Signaling efficiency of $G_\text{aq}$ through its effectors p63RhoGEF and GEFT depends on their subcellular location

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The p63RhoGEF and GEFT proteins are encoded by the same gene and both members of the Dbl family of guanine nucleotide exchange factors. These proteins can be activated by the heterotrimeric G-protein subunit $G_\text{aq}$. We show that p63RhoGEF is located at the plasma membrane, whereas GEFT is confined to the cytoplasm. Live-cell imaging studies yielded quantitative information on diffusion coefficients, association rates and encounter times of GEFT and p63RhoGEF. Calcium signaling was examined as a measure of the signal transmission, revealing more efficient signaling through the membrane-associated p63RhoGEF. A rapamycin dependent recruitment system was used to dynamically alter the subcellular location and concentration of GEFT, showing efficient signaling through GEFT only upon membrane recruitment. Together, our results show efficient signal transmission through membrane located effectors, and highlight a role for increased concentration rather than increased encounter times due to membrane localization in the $G_\text{aq}$ mediated pathways to p63RhoGEF and PLC$_\beta$.

$G$-protein coupled receptors (GPCRs) comprise the largest family of receptors at the plasma membrane, with over 800 genes in the human genome encoding a GPCR. These seven transmembrane proteins can perceive a wide variety of extracellular signals including light, hormones, ions and neurotransmitters. Activation of the receptor induces a conformational change, which allows for nucleotide exchange factor (GEF) activity acting on the heterotrimeric G-protein complex. The GEF activity catalyzes the release of GDP from the $G_\alpha$ subunit, which is replaced by GTP. Subsequently, the GTP-bound activated $G_\alpha$ subunit interacts with downstream effectors.

Almost twenty different $G_\alpha$ subunits can be discerned and these are grouped in four classes; Gi/Go, Gs, Gq and G12/G13. The classical effector of the Gq class is phospholipase C-beta (PLC$_\beta$). PLC$_\beta$ is a 150 kDa enzyme that catalyzes the hydrolysis of phosphoinositide PtdIns(4,5)P$_2$ located in the plasma membrane into diacylglycerol (DAG) and Ins(1,4,5)P$_3$. The latter diffuses into the cytosol and releases calcium from intracellular stores.

Recently, several studies, using biochemical and structural approaches, have shown that p63RhoGEF (and its homologues, Trio and Kalirin) can physically interact with $G_\text{aq}$. This interaction stimulates the GEF activity of p63RhoGEF, which catalyzes nucleotide exchange on Rho family GTPases, suggesting that p63RhoGEF links $G_\text{aq}$ with the actin cytoskeleton.

p63RhoGEF is a protein of 580 amino acids, belonging to the Dbl family of guanine nucleotide exchange factors (GEFs) based on homology. The members of this family are characterized by a tandem domain structure consisting of a Dbl homology (DH) domain directly followed by a pleckstrin homology (PH) domain. The DH domain has intrinsic GEF activity, which is regulated by the adjacent PH domain. The PH domain of p63RhoGEF contains a C-terminal extension that was identified to be required for interaction with $G_\text{aq}$. Deletion of the PH domain increases the GEF activity of the DH domain, indicating an autoinhibitory role for the PH domain. Further studies on the activation mechanism have revealed that $G_\text{aq}$ acts allosterically by binding to the C-terminal extension of the PH domain, relieving the DH domain from autoinhibition. The crystal structure of the $G_\text{aq}$-p63RhoGEF complex supports this mode of activation.

The gene encoding p63RhoGEF also encodes a splice variant, known as GEFT. This variant lacks the first 105 amino acids. It was recently reported that the p63RhoGEF has cysteine residues in the N-terminal region that are palmitoylated, conferring plasma membrane localization. Whereas GEFT lacks the N-terminal lipidation motif, it does contain the full DH/PH region including the complete $G_\text{aq}$ interaction surface. It is unclear whether these variants interact similarly with $G_\text{aq}$ in living cells, or alternatively, whether one is preferred over the other.
study the effect of palmitoylation of p63RhoGEF and subcellular location of p63RhoGEF and GEFT on the interaction with Gαq. We investigated the interaction in living cells using a variety of fluorescence techniques. We reveal that the plasma membrane located p63RhoGEF interacts efficiently with Gαq, while cytoplasmic GEFT does not.

