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The N-Terminal Pleckstrin, Coiled-Coil, and IQ Domains of the Exchange Factor Ras-GRF Act Cooperatively To Facilitate Activation by Calcium

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We have recently shown that the neuronal exchange factor p140 Ras-GRF becomes activated in vivo in response to elevated calcium levels [C. L. Farnsworth, N. W. Freshney, L. B. Rosen, A. Ghosh, M. E. Greenberg, and L. A. Feig, Nature (London) 376:524–527, 1995]. Activation is mediated by calcium-induced calmodulin binding to an IQ domain near the N terminus of Ras-GRF. Here we show that the adjacent N-terminal pleckstrin homology (PH), coiled-coil, and IQ domains function cooperatively to allow Ras-GRF activation. Deletion of the N-terminal PH domain redistributes a large percentage of Ras-GRF from the particulate to the cytosolic fraction of cells and renders the protein insensitive to calcium stimulation. A similar cellular distribution and biological activity are observed when only the core catalytic domain is expressed. Although the PH domain is necessary for particulate association of Ras-GRF, it is not sufficient for targeting the core catalytic domain to this cellular location. This requires the PH domain and the adjacent coiled-coil and IQ sequences. Remarkably, this form of Ras-GRF is constitutively activated. The PH and coiled-coil domains must also perform an additional function, since targeting to the particulate fraction of cells is not sufficient to allow Ras-GRF activation by calcium. A Ras-GRF mutant containing the PH domain from Ras-GTPase-activating protein in place of its own N-terminal PH domain localizes to the particulate fraction of cells but does not respond to calcium. Similar phenotypes are seen with mutant Ras-GRFs containing point mutations in either the PH or coiled-coil domain. These findings argue that the N-terminal PH, coiled-coil, and IQ domains of Ras-GRF function together to connect Ras-GRF to multiple components in the particulate fractions of cells that are required for responsiveness of the protein to calcium signaling.

A wide variety of extracellular signaling molecules influence intracellular events, at least in part, by promoting the active GTP-bound state of Ras proteins (for a review, see reference 20). Once activated, Ras is known to bind and contribute to the stimulation of at least three classes of downstream signaling molecules: (i) serine/threonine protein kinases of the Raf family (for a review, see reference 39), (ii) lipid kinases that phosphorylate the 3′ position of phosphoinositides (29), and (iii) the guanine nucleotide exchange factor Ral-GDS, which activates the Ral GTPases (16, 18, 32, 36). Ras also activates the Rho family of GTPases, although the mechanisms involved have not yet been revealed (27, 28). Presumably, these effector molecules contribute to the ability of Ras to promote cell proliferation, differentiation, or expression of differentiated function, depending upon the cellular environment.

Many signaling molecules, including growth factors and neurotrophins, activate Ras by binding to tyrosine kinase receptors or to receptors that activate associated intracellular tyrosine kinases. Other ligands, such as bradykinin, lysophosphatidic acid, and muscarinic agonists, activate Ras by binding to receptors that are coupled to heterotrimeric G proteins (for a review, see reference 2). The most commonly used pathway from receptors to Ras is through activation of the Son of Sevenless (SOS) guanine nucleotide exchange factor (3). Exchange factors promote the release of GDP bound to Ras, allowing it to be replaced with GTP. Tyrosine kinase receptors autophosphorylate or transphosphorylate docking proteins such as Shc (26). These events generate binding sites for the SH2-containing protein Grb2. Grb2 is bound, via its SH3 domain, to a proline-rich region at the C terminus of SOS. In this way, it is thought that SOS is targeted to its substrate Ras, which is present at the plasma membrane (for a review, see reference 23). Some G protein-coupled receptors also activate Shc (37). In some of these cases, receptor-induced elevation of calcium activates the Pyk tyrosine kinase, which phosphorylates Shc (19).

In brain neurons, elevated calcium levels can activate Ras without the involvement of tyrosine kinases. This newly identified pathway occurs through calmodulin binding to the neuronal p140 Ras-GRF exchange factor (8). Ras-GRF and SOS have similar catalytic domains near their C termini (1a). Both proteins also contain adjacent Dbl homology and pleckstrin homology (PH) domains upstream from the Ras-activating domain. Dbl homology domains in other proteins have been shown to have exchange factor activity on Rho GTPases (14), although this has yet to be shown for Ras-GRF or SOS. Unlike SOS, GRF does not contain an SH3 binding site at its C terminus and thus does not respond to tyrosine kinase-induced signals (31). Instead, Ras-GRF contains unique sequences at its N terminus, including a second PH domain, followed by a putative coiled coil and then a calmodulin-binding IQ domain (6, 8).

PH domains of about 100 amino acids are found on numerous signaling molecules (15, 22, 24). These domains have been suggested to target proteins to membranes (7). In some cases, this appears to be due to their ability to bind lipids, such as phosphoinositides and their head groups (10, 13), or to pro-

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teins, such as the βγ subunits of heterotrimeric G proteins (33). Coiled coils are formed by two or three amphipathic α-helices in parallel. They display a pattern of hydrophilic and hydrophobic amino acids that is repeated every seven residues. They can bind to coiled coils on other proteins, generating either homo- or heterodimers (21). In this report, we document that the N-terminal PH domain of Ras-GRF functions as part of a unit with neighboring coiled-coil and IQ domains to allow proper association of the protein with membranes and with as yet unidentified cellular components that are required for calcium-induced activation.

MATERIALS AND METHODS

Cell culture and transfection. 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% iron-enriched calf serum (Hyclone) at 37°C in 5% CO_2. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Hyclone) and 400 μg of G418 (Gibco) per ml when appropriate. 293T cells were plated at a density of 3×10^5 cells per 60-mm-diameter plate 1 day before transfection. Adenovirus major late promoter-based expression vector pMT3 was used for transient expression of wild-type and mutant Ras-GRF in 293T cells. DNA was introduced into these cells by a modified calcium phosphate precipitation procedure (8). NIH 3T3 cells were transfected by the standard calcium phosphate protocol, using a Moloney murine leukemia virus long terminal repeat promoter-based expression vector pJ4, along with pZipneoSV(X) to provide neomycin resistance (9). Stable transformants were selected in G418-containing medium and pooled.

