**Characterization of Graf, the GTPase-activating Protein for Rho Associated with Focal Adhesion Kinase**

**PHOSPHORYLATION AND POSSIBLE REGULATION BY MITOGEN-ACTIVATED PROTEIN KINASE**

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Graf is a GTPase-activating protein for Rho that interacts with focal adhesion kinase and co-localizes with the actin cytoskeleton (Hildebrand, J. D., Taylor, J. M. and Parsons, J. T. (1996) Mol. Cell. Biol. 16, 3169–3178). We examined the expression and regulation of Graf as a prelude to understanding the role of Graf in mediating signal transduction in *vivo*. We demonstrated that Graf is a ubiquitously expressed 95-kDa protein with high levels observed in heart and brain and cells derived from these tissues. Stimulation of PC12 cells with epidermal growth factor or nerve growth factor induced a phosphatase-reversible mobility shift upon gel electrophoresis, indicative of phosphorylation. In *vivo*, purified mitogen-activated protein (MAP) kinase catalyzed the phosphorylation of Graf on serine 510, suggesting that Graf phosphorylation may be mediated through MAP kinase signaling. In addition, the mutation of serine 510 to alanine inhibited the epidermal growth factor-induced mobility shift of mutant Graf protein *in vivo*, consistent with serine 510 being the site of *in vivo* phosphorylation. Based on these data we suggest that phosphorylation of Graf by MAP kinase or related kinases may be a mechanism by which growth factor signaling modulates Rho-mediated cytoskeletal changes in PC12 and perhaps other cells.

Rho-related GTP-binding proteins constitute a functionally distinct group within the small molecular weight (smw) G protein family, which includes Rho A, B, C, and G, Rac1 and Rac2, Cdc42, and TC10 (1). These proteins are 30% identical to Ras and 55% identical to each other. The Rho family members have been linked to a variety of cellular effects, including changes in cytoskeletal dynamics (actin polymerization and reorganization), gene expression (p38/Jun NH₂-terminal kinase and serum response factor activity), cleavage furrow formation, G₁ cell cycle progression, endocytosis, exocytosis, and superoxide production (2).

Regulation of the cytoskeleton by Rho family members seems to be dependent on a cascade of events within the actinomysosin system (2). In response to a variety of stimuli (including bradykinin, tumor necrosis factor-α, and interleukin-1) cells send out exploratory filopodia, a process regulated by Cdc42. Activation of Rac (by Cdc42-mediated signaling or in response to stimulation with platelet-derived growth factor, EGF, bombesin, or insulin) results in extensions of broad sheet-like lamellipodia. Finally, activation of Rho (by Rac-mediated signaling or by stimulation with bombesin or lysophosphatidic acid) results in the formation of actin stress fibers and focal adhesions (3–8). Each of these structures is formed by the ordered polymerization of F-actin and presumably serve to mediate specialized cellular functions, such as endocytosis, cell migration, and cell division (9).

Like all smw G proteins, Rho family members exist in an inactive GDP-bound form and an active GTP-bound form. The rate of conversion between the GDP-bound form and GTP bound form is modulated by guanine nucleotide dissociation inhibitors, which inhibit GDP dissociation, guanine nucleotide exchange factors (GEFs), which stimulate the replacement of GDP by GTP, and by GTPase-activating proteins (GAPs), which stimulate the rate of intrinsic GTP hydrolysis by the GTPase (10). To date, at least seven mammalian GEFs and 10 mammalian GAPs have been identified for the Rho GTPase family (11–14). The existence of multiple GAP proteins suggests that these proteins may act on different subcellular pools of Rho proteins or may have different effector-functions upon association with Rho proteins. Although GAP proteins down-regulate the signal input by rapidly converting the G protein to its inactive GDP bound state, certain GAP proteins, including p120Ras GAP, n-chimaerin, and phospholipase C (a GAP for heterotrimeric G proteins) simultaneously send a signal that is required for downstream signaling from the G protein (15–17). 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 (16). Apparently, the binding of n-chimaerin to activated Rac enhances a functional interaction between n-chimaerin and other unknown protein(s) to regulate cellular morphology.

