Activation of the exchange factor Ras-GRF by calcium requires an intact Dbl homology domain

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Abstract Ras-GRF is a guanine nucleotide exchange factor that activates Ras proteins. Its activity on Ras in cells is enhanced upon calcium influx. Activation follows calcium-induced binding of calmodulin to an IQ motif near the N-terminus of Ras-GRF. Ras-GRF also contains a Dbl homology (DH) domain C-terminal to the IQ motif. In many proteins, DH domains act as exchange factors for Rho-GTPase family members. However, we failed to detect exchange activity of this domain on well characterized Rho family members. Instead, we found that mutations analogous to those that block exchange activity of Dbl prevented Ras-GRF activation by calcium/calmodulin in vivo. All DH domains are followed immediately by a pleckstrin homology (PH) domain. We found that a mutation at a conserved site within the PH domain following the DH domain also prevented Ras-GRF activation by calcium in vivo. These results suggest that in addition to playing a role as activators of Rho proteins, DH domains can also contribute to the coupling of cellular signals to Ras activation.

Key words: Ras; Calcium; Calmodulin; Dbl homology domain; GTPase; Neuronal signaling

1. Introduction

Ras proteins are members of a superfamily of GTPases that cycle between the active GTP and inactive GDP bound states. These proteins become activated upon interaction with guanine nucleotide exchange factors (GEFs), which promote the release of GDP from Ras and allow its replacement with activating GTP. Ras proteins become deactivated when they hydrolyze GTP to GDP, a process that is enhanced by the interaction with GTPase activating proteins (GAPs) (for review see [1]).

Depending on the cell type and stimulus, Ras proteins can influence cell proliferation, differentiation or functions associated with non-dividing fully differentiated cells [2]. Ras proteins perform these functions by coupling signals originating at the cell surface to intracellular signaling cascades. At present, three classes of downstream effector proteins are known to be regulated upon binding to activated Ras in cells: (1) Raf protein kinases, which initiate a cascade of kinase activations including MEK and ERK [3]; (2) a phosphatidylinositol 3-kinase (PI 3-kinase), whose lipid product activates the Akt kinase [4–6] and non-conventional PKC isoforms [7,8]; and (3) a guanine nucleotide exchange factor that activates the Ral-GTPases [9–12].

In most cases studied to date, Ras proteins become activated in response to an increase in the activity of GEFs (for review see [13]), although examples of inhibition of GAP activity also have been documented [14]. In mammalian cells, regulatory pathways involving two classes of exchange factors, termed SOS and Ras-GRF (or CDC25 Mm), have been identified. SOS mediates tyrosine kinase receptor activation of Ras through its binding to the adapter protein Grb2 (for review see [13]). Some G protein-linked receptors are also thought to activate Ras via SOS [15]. One characterized pathway involves activation of the cytoplasmic PYK2 tyrosine kinase in response to elevated calcium levels [16], while others involve Src family tyrosine kinases [17–19].

Calcium can also activate Ras without the involvement of tyrosine kinase stimulation via the exchange factor Ras-GRF [20]. Such a mechanism may account for Ras activation associated with depolarization of primary cortical neurons [20]. Ras-GRF does not have a Grb2 binding site, but instead contains a calmodulin-binding IQ domain near its N-terminus. Ras-GRF activity is enhanced in cells upon elevated calcium levels, and this activation is dependent upon calmodulin binding to the IQ domain [20]. Ras-GRF activity has also been shown to be enhanced upon M1 and M2 muscarinic receptor activation, apparently through the liberation of βγ subunits of activated G proteins [21]. In this case the activating event is thought to be phosphorylation.

Ras-GRF contains additional conserved domains that are found in many other signaling molecules. N-terminal to the catalytic domain that activates Ras is a domain which shares homology with the proto-oncogenic protein Dbl [22,23]. A similar domain is also found in SOS [1]. Dbl is a GEF for the Rho A and CDC42 GTPases [24,25]. Along with Rac, Rho G and TC10, these proteins make up the Rho family of GTP binding proteins, some of which have been shown to regulate the actin cytoskeleton [26] distinct protein kinase cascades (for review see [27]). Many other proteins containing Dbl homology (DH) domains exhibit in vitro nucleotide exchange activity towards discrete sets of Rho family GTPases. For example, Ost activates RhoA and CDC42 [28], Lbc activates Rho A, B and C [29], Tiam1 activates Rac, RhoA and CDC42 [30], Abr and BCR activate CDC42, Rac and Rho [31], and CDC24 activates CDC42 [32]. Many Dbl-containing proteins have oncogenic potential presumably by virtue of their ability to activate Rho family proteins in vivo.

