Differential inhibitor of Gβγ-signaling to AKT and ERK derived from Phosducin-like protein: Effect on sphingosine-1-phosphate-induced endothelial cell migration and in vitro angiogenesis

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Running title: PhLP-M1-G149: a differential inhibitor of Gβγ-signaling to AKT

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SUMMARY

Differential inhibitors of Gβγ-effector regions are required to dissect the biological contribution of specific Gβγ-initiated signaling pathways. Here, we characterize PhLP-M1-G149, a Gβγ-interacting construct derived from Phosducin-like protein 1 (PhLP) as a differential inhibitor of Gβγ, which, in endothelial cells, prevented sphingosine-1-phosphate (S1P)-induced phosphorylation of AKT, GSK-3β, cell migration and tubulogenesis, while having no effect on ERK phosphorylation or hepatocyte growth factor (HGF)-dependent responses. This construct attenuated the recruitment of PI3Kγ to the plasma membrane and the signaling to AKT in response to Gβγ overexpression. In coimmunoprecipitation experiments, PhLP-M1-G149 interfered with the interaction between PI3Kγ and Gβγ. Other PhLP derived constructs interacted with Gβγ but were not effective inhibitors of Gβγ signaling to AKT or ERK. Our results indicate that PhLP-M1-G149 is a suitable tool to differentially modulate the Gβγ-initiated pathway linking this heterodimer to AKT, endothelial cell migration and in vitro angiogenesis. It can be also useful to further characterize the molecular determinants of Gβγ-PI3Kγ interaction.

INTRODUCTION

Heterotrimeric G protein signaling depends on the actions of GTP-loaded Go and free Gβγ, the two functional components of the heterotrimer, leading to the generation of second messengers and cell specific functional events (1,2). Differential inhibitors of Gβγ are required to dissect the biological impact of different Gβγ-dependent effectors. Gβγ actions can be blocked by competition with peptides derived from its effectors. For example, the effect of Gβγ on adenylyl cyclase II, G protein-activated inward rectifier K+ channel (GIRK), GRK-2, and PLCβ3, is attenuated by a peptide from adenylyl cyclase II (3). In addition, RACK1 (receptor for activated C kinase 1) selectively inhibits the effect the chemokine receptor CXCR2 on the activation of phospholipase Cβ2 and adenylyl cyclase II in HEK293 cells, without affecting other functions of Gβγ (4). Recently, Smrcka and colleagues characterized the effect of small molecule inhibitors of Gβγ, suggesting their potential application in therapeutic strategies targeting particular Gβγ-dependent pathways (5). Emerging possibilities to target this heterodimer in pathological situations such as inflammation and angiogenesis are based on the role of Gβγ in cell survival and chemotaxis. To the best of our knowledge, no molecular tool is yet available to differentially inhibit Gβγ signaling to AKT.

Gβγ is a key transducer of sphingosine-1-phosphate (S1P)-elicited angiogenic signals promoting endothelial cell migration, proliferation and survival (6-12). Multiple Gβγ-dependent effectors are potentially involved in the molecular

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events required for endothelial cell migration. These include lipid kinases such as PI3Kγ and PI3Kβ (13), and a novel family of Rac guanine nucleotide exchange factors, represented by P-REX1, which is activated by Gβγ and phosphatidylinositol 3,4,5-trisphosphate (14-16). Gβγ signaling is frequently attributed to pertussis toxin-sensitive Gi coupled receptors and it has been consistently revealed by the antagonistic effect of the carboxy-terminal region of GRK-2, which sequesters Gβγ thereby inhibiting all its intracellular actions (17). In addition, mutational analysis of Gβ revealed that different residues, all of them mapping to the interface of contact between Gβγ and Ga, are important for the activation of distinct Gβγ effector molecules (18).

Phosducin was originally identified as a phosphoprotein restricted to the retina and pineal gland forming a complex with Gβγ (19,20). It was considered a protein kinase A-sensitive regulator of G-protein mediated signaling (21,22). Further studies identified a family of phosducin-like proteins (PhLPs) (23,24). Phosducin and Ga share affinity for the same region of Gβγ, as revealed by the structural analysis of Gβγ in complex with Ga or phosducin and by in vitro binding experiments (25). This area of interaction includes some of the residues considered necessary for the activation of Gβγ-dependent effectors (18,26). It was initially postulated that phosducin and related proteins, by interfering with the availability of free Gβγ, exert an inhibitory role on Gβγ signaling. However, recent genetic evidence raised an apparently conflicting situation; the knockout of PhLP in fungi resulted in a phenotype equivalent to the absence of Gβγ, contrary to its expected role as an inhibitor (27). Novel experimental evidence indicated that PhLP has a positive effect on Gβγ signaling due to its participation in the assembly of the heterodimer, helping to stabilize free Gβ subunits leaving the ribosome after synthesis (28-31).

Despite the positive role of full length PhLP in the assembly of Gβγ heterodimers, it is still possible that different fragments of this protein, which could retain their interaction with distinct regions of Gβγ, might function as inhibitors of Gβγ signaling. Accordingly, we characterized here the effect of different PhLP-derived constructs on the signaling pathways elicited by S1P or HGF in endothelial cells. In addition, we explored the mechanism by which PhLP-M1-G149 interferes with Gβγ preventing the activation of AKT.

EXPERIMENTAL PROCEDURES

Bioinformatic tools. The structure of Gβγ/Phosducin (25,32,33) was analyzed with the CN3D program, available from NCBI’s web page, http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml. This program was used to identify the amino acids at the Gβγ/Phosducin-contact interface within 3-3.5 angstroms.