It is currently unclear how the Rho cascade is activated and how the signal progresses from one smw G protein to another.
It is possible that one smw G protein may alter the GEF or GAP activity toward another smw G protein to modify signal transduction. Indeed, in yeast, Cdc42 directly links the smw G protein Bud1 and Cdc42 by being a binding target for Bud1 and a GEF for Cdc42 (8). In mammalian cells a putative Rac effector, Ost, is a GEF for Cdc42 and Rac (13). Also, RasGAP associates with p190RhoGAP, suggesting a possible interplay between these two G protein activators (18). Alternatively, GEFs or GAPs may be directly regulated by external stimuli resulting in altered activity or subcellular localization. For example, the activity of the Ras GEF Cdc25S is stimulated by muscarinic receptor-mediated phosphorylation in vivo (19).

Also, the in vitro activity of the Rac GAP n-chimaerin is stimulated by phosphatidylserine and phosphatidic acid and inhibited by phospholipids such as lysophosphatidic acid (20).

Recently, our laboratory identified Graf, a GEF for Rho associated with focal adhesion kinase (FAK) following screening a chicken embryo Agt11 expression library with a radiolabeled carboxy-terminal domain of FAK (21). This protein contains a centrally located GAP domain followed by a serine/proline-rich domain and a carboxyl-terminal SH3 domain. The Graf SH3 domain was shown to specifically bind to a proline-rich region in the COOH terminus of FAK. In vitro analysis revealed that the Graf GAP domain enhanced GTP hydrolysis of Cdc42 and RhoA but not Rac or Ras.

The experiments presented herein examine the expression and regulation of Graf as a prelude to understanding the role of Graf in mediating signal transduction in vivo. We demonstrate that Graf is ubiquitously expressed with high levels observed in heart and brain. Somewhat surprisingly, Graf phosphorylation is mediated by EGFR/NGF signaling through MEK/MAP kinase. In vitro, purified MAP kinase catalyzes the phosphorylation of Graf on Ser^{510}, a residue within the serine/proline-rich domain. The mutation of Ser^{510} to Ala inhibits EGFR-induced phosphorylation of mutant Graf protein in vivo. Phosphorylation of Ser^{510} causes a slower mobility of Graf upon SDS-PAGE, suggestive of a conformational change in Graf. Together, these data indicate that Graf may be a site of convergence of growth factor signaling with Rho-mediated cytoskeletal changes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cardiomyocytes from adult chicken ventricles were isolated by collagenase digestion using a Langendorf perfusion apparatus as described previously (22). Cells were lysed immediately for immunoprecipitation or plated on laminin-coated glass coverslips and maintained in serum-free Dulbecco’s modified Eagle’s medium:F-12 (1:1) containing 10% fetal calf serum, 5% horse serum, and 1% penicillin/streptomycin solution. HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium:F-12 (1:1) containing 10% fetal calf serum and 1% penicillin/streptomycin.

**Construction of Epitope-tagged Graf, Mutant Graf Proteins, GST Fusion Proteins, and Polyclonal Antibodies**—The cDNA construct encoding the NH2-terminal Flag-tagged variant of Graf (F-Graf; amino acid residues 1–584) was generated by PCR using primers that generated 5’ HindIII and 3’ XhoI restriction sites. The resultant PCR product was digested with HindIII and XhoI and ligated with HindIII and XhoI digested pcDNA3 DNA containing sequences encoding a 7 amino acid NH2-terminal epitope tag with the sequence, DYKDDDK. Mutations in digested pcDNA3 DNA containing sequences encoding a 7 amino acid were digested with 8064 and ligated with pGEX4T2 DNA digested with BamHI and XhoI. For antigen production, the GrafSH3 and GrafGAP fusion proteins were expressed in Escherichia coli and the fusion proteins purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech). The hybrid proteins were digested with 20 μl of trypsin (in 0.1% Tri-HCl, pH 8.0, 0.12 μM NaCl), and dialyzed against PBS (10 mM NaHPO4, 0.15 mM NaCl, 2.7 mM KCl, 1.2 mM KH2PO4). Rabbit polyclonal antisera to GST-GrafGAP (αGGAP) and GST-GrafSH3 (αGSH3) were generated in New Zealand White female rabbits. The serum was affinity-purified using purified antigen coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech).

