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Gα_q allosterically activates and relieves autoinhibition of p63RhoGEF

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Abstract

Gα_q directly activates p63RhoGEF and closely related catalytic domains found in Trio and Kalirin, thereby linking G_q-coupled receptors to the activation of RhoA. Although the crystal structure of Gα_q in complex with the catalytic domains of p63RhoGEF is available, the molecular mechanism of activation has not yet been defined. In this study, we show that membrane translocation does not appear to play a role in Gα_q-mediated activation of p63RhoGEF, as it does in some other RhoGEFs. Gα_q instead must act allosterically. We next identify specific structural elements in the PH domain that inhibit basal nucleotide exchange activity, and provide evidence that Gα_q overcomes this inhibition by altering the conformation of the α6-αN linker that joins the DH and PH domains, a region that forms direct contacts with RhoA. We also identify residues in Gα_q that are important for the activation of p63RhoGEF and that contribute to Gα subfamily selectivity, including a critical residue in the Gα_q C-terminal helix, and demonstrate the importance of these residues for RhoA activation in living cells.

Keywords

Gα_q; p63RhoGEF; RhoA; Dbl homology domain; pleckstrin homology domain; autoinhibition; allosteric activation

1. INTRODUCTION

Rho guanine nucleotide triphosphatases (GTPases)² belong to the Ras superfamily of small GTP-binding (G) proteins, and serve as master regulators of filamentous actin structure and

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cell morphology. In response to the activation of tyrosine kinase, semaphorin, and G protein-coupled receptors (GPCRs), they control processes that include smooth muscle contraction, cell proliferation, adhesion, and migration, and axon guidance. Because many of these processes are involved in the progression of cancer, Rho signaling is thought to play an important role in tumorigenesis, metastasis, and tissue invasion [1-4].

Like other G proteins, Rho GTPases are activated upon binding GTP, which stabilizes a conformation of the enzyme that can interact with and regulate downstream effectors. Three classes of accessory proteins control the cycling of Rho GTPases between their GDP and GTP bound states [5]. Rho guanine nucleotide exchange factors (RhoGEFs) stimulate the release of GDP and the binding of GTP, which exists at higher concentrations than GDP in the cell. GTPase-activating proteins enhance the rate of GTP hydrolysis, and Rho guanine nucleotide dissociation inhibitors stabilize the inactive GDP-bound state.

The largest and best characterized family of RhoGEFs in the human genome contain a ~200 amino acid catalytic domain known as the Dbl homology (DH) domain [2]. The DH domain is responsible for binding Rho GTPases in a conformation that disfavors the binding of Mg^{2+} and guanine nucleotides. Nearly all Dbl family RhoGEFs have a ~140 amino acid pleckstrin homology (PH) domain that immediately follows the DH domain in the primary sequence. PH domains are found in many peripheral membrane proteins, where they have been shown to bind phospholipids or mediate protein-protein interactions. In the context of Dbl family RhoGEFs, PH domains can play either or both of these roles. A flexible linker of variable length joins the DH and PH domains, allowing the relative orientation of the two domains to vary.

The RhoGEF PH domain can have either a positive, neutral, or negative impact on *in vitro* exchange activity. In GTPase-bound structures of Dbl's big sister (Dbs) [6], the N-terminal DH/PH domains of Trio (TrioN) [7], leukemia-associated RhoGEF (LARG) [8], and PDZ-RhoGEF [9], the PH domains adopt a similar orientation with respect to the DH domain, directly contact the bound GTPase, and promote GEF activity. In the Vav-Rac1 complex [10,11], a C-terminal zinc finger-like domain bridges the DH and PH domains and is required

²The abbreviations used are:

AF	Alexa Fluor 532
Dbs	Dbl's big sister
DH	Dbl homology
FCPIA	flow cytometry protein interaction assay
FP	fluorescence polarization
GFP	green fluorescent protein
GRK	G protein-coupled receptor kinase
GPCR	G protein-coupled receptor
MFI	median fluorescence intensity
mP	millipolarization
PH	pleckstrin homology
RH	regulator of G protein signaling homology
Sos	Son of Sevenless
WT	wild-type

for the formation of the most active, stable form of the RhoGEF. In structures of Tiam1 [12], intersectin [13], and collybistin [14], the PH domains adopt unique orientations relative to the DH domain, do not contact the bound GTPase, and do not appear to contribute to *in vitro* GEF activity. In the structure of autoinhibited Son of Sevenless (Sos), the PH domain masks the GTPase binding site of the DH domain and thereby inhibits GEF activity [15].

p63RhoGEF, and the closely related C-terminal DH/PH domains of Trio (TrioC) and Kalirin (KalirinC), also have a PH domain that inhibits intrinsic GEF activity [16,17]. These enzymes are activated upon binding $G\alpha_q$ subunits [18-20], establishing a signal transduction pathway linking $G\alpha_q$ -coupled receptors to the activation of RhoA [21,22]. In *C. elegans*, this pathway is important for smooth muscle function, egg laying, and growth, and operates in parallel to the second known $G\alpha_q$ pathway, *i.e.* that of Egl-8, a nematode homolog of phospholipase C β [23]. Another well established pathway that links GPCRs to RhoA instead depends on $G\alpha_{13}$, which binds to the regulator of G protein signaling homology (RH) domain found in p115RhoGEF, PDZ-RhoGEF, and LARG [24-27]. The mechanism of $G\alpha_{13}$ -mediated activation in this subfamily is not yet clear, but likely involves membrane recruitment in addition to multiple interactions formed among the various domains of the RhoGEF and/or with other proteins at the cell membrane [28,29].

In the present study, we show that recruitment to the cell membrane does not appear to be part of the activation mechanism of p63RhoGEF. We go on to demonstrate that $G\alpha_q$ not only relieves autoinhibition mediated by residues in the p63RhoGEF PH domain, but also activates the DH domain via an independent, allosteric mechanism. Finally, we assess the impact of mutations of residues within the subunit interfaces of the $G\alpha_q$ -p63RhoGEF complex *in vitro* and in living cells, providing further insight into the molecular determinants for effector specificity in the $G\alpha_{q/11}$ subfamily of heterotrimeric G proteins.

