

# Characterization of G $\alpha_{13}$ -dependent plasma membrane recruitment of p115RhoGEF

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The Ras homology (Rho) guanine nucleotide exchange factor (GEF), p115RhoGEF, provides a direct link between the G-protein  $\alpha$  subunit,  $\alpha_{13}$ , and the small GTPase Rho. In the present study, we demonstrate that activated mutants of  $\alpha_{13}$  or  $\alpha_{12}$ , but not  $\alpha_q$ , promote the redistribution of p115RhoGEF from the cytoplasm to the plasma membrane (PM). We also show that the PM translocation of p115RhoGEF is promoted by stimulation of thromboxane A<sub>2</sub> receptors. Furthermore, we define domains of p115RhoGEF required for its regulated PM recruitment. The RhoGEF RGS (regulators of G-protein signalling) domain of p115RhoGEF is required for PM recruitment, but it is not sufficient for strong  $\alpha_{13}$ -promoted PM recruitment, even though

it strongly interacts with activated  $\alpha_{13}$ . We also identify the pleckstrin homology domain as essential for  $\alpha_{13}$ -mediated PM recruitment. An amino acid substitution of lysine to proline at position 677 in the pleckstrin homology domain of p115RhoGEF inhibits Rho-mediated gene transcription, but this mutation does not affect  $\alpha_{13}$ -mediated PM translocation of p115RhoGEF. The results suggest a mechanism whereby multiple signals contribute to regulated PM localization of p115RhoGEF.

**Key words:** heterotrimeric G-protein, plasma membrane translocation, regulators of G-protein signalling, signal transduction.

## INTRODUCTION

Heterotrimeric G-proteins ( $\alpha$ ,  $\beta$  and  $\gamma$  subunits) transduce extracellular stimuli into discrete intracellular messages. Heterotrimeric G-proteins undergo cycles of guanine nucleotide exchange and hydrolysis and thus act as molecular switches to turn on or turn off signalling events. Activated G-protein-coupled receptors (GPCRs) interact with and cause a conformational change in G $\alpha$ , which promotes exchange of GDP for GTP. GTP-bound G $\alpha$  dissociates from G $\beta\gamma$ , and activates downstream effector molecules. G $\alpha$  can be divided into four families,  $\alpha_s$ ,  $\alpha_i$ ,  $\alpha_q$  and  $\alpha_{12}$ , on the basis of sequence and functional similarities. The  $\alpha_{12}$  family consists of  $\alpha_{12}$  and  $\alpha_{13}$  which exhibit 65% amino acid identity and have been shown to regulate pathways involved in cell growth, oncogenesis and cell-shape changes [1].

Activation of  $\alpha_{12}$  and  $\alpha_{13}$  leads to activation of the small GTPase Rho (Ras homology) [2]. Rho proteins are members of the Ras superfamily of small GTPases, and they control a wide variety of cellular processes including cytoskeletal rearrangement, cell-cycle progression, gene transcription and cytokinesis [3,4]. The Rho family is divided into three subfamilies: Rho, Rac and Cdc42. In fibroblasts, Rho promotes actin stress fibre formation, Rac induces formation of lamellipodia and Cdc42 induces filopodia formation. Rho activity is tightly regulated by guanine nucleotide exchange factors (GEFs), which stimulate GTP loading, GTPase-activating proteins (GAPs), which catalyse GTPase activity, and guanine nucleotide dissociation inhibitors, which antagonize both GEFs and GAPs. Members of the RhoGEF family [5] use their Dbl homology (DH) domain to turn on Rho activity by catalysing the exchange of GDP for GTP on Rho. Most RhoGEFs also contain a pleckstrin homology (PH) domain immediately C-terminal to the DH domain. In some cases [6], it has been demonstrated that the PH domain is required for

biological activity. The PH domain is probably necessary for membrane attachment and/or assisting the DH domain in guanine nucleotide exchange activity.

The first link between heterotrimeric G-proteins and the monomeric small GTPase Rho was established when p115RhoGEF was identified [7,8]. P115RhoGEF binds to both  $\alpha_{12}$  and  $\alpha_{13}$ , but, in elegant experiments using reconstitution of purified proteins, only  $\alpha_{13}$  was capable of stimulating the Rho guanine nucleotide exchange activity of p115RhoGEF [7,8]. Two additional RhoGEFs, PDZ-RhoGEF (where PDZ stands for *post synaptic density protein, discs large protein, zonula occludens*; also called KIAA0380 and GTRAP48) [9,10] and LARG (leukaemia-associated RhoGEF) [11,12], were identified as members of the p115RhoGEF family according to sequence similarity.  $\alpha_{13}$  shows little [13] or no [14] ability to stimulate the Rho guanine nucleotide exchange activity of PDZ-RhoGEF, whereas LARG remains to be tested. P115RhoGEF, PDZ-RhoGEF and LARG all contain an N-terminal regulators of G-protein signalling (RGS) domain. In p115RhoGEF [7,8], the RGS domain is not only a binding site for  $\alpha_{12}$  and  $\alpha_{13}$  but also serves as a GAP for  $\alpha_{12}$  and  $\alpha_{13}$ . More than 20 RGS proteins have been identified, and they share an approx. 120 amino acid conserved domain called the RGS box [15]. The RGS domains of p115RhoGEF, PDZ-RhoGEF and LARG show very weak identity to other RGS boxes, and crystal structures of RGS domains from p115RhoGEF [16] and PDZ-RhoGEF [17] show that these RGS domains require an additional approx. 60 amino acids to form the structural RGS domain. For these reasons, the RGS domains from the RhoGEFs have been termed RGS-like, or rgRGS for RhoGEF RGS domains.

Little is known regarding regulation of p115RhoGEF in cells. One report showed that removal of approx. 150 amino acids at the C-terminus of p115RhoGEF greatly decreases Rho guanine

Abbreviations used:  $\alpha_{13}$ QL (etc.), constitutively active  $\alpha_{13}$  (etc.); COS, cellular origin of SV-40; DH, Dbl homology; DMEM, Dulbecco's modified Eagle's medium; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HA, haemagglutinin; LARG, leukaemia-associated RhoGEF; P fraction, particulate fraction; PDZ, *post synaptic density protein, discs large protein, zonula occludens*; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; p115GFP, GFP-tagged p115RhoGEF; PM, plasma membrane; RGS, regulators of G-protein signalling; S fraction, soluble fraction; SRF, serum response factor; TP, thromboxane A<sub>2</sub> receptor; wt, wild-type.

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nucleotide exchange activity in a cell-free system. In contrast, the C-terminally truncated p115RhoGEF displays enhanced signalling in transfected cells [18]. The authors postulated that the C-terminus of p115RhoGEF binds an inhibitory protein in cells. A domain from HIV-1 gp41 was found to interact with p115RhoGEF [19], but other p115RhoGEF interacting proteins need to be discovered.

In addition to the binding of regulatory proteins, many signalling proteins are regulated by reversible changes in subcellular localization. We demonstrated previously that constitutively active  $\alpha_{13}$  ( $\alpha_{13}$ QL) induces recruitment of p115RhoGEF to the plasma membrane (PM) from the cytoplasm [20], and treatment of cells with lysophosphatidic acid can increase the membrane-bound fraction of p115RhoGEF [18]. This regulated PM targeting of p115RhoGEF is intriguing because activation of certain GPCRs causes translocation of RhoA from the cytoplasm to the PM [21,22]. Therefore it is probable that a crucial step in Rho signalling is to bring p115RhoGEF and RhoA together at cellular membranes. However, the mechanisms that control PM recruitment of p115RhoGEF have not been addressed. The purpose of the studies presented here is to test the specificity in terms of G-protein  $\alpha$  subunits capable of promoting PM recruitment of p115RhoGEF and to define crucial domains of p115RhoGEF that mediate its PM recruitment. In the present study, we show that the PM targeting of p115RhoGEF is induced specifically by  $\alpha_{12}$ QL or  $\alpha_{13}$ QL, but not by activated  $\alpha_q$ QL. Moreover, agonist activation of thromboxane  $A_2$  receptor (TP) stably expressed in HEK-293 cells promotes rapid PM recruitment of endogenous p115RhoGEF. Lastly, we demonstrate that the rgRGS domain of p115RhoGEF is necessary but not sufficient to mediate strong PM recruitment in response to activated  $\alpha_{13}$ , and show that the PH domain is required in concert with the rgRGS domain for efficient PM localization.

## EXPERIMENTAL

### Plasmids and expression vectors

The N-terminal Myc epitope (MEQKLISEED)-tagged, pEXV-Myc-p115RhoGEF, was a gift from M. J. Hart (Onyx Pharmaceuticals, Richmond, CA, U.S.A.) [23]. This cDNA encoding Myc epitope-tagged p115RhoGEF was subcloned into the mammalian expression vector pcDNA3 using the PCR. Myc epitope-tagged fragments of p115RhoGEF were amplified with an N-terminal *Hind*III site and C-terminal *Xba*I site for cloning into pcDNA3. The haemagglutinin (HA) epitope-tagged (DVPDYA) pcDNA3HA  $G\alpha_{13}$ wt (where wt stands for wild-type) and pcDNA3HA  $G\alpha_{13}$ QL were gifts from J. S. Gutkind (Oral and Pharyngeal Cancer Branch, MD, U.S.A.) [9]. The cDNA encoding non-tagged  $G\alpha_{12}$ QL in pcDNA1 was a gift from N. Dhanasekaran (Temple University, Philadelphia, PA, U.S.A.). The cDNA encoding HA epitope-tagged  $G\alpha_q$ QL in pcDNA1 was purchased from A.T.C.C. (Manassas, VA, U.S.A.) and was subcloned into pcDNA3. The reporter plasmid that expresses the luciferase gene under the control of a mutant serum response element lacking a ternary complex factor binding site, termed pSRF-Luc (where SRF stands for serum response factor), was purchased from Stratagene (La Jolla, CA, U.S.A.). The plasmid carrying the  $\beta$ -galactosidase gene (pCMV- $\beta$ -gal) was obtained from P. Tschlis (Kimmel Cancer Institute, Philadelphia, PA, U.S.A.). The expression vector for Myc epitope-tagged C3-transferase, pEF-Myc-C3-transferase, was a gift from A. Hall (University College London, London, U.K.) [24]. The correct DNA sequence of the mutants was confirmed by DNA sequencing of the entire open reading frame (Kimmel Cancer Institute Nucleic Acid Facility).

