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Reversible Translocation of p115-RhoGEF by $G_{12/13}$-coupled Receptors

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Abstract G protein-coupled receptors (GPCRs) are important targets for medicinal agents. Four different G protein families, G_s, G_i, G_q, and G_{12}, engage in their linkage to activation of receptor-specific signal transduction pathways. G_{12} proteins were more recently studied, and upon activation by GPCRs they mediate activation of RhoGTPase guanine nucleotide exchange factors (RhoGEFs), which in turn activate the small GTPase RhoA. RhoA is involved in many cellular and physiological aspects, and a dysfunction of the $G_{12/13}$-Rho pathway can lead to hypertension, cardiovascular diseases, stroke, impaired wound healing and immune cell functions, cancer progression and metastasis, or asthma. In this study, regulator of G protein signaling (RGS) domain-containing RhoGEFs were tagged with enhanced green fluorescent protein (EGFP) to detect their subcellular localization and translocation upon receptor activation. Constitutively active $G\alpha_{12}$ and $G\alpha_{13}$ mutants induced redistribution of these RhoGEFs from the cytosol to the plasma membrane. Furthermore, a pronounced and rapid translocation of p115-RhoGEF from the cytosol to the plasma membrane was observed upon activation of several $G_{12/13}$-coupled GPCRs in a cell type-independent fashion. Plasma membrane translocation of p115-RhoGEF stimulated by a GPCR agonist could be completely and rapidly reversed by subsequent application of an antagonist for the respective GPCR, that is, p115-RhoGEF relocated back to the cytosol. The translocation of RhoGEF by $G_{12/13}$-linked GPCRs can be quantified and therefore used for pharmacological studies of the pathway, and to discover active compounds in a $G_{12/13}$-related disease context. J. Cell. Biochem. 104: 1660–1670, 2008.

Key words: G protein-coupled receptor (GPCR); $G_{12/13}$; RhoGTPase guanine nucleotide exchange factor (RhoGEF); regulator of G protein signaling (RGS)

GPCRs constitute the largest family of transmembrane cell-surface proteins involved in signal transduction, and they recognize signals as diverse as light, odorants, hormones, growth factors, and neurotransmitters. They interact with heterotrimeric guanine nucleotide-binding proteins, so-called G proteins, which transmit receptor signal to different effector molecules. Many GPCRs couple to more than one of the four G protein families: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$, whereof $G_{12/13}$ signaling is more recently studied [Offermanns, 2003]. G_{12} and G_{13} are ubiquitously expressed, and generally activated by receptors also coupling to other G proteins like, for example, $G_{i/o}$ or $G_{q/11}$. $G_{12/13}$ link GPCRs to unique signaling pathways with physiological relevance. $G_{12/13}$-mediated activation of RhoA, a small GTPase of the Rho family, leads to the activation of several downstream effectors such as Rho kinase (ROCK), protein kinase N (PKN), Jun kinase (JNK), citron kinase, phospholipase D (PLD), LIM kinase (LIMK), Diaphanous (Dia)1, rhophilin, and rhotekin [Bishop and Hall, 2000; Sah et al., 2000; Jaffe and Hall, 2005]. The RhoA pathway is further involved in multiple cellular functions, including regulation of the actin cytoskeleton, cell shape, cell polarity, microtubule dynamics, membrane

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transport pathways, gene transcription, neurite retraction, cell adhesion and migration, and cell growth [Bhattacharyya and Wedegaertner, 2003; Burridge and Wennerberg, 2004; Jaffe and Hall, 2005]. Disbalanced GPCR-regulated $G_{12/13}$-signaling can lead to diseases, and examples are: (i) vasoconstriction mediated by receptors for thrombin (protease-activated receptor; PAR-1), thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$) endothelin, angiotensin II, and vasopressin, which can cause hypertension, heart failure, stroke, or asthma, (ii) LPA receptor-mediated neurite retraction, which induces increases of Alzheimer's disease-like Tau phosphorylation in neuroblastoma cells, or (iii) cancer progression and metastasis mediated by receptors for thrombin or lysophosphatidic acid (LPA) [Sayas et al., 1999; Gohla et al., 2000; Sahai and Marshall, 2002; Seasholtz and Brown, 2004; Watterson et al., 2005; Wettschureck and Offermanns, 2005; Kelly et al., 2007].

The discovery of p115-RhoGEF provided a first direct link between the activation of the G_{12}-family G proteins and the small GTPase RhoA [Hart et al., 1996]. Subsequently, other $G_{12/13}$-regulated RhoGEFs were discovered, namely PDZ-RhoGEF and leukemia-associated RhoGEF (LARG), which link $G\alpha_{12/13}$ to Rho [Fukuhara et al., 1999; Kourlas et al., 2000]. All three RGS-containing RhoGEFs are widely expressed among tissues, although high levels of p115-RhoGEF and PDZ-RhoGEF are found in hematopoietic cells and in the nervous system, respectively. They interact with their N-terminal RGS domain with $G\alpha_{12/13}$ proteins, and specifically stimulate the intrinsic GTPase activity of $G\alpha_{12}$ and $G\alpha_{13}$ [Aasheim et al., 1997; Kozasa et al., 1998; Fukuhara et al., 1999, 2000]. Common to all RhoGEFs are a Dbl homology (DH) domain and a pleckstrin homology (PH) domain. The DH domain catalyses the exchange of GDP for GTP of RhoGTPases, whereas the PH domain is involved in subcellular localization and is essential for full exchange activity [Rossman et al., 2005]. RGS-containing RhoGEFs are therefore capable of acting as both negative regulators and downstream effectors of $G\alpha_{12/13}$ [Fukuhara et al., 2001]. More recently, a fourth GPCR-activated RhoGEF, Lbc-RhoGEF, was described. Lbc-RhoGEF contains a putative RGS-like domain near its C-terminus, and links $G\alpha_{12/13}$ to activation of RhoA [Dutt et al., 2004]. Various splice variants of Lbc-RhoGEF are present in different tissues, and for example A-kinase anchoring protein (AKAP)-Lbc is highly expressed in the heart [Diviani et al., 2001]. RGS domains of p115-RhoGEF, PDZ-RhoGEF, and LARG bind to active, GTP-bound $G\alpha_{12/13}$, and are thought to relocate from the cytosol to the plasma membrane upon activation of $G\alpha_{12/13}$. Such relocation was demonstrated for p115-RhoGEF when co-expressed with constitutively active $G\alpha_{12/13}$ mutants, or upon activation of the TXA_2 receptor [Bhattacharyya and Wedegaertner, 2003]. RGS domain-containing RhoGEFs belong to the large and diverse family of RGS proteins initially identified as GTPase activation proteins (GAPs) of heterotrimeric G proteins, and other members show a similar relocation such as RGS3 upon activation of $G\alpha_{11}$ [Dulin et al., 1999; Willars, 2006].

