphorylated p190 to \( p120^{RasGAP} \) and stimulated \( p120^{RasGAP} \)-associated RhoGAP activity. Furthermore, these events required the catalytic activity of v-Src and were associated with actin stress fibre disruption during the morphological changes induced by the oncoprotein, but were not a consequence of mitogenesis. We further showed that these effects were rapidly reversible, since inactivation of ts v-Src by switch to the restrictive temperature led to dissociation of the \( p190/p120^{RasGAP} \) complex, inactivation of \( p120^{RasGAP} \)-associated RhoGAP activity and re-induction of actin stress fibres. Moreover, we demonstrated that v-Src-induced stress fibre loss is suppressed by transfection of a constitutively activated form of RhoA, Val\(^{14} \)-Rho A, indicating that v-Src-induced Rho inactivation is a prerequisite for its actin-disrupting activity. Thus, p190 is a strong candidate effector of v-Src-induced cytoskeletal changes, most likely mediated by v-Src-induced phosphorylation and antagonism of the cellular function of Rho A and its downstream effectors.

**MATERIALS AND METHODS**

**Growth of CEF expressing v-Src mutants and induction of mitogenesis**

Primary CEF cultures were grown in DMEM supplemented with 5% newborn calf serum and tryptose phosphate. Low density cultures were transfected with replication competent avian retroviral RAV-src constructs (5 \( \mu \)g per 25-cm\(^2 \) flask), or with vector alone, by the DOTAP method (Boehringer Mannheim Corp.) and sub-cultured at the permissive temperature of 35°C until the cells were uniformly infected and expressing Src protein (judged by protein immunoblotting). The generation of retrovirus encoding ts LA29 v-Src has been described (Welham and Wyke, 1989). Retrovirus encoding the kinase-defective variant of ts LA29 v-Src was generated by converting the ATP binding site at position 295 from lys to arg using PCR mutagenesis. The mutant sense oligonucleotide was 5'-GAG TGG CCA TAC G-3'. Cell cultures infected with retrovirus encoding ts v-Src mutants, or with retrovirus alone, were grown either at restrictive (41°C) or permissive temperature and were buffered with 5% CO\(_2\). When required, infected cultures were made quiescent by incubating in low serum containing medium (0.2%) for 48 hours at 41°C and were stimulated to re-enter cell cycle either by reactivating v-Src at the permissive temperature or by addition of 10% newborn calf serum. S-phase entry was checked by \([\text{H}]\)thymidine uptake.

**Antibodies**

Antiserum was prepared in rabbits against a trp-E-GAP bacterial fusion protein containing amino acid residues 171-448 from the amino-terminal region of human GAP. The use of an antisem to \( p120^{RasGAP} \) prepared in a similar manner has been reported (Ellis et al., 1990). The antiserum to \( pp125^{FAK} \) that was used here as a control was prepared by immunising rabbits with the carboxy-terminal 14 amino acids of avian pp125\(^{FAK} \) (C-DQARLKMISQSPH). The addition of the amino-terminal Cys residue allowed coupling to keyhole limpet haemocyanin (KLH) to give a peptide-carrier ratio of about 200:1. Antisera specific for an epitope in the carboxy-terminal region of p190 was obtained commercially (Transduction Laboratories).

**Immunoprecipitation and immunoblotting**

For protein analyses, dishes of cells were washed with cold phosphate buffered saline (PBS), drained and frozen at \(-70°C\). For immunoprecipitation (IP), monolayers were thawed, lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 5 mM EGTA, 0.1% SDS, 1% NP40 and 1% deoxycholate) with inhibitors (10 mM pyrophosphate, 100 mM sodium fluoride, 1 mM PMSF, 10 \( \mu \)g/ml aprotinin (Sigma), 100 \( \mu \)M sodium vanadate, 10 \( \mu \)g/ml leupeptin (Sigma) and 10 \( \mu \)g/ml benzamide (Sigma)), clarified by high speed spin at 4°C and pre-cleared with normal IgG and Protein A-Sepharose (Sigma Chemical Co.). 500-750 \( \mu \)g cell lysate (measured by the Micro BCA Protein Assay Kit; Pierce) was immunoprecipitated with 5 \( \mu \)l anti-\( p120^{RasGAP} \) or 5 \( \mu \)l anti-\( pp125^{FAK} \) serum. Immune-complexes were collected on anti-mouse IgG-coated Protein A-Sepharose beads, washed four times with RIPA buffer, once with 0.6 M lithium chloride, eluted using high SDS-containing buffer at high temperature and separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Immunoprecipitated proteins were transferred to nitrocellulose and probed with either anti-phosphotyrosine (PY20 at 1:1000; Upstate Biotechnology Inc.) or with anti-\( p120^{RasGAP} \) or anti-p190 (both at 1:1000). Detection was by incubation with horse radish peroxidase-conjugated secondary antibody and visualisation was by enhanced chemiluminescence (Amersham).

