FEBS 26745

FEBS Letters 531 (2002) 565–569

Activated Gαq family members induce Rho GTPase activation and Rho-dependent actin filament assembly

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Received 14 October 2002; accepted 17 October 2002

First published online 1 November 2002

Edited by Jesus Avila

Abstract **Rho GTPase is required for actin filament assembly and serum response element (SRE)-dependent gene transcription. Certain G protein-coupled receptors (GPCRs) induce Rho-dependent responses, but the intermediary signaling steps are poorly understood. The heterotrimeric Gα12 family can induce Rho-dependent responses. In contrast, there are conflicting reports on the role of the Gαq family in Rho signaling. We report that expression of activated Gαq members, or activation of endogenous Gαq via GPCR stimulation, induces SRE reporter activation via Rho, and increased GTP-Rho levels. Moreover, microinjection of activated Gαq in fibroblasts induces actin stress fiber formation via Rho. Gαq functionally cooperates with Lbc Rho guanine nucleotide exchange factor. Overall, these findings indicate that Gαq family signals are sufficient to induce Rho-dependent cellular responses.**

Key words: Rho; Gαq; G protein-coupled receptor; Signaling; Serum response element; Actin filament

1. Introduction

The ubiquitous Rho small GTPase is required for multiple cellular responses such as cell growth, contraction, retraction and migration [1]. Rho controls a distinct signaling pathway that regulates actin filament assembly [2] and serum response factor (SRF)-dependent gene transcription [3]. Moreover, defective Rho signaling is implicated in diseases such as cancer and cardiovascular defects [4–6]. However, early regulatory steps in the Rho pathway are poorly understood.

Rho signaling is rapidly induced by serum, and many of the stimulatory serum components are G protein-coupled receptor (GPCR) agonists such as lysophosphatidic acid, thrombin, and agonists for the α1-adrenergic and M1 muscarinic GPCRs which induce actin polymerization and serum response element (SRE) transcriptional reporter activation via Rho [7]. Moreover, it is now clear that the mechanisms of GPCR-induced Rho responses involve Gα subunits of heterotrimeric GTPases [7]. For example, actin filament assembly, a Rho-dependent cytoskeletal response [8], is induced by microinjection of activated forms of the Gα12 family, Gα12QL or Gα13QL, into fibroblasts [9]. Based on these and other studies, it is now well established that Gα12 and Gα13 signals induce Rho activation and subsequent cellular responses. Moreover, the mechanism by which Gα13 activates Rho has been defined in vitro, and requires a Rho guanine nucleotide exchange factor (GEF). Rho GEFs directly activate Rho by inducing GDP–GTP exchange in response to extracellular stimuli [10]. Activated Gα13, but not Gα12 or Gαq, directly stimulates the intrinsic activity of p115 Rho GEF, leading to Rho activation [11].

In contrast to the Gα12 family, there is conflicting evidence concerning the role of Gαq in Rho stimulation. The ubiquitous Gαq is a key GTPase that activates phospholipase Cβ, resulting in Ca^{2+} influx and protein kinase C activation [12] in response to many stimuli. The Gαq family includes αq/11, α14 and α15, of which α14 and α15 show restricted tissue expression [13]. Originally, activated Gαq was reported to be inactive in inducing actin stress fiber formation in fibroblasts [9] and SRF activation [14]. In contrast, others observed that Gαq signals could induce Rho-dependent responses such as neurite retraction [15], astrocytoma cell rounding [16] and SRF activation [17].

In addition to these conflicting findings, there are contrasting reports on potential mechanisms of Gαq-induced Rho responses. One unresolved issue has been whether Gαq can induce direct Rho activation, e.g. GTP-Rho formation in vivo, since some reports found that Gαq induces weak or no GTP-Rho formation [16,18], while others report significant GTP-Rho formation following Gαq expression [19]. Further uncertainty concerns the role of Rho GEFs in Gαq-induced Rho signals. Gαq does not stimulate p115 Rho GEF as does Gα13 [11]; therefore, it must induce Rho signals by a different pathway(s), possibly via a different Rho GEF. However, the precise involvement of Rho GEFs in Gαq signals remains to be defined.

The aim of this study is to clarify the role of Gαq in Rho signaling. By using three independent assays, our results show that expression of Gαq family members is sufficient to induce Rho activation and Rho-dependent cellular responses, including, for the first time, actin stress fiber formation in fibroblasts.