Phosducin-Like Protein derived Constructs. Based on the structure of the complex formed by Gβγ and Phd (25,32,33), and considering the homology between Phd and PhLP, five PhLP-derived constructs were obtained by PCR using as template the cDNA of human PhLP (24) (NCBI accession AF076463, kindly provided by Cheryl Craft, University of Southern California, Los Angeles). According to the structural analysis, these constructs would interact with different amino acids at the effector region of Gβγ. The corresponding cDNAs were cloned into the mammalian expression vectors pCEFL-EGFP, pCEFL-GST or pCEFL-EGFP-CAAX. EGFP or GST were expressed at the amino terminus of the constructs whereas in the case of the EGFP-CAAX, the CAAX box was at the carboxyl terminus. All the constructs were cloned with BamHI and EcoRI restriction sites (5’ and 3’ respectively) that were included in the corresponding primers. The sequences of the oligonucleotides are as follows: PhLP (M1-E283) sense, atagGTCCatgcgcagagcttgacagc and antisense, atagGAATTCTcattcacaatcctctctct; (PhLP-M1-G149) sense, atagGTCCatgcgcagagcttgacagcc and antisense, atagGAATTCCcataattctagcccaaaactc; (PhLP-Q94-E283) sense atagGTCCcagatgagatgaaagagcc and antisense atagGAATTCCcattctagcccaaaactc; (PhLP-Q94-G149) sense atagGTCCcagatgagatgaaagagcc and antisense atagGAATTCCcattctagcccaaaactc; (PhLP-M1-K102) sense atagGTCCcagatgagatgaaagagcc and antisense atagGAATTCCcattctagcccaaaactc; and
Expression of proteins and the putative amino terminal tags. Briefly, cells were washed three times with lysis buffer and lysed at 4°C in TBS-T buffer containing 50 mM Tris, pH 7.5, 0.15 M NaCl, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, and insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4°C. His6-tagged Gβγ was isolated by incubating with 40 µl of Talon beads (Metal Affinity Resin, Clontech) for 2 h at 4°C. Sepharose beads were washed three times with lysis buffer and boiled for 5 min in 1X Laemmli sample buffer. Expression of proteins and the putative interactions between them were revealed by western blot using anti-His6 monoclonal antibodies (Sigma) or GFP monoclonal antibodies Covance). Immunoblots were visualized by enhanced chemiluminescence detection (Pierce). Similar experiments were done to investigate the effect of PhLP-Q94-G149 construct in the interaction between PhLP-M1-G149 and Gβγ. Briefly, HEK 293T cells in 10-cm dishes were transfected with 1.0 µg of PhLP-M1-G149, 0.3 µg each His6-Gβγ, and increasing concentration of the plasmid coding for PhLP-Q94-G149 construct (0.3, 1 and 3 µg). Pull down assays were performed two days after transfection.

For experiments assessing the interaction between Gβ1 mutants and PhLP constructs the following Gβ mutants were used: Gβ1(L55A) (residue at the NH2-terminal interface (18), Gβ1(K57A), Gβ1(S98A), Gβ1(T143A), and Gβ1(W332A) (all of them at the switch interface) (18) were obtained by the site directed mutagenesis system (QuikChange Kit, Stratagene). Their interaction with PhLP, PhLP-M1-G149 or PhLP-Q94-E283 was assessed in HEK293T cells cotransfected with 1 µg of the different pCEFL-HA-Gβ1 mutants (as indicated in Figure 2) or pCEFL-HA Gβ1, and 1 µg pCEFL-GT-Gβ1, 2 µg pCEFL-GST-PhLP, or 3 µg pCEFL-GST-PhLP-M1-G149 or 3 µg pCEFL-GST-PhLP-Q94-E283. Pull-down experiments were done two days after transfection. The interaction between different Gβγ heterodimers and PhLP constructs was assessed in HEK293T cells. Briefly, Flag-tagged Gβ subunits (in pCDNA3, obtained from the Guthrie cDNA Resource Center) were cotransfected with pCEFL-HA-Gβ1 mutants and PhLP constructs the following constructs were isolated by pull-down using Glutathione sepharose beads, the presence of associated Gβ was revealed by western blot. Cells were lysed with TNTE buffer (50 mM Tris–HCl buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA containing 1% Triton X-100) plus of the previously indicated protease inhibitors, cell lysates were incubated with 40µl of glutathione-Sepharose 4B beads (Amersham Biosciences) rocking for 1h at 4°C. Sepharose beads were collected by centrifugation and washed three times with TNTE buffer. Bound proteins were resolved by SDS-PAGE and detected by western blot using anti-HA (Covance), anti-Flag (Sigma) or anti-GST

**Cell Lines and Transfections.** Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10 % fetal bovine serum (FBS, Terra Cell International). For transient transfections, tissue culture plates were treated for 10 min with phosphate-buffered saline (PBS) containing 5 µg/ml poly-D-lysine before seeding the cells to prevent them from detaching from the plates during the transfection procedure and thereafter. Cells (2.2 X 10⁶) were seeded the day before transfection in 100 mm dishes in DMEM supplemented with 10 % FBS. HEK 293T cells were routinely transfected with DNA plasmids premixed with LipofectAMINE Plus reagent according to the manufacturer’s instructions and were then incubated overnight at 37°C in DMEM containing 10% FBS. The total amount of DNA in all transfections was 4µg/plate. When required, empty pCEFL vector was used to maintain a constant amount of DNA.

**Interactions between Gβγ and PhLP constructs.** The interaction between Gβγ and PhLP-derived constructs was assessed in HEK293T as indicated in figure legends. Gβγ pull-down experiments were done using both subunits fused to His-6 tag. Expression of fusion proteins and the putative interactions between them were revealed by western blot using anti-His6 monoclonal antibodies (Sigma) or GFP monoclonal antibodies (PhLP-R147-E283) sense atagatgtttgtgtgtagc and antisense atagatgtttgtgtgtagc. For the EGFP-CAAX constructs, the stop codon in the antisense oligonucleotides was omitted.
(Santa Cruz) monoclonal antibodies. Whole cell lysates (25 µg of protein per lane) were used for western blots, whereas ~2 mg of protein were used for pull-down assays. Data showed are representative of at least 3 independent experiments.

The influence of PhLP constructs on the expression of different Gβ subunits was assessed in HEK293T cells cotransfected with Flag-tagged Gβ subunits, Gγ2, and PhLP, PhLP-M1-G149 or PhLP-Q94-E283. Two days after transfection, cells were harvested and 10 µg of total cell lysates were used for SDS-PAGE and the expression of the different Gβ subunits was detected by western blot using anti-Flag antibodies.

