The EMBO Journal vol.12 no.8 pp.3073–3081, 1993

The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion

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Communicated by J.Schlessinger

Ras GTPase activating protein (GAP) possesses a C-terminal domain that interacts with GTP-bound Ras, and an N-terminal region containing two SH2 domains and an SH3 domain. In addition to its association with Ras, GAP binds stably to autophosphorylated βPDGF receptors, and to two cytoplasmic phosphoproteins: p62, an RNA binding protein, and p190, which possesses GAP activity towards small guanine nucleotide binding proteins in the Rho/Rac family. To define the region of GAP that mediates these interactions with cellular phosphoproteins, and to investigate the biological significance of these complexes, a truncated GAP polypeptide (GAP-N) containing residues 1–445 was stably expressed in Rat-2 fibroblasts. GAP-N contains the SH2 and SH3 domains, but lacks the Ras GTPase activating domain. Stimulation of cells expressing GAP-N with PDGF induced association of GAP-N with the βPDGF receptor, and phosphorylation of GAP-N on tyrosine, consistent with the notion that GAP SH2 domains direct binding to the autophosphorylated βPDGF receptor *in vivo*. GAP-N bound constitutively to p190 in both serum-deprived and growth factor-stimulated cells. This GAP-N–p190 complex had Rho GAP activity *in vitro*. The expression of GAP-N in Rat-2 cells correlated with changes in the cytoskeleton and in cell adhesion, typified by the disruption of action stress fibres, a reduction in focal contacts, and an impaired ability to adhere to fibronectin. These results suggest that the N-terminal domain of GAP can direct interactions with cellular phosphoproteins *in vivo*, and thereby exert an effector function which modulates the cytoskeleton and cell adhesion. This effect of GAP-N on the cytoskeleton correlates with its association with p190, and may therefore result from regulation of Rho/Rac GTPases by the GAP–p190 complex. GAP may therefore couple growth factors to control of cell shape and attachment.

Key words: cell adhesion/cytoskeleton/GAP

Introduction

Ras GTPase activating protein (GAP) specifically recognizes the GTP-bound form of $p21^{ras}$ (Ras), and markedly accelerates the hydrolysis of Ras-associated GTP, thereby converting Ras from an active to an inactive state (Trahey *et al.*, 1988; Vogel *et al.*, 1988). GAP is composed of multiple domains, with entirely distinct structural and functional properties. The C-terminal region of GAP is both necessary and sufficient for its interaction with Ras, and for stimulation of Ras GTPase activity (Marshall *et al.*, 1989). This C-terminal region of GAP is structurally related to sequences within the *IRA1* and *IRA2* gene products of *Saccharomyces cerevisiae*, and to human neurofibromin, which also stimulate Ras GTPase activity (Martin *et al.*, 1990, 1992; Tanaka *et al.*, 1990; Xu *et al.*, 1990). Since Ras is active in intracellular signalling in the GTP-bound state, and is rendered inactive by conformational changes invoked by GTP hydrolysis, GAP might be anticipated to function as a negative regulator of Ras. Indeed over-expression of full-length GAP, or of a fragment of GAP containing only the C-terminal GAP domain, increases the fraction of cellular Ras in the inactive GDP-bound state. GAP can thereby block the transforming activity of c-Ras proteins, or of the v-Src tyrosine kinase which requires Ras activation for its mitogenic effect (DeClue *et al.*, 1991; Nori *et al.*, 1991).

Oncogenic Ras variants, which are locked in the GTP-bound form, still interact with GAP, although they are resistant to its GTPase activating function (Trahey and McCormick, 1987; Vogel *et al.*, 1988). Amino acid substitutions within the effector region of Ras which impair Ras transforming activity, presumably by blocking the interactions of Ras with its targets, can also compromise the recognition of Ras–GTP by GAP (Adari *et al.*, 1988; Cales *et al.*, 1988). These observations have engendered the hypothesis that the interaction of Ras with GAP might also activate a downstream signalling pathway, and thereby induce a cellular response to GTP-bound Ras.

If GAP were a Ras target, or were otherwise involved in stimulation of a biochemical pathway, it might be expected to have additional sequences that bind cellular proteins other than Ras itself. The N-terminal region of GAP has two Src homology 2 (SH2) domains, and an intervening SH3 domain, which could potentially direct such interactions. SH2 domains bind with high affinity to specific phosphotyrosine-containing sites, and are found in a variety of cytoplasmic signalling proteins that interact with autophosphorylated growth factor receptors, and other tyrosine phosphorylated proteins (Koch *et al.*, 1991). GAP binds through its SH2 domains to a specific autophosphorylation site within the activated β receptor for platelet-derived growth factor (PDGFR) (Kazlauskas *et al.*, 1990); indeed GAP is itself

a substrate for tyrosine phosphorylation in cells stimulated with growth factors such as EGF or PDGF, or transformed by oncogenic tyrosine kinases (Anderson *et al.*, 1990; Ellis *et al.*, 1990; Kaplan *et al.*, 1990). SH3 domains also appear to control protein−protein interactions, as typified by the Abl SH3 domain, which binds *in vitro* to a proline-rich sequence in a protein with homology to GAPs for the Rho/Rac family of small guanine nucleotide binding proteins (Cicchetti *et al.*, 1992).

In cells stimulated with EGF, or transformed by v-Src, GAP forms distinct complexes with two cytoplasmic phosphoproteins, p62 and p190 (Ellis *et al.*, 1990; Moran *et al.*, 1991). In v-*src*-transformed cells, a minor species of p62 is highly tyrosine phosphorylated, and associated with GAP SH2 domains (Koch *et al.*, 1992). A non-phosphorylated form of p62, which is structurally related to a group of RNA binding proteins and associates with RNA *in vitro*, is also complexed with GAP (Wong *et al.*, 1992). Although GAP is primarily monomeric in normal, unstimulated rat fibroblasts a majority of GAP in EGF-stimulated or v-*src*-transformed cells is associated with p190 (Ellis *et al.*, 1990; Moran *et al.*, 1991). p190 contains a small amount of phosphotyrosine, but is principally phosphorylated on serine (Ellis *et al.*, 1990). Denatured p190 will associate *in vitro* with a bacterial fusion protein containing GAP SH2 and SH3 domains, suggesting that it binds within the N-terminal region of GAP. cDNAs for rat p190 have recently been isolated, and shown to encode a polypeptide with an N-terminal region related to guanine nucleotide binding proteins and a central region which is virtually identical to a putative transcriptional regulator (Settleman *et al.*, 1992b). The C-terminal domain of p190 is homologous to proteins such as BCR and N-chimerin which have GAP activity towards the Rho/Rac family of small guanine nucleotide binding proteins, and functions as a Rho/Rac GAP *in vitro* (Settleman *et al.*, 1992a).