**Northern Blot Analysis**—RNA was isolated from chicken or rat tissues by phenol chloroform extraction using RNAzol (Tel-Test, Friendswood, TX), and mRNA was selected using oligo(dT)-Sepharose chromatography (Qiagen, Chatsworth, CA). RNA was resolved on a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose filters, and Northern blots containing chicken RNA were probed with a 1.5-kb Graf cDNA probe (nucleotides 285–1804). The mouse multiple tissue Northern blot (2 μg of poly(A-) RNA per lane, CLONTECH) and the rat Northern blot (containing 20 μg of total RNA) were probed with a 32P-labeled 0.9-kb mouse cDNA probe (corresponding to nucleotides 498–1400 of the chicken Graf sequence). The probes were labeled, hybridized, and the Northern blots washed as described previously (21, 24).

**In Vitro Phosphorylation of Graf**—Recombinant wild-type p42MAPK was purified to apparent homogeneity as described previously (26). Equal amounts of GST or GST-Graf fusion proteins (approximately 2–6 μg) were incubated with mAb (13B8B; gift from Dr. Michael Weber) as described above. The immune complexes were resuspended in MAP kinase reaction buffer (25 mM Hepes, pH 7.5, 10 mM magnesium acetate, and 50 μM unlabeled ATP) containing myelin basic protein (2 μg) and [γ-32P]ATP (1 μCi) and incubated at 30 °C for 20 min. The reaction mixtures were boiled in SDS sample buffer, resolved by 15% SDS-PAGE, and visualized by autoradiography.

In Vitro Phosphorylation of Graf—Recombinant wild-type p42MAPK-specific mAb (13B8B; gift from Dr. Michael Weber) as described above. The immune complexes were resuspended in MAP kinase reaction buffer (25 mM Hepes, pH 7.5, 10 mM magnesium acetate, and 50 μM unlabeled ATP) containing myelin basic protein (2 μg) and [γ-32P]ATP (1 μCi) in MAP kinase reaction buffer. After incubation for 20 min at 30 °C, the reaction mixtures were boiled in SDS sample buffer resolved by 15% SDS-PAGE and visualized by autoradiography.

**Phosphopeptide Mapping and Peptide Sequencing**—GST-Graf fusion proteins (phosphorylated by MAP kinase as described above) were digested with chymotrypsin as described previously (27). Phosphopeptides were spotted onto thin-layer chromatography (TLC) plates, resolved by high-voltage electrophoresis (40 min at 1,000 V in pH 8.9 buffer containing 0.295 M ammonium bicarbonate), followed by chroma-
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RESULTS

Analysis of Graf mRNA and Protein Expression—Northern blot analysis using a chicken-Graf cDNA probe revealed a 5.0-kb Graf RNA in chicken brain and liver, consistent with previously published data (Fig. 1A; Ref. 21). In contrast, Northern analysis using a mouse Graf cDNA probe directed to the SH3 domain revealed a major 8.5-kb RNA species in most rodent tissues (Fig. 1B). The 8.5-kb transcript was most abundant in mouse heart and brain with lower levels expressed in other mouse tissues, including a ubiquitous RNA species of approximately 4.5 kb and more restricted expression of RNAs of approximately 7, 2.5, and 2 kb. Similar results were observed when the blot was hybridized with a mouse Graf GAP domain cDNA probe (data not shown). As observed in Fig. 1, the major RNA species expressed in chicken co-migrates with a RNA expressed in rat (A) and may thus correspond to the 4.5-kb RNA species observed in the mouse Northern blot (B). Further evidence for alternative RNA processing arises from the identification of a variant of Graf (which contains sequences encoding a unique NH2-terminal domain) in a chicken brain cDNA library. A cDNA probe complementary to this alternative NH2-terminal sequence specifically hybridized with a transcript of approximately 2 kb, which was highly expressed in liver relative to brain, indicating that at least the 2-kb RNA species in liver may arise by alternative splicing. RNA from several cells lines was analyzed by Northern analysis, including RNA from REF52 (rat embryo fibroblasts), chicken embryo fibroblasts, Madin-Darby canine kidney epithelial cells, rat