Influence of PhLP constructs on the interaction between Gβγ and PI3Kγ. The interaction between Gβγ and PI3Kγ was assessed by coimmunoprecipitation experiments in HEK 293T cells transfected with plasmids expressing 3X-Flag Gβ1, Gγ2, PI3Kγ (EE-tagged p110 and EE-tagged p101), kindly provided by L. R. Stephens (34) and PhLP constructs as indicated in Figure 9. Briefly, two days after transfection, cells, grown in 10 cm dishes, were placed on ice, rinsed once with phosphate-buffered saline and lysed in 1 ml of ice-cold TNTE buffer containing protease inhibitors, insoluble material was removed by centrifugation. After clearing, 3 µl of EE antibody (Covance) was added to the supernatant and rotated overnight at 4°C. Next day, 25 µl of protein A-Sepharose was added and rotated again for 2 hours at 4°C. Immunoprecipitates were washed three times with 1 ml of lysis buffer and resuspended in 1 X protein sample buffer. Lysates containing ~50 µg of total cellular protein or immunoprecipitates were analyzed by western blotting after SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence detection (Millipore).

The influence of PhLP constructs on the recruitment of PI3Kγ to the membrane in response to Gβγ overexpression was determined by immunofluorescence in PAE cells. Cells seeded on fibronectin (20 µg/ml) coated coverslips were transfected with 100 ng each, of His6-Gβ1γ2, EE-PI3Kγ (p101 and p110) and 200 ng of PhLP constructs or 100 ng of EGFP-CAAX as indicated in figure 9. Serum starved cells were processed for immunofluorescence, two days after transfection, using previously described conditions (35). Briefly, cells were washed with PBS, fixed with fresh 4% paraformaldehyde in PBS, pH 7.4, for 20 min, washed five times with PBS and permeabilized with 100 % methanol for 6 min at –20°C, and incubated with 1 % BSA for 30 min at 37°C. PI3Kγ was detected with EE monoclonal antibody (Covance) used at 1/500 in 0.5% BSA for 1 hr at 37°C, washed and followed by incubation with Cy5 secondary antibody (Jackson) used at 1/200 in 1% BSA for 40 min at room temperature. Cells were mounted with Vectashield (Vector Laboratories) and images were acquired with a DMIRE2 confocal laser-scanning microscope (Leica Microsystems, Deerfield, IL) by the use of a 63X, numerical aperture 1.4 oil immersion objective and a zoom of 2.

Stably Transfected Endothelial Cells. All EGFP-tagged constructs derived from PhLP were transfected into porcine aortic endothelial (PAE) cells using Polyfect reagent (Qiagen); control cells were transfected with pCEFL-EGFP. Clones were selected 48 hrs after transfection with 2.0 mg/ml of G418 disulfate salt (Genetecin 418, Sigma) in DMEM supplemented with penicillin/streptomycin (Biosource, International) and 10% FBS. Expression of the transfected constructs was determined by FACS, immunoblot and fluorescence microscopy.

ERK, AKT and GSK3β Assays. Stably transfected endothelial cells were cultured in DMEM including 10 % serum and G418 antibiotic until they reached confluence. In brief, cells were serum-starved for 8 h and either stimulated with 1 µM S1P, 10 ng/ml HGF, or vehicle for the indicated period. Cells were washed and lysed with 1 X boiling Laemmli sample buffer. Total cell lysates were used for western blots as indicated previously. Phosphorylation of AKT, ERK 1/2 or GSK3β and expression of these proteins was detected using the following rabbit antibodies: phospho-AKT-1/2/3 (Ser 473) antibody (Santa Cruz Biotechnology); phospho-p44/42 Map Kinase (Thr202/Tyr204) antibody (Cell Signaling) and phospho-GSK3β (Ser9)
antibody (Cell Signaling). Protein expression was analyzed using: mouse monoclonal anti-protein kinase B (PKB/AKT1, Sigma), rabbit polyclonal anti-mitogen-activated protein kinase (MAPK, Santa Cruz Biotechnology) and rabbit polyclonal anti-Glycogen synthase kinase-3 (GSK-3, Cell Signaling).

The effect of Gβγ overexpression on AKT and ERK phosphorylation was assessed in Cos7 cells. Briefly, cells were cultured in 6 well plates in DMEM with 10% fetal bovine serum until they reached a 70% of confluence. Cells were then transfected with 0.5 µg of plasmids coding for different Gβ subunits (1-5), Gγ2 and PhLP constructs as indicated in figure legends. Next day, cells were serum starved, and the day after they were lysed in 200 µl of ice-cold TBS-T buffer containing the previously indicated protease inhibitors and phosphatase inhibitors (20 mM β-glycerophosphate, 1 mM sodium vanadate), insoluble material was removed by centrifugation. Phosphorylation of AKT, or ERK 1/2 was detected as described above.

Effect of PI3K inhibitors in endothelial cells. The effect of PI3Kγ and PI3Kβ inhibitors (36) on the activation of AKT in PAE cells was assessed in serum starved confluent cultures grown in DMEM in 6 well plates. In brief, cells were serum-starved the night before the experiment. Next day, media was replaced before incubation with the inhibitors, either PIK93 for PI3Kγ (IC50, 0.016 µM), TGX115 (ZK 28) for PI3Kβ (IC50, 0.13 µM), or vehicle, which were incubated in 1 ml of serum free media before stimulation as indicated in Figure 5. The maximum concentration used in our experiments was 0.1 µM. Stimulated or control cells were washed and lysed with boiling 1 X Laemmli sample buffer. Total cell lysates were used for western blots to detect the phosphorylation of AKT using anti-phospho-AKT (Ser 473) antibody (Cell Signaling, 9271). The experiments using PI3K inhibitors were done by MLGH in Tamas Balla’s lab at NICHD-NIH using inhibitors kindly provided by Zaccharary Knight and Kevan Shokat (UCSF).

Cell Migration Assays. Migration assays of stably transfected endothelial cells were performed in Boyden chambers. Filters with 8 µm pores were coated overnight with 10 µg/ml fibronectin. The bottom wells of the chamber were filled with 150 µl of medium containing (or lacking) chemoattractant. Stably transfected endothelial cells were harvested by trypsinization, washed by centrifugation and resuspended 1 X 10⁶ cells in 100 µl of fresh DMEM. After the cells were inoculated into the upper chamber and incubated for 6 hours at 37°C. The migrated cells on the bottom of the chamber were fixed with methanol and stained with 10 mg/ml of crystal violet. The cells that remained on the top side of the filter (those that did not migrate) were removed with a cotton swab. The level of migration was determined by densitometric quantitation of the stained filters, using ImageJ software.

