Cytoskeletal changes induced by GRAF, the G\textsuperscript{TPase} regulator associated with focal adhesion kinase, are mediated by Rho

Joan M. Taylor, Marlene M. Macklem and J. Thomas Parsons*

Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, Virginia, 22908, USA

*Author for correspondence (e-mail: jtp@virginia.edu)

Accepted 12 November; published on WWW 21 December 1998

SUMMARY

Graf, the G\textsuperscript{TPase} regulator associated with focal adhesion kinase was previously shown to have GAP activity for Rho A and Cdc42 in vitro (Hildebrand et al 1996 Mol. Cell Biol. 16: 3169-3178). In this study we sought to determine whether Graf acted at the level of Cdc42, Rho, or both in vivo and whether Graf was a signal terminator or transducer for these proteins. Microinjection of Graf cDNA into subconfluent Swiss 3T3 cells (in the presence of serum) has marked effects on cell shape and actin localization. Graf expression causes clearing of stress fibers followed by formation of long actin based filopodial-like extensions. Similar phenotypes were observed following injection of the Rho-inhibitor, C3 into these cells. The Graf response was dependent on GAP activity, since injection of Graf cDNA containing point mutations in the GAP domain (R236Q or N351V) which block enzymatic activity, does not confer this phenotype. Injection of Graf into Swiss 3T3 cells in which Rho has been down-regulated by serum starvation has no effect on cell morphology. Using this system, we demonstrate that Graf blocks sphingosine-1-phosphate (SPP) stimulated (Rho-mediated) stress fiber formation. Conversely, Graf expression does not inhibit bradykinin stimulated (Cdc42-mediated) filopodial extensions. These data indicate that Graf is a GAP for Rho in vivo. To further substantiate these results we examined the effect of Graf over-expression on Rho-mediated neurite retraction in nerve growth factor (NGF)-differentiated PC12 cells. In PC12 cells, which express relatively high levels of endogenous Graf, overexpression of Graf (but not Graf containing the R236Q mutation) enhances SPP-induced neurite retraction. These data indicate the possibility that Graf may be an effector for Rho in certain cell types.

Key words: Rho, GAP, Cytoskeleton

INTRODUCTION

The small molecular weight GTP-binding proteins (smw G proteins) Rho A, B, C, E and G, Rac 1 and Rac 2, Cdc42 and TC10 are a family of molecular switches which are 30% identical to Ras and 55% identical to each other (Boguski and McCormick, 1993). The Rho-family members regulate a variety of cellular responses including actin polymerization and reorganization, gene expression (p38/JNK and SRF activity), cleavage furrow formation, G1 cell cycle progression, endocytosis, exocytosis, and superoxide production (Ridley, 1996).

Cytoskeletal rearrangement is one of the best characterized responses of Rho-family members. Studies employing quiescent Swiss 3T3 cells have indicated that Rho-dependent regulation of the cytoskeleton involves signaling through Cdc42, Rac and Rho (Hall, 1998). In these cells activation of Cdc42 (by bradykinin, tumor necrosis factor-\textalpha\ (TNF-\textalpha\) and IL-1 (interleukin-1)) stimulates the formation of finger-like projections of actin filaments called filopodia. Activation of Rac (either indirectly by Cdc42-mediated signaling or directly by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), bombesin, or insulin) results in extensions of broad sheet-like structures called lamellipodia. Finally, activation of Rho (indirectly by Rac-mediated signaling or directly by bombesin; sphingosine 1-phosphate (SPP) or lysophosphatidic acid (LPA) results in the formation of actin stress fibers and focal adhesions (Nobes and Hall, 1995; Ridley and Hall, 1992, 1994; Ridley et al., 1992; Chant and Stowers, 1995). The cyclical production of these actin-based structures is proposed to be important for cellular motility. In fact, activated Cdc42 and Rac increase the motility and invasiveness of mammary carcinoma cells (Keely et al., 1997) and a dominant-interfering mutant for Rac blocks PDGF-stimulated migration of Rat-1 fibroblasts (Anand-Apte et al., 1997).

The downstream effectors of Cdc42, Rac and Rho which lead to the assembly of filopodia, lamellipodia and stress fibers are not fully understood; however, some potential candidates have been recently identified. Some evidence suggests that smw G proteins utilize protein kinases to elicit their effects on actin polymerization. Both Cdc42 and Rac activate the tyrosine kinase Ack as well as the serine/threonine kinase PAK. Rho activates several serine/threonine kinases including protein kinase N, p164 Rho kinase (ROK\textalpha\ or ROCK) and PRK2 kinase (which is also activated by Rac) (Vincent and
Settleman, 1997; Leung et al., 1996; Amano et al., 1996b; Watanabe et al., 1996). In addition to protein kinase signaling, Rho binds to and stimulates a lipid kinase, phosphatidylinositol 4-phosphate-5-kinase (PIP5-K), which converts phosphatidylinositol-4-phosphate (PIP) to polyphosphatidyl inositol 4,5-bisphosphate (PIP2; Ren et al., 1996). PIP2 binds to and regulates a number of actin binding proteins resulting in enhanced actin polymerization (Taylor et al., 1998a).