2 J. M. Taylor and J. T. Parsons, unpublished observation.

![Fig. 1](https://example.com/fig1.png) Northern blot analysis of Graf RNA expression. A, poly(A+) RNA (2 μg) prepared from adult chicken brain (lane 1) and liver (lane 2) or total RNA from rat brain (lane 3; 20 μg) were subjected to Northern analysis using a 1.5-kb Graf-specific chicken cDNA probe (nucleotides 285–1804; lanes 1 and 2) or a 0.9-kb mouse cDNA probe (corresponding to nucleotides 498–1400 of the chicken Graf sequence, lane 3). The arrows indicate the positions of the 28 and 18 S ribosomal RNAs. B, a mouse multiple tissue Northern blot (2 μg of poly(A+) RNA/lane, CLONTECH) was probed with a 0.9-kb mouse cDNA probe (corresponding to nucleotides 498–1400 of the chicken Graf sequence, upper panel). The lower panel was hybridized with a 2.0-kb human β-actin cDNA probe to control for mRNA loading. Filters were washed under high stringency conditions and exposed to Kodak XAR film for 18 h as described under “Experimental Procedures.” Tissues include: heart (H), brain (B), spleen (S), lung (Lu), liver (Li), skeletal muscle (M), kidney (K), and testis (T). The filled circles indicate the 7- and 2.5-kb transcripts, and the ▲ indicates a possible alternatively spliced 2 kb product. Molecular size markers (kb) are shown at the left.

![Fig. 2](https://example.com/fig2.png) Tissue distribution of Graf protein. Graf was immunoprecipitated from chicken tissue extracts (2 mg/lane, A) or rat tissue extracts (2 mg/lane, C) using the Graf-GAP-specific aGGAP Ab (+) or preimmune anti serum (−). Western blots were performed using a polyclonal antibody generated from a Graf-SH3 GST fusion protein (αGSH3). Tissues include: heart (H), brain (B), lung (Lu), liver (Li), skeletal muscle (M), kidney (K), and testis (T). B, equal amounts of GST, GST-CT (a fusion protein containing the C-terminal domain of FAK, aa 686–1053), or GST-P878A (GST-CT with a Pro878 → Ala mutation) were incubated with chicken heart lysates (2 mg) for 1 h at 4 °C. Protein complexes were collected on glutathione beads, pelleted, washed, and the proteins eluted and separated by SDS-PAGE and visualized by Western blotting as described under “Experimental Procedures.”
vascular smooth muscle cells, bovine endothelial cells, and PC12 rat pheochromocytoma cells. Of these, only PC12 cells expressed detectable levels of Graf RNA (data not shown). These results indicate that Graf transcription is broadly detectable in tissues with highest levels in brain and heart, but that its expression in tissue cultured cell lines is quite restricted.

Two polyclonal antibodies were used to determine the expression patterns and size of endogenous Graf protein in various tissues. The antibodies were generated against GST fusion proteins to the GAP domain (αGGAP) and the SH3 domain (αGSH3) of Graf. Immunoprecipitation and Western blotting with αGGAP and αGSH3, respectively, revealed an immunoreactive band of approximately 95 kDa in both chicken and rat tissues with high levels detected in brain and heart in agreement with the levels of transcripts detected in the Northern blot analysis (Fig. 2, A and C). Significant levels of Graf protein were also observed in lung, liver, and testis. We previously determined that ectopically expressed Graf bound to a FAK COOH-terminal GST fusion protein (CT), but did not bind the FAK COOH-terminal fusion protein containing a P878A mutation (CT-P878A). Fig. 2B shows that the 95-kDa immunoreactive band also binds efficiently to CT, but not to CT-P878A, consistent with this 95-kDa protein being endogenous Graf. A higher migrating band (approximately 190 kDa) was also observed in the rat brain lysate (Fig. 2C). The significance of this form is not known, but it appears not to be expressed in chicken brain (Fig. 2A).