### Cell culture and transient expression

HEK-293 and cellular origin of simian virus-40 (COS) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal bovine serum, penicillin and streptomycin. HEK-293 cells stably expressing HA-tagged TP $\beta$  were kindly provided by J. Benovic (Kimmel Cancer Institute, Philadelphia, PA, U.S.A.) [25]. Unless otherwise mentioned, cells were plated in 6-well plates at  $7.0 \times 10^5$  cells/well and grown for 24 h before transfection. The total expression plasmids (1  $\mu$ g) were transfected into the cells using FuGene 6 (Roche Applied Science, Indianapolis, IN, U.S.A.). Expression of the proteins was detected by immunoblotting the lysates of the transfected cells. We used anti-HA monoclonal antibody 12CA5 (Roche Applied Science) and anti-Myc monoclonal antibody 9E10 (BabCo) to detect HA and Myc epitope-tagged proteins respectively. Polyclonal anti-p115RhoGEF antibody (anti-p115) was a gift from G. Bollag (Onyx Pharmaceutical, CA, U.S.A.), and was used to detect endogenous p115RhoGEF. Anti- $G\alpha_{12}$  polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was used to detect expression of  $G\alpha_{12}$ .

### Subcellular fractionation

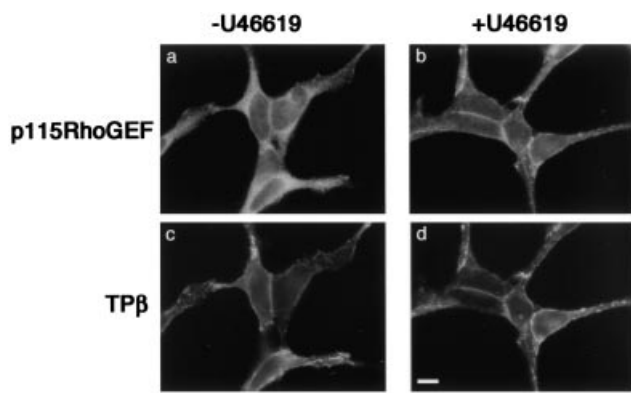
HEK-293 cells were plated at  $2 \times 10^6$  cells in 6 cm plates, grown for 24 h and transfected with 3  $\mu$ g of expression plasmids. Cells were re-plated into 10 cm plates, 24 h post-transfection. The cells were lysed 24 h later and separated into membrane-rich particulate (P) and cytoplasmic soluble (S) fractions after centrifuging the lysates at 100 000 *g* for 20 min as described earlier [20]. Equal volumes (i.e. equivalent cell fractions) of P and S fractions were loaded on SDS/PAGE for immunoblotting.

### Fluorescence microscopy

HEK-293 cells were grown on cover-slips placed in 6-well plates and were transfected with appropriate plasmids as described in the Figure legends. The cells, 24 h after transfection, were fixed with 3.7% (v/v) formaldehyde in PBS for 15 min and permeabilized by incubation in blocking buffer [2.5% (w/v) non-fat milk and 1% (v/v) Triton X-100 in Tris-buffered saline] for 30 min. Cells were then incubated with appropriate primary antibody, as mentioned in each Figure legend, in blocking buffer for 1 h. Anti-Myc monoclonal antibody 9E10 and anti-HA monoclonal antibody 12CA5 were used at 5  $\mu$ g/ml, whereas anti-p115 polyclonal anti-sera was used at a dilution of 1:100. The cells were washed with blocking buffer and incubated with a 1:100 dilution of a fluorescently labelled secondary antibody. Goat anti-mouse antibodies labelled with Alexa 488 and goat anti-rabbit antibodies labelled with Alexa 594 (Molecular Probes, Eugene, OR, U.S.A.) were used in our immunofluorescent labelling. The cover-slips were washed with 1% Triton X-100/Tris-buffered saline, rinsed in distilled water and mounted on glass slides with 10  $\mu$ l of Prolong Antifade reagent (Molecular Probes). Images were viewed with an Olympus BX60 microscope equipped with a  $\times 60$ /NA1.4 objective. Images were recorded with a Sony DKC-5000 digital camera and transferred to Adobe Photoshop for digital processing.

### SRF-mediated luciferase gene transcription assay

HEK-293 cells ( $7.0 \times 10^5$ ) were plated in 6-well plates and grown in 10% (v/v) serum-supplemented DMEM for 24 h. Cells were then switched to serum-free DMEM (with antibiotics) and were transfected, using FuGene 6, with pCMV- $\beta$ -gal (0.1  $\mu$ g), pSRF-



**Figure 1** Stimulation of TP $\beta$  induces PM recruitment of endogenous p115RhoGEF

TP $\beta$ -stable cells were grown in serum-free medium and were either not treated (a, c) or treated (b, d) with 100 nM U46619 for 2 min. Cells were fixed and simultaneously stained with anti-p115 antisera and monoclonal anti-HA antibody. Anti-p115 antibody and Alexa-488-conjugated anti-rabbit antibody detected distribution of endogenous p115RhoGEF (a, b). Anti-HA monoclonal antibody followed by Alexa-594-conjugated anti-mouse antibody-stained HA epitope-tagged TP $\beta$  (c, d). Scale bar, 10  $\mu$ m.

Luc (0.1  $\mu$ g), pcDNA3 (0.75  $\mu$ g) and other plasmids (0.05  $\mu$ g) as described in the Figure legends. The cells, 24 h after transfection were carefully washed with cold PBS and were lysed using reporter lysis buffer (Promega) according to the manufacturer's instructions. Lysates (20  $\mu$ l) were mixed at room temperature (25  $^{\circ}$ C) with 50  $\mu$ l of luciferase substrate (Promega), and luciferase activities were measured as described previously [20]. The  $\beta$ -galactosidase activities were measured by a colorimetric method as described in [20,26], and were used to normalize transfection efficiency.

#### Co-immunoprecipitation and Western-blot analysis

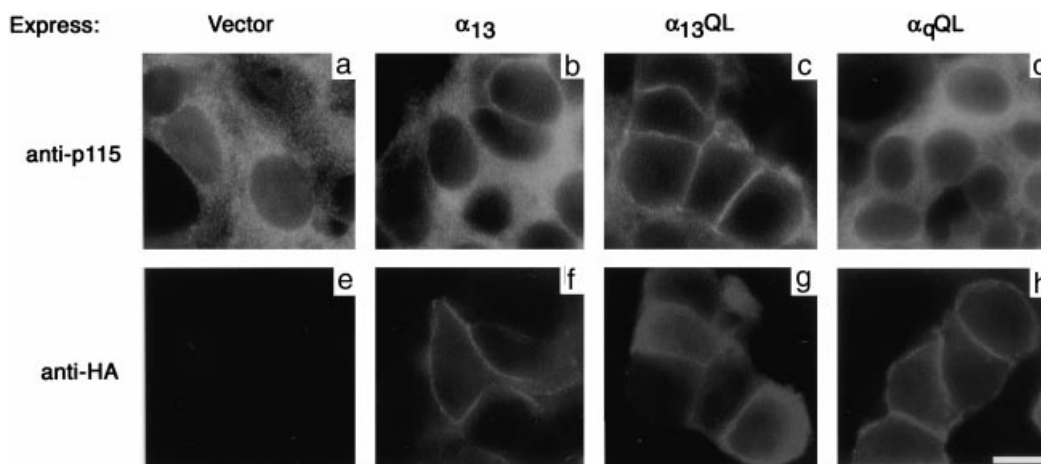
COS cells ( $2 \times 10^6$ ) were plated in 6 cm plates and grown for 24 h. The cells were transfected with plasmids carrying Myc epitope-

tagged p115 or its mutants and with HA epitope-tagged  $\alpha_{13}$ . The cells, 24 h after transfection, were re-plated into 10 cm plates and were grown for another 48 h. The cells were then washed twice with cold PBS and the cell lysates were obtained by suspending the cells in 500  $\mu$ l lysis buffer [25 mM Hepes (pH 7.5)/150 mM NaCl/2.5 mM MgCl<sub>2</sub>/1 mM EDTA/1% Triton X-100/0.5% sodium deoxycholate/1 mM PMSF/20  $\mu$ g/ml of aprotinin/20  $\mu$ g/ml of leupeptin/20 mM  $\beta$ -glycerophosphate/1 mM sodium vanadate] and placing the suspension on ice for 1 h. The suspensions were centrifuged at 1500 g for 5 min at 4  $^{\circ}$ C. The resultant supernatants were divided into two aliquots. One aliquot was treated with 20  $\mu$ M AlCl<sub>3</sub> and 20 mM NaF (+AlF<sub>4</sub><sup>-</sup>) and the other part was untreated (-AlF<sub>4</sub><sup>-</sup>). Myc epitope-tagged proteins were immunoprecipitated from treated and untreated cell lysates by incubating with anti-Myc monoclonal antibody for 1 h at 4  $^{\circ}$ C. Similarly, HA epitope-tagged  $\alpha_{13}$  was immunoprecipitated by anti-HA monoclonal antibody. The immunocomplexes were recovered using Protein A/G-agarose (Santa Cruz Biotechnology) as described previously [20]. The lysates and the immunocomplexes were analysed by immunoblotting.