Plasmid construction. The cloning of Glu epitope-tagged Ras-GRF and Ras-GRF-IQ$^{(-)}$ into pMT3 has been described previously (8). Ras-GRF-CC$^{(-)}$, containing two alanines inserted after Val-168 and Ile-170, was constructed by overlap PCR (17). In particular, a pair of mutagenic oligonucleotides were used together with external primers to recreate a Glu epitope at the 5′ end of the gene and ~900 bp of GRF coding sequence. This PCR product was digested with *Not*I and *Bgl*II and then inserted in place of the wild-type Ras-GRF fragment in pBluescript (pBS). Mutated Ras-GRF was then removed from pBS by *Not*I and *Kpn*I digestion and inserted into pMT3. N-terminal PH domain deletion mutants were made by overlap PCR using external primers to generate a *Bam*HI site preceding various segments of the N-terminal PH domain. PCR products were digested with *Bam*HI and *Bgl*II and then inserted in place of the wild-type GRF fragment in pBS. Ras-GRF was removed by *Not*I-*Kpn*I digestion and inserted into pMT3. Overlap PCR and a similar cloning strategy were used to make Ras-GRF-PH$^{(-)}$, a mutant with alanines substituted for the conserved Trp-119 and semiconserved Ile-123 in the N-terminal PH domain. Overlap PCR was used to fuse the PH domain of Ras-GAP (amino acids [aa] 440 to 618) with Ras-GRF at aa 171. This PCR product was digested with *Bam*HI-*Bgl*II and used to replace the natural 900-bp fragment of Ras-GRF in pBS. This form of Ras-GRF was then removed as a *Not*I-*Kpn*I fragment and cloned into pMT3. Overlap PCR and ligation into pMT3 by ligation of *Not*I-*Kpn*I digests were used to create deletion mutants of Ras-GRF with N-terminal domains juxtaposed next to the minimum C-terminal catalytic domain of Ras-GRF. Cat was constructed by subcloning an *Nsi*-*Kpn*I fragment of Ras-GRF (containing aa 958 to 1440) into pMT3 with oligonucleotide linkers.

Cellular fractionation. Sixty hours after transient transfection (293T cells), or when cells reached confluence (NIH 3T3 cells), cells were washed in phosphate-buffered saline (PBS), pelleted, resuspended in isotonic buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) containing protease inhibitors (10 μg of aprotinin per ml, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride), and lysed by sonication. The sample was centrifuged at 10,000 × *g* for 10 min to remove partially disrupted cells. The supernatant was then removed and centrifuged at 100,000 × *g* for 1 h to separate cytosol from particulate fractions. The particulate fractions were resuspended in isotonic buffer by sonication. Equal amounts of protein, determined by Lowry assay, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunoblotted. Full-length Ras-GRF, ΔN158Ras-GRF, ΔN103Ras-GRF, ΔN59Ras-GRF, PH1$^{(-)}$-Ras-GRF, CC$^{(-)}$-Ras-GRF, IQ$^{(-)}$Ras-GRF, and GAP-Ras-GRF proteins were detected with anti-GRF antibody 2544 (30). Cat, PCat, PCCat, PCQCat, PCQ$^{(-)}$ Cat, P$^{(-)}$CQCat, and PC$^{(-)}$QCat proteins were detected by anti-Ras-GRF antibody C-20 (Santa Cruz Biotechnology), which recognizes the C-terminal 20 aa of Ras-GRF. Immune complexes were detected by enhanced chemiluminescence (Dupont NEN) using horseradish peroxidase-conjugated anti-rabbit immunoglobulin.

Calmodulin binding. 293T cells were transiently transfected with wild-type or mutant Ras-GRF. Sixty hours after transfection, cells (from dishes that were 4 by 100 mm) were washed, pelleted in PBS, and then lysed in 200 μl of buffer M (1% Triton X-100 in 20 mM Tris HCl [pH 7.4], 125 mM NaCl, 1 mM $MgCl_2$) with leupeptin. After centrifugation at 10,000 × *g* for 10 min to remove insoluble material, supernatants were incubated at 4°C for 2 h with 25 μl of protein A-Sepharose (Pharmacia) and 2 μl of anti-Glu epitope antibody (for Glu epitope-tagged constructs) or 2 μl of anti-GRF antibody 2544 (for ΔN158Ras-GRF, ΔN103Ras-GRF, and ΔN59Ras-GRF). Precipitated proteins were washed in buffer M containing 0.1% Triton X-100 and 100 μM $CaCl_2$ and boiled in Laemmli loading buffer. Aliquots of precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-GRF or anticalmodulin antibodies (Upstate Biotechnology, Inc.).

Nucleotide exchange assays. Sixty hours after 293T cells were transiently transfected with empty vector or vector containing wild-type or mutant Ras-GRF, cells were extracted in buffer M (10^6 cells in 200 μl). After centrifugation at 10,000 × *g* to remove insoluble material, supernatant fractions were assayed for nucleotide exchange activity on a glutathione *S*-transferase (GST)–RasH fusion protein immobilized on glutathione beads. After being washed in exchange buffer (20 mM Tris [pH 7.5], 1 mM dithiothreitol, 40 μg of bovine serum albumin per ml, 50 mM NaCl) with 0.1% Triton X-100, glutathione-bound GST-Ras was loaded with 2 μM [^{3}H]GDP (NEN; 5 to 15 Ci/mmol) in exchange buffer with 1 mM EDTA at 30°C. Loading was stopped with 6 mM $MgCl_2$, and beads were washed extensively in exchange buffer with 1 mM $MgCl_2$ and kept on ice. In exchange reactions, lysates were incubated with 0.3 μM [^{3}H]GDP–GST–Ras for 15 min at 30°C in 50-μl volumes in exchange buffer containing 1 mM $MgCl_2$, 100 μM $CaCl_2$, and 200 μM cold GTP. After brief centrifugation, radioactivity in aliquots of reaction supernatant were quantitated by scintillation counting to measure release of bound [^{3}H]GDP.

Erk-kinase assays. 293T cells were cotransfected with Myc-epitope-tagged Erk-1 together with an empty pMT3 vector or a vector containing wild-type or mutant Ras-GRF that did not contain a Glu epitope tag. This tag was found to partially suppress calcium-induced Ras-GRF activation. At 48 h after transient transfection, cells were serum starved for 16 h and then stimulated with 5 μM ionomycin (Calbiochem) in dimethyl sulfoxide or with dimethyl sulfoxide only for 5 min at 37°C, washed in PBS, and scraped in 200 μl of mild buffer M. After removal of insoluble material by centrifugation at 10,000 × *g* for 10 min, supernatants were incubated at 4°C for 2 h with 25 μl of protein A-Sepharose beads (50% slurry; Pharmacia) and 10 μl of ascites fluid containing anti-Myc antibody 9E10 to precipitate expressed Myc-Erk. Supernatant aliquots were reserved for protein quantitation by Lowry assay and for SDS-PAGE, nitrocellulose transfer, and immunoblotting to verify transfection expression. Beads were washed three times in PBS with 1% Triton X-100 and once in kinase buffer (50 mM Tris HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol). For kinase assay, beads were resuspended in 30 μl of kinase buffer containing 25 μM [^{32}P]ATP (0.1 μCi/μl; <60 Ci/mmol) and 50 μg of myelin basic protein (Sigma) and incubated at 25°C for 15 min. After addition of 10 μl of 4× Laemmli sample buffer, samples were electrophoresed on an SDS–15% polyacrylamide gel. Phosphorylated myelin basic protein was identified and quantified with a PhosphorImager.