2. MATERIALS AND METHODS

2.1 Mutagenesis, protein purification, and expression vectors

Site-directed mutations were introduced into expression vectors using the QuikChange mutagenesis protocol (Stratagene). Wild-type (WT) and variant human p63RhoGEF DH/PH (residues 149-502) proteins were expressed using the pMCSG9 vector and the p63RhoGEF DH domain (residues 149-338) and RhoA proteins were expressed using the pMALc2H₁₀T vector and purified from *E. coli* lysates as previously described [19]. Both PH477 (residues 351-477 of p63RhoGEF) and PH502 (residues 351-502 of p63RhoGEF) were expressed in *E. coli* as maltose binding protein-hexahistidine-tagged fusion proteins using the pMALc2H₁₀T vector, and purified by Ni-NTA affinity and then size exclusion chromatography. $G\alpha_{i/q}$ chimera and the $G\alpha_{i/q}$ -Y356A variant were produced in High Five insect cells as described previously [30]. $G\alpha_{i/q}$ contains the N-terminal helix (residues 1-28) of $G\alpha_{i1}$, an engineered Arg and Ser linker, followed by the Ras-like and helical domains (residues 37-359) from mouse $G\alpha_q$. Rat RGS4 was purified as described previously [31]. The construction of plasmids encoding myc-tagged p63RhoGEF DH/PH domains in the pCMV-Tag3B vector was reported before [16, 19]. Expression vectors for $G\alpha_q$, $G\alpha_{13}$, and their constitutive active mutants subcloned into pCDNA3 were from the Missouri S&T cDNA Resource Center. The pEGFP-p63RhoGEF-2xPLC δ 1 PH vector encoding p63RhoGEF with two C-terminal tandem PLC δ 1 PH domains was created by cloning p63RhoGEF into the restriction sites *Xho*I and *Hind*III of a pEGFP-C1 vector that already contained PLC δ 1-2xPH [28]. All plasmids encoding mutant and fusion proteins were verified by DNA sequencing.

2.2 Fluorescence polarization Assay

The rate of nucleotide exchange of RhoA in the presence or absence of p63RhoGEF and/or $G\alpha_{i/q}$ was measured by following the change in fluorescence anisotropy of 1 μ M BODIPY FL GTP γ S (Invitrogen) as it binds RhoA, which was measured on a PHERAstar plate reader, as previously described [19].

2.3 Flow cytometry protein interaction assay (FCPIA)

All experiments used a Luminex 96-well plate bead analyzer to monitor the equilibrium binding of p63RhoGEF variants or RhoA labeled with Alexa Fluor 532 (AF), which has excitation/emission maxima of ~531/554 nm (Invitrogen), to xMap LumAvidin microspheres (Luminex) linked to either biotinylated $G\alpha_{i/q}$ or RhoA. Binding experiments with $G\alpha_{i/q}$ were performed as described previously [19]. Binding experiments with RhoA were performed in 20 mM HEPES pH 8.0, 150 mM NaCl, 0.1% lubrol, 2 mM DTT, 1% BSA supplemented with either 10 mM EDTA (to measure total binding) or 1.25 mM $MgCl_2$ and 50 μ M GDP (to measure nonspecific binding). In competition experiments, increasing concentrations of p63RhoGEF variants were incubated with a fixed amount of an AF-labeled DH/PH fragment for binding to either bead bound biotinylated $G\alpha_{i/q}$ or RhoA. K_I values were determined from competition curves that were fitted using the one-site competition equation in GraphPad Prism. To see the effect of $G\alpha_{i/q}$ on p63RhoGEF binding to nucleotide free RhoA, bead bound $G\alpha_{i/q}$ was added to increasing amounts of AF-labeled RhoA in the presence of 400 nM of each p63RhoGEF variant, 20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% lubrol, 20 μ M $AlCl_3$, 10 mM NaF, 1% BSA, and 5 mM DTT. To measure nonspecific binding, p63RhoGEF fragments were omitted from the wells.

2.4 p63RhoGEF pull-down of $G\alpha_q$ variants

Mutations in $G\alpha_q$ were generated in the cDNA of mouse $G\alpha_q$ in pCMV5 and the mutants were expressed in HEK293T cells as described previously [30]. The cells were lysed with 1 ml of lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 10 μ M GDP, 0.5% Triton X-100, and protease inhibitors) and incubated on ice for 20 min. The samples were then centrifuged at 15,000g for 20 min at 4 °C and the supernatants collected. The DH/PH domains of either p63RhoGEF or RGS4 (serving as a positive control) were biotinylated by incubating 80 μ g of the protein with equimolar amounts of biotinamidohehexanoyl-6-amino-hexanoic acid N-hydroxy-succinimide ester (Sigma) on ice for 1 hr. The conjugate was then filtered through a 0.5 ml Zeba™ desalt spin column. 100 μ l of the lysates from cells expressing each $G\alpha_q$ variant were incubated with 850 ng of biotinylated DH/PH or RGS4 and streptavidin beads (Invitrogen) either in the presence or absence of AlF_4^- for 3 hr at 4 °C. The beads were washed three times with 500 μ l of the lysis buffer, with or without AlF_4^- as appropriate. The beads were treated with 5 μ l of 4x SDS-PAGE loading buffer and $G\alpha_q$ was detected by immunoblot analysis.

2.5 Cell culture and transfection

HEK293 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine at 37°C in an atmosphere of 5% CO_2 . If not otherwise indicated, DNA transfections were performed with PolyFect (Qiagen) according to manufacturer's recommendations. After transfection, the cells were maintained in serum-reduced (0.5%) DMEM.

2.6 SRF activation assay

Luciferase reporter gene assays were performed with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Briefly, HEK293 cells were

seeded into 96-well plates and co-transfected with the indicated plasmids together with pSRE.L encoding firefly luciferase reporter plasmid (kindly provided by Dr J. Mao and Dr. D. Wu, Rochester, NY) and pRL-TK (Promega) encoding *Renilla* luciferase control vector. The transfected cells were maintained in DMEM with 0.5% FCS for 48 h. Afterwards, cells were lysed with passive lysis buffer (Promega), and luciferase activities were determined with an EnVision plate reader (Perkin Elmer) using a white 96-well plate.

2.7 Immunoblot analysis

Proteins were separated by discontinuous SDS-PAGE (10% acrylamide) and subsequently transferred onto a nitrocellulose membrane. For detection of specific proteins, the following antibodies were used: anti-c-Myc antibody (clone 9E10, Roche, 0.2 µg/ml) for N-terminally Myc-tagged p63RhoGEF variants, anti-eGFP antibody (SC-9996, Santa Cruz, 0.2 µg/ml) for N-terminally eGFP-tagged p63RhoGEF variants, and anti-Gα_{q/11} antibody (C-19, Santa Cruz, 0.2 µg/ml) for Gα_q variants.