Results

p63RhoGEF is located at the plasma membrane while GEFT is in the cytoplasm. To address the localization and dynamics of p63-RhoGEF and its splice variant GEFT in living cells, fusions with fluorescent proteins were made (Figure 1). The full-length p63-RhoGEF fused to the YFP variant mVenus16,17, denoted as YFP-p63, displayed clear plasma membrane localization in living HeLa cells as can be inferred from confocal images depicted in Figure 2A and as was observed previously18. Plasma membrane localization was observed when YFP was fused to the C-terminus of p63RhoGEF (p63-YFP) (Figure 2A), indicating that plasma membrane labeling was independent of the location of the fluorescent protein. In contrast, the splice variant, GEFT, did not localize at the membrane but was located in the cytoplasm and was largely excluded from the nucleus (Figure 2A). To exclude a cell-to-cell variability with respect to the localization, GEFT and p63RhoGEF were co-expressed in the same cell, again showing non-overlapping localization with p63RhoGEF at the plasma membrane and GEFT in the cytoplasm (Figure 2B).

The clear difference in localization between GEFT and p63RhoGEF is due to the lack of the N-terminal 105 amino acids in case of GEFT. To examine the minimal stretch of amino acids that is sufficient for membrane localization, several variants comprising the first 154, the first 106 or the first 29 amino acids of p63RhoGEF were made, which all localized at the plasma membrane of living cells (Figure 2C and 2D). Together, these results show that the first 29 amino acids at the N-terminus of p63RhoGEF are required and sufficient to confer its plasma membrane localization.

Membrane binding of p63RhoGEF requires cysteines in the N-terminus. It has been shown that cysteines in the N-terminus of p63RhoGEF are palmitoylated19 and that cysteine mutants do not localize to the plasma membrane. To verify this, we mutated several of the cysteines into serines. Three constructs were made as depicted in Figure 3A. The distribution of these constructs in living cells is shown in Figure 3B. Only the p63-C2S construct, with mutations C105S and C12S, displayed membrane binding. The other two mutants showed cytoplasmic and nuclear localization. These results suggest that cysteine residues 10 and 12 are dispensible for membrane localization of p63RhoGEF. Our results are in line with previous observations in a different cell type20.

The diffusion time of membrane associated proteins is strongly reduced when compared to the diffusion time of cytosolic proteins21. Fluorescence Recovery After Photobleaching (FRAP) can be used to quantify the diffusion of fluorescent biomolecules in cells22,23. Previously, we have used FRAP to detect the lipidation-dependent interaction of the plant Gz subunit GPz1 with the plasma membrane24. In order to examine membrane interaction of p63RhoGEF, FRAP was performed on the basal membrane of cells expressing p63RhoGEF. The recovery of p63RhoGEF was characterized by a diffusion time of 0.095 μm²/s (Figure 3C), corresponding to values previously reported for palmitoylated proteins25. The recovery of p63(1–29)-YFP was similar with a diffusion time of 0.13 μm²/s (data not shown). These results are in agreement with the observation that the first 29 amino acids are required and sufficient for membrane binding. Next, the recoveries of the cysteine mutants were analyzed. The p63-C3S mutant showed a fast recovery (Figure 3D) which did not allow accurate fitting of the recovery curve. The diffusion time was estimated to be >1 μm²/s. The FRAP recovery curve of p63-C5S was identical to that of p63-C3S (data not shown). However, the mutant p63-C2S, carrying Cys10Ser and Cys12Ser, showed a slow recovery with a diffusion time of 0.24 μm²/s. Hence, the diffusion of this mutant is approximately two-fold increased compared to the diffusion time of wild-type p63RhoGEF. These results agree with the observed localization of the cysteine mutants (Figure 3B) and previous observations25 and indicate that the stretch comprising cysteine residues 23, 25 and 26 is required for membrane localization of p63RhoGEF, but that Cys10 and Cys12, while not sufficient for membrane binding, confer affinity for the membrane.