**Graf Expression and Phosphorylation in Cardiomyocytes and Neuronal Cells**—Since whole heart extracts contain high levels of Graf protein, we isolated cardiomyocytes to determine whether the expression in heart is due to the presence of Graf in these contractile cells. Both immunocytochemistry and immunoprecipitation revealed the expression of Graf in isolated cardiomyocytes. Endogenous Graf in cardiomyocytes was localized in punctate structures along the actin stress fibers (data not shown), similar to the localization observed when Graf was ectopically expressed in chicken embryo fibroblasts (21). Western blot analysis revealed that Graf, immunoprecipitated from freshly isolated cardiomyocytes, exhibited a retarded mobility on SDS-PAGE compared with Graf immunoprecipitated from whole heart extracts (Fig. 3A). The mobility shift was reversed by treatment of Graf cardiomyocyte immunoprecipitates with calf intestinal alkaline phosphatase (CIP; Fig. 3B). These data indicate that Graf is phosphorylated in isolated cardiomyocytes and that phosphorylation appears to induce a change in mobility on SDS-PAGE.

Interestingly, when Graf was immunoprecipitated from PC12 cells, it co-migrated with the major (presumably non-phosphorylated) form of Graf from whole heart extracts. However, treatment of PC12 cells with either EGF (100 ng/ml) or NGF (100 ng/ml) for 10 min induced a change in mobility of Graf, consistent with EGF- or NGF-induced phosphorylation (Fig. 3C). In some experiments two slower migrating forms of Graf were detected after EGF stimulation or upon isolation of cardiomyocytes. We found that Graf phosphorylation after treatment with EGF and NGF was not on tyrosine residues as determined by the lack of anti-Tyr(P) immunoreactivity (data not shown). These data indicate that Graf is phosphorylated on serine and/or threonine and that phosphorylation is regulated in PC12 cells. These data further indicate that the slower mobility of Graf observed in freshly isolated cardiomyocytes may be due to phosphorylation which was induced during the isolation procedure.

Upon analysis of the time course of EGF and NGF-stimulated band shift of Graf, we observed a more rapid phosphorylation of Graf with EGF than NGF treatment. Thus, the temporal order of Graf phosphorylation was reminiscent of the temporal activation of MAP kinase by these agents in PC12 cells (30, 31). Fig. 4 shows a correlation between the time course of MAP kinase activation and Graf phosphorylation (both measured by the mobility shift on SDS-PAGE) after stimulation of PC12 cells with EGF and NGF. A complete mobility...
shift of MAP kinase was observed 4 min after EGF treatment and 8 min after NGF treatment (A). The mobility shift of Graf lagged slightly behind, with a nearly complete shift observed 8 min after EGF treatment and 20 min after NGF treatment. The duration of the MAP kinase band shift was prolonged with NGF compared with EGF treatment (A). Similarly, the Graf mobility shift remained complete 45 min after NGF treatment, but was reduced 45 min after EGF treatment. These data indicate the possibility that EGF/NGF-induced Graf phosphorylation is mediated by signaling though MEK/MAP kinase.

The MEK inhibitor PD098059 was used to determine whether the MAP kinase pathway was involved in Graf phosphorylation. Fig. 5 shows that pretreatment of PC12 cells with PD098059 blocked the ability of EGF to induce the mobility shift of Graf (A) and inhibited the activation of MAP kinase as measured by MAP kinase mediated MBP phosphorylation (B). Thus the inhibition of MAP kinase activity correlated with the inhibition of Graf phosphorylation as measured by mobility shift.