## RESULTS

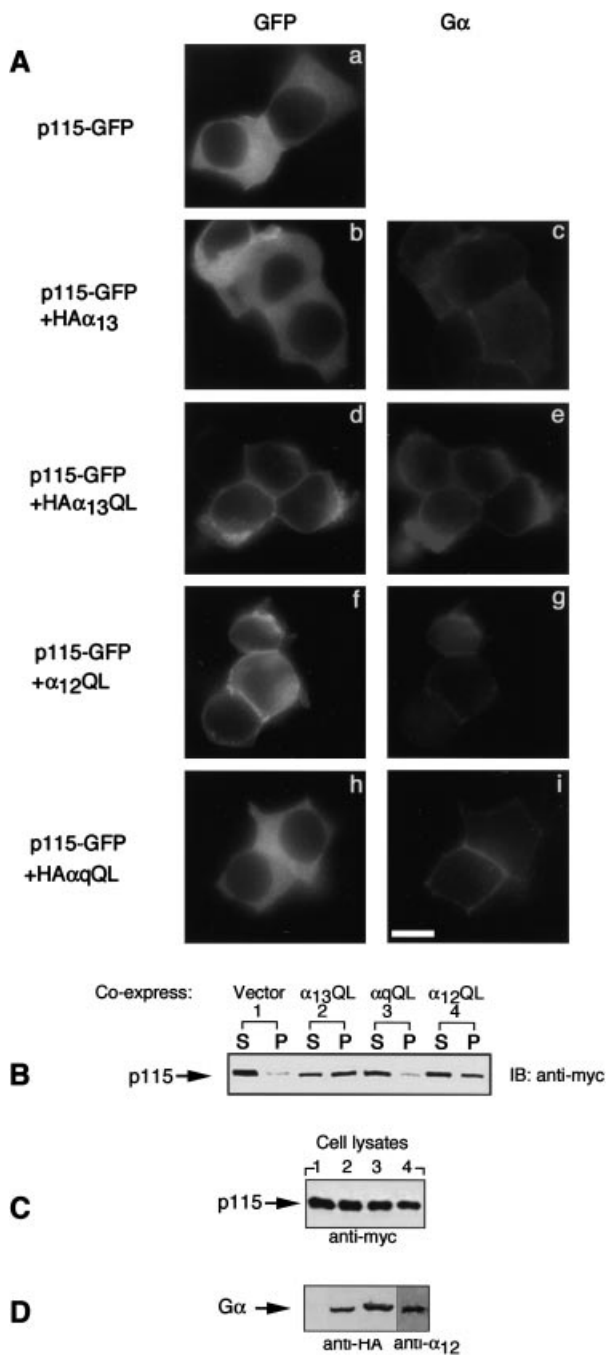
### TP activation induces PM translocation of endogenous p115RhoGEF

We reported previously that activated  $\alpha_{13}$  induces translocation of expressed p115RhoGEF to the PM of HEK-293 cells [20]. Here we examined the effect of receptor activation on subcellular localization of endogenous p115RhoGEF. TP has been demonstrated to couple with  $\alpha_{13}$  [27], in addition to  $\alpha_q$  [28] and  $\alpha_i$  [29]. In these experiments, we utilized HEK-293 cells stably expressing HA epitope-tagged TP $\beta$  isoform [25]. Subcellular distribution of endogenous p115RhoGEF in TP $\beta$ -stable cells was detected by immunofluorescence microscopy after staining with a previously described polyclonal antisera, anti-p115 [23]. Anti-p115 successfully detected endogenous p115RhoGEF in an immunoblot of HEK-293 cell lysates (results not shown) and by immunofluorescence microscopy of fixed cells (Figures 1 and 2). p115RhoGEF was distributed throughout the cytoplasm of TP $\beta$ -stable cells (Figure 1a). When TP $\beta$ -stable cells were treated



**Figure 2** Immunofluorescence microscopy of endogenous p115RhoGEF

HEK-293 cells were transfected either with vector alone (a, e) or with an expression plasmid encoding wt  $\alpha_{13}$  (b, f),  $\alpha_{13}$ QL (c, g) or  $\alpha_q$ QL (d, h). Cells were subjected to immunofluorescence microscopy, as described in the Experimental section, after probing simultaneously with anti-p115 polyclonal antisera (a-d) and anti-HA monoclonal antibody (e-h). Scale bar, 10  $\mu$ m.



**Figure 3** PM translocation of p115RhoGEF is driven by  $\alpha_{12}$ QL and  $\alpha_{13}$ QL, but not by  $\alpha_q$ QL

(A) A plasmid expressing C-terminal GFP-tagged p115RhoGEF (p115GFP) (0.1  $\mu$ g) was transiently transfected in HEK-293 cells, together with pcDNA3 alone (a) or with 0.5  $\mu$ g each of pcDNA3 encoding wt  $\alpha_{13}$  (b, c),  $\alpha_{13}$ QL (d, e),  $\alpha_{12}$ QL (f, g) or  $\alpha_q$ QL (h, i). The cells, 24 h after transfection, were processed for immunofluorescence, as described in the Experimental section. Localization of p115RhoGEF was determined by visualization of GFP (a, b, d, f, h) using fluorescence microscopy. Cells were probed with anti-HA monoclonal antibody to detect distribution of HA epitope-tagged  $\alpha_{13}$  (c),  $\alpha_{13}$ QL (e) and  $\alpha_q$ QL (i). Anti-G $\alpha_{12}$  polyclonal antibody was used to detect the distribution of  $\alpha_{12}$ QL (g). (B) HEK-293 cells expressing Myc epitope-tagged p115RhoGEF (p115) alone (vector) or with  $\alpha_{13}$ QL,  $\alpha_q$ QL or  $\alpha_{12}$ QL, were lysed and separated into P and S fractions as described in the Experimental section. The P and the S fractions were immunoblotted (IB), using anti-Myc monoclonal antibody to detect distribution of p115RhoGEF. (C) Cell lysates from (B) were immunoblotted and probed with anti-Myc antibody to detect expression of p115RhoGEF. (D) Cell lysates from (B) were immunoblotted and probed with anti-HA monoclonal antibody (lanes 1–3) or with anti- $\alpha_{12}$  polyclonal antibody (lane 4) to confirm expression of G $\alpha$  subunits. Scale bar, 10  $\mu$ m.

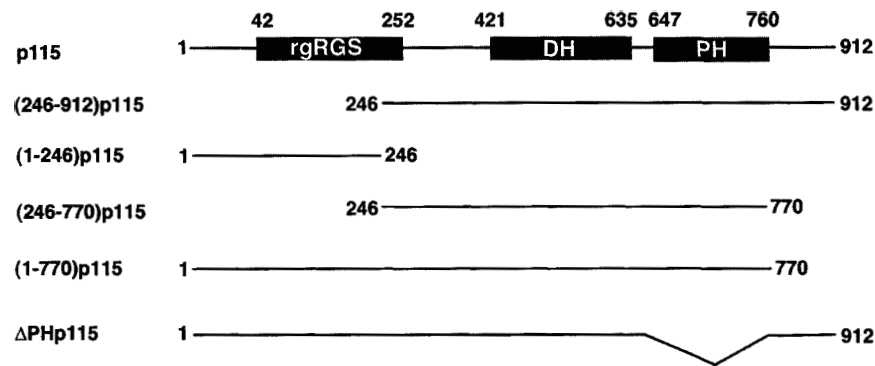
with the TP agonist U46619 for 2 min, endogenous p115RhoGEF was recruited to the PM, as visualized by sharp staining at the cell periphery (Figure 1b). TP $\beta$  was detected at the PM (Figure 1c), and this localization was relatively unchanged after brief agonist treatment (Figure 1d), as expected [30]. In naïve HEK-293 cells, which have little or no TP [31], endogenous p115RhoGEF remained in the cytoplasm regardless of TP agonist treatment (results not shown). To our knowledge, this is the first demonstration by immunofluorescence microscopy of PM recruitment of p115RhoGEF in response to GPCR activation.

#### Activated $\alpha_{12}$ and $\alpha_{13}$ , but not $\alpha_q$ induce PM translocation of p115RhoGEF

TP couples with both  $\alpha_{12/13}$  [32] and  $\alpha_q$  families of G-protein  $\alpha$  subunits. We therefore examined the effect of individual  $\alpha$  subunits on activation-dependent PM recruitment of p115RhoGEF.  $\alpha_q$ ,  $\alpha_{12}$  and  $\alpha_{13}$  can all stimulate Rho-mediated signalling [26,33–35] but only  $\alpha_{12}$  and  $\alpha_{13}$  have been shown to interact with p115RhoGEF [8]. Furthermore,  $\alpha_{13}$ , but not  $\alpha_{12}$  or  $\alpha_q$ , can directly activate p115RhoGEF [7]. We tested the ability of constitutively active forms of  $\alpha_{13}$ ,  $\alpha_{12}$  and  $\alpha_q$  to promote PM recruitment of endogenous p115RhoGEF or a green fluorescent protein (GFP)-tagged version of p115RhoGEF (p115GFP). Endogenous p115RhoGEF appears to be distributed throughout the cytoplasm (Figure 2a) with no detectable staining at the cell periphery or at the nucleus. Moreover, endogenous p115RhoGEF was found almost exclusively in the S fraction after subcellular fractionation (results not shown). Endogenous p115RhoGEF displayed cytoplasmic distribution (Figure 2b) when wt  $\alpha_{13}$  (Figure 2f) was transfected in HEK-293 cells but showed sharp PM staining (Figure 2c) when  $\alpha_{13}$ QL (Figure 2g) was expressed. Similarly, expression of  $\alpha_{12}$ QL induced strong PM staining of endogenous p115RhoGEF (results not shown). Expression of  $\alpha_q$ QL did not alter the predominant cytoplasmic distribution of endogenous p115RhoGEF in HEK-293 cells (Figure 2d). Expressed G $\alpha$  subunits, when co-stained with anti-HA monoclonal antibody, were predominantly PM associated (Figures 2f–2h), as expected.

