BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 41–49
© 2009 The Author
Journal compilation © 2009 The British Pharmacological Society All rights reserved
0007-1188/09
www.brjpharmacol.org

THEMED ISSUE: GPCR

REVIEW

Regulation of RhoGEF proteins by $G_{12/13}$-coupled receptors

Sandra Siehler

Novartis Institutes for BioMedical Research Basel, Center for Proteomic Chemistry, Novartis Pharma AG, Basel, Switzerland

G protein-coupled receptors (GPCRs) represent a large family of seven transmembrane receptors, which communicate extracellular signals into the cellular lumen. The human genome contains 720–800 GPCRs, and their diverse signal characteristics are determined by their specific tissue and subcellular expression profiles, as well as their coupling profile to the various G protein families (G_s, G_i, G_q, G_{12}). The G protein coupling pattern links GPCR activation to the specific downstream effector pathways. $G_{12/13}$ signalling of GPCRs has been studied only recently in more detail, and involves activation of RhoGTPase nucleotide exchange factors (RhoGEFs). Four mammalian RhoGEFs regulated by $G_{12/13}$ proteins are known: p115-RhoGEF, PSD-95/Disc-large/ZO-1 homology-RhoGEF, leukemia-associated RhoGEF and lymphoid blast crisis-RhoGEF. These link GPCRs to activation of the small monomeric GTPase RhoA, and other downstream effectors. Misregulated $G_{12/13}$ signalling is involved in multiple pathophysiological conditions such as cancer, cardiovascular diseases, arterial and pulmonary hypertension, and bronchial asthma. Specific targeting of $G_{12/13}$ signalling-related diseases of GPCRs hence provides novel therapeutic approaches. Assays to quantitatively measure GPCR-mediated activation of $G_{12/13}$ are only emerging, and are required to understand the $G_{12/13}$-linked pharmacology. The review gives an overview of $G_{12/13}$ signalling of GPCRs with a focus on RhoGEF proteins as the immediate mediators of $G_{12/13}$ activation.
British Journal of Pharmacology (2009) **158,** 41–49; doi:10.1111/j.1476-5381.2009.00121.x; published online 18 February 2009

This article is part of a themed issue on GPCR. To view this issue visit
http://www3.interscience.wiley.com/journal/121548564/issueyear?year=2009

Keywords: G protein-coupled receptor; $G_{12/13}$; RhoGTPase nucleotide exchange factor; RhoA; Rho kinase; fluorescence imaging

Abbreviations: AKAP, A-kinase anchoring protein; CFP, cyan fluorescent protein; DH, Dbl-homology; ERM, ezrin/radixin/moesin; FAK, focal adhesion kinase; FRET, fluorescence resonance energy transfer; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; JNK, c-Jun N-terminal kinase; LARG, leukemia-associated RhoGEF; Lbc, lymphoid blast crisis; LIMK, LIM kinase; LPA, lysophosphatidic acid; mDia, Diaphanous; MLC, myosin light chain; PAR, protease-activated receptor; PDZ, PSD-95/Disc-large/ZO-1 homology; PH, pleckstrin-homology; PKA, protein kinase A; PKC, protein kinase C; RBD, Rho binding domain; RGS, regulator of G protein signalling; ROCK, Rho kinase; SMC, smooth muscle cell; SPA, scintillation proximity assay; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; YFP, yellow fluorescent protein

Activation of RhoGEF by G_{12}-coupled G protein-coupled receptors (GPCRs)

Coupling to $G_{12/13}$ proteins is being found for an increasing number of GPCRs, but remains to be elucidated for many others. Known GPCRs are purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), M_1 and M_3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$), lysophosphatidic acid (LPA_1, LPA_2, LPA_3), angiotensin II (AT_1), serotonin ($5\text{-}HT_{2c}$ and $5\text{-}HT_4$), somatostatin (sst_5), endothelin (ET_A and ET_B), cholecystokinin (CCK_1), V_{1a} vasopressin receptors, D_5 dopamine receptors, fMLP formyl peptide receptors, GAL_2 galanin receptors, EP_3 prostanoid receptors, A_1 adenosine receptors, α_1 adrenergic receptors, BB_2 bombesin receptors, B_2 bradykinin receptors,

Correspondence: Sandra Siehler, Novartis Institutes for BioMedical Research Basel, WSJ-88.2.05, Novartis Pharma AG, 4002 Basel, Switzerland. E-mail: sandra.siehler@novartis.com
Received 11 September 2008; revised 17 November 2008; accepted 1 December 2008

calcium-sensing receptors, KSHV-ORF74 chemokine receptors, NK_1 tachykinin receptors and thyroid-stimulating hormone (TSH) receptors (Katoh *et al.*, 1996; Sauzeau *et al.*, 2000; Komatsuzaki *et al.*, 2001; Thevananther *et al.*, 2001; Greenberg *et al.*, 2003; Zheng *et al.*, 2003; Riobo and Manning, 2005; Hains *et al.*, 2006; Alexander *et al.*, 2008). $G_{12/13}$ coupling can be predicted using a hidden Markov model algorithm based on the presence of certain amino acid sequences (Sgourakis *et al.*, 2005). All known $G_{12/13}$-linked GPCRs signal additionally through other types of G proteins such as $G_{i/o}$ and/or $G_{q/11}$, but GPCRs uniquely coupling to $G_{12/13}$ may have been missed in the past as assay technologies for their readout evolved only recently (Riobo and Manning, 2005; Siehler, 2008). Activation of $G_{12/13}$-coupled GPCRs causes activation of heterotrimeric G_{12} and/or G_{13} proteins (Figure 1). The activated and guanosine triphosphate (GTP)-bound α subunit is thought to dissociate from the βγ dimer, and its activity is terminated upon GTP hydrolysis (Hepler and Gilman, 1992). $G\alpha_{12}$ and $G\alpha_{13}$ were discovered in 1991 as a fourth class of Gα proteins. They are ubiquitously expressed and mouse isoforms reveal 67% homology (Strathmann and Simon, 1991; Offermanns, 2003). N terminal palmitoylation is relevant for plasma membrane attachment and receptor interactions. Their slow guanosine diphosphate (GDP)/GTP exchange rate indicates their importance in prolonged signalling (Dhanasekaran and Dermott, 1996).

'Regulator of G protein signalling' (RGS) proteins further regulate the activity of G proteins, mostly as GTPase-activating proteins (GAPs) accelerating G protein inactivation, or they can enhance G protein function (Zhong and Neubig, 2001; Xie and Palmer, 2007). RGS proteins for $G_{12/13}$ proteins are RhoGEFs, four of which are known to be regulated by G_{12}-type proteins: p115-RhoGEF, PSD-95/Disc-large/ZO-1 homology (PDZ)-RhoGEF, leukemia-associated RhoGEF (LARG) and lymphoid blast crisis (Lbc)-RhoGEF. They function as GAPs towards $G\alpha_{12/13}$, and binding to $G\alpha_{12/13}$ stimulates their guanine nucleotide exchange factor (GEF) activity (Fukuhara *et al.*, 2001; Dutt *et al.*, 2004). RhoGEF catalyses the exchange of GDP for GTP by promoting an active conformation of the small monomeric GTPase RhoA (Sah *et al.*, 2000; Wheeler and Ridley, 2004). Upon activation by $G\alpha_{12/13}$ RhoGEF translocates from the cytosol to the plasma membrane (Meyer *et al.*, 2008). Oligomerization of RhoGEF negatively regulates its activity, which may prevent interaction with RhoA (Chikumi *et al.*, 2004; Baisamy *et al.*, 2005). Activation of RhoA is further regulated by guanine nucleotide dissociation inhibitors (GDIs), which bind to the C terminus of RhoA, where they mask the geranylgeranyl residue responsible for membrane association. RhoGDI release is required for RhoA activation, and it extracts RhoA from membranes for subsequent inactivation. Moreover, RhoGAP proteins inactivate RhoA by accelerating intrinsic GTPase activity (Fukuhara *et al.*, 2001; Wheeler and Ridley, 2004). RhoA regulates multiple downstream effectors including many cytoskeletal proteins, and most of these have not been studied in detail. Research has elucidated RhoA-mediated activation of Rho

