Maintenance of Cdc42 GDP-bound State by Rho-GDI Inhibits MAP Kinase Activation by the Exchange Factor Ras-GRF

EVIDENCE FOR Ras-GRF FUNCTION BEING INHIBITED BY Cdc42-GDP BUT UNAFFECTED BY Cdc42-GTP*

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The function of the Ras guanine nucleotide exchange factor Ras-GRF/cdc25Mn is subject to tight regulatory processes. We have recently shown that the activation of the Ras/MAPK pathway by Ras-GRF is controlled by the Rho family GTPase Cdc42 through still unknown mechanisms. Here, we report that retaining Cdc42 in its GDP-bound state by overexpressing Rho-GDI inhibits Ras-GRF-mediated MAPK activation. Conversely, Ras-GRF basal and LPA- or ionomycin-stimulated activities were unaffected by a constitutively active GTP-bound Cdc42. Moreover, the Cdc42 downstream effectors MLK3, ACK1, PAK1, and WASP had no detectable influence on Ras-GRF-mediated MAPK activation. In contrast, promoting GDP release from Cdc42 with the Rho family GTPase Dbl or with ionomycin suppressed the restraint exerted by Cdc42 on Ras-GRF activity. We conclude that Cdc42-GDP inhibits Ras-GRF-induced MAPK activation, but neither Cdc42-GTP nor the Cdc42 downstream effectors affect Ras-GRF performance. Interestingly, the loss of the GDP-bound state by Cdc42 abolishes its inhibitory effects on Ras-GRF function. These results suggest that the Cdc42 mechanism of action may not be solely restricted to activation of downstream signaling cascades when GTP-loaded. Furthermore, the GDP-bound form may be acting as an inhibitory molecule down-modulating parallel signaling routes such as the Ras/MAPK pathway.

Small GTP-binding proteins act as key molecular switches in signal transduction routes through which stimuli received through cell surface receptors are conveyed to the nucleus. The hallmark of small GTP-binding protein function, the transition from an inactive GDP-bound state to an activated GTP-bound state, is tightly regulated by three types of regulatory proteins: GTPase-activating proteins (GAPs),1 potentiators of the intrinsic capacity of small G proteins to hydrolyze GTP; guanine nucleotide dissociation inhibitors (GDIa) that regulate the nucleotide interchange process by preventing GDP release; and guanine nucleotide exchange factors (GEFs) that initiate the nucleotide replacement cycle by catalyzing the exchange of GDP for GTP (1).

Ras-GRF/Cdc25Mn is a GEF for the Ras family of small GTP-binding proteins cloned by virtue of its homology with the CDC25 gene product that stimulates nucleotide exchange on Saccharomyces cerevisiae Ras (2–5). The primary structure of Ras-GRF reveals the presence of a number of regulatory motifs presumably involved in diverse signaling control mechanisms and protein-protein interactions. These include a Dbl homology (DH) domain (6), generally present in GEFs for the Ras family of small G proteins (7). The DH domain is flanked by two Pleckstrin homology (PH) domains, the function of which remains largely unclear, although it has been suggested that it may play a role in membrane targeting (8).

With regard to regulation, it is known that Ras-GRF exchange activity over Ras is augmented by G protein-coupled receptors but is largely unaffected by receptors of the tyrosine kinase type (9–13). Calcium can also regulate Ras-GRF activity by a mechanism mediated through a calmodulin-binding motif (IQ domain) present in its N terminus (14, 15). As such, calcium ionophores, like ionomycin, can greatly enhance the activation of the MAPK pathway by Ras-GRF (15, 16). Interestingly, we have recently shown that the ability of Ras-GRF to activate the Ras/MAPK pathway is regulated by the Rho family GTPase Cdc42 that apparently controls the translocation of Ras-GRF to the membrane (17).

