MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 3169–3178 Vol. 16, No. 6
0270-7306/96/$04.00+0

An SH3 Domain-Containing GTPase-Activating Protein for Rho and Cdc42 Associates with Focal Adhesion Kinase

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Received 4 December 1995/Returned for modification 21 January 1996/Accepted 28 March 1996

The integrin family of cell surface receptors mediates cell adhesion to components of the extracellular matrix (ECM). Integrin engagement with the ECM initiates signaling cascades that regulate the organization of the actin-cytoskeleton and changes in gene expression. The Rho subfamily of Ras-related low-molecular-weight GTP-binding proteins and several protein tyrosine kinases have been implicated in mediating various aspects of integrin-dependent alterations in cell homeostasis. Focal adhesion kinase (FAK or pp125^{FAK}) is one of the tyrosine kinases predicted to be a critical component of integrin signaling. To elucidate the mechanisms by which FAK participates in integrin-mediated signaling, we have used expression cloning to identify cDNAs that encode potential FAK-binding proteins. We report here the identification of a cDNA that encodes a new member of the GTPase-activating protein (GAP) family of GTPase regulators. This GAP, termed Graf (for GTPase regulator associated with FAK), binds to the C-terminal domain of FAK in an SH3 domain-dependent manner and preferentially stimulates the GTPase activity of the GTP-binding proteins RhoA and Cdc42. Subcellular localization studies using Graf-transfected chicken embryo cells indicates that Graf colocalizes with actin stress fibers, cortical actin structures, and focal adhesions. Graf mRNA is expressed in a variety of avian tissues and is particularly abundant in embryonic brain and liver. Graf represents the first example of a regulator of the Rho family of small GTP-binding proteins that exhibits binding to a protein tyrosine kinase. We suggest that Graf may function to mediate cross talk between the tyrosine kinases such as FAK and the Rho family GTPases that control steps in integrin-initiated signaling events.

Integrin-mediated cell adhesion to the extracellular matrix (ECM) has both structural and biochemical ramifications for cell homeostasis (31). Integrins bind components of the ECM via large extracellular domains. These sites of integrin-ECM interaction trigger the formation of protein complexes on the cytoplasmic face of the plasma membrane termed focal adhesions. Focal adhesions contain several proteins with the capacity to simultaneously bind the short cytoplasmic tails of integrins and actin filaments. Thus, focal adhesions serve to anchor the actin cytoskeleton to the plasma membrane and directly link the extracellular and intracellular environments (10). Besides playing a structural role, the binding of integrins to ECM results in the initiation of intracellular signaling cascades (17, 49). Activation of these cascades has been proposed to regulate gene expression (22, 66), cell survival (8, 54), cell migration and adhesion, and differentiation (31). Recent studies have generated an extensive list of potential mediators of integrin signaling, including the cytoplasmic protein kinases focal adhesion kinase (pp125^{FAK} or FAK) (55), pp60^{src} (18, 35, 55), pp59^{fyn} (18), C-terminal Src kinase (Csk) (4), and protein kinase C isoforms α and δ (2, 32); the adaptor molecules Crk and Grb2 (5, 61); the p21 Ras guanine nucleotide exchange factors C3G and Sos (61, 65); and phosphoinositol 3-kinase (PI 3-K) (15, 24). FAK has been predicted to play a critical role in initiating cytoplasmic signals from focal adhesions (49, 59). Following cell adhesion to ECM components, FAK displays increases in both phosphotyrosine (pTyr) content and FAK-associated tyrosine kinase activity (23, 26, 40, 56). Phosphorylation of FAK on Tyr^{397} creates a high-affinity binding site for the Src homology 2 (SH2) domains of Src family kinases Src and Fyn (18, 57). The binding of activated Src family kinases to FAK leads to increased tyrosine phosphorylation of FAK (12) and other focal adhesion proteins, including paxillin (11), tensin (6), and p130^{CAS} (26a). When tyrosine phosphorylated, these proteins may serve to recruit SH2 domain-containing signaling molecules such as Grb2, Crk, and PI 3-K p85 subunit to focal adhesions (5, 12, 15, 60, 61).

FAK exhibits a unique domain structure, possessing a centrally located catalytic domain flanked by N- and C-terminal domains of 415 and 378 amino acid residues, respectively, that appear to serve as binding sites for potential regulators, effectors, and activators of FAK. In vitro, the N-terminal domain of FAK directly binds peptides that mimic the cytoplasmic domains of β_1, β_2, and β_3 integrins (58), while in vivo, the N-terminal domain also binds SH2 domains of Src family kinases (18, 57). Similarly, the C-terminal domain has been shown to bind the focal adhesion proteins paxillin (28), p130^{CAS} (26a), and talin (14), as well as the adaptor molecules Grb2 (61) and the p85 subunit of PI 3-kinase (15, 24). Finally, C-terminal residues 904-1041 of FAK contain a focal adhesion targeting (FAT) sequence, a domain that is both necessary and sufficient for focal adhesion localization (27). Although the paxillin binding site extensively overlaps the FAT sequence, the FAK-paxillin interaction does not appear to be sufficient to mediate efficient localization of FAK to focal contacts (28).

Integrin-mediated formation of focal adhesions and phosphorylation of FAK are largely dependent on the ability of a cell to form an intact cytoskeleton. Perhaps the most significant regulators of actin cytoskeleton are the small GTP-binding proteins of the Rho subfamily (25). In fibroblasts, it has been shown that Rho family members Cdc42, Rac1, and RhoA

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function to organize the actin cytoskeleton, including filopodium extension, lamellipodium formation, and generation of actin stress fibers and focal adhesions, respectively (37, 45, 50–52). Furthermore, these three GTPases function in a cascade, such that activation of Cdc42 leads to activation of Rac1, which in turn activates Rho (35, 43). The observed effects of Rho on the cytoskeletal organization may be mediated in part by PIP_2. Activation of Rho leads to an increase in phosphatidylinositol 4,5-bisphosphate (4,5-PIP_2) (16), which has been shown to regulate several actin-binding proteins (21, 33, 39, 67). Recently, these GTPases have also been implicated in regulating kinase cascades and gene expression, as activated Rac1 and Cdc42 stimulate c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) (20, 44), and activated Rac1, RhoA, and Cdc42Hs stimulate serum-response factor (SRF) (29). Similarly, GTP-bound forms of Rac1 and Cdc42 have been shown to bind and activate the cytoplasmic protein kinases $p65^{PAK}$ and $p120^{ACK}$, further linking this family of GTPases to kinase cascades (42, 43). Rho family GTPases also play a role in regulating growth control. Activated mutants of Rho GTPases and activators of Rho GTPases can mediate cellular transformation. Furthermore, Rac has been shown to mediate aspects of Ras-induced transformation (46). Finally, it is well documented that the ability of Rho family GTPases to participate in signaling events is determined by their nucleotide-bound state and subcellular localization (7). The cycling of GTPases between active GTP-bound states and inactive GDP-bound states is regulated by numerous GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDIs) (25).