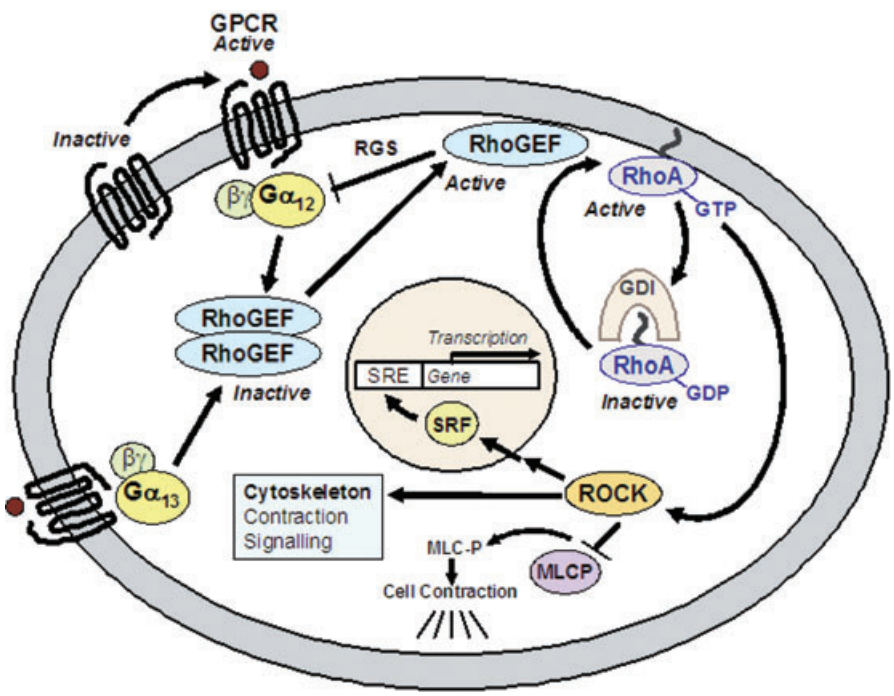

Figure 1 $G_{12/13}$-RhoGEF-RhoA signalling pathway. GPCR-activated $G_{12/13}$-proteins mediate translocation of RhoGEF from the cytosol to the plasma membrane, and stimulate its GEF activity. RhoGEFs oligomerize in their inactive form, and active monomers bind via their RGS domains to active $G\alpha_{12/13}$ at the plasma membrane. RhoGEF reveals GAP activity towards $G\alpha_{12/13}$, and induces downstream activation of the monomeric GTPase RhoA. Active GTP-bound RhoA is released from its inhibitory protein RhoGDI, and anchors to the plasma membrane through its geranylgeranyl-residue. Downstream targets of RhoA include ROCK, which mediates cell contraction through inhibition of MLC phosphatase, and increased SRF-dependent gene transcription. GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; MLC, myosin light chain; RGS, regulator of G protein signaling; ROCK, Rho kinase; SRF, serum response factor.

kinases (ROCK1/2) and certain downstream pathways. ROCK phosphorylates the focal adhesion kinase (FAK), which leads to formation of actin stress fibres, and to activation of the 'serum response' transcription factor SRF. SRF binds in the nucleus to serum response element (SRE) sequences in the promoter of specific genes, and regulates their transcription (Narumiya *et al.*, 1997; Treisman *et al.*, 1998; Riento and Ridley, 2003). ROCK causes on the other hand inhibition of myosin light chain (MLC) phosphatase and direct phosphorylation of MLCs. The increase of phosphorylated MLCs in the cytoskeleton induces cell contraction (Narumiya *et al.*, 1997; Riento and Ridley, 2003). Described cellular phenomena caused by the $G_{12/13}$-RhoGEF-RhoA pathway of GPCRs hence often involve signalling or contraction through cytoskeletal proteins.

$G_{12/13}$-regulated RhoGEF proteins

GPCRs coupled to $G_{12/13}$ proteins activate the RhoGEF family members p115-RhoGEF, PDZ-RhoGEF, LARG and Lbc-RhoGEF (Fukuhara *et al.*, 2001; Dutt *et al.*, 2004). RhoGEFs are large proteins, and human p115-RhoGEF, PDZ-RhoGEF and LARG consist of 927, 1522 and 1544 amino acids respectively. Lbc-RhoGEF has multiple splice variants present in various tissues, and the unspliced form consists of 893 amino acids (Table 1). The amino acid identity of human p115-RhoGEF and human PDZ-RhoGEF, LARG and Lbc-RhoGEF is 36%, 39% and 31% respectively. All four RhoGEFs are widely expressed in mammals (Fukuhara *et al.*, 2001; Dutt *et al.*, 2004). High transcript levels of p115-RhoGEF are present in hematopoietic cells, and of PDZ-RhoGEF in the central nervous system (Kuner *et al.*, 2002). The Lbc-RhoGEF splice variant A-kinase anchoring protein (AKAP)-Lbc is highly expressed in the heart. AKAPs bind the regulatory subunit type II of the cyclic adenosine monophosphate-dependent protein kinase A (PKA) anchoring it at specific subcellular sites, but additionally function as scaffold proteins for other signalling complexes (Diviani *et al.*, 2001; 2004).

All four RhoGEFs contain an RGS domain to directly interact with $G_{12/13}$ and to exert GAP activity towards $G_{12/13}$ The RGS sequence homology to classical RGS proteins is rather low. Whereas p115-RhoGEF, PDZ-RhoGEF and LARG contain the RGS domain in the N terminal sequence, Lbc-RhoGEF has a C terminal region sharing 39% amino acid identity to the consensus RGS domain and hence called 'RGS-like'. Nevertheless, the homology of the RGS domain of p115-RhoGEF with those of PDZ-RhoGEF and LARG is also only 32% and 41% respectively (Hart *et al.*, 1996; Kozasa *et al.*, 1998; Wells *et al.*, 2002; Dutt *et al.*, 2004). Two other common motifs of RhoGEF proteins in the C terminal region are the Dbl-homology (DH) and pleckstrin-homology (PH) domains. The DH domain specifically binds to and stabilizes nucleotide- and Mg^{2+}-free RhoA transition states to enhance its nucleotide exchange. Cells contain higher levels of GTP as compared with GDP, and the RhoGEF DH domain therefore induces loading of RhoA with GTP, and its transition into an active state (Rossman *et al.*, 2005). The PH domain is essential for full GEF activity, and moreover anchors RhoGEF to other signalling proteins to trigger specific subcellular localizations (Fukuhara *et al.*, 2001; Dutt *et al.*, 2004). PDZ-RhoGEF and LARG additionally contain an N terminal PDZ domain, which enables coupling to cell surface receptors such as plexins, insulin-like growth factor receptors or GPCRs (Fukuhara *et al.*, 2001; Taya *et al.*, 2001; Chikumi *et al.*, 2004; Dutt *et al.*, 2004; Yamada *et al.*, 2005; Grabocka and Wedegaertner, 2007; Kelly *et al.*, 2007). The G protein-coupled LPA_1 and LPA_2 receptors were shown to directly interact through their C terminal PDZ domain-binding motifs with the PDZ domain of LARG and PDZ-RhoGEF to activate RhoA (Yamada *et al.*, 2005), and it remains to be seen whether further GPCRs can directly interact with these RhoGEFs.