In this study we have taken a step further in the investigation of the mechanisms by which Cdc42 regulates the activation of the Ras/MAPK pathway by Ras-GRF. We show that Cdc42, retained in a GDP-bound state by the action of the guanine dissociation inhibitor Rho-GDI, acts as a down-regulator of Ras-GRF. Conversely, ectopic expression of either a constitutively activated, GTP-loaded Cdc42 or Cdc42 downstream effector proteins does not affect Ras-GRF-mediated activation of MAPK. Interestingly, inducing GDP release from Cdc42 by the overexpression of Dbl, a bona fide Cdc42 exchange factor, relieves the inhibitory effects exerted by Cdc42 on the activation of the MAPK pathway by Ras-GRF.

MATERIALS AND METHODS

Constructs—Plasmids encoding WASP and MLK3 were provided by J. S. Gutkind and PAK1 (L83, L86) by J. Field. A constitutively activated mutant (L543F) of ACK1 and Rho-GDI160 were generated by polymerase chain reaction-directed mutagenesis and subcloned in pCDNA3. The sequences of the oligonucleotides utilized are available upon request. All constructs used the CMV promoter (18).

Cell Culture—COS-7 cells were regularly grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Subconfluent cells were transfected by the DEAE-dextran technique (18). Total amount of transfected plasmid DNA was equalized with vector DNA when necessary.
Kinase Assays—MAPK and JNK kinase activities were determined as described previously (19) in anti-HA immunoprecipitates using myelin basic protein (MBP) (Sigma) or GST-ATF2 as substrates for MAPK or JNK, respectively. Reactions were terminated by the addition of 5× Laemmli buffer and boiled and electrophoresed in 12% SDS-polyacrylamide gels. Gels were visualized by autoradiography and quantitated by phosphorimager.

Immunoblotting—Immunoprecipitations were performed as described previously (20). Total lysates and immunoprecipitates were fractionated in SDS-polyacrylamide gels and transferred onto nitrocellulose filters. Immunocomplexes were visualized by ECL detection (Amersham Pharmacia Biotech) using horseradish peroxidase-conjugated secondary antibody (Cappel). Mouse monoclonal anti-AU5, anti-HA, and anti-Myc antibodies were from Babco. Rabbit polyclonal antibodies anti-Ras-GRF, anti-Cdc42, anti-MLK3, anti-Dbl, and anti-RHO-GDI were from Santa Cruz Laboratories. Subcellular fractionation was performed in 20 mM HEPES, pH 7.4 buffer, as described (17).

**Ras GTP Loading**—Ras GTP loading was performed basically as described by Taylor and Shalloway (21). Briefly, cells cotransfected with AU5-H-Ras and the indicated plasmids were lysed in HEM buffer (25 mM HEPES, pH 7.3, 10 mM MgCl2, 150 mM NaCl, 0.5 mM EGTA, 20 mM β-glycerophosphate, 0.5% Nonidet-P40, 4% glycerol, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin. AU5-H-Ras was affinity sequestered with bacterially synthesized GST-Raf Ras Binding Domain (RBD) (amino acids 1–149). Immunoblots were performed as described above using anti-AU5 antibody and quantitated by densitometry, using the program NIH Image 1.60. Ras GTP levels were related to the total Ras protein levels as determined by anti-AU5 immunoblotting in the corresponding cellular lysates.

**GTP-Cdc42 Pull-down Assay**—AU5-Cdc42-transfected cells were lysed as above. GTP-bound AU5-Cdc42 was affinity sequestered with bacterially synthesized GST-PAK CRIB (Cdc42/Rac Interaction Binding) domain (amino acids 70–106) basically as described (22). Immunoblots were performed as described above using anti-AU5 antibody.

**RESULTS AND DISCUSSION**

MAPK Activation by Ras-GRF Is Inhibited by Rho-GDI—We have recently demonstrated that the activation of Ras/MAPK by Ras-GRF could be efficiently prevented by the Cdc42 dominant interfering mutant, Cdc42 N17 (17). It is currently believed that GTPases N17 mutants work by competing with the endogenous proteins for binding to GEFs, thus retaining them in an inactive, GDP-bound form. However, potential pitfalls exist when interpreting results obtained with N17 mutants (23). Because of this, we tested whether other means of maintaining Cdc42 in its GDP-bound state would also restrain Ras-GRF function. For this purpose, we utilized the guanine nucleotide dissociation inhibitor Rho-GDI (24) that has been shown to prevent GDP dissociation from Cdc42 and other Rho GTPases (25, 26).