The three RGS domain-containing RhoGEFs, p115-RhoGEF, PDZ-RhoGEF, and LARG, were either N- or C-terminally tagged with EGFP. Pronounced translocation from the cytosol to the plasma membrane was observed for all three RhoGEFs in Madin–Darby canine kidney (MDCK) cells when co-expressed with constitutively active $G\alpha_{12}$. Furthermore, the translocation of p115-RhoGEF-EGFP was induced by activation of the $G_{12/13}$-coupled receptors, $S1P_2$, $S1P_3$, LPA_2, and PAR-1, when co-expressed with wild-type $G\alpha_{12}$ or $G\alpha_{13}$. Translocation of p115-RhoGEF from the cytosol to the plasma membrane was reversible upon addition of respective GPCR-specific antagonists, and provides a quantitative readout for the activation of $G\alpha_{12}$ or $G\alpha_{13}$ by GPCRs.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), MEM, McCoy's medium, OptiMEM, fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin–streptomycin, Na^+-pyruvate, non-essential amino acids, Lipofectamine 2000, HEPES, and HEPES-buffered saline solution (HBSS) were obtained from Invitrogen. Fatty acid-free bovine serum albumin (FA-BSA) was purchased from Calbiochem. JTE-013, TFLLR-NH_2, and SCH79797 were purchased from Tocris, and S1P and LPA from Biomol. The rabbit anti-EGFP polyclonal antibody was obtained from Chemicon, and the goat anti-rabbit IgG (H + L)

horseradish peroxidase conjugate from Biorad. Protease inhibitors (Complete) and Lumi Light Plus were obtained from Roche.

Plasmids

Human p115-RhoGEF (NM_199002.1), PDZ-RhoGEF (NM_014784.2), and LARG (NM_015313.1) were amplified from cDNA prepared from HUVEC cells, and cloned into the pCMV/myc/cyto expression vector (Invitrogen) with either N- or C-terminal fusion of EGFP and a GSGSG linker. The human $S1P_2$ receptor (AF034780) and human $S1P_3$ receptor (X83864) were cloned into the pcDNA3.1 expression vector (Invitrogen), whereas the human LPA_2 receptor (NM_004720) was cloned into a pcDNA3.1 myc.His A expression vector (Invitrogen). The human PAR-1 receptor (NM_001992.2) was cloned into the pCMV-Tag1 expression vector (Stratagene). Wild-type $G\alpha_{12}$ and $G\alpha_{13}$, and constitutively active $G\alpha_{12}QL$, $G\alpha_{13}QL$, and $G\alpha_qQL$ in pcDNA3.1 were purchased from the UMR cDNA Resource Center.

Cell Culture and Transfection

Parental HeLa cells (ATCC, CCL2), MDCK cells (ATCC, NBL-2), and U-2 OS (ATCC, HTB-96) cells were cultured at 37°C, 5% CO_2, and 95% relative humidity. The culture medium used were: for HeLa cells, DMEM, 10% FBS and 100 U/ml penicillin, 0.1 mg/ml streptomycin, for MDCK cells, MEM, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM Na^+-pyruvate, 1% non-essential amino acids, and for U-2 OS cells, McCoy's, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin. Cell lines were transfected using Lipofectamine 2000 (LF) and OptiMEM medium (OMEM). For transient transfection, cells were seeded at 25,000 cells/Lab-Tek II 8 well (Nunc) in 0.25 ml medium 20–24 h before transfection to achieve a 80–90% confluency. Culture medium was replaced by OMEM approximately 20 min before addition of the transfection solution to remove FBS and penicillin–streptomycin. Plasmid cDNA and LF were diluted separately in OMEM, gently mixed, and incubated for 5 min at RT. Diluted cDNA and LF solutions were combined, gently mixed, and incubated for 20 min at room temperature (RT). The mix was added to the cells, and cells further incubated at 37°C, 5% CO_2, and 95% relative humidity. The transfection solution was replaced after 4–6 h by culture medium containing no serum for overnight starvation.