p63RhoGEF and GEFT interact with Gqα-GTP in living cells. The interaction between Gqα and p63RhoGEF has been thoroughly studied using purified proteins26,27 or proteins in cell extracts28, demonstrating a direct interaction between GTP-bound Gqα and p63RhoGEF in vitro. However, interaction data from intact living cells is lacking. It has been shown that overexpressed, wild-type Gqα...
is able to recruit a palmitoylation-deficient p63RhoGEF to the plasma membrane\(^{15}\). This would suggest an interaction between wild-type Gaq and p63RhoGEF. Since wild-type Gaq is predominantly GDP bound due to intrinsic GTPase activity\(^{23}\) this observation contrasts previous findings from in vitro studies in which only active Gaq\(_{\text{GTP}}\) activates p63RhoGEF\(^{9}\). To examine this in more detail, we co-expressed a CFP-Gaq that is fully functional\(^{24}\) with a wild-type version and a palmitoylation-deficient p63-C5S. Control samples in which p63RhoGEF and Gaq were co-expressed, show that both proteins are located at the plasma membrane (figure 4A). When the palmitoylation-deficient p63-C5S is co-expressed with Gaq, the RhoGEF is confined to the cytoplasm and the Gaq is located at the plasma membrane (67/72 cells show plasma membrane localization of Gaq). Similar results were obtained when GEF is co-expressed with Gaq, although we observed in cells highly expressing GEF that Gaq shows a more cytoplasmic localization (28 out of 124 cells show cytoplasmic localization of Gaq). Together, these results suggest that there is little or no interaction between over-expressed wild-type Gaq and GEF or p63RhoGEF in HeLa cells.

To examine whether and where an interaction occurs between Gaq and p63RhoGEF in living cells, we made use of YFP-Gaq\(_{\text{Q209L}}\), which is a YFP labeled Gaq made constitutively active by a single point mutation (Q209L). This mutant is GTPase deficient and therefore is locked in the active GTP-bound form. The Gaq\(_{\text{Q209L}}\) mutant is predominantly located in the cytoplasm (figure 4B) which might be due to diminished interaction with the G\(\beta\)\(\gamma\) dimer, thereby reducing plasma membrane affinity. Remarkably, upon co-expression of p63-mCherry, the YFP-Gaq\(_{\text{Q209L}}\) is located at the plasma membrane (figure 4B), suggesting that the two proteins interact at the plasma membrane. It was previously shown that the L475A point mutation in the extension of the PH domain of p63RhoGEF abolishes the interaction\(^{9}\) in vitro. The localization of p63(L475A)-YFP is identical to that of p63-YFP. However, the co-expressed constitutively active Gaq remains cytoplasmic in the presence of p63(L475A)-YFP (figure 4B), confirming in living cells that the interaction between p63 and Gaq is strictly dependent on the L475A mutation in p63RhoGEF.

Since GEF and the constitutive active Gaq are both located in the cytoplasm, it is unclear from their steady-state localization whether these two proteins interact. To examine whether these proteins interact in intact living cells we performed a co-recruitment assay.
Co-recruitment is a strong indicator of a physical interaction or complex formation with the advantage that it can be performed in single, intact, living cells. We have recently used this method to demonstrate the interaction between Gqα and PLCβ in cells. To this end, GEFT and FKBP12-Gqα were co-expressed. The FKBP12-Gqα is cytoplasmic due to removal of the lipidation motif. The FKBP12 domain strongly binds a FRB domain after addition of the cell-permeant heterodimerizer rapamycin. By targeting the FRB domain to the plasma membrane, the FKBP12-Gqα can be recruited to the plasma membrane. We performed the co-recruitment with both the wild-type Gqα and the GTPase deficient Gqα. Recruitment of YFP-GEFT was only observed when active, GTP-bound, Gqα was relocalized (figure 4C). Quantification of the translocation of YFP-GEFT observed in multiple cells induced by recruitment of mRFP-Gqα-Q209L or mRFP-Gqα is shown in figure 4D. Together our data suggest that GEFT and p63RhoGEF both interact with Gqα-Q209L, while GDP-bound Gqα does not have affinity for p63RhoGEF and GEFT.