p115-RhoGEF localizes throughout the cytosol, and rapidly translocates to the plasma membrane upon GPCR-activation of $G\alpha_{12/13}$, or expression of constitutively active $G\alpha_{12/13}$ mutants (Bhattacharyya and Wedegaertner, 2003a; Meyer *et al.*, 2008). An E229L mutation of $G\alpha_{13}$ disrupted p115-RhoGEF interaction and recruitment to the plasma

Table 1 Characteristics of $G_{12/13}$-regulated RhoGEF Proteins

	p115-RhoGEF	*PDZ-RhoGEF*	*LARG*	*Lbc-RhoGEF*
Expression	Blood cells, wide at low levels	CNS, wide at low levels	Ubiquitous	Wide (AKAP-Lbc in heart)
Size (human)	927 aa	1522 aa	1544 aa	Variable (splice variants)
RGS domain	Yes	Yes	Yes	RGS-like (C-terminal)
PDZ domain	No	Yes	Yes	No
DH domain	Yes	Yes	Yes	Yes
PH domain	Yes	Yes	Yes	Yes
Localization inactive form	Cytoplasm	Cytoplasm or PM (cell type)	Cytoplasm or PM (cell type)	Cytoplasm
Localization active form	Plasma membrane	Plasma membrane	Plasma membrane	Plasma membrane
$G\alpha_{12}$-induced GEF activation	No	No	Yes	Yes
$G\alpha_{13}$-induced GEF activation	Yes	Yes	Yes	Yes
Regulation of GEF activity	PKC (+), PAK (–)	Tec, FAK (+)	Tec, FAK (+)	PKA (-) for AKAP-Lbc
Oligomerization	Homo-oligomers	Homo- and hetero-oligomers	Homo- and hetero-oligomers	Homo-oligomers (AKAP-Lbc)
Knockout mice	Yes	No	No	No

AKAP, A-kinase anchoring protein; CNS, central nervous system; DH, Dbl-homology; FAK, focal adhesion kinase; GEF, guanine nucleotide exchange factor; LARG, leukemia-associated RhoGEF; Lbc, lymphoid blast crisis; PAK, p21-activated kinase; PDZ, PSD-95/Disc-large/ZO-1 homology; PH, pleckstrin-homology; PKA, protein kinase A; PKC, protein kinase C; PM, plasma membrane; RGS, regulator of G protein signalling; Tec, tyrosine kinase expressed in hepatocellular carcinoma.

membrane (Grabocka and Wedegaertner, 2005). E27 and E29 in the acidic-rich N terminus of p115-RhoGEF are besides the RGS and PH domains required for binding to activated $G\alpha_{13}$, but not for plasma membrane translocation (Bhattacharyya and Wedegaertner, 2003a,b). LARG is reported to be distributed throughout the cytoplasm in most cells, except of Madin-Darby canine kidney cells, in which LARG is localized at lateral membranes. In fibroblasts, LARG was reported to localize to the microtubule-organizing centre and along microtubule tracks to contribute to cell polarity. PDZ-RhoGEF localizes in some cell types also in the cytosol, but in others at the cell periphery at or near the plasma membranes where it interacts with cortical actin. In polarizing neutrophils, PDZ-RhoGEF localizes to the back of the cells. Activation of PDZ-RhoGEF and LARG by $G\alpha_{12/13}$ induces their translocation to the plasma membrane (Banerjee and Wedegaertner, 2004; Wong *et al.*, 2007; Goulimari *et al.*, 2008; Meyer *et al.*, 2008). The subcellular localization of LARG and PDZ-RhoGEF might depend on PDZ-interacting proteins present in a cell. $G\alpha_{12}$ and $G\alpha_{13}$ specifically interact with distinct splice variants of Lbc-RhoGEF, and cause redistribution of Lbc-RhoGEF from the cytosol to the plasma membrane (Diviani *et al.*, 2001; 2004). The localization of all RhoGEFs specifically controls the spacial distribution of RhoA activity in cells.

$G\alpha_{13}$, but not $G\alpha_{12}$, was found to stimulate the GEF activity of p115-RhoGEF and PDZ-RhoGEF (Hart *et al.*, 1998; Tanabe *et al.*, 2004). $G\alpha_{12}$ is reported to enhance the GEF activity of tyrosine-phosphorylated LARG, but not of unphosphorylated LARG. $G\alpha_{13}$ on the other hand enhances more weakly both phosphorylated and unphosphorylated forms of LARG (Suzuki *et al.*, 2003). $G\alpha_{12/13}$ enhance the GEF activity of the Lbc-RhoGEF splice variant AKAP-Lbc through triggering the release of PKA, which inhibits its GEF activity by phosphorylation and concomitant recruitment of 14-3-3 (Diviani *et al.*, 2001; Kurose, 2003; Diviani *et al.*, 2004). Stimulation of RhoGEF activities by kinases include tyrosine phosphorylation of PDZ-RhoGEF and LARG by FAK or Tec (Fukuhara *et al.*, 2001; Suzuki *et al.*, 2003; Chikumi *et al.*, 2004), and serine phosphorylation of p115-RhoGEF by protein kinase C (PKC)α (Holinstat *et al.*, 2003). p21-activated kinase (PAK)1 can bind to the DH/PH domains of p115-RhoGEF (but not of LARG or PDZ-RhoGEF), and mediate Rac-induced inhibition of RhoA activation (Rosenfeldt *et al.*, 2006).

RhoGEFs were recently found to oligomerize through their C terminus, which inhibits their GEF activity and interaction with RhoA. Oligomerization was not affected by interaction with $G\alpha_{12/13}$. p115-RhoGEF and AKAP-Lbc form homooligomers, whereas PDZ-RhoGEF and LARG can form homo- and heterooligomers. Blockade of AKAP-Lbc oligomerization abolished the inhibitory effect of PKA and 14-3-3 (Chikumi *et al.*, 2004; Baisamy *et al.*, 2005). RhoGEFs can compete with other RGS proteins for binding to $G\alpha_{12/13}$, which can prevent RhoA activation (Kelly *et al.*, 2007). Examples are axin which blocks $G\alpha_{12}$-p115-RhoGEF interaction, and RGS16 that prevents $G\alpha_{13}$-p115-RhoGEF interaction (Johnson *et al.*, 2003; Stemmle *et al.*, 2006). Mutant RhoGEFs lacking GEF activity function as dominant-negative inhibitors of GPCR- or $G_{12/13}$-mediated responses, whereas constitutively active p115-RhoGEF, lacking the N and C terminal regulatory domains, activates RhoA (Mao *et al.*, 1998; Fukuhara *et al.*, 1999; Lee *et al.*, 2004). $G_{12/13}$-regulated RhoGEFs all activate RhoA, but are not completely redundant. Using small interfering ribonucleic acid, the thrombin receptor was found to signal through LARG to activate RhoA, whereas the LPA receptor utilized PDZ-RhoGEF (Wang *et al.*, 2004).