**RhoGAP assays**

Sub-confluent dishes of cells were lysed for 15 minutes at 4°C in 50 mM Hepes, pH 7.6, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride, 1 mM EGTA and inhibitors (10 mM sodium pyrophosphate, 5 mM sodium fluoride, 0.1 mM PMSF, 10 \( \mu \)g/ml aprotinin (Sigma) and 10 \( \mu \)g/ml leupeptin). The lysates were cleared by spinning at 14,000 rpm in a Sorvall SS34 rotor for 30 minutes at 4°C. 500-750 \( \mu \)g protein was immunoprecipitated with an excess of anti-\( p120^{RasGAP} \) and collected on Protein A-Sepharose beads. IPs were washed three times in lysate buffer and twice in GAP assay buffer (50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA. 10 mM magnesium chloride, 1 mM DTT) and re-suspended in 97 mM GAP assay buffer containing 5 mM EDTA for 10 minutes at 30°C. Magnesium chloride was added to a final concentration of 17 mM on ice. 3 \( \mu \)l of the Rho-GTP\[^{32}P\] mix and 0.5 mM unlabelled GTP was added to anti-\( p120^{RasGAP} \) IPs and the reactions mixed at room temperature for 15 minutes (reaction kinetics were checked by removal of aliquots at various times). Reactions were diluted in wash buffer (50 mM Tris-HCl, pH 7.6, 5 mM magnesium chloride, 50 mM sodium chloride and filtered through 0.45 \( \mu \)m nitrocellulose filter discs using a Millipore filtration unit. Filters were washed with 10 ml wash buffer and the radioactivity remaining as GT\[^{32}P\] determined by scintillation counting. RhoGAP activity was expressed as % GTP hydrolysed defined as (100-%GT\[^{32}P\]) remaining.

**Visualisation of actin stress fibres and cellular morphology**

Cells were grown on glass coverslips, fixed at 4°C for 15 minutes with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 and incubated with 5 \( \mu \)g/ml TRITC-conjugated phalloidin (Sigma). Cells were visualised using a confocal microscope (model MRC 600; Bio-Rad) and images were printed on a dye sublimation printer (Kodak).

**Transient transfection of activated RhoA and green fluorescence protein (GFP)**

A cDNA encoding Rho A with an F25N stabilizing mutation was obtained from Alan Hall (UCL, London). We mutated codon-14 to...
encode a valine which gives rise to a constitutively activated form of Rho A (Val¹⁴-Rho A) that has been used previously to demonstrate the function of Rho A in inducing actin stress fibre assembly (Paterson et al., 1990; Ridley and Hall, 1992). Val¹⁴-Rho A cDNA was subcloned into the splice-acceptor positive version of pSFCV (Fuerstenberg et al., 1990) to produce pSFCV-Sa⁺-Val¹⁴-Rho A. GFP encoded in pcDNA3.1 (Invitrogen) was provided to us by Gordon McLean (Beatson Institute, Glasgow). Low density chicken embryo fibroblasts expressing tsLA 29 v-Src were grown on glass coverslips and transfected with either 1 μg pcDNA3.1-GFP alone as control, or with 1 μg pcDNA3.1-GFP plus 5 μg pSFCV-Sa⁺-Val¹⁴-Rho A. The liposomal transfection reagent DOTAP (Boehringer Mannheim) was used according to the manufacturer’s instructions. After transfection, the cells were maintained at 41°C (restrictive temperature) overnight and either kept at 41°C (controls) or shifted to 35°C (permissive temperature) for various times.

