The Ras guanine-nucleotide exchange factor Ras-GRF/Cdc25
harbors a complex array of structural motifs that include a Dbl-homology (DH) domain, usually
found in proteins that interact functionally with the
Rho family GTPases, and the role of which is not yet
fully understood. Here, we present evidence that Ras-
GRF requires its DH domain to translocate to the mem-
brane, to stimulate exchange on Ras, and to activate
mitogen-activated protein kinase (MAPK). In an unprec-
edented fashion, we have found that these processes are
regulated by the Rho family GTPase Cdc42. We show
that GDP- but not GTP-bound Cdc42 prevents Ras-GRF
recruitment to the membrane and activation of Ras/
MAPK, although no direct association of Ras-GRF with
Cdc42 was detected. We also demonstrate that cata-
lyzing GDP/GTP exchange on Cdc42 facilitates Ras-
GRF-induced MAPK activation. Moreover, we show that
the potentiating effect of ionomycin on Ras-GRF-mediated
MAPK stimulation is also regulated by Cdc42.
These results provide the first evidence for the involve-
ment of a Rho family G protein in the control of the activity
of a Ras exchange factor.

Small GTP-binding proteins function as key molecular
switches in signal transduction routes that convey stimuli re-
ceived in cell surface receptors to the nucleus. The Ras family of
small GTP-binding proteins plays an essential role in the
regulation of cell growth and differentiation. Distributing sig-
als to downstream effector pathways, mainly those mediated
by mitogen-activated protein kinase (MAPK),1 phosphatidy-
inositol 3-kinase, and Ral-GDS (1). Ras GTPases cycle between
an inactive, GDP-bound state and an active GTP-bound state,
and it is known that this mechanism is controlled by at least
two types of regulatory proteins directly acting on Ras: GAPs
(GTPase-Activating Proteins), potentiators of the capacity of
Ras to hydrolyze GTP, and GEFs (Guanine nucleotide Ex-
change Factors) that catalyze the exchange of GDP for GTP,
thus promoting its activation (2).

Ras-GRF/Cdc25
is a GEF for the Ras family of small
GTP-binding proteins cloned by virtue of its homology with the
Saccharomyces cerevisiae CDC25 gene product that stimulates
nucleotide exchange on S. cerevisiae RAS (3–6). In mammali-
ans, Ras-GRF is expressed at high levels in brain (3, 5), but it
is also detected in other tissues and cell lines (7). Analysis of
Ras-GRF primary structure reveals the presence of a number
of functional motifs presumably involved in diverse signaling
control mechanisms and protein-protein interactions. As such,
the carboxyl-terminal Cdc25 domain has strong homology with
S. cerevisiae CDC25, thus its name, and it has been shown to
catalyze nucleotide exchange on Ras. This domain is also pre-
sent in other GEFs for the Ras family (8). In its amino terminus,
Ras-GRF contains a Dbl homology domain (DH) (9) that bears
strong resemblance to the oncoprotein Dbl, a GEF for the
GTPase Cdc42 (10). The DH domain is generally present in
GEFs for the Rho family of small G proteins (11) and other
proteins such as Rap1 (12) of yet unidentified function but also
believed to be involved in the regulation of the Rho family
GTPases. In Ras-GRF, the DH domain is flanked by two Pleck-
strin homology domains (PH) also present in Rho family GEFs
and other unrelated proteins. Although suggested to bind phos-
pholipids and play a role in membrane targeting, the function
of the PH domain remains largely unknown (13).

With regards to the biological role of Ras-GRF and the mecha-
isms governing it, it has been shown that Ras-GRF is capa-
ble of inducing cellular transformation in fibroblast (14). Ras-
GRF is also involved in conveying signals from G protein-
coupled receptors to Ras (15–17). Calcium can also regulate
Ras-GRF activity by a mechanism mediated through a cal-
modulin-binding motif (IQ domain) present in its amino termi-
nus (18, 19). As such, calcium ions act as Ras-GRF activators and
ionomycin can enhance Ras-GRF-mediated activation of the MAPK
pathway (19, 20). Interestingly, mutations within the DH domain inhibit
ionomycin-induced MAPK activation (21), suggesting that the
Ras-GRF DH domain may somehow function in the regulation
of this signaling route.

In this study we have investigated the role of Ras-GRF DH
domain in the activation of the Ras/MAPK pathway. We pre-
sent evidence that Ras-GRF requires its DH domain to translo-
cate to the membrane and, in so doing, become activated. We
also demonstrate that, in an unprecedented fashion, this process is regulated by the GTPase Cdc42 that when GDP-bound precludes Ras-GRF recruitment to the membrane and the subsequent activation of the Ras/MAPK pathway.