These findings raise the possibility that the N-terminal SH2/SH3 region of GAP, through interactions with proteins such as p190 and p62, might be positively involved in signal transduction initiated by tyrosine kinases. Consistent with this notion, the ability of GAP to block the opening of K^+ channels in response to muscarinic stimulation of atrial membranes *in vitro*, which normally requires Ras, can be mimicked by a fragment containing only the N-terminal GAP SH2 domain and the SH3 domain, which is no longer dependent on Ras (Martin *et al.*, 1992). We have explored the binding properties of the N-terminal region of GAP *in vivo*, and have investigated its biological activity. Our results suggest that GAP is involved through its interactions with p190 in the control of cytoskeletal organization and in cell adhesion.

Results

Expression of the N-terminal region of GAP

To assess the function of the N-terminal region of GAP, the codon for Gln446 of human type I GAP was converted to a translational termination codon. This mutated cDNA encodes a truncated 49 kDa polypeptide (GAP-N) that contains only the hydrophobic sequence at the extreme N-terminus of GAP, the two SH2 domains and the SH3 domain (Figure 1A). The GAP-N protein terminates immediately after the C-terminal SH2 domain, and hence is lacking the

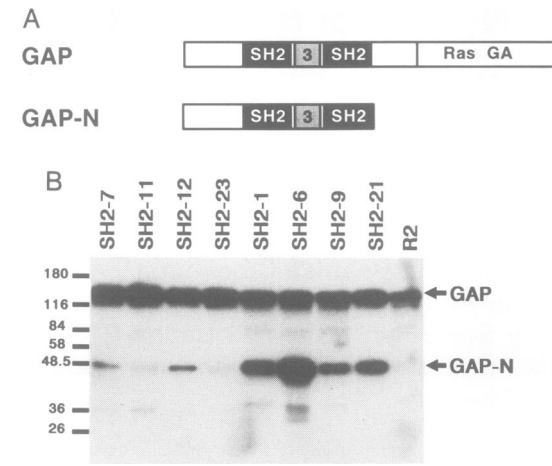

Fig 1. Structure and expression of the GAP-N protein. (**A**) Schematic representation of GAP and GAP-N proteins. Amino acid 446 of human GAP was changed to a stop codon by site directed mutagenesis to generate the GAP-N protein. The positions of the SH2, SH3 and GTPase activating domains (GA) of GAP and GAP-N are shown. (**B**) Expression of GAP-N in fibroblasts. Rat-2 fibroblasts (R2) were transfected with the plasmid pECE-GAP-N and stable cell lines isolated. The expression of the GAP-N protein in eight separate lines was analysed by immunoblotting whole cell lysates with anti-GAP antibodies followed by [^{125}I]protein A. Both endogenous GAP and GAP-N are recognized by the polyclonal GAP antisera. The amount of GAP-N expressed relative to endogenous GAP was determined using Phosphorimager analysis of immunoblots. The molecular weights ($\times 10^{-3}$) of protein markers are indicated.

major physiological site of tyrosine phosphorylation, located at Tyr460 (Liu and Pawson, 1991), the central region, and the entire GTPase activating domain. A mammalian expression vector containing the mutant GAP cDNA was transfected into Rat-2 fibroblasts, and cell clones were isolated by resistance to G418. To screen for expression of the truncated GAP-N polypeptide, we used anti-GAP antibodies raised to the N-terminal region, which therefore recognize both the mutant and endogenous wild-type GAP proteins (Ellis *et al.*, 1990). Eight cell lines, designated SH2 cells, were identified that expressed varying amounts of the GAP-N protein, at levels ranging from 0.01 to 3-fold of endogenous GAP (Figure 1B). The highest level of GAP-N expression was exhibited by the SH2-6 line.

The truncated GAP-N protein binds constitutively to p190

The binding properties of bacterial polypeptides containing the GAP SH2 and SH3 domains have suggested that this N-terminal region of GAP participates in a series of protein−protein interactions in cells stimulated with growth factors, or transformed by v-*src* (Anderson *et al.*, 1990; Koch *et al.*, 1992). The association of GAP-N with cytoplasmic cellular proteins was examined initially by metabolic labelling with [^{35}S]methionine (Figure 2A and B). In parental Rat-2 cells growing in 10% serum, anti-GAP antibodies precipitated the endogenous 120 kDa GAP protein, with a barely detectable level of associated p190. In contrast, an anti-GAP immunoprecipitate from SH2-6 cells contained not only the endogenous GAP and mutant GAP-N proteins, but also a high level of p190. Anti-GAP immunoprecipitates from [^{35}S]methionine-labelled SH2-1

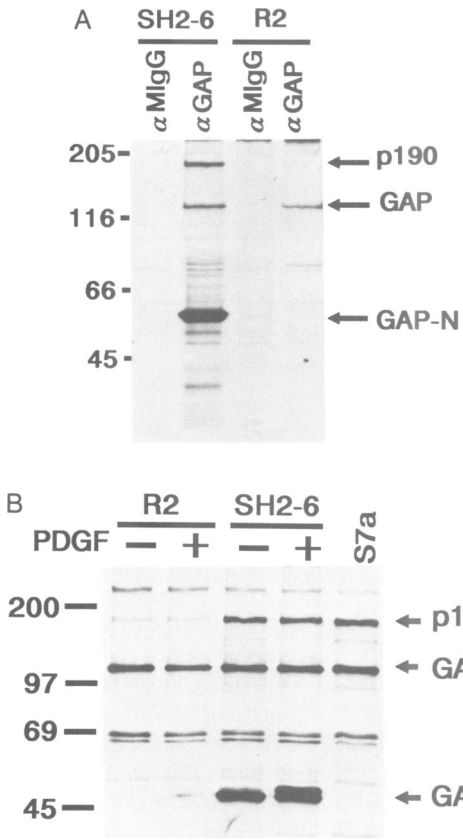

Fig 2. GAP-N binds constitutively to p190. (**A**) R2 and SH2-6 cells were metabolically labelled with [^{35}S]methionine for 16 h. Cell lysates were immunoprecipitated with either anti-GAP antiserum (α GAP) or rabbit anti-mouse antibody as a control (αMIgG) and analysed by 10% SDS−PAGE. The molecular weights (× 10^{-3}) of protein markers are indicated. (**B**) R2, SH2-6 or R2 cells transformed by v-*src* (S7a) were metabolically labelled with [^{35}S]methionine for 16 h in 0.5% (R2 and SH2-6) or 5% (S7a) dialysed calf serum. At the end of the labelling period R2 or SH2-6 cells were stimulated (+) or not (−) with 75 ng/ml PDGF for 15 min. Cell lysates were immunoprecipitated with anti-GAP antiserum and analysed by 10% SDS−PAGE. The GAP-N polypeptide precipitated from PDGF-stimulated cells (+) migrates as a doublet, presumably due to a phosphorylation-induced mobility shift. The molecular weights (× 10^{-3}) of protein markers are indicated.

cells, which express a lower level of GAP-N than SH2-6 cells, had an intermediate level of p190 (data not shown). Comparative V8 protease mapping showed that the 190 kDa protein that co-precipitated with GAP and GAP-N from SH2-6 cells was indistinguishable from the GAP-associated p190 isolated from v-*src*-transformed cells (data not shown).