The interaction of p63RhoGEF with Gqα is dynamic and reversible. Although co-recruitment and co-localization strongly suggest interaction, it is not direct proof. To study the interaction more directly we made use of Förster Resonance Energy Transfer (FRET) which is highly distance dependent and is therefore suited to monitor proximity of molecules in living cells. To examine whether the interaction between Gqα and p63RhoGEF can be observed in living cells, a Gqα subunit tagged with mTurquoise, CFP-Gqα, and p63RhoGEF tagged with YFP were co-expressed. Both proteins are located at the plasma membrane (figure 5A). In case of interaction, it is expected that the YFP over CFP ratio increases. Upon addition of histamine, a significant increase in the YFP/CFP ratio was observed (figure 5B). When an H1R antagonist, perylamine, was added, the ratio decreased and returned to the baseline value. Kinetic analysis of the interaction yielded a kon of 0.23 s⁻¹ and a koff of 0.045 s⁻¹, corresponding to half-times of 3 and 15 seconds, respectively. To examine the specificity of the interaction, the mutant p63(L475A)-YFP was used. We hardly observed any change in YFP/CFP ratio when histamine receptors were activated (fig. 5B), indicating that the interaction between Gqα and the L475A mutant is strongly reduced.

p63RhoGEF rather than GEFT inhibits PLCβ-dependent calcium signaling. The different location of the Gqα effectors, i.e. membrane associated p63RhoGEF versus cytoplasmic GEFT, comprises an excellent model to address the effects of location on interaction efficiency and signal transmission. We took advantage of the well-characterized signaling pathway in which Gqα activation of PLCβ results in intracellular calcium increases. First, we examined whether the Gqα-p63RhoGEF interaction competes significantly with PLCβ activation. In HeLa cells, the Gqα-PLCβ pathway is activated upon addition of histamine, leading to an increase in the intracellular calcium concentration. Calcium imaging was performed on a population of cells in which the cells expressing YFP-tagged p63RhoGEF could be easily identified based on their YFP fluorescence. The non-transfected cells were used as a control for timing of the onset of the calcium response. When the calcium...
increase in cells expressing p63RhoGEF was analyzed, a marked delay was observed (Figure 6A), suggesting that p63RhoGEF is capable of inhibiting calcium signaling by competing with PLCβ for interaction with Gqα. A similar experiment with the L475A mutant demonstrated no significant delay in calcium signaling (Figure 6B). These results correlate membrane binding with the efficiency of delaying calcium signaling, the latter suggesting competition between p63RhoGEF and PLCβ.

**Discussion**

In this study, we used fluorescent protein tagged p63RhoGEF and GEFT to study its subcellular location and interaction with Gqα. The heterotrimeric G-protein complex itself is predominantly located at the plasma membrane by lipid modification of the Gz and Gγ subunits. Both GFT and p63RhoGEF contain the entire region which is necessary for Gqα binding. However, while p63RhoGEF localizes almost exclusively to the plasma membrane, GEFT is confined to the cytoplasm. Therefore, these signaling components comprise an excellent model to address the effects of location on interaction efficiency and signal transmission.

The p63RhoGEF and GEFT do not differ in their Gqα interaction domain, encompassing roughly residues 149–502, which has an affinity for active Gqα (Gqα-GTP) of <0.1 μM10 or 1 μM depending on the assay. Our data confirm that active GTP-bound Gqα (Gqα-Q209L) is capable of interacting with both GFT and p63RhoGEF in cells, while the wildtype Gqα, which is predominantly GDP-bound, has hardly any affinity.