**MATERIALS AND METHODS**

**Constructs**—Ras-GRF mutants: ΔPH1 (bp 74–460 deleted), ΔDH (bp 578–1519), ΔPH2 (bp 1417–1993), and ΔCdc25 (bp 1993–3790) were obtained by standard restriction enzyme digestions and polymerase chain reaction-directed mutagenesis. Sequences of the oligonucleotides used and the mutation strategies followed are available upon request. All constructs were in the pCEV mammalian expression vector background (22).

**Cell Culture**—COS-7 cells were regularly grown in DMEM supplemented with 10% fetal calf serum. Subconfluent cells were transfected by the DEAE-dextran technique (22). The total amount of plasmid DNA used was adjusted to 3–4 μg per plate with vector DNA when necessary.

**Transformation Assays**—NH373 cells were cultured in DMEM supplemented with 10% calf serum DNA and transfected by the calcium phosphate precipitation technique (23). After 10–15 days in culture plates, the cells were stained in 5% Giemsa, and transformed foci were scored.

**Kinase Assays**—MAPK and JNK kinase activities were determined as described previously (24) in anti-HA immunoprecipitates using myelin basic protein (Sigma) or GST-ATF2 as substrates for MAPK or JNK, respectively, and incubated at 30 °C for 30 min. Reactions were terminated by addition of 5× Laemmli buffer, boiled, and electrophoresed in 12% SDS-polyacrylamide gel electrophoresis gels. The gels were visualized by autoradiography and quantitated by PhosphorImager (Molecular Dynamics).

**Immunoblotting**—Total lysates were fractionated in SDS-polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose filters. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech), using horseradish peroxidase-conjugated secondary antibody (Cappel). Mouse monoclonal anti-HA antibody was from Babco. Rabbit polyclonal antibodies anti-AU5 antibody was from Babco. Rabbit polyclonal antibodies anti-phospho-AU5 antibody was from Babco. Rabbit polyclonal antibodies anti-HA antibody was from Babco. Rabbit polyclonal antibodies anti-AU5 antibody was from Babco. Rabbit polyclonal antibodies anti-HA antibody was from Babco.

**Nucleotide Exchange Reactions**—In vivo nucleotide exchange was determined in COS-7 cells cotransfected with AU5-tagged GTPases and the different activating constructs as described (25, 26). Briefly, cells were cultured for 48 h, serum-starved in phosphate-free DMEM for 18 h, labeled with [32P]orthophosphate (100 μCi/ml) for 2–6 h and disrupted in lysis buffer (50 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotonin). Lysates were immunoprecipitated with anti-AU antibody for 1 h, and immunocomplexes were recovered using protein G-Sepharose beads, washed only in lysis buffer, washed twice in 50 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 500 mM NaCl, and resuspended in 1 mM KH2PO4, 5 mM EDTA (pH 8.0). Bound nucleotides were released by heating and fractionated using polyethyleneimine thin layer chromatography plates.

**Subcellular Fractionation**—This was performed in 20 mM HEPES, pH 7.4, buffer, basically as described previously (21).

**GTPase Pull-down Assays**—Bacterially synthesized GST-fusion proteins were purified using standard procedures. 20 μg were incubated with lysates from cells transfected with Ras-GRF, basically as described previously (27).

**GTP-Cdc42 Pull-down—** AU5 Cdc42-transfected cells were lysed in: 25 mM HEPES (pH 7.3), 10 mM MgCl2, 150 mM NaCl, 0.5 mM EGTA, 20 mM β-glycero-phosphate, 0.5% Nonidet P-40, 4% glycerol, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotonin. GST-bound AU5 Cdc42 was affinity-sequestered with bacterially synthesized GST-PAK Rac/Cdc42 binding domain (RBD) (70–106 amino acids) basically as described (28). Immunoblots were performed as described above using anti-AU5 antibody.

**RESULTS**

**Ras and MAPK Activation Induced by Ras-GRF Deletion Mutants**—To investigate the role of Ras-GRF DH domain in the activation of the Ras/MAPK pathway, we utilized transient overexpression as an experimental approach, because most of the available data on Ras-GRF biochemistry has been obtained by this method. To begin with, we generated a series of expression plasmids encoding for deletion mutants of the different domains present in Ras-GRF, and their expression in COS-7 cells was ascertained by immunoblotting (Fig. 1A, top panel). Because the anti-GRF antibody utilized (Santa Cruz) recognizes a carboxyl terminus epitope absent in the Cdc25 deletion mutant (ΔCdc25), we tagged this mutant with an amino-terminal FLAG epitope to enable its detection (Fig. 1A, right panel).