We have used an expression cloning technique to identify binding partners for the C-terminal domain of FAK. We report here the identification of a cDNA that encodes a FAK-binding protein. The predicted amino acid sequence of this cDNA revealed two homology domains: a centrally located GAP domain and a distally located SH3 domain. In vitro analysis indicates that the protein encoded by this cDNA is a functional GAP for Cdc42 and RhoA and the SH3 domain binds to a proline-containing amino acid sequence in FAK. We refer to this molecule as Graf (GTPase regulator associated with FAK). Graf mRNA is expressed in a variety of avian tissues and is relatively abundant in brain and liver. Exogenous expression of the *Graf* cDNA in chicken embryo (CE) cells revealed that Graf can associate with actin-based cytoskeletal structures and may play a role in organizing cytoskeletal architecture.

MATERIALS AND METHODS

Cells and viruses. CE cells were prepared and maintained as described previously (9). For expression of Graf and FAK in CE cells, cDNAs encoding these proteins were cloned into RCAS retroviral vectors (subgroup A for FAK expression or subgroup B for Graf expression) (27, 28, 30) and transfected into primary CE cells by the calcium phosphate method (9, 48). Following transfection, cells were passaged for 7 to 9 days to allow for viral propagation and infection of the cell population. Typically, by day 5 posttransfection, transfected cell populations were resistant to superinfection by a virus of the same subgroup, indicating efficient transfection and virus propagation.

Screening of cDNA libraries. A λgt11 cDNA library derived from CE mRNA (Clontech, Palo Alto, Calif.) was screened by using a GST-FAK fusion protein, GST-Cterm (consisting of amino acid residues 686 to 1053 of avian FAK) that had been labeled in vitro with ^{32}P (28). Generation of ^{32}P-labeled GST-FAK was as follows. GST-Cterm was expressed in *Escherichia coli*, affinity purified by using glutathione-Sepharose, and phosphorylated in vitro using cyclic AMP-dependent protein kinase catalytic subunit (PKA, Sigma, St. Louis, Mo.) as described (28, 34). Briefly, GST-Cterm fusion protein (approximately 3 μg) coupled to glutathione-Sepharose was washed in heart muscle kinase (HMK) buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 12 mM $MgCl_2$), resuspended in 50 μl of HMK buffer containing 50 U of PKA, 100 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont NEN, Wilmington, Del.) and 1 mM dithiothreitol (DTT), and incubated for 45 min at room temperature (RT). Beads were subsequently washed once with 1 ml of HMK stop buffer (10 mM sodium phosphate [pH 8.0], 10 mM sodium pyrophosphate, 10 mM EDTA) and five times with 1 ml of Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 7.5], 150 mM NaCl). GST-Cterm was eluted twice at RT with constant rotation in 10 bed volumes of glutathione solution (20 mM reduced glutathione, 50 mM Tris-HCl [pH 8.0], 120 mM NaCl).

Screening of λgt11 expression libraries has been previously described (34, 64). Briefly, approximately 10^6 PFU were plated on 20 150-mm plates and grown for 4 h at 37°C. Plates were covered with nitrocellulose filters (B85; Schleicher & Schuell) that had been soaked in isopropyl-β-D-thiogalactopyranoside (IPTG) and returned to 37°C for an additional 8 h of growth. Filters were removed, denatured and renatured, and blocked in hybridization solution as follows. For denaturation and renaturation, filters were incubated twice for 5 min in 100 ml of Hyb75 (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.7], 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% Nonidet P-40) (34) supplemented with guanidine hydrochloride (GuHCl) to a final concentration of 6 M. The filters were then washed four times for 10 min per wash in 100 ml of Hyb75 containing 3 M GuHCl (a 1:1 dilution of the 6 M GuHCl solution). This step was repeated four times, each time using Hyb75 containing a twofold dilution of the GuHCl from the previous wash step. Filters were transferred to new dishes and washed twice for 30 min in 100 ml of Hyb75. Filters were then blocked for 30 min in 50 to 75 ml of Hyb75 plus 5% milk and then 30 min in 50 to 75 ml of hybridization solution plus 5% milk. Hybridization solution was generated as described previously (34). Briefly, DH5 *E. coli* cells harboring the parental pGex2TK vector were induced to express GST with IPTG (0.1 mM final concentration) for 2 h at 37°C. Following induction, bacteria were pelleted, resuspended in 1/20 the original volume in Hyb75, and lysed on ice by sonication. Lysates were clarified by centrifugation at 12,000 × *g* for 15 min at 4°C. Hybridization buffer was stored at −20°C and diluted 1:5 prior to use. Following blocking, ^{32}P-labeled fusion protein was added to a final concentration of 10^6 cpm/ml of hybridization buffer. Filters were hybridized for 12 h at 4°C. Following hybridization, filters were washed three times for 15 min per wash at RT in 100 ml of Hyb75 plus 1% milk. Filters were then exposed to film for 12 h at −70°C with an intensifying screen. Positive clones were purified by successive rounds of plaque purification. cDNA inserts were cloned into pBluescript (Stratagene, La Jolla, Calif.) and sequenced on both strands by dideoxy sequencing. Genetics Computer Group sequence analysis software was used to analyze DNA and amino acid sequences.

Mutagenesis, epitope-tagged Graf, and fusion proteins. For expression of GST-GrafSH3, clone 11A cDNA sequences (see Fig. 1) encoding the C-terminal 113 amino acid residues (471 to 584) were cloned into pGex3X. Vectors encoding GST-AblSH3, and GST-Grb2, GST-ScrSH3, and GST-CasSH3 were generously provided by A. M. Pendergast, A. Pawson, J. Brugge, and A. H. Bouton, respectively. pGex2TK-cterm encodes the fusion protein GST-Cterm and was constructed by cloning *FAK* sequences that encode amino acid residues 686 to 1053 into pGex2TK. GST-GrafGAP was engineered by cloning 11A cDNA sequences that encode residues 171 to 404 into pGex3X. Mutations resulting in FAK point mutants pmP878A and pmP881A were generated in the *FAK* cDNA by site-directed mutagenesis (Altered Sites; Promega, Madison, Wis.) and confirmed by dideoxy sequencing. *FAK* sequences encoding the proline to alanine mutants were cloned into pGex2TK-Cterm for in vitro expression and evaluation.