To examine the effects on signal transmission, we used calcium imaging as an output signal for GPCR activation. Interaction of Gqα with its RhoGEF effector leads to a reduction in the calcium signal. The overexpression of membrane located p63RhoGEF strongly inhibited calcium signaling. In contrast, the effect of overexpressed cytoplasmic cysteine mutants and GEFT are far less efficient in inhibiting calcium signaling. This indicates that the interaction between Gqα and membrane located p63RhoGEF is stronger. Our data fit with our previous observations that Gqα activation and interaction with PLCβ occurs predominantly at the plasma membrane.24,25,29 Since p63RhoGEF with L475A did not interfere with calcium signaling, the interaction between Gqα and p63RhoGEF is required to delay calcium signaling. Still, it is possible that some other mechanism than competition between p63RhoGEF and PLCβ inhibits the calcium signal, e.g. downregulation of PLCβ activity by p63RhoGEF or modulation of calcium channels by p63RhoGEF. Further experiments are included as supplemental information (figure S1). These results show that recruitment of GEFT to the membrane attenuates calcium signals, suggesting that GEFT competes dynamically with PLCβ for Gqα binding.
required to observe directly the competition between PLCβ and p63RhoGEF for Gq, for instance by multiplex FRET measurements. Since our experiments employ overexpressed proteins, it is necessary to understand whether p63RhoGEF competes with PLCβ under physiological conditions. Recently, Momotami et al. detected relative high expression of p63RhoGEF in vascular smooth muscle cells and especially portal veins. Previous studies have revealed a role for Gq-PLCβ in portal vein myocytes. Therefore further studies using knock-down approaches may shed light on the balance between the PLCβ and p63RhoGEF pathway in smooth muscle cells.

How does membrane localization of the RhoGEF result in a more efficient interaction with Gq? Two effects have been brought forward which are (i) a decrease in time necessary for a productive interaction and (ii) an increased concentration of the interactors. Theoretical analysis of interacting membrane components favors the second idea. The encounter time between Gq and p63RhoGEF, calculated based on the p63RhoGEF diffusion time of 0.1 μm²/s² that we measured, is about 0.3 s for a cell expressing 10,000 copies of each molecule. The encounter time probably reflects a minimum as we can easily obtain concentrations around 1 μM in transient expression systems, which would correspond to 1 million copies and hence a shorter encounter time. The actual half time of association between p63RhoGEF and Gq as judged from dynamic FRET data, is 3 s, which is at least an order of magnitude slower than the encounter time. Since non-membrane bound p63RhoGEF moves with a diffusion coefficient that is an order of magnitude faster, the encounter rate with Gq will be even faster. Still, our

Figure 6 | Overexpressed p63RhoGEF inhibits Gq mediated calcium signaling. (A) Single cell traces of calcium release measured with Fura Red in HeLa cells stimulated with histamine. The time delay in the calcium release between control HeLa cells (dashed line) and cells expressing p63RhoGEF-YFP (solid line) was quantified. (B) Bar graph that displays the delay in calcium signaling in cells expressing YFP fused to p63RhoGEF, p63RhoGEF-L475A, GEFT or the indicated cysteine mutants. Average values (± s.e.m.) of at least three independent experiments with a total of at least 10 cells are shown. The P-values were calculated with an unpaired two-tailed Student’s t-test: P < 0.0002 (**), P < 0.004 (*) and P < 0.05 (#).

Figure 7 | Dynamic inhibition of calcium oscillations by recruitment of GEFT. (A) Cartoon illustrating the principle of the experiment. Rapamycin recruits the FKBP12-GEFT (F12-GEFT) fusion to the FRB domain located at the plasma membrane, effectively competing with PLCβ for activated Gq, thereby attenuating calcium signals. (B) Single cell calcium imaging data showing oscillations due to GPCR activation by histamine. Subsequent recruitment of mCherry-FKBP12-GEFT strongly attenuates the signal. (C) Single cell calcium imaging data from a control cell expressing mCherry-FKBP12. Recruitment induced by rapamycin does not alter the frequency or amplitude of the calcium spiking.
experiments show that Gqα and p63RhoGEF interact more efficiently. These considerations support the idea that concentration rather than encounter time is the factor that determines signaling efficiency. This hypothesis can be tested by controlling the local concentration in a cell and compare the situation before and after the manipulation. Experiments with the recruitment of GEF under influence of rapamycin increase the effective concentration of the protein at the plasma membrane22,23, and have a strong effect on the signal transmission. Therefore, our observations support the concept that the increased concentration is the dominant factor.