All known DH domains are followed directly by a pleck-

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Abbreviations: Ras-GRF, Ras-guanine nucleotide releasing factor; DH, dbl homology; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; ERK, extracellular regulated kinase; SOS, son of sevenless; GST, glutathione *S*-transferase

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strin homology (PH) domain [27]. Although the PH domain of Dbl is not required for exchange activity in vitro, it is required for in vivo transforming activity of Dbl [27]. PH domains without neighboring DH domains are also found in a wide variety of signaling molecules such as protein kinases (βARK-1, Akt/PTB and BTK), GTPases (dynamin) and cytoskeletal proteins (β-spectrin) [33]. PH domains are about 100 amino acids in length and may contain binding sites for lipids, such as PIP_2 (for review see [34]) and part of a binding site for proteins, such as the βγ subunits of G proteins [35]. These interactions may target PH domain-containing proteins to membranes.

The aim of this work was to investigate the role of the DH and its associated PH domain in Ras-GRF function. Initially, we screened a wide range of Rho family GTPases as potential downstream targets for Ras-GRF. To our surprise, Ras-GRF did not function as an exchange factor for any of the GTPases tested. However, we did find that intact DH and PH2 domains were required for the efficient calcium-mediated activation of Ras-GRF. Although we cannot rule out that this DH domain is an activator of an untested Rho family GTPase, the results of this work suggest that it plays an important role in the ability of Ras-GRF to activate Ras in response to calcium.

2. Materials and methods

2.1. Plasmids and recombinant DNA

Constructions of expression vectors for Ras-GRF, NGlu-Ras-GRF, Ras-GRF(IQ−), pJ3M-ERK1, and pMT3-Ras17N have been described previously [20]. NGlu-Ras-GRF(DH−) was constructed by overlap PCR using outside primers encoding the Glu tag and the amino terminus of Ras-GRF [20] and codons 570–575, and internal primers introducing IIIRDII at codons 390–396. The PCR product was cut with *Not*I and *Afl*II and subcloned into full length NGlu-Ras-GRF at the same sites. NGlu-Ras-GRF(PH2−) was constructed in a similar manner except that the outside primers were at codons 218–231 and 689–695 and the inside primers introduced W to A and I to A at codons 573 and 577 respectively. The PCR product was digested with *Bgl*II and *Pfl*mI and ligated into wild type Ras-GRF previously cut at the same sites. DNA sequencing confirmed the expected nucleotide alterations.

2.2. Cell culture and transfections

293T and COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL) and 10% iron-supplemented defined bovine calf serum (Hyclone). Cells were seeded at a density of $1–2\times10^5$ cells per 60 mm^2 dish 24 h prior to transfection. Cells were transfected with the indicated amounts of DNA by calcium phosphate precipitation (for 293T cells) or by the DEAE-dextran method (for Cos cells).

2.3. Cell stimulation, lysis and immunoprecipitation

Cells were serum starved for 16 h where indicated. 60 mm^2 dishes were incubated in 1 ml DMEM containing either 5 μM ionomycin (Calbiochem) for 5 min or buffer alone. After stimulation, the dishes were placed on ice, washed in 3 ml of ice cold phosphate-buffered saline (PBS) and scraped into 1 ml of PBS. The cells were collected in a microfuge at 5000 rpm for 4 min, resuspended in 250 μl of mild buffer (20 mM Tris-HCl pH 7.4, 125 μM NaCl, 1 mM $MgCl_2$) containing 1% Triton X-100, 50 mM sodium fluoride, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin and left on ice for 10 min with occasional vortexing. The extracts were centrifuged at $15\,000\times g$ for 10 min at 4°C and the supernatant removed. 50 μl of each supernatant was removed for immunoblotting and total protein determination and epitope-tagged proteins were immunoprecipitated by incubating 200 μl of each extract for 2 h at 4°C with 20 μl (1:1 slurry) of protein A Sepharose (Pharmacia) and the designated antibody (1:40 dilution of anti-myc 9E10 hybridoma supernatant; 1:100 dilution of anti-Glu hybridoma supernatant).