The cDNA construct encoding the C-terminal epitope-tagged variant of Graf, cGraf, was generated as follows. The coding region of clone 11A was amplified by PCR using primers that generated 5′ *Nhe*I and 3′ *Cla*I restriction sites. The resulting PCR product was digested with *Nhe*I and *Cla*I and ligated into *Xba*I/*Cla*I-digested pBSctag (27, 28, 56) to generate pBS*Graf*-ctag. To facilitate cloning of *Graf*-ctag into RCAS B, *Graf*-ctag was cloned into the Cla12Nco adaptor plasmid (30). pBS*Graf*-ctag was digested with *Nco*I and *Sal*I and ligated into similarly digested Cla12Nco. The *Nco*I-*Sal*I fragment derived from *Graf*-ctag encodes Graf amino acid residues 92 to 584 fused in frame to the 11-amino-acid epitope tag consisting of the amino acid sequence KPPTPPPEPET. The *Graf*-ctag expression cassette was subsequently cloned from Cla12Nco into RCAS B as a *Cla*I fragment. The *Nco*I sites of clone 11A and Cla12Nco provide a eukaryotic initiation codon for expression in CE cells (30, 36).

In vitro association. For in vitro binding experiments, approximately 5 μg of GST, GST-AblSH3, GST-CasSH3, GST-Grb2, GST-SrcSH3, GST-GrafSH3, GST-Cterm, GST-CtermP878A, or GST-CtermP881A was added to either 1.0 mg of total cell protein (concentration of 1.0 mg/ml) derived from normal CE cells or 500 μg of total cell protein (concentration of 0.5 mg/ml) derived from CE cells expressing cGraf that had been lysed in radio immunoprecipitation assay buffer (RIPA). Protein complexes were washed three times with RIPA and twice with TBS, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38), and transferred to nitrocellulose. FAK and cGraf were detected by immunoblotting using the FAK-specific monoclonal antibody (MAb) 2A7 (55) or the epitope tag-specific MAb KT3 (which recognizes the peptide KPPTPPPEPET) at a concentration of 2.5 μg/ml (28, 41).

GTPase activation assays. RhoA, Rac1, Cdc42 (gifts from Alan Hall), Ras (a gift from Michael Weber), and the Graf GAP domain were expressed as GST fusion proteins in *E. coli* and purified by protease cleavage as described previously (50, 52). First, 40 ng of purified RhoA, Rac1, Cdc42, and Ras were loaded with 15 μCi of [γ-^{32}P]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 of bovine serum

albumin [BSA] per ml, 1 mM DTT) for 15 min at RT in a total volume of 30 μl. $MgCl_2$ was added to a final concentration of 10 mM, and the GTPase mixture was placed on ice. Then, 5 μl of each loaded GTPase was diluted to a final concentration of 5 nM in GTPase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mg of BSA per ml, 1 mM DTT) in the presence or absence of 1, 5, 10, or 20 nM Graf GAP domain and incubated for 15 min at RT. Following incubation, 1 ml of wash buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 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, air dried, and counted to determine the amount of [γ-^{32}P]GTP remaining (63). For analysis of in vivo-expressed Graf, cGraf was immunoprecipitated from 500 μg of total cell protein (concentration of 0.5 mg/ml) in RIPA using MAb KT3 at a concentration of 10 μg/mg of cellular protein. Immune complexes were washed three times with RIPA, once with TBS, and once with GAP buffer and resuspended in 100 μl of GAP buffer. GTPases preloaded with [γ-^{32}P]GTP were added to a final concentration of 5 nM, and the mixture was incubated for 15 min at RT with constant rotation. Following incubation, 1 ml of wash buffer was added. The immune complexes were pelleted, and the supernatants were removed and filtered as described above. Filters were washed and counted as described above. Before the immune complexes were washed in GAP buffer, one-fifth of each immunoprecipitate was removed and subjected to immunoblot analysis using MAb KT3.

Immunofluorescence. CE cells expressing cGraf alone or coexpressing FAK and cGraf were plated on glass cover slides and grown overnight. Cells were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS) and fixed using 3.7% paraformaldehyde in PBS for 20 min at RT. Cells were washed three times in PBS and permeabilized with 0.4% Triton X-100 in PBS for 3 min at RT. Slides were washed three times in PBS to remove Triton. For simultaneous detection of cGraf and actin, cells were incubated with MAb KT3 at a concentration of 10 μg/ml in PBS and TRITC-phalloidin (for detection of actin) (Sigma, St. Louis, Mo.) (1:5,000 dilution in PBS) for 1 h at RT. Slides were washed three times in PBS to remove excess antibody. Primary antibody was detected by using fluorescein isothiocyanate-conjugated goat anti-mouse Ab (Jackson ImmunoResearch, West Grove, Pa.) at a concentration of 5 μg/ml for 1 h at RT. For costaining of FAK and cGraf, cells were incubated with a mixture of MAb KT3 (10 μg/ml in PBS) and protein A-purified FAK-specific polyclonal antibody BC3 (1:400 dilution in PBS) (55) for 1 h at RT. Primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse Ab and Texas red-conjugated donkey anti-rabbit Ab (Jackson ImmunoResearch, West Grove, Pa.). Coverslips were washed in PBS and mounted on microscope slides for observation and photography.

Northern (RNA) blot analysis. RNA was isolated from 17-day CE tissues by phenol-chloroform extraction using RNAzol (Tel-Test, Friendswood, Tex.) and poly(A)-containing RNA was selected by using oligo(dT)-Sepharose (Qiagen, Chatsworth, Calif.). The GRAF cDNA probe (bp 285 to 1804) was radiolabeled with [α-^{32}P]CTP by nick translation using random primers. The probe (5×10^8 cpm/μg) was hybridized with poly(A)-RNA-containing filters overnight at 42°C and washed twice (15 min) in 0.2× SSC–0.1% SDS at 42°C and twice (15 min) in 0.1× SSC–0.1% SDS at 68°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Autoradiograms were obtained by exposure of blots to Kodak XAR film for 18 to 72 h.

Nucleotide sequence accession number. The DNA sequence of clone 11A has been deposited in GenBank under accession no. U36309.

RESULTS

Cloning of FAK binding proteins. We have previously described a C-terminal domain of FAK that is both necessary and sufficient for focal adhesion localization (27). In an effort to identify proteins that bind either the FAT sequence or other regions of the FAK C terminus, we used the entire C-terminal domain of FAK (amino acid residues 686 to 1053) to screen a λgt11 expression library derived from CE cDNA as described in Materials and Methods. Of the 10^6 PFU screened, one clone (clone 11A) was positively identified and purified. The translated amino acid sequence of clone 11A predicted a 584-amino-acid open reading frame contiguous with β-galactosidase encoded by the phage vector (Fig. 1A).

Analysis of the predicted amino acid sequence indicated that 11A encoded a novel SH3 domain-containing regulator of Ras-related low-molecular-weight GTP-binding proteins. Centrally located residues 213 to 363 showed extensive homology to GAPs for members of the Rho family of GTPases (Fig. 1B), showing highest homology to β-chimaerin. C-terminal amino acid residues 533 to 582 shared homology with SH3 domains of several proteins (Fig. 1C). On the basis of the limited degree of amino acid sequence similarity, this cDNA does not appear to encode the chicken homolog of a previously identified GAP or SH3 domain-containing protein. Finally, amino acid residues 396 through 527 comprise a proline/serine-rich motif in which the proline and serine content is approximately 43%. We refer to the protein encoded by this cDNA as Graf, for GTPase regulator associated with FAK.