We first assayed the ability of the deletion mutants to catalyze GDP/GTP exchange on Ras. For this purpose, the different Ras-GRF constructs were cotransfected into COS-7 cells together with an AU5-tagged H-Ras (26), cells were incubated with [32P]orthophosphate, and nucleotide exchange was scored in anti-AU5 immunoprecipitates after thin-layer chromatography (see “Materials and Methods”). As shown in Fig. 1A (lower panel), deletion of the PH1 and PH2 domains (ΔPH1, ΔPH2) had no effect on the capacity of Ras-GRF to induce GDP/GTP exchange on Ras, but the disruption of the DH domain (ΔDH) decreased Ras-GRF exchange activity to an extent similar to that caused by deleting the catalytic Cdc25 domain (ΔCdc25). Consequently, we then determined how the different deletions affected Ras-GRF-induced MAPK activation, by cotransfecting the mutants with an HA epitope-tagged form of ERK2 (29). In full agreement with the data above, we found that there were no significant differences on the activation of MAPK elicited by the wild type, ΔPH1, and ΔPH2 mutants. On the other hand, MAPK activation was severely diminished on those cells transfected with the DH domain deletion mutant (Fig. 1B).

It would be conceivable that the observed inactivity of the ΔDH mutant could be due to the deletion of the DH domain resulting in a misfolded, nonfunctional protein. To rule out this possibility, we assayed the activation of MAPK by another Ras-GRF mutant (DH−) in which the conserved LTLHELL motif within the DH domain had been substituted (21) and where no misfolding should be expected. We found that DH− was also impaired in its ability to activate MAPK (Fig. 1C). Moreover, both the ΔDH and DH− mutants could interact in vitro with H-Ras as efficiently as did the wild type protein (Fig. 1D). This ruled out a misfolded protein incapable of interacting with Ras as the reason for the inability of the ΔDH mutant to stimulate the Ras/MAPK pathway.

**Effects of the DH Domain Deletion on Cellular Transformation by Ras-GRF**—The fact that Ras-GRF has been shown to transform murine fibroblasts (14, 30) prompted us to investigate how the removal of the DH domain would affect the Ras-GRF ability to induce focus formation. To do so, we utilized the standard focus-formation assay in NIH 3T3 murine fibroblasts (23). The colonies arising after transfection of NIH 3T3 fibroblasts with Ras-GRF showed a typical “Ras” phenotype (Fig. 2 and data not shown). Nevertheless, Ras-GRF transforming potential, as ascertained by the number of foci generated upon transfection, was 20-fold less than that exhibited by Ras V12. Deletion of the PH1 domain did not alter focus-formation induced by Ras-GRF. Surprisingly, ΔPH2 which, as shown above, exhibited an unaltered capacity for inducing Ras GDP/GTP exchange and MAPK stimulation, was entirely inhibited in its focus-forming activity. On the other hand, the absence of the DH domain completely abolished Ras-GRF-induced transformation, just as much as deleting the catalytic Cdc25 domain (Fig. 2). Overall, these results indicate that the DH domain is essential for Ras-GRF activation of the Ras/MAPK pathway and subsequent biological activity.

**Cdc42 Is Required for Ras-GRF Signaling**—Because deletion of Ras-GRF DH domain had dramatic consequences on the activation of the Ras/MAPK pathway and because the DH domain is a common structural feature of proteins that interact with Rho family G proteins (10), we next explored whether the participation of a Rho family GTPase would be necessary for...
Ras activation induced by Ras-GRF. As an approach, we utilized the Rho family dominant inhibitory mutants analogous to Ras N17 (31). It was found that Cdc42 N17 caused a 60% reduction on Ras-GRF-induced GDP/GTP exchange over Ras, almost as much as Ras N17 (Fig. 3A). But Rac and Rho dominant inhibitory mutants, otherwise capable of blocking JNK activation in COS-7 and 293T cells respectively (22, 32 and data not shown), were ineffective. As an additional control, none of these inhibitory mutants affected MAPK stimulation by an activated MEK mutant (MEK E) known to act downstream of Ras (33) (Fig. 3B). These results suggested that Cdc42 could be participating in the activation of the Ras/MAPK pathway by Ras-GRF. Interestingly, the inhibitory effect exerted by Cdc42 dominant interfering mutant was specific for Ras-GRF, because it did not affect the activation of MAPK by another Ras exchange factor such as SOS (Fig. 3C) under the same experimental setting.