2. Materials and methods

2.1. Cell lines and reagents

HEK293T cell line was from ATCC. Quiescent serum-starved Swiss 3T3 fibroblasts were prepared as described [20]. Carbachol and iso-

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PII: S0014-5793(02)03625-6

proterenol were from Sigma; Rho kinase inhibitor Y27632 was from Wilfide Co. (Osaka, Japan).

2.2. Plasmids

Wild-type and activated Gαq Q209L, Gα11 Q209L, Gα14 Q205L, Gα15 Q212L, Gα12 Q231L, Gα13 Q226L, Gαs Q213L, and M1 muscarinic and β2-adrenergic receptor cDNAs in pcDNA3 vector were obtained from Guthrie cDNA Resource. pBN112-Gαq Q209L expression vector was obtained from ATCC. The SRE.L luciferase reporter, pEFC3 transferase, pGEX2T Rhotekin RBD and p115 Rho GEF plasmids were gifts. pEXVRhoV14, pEXVRacL61 and pSR-wt-Lbc:Flag are described in [8,21].

2.3. Antibodies

Anti-RhoA and anti-Gα-subunit antibodies were from Santa Cruz.

2.4. Cell transfection

Cells at 70% confluence (six well dishes for reporter assays, 100 mm dishes for RBD assays) were transfected using Lipofectamine Plus (Gibco BRL).

2.5. Immunoblotting

Cellular material was resolved by 10% SDS–PAGE. Immunoblotting was carried out as described in [16].

2.6. Dual luciferase reporter assay

SRE.L luciferase reporter plasmid, which encodes a mutant SRE that contains SRF binding sites, but eliminates the ternary complex factor binding site [3], was used. Twenty-four hours following HEK293T transfection, luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Results were normalized by sequential measurement of the internal control *Renilla* and experimental firefly luciferase levels by luminometer. Each point was performed in triplicate.

2.7. RBD assay

HEK293T were transfected with plasmids and serum-starved overnight before harvesting. After 24 h, a portion of the cell lysate was incubated with affinity gel-bound GST-Rhotekin Rho binding domain (RBD) fusion protein which specifically binds GTP-Rho, and affinity purification was performed as described [22]. Total cell lysate or pull-down material was resolved by 10% SDS–PAGE, followed by immunoblotting with anti-RhoA antibody.

2.8. Microinjection

Quiescent Swiss 3T3 fibroblasts were injected with expression plasmids for Gα12 Q205L, Gαq Q209L or Gα13 Q226L (16–50 μg/ml). Two to four hours after injection, cells were fixed and actin visualized by TRITC–phalloidin staining as described [20]. Injected cells were identified by coinjection of an injection marker. For some experiments, C3 transferase expression vector was coinjected, or cells were treated with 5 μM of Rho kinase inhibitor Y27632 from the time of injection.

3. Results and discussion

3.1. Induction of SRE reporter activation by Gαq members

Since activation of SRF by extracellular factors requires Rho function [3], we tested the ability of Gαq members to induce SRE.L luciferase reporter activity. As shown in Fig. 1A, expression of activated mutant Gαq family members αq, α11, α14, and α15 induced substantial SRE luciferase reporter activity. In contrast, expression of activated Gαs had no effect, whereas activated Gα12/13 led to robust luciferase reporter induction. Furthermore, Fig. 1A shows that coexpression of C3 transferase, which inactivates Rho, blocked Gαq member-induced SRE reporter activation, demonstrating that the response is Rho-dependent in each case. A positive control was provided by expression of activated RhoV14 which induced C3-sensitive luciferase reporter activity. In contrast, SRE reporter induction by activated RacL61 was not blocked by C3 transferase coexpression, indicating the target selectivity of C3 to Rho. The finding that Gαq members induce substantial SRE.L reporter activity via Rho is in agreement with earlier reports [16,17].