Phosphorylation of Graf in Vitro and in Vivo on Ser510—The amino acid sequence of Graf predicts four consensus sites for MAP kinase phosphorylation, Pro-X-Ser/Thr-Pro, each located within the serine/proline-rich region (Fig. 6A). To determine whether MAP kinase catalyzed phosphorylation of Graf directly, a GST fusion protein (GAPSH; Fig. 6A) containing the four phosphorylation consensus sites was incubated with purified MAP kinase and [γ-32P]ATP. Fig. 6B shows that GAPSH was efficiently phosphorylated by MAP kinase in vitro. A fusion protein containing the three COOH-terminal consensus sites...
(SH3; Fig. 6A) was also phosphorylated by MAP kinase in vitro. In contrast, neither GST alone nor a Graf fusion protein containing the NH₂-terminal most consensus site (GAP; Fig. 6A) was phosphorylated by MAP kinase.

Based on these observations, we mutated each of the serines within the three COOH-terminal MAP kinase consensus sites (Ser⁴⁹⁶, Ser⁴⁹⁹, and Ser⁵¹⁰) to alanine to determine which if any of these sites was phosphorylated by MAP kinase in vitro. Purified GAPSH with either the S⁴⁹⁶A or S⁴⁹⁹A mutation was phosphorylated by MAP kinase to the same extent as wild-type GAPSH. In addition, each of these mutant proteins exhibited the same pattern of phosphochymotryptic peptides as wild-type GAPSH (data not shown). In contrast, the ability of MAP kinase to phosphorylate GAPSH with the point mutation S⁵¹⁰A was dramatically reduced (Fig. 7A). Mutation of Ser 510 to Ala consistently resulted in a reduced yield of full-length GST fusion protein when expressed in E. coli. However, addition of three times more mutant protein resulted in equal quantities of full-length GAPSH and S⁵¹⁰A fusion proteins (as observed by Coomassie stain, Fig. 7A, left). Under these conditions, wild-type protein (GAPSH) was phosphorylated by MAP kinase, whereas the full-length mutant protein (S⁵¹⁰A) was not. However, a smaller product (presumably a breakdown product of S⁵¹⁰A) was phosphorylated albeit at approximately 25% of control level. The inability of the full-length S⁵¹⁰A fusion protein to be phosphorylated by MAP kinase strongly suggests that Ser⁵¹⁰ is a target for MAP kinase.

To confirm these data, the phosphorylated GAPSH and mutant fusion proteins were subjected to phosphopeptide mapping. Chymotrypsin digestion of the wild-type GAPSH fusion protein following incubation with purified MAP kinase and [γ-³²P]ATP revealed seven major phosphopeptides (Fig. 7B). In contrast, the phosphopeptide map of the smaller radiolabeled product containing the S⁵¹⁰A mutation revealed three major phosphopeptides that co-migrated with the phosphopeptides labeled 5, 6, and 7 of wild-type GAPSH (Fig. 7B). The four phosphopeptides in GAPSH, which are absent in the S⁵¹⁰A map, were isolated and sequenced by automated Edman degradation to determine the position(s) of the phosphorylated residues. In the case of phosphopeptides 1 and 2, label was released after 5 cycles, the position expected upon phosphorylation of Ser⁵¹⁰ and cleavage by chymotrypsin after Phe⁵⁰⁵ (Fig. 7C). For phosphopeptides 3 and 4, ³²P was recovered after 6 cycles, consistent with the phosphorylation of Ser⁵¹⁰ and chymotrypsin cleavage after Met⁵⁰⁴ (Fig. 7C). Chymotrypsin preferentially catalyzes the hydrolysis of peptide bonds COOH-terminal to Phe, Tyr, and Trp residues; however, cleavage can also occur COOH-terminal to Met, Glu, Asp, and Leu residues (32, 33). Therefore, the data are consistent with the two separate phosphopeptides (1, 2 and 3, 4) generated by different sites of COOH-terminal cleavage by chymotrypsin. A longer incubation time with chymotrypsin resulted in a reduced yield of