The direct method for analyzing the activity of an exchange factor is by assaying its capacity to catalyze nucleotide exchange on its target GTPase. For this purpose Ras-GRF was cotransfected with AU5-tagged H-Ras into COS-7 cells, in the presence of Cdc42 N17 or Rho-GDI, and Ras-GTP was affinity-purified from the resulting cellular lysates with the aid of GST-Raf RBD (see “Materials and Methods”). As shown in Fig. 1A, Ras-GRF induced a potent incorporation of GTP into Ras. However Ras-GTP levels dropped over 60% when Ras-GRF was cotransfected with Cdc42 N17 or Rho-GDI, indicating that these proteins were effectively blocking Ras-GRF-induced nu-
cleotide exchange on Ras. These results closely matched those obtained when MAPK activation was used to monitor the effects of Rho-GDI on Ras-GRF function. As shown in Fig. 1B, Rho-GDI blocked Ras-GRF-induced MAPK activation almost as efficiently as Cdc42 N17 when expressed at similar levels. On the other hand, the activation of MAPK by RasV12 was unaltered by Rho-GDI (Fig. 1B), thereby demonstrating that Rho-GDI is affecting the activation of the Ras/MAPK pathway upstream from Ras. These data, together with our previous findings (17), clearly demonstrate that the results obtained by direct measurement of Ras GDP/GTP exchange are identical to those obtained by assaying the activation of MAPK. Thus, we have chosen MAPK activation as a valid readout for further experimentation.

Calcium ionophores and certain agents acting through G protein-coupled receptors such as lysophosphatidic acid (LPA) have been shown to boost Ras-GRF-mediated MAPK activation (9, 15, 16). Thus, we also tested the effects of Rho-GDI on the activity of Ras-GRF under the influence of external stimuli. In agreement with our previous observations, Rho-GDI potently inhibited Ras-GRF-mediated MAPK activation when stimulated by ionomycin or LPA but had no effects on MAPK activation elicited by EGF (Fig. 1C), which is known to be Ras-GRF-independent (10, 11).

An alternative mechanism through which a G protein can be kept in its GDP form is by potentiating its GTPase activity, which can be accomplished by the overexpression of adequate GAP proteins. Therefore, we determined if p50Rho-GAP (27), known to have a potent GAP activity against Cdc42 (28), could also prevent the activation of MAPK by Ras-GRF. As expected, p50Rho-GAP could block the stimulation of MAPK by Ras-GRF just as efficiently as Rho-GDI (Fig. 1D). These effects could not be attributed to differences in the protein levels of the HA-MAPK cotransfected in these assays, since the protein was equally well expressed in all cases (Fig. 1C, lower panel).

In summary, our results showing that Cdc42 N17, Rho-GDI, and p50Rho-GAP can clearly abrogate the activation of MAPK by Ras-GRF, strongly point to the maintenance of Cdc42 in its GDP-bound state as the underlying cause of the Cdc42 inhibitory effects on Ras-GRF activity. Also, these results suggest that the effects of Cdc42 N17 on Ras-GRF function are not due to other indirect mechanisms. It should be noted that both Rho-GDI and p50Rho-GAP are also active on the Rho family GTPases Rho and Rac (25, 26, 28). However, we have previously described that unlike Cdc42, the dominant inhibitory mutants of Rho and Rac have no detectable effects on the activation of MAPK induced by Ras-GRF (17). In light of this data, it is reasonable to attribute the effects of Rho-GDI and p50Rho-GAP on Ras-GRF function, mainly to their influence on Cdc42.