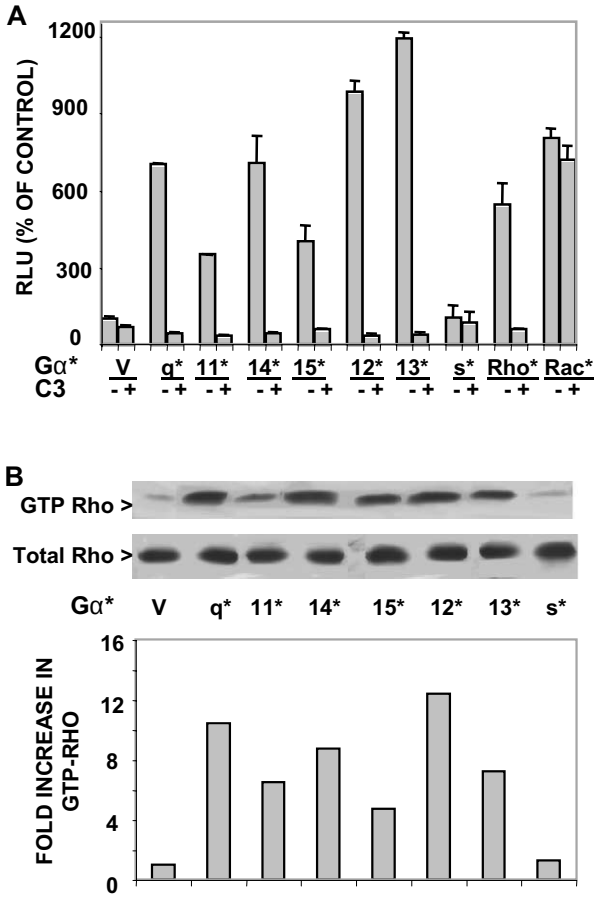

Fig. 1. Gαq members stimulate SRE.L luciferase reporter activity, and GTP-Rho formation. A: Activated Gαq members were coexpressed in HEK293T with SRE.L luciferase reporter, and dual luciferase assay carried out. RLU = relative luciferase units. * indicates activated mutant GTPase forms described in Section 2.2; V = vector. Results shown are mean ± S.D. B: Following activated Gαq member expression in HEK293T, GTP-Rho pull-down was carried out. RhoA immunoblot of the GTP-Rho pull-down (upper panel) and total cellular Rho (lower panel) from a representative experiment are shown. Graph shows mean fold increase in GTP-Rho densitometric values normalized to vector from at least two experiments.

3.2. Induction of GTP-Rho formation by Gαq members

To further investigate the observed response, the effect of expression of Gαq members on Rho activity was determined by measuring changes in GTP-Rho levels in HEK293T cells by the GTP-Rho pull-down assay. As shown in Fig. 1B, expression of activated Gαq, α11, α14 or α15 reproducibly caused increased GTP-Rho levels in vivo of at least four-fold. As expected, activated Gα12 or Gα13 expression also led to increased GTP-Rho levels. In contrast, expression of activated Gαs, which does not stimulate Rho responses, had no effect. In contrast to some previous studies [16,18], our finding of Gαq-induced increases in GTP-Rho levels is consistent with the observed SRE reporter activation via Rho, and in agreement with the recent finding of Chikumi et al. [19].