2.8 Confocal microscopy

HEK293T cells, maintained in 10% new born calf serum (NCS) with penicillin/streptomycin, were seeded onto poly-D lysine coated cover slips in 12 well plates. After attachment, cells were transfected with the indicated plasmids in 10% NCS with no antibiotics using lipofectamine 2000 (Invitrogen). 6 h later, the medium was changed to 10% NCS containing penicillin/streptomycin. 24 h after transfection, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and incubated in 50 mM glycine/PBS as a quench. Coverslips were subsequently mounted on glass slides using Vectashield mounting medium (Vector Laboratories). Images were captured using an Olympus FluoView 500 laser scanning confocal microscope (Olympus America, Inc.) with a 60x oil immersion objective (Nikon, Japan) and then imported into Adobe Photoshop for processing.

3. RESULTS

3.1 Membrane localization of p63RhoGEF

Many RhoGEFs are cytoplasmic, and recruitment to the cell membrane is believed to be an essential aspect of their activation mechanism. For example, the addition of membrane targeting domains to p115RhoGEF, LARG, or Dbs enhances GEF activity [28,32,33]. To test if Gα_q can target p63RhoGEF to cell membranes, we created GFP fusions of p63RhoGEF so we could examine the cytosolic distribution of the expressed protein in the presence or absence of Gα_q by confocal microscopy. We also created a variant that contained two C-terminal tandem PH domains of PLCδ1, which bind to phosphatidylinositol 4,5-bisphosphate and constitutively localizes proteins to the plasma membrane. (Fig. 1a) [28,32,34-36].

Confocal microscopy of HEK293T (Fig. 1b) or COS cells (data not shown) transfected with p63RhoGEF fusion proteins revealed that the WT protein is already at or near the plasma membrane in the basal state. Apparently, this close association with the plasma membrane is independent of the capability of p63RhoGEF to bind Gα_q, because the Gα_q-binding defective mutant F471E [19] exhibits the same localization as the WT protein (Fig. 1b). No obvious change in localization was observed for the PLCδ1 PH fusion proteins. The WT and PLCδ1 PH fusion protein variants of GFP-fused p63RhoGEF were similarly active. As shown in Fig. 1c, they all induce similar basal SRE-dependent luciferase expression, with each being activated by Gα_q-R183C about 10-fold. Thus, Gα_q does not appear to play a role in membrane targeting of p63RhoGEF, and its ability to enhance GEF activity thus appears to be entirely allosteric.

3.2 Molecular basis for inhibition of basal p63RhoGEF activity

The crystal structure of the $G\alpha_{i/q}$ -p63RhoGEF-RhoA complex revealed that $G\alpha_{i/q}$ forms extensive interactions with both the PH and DH domains of p63RhoGEF [19]. Because the structure of the basal state of p63RhoGEF is not known, the available data do not provide a mechanism for how allosteric activation of GEF activity is achieved. Inspection of the structure does, however, suggest several hypotheses. The DH and PH domains of p63RhoGEF adopt a dramatically different conformation than those of Dbs and TrioN [7,13], the closest homologs of known structure based on their primary sequence. When the DH domain of the $G\alpha_{i/q}$ -p63RhoGEF-RhoA structure is superimposed with that of Dbs-RhoA, the p63RhoGEF PH domain is rotated by $\sim 50^\circ$ around the $\alpha 6$ - αN helical linker relative to that of Dbs (Fig. 2). Based on their high homology, it therefore seems reasonable to conjecture that the PH domain of p63RhoGEF will adopt a more Dbs-like conformation in the basal state, and that this configuration somehow interferes with RhoA binding. Activation by $G\alpha_q$ could then occur by constraining the PH domain away from the RhoA binding site in a more permissive conformation. Second, it is known in Dbs and other RhoGEFs that the interactions of the helix joining the DH and PH domains (the $\alpha 6$ - αN linker region) can play an important role in modulating GEF activity, presumably via its direct interactions with switch II of the bound GTPase. We used site-directed mutagenesis and biochemical assays to test these hypotheses and define residues and structural elements involved in autoinhibition and allosteric regulation by $G\alpha_q$.

In autoinhibited Sos, the PH domain binds to the DH domain in a manner that sterically blocks the GTPase binding site [15]. To test if the PH domain of p63RhoGEF can inhibit basal activity via a similar mechanism, we measured the GEF activity of the isolated DH domain in the presence of increasing concentrations of p63RhoGEF PH domain added *in trans*. However, neither the isolated PH domain of p63RhoGEF (residues 351-502) nor a more C-terminally truncated variant (residues 351-477), which lacks critical $G\alpha_q$ -binding residues, could inhibit nucleotide exchange (Fig. 3). These data demonstrate that PH domain-mediated inhibition of p63RhoGEF is not mediated by direct interactions between residues in the PH and DH domains, and apparently requires the $\alpha 6$ - αN linker region joining the domains.

The $\alpha 6$ - αN linker regions of Dbs and TrioN promote GEF activity in a PH domain-dependent manner by forming bridging interactions between residues in the PH domain and residues in switch II of the bound GTPase [6,7] (Fig. 4a). Residues in the $\alpha 6$ - αN linker are not well conserved between Dbs and p63RhoGEF, suggesting that they could instead directly hinder RhoA binding (Fig. 4b). In particular, if the p63RhoGEF DH and PH domains are modeled in a Dbs-like conformation (Fig. 4c), a steric and electrostatic clash may occur between the side chains of RhoA-Arg⁶⁸ and p63RhoGEF-Arg³⁴¹. Alternatively, the extended $\beta 3$ - $\beta 4$ loop of the PH domain, although disordered in the $G\alpha_{i/q}$ -p63RhoGEF-RhoA structure, could block the RhoA binding site when the PH domain is in a more Dbs-like configuration. To test these ideas, we introduced site-directed mutations in the p63RhoGEF DH/PH domains at four positions that are conserved among p63RhoGEF, TrioC, and KalirinC (Fig. 4b), converting them to their equivalents in Dbs: P330L, M336S, G340I, and R341A. We also deleted residues 397-402 in the $\beta 3$ - $\beta 4$ loop of the PH domain to create the $\Delta 397$ -402 variant. If the basal p63RhoGEF activity were increased in any of these variants, then it would indicate that the altered residues contribute to autoinhibition. Indeed, the G340I and R341A mutants had higher basal rates of GEF activity (1.9 and 1.6-fold over WT, respectively) under the assay conditions (Table 1). Analysis of these mutants at varying p63RhoGEF concentrations revealed them to be 8.5- and 3.8-fold more active than WT, respectively, although still not as active as the DH fragment alone (45-fold more active than WT) (Fig. 5a). The P330L mutant was impaired in basal GEF activity, suggesting that it plays a structural role, whereas the M336S and $\Delta 397$ -402 mutations had no significant effect (Table 1).