Analysis of Graf mRNA expression. To determine the tissue distribution of Graf mRNA expression, poly(A)-containing RNA was prepared from individual tissues from 17-day CEs. Northern blot analysis using a probe directed to the GAP domain revealed a major 5.0-kb RNA species in most tissues (Fig. 2B). The 5.0-kb RNA species was most abundant in brain and liver (Fig. 2B). On the basis of the size of the major RNA species detected by Northern blot analysis and the total length of overlapping cDNAs isolated to date (approximately 3.0 to 3.2 kb), there remains approximately 1.8 to 2.0 kb of unidentified 5′ and 3′ sequence.

Graf is GAP for RhoA and Cdc42. Sequence analysis indicated that Graf should function as a GAP for members of the Rho subfamily of small GTP-binding proteins. To assess the GAP activity of Graf, a portion of Graf containing the GAP domain (residues 171 to 404) was expressed as a GST fusion protein, and the GST fusion protein was assayed for the ability to activate the intrinsic GTPase activity of GTP-bound Rac1, Cdc42, and RhoA. As shown in Fig. 3A, the Graf GAP domain was able to stimulate the intrinsic GTPase activity of RhoA and Cdc42 but not that of Rac1 or Ras (data not shown). In a parallel analysis, the GAP domain of p190 stimulated the GTPase activity of RhoA, Cdc42, and Rac1, showing highest activity towards Rho (16-fold activation of Rho GTPase activity; data not shown) in agreement with previously published data (63). These results indicate that Cdc42 and RhoA may be the preferred substrates for Graf.

To assess the GAP activity of Graf expressed in vivo, Graf was expressed in CE cells and assayed for activity towards RhoA, Rac1, Cdc42, and Ras (Fig. 3B). An epitope-tagged variant of Graf, cGraf was expressed in CE cells and immunoprecipitated by using the epitope-specific MAb KT3. One-fifth of each immunoprecipitate was removed and analyzed by immunoblotting to verify that equal amounts of cGraf were present in each experimental sample and that no cGraf was present in the negative control samples (inset, Fig. 3B). The remainder of each immunoprecipitate was then assayed for GAP activity. Figure 3B (inset) shows that comparable amounts of cGraf were recovered in each KT3 precipitation (+ lanes) used in the GAP assay, while there was no detectable cGraf in the control precipitations (− lanes). Immune complexes containing cGraf exhibited a six- to sevenfold increase in GAP activity towards Cdc42 and RhoA compared to immune complexes isolated from nontransfected cells. Immune complexes from cGraf-transfected cells showed little GAP activity with GTP-Rac or Ras. These results confirm the activity of Graf towards Rho and Cdc42 and suggest that the activity towards Rho and Cdc42 in vivo may be comparable. Considering that the Graf GAP domain exhibited highest sequence similarity to α- and β-chimaerins, GAPs that function exclusively on Rac GTPases (1), the lack of RacGAP activity associated with Graf was surprising.

The SH3 domain of Graf mediates binding to FAK in vitro. The experimental approach used to identify clone 11A indicated an interaction of Graf with the C-terminal domain of FAK. Since SH3 domains have been shown to mediate protein-protein interactions (19) we assayed the ability of the Graf SH3 domain to bind FAK in vitro. Portions (5 μg) of GST, GST-GrafSH3, GST-AblSH3, GST-SrcSH3, GST-CasSH3, or GST-