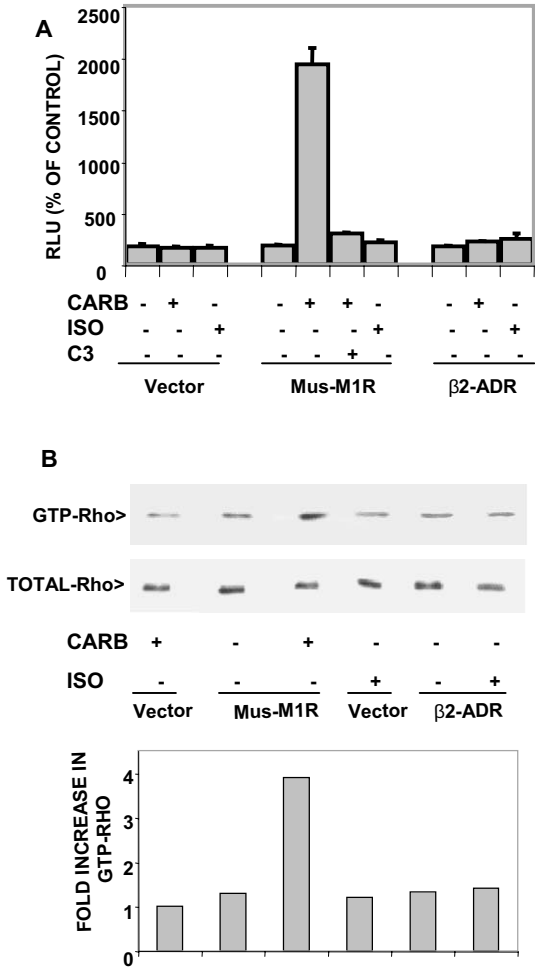

Fig. 2. Gαq-linked GPCR stimulation induces SRE reporter activation and GTP-Rho formation. A: HEK293T cells were transfected with SRE.L luciferase reporter and either vector, pcDNA:M1 muscarinic receptor (Mus-M1R) or pcDNA:β2-adrenergic receptor (β2-ADR). Following stimulation with 100 μM carbachol (CARB) or 10 μM isoproterenol (ISO) for 6 h, dual luciferase assay was carried out. Results shown are mean ± S.D. B: HEK293T cells were transfected with 1 μg of vector or GPCR plasmid. After overnight serum starvation, cells were stimulated with 100 μM carbachol for 10 min, followed by GTP-Rho pull-down. RhoA immunoblot of the GTP-Rho pull-down (upper panel) and total cellular Rho (lower panel) from a representative experiment are shown. Graph shows mean fold increase in GTP-Rho densitometric values normalized to vector from at least two experiments.

3.3. Induction of GTP-Rho formation by Gαq-linked GPCR

We next tested whether activation of endogenous Gαq signals results in Rho signaling and activation by expressing M1 muscarinic receptor cDNA, a specific Gαq-linked GPCR [23], in HEK293T, and stimulating with the agonist carbachol. Fig. 2A shows that carbachol treatment led to substantial SRE reporter activation which was blocked by C3 transferase coexpression. Next, the effect of M1 muscarinic receptor stimulation on GTP-Rho levels was measured. Fig. 2B shows that compared to non-stimulated cells, carbachol stimulation of cells expressing M1 muscarinic receptor led to increased GTP-Rho levels. In contrast, stimulation of cells expressing a Gαs-linked GPCR, β2-adrenergic receptor by the agonist isoproterenol, had no effect on GTP-Rho levels. Taken together, the findings that exogenous expression of activated Gαq, or stimulation of endogenous Gαq signals, leads to Rho signals and GTP-Rho formation support the notion that Gαq signals are sufficient to activate Rho.

3.4. Induction of Rho-dependent actin filament assembly by Gαq

We next investigated the effect of Gαq actin cytoskeletal responses. As shown in Fig. 3A,B, expression of activated pBN-Gαq Q209L in quiescent Swiss 3T3 fibroblasts by microinjection induced a very strong stress fiber response in 75–100% of the injected cells within 2–4 h of injection, while there was no response in control-injected cells (not shown). To ensure that stress fiber induction resulted from the Gαq Q209L sequence, the Gαq Q209L cDNA was subcloned into pcDNA3 and resequenced. Microinjection of pcDNA3-Gαq Q209L gave identical results to pBN-Gαq Q209L expression.

To investigate the role of Rho in Gαq-induced stress fiber formation we coinjected the pEFC3 transferase plasmid with Gαq Q209L. Upon coexpression stress fiber formation was

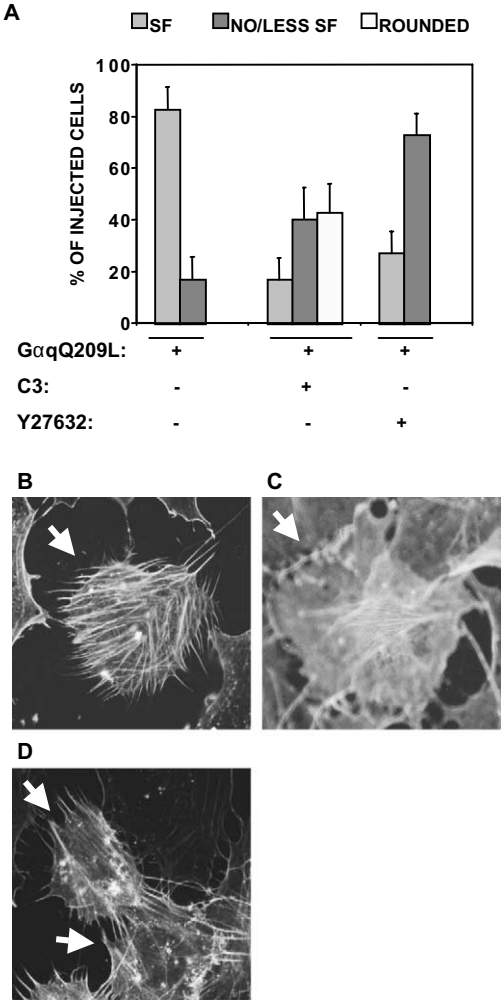

Fig. 3. Activated Gαq expression induces actin stress fiber formation. A: Confluent quiescent Swiss 3T3 cells were injected with indicated expression vectors with or without Y27632, and the actin cytoskeleton visualized. Percentages of injected cells exhibiting strong stress fiber (SF) formation are shown as mean ± S.D. of at least three experiments each examining 100 injected cells. Typical morphologies of cells expressing Gαq Q209L (B); Gαq Q209L and C3 (C); and Gαq Q209L+Y27632 (D). Arrows indicate injected cells.