In Vitro Angiogenesis Assay. Stably transfected endothelial cells were serum starved 15 h before the experiment, detached by gently trypsinization, washed, counted, and resuspended in DMEM. Previously, matrigel basement membrane matrix (BD, Bioscience 354230) diluted 1:2 in cold serum-free DMEM was plated into 96-well tissue culture plates and incubated for 1 hour at 37°C before seeding the cells. Then, 10 X 10³ cells were added on top the matrigel in the absence or presence of different stimuli (1 µM S1P or 0.5ng/ml HGF). After 9 h of incubation, the experiment was stopped, and the results were assessed morphologically using light microscopy. The length of the tubes was measured using ImageJ software.

Statistical analysis. Test and control samples in the functional assays were compared for statistical significance by using Student’s t test, a p <0.05 was considered significant.

RESULTS

Structural analysis of Gβγ/Phosducin complex and interaction between Gβγ and constructs derived from PhLP

In this study, we tested the possibility that constructs derived from PhLP, a Gβγ-interacting protein, differentially inhibit Gβγ signaling to AKT and ERK. In order to identify the sites of interaction between different regions of Phosducin and Gβγ, we analyzed the crystal structure of Phosducin/Gβγ shown in Figure 1A
According to the analysis of three different structures of Gβγ-Phosducin complexes: 1B9Y (33), 2TRC (25), and 1AOR (32), and assuming that Phosducin and PhLP interact with the same residues in Gβγ, the amino acids shown in white color in Figure 1B, bottom right, are those in Gβγ interacting with PhLP amino terminal region (PhLP-M1-K102): K57, N230, T274, and D290; those shown in white color in the left panel, interact with PhLP central region (PhLP-Q94-G149): Q75, S98 and W332 (W332 within a distance of 3.5 Å) and those shown in white color in the upper right panel, interact with PhLP carboxyl terminal region (PhLP-R147-E283), within a distance of 3.0 Å, (25,32): Q44, R46, T47, R304, and H311. Previous studies with Gβ point mutants, revealed that some of these residues are required for the activation of different effectors (13). In particular, the residues of Gβγ involved in the interaction with phosphoinositide-3-kinase (PI3K), a known effector of Gβγ, the amino acids shown in Figure 2, wild type Gβγ interacted with PhLP and the constructs PhLP-M1-G149 or PhLP-Q94-E283 in pull-down experiments using PhLP constructs fused to GST. Interestingly, Gβ1Ser98Ala was unable to interact with full length PhLP, indicating that the interacting interface formed by GβSer98 with the central region of PhLP is critical for the interaction. In Phd, the amino acids that interact with Gβ1Ser98 are Phd-E85, Phd-L90 and Phd-R94, establishing a region of electrostatic interactions between Gβγ and Phd (25,32,33). The importance of Gβ1Ser98 and Gβ1W332 was analyzed with the NCBI’s CN3D program, and the predicted interface is highlighted in the structure of the complex formed by Phosducin and Gβγ (25) shown in Figure 2, lower panel.

The experiments with Gβ point mutants and those comparing the interaction between Gβγ and different PhLP constructs suggested the importance of the central region of PhLP on the interaction with Gβγ. Thus, we tested the ability of the construct corresponding to this region, PhLP-Q94-G149, to compete with the construct PhLP-M1-G149, that includes the central region and extends towards the amino terminus. As shown in Figure 3, top panel, the central region of PhLP competed with PhLP-M1-G149 for the interaction with Gβγ, confirming the importance of the PhLP fragment Q94-G149 in the interaction with Gβγ (Figure 3).
The interaction between different Gβγ heterodimers and PhLP constructs was analyzed by pull down experiments using PhLP constructs fused to GST. As shown in Figure 4, Gβγ heterodimers containing either Gβ1, Gβ2, Gβ3 or Gβ4, all interacted with PhLP, PhLP-M1-G149 or PhLP-Q94-E283. Interestingly, PhLP-Q94-E283 also interacted with Flag-tagged Gβγ. The interaction was specific as shown in the pull downs in which GST and Flag-Gβγ were used as negative controls.

**Effect of PTX and PhLP-derived constructs on S1P and HGF signaling to AKT and ERK in endothelial cells**

We next tested whether PhLP constructs can differentially interfere with Gβγ signaling in endothelial cells stimulated with S1P or HGF. Both agonists are known to induce activation of PI3K-AKT, ERK and chemotactic migration in endothelial cells (10,12,37). The action of S1P activating these pathways is known to be mediated through Gi-coupled receptors (10,12,38). Here, using antibodies against the phosphorylated forms of AKT and ERK, we demonstrated that 1 µM S1P and 10 ng/ml HGF induced a rapid phosphorylation of AKT and ERK in PAE cells (Figure 5A). Pertussis toxin (PTX) differentially inhibited the effect of S1P, but not of HGF, on AKT and ERK phosphorylation (Figure 5B). These data indicated that S1P regulates signaling to AKT and ERK through a Gi heterotrimeric protein in PAE cells. In addition, S1P and HGF signal to AKT via the intervention of different PI3K isoforms as demonstrated by a differential effect of specific inhibitors. As shown in Figure 5C, S1P signaling to AKT depends on the activity of PI3Kγ and the effect of HGF is independent of this isoform. The potential effect of the different PhLP constructs as modulators of Gβγ signaling to AKT and ERK was determined in stably transfected PAE cells stimulated with S1P or HGF. PhLP-M1-G149, the construct corresponding to the amino plus the central regions of PhLP, prevented the activation of AKT in cells stimulated with S1P while having no effect on the activation of ERK (Figure 5D and E). Other PhLP-derived constructs did not interfere with the ability of S1P to promote the phosphorylation of AKT and ERK (Figure 5D and E). We noticed a slight increase in the basal phosphorylation of ERK in cells expressing PhLP-M1-G149 and PhLP-Q94-E283, but these constructs did not affect the agonist dependent activation of ERK. On the other hand, cells expressing the smaller constructs, corresponding to the amino, central and carboxyl terminal regions of PhLP did not show differences, respect to the control in the activation of AKT and ERK (supplementary Fig.1). The effect of S1P on AKT and ERK in cells expressing PhLP-M1-G149 was still sensitive to inhibition by PTX (Figure 5F), indicating that the coupling properties of S1P receptors in these cells were not modified by expression of the PhLP-M1-G149 construct. Neither PhLP-derived constructs nor PTX inhibited the effect of HGF (Figure 5 D-F).