Together, our data imply that, in our system, GEF is under hardly any or no control of Gαq and suggest that p63RhoGEF mediates Gαq dependent remodeling of the cytoskeleton24,25. Still, it is possible that the location of GEF is under control of other, unknown, factors and therefore GEF may play a role in signaling through Gαq under conditions where it is recruited to the plasma membrane26.

The observation that signal transmission is dominated by membrane-bound proteins has important implications for subsequent signaling steps. Both Gαq and p63RhoGEF are palmitoylated, a post-translational modification that is dynamically and spatially regulated27. Since p63RhoGEF and Gαq are implicated as oncoproteins28,29 it will be relevant to study whether the malignant activity of these proteins can be reduced by small molecules that interfere with palmitoylation30.

We show directly that the subcellular location is a key determinant for the interaction between Gαq and its effector, with membrane located p63RhoGEF interacting strongly with Gαq. For several other RhoGEFs, it was shown that they translocate to the plasma membrane upon stimulation of cells31,32. These data suggest that the plasma membrane is an important platform for RhoGEF activity, possibly by activating a specific subset of RhoGTPases. Hence, it will be key to study where the RhoGTPases and regulatory proteins, such as mixed lineage kinase 3 (MLK3)33 are located to understand signal transduction further down the cascade.

Methods

Materials. Fura Red/AM and lipofectamine 2000 were from Invitrogen (Bleiswijk, The Netherlands). Rapamycin was from LC Laboratories (Woburn, USA).

Construction of fluorescent protein fusions. Mammalian expression vectors were based on the pEGFP-N1 and pEGFP-C1 plasmids in which EGFP was replaced by the pEGFP-N1 and pEGFP-C1 plasmids in which EGFP was replaced by the length human p63RhoGEF9 was used as a template for PCR. To construct FGFT under manipulation. Experiments with the recruitment of GEFT under transduction further down the cascade.

Microscopy. Confocal laser scanning microscopy was performed with a Zeiss LSM510 microscope equipped with a 63x oil immersion objective (Plan-Neofluar, NA 1.4) and the pinhole set to a 1 airy unit to obtain optical slices (~0.8 μm). For YFP fluorescence, excitation was at 514 nm and the emission light was filtered through a 530–600 nm bandpass filter. For co-imaging of green and red fluorophores, excitation wavelengths of 488 nm and 543 nm were used and emission filters were 505–530 nm and LP560 nm or LP685 nm. For co-imaging of cyan and yellow fluorophores, excitation wavelengths of 458 nm and 514 nm were used, and emission filters were 470–500 nm and 520–555 nm. To avoid bleed-through, images were acquired in multitrack mode. Calcium imaging with Fura Red (figure 6) was performed as described26. All images are representative of multiple experiments, i.e. multiple transfections performed on different days.

FRET ratio imaging. The calcium imaging experiments reported in figure 7 were performed using FRET ratio imaging of yellow cameleon 3.60. FRET ratio-imaging was performed as described24,25 to measure diffusion in the basal membrane of cells. A 1.4 μm wide strip (40 pixels, zoom 8) was bleached by two scans at maximum excitation power (AOTF at 100% transmission) of the 488 nm and 514 nm laser line. Ten pre-bleach and 100 post-bleach images were acquired with a 500 ms interval. The resulting mean fluorescence from the strip was quantified. The recovery was fit to the empirical formula described previously34,35 to yield the effective diffusion time. At least ten cells were measured for each construct.

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Author contributions

J.G., J.v.U., M.J.W.A. and T.W.J.G. designed experiments; J.G., J.v.U. and M.J.W.A. conducted experiments and analysed data; J.G. aided by the other authors, wrote the paper; All authors reviewed the manuscript.

Additional information

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