RESULTS

v-Src induces complexing of tyrosine phosphorylated p190 to p120RasGAP in CEF

Since p190 was first identified as a tyrosine phosphorylated species co-precipitating with p120RasGAP in rodent fibroblasts transformed by tyrosine kinase oncoproteins (Ellis et al., 1990), we first confirmed that activation of ts LA29 v-Src by switch to the permissive temperature also induced association of tyrosine phosphorylated p190 with p120RasGAP in CEF. Immunoprecipitation with anti-p120RasGAP followed by immunoblotting with anti-phosphotyrosine, demonstrated that v-Src-induced association of a 190 kDa tyrosine phosphorylated species with p120RasGAP (Fig. 1A). The

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**Fig. 1.** Association of tyrosine phosphorylated p190 with p120RasGAP induced by v-Src. (A) p120RasGAP was immunoprecipitated (IP) from lysates of CEF expressing ts LA29 v-Src at either restrictive temperature (0 hours), or shifted to the permissive temperature (35°C) for 1, 2, 4 or 24 hours. Precipitated proteins were immunoblotted using anti-phosphotyrosine (anti-p-tyr). The positions of molecular mass markers are indicated on the left vertical axis and the positions of p190 and an unidentified co-precipitating protein (p150) are indicated on the right vertical axis. (B) The amount of p120RasGAP was determined by probing anti-p120RasGAP immunoprecipitations with anti-p120RasGAP. (C) The amount of cellular p190 was determined by immunoblotting whole cell lysates with anti-p190. (D) Lysates of cells that were maintained at restrictive temperature (0 hours) or were shifted to the permissive temperature (for 1, 2, 4 or 8 hours) were depleted by one round of immunoprecipitation (IP) with anti-p120RasGAP, or with normal rabbit serum (NRS) as control, and post IP lysates were immunoblotted with anti-p190.
identity of this species as p190 was confirmed by blotting with anti-p190 (shown later in Fig. 3B). An unidentified tyrosine phosphorylated species of around 150 kDa (p150) also associated with p120RasGAP in some experiments (Fig. 1A), although this species was not consistently observed. In contrast to previous studies in rodent fibroblasts (Ellis et al., 1990), we did not detect v-Src-induced tyrosine phosphorylation of either p120RasGAP or its associated protein, p62 (Figs 1A and 3A).

We showed that the amount of p120 RasGAP immunoprecipitated after v-Src activation, and the cellular level of p190, were not substantially altered after activation of v-Src (Fig. 1B and C, respectively), indicating that the increase in tyrosine phosphorylated p190 co-precipitating with p120RasGAP was not a consequence of changes in the expression of either protein. Furthermore, removal of p120RasGAP from cell lysates by immunodepletion led to co-depletion of p190 (Fig. 1D), indicating that v-Src induced complexing of p190 with p120RasGAP and not tyrosine phosphorylation of a pre-existing complex. Thus, activation of ts LA29 v-Src in CEF induced association of p190, in a tyrosine phosphorylated form, with p120RasGAP that was not itself phosphorylated on tyrosine.

The association of tyrosine phosphorylated p190 with p120RasGAP is not a consequence of mitogenesis

v-Src induces both morphological transformation and mitogenesis of quiescent CEF (Bell et al., 1975; Catling et al., 1993; Frame et al., 1994). We, and others, have studied the pathways by which v-Src induces Go/G1 to S-phase transition in serum-deprived fibroblasts and have been unable to distinguish potential mediators from those involved in growth factor-induced mitogenesis. In particular, the signalling pathway that links p21 Ras to the mitogen-activated protein (MAP) kinases and their targets, including the transcription factor AP-1, are likely mediators of both v-Src (Frame et al., 1994; Wyke et al., 1995) and serum growth factor-induced Go/G1 to S-phase progression. Thus, we tested whether the association of tyrosine phosphorylated p190 with p120RasGAP was linked to the ability of v-Src to stimulate mitogenic signalling. CEF made quiescent by serum-deprivation were stimulated by activation of ts LA29 v-Src, or its kinase-defective derivative ts LA29 v-Src-KD) were maintained at the restrictive temperature (0 hours) or shifted to the permissive temperature for 1, 2 or 24 hours. Lysates were immunoprecipitated with anti-p120RasGAP and immunoblotted with anti-phosphotyrosine (anti-p-tyr). The 97 kDa molecular mass marker is shown.