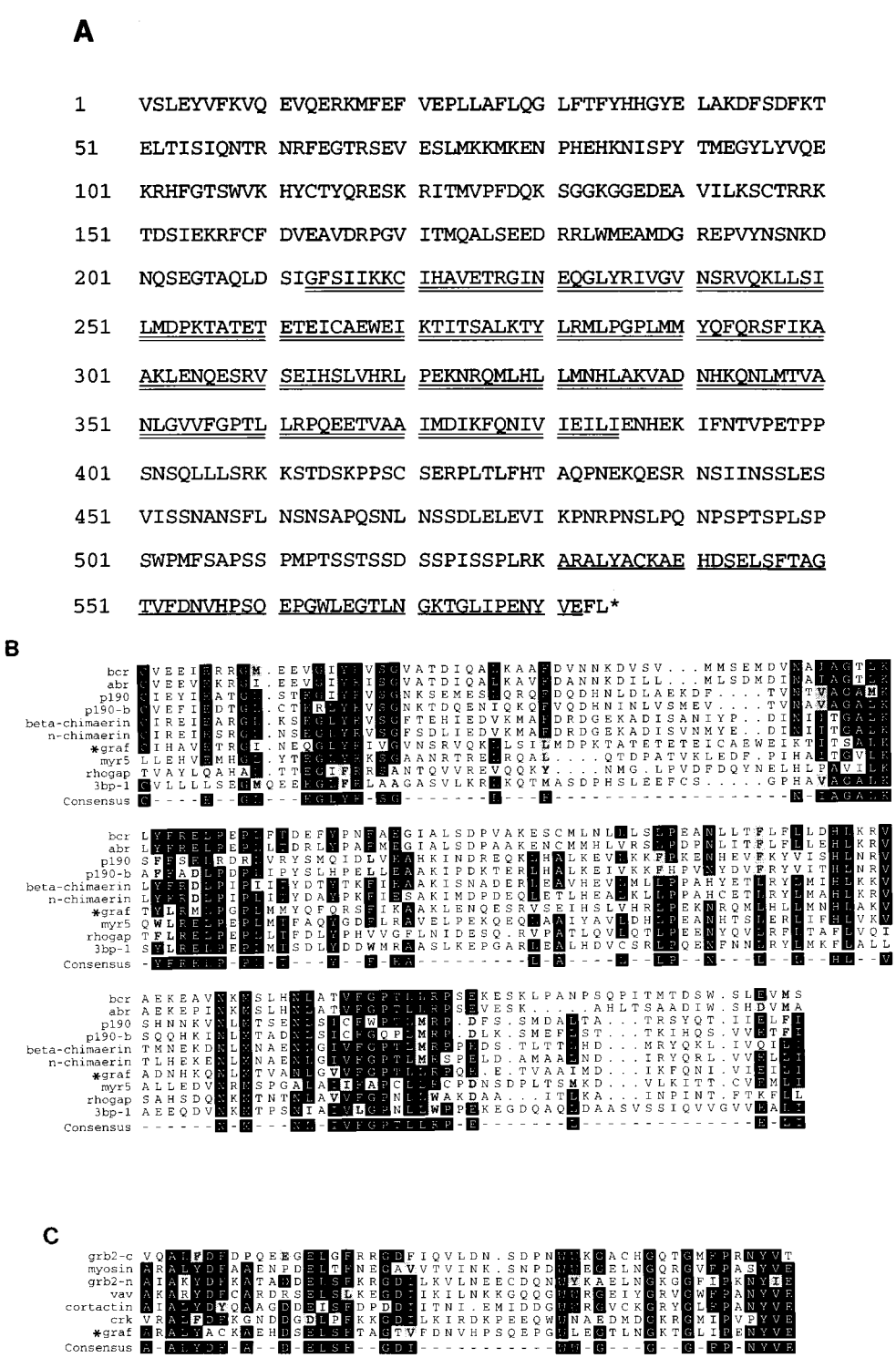

FIG. 1. Amino acid sequence of clone 11A and alignments of homology domains. (A) Predicted amino acid sequence of clone 11A. The putative GAP domain is underlined twice; the SH3 domain is underlined once; *, termination codon. (B) Alignment of the predicted Graf GAP domain (*) with other defined GAP domains. (C) Alignment of the predicted Graf SH3 domain (*) with other identified SH3 domains. For panels B and C, identical residues are boxed in black, while residues representing conserved substitutions are boxed in gray. Periods indicate where spaces were added in the sequence to achieve optimal alignments; dashes indicate there is no consensus amino acid at that position. Alignments were determined using the program Pileup from the Genetics Computer Group.

Grb2 fusion proteins coupled to glutathione-Sepharose were mixed individually with 1 mg of total cell protein (concentration of 1.0 mg/ml in RIPA) derived from CE cells. The GST complexes were recovered and assayed for the amount of bound FAK by immunoblotting the precipitated protein complexes with MAb 2A7. As shown in Fig. 4, the Graf SH3 fusion protein precipitated approximately 20% of the total FAK present in the lysate (compare lanes 1 and 3; lane 1 represents

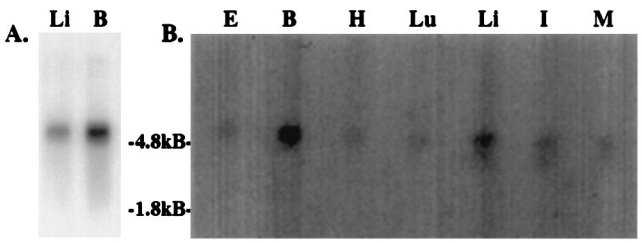

FIG. 2. Northern blot analysis of Graf mRNA. Poly(A)-containing RNA (2 μg), prepared from individual tissues of a 17-day-old embryo, were subjected to Northern blot analysis using an approximately 1.5-kb Graf-specific cDNA probe (nucleotides 285 to 1804). Panel A shows the analysis of RNAs prepared from liver (Li) and brain (B); panel B shows the analysis of RNAs prepared from eye (E), brain (B), lung (Lu), liver (Li), intestine (I), or skeletal muscle (M). The blot in panel B is overexposed to reveal the presence of low levels of Graf RNA in eye, heart, lung, intestine, and skeletal muscle. The positions of 28S (4.8 kb) and 18S (1.8 kb) RNAs are indicated.

the total amount of immunoreactive FAK in 100 μg of lysate). Conversely, GST (lane 2), AblSH3 (lane 4), and SrcSH3 (lane 5) bound no detectable FAK. The SH3 domain of $p130^{CAS}$ efficiently bound FAK in the lysate (lane 6), whereas the Grb2 fusion protein bound approximately 0.5 to 1% of the FAK in the lysate (lane 7). Since the GST-Grb2 fusion protein contains an SH2 domain as well as two SH3 domains, it is unclear that the observed interaction is mediated solely by the Grb2 SH3 domains.

The C terminus of FAK contains two proline-rich regions that may function as ligands for SH3 domains. The first proline-rich motif spans residues 712 to 721 and includes the sequence P^{712}PRPSRPGYP. This proline-rich sequence of FAK was dispensable for the Graf-FAK interaction, as a GST-FAK fusion protein consisting of residues 721 to 941 bound the GrafSH3 fusion protein in a far Western blot and bound cGraf in solution (data not shown). The second proline motif spans residue 875 to 884 and contains the sequence P^{875}KKPPR PGAP. To ascertain the role of this proline-rich sequence in FAK-Graf interactions, *FAK* cDNA sequences encoding the proline motif PKKPPRPGAP were mutated to encode either PKK$\underline{A}^{878}$PRPGAP or PKKPPR$\underline{A}^{881}$GAP and cloned into pGex2TK-Cterm. The resulting Cterm mutants, CtermP878A and CtermP881A, were expressed in *E. coli* and assayed for the ability to bind cGraf expressed in CE cells. As shown in Fig. 5B, Cterm (lane 4) and CtermP881A (lane 6) efficiently bound to cGraf. In contrast, CtermP878A showed substantially reduced binding (lane 5). As expected, GST alone showed no binding to cGraf (lane 2), and no KT3-reactive proteins were precipitated by Cterm in mock-transfected CE lysate (lane 3). These data indicate that the proline-containing sequence P^{875}KKPPR is sufficient for Graf binding to FAK, with Pro^{878} being an essential residue in the Graf SH3 ligand. Of interest in these experiments is the observation that CtermP881A reproducibly bound more cGraf than wild-type Cterm (compare lanes 4 and 6). The sequences encompassing amino acid residues 875 to 884 contain three overlapping PXXP motifs, the core sequence required for SH3 binding. Two of these are eliminated by the P881A mutation. Therefore, it is possible that normally other proteins in the cell lysate compete with Graf for binding to the 875 to 884 proline-rich sequence and that the P881A mutation relieves this competition. Support for this hypothesis comes from the observation of Guinebault et al. that the SH3 domain of the p85 subunit of PI 3-K binds to this proline-rich region in human FAK (24).

Subcellular localization of Graf. To investigate the potential role of Graf in vivo, an epitope-tagged variant of Graf was