2.4. Protein kinase assays

ERK1 kinase assays were performed as previously described [20]. Briefly, immunoprecipitates were washed twice with PBS containing 1% Triton X-100 and twice with kinase buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM DTT). Immune complex phosphorylation reactions were performed at 20°C in 30 μl of kinase buffer containing 50 mM sodium fluoride, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 25 μM ATP, 0.1 μCi/μl [γ-^{32}P]ATP and 50 μg/ml of myelin basic protein. Reactions were stopped by the addition of 10 μl 4×Laemmli sample buffer and phosphorylated proteins were visualized and quantitated after SDS-PAGE using a phosphorimager (Molecular Dynamics).

2.5. Preparation of GDP-bound GST-Ras

GST-Ras was overexpressed and purified from bacteria. The fusion protein was eluted from glutathione beads by washing in exchange buffer (20 mM Tris-HCl pH 8, 100 mM NaCl, 40 μg/ml BSA, 1 mM $MgCl_2$, 2 mM DTT) containing 10 mM glutathione. The eluate was dialyzed overnight at 4°C against exchange buffer containing 2 μM GDP. An aliquot of the GST-Ras was visualized with Coomassie brilliant blue after SDS-PAGE to estimate the protein concentration. The GTPase was adjusted to a final concentration of 3 μM and 100 μl of each were incubated on ice for 15 min with 100 μl of exchange buffer containing 1.5μM GDP.

2.6. In vitro Ras exchange assay

10×100 mm^2 dishes of subconfluent 293T cells were transfected with 10 μg of either pMT3 NGluRas-GRF, NGluGRF(DH−) or NGluGRF(PH2−). Cell extracts were prepared as described above, except that each dish was extracted into 100 μl of lysis buffer. NGluGRF was immunoprecipitated from the extracts by incubation for 2 h at 4°C with 100 μl of a 1:1 slurry of protein A Sepharose which had been pre-bound to 10 μl of anti-Glu antibody. Immunoprecipitated NGluGRF was washed three times in mild buffer containing 0.5 mM $CaCl_2$ and 1% Triton X-100, and three times in mild buffer containing 0.5 mM $CaCl_2$ and 0.1% Triton X-100. Purified NGluGRF was eluted from the immune complex by incubation for 1 h at 4°C with a peptide of the Glu epitope (0.2 mg/ml). 5 μl of the GDP-loaded GST-Ras described above was incubated at 30°C for the indicated times with 35 μl of exchange buffer containing 2 μCi of [α-^{32}P]GTP and 100 ng of wild type or mutant NGluGRF or buffer alone containing Glu peptide. To determine the maximal binding of [α-^{32}P]GTP to GDP-Ras, 4.5 mM EDTA was added instead of NGluGRF. The mixture was incubated at 30°C for 5 min, $MgCl_2$ was added to a final concentration of 6 mM and incubated at 30°C for a further 5 min. 10 μl of each assay was spotted onto 1 cm^2 square pre-moistened nitrocellulose membrane and was washed with 4 ml of Tris-HCl-buffered saline containing 5 mM $MgCl_2$. The amount of [α-^{32}P]GTP bound to the membrane was quantitated by Cerenkov counting.

2.7. Subcellular fractionation of 293T cells

293T cells were transiently transfected with 3 μg of pMT3 Ras-GRF or Ras-GRF mutants in each 60 mm^2 dish. Cells were harvested as described above except that the cell pellets were resuspended in 125 μl of isotonic lysis buffer (20 mM Tris-HCl pH 7.5, 125 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin, 50 mM sodium fluoride, 25 mM β-glycerophosphate and 100 μM sodium orthovanadate) and sonicated. Samples were then microfuged at $10\,000\times g$ for 10 min at 4°C. The supernatants were removed and centrifuged at $100\,000\times g$ for 45 min at 4°C. The supernatant was removed and the pellet resuspended in 120 μl of lysis buffer containing 1% Triton X-100. 10 μg of each extract was separated by SDS-PAGE and Ras-GRF was identified by immunoblotting using a peptide antiserum against the C-terminal 20 residues of the protein (Santa Cruz).