These data suggested that expression of GAP-N markedly increased the amount of GAP-associated p190 in Rat-2 cells, in a fashion that was proportional to GAP-N expression. To test whether the increased level of co-precipitating p190 resulted from a direct interaction with the truncated GAP-N, SH2-6 cell lysates were immunoprecipitated with anti-p190 antibodies. Immunoblotting of the anti-p190 immune complexes from SH2-6 cells with anti-GAP antibodies revealed that the mutant GAP-N protein was physically associated with p190 (Figure 3).

The GAP-N polypeptide was associated with a high level of p190 in SH2-6 cells, regardless of whether the cells were maintained in high (10%) or low (0.5%) serum, or were

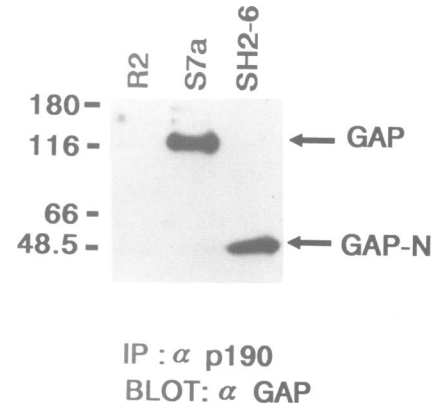

Fig 3. GAP-N interacts directly with p190. Cell lysates from R2, SH2-6 or v-*src* transformed R2 cells (S7a) were immunoprecipitated with anti-p190 antiserum. Immunoprecipitates were separated by SDS−PAGE, transferred to nitrocellulose and immunoblotted with anti-GAP antiserum followed by [^{125}I]protein A. The molecular weights (× 10^{-3}) of protein markers are indicated.

acutely stimulated with PDGF (Figure 2). The amount of ^{35}S-labelled p190 that co-precipitated with GAP-N and GAP from serum-deprived SH2-6 cells was equivalent to the amount of p190 complexed with endogenous GAP in v-*src*-transformed cells (Figure 2B). We have not observed a marked increase in p190 association with endogenous GAP in PDGF-stimulated cells (Figure 2B), although EGF, like v-Src, stimulates formation of the GAP−p190 complex (Ellis *et al.*, 1990).

GAP-associated p190 isolated from v-*src*-transformed or growth factor-stimulated cells contains phosphotyrosine. GAP-N-associated p190 from SH2-6 cells cultured in low serum was also phosphorylated on tyrosine, as detected by immunoblotting of anti-GAP immunoprecipitates with anti-phosphotyrosine antibodies (Figure 4A). Taken together these results indicate that the truncated GAP-N protein associates constitutively with p190 in Rat-2 cells, even under conditions of serum deprivation. Hence, by overexpressing GAP-N, the amount of GAP-associated p190 and its phosphorylation on tyrosine were increased to a level normally only observed in v-*src*-transformed cells.

The N-terminal region of GAP interacts inducibly with the PDGF receptor in vivo

To test the ability of GAP-N to associate with specific phosphotyrosine-containing proteins *in vivo*, other than p190, we examined its interactions with the PDGFR. Cells expressing GAP-N were serum-starved and then stimulated with PDGF; cell lysates were immunoprecipitated with anti-PDGFR antibodies, and the immune complexes were then immunoblotted with anti-GAP or anti-phosphotyrosine antibodies. GAP-N co-precipitated with the PDGFR from lysates of PDGF-stimulated cells, but not from unstimulated cells (Figure 4B). These results indicate that the stably expressed GAP-N protein contains all of the information required for the formation of a complex with the autophosphorylated PDGFR *in vivo*. The interaction of GAP-N with the PDGFR differed from its association with p190, in the sense that receptor binding was entirely dependent on PDGF stimulation, presumably due to a requirement for receptor autophosphorylation, whereas p190 binding was constitutive.

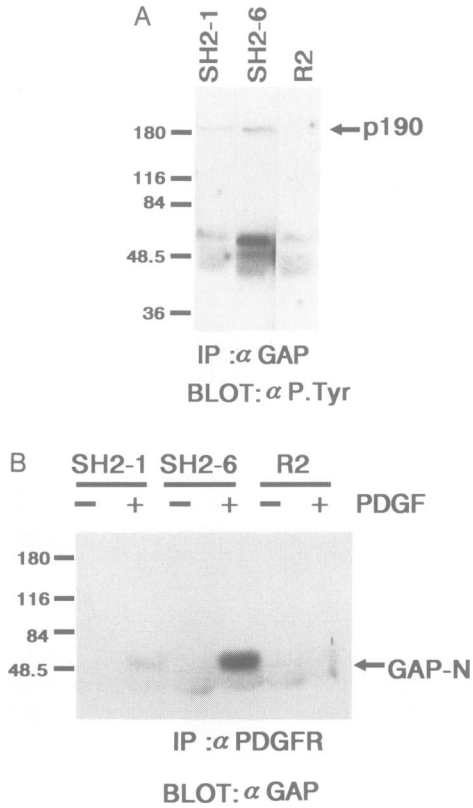

Fig 4. Binding of GAP-N to tyrosine phosphorylated p190 and activated PDGFR *in vivo*. (**A**) Tyrosine phosphorylation of p190. R2, SH2-1 and SH2-6 cells. Cells were lysed following 48 h in 0.5% FBS and immunoprecipitated with anti-GAP antibodies, separated by SDS−PAGE and immunoblotted with affinity purified anti-phosphotyrosine antibodies followed by [^{125}I]protein A. The molecular weights ($\times 10^{-3}$) of protein markers are indicated. (**B**) GAP-N binds activated PDGFR *in vivo*. The parental R2 cell line and GAP-N expressing lines SH2-1 and SH2-6 were stimulated with 75 ng/ml PDGF for 5 min (+) following 48 h in 0.5% FBS (−). Lysates were made from starved or stimulated cells and immunoprecipitated with anti-PDGFR antibodies (αPR4). Immunoprecipitates were separated by SDS−PAGE, transferred to nitrocellulose and immunoblotted with anti-GAP antiserum followed by [^{125}I]protein A. The molecular weights ($\times 10^{-3}$) of protein markers are indicated.

Anti-phosphotyrosine antibody blotting of anti-GAP immunoprecipitates showed that the GAP-N protein becomes phosphorylated on tyrosine following PDGF stimulation of SH2-1 and SH2-6 cells (data not shown). PDGF stimulation also induced approximately half of the GAP-N immunoprecipitated from [^{35}S]methionine-labelled cells to migrate more slowly, indicative of a phosphorylated species (Figure 2B). Therefore, GAP-N not only binds the PDGFR, but is a relatively efficient *in vivo* substrate for a tyrosine kinase, presumably the activated receptor itself. Since GAP-N lacks the principal site of phosphorylation of wild-type human GAP, Tyr460 (Liu and Pawson, 1991), it appears that tyrosine sites in GAP-N, which are poorly phosphorylated in full-length GAP, become accessible for phosphorylation by the activated receptor.