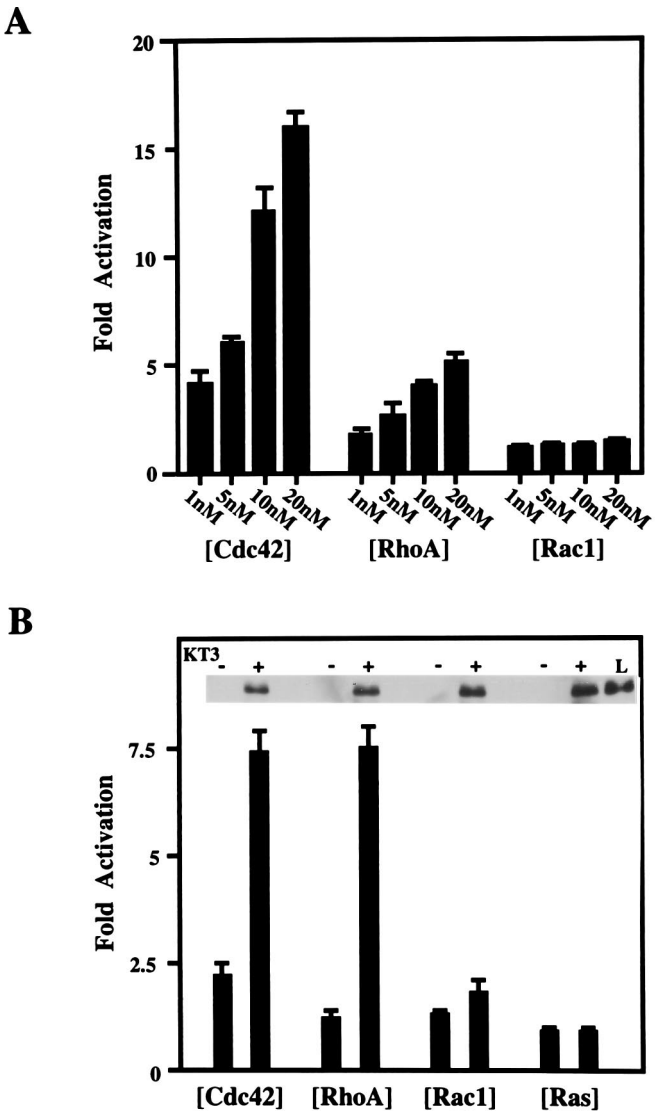

FIG. 3. GAP activity of Graf towards Rho family GTPases. RhoA, Rac1, Cdc42, and Ras GTPases were expressed in *E. coli* and purified as described in Materials and Methods. (A) Equal amounts (in moles) of each GTPase were preloaded with [γ-^{32}P]GTP and incubated in either the absence or presence of increasing concentrations of purified Graf GAP domain. The amount of [γ-^{32}P]GTP remaining bound to the GTPase was determined by a nitrocellulose filtration assay (see Materials and Methods) (63). Fold activation represents the ratio of radioactivity remaining following incubation in the absence of GAP domain versus the amount of radioactivity remaining following incubation in the presence of GAP domain. (B) cGraf was immunoprecipitated from 500 μg of total cell protein with either MAb KT3 (+) or a nonspecific MAb (−). One-fifth of each immunoprecipitate was evaluated for the presence of cGraf by immunoblotting with MAb KT3 (inset). The remainder of each precipitation was subsequently incubated with purified [γ-^{32}P]GTP-loaded GTPase (indicated across the bottom). Following incubation, the supernatants were removed and assayed by nitrocellulose filtration as described above. Each lane of the inset corresponds to the vertical data bar directly beneath it. For both panels A and B, all datum points represent the average value of at least three independent experiments, while the immunoblot of cGraf in panel B is representative of one such experiment.

generated and expressed in CE cells. CE cells expressing cGraf were grown overnight on glass cover slides and stained by using MAb KT3. As shown in Fig. 6A and B, some cells overexpressing Graf displayed a diffuse cytoplasmic staining. However, a significant portion of the Graf staining was localized to the

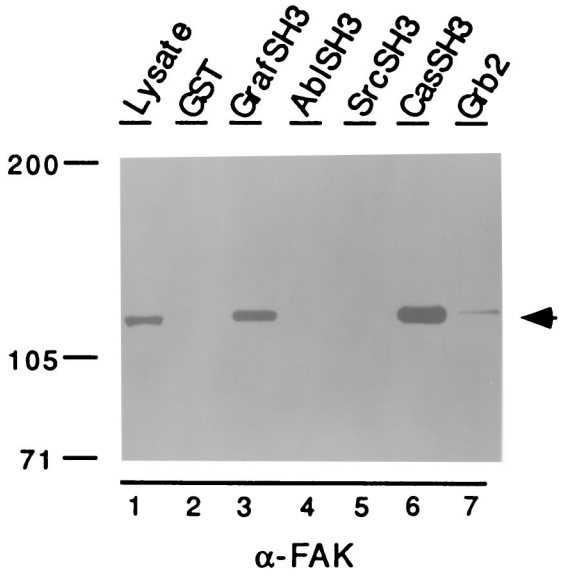

FIG. 4. In vitro binding of the Graf SH3 domain to FAK. GST-fusion proteins (5 μg) coupled to glutathione-Sepharose were added to 1.0 mg of total cell protein derived from CE cells and incubated for 1 h. Protein complexes associated with the beads were pelleted, washed, resolved by SDS-PAGE, transferred to nitrocellulose, and assayed by immunoblotting with the FAK-specific MAb 2A7 as described in Materials and Methods. Lysate (lane 1) depicts the amount of immunoreactive FAK in 100 μg of total cell protein and serves as a 10% loading control. GST fusion proteins used for each sample are indicated across the top.

actin cytoskeleton, including both actin stress fibers and cortical actin (open arrows, panels A and B). To assess the possible colocalization of Graf and filamentous actin, CE cells expressing cGraf were costained with MAb to the KT3 tag (panels C, E, and G) and TRITC-phalloidin to detect actin stress fibers (panels D, F, and H). There was a significant colocalization of cGraf with both actin stress fibers (C versus D and E versus F) and cortical actin (G versus H). The cells depicted in Fig. 6 also show that Graf overexpression may affect cytoskeleton integrity. The cell displayed in panels C and D had relatively low levels of cGraf expression and exhibited well-defined actin stress fibers and normal cell shape. In contrast, the cells shown in panels G and H displayed intense staining with KT3 (G), contained few actin stress fibers (H), and exhibited an abnormal cell shape.

Colocalization of FAK and cGraf. The ability of FAK and Graf to associate in vivo would indicate a possible role for this interaction in integrin-mediated signaling events. Thus, CE cells overexpressing both FAK and cGraf were immunostained by using MAb KT3 alone (Fig. 7A) or in combination with the FAK-specific polyclonal sera BC3 (Fig. 7B and C). In CE cells expressing both FAK and cGraf, cGraf localized to structures similar to focal adhesions (panel A). To further test if these cellular structures were focal contacts, cells were costained for the detection of cGraf (panel B) and FAK (panel C). As shown in panels B and C, there was colocalization of cGraf and FAK in focal adhesion structures (open arrows). In these cells, cGraf also colocalized with actin fibers, whereas the FAK-specific antibody, BC3, stained predominantly focal adhesions, indicating that cGraf expression did not alter FAK localization.

DISCUSSION

Using expression cloning, we have identified a novel GAP for the Rho family GTPases that we refer to as Graf. Graf contains several readily identifiable domains, including a centrally located GAP domain, a serine-proline-rich domain, and a C-terminally located SH3 domain. To date, we have been unable to identify the authentic 5′ end of the Graf cDNA; thus, additional domains may reside N terminal to the consensus GAP domain. In addition, the presence of a serine-proline-rich domain predicts that Graf may be the target of proline-directed serine/threonine kinases, perhaps phosphorylating Graf in a cell cycle-specific fashion. Biochemical characterization of Graf demonstrates that Graf can function as a negative regulator of RhoA and Cdc42. Graf also preferentially binds to the C-terminal domain of FAK via a proline-rich motif in FAK and the SH3 domain of Graf. The potential localization of a GAP for Cdc42 and/or Rho to cytoskeletal structures, including focal adhesions and actin stress fibers, is intriguing in light of the recent work implicating Rho family GTPases in regulating cytoskeletal organization and architecture (including stress fiber and focal adhesion formation, membrane ruffling, and filopodium outgrowth), kinase cascades, and gene expression (20, 29, 37, 44, 45, 50, 52, 62).