RESULTS

PH1 and coiled-coil domains are required for calcium activation of Ras-GRF. We have recently documented that the N terminus of Ras-GRF contains a calmodulin-binding IQ motif that mediates calcium-induced activation of Ras-GRF. To assess the functions of the PH and coiled-coil domains that reside immediately N terminal to the IQ motif (Fig. 1), we analyzed the activity of a set of Ras-GRF mutants containing alterations in these regions. Ras-GRF activity was measured in transiently transfected 293T cells by its ability to activate Erk-1, a mitogen-activated protein kinase that lies on a signaling pathway downstream of Ras. Myc epitope-tagged Erk-1 was transfected into 293T cells with wild-type or mutant Ras-GRFs. The cells were then exposed to the calcium ionophore ionomycin, and Erk activity was measured after immunoprecipitation by an in vitro kinase assay with myelin basic protein as a substrate (Fig. 2A). As shown previously (8), in cells lacking Ras-GRF, exposure to ionomycin increased Erk-1 activity marginally. However, in Ras-GRF-expressing cells, exposure to ionomycin increased Erk-1 activity ~14-fold, indicative of Ras-GRF activation. Also as shown previously, ionomycin failed to activate a mutant Ras-GRF with point mutations in its IQ domain that blocked calmodulin binding [IQ$^{(-)}$-Ras-GRF].

N-terminal mutants revealed the critical importance of the PH1 domain in Ras-GRF activation by calcium. Deletion of the first 158 aa of Ras-GRF, containing the entire PH1 domain (ΔN158), blocked the protein's ability to respond to calcium influx (Fig. 2A). Similar results were obtained when Ras-GRF activity was assessed by observing its ability to increase the

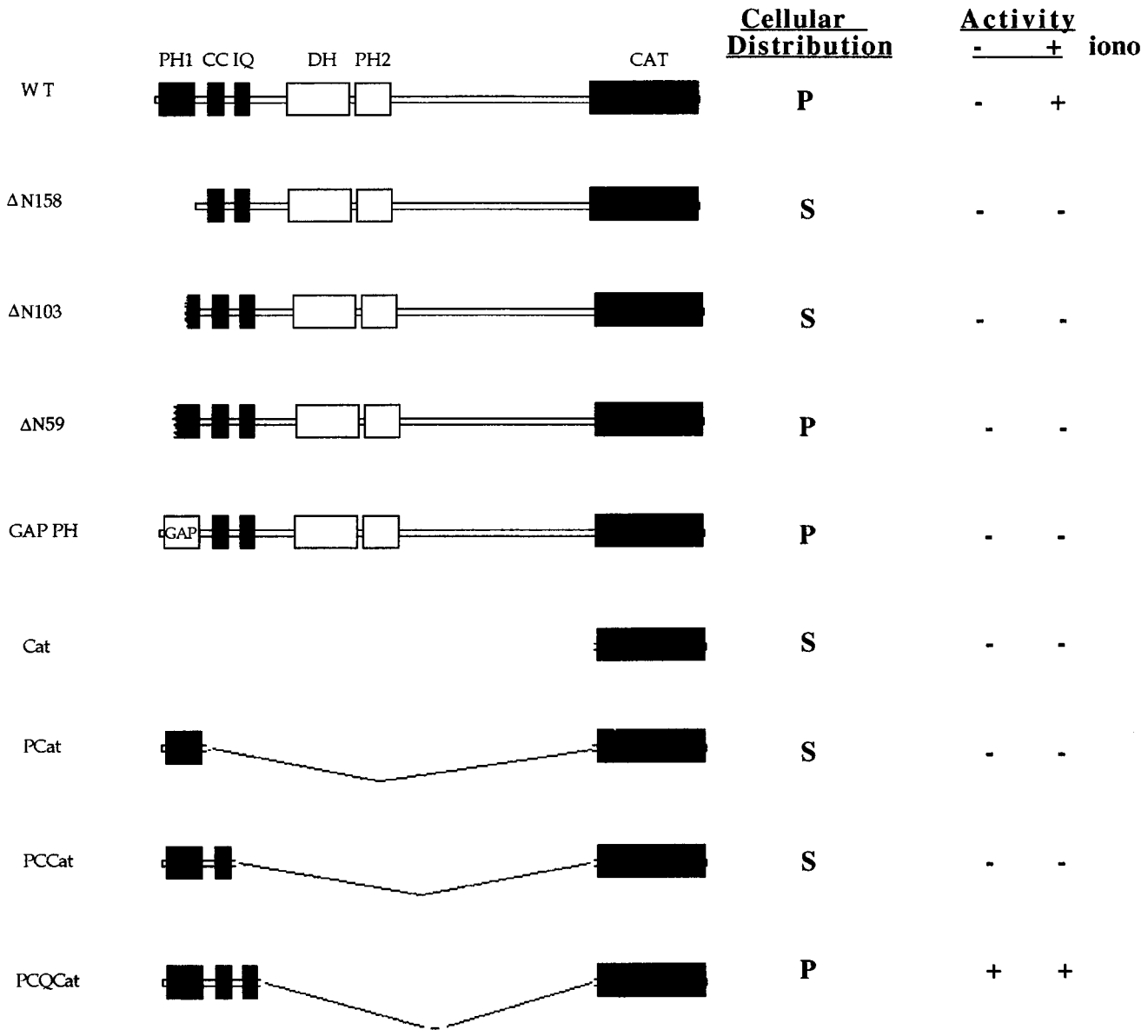

FIG. 1. Schematic diagram of wild-type (WT) and mutant Ras-GRFs highlighting functional domains. The N terminus of Ras-GRF contains a PH domain (PH1) (aa 17 to 133) followed by a putative coiled coil (CC) (aa 150 to 185) and then a calmodulin-binding IQ motif (aa 204 to 225). Closer to the C terminus is a Dbl homology domain (DH) (aa 238 to 450) followed by a second PH domain (PH2) (aa 450 to 586). Finally, the catalytic domain that activates Ras (CAT) is present at the C terminus (aa 958 to 1244). ΔN158 is a deletion mutant that lacks the first 158 aa encompassing the entire PH1 domain. ΔN103 and ΔN59 lack the first 103 and 59 aa within PH1, respectively. GAP^{PH} contains the PH domain of Ras-GAP in place of PH1 of Ras-GRF. Cat contains the C-terminal 286 aa of Ras-GRF that is approximately the minimal region needed for Ras nucleotide exchange activity. PCat, PCCat, and PCQCat are deletion mutants that contain (i) PH1, (ii) PH1 and CC, or (iii) PH1, CC, and IQ fused to Cat, respectively. The properties of all of the mutants are summarized. P, wild-type partitioning mostly in the particulate fraction; S, excessive cytoplasmic partitioning. Activity, activity in the absence (−) and presence (+) of ionomycin (iono).

level of Ras-GTP in cells (8) (data not shown). Structural studies of other PH domains have revealed that they consist of a seven-stranded β-sandwich consisting of two antiparallel sheets followed by an α-helix (for a review, see reference 11). Phosphoinositide binding sites in the phospholipase Cδ PH domain have been detected in the loops between the first four strands forming the N-terminal antiparallel sheet (10). Part of the binding sites for the βγ subunits of G proteins have been shown to reside in the remaining sequences of a variety of PH domains (33, 34). Thus, we studied partial PH1 deletion mutants that lacked either the region analogous to the lipid binding site of other PH domains (ΔN59) or the entire β-sandwich (ΔN103) (Fig. 1). Both of these mutants failed to be activated by calcium in transiently transfected 293T cells (Fig. 2A). A mutant containing alanines in place of the highly conserved tryptophan at position 120 and semiconserved leucine at position 125 [$PH^{(-)}$] in the C-terminal α-helix of PH1 was also unresponsive to calcium. Finally, a mutant containing the PH domain from Ras-GTPase-activating protein (Ras-GAP) in place of the PH1 domain of Ras-GRF (GAP^{PH}) was also not activated by calcium in vivo. Again, similar results were obtained when Ras-GRF activity was assessed by observing its ability to increase the level of Ras-GTP in cells (data not shown). Figure 2B shows that all of the mutants were ex-