Similar results were obtained when p115GFP was visualized. p115GFP was diffusely distributed throughout the cytoplasm and not detected at the PM when expressed alone (Figure 3A, a) or when co-expressed with wt  $\alpha_{13}$  (Figure 3A, b). However, p115GFP was detected predominantly at the PM when expressed with constitutively active  $\alpha_{13}$ QL (Figure 3A, d). Strong PM localization of p115GFP was also observed in viable HEK-293 and NIH3T3 cells when co-expressed with  $\alpha_{13}$ QL (results not shown). Expression of  $\alpha_{12}$ QL also induced PM localization of p115GFP (Figure 3A, f). In contrast, p115GFP remained in the cytoplasm when expressed with  $\alpha_q$ QL (Figure 3A, h). Over-expressed  $\alpha_{13}$ wt,  $\alpha_{13}$ QL,  $\alpha_{12}$ QL and  $\alpha_q$ QL were all localized to the PM (Figures 3A, c, e, g and i).

The fluorescence microscopy observations were confirmed by performing a fractionation analysis. A Myc epitope-tagged p115RhoGEF (p115) was expressed with empty vector,  $\alpha_{13}$ QL,  $\alpha_q$ QL or  $\alpha_{12}$ QL. Cells were lysed and separated into S and membrane-rich P fractions by high-speed centrifugation. The distribution of p115RhoGEF was determined by immunoblotting with anti-Myc antibody. p115 was detected almost exclusively in the S fractions when expressed with empty vector (Figure 3B, lanes 1S and 1P) or with  $\alpha_q$ QL (Figure 3B, lanes 3S and 3P). However, a significant amount of Myc-p115 was distributed into the P fractions when expressed with  $\alpha_{13}$ QL (Figure 3B, lanes 2S and 2P), as expected [20], or with  $\alpha_{12}$ QL (Figure 3B, lanes 4S and 4P). Thus both fluorescence microscopy and subcellular fraction-



**Figure 4** Constructs of p115RhoGEF used in this study

Schematic representation of p115RhoGEF and deletion mutants utilized in the experiments. All constructs contain a Myc epitope tag at their N-terminus. Amino acid numbering and the location of the critical domains are indicated. The rgRGS domain is defined by structural analysis [16], whereas the DH and PH domains are defined by sequence comparisons [23] and functional analysis [18].

ation demonstrate that  $\alpha_{13}$ QL and  $\alpha_{12}$ QL, but not  $\alpha_4$ QL, promote recruitment of p115RhoGEF from the cytoplasm to the PM.

#### Analysis of domains required for $\alpha_{13}$ -mediated PM translocation of p115RhoGEF

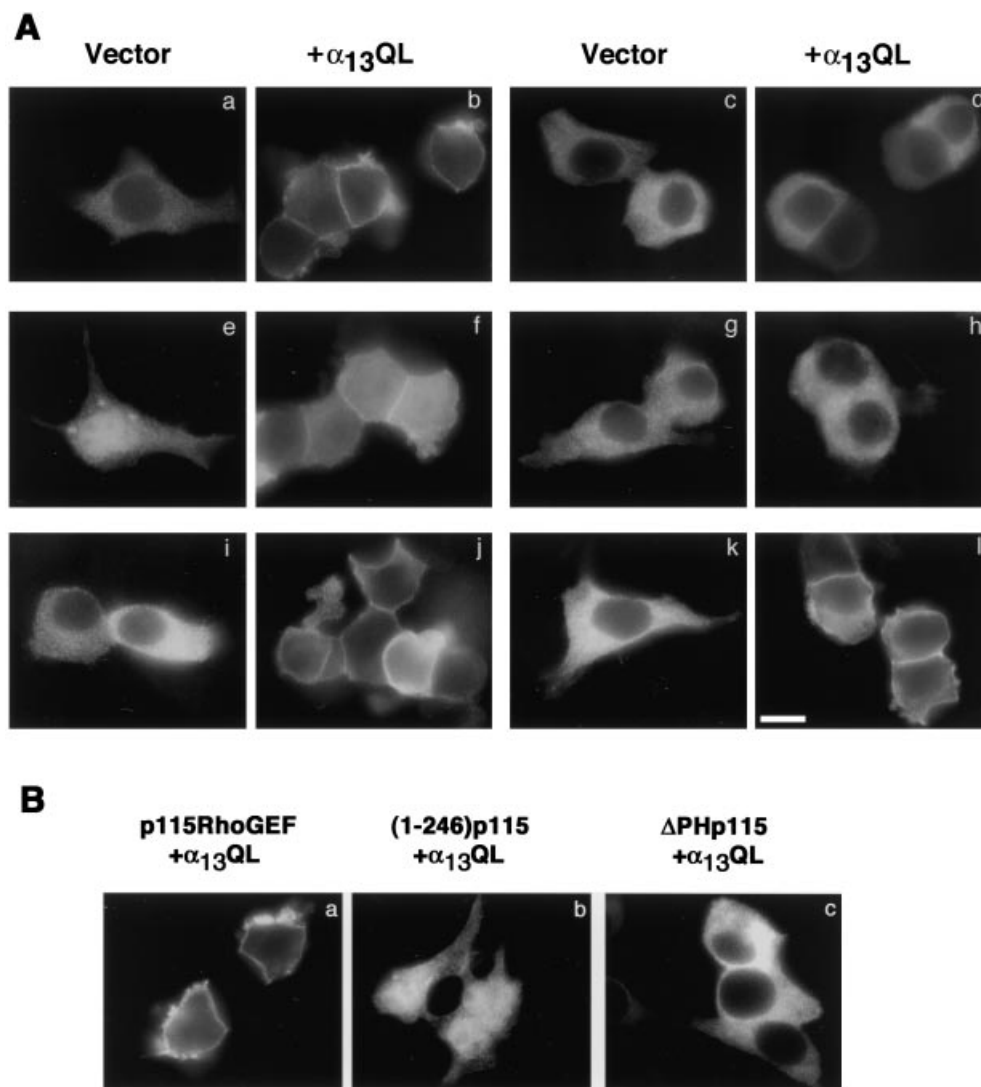
To identify the domain(s) responsible for p115RhoGEF's regulated PM targeting, we generated several deletion mutants of p115RhoGEF (Figure 4). The mutants were expressed in HEK-293 cells with or without  $\alpha_{13}$ QL and examined by immunofluorescence microscopy (Figure 5) and subcellular fractionation (Figure 6A). All mutants and  $\alpha_{13}$ QL were expressed to similar extents (Figures 6B and 6C). Full-length p115RhoGEF was detected almost exclusively in the cytoplasm (Figure 5A, a) and the S fraction (Figure 6A) [20]. When expressed with  $\alpha_{13}$ QL, p115RhoGEF showed a predominant PM distribution (Figures 5A, b and 5B, a) and a substantial shift (40–50%) into the P fraction (Figure 6A), as was seen earlier [20].

First, the importance of the rgRGS domain [16] of p115RhoGEF was examined by deletion of the rgRGS domain to create (246–912)p115 (Figure 4) and by creating a construct consisting primarily of the rgRGS domain, (1–246)p115 (Figure 4). (246–912)p115 was entirely cytoplasmic and soluble when expressed without (Figures 5A, c and 6A) or with  $\alpha_{13}$ QL (Figures 5A, d and 6A). The rgRGS domain alone, (1–246)p115, was detected in the cytoplasm and nucleus when expressed alone (Figure 5A, e). The nuclear localization of (1–246)p115 is probably due to its small size. (1–246)p115 was almost entirely in the S fraction when assayed by subcellular fractionation (Figure 6A). (1–246)p115 showed both cytoplasmic and some PM distribution (Figure 5A, f) when co-expressed with  $\alpha_{13}$ QL. Compared with full-length p115RhoGEF, (1–246)p115 displayed much weaker recruitment to the PM by  $\alpha_{13}$ QL (cf. Figures 5A, b and f). In fact,  $\alpha_{13}$ QL-induced PM recruitment of (1–246)p115 was only observed when very low amounts of (1–246)p115-expressing DNA (0.05  $\mu$ g) were transfected. When 10-fold more DNA (0.5  $\mu$ g) was co-transfected with  $\alpha_{13}$ QL (Figure 5B, b), (1–246)p115 showed very little or no PM recruitment, whereas PM recruitment of full-length p115 was still strongly seen when a higher amount of DNA was transfected (Figure 5B, a). The relative expression levels of full-length p115RhoGEF or (1–246)p115 were similar when compared with each other for either the

low or high amount of transfected DNA (results not shown, Figure 6B). Moreover, (1–246)p115 showed very weak or no ability to partition into the P fraction when co-expressed with  $\alpha_{13}$ QL (Figure 6A), providing further evidence that the rgRGS domain alone is not sufficient for  $\alpha_{13}$ QL-mediated membrane recruitment. The lack of association with the P fraction when (1–246)p115 is co-expressed with  $\alpha_{13}$ QL is also consistent with a weak PM association that is disrupted by the dilution caused by cell lysis. Thus the results described above indicate that the 1–246 rgRGS region appears to be required for  $\alpha_{13}$ QL-promoted PM translocation. However, the rgRGS region by itself is not sufficient to mediate strong PM translocation.