**Analysis of Rho-GDI/Cdc42 Interactions**—It has been shown that in vitro Rho-GDI is able to interact equally well with the inactive GDP-bound state of Rho proteins and the active GTP-bound form (29). This circumstance should be taken into account when interpreting the effects of Rho-GDI on the functional relationship between Ras-GRF and Cdc42. To clarify this point, we investigated the interaction of Rho-GDI and Cdc42 in our cellular system. COS-7 cells were transfected with wild-type AU5-tagged Cdc42 that is GDP-bound under serum-starvation conditions or with AU5-Cdc42 Q61L (QL) found in a GTP state under the same experimental conditions. Upon anti-AU5 immunoprecipitation, it was found that endogenous Rho-GDI associated strongly with Cdc42 wt but was undetected in Cdc42-QL immunoprecipitates (Fig. 2A). This may reflect the observation that association of Rho-GDI with the GTP-bound form of Rho GTPases takes place mainly in vitro (29). Other reports indicate that Rho-GDI interacts with the GTP-bound form of its cognate proteins much less efficiently than with the GDP-bound form (30), quite in line with our results. Moreover, in absolute agreement with our observations, a recent in vivo study undertaken in COS-1 cells clearly demonstrates that Rho-GDI does not bind to Cdc42-QL (31). Although it cannot be
excluded that under certain physiological circumstances Rho-GDI could bind to Cdc42-GTP, under our experimental conditions and in our cellular environment, Rho-GDI is preferentially associated to Cdc42-GDP.

In addition to its capability of inhibiting GDP release from Rho family G-proteins, Rho-GDI can also bring about the extraction of Rho proteins from cellular membranes, thus rendering the GTPases cytoplasmic and therefore inactive (24, 25, 32). It was of interest to determine whether Rho-GDI effects on Ras-GRF could be somehow associated with this feature. For this purpose, AU5-Cdc42 was coexpressed with Myc-tagged Rho-GDI, and its cellular distribution was examined. Subcellular fractionation of the COS-7 lysates demonstrated that in the absence of exogenous Rho-GDI, Cdc42 was found in the cell particulate fraction as expected (Fig. 2B). However, despite high levels of expression, as verified by anti-Myc immunoblotting, Rho-GDI did not alter the cellular distribution of Cdc42, because no Cdc42 could be detected in the soluble fraction (Fig. 2B). This demonstrates that in our cellular system and under our experimental conditions, Rho-GDI did not provoke the release of Cdc42 from the cell membranes. The fact that Rho-GDI effectively solubilizes Cdc42 in other cell types such as human placental cells and epidermal carcinoma cells (25) suggests that other unknown cell-specific factors may determine whether Rho-GDI membrane-extracting activity is functional, depending on the cell type and/or physiological circumstances. A recent report suggests that the solubilization of Rho GTPases is strictly dependent on the stoichiometric relationship between Rho-GDI and its cognate GTPases (31). It is thus conceivable that the concentrations of Rho-GDI utilized in our assays, although sufficient to inhibit nucleotide exchange, are not high enough to solubilize Cdc42. Indeed, we have observed that steadily increasing the concentration of Rho-GDI brings about a gradual solubilization of Cdc42 (data not shown).

The possibility exists that Rho-GDI could inhibit Ras-GRF function through a direct interaction by sequestering it unproductively in the cytoplasm. To test this hypothesis, we cotransfected AU5-Rho-GDI with Ras-GRF and assayed its ability to coinmunoprecipitate. As shown in Fig. 2C, no Ras-GRF was detected in association with anti-AU5 immunoprecipitates, either under basal or ionomycin-stimulated conditions, indicating that no direct interaction exists between Rho-GDI and Ras-GRF.

On the other hand, it was found that Rho-GDI markedly inhibited Cdc42 GDP/GTP exchange induced by the GEF Dbl or by stimulation with ionomycin (data not shown) as determined by Cdc42-GTP pull-down assays using the GST-PAK CRIB domain (see “Materials and Methods”). To further substantiate the notion that Rho-GDI down-regulatory effects over Ras-GRF were mainly due to its function as an inhibitor of Cdc42 nucleotide exchange, we made use of Rho-GDIΔ60. This mutant lacks the 60 N-terminal amino acids, a region that contributes little to binding but is necessary to inhibit nucleotide dissociation from Cdc42 (33). As shown in Fig. 2D, the activation of MAPK brought about by Ras-GRF was clearly inhibited by AU5-Rho-GDI, but was largely unaffected by AU5-Rho-GDIΔ60 when expressed at similar levels. These results clearly demonstrate that Rho-GDI inhibitory effects over Ras-GRF reside with its ability to repress the dissociation of GDP from Cdc42.