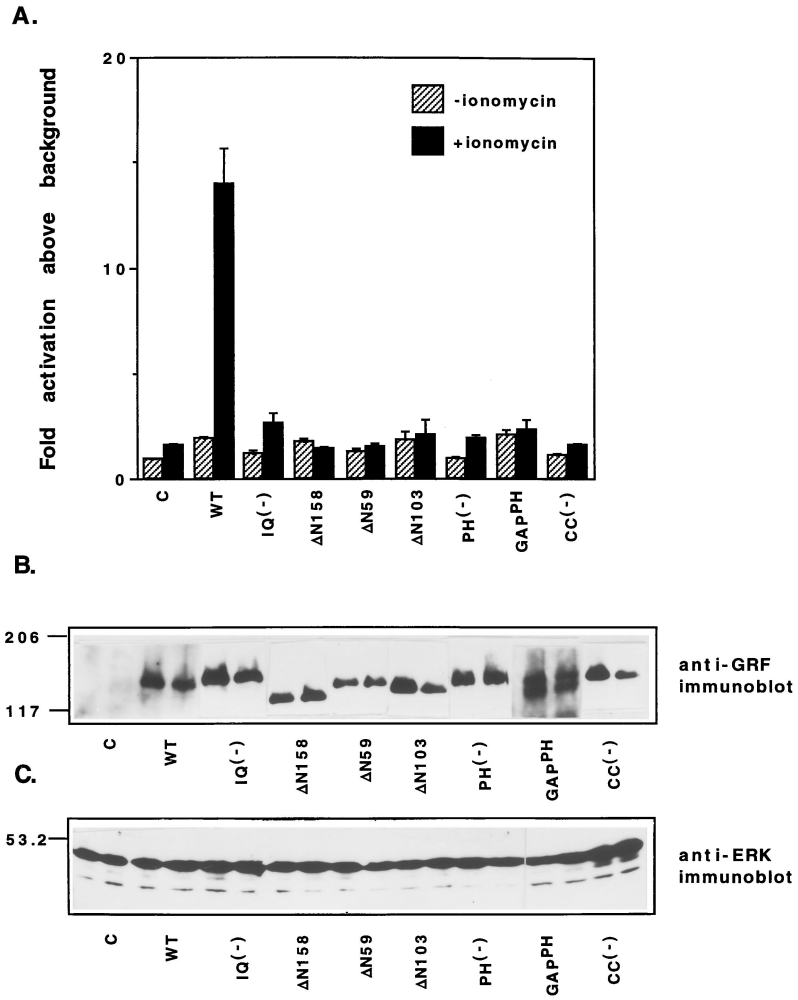

FIG. 2. Activity of Ras-GRF and N-terminal mutants in vivo. (A) Wild-type (WT) or mutant Ras-GRF was transiently cotransfected with epitope-tagged Erk into 293T cells. Cells were then stimulated with either ionomycin or medium alone for 5 min. Erk was then immunoprecipitated from cell lysates and assayed with myelin basic protein as a substrate. Fold activation was calculated from the ratio of the specific activity of samples from cells transfected with Ras-GRF to those from unstimulated cells transfected with an empty expression vector (marked C). Results represent the mean ± the standard deviation of the mean from at least three experiments. (B and C) Expression of Ras-GRF and Erk in cotransfected cell lysates. Equal amounts of protein from lysates were separated by SDS-PAGE and immunoblotted with anti-GRF antiserum (B) or anti-Erk antiserum (C). Antibody interaction was detected by a chemiluminescence assay with horseradish peroxidase-conjugated anti-rabbit immunoglobulin. The numbers on the left are molecular sizes in kilodaltons.

pressed at similar levels, and Fig. 2C shows that Erk levels in various transfected cells were also similar. These data demonstrate that an intact PH1 domain is required for Ras-GRF activation by calcium and that Ras-GRF PH1 has a specific function that is not common to all PH domains.

A putative coiled coil, containing at least four α-helices, resides almost immediately distal to the PH1 domain and proximal to the calmodulin IQ motif (Fig. 1). To investigate its contribution to Ras-GRF activation by calcium, an alanine was inserted in the middle of each of two predicted α-helices (after V-169 and I-171) [$CC^{(-)}$-Ras-GRF]. In this way, the helical structure of this region should have been maintained. However, the coiled-coil structure should have been destroyed, because the hydrophobic amino acids that originally formed a heptad repeat would no longer be predicted to reside on the same face of the helix. Figure 2A shows that the presumed loss of coiled-coil structure blocked Ras-GRF activation by calcium.

A simple explanation for the loss of responsiveness of these mutants to calcium could be that they can no longer bind calmodulin. This was not the case, because immunoprecipitates of these mutants obtained from stimulated cells contained bound calmodulin to a degree comparable to that found for wild-type Ras-GRF (Fig. 3). A loss of intrinsic nucleotide exchange activity was also not involved. Nucleotide exchange assays with soluble Ras, using lysates of cells transfected with various mutants, revealed little difference between mutant and wild-type Ras-GRFs (Fig. 4).

The PH1 domain promotes particulate association of Ras-GRF and plays an additional role. Since PH domains have been suggested to promote membrane association of proteins, we assessed whether changes in subcellular distribution could account for the loss of activities of these Ras-GRF mutants in cells. Wild-type and mutant Ras-GRFs were either stably expressed in NIH 3T3 cells or transiently transfected into 293T cells, as described above. Unstimulated cells were fractionated into cytosolic and particulate fractions by ultracentrifugation and probed for Ras-GRF by immunoblotting (Fig. 5). As shown previously for Ras-GRF in the brain (30), the majority of wild-type protein was present in the particulate fraction of

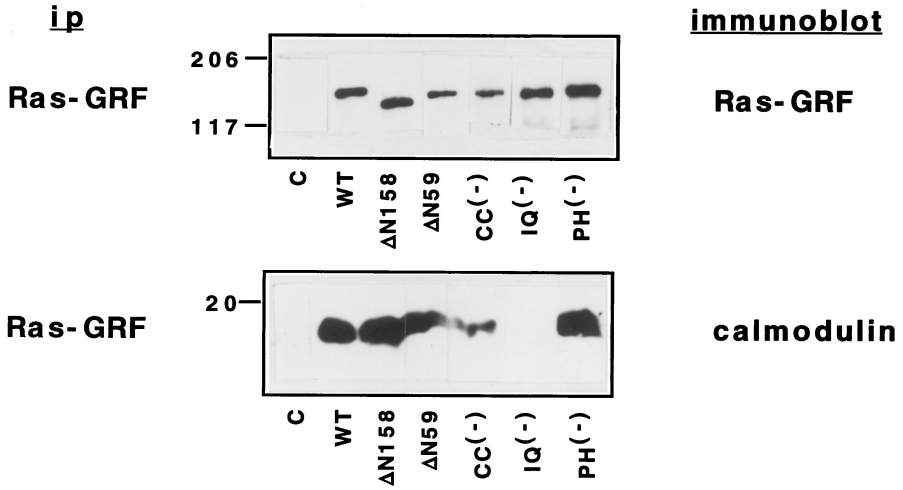

FIG. 3. Calmodulin binding of GRF N-terminal mutants. Wild-type (WT) or mutant Ras-GRF was transiently transfected into 293T cells, immunoprecipitated (ip), and immunoblotted with anti-GRF antiserum (top) or monoclonal anticalmodulin antibody (bottom) (Upstate Biotechnology, Inc.). Lanes C, control (untransfected cells).