Thus, both Gly³⁴⁰ and Arg³⁴¹ contribute to the low basal activity of p63RhoGEF. Substitution of these residues with their equivalents in Dbs would therefore be expected to increase the binding affinity for RhoA. Consistent with this hypothesis, the R341A mutant enhanced binding (~2-3-fold over WT) as measured by either direct binding or competition FCPIA assays (Fig. 6a, Table 2). The G340I mutant was poorly behaved in the competition assay, preventing us from drawing conclusions on how this mutation affects RhoA binding. Because both of these residues are located within the $\alpha 6$ - αN linker, they could modulate p63RhoGEF activity either through direct contacts with RhoA, or by influencing the conformation of the linker region. To test for a direct interaction between Arg³⁴¹ and Arg⁶⁸ of RhoA, we measured the rate of nucleotide exchange catalyzed on RhoA-WT, RhoA-R68A, and RhoA-R68E by the p63RhoGEF DH/PH, DH/PH-R341A, and DH/PH-R341E variants. If RhoA-Arg⁶⁸ and DH/PH-Arg³⁴¹ repel each other in the basal state, then one would expect to see the highest activity for DH/PH-R341E compared to the other variants when RhoA-WT is used as the substrate, and for DH/PH-WT when RhoA-R68E is used as the substrate. Indeed, these combinations yielded the highest measured rates for each of the RhoA variants tested (Fig 5b, c, d). Moreover, the R341E mutation lowered the K_D for RhoA-WT ~7-fold (Table 2). However, the DH/PH-R341E mutant had similar activity towards both RhoA-WT and RhoA-R68A, and DH/PH-WT was just as effective as DH/PH-R341E at catalyzing exchange on RhoA-R68E (Fig. 5b, c, d), indicating that Arg³⁴¹ in p63RhoGEF does not interact directly with RhoA. Instead, our results are most consistent with substitutions at Arg³⁴¹ (and presumably Gly³⁴⁰) altering either the conformation or dynamics of the $\alpha 6$ - αN linker in a manner that leads to inhibition of nucleotide exchange in the basal state.

3.3 Molecular basis for activation of p63RhoGEF by $G\alpha_q$

$G\alpha_q$ binding is expected to increase the affinity of the DH domain for RhoA. To measure this effect, $G\alpha_{i/q}$ was attached to beads, and then incubated with saturating DH/PH fragment and increasing amounts of fluorescently labeled RhoA (Fig. 6b). $G\alpha_{i/q}$ reduced the dissociation constant for RhoA ~23-fold to 17 ± 2.4 nM (Table 2). If the mechanism of $G\alpha_q$ activation is simply to relieve the autoinhibition imposed on the basal state by residues in the $\alpha 6$ - αN linker region of p63RhoGEF, then its effect should be blunted by mutation of these residues to their equivalents in Dbs (Fig. 4b), whose $\alpha 6$ - αN linker instead promotes nucleotide exchange. The P330L variant bound $G\alpha_{i/q}$ with a similar binding affinity as WT, but exhibited only very mild activation by $G\alpha_{i/q}$. Although this result could indicate that a proline at this position is important for the activation mechanism, the relatively low basal GEF activity of DH/PH-P330L suggests that a significant fraction of the expressed protein was nonfunctional. The M336S and $\Delta 397$ -402 mutations had little effect on $G\alpha_{i/q}$ -activation or on binding affinity. In contrast, the G340I and R341A variants had markedly higher $G\alpha_{i/q}$ -stimulated rates (5.2 and 2.2-fold over WT, respectively), despite having lower apparent affinities for $G\alpha_{i/q}$ (Table 1). Analysis at varying p63RhoGEF concentrations in the presence of 800 nM $G\alpha_{i/q}$ (Fig. 5a) showed that the activity of the p63RhoGEF DH/PH protein is enhanced ~20-fold by $G\alpha_{i/q}$. However, instead of blunting the effect of $G\alpha_{i/q}$, the G340I and the R341A mutants were similarly enhanced by the G protein (~30-fold). Moreover, in the presence of $G\alpha_{i/q}$, the G340I, R341A, and R341E variants exhibit affinities for RhoA not significantly different from WT (Fig. 6b, Table 2). Therefore, although Gly³⁴⁰ and Arg³⁴¹ play a role in controlling the basal rate of nucleotide exchange, they do not appear to play a role in the mechanism of $G\alpha_{i/q}$ -mediated activation.

Based on our structural and biochemical data thus far, it seemed most likely that $G\alpha_q$ uses its bridging interactions with the p63RhoGEF DH and PH domains to constrain the $\alpha 6$ - αN linker in a conformation better suited for nucleotide exchange. If so, then extension of the linker by insertion of residues with a helical propensity might be expected to ablate the ability of $G\alpha_q$ to activate the enzyme. To test this, we inserted 1-7 alanine residues ("1A"- "7A") after Thr³³⁸ at the end of the $\alpha 6$ helix of the DH domain in the p63RhoGEF DH/PH fragment.

Assuming these additions form a continuous helical extension of $\alpha 6$ (as opposed to “looping out”), then the PH domain would be rotated by $\sim 100^\circ$ and translated $\sim 1.5 \text{ \AA}$ away from the DH domain upon insertion of each alanine (Fig. 7a). The PH domain would be closest to the same orientation with respect to the DH domain as WT in the 4A and 7A variants, although translated away from the DH domain by 6 and $10.5 \text{ \AA}$, respectively. In nearly all the insertion mutants, the basal GEF activity of the DH/PH fragment was enhanced by ~ 2 fold under the conditions tested, consistent with the idea that the PH domain contributes to inhibition of the basal state (data not shown). However, only the 1A, 2A, and 3A insertion mutants showed any activation by $G\alpha_{i/q}$, although at levels 50% or less than WT (Fig. 7b). All alanine insertion mutants except the 4A mutant exhibited decreased affinity for $G\alpha_{i/q}$, with the 1A, 3A, and 7A mutants exhibiting intermediate loss of affinity. The changes in affinity therefore mimic the intended helical periodicity of the insertion mutants, with higher affinity binding occurring only when the DH and PH domains adopt an orientation roughly similar to that of WT. The relative amount of translation imposed by the alanine insertions seems to be less critical for binding, as might be expected from the fact that the linker joining the DH and PH domains is flexible. Notably, the 4A mutant lacked the ability to be activated by $G\alpha_{i/q}$ despite retaining WT affinity. Therefore, the interface between $G\alpha_q$ and the DH domain *per se* is not the source of allosteric activation, and the results are most consistent with $G\alpha_q$ altering the conformation of the $\alpha 6$ - αN linker region and their direct contacts with the bound GTPase (Fig. 4). Insertion of more than three additional residues in the linker (as in the “4A” insertion mutant) is apparently enough to completely decouple high affinity binding of $G\alpha_q$ from activation of this conformational switch.