Another potential pathway whereby Rho family members could regulate down-stream signaling is through their GTPase activating proteins (GAPs). Although GAP proteins down-regulate the signal input by rapidly converting the active GTP bound G protein to the inactive GDP bound state, certain GAP proteins including p120Ras GAP, n-chimaerin, and PLC (a GAP for heterotrimeric G proteins) simultaneously send a signal which is required for downstream signaling from the G protein (Duchesne et al., 1993; Boguski and McCormick, 1993; Kozma et al., 1996). For example, microinjection of the Rac GAP n-chimaerin (like activated Rac) induces the formation of lamellipodia, an effect that is dependent on Rac, but not dependent on the GAP activity of n-chimaerin (Kozma et al., 1996). Apparently, the binding of n-chimaerin to activated Rac enhances a functional interaction between n-chimaerin and another unknown protein(s) to regulate cellular morphology.

Graf (GTPase regulator associated with focal adhesion kinase) is a recently identified GTPase activating protein containing a centrally located GAP domain, followed by a serine/proline rich domain and a carboxy-terminal Src-homology 3 (SH3) domain. In vitro, the Graf GAP domain enhances GTP hydrolysis of both Cdc42 and RhoA but not Rac1 or Ras. Graf is a binding partner for focal adhesion kinase (FAK) in that the SH3 domain of Graf binds to PXXP motifs within the C-terminal domain of FAK (Hildebrand et al., 1996). The aim of this study was to characterize Graf activity in vivo. More specifically, we tested whether in vivo, Graf acts at the level of Cdc42, Rho, or both and whether Graf is a signal terminator or transducer for these proteins. We show herein that ectopic expression of Graf has marked effects on cell shape and actin localization in growing Swiss 3T3 cells. The Graf-injected cells extend long stable filopodia, an effect that is dependent on Graf’s GAP activity and is similar to the effects of the Rho inhibitor, C3 exoenzyme. The ability of Graf to block SPP-mediated (but not bradykinin-mediated) signaling in quiescent cells suggests that Graf is an in vivo regulator of Rho. Interestingly, overexpression of Graf in PC12 cells enhances SPP-induced (Rho-mediated) neurite retraction, indicating that Graf may be a down-stream effector for Rho in certain cell types.

MATERIALS AND METHODS

Epitope-tagged Graf, deletion constructs and mutagenesis
All Cdc42, Rac, Rho and C3 constructs were generous gifts from Alan Hall. The cDNA construct encoding the N-terminal Flag-tagged variant of Graf (F-Graf; aa 1-584) was generated by PCR using primers that engineered 5’ Hin dIII and 3’ XhoI restriction sites. The PCR product was digested with Hin dIII and XhoI and ligated into HindIII and XhoI digested pcDNA3 containing a 7 amino acid N-terminal epitope tag with the sequence, DYKDDDK. The Flag-tagged N-terminal Graf construct (F-NT; aa 1-241) was generated by digesting F-Graf with XhoI and religated to remove the C-terminal GAP and SH3 domains. The Flag-tagged GAP construct (F-GAP; aa 1-422) was generated by digesting F-Graf with HindIII and NotI and ligating into HindIII and NotI digested pcDNA3 to remove the C-terminal SH3 domain. The Flag-tagged SH3 construct (F-SH3; aa 472-584) was generated by digesting F-Graf with EcoRI and XhoI and ligating into EcoRI and XhoI digested pcDNA3. The C-terminal green fluorescent protein (GFP)-tagged variants of Graf were obtained by digesting F-Graf or R236Q with HindIII and XhoI and ligating into HindIII and SalI digested pEGFP-N1 (Clontech). Mutations resulting in Graf point mutants R236Q, N351V and S510A were generated in F-Graf using PCR based site-directed mutagenesis (Quick Change, Stratagene). The sequence of all constructs was verified by direct DNA sequencing.

Cell culture and transfection
Swiss 3T3 (ATCC) and COS-1 (ATCC) cells were maintained in DMEM containing 10% FCS, and a 1% penicillin-streptomycin solution. For transfections, COS-1 cells were plated at 2x105 cells/ml and transfected with the Superfect reagent (Qiagen) using 10 μg plasmid DNA per 100 cm dish. Cells were incubated with the transfection mixture for 4 hours in the presence of serum and antibiotics. Cells were rinsed and incubated in serum-containing medium for 24-48 hours prior to harvesting. PC12 cells (ATCC) were maintained in DMEM containing 10% HS, 5% FCS and a 1% penicillin-streptomycin solution. For transfections, PC12 cells were plated at 2x104 cells/ml on 35 mm dishes coated with laminin (10 μg/ml). Cells were transfected with the Superfect reagent (Qiagen) using 2 μg plasmid DNA per 35 mm dish as described above. After 24 hours, cells were treated with 100 nM NGF for a 72 hour period to induce neuronal differentiation.