Fig. 2. Activation of v-Src, but not addition of serum, stimulates tyrosine phosphorylated p190 to associate with p120RasGAP. CEF expressing ts LA29 v-Src were made quiescent by maintaining in 0.2% serum for 3 days at the restrictive temperature (0 hours). The cells were stimulated either by activation of ts v-Src (by switch to the permissive temperature) or by re-feeding with 10% serum for 1, 2 or 4 hours. Lysates were immunoprecipitated with anti-p120RasGAP and immunoblotted with anti-phosphotyrosine (anti-p-tyr). The 97 kDa molecular mass marker is shown.

Fig. 3. The binding of tyrosine phosphorylated p190 to p120RasGAP requires v-Src kinase activity. CEF expressing ts LA29 v-Src, or its kinase-defective derivative ts LA29 v-Src-KD) were maintained at the restrictive temperature (0 hours) or shifted to the permissive temperature for 1, 2 or 24 hours. Lysates were immunoprecipitated with anti-p120RasGAP and immunoblotted with (A) anti-phosphotyrosine (anti-p-tyr), or (B) anti-p190. As control, lysates of cells expressing ts LA29 v-Src that had been switched to permissive temperature for 24 hours were immunoprecipitated with normal rabbit serum (NRS).
p120RasGAP is more likely linked to v-Src-induced cytoskeletal re-arrangements and morphological transformation.

The association of p190 with p120RasGAP requires the catalytic activity of v-Src and is linked to actin stress fibre loss during transformation

Since ts LA29, the conditional mutant v-Src protein used here and in previous studies (Ellis et al., 1990), is defective for both actin-dependent translocation to the cell periphery and kinase activity at the non-permissive temperature (Fincham and Frame, 1998), we addressed whether translocation was sufficient or whether Src catalytic activity was required for the association of tyrosine phosphorylated p190 with p120RasGAP. For this we used a constitutively kinase-defective mutant of, ts LA29 v-Src-KD, that we have previously shown to be temperature-dependent for translocation to the cell periphery, but to be impaired in inducing the turnover of focal adhesions required for cell motility (Fincham and Frame, 1998). Upon shifting CEF expressing ts LA29 v-Src-KD to the permissive temperature (35°C) was compared to that induced by ts LA29 v-Src-KD. Also shown are RhoGAP activity measurements of anti-p120RasGAP immunoprecipitations of lysates made from CEF expressing RAV and NRS precipitations of lysates from CEF expressing ts LA29 v-Src at both temperatures.
v-Src induces p120RasGAP-associated RhoGAP activity

Since p190 contains a GAP domain at its carboxy-terminus and possesses GAP activity for Rho family members (Settleman et al., 1992a,b), with a particular preference for Rho A (Ridley et al., 1993), we tested whether the v-Src-induced association of tyrosine phosphorylated p190 with p120RasGAP was accompanied by a change in p120RasGAP-associated RhoGAP activity. Recombinant Rho A protein was pre-loaded with [γ-32P]GTP and immunoprecipitates were tested for their ability to induce release of 32P. Although the RhoGAP activity of anti-p120RasGAP immunoprecipitates was negligible at the restrictive temperature and was similar to normal serum immunoprecipitation controls, p120RasGAP-associated RhoGAP activity was substantially elevated after activation of v-Src (Fig. 5A). We were unable to measure cellular p190 RhoGAP activity directly since the p190-specific antibody available to us, which we used for immunoblotting (Figs 1 and 3), only immunoprecipitated p190 in the presence of detergent (not shown), conditions which were not permissive for GAP activity measurements. However, since most of the cellular p190 is bound to p120RasGAP after v-Src has been switched on for 8 hours (Fig. 1D), it seems likely that most p190 RhoGAP activity is associated with p120RasGAP in v-Src transformed cells.

pp125FAK is a likely mediator of the morphological transforming activity of v-Src and can bind to an SH3 domain protein with GAP activity towards Rho and Cdc42, termed Graf (Hildebrand et al., 1996). We also tested whether pp125FAK associated with a RhoGAP activity after activation of v-Src. Immunoprecipitated pp125FAK did not support RhoGAP activity (Fig. 5A). This is consistent with the reported absence of endogenous Graf in CEF (Hildebrand et al., 1996) and confirmed that pp125FAK does not associate with any detectable RhoGAP activity during v-Src transformation of CEF.