Next we examined the C-terminal region of p115RhoGEF. This region has been proposed to regulate activity of p115RhoGEF possibly via interaction with inhibitory cellular proteins [18]. A mutant of p115RhoGEF, lacking both the rgRGS and C-terminal domains, (246–770)p115, and another mutant lacking only the C-terminus, (1–770)p115 (Figure 4), were entirely cytoplasmic and soluble when expressed alone (Figures 5A, g, i and 6A). (246–770)p115 remained distributed throughout the cytoplasm and remained in the S fraction when expressed with  $\alpha_{13}$ QL (Figures 5A, h and 6A). However, a majority of (1–770)p115 was detected at the PM when co-expressed with  $\alpha_{13}$ QL (Figure 5A, j), and (1–770)p115 displayed a shift to the P fraction (Figure 6A). These results suggest that the C-terminal region (amino acids 771–912) is not involved in mediating PM recruitment of p115RhoGEF.

Finally, we expressed ΔPHp115 (Figure 4), in which the entire PH domain (amino acids 647–760) was internally deleted. PH domains often mediate membrane localization by binding membrane lipids and other proteins [36]. ΔPHp115 displayed cytoplasmic distribution (Figure 5A, k) and partitioned into the S fraction (Figure 6A) when expressed in HEK-293 cells. When expressed together with  $\alpha_{13}$ QL, ΔPHp115 showed both cytoplasmic and weak PM distribution (Figure 5A, l). ΔPHp115 displayed a much reduced ability, compared with full-length p115RhoGEF, to localize to the PM when a low amount (0.05  $\mu$ g) of ΔPHp115-expressing DNA was co-transfected with  $\alpha_{13}$ QL. Similar to (1–246)p115, no PM staining was observed when 10-fold more DNA encoding ΔPHp115 was co-expressed with  $\alpha_{13}$ QL (Figure 5B, c). In agreement with the decreased PM recruitment, ΔPHp115 remained almost exclusively in the S fraction when expressed with  $\alpha_{13}$ QL, showing a very weak



**Figure 5 Immunofluorescence microscopy of p115RhoGEF and its mutants**

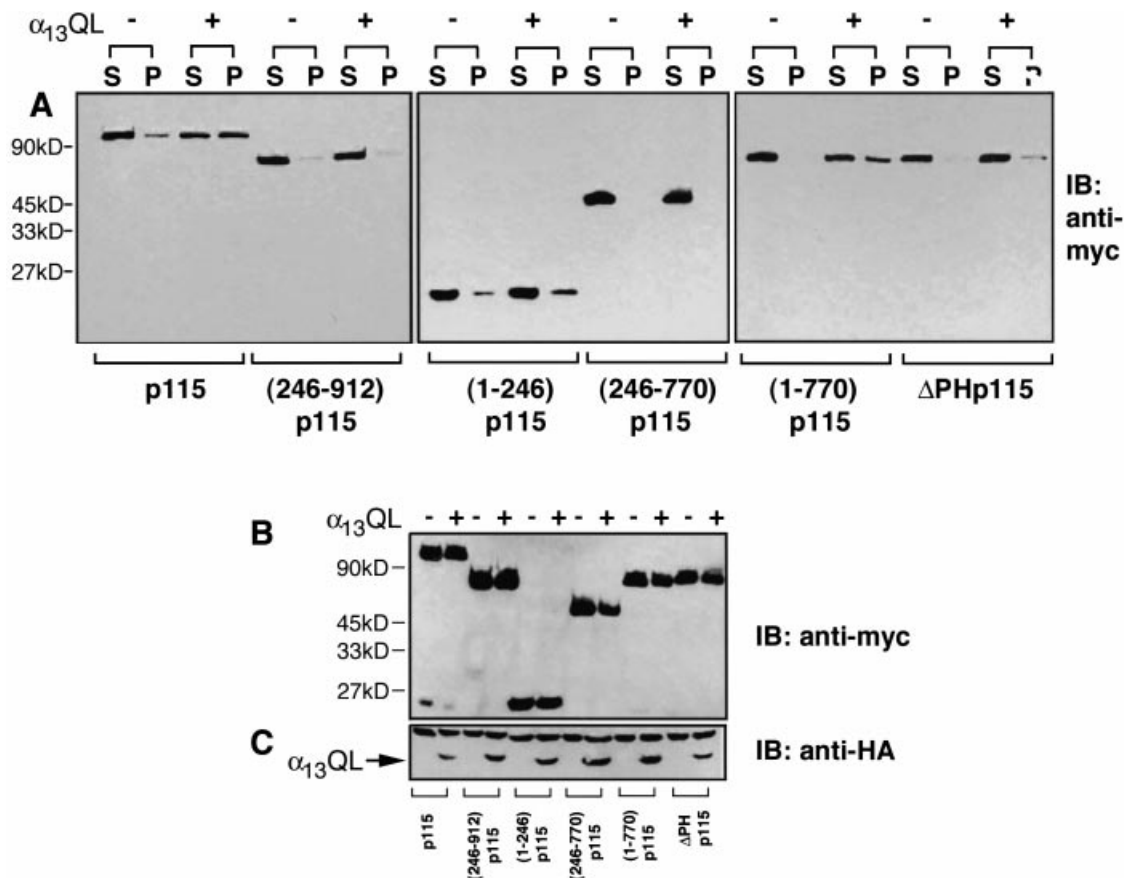
(A) HEK-293 cells were transiently transfected with 0.05  $\mu$ g expression plasmids for p115RhoGEF (a, b), (246–912)p115 (c, d), (1–246)p115 (e, f), (246–770)p115 (g, h), (1–770)p115 (i, j) or  $\Delta$ PHp115 (k, l) without (a, c, e, g, i, k) or with (b, d, f, h, j, l) co-transfecting 0.5  $\mu$ g DNA encoding  $\alpha_{13}$ QL. Cells, 24 h after transfection, were fixed and processed for immunofluorescence as described in the Experimental section. An anti-Myc monoclonal antibody followed by an Alexa-488-conjugated anti-mouse antibody was used to detect distribution of p115RhoGEF and its mutants. An anti-HA polyclonal antibody and an Alexa-594-conjugated anti-rabbit antibody detected cells expressing HA epitope-tagged  $\alpha_{13}$ QL (results not shown). Only cells expressing  $\alpha_{13}$ QL (results not shown) were chosen to identify distribution of p115RhoGEF and its mutants in  $\alpha_{13}$ QL co-expression experiments. More than 100 cells were examined in at least three separate experiments and representative images are presented. Scale bar, 10  $\mu$ m. (B) Expression plasmids (0.5  $\mu$ g) for p115RhoGEF (a), (1–246)p115 (b) or  $\Delta$ PHp115 (c) were transfected in HEK-293 cells with 0.5  $\mu$ g of DNA encoding  $\alpha_{13}$ QL. Cells were fixed after 24 h transfection and processed for immunofluorescence microscopy as described above.

partitioning to the P fraction (Figure 6A). These results suggest that the PH domain participates in mediating regulated PM localization of p115RhoGEF. Overall, the results with the various deletion mutants indicate that both the rgRGS and the PH domains play important roles in  $\alpha_{13}$ -mediated PM translocation of p115RhoGEF.

#### Interaction of $\alpha_{13}$ with p115RhoGEF and its mutants

For most RGS proteins, an approx. 120 amino acid RGS core domain mediates binding to G $\alpha$ . Moreover, some RGS core domains have been shown to bind preferentially AIF $_4^-$ -activated G $\alpha$ , a condition that experimentally mimics the transition state

of GTP hydrolysis [37–39]. Previously, we demonstrated that  $\alpha_{13}$  co-immunoprecipitates with p115RhoGEF from cell lysates treated with AIF $_4^-$  [20]. We now tested the ability of the mutants of p115RhoGEF (Figure 4) to interact with  $\alpha_{13}$ , as measured by AIF $_4^-$ -dependent co-immunoprecipitation. Co-immunoprecipitations were performed in a reciprocal manner. Immunoprecipitation with anti-Myc antibody tested the ability of p115RhoGEF or the various deletion mutants to ‘pull down’  $\alpha_{13}$  (Figure 7A), whereas immunoprecipitation using anti-HA antibody tested the ability of  $\alpha_{13}$  to ‘pull down’ p115RhoGEF or its mutants (Figure 7B). Full-length p115RhoGEF co-immunoprecipitated with  $\alpha_{13}$  from cell lysates treated with AIF $_4^-$  (+) only (Figures 7A and 7B). However, (246–912)p115 did not co-