Only limited genetic mouse models and genetic linkages are known for the four $G_{12/13}$-regulated RhoGEFs. Knockout mice are described for p115-RhoGEF, but not yet for PDZ-RhoGEF, LARG and Lbc-RhoGEF. The murine homologue of human p115-RhoGEF, Lsc, is solely expressed in hematopoietic cells. Lsc-knockout mice reveal impaired B/T lymphocyte proliferation, and B cell homing (Francis *et al.*, 2006). Moreover, a mutant form of LARG has been found in some human cancers such as acute myeloid leukemia (Kourlas *et al.*, 2000). In the worm species *Caenorhabditis elegans* only one RGS-containing RhoGEF exists, RHGF-1, that is a homologue of the mammalian p115-RhoGEF. RHGF-1 is regulated by the $G\alpha_{12}$ homologue GPA-12, and causes activation of the RhoA homologue RHO-1. RHO-1 has been characterized to regulate neuronal activity such as acetylcholine release and locomotion (McMullan and Nurrish, 2007).

Downstream signalling of RhoGEF proteins

The $G_{12/13}$-regulated RhoGEFs, p115-RhoGEF, PDZ-RhoGEF, LARG and Lbc-RhoGEF, cause activation of RhoA. Three closely related isoforms of Rho exist: RhoA, RhoB and RhoC, which are ubiquitously expressed, share 85% amino acid sequence identity, and have a C terminal geranylgeranyl residue. RhoA plays a key role in the regulation of the actin cytoskeleton, cell shape, cell polarity, microtubule dynamics, membrane transport pathways, gene transcription, cell adhesion, cell migration, neurite extension/retraction and cell growth. The roles of RhoB and RhoC are less clear. Activation of RhoA by a $G_{12/13}$-linked GPCR induces its redistribution to the plasma membrane, which is reversed by injection of RhoGDI (Michaelson *et al.*, 2001; Bhattacharya *et al.*, 2004; Wheeler and Ridley, 2004; Wennerberg and Der, 2004; Yonemura *et al.*, 2004; Jaffe and Hall, 2005; Rossman *et al.*, 2005). Three RhoGDI proteins are described, and only RhoGDIα is ubiquitously expressed and has the highest affinity for RhoA. Phosphorylation of RhoGDIα by PKCα causes activation of RhoA, whereas phosphorylation by PKA causes RhoA inhibition (Mehta *et al.*, 2001; Jaffe and Hall, 2005). Binding of GTP to RhoA causes its conformational change, and subsequent interaction with effector proteins (Bhattacharya *et al.*, 2004). C3 toxin specifically ADP-ribosylates RhoA at N41 in the effector domain and inactivates RhoA. It is utilized to detect involvement of RhoA in signalling (Jaffe and Hall, 2005). The RhoA-S19N dominant-negative mutant has decreased affinity for GTP, and increased affinity for RhoGEFs. The GTPase activity of RhoA is impaired by G14V or G63L mutations, and these result in a constitutively active mutant (Seasholtz *et al.*, 1999). Mutant RhoA forms do not bind RhoGDI, and localize at the plasma membrane and internal membranes (Jaffe and Hall, 2005).

Many effector proteins of RhoA have been found, but only a few have been further studied. The best characterized RhoA

effectors, ROCK1/2, are ubiquitously expressed serine/threonine kinases, which translocate from the cytosol to the plasma membrane in the presence of active RhoA (Narumiya *et al.*, 1997). Binding to RhoA disrupts the interaction between the ROCK kinase domain and its autoinhibitory C terminal domain. ROCK-mediated effects can be prevented by the selective and adenosine triphosphate-competitive ROCK inhibitor fasudil (Y27632) (Sah *et al.*, 2000). ROCK phosphorylates many substrates such as FAK, c-Jun N-terminal kinase (JNK), MLC phosphatase, ezrin/radixin/moesin (ERM), LIM kinase (LIMK), Diaphanous (mDia), rhophilin, rhotekin, citron kinase and microtubule-associated Tau. In neurons, ROCK inactivates collapsin response mediator protein-2 (CRMP-2), which leads to blockade of microtubule assembly and hence neurite retraction (Sah *et al.*, 2000; Jaffe and Hall, 2005). Phosphorylated FAK enhances formation of focal extracellular matrix adhesions of cells, complexes additionally containing paxillin, talin, α-actinin and vinculin, among other proteins. Focal adhesions link actin stress fibres to certain integrins at the inner surface of the plasma membrane, and p115-RhoGEF, PDZ-RhoGEF and LARG activation were demonstrated to be involved in focal adhesion formation and movement (Dubash *et al.*, 2007; Iwanicki *et al.*, 2007). Phosphorylated active JNK causes downstream phosphorylation of the transcription factors Jun and ATF2, and hence regulates gene transcription (Kurose, 2003). As previously described, ROCK inhibits MLC phosphatase by phosphorylation of the regulatory myosin-binding subunit of myosin phosphatase (MYPT1), which results in enzymatic inhibition. The resulting increased levels of the phosphorylated regulatory light chain MLC20 of myosin II enhances actomyosin crossbridging which leads to cell contraction in different cell types (Narumiya *et al.*, 1997). The ERM protein family links plasma membrane proteins with the actin cytoskeleton. ERM proteins are relevant in cell adhesion, cell migration and cell division, and are stabilized by ROCK through phosphorylation (Kurose, 2003; Kelly *et al.*, 2007).

Serum response factor-dependent gene transcription is controlled by two RhoA-dependent pathways: (i) activated ROCK/LIMK stabilize F-actin by blocking the depolymerizing factor cofilin; and (ii) active mDia1/vasodilator-stimulated phosphoprotein enhance F-actin assembly by promoting filament nucleation. The resulting G-actin depletion induces activation of SRF. The transcription factor SRF binds as a dimer to SREs together with a co-transcription factor ['ternary complex factor' (TCF) like e.g. Elk-1] regulated by the Ras/Raf/extracellular signal-regulated kinase pathway. SRF controls expression of immediate-early and muscle-specific genes, including the cytoskeletal proteins β-actin and vinculin (Marinissen and Gutkind, 2005). A SRF-dependent reporter gene assay has been invented by mutation of the TCF-binding site in the c-fos SRE, and has been widely used to indirectly detect RhoA activation. The canonical SRE core sequence $CC[A/T]_6GG$ represents the DNA-binding site for SRF. Another SRF cofactor, MAL, dissociates from cytosolic actin monomers to translocate to the nucleus upon RhoA activation. The transcription of different SRF target genes requires specific SRF cofactors (Treisman *et al.*, 1998; Jaffe and Hall, 2005). SRF is activated not only by $G_{12/13}$-linked GPCRs, active $G\alpha_{12/13}$ proteins and RhoA, but also by overexpression of p115-RhoGEF, PDZ-RhoGEF and LARG (Seasholtz *et al.*, 1999; Chikumi *et al.*, 2004; Wang *et al.*, 2004).

Gene deletion studies in mice revealed differences of G_{12} versus G_{13} (Offermanns, 1999; Kelly *et al.*, 2007); however, no effector has been identified that is uniquely activated by either G protein. A few GPCRs apparently couple only to either G protein, such as 5-HT_4 and LPA_2 receptors linked to G_{13}, or the PAR-1 receptor linked to G_{12}. PAR-1 in another cell type, however, coupled also to G_{13}, and these apparent specificities might be cell type or assay-dependent (Gu *et al.*, 2002; Moers *et al.*, 2003; Yamaguchi *et al.*, 2003; Riobo and Manning, 2005). Differences in ligand potencies and efficacies dependent on G_{12} versus G_{13} were reported for the 'thromboxane' TXA_2 receptor in a Gα subunit-specific guanosine-5′-*O*-(3′-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding assay. Ligands were only weakly active or inactive at the TXA_2 receptor upon coexpression or receptor fusion of $G\alpha_{12}$, but potent in the presence of $G\alpha_{13}$ (Zhang *et al.*, 2006). Not only G_{12} versus G_{13} activation, but also RhoGEF and downstream effector activation differences might be explained by ligand-effector trafficking, i.e. selective ligand potencies towards specific effector pathways.