Recent evidence shows that the Rho family GTPases Cdc42, Rac, and Rho act in a cascade to regulate the formation of filopodia, lamellipodia, actin microfilaments, and focal adhesions (37, 45, 50, 52). The mechanism(s) by which integrin-ECM interactions trigger the activation of these GTPases is unclear. Despite the fact that several potential effectors of Cdc42, Rac, and Rho have been characterized, the mechanisms by which these enzymes act to influence cytoskeletal architecture largely remains unknown. It has been reported that activated Rho regulates the production of 4,5-PIP_2 by stimulating the activity of a phosphatidylinositol 5′-kinase (PI 5-K) (16). In turn, 4,5-PIP_2 has been shown to regulate the

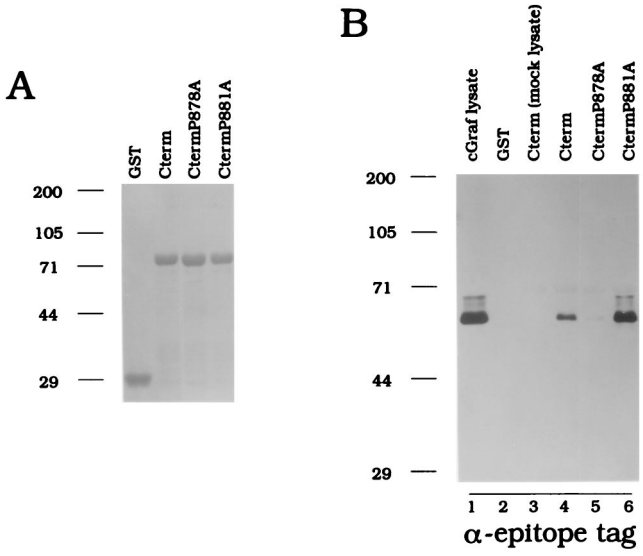

FIG. 5. Pro^{878} in the FAK C terminus is essential for efficient binding to the Graf SH3 domain. (A) To ensure that equal amounts of fusion protein were added to each sample, equal volumes of each GST-Cterm fusion protein were subjected to SDS-PAGE and Coomassie blue staining. (B) Equal amounts (5 μg) of GST, GST-Cterm, GST-CtermP878A, and GST-CtermP881A (indicated across the top) coupled to glutathione-Sepharose were added to 500 μg of total cell protein derived from CE cells expressing cGraf (lanes 2 and 4 to 6) and incubated for 1 h. Following incubation, protein complexes associated with the beads were pelleted, washed, resolved by SDS-PAGE, transferred to nitrocellulose, and assayed by immunoblotting with MAb KT3 as described in Materials and Methods. Lane 1 depicts the amount of cGraf in 50 μg of total cell protein and serves as a 10% loading control. For lane 3, cell lysate derived from mock-transfected CE cells was used as a negative control.

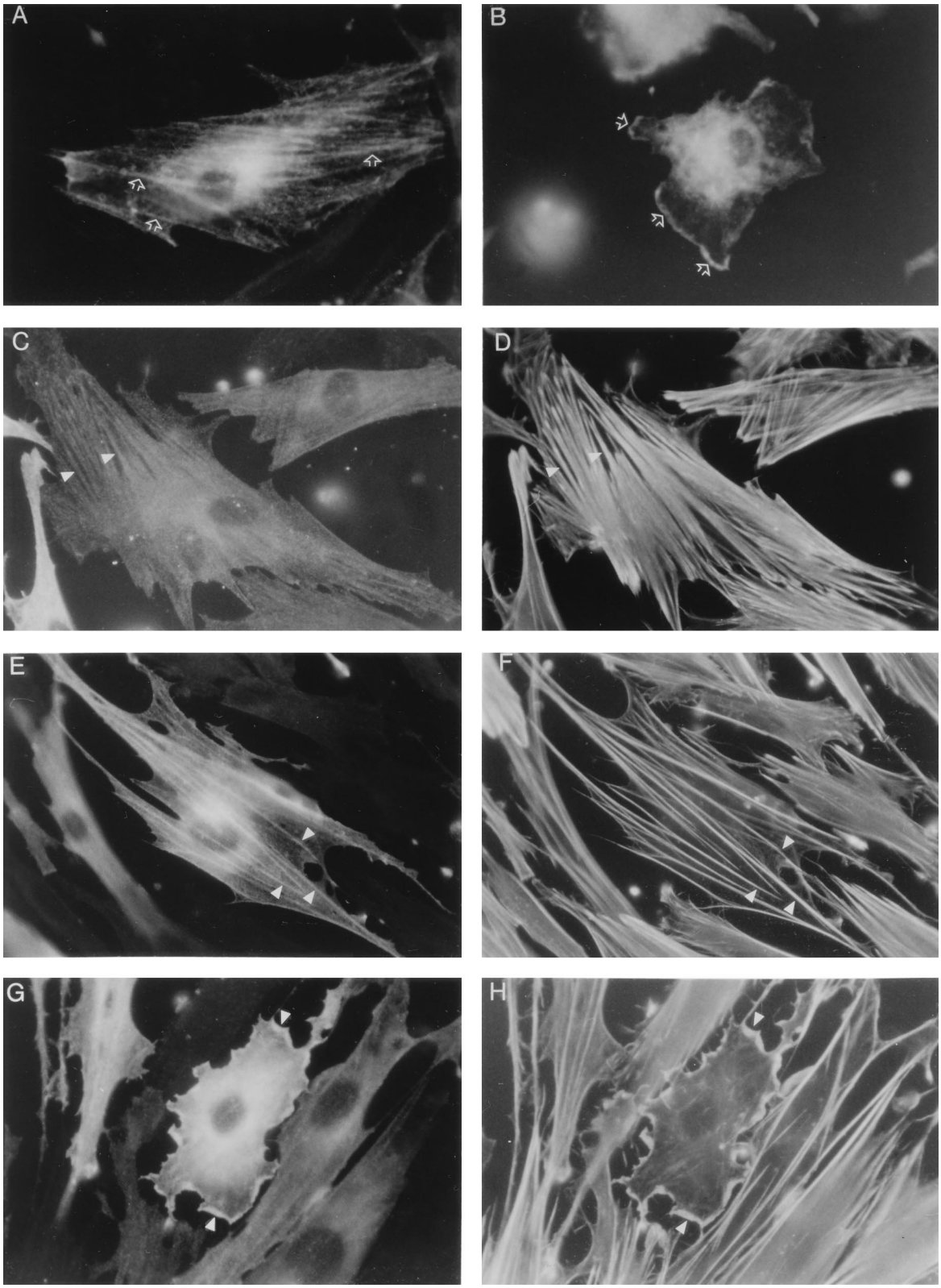

FIG. 6. Colocalization of Graf with the actin cytoskeleton. (A and B) CE cells were grown on glass cover slides and stained with MAb KT3 for detection of cGraf. Arrows indicate putative actin stress fibers and cortical actin. (C to H) Cells were costained with MAb KT3 to detect cGraf (C, E, and G) and phalloidin to detect filamentous actin (D, F, and H). Arrowheads indicate regions of cGraf and actin colocalization.