Cell Fractionation and Immunoblot Analysis

1 million HeLa cells were plated on 10 cm cell culture plates in 10 ml medium, cultivated for 24 h, and transfected with p115-RhoGEF-EGFP, and either an empty vector, $G\alpha_{13}QL$, or $G\alpha_qQL$ with a DNA ratio of 1:2. Twenty four hours post-transfection, cells were trypsinized, collected in 50 ml falcon tubes and centrifuged at 500*g* for 3 min. Cells were resuspended in 1 ml PBS, transferred into 2 ml Eppendorf tubes, and centrifuged at 1,000*g* for 2 min. Supernatant was discarded, and if required, cell pellets stored at −80°C. Cell pellets were thawed on ice, and resuspended in 300 μl lysis buffer (50 mM Tris/pH 7.2, 100 mM NaCl, 2 mM EDTA, protease inhibitors). Cells were lysed on ice with a Polytron homogenizer at 19,000 rpm for 15 s, and centrifuged for 5 min at 1,000*g* and 4°C. The supernatant was transferred into a precooled centrifugation tube (OakRide, Nalgene). The remaining pellet was resuspended in 300 μl lysis buffer, again homogenized (19,000 rpm, 15 s, on ice), and centrifuged for 5 min at 1,000*g* and 4°C. The supernatant was transferred to the same tube as before. To separate the membrane fraction from the cytosolic fraction samples were centrifuged at 45,000*g* for 30 min at 4°C. Supernatant and the pellet were resupended in 300 μl lysis buffer and stored at −80°C. Protein concentrations were determined using the Pierce BCA protein quantification kit. Equal quantities of protein (1–2 μg) were loaded on SDS/PAGE for immunoblotting. Blots were incubated with a rabbit anti-EGFP polyclonal antibody (1:500 dilution in blocking buffer; PBS/5% milk/0.05% Tween-20) overnight at RT, followed by incubation of a secondary goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (1:1,000 dilution in blocking buffer), and enhanced chemiluminescence (ECL) detection using Lumi-Light PLUS.

Confocal Microscopy

Cells were seeded into Lab-Tek II 8-well plates (Nunc) using 25,000 cells/well in 250 μl media for imaging. Cells were transfected on the next day, and serum-starved over night with culture medium containing 0% FBS. Before imaging, cells were incubated 10–15 min with assay buffer (1× HBSS, 20 mM HEPES, 0.2%

FA-BSA). After staining, buffer was replaced with 120 μl assay buffer (1× HBSS, 20 mM HEPES, 0.2% FA-BSA). Ligands were manually added while recording a time series. One hundred twenty microliters of twofold concentrated ligand solution were added to 120 μl assay buffer to stimulate the cells. When applying a second ligand, 120 μl of threefold concentrated ligand were applied to reach the desired final concentration. Confocal micrographs were recorded on a Zeiss laser scanning confocal microscope (LSM) 510 META (Carl Zeiss AG) with a C-Apochromat 63×/1.2 W corr objective. EGFP was excited at 488 nm, and detected using a 505–530 nm emission filter. To quantify the translocation of p115-RhoGEF-EGFP, the ratio of the average EGFP fluorescence intensity in the plasma membrane over the average EGFP fluorescence intensity in the cytosol was calculated for single cells. A region of interest was drawn over a section of the plasma membrane or the cytosol, respectively, and the average intensities were calculated using the Zeiss LSM 510 software.

Online Supplemental Material

Video 1 shows a time series of the translocation events of p115-RhoGEF-EGFP in HeLa cells co-expressing $G\alpha_{12}$ and $S1P_2$ receptors.

RESULTS

Subcellular Translocation of RhoGEFs Induced by Constitutively Active $G\alpha_{12}$

RGS domain-containing RhoGEF proteins were reported to bind to GTP-bound, and therefore active $G\alpha_{12}$ or $G\alpha_{13}$. To investigate the subcellular localization of these RhoGEFs in the presence or absence of active $G\alpha_{12}$ or $G\alpha_{13}$, p115-RhoGEF, PDZ-RhoGEF and LARG, were cloned, and EGFP was fused to its N-terminus or C-terminus. The resulting EGFP fusion proteins were transiently transfected into MDCK cells, and their subcellular localization analyzed by confocal microscopy (Fig. 1). Both, EGFP-tagged p115-RhoGEF and LARG revealed a homogenous cytosolic localization, whereas PDZ-RhoGEF localized also to intracellular structures. The nature of these intracellular structures is not known and has not been further investigated. Since RGS domain-containing RhoGEF proteins reveal GAP activity towards $G\alpha_{12}$ and $G\alpha_{13}$, they were expected to localize at the plasma membrane in the presence of constitutively active $G\alpha_{12/13}$ mutants. MDCK cells were transiently co-transfected with either EGFP-tagged RhoGEF and constitutively active $G\alpha_{12}$QL. Introduction of $G\alpha_{12}$QL caused a plasma membrane localization of N- and C-terminally tagged p115-RhoGEF, which indicates that p115-RhoGEF indeed interacts with active $G\alpha_{12}$. In addition, PDZ-RhoGEF and LARG localized to the plasma membrane of MDCK cells upon co-expression of $G\alpha_{12}$QL. No significant differences of N- versus C-terminally tagged RhoGEFs were apparent. C-terminally EGFP-tagged p115-RhoGEF was chosen for further studies. p115-RhoGEF does not contain a PDZ domain like PDZ-RhoGEF and LARG, which might interact directly with other membrane proteins [Taya et al., 2001; Driessens et al., 2002].

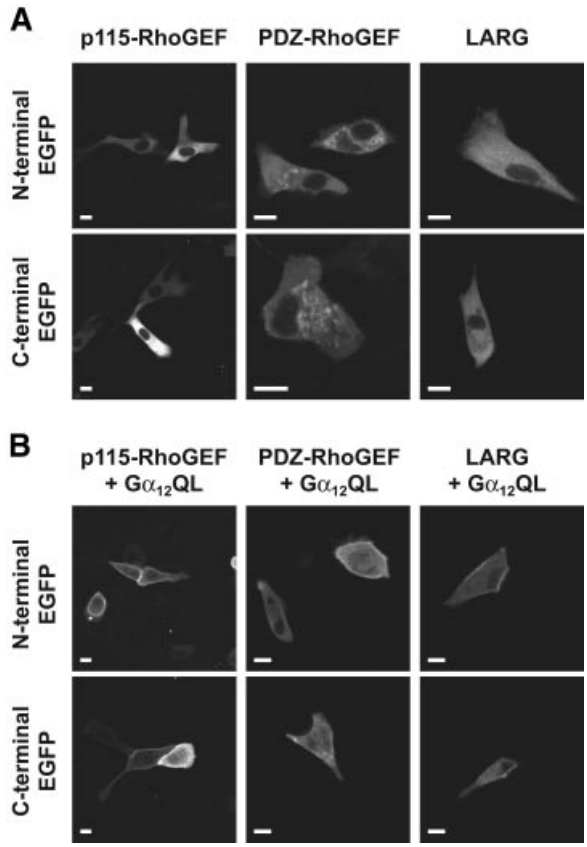

Fig. 1. $G\alpha_{12}$-induced translocation of p115-RhoGEF, PDZ-RhoGEF, and LARG. Fluorescence confocal micrographs showing N- and C-terminally EGFP-tagged p115-RhoGEF, PDZ-RhoGEF or LARG transiently expressed in MDCK cells together with either (**A**) empty vector, or (**B**) $G\alpha_{12}$QL, and using a cDNA ratio of 1:2. Scale bars represent 10 μm.