Measurement of $G_{12/13}$-dependent RhoGEF activation

Multiple assay technologies are available since many years to measure G_s-, G_i- or G_q-type signalling of GPCRs. In contrast, readouts to measure modulation of the $G_{12/13}$-RhoGEF-RhoA pathway emerged only recently (Siehler, 2008). Initially, RhoA antibodies have been used to immunoprecipitate RhoA, and to measure [^{35}S]GTPγS or [^{32}P]GTP binding. This was hampered by the lack of specificity of available RhoA antibodies versus other small RhoGTPases, and by the small signals due to a high GTP hydrolysis rate of RhoA (Seasholtz *et al.*, 1999). Similar assays were developed to directly detect activation of $G_{12/13}$ by using specific antibodies. Stimulated cells are lysed, and [^{35}S]GTPγS binding is measured either at immunoprecipitated $G\alpha_{12/13}$, or at $G\alpha_{12/13}$ immunocaptured with antibody-linked scintillation proximity assay (SPA) beads (Barr *et al.*, 1997; Milligan, 2003; DeLapp, 2004) (Figure 2). All these methods were either not quantitative, or in the case of the [^{35}S]GTPγS SPA assay the assay window was only limited. Another method to detect RhoA activation uses the Rho binding domain (RBD) of the effector Rhotekin to specifically extract active RhoA-GTP, and to detect levels by immunoblot detection (Ren and Schwartz, 2000). The rapid GTP hydrolysis rate and immunodetection do not allow a true quantification of signals to characterize GPCR-related pharmacology.

A quantitative method was invented using an SRF-specific reporter gene readout with a mutated c-fos promoter SRE that only binds SRF dimers but no co-transcription factor (Mao *et al.*, 1998; Treisman *et al.*, 1998). Nevertheless, the assay window is limited, and the RhoA-dependent activation of SRF is also modulated by other pathways. Fluorescent biosensors were developed to measure RhoA activation by detection of RhoA-GTP. One biosensor consists of four fused proteins,

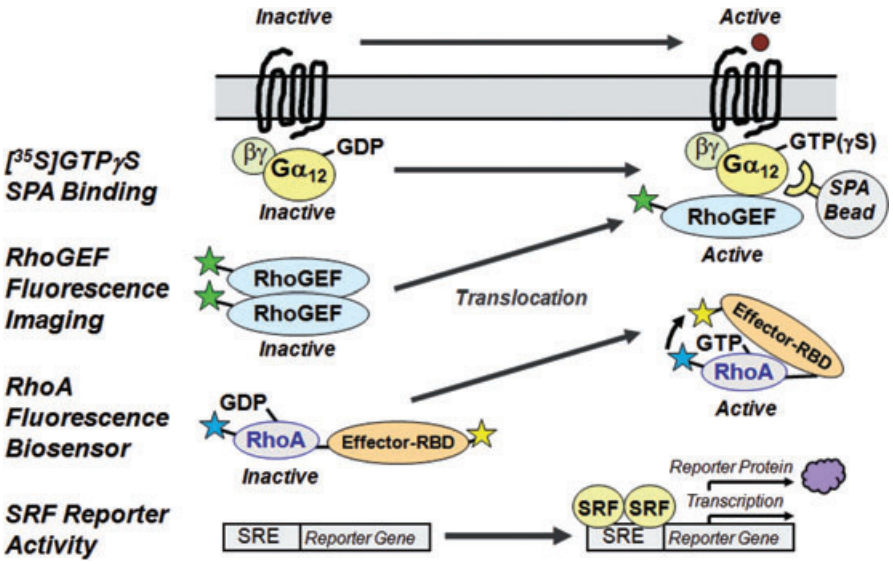

Figure 2 Quantitative Bioassays for Activation of the $G_{12/13}$-RhoGEF-RhoA Axis. Activation of $G_{12/13}$ proteins is reflected by enhanced [^{35}S]GTPγS binding to their α subunits, which are captured by antibody-linked SPA beads. GPCR-activated $G_{12/13}$ mediate redistribution of RhoGEF from the cytosol to the plasma membrane, which can be visualized by tagging of RhoGEF with, for example, EGFP (or by immunostaining). Fluorescence subcellular imaging allows quantification of cytosolic and plasma membrane-localized RhoGEF using suitable algorithms. Fluorescent biosensors contain CFP-labelled RhoA fused to the Rho binding domain (RBD) of an effector protein labelled with YFP. FRET signals between the donor CFP and acceptor YFP occur upon binding of the effector RBD to activated (GTP-bound) RhoA, and can be quantified using fluorescence imaging. Activation of RhoA can be indirectly measured in an SRF-dependent reporter gene assay. CFP, cyan fluorescent protein; EGFP, enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; GTPγS, guanosine-thio triphosphate; SPA, scintillation proximity assay; SRF, serum response factor; YFP, yellow fluorescent protein.

namely yellow fluorescent protein (YFP), the RBD of protein kinase N, truncated RhoA (amino acids 1-189) and cyan fluorescent protein (CFP). Active RhoA within the fusion protein binds the RBD of protein kinase N, and the proximity of the two fluorophores leads to an intramolecular fluorescence resonance energy transfer (FRET) signal that can be measured using subcellular confocal imaging. Another biosensor contains fused Rhotekin-RBD, CFP, YFP and C-terminal full-length RhoA, that is, the fluorophores were placed in the centre to allow RhoA to bind to RhoGDI (Yoshizaki *et al.*, 2003; Pertz *et al.*, 2006). These biosensors have the potential for quantitative FRET measurements using high content screening; however, only single cell analyses have been demonstrated so far.

A quantitative drug screening assay for $G_{12/13}$ activation by GPCRs was recently established, and detects translocation of 'enhanced green fluorescent protein'-tagged p115-RhoGEF. Alternatively, p115-RhoGEF can be visualized by immunostaining using a specific antibody. p115-RhoGEF translocates from the cytosol to the plasma membrane upon activation by $G_{12/13}$-linked GPCRs, and signals can be recorded in a kinetic fashion. Translocation signals are measured using subcellular confocal imaging, and signals are quantified using suitable software algorithms for data analysis. All tested $G_{12/13}$-coupled GPCRs mediated translocation of p115-RhoGEF. Co-expression of wildtype $G\alpha_{12}$ or $G\alpha_{13}$ allows to distinguish between G_{12}- versus G_{13}-dependent p115-RhoGEF translocation. The RhoGEF translocation assay enables compound screening and the discovery of GPCR ligands active in a $G_{12/13}$-linked disease context (Meyer *et al.*, 2008).