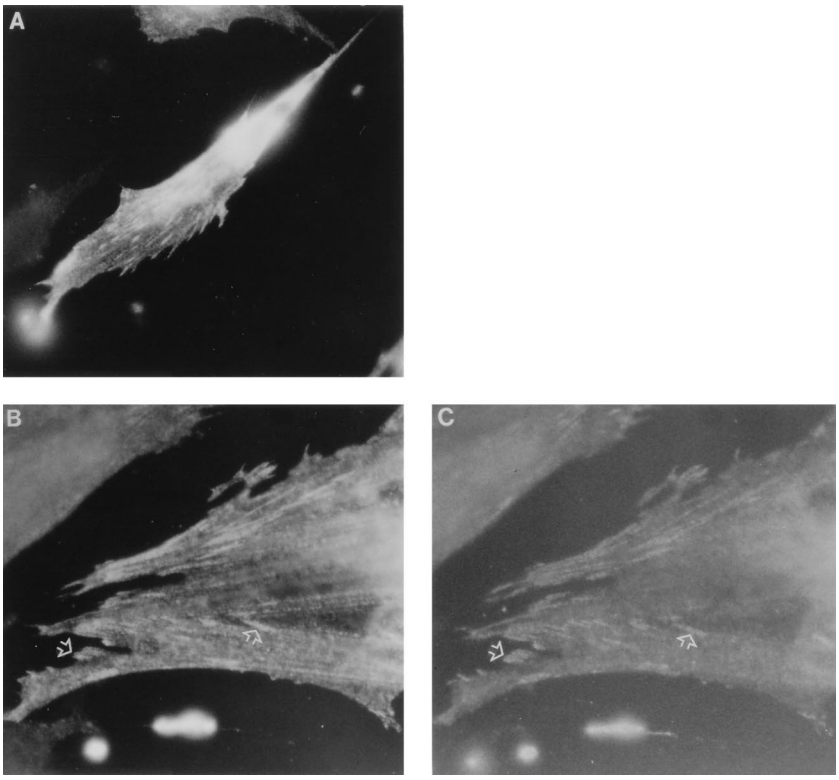

FIG. 7. Colocalization of FAK and cGraf. CE cells expressing cGraf and FAK were grown on glass cover slides and stained with either MAb KT3 alone (A) or a combination of KT3 and polyclonal serum BC3 for simultaneous detection of cGraf and FAK (B and C). Arrows indicate sites of colocalization of cGraf and FAK.

function of several actin-binding proteins that are predicted to modulate the polymerization and depolymerization of actin filaments (21, 33, 39, 67). Thus, Rho may act in part to regulate actin microfilament assembly by regulating the production of PIP_2.

The evidence presented herein indicating an association of Graf, a negative regulator of both Cdc42 and RhoA, and FAK raises the possibility that Graf may have pleiotropic effects on integrin-mediated cell signaling. Unfortunately, to date we have been unable to detect endogenous Graf in fibroblasts or other tissue extracts because of the poor immunoreactivity of current antibodies. Thus, we have been unable to establish definitively an in vivo interaction between endogenous Graf and FAK. Nonetheless, the in vitro association of the Graf SH3 domain with FAK, the apparent specificity of this interaction, and the localization of overexpressed Graf to focal adhesion structures suggest a potential interaction, a possibility that must be assessed by using better reagents.

It is intriguing to speculate that Graf may function to downregulate the Cdc42-Rac-Rho GTPase cascade, perhaps attenuating GTPase-mediated cellular responses to integrin-ECM interactions, including actin reorganization, tyrosine kinase cascades, and gene expression. Thus, the recruitment of Graf to newly assembled focal complexes (or other FAK-containing structures) may serve to place Cdc42 in the GDP-bound state, resulting in the attenuation of formation of filopodia, lamellipodia, actin stress fibers, and focal adhesions. These possibilities are testable and are currently under investigation.

To date, many proteins exhibiting RhoGAP activity have been identified. In vivo, GAPs may exhibit exquisite substrate specificity, acting only on one subtype of GTPase (53); thus, multiple GAPs would be needed to regulate the large number of small GTPases identified to date. Alternatively, individual GAPs may be expressed in specific cell types or at specific stages of development, providing overlapping specificity for different small GTPases. Finally, individual GAPs may be regulated spatially, biochemically, and/or temporally. In this scenario, a GAP may be targeted (perhaps by phosphorylation) to a specific subcellular compartment or regulated by specific environmental stimuli. While there are no reports of RhoGAPs that possess regulated GAP activity, there is evidence for the directed subcellular localization of GAPs; Myr5 and p120 RasGAP-associated p190 may associate with the actin cytoskeleton in vivo. Specifically, Myr5 demonstrates actin binding activity (47) and p190 shows colocalization with the actin cytoskeleton (13). Graf provides another example of a GAP that may be targeted to the actin cytoskeleton. Since the SH3 domain has been shown to bind FAK directly, one might predict that the SH3 domain of Graf is also responsible for mediating its association with other components of the actin cytoskeleton (3), thus targeting Graf to cortical actin and actin stress fibers.

Whereas only preliminary experiments address the tissue- or cell type-specific expression of Graf, it appears that Graf is evolutionarily conserved. Two expressed sequence tag sequences, derived from *Caenorhabditis elegans* mRNA, contain a predicted open reading frame with significant similarity to avian Graf (accession no. D36250 and D36080; submitted by Yuji Kohara et al.). Sequence comparison of the Graf peptide sequence to the DNA database of the *C. elegans* genome project reveals two cosmid clones, zk328 and t04c9, that, when translated, also display high sequence similarity to Graf. The homology between avian Graf and the predicted open reading frames of these cosmids and cDNAs spans the amino-terminal

domain, the GAP domain, and the SH3 domain, with an overall identity of 53%. Thus, the conservation of Graf between vertebrates and invertebrates indicates that Graf may regulate common biological processes that have been conserved through evolution.

ACKNOWLEDGMENTS

We thank A. Richardson, M. Schaller, and Rajesh Malik for helpful discussions and C. Borgmann for technical support.

This work was supported by DHHS grants CA40042 and CA29243.

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