Ras-GRF Does Not Interact Directly with Cdc42—The above results suggested that there could exist a functional relationship between Cdc42 and Ras-GRF. This led us to investigate the existence of a direct interaction between Ras-GRF and Cdc42. In vitro experiments have revealed that Ras-GRF cannot stimulate guanine nucleotide exchange in Rho family proteins, including Cdc42 (4). To confirm if this was the case in vivo, we tested whether Ras-GRF could promote nucleotide exchange on Cdc42 in COS-7 cells, by scoring 32P-labeled GDP/GTP uptake on cotransfected AU5-Ras and Cdc42 (Fig. 4A, top panel). Thus, verifying in vivo that Ras-GRF is not an exchange factor for Cdc42. This result was further substantiated, as Cdc42 is a potent activator of Jun amino-terminal kinase (JNK) in COS-7 cells (22). However, Ras-GRF was unable to induce any JNK activation, whereas under the same experimental conditions, two exchange factors known to act on Cdc42, Dbl or Ost (34), markedly stimulated JNK (Fig. 4A, lower panel). Also, we could not detect any physical association in vitro between Ras-GRF and Cdc42, when nucleotide-free glutathione S-transferase (GST) fusions of the Rho family GTPases were incubated with Ras-GRF under the same experimental setting.
ship between Ras-GRF and Cdc42.

Cdc42 and the DH Domain Regulate Ras-GRF Recruitment to the Membrane Fraction—It has been previously demonstrated that the amino-terminal region of Ras exchange factors is critical for determining their membrane localization (27, 35). Therefore, we then examined how the deletion of the DH domain affected the subcellular localization of Ras-GRF. As shown in Fig. 5A, in transfected COS-7 cells Ras-GRF was evenly distributed between the cytosolic and particulate fractions. However, Ras-GRF ΔDH was almost completely absent from the membranous fraction. Strikingly, whereas Rac N17 and Rho N19 inhibitory mutants did not affect Ras-GRF cellular distribution, cotransfecting Cdc42 N17 together with Ras-GRF mimicked the effect of deleting the DH domain, by preventing the presence of Ras-GRF in the particulate fraction. Wild type Cdc42, which similar to Cdc42 N17 is preferentially GDP-bound under serum starvation conditions utilized in this assay (36), also diminished Ras-GRF recruitment to the particulate fraction. On the other hand, a constitutively active, GTP-bound, Cdc42 QL (22) did not alter Ras-GRF distribution (Fig. 5A). The cellular distribution of Ras-GRF under these circumstances was further substantiated with the aid of enhanced green fluorescent protein chimeras in which the Cdc25 domains of wild type and ΔDH Ras-GRF were substituted by enhanced green fluorescent protein, generating proteins clearly visible under the microscope. By this method we could verify the above-mentioned results: the presence in the membrane of Ras-GRF wt and its disappearance from this structure upon cotransfection with Cdc42 N17, resembling the distribution of the ΔDH mutant present solely in the cytoplasm (data not shown).

Interestingly, the effects of the different Cdc42 proteins on Ras-GRF localization in the particulate fraction, matched their influence over the Ras-GRF-induced activation of MAPK: Cdc42 N17 caused a 60% reduction, Cdc42 wild type diminished it by 30%, and MAPK activation was unaffected by Cdc42 QL (Fig. 5B). These effects could not be attributed to their expression levels, because they were similarly expressed (inset in Fig. 5B). The inhibitory effect of Cdc42 wild type was dose-dependent, because increasing the amount of cotransfected Cdc42 wild type gradually decreased Ras-GRF-induced MAPK activation, concomitantly with the disappearance of Ras-GRF from the particulate fraction and its accumulation in the cytoplasm (data not shown).

These results suggested that the DH domain might be required for positioning Ras-GRF in the particulate fraction and, in so doing, bringing about the activation of the Ras/MAPK pathway. As such, we reasoned that targeting inactive Ras-GRF ΔDH to the membrane might restore its ability to activate MAPK. To ascertain this point, we engineered a membrane-bound Ras-GRF ΔDH by the addition of an H-Ras CAAX box (37) (ΔDH CAAX). It was found that this protein, verified to be preferentially located in the membrane fraction, was capable of potently activating MAPK (Fig. 5C). Thereby, this proved that the DH domain is involved in Ras-GRF recruitment to the membrane, a requirement for activating MAPK.

In the same line, we hypothesized that, if Cdc42 N17 would be inhibiting Ras-GRF-mediated MAPK activation by precluding its recruitment to the particulate fraction, a membrane-targeted Ras-GRF should be insensitive to Cdc42 N17 restraint. As reasoned, we found that activation of MAPK by the membrane-bound Ras-GRF CAAX, mainly located in the particulate fraction, was unaffected by Cdc42 N17 (Fig. 5D). Therefore, this supports the notion that Cdc42 has a role in directing Ras-GRF to the membrane.