Microinjection
For most microinjection experiments, Swiss 3T3 cells (5x104 cells/35 mm dish) were plated in the presence of serum onto gridded glass coverslips and allowed to attach and spread overnight. Serum-starved Swiss 3T3 cells were prepared as described previously (Lamarche et al., 1996). Briefly, 1x105 cells/60 mm dish were plated in serum and incubated for 7-10 days prior to serum starving for 16 hours. Cells were then trypsinized and resuspended in serum-free DMEM containing 0.5 mg/ml soybean trypsin inhibitor. Cells were centrifuged, resuspended in serum-free medium and replated onto gridded glass coverslips (6x103 cells/35 mm dish) pre-coated with fibronectin (10 μg/ml). Eukaryotic expression vectors encoding the cDNAs for Graf (in Flag-pCDNA3 or myc-PRK5) CDC42 (L61), Rac (L61) and Rho (L63: in myc-PRK5) or C3 (in pEF) were diluted into TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.5) and microinjected into the nucleus of cells using a Zeiss micromanipulator. Certain constructs were injected with the cDNA for GFP (pEGFP-N1; 5 μg/μl) to aid in the detection of injected cells. Routinely 50-70 cells were injected within a period of 15 minutes. Injected cells were then incubated at 37°C in 5% CO2 for 1-4 hours before fixation.

Immunocytochemistry
For immunofluorescence staining, cells were washed three times with phosphate-buffered saline (PBS, calcium and magnesium free) and fixed using 4% paraformaldehyde in PBS for 20 minutes at RT. Cells were washed three times in PBS and permeabilized with 0.4% Triton X-100 in PBS for 3 minutes at RT. Slides were washed three times in PBS to remove the detergent and were incubated with either the primary anti-Flag monoclonal antibody, M5 (5 μg/ml) or anti-myc monoclonal antibody, 9E10 (1 μg/ml) for 1 hour followed by Texas Red-conjugated goat anti-mouse antibody (Ab; 5 μg/ml) for 1 hour.
A fluorescent phalloidin (1:1000 fluorescein or Texas Red conjugated; Molecular Probes) was used to visualize filamentous actin.

**Immunoprecipitation and western blots**

COS-1 cells overexpressing F-Graf or F-Graf variants were lysed by scraping cells into 2× Laemmli sample buffer. Proteins were separated using 10-12% sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE). Proteins were transferred to nitrocellulose, and western blots were performed using the Flag-tag specific monoclonal antibody M5 at a 1/1000 dilution. Blots were then incubated with HRP-conjugated donkey anti-mouse Ab at a 1/1000 dilution, followed by visualization by chemiluminescence (ECL, Amersham).

**GTPase activation assays**

For GTPase activation analysis of in vivo expressed Graf constructs, F-Graf and the GAP domain mutants were immunoprecipitated from 1 mg of over-expressing COS-1 cells using the Flag-tag specific monoclonal antibody M5 (10 µg/ml). Immune complexes were washed twice with radioimmunoprecipitation assay buffer (RIPA), once with TBS and once with GAP buffer and resuspended in 100 µl GAP buffer. Cdc42 or Rho (gifts from Alan Hall) were expressed as GST fusion proteins in *E. coli* and purified by thrombin cleavage as previously described (Self and Hall, 1995). For the GAP assays, 1 µg of purified Cdc42 or Rho were loaded with 20 µCi of [γ-32P]GTP (6,000 Ci/mmol; Dupont NEN) in loading buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mg/ml BSA, 1 mM DTT) for 15 minutes at RT. MgCl2 was added to a final concentration of 10 mM to stop the exchange reaction. The labeled G protein was diluted to 5 nM in GTPase buffer (50mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mg/ml BSA, 1 mM DTT) combined with the Graf immunoprecipitates, then incubated for 15 minutes at RT. Following incubation, 1 ml of wash buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT) was added and the mixture was filtered through nitrocellulose membranes (B85; Schleicher & Schuell). Membranes were washed with 10 ml of wash buffer and counted to determine the amount of [γ-32P]GTP remaining. To ensure equal loading, one-fifth of each immunoprecipitate was removed and subjected to immunoblot analysis using the M5 mAb.