Rho/Rac GAP activity associated with GAP-N

Having established the ability of GAP-N to bind p190 and the PDGFR, we wished to ascertain whether these interactions might have functional consequences. The sequence of p190 suggests that it might function as a GAP

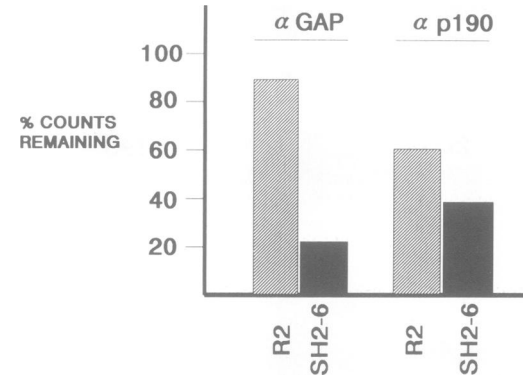

Fig 5. Rho GAP activity is associated with GAP-N. Anti-GAP or anti-p190 immunoprecipitates from R2 or SH2-6 cells were incubated with purified RhoA protein which had been loaded with [γ-^{32}P]GTP and the radioactivity remaining on RhoA after 10 min was determined in a filter binding assay. The percentage of counts remaining is relative to the initial radioactivity of RhoA added to each sample. The results presented are the average of three independent experiments.

towards members of the Rho/Rac family of small GTPases, and this prediction has been verified experimentally (Settleman *et al.*, 1992a). To test the possibility that the p190 associated with GAP-N stimulates Rho/Rac GTPase activity, anti-GAP immunoprecipitates from parental Rat-2 cells and SH2-6 cells were tested for their ability to enhance the GTPase activity of RhoA. Anti-GAP precipitates from the SH2-6 line showed an increase in the hydrolysis of GTP bound to RhoA, compared with anti-GAP immunoprecipitates from the parental cell line (Figure 5). These results indicate that the association of p190 with the truncated GAP-N protein is accompanied by the presence of Rho/Rac GAP activity in the immune complex.

Anti-p190 immunoprecipitations from GAP-N cells showed only a small increase in GAP activity compared with immunoprecipitates from R2 cells (Figure 5). This suggests that no significant increase in the specific activity of total cellular p190 has occurred. However, changes in p190 function *in vivo* related to its access to substrates or its interactions with other molecules, may not be detectable in an *in vitro* assay of this type.

Disruption of the actin cytoskeleton and focal contacts by GAP-N

To investigate whether expression of the N-terminal region of GAP had any phenotypic effects, the proliferative and morphological properties of cell lines expressing GAP-N were examined. DNA synthesis following serum stimulation of cells expressing GAP-N was similar to that in parental Rat-2 cells (data not shown). GAP-N expression did not have a pronounced effect on the rate of cell doubling, nor did it reproducibly promote growth in soft agar. The level of Fos protein, taken as a typical early gene product which is induced by activated Ras, was also normal in Rat-2 cells expressing GAP-N (R. Bravo, personal communication). These data suggest that stable expression of the N-terminal region of GAP does not, of itself, constitutively activate a mitogenic signalling pathway.

In contrast, in an analysis of eight cell lines expressing varying levels of GAP-N, we have observed pronounced changes in cytoskeletal organization and cell adhesion. Rat-2 cells cultured in 10% FBS possessed a regular array of actin

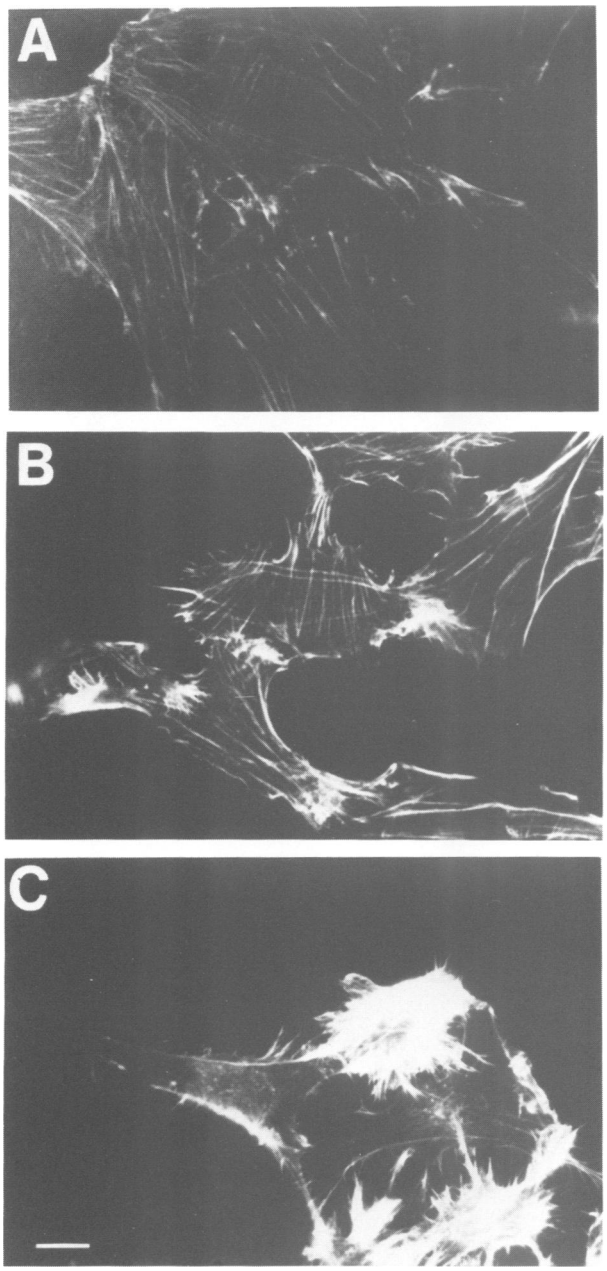

Fig 6. GAP-N overexpression in Rat-2 cells causes increased ruffling and loss of actin filaments. Cells were grown in coverslips in 10% FBS, fixed in 3.7% paraformaldehyde, permeabilized by a 0.5% Triton X-100 buffer and stained for actin with rhodamine phalloidin (see Materials and methods). The cells were viewed using epifluorescence microscopy. (**A**) R2, (**B**) SH2-4 and (**C**) SH2-6. Bar = 20 μm.

stress fibres, running the length of the cell (Figure 6A). However, staining for F-actin revealed that the actin fibres in cells expressing GAP-N were irregular and branched, did not extend throughout the cell, and were concentrated at the membrane (Figure 6B and C). This phenotype was most pronounced in SH2-6 cells, which express the highest level of GAP-N, but was readily detected in several lines with lower amounts of GAP-N such as SH2-4, as shown in Figure 6B. The disruption of actin stress fibres in cells expressing GAP-N was greatly enhanced by incubating the cells in (0.5%) serum. Under these conditions normal Rat-2 cells retained a regular array of actin fibres, whereas the F-actin in cells expressing GAP-N retracted into bundles associated with membranes (data not shown).