Cdc42 GDP/GTP Exchange Facilitates Ras-GRF-mediated MAPK Activation—The experiments described above demonstrated that Cdc42 wild type and N17, but not QL, blocked MAPK activation by Ras-GRF. This implied that Cdc42 would be acting as a blocker in the GDP-bound but not in the GTP-bound state. If this were to be the case, induction of GDP/GTP exchange on Cdc42 should facilitate Ras-GRF-mediated MAPK activation. To ascertain this hypothesis, we cotransfected the Rho family GEFs Dbl and Vav with suboptimal concentrations of Ras-GRF, not enough to yield MAPK per se. High concentrations of Cdc42 wt were also cotransfected in order to make more remarkable any effect exerted by the exchange factors. As shown in Fig. 6A, Dbl markedly potentiated Ras-GRF-mediated MAPK activation, whereas Vav, a Rac-1-specific exchange factor (26), was largely ineffective. On the other hand, JNK activation by the exchange factors was unaffected by the presence of Ras-GRF (Fig. 6A). This result suggested that decreasing the amount of GDP-bound Cdc42 promoted MAPK stimulation by Ras-GRF.

The calcium ionophore ionomycin has been shown to greatly potentiate Ras-GRF activity (19). Our results suggest that GDP-bound Cdc42 inhibits the activation of Ras-GRF. Therefore, to stimulate Ras-GRF, ionomycin would be expected to alleviate the Cdc42-mediated restraint by diminishing the levels of GDP-Cdc42, something that could be accomplished by the induction of GDP/GTP exchange over Cdc42. To test this hypothesis, we assessed the ionomycin-induced generation of GTP-bound Cdc42 by specifically capturing it with the aid of a GST-PAK Rac/Cdc42 binding domain (RBD) fusion (28). For this purpose, COS-7 cells transfected with AUS-Cdc42 were stimulated with ionomycin for 1 min, and this treatment in-
duced the formation of GTP-Cdc42 to the same extent as in those cells transfected with the Cdc42 exchange factor Dbl (Fig. 6B). Moreover, the Cdc42 N17 dominant interfering mutant could markedly diminish Ras-GRF-mediated MAPK activation induced by ionomycin in a specific fashion, because Rac and Rho inhibitory mutants failed to affect it (Fig. 6C). This proves that the stimulatory effect of ionomycin over Ras-GRF activity is dependent on Cdc42.

DISCUSSION

In this study we have investigated the role of the DH domain in Ras-GRF biochemical and biological functions. Using a set of deletion mutants for the different domains harbored in the Ras-GRF regulatory domain, we show that the amino-terminal PH1 domain is dispensable for the stimulation of nucleotide exchange on Ras, MAPK activation, and cellular transformation. This amino-terminal PH domain is a unique feature in Ras-GRF and has been implicated in the responsiveness of the protein to calcium signaling. The disruption of this domain makes Ras-GRF insensible to stimulation by calcium ionophores (20). However, based in previous (14) and our present observations, it would not be essential for Ras-GRF basal biochemical activity.

Despite being involved in membrane and cytoskeletal targeting (35, 38), the function of the second PH domain (PH2), common to all Dbl family proteins, remains largely unknown. We have found that the deletion of PH2 does not alter the ability of Ras-GRF to stimulate nucleotide exchange on Ras or induce MAPK activation. However, it completely abolishes Ras-GRF focus-forming potential. In agreement, mutations on SOS PH domain reduce its focal transforming activity, although they also slightly affect its exchange potential (39). A similar effect is observed in Dbl, where a PH deletion abrogates its transforming ability without affecting its in vitro catalytic activity as an exchange factor for Rho and Cdc42 (38). Moreover, an isolated SOS PH domain induces germinal vesicle breakdown in Xenopus oocytes in a cooperative fashion with Ras (40), suggesting that the PH domain might be mediating GTPase-independent events necessary for inducing proliferation and/or transformation.

Ras-GRF localizes in the particulate fraction (4, 20 and this study). A recent report indicates that a mutation on a highly conserved residue of the PH2 domain, which impairs Ras-GRF responsiveness to calcium, does not affect the cellular localization of the protein (21). All these observations lead to speculation that the PH domain could be much more than a targeting signal, implicated somehow in processes of key importance in the upbringing of cellular transformation by mechanisms cur-
recently under investigation.

We have observed that deleting the complete DH domain results in a biologically inactive Ras-GRF, incapable of catalyzing GTP incorporation on Ras in vivo, activating MAPK, nor inducing cellular transformation. Accordingly, a recent report (41) indicates that a mutation in a critical residue within the DH domain markedly diminishes the ability of Ras-GRF to induce focus formation and to activate Ras, by precluding Ras-GRF oligomerization. Moreover, Dbl exchange activity and