Plasma Membrane Translocation of p115-RhoGEF Induced by Active $G\alpha_{12}$ or $G\alpha_{13}$

Both, $G\alpha_{12}$QL or $G\alpha_{13}$QL induced a translocation of p115-RhoGEF in HeLa and U2-OS cells (Fig. 2). GPCRs linked to $G_{q/11}$ generally additionally couple to $G_{12/13}$. To examine whether p115-RhoGEF translocation is specifically induced by $G\alpha_{12/13}$, HeLa cells were transiently transfected with p115-RhoGEF-EGFP and $G\alpha_q$QL. $G\alpha_q$QL did not induce any translocation of p115-RhoGEF. To confirm these results, transfected cells were lysed and fractionated into cytosolic and membrane fractions. Detection of p115-RhoGEF-EGFP with an antibody against EGFP revealed an accumulation of p115-RhoGEF-EGFP at the plasma membrane in cells expressing $G\alpha_{13}$QL, but not in control cells or cells expressing $G\alpha_q$QL. These data show that the redistribution of p115-RhoGEF-EGFP does not depend on active $G\alpha_q$, and is in agreement with previous findings [Kozasa et al., 1998; Bhattacharyya and Wedegaertner, 2003].

$S1P_2$-Mediated Translocation of p115-RhoGEF Requires Sufficient Levels of $G\alpha_{12/13}$

p115-RhoGEF-EGFP was present in the cytosol when co-expressed with wild-type $G\alpha_{12}$ or $G\alpha_{13}$, and $G\alpha_{12}$QL or $G\alpha_{13}$QL induced a plasma membrane localization. HeLa cells were next transiently transfected with p115-RhoGEF-EGFP and the $G_{12/13}$-coupled receptor $S1P_2$ to investigate relocation of p115-RhoGEF upon receptor stimulation. As a control, no translocation was observed when HeLa cells expressing p115-RhoGEF-EGFP, but not $S1P_2$, were stimulated with S1P (Fig. 3). Surprisingly, stimulation of cells co-expressing p115-RhoGEF-EGFP and $S1P_2$ did not lead to translocation of p115-RhoGEF-EGFP either. EGFP-tagged p115-RhoGEF did only translocate to the plasma membrane after receptor stimulation with S1P in the presence of either wild-type $G\alpha_{12}$ or $G\alpha_{13}$. This demonstrated the requirement of recombinant co-expression of $G\alpha_{12}$ or $G\alpha_{13}$ upon recombinant expression of p115-RhoGEF-EGFP and $S1P_2$. Co-expression of $G\alpha_{12}$ or $G\alpha_{13}$ without transfected $S1P_2$ resulted in a weak S1P-stimulated translocation of p115-RhoGEF-EGFP through endogenous S1P receptors. Furthermore, $S1P_2$-induced translocation of p115-RhoGEF-EGFP was seen in U-2 OS and MDCK cells, and the co-expression of $G\alpha_{12}$ or $G\alpha_{13}$ was also required (data not shown).

Translocation of p115-RhoGEF Through $S1P_2$ Receptors is Rapid and Reversed by a Receptor Antagonist

In order to retrieve kinetics for translocation of p115-RhoGEF-EGFP, live cells were recorded using a confocal microscope. HeLa cells were transiently transfected with $S1P_2$, $G\alpha_{12}$, and p115-RhoGEF-EGFP, and serum starved over night. Stimulation of $S1P_2$ receptors with the