Actin filaments terminate at focal contacts, which represent specialized structures containing specific cytoskeletal proteins, such as vinculin, and transmembrane proteins which mediate attachment to extracellular substrates, notably the integrins (Burridge *et al.*, 1988). Normally, cells grown in culture develop focal contacts both along their edges and over the cell surface where they adhere to the substratum. Staining of parental Rat-2 cells for vinculin showed focal contacts at the edges and throughout the cell (Figure 7A). In contrast, immunofluorescence staining of vinculin in cells expressing GAP-N showed an increasing loss of focal contacts in the middle of the cell as GAP-N overexpression increased (Figure 7B and C). The most extreme GAP-N overexpression caused vinculin to aggregate at a few specific locations on the cell membrane and induced cytoplasmic vinculin staining. Again, serum starvation augmented the loss of focal contacts associated with GAP-N expression (data not shown). Consistent with these observations, interference reflection microscopy showed a total loss of dark areas associated with cell adherence, in the SH2-6 GAP-N overexpressing cells (data not shown). These results suggest that the GAP-N polypeptide induces the disassembly of focal contacts.

GAP-N expression results in decreased fibronectin binding and cell adhesion

The extracellular matrix can affect cell growth, differentiation and cell shape. Fibronectin is one of the principal glycoprotein components of the extracellular matrix, and is important in cell adhesion to the substratum. Extracellular fibronectin is indirectly coupled to intracellular actin fibres at focal contacts through integrins (Burridge *et al.*, 1988). Normally, fibronectin fibrils are organized in register with actin microfilaments and focal contacts, as detected by immunofluorescent staining. As expected, the parent Rat-2 cell line displayed normal fibronectin staining in between cells, and showed a co-linear arrangement with actin microfilaments (Figure 8A). However, as GAP-N expression increased, all fibronectin staining in the middle of the cells was lost and only fibronectin staining between the cells remained (Figure 8B and C). This pattern of fibronectin staining did not change with cell density. These results could be explained either by inhibition of intracellular fibronectin synthesis, or by reduced fibronectin binding. Cells in which fibronectin synthesis is blocked can be restored to a flattened and more adherent morphology, with increased focal contacts, if exogenous fibronectin is added (Chen *et al.*, 1986). However, when 100 μg/ml of cell culture fibronectin was added to SH2-6 cells, and the cells monitored at 0.5, 1 and 14 h after addition, no morphological change or restoration of actin fibres was evident (data not shown). These results suggest that fibronectin binding by these GAP-N overexpressors is inhibited.

The observation that cells expressing GAP-N are impaired in their ability to form focal contacts, and to retain fibronectin, suggested that their capacity to adhere to extracellular matrix substrates might be compromised. Indeed, SH2-6 cells passaged into low serum became progressively more round and refractile (Figure 9). The majority of cells placed in low serum detached from the tissue culture dish within ~3 days. These detached cells

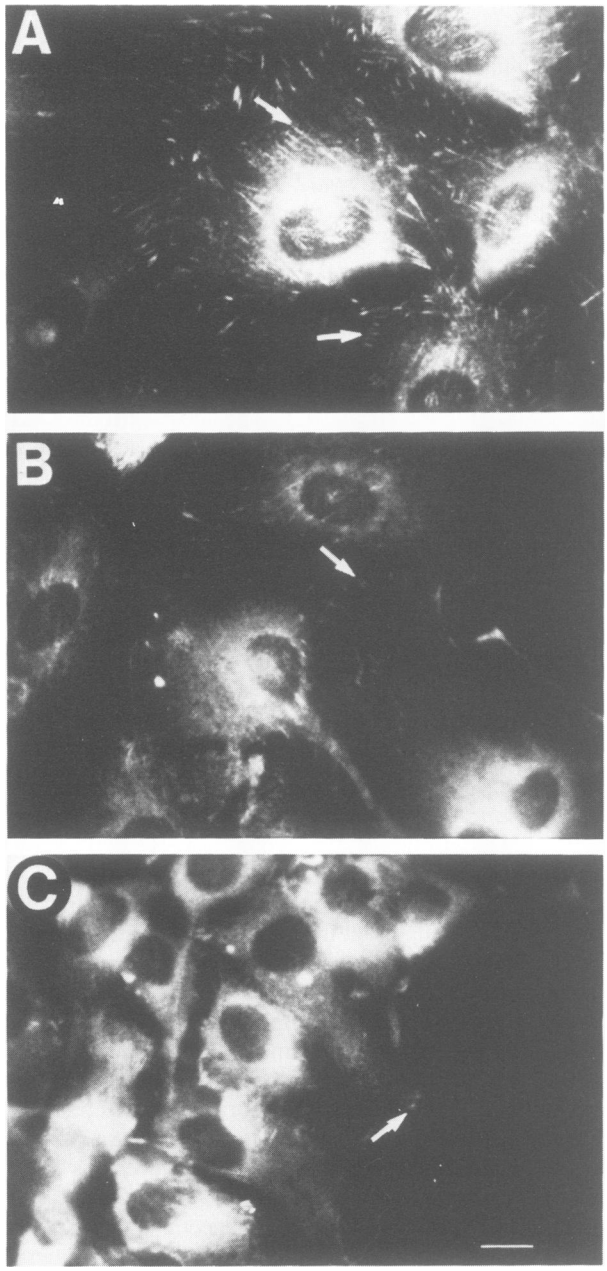

Fig 7. GAP-N overexpression correlates with decreased, focal adhesions. Rat-2 parental and GAP-N transfected cells were grown on coverslips in 10% FCS, fixed with methanol at 20°C, indirectly immunofluorescently stained for vinculin, and viewed with epifluorescence microscopy. Arrows show focal contacts. (**A**) Parental cell line, (**B**) SH2-1 and (**C**) SH2-6. Bar = 20 μm.