Fig. 5. Regulation of Ras-GRF cellular distribution and function by the DH domain and Cdc42. A, subcellular distribution of Ras-GRF proteins in COS-7 cells transfected with Ras-GRF wild type (wt) or Ras-GRF DH deletion mutant (ΔDH), in addition to the constructs encoding for the indicated Rho family proteins, as detected by anti-Ras-GRF immunoblotting. (S) S100 soluble fraction; (P) P100 particulate fraction. B, effects of Cdc42 wild type (wt), N17, and QL on MAPK activation induced by Ras-GRF and Ras V12. Data shows average ± S.E. of three independent experiments. Inset, Cdc42 protein expression in the corresponding cellular lysates. C, activation of MAPK by Ras-GRF ΔDH-CAAX. Top, subcellular distribution of Ras-GRF CAAX. Middle, MAPK activation by the indicated Ras-GRF constructs, including ΔDH-CAAX. Bottom, expression of the different Ras-GRF proteins. D, effects of Cdc42 N17 on MAPK activation by Ras-GRF CAAX. Top, subcellular distribution of Ras-GRF CAAX. Middle, MAPK activation by Ras-GRF wt and Ras-GRF CAAX in the absence (−) or presence (+) of Cdc42 N17. Bottom, expression levels of Ras-GRF wt and Cdc42 N17.

Fig. 6. Effects of Cdc42 GDP/GTP exchange on Ras-GRF-mediated MAPK activation. A, effects of Rho family GEFs on Ras-GRF-mediated MAPK (top) and JNK (bottom) activation: Dbl and Vav (1 μg each) were cotransfected without (−) or with (+) suboptimal concentrations of Ras-GRF (100 ng) in the presence of Cdc42 (3 μg). B, nucleotide exchange on Cdc42 induced by ionomycin. Cells transfected with AU5-tagged Cdc42 wild type and QL, as indicated, were stimulated for 1 min with 5 μM ionomycin or by cotransfection with Dbl. GTP-bound AU5 Cdc42 was pulled-down with GST-PAK RBD (70–106). C, effects of the Rho family dominant inhibitory mutants on ionomycin-induced MAPK activation: constructs encoding for the different GTPases inhibitory mutants and Ras V12 as indicated, were cotransfected with (+) or without (−) suboptimal concentrations of Ras-GRF (100 ng) and stimulated, where indicated, with 5 μM ionomycin for 1 min.
transformation are impaired by conservative substitutions in a region of seven amino acids that is highly conserved in most DH domains (42). Also in perfect agreement with our observations, an in vivo study (39) shows that a similar cluster of mutations was found to diminish MAPK activation and cellular transformation by SOS. When introduced in Ras-GRF, however, these same mutations do not affect its exchange activity over Ras in vitro (21). Although apparently conflicting with our results, in vitro settings may not always reflect physiological conditions. Indeed, we show that mutations within the DH domain that do not affect Ras-GRF interaction with Ras in vitro, are biologically inactivating in vivo. On the other hand, the DH domain seems to be dispensable for the activation of MAPK by Ras-GRF2 (43), but in this case the DH domain is neither necessary for mediating ionomycin activation, as opposed to Ras-GRF DH domain (21). So, it is conceivable that despite their high similarities these two isoforms may be differentially regulated.

Interestingly, we show that ablation of the DH domain prevents Ras-GRF recruitment to the particulate fraction. But the addition of a CAAX membrane-targeting signal restores the capability of this mutant to activate MAPK. These results are suggestive of the DH domain being involved in Ras-GRF recruitment to the membrane, which would be a requisite for activating the Ras/MAPK pathway. In agreement with our data, biologically inactivating mutations within the Vav3 DH domain render cytoplasmic an otherwise membrane-associated protein (44). The DH domain is a complex motif composed of three conserved regions interspaced by heterogeneous sequences (9). Thus, it cannot be discounted that, in addition to catalyzing exchange on Rho proteins, other regions within this domain could also be involved in up to now unveiled processes necessary for the biological activity of the exchange factors, such as regulating cellular localization, as our results suggest. This can be envisioned by our data showing that the complete deletion of the DH domain results in a transformation-defective Ras-GRF. However, a partial DH deletion maintaining the last 36 residues retains its transforming potential (14), indicating that this region, in which no known consensus motifs are found, may entail some functions necessary for Ras-GRF optimal activity. The portion of the DH domain responsible for targeting Ras-GRF to the membrane is being investigated.

To date, all Dbl-related proteins are connected, one way or another, to the Rho family GTPases (11). Our results indicate that Ras-GRF is no exception, because Cdc42 can profoundly affect its biochemical activity. We show that wild type and N17 Cdc42 preclude the Ras-GRF presence in the particulate fraction and activation of the Ras/MAPK pathway. The fact that a membrane-bound Ras-GRF circumvents Cdc42 N17 inhibitory effects suggests that Cdc42 plays an essential role in the recruitment of Ras-GRF to the membrane. In this context, we have not been able to detect any direct binding of Ras-GRF to Cdc42 in vitro. This however, may be due to the requirement for other components to form a stable complex and does not rule out that such an interaction takes place in vivo.