3. Results

3.1. Functional DH and PH2 domains of Ras-GRF are required for activation by calcium

By virtue of its sequence similarity to Dbl, the DH domain of Ras-GRF has been proposed to be an exchange factor for Rho family GTPases. However, we have found that Ras-GRF

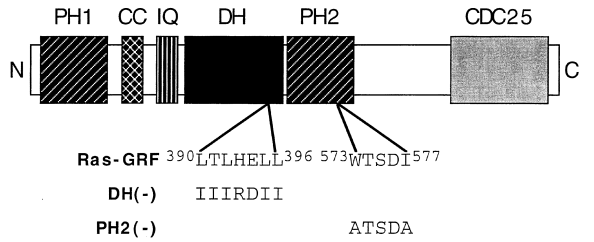

Fig. 1. Domain structure of wild type Ras-GRF indicating DH(−) and PH2(−) mutants. The N-terminus of Ras-GRF contains two domains which share homology with pleckstrin (PH1 and PH2), and one domain which shares homology with the oncoprotein Dbl (DH). To investigate the role of these conserved regions in Ras-GRF activation, we made mutations as indicated by DH(−) and PH2(−). The corresponding residues of wild type Ras-GRF are also shown. The C-terminus of the protein contains the catalytic domain (CDC25) which induces nucleotide exchange on Ras. In the N-terminus of the molecule is an IQ domain which binds calmodulin and a putative coiled-coil region (CC).

or the isolated DH domain of Ras-GRF purified from either insect or mammalian cells was unable to promote nucleotide exchange on RhoA, B, C, G, Rac1, CDC42 or TC21, in vitro (data not shown). We cannot rule out the possibility that the DH domain of Ras-GRF activates other unidentified or less well-defined members of the Rho family of GTPases, or that we have failed to isolate the protein in a form that allows it to express this activity in vitro.

Subsequently, we tested whether the DH domain of Ras-GRF might perform a different function, by contributing to the regulation of the C-terminal Ras exchange (CDC25) domain of the protein. Therefore, we investigated the effect of mutating the DH domain on the ability of Ras-GRF to activate Ras. Hart et al. showed that several non-transforming mutants of Dbl also had impaired nucleotide exchange activity towards CDC42 [36]. One of these Dbl mutants contained conservative substitutions in a region of seven amino acids (640–646) which is highly conserved in Ras-GRF and other DH-containing proteins. Therefore, we made analogous mutations in this region of Ras-GRF to examine the effect on enzyme activity (see DH(−) Ras-GRF in Fig. 1).

Using a similar strategy, we also investigated the role of the adjacent PH (PH2) domain in Ras-GRF activation by mutating a highly conserved tryptophan (W^{573}) to alanine and a nearby isoleucine to alanine (see (PH2(−)) Ras-GRF in Fig. 1). Ras-GRF activity was measured indirectly by its ability to activate ERK1, which lies downstream of Ras in the pathway (Fig. 2). Epitope tagged ERK1 was transiently expressed in 293T cells with wild type or mutant Ras-GRF, or with vector alone. Following stimulation with the calcium ionophore, ionomycin, ERK1 was immunoprecipitated from the cells and its activity was measured in an in vitro kinase assay. In cells lacking Ras-GRF, ionomycin produced a small (~3-fold) increase in ERK1 activity. As shown previously [20], the expression of Ras-GRF magnified the calcium effect, demonstrating Ras-GRF activation. In particular, Ras-GRF expression increased ERK1 activity ~3-fold in the absence of stimulation and 27-fold in response to the addition of ionomycin. As previously shown, Ras-GRF functioned through Ras to activate ERK, since co-expression of dominant negative 17N Ras suppressed Ras-GRF activity in the presence and absence of calcium influx.