inhibited in approximately 45% of the cells (Fig. 3A,C), while 10% of cells retained stress fibers. Thus, we show here for the first time that expression of activated Gαq is capable of inducing robust actin stress fiber formation in fibroblasts via Rho. This is in contrast to previous reports that Gαq is inactive in stress fiber formation [9,24]. While the reason for the discrepancy between these studies and ours is not clear, it should be noted that Gohla et al. [24] used a different activated Gαq mutant (Gαq R183C) and that Buhl et al. [9] did note some subtle differences in the appearance of the actin cytoskeleton upon Gαq Q209L expression. In addition our results are consistent with reports on Gαq-induced neurite retraction and astrocytoma cell rounding [15,16], which both require actin cytoskeletal rearrangements. In addition, in quiescent fibroblasts, C3 and Gαq Q209L coexpression resulted in rounding up of about 45% of the cells (Fig. 3A), although C3 alone or in combination with Gα13 Q226L did not (not shown). The specific coexpression of Gαq Q209L and C3 in serum-starved fibroblasts may cause this effect, but its molecular basis remains to be defined.

Next, the role of the Rho downstream effector Rho kinase in Gαq-induced stress fiber formation was determined. Treatment of cells with the Rho kinase inhibitor Y27632 inhibited the internal stress fiber formation induced by Gαq, resulting in stress fiber inhibition in 75% of the cells (Fig. 3A,D). We conclude that activated Gαq is a potent inducer of actin stress fiber formation in quiescent fibroblasts through a Rho- and Rho kinase-dependent pathway. While Y27632 caused an almost total disappearance of internal stress fibers in Gαq-expressing cells, the cells still appeared to be contracted along the cortical rim with several actin-rich spikes or retraction fibers occurring. In light of reports that myosin light chain kinase (MLCK) activity is associated with actin fiber formation at the periphery/cortical border of the cell, while Rho kinase is primarily involved in internal stress fiber formation [25,26], it can be speculated that Gαq may induce both MLCK and Rho kinase activation.

3.5. Involvement of Rho GEFs in Gαq-induced Rho signals

We next analyzed potential mechanisms of Gαq-induced Rho signals by assessing the ability of two Rho-specific GEFs, Lbc and p115 Rho GEF, to functionally cooperate in Gαq family-induced signals. Fig. 4A shows that coexpression of full-length wild-type Lbc with Gαq, Gα14, or Gα15 results in increased SRE luciferase reporter activity of up to three-fold, without changes in Gαq subunit expression. In