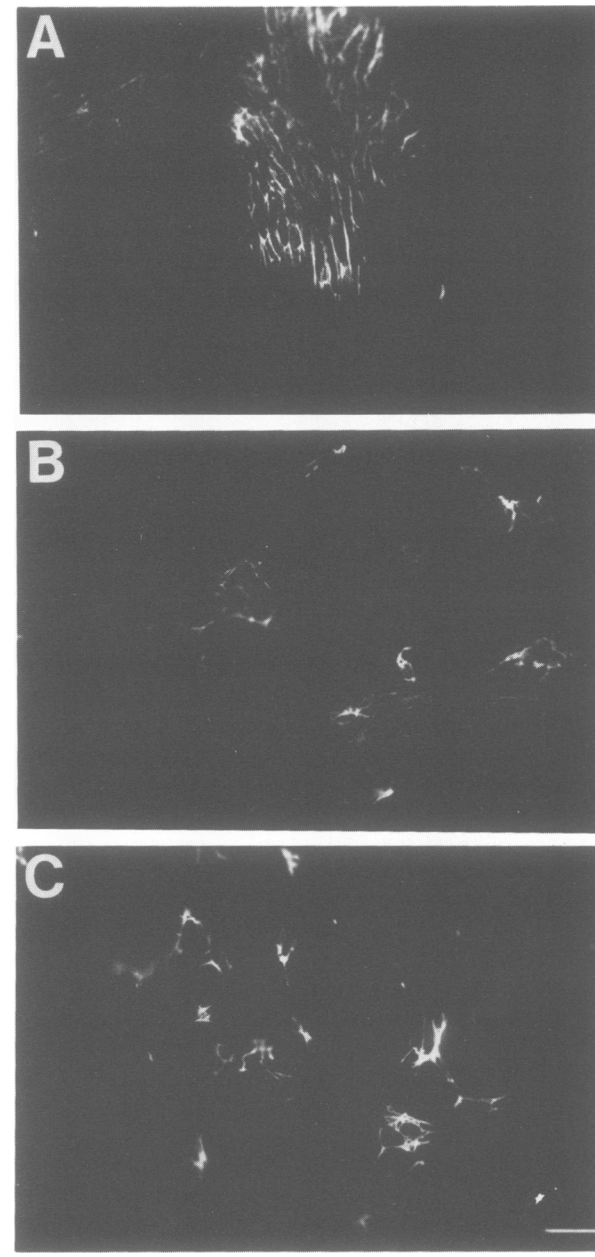

Fig 8. GAP-N overexpression correlates with decreased fibronectin binding. Cells were grown in 10% FBS on coverslips, fixed in 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100, indirectly stained for fibronectin by immunofluorescence and viewed with epifluorescence microscopy. (**A**) R2, (**B**) SH2-1 and (**C**) SH2-6. Bar = 20 μm.

remained viable, as judged by trypan blue exclusion, and reattached to the substrate and resumed proliferation upon the addition of 10% serum. We quantitated this apparent change in cell adhesion by measuring cell attachment to a fibronectin surface in serum-free conditions. Compared with Rat-2 cells, SH2-6 cells attached poorly to fibronectin; SH2-1 cells, which have a lower level of GAP-N expression, were intermediate in their attachment to fibronectin (Figure 10). The loss of fibronectin staining in GAP-N cells, and their reduced ability to attach to fibronectin, is consistent with a defect in fibronectin binding. Despite the decrease in fibronectin binding and responsiveness, there was no significant difference in the expression of integrins in GAP-N cells compared with parental Rat-2 cells (data not shown).

Discussion

Protein–protein interactions mediated by the N-terminal region of GAP

By stably expressing high levels of a GAP fragment containing only the N-terminal hydrophobic sequence, and the SH2 and SH3 domains, we have been able to examine the protein interactions mediated by these non-catalytic elements of GAP *in vivo*. Stimulation of Rat-2 cells

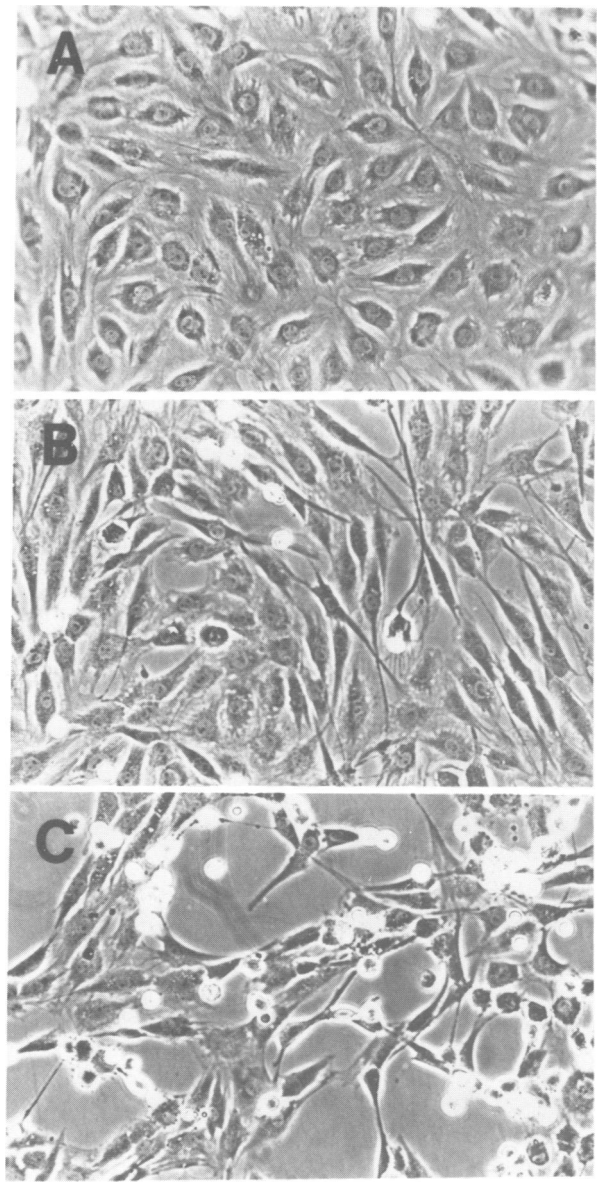

Fig 9. GAP-N overexpression causes morphological changes in low serum conditions. (**A**) R2, (**B**) SH2-1 or (**C**) SH2-6 cells were plated on DMEM with 10% FCS. After 18 h the medium was replaced with DMEM plus 0.5% FCS. Phase-contrast micrographs were taken following 5 days in culture.

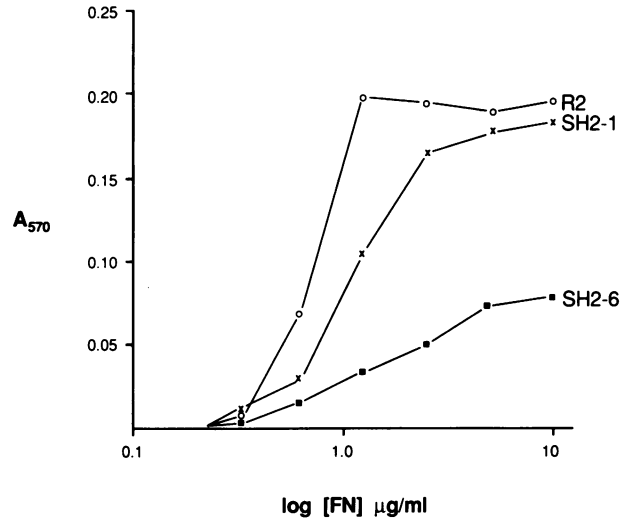

Fig 10. GAP-N overexpression results in decreased adhesion to fibronectin. R2, SH2-1 or SH2-6 cells were plated on microtitre plates coated with increasing concentrations of fibronectin (μg/ml). Cells were allowed to attach for 1 h, the plates were washed and then attachment was quantitated by toluidine blue staining and measurement of absorbance at 570 nm.