Dysfunction of the $G_{12/13}$-RhoGEF pathway

Misregulation of $G_{12/13}$ signalling of GPCRs causes multiple pathophysiological conditions, which underlines the important role of the $G_{12/13}$-RhoGEF-RhoA pathway in physiological processes (Siehler, 2007; Worzfeld *et al.*, 2008). Some of these are reviewed in more detail. In many types of smooth muscle cells (SMCs) (vascular, coronary, bronchial, cerebral, visceral and gastric) the G_{12}-RhoA-ROCK axis causes sustained contraction through MLC phosphatase inhibition, whereas Ca^{2+}-MLC kinase regulates transient contraction. ROCK inhibitors act on vascular SMCs to prevent vasoconstriction, and are in the clinic for arterial hypertension therapy (Watterson *et al.*, 2005; Wettschureck and Offermanns, 2005; Brown *et al.*, 2006). Development of arterial hypertension in a salt-induced mouse model was shown to require $G_{12/13}$-LARG signalling in vascular SMCs (Wirth *et al.*, 2008). Moreover, ROCK inhibitors are effective for treatment of cerebral vasospasm, pulmonary arterial hypertension and asthma by preventing contraction of cerebral and bronchial SMCs respectively (Calo and Pessina, 2007). Other data indicate a role of ROCK in coronary artery spasm and hence ischemic heart disease due to constriction of human coronary artery SMCs (Ohmori *et al.*, 2003). In myocardial infarction, RhoA and ROCK expression is up-regulated and causes cardiomyocyte apoptosis, and RhoA activation induces hypertrophy of primary cardiomyocytes (Brown *et al.*, 2006; del Re *et al.*, 2007). Dominant-negative p115-RhoGEF blocks hypertrophic responses in cardiac myocytes (Kurose, 2003). Transgenic expression of active $G\alpha_{13}$ or of RhoA in the heart causes cardiac hypertrophy, and p115-RhoGEF is up-regulated in

hypertrophic cardiomyocytes (Wettschureck and Offermanns, 2005; Brown *et al.*, 2006; Porchia *et al.*, 2008).

In the immune system, the $G_{12/13}$-RhoA axis is relevant in the regulation of lymphocyte adhesion and migration, and neutrophil chemokinesis and chemotaxis. Lsc/p115-RhoGEF-knockout mice reveal defective B-cell homing and T- and B-cell proliferation (Wettschureck and Offermanns, 2005; Francis *et al.*, 2006). $G_{12/13}$ signalling is required for splenic B cell maturation, migration and polarization (Rieken *et al.*, 2006). G_{13} and RhoA are further required for platelet activation, shape change and aggregation, and hence for wound healing (Moers *et al.*, 2003; Kelly *et al.*, 2007). Constitutively active $G\alpha_{12/13}$ proteins were found to be oncogenes in various cell types. No $G\alpha_{12/13}$ mutations have been identified in various tumours of cancer patients, but instead increased expression of $G\alpha_{12/13}$ and RhoA could be detected breast, prostate and colon tumours. RhoA is involved in carcinogenesis and cancer progression. Cell adhesion is reduced, which promotes cell migration and invasion of breast, prostate and colon cancer cells, and ultimatively leads to metastatic tumour progression (Kurose, 2003; Wettschureck and Offermanns, 2005; Kelly *et al.*, 2006; 2007). Further literature data support a role of the $G_{12/13}$-RhoA axis in ovarian and lung cancer (Kelly *et al.*, 2007; Touge *et al.*, 2007).

The $G_{12/13}$-RhoGEF-RhoA pathway of GPCRs therefore is implicated in many diseases including cardiac hypertrophy, cardiac failure, ischemic heart disease, arterial and pulmonary hypertension, bronchial asthma, inflammatory diseases, stroke, cancer progression and metastasis.

Conclusions

$G_{12/13}$-signalling of GPCRs represents an emerging research area. Whereas aspects of $G_{12/13}$ have been studied in greater detail in cellular systems and various tissues over the past years (Riobo and Manning, 2005; Worzfeld *et al.*, 2008), the coupling potential of many GPCRs to this class of G proteins has often not been examined. Even less clear is the involvement of specific RhoGEF proteins and effectors in downstream signalling events. A few GPCRs were found to couple solely to G_{12} or G_{13} (Gu *et al.*, 2002; Moers *et al.*, 2003; Yamaguchi *et al.*, 2003), although these specificities have been neither studied in various cell types, nor at the level of various downstream readouts. All four known $G_{12/13}$-regulated RhoGEFs, p115-RhoGEF, PDZ-RhoGEF, LARG and Lbc-RhoGEF, are activated by $G_{13.}$ p115-RhoGEF and PDZ-RhoGEF on the other hand also translocate in the presence of GPCR-activated G_{12}, but do not reveal any GEF activation by G_{12} (Hart *et al.*, 1998; Tanabe *et al.*, 2004; Meyer *et al.*, 2008). Other factors such as kinases regulate the activity of RhoGEFs. Besides specificities towards G_{12} versus G_{13} regulation of RhoGEFs, two $G_{12/13}$-linked GPCRs have been discovered to signal specifically through one RhoGEF versus another (Wang *et al.*, 2004). On the other hand, no specific effector protein uniquely activated by either G_{12} or G_{13}, or by either RhoGEF protein is known so far.

Understanding the complexity of the specific regulation of $G_{12/13}$, RhoGEF proteins and RhoA-linked effectors is required to interpret pharmacological differences of $G_{12/13}$-coupled GPCRs in various primary cell systems and tissues. Future research on the $G_{12/13}$-RhoGEF-RhoA pathway of GPCRs is needed to further elucidate molecular pathways and their physiological context, which will facilitate the development of innovative therapies for patients.

Acknowledgement

The author is an employee of the Novartis Institutes for BioMedical Research.

Conflict of interest

The author declares no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. (2008 revision). *Br J Pharmacol* **153**: S1–S209.

Baisamy L, Jurisch N, Diviani D (2005). Leucine zipper-mediated homo-oligomerization regulates the Rho-GEF activity of AKAP-Lbc. *J Biol Chem* **280**: 15405–15412.

Banerjee J, Wedegaertner PB (2004). Identification of a novel sequence in PDZ-RhoGEF that mediates interaction with the actin cytoskeleton. *Mol Biol Cell* **15**: 1760–1775.

Barr AJ, Brass LF, Manning DR (1997). Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor-G protein coupling. *J Biol Chem* **272**: 2223–2229.

Bhattacharya R, Babwah AV, Ferguson SS (2004). Small GTP-binding protein-coupled receptors. *Biochem Soc Trans* **32**: 1040–1044.

Bhattacharyya R, Wedegaertner PB (2003a). Characterization of Ga_{13}-dependent plasma membrane recruitment of p115RhoGEF. *Biochem J* **371**: 709–720.

Bhattacharyya R, Wedegaertner PB (2003b). Mutation of an N-terminal acidic-rich region of p115-RhoGEF dissociates a_{13} binding and a13-promoted plasma membrane recruitment. *FEBS Lett* **540**: 211–216.

Brown JH, Del Re DP, Sussman MA (2006). The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. *Circ Res* **98**: 730–742.

Calo LA, Pessina AC (2007). RhoA/Rho-kinase pathway: much more than just a modulation of vascular tone. Evidence from studies in humans. *J Hypertens* **25**: 259–264.

Chikumi H, Barac A, Behbahani B, Gao Y, Teramoto H, Zheng Y *et al.* (2004). Homo- and hetero-oligomerization of PDZ-RhoGEF, LARG and p115RhoGEF by their C-terminal region regulates their in vivo Rho GEF activity and transforming potential. *Oncogene* **23**: 233–240.

Del Re DP, Miyamoto S, Brown JH (2007). RhoA/Rho kinase up-regulate Bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. *J Biol Chem* **282**: 8069–8078.

DeLapp NW (2004). The antibody-capture [^{35}S]GTPγS scintillation proximity assay: a powerful emerging technique for analysis of GPCR pharmacology. *Trends Pharmacol Sci* **25**: 400–401.

Dhanasekaran N, Dermott JM (1996). Signaling by the G_{12} class of G proteins. *Cell Signal* **8**: 235–245.

Diviani D, Soderling J, Scott JD (2001). AKAP-Lbc anchors protein kinase A and nucleates $G\alpha_{12}$-selective Rho-mediated stress fiber formation. *J Biol Chem* **276**: 44247–44257.