**RESULTS**

**Graf regulation of actin polymerization**

We have previously shown that Graf stimulates GTPase activity of both Cdc42 and Rho in vitro (Hildebrand et al., 1996). To determine whether Graf exhibited a preference in vivo for one of these GTPases we examined the morphology of Swiss 3T3 cells upon microinjection of Graf cDNA. Previous studies have shown that microinjection of each of these smw G proteins has profound effects on the cytoskeletal organization of cells; Cdc42 stimulates filopodial extensions whereas Rho stimulates the formation of stress fibers. Microinjection of Graf cDNA into Swiss 3T3 cells (in the presence of serum) caused clearing of stress fibers followed by formation of long actin based filopodial-like extensions (Fig. 1A-F). The percentage of F-Graf-injected cells that exhibited the extended phenotype (as assessed by co-injecting green fluorescent protein (GFP) cDNA and scoring for GFP fluorescence) was approximately 57% (Fig. 3C). When cells were injected with F-Graf in the absence of GFP and injected cells were scored by anti-Flag staining, the percentage of cells exhibiting Graf induced cytoskeletal changes was 76%. A similar phenotype was observed when Graf was overexpressed in other fibroblasts including REF 52 and 10T1/2 cells (data not shown).

We microinjected cDNAs encoding activated Rho, C3 (a Rho-inhibitor) and the activated and dominant-negative forms of Cdc42 and Rac into growing Swiss cells to determine which of these constructs could mimic the effects of Graf microinjection. Microinjection of activated Cdc42 (L61) caused the formation of small microspikes (data not shown), an effect similar to, although not as robust as, what has been previously reported following microinjection of activated Cdc42 in quiescent cells. The Cdc42 (L61) induced microspikes are distinct from the much broader and larger extensions we observed after microinjection of Graf into Swiss 3T3 cells. Microinjection of the dominant-negative Cdc42 (N17), activated or dominant negative Rac (L61 or N17) and activated Rho (L63) into the growing cells had little, if any, effect on overall cellular structure as assessed by fluorescein-phalloidin staining (data not shown). However, an increase in stress fibers was observed upon microinjection of the activated Rac (L61) and Rho (L63) constructs (data not shown). Interestingly, inhibition of Rho activity by microinjection of the C3 exoenzyme resulted in phenotypes similar to those observed following injection of Graf cDNA. Microinjection of C3 caused clearing of stress fibers following by formation of long actin based filopodial-like extensions (Fig. 1G and H). These data suggest that Graf, like C3, downregulates Rho activity which, in turn, stimulates (or stabilizes) cellular filopodial-like extensions in growing cells.

To investigate the dynamics of the Graf-mediated filopodial extensions, cells were monitored by video microscopy following microinjection. As shown in Fig. 2, the extended filopodial-like extensions induced by Graf injection resulted from the continuous formation of filopodia and lamellipodial-like structures and was not due to mere contraction of the cell body. The Graf-induced projections grew continuously up to 4 hours post-injection. Interestingly, immunofluorescent staining of the Graf injected cells indicates that Graf is localized in punctate structures along the protruding actin-based extensions (Fig. 1E-F).

To confirm that the effect of Graf on Swiss 3T3 morphology was dependent on its ability to act as a GTPase activating protein, we examined the effects of Graf domain deletion mutations. Graf contains a centrally located GAP domain followed by a Ser/Pro rich domain and a carboxy-terminal SH3 domain (Fig. 3). Microinjection of wild-type Graf protein (F-Graf; 50 ng/µl) or a construct containing the GAP domain without the SH3 domain (F-GAP; 50 ng/µl) along with GFP (5 ng/µl) resulted in filopodial-like extensions in approximately 57% and 55% of GFP-expressing cells, respectively. In contrast, microinjection of the amino-terminal (F-NT) or SH3 (F-SH3) domains with GFP had no effect on cellular morphology (Fig. 3C).

As observed in Fig. 3B, overexpression of F-Graf results in the production of two protein bands. Previous experiments have shown that the slower migrating band results from phosphorylation of Ser 510 by p42 mitogen-activated protein (MAP) kinase (Taylor et al., 1998b). To determine whether phosphorylation of this site modulates the Graf-mediated cytoskeletal changes of Swiss 3T3 cells, we microinjected F-Graf with a point mutation (S510A) which prevents the in vivo phosphorylation of Graf at Ser 510.
phosphorylation of Graf. Microinjection of this mutant resulted in the extended phenotype in 51% of GFP-expressing cells (as scored in 5 separate experiments), suggesting that phosphorylation does not significantly affect the GAP activity of Graf (data not shown).

To confirm that GAP activity is required for the Graf-mediated cytoskeletal changes, we created point mutations in the GAP domain which rendered Graf enzymatically inactive. Sequence alignment of the Rho-GAP family members reveals that nine residues within the GAP domain of known Rho-GAPs are completely conserved (Fig. 4A; Barrett et al., 1997). In light of recent evidence provided by the crystal structure of the p50 Rho-GAP, we chose to individually mutate two conserved residues within the GAP domain of Graf which form part of a hydrophilic core in p50 Rho-GAP.