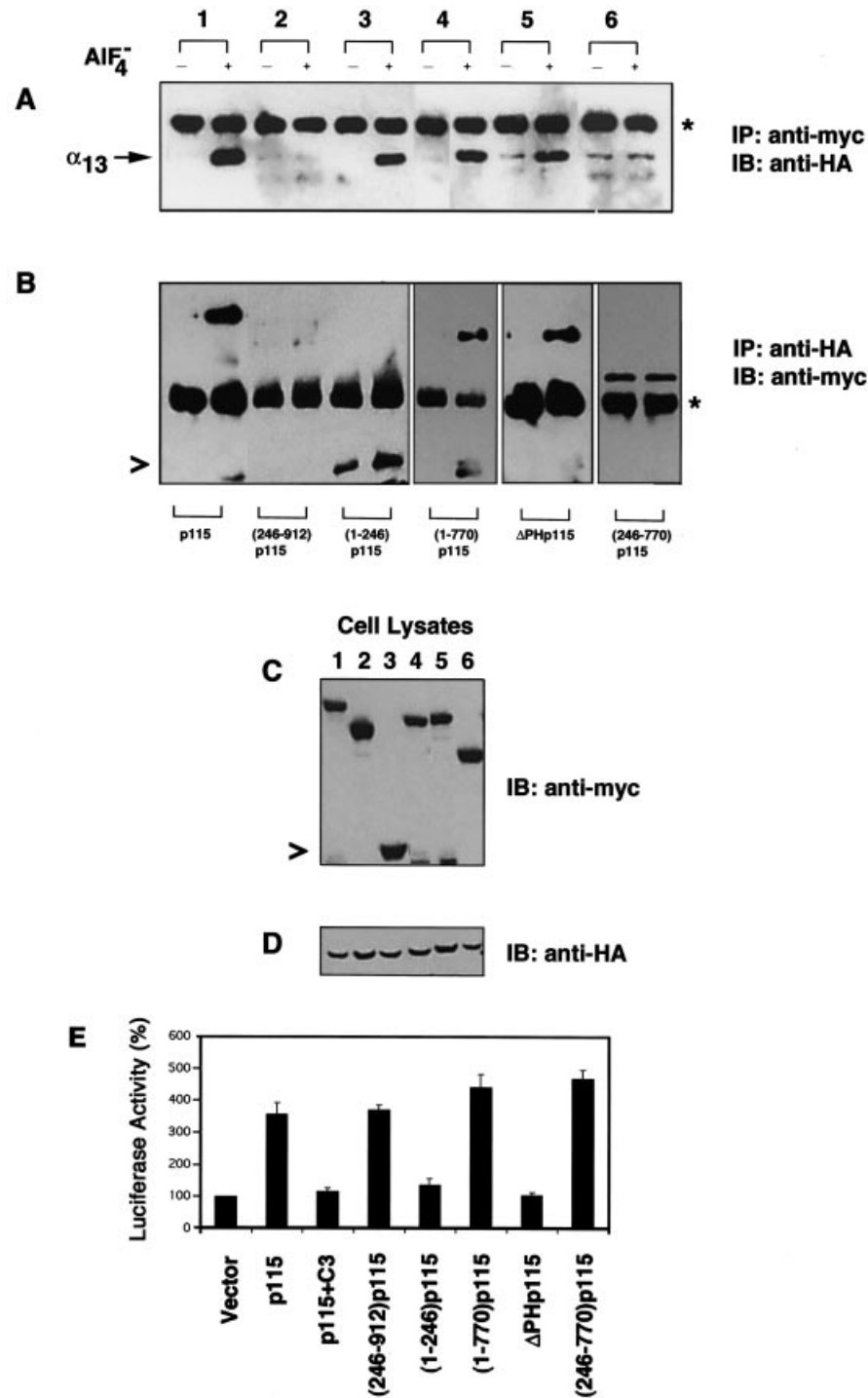
**Figure 6** Fractionation of cells expressing p115RhoGEF and its mutants

(A) HEK-293 cells were transiently transfected with plasmids encoding Myc epitope-tagged p115RhoGEF, (246–912)p115, (1–246)p115, (246–770)p115, (1–770)p115 or ΔPHp115, without (–) or with (+) co-transfection of α<sub>13</sub>QL, as indicated. Cells were lysed and separated into S and P fractions, as described in the Experimental section. The fractions were immunoblotted (IB) with an anti-Myc monoclonal antibody. (B) Expression of p115 and its mutants in the corresponding cell lysates was determined by probing with anti-Myc monoclonal antibody. (C) Expression of α<sub>13</sub>QL detected by probing lysates with anti-HA monoclonal antibody.

immunoprecipitate with α<sub>13</sub> (Figures 7A and 7B), as expected since it lacks the rgRGS domain. The rgRGS domain of the mutant, (1–246)p115, interacted well with activated α<sub>13</sub> in an AIF<sub>4</sub><sup>–</sup>-dependent manner (Figures 7A and 7B). Interestingly, (1–246)p115 also showed a consistent interaction with α<sub>13</sub> in the absence of AIF<sub>4</sub><sup>–</sup> (Figure 7B), although this was only observed when the anti-HA antibody was used to immunoprecipitate α<sub>13</sub>. The reason for this higher basal interaction is unclear. Others have also observed considerable co-immunoprecipitation of α<sub>13</sub> and the rgRGS domain of p115RhoGEF in the absence of AIF<sub>4</sub><sup>–</sup> [40]. Nonetheless, co-immunoprecipitation of α<sub>13</sub> and (1–246)p115 was always increased by the addition of AIF<sub>4</sub><sup>–</sup> (Figures 7A and 7B). The C-terminal deletion mutant (1–770)p115 and ΔPHp115 co-immunoprecipitated with AIF<sub>4</sub><sup>–</sup>-activated (+) α<sub>13</sub> (Figures 7A and 7B) to an extent very similar to that of wt p115 (Figures 7A and 7B). Although (1–246)p115 and ΔPHp115 showed impaired α<sub>13</sub>QL-mediated PM translocation (Figures 5 and 6), they both appear to interact well with activated α<sub>13</sub> (Figures 7A and 7B). These results thus indicate that the regulated PM association requires more than just binding to α<sub>13</sub>. Interestingly, (246–770)p115, lacking both the rgRGS and the C-terminal domains, displayed weak but consistent co-immunoprecipitation with α<sub>13</sub> both in the absence (–) or presence

(+) of AIF<sub>4</sub><sup>–</sup>, suggesting the presence of a secondary non-RGS binding domain for α<sub>13</sub>, in agreement with a previous study [40].

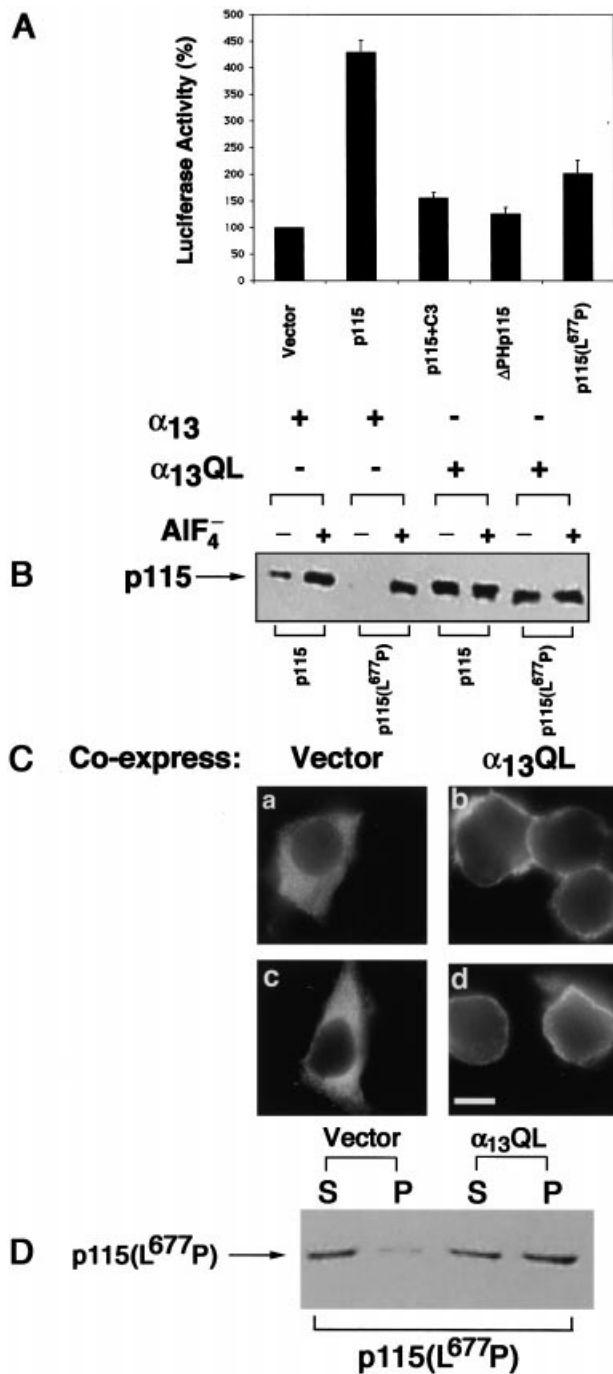
We confirmed that all mutants with intact tandem DH/PH domains were capable of Rho-dependent signalling (Figure 7E), using a transient transfection assay employing a reporter plasmid containing a luciferase gene under the control of a serum response element that binds to the SRF [20,26]. Expression of full-length p115RhoGEF (p115) stimulated SRF-mediated transcription, and this was blocked by C3 transferase (Figure 7E). C3 transferase is an exoenzyme of *Clostridium botulinum* which inhibits Rho GTPase by ADP-ribosylating a critical amino acid (Asn<sup>41</sup>) in the effector binding region and, thus, it prevents effector interaction and downstream signalling [41]. Inhibition of p115RhoGEF-induced SRF-mediated transcription by C3 transferase confirms that p115RhoGEF activation of SRF-mediated gene transcription is Rho-dependent. The DH/PH domain-containing mutants (246–912)p115, (246–770)p115 and (1–770)p115 all promoted SRF-mediated transcription (Figure 7E), very similar to full-length p115RhoGEF. As expected, (1–246)p115 did not stimulate SRF-mediated transcription (Figure 7E). ΔPHp115 also did not induce SRF-mediated transcription (Figure 7E), indicating that an intact PH domain is required for Rho signalling. α<sub>13</sub>QL stimulated SRF-mediated transcription, but we