Expression of either the DH(−) or PH2(−) Ras-GRF mutants also increased ERK1 activity, in the absence of stimulation, although this effect was consistently smaller than that found for wild type Ras-GRF. Strikingly, activation of either DH(−) or PH2(−) Ras-GRF by calcium influx was severely suppressed. Immunoblotting of the cell extracts revealed that the protein levels of ERK1 and Ras-GRF proteins did not vary significantly between each assay (data not shown). Thus, functional DH and PH domains are required for calcium induced activation of Ras-GRF.

3.2. *Mutations in the DH and PH2 domains do not affect the activity of Ras-GRF in vitro*

Next, we investigated whether the inhibitory effects of the mutations in the DH and PH2 domains of Ras-GRF were due to a reduction in the intrinsic catalytic activity of the enzyme. To answer this question, we measured the activity of mutant Ras-GRF in vitro. Epitope tagged wild type and mutant Ras-GRFs were purified from 293T cells transiently expressing the proteins. Their exchange activities were measured by their ability to induce GDP release from GST-Ras and subsequent binding of [α-^{32}P]GTP. Both DH(−) and PH2(−) mutants had exchange activities that were very similar to that of wild type Ras-GRF (Fig. 3).

3.3. *Mutations in the DH and PH2 domains do not affect the ability of Ras-GRF to bind calmodulin*

We have previously shown that the calcium-dependent binding of calmodulin to a conserved motif in the N-terminus of Ras-GRF is an essential event for Ras-GRF activation [20]. Therefore we examined whether or not the mutations in the DH and PH2 domains impaired the interaction between calmodulin and Ras-GRF. Epitope tagged wild type and mutant Ras-GRF were transiently expressed in 293T cells, immunoprecipitated and identified by immunoblotting following SDS-PAGE (Fig. 4A, upper panel). Co-precipitating calmo-

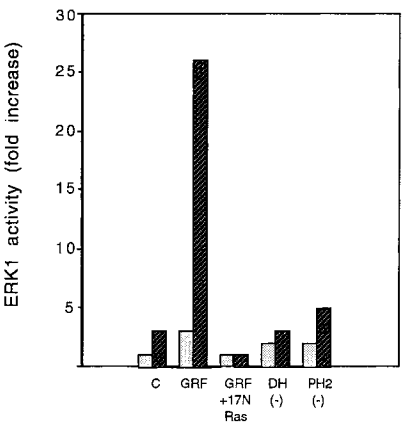

Fig. 2. Mutations in the DH and PH2 domains of Ras-GRF inhibit its activation by ionomycin–ERK1 activation assay. 60 mm^2 dishes of 293T cells were transiently transfected with 2 μg of pJ3M myc-ERK1 and 0.5 μg of either wild type or mutant Ras-GRF, or vector alone as indicated. In some experiments, 2 μg of 17N Ras was also co-expressed. Following serum starvation, cells were stimulated for 5 min with 5 μM ionomycin (black bars) or vehicle alone (grey bars). ERK1 activity was measured by immune complex kinase assays using myelin basic protein as substrate. Phosphorylated substrate was quantitated by a phosphorimager following SDS-PAGE. ERK1 extracted from resting cells was given an arbitrary protein kinase activity value of 1. Results represent the average of three experiments ± S.E.M.