Diviani D, Abuin L, Cotecchia S, Pansier L (2004). Anchoring of both

PKA and 14-3-3 inhibits the Rho-GEF activity of the AKAP-Lbc signaling complex. *EMBO J* **23**: 2811–2820.

Dubash AD, Wennerberg K, Garcia-Mata R, Menold MM, Arthur WT, Burridge K (2007). A novel role for Lsc/p115 RhoGEF and LARG in regulating RhoA activity downstream of adhesion to fibronectin. *J Cell Sci* **120**: 3989–3998.

Dutt P, Nguyen N, Toksoz D (2004). Role of Lbc RhoGEF in $G\alpha_{12/13}$-induced signals to Rho GTPase. *Cell Signal* **16**: 201–209.

Francis SA, Shen X, Young JB, Kaul P, Lerner DJ (2006). Rho GEF Lsc is required for normal polarization, migration, and adhesion of formyl-peptide-stimulated neutrophils. *Blood* **107**: 1627–1635.

Fukuhara S, Chikumi H, Gutkind JS (2001). RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? *Oncogene* **20**: 1661–1668.

Fukuhara S, Murga C, Zohar M, Igishi T, Gutkind JS (1999). A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to. *Rho. J Biol Chem* **274**: 5868–5879.

Goulimari P, Knieling H, Engel U, Grosse R (2008). LARG and mDia1 link $G\alpha_{12/13}$ to cell polarity and microtubule dynamics. *Mol Biol Cell* **19**: 30–40.

Grabocka E, Wedegaertner PB (2007). Disruption of oligomerization induces nucleo-cytoplasmic shuttling of leukemia-associated RhoGEF. *Mol Pharmacol* **72**: 993–1002.

Grabocka E, Wedegaertner PB (2005). Functional consequences of $G\alpha_{13}$ mutations that disrupt interaction with p115RhoGEF. *Oncogene* **24**: 2155–2165.

Greenberg DL, Mize GJ, Takayama TK (2003). Protease-activated receptor mediated RhoA signaling and cytoskeletal reorganization in LNCaP cells. *Biochemistry* **42**: 702–709.

Gu JL, Muller S, Mancino V, Offermanns S, Simon MI (2002). Interaction of $G\alpha_{12}$ with $G\alpha_{13}$ and $G\alpha_q$ signaling pathways. *Proc Natl Acad Sci USA* **99**: 9352–9357.

Hains MD, Wing MR, Maddileti S, Siderovski DP, Harden TK (2006). $G\alpha_{12/13}$- and rho-dependent activation of phospholipase C-ε by lysophosphatidic acid and thrombin receptors. *Mol Pharmacol* **69**: 2068–2075.

Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG *et al.* (1998). Direct stimulation of the guanine nucleotide exchange activity of p115RhoGEF by $G\alpha_{13}$. *Science* **280**: 2112–2114.

Hart MJ, Sharma S, elMasry N, Qiu RG, McCabe P, Polakis P *et al.* (1996). Identification of a novel guanine nucleotide exchange factor for the Rho GTPase. *J Biol Chem* **271**: 25452–25458.

Hepler JR, Gilman AG (1992). G proteins. *Trends Biochem Sci* **17**: 383–387.

Holinstat M, Mehta D, Kozasa T, Minshall RD, Malik AB (2003). Protein kinase Cα-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. *J Biol Chem* **278**: 28793–28798.

Iwanicki MP, Vomastek T, Tilghman RW, Martin KH, Banerjee J, Wedegaertner P *et al.* (2007). FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts. *J Cell Sci* **121**: 895–905.

Jaffe AB, Hall A (2005). Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* **21**: 247–269.

Johnson EN, Seasholtz TM, Waheed AA, Kreutz B, Suzuki N, Kozasa T *et al.* (2003). RGS16 inhibits signalling through the $G\alpha_{13}$-Rho axis. *Nat Cell Biol* **5**: 1095–1103.

Katoh H, Negishi M, Ichikawa A (1996). Prostaglandin E receptor EP_3 subtype induces neurite retraction via small GTPase Rho. *J Biol Chem* **271**: 29780–29784.

Kelly P, Moeller BJ, Juneja J, Booden MA, Der CJ, Daaka Y *et al.* (2006). The G_{12} family of heterotrimeric G proteins promotes breast cancer invasion and metastasis. *Proc Natl Acad Sci USA* **103**: 8173–8178.

Kelly P, Casey PJ, Meigs TE (2007). Biologic functions of the G_{12} subfamily of heterotrimeric G proteins: growth, migration, and metastasis. *Biochemistry* **46**: 6677–6687.

Komatsuzaki K, Terashita K, Kinane TB, Nishimoto I (2001). Somatostatin type V receptor activates c-Jun N-terminal kinases via $G\alpha_{12}$ family G proteins. *Biochem Biophys Res Commun* **289**: 1211–1217.

Kourlas PJ, Strout MP, Becknell B, Veronese ML, Croce CM, Theil KS *et al.* (2000). Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia. *Proc Natl Acad Sci USA* **97**: 2145–2150.

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG *et al.* (1998). p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science* **280**: 2109–2111.

Kuner R, Swiercz JM, Zywietz A, Tappe A, Offermanns S (2002). Characterization of the expression of PDZ-RhoGEF, LARG and $G\alpha_{12}$/$G\alpha_{13}$ proteins in the murine nervous system. *Eur J Neurosci* **16**: 2333–2341.

Kurose H (2003). $G\alpha_{12}$ and $G\alpha_{13}$ as key regulatory mediator in signal transduction. *Life Sci* **74**: 155–161.

Lee YN, Malbon CC, Wang HY (2004). Ga_{13} signals via p115RhoGEF cascades regulating JNK1 and primitive endoderm formation. *J Biol Chem* **279**: 54896–54904.

McMullan R, Nurrish SJ (2007). Rho deep in thought. *Genes Dev* **21**: 2677–2682.

Mao J, Yuan H, Xie W, Simon MI, Wu D (1998). Specific involvement of G proteins in regulation of serum response factor-mediated gene transcription by different receptors. *J Biol Chem* **273**: 27118–27123.

Marinissen MJ, Gutkind JS (2005). Scaffold proteins dictate Rho GTPase-signaling specificity. *Trends Sci* **30**: 423–426.

Mehta D, Rahman A, Malik AB (2001). Protein kinase C-α signals rho-guanine nucleotide dissociation inhibitor phosphorylation and rho activation and regulates the endothelial cell barrier function. *J Biol Chem* **276**: 22614–22620.

Meyer BH, Freuler F, Guerini D, Siehler S (2008). Reversible translocation of p115-RhoGEF by $G_{12/13}$-coupled receptors. *J Cell Biochem* **104**: 1660–1670.

Michaelson D, Silletti J, Murphy G, D'Eustachio P, Rush M, Philips MR (2001). Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol* **152**: 111–126.

Milligan G (2003). Principles: extending the utility of [^{35}S]GTPγS binding assays. *Trends Pharmacol Sci* **24**: 87–90.

Moers A, Nieswandt B, Massberg S, Wettschureck N, Gruener S, Konrad L *et al.* (2003). G_{13} is an essential mediator of platelet activation in hemostasis and thrombosis. *Nat Med* **9**: 1418–1422.

Narumiya S, Ishizaki T, Watanabe N (1997). Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett* **410**: 68–72.

Offermanns S (1999). New insights into the in vivo function of heterotrimeric G-proteins through gene deletion studies. *Naunyn Schmiedebergs Arch Pharmacol* **360**: 5–13.

Offermanns S (2003). G-proteins as transducers in transmembrane signalling. *Prog Biophys Mol Biol* **83**: 101–130.