3.4 Analysis of the $G\alpha_q$ interface with p63RhoGEF

If the bridging model proposed above were correct, then mutation of residues in $G\alpha_q$ that contact either PH or DH domain should inhibit activation of p63RhoGEF. Mutation of residues that line the canonical effector-docking site, formed between the switch II and the $\alpha 3$ helices of $G\alpha_q$, are anticipated to abrogate binding, as was previously shown for mutation of residues of p63RhoGEF in the PH domain that dock in this site [19,20]. If these residues were also unique to the $G\alpha_{q/11}$ subfamily, then they would help explain the selectivity of p63RhoGEF for $G\alpha_{q/11}$ subunits. We examined the sequence conservation of residues found in the $G\alpha_q$ interface with p63RhoGEF PH domain (Fig. 8a) and identified three positions, Ala²⁵³, Tyr²⁶¹, and Trp²⁶³, that appeared poised to dictate selectivity (Fig 8b). These positions were mutated to their equivalents in other $G\alpha$ families and then tested for their ability to bind p63RhoGEF in a pull-down assay. The structural integrity of each variant was confirmed by its ability to bind RGS4 in an activation dependent manner (Fig. 8c). The $G\alpha_q$ -A253K variant exhibited almost no binding to p63RhoGEF, whereas the $G\alpha_q$ -Y261N and -W263D mutants were impaired. The T257E mutant, a positive control designed to block the effector-docking site, abrogated binding just as it did for GRK2 [30] and to the same extent as $G\alpha_q$ -A253K. The ability of $G\alpha_q$ and its mutants to induce p63RhoGEF DH/PH-mediated SRE-dependent luciferase expression in HEK293 cells was consistent with that measured in the *in vitro* pull-down assays (Fig. 8d). Like $G\alpha_q$ -T257E, the A253K and W263D mutants were severely impaired in their ability to activate p63RhoGEF. Thus, Ala²⁵³ and Trp²⁶³ contribute to the selectivity of p63RhoGEF for $G\alpha_q$ subfamily members. Tyr²⁶¹ is substituted by Leu in $G\alpha_{16}$, which also binds p63RhoGEF [37], and its mutation to Asn did not significantly inhibit its ability to activate p63RhoGEF in cells (Fig. 8d). Thus, this position is of relatively minor importance with regards to the binding and activation of p63RhoGEF.

We additionally examined the role of two residues in $G\alpha_q$ that contact the DH domain: $G\alpha_q$ -Tyr³⁵⁶ and -Asp³²¹. Tyr³⁵⁶ was of particular interest because it makes extensive contacts with the DH domain and resides in the C-terminal helix of $G\alpha_q$ (Fig. 8a), a region that has previously not been recognized as being important for effector interactions. To test its role, the $G\alpha_{i/q}$ -

Y356A mutant was expressed in insect cells, purified to homogeneity, and assayed for p63RhoGEF binding and activation (Fig. 9). $G_{\alpha_{i/q}}$ -Y356A bound to AF-labeled p63RhoGEF with similar affinity as WT (Fig. 9a), but failed to activate p63RhoGEF in the FP assay (Fig. 9b). This result suggests that the interaction of the G_{α_q} C-terminal helix with the DH domain is a critical determinant for fixing the relative orientation of the DH and PH domains, and hence the conformation of the $\alpha 6$ - αN linker. Both the Y356A and Y356F (a $G_{\alpha_{14}}$ specific substitution) variants expressed in mammalian cells exhibited similar affinity for p63RhoGEF as WT as measured by a pull-down assay, yet had no effect on the binding of GRK2 (Fig. 9c), as expected from the structure of the G_{α_q} -GRK2 complex in which the C-terminus of G_{α_q} is disordered. The Y356A mutant was also significantly impaired in its ability to activate the p63RhoGEF DH/PH fragment in an SRE-dependent luciferase assay (Fig. 9d). In contrast, the G_{α_q} -D321A mutant, which was designed to disrupt a potential salt-bridge with p63RhoGEF-Arg²⁴⁵, had similar binding properties and cell-based activity as WT (Fig. 8c, d) and thus is apparently not of functional importance.

4. DISCUSSION

In this study, we sought to characterize the activation mechanism by which G_{α_q} is able to stimulate the activity of its recently identified effector protein p63RhoGEF [18-20]. Because interaction between multiple domains, phospholipids, or other proteins at the membrane surface have been proposed to be important for the regulation of other heterotrimeric G protein-regulated RhoGEFs [29], we first tested whether active G_{α_q} is required for membrane translocation. The data obtained revealed that p63RhoGEF is predominantly localized to the cell membrane regardless of its ability to bind active G_{α_q} , and that an artificially membrane targeted version of p63RhoGEF can still be allosterically activated by G_{α_q} to the same extent as WT. This data refutes the hypothesis that membrane targeting is involved in the regulation of p63RhoGEF.

We therefore studied possible mechanisms involved the allosteric regulation of the exchange activity of p63RhoGEF. Its PH domain has a pronounced inhibitory effect on the exchange activity catalyzed by the DH domain [16,19]. The DH/PH domains of Dbs and TrioN are the closest homologs of known structure, but their PH domains instead have a stimulatory effect. Crystallographic analysis indicates that the Dbs and TrioN DH and PH domains adopt a similar conformation in both GTPase-bound and free states [7,13,38,39], and that their PH domains directly contact the bound GTPase. However, the interdomain contacts of residues in their $\alpha 6$ - αN linkers were found to be the most important contributors to the PH domain-mediated enhancement of GEF activity [6]. For example, Dbs-His⁸¹⁴, located at the end of the $\alpha 6$ helix of the DH domain, forms important bridging hydrogen bonds between RhoA-Asp65 in switch II and Dbs-Tyr⁸⁸⁹ in the PH domain (Fig. 4a) [6]. In the structure of the $G_{\alpha_{i/q}}$ -p63RhoGEF-RhoA complex, the PH domain of p63RhoGEF makes no contact with RhoA and is rotated with respect to the DH domain by $\sim 50^\circ$ around the axis of the $\alpha 6$ helix relative to those of Dbs and TrioN (Fig 2). Furthermore, despite high sequence homology with Dbs and TrioN, p63RhoGEF lacks a histidine that could play the same role as Dbs-His⁸¹⁴ (Fig 4).