both cell types. In contrast, ΔN158, which lacks the entire PH1 domain, and ΔN103, which lacks the β-sandwich but retains the α-helix, distributed approximately equally between the particulate and cytosolic fractions of both NIH 3T3 and 293T cells. A similar distribution of protein was observed when the core catalytic domain of Ras-GRF (Cat [Fig. 1]) was expressed on its own (Fig. 5). These results argue that the PH1 domain plays a major role in localizing Ras-GRF to the particulate fraction of cells and suggest that these mutants are unresponsive to calcium signaling because they are mislocalized.

Interestingly, the ΔN59 mutant, which lacks the N-terminal β-antiparallel sheet that contains the binding site for lipid in other PH domains, retained predominant particular localization in both cell types (Fig. 5), despite the fact that this mutant was unresponsive to calcium in 293T cells (Fig. 2). Similar subcellular distributions were obtained for the unresponsive Ras-GRF mutants containing either amino acid substitutions in conserved residues in the C-terminal α-helix of the PH1 domain [$PH^{(-)}$] or the PH domain from Ras-GAP in place of its own N-terminal PH1 domain (GAP^{PH}) (Fig. 5). Thus, in addition to targeting Ras-GRF to the particulate fraction, which can be accomplished by other PH domains, PH1 plays a more specific role that participates in the activation of Ras-GRF by elevated calcium levels.

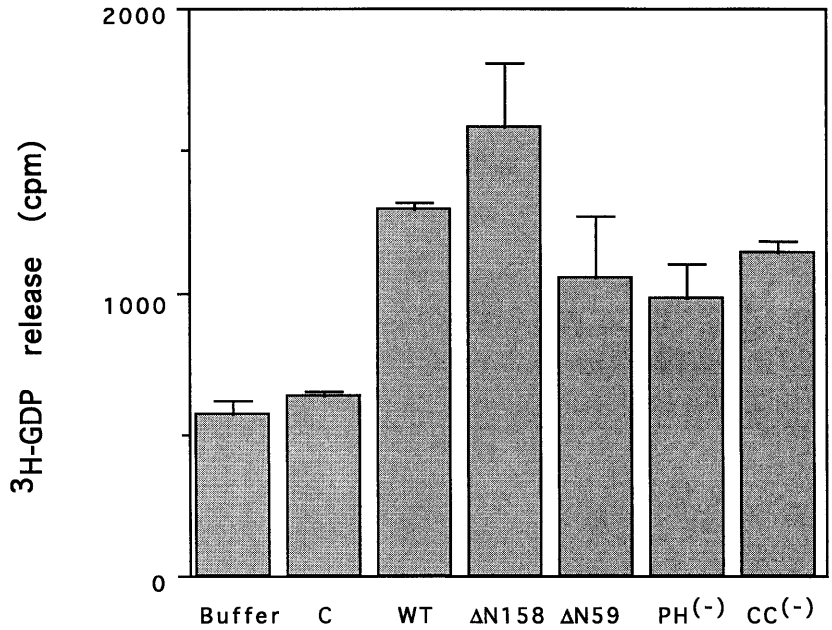

FIG. 4. Intrinsic nucleotide-exchange activity of GRF N-terminal mutants. Wild-type (WT) or mutant GRF was transiently transfected into 293T cells. Cell lysates were normalized for equivalent Ras-GRF expression by Western blotting (immunoblotting) prior to assay. Lysates were incubated with glutathione-Sepharose-bound GST-Ras preloaded with [^{3}H]GDP. Nucleotide exchange activity was measured by the ability to promote release of [^{3}H]GDP from the beads. Baseline release was determined with lysis buffer alone. Control C represents release promoted by lysates of cells transfected with an empty expression vector. Maximum exchange on Ras, quantitated by exposure to 2 mM EDTA, was found to be 19-fold above background. Results represent the mean (± standard error of the mean) from at least two experiments performed in triplicate.

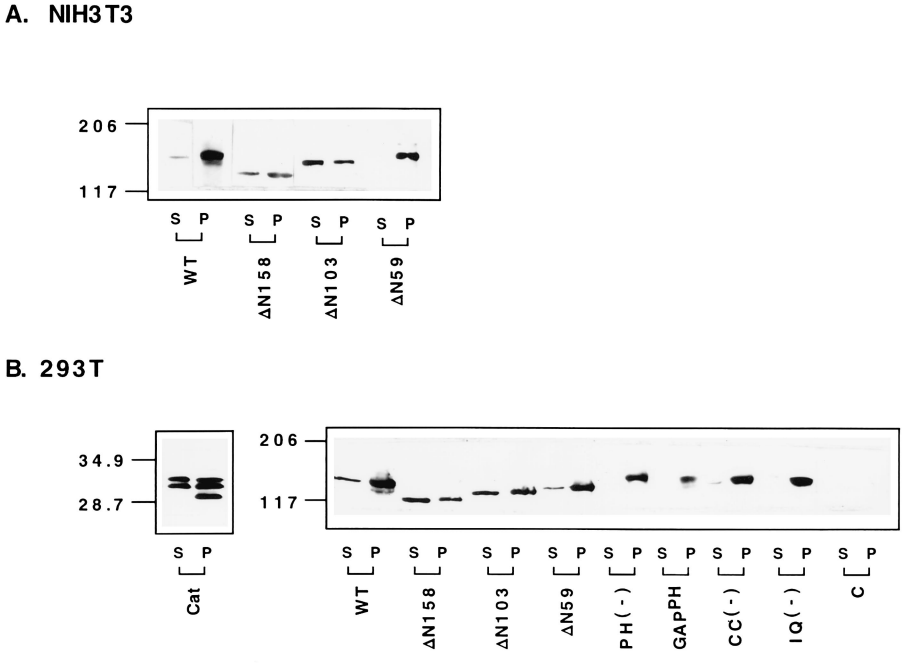

FIG. 5. Subcellular fractionation of Ras-GRF and N-terminal mutants. Transfected cells were lysed by sonication. Cytoplasmic and particulate fractions were separated by centrifugation at 100,000 × *g* for 1 h. The protein content of each fraction was determined by the Lowry assay, and equal amounts of protein were separated by SDS-PAGE and immunoblotted with anti-GRF antiserum. The uppermost band of Cat represents the full-length protein. The smaller bands presumably represent partially degraded fragments. Lanes S, the soluble cytoplasmic fraction; lanes P, the particulate fraction. (A) Stably transfected NIH 3T3 cell lines; (B) transiently transfected 293T cells. Lanes C, untransfected 293T cells. WT, wild type.