It could be argued that the inhibitory effects of Cdc42 N17 could be very indirect by inducing profound alterations in the cell (e.g. in the actin cytoskeleton), in a way that would prevent Ras-GRF recruitment to the membrane and subsequent Ras/MAPK activation. This is unlikely, because Rho and Rac dominant interfering mutants that should also be expected to induce major cytoskeletal alterations do not affect Ras-GRF function. More importantly, MAPK activation by SOS is completely unaffected by Cdc42 N17, which speaks in favor of the described Cdc42 inhibitory effects being specific for Ras-GRF.

The Ras-GRF relationship with Cdc42 does not imply, in any case, that Ras-GRF should promote nucleotide exchange on the GTPase. As previously shown in vitro (4, 21) our results provide evidence that Ras-GRF is not an exchange factor for Cdc42 in living cells. In agreement, recent reports (45, 46) indicate that serpentine receptor by subunits and Src induce nucleotide exchange activity of Ras-GRF over Rac-1 but not over Cdc42.

Surprisingly, although Cdc42 wild type and N17 act as effective blockers of Ras-GRF membrane localization and MAPK activation, the constitutively activated mutant Cdc42 QL does not. This implies that Cdc42 acts as an interfering molecule only when GDP-bound, but not in the GTP-bound form. In agreement, we show that stimulating the formation of GTP-Cdc42 with a Cdc42 exchange factor such as Dbl potentiates Ras-GRF-mediated MAPK activation. The fact that Dbl cannot activate MAPK per se excludes the possibility of this effect being due to a Cdc42 > Pak3 > Raf > MEK > MAPK connection (47) activated by Dbl itself. Moreover, ionomycin, a stimulator of Ras-GRF (21), induces nucleotide exchange on Cdc42, suggesting that relieving the blockade exerted by GDP-Cdc42 is necessary for activating Ras-GRF. Furthermore, Ras-GRF-mediated MAPK activation stimulated by ionomycin is specifically blocked by Cdc42 N17, verifying that the effects of ionomycin over Ras-GRF are Cdc42-dependent.

Overall, and based on our presented data, we propose a hypothetical model that may help to envisage the role of Cdc42 in Ras-GRF activation: GDP-bound Cdc42 may be sequestering a yet unidentified “membrane anchor” protein that Ras-GRF would require to localize in the membrane. Upon loading Cdc42 with GTP, this interaction would be relieved thus allowing Ras-GRF to gain access to its membrane attachment and to activate Ras. Based on our data, the association between Ras-GRF and its membrane anchor could be mediated by the DH domain.

If found to be true, this model may help explain a long-lasting, apparent inconsistency: Why are most Rho family GEFs potent oncopogenes, if their only known effectors, the Rho GTPases, are not, even when mutationally activated (11)? In light of our current hypothesis, this could be explained by Rho GEFs being capable of promoting a certain amount of Ras activation upon inducing nucleotide exchange on the Rho GTPases. This relieves the restraint that GDP-bound Rho family proteins may be exerting over Ras-specific exchange factors by sequestering their membrane attachments. In fact, we show that in the presence of suboptimal concentrations of Ras-GRF, Dbl is capable of inducing the activation of the Ras/MAPK pathway. Whether this model is true and applicable to other Ras exchange factors and Rho family proteins is under current investigation as is the determination of the identity of the putative membrane anchoring proteins.