**Effect of PhLP-M1-G149 on GSK3β phosphorylation in endothelial cells**

Glycogen synthase kinase 3β (GSK3) is a target of the PI3K signaling pathway, being phosphorylated by AKT and p70S6 kinase (39,40). To assess the role of Gi in the phosphorylation of GSK3β in endothelial cells responding to S1P or HGF, PTX-treated cells were stimulated with these agonists as indicated in Figure 6A. Both, S1P and HGF promoted the phosphorylation of GSK3β, an effect that was sensitive to PTX in S1P-stimulated cells (Figure 6A, upper panel). To further assess whether PhLP-M1-G149 prevents the phosphorylation of AKT substrates, we evaluated the phosphorylation of GSK3β in cells expressing PhLP-M1-G149 and stimulated with S1P or HGF. The results, shown in Figure 6B and C, indicated that PhLP-M1-G149 prevented the functionality of AKT (assessed by its ability to phosphorylate GSK3β) in cells responding to S1P. Full length PhLP or PhLP-Q94-E283 did not interfere with the phosphorylation of GSK3β in cells responding to S1P or HGF (Figure 6B and C).

**Effect of PhLP-M1-G149 on endothelial cell migration**

In order to examine the effect of PhLP or PhLP-M1-G149 on endothelial cell migration, we assessed the chemotactic response to S1P or HGF of endothelial cells expressing these constructs or control cells expressing EGFP. Interestingly, cells expressing PhLP-M1-G149 showed a notably reduced migration in response to S1P, while the effect of HGF was not affected (Figure 7A and B). Full length PhLP did not interfere with the
migration of endothelial cells in response to S1P or HGF, in fact, an increase in the effect of S1P was detected (Figure 7A and B).

**Effect of PhLP-M1-G149 on in vitro angiogenesis.**

Considering the angiogenic properties of S1P, we tested if the expression of PhLP-M1-G149 affects this response. We assessed the effect of S1P and HGF on the in vitro angiogenic response of PhLP-M1-G149-transfected endothelial cells. Our results indicated that PhLP-M1-G149 was able to interfere with S1P-induced angiogenesis, while the effect of HGF was not affected. In parallel, cells transfected with EGFP or EGFP-tagged PhLP showed normal angiogenic responses to S1P or HGF (Figure 8A and B).

**Molecular mechanism of PhLP-M1-G149 mediated inhibition**

In order to assess the molecular mechanism by which PhLP-M1-G149 affects Gβγ signaling to AKT, we tested the hypothesis that this construct interferes in the ability of Gβγ to interact with PI3Kγ and the ability of this heterodimer to recruit PI3Kγ to the plasma membrane. As shown in Figure 9A, Flag-tagged Gβ1γ2 interacted with PI3Kγ in coimmunoprecipitation experiments. This interaction was significantly reduced in cells expressing PhLP-M1-G149, but not in cells expressing full length PhLP or PhLP-Q94-E283. In addition, the association of PI3Kγ to the plasma membrane in response to the overexpression of Gβγ was attenuated by PhLP-M1-G149, expressed in endothelial cells as a GFP-tagged protein with a CAAX box at the carboxyl terminus to restrict its expression to the membrane. Full length PhLP or the carboxyl terminal construct did not interfere with the recruitment of PI3Kγ to the membrane (Figure 9B). In experiments using higher amounts of transfected Gβγ, PhLP-M1-G149 lost its ability to compete with PI3Kγ (not shown), further indicating that it recognizes the same interacting interface in Gβγ.

Intriguingly, our results showed that full length PhLP did not interfere with Gβγ signaling. Since it has been recently reported that PhLP contributes to Gβ expression (31), we tested whether PhLP, PhLP-M1-G149 or PhLP-Q94-E283 could affect the expression of different Gβγ heterodimers. As shown in Figure 10, with the exception of Gβ3, the expression of all other Gβ subunits, in particular Gβ1 and Gβ2, increased in cells transfected with PhLP. Also, cells expressing PhLP-M1-G149 or PhLP-Q94-E283 showed a slight effect compared to the respective controls.

**Effect of PhLP-M1-G149 on the activation of AKT and ERK in response to the overexpression of different Gβγ heterodimers.**

In order to test the effect of PhLP-M1-G149 on the action of different Gβγ heterodimers, Cos7 cells were transfected with different Gβ subunits and Gγ2. The results shown in Figure 11A indicated that, by overexpression, only Gβ1γ2 and Gβ2γ2 were able to provoke a significant increase in the activation of both AKT and ERK. Thus, we tested the effect of PhLP-M1-G149 or full length PhLP on the phosphorylation of AKT and ERK in response to Gβ1γ2 or Gβ2γ2 overexpression. As shown in Figure 11B and C, PhLP-M1-G149 significantly attenuated the ability of Gβ1γ2 or Gβ2γ2 to promote the phosphorylation of AKT, while full length PhLP showed just a non significant, slight attenuation on the effect of Gβγ overexpression on AKT phosphorylation. Neither construct showed a significant reduction on the activation of ERK in response to Gβγ overexpression.

**DISCUSSION**

Differential inhibitors of Gβγ could help to determine the cellular roles of different Gβγ effectors, thus providing the basis for future therapeutic strategies. Here, we demonstrate that PhLP-M1-G149, a construct corresponding to the amino terminal region of PhLP1, has a differential effect in endothelial cells stimulated with S1P, attenuating the activation of AKT without affecting signaling to ERK, or the activation of both pathways in response to HGF. Both angiogenic factors known to activate AKT, ERK and endothelial cell migration (41-45). The effect of PhLP-M1-G149 seems to be due to a blockade of Gβγ signaling by a direct interference with the effector interface of Gβγ involved in the activation of PI3Kγ. Accordingly, this construct interfered with the interaction between Gβγ and PI3Kγ and the recruitment of this phosphoinositide kinase to the membrane in response to Gβγ overexpression.
Effect that is diminished when the amount of transfected Gβγ increases, supporting that the mechanism of inhibition is due to a competition for the effector interface. Considering these findings, an inherent limitation on the use of PhLP-M1-G149 as an inhibitor of Gβγ is the fact that this heterodimer may utilize additional mechanisms to activate AKT in a cell-specific fashion. These include an effect mediated by PI3Kβ (46), growth factor receptor transactivation (47,48) and a recently described β-arrestin dependent mechanism (49). In this regard, PhLP-M1-G149 prevents the activation of AKT in response to S1P in endothelial cells but it has no activity in the response to this agonist in S1P1-transfected HEK293 cells (not shown), in which Gβγ mainly uses PI3Kβ to lead to AKT activation (46). A similar, cell specific effect for a Gβγ inhibitor has been reported by Ham’s group showing the inhibitory potential of RACK1, a Gβγ-partner that is unable to inhibit RACK1-Gβγ dependent cell migration in HEK293 (50), but attenuates CXCR-2 dependent migration of Jurkat cells (51).