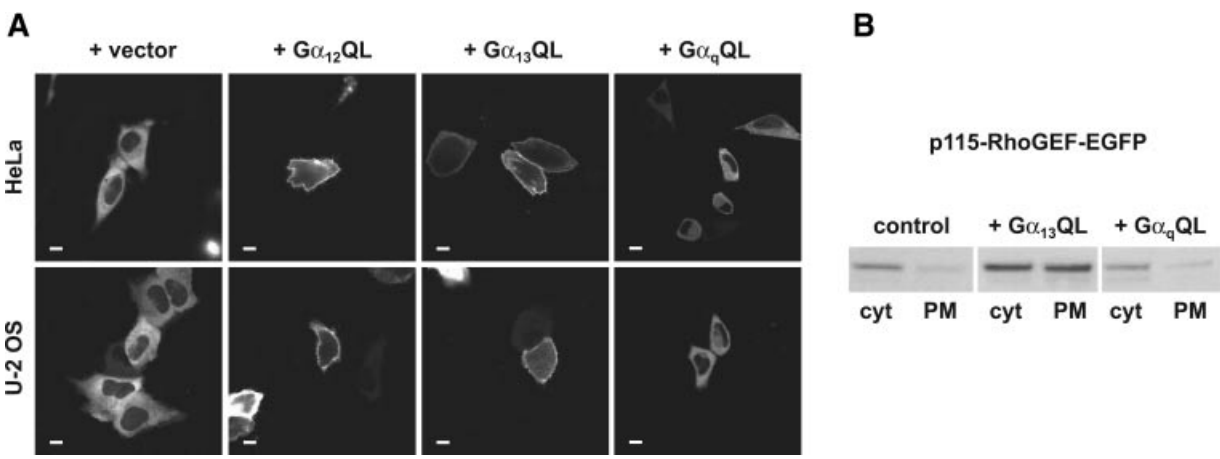

Fig. 2. $G\alpha_{12/13}$-induced translocation of p115-RhoGEF in other cell types. C-terminally EGFP-tagged p115-RhoGEF was transiently co-expressed together with empty vector, $G\alpha_{12}$QL, $G\alpha_{13}$QL, or $G\alpha_q$QL, respectively, in HeLa and U-2 OS cells using a cDNA ratio of 1:2. **A**: Fluorescence confocal micrographs representing EGFP fluorescence. Scale bars represent 10 μm. **B**: Western blot analysis of fractionated (cyt, cytosol; PM, plasma membrane) HeLa cells transiently expressing p115-RhoGEF-EGFP and empty vector, $G\alpha_{13}$QL, or $G\alpha_q$QL, respectively. p115-RhoGEF-EGFP was detected with an antibody against EGFP.

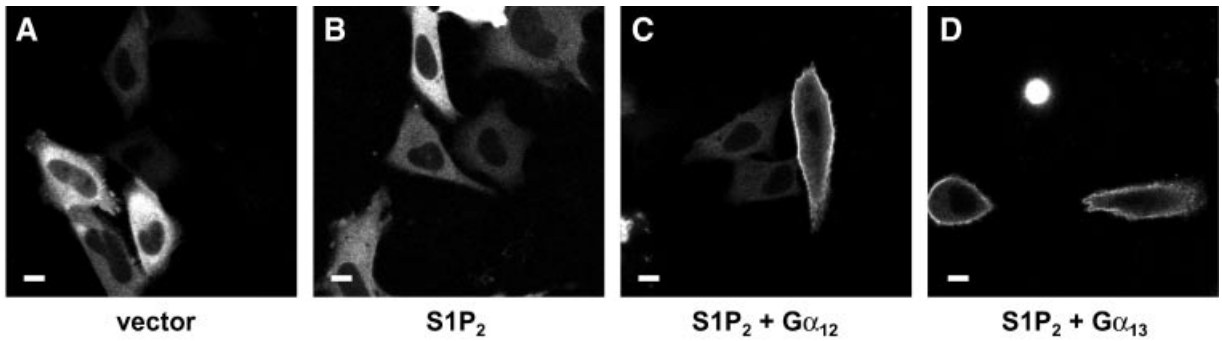

Fig. 3. Expression of $G\alpha_{12/13}$ is required for $S1P_2$ receptor-mediated translocation of p115-RhoGEF. Fluorescent confocal micrographs of HeLa cells expressing (**A**) p115-RhoGEF-EGFP alone, or together with (**B**) $S1P_2$, (**C**) $S1P_2$, and $G\alpha_{12}$, or (**D**) $S1P_2$ and $G\alpha_{13}$. A DNA ratio of 1:2:2 was used for transient transfection of p115-RhoGEF-EGFP, $S1P_2$ receptor, and $G\alpha_{12}$ or $G\alpha_{13}$ (or empty vectors). All cells were stimulated with 2 μM S1P. Scale bars represent 10 μm.

agonist S1P led to a rapid and pronounced translocation of p115-RhoGEF-EGFP from the cytosol to the plasma membrane (Fig. 4; Video 1 in online supplemental material). JTE-013, a commercially available $S1P_2$-selective receptor antagonist [Osada et al., 2002], reversed the translocation of p115-RhoGEF-EGFP from the plasma membrane back to the cytosol by terminating the $S1P_2$ receptor-induced activation of $G\alpha_{12/13}$. Kinetic readouts were generated by recording time courses of living cells while sequentially adding the agonist and antagonist. A ratio of the average EGFP fluorescence intensity in the cytosol over the intensity at the plasma membrane of single cells was calculated to obtain the kinetic ratio. Within 1–2 min S1P stimulated maximal translocation of p115-RhoGEF-EGFP to the plasma membrane, which could be rapidly reversed by the subsequent addition of the antagonist JTE-013. The plasma membrane versus cytosolic fluorescence intensity ratio returned to baseline within approximately 0.8–1.0 min following addition of the antagonist. In contrast, plasma membrane localization of p115-RhoGEF-EGFP remained stable for at least 30 min when cells were only treated with the agonist (data not shown).

Translocation of p115-RhoGEF by Other $G_{12/13}$-Linked GPCRs

Other known $G\alpha_{12/13}$-coupled receptors, such as $S1P_3$, PAR-1 and LPA_2 receptors, were tested for translocation of p115-RhoGEF-EGFP. Each