The mutant GRF that contains alanine insertions in its putative coiled-coil domain [$CC^{(-)}$] also retained a predominantly particulate distribution in cells, even though the protein was unresponsive to calcium. Thus, like the PH1 domain, the coiled-coil region appears to play a role in Ras-GRF activation that is distinct from ensuring particulate localization. Finally, Ras-GRF with a mutation in the IQ domain that prevents calmodulin binding also distributed primarily in the particulate fraction of cells, demonstrating that calmodulin binding is not required for proper distribution of Ras-GRF between cytosolic and particulate fractions.

The PH1 domain is not sufficient to target the catalytic domain of Ras-GRF to the particulate fraction of cells. Since the PH1 domain appeared to be critical for the particulate localization of Ras-GRF (Fig. 5), we investigated whether it was sufficient to localize the core catalytic domain. A fusion protein containing PH1 and Cat (PCat [Fig. 1]) was constructed and transfected transiently into 293T cells. Cytosolic and particulate fractions were prepared and immunoblotted with anti-GRF antisera. Figure 6A shows that this fusion protein had a distribution similar to that of Cat, indicating that PH1 alone was not sufficient for targeting the protein to the particulate fraction. Next, we fused both the PH1 and coiled-coil domains to the core catalytic domain (PCCat [Fig. 1]). This fusion protein also was present excessively in the cytosol (Fig. 6A). However, when the IQ domain was also included (PCQCat [Fig. 1]), preferential association with the particulate fraction was restored (Fig. 6A).

To explore how these domains might contribute to the localization of PCQCat in cells, previously described point mutations were introduced into the PH1, coiled-coil, and IQ motifs of this version of Ras-GRF. None of these mutations altered the protein's distribution in cells (Fig. 6A). Thus, although sequences distal to PH1 were needed for membrane targeting of PCQCat, the calmodulin binding property of the IQ motif and the coiled-coil structure of the neighboring region were not involved.

When these sets of mutants were tested for biological activity, we found that Cat, PCat, and PCCat-Ras-GRFs were unresponsive to calcium (Fig. 6B). Remarkably, the particulate PCQCat displayed elevated basal activity in cells (approximately sevenfold) that was not stimulated further by calcium influx. The enhanced activity of this mutant in vivo was not a consequence of elevated intrinsic nucleotide exchange activity, since its activity against Ras in vitro was similar to that of wild-type Ras-GRF (data not shown). It also bound calmodulin only in the presence of calcium (data not shown). Moreover, the requirements for intact PH1, coiled-coil, and IQ domains remained in this constitutively activated mutant. Point mutations in either the PH1, coiled-coil, or IQ domain suppressed the activity of PCQCat under both basal and elevated calcium conditions. The localization and calcium activation properties of all of the mutants are summarized in Fig. 1.

DISCUSSION

Calcium-induced stimulation of Ras-GRF, mediated by calmodulin binding, represents a newly discovered mode of Ras

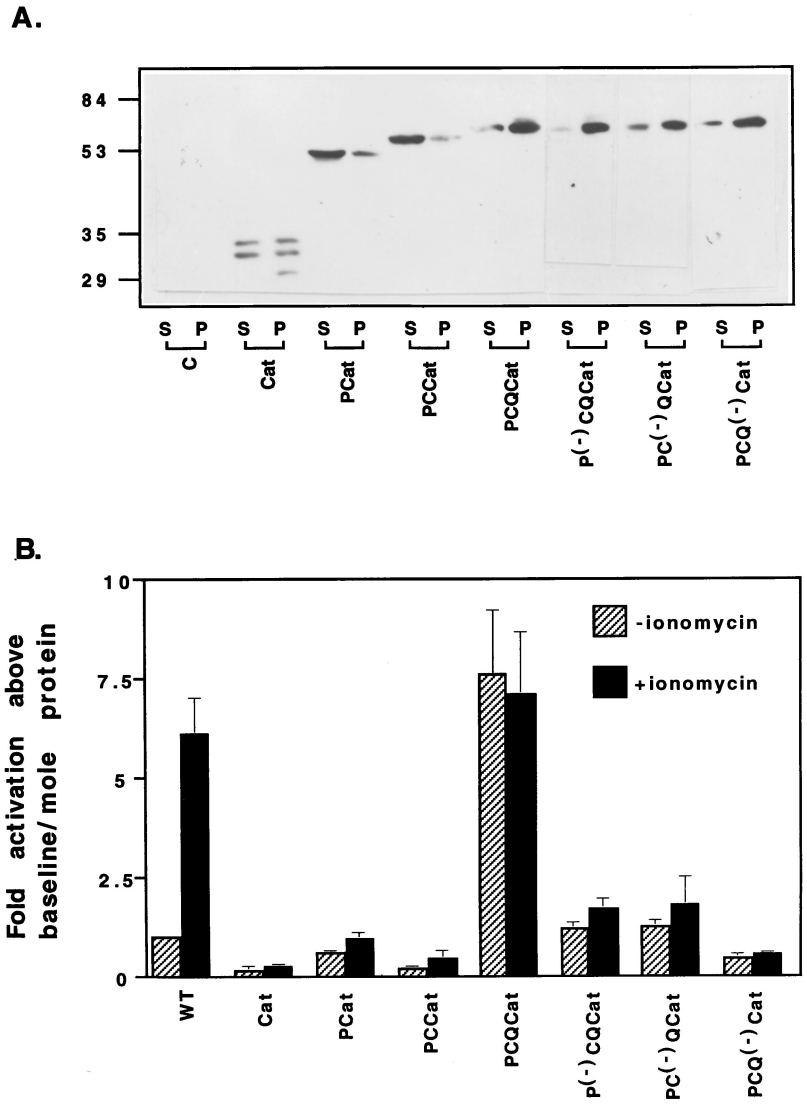

FIG. 6. Subcellular fractionation and biological activity of Ras-GRF deletion mutants. (A) Deletion mutants of GRF were transiently transfected into 293T cells. Cell lysates were fractionated into cytoplasmic and particulate fractions as described in Fig. 5. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with anti-GRF antibody C20 (Santa Cruz), which recognizes the catalytic domain of GRF. Lanes S, the soluble cytoplasmic fraction; lanes P, the particulate fraction. Numbers on the left show molecular sizes in kilodaltons. (B) Wild-type (WT) and deletion mutant Ras-GRFs were transiently transfected into 293T cells. Ras-GRF activity in vivo was detected by its ability to activate cotransfected Myc-tagged Erk as described in the legend to Fig. 2. Since the deletion mutants had molecular weights different from that of wild-type Ras-GRF and may have transferred to nitrocellulose with different efficiencies, the expression levels of the proteins were quantitated by comparing immunoblot signals with known amounts of purified samples of either wild-type Ras-GRF or PCQCat. Activities of Ras-GRF and mutants were normalized on the basis of molarity in extracts and expressed as fold activation above wild-type Ras-GRF in unstimulated cells. +, stimulation with ionomycin for 5 min; −, stimulation with medium alone. Results represent the mean (± standard error of the mean) from at least two experiments.

activation that occurs in neurons. Our previous work failed to detect an increase in the in vitro nucleotide exchange activity of Ras-GRF upon calmodulin binding, implying that the activation mechanism involves the interaction of Ras-GRF with additional cellular components (8). These components could participate in redistributing the catalytic domain of Ras-GRF to Ras or in increasing the intrinsic nucleotide exchange activity of Ras-GRF in cells in response to increased calcium. This study suggests that the PH1 and coiled-coil domains that neighbor the calmodulin-binding IQ domain of Ras-GRF interact with at least some of these undefined components. This hypothesis is based on the observation that perturbation of either of these domains blocked exchange factor activation by calcium without interfering with calmodulin binding or intrinsic nucleotide exchange activity of Ras-GRF measured in vitro.