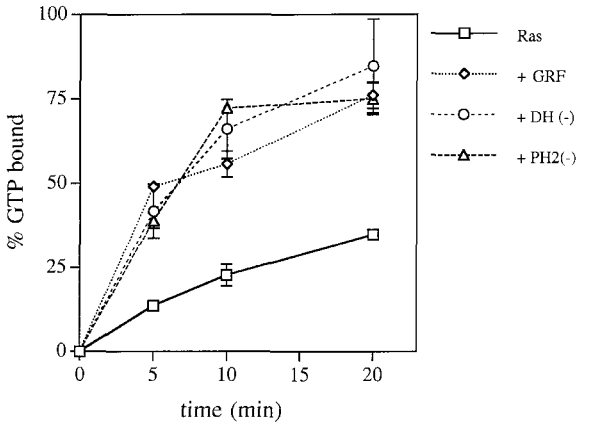

Fig. 3. Mutations in the DH and PH2 domains of Ras-GRF do not affect its activity in vitro. 250 ng of GDP-bound GST-Ras was incubated at 30°C for the indicated times alone (□) or with approximately 150 ng of wild type (◇) or mutant epitope-tagged Ras-GRF (○ DH−; △ PH2−) in the presence of 2 μCi of [α-^{32}P]GTP. NGlu-tagged Ras-GRF had been purified from 293T cells transiently expressing the protein and GST-Ras had been purified from bacteria. An aliquot of each reaction mixture was spotted onto a nitrocellulose membrane, washed and the amount of [α-^{32}P]GTP bound to each GTPase was determined by Cerenkov counting. 100% represents maximal binding of [α-^{32}P]GTP to each GTPase as measured by incubating the proteins at 30°C for 10 min with 4.5 mM EDTA to induce nucleotide exchange. Data are representative of two experiments each performed in duplicate.

dulin was detected after immunoblotting with an antibody against the protein (Fig. 4A, lower panel). As expected, wild type Ras-GRF bound calmodulin, while the IQ− mutant did not. Calmodulin co-precipitated with both the Ras-GRF DH(−) and the PH2(−) mutants.

3.4. *Mutations in the DH and PH2 domains do not affect the cellular localization of Ras-GRF*

Ras-GRF is localized preferentially to the particulate fraction even in unstimulated cells. We have recently shown that this localization is necessary but not sufficient for calcium to enhance Ras-GRF's ability to activate Ras in vivo [37]. Thus, we investigated whether altered cellular distribution might account for the loss in responsiveness of the DH and PH2 mutants. Immunoblot analysis of cytosolic and crude particulate fractions from 293T cells transiently expressing either wild type or mutant Ras-GRFs failed to demonstrate a significant difference among them (Fig. 4B). A recent report [38] showed that the PH domain of Dbl, which is essential for transformation by this protein, may enable it to bind to cytoskeletal components found in the Triton X-100 insoluble fraction of cells. While some wild type Ras-GRF exists in the Triton X-100 insoluble fraction, mutations in the DH and PH2 domains did not alter its association with this fraction (data not shown).

4. Discussion

Dbl and many other DH domain-containing proteins act as nucleotide exchange factors for Rho family GTPases. Since Ras-GRF possesses a DH domain in addition to its Ras exchange domain, it was proposed that Ras-GRF might function as a dual specificity exchange factor [22,23]. However, we could not detect such activity against well-characterized members of the Rho GTPase family. Thus, if the Ras-GRF DH domain functions as an exchange factor, we have not been able to isolate the protein in an active form, or its substrate is an untested Rho family member.

Instead, we have shown that the DH domain participates in Ras activation by Ras-GRF. Ras-GRF activity in vivo was measured by its ability to activate both its target Ras and ERK1, a kinase that lies downstream from Ras. In both assays, the DH mutation severely suppressed Ras-GRF's activation by calcium influx. In the ERK1 assay we also detected a small but reproducible decrease in the ability of the mutant Ras-GRF to activate Ras in the absence of calcium stimulation. Since we could not detect a defect in the intrinsic nucleotide exchange activity of this mutant as assayed in vitro, these findings suggest that the catalytic domain of the mutant may be incorrectly targeted to Ras in cells. This defect must be subtle because we could not detect a significant change in the distribution of the mutant between the cytosolic and particulate fractions of cells. Since the mutant also bound calmodulin as well as the wild type protein did, the DH domain may contribute a novel function, which is essential for proper Ras-GRF activity.