Our findings suggest that the effector interface of Gβγ linking to PI3Kγ lies at the region of Gβγ interacting with the amino terminal part (amino acids M1-K102) of the PhLP-M1-G149 construct. This possibility from the different effects of PhLP-M1-G149 and PhLP-Q94-E283, both constructs share the amino acids Q94-G149, but they do not have the same inhibitory effects. According to the available structures of Phd-Gβγ (25,32,33) Gβ1 that interact with the amino terminal region of Phd, within a distance of 3Å, are K57, N230, T274 and D290. At this point, the hypothesis linking these residues to PI3Kγ activation is speculative. However, since the crystal structure of this phosphoinositide kinase in complex with Gβγ is not available, it will be interesting to explore this possibility in future studies. In agreement with these possibilities, Hamm’s group recently published that RACK1 interferes in in vitro assays with the ability of Gβγ to activate PI3Kγ (51). The residues of Gβγ interacting with RACK1 are not known at the structural level, however, the ability of RACK1 to interact with both Gβ1γ2 and Gβ3γ2 (52) suggests that the interacting residues correspond to those shared between these Gβ subunits. In addition, since Gβ5γ2, contrary to the action of other heterodimers, is not able to activate PI3Kγ (53), it can be speculated that while the RACK1-Gβ interface corresponds to residues shared between Gβ1 and Gβ3 (52), the effector interface linking Gβ1 to PI3Kγ (putatively recognized by both RACK1 (51) and PhLP-M1-K102) corresponds to non conserved residues. In this regard, it is interesting to notice that the amino acids of Gβ1 that interact with PhLP-M1-K102 are either not conserved between Gβ1 and Gβ5 (T274 and D290) or located in a region of Gβ5 in which the adjacent residues are not conserved (K57 and N230 are conserved in Gβ5 but A56, I58 and A231 are not).

The putatively critical residues for the interaction between Gβγ and PI3Kγ are conserved in Gβ1, Gβ2, Gβ3 and Gβ4, all of them able to activate PI3Kγ (supplementary Figure 2) (53).

Our results also indicate that the central region of PhLP (amino acids 94-149) is important to support a stable interaction between PhLP and Gβγ. Independent evidences pointing to this conclusion are derived from the characterization, in transfected cells, of the interaction between PhLP derived constructs and Gβγ. The shortest PhLP derived construct that shows an important interaction with Gβγ corresponds to this central region, which also competes in the interaction between PhLP-M1-G149 and Gβγ. In addition, according to the structure of the complex between Gβ1γ2 and Phd (25,32,33) Gβ1(S98) and Gβ1(W332) are residues involved in the interaction between Gβγ and the central region of Phosducin. In agreement, we found that Gβ1(S98A)γ2 does not interact with PhLP and decreases its interaction with PhLP-M1-G149, while it interacts with PhLP-Q94-E283 as effectively as wild type Gβ1γ2. These data also suggest that the PhLP derived constructs have conformational adjustments that allow them to interact with Gβ mutants or isoforms with a different affinity compared to full length PhLP. Another interesting difference regarding the interacting properties of PhLP derived constructs is related to their ability to recognize different Gβγ heterodimers. While both, full length PhLP and PhLP-M1-G149 interact with heterodimers containing Gβ1, Gβ2, Gβ3 and Gβ4, they lack the ability to interact with Gβ5. In contrast, PhLP-Q94-E283 recognizes all the heterodimers, including Gβ5γ2. These data also
suggest the existence of conformational differences between Gβ5 and other Gβ subunits, which, as mentioned, share the ability to activate PI3Kγ (53). In addition, the ability of PhLP-M1-G149 to interact with different Gβγ heterodimers containing the Gβ subunits known to be able to activate PI3Kγ (53), and interfere with signaling to AKT by Gβ1γ2 or Gβ2γ2, support the possibility that PhLP-M1-G149 can be used to characterize the signaling from different G protein coupled receptors that utilize these different Gβγ heterodimers to activate the PI3Kγ/AKT signaling pathway.

Intriguingly, while PhLP-M1-G149 inhibits S1P signaling to AKT, full length PhLP is not inhibitory. Based on our results, two alternative possibilities can be proposed. First, we observed that full length PhLP has a positive effect contributing to the expression of different Gβγ heterodimers, in particular those containing Gβ1 or Gβ2, suggesting that cells that express full length PhLP also express a higher content of Gβγ heterodimers that are then available to interact with PhLP but also with PI3Kγ and other effectors. Previous reports assigning an inhibitory role to phosducin and PhLP on Gβγ signaling were mainly based on in vitro assays or overexpression experiments (54). However, recent findings assign a positive role for PhLP in Gβγ function, (31). In this scenario, Gβγ folding and heterodimer formation is positively influenced by PhLP (28,55). Our results confirm these findings and extend the observations to different Gβγ heterodimers, suggesting that, excluding Gβ5, the expression of all other Gβ subunits is, to some extent, positively regulated by full length PhLP. The second possibility that helps to explain why full length PhLP is not inhibitory emerges from the observed differences in the interacting properties of full length PhLP and PhLP-M1-G149 with Gβ mutants or different heterodimers. These differences can be interpreted as an indication of conformational differences that, in theory, can confer to the PhLP-M1-G149 construct a better ability to recognize and interfere with the Gβγ-PI3Kγ effector interface.

All together, our results indicate that PhLP-M1-G149 interacts with Gβγ thereby inhibiting its effector region involved in the activation of PI3Kγ/AKT, cell migration and tubulogenesis, while the effector region leading to ERK activation remains available but does not participate in these S1P-dependent cellular events. In conclusion, PhLP-M1-G149 may represent a suitable tool to differentially modulate Gβγ in the pathway linking this heterodimer to AKT signaling, polarized cell migration and in vitro angiogenesis.