PH domains have been found in a wide variety of signaling molecules, and for some, such as the insulin receptor substrate 1 and the Btk kinase, the PH domains have been shown to be necessary for proper protein function in vivo (25, 38). Although a common activity for all PH domains has yet to be elucidated, individual PH domains have been shown to possess distinct biochemical properties. Some PH domains participate in binding to other proteins, such as the βγ subunits of heterotrimeric G proteins (33). The binding site for βγ resides in the carboxyl portion of the PH domain, plus 25 immediately distal amino acids. For the PH domain of β-adrenergic receptor kinase (βark), this binding activity is thought to localize the

kinase to its substrate in the plasma membrane. PH domains can also contain a second binding activity. The Btk kinase can bind to protein kinase C at the amino-terminal end of its PH domain, as well as to βγ subunits at the carboxyl end (35, 40). Some other PH domains bind lipids at their amino end. For example, the PH domains of spectrin, phospholipase Cγ, and βark bind phosphatidylinositol-4,5-bisphosphate (PIP2) and Akt binds phosphatidylinositol-3-phosphate (PIP3) (7, 10, 12, 13). For βark, PIP2 binding at the carboxyl-terminal end of the domain cooperates with βγ binding at the C-terminal end to target βark to its substrate β-adrenergic receptor (34). For Akt, PIP3 binding leads to activation of the enzyme (5, 12).

The data presented here suggest that the PH1 domain of Ras-GRF also plays multiple roles. Since deletion of PH1 redistributes a large percentage of Ras-GRF to the cytosol and renders the protein unresponsive to calcium, one key function of PH1 is to ensure that Ras-GRF is present mainly in the particulate fraction of cells. Although a detailed analysis of Ras-GRF subcellular localization has not yet been performed, much of the particulate Ras-GRF is membranous, since it can be solubilized with detergent (unpublished data). This localization distinguishes Ras-GRF from the SOS exchange factor, which has been reported to be primarily in the cytoplasmic fraction of unstimulated cells (1, 4). Unlike SOS, which redistributes to the particulate fraction of cells upon cell stimulation (4), Ras-GRF appears to be present in this compartment constitutively. If redistribution of Ras-GRF to Ras is a component of Ras-GRF activation, it must be a subtle change that is not detectable by the simple cell fractionation techniques used here.

Although PH1 was necessary for normal subcellular distribution of Ras-GRF, it was not sufficient. It failed to restore particulate association to the core catalytic domain of Ras-GRF. Only when sequences containing the coiled-coil and IQ domains were added to PH1 was predominant particulate association restored. However, the coiled-coil structure and calmodulin binding activity of these sequences were not necessary for this activity, since mutations in these regions had no observable effect on localization of the protein. Whether these sequences serve a second specific function in localizing Ras-GRF or merely as a spacer between the PH1 and the catalytic domain remains to be determined.

PH1 likely plays an additional role that is more specific than targeting the protein to the particulate fraction of cells, since the PH domain of Ras-GAP could substitute for PH1 in localizing Ras-GRF but could not support activation by calcium. Moreover, mutant Ras-GRFs that contained a deletion lacking the first 59 amino acids of PH1 or point mutations in the α-helix at the carboxyl end of the domain displayed similar phenotypes. Previous work with other PH domains suggests that PH1 promotes particulate association and calcium-induced activation by interacting with proteins and/or lipids. It has already been reported that the Ras-GRF PH1 domain plus 25 additional distal amino acids can bind to the βγ subunits of heterotrimeric G proteins in vitro, although the binding was much weaker than that observed for βark (33). It remains to be determined whether these proteins contribute to calcium-induced activation of Ras-GRF. In addition, we are presently investigating whether the PH1 domain binds to specific lipids.

We have also demonstrated the importance of a putative coiled coil that resides in ~30 aa between the βγ binding region and the IQ domain of Ras-GRF. Mutations which should prevent coiled-coil formation blocked Ras-GRF activation by calcium without influencing subcellular distribution, calmodulin binding, or intrinsic nucleotide exchange activity of Ras-GRF. Coiled coils are known to be sites of protein-protein interaction. Since the phenotype of the mutation in the coiled-coil domain was identical to that of point mutations and small deletions in the PH1 domain, it is possible that the two domains cooperate to allow particulate Ras-GRF to interact with membrane components that contribute to the activation mechanism.

We observed that a protein containing the PH1, coiled-coil, and IQ domains fused to the catalytic domain of Ras-GRF (Fig. 1) (PCQCat) was constitutively activated in cells. Its basal activity was similar to that of calcium activated wild-type Ras-GRF, and this activity was not enhanced further by calcium influx. This finding suggests that sequences that normally reside between the IQ and Cat regions of Ras-GRF function to suppress basal Ras-GRF activity in vivo. Simple explanations for enhanced activity of PCQCat, such as enhanced intrinsic nucleotide exchange activity or the ability to bind calmodulin in the absence of calcium, were not supported by experimentation.

Mutations in PH1, coiled-coil, and IQ domains that inhibited calcium activation of wild-type Ras-GRF also suppressed the elevated basal activity of PCQCat and prevented its reactivation by calcium. Apparently, the mutant continues to depend upon interactions with the same set of cellular components as wild-type Ras-GRF, although these interactions may be constitutive. Thus, studies on PCQCat may help identify these missing components involved in Ras-GRF activation. The mutant may also be useful for evaluating the effect of elevated Ras-GRF activity on neuronal function in vivo.

REFERENCES

1. **Aronheim, A., D. Engelberg, N. Li, N. Al-Alawi, J. Schlessinger, and M. Karin.** 1994. Membrane targeting of the nucleotide exchange factor SOS is sufficient for activating the Ras signalling pathway. Cell **78:**949–961.

1a. **Boguski, M. S., and F. McCormick.** 1993. Proteins regulating Ras and its relatives. Nature (London) **366:**643–654.