Ohmori T, Yatomi Y, Osada M, Kazama F, Takatuta T, Ikeda H *et al.* (2003). Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S. *Cardiovasc Res* **58**: 170–177.

Pertz O, Hodgson L, Klemke RL, Hahn KM (2006). Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* **440**: 1069–1072.

Ren XD, Schwartz MA (2000). Determination of GTP loading on. *Rho. Methods Enzymol* **325**: 264–272.

Porchia F, Papucci M, Gargini C, Asta A, de Marco G, Agretti P *et al.* (2008). Endothelin-1 up-regulates p115RhoGEF in embryonic Rat cardiomyocytes during the hypertrophic response. *J Recept Signal Transduct Res* **28**: 265–283.

Rieken S, Sassmann A, Herroeder S, Wallenwein B, Moers A, Offermanns S *et al.* (2006). G_{12}/G_{13} family G proteins regulate marginal zone B cell maturation, migration, and polarization. *J Immunol* **177**: 2985–2993.

Riento K, Ridley AJ (2003). Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* **4** (6): 446–456.

Riobo NA, Manning DR (2005). Receptors coupled to heterotrimeric G proteins of the G_{12} family. *Trends Pharmacol Sci* **26**: 146–154.

Rosenfeldt H, Castellone MD, Randazzo PA, Gutkind JS (2006). Rac inhibits thrombin-induced Rho activation: evidence of a Pak-dependent GTPase crosstalk. *J Mol Signal* **1**: 1–10.

Rossman KL, Der CJ, Sondek J (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* **6**: 167–180.

Sah VP, Seasholtz TM, Sagi SA, Brown JH (2000). The role of Rho in G protein-coupled receptor signal transduction. *Annu Rev Pharmacol Toxicol* **40**: 459–489.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Vaillant N, Gadeau AP, Desgranges C *et al*. (2000). $P2Y_1$, P2Y2, $P2Y_4$, and $P2Y_6$ receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am J Physiol Heart Circ Physiol* **278**: H1751–1661.

Seasholtz TM, Majumdar M, Brown JH (1999). Rho as a mediator of G protein-coupled receptor signaling. *Mol Pharmacol* **55**: 949–956.

Sgourakis NG, Bagos PG, Hamodrakas SJ (2005). Prediction of the coupling specificity of GPCRs to four families of G-proteins using hidden Markov models and artificial neural networks. *Bioinformatics* **21**: 4101–4106.

Siehler S (2007). $G_{12/13}$-dependent signaling of G protein-coupled receptors: disease context and impact on drug discovery. *Expert Opin Drug Discov* **2**: 1591–1604.

Siehler S (2008). Cell-based assays in GPCR drug discovery. *Biotechnology J* **4**: 471–483.

Stemmle LN, Fields TA, Casey PJ (2006). The regulator of G protein signaling domain of axin selectively interacts with $G\alpha_{12}$ but not $G\alpha_{13}$. *Mol Pharmacol* **70**: 1461–1468.

Strathmann MP, Simon MI (1991). $G\alpha_{12}$ and $G\alpha_{13}$ subunits define a fourth class of G protein alpha subunits. *Proc Natl Acad Sci USA* **88**: 5582–5586.

Suzuki N, Nakamura S, Mano H, Kozasa T (2003). $G\alpha_{12}$ activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proc Natl Acad Sci USA* **100**: 733–738.

Tanabe S, Kreutz B, Suzuki N, Kozasa T (2004). Regulation of RGS-RhoGEFs by $G\alpha_{12}$ and $G\alpha_{13}$ proteins. *Methods Enzymol* **390**: 285–294.

Taya S, Inagaki N, Sengiku H, Makino H, Iwamatsu A, Urakawa I *et al*. (2001). Direct interaction of insulin-like growth factor-1 receptor with leukemia-associated RhoGEF. *J Cell Biol* **155**: 809–820.

Thevananther S, Rivera A, Rivkees SA (2001). A1 adenosine receptor activation inhibits neurite process formation by Rho kinase-mediated pathways. *Neuroreport* **12**: 3057–3063.

Touge H, Chikumi H, Igishi T, Kurai J, Makino H, Tamura Y *et al*. (2007). Diverse activation states of RhoA in human lung cancer cells: contribution of G protein coupled receptors. *Int J Oncol* **30**: 709–715.

Treisman R, Alberts AS, Sahai E (1998). Regulation of SRF activity by Rho family GTPases. *Cold Spring Harb Symp Quant Biol* **63**: 643–651.

Wang Q, Liu M, Kozasa T, Rothstein JD, Sternweis PC, Neubig RR (2004). Thrombin and lysophosphatidic acid receptors utilize distinct RhoGEFs in prostate cancer cells. *J Biol Chem* **279**: 28831–28834.

Watterson KR, Ratz PH, Spiegel S (2005). The role of sphingosine-1-phosphate in smooth muscle contraction. *Cell Signal* **17**: 289–298.

Wells CD, Liu MY, Jackson M, Gutowski S, Sternweis PM, Rothstein JD *et al*. (2002). Mechanisms for reversible regulation between G_{13} and Rho exchange factors. *J Biol Chem* **277**: 1174–1181.

Wennerberg K, Der CJ (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). *J Cell Sci* **117**: 1301–1312.

Wettschureck N, Offermanns S (2005). Mammalian G proteins and their cell type specific functions. *Physiol Rev* **85**: 1159–1204.

Wheeler AP, Ridley AJ (2004). Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp Cell Res* **301**: 43–49.

Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S *et al*. (2008). G_{12}-G_{13}–LARG–mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nature Med* **14**: 64–68.

Wong K, Van Keymeulen A, Bourne H (2007). PDZRhoGEF and myosin II localize RhoA activity to the back of polarizing neutrophil-like cells. *J Cell Biol* **179**: 1141–1148.

Worzfeld T, Wettschureck N, Offermanns S (2008). G_{12}/G_{13}-mediated signalling in mammalian physiology and disease. *Trends Pharmacol Sci* **29**: 582–589.

Xie GX, Palmer PP (2007). How regulators of G protein signaling achieve selective regulation. *J Mol Biol* **366**: 349–365.

Yamada T, Ohoka Y, Kogo M, Inagaki S (2005). Physical and functional interactions of the lysophosphatidic acid receptors with PDZ domain-containing Rho guanine nucleotide exchange factors (RhoGEFs). *J Biol Chem* **280**: 19358–19363.

Yamaguchi Y, Katoh H, Negishi M (2003). N-terminal short sequences of alpha subunits of the G_{12} family determine selective coupling to receptors. *J Biol Chem* **278**: 14936–14939.

Yonemura S, Hirao-Minakuchi K, Nishimura Y (2004). Rho localization in cells and tissues. *Exp Cell Res* **295**: 300–314.

Yoshizaki H, Ohba Y, Kurokawa K, Itoh RE, Nakamura T, Mochizuki N *et al*. (2003). Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. *J Cell Biol* **162**: 223–232.

Zhang L, DiLizio C, Kim D, Smyth EM, Manning DR (2006). The G_{12} family of G proteins as a reporter of thromboxane A2 receptor activity. *Mol Pharmacol* **69**: 1433–1440.

Zheng S, Yu P, Zeng C, Wang Z, Yang Z, Andrews PM *et al*. (2003). G_{12}- and G_{13}-protein subunit linkage of D5 dopamine receptors in the nephron. *Hypertension* **41**: 604–660.

Zhong H, Neubig RR (2001). Regulator of G protein signaling proteins: novel multifunctional drug targets. *J Pharmacol Exp Ther* **297**: 837–845.