In co-crystals of Cdc42 and p50RhoGAP, amino acid R85 in p50RhoGAP (R236 in Graf) was shown to make direct contacts with Gly12 of Cdc42 and has been proposed to stabilize a transition state of the GTP-bound Cdc42 (Barrett et al., 1997; Rittenger et al., 1997). Other Rho-GAP residues which made contacts with the switch 1 region of Cdc42 include Lys 189, Thr 191 and Asn 194 (Lys 346, Thr 348 and...
Asn 351 in Graf; Rittenger et al., 1997). Of these, only Asn 194 is conserved among the Rho-GAPs with the exception of p85 PI3 kinase which contains Val at this position and has a non-functional GAP domain. We therefore mutated Arg 236 in Graf to Gln and Asn 351 to Val in an attempt to create a GAP-deficient Graf. The R236Q and N351V mutants were catalytically inactive failing to enhance GTPase activity of Rho protein in vitro (data not shown). In addition, microinjection of the R236Q and N351V Graf mutants failed to induce cellular extensions (Figs 4 and 5) indicating that GAP activity is required for this effect. To rule out dosage effects, these cDNA constructs were injected at concentrations of up to 200 μg/ml with no effect on cell morphology (data not shown).

To more precisely determine which of the Rho family of small G protein(s) is regulated by Graf, we examined the ability of Graf to block SPP-mediated Rho-induced stress fiber formation and bradykinin-mediated Cdc42-induced microspike formation in serum starved Swiss 3T3 cells. Serum starvation leads to a loss of stress fibers, lamellipodia and membrane ruffles presumably due to a down-regulation of the Rho-related G proteins in these cells (Fig. 6A; Ridley and Hall, 1992). Microinjection of Graf cDNA into serum starved Swiss 3T3 cells did not significantly alter cellular morphology within 4 hours (data not shown). A 30 minute treatment with SPP induced stress fiber formation in nearly all of the quiescent cells (Fig. 6B). As shown in Fig. 6C-F, Graf-expression blocked the SPP-induced re-organization of actin into stress fibers. In some Graf injected cells, SPP enhanced F-actin staining, as observed by the relatively intense staining of arc-like structures of polymerized actin, but these actin-rich filaments did not assemble into stress-fibers. In accordance with these data, injection of activated (L63)Rho along with FGraf in serum containing Swiss 3T3 cells resulted in the extended phenotype in less then 5% of the injected cells, indicating that Rho reversed the effects induced by Graf (data not shown).

To investigate the effects of Graf expression on bradykinin-induced microspikes, we injected N17Rac cDNA to block endogenous Rac activity prior to treatment with bradykinin (Fig. 7A and B). Since activation of Cdc42 leads to activation of Rac followed by Rho in these cells, N17Rac provides a means to block the activation of downstream G proteins and their subsequent effects on actin polymerization (compare the bradykinin-induced microspikes in the N17Rac injected cells in Fig. 7B vs the bradykinin-induced stress fibers in the uninjected cells in Fig. 7D and F). When Graf cDNA and N17Rac cDNA were coinjected into the quiescent cells and the cells were treated with bradykinin, the cells produced numerous microspikes indicating that Graf expression does not block Cdc42 signaling (Fig. 7C-F). These data indicate that Graf can block Rho-induced assembly of stress fibers, but not Cdc42-induced filopodia.

To further substantiate the specificity of Graf as a GAP for Rho in vivo, we examined the effect of Graf over-expression
in PC12 cells which typically express relatively high levels of endogenous Graf. Previous reports have shown that expression of C3 or the dominant-interfering mutant for Rho (N19) induces neurite outgrowth in these cells (Jalink et al., 1994; Gebbink et al., 1997). In addition, activation of Rho (by SPP or LPA) in both NGF-differentiated PC12 cells and NIE-115 cells induces neurite retraction and cell rounding (Postma et al., 1996; Tigyi et al., 1996). Unlike C3 and N19Rho, expression of GFP-Graf was not sufficient to stimulate neurite outgrowth in PC12 cells (data not shown).

To examine the effect of Graf overexpression on Rho-mediated neurite retraction, PC12 cells were transfected with GFP-Graf and treated with NGF for 72 hours. Under these conditions, neurite outgrowth was observed in a significant percentage of GFP-Graf expressing cells (data not shown). Fig. 8A and B show that SPP-induced neurite retraction is not blocked in PC12 cells overexpressing GFP-Graf. In fact, the GFP-Graf expressing cells actually appear to have an enhanced rate of SPP-induced neurite retraction compared to control cells. Neurites from GFP-Graf expressing cells retract as early as 6 minutes after application of SPP, whereas adjacent control cells require 20 minutes for a similar extent of retraction. In cells expressing GFP-Graf R236Q the rate of SPP-induced neurite retraction appeared similar to, if not slower than, neurite retraction in adjacent control cells (Fig. 8C and D). These data indicate that the Graf-mediated response is dependent on GAP activity and indicate the possibility that Graf may be an effector for Rho in certain cell types.