Thus, the stimulation of p120RasGAP-associated RhoGAP activity correlated with binding of tyrosine phosphorylated p190, but not with tyrosine phosphorylation of p120RasGAP itself (Figs 1, 2 and 3). In addition, stimulation of p120RasGAP-associated RhoGAP activity required the catalytic activity of v-Src, since the kinase-defective variant of ts LA29 v-Src did not induce substantial p120RasGAP-associated RhoGAP activity upon switch to the permissive temperature (Fig. 5B).

Reversal of v-Src-induced stimulation of p120RasGAP-associated RhoGAP activity is associated with stress fibre re-induction

Inactivation of ts LA29 v-Src reverses the transformed phenotype and induces re-spreading of rounded cells by 2 hours (Fincham et al., 1995). To determine whether v-Src-induced stimulation of p120RasGAP-associated RhoGAP activity was reversed during restitution of normal morphology, we switched transformed CEF to the restrictive temperature (41°C). As with re-spreading, p120RasGAP-associated RhoGAP activity returned to the normal level within 2 hours (Fig. 6A). In addition, p190 dissociated from the p120RasGAP complex upon inactivating v-Src, demonstrating that maintenance of the p190/p120RasGAP complex was dependent on v-Src activity (Fig. 6B). Furthermore, dissociation of p190 from the p120RasGAP complex and inactivation of p120RasGAP-associated RhoGAP activity were accompanied by dramatic re-induction of actin stress fibres with similar kinetics (not shown). These data imply
that v-Src perturbs the normal dynamic regulation of actin stress fibre assembly and disassembly and that its reversible effects on p190 RhoGAP activity in association with p120^{RasGAP} may mediate these effects.

v-Src-induced loss of actin-stress fibres and reduction in cell size require the inactivation of Rho

To address whether or not cytoskeletal disruption induced by v-Src requires GAP-induced inactivation of Rho-mediated cellular changes, we transfected CEF with a form of Rho A that is insensitive to GAPs and is constitutively active (Val^{14}-RhoA; Paterson et al., 1990; Ridley and Hall, 1992). As a reporter for transiently transfected cells, we co-transfected a plasmid expressing GFP (at a ratio of Val^{14}-Rho A:GFP of 5:1) and monitored phallolidin-staining actin in transfected and untransfected cells after v-Src had been switched on for 24 hours (Fig. 7A). Transfected cells (visualised by GFP expression in Fig. 7A, right panels) generally retained actin stress fibres and maintained normal cell shape and size when Val^{14}-RhoA was co-transfected (stress fibres pointed out by arrows in Fig. 7A, left panels). In contrast, cells that were untransfected (shown by broken arrows in Fig. 7A) had no detectable stress fibres with only punctate actin staining and these cells had become smaller and lost normal shape by 24 hours after v-Src activation (Fig. 7A, left panels). Visualisation of individual ts v-Src expressing cells that were transfected with only the GFP-expressing plasmid, confirmed that GFP itself had no effect on cellular stress fibres at the restrictive temperature (Fig. 7B, 41°C) or on stress fibre loss and cell size reduction induced by 24 hours after switch to the permissive temperature (Fig. 7B, 35°C). The effect of transfecting Val^{14}-RhoA was quantitated by counting GFP-positive cells with obvious residual stress fibres at various times after ts v-Src activation (Fig. 7C, darker bars). When compared with cells that were transfected with only the GFP-expressing plasmid (Fig. 7C, lighter bars), it was evident that Val^{14}-RhoA induced cellular resistance to v-Src induced stress fibre loss and cell size reduction normally seen in transformed cells. Thus, v-Src-induced stress fibre loss is suppressed by the activity of a form of RhoA that is not sensitive to inactivation by RhoGAPs, indicating that v-Src induces its effects on the actin cytoskeleton by antagonising the cellular function of RhoA.

