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$G\alpha_q$ allosterically activates and relieves autoinhibition of p63RhoGEF

Aruna Shankaranarayanan [a,b,1], Cassandra A. Boguth [a], Susanne Lutz [c], Christiane Vettel [c], Franca Uhlemann [c], Mohamed Aittaleb [a], Thomas Wieland [c], John J.G. Tesmer [a,d,*]

[a] Life Sciences Institute, University of Michigan, Ann Arbor, MI 48109-2216, USA
[b] Institute for Cellular and Molecular Biology, Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, TX 78712, USA
[c] Institute of Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, University of Heidelberg, Maybachstrasse 14, D-68169 Mannheim, Germany
[d] Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109-2216, USA

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ABSTRACT

$G\alpha_q$ 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 $G\alpha_q$ 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 $G\alpha_q$-mediated activation of p63RhoGEF, as it does in some other RhoGEFs. $G\alpha_q$ instead must act allosterically. We next identify specific structural elements in the PH domain that inhibit basal nucleotide exchange activity, and provide evidence that $G\alpha_q$ 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 $G\alpha_q$ that are important for the activation of p63RhoGEF and that contribute to Gα subfamily selectivity, including a critical residue in the $G\alpha_q$ C-terminal helix, and demonstrate the importance of these residues for RhoA activation in living cells.

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 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 contains 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 for the formation of the most active and 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

Abbreviations: 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.

* Corresponding author. 210 Washtenaw Avenue, Ann Arbor, MI 48109-2216, USA. Tel.: +1 734 615 9544; fax: +1 734 763 6492.
E-mail address: johntesmer@umich.edu (J.J.G. Tesmer).
[1] Present address: Department of Structural Biology, D138 Fairchild Science Building, Stanford University School of Medicine, Stanford, CA 94305-5126, USA.

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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 *Caenorhabditis 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 Cβ [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 pMALc2H$_{10}$T vector and purified from *Escherichia 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–decahistidine-tagged fusion proteins using the pMALc2H$_{10}$T 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–2xPLCδ1 PH vector encoding p63RhoGEF with two C-terminal tandem PLCδ1 PH domains was created by cloning p63RhoGEF into the restriction sites *Xho*I and *Hind*III of a pEGFP-C1 vector that already contained PLCδ1–2xPH [28]. All plasmids encoding mutant and fusion proteins were verified by DNA sequencing.

2.2. Fluorescence polarization assay

The rate of nucleotide exchange on 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 μM BODIPY FL GTPγS (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 μM GDP (to measure non-specific binding). In competition experiments, increasing concentrations of unlabeled 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 μM $AlCl_3$, 10 mM NaF, 1% BSA, and 5 mM DTT. To measure non-specific 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 μM GDP, 0.5% Triton X-100, and protease inhibitors) and incubated on ice for 20 min. The samples were then centrifuged at 15,000 ×*g* for 20 min at 4 °C and the supernatants collected. The p63RhoGEF DH/PH domains and RGS4 (serving as a positive control) were biotinylated by incubating 80 μg of the protein with equimolar amounts of biotinamidohexanoyl-6-amino-hexanoic acid N-hydroxy-succinimide ester (Sigma) on ice for 1 h. The conjugate was then filtered through a 0.5 ml Zeba™ desalt spin column. 100 μl 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 h at 4 °C. The beads were washed three times with 500 μl of the lysis buffer, with or without AlF_4^- as appropriate. The beads were treated with 5 μl of 4× 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 μg/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)