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ACKNOWLEDGMENTS
We acknowledge Sheryl Craft who kindly provided PhLP cDNA, and Tamas Balla who allowed MLGH to do the experiments using PI3K inhibitors in his lab at NICHD-NIH using inhibitors kindly provided to him by Zacchary Knight and Kevan Shokat (UCSF). This work was supported by grants from CONACyT (61127 to J.V.P. and 45957 to G.R.C.) and FIC-NIH (R01TW006664 to J.V.P). M.L.G.H., J.C.O., R.H.G., and A.G.R. are graduate students supported by fellowships from CONACyT. Technical assistance provided by Estanislao Escobar Islas and David Pérez is acknowledged. The authors declare no conflict of interest.

FOOTNOTES
The abbreviations used: PhLP, Phosducin like protein; PI3K, Phosphatidylinositol 3-kinase; AKT, serine/threonine kinase with a pleckstrin homology domain; S1P, sphingosine-1-phosphate.
FIGURE LEGENDS

Figure 1. Structural analysis of Gβγ/Phosducin complex and interaction between Gβγ and constructs derived from PhLP. A) Structure of the heterotrimer formed by Gβ (light grey), Gγ (dark grey) and Phosducin (white) (25). B) Phosducin-interacting aminoacids (within 3-3.5 angstroms) highlighted in white in Gβγ as detected with the NCBI’s CN3D program. Those interacting with the different regions of Phosducin are shown as white residues in structures of Gβγ next to the corresponding interacting region of Phd. C) Based on the structure of Phosducin, constructs corresponding to the different regions of PhLP were generated. The amino terminal domain (M1-K102) corresponds to the dark grey region, the central region (Q94-G149) corresponds to the light grey region and the carboxyl terminal domain (R147-E283) corresponds to the black part of the structure; right panel. The interaction between PhLP derived constructs and Gβγ was assessed in HEK-293T transiently transfected with His6-tagged Gβ1γ2 and EGFP-tagged PhLP constructs. His6-Gβ1γ2 was isolated with a cobalt-based metal affinity resin and interacting PhLP derived constructs were detected by western blotting using anti-GFP antibodies. As a control, a construct corresponding to the carboxyl-terminal region of GRK-2 fused to GFP was used. The expression of transfected EGFP-tagged PhLP or the indicated constructs is shown in total lysates and those interacting with His6-Gβ1γ2 are shown in the pulldown (PD: His). His6-Gβ1γ2 is shown in the bottom panel.

Figure 2. Analysis of the interaction between Gβγ mutants and constructs derived from PhLP. Upper panel) Gβ1 with the indicated point mutations at the interface known to interact with Gα or effectors (18) was cotransfected with Gγ2 and PhLP constructs in HEK293T cells. Interaction between Gβγ (wild type Gβ or the indicated mutant) and PhLP constructs, fused to GST was detected by pull-down assays. The presence of Gβ or the indicated mutants interacting with PhLP or the constructs PhLP-M1-G149 or PhLP-Q94-E283 was detected by western blot using HA antibodies that recognized this tag fused at the amino terminal of Gβ. As a negative control, a construct corresponding to GST and wild type Gβγ was used. The expression of transfected GST-tagged PhLP is shown in the pull-downs and the expression of Gβ and the indicated mutants is shown in total cell lysates (TCL). Bottom panel) The importance of Gβ1 Ser98 and Gβ1 W332, analyzed with the NCBI’s CN3D program, is highlighted in the structure of the complex formed by Phosducin and Gβγ (25); as shown, these amino acids establish two interacting spots with the central region of Phosducin. According to the pull-down experiments shown in the upper panel, the first interacting contact, depending on Gβ1 Ser98, is critical for the interaction with full length PhLP and is also important for PhL-M1-G149.

Figure 3 Importance of the central region of PhLP in the interaction with Gβγ. Pull down experiments as those described in Figure 1C were done in order to test the effect of increasing concentration of PhLP-Q94-G149 in the interaction between PhLP-M1-G149 and Gβγ. As shown in the upper panel, the central region of PhLP competes in the interaction of PhLP-M1-G149 with Gβγ, pointing out to the critical role of the central region of PhLP in the interaction with Gβγ. The expression of the transfected proteins was verified by western blot in total cell lysates (TCL, lower panel). As a negative control, EGFP was used in the pull downs along with PhLP-M1-G149 (tagged with EGFP) and Gβγ.

Figure 4. Analysis of the interaction between different Gβγ heterodimers and constructs derived from PhLP. A) The indicated Flag-tagged Gβ subunits were cotransfected with Gγ2 and the indicated PhLP construct in HEK293T cells. The interaction with PhLP or the indicated constructs, fused to GST was detected by pull-down assays. The presence of Gβ interacting with PhLP or the constructs PhLP-M1-G149 or PhLP-Q94-E283 was detected by western blot using Flag antibodies that recognized this tag fused at the amino terminal of Gβ (upper panel). Cells transfected with GST and Flag-Gβ1γ2 were used as negative controls (C). The expression of transfected GST-tagged PhLP and the indicated constructs is shown in the pull-downs (lower panel) and the expression of transfected Flag-Gβ is shown in total cell lysates (TCL, middle panel).
Figure 5. Effect of PTX and PhLP-derived constructs on S1P or HGF-induced activation of AKT and ERK in endothelial cells. A) Upper panels: endothelial cells were stimulated with 1 μM S1P or 10 ng/ml HGF for the indicated time. After stimulation, phosphorylated AKT and ERK (pAKT and pERK) were detected by western blotting using phospho-specific antibodies. As a reference, the expression of AKT and ERK was detected in parallel using antibodies that recognize the kinases regardless of their phosphorylation state (bottom panels). B) Effect of pertussis toxin (PTX, 100 ng/ml) on S1P or HGF signaling to AKT and ERK in EGFP transfected endothelial cells. C) Effect of PI3Kγ and PI3Kβ inhibitors on the activation of AKT elicited by S1P or HGF. D and E) Effect of PhLP-derived constructs on S1P or HGF signaling to AKT and ERK in endothelial cells. The activation of AKT and ERK (pAKT and pERK) was examined with phospho-specific antibodies, and the expression of AKT and ERK was detected in parallel. EGFP transfected cells were used as control. Bars represent the mean densitometric values of three independent experiments, as the one shown below the graph, normalized to the maximal agonist dependent effect (100%) obtained in HGF-stimulated cells; in the case of PhLP-M1-G149 transfected cells, the results correspond to 6 independent experiments; vertical lines represent the S.E.M. (*p<0.05 respect to control). F) Effect of pertussis toxin (PTX) on S1P and HGF signaling to AKT and ERK in PhLP-M1-G149-transfected endothelial cells.