![Fig. 7. MAP kinase phosphorylates Ser⁵¹⁰ in vitro. A. wild-type GAPSH (2 µg) or GAPSH with the S⁵¹⁰A mutation (6 µg) were incubated with purified MAP kinase as described under “Experimental Procedures.” Samples were analyzed by SDS-PAGE, and the gel was transferred to nitrocellulose, stained with Ponceau S (left), and exposed to Kodak XAR film for 2 h (right). The arrowhead denotes the position of full-length GST fusion protein. B, the radioactive bands from A (indicated by the asterisk) were excised, digested with chymotrypsin, and subjected to two-dimensional peptide mapping as described under “Experimental Procedures.” The mix denotes a sample comprised of equal cpms of peptides from wild-type GAPSH and S⁵¹⁰A digests. The phosphochymotryptic peptides are designated 1–7. C, the phosphochymotryptic peptides (designated PP1, PP2, PP3, and PP4) were isolated from the TLC plate and subjected to automated sequence analysis as described under “Experimental Procedures.” Each cycle was monitored for the release of ³²P by liquid scintillation counting. The sequence surrounding Ser⁵¹⁰ is shown and possible sites of chymotrypsin cleavage are indicated by the arrows. The amount of labeled peptide (counts/min) bound to the column matrix were 681, 93, 439, and 498 for phosphopeptides PP1–PP4, respectively.](http://www.jbc.org/DownloadedFrom)
and cell lines. Notably, Graf is not found in cells wherein FAK has been shown to be important in adhesion-dependent signaling (e.g. fibroblasts); nor is Graf regulated (as assessed by tyrosine phosphorylation) by adhesion-dependent signaling or overexpression of FAK. In contrast, Graf phosphorylation appears to be regulated by EGF and NGF in PC12 pheochromocytoma cells, an event that correlates with the activation of MEK and MAP kinase.

The time course of Graf phosphorylation, inhibition of phosphorylation by PD98059, and the fact that MAP kinase phosphorylates Graf in vitro on sites that are also phosphorylated after EGF treatment in vivo all support the possibility that Graf is an in vivo substrate for MAP kinase in PC12 cells. The observation that Graf was phosphorylated in freshly isolated cardiomyocytes suggests that MAP kinase (or a related kinase) mediates this process. Interestingly, despite the fact that adult cardiomyocytes are terminally differentiated cells, these cells have been shown to contain significant levels of MAP kinase (34). In addition, increased coronary pressure can activate the MAP kinase cascade in these cells (34), an event that may occur during the cell isolation procedure upon reperfusion of the heart.

Although the data presented here are consistent with Graf being an in vivo target for MAP kinase, it is also possible that another MEK or MAP kinase activated kinase is responsible for phosphorylating Graf. MAP kinase phosphorylates and activates p90RSK as well as two recently identified Ser/Thr kinases, Mnk1 and Mnk2 (35–37). The time course of Graf phosphorylation and inhibition of phosphorylation by PD98059 would not rule out these candidate kinases. Notably Mnk2 is abundant in heart, but both Mnk1 and Mnk2 are expressed in very low levels in brain (36, 37).

We identified a major site of EGF-stimulated Graf phosphorylation as Ser510. Phosphorylation of this site correlates with a mobility shift of Graf on SDS-PAGE, suggestive of a conformational change in the protein. EGF may result in phosphorylation of a second site, since two slower migrating forms of Graf were detected in some experiments after EGF stimulation. The inability of the ectopically expressed S510A mutant to undergo a mobility shift after EGF treatment suggests that the second site of phosphorylation may be dependent on prior phosphorylation of Ser510. Interestingly, Ser510 resides in a proline-rich region adjacent to the carboxyl-terminal SH3 domain. An attractive possibility to explain the mobility shift is that the SH3 domain exhibits an intramolecular interaction with prolines in this region (e.g. closed form), and upon phosphorylation, the SH3 domain becomes conformationally altered (e.g. open form). Although there are no type I or II SH3 consensus binding sites in this proline-rich region (RXLPXP and PPLPXK, boldface letters represent conserved residues, respectively), there are six PXXP motifs that form the core of the polypeptide “type II” helix to which SH3 domains bind (38). Interestingly, the crystal structure of Src revealed that its linker region (which does not contain a consensus SH3 binding site) forms a polypeptide helix that is sufficient for an intramolecular interaction with the Src SH3 domain (39). Thus, formation of a polypeptide helix within the proline-rich region in Graf could direct an intramolecular interaction with the SH3 domain. Phosphorylation of Ser510 could relieve the intramolecular interaction and result in an open conformation with an exposed SH3 domain.