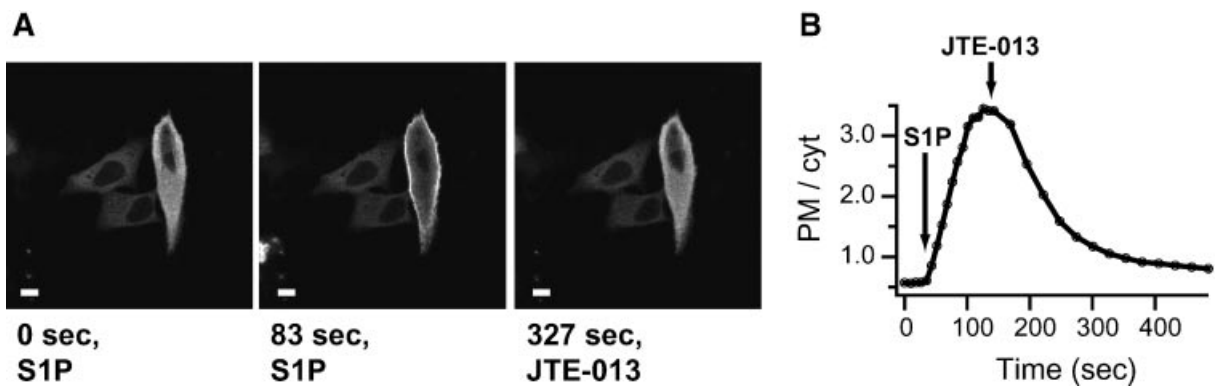

Fig. 4. Rapid and reversible translocation of p115-RhoGEF through $S1P_2$ receptors. **A**: Fluorescence confocal micrographs presenting HeLa cells co-expressing p115-RhoGEF-EGFP, $G\alpha_{12}$ and $S1P_2$ receptors using a DNA ratio of 1:2:2 for transient transfection. $S1P_2$ receptors were stimulated with 2 μM of the agonist S1P, and subsequently challenged with a 20 μM concentration of the $S1P_2$-selective antagonist JTE-013. The first two images were taken zero and 83 s after agonist addition. The third image was taken 327 s after antagonist addition. Scale bars represent 10 μm (Video 1 in online supplemental material). **B**: Time traces of the ratio of the average EGFP fluorescence intensity at the plasma membrane (PM) over the intensity in the cytosol (cyt).

receptor was transfected with p115-RhoGEF-EGFP, and either $G\alpha_{12}$ or $G\alpha_{13}$. Stimulation of human $S1P_3$ receptors with S1P led in the presence of $G\alpha_{13}$ to a maximal translocation of p115-RhoGEF-EGFP to the plasma membrane within 1–2 min similarly to $S1P_2$ receptors (Fig. 5). LPA stimulation of human LPA_2 receptors induced maximal translocation of p115-RhoGEF-EGFP within one min. In addition, stimulation of human PAR-1 receptors with TFLLR-NH_2 induced maximal plasma membrane localization of p115-RhoGEF-EGFP in the presence of $G\alpha_{12}$ within 2 min. p115-RhoGEF-EGFP rapidly relocated to the cytosol after addition of the PAR-1 selective antagonist SCH 79797. Stimulation of four $G_{12/13}$-coupled receptors caused a pronounced plasma membrane localization of p115-RhoGEF-EGFP, which demonstrates that p115-RhoGEF ubiquitously translocates upon activation of $G\alpha_{12/13}$ by $G_{12/13}$-coupled GPCRs. In summary, stimulation of $S1P_2$, $S1P_3$, or LPA_2 receptors leads to the translocation of p115-RhoGEF to the plasma membrane in a similar time range when co-expressed with either $G\alpha_{12}$ or $G\alpha_{13}$ (some data not shown). In contrast, no translocation could be observed for PAR-1 receptors co-expressed with $G\alpha_{13}$.

DISCUSSION

$G\alpha_{12}$ and $G\alpha_{13}$ are N-terminally palmitoylated, which is relevant for their plasma membrane attachment and receptor interactions