The isolated PH domain of p63RhoGEF does not inhibit the nucleotide exchange activity of the DH domain when added *in trans* (Fig. 3) which appears to rule out an autoinhibitory mechanism like that of the PH domain of Sos [15]. Instead, an intact linker between the DH and PH domains appears critical for negative regulation mediated by the PH domain. We then showed that residues Gly³⁴⁰ and Arg³⁴¹, which are uniquely conserved in the $\alpha 6$ - αN linker region of p63RhoGEF, TrioC, and KalirinC, contribute to the autoinhibition imposed by the PH domain (Fig. 4-6). These residues most likely act by altering the conformation of the linker region, and not through direct contacts with RhoA. The mechanism by which these residues

inhibit activity, however, awaits further study, ideally through the structure determination of p63RhoGEF in its basal state.

$G\alpha_q$ binding relieves this autoinhibition, as evidenced by the fact that substitutions at these sites have no effect on p63RhoGEF affinity for RhoA in the $G\alpha_{i/q}$ -bound state (Fig. 6). Mutation of Gly³⁴⁰ and Arg³⁴¹ to their equivalents in Dbs also does not reduce the ability of $G\alpha_{i/q}$ to stimulate activity (Fig 5), indicating that $G\alpha_q$ acts allosterically via a mechanism that does not directly involve these residues. One major component of this mechanism obviously involves the domain-bridging interactions of $G\alpha_q$, which we propose leads to alterations in the $\alpha 6$ - αN linker region. This mechanism is consistent with the fact that we can readily decouple high affinity $G\alpha_q$ binding, which presumably requires interactions with both DH and PH domains (Fig. 9 and [19]), from activation. For example, the alanine insertion mutant A4 of p63RhoGEF is able to bind $G\alpha_{i/q}$ with the same affinity as WT, but its ability to be activated by $G\alpha_{i/q}$ is abrogated (Fig 7). Similarly, mutation of $G\alpha_q$ -Tyr³⁵⁶ to alanine, which disrupts a contact with the DH domain (Fig. 8a), eliminated activation of p63RhoGEF but not high affinity binding (Fig. 9). Because the DH domain alone is very active compared to the DH/PH tandem domains (*i.e.* not in need of allosteric activation), it is unlikely that the interaction of Tyr³⁵⁶ on its own significantly alters the conformation of the DH domain. Rather, our results more likely imply that the Y356A substitution leads to a defect in maintaining the DH and PH domains in a configuration that allows more productive contacts between the $\alpha 6$ - αN linker region and RhoA. Consistent with a role for the C-terminus of $G\alpha_q$ in activating p63RhoGEF, an Ile is substituted for Tyr³⁵⁶ in $G\alpha_{16}$, which binds to but does not activate p63RhoGEF [37]. As if to underscore the importance of this interaction, the Tyr³⁵⁶ side chain directly interacts with p63RhoGEF-Ile²⁰⁵, a residue whose missense mutation in the *C. elegans* ortholog of p63RhoGEF, UNC-73, leads to a dramatic loss of $G\alpha_q$ signaling to RhoA [23].

Taken together, it appears that the $\alpha 6$ - αN linker of RhoGEFs can either positively or negatively regulate GEF activity, and as such it represents a conformational switch that can readily be manipulated by the interactions of the RhoGEF with other proteins, such as $G\alpha_q$ or, presumably, with phospholipid bilayers. If the $\alpha 6$ - αN conformational switch is of regulatory importance across the Dbl family, then it helps to explain why PH domains are almost always found immediately C-terminal to DH domains.

5. CONCLUSION

Our study provides new insights into the mechanism of regulation of p63RhoGEF activity. Although membrane targeting is believed to be an important component for the activation of many RhoGEFs, p63RhoGEF appears to be constitutively membrane bound, implying that $G\alpha_q$ activates p63RhoGEF *in vivo* primarily through an allosteric mechanism. We show that the most likely mechanism for inhibition of basal activity and for activation by $G\alpha_q$ is via conformational changes in the $\alpha 6$ - αN linker connecting the DH and PH domains. Different residues in the linker appear to be involved in autoinhibition and in $G\alpha_q$ -mediated activation. We also identified residues on $G\alpha_q$ that were important for interactions with p63RhoGEF, including Tyr³⁵⁶ in the C-terminal $\alpha 5$ helix, a region formerly thought of as being solely utilized for coupling with GPCRs. These residues help define the selectivity of p63RhoGEF for $G\alpha_q$ subfamily members.

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Figure 1. p63RhoGEF is constitutively localized to the plasma membrane independently of active Gα_q

(A) p63RhoGEF constructs fused C-terminal to eGFP for use in confocal microscopy. (B) Confocal images of HEK293T cells transfected with the various eGFP fusions of p63RhoGEF, as indicated. The scale bar represents 10 μm. (C) Luciferase production was measured in HEK293 cells transfected with a mixture (25 ng) of the reporter plasmids, 50 ng plasmids encoding the indicated N-terminal eGFP-p63RhoGEF fusion proteins (open bars) either alone or in combination with 50 ng plasmids encoding Gα_q-R183C (black bars). Activity was measured 48 h post transfection. Values are given as means ± S.E.M. (n=4). A representative immunoblot analysis of eGFP-p63RhoGEF fusion protein expression in HEK293 cell lysates is shown in the inset.

Figure 2. Comparison of the conformation of the DH/PH domains of p63RhoGEF and Dbs
 The DH and PH domains of p63RhoGEF are closely related in sequence and structure to those of Dbs, yet their DH/PH domains adopt dramatically different conformations. (A) Crystal structure of $G\alpha_{i/q}$ -p63RhoGEF-RhoA complex (PDB ID: 2RGN). Compared to Dbs, the PH domain of p63RhoGEF in complex with $G\alpha_{i/q}$ is rotated away from the RhoA binding site by $\sim 50^\circ$ around the long axis of the $\alpha 6$ helix of the DH domain. The DH and PH domains of p63RhoGEF are colored yellow and purple, respectively. RhoA is shown in green with gold β strands and its switch regions (SwI and SwII) are highlighted in red. $G\alpha_{i/q}$ is colored grey and $Mg^{2+} \cdot GDP \cdot AlF_4^-$ in the active site is shown as a ball and stick model. The relative positions of Arg³⁴¹ in the PH domain of p63RhoGEF and Arg⁶⁸ in SwII of RhoA are shown. (B) Crystal structure of the Dbs-RhoA complex (PDB ID: 1LB1). The color scheme is same as in panel A. Ala⁸¹⁷ in the PH domain is analogous to Arg³⁴¹ of p63RhoGEF. In each panel, the expected membrane surface runs parallel to the top of the figure.

Figure 3. The PH domain of p63RhoGEF does not significantly inhibit DH domain activity *in trans*
 The GEF activity of 200 nM p63RhoGEF DH domain (residues 149-338) on 2 μ M RhoA was measured using a FP assay either alone or in the presence of increasing amounts of two p63RhoGEF PH domain fragments, PH502 (A) or PH477 (B). PH477 lacks the α C helix extension that docks into the effector binding site of $G\alpha_q$.