expressing GAP-N with PDGF induced association of GAP-N with the autophosphorylated PDGFR. This is consistent with the suggestion, based on *in vitro* binding experiments, that the GAP SH2 domains mediate receptor binding. Since abolition of the GAP binding site on the human or mouse βPDGFR reportedly has no obvious effect on cell phenotype (Kashishian *et al.*, 1992), the function of the interaction between GAP and the PDGFR is unclear. The activated βPDGFR induces actin reorganization and cell migration (Bockus and Stiles, 1984), which potentially might involve GAP. These results demonstrate that the N-terminal region of GAP contains all of the information required for binding to the PDGFR in stably expressing cells stimulated with PDGF.

In contrast to the PDGF-dependent binding of GAP-N to the PDGFR, presumably due to the requirement for receptor autophosphorylation for SH2 binding, GAP-N associated constitutively with a phosphotyrosine-containing form of p190. This confirms the previous suggestion that p190 binds to a site within the GAP N-terminal region, although the precise GAP residues required for p190 binding remain to be defined. Since GAP-associated p190 contains phosphotyrosine, it is possible that the GAP SH2 domains recognize a phosphorylated site on p190. However, the p190−GAP interaction differs from the binding of GAP to both activated receptors and tyrosine phosphorylated p62, in the sense that it cannot readily be reconstructed *in vitro* using bacterially expressed GAP SH2 domains. The primary p190 binding site on GAP may lie outside the SH2 domains; this initial interaction might be stabilized by subsequent binding of a GAP SH2 domain to a p190 tyrosine phosphorylation site, which in turn would be protected from dephosphorylation. This scheme could explain the increased tyrosine phosphorylation of p190 associated with GAP-N in serum deprived cells, if p190 were normally basally phosphorylated on tyrosine, but rapidly dephosphorylated in the absence of GAP binding.

The binding of endogenous GAP to p190 in rat fibroblasts, and the tyrosine phosphorylation of GAP-associated p190, is normally stimulated by the EGF receptor or v-Src (Ellis *et al.*, 1990; Moran *et al.*, 1991). These results suggest that these tyrosine kinases modify GAP, either directly or indirectly, in a fashion that promotes p190 binding. The GAP-N protein may mimic this activated form of GAP, and hence bind constitutively to p190. Two mechanisms by which tyrosine kinases might normally elicit a conformational change in GAP, thereby promoting p190 binding can be envisaged. Circumstantial evidence suggests an interaction between the N- and C-terminal regions of GAP (Gideon *et al.*, 1992). Elevation of Ras−GTP levels by tyrosine kinases, and consequent binding of Ras−GTP to the C-terminal GAP domain, could free the N-terminal region for p190 binding. Alternatively, direct tyrosine phosphorylation of GAP, which normally occurs at a site just C-terminal to

the GAP-N sequence, might alter GAP conformation and promote p190 binding. These potential mechanisms are not necessarily mutually exclusive, and could in principle act in concert. This scheme reflects the model proposed by Martin *et al.* (1992) to explain the *in vitro* effects of GAP proteins on K^+ channels in atrial membranes.

GAP–p190 complexes regulate cell cytoskeleton and adhesion

Rat-2 cells expressing GAP-N did not show any profound changes in DNA synthesis or cell proliferation. However, expression of GAP-N did correlate with striking effects on actin stress fibres, focal contacts, fibronectin binding, organization of fibronectin fibrils, cell adhesion and cell morphology. Cells expressing GAP-N had a disorganized actin network, reduced focal contacts and bound poorly to fibronectin. The extent of these changes correlated, in general, with the level of GAP-N expression. In low serum, these phenotypes were aggravated, and cells expressing higher levels of GAP-N underwent marked changes in morphology and adhesion. A potential explanation for these findings is that the constitutive association of GAP-N with p190 is directly responsible for the disruptions in cytoskeletal architecture, focal contacts and cell adhesion detected in GAP-N expressing cells. How might these changes be brought about?

Recent evidence suggests that activation of the Rho proteins by GTP binding induces rapid formation of actin stress fibres, and the redistribution of vinculin into focal contacts (Ridley and Hall, 1992). Similarly, activation of the related Rac proteins induces membrane ruffling (Ridley *et al.*, 1992). Furthermore, conversion of Rho and Rac to the GTP-bound form is apparently required for the induction of actin stress fibres, focal contacts and membrane ruffling by serum (Ridley and Hall, 1992; Ridley *et al.*, 1992). p190 has a Rho/Rac GAP domain, which can inactivate Rho/Rac family members converting them to the GDP-bound form (Settleman *et al.*, 1992a). We have shown that the GAP-N–p190 complex has Rho/Rac GAP activity *in vitro*. The phenotype of GAP-N cells could be explained if the binding of GAP-N to p190 stimulates p190 Rho/Rac GAP activity, either directly or by providing p190 access to its substrates. The conversion of Rho/Rac proteins to the inactive GDP-bound form would be anticipated to precipitate the observed loss of actin stress fibres and decreased cell adhesion. Serum may antagonize these effects by stimulating the exchange of GDP for GTP on Rho/Rac proteins, or blocking GTP hydrolysis, thereby explaining the exaggerated phenotype induced by reducing the serum concentration.

GAP is a multi-functional protein

GAP is composed of several domains, with quite distinct functions. The C-terminus has Ras GAP activity, in common with other proteins found in yeast, flies and mammals, and acts as a negative regulator of Ras. The N-terminus contains SH2 and SH3 domains, and participates in growth-factor-dependent interactions with several phosphoproteins, including activated βPDGFR, p190 and p62. Here, we have shown that the N-terminal region of GAP has pronounced effects on cytoskeletal architecture and fibronectin binding, probably through its association with p190. GAP may therefore be an effector that couples tyrosine kinases and Ras to control of the actin cytoskeleton, and cell adhesion to the extracellular matrix.

Modifications of cytoskeletal architecture and cell–matrix interactions are necessary elements of mitosis, and are also important in cell migration. We did not observe an effect of GAP-N on DNA synthesis. We cannot exclude the possibility that the central region of GAP, which is absent from the GAP-N polypeptide, is involved in control of cell proliferation, or that GAP-N itself has a mitogenic function not revealed in our experiments. However, another possibility is that Ras has multiple targets, and that an effector other than GAP is involved in regulation of DNA synthesis.

Materials and methods

Construction of GAP-N expression plasmid

An *Eco*RI–*Sca*I fragment of the human GAP cDNA was subcloned into M13mp18, and amino acid 446 was converted to a translation termination codon (CAA to TAA) by site-directed mutagenesis. The mutated cDNA was then subcloned into the mammalian expression plasmid pECE.