**DISCUSSION**

We previously reported that Graf has GAP activity for Rho A and Cdc42 in vitro but not Rac1 or Ras (Hildebrand et al., 1996). In this study we show that Graf specifically down-regulates Rho activity in vivo. Microinjection of Graf cDNA (or cDNA for the Rho-inhibitor C3) into subconfluent Swiss 3T3 cells, in the presence of serum, causes clearing of stress fibers followed by formation of long actin based filopodial-like extensions. In contrast, microinjection of Graf cDNA containing mutations in the GAP domain which render the protein enzymatically inactive have no effect on cellular morphology. Using serum starved Swiss 3T3 cells (in which Rho is down-regulated) we have shown that Graf blocks SPP stimulated (Rho-mediated) stress fiber formation, but not bradykinin stimulated (Cdc42-mediated) filopodial extensions. Although purified Graf can accelerate the GTP hydrolysis of...
both Cdc42 and Rho in vitro, our observations clearly show that Graf exhibits specificity for Rho-mediated signaling in vivo.

The filopodial extensions observed following microinjection of either Graf or C3 into Swiss 3T3 cells are likely due to inactivation of Rho GTPase activity and the subsequent un-opposed activation of Cdc42 and Rac. Since in these experiments the cells are cultured in complete medium containing serum, the initial activation of Cdc42 and Rac could be due to activating factors in the serum, or to positive feedback signaling resulting from downregulation of Rho. The later has been suggested to occur in the NIE-115 neuroblastoma cells whereby in serum starved cells, C3-mediated neurite outgrowth was inhibited by either dominant negative Cdc42 (T17N) or Rac (T17N) (Kozma et al., 1997). This apparent feedback signaling may be due to the disruption of the balance among the regulatory factors of these smw G proteins. The rate of conversion between the GDP-bound form and GTP bound form of smw G proteins is modulated by guanine nucleotide dissociation inhibitors (GDIs) which inhibit GDP dissociation, guanine nucleotide exchange factors (GEFs) which stimulate the replacement of GDP by GTP, and by GAPs which stimulate the rate of intrinsic GTP hydrolysis by the GTPase. Thus, Graf, by virtue of it being a GTPase activator for Rho would be expected to increase the level of GDP bound Rho and thus increase the amount of Rho available for binding to the Rho GDI. Since Rho GDI binds indiscriminately to Rho, Cdc42 and Rac, the equilibrium of GDI-bound Cdc42 and Rac could be shifted to favor release of these proteins from the GDI and subsequent activation by their respective guanine nucleotide exchange factors (Boguski and McCormick, 1993).

The specificity of Graf as a GAP for Rho, but not Cdc42 is intriguing, in light of the ability of Graf to enhance the rate of GTP hydrolysis for both smw G proteins in vitro (Hildebrand et al., 1996). The data supporting the conclusion that Graf is a Rho-specific GAP include the similar phenotypes observed after Graf and C3 injection into serum-containing Swiss 3T3 cells, and the ability of Graf to block SPP stimulated stress fibers, but not bradykinin stimulated actin projections. SPP signals through the recently identified EDG-1 receptor, a heterotrimeric G-protein coupled receptor which is linked (by a poorly understood mechanism) to Rho activation and Rho-dependent formation of stress fibers and focal adhesions (Lee et al., 1998). SPP treatment has also been shown to transiently increase the total F-actin pool in starved cells although this ‘pool’ is not readily incorporated into stress fibers (Machesky and Hall, 1997). We show here that in the presence of Graf, SPP induces an increase in actin staining without the bundling of these filaments into stress fibers. Interestingly, a recent report showed that LPA-stimulated (Rho-mediated) stress fiber formation was inhibited by the contraction inhibitor, 2,3-butanedione 2-monoxime, whereas LPA-induced actin

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**Fig. 4.** Filopodial extensions in cells expressing catalytically inactive Graf. (A) The sequence alignment of the GAP domains of Graf, p50RhoGap, and p85 PI3Kinase (p85) is shown. The nine conserved residues within the GAP domain of known Rho-GAPs are indicated in shaded boxes. The mutations introduced into the GAP domain are indicated by asterisks (residues R236 and N351 in Graf). (B) F-Graf, or F-Graf(R236Q) or N351V were expressed in COS-1 cells as described in Materials and Methods. The cells were lysed in sample buffer analyzed by SDS-PAGE and western blotting using the M5 Flag-specific Ab as described in Materials and Methods. (C) Subconfluent Swiss 3T3 cells were microinjected with either F-Graf, R236Q or N351V cDNA (50 ng/μl) along with GFP cDNA (5 ng/μl). The cells were incubated for 3 hours prior to fixation and incubation with Texas Red conjugated phalloidin to visualize filamentous actin. The data are presented as the percentage of injected cells (as assessed by GFP fluorescence) that exhibit the extended phenotype. The number of GFP-expressing cells for each construct was: F-Graf, 69 cells in 3 separate experiments; R236Q, 67 cells in 5 separate experiments and N351V, 77 cells in 4 separate experiments.
polymerization was unaffected by this inhibitor (Chrzanowska-Wodnicka and Burridge, 1996). These data led the authors to propose that Rho regulates the actin cytoskeleton by two synergistic pathways; one involving a kinase cascade resulting in contraction and stress fiber formation and the other possibly involving activation of PIP2 leading to actin polymerization (Chrzanowska-Wodnicka and Burridge, 1996). Due to the divergent sensitivity of these endpoints to Graf, our data suggest that the kinase/contractility pathway is dependent on active (GTP bound) Rho, whereas the PIP2/actin polymerization pathway is not.