Overall, our results demonstrate that under our experimental conditions: (i) Rho-GDI does not associate with Cdc42 when in a GTP-bound state; (ii) Rho-GDI is ineffective in rendering Cdc42 cytoplasmic; and (iii) Rho-GDI does not interact directly with Ras-GRF. Furthermore, our data prove that the effects of Rho-GDI on Ras-GRF function are primarily due to its role as a blocker of Cdc42 nucleotide exchange, thus to maintain Cdc42 in a GDP-bound form.

**Rho-GDI Blocks Ras-GRF Recruitment to the Membrane**—Our previous findings demonstrate that the inhibition of Ras-GRF-mediated MAPK activation by Cdc42 N17 correlates with the reduction of Ras-GRF protein levels in the cell particulate fraction (17), suggesting that Cdc42, when GDP-bound, would be interfering with Ras-GRF translocation to the cell membranes. If this was the case, Rho-GDI, which as shown before retains Cdc42 in its GDP-bound form, would be expected to prevent a Ras-GRF presence in the membrane fraction. To test this point, we analyzed Ras-GRF cellular distribution upon cotransfection with Rho-GDI. As shown in Fig. 3, in the absence of ectopic Rho-GDI, Ras-GRF was evenly distributed between the particulate and soluble fractions. However, cotransfection of Rho-GDI markedly diminished Ras-GRF levels in the membrane fraction. This effect clearly correlated with the inhibition of Ras-GRF-mediated MAPK activation by Rho-GDI (Fig. 3). On the other hand, the activation of MAPK induced by a membrane-bound Ras-GRF-CAAX (17) was unaffected by Rho-GDI. Likewise, the cellular distribution of this membrane-targeted Ras-GRF was unaltered by Rho-GDI (Fig. 3). These results are in perfect agreement with our previous observations using Cdc42 N17 (17) and suggest that Cdc42 in its GDP state, regardless of the mechanism by which this state is achieved, abrogates Ras-GRF activation by preventing its localization to the cell particulate fraction.

**Ras-GRF Function Is Unaffected by Cdc42-GTP or by Cdc42 Downstream Effectors**—A vast collection of data gathered hitherto indicates that most biological processes that are inhibited or diminished by the action of a “switched off” GDP-bound GTPase are activated or potentiated by the “switched on” GTP-bound form of the GTPase. Thus, it would be conceivable that the activation of MAPK by Ras-GRF, which is inhibited by Cdc42-GDP, should be potentiated by Cdc42-GTP. To verify this point, we tested whether the Cdc42 constitutively active mutant, Cdc42-QL, should be capable of boosting the MAPK response induced by Ras-GRF. For this purpose Ras-GRF was cotransfected with increasing amounts of Cdc42-QL into COS-7 cells. It was found that Cdc42-QL strongly activated JNK (Fig. 4A, middle panel), known to be a downstream target of Cdc42 (18). However, it did not affect MAPK activation elicited by different concentrations of Ras-GRF (Fig. 4A, top and lower panels), thus suggesting that basal Ras-GRF-induced MAPK activation is independent of Cdc42-GTP levels. In a similar fashion, overexpression of Cdc42-QL did not alter Ras-GRF-mediated MAPK activation in cells treated with ionomycin or LPA (Fig. 4B), indicating that the externally stimulated activation of MAPK mediated by Ras-GRF is also largely insensitive to the amount of Cdc42-GTP present in the cell.
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To further substantiate this point, we also tested whether several proteins known to be activated by Cdc42-GTP and well proven as bona fide Cdc42 effectors (7) would be capable of potentiating Ras-GRF function. Hyperactive forms of ACK1 (L543F) (94), MLK3 (35), PAK1 (L83, L86) (36), and WASP (37) were found to be well expressed and/or to potently activate JNK upon transfection into COS-7 cells (Fig. 4C, middle and lower panels). However, they were unable to augment even slightly the activation of MAPK induced by Ras-GRF in an experimental setting in which ionomycin strongly potentiated Ras-GRF-mediated MAPK activation (Fig. 4C, top panel).