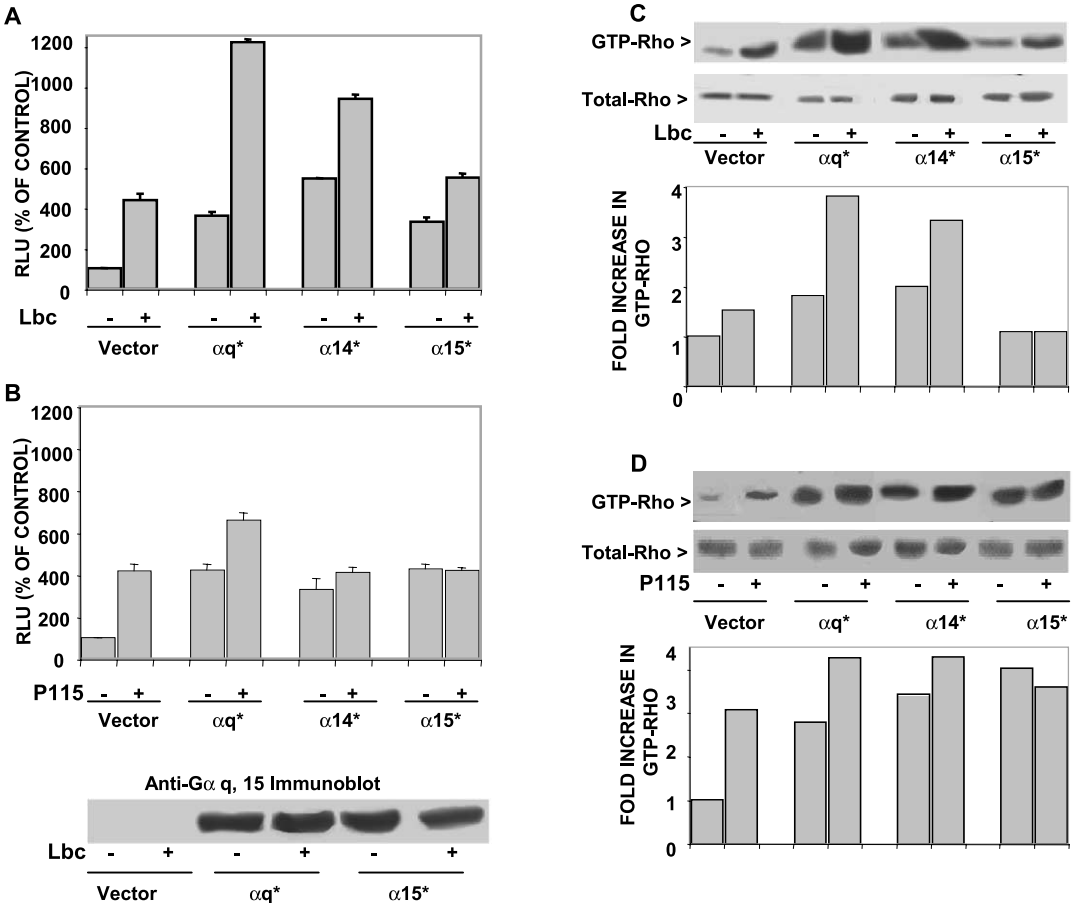

Fig. 4. Functional cooperativity of Gαq members with Lbc Rho GEF. A,B: Activated Gαq members were coexpressed with either wild-type Lbc (A) or p115 Rho GEF (B) in HEK293T with SRE.L luciferase reporter, and dual luciferase assay carried out. RLU = relative luciferase units. Results shown are mean ± S.D. Lower panel is an immunoblot of total cellular Gαq and Gα15 expression levels in the presence of Lbc. C,D: Activated Gαq members were coexpressed with either wild-type Lbc (C) or p115 Rho GEF (D) in HEK293T, and GTP-Rho pull-down carried out. RhoA immunoblot of the GTP-Rho pull-down (upper panel) and total cellular Rho (lower panel) from a representative experiment are shown. Graphs below the immunoblot panels show mean fold increase in GTP-Rho densitometric values normalized to vector from at least two experiments.

contrast, coexpression of full-length p115 Rho GEF with Gαq members results in more modest cooperativity. Furthermore, Fig. 4B shows that coexpression of Lbc with Gαq or Gα14 caused increased GTP-Rho formation in vivo as measured by RBD pull-down experiments. This result is consistent with the signaling cooperativity observed in SRE reporter activation. In comparison, p115 Rho GEF coexpression with Gαq members led to little or no cooperativity in inducing GTP-Rho formation.

These results suggest that Gαq or Gα14 signals show a greater enhancement with Lbc as compared to p115 Rho GEF. Gα15 showed less cooperativity with Lbc compared to Gαq or Gα14; while the reason for this is unclear, it may reflect differences between Gαq family members and their potential cognate Rho GEFs, analogous to the finding that p115 Rho GEF is only stimulated by Gα13, but not by its close relative Gα12 [11]. The observed functional cooperativity is consistent with the previous finding that while Lbc physically associates with Gαq [16], p115 GEF neither directly associates with, nor transduces Gαq signals [11]. Gαq also functionally cooperates and associates with LARG GEF [27], presenting the possibility that Gαq may induce Rho signals via a Rho GEF such as Lbc and/or LARG GEF in vivo, and this remains to be directly demonstrated. Whether or not the precise mechanistic link between Gαq and Rho GEFs will have the same basis as the Gα13-activated p115 Rho GEF paradigm remains to be determined. In summary, our findings definitively extend the signaling pathways of Gαq-linked GPCRs to include Rho activation, and Rho-dependent transcriptional and actin cytoskeletal responses.

Acknowledgements: We thank G. Bollag, K. Kaibuchi, S. Offermanns, M. Schwartz, and R. Treisman for plasmids. Funding was provided by NIH T32 DK07542 to P.D., EMBO and the Danish Cancer Society to L.K., Cancer Research U.K. to A.H., and NCI CA62029 and American Heart Association 0050915T to D.T.

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