The candidate kinases which could be involved in Rho-mediated stress fiber formation include protein kinase N, PRK2 kinase and a family of RhoA binding kinases termed p160ROKα (rat homologue) or ROCK (mouse homologue), or p164 Rho kinase (bovine homologue). Microinjection studies have revealed that both ROKα and PRK2 can affect Rho-dependent stress fiber formation, suggesting that these kinases phosphorylate substrates which mediate the Rho-dependent effects on actin rearrangement (Vincent and Settleman, 1997; Amano et al., 1997). Interestingly, a substrate for the Rho-stimulated kinase, ROCK is the myosin-binding, regulatory subunit of the myosin phosphatase PP1-M. Phosphorylation of PP1-M by ROCK inactivates the phosphatase and in turn enhances myosin-light chain phosphorylation, an effect that stimulates actin-myosin mediated contraction (Amano et al., 1996a; Kimura et al., 1996). Since the formation of focal adhesions and stress fibers has been linked to actin-myosin contractility, ROCK

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**Fig. 5.** Morphology of cells expressing Graf GAP domain variants. Subconfluent Swiss 3T3 cells were microinjected with either F-GAP (50 ng/µl; coinjected with 5 ng/µl GFP, A and B), R236Q (50 ng/µl; C and D) or N351V cDNA (50 ng/µl; E and F). The cells were cultured for 3 hours prior to fixation and staining. The injected cells were visualized by either GFP fluorescence (A) or with an anti-Flag antibody (C and E) as described in Materials and Methods. Filamentous actin was visualized with fluorescently tagged phalloidin (B,D,F). Injected cells are indicated by the arrows, un-injected neighboring control cells are denoted by asterisks.
Graf regulates Rho signaling could be also be involved in mediating the effects of Rho on the cytoskeleton (Burridge and Chrzanowska-Wodnicka, 1996).

To date, the only candidate protein proposed to be involved in Rho-stimulated actin polymerization is the lipid kinase, phosphatidylinositol 4-phosphate-5-kinase, PIP5-K which converts phosphatidylinositol-4-phosphate (PIP) to PIP2 (Ren et al., 1996). The ability of PIP2 to bind and regulate several actin binding proteins including those involved in filament capping, cross-linking and severing (Gilmore and Burridge, 1996; Fukami et al., 1992; Hirao et al., 1996; Lassing and Lindberg, 1985) suggests that the synthesis of PIP2 may be a critical regulatory step in Rho-mediated actin polymerization and assembly of stress fibers and focal adhesions. Interestingly, Rho binds to and stimulates PIP5-K in a GTP independent fashion (Ren et al., 1996), consistent with the lack of effect of Graf on this cellular process.

In contrast to the results in Swiss 3T3 cells, we show that Graf appears to enhance SPP-induced Rho-mediated effects in PC12 cells. Evidence using NGF-differentiated PC12 cells and other neuroblastoma cells (NIE-115 and NG108) indicates that the Rho family members control the actin-based axonal extensions within the growth cones of these cells. Activation of Rac and Cdc42 has been shown to promote the formation of lamellipodia and filopodia along the neurite extensions in NIE-115 cells (Kozma et al., 1997). Activation of Rho in both NGF-differentiated PC12 cells and NIE-115 cells induces neurite retraction and cell rounding (an effect that is mediated by Rho-kinase) (Postma et al., 1996; Tigyi et al., 1996; Katoh et al., 1998). Since Graf blocked SPP-induced stress fiber formation in Swiss 3T3 cells, we predicted that

Fig. 6. Graf inhibits SPP-induced Rho-mediated stress fiber formation in serum-starved Swiss 3T3 cells. Serum starved quiescent Swiss 3T3 cells, either uninjected controls (A and B) or cells microinjected with 200 μg/ml Graf cDNA (in PRK5-myc; C-F), were cultured for 4 hours prior to treatment with vehicle (A) or 5 μM SPP (B-F) for 30 minutes. Injected cells were visualized using an anti-myc antibody (9E10) as described in Materials and Methods (C and E). Filamentous actin was visualized with fluorescently tagged phalloidin (A,B,D). Injected cells are indicated by the arrows, un-injected neighboring control cells are denoted by asterisks. The phenotype shown was observed in 35/46 myc-positive cells.
overexpression of Graf in NGF-differentiated PC12 cells would attenuate SPP-induced neurite retraction. Somewhat surprisingly, we found that overexpression of Graf enhanced the apparent rate of Rho-mediated neurite retraction in PC12 cells. In cells expressing the GAP-deficient R236Q mutant, neurite retraction was virtually indistinguishable from control cells. One might expect that relatively high levels of expression of the R236Q mutant would be sufficient to have a dominant-interfering effect on endogenous Graf signaling and therefore block neurite retraction. However, such levels may not be consistently maintained in transfected PC12 cells at the time point at which experiments were carried out (96 hours after transfection). Nonetheless, these data are consistent with the requirement for GAP activity for Graf-mediated increases in neurite retraction and suggest that Graf may act to transmit rather than inhibit Rho signaling in PC12 cells.