Overall, these results clearly demonstrate that the activation of MAPK by Ras-GRF, although inhibited by Cdc42-GDP, is unaffected either by GTP-loaded Cdc42 or by Cdc42 effector proteins. These data strongly support our initial observations that Cdc42-QL does not affect the presence of Ras-GRF in the cell particulate fraction (17), although they conflict with a recent report in which ACK1 was found to synergize with Ras-GRF in the activation of MAPK (34). However, the cellular and/or experimental conditions utilized in the last study was such that Ras-GRF activity was affected by stimuli signaled by receptors of the tyrosine kinase type such as EGF, in contrast to previous results (9–11).

Inhibition of Ras-GRF by Cdc42-GEFs—We have previously shown that the intensifying effect of ionomycin over Ras-GRF-mediated MAPK activation can be blocked by Cdc42 N17. We have also demonstrated that ionomycin induces nucleotide exchange on Cdc42 (17). This means that relieving the blockade that Cdc42-GDP exerts on Ras-GRF could be a requisite for fully enhancing Ras-GRF potential to activate the Ras/MAPK pathway. To test this hypothesis, we tested whether exchange factors for Cdc42 would be capable of rescuing the restraint on Ras-GRF activity caused by GDP-bound Cdc42. We have previously reported that high concentrations of Cdc42-wt, mainly GDP-loaded under the serum-starved conditions used in our assays, can be as efficient as Cdc42 N17 for inhibiting Ras-GRF (17). Indeed, MAPK activation by Ras-GRF was remarkably diminished upon cotransfection with 1 μg of Cdc42-wt (Fig. 5A). Interestingly, MAPK activation levels were completely restored by coexpression of the GEF Ost (38) (Fig. 5A), in agreement with our previous findings in which Dbl was shown to be capable of potentiating Ras-GRF-induced MAPK activation (17). This effect could not be attributed to the stimulation of MAPK through alternative routes, such as the PAK→MEK→MAPK connection (39), as Dbl did not affect MAPK when acting alone (Ref. 17 and data not shown). Likewise, ionomycin was also capable of completely rescuing Ras-GRF activity. On the other hand, the GEF Ost and Cdc42-QL were incapable of overcoming the inhibitory effects of Cdc42-wt (Fig. 5A). The ineffectiveness of Ost, despite high expression levels (not shown), is noteworthy, as Ost has been reported to act as a Cdc42 GEF in vitro (40). A likely explanation could be that Ost is not a GEF for Cdc42 in vivo, at least in COS-7 cells. In a similar fashion, other Rho family exchange factors such as Vav, which is not active over Cdc42 (41), also fail to affect Ras-GRF activity (17).

Finally, we explored whether GEFs for Cdc42 could also alleviate the inhibition of Ras-GRF activity brought about by Rho-GDI. For this purpose, Ras-GRF was cotransfected with equal concentrations of Cdc42-wt and Rho-GDI. Both constructs were able to markedly diminish the activation of MAPK induced by Ras-GRF (Fig. 5B). The coexpression of increasing concentrations of Dbl, gradually counteracted the repression on Ras-GRF caused by Cdc42-wt. This effect was also observed in Rho-GDI-transfected cells, although in this case, the amount of Dbl necessary to initiate the rescue of Ras-GRF activity was 2-fold higher (Fig. 5B). This can be explained as Rho-GDI induces a potent inhibitory effect on GDP release catalyzed by Dbl, which can only be overcome by high concentrations of this GEF (25). On the other hand, the wild-type version of Dbl, proto Dbl, known to have a weaker nucleotide exchange activity on Cdc42 (38) was less effective for rescuing Ras-GRF-mediated MAPK activation (Fig. 5B).