Tissue culture

Rat-2 (R2) cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10 or 0.5% fetal calf serum (FCS). Cell lines expressing the GAP-N protein were created by cotransfection of R2 cells with pECE-GAP-N and pSV2Neo and selection in 400 μg/ml G418. Resistant colonies were expanded and the expression of the GAP-N protein was analysed by Western blotting of whole cell lysates using anti-GAP antibodies. Stable GAP-N cell lines were continuously cultured in DMEM containing 10 or 0.5% FCS and 40 μg/ml G418. PDGF stimulations of previously serum starved cells (48 h in 0.5% FCS) were carried out using 75 ng/ml PDGF for 5 min at 37°C. R2 cells overexpressing v-*src* (S7a) were grown in DMEM with 5% calf serum and have been previously described (Brooks-Wilson *et al.*, 1989; Koch *et al.*, 1992).

Immunoprecipitations and immunoblotting

Immunoprecipitations were carried out essentially as described (Moran *et al.*, 1991). Confluent monolayers of cells were lysed in PLC lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaFl, 10 mM $NaPP_i$, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride with aprotinin and leupeptin at 10 μg/ml], except for metabolically labelled cells where 0.1% SDS was added. Lysates were preincubated with protein A–sepharose and then incubated with 10 μl of anti-GAP antiserum (Ellis *et al.*, 1990), 5 μl of the anti-PDGFR antiserum (PR4) (generously provided by S.Courtneidge), or 2 μl of an anti-p190 antiserum (Settleman *et al.*, 1992b). Whole cell lysates were prepared by adding SDS sample buffer [50 mM Tris pH 6.8, 10% glycerol, 2% SDS (w/v), 0.5% β-mercaptoethanol (v/v)] directly to cell monolayers after they had been briefly washed with PBS. Protein concentrations were determined and 25 μg of protein were analysed.

For Western blotting, samples were resolved by SDS–PAGE and transferred to nitrocellulose with a semi-dry transfer apparatus. Blots were blocked and then immunoblotted with 1:500 dilution of anti-GAP antiserum, 1 μg/ml affinity purified rabbit anti-phosphotyrosine antibody as previously described (Anderson *et al.*, 1990). Blots were then probed with ^{125}I-labelled protein A (35 μCi/ml) and exposed to Kodak XAR-5 film.

Metabolic labelling

Cells were labelled with [^{35}S]methionine (150 μCi/ml) for 16 h in DMEM containing 0.5% dialysed fetal calf serum unless otherwise noted. Cells were lysed and immunoprecipitations performed as described above. Samples were analysed by SDS–PAGE and gels were treated with EN^{3}HANCE (Amersham), dried and exposed to Kodak XAR-5 film.

GAP assay

RhoA was purified as a GST fusion protein using glutathione sepharose (Sigma) followed by thrombin cleavage as described previously (Settleman *et al.*, 1992a). Purified RhoA was bound to [γ-^{32}P]GTP by incubation in 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mg/ml BSA, 1 mM DTT and 1 μM [γ-^{32}P]GTP for 10 min at 37°C, followed by addition of $MgCl_2$ to a final concentration of 10 mM. p190 and GAP proteins were immunoprecipitated as described above, then washed and resuspended in 100 μl of 50 mM Tris, pH 7.5, 1 mg/ml BSA, 10 mM $MgCl_2$, 1 mM DTT. RhoA–GTP was added to the immunoprecipitates and the samples were incubated for 10 min at 25°C, brought to 500 μl with 500 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and filtered through nitrocellulose

(BA85, S&S). Filters were washed with the same buffer and radioactivity remaining on the filters was determined.

Immunofluorescence

Cells were grown on coverslips in 20 μg/ml G418 in DMEM with either 10 or 0.5% FBS. Coverslips stained for F-actin or fibronectin were fixed in paraformaldehyde solution (3.7% paraformaldehyde in PBS pH 7.4) for 20 min, washed in PBS and permeabilized in a Triton solution for 5 min (0.5% Triton X-100, 50 mM NaCl, 10 mM HEPES, 300 mM sucrose pH 7.5). F-actin was illuminated by incubating fixed cells with 266 ng/ml rhodamine phalloidin and incubation in a humid chamber at 37°C for 20 min. Fibronectin was indirectly immunofluorescently labelled with a 1:4000 dilution of mouse monoclonal antibody F6140 (Sigma) for 60 min in a humid chamber at 37°C followed by a 30 min incubation with a 1:30 dilution of fluoresceinated goat anti-mouse antibody (Sigma) at 37°C. Cells immunofluorescently labelled for vinculin were fixed in methanol at −20°C for 5 min, washed with PBS, and incubated for 60 min at 37°C with 1:100 dilution of mouse monoclonal antibody against vinculin (vin-11-5, Sigma), followed by a 30 min incubation at 37°C with a 1:30 dilution of fluoresceinated goat anti-mouse antibody (Sigma). The coverslips were mounted on to microscope slides with a 50% gelatin/gelvaltol + 10% w/w *n*-propyl gallate mixture. Slides were viewed with a Zeiss Axiophot microscope equipped with epifluorescence and interference reflection microscopy optics and a 100 W mercury lamp. Photographs were taken using Kodak T_{max}-400 (pushed twice), Ilford XP1-400 and Kodak Ektar 1000/31 film.

Cell attachment assays

Attachment of cells to fibronectin (Telios Pharmaceuticals, La Jolla, CA) was carried out in 96-well microtitre plates. Wells were coated in triplicate with increasing concentrations of fibronectin. After overnight incubation at 4°C the unbound substrate was washed away with PBS and the wells were blocked with DMEM containing 2.5 mg/ml BSA for 2 h at 37°C. At this point, the cells were harvested from culture, washed three times in serum-free DMEM, and resuspended in DMEM containing 2.5 mg/ml BSA at a concentration of 3×10^5 cells/ml. Cells (0.1 ml) were then applied to each well and cell attachment carried out at 37°C for 1 h. Unattached cells were removed and the wells washed with PBS. Attached cells were fixed with paraformaldehyde solution for 30 min followed by overnight staining with toluidene blue (0.5%) in 3.7% paraformaldehyde. The excess stain was washed away with PBS and cell attachment was quantitated by measuring absorbance at 470 nm using an automated microtiter well plate reader.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (NCIC), with funds made available by the Canadian Cancer Society, and by an International Research Scholar Award from the Howard Hughes Medical Institute (to T.P.), and by a grant from the Ontario Cancer Treatment Research Foundation (to S.D.). J.M. is supported by a fellowship from the NCIC; T.P. is a Terry Fox Cancer Research Scientist of the NCIC. B.B. was supported by a grant from the NIH in General Medicine #5RO1 GM38318 8195206.

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Received on March 8, 1993; revised on April 27, 1993