2. **Bourne, H. R.** 1995. Team blue sees red. Nature (London) **376:**727–729.
3. **Bowtell, D., P. Fu, M. Simon, and P. Senior.** 1992. Identification of murine homologues of the Drosophila Son of sevenless gene: potential activators of Ras. Proc. Natl. Acad. Sci. USA **89:**6511–6515.
4. **Buday, L., and J. Downward.** 1993. Epidermal growth factor regulates p21Ras through the formation of a complex of receptor, Grb2 adaptor protein, and SOS nucleotide exchange factor. Cell **73:**611–620.
5. **Burgering, B. M. T., and P. J. Coffer.** 1995. Protein kinase B (c-Akt) in phosphoinositol-3-OH kinase signal transduction. Nature (London) **376:** 599–602.
6. **Cheney, R. E., and M. S. Mooseker.** 1994. p140 Ras-GRF contains an IQ motif and binds to calmodulin. Mol. Biol. Cell **5**(Suppl.)**:**21a.
7. **Davis, L. H., and V. J. Bennet.** 1994. Identification of two regions of βγ spectrin that bind to distinct sites in brain membranes. J. Biol. Chem. **269:** 4409–4416.
8. **Farnsworth, C. L., N. W. Freshney, L. B. Rosen, A. Ghosh, M. E. Greenberg, and L. A. Feig.** 1995. Calcium activation of Ras mediated by the neuronal exchange factor Ras-GRF. Nature (London) **376:**524–527.
9. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH 3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. Mol. Cell. Biol. **8:**3235–3243.
10. **Ferguson, K. M., M. A. Lemmon, J. Schlessinger, and P. B. Sigler.** 1995. Structure of the high affinity complex of inositol triphosphate with a phospholipase C pleckstrin homology domain. Cell **83:**1037–1046.
11. **Ferguson, K. M., M. A. Lemmon, P. B. Sigler, and J. Schlessinger.** 1995. Scratching the surface with the PH domain. Nat. Struct. Biol. **2:**715–718.
12. **Franke, T. F., S. I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis.** 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell **81:**727–736.
13. **Harlan, J. E., P. J. Hajduk, H. S. Yoon, and S. W. Fesik.** 1994. Pleckstrin homology domains bind to phosphoinositol-4,5-bisphosphate. Nature (London) **371:**168–170.
14. **Hart, M. J., A. Eva, T. Evans, S. A. Aaronson, and R. A. Cerione.** 1991. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. Nature (London) **354:**311–313.
15. **Haslam, R. J., H. B. Koide, and B. A. Hemmings.** 1993. Pleckstrin domain homology. Nature (London) **363:**309–310.
16. **Hofer, F., S. Fields, C. Schneider, and G. S. Martin.** 1994. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proc. Natl.

Acad. Sci. USA **91:**11089–11093.
17. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61–68.
18. **Kikuchi, A., S. D. Demo, Z.-H. Ye, Y.-W. Chen, and L. T. Williams.** 1994. ralGDS family members interact with the effector domain of *ras* p21. Mol. Cell. Biol. **14:**7483–7491.
19. **Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J. M. Musacchio, G. D. Plowman, B. Rudy, and J. Schlessinger.** 1995. Protein tyrosine kinase PYK2 involved in Ca2+-induced regulation of ion channel and MAP kinase functions. Nature (London) **376:**737–745.
20. **Lowy, D. R., and B. M. Willumsen.** 1993. Function and regulation of Ras. Annu. Rev. Biochem. **62:**851–891.
21. **Lupas, A., M. Van Dyke, and J. Stock.** 1991. Predicting coiled coils from protein sequences. Science **252:**1162–1164.
22. **Mayer, B. J., R. Ren, K. L. Clark, and D. Baltimore.** 1993. A putative modular domain present in diverse signaling proteins. Cell **73:**629–630.
23. **McCormick, F.** 1993. How receptors turn Ras on. Nature (London) **363:**15–16.
24. **Musacchio, A., T. Gibson, P. Rice, J. Thompson, and M. Saraste.** 1993. The PH domain: a common piece in the structural patchwork of signalling proteins. Trends Biochem. Sci. **18:**343–348.
25. **Myers, M. G., T. C. Grammer, J. Brooks, E. M. Glasheen, L. M. Wang, X. J. Sun, J. Blenis, J. H. Pierce, and M. F. White.** 1995. The pleckstrin homology domain in insulin receptor substrate-1 sensitizes insulin signaling. J. Biol. Chem. **270:**11715–11758.
26. **Pellicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicolletti, F. Grignani, T. Pawson, and P. G. Pelicci.** 1992. A novel transformin protein (Shc) with and SH2 domain is implicated in mitogenic signal transduction. Cell **70:**93–104.
27. **Ridley, A., and A. Hall.** 1992. The small GTP-binding protein rho regulates the assembly of focal adhesion and actin stress fibers in response to growth factors. Cell **70:**389–399.
28. **Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall.** 1992. The small GTP binding protein rac regulates growth factor-induced membrane ruffling. Cell **70:**401–410.
29. **Rodriguez-Viciana, P., P. H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M. J. Fry, M. D. Waterfield, and J. Downward.** 1994. Phophatidylinositol-3OH kinase as a direct target of Ras. Nature (London) **370:**527–532.
30. **Shou, C., C. L. Farnsworth, B. G. Neel, and L. A. Feig.** 1992. Molecular cloning of cDNAs encoding a guanine-nucleotide releasing factor for Ras p21. Nature (London) **358:**351–354.
31. **Shou, C., A. Wurmser, K. L. Suen, M. Barbacid, and L. A. Feig.** 1995. Differential regulation of two Ras activator proteins, Ras-GRF and SOS by EGF receptor and Grb2. Oncogene **10:**1887–1893.
32. **Spaargaren, M., and J. R. Bischoff.** 1994. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-Ras, N-Ras, K-Ras and Rap. Proc. Natl. Acad. Sci. USA **91:**12609–12613.
33. **Touhara, K., J. Inglese, J. A. Pitcher, G. Shaw, and R. J. Lefkowitz.** 1994. Binding of G protein by subunits to pleckstrin homology domains. J. Biol. Chem. **269:**10217–10220.
34. **Touhara, K., W. J. Koch, B. Hawes, and R. J. Lefkowitz.** 1995. Mutational analysis of the pleckstrin homology domain of the β-adrenergic receptor kinase. J. Biol. Chem. **270:**17000–17005.
35. **Tsukada, S., M. I. Simon, O. N. Witte, and A. Katz.** 1994. Binding of the beta gamma subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. Proc. Natl. Acad. Sci. USA **91:**11256–11260.
36. **Urano, T., R. Emkey, and L. A. Feig.** 1996. Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. EMBO J. **16:**810–816.
37. **van Biesen, T., B. E. Hawes, D. K. Luttrell, K. M. Krueger, K. Touhara, E. Porfiri, M. Sakaue, L. M. Luttrell, and R. J. Lefkowitz.** 1995. Receptor-tyrosine kinase- and Gβγ-mediated MAP kinase activation by a common signalling pathway. Nature (London) **376:**781–784.
38. **Voliovitch, H., D. G. Schindler, Y. R. Hadari, S. I. Taylor, D. Accili, and Y. Zick.** 1995. Tyrosine phosphorylation of insulin receptor substrate-1 in vivo depends upon the presence of its pleckstrin homology domain. J. Biol. Chem. **270:**18083–18087.
39. **Williams, N. G., and T. M. Roberts.** 1994. Signal transduction pathways inducing the Raf proto-oncogene. Cancer Metastasis Rev. **13:**14553–14556.
40. **Yao, L., Y. Kawakami, and T. Kawakami.** 1994. The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. Proc. Natl. Acad. Sci. USA **91:**9175–9179.