In conclusion, our data demonstrate that Cdc42 in its GDP-bound form, acts as a potent down-regulator of Ras-GRF-induced activation of the Ras/MAPK pathway. However, Cdc42 in a GTP-bound state does not have the expected potentiating effect on Ras-GRF function. This is clearly envisioned by the unresponsiveness of Ras-GRF to Cdc42-activated downstream effector proteins and to hyperactive Cdc42-QL, and by the
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If this were the case, the mode of action of Cdc42 would not be restricted solely to the stimulation of downstream effector proteins, such as those bearing CRIB domains (7), when in an activated, GTP-bound state. In addition, when GDP-loaded, Cdc42 may be acting as a down-regulator of the Ras/MAPK pathway by inhibiting the activity of Ras-specific GEFs. This double mechanism of action, although hitherto unprecedented among small GTP-binding proteins, is no stranger in the world of G proteins. Indeed, heterotrimeric G proteins function in that way. As such, Ga subunits when GTP-loaded directly stimulate downstream effectors like PLCβ or adenylate cyclase (42). Also, when in a GDP-bound state, Ga subunits act by sequestering Gβγ dimers, thereby preventing this component from interacting with its own effectors (43). It is interesting to note that one of the main pathways triggered by Gβγ dimers, thus inhibited by GDP-bound Ga subunits and unaffected by the ectopic expression of constitutively active Ga subunits, is the Ras/MAPK pathway (20). It is also noteworthy that in the case of heterotrimeric G proteins the connection between the Gβγ complex and Ras exchange factors is not direct and implies the presence of many intermediaries (44).

At present, whether this model can be applicable to Cdc42 needs to be fully validated. As yet, we ignore the precise mechanism by which Cdc42 inhibits Ras-GRF. Our previous results (17) do not indicate a direct physical interaction between Ras-GRF and Cdc42, at least in vitro. That suggests the participation of additional components, in a fashion also reminiscent of heterotrimeric G-proteins. Elucidating the exact mechanism of action and the identity of these components is necessary in future investigations.

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fig. 5. Inhibition of Ras-GRF by Cdc42 can be rescued by Rho-family GEFs. A, effects of Rho-family GEFs on Ras-GRF-induced MAPK activation when down-regulated by Cdc42-wt. MAPK activity levels in COS-7 cells transfected with Ras-GRF and Cdc42 wt (1 μg each) as shown (+), in addition to Dbl, Ost, Cdc42-QL, or empty vector (−) (1 μg each) and treated with 1 μM ionomycin for 5 min where indicated. Data show average ± S.E. of three independent experiments, expressed relative to the MAPK levels detected in cells transfected with Ras-GRF only. B, effects of Dbl on Ras-GRF-mediated MAPK activation when inhibited by Cdc42-wt or by Rho-GDI. Ras-GRF (1 μg) (+) was cotransfected with Cdc42 wt or Rho-GDI (1 μg each), and increasing concentrations (0.5, 1, and 2 μg) of Dbl or 2 μg of protoDbl were also transfected where indicated. Bottom, expression levels of Dbl and proto-Dbl.

inability of this mutant to rescue the restraint exerted on Ras-GRF activity by the overexpression of Cdc42-wt. Despite this, we show that the stimulation of GDP release from Cdc42, with the aid of agents that promote GDP/GTP exchange such as an external stimulus like ionomycin or the ectopic expression of a specific GEF for Cdc42, strongly facilitates the activation of MAPK by Ras-GRF.

The most plausible model that emerges from these data is one in which the loss of Cdc42 GDP-bound structural characteristics rather than the acquisition of a GTP-bound structural conformation, would be the key step for relieving the inhibitory effect that Cdc42 imposes on Ras-GRF function. Based on this hypothesis, the conformational features of Cdc42 when GDP-loaded would enable it to exert an inhibitory effect over Ras-GRF activity by still unknown mechanisms. This inhibition would be unaffected by the presence of ectopic Cdc42-GTP or Cdc42-activated effectors. However, catalyzing GDP release from Cdc42 would bring about the structural change, concomitant with the acquisition of a GTP-bound conformation, which would relieve the restraint exerted over Ras-GRF functions.
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