**Figure 7** Co-immunoprecipitation of p115RhoGEF and its mutants with  $\alpha_{13}$

COS cells were transfected with plasmids encoding Myc epitope-tagged p115RhoGEF, (246–912)p115, (1–246)p115, (1–770)p115,  $\Delta$ PHp115 or (246–770)p115 along with a plasmid encoding HA epitope-tagged  $\alpha_{13}$ . Cells were lysed as described in the Experimental section and were either treated (+) or untreated (–) with  $\text{AIF}_4^-$ . Both treated (+) and untreated (–) lysates were subjected to immunoprecipitation (IP) either by using anti-Myc monoclonal antibody to precipitate p115RhoGEF and its mutants (**A**), or by using anti-HA monoclonal antibody to precipitate  $\alpha_{13}$  (**B**). The precipitates were immunoblotted (IB) with anti-HA monoclonal (**A**) and with anti-Myc monoclonal (**B**) antibodies. Bands marked with (\*) represent immunoglobulins precipitated from each immunoprecipitation. In (**B**, **C**), the arrowhead indicates the position of (1–246)p115. All other p115RhoGEF mutants are located above the immunoglobulin band. (**C**) The corresponding total cell lysates were immunoblotted and probed with anti-Myc monoclonal antibody to compare expression levels of p115RhoGEF (lane 1), (246–912)p115 (lane 2), (1–246)p115 (lane 3), (1–770)p115 (lane 4),  $\Delta$ PHp115 (lane 5) and (246–770)p115 (lane 6). (**D**) Cell lysates were also immunoblotted with anti-HA monoclonal antibody to compare expression levels of  $\alpha_{13}$  (lanes 1–6) in each sample. (**E**) SRF-mediated gene transcription by p115RhoGEF and its deletion mutants. pSRF-Luc and pCMV- $\beta$ -Gal plasmids were transfected into HEK-293 cells along with pDNA3 (Vector) or expression vectors for the indicated proteins. Cells were lysed 24 h after transfection and cell extracts were assayed for luciferase activity and for  $\beta$ -galactosidase, as described in the Experimental section. Luciferase activities were normalized by  $\beta$ -galactosidase activity present in each lysate. Results are expressed as percentage activity with respect to control cells (Vector), and are the means  $\pm$  S.E.M. of triplicate transfections. Similar results were obtained in three other independent experiments.





**Figure 8** An L677P mutation in the PH domain of p115RhoGEF severely reduces its activity but retains  $\alpha_{13}$ QL-mediated PM targeting and binding to  $\alpha_{13}$

(A) pSRF-Luc and p-CMV- $\beta$ -galactosidase plasmids were co-transfected in HEK-293 cells along with pcDNA3 (Vector) or with plasmids encoding p115RhoGEF,  $\Delta$ PHp115 or p115(L<sup>677</sup>P). Cells were lysed 24 h after transfection and luciferase activity and  $\beta$ -galactosidase activity were measured as described in the Experimental section, and the activities were plotted as described in Figure 7. Data are expressed as percentage activity with respect to control cells (Vector), and are the means  $\pm$  S.E.M. of triplicate transfections. (B) COS cells co-expressing p115 or p115(L<sup>677</sup>P) along with wt  $\alpha_{13}$  or with  $\alpha_{13}$ QL. HA epitope-tagged  $\alpha_{13}$  and  $\alpha_{13}$ QL were immunoprecipitated from cell lysates and were either not treated (-) or treated (+) with AIF<sub>4</sub><sup>-</sup>, and immunoblotted using an anti-Myc monoclonal antibody. (C) HEK-293 cells were transfected with plasmids encoding Myc epitope-tagged p115 (a, b) or p115(L<sup>677</sup>P) (c, d) along with either pcDNA3 (Vector) (a, c) or with a plasmid encoding HA epitope-tagged  $\alpha_{13}$ QL ( $\alpha_{13}$ QL) (b, d). Cells were processed for indirect immunofluorescence 24 h after transfection,

were not able to observe reproducible synergy between expressed  $\alpha_{13}$ QL and p115RhoGEF (results not shown).

#### A signalling defective PH domain mutant of p115RhoGEF retains $\alpha_{13}$ -mediated PM recruitment

The results described so far indicate an important role for the PH domain in mediating PM recruitment of p115RhoGEF; however, the observed defect in  $\alpha_{13}$ -mediated PM translocation of  $\Delta$ PHp115 could be due to an inability to stimulate Rho signalling (Figure 7E). To test the requirement for Rho signalling, we analysed a novel PH domain mutation in which the leucine residue at position 677 was changed to proline. This mutation was fortuitously identified during mutagenesis of p115RhoGEF (R. Bhattacharyya and P. B. Wedegaertner, unpublished work). Similar to  $\Delta$ PHp115, p115L677P showed a greatly decreased ability to stimulate SRF-mediated gene transcription (Figure 8A) and displayed strong co-immunoprecipitation with both AIF<sub>4</sub><sup>-</sup>-activated  $\alpha_{13}$  and with  $\alpha_{13}$ QL (Figure 8B). However, in contrast with  $\Delta$ PHp115, the point mutant p115L677P displayed strong  $\alpha_{13}$ QL-mediated PM recruitment (Figure 8C, d), very similar to the wt p115 (Figures 8C, b and 5B, a). Subcellular fractionation also revealed that p115L677P is distributed predominantly into the S fraction when expressed alone but partitioned to the P fraction when co-expressed with  $\alpha_{13}$ QL (Figure 8D). Thus p115L677P retains  $\alpha_{13}$ QL-mediated PM recruitment although its signalling function is defective. By inference, the PM recruitment defect of  $\Delta$ PHp115 is not solely due to loss of signalling function. The above observations suggest that mutation of Leu<sup>677</sup> to Pro can separate PH domain effects on Rho-dependent signalling from PH domain effects on regulated PM recruitment.

#### DISCUSSION

A variety of cell-surface GPCRs that couple with  $\alpha_{12/13}$  and/or  $\alpha_q$  can initiate Rho-dependent signalling, although the mechanisms of Rho activation are not yet well understood. p115RhoGEF appears to link  $\alpha_{13}$  to Rho directly. Activated  $\alpha_{13}$  can stimulate the RhoGEF activity of p115RhoGEF [7], and p115RhoGEF can be recruited to the PM by constitutively active  $\alpha_{13}$ QL [20]. In the present study, we demonstrated that endogenous p115RhoGEF is also recruited to the PM on stimulation of TP $\beta$ , a GPCR known to couple with  $\alpha_{13}$ . We further characterized the PM recruitment of p115RhoGEF by demonstrating that  $\alpha_{12}$ QL and  $\alpha_{13}$ QL, but not  $\alpha_q$ QL, promote PM targeting of p115RhoGEF. In addition, this study indicates that at least two distinct membrane-targeting signals, rgRGS domain-mediated interaction with  $\alpha_{13}$  and PH domain interaction with unknown ligands, function together to mediate  $\alpha_{13}$ -induced PM recruitment of p115RhoGEF.

#### $\alpha_{13}$ QL and $\alpha_{12}$ QL, but not $\alpha_q$ QL, promote PM targeting of p115RhoGEF

Previous studies have demonstrated that p115RhoGEF can interact directly with  $\alpha_{12}$  and  $\alpha_{13}$ , but only  $\alpha_{13}$  can stimulate the Rho guanine nucleotide exchange activity of p115RhoGEF [7,8]. In the present study, we demonstrate that a GPCR, TP $\beta$ ,

using an anti-Myc monoclonal antibody. Scale bar, 10  $\mu$ m. (D) HEK-293 cells were transfected with plasmids encoding p115(L<sup>677</sup>P) along with pcDNA3 (Vector) or plasmid encoding  $\alpha_{13}$ QL ( $\alpha_{13}$ QL). Cells were fractionated into S or P fractions as described in the Experimental section. Fractions were immunoblotted using an anti-Myc monoclonal antibody to detect Myc epitope-tagged p115(L<sup>677</sup>P).

which couples with  $\alpha_{12}$  and  $\alpha_q$  families of G-proteins, induces PM association of endogenous p115RhoGEF (Figure 1). Moreover, both  $\alpha_{12}$ QL and  $\alpha_{13}$ QL promote PM translocation of p115RhoGEF. These results indicate that the PM targeting of p115RhoGEF alone may not be sufficient for activation, since both  $\alpha_{12}$ QL and  $\alpha_{13}$ QL promote PM targeting of p115RhoGEF but only  $\alpha_{13}$ QL can stimulate its RhoGEF activity. A recent report suggested that  $\alpha_{13}$  undergoes protein kinase A-mediated phosphorylation which stabilizes coupling with TP $\beta$  and inhibits Rho activation. In contrast,  $\alpha_q$  does not undergo protein kinase A-mediated phosphorylation and therefore its coupling with TP is unaffected by protein kinase A activation [42]. This clearly suggests critical differences between  $\alpha_{13}$ - and  $\alpha_q$ -mediated cellular signalling. Our results reveal that PM recruitment of p115RhoGEF is another process specifically promoted by  $\alpha_{12}$  and  $\alpha_{13}$  but not by  $\alpha_q$ .

Activated  $\alpha_q$  and several GPCRs that couple with  $\alpha_q$  can activate Rho and Rho-dependent signalling [43–45]; however, it is not yet clear whether  $\alpha_q$  directly binds to and activates a RhoGEF. The current literature contains conflicting reports regarding physical and functional interactions of  $\alpha_q$  with different RhoGEFs [43–46]. Although  $\alpha_q$ QL can activate Rho signalling,  $\alpha_q$ QL does not induce PM recruitment of either endogenous (Figure 2) or transfected p115RhoGEF (Figure 3). In contrast,  $\alpha_{13}$ QL can promote PM translocation of both endogenous (Figure 2) and transfected (Figure 3) p115RhoGEF. These results show the specificity of regulated PM translocation of p115RhoGEF and suggest that p115RhoGEF is unlikely to mediate  $\alpha_q$ -mediated activation of Rho.