Given the above model for Graf regulation, one would predict that serine phosphorylation of Graf would enhance its binding efficiency for FAK. In the case of Sos1, serine phosphorylation has been shown to alter the affinity of Sos1 binding to the Grb2 SH3 binding domain (40). However, the low affinity interaction...
observed between FAK and Graf, and the lack of sensitivity of the in vitro association assays, have to date precluded us from thoroughly investigating this possibility. It is also possible that phosphorylation could modulate the ability of Graf to associate with another yet unidentified SH3 binding partner. Graf has been shown to bind the FAK family member Pyk2, in vitro, via a PXPF motif; however, the low level of Pyk2 in heart suggests that this may not be a physiologically relevant binding partner.3

That a mitogenic signal may regulate Rho-mediated cytoskeletal changes is intriguing in light of the observation that MAP kinase itself has not been directly implicated in mediating cytoskeletal changes. In fact Ras-mediated membrane ruffling in fibroblasts and Ras-mediated cytoskeletal changes in cardiomyocytes do not require MAP kinase signaling (41, 42). However, MAP kinase is required for differentiation of PC12 cells into neuronal cells, a process that requires continued cytoskeletal changes (43, 44). EGF and NGF stimulate PC12 cell filopodial projections and membrane ruffling with a time course similar to that of MAP kinase activation (45, 46). Interestingly, inactivation of Rho by ADP-ribosylation with C3 results in filopodial extensions in the neuroblastoma NEI-115 cell line (47). We are currently investigating whether EGF-mediated MAP kinase activation and subsequent phosphorylation of Graf could result in activation of Graf, down-regulation of Rho, and enhancement of neurite extensions.

The elevated levels of Graf in isolated cardiomyocytes are interesting in light of recent evidence that implicates Rho signaling in cardiac hypertrophy (48, 49). When embryonic cardiomyocytes are treated with the α1-adrenergic agonist phenylephrine, cells undergo drastic morphological changes that include complete reorganization of the actin cytoskeleton in addition to various changes in gene expression. The changes in gene expression that accompany hypertrophy have been reported to require Rho signaling, since C3 can block the phe-nylphrine-induced production of atrial natriuretic factor and the contractile protein, myosin light chain-2 (49). We are currently testing whether Graf can modulate this hypertrophic response in vivo.

The physiological function of Graf is presently unknown. However, the data presented above indicate that phosphorylation may play a role in modulating Graf activity or interactions in vivo. Preliminary experiments indicate that phosphorylation of Graf does not effect enzymatic activity, since microinjection of the Ser510→Ala mutant into Swiss 3T3 cells produces the same dramatic cytoskeletal effects as wild-type Graf, an effect which is dependent on GAP activity.2 Therefore, we hypothesize that phosphorylation may regulate the ability of Graf to interact with SH3 binding partners. Importantly, SH3 domain-containing interactions are often responsible for the intracellular targeting of proteins (38). It will be of interest to determine whether the subcellular localization of Graf is altered after mitogenic stimulation.

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Characterization of Graf, the GTPase-activating Protein for Rho Associated with Focal Adhesion Kinase: PHOSPHORYLATION AND POSSIBLE REGULATION BY MITOGEN-ACTIVATED PROTEIN KINASE

Joan M. Taylor, Jeffrey D. Hildebrand, Christopher P. Mack, Michael E. Cox and J. Thomas Parsons

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