DISCUSSION

The cellular adhesion and cytoskeletal network is subject to dynamic regulation during routine cell behaviour. Cell motility, in particular, is driven by the intracellular regulators that control the making and breaking of cellular adhesions and the assembly and disassembly of the organised actin cytoskeleton. These ongoing changes are mediated, at least in part, by the Rho family of small GTPases, including Rho A, which controls the formation of focal adhesions and F-actin bundles known as stress fibres (Ridley and Hall, 1992). Other Rho proteins, including Rac and Cdc42, which control the stimulus-induced formation of other types of actin structures that lead to assembly of lamellipodia and filopodia, respectively (Nobes and Hall, 1995) are also involved in the dynamic regulation of the actin cytoskeleton. The activity of these proteins is dependent on the rate of cycling between their GTP- and GDP-bound states, which is in turn partly controlled by GAPs that stimulate their intrinsic GTPase activities.

The cloning of p190, revealed that it contained a GAP domain at its carboxy terminus (Settleman et al., 1992a), with specificity towards the Rho family of small G-proteins (Settleman et al., 1992b), particularly Rho A (Ridley et al., 1993). Microinjection of the p190 GAP domain into Swiss 3T3 cells blocked Rho-mediated stress fibre formation (Ridley et al., 1993), indicating that the p190 GAP domain can antagonise the function of Rho A in vivo. Furthermore, expression of the amino-terminal region of p120^{RasGAP}, which encodes its Src homology (SH)-3 and SH2 domains, binds constitutively to p190 and induces disruption of actin stress fibres, implying that the p120^{RasGAP}/p190 complex modulates the actin cytoskeleton (McGlade et al., 1993). Thus, a number of lines of evidence implicate p190 in the disruption of actin stress fibres, mediated by negative regulation of Rho A.

The dynamic regulation of the assembly and disassembly of actin stress fibres is severely perturbed by the v-Src oncoprotein by mechanisms that are not yet fully understood. The conditional ts mutants of v-Src are therefore useful tools to study the early events associated with disruption of the organised actin cytoskeleton. Since p190 has been cloned or identified as a tyrosine phosphorylated species associating with p120^{RasGAP} in Src-transformed rodent fibroblasts (Ellis et al., 1990), we considered it a likely mediator of v-Src-induced changes to the actin cytoskeleton. However, this earlier study was carried out before p190 had been identified as a putative RhoGAP and did not address the determinants of v-Src-induced binding of tyrosine phosphorylated p190 to p120^{RasGAP} or whether this was associated with altered p120^{RasGAP}-associated RhoGAP activity and v-Src-induced cytoskeletal disruption.