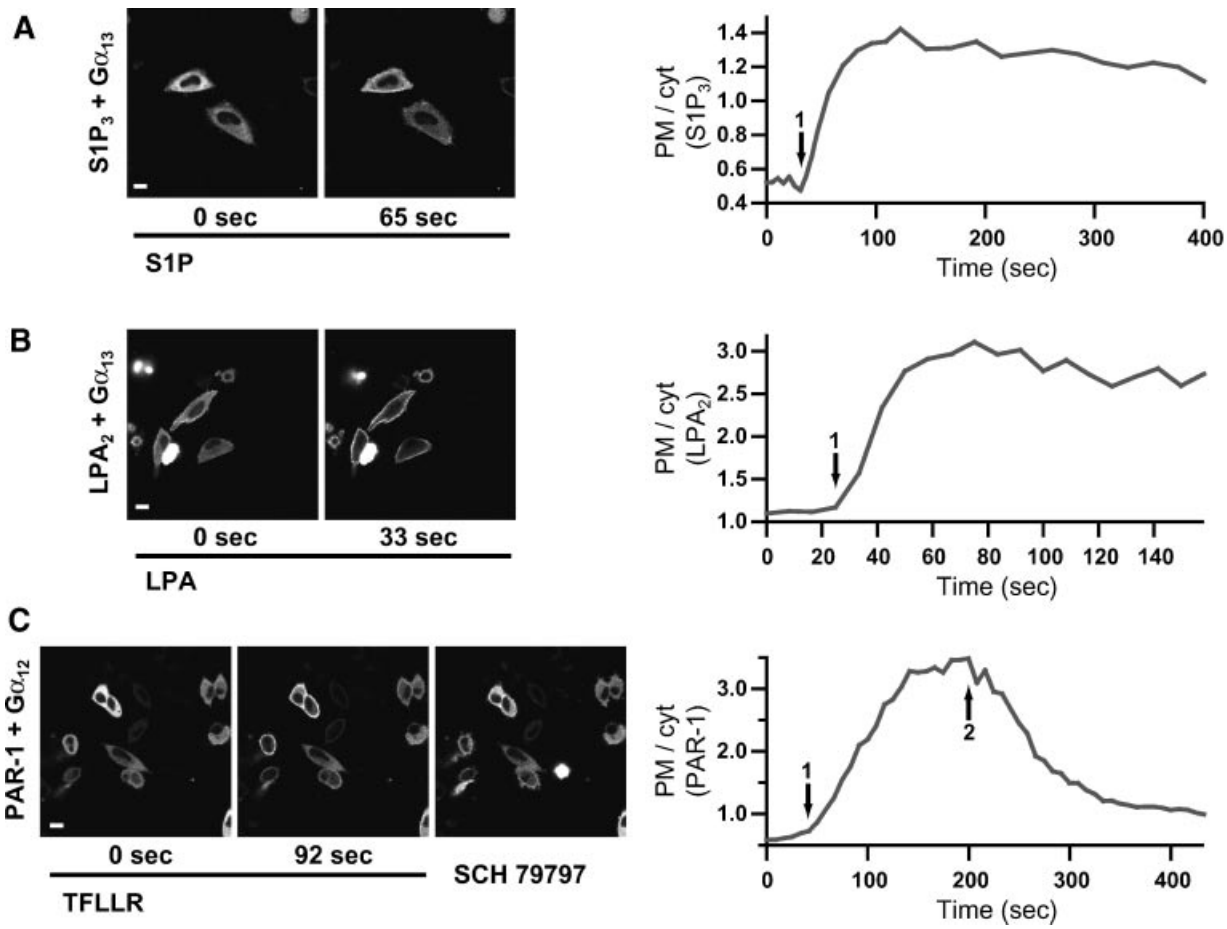

Fig. 5. Translocation of p115-RhoGEF by other $G_{12/13}$-coupled GPCRs. Fluorescence confocal micrographs showing the translocation of p115-RhoGEF-EGFP mediated by three different GPCRs. A DNA ratio of 1:2:2 was used for transient transfection of p115-RhoGEF-EGFP, receptor, and G protein. **A**: Translocation of p115-RhoGEF-EGFP in HeLa cells expressing $S1P_3$ receptors and $G\alpha_{13}$ upon stimulation with 10 μM S1P. **B**: Translocation of p115-RhoGEF-EGFP mediated by LPA_2 receptors and $G\alpha_{13}$ upon stimulation with 10 μM LPA. **C**: Translocation of p115-RhoGEF-EGFP induced by PAR-1 receptors and $G\alpha_{12}$ upon stimulation with 10 μM of the PAR-1 agonist TFLLR-NH_2, and subsequent addition of 40 μM of the PAR-1 antagonist SCH 79797. Scale bars represent 10 μm. Kinetic curves reveal the ratio of fluorescence intensities at the plasma membrane (PM) over the cytosol (cyt) after addition of agonist (1) and antagonist (2).

[Dhanasekaran and Dermott, 1996]. In comparison, RhoGEF proteins are not described to contain posttranslational residues relevant for membrane attachment, and translocation to the plasma membrane coincides with interaction with membrane-associated proteins. There are only a few reports describing the subcellular localization of RGS domain-containing RhoGEFs, and cell-type specific differences are reported [Togashi et al., 2000; Taya et al., 2001; Driessens et al., 2002; Bhattacharyya and Wedegaertner, 2003; Banerjee and Wedegaertner, 2004]. In the case of PDZ-RhoGEF and LARG it was shown that the PDZ domain can direct the proteins towards the plasma membrane by interacting with other proteins such as plexin-B1, plexin-B2 or the insulin-like growth factor-1 receptor [Taya et al., 2001; Driessens et al., 2002]. Also, a proline-rich motif in PDZ-RhoGEF was found to lead to plasma membrane localization of PDZ-RhoGEF [Togashi et al., 2000; Banerjee and Wedegaertner, 2004]. In contrast, in our study EGFP-tagged PDZ-RhoGEF transiently expressed in MDCK cells was cytosolic and present in some intracellular structures, whereas EGFP-tagged LARG showed a homogenous cytosolic localization. It seems that PDZ-RhoGEF and LARG do not necessarily localize at the plasma membrane, which might be due to over-expression or a lack of interaction partners. PDZ-RhoGEF and LARG translocated to the plasma membrane when co-expressed together with constitutively active $G\alpha_{12}$.

In the inactive state, p115-RhoGEF localizes in the cytosol as shown by either immunostaining or EGFP-tagging of p115-RhoGEF. Co-expression of constitutively active $G\alpha_{12}$ or $G\alpha_{13}$ resulted in plasma membrane localization of p115-RhoGEF [Bhattacharyya and Wedegaertner, 2003]. This change in localization could be confirmed using EGFP-tagged p115-RhoGEF. Neither N- or C-terminal tagging of p115-RhoGEF interfered with plasma membrane localization triggered by active $G\alpha_{12/13}$ mutants. In addition, two other cell lines, HeLa and U-2 OS, showed the same localization pattern of p115-RhoGEF-EGFP. The expression of constitutively active $G\alpha_q$ did not lead to a plasma membrane localization of p115-RhoGEF-EGFP, which further confirmed the specific interaction of p115-RhoGEF with active $G\alpha_{12/13}$ proteins. G_q was initially described to also activate the RhoA pathway. In certain cell types, high ligand concentrations could have some effects on RhoA activation by G_q, which may be explained by the positive regulation of for example p115-RhoGEF through PKCα [Holinstat et al., 2003]. However, studies using embryonic fibroblasts from $G\alpha_{12}/G\alpha_{13}$ or $G\alpha_q/G\alpha_{11}$ double knockout mice demonstrated that thrombin activates RhoA by two orders of magnitude more potent through $G_{12/13}$ as compared to G_q [Vogt et al., 2003].

Results described here show that endogenous levels of $G\alpha_{12/13}$ are not sufficient to promote significant translocation of recombinant p115-RhoGEF-EGFP, because it was necessary to co-express either $G\alpha_{12}$ or $G\alpha_{13}$ in order to observe a receptor-dependent translocation of p115-RhoGEF-EGFP from the cytosol to the plasma membrane. It has been shown that the RGS domain-containing p115-RhoGEF interacts with active $G\alpha_{12}$ and $G\alpha_{13}$ to catalyze their intrinsic hydrolysis of GTP to GDP [Kozasa et al., 1998]. Recently, a complex between $G\alpha_{13}$ and the RGS domain of p115-RhoGEF has been crystallized [Chen et al., 2005]. The crystal structure of the complex with the N-terminal end of p115-RhoGEF inserted into the GTPase site of $G\alpha_{13}$ suggests a 1:1 stoichiometry between G_{12}-family G proteins and p115-RhoGEF. To observe a significant translocation of p115-RhoGEF-EGFP to the plasma membrane, the expression level of $G\alpha_{12/13}$ presumably needs to be at least as high as the expression level of p115-RhoGEF-EGFP. Therefore, a 1:1 stoichiometry may explain the necessity of over-expression of wild-type $G\alpha_{12/13}$ to increase their protein levels. S1P caused a weak translocation of p115-RhoGEF-EGFP upon over-expression of $G\alpha_{12}$ or $G\alpha_{13}$ in HeLa cells without any transfected receptor. HeLa cells contain low levels of endogenous $S1P_2$ and $S1P_3$ [Siehler and Guerini, 2006]. Endogenous GPCRs may therefore be monitored, but require a sufficient native expression level as compared to transfected p115-RhoGEF-EGFP. Bhattacharyya and Wedegaertner [2003] showed a plasma membrane localization of p115-RhoGEF through stably expressed TXA_2 receptors without co-expressing $G\alpha_{12}$ or $G\alpha_{13}$, but in their experiment they stained for endogenous p115-RhoGEF rather than recombinant p115-RhoGEF. Cellular relocation events have been described for other RGS proteins such as RGS3, which translocates from the cytosol to the plasma membrane upon activation of $G\alpha_{11}$