Figure 4. Comparison of the $\alpha 6$ - αN linker region of Dbs and p63RhoGEF modeled in a Dbs-like conformation

(A) Detailed view of residues in the $\alpha 6$ - αN linker between the DH and PH domains of Dbs that interact with the SwII region of RhoA. Dashed lines indicate hydrogen bonds. (B) Structure-based sequence alignment of the $\alpha 6$ - αN linker region in Dbs, TrioN, p63RhoGEF, TrioC, and KalirinC. Positions mutated in this study are highlighted. Secondary structure is represented using cylinders and arrows for α -helices and β -strands, respectively. The alignment was generated by the Promals3D server using sequences of human p63RhoGEF (GenBank ID NM_182947), human Kalirin (Q9Y2A5), human Trio (AAC34245), and mouse Dbs (Q64096), and the structures of the DH/PH domains in p63RhoGEF (PDB ID: 2RGN), Dbs (1LB1), and

TrioN (1NTY). (C) Detailed view of residues in the $\alpha 6$ - αN linker between the DH and PH domains of p63RhoGEF modeled in Dbs-like conformation. In this conformation, potential steric clashes are formed between Arg³⁴¹ and the $\beta 3$ - $\beta 4$ loop of the PH domain with the bound GTPase.

Figure 5. Mutational analysis of residues in the $\alpha 6$ - αN linker region of p63RhoGEF

(A) $k_{app}/[E_0]$ for BODIPY FL GTP γ S binding to RhoA catalyzed by p63RhoGEF DH and DH/PH fragments in the presence or absence of 800 nM G $\alpha_{i/q}$. Values are the slope of a line fitted for GEF activity at three different concentrations of the RhoGEF fragment. The Y intercept was fixed to be the rate of nucleotide exchange for RhoA alone. Concentrations typically used were 10, 20, and 40 nM for the DH domain; 100, 200, and 400 nM for DH/PH; 25, 50, and 100 nM for DH/PH in the presence of G $\alpha_{i/q}$; 100, 200, and 400 nM for DH/PH-G340I, 2.5, 5, and 10 nM for DH/PH-G340I in the presence of G $\alpha_{i/q}$; 100, 200, and 400 nM for DH/PH-R341A, 10, 20, and 40 nM for DH/PH-R341A in the presence of G $\alpha_{i/q}$. Values represent the average $\pm$ standard deviation of five independent experiments. (B-D) Basal activity of

p63RhoGEF DH/PH variants on RhoA-WT, RhoA-R68A, and RhoA-R68E. Each value represents six independent measurements of 100 nM DH/PH variant in the presence of each of the indicated RhoA variants. Asterisks indicate a significant difference at the $P < 0.01$ level compared to DH/PH-WT.

Figure 6. RhoA binding properties of p63RhoGEF DH/PH domain variants

(A) Binding of p63RhoGEF variants to RhoA. Various concentrations of AF-labeled p63RhoGEF DH/PH-WT, R341A, or R341E mutants were incubated with bead-bound biotinylated RhoA in the presence of 10 mM EDTA, and then the bead-bound fluorescence was measured by FCPIA. For each curve, non-specific binding was measured by including Mg^{2+} and GDP instead of EDTA. For clarity, only the DH/PH-WT non-specific binding curve is shown, which is representative of those from all experiments. See Table 1 for fitted statistics. (B) Binding of RhoA to DH/PH variants in the presence of $\text{G}\alpha_{i/q}$. The binding of 5 nM bead-bound AlF_4^- activated $\text{G}\alpha_{i/q}$ to increasing concentrations of AF-labeled RhoA in the presence of 400 nM unlabeled p63RhoGEF DH/PH domains was measured by FCPIA. The DH/PH domains were excluded to obtain the curve for non-specific binding (bottom, linear plot). For each DH/PH variant, activated $\text{G}\alpha_{i/q}$ decreased the K_D of the p63RhoGEF-RhoA complex to ~15 nM.

Figure 7. Consequences of extending the helical $\alpha 6$ - αN linker between the DH and PH domains
 (A) Cartoon depicting the changes in the relative orientation of DH and PH domains by insertion of a series of 1-7 alanine residues (“1A-7A”) at the C-terminus of $\alpha 6$ helix of the DH domain. A surface model of p63 DH/PH as viewed down the $\alpha 6$ helix was used to outline the domain boundaries of DH and PH domains. Assuming a helical progression and no domain collisions, the PH domain would be rotated by 100° and translated by $1.5 \text{ \AA}$ away from the DH domain with each additional alanine residue. (B) $G\alpha_{i/q}$ binding and activation properties of the alanine insertion mutants. Increasing concentrations of the WT DH/PH domains and alanine insertion variants were used to compete against the binding of 100 nM AF-labeled DH/PH domains to bead-bound, AlF_4^- -activated $G\alpha_{i/q}$ (average of 3 experiments $\pm$ standard deviation, with each concentration measured in duplicate in each experiment). The K_1 value was calculated from the IC_{50} value using the Cheng-Prusoff equation. The basal GEF activity of 400 nM DH/PH variant and its activation by 800 nM $G\alpha_{i/q}$ were measured using the FP nucleotide exchange assay. Data is plotted as fold activation by $G\alpha_{i/q}$ over the rate of DH/PH variant alone. The kinetic data for the alanine insertion mutants is the average of 3-5 independent experiments $\pm$ standard deviation, with individual curves measured in triplicate.