Several GAP proteins including p120Ras GAP, n-chimaerin, PLCβ and the γ subunit of phosphodiesterase have been shown to transmit signals down-stream of their respective G proteins (Duchesne et al., 1993; Boguski and McCormick, 1993; Kozma et al., 1996). In the case of p120Ras GAP this signal may involve targeting other signaling molecules to Ras via protein-interactions directed by the SH3 domain located in the N terminus of RasGAP. Evidence supporting this hypothesis include the ability of a monoclonal antibody which was found to recognize the SH3 domain in RasGAP and synthetic peptides corresponding to this region to block activated Ras (K12) induced germinal vesicle breakdown in *Xenopus* oocytes (Duchesne et al., 1993). Presumably, p120Ras GAP is directed to an additional binding partner(s) upon binding GTP-Ras. Thus it is possible that Graf, like p120Ras GAP, transmits downstream signals through SH3 domain-mediated protein interactions.

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**Fig. 7.** Graf does not block bradykinin induced Cdc42-mediated microspikes in serum-starved Swiss 3T3 cells. Serum starved quiescent Swiss 3T3 cells microinjected with 200 μg/ml N17Rac cDNA (in PRK5-myc) in the absence (A and B) or presence of 200 μg/ml Graf cDNA (in PRK5-myc; C-F). The cells were cultured for 4 hours prior to treatment with 100 ng/ml bradykinin (B,E-H) for 15 minutes. Injected cells were visualized using an anti-myc antibody (9E10) as described in Materials and Methods (A,C,E). Filamentous actin was visualized with fluorescently tagged phalloidin (B,D,F). Injected cells are indicated by the arrows, un-injected neighboring control cells are denoted by asterisks. The phenotypes shown were observed in 24/30 myc-positive N17Rac injected cells and 33/42 myc-positive cells coinjected with N17Rac and Graf.
Graf regulates Rho signaling

The data presented in this paper indicate that Graf specifically regulates Rho activity in vivo. We have shown that Graf downregulates Rho activity in Swiss 3T3 cells but apparently enhances Rho-dependent effects in PC12 cells. The ability of Graf to be a signal terminator in Swiss 3T3 cells but a signal transmitter in PC12 cells could be due to the different levels of endogenous Graf protein (and Graf binding partners) in these cell lines. We have previously shown that Graf is not expressed at detectable levels in Swiss 3T3 cells (or other fibroblasts) but is highly expressed in PC12 cells (Taylor et al., 1998b). Since the ability of Graf to act as a signal transducer is likely due to protein-protein interactions, it is possible that the down-stream target for Graf in PC12 cells is also lacking in Swiss 3T3 cells. Thus, in Swiss 3T3 cells ectopic expression of Graf merely inactivates Rho and does not transmit further signaling. Since Graf is enriched in the brain, and appears to regulate Rho-mediated neurite retraction, Graf may play an important role in neuronal cell morphology. However, further investigation is required to define the precise role of Graf in this context.

This work was supported in part by Grants CA29243 and CA40042 from the DHHS-NCI and Grant 4491 from the Council for Tobacco Research, Inc. J.M.T. was supported by National Research Award H1-F32-GM18297-01. We thank Dr Alan Hall for providing the C3, Cdc42, Rac and Rho constructs. We thank Dr Scott Weed for

Fig. 8. Graf enhances SPP-induced neurite retraction in NGF-differentiated PC12 cells. PC12 cells were transfected with GFP-Graf (A and B) or the GFP-Graf R236Q variant (C and D) as described in Materials and Methods. Cells were treated with NGF for 72 hours to induce neurite extensions. Cells were treated with SPP (100 nM) and analyzed by video microscopy for 30 minutes. Selected frames from four separate experiments were captured using the ISee computer imaging program. GFP-Graf transfected cells were visualized by fluorescence microscopy (left panels). Phase contrast micrographs are shown at 0, 6, 12 and 20 minutes after treatment with SPP. The arrows denote neurites from GFP-Graf expressing cells and the asterisks denote neurites from adjacent control cells. Data are representative of cells analyzed in 13 (GFP-Graf) or 10 (GFP-Graf-R236Q) fields from 4 or 5 separate transfections, respectively.
providing the Flag-tagged cloning vectors, Andrei Karginov for cloning the GFP-Graf variants and both Dr Weed and YuniRui Du for helpful suggestions. We also thank Stephanie Morton for technical assistance.

REFERENCES