by aluminium fluoride, or through stimulation with endothelin-1 [Dulin et al., 1999]. Another example is RGS7, which binds to $G\beta_5$ subunits, and relocates from the cytosol to the plasma membrane upon co-expression with constitutively active $G\alpha_o$ [Takida et al., 2005]. On the other hand, RGS2 and RGS4 localized at the plasma membrane independent of the activation state of the corresponding G proteins [Roy et al., 2003]. Moreover, RGS proteins other than RhoGEFs can bind to G_{12}-proteins, like for example RGS1 and axin to $G\alpha_{12}$, and RGS16 to $G\alpha_{13}$, and axin and RGS16 can block association of p115-RhoGEF with $G\alpha_{12}$ or $G\alpha_{13}$, respectively [Kelly et al., 2007].

To our knowledge, there is no report about the relocation of RGS or RGS-like proteins, after inactivation of a GPCR by an antagonist. This report describes for the first time the kinetics of translocation and relocation events of an RGS protein. Interestingly, the addition of JTE-013, a $S1P_2$-selective antagonist [Osada et al., 2002], led to a rapid relocation of p115-RhoGEF-EGFP to the cytosol. Receptor-activated $G\alpha_{12/13}$ appears to activate p115-RhoGEF over a prolonged period, because p115-RhoGEF retained at the plasma membrane for at least 30 min in the presence of the agonist. The slow nucleotide exchange rate of $G\alpha_{12/13}$ in comparison to other $G\alpha$ proteins indicates a prolonged signaling mediated by these $G\alpha$ subunits [Dhanasekaran and Dermott, 1996]. The rate of relocation of p115-RhoGEF from the cytosol to the plasma membrane after addition of an antagonist can be estimated from the kinetic curves in Figures 4 and 5, and are in the range of 0.8–1.0 min. Interestingly, these rates are in the range of GTP hydrolysis by $G\alpha_{12}$ in presence of the RGS domain of p115-RhoGEF, which were determined to be three- to fivefold higher than for $G\alpha_{12}$ alone with a hydrolysis rate of 0.12 min^{-1} at 21°C [Stemmle et al., 2006]. Agonist stimulation of a GPCR generally causes a rapid densensitization of signals through internalization of the receptor [Kahout and Lefkowitz, 2003]. Receptor internalization would not explain the inhibitory action of the subsequently added $S1P_2$-selective antagonist JTE-013. JTE-013 is not an inverse agonist in other assay systems and in vivo [Osada et al., 2002], and even an inverse agonist would not bind to internalized $S1P_2$ receptors. In addition, a known PAR-1 selective antagonist evoked the same p115-RhoGEF relocation effect through PAR-1 receptors. An explanation remains elusive at this point, however, GPCR/$G\alpha_{12/13}$/p115-RhoGEF signaling complexes may be rather resistant to internalization, which would leave the receptor accessible to an antagonist. We have not investigated the addition of an antagonist following an agonist exposure for longer than for a few minutes, and receptors could eventually internalize.

In this study, all four examined GPCRs, namely $S1P_2$, $S1P_3$, PAR-1, and LPA_2, were found to trigger the translocation of p115-RhoGEF from the cytosol to the plasma membrane. $S1P_2$, $S1P_3$, and LPA_2-mediated translocation of p115-RhoGEF-EGFP could be observed when co-expressed with either $G\alpha_{12}$ or $G\alpha_{13}$. In contrast, no translocation was observed for PAR-1 in the presence of $G\alpha_{13}$. Similarly, a literature report describes selective activation of $G\alpha_{12}$ by PAR-1 receptors using a phosphatase PP5 pull-down assay and transfection of HEK293T cells [Yamaguchi et al., 2003]. In contrast, the selectivity of LPA_2 receptors for $G\alpha_{12}$ described in the same article could not be confirmed in the p115-RhoGEF translocation assay. For $S1P_2$ and PAR-1 receptors, for which antagonists were available, the translocation could be reversed by subsequent addition of a receptor-selective antagonist, and p115-RhoGEF relocated to the cytosol. Agonist and antagonist effects on the subcellular localization pattern of p115-RhoGEF-EGFP clearly showed the involvement of the respective GPCR. The translocation of p115-RhoGEF was demonstrated in MDCK, HeLa, and U-2 OS cell lines. The p115-RhoGEF translocation assay is therefore broadly applicable to investigate activation of the $G_{12/13}$ signaling pathway by GPCRs, and the assay will enable further mechanistic and pharmacological studies of this pathway in the context of GPCR modulation. Quantification of signals can be performed using subcellular imaging devices and suitable algorithms, and new kinetic insights can be retrieved. Furthermore, the co-expression of p115-RhoGEF with $G\alpha_{12}$ or $G\alpha_{13}$ allows to distinguish G_{12} versus G_{13} activation by GPCRs.

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