So far, we have not been able to demonstrate clearly that regulated PM recruitment of p115RhoGEF is essential for the activation of Rho signalling in response to  $\alpha_{13}$  activation. Because expression of p115RhoGEF and various deletion mutants constitutively activate Rho-dependent signalling (Figure 7E) [18,19,43,47], it is difficult to demonstrate conclusively that the expression of an activated  $\alpha_{13}$  positively regulates p115RhoGEF activity in cells. One study showed synergistic activation of SRF-mediated transcription upon co-expression of  $\alpha_{13}$ QL and p115RhoGEF [47]; however, our studies (results not shown) and those of others [43] failed to observe such synergy between  $\alpha_{13}$ QL and p115RhoGEF. Moreover, we also examined a role for PM localization in p115RhoGEF signalling by constructing a mutant p115RhoGEF-CAAX, where a 20-amino-acid CAAX box from H-Ras was fused to the C-terminus of p115RhoGEF, resulting in constitutive PM localization. The experiment revealed that p115RhoGEF-CAAX did not significantly enhance signalling compared with full-length p115RhoGEF in transfected cells (results not shown). On the other hand, it is possible that p115RhoGEF translocates to and signals at specific PM microdomains, but that p115RhoGEF-CAAX is found in different membrane microdomains. Such a possibility remains to be tested. Although a definitive demonstration of the requirement for PM recruitment of p115RhoGEF in GPCR to Rho signalling awaits a further mechanistic understanding, our observation that activation of TP promotes rapid PM recruitment of endogenous p115RhoGEF is at least consistent with physiological relevance.

#### The rgRGS domain is required but not sufficient for strong PM targeting of p115RhoGEF

RGS proteins are known to interact with activated  $\alpha$  subunits through their RGS or rgRGS core domains. Likewise, the N-terminal rgRGS domain of p115RhoGEF, amino acids 1–246, interacts with activated forms of  $\alpha_{13}$ , as assayed by co-immunoprecipitation (Figure 6) [40] and binding of purified

proteins [40]. We asked whether this rgRGS– $\alpha_{13}$  interaction was necessary and sufficient for  $\alpha_{13}$ QL-promoted PM recruitment of p115RhoGEF. When amino acids 1–246 were deleted from the N-terminus of p115RhoGEF,  $\alpha_{13}$ QL had no effect on the subcellular localization of this rgRGS domain-lacking mutant. (246–912)p115RhoGEF remained cytoplasmic as assayed by immunofluorescence microscopy (Figure 5) and soluble, based on subcellular fractionation (Figure 6), regardless of the presence of activated  $\alpha_{13}$ QL. Thus the rgRGS domain is required for  $\alpha_{13}$ QL-promoted PM targeting of p115RhoGEF.

In addition, the rgRGS domain appears to be required for  $\alpha_{13}$ QL-promoted PM recruitment of p115RhoGEF even when a potential secondary  $\alpha_{13}$  binding site on p115RhoGEF is revealed by N- and C-terminal truncation. A previous report [40] showed that a p115RhoGEF construct consisting of amino acids 288–760 strongly interacted with purified AIF $_4^-$ -activated  $\alpha_{13}$ , but not with unactivated  $\alpha_{13}$ , in spite of the lack of the rgRGS domain. However, in co-immunoprecipitation experiments (288–760)-p115RhoGEF appeared to interact less well with  $\alpha_{13}$  when compared with full-length p115RhoGEF and, importantly, the co-immunoprecipitation was activation-independent [40]. In agreement with those results, (246–770)p115RhoGEF (Figure 4) displayed a weak, but reproducible ability to co-immunoprecipitate with both active (+AIF $_4^-$ ) and inactive (–AIF $_4^-$ )  $\alpha_{13}$  (Figure 7). In spite of this interaction with  $\alpha_{13}$ , (246–770)p115RhoGEF was completely refractory to  $\alpha_{13}$ QL-promoted membrane recruitment (Figures 5 and 6). Thus the presence of this potential secondary binding site for  $\alpha_{13}$  is not sufficient to overcome the loss of the rgRGS domain and promote membrane binding.

Although the rgRGS domain is required for binding to activated  $\alpha_{13}$  and for  $\alpha_{13}$ QL-promoted PM localization, the rgRGS domain alone, (1–246)p115, displayed only limited association with membranes when expressed with  $\alpha_{13}$ QL (Figures 5 and 6). Partial PM localization of (1–246)p115 could only be observed when its DNA was transfected with  $\alpha_{13}$ QL expressing DNA at a ratio of 1:10 (Figure 5), whereas strong PM localization of full-length p115RhoGEF could be readily observed using equimolar ratio of p115RhoGEF and  $\alpha_{13}$ QL DNA. Moreover, (1–246)p115 PM localization was detected in fewer cells compared with full-length p115RhoGEF, and (1–246)p115 was less efficiently recruited to PM by  $\alpha_{13}$ QL as indicated by cytoplasmic staining and a lack of observable PM staining in cells transfected with a greater amount of DNA (Figure 5B) and the presence of substantial cytoplasmic staining in addition to PM staining in cells transfected with a lower amount of DNA (Figure 5A). Lastly, when assayed by subcellular fractionation (Figure 6), (1–246)p115 exhibited little or no recruitment to a particulate membrane fraction upon co-expression of  $\alpha_{13}$ QL. Taken together, these results indicate that the rgRGS domain of p115RhoGEF is not sufficient for strong PM localization and membrane binding in response to activated  $\alpha_{13}$ . This suggests that additional regions of p115RhoGEF mediate translocation to the PM.

#### The PH domain is required for $\alpha_{13}$ -mediated PM targeting of p115RhoGEF

Activation-induced PM targeting of a number of signalling proteins has been found to be mediated by the binding of PH domains to phosphoinositides [36,48], and PH domain-dependent membrane binding of several RhoGEFs have been reported [5,49]. Our examination of  $\Delta$ PHp115 (Figure 4), in which the PH domain is internally deleted, suggests that the PH domain in p115RhoGEF functions in regulated PM localization. By

immunofluorescence microscopy,  $\Delta$ PHp115 displayed a weak ability, only observed when relatively low amounts of DNA were transfected, to translocate to PM when co-expressed with  $\alpha_{13}$ QL (Figures 5A and 5B). Likewise, little or no increase in membrane recruitment of  $\Delta$ PHp115 when co-expressed with  $\alpha_{13}$ QL was detected by subcellular fractionation (Figure 6). These results are thus consistent with a role for the PH domain in facilitating PM localization of p115RhoGEF. Our preliminary results, however, suggest that PH domain-facilitated PM localization is not mediated by binding to phosphoinositide products of phosphoinositide 3-kinase (PI 3-kinase). The PI 3-kinase inhibitor, wortmannin, did not affect the  $\alpha_{13}$ QL-promoted PM translocation of p115RhoGEF (results not shown), and expression of a constitutively active membrane targeted PI 3-kinase failed to promote PM recruitment of p115RhoGEF (results not shown). In addition, Wells et al. [18] reported that phosphatidylinositol 4,5-bisphosphate had no effect on the activity of p115RhoGEF. The identification of relevant lipid or protein ligands for the PH domain will be important for further understanding the mechanisms of p115RhoGEF regulation and subcellular localization.

The PH domain of p115RhoGEF is apparently not sufficient by itself to mediate membrane localization. In the absence of  $\alpha_{13}$  activation, endogenous p115RhoGEF, Myc-tagged p115RhoGEF and GFP-tagged p115RhoGEF viewed in viable cells all appeared to be distributed throughout the cytoplasm with no detectable PM localization. Furthermore, p115RhoGEF is a soluble protein as determined by fractionation of HEK-293 cell lysates. In our experiments, deletions from the N- and C-termini, which might enhance the accessibility of the PH domain to potential ligands, failed to reveal any membrane localization of p115RhoGEF in the absence of  $\alpha_{13}$  activation. In contrast, another study found a substantial amount of overexpressed p115RhoGEF or various mutants in the membrane fraction [18], although the subcellular localization of this P fraction was not identified by microscopy. Whether this conflicting result represents differences in expression levels or cell type specific differences is unclear.

The PH domain of p115RhoGEF is required for Rho activation *in vitro* and *in vivo* [18], indicating a function beyond its role in membrane targeting. Indeed, several reports have indicated that for other RhoGEFs, the PH domain, located in tandem with the DH domain, is required for GEF activity [5]. The crystal structure of the DH/PH fragment of Dbs (a Dbl-related oncoprotein) indicated that residues present in the PH domain directly contact Cdc42 and assist in guanine nucleotide exchange [50]. Consistent with dual roles for PH domains in RhoGEFs, we identified a mutation, L677P (Lys<sup>677</sup> → Pro), in the PH domain of p115RhoGEF that decreased Rho signalling activity in cells but, in contrast with the PH domain deletion, did not affect  $\alpha_{13}$ -promoted PM recruitment (Figure 8).

#### Multiple interactions contribute to regulated PM localization of p115RhoGEF

Taken together, the results presented here suggest a mechanism for PM recruitment of p115RhoGEF. Activation of  $\alpha_{13}$  or  $\alpha_{12}$  by an appropriate GPCR provides a PM localized binding site for the initially cytoplasmic p115RhoGEF. Although  $\alpha_{13}$  GAP activity in p115RhoGEF may rapidly inactivate  $\alpha_{13}$ , weak or transient interaction with  $\alpha_{13}$  may be sufficient to serve as an initial membrane-targeting signal for p115RhoGEF. Once p115RhoGEF interacts with  $\alpha_{13}$  and translocates to the PM, the PH domain probably fulfils the role of a second membrane-targeting signal to allow more stable PM binding. Probably, additional membrane or protein interactions contribute to regu-

lated PM recruitment of p115RhoGEF and the initiation of Rho activation. It will also be important to determine how the spatial and temporal characteristics of Rho activation compare with p115RhoGEF PM recruitment inside the cell.

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