All known DH domains are followed by PH domains, suggesting that they function coordinately in cells. In Dbl, a mutation in the PH domain did not alter the protein's intrinsic nucleotide exchange activity, but it did block the protein's ability to oncogenically transform cells [38]. Similarly, mutations in the PH2 domain of Ras-GRF blocked the protein's ability to respond to calcium influx in vivo. The mutations did

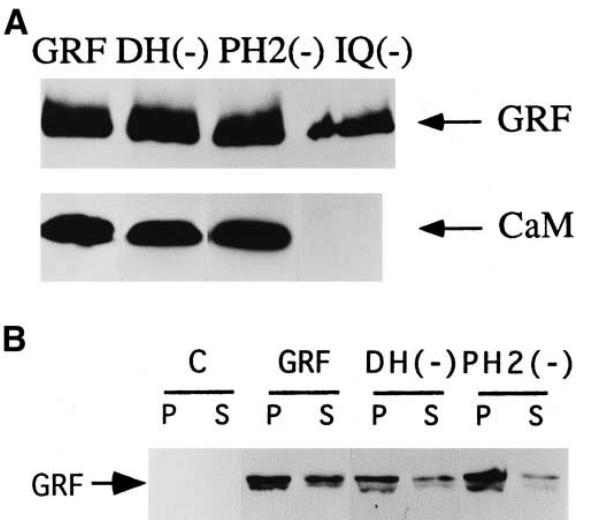

Fig. 4. The DH(−) and PH2(−) mutations do not inhibit calmodulin binding to Ras-GRF and do not affect its subcellular distribution. A: Calmodulin binding: 100 mm^2 dishes of 293T cells were transiently transfected with 10 μg of wild type or mutant epitope-tagged NGlu Ras-GRF as indicated. Following cell lysis, Ras-GRF was immunoprecipitated from the cells using an antibody against the epitope which had been pre-bound to protein A Sepharose. The upper panel shows an immunoblot of immunoprecipitated Ras-GRF and the lower panel shows an immunoblot of co-precipitating calmodulin. B: Subcellular distribution: 60 mm^2 dishes of 293T cells were transiently transfected with 3 μg of wild type or mutant pMT3 Ras-GRF as indicated. Cells were lysed in an isotonic buffer followed by sonication. Unbroken cells and debris were removed by a 10000×g spin, and the extracts were fractionated by ultracentrifugation at 100000×g to yield supernatant (S) and particulate (P) fractions. 10 μg of each extract was purified by SDS-PAGE and Ras-GRF was identified following immunoblotting.

not dramatically affect the protein's intrinsic nucleotide exchange activity against Ras in vitro, its cellular localization, or its ability to bind to calmodulin in vivo. Thus, this domain may function with the DH domain to provide a distinct function required for proper activation of Ras by Ras-GRF.

Interestingly, we have been unable to detect any difference between the in vitro exchange activities of Ras-GRF purified from either resting or ionomycin-stimulated cells. Furthermore, the presence of calmodulin does not alter the exchange activity of the enzyme in vitro [22]. Thus, additional cellular components may be required for full Ras-GRF activity. Such components could bind to the DH and PH2 domains. Such a component may fail to co-purify with the Ras-GRF, rendering it unresponsive to calmodulin binding vitro. Alternatively, mutations in either of these domains could indirectly inhibit the function of other domains that are known to be critical for Ras-GRF activation, such as the first PH domain or the coiled coil at the N-terminus of the protein [37]. Such a possibility could be tested in the future, if specific functions for the regions are elucidated.

Previous experiments on SOS demonstrated that its DH and PH domains contribute to its ability to activate Ras. In particular, deletion of both of these domains in *Drosophila* SOS prevented it from functioning properly in a differentiation pathway required for eye development [39]. Moreover, deletion of both the DH and PH domains of mammalian SOS inhibited the protein from activating Ras in transiently transfected COS cells [40]. However, deletion of either the PH or DH domain of SOS individually had no effect on the basal activity of SOS [40]. Here, we show that point mutations in either of these domains have a small inhibitory effect on the basal activity of Ras-GRF in vivo, and a large inhibitory effect on the protein's response to calcium influx. Analogous experiments on tyrosine kinase activation of SOS in the previous study were not performed, so it remains to be determined whether the DH and PH domains play similar roles in these two Ras exchange factors.

Exchange factors for Ras have turned out to be complex macromolecules with multiple functional domains. This may potentially allow them to respond to and influence diverse cellular signals. The results presented here add support to the idea that DH domains may themselves be multifunctional. In addition to acting as activators of Rho-family GTPases, they may also contribute to Ras activation by participating in the coupling of extracellular signals to activation of Ras exchange factor domains.

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