Figure 8. Analysis of the $G\alpha_q$ interface with p63RhoGEF

(A) Atomic model of $G\alpha_{i/q}$ (Ras-like domain) in complex with the DH/PH domains of p63RhoGEF. The DH/PH domains were rendered transparently for clarity. Side chains of residues in $G\alpha_q$ that were targeted by site-directed mutagenesis studies are shown as ball and stick models. (B) Sequence alignment of $G\alpha_q$ subfamily members with $G\alpha_{i1}$, $G\alpha_s$, and $G\alpha_{13}$. Sequences comprising switch II and switch III are colored orange-red. $G\alpha_q$ residues targeted for site-directed mutagenesis are highlighted in green. The sequences correspond to rat $G\alpha_{i1}$ (GI: 121020), bovine $G\alpha_t$ (GI: 121031), bovine $G\alpha_s$ (GI: 121000), mouse $G\alpha_q$ (GI: 84662745), mouse $G\alpha_{13}$ (GI: 120984), mouse $G\alpha_{i1}$ (GI: 6754004), mouse $G\alpha_{14}$ (GI: 160298199), and human $G\alpha_{16}$ (GI: 182891). (C) Mutational analysis of $G\alpha_q$ residues involved in p63RhoGEF binding. Pull-down assays were performed by incubating the lysates of HEK293T cells expressing $G\alpha_q$ variants with either biotinylated p63RhoGEF DH/PH domains or biotinylated RGS4 (control) immobilized on streptavidin beads, either in the presence or absence of AIF_4^- . Bound $G\alpha_q$ was detected by immunoblot. All $G\alpha_q$ variants expressed at similar level, as determined by western analysis (bottom panel), and could bind RGS4 equally well, indicating that all of the proteins were properly folded. (D) Activation of the p63RhoGEF DH/PH domains by $G\alpha_q$ variants in cells. Luciferase production was measured in HEK293 cells transfected with a mixture (25 ng) of the reporter plasmids pSRE.L and pRL.TK encoding the firefly and *Renilla* luciferases respectively, 50 ng control vectors or plasmids encoding p63RhoGEF DH/PH, and 50 ng $G\alpha_q$ variant. Activity was measured 48 h post transfection. Values are given as means $\pm$ S.E.M. (n=6-10). Highly significant ($p < 0.01$) and significant ($p < 0.05$) alterations are indicated by dark and middle gray colors of the bars, respectively. Light grey bars indicate alterations that are not significant. (E) Representative immunoblot analysis of $G\alpha_q$ expression in HEK293 cell lysates used in the luciferase activity assay.

Figure 9. Role of $G\alpha_q$ -Tyr³⁵⁶ in p63RhoGEF regulation

(A) Equilibrium binding of $G\alpha_{i/q}$ or $G\alpha_{i/q}$ -Y356A to AF-DH/PH measured using FCPIA. In this experiment, $K_D = 63 \pm 9$ and 49 ± 5 nM for the p63RhoGEF- $G\alpha_{i/q}$ and $G\alpha_{i/q}$ -Y356A complexes, respectively. (B) $G\alpha_{i/q}$ -Y356A does not activate p63RhoGEF DH/PH *in vitro*. The GEF activity of 200 nM DH/PH was measured using FP in the presence of either 800 nM $G\alpha_{i/q}$ -Y356A or WT $G\alpha_{i/q}$. (C) Pull-down of $G\alpha_q$ (WT, Y356A, or Y356F) expressed in HEK293T cells with either biotinylated p63RhoGEF DH/PH or biotinylated GRK2 in the presence or absence of AIF₄⁻. Bound $G\alpha_q$ was detected by immunoblot. The Y356A and Y356F pull-downs are representative of 3 and 2 experiments, respectively. (D) Luciferase production in HEK293 cells transfected with 25 ng of a mixture containing the reporter plasmids pSRE.L and pRL.TK, 50 ng control vectors or plasmids encoding p63RhoGEF DH/PH in the presence

of either 50 ng WT $G\alpha_q$ or $G\alpha_q$ -Y356A. Activity was measured 48 h post transfection. Values are given as means $\pm$ S.E.M. (n=6, *p<0.05 vs. WT). Equal expression of $G\alpha_q$ -WT and $G\alpha_q$ -Y356A was verified by immunoblot (inset).

TABLE 1
Activity of p63RhoGEF DH/PH variants and their affinity for $G\alpha_{i/q}$

Fold GEF activation is the rate of 400 nM p63RhoGEF DH/PH domain catalyzed guanine nucleotide exchange on RhoA divided by the intrinsic exchange rate of RhoA, as measured by rate of change in FP of BODIPY FL GTP γ S as it binds RhoA. The average apparent rate constant for these experiments was $0.014 \pm 0.002 \text{ min}^{-1}$ (n=10). Fold $G\alpha_{i/q}$ activation is the exchange rate of 400 nM DH/PH domains in the presence of 800 nM $G\alpha_{i/q}$ divided by that of the DH/PH domain alone. The inhibition constant for each variant (K_I) was measured using FCPIA to monitor competition with 100 nM AF-labeled DH/PH binding to bead-bound biotinylated $G\alpha_{i/q}$.

P63RhoGEF variant	Fold GEF activation	Fold $G\alpha_{i/q}$ activation	K_I (nM)
DH/PH-wt	1.4 ± 0.13 (10)	3.5 ± 0.70 (10)	46 ± 14 (9)
DH/PH-P330L	1.1 ± 0.07 (4)	1.2 ± 0.03 (4)	$40. \pm 6.9$ (3)
DH/PH-M336S	1.6 ± 0.17 (4)	2.2 ± 0.16 (4)	68 ± 29 (3)
DH/PH-G340I	2.6 ± 0.43 (3)	17 ± 9.1 (3)	670 ± 490 (4)
DH/PH-R341A	2.2 ± 0.25 (5)	7.2 ± 1.9 (5)	75 ± 33 (4)
DH/PH-R341E	-	-	120 ± 38 (3)
DH/PH- Δ (397-402)	1.3 ± 0.13 (4)	4.0 ± 0.54 (9)	48 ± 8.3 (3)

TABLE 2
Affinity of p63RhoGEF variants for RhoA

RhoA direct binding experiments contained bead-bound biotinylated RhoA with increasing amounts of AF-labeled p63RhoGEF variants. Competition experiments contained bead-bound biotinylated RhoA, a fixed amount of an AF-labeled p63RhoGEF variant, and increasing amounts of each unlabeled p63RhoGEF variant. The experiments testing the effect of $G\alpha_{i/q}$ on p63RhoGEF binding to RhoA contained bead-bound biotinylated $G\alpha_{i/q}$, increasing amounts of AF-labeled RhoA, and 400 nM p63RhoGEF variant. Values shown represent the mean $\pm$ standard deviation (number of experiments) for either direct binding of AF-labeled protein (K_D) or competition with AF-labeled protein (K_I) by the indicated variant. Each data point in each curve was measured in duplicate.

P63RhoGEF variant	K_D (nM)	K_I (nM)
DH/PH-wt	390 $\pm$ 95 (6)	700 $\pm$ 410 (7)
DH/PH-wt + $G\alpha_{i/q}$	17 $\pm$ 2.4 (4)	-
DH/PH-R341A	210 $\pm$ 91 (10)	190 $\pm$ 47 (10)
DH/PH-R341A + $G\alpha_{i/q}$	16 $\pm$ 6 (5)	-
DH/PH-R341E	160 $\pm$ 34 (5)	94 $\pm$ 16 (5)
DH/PH-R341E + $G\alpha_{i/q}$	15 $\pm$ 3 (4)	-
DH/PH-G340I	-	1200 $\pm$ 650 (8)
DH/PH-G340I + $G\alpha_{i/q}$	16 $\pm$ 9 (3)	-
DH	-	300 $\pm$ 79 (2)