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REFERENCES
1. Katz, M. E., and McCormick, F. (1997) Curr. Opin. Genet. Dev. 7, 75–79
2. Boguski, M. S., and McCormick, F. (1993) Nature 366, 643–654
3. Martegani, E., Vanoni, M., Zippel, R., Cocetti, P., Brambilla, R., Ferrari, C., Sturani, E., and Algerhina, L. (1992) EMBO J. 11, 2151–2157
4. Shou, C., Farnsworth, C. L., Neel, B. G., and Feig, L. A. (1992) Nature 358, 351–354
5. Wei, W., Mosteller, R. D., Sanyal, P., Gonzales, E., McKinney, D., Dasgupta, C., Li, P., Liu, B., and Brock, D. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 7100–7104
6. Cen, H., Papageorge, A. G., Zippel, R., Lowy, D. R., and Zhang, K. (1992) EMBO J. 11, 4007–4015
7. Guererro, C., Rojas, J. M., Chedid, M., Esteban, L. M., Zimonjic, D. B., Popescu, N. C., Font de Mora, J., and Santos, E. (1996) Oncogene 12, 1097–1107
8. Feig, L. A. (1994) Curr. Opin. Cell Biol. 6, 204–211
9. Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) Biochim. Biophys. Acta 1332, F1–F23
10. Cerrone, R. A., and Zheng, Y. (1996) Curr. Biol. 6, 216–222
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11. Van Aelst, L., and D’Souza-Schorey, C. (1997) Genes Dev. 11, 2295–2322
12. Miki, T., Smith, C. L., Long, J. E., Eva, A., and Fleming, T. P. (1993) Nature 362, 462–465
13. Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996) Cell 85, 621–624
14. Ando, H., Papageorge, A. G., Vass, W. C., Zhang, K., and Lowy, D. R. (1993) Mol. Cell. Biol. 13, 7718–7724
15. Martinghö, R. R., and Macara, I. G. (1996) Nature 382, 268–272
16. Shou, C., Wurmser, A., Ling, K., Barbacid, M., and Feig, L. A. (1995) Oncogene 10, 1887–1893
17. Zippel, R., Orecchia, S., Sturani, E., and Martegani, E. (1996) Oncogene 12, 2697–2703
18. Cheney, R. E., and Mooseker, M. S. (1994) Mol. Biol. Cell (suppl.) 5, 21a
19. Farnsworth, C. L., Freshney, N. W., Rosen, L. B., Gosh, A., Greenberg, M. E., and Feig, L. A. (1995) Nature 376, 524–527
20. Buchsbaum, R., Telliez, J., Gooneskera, S., and Feig, L. (1996) Mol. Cell. Biol. 16, 4888–4896
21. Freshney, N. W., Gooneskera, S. D., and Feig, L. A. (1997) FEBS Lett. 407, 111–115
22. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) Cell 81, 1137–1146
23. Wigler, M. S., Silverstein, L. S., Lee, S., Pellicer, A., Cheng, Y., and Axel, R. (1977) Cell 11, 235–232
24. Ajenjo, N., Aaronson, D. S., Galle, E., LeBrasseur, C., Lein, J., and Crespo, P. (2000) J. Biol. Chem. 275, 7189–7189
25. Laudanna, C., Campbell, J. A., and Butcher, E. (1996) Science 271, 981–983
26. Crespo, P., Schuebel, K. E., Ostrem, A. A., Gutkind, J. S., and Bustelo, X. R. (1997) Nature 385, 169–172
27. Byrne, J. L., Paterson, H. F., and Marshall, C. J. (1996) Oncogene 13, 2055–2065
28. Marsic, E., Lo, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1999) Mol. Cell. Biol. 1, 183–192
29. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) Nature 369, 418–420
30. Khosravi-Far, R., Chrzanowska-Wodnicka, M., Soltski, P. A., Eva, A., Burridge, K., and Der, C. J. (1994) Mol. Cell. Biol. 14, 6848–6857
31. Feig, L. A., and Cooper, G. M. (1988) Mol. Cell. Biol. 8, 3235–3243
32. Teramoto, H., Crespo, P., Coso, O. A., Igishi, T., Xu, N., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 25731–25734
33. Crews, C. M., Alessandri, M. A., and Erikson, R. L. (1992) Science 257, 478–480
34. Hori, Y., Beeler, J. F., Sakaguchi, K., Tachibana, M., and Miki, T. (1994) EMBO J. 13, 4776–4786
35. Chen, R. H., Corbalan-Garcia, S., and Bar-Sagi, D. (1997) EMBO J. 16, 1351–1359
36. Feig, L. A. (1999) Nat. Cell Biol. 1, 25–27
37. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
38. Zheng, Y., Zangrilli, D., Cézine, R. A., and Eva, A. (1996) J. Biol. Chem. 271, 19017–19020
39. Qian, X., Vass, W. C., Papageorge, A. G., Anborgh, P. H., and Lowy, D. R. (1998) Mol. Cell. Biol. 18, 771–778
40. Font de Mora, J., Guerrero, C., Mahadevan, D., Coule, J. R., Rojas, M., Esteban, L. M., Rebecchi, M., and Santos, E. (1996) J. Biol. Chem. 271, 18272–18276
41. Anborgh, P. H., Qian, X., Papageorge, A. G., Vass, W. C., DeClue, J. E., and Lowy, D. R. (1999) Mol. Cell. Biol. 19, 4611–4622
42. Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cézine, R. A., and Zheng, Y. (1994) J. Biol. Chem. 269, 62–65
43. Fan, W., Koch, C. A., de Hoog, C. L., Fam, N. P., and Moran, M. F. (1998) Curr. Biol. 8, 935–938
44. Movilla, N., and Bustelo, X. R. (1999) Mol. Cell. Biol. 19, 7870–7885
45. Kiyono, M., Satoh, T., and Kaziro, Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4826–4831
46. Kiyono, M., Kaziro, Y., and Satoh, T. (2000) J. Biol. Chem. 275, 5441–5446
47. King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marshall, M. S. (1998) Nature 396, 180–183
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