Here, in experiments designed to address these issues, we have demonstrated that v-Src-induced binding of tyrosine phosphorylated p190 to p120^{RasGAP}, the concomitant reduction in uncomplexed cellular p190 (Fig. 1) and stimulation of p120^{RasGAP}-associated RhoGAP activity (Fig. 5), were linked to v-Src-induced cytoskeletal changes, but not mitogenesis of CEF (Fig. 2). The binding of p190 in a tyrosine phosphorylated form is consistent with the reported binding of p190 to the SH2 domains of p120^{RasGAP} (Bryant et al., 1995), an interaction that induces conformational changes in p120^{RasGAP} resulting in an apparent increased accessibility of the SH3 domain (Hu and Settleman, 1997). We also demonstrated that translocation of v-Src to the cell periphery was not sufficient to induce binding of p190 to p120^{RasGAP} or p120^{RasGAP}-associated RhoGAP activity, since a kinaseinactive variant was unable to induce these changes upon switch to the permissive temperature (Figs 3 and 5), although it translocated to the cell periphery (Fincham and Frame, 1998). Thus, the catalytic activity of v-Src is necessary, suggesting that tyrosine phosphorylation of p190 is required for complexing with p120^{RasGAP}. However, recent work by van der Geer et al. (1997) has shown that normal tyrosine phosphorylation of p190 is suppressed in fibroblasts derived from p120^{RasGAP}→− embryos, indicating that the regulation of p190 binding to p120^{RasGAP} is complex. Our finding that the catalytic activity of v-Src is necessary to induce tyrosine phosphorylation of p120^{RasGAP}-associated p190 suggests that p190 may be a direct target of v-Src. Consistent with this, p190 has been inferred as a preferred substrate of c-
Fig. 7. Transient transfection of Val14-RhoA suppresses v-Src-induced stress fibre loss. CEF expressing tsLA29 v-Src were transiently transfected with expression plasmids encoding Val14-RhoA and GFP at a ratio of 5:1 (Val14-RhoA-GFP) (or GFP alone as control) and maintained at the restrictive temperature (41°C) for at least 16 hours before switching to the permissive temperature (35°C) for various times. Transfected cells were visualised for GFP expression and polymerised actin by TRITC-phalloidin staining. (A) Phalloidin staining (left panels) and GFP expression (right panels) of three fields containing both transfected and untransfected cells (as judged by GFP expression) that had been switched to 35°C for 24 hours. Bars, 25 μm. (B) Phalloidin staining (left panels) and GFP expression (right panels) of cells that had been transfected with GFP plasmid alone and maintained at 41°C (upper panel), or switched to 35°C for 24 hours (lower panel). Bars, 25 μm. (C) Cells that had been transfected with GFP plasmid alone (lighter bars) or Val14-RhoA and GFP (darker bars) were examined at various times after ts LA29 v-Src activation by shift to 35°C. 20 GFP-expressing cells for each time point were examined and the number with residual stress fibres was recorded.
Src during EGF-dependent actin cytoskeletal re-organisation in murine fibroblasts (Chang et al., 1995). Thus, the catalytic activity of both v-Src and c-Src, although not required for translocation of these kinases to the cell periphery (Fincham and Frame, 1998; Kaplan et al., 1994), is required for phosphorylation of p190 and the associated cytoskeletal changes (Fig. 4 and Chang et al., 1995). In addition, the effects of v-Src were rapidly reversible. Switching off the v-Src kinase by transfer to restrictive temperature led to concomitant dissociation of the p190/p120RasGAP complex, reduction of p120RasGAP-associated RhoGAP activity to basal levels (Fig. 6) and the re-induction of organised actin stress fibres that accompanies restitution of normal morphology.

Thus, the data presented here show that the reversible switch off and on of p120RasGAP-associated RhoGAP activity in response to inactivation and activation of v-Src respectively, correlates with induced assembly and disassembly of actin stress fibres in CEF. It seems likely that p120RasGAP-associated RhoGAP activity is a function of p190, since p120RasGAP itself has only minimal activity towards Rho A-GTP and is not subject to v-Src-induced tyrosine phosphorylation in CEF (Fig. 1). This, together with the previous reports that the p120RasGAP-p190 complex mediates cytoskeletal disruption (McGlade et al., 1993), implies that v-Src induces actin stress fibre loss in CEF by stimulating p120RasGAP-associated RhoGAP activity via tyrosine phosphorylation and binding of p190 to p120RasGAP. We cannot distinguish between the stimulation of p120RasGAP-associated RhoGAP activity occurring as a consequence of induced binding of already pre-activated p190, or as a direct result of activation of p190 mediated either by tyrosine phosphorylation or a conformational switch upon binding to p120RasGAP.

In conclusion, the reversible effects of the v-Src tyrosine kinase on p190 are likely contributors to the v-Src-induced perturbations of the normal dynamic regulation of actin stress fibre assembly and disassembly. Our data provide the first demonstration that expression of an activated form of Rho A suppresses v-Src-induced cytoskeletal disruption, indicating that the activated tyrosine kinase normally antagonises the cellular function of Rho A and perturbs the actin assembly/disassembly cycle as a consequence. Our data presented here also provide the first demonstration that tyrosine phosphorylation of p190, and its binding to p120RasGAP, are associated with stimulation of p120RasGAP-RhoGAP activity and stress fibre disassembly, consistent with the previously proposed role for the p120RasGAP-p190 complex (McGlade et al., 1993). Thus, our work implicates p190, in association with p120RasGAP, as a candidate effector of Src-induced morphological transformation, most likely mediated by its effects on inactivation of cellular Rho A.

We are grateful to John Wyke and Val Brunt for reading the manuscript, Alan Hall for the vector encoding Rho A, Gordon McLean for pcDNA 3.1 encoding GFP, Tony Pawson for the vector encoding trp-E-GAP and to Kate Nobes for advice on RhoGAP assays. This work was supported by the Cancer Research Campaign (UK).

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