Figure 6. Effect of PTX, PhLP-M1-G149, PhLP, or PhLP-Q94-E283 on the phosphorylation of GSK-3β induced by S1P or HGF in endothelial cells. A) Effect of pertussis toxin (PTX, 100 ng/ml) on S1P or HGF-induced phosphorylation of GSK-3β (pGSK-3β, an AKT substrate) in EGFP transfected endothelial cells. EGFP expressing PAE cells were stimulated for 5 min either with 1 μM S1P or 10 ng/ml HGF, phosphorylation of GSK-3β was detected in total cell lysates using antibodies recognizing the kinase phosphorylated in Ser-9. As a reference, the expression of GSK-3β was detected in parallel using antibodies that recognize the kinase regardless of its phosphorylation state (bottom panel). B) Effect of PhLP, PhLP-M1-G149 or PhLP-Q94-E283 on S1P or HGF-induced phosphorylation of GSK-3β in stably transfected endothelial cells; PAE cells expressing EGFP were used as control. After stimulation, total cell lysates were analyzed by western blotting using phospho-specific antibodies against GSK-3β or antibodies recognizing the kinase regardless of its phosphorylation state. C) Bars represent the mean densitometric values of phospho-GSK-3β from three independent experiments, as the one shown in B, normalized to the maximal effect (100%) obtained in HGF-stimulated cells; vertical lines represent the S.E.M. (*p<0.05 respect to control).

Figure 7. Effect of PhLP-M1-G149 or PhLP on S1P- or HGF-induced migration of endothelial cells. Stably transfected endothelial cells expressing EGFP, PhLP (full length M1-E283) or PhLP-M1-G149 were subjected to migration assays in Boyden chambers containing either vehicle, 1 μM S1P or 10 ng/ml HGF in the bottom wells; cells that migrated through fibronectin-coated polycarbonate filters (with 8μM pores) were fixed and stained. A) A representative filter showing cells that migrated under the different conditions. B) Bars represent the mean densitometric values of stained filters from six independent migration assays, as the one shown in A, normalized to the migration obtained in HGF-stimulated cells; vertical lines represent the S.E.M. (**p<0.05 respect to control).

Figure 8. Effect of PhLP-M1-G149 or PhLP on S1P or HGF-induced in vitro angiogenesis. Stably transfected endothelial cells expressing EGFP, PhLP-M1-G149 or PhLP (full length M1-E283) were subjected to in vitro angiogenesis assay in 96-well tissue culture plates containing matrigel. These cells were stimulated with vehicle, 1 μM S1P or 0.5ng/ml HGF in serum-free DMEM. A) A representative area showing cells that form tube-like structures under 1 μM S1P and 0.5 ng/ml HGF. B) Bars represent the mean capillary tube length from three independent assays, as the one shown in A, normalized to the tube formation obtained in HGF-stimulated cells; vertical lines represent the S.E.M.
Figure 9. Molecular mechanism of PhLP-M1-G149 mediated inhibition. A) The effect of PhLP-M1-G149, PhLP, or PhLP-Q94-E283 on the interaction between EE-tagged PI3Kγ and Flag-Gβγ was assessed by immunoprecipitation in transfected HEK293T cells (upper panel). The expression of the transfected proteins was verified in total cell lysates (TCL, lower panel). The graph on the right panel shows the average of 5 independent experiments normalized to the result obtained in EGFP transfected cells, vertical lines represent the S.E.M, (*p<0.05 respect to control). B) The effect of PhLP, PhLP-M1-G149 or PhLP-Q94-E283 on the recruitment of PI3Kγ to the membrane in response to Gβγ overexpression was assessed by immunofluorescence in endothelial cells. Confocal images show the localization of PI3Kγ detected with antibodies against the EE tag fused to p101 and p110 subunits (middle and bottom panels). The expression of PhLP constructs, fused to EGFP and a carboxyl terminus CAAX box is shown in the upper panel. EGFP-CAAX was used as control.

Figure 10. Effect of PhLP, PhLP-M1-G149 or PhLP-Q94-E283 on the expression of different Gβγ heterodimers. Since it has been recently reported that PhLP acts as a chaperone contributing to the expression of Gβ (31), we tested if PhLP, PhLP-M1-G149 or PhLP-Q94-E283 affected the expression of different Gβγ heterodimers. The indicated Flag-tagged Gβ subunits were cotransfected with Gγ in HEK-293T cells, their expression were detected in total cell lysates using Flag antibodies that recognized this tag fused at the amino terminal of Gβ. Untransfected cells or cells transfected with GST and the different Gβγ heterodimers were used as negative controls. Total AKT was detected in the same lysates as a loading control.

Figure 11. Effect of PhLP-M1-G149 on the activation of AKT and ERK in response to the overexpression of different Gβγ heterodimers. A) The effect of overexpressing different Gβγ heterodimers on the activation of AKT and ERK in Cos7 cells was detected by western blot using antibodies that recognized the phosphorylated forms of these kinases. The expression of total AKT and ERK was detected with antibodies that recognize these kinases regardless of their phosphorylation status. B and C) The effect of PhLP or PhLP-M1-G149 on the activation of AKT and ERK in response to Gβ1γ2 or Gβ2γ2 overexpression (those that were able to activate both AKT and ERK in the overexpression experiments shown in A) was determined by western blot using phosphospecific antibodies as described in A. Cells transfected with EGFP and the indicated Gβγ heterodimers were used as controls. Bars represent the average results of 8 independent experiments, vertical lines represent the S.E.M, (*p<0.05 respect to control).

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Figure 1
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Figure 6

(A) PTX treatment with different stimuli on GSK-3β and p-GSK-3β.

(B) Western blot analysis showing GSK3β and p-GSK3β levels.

(C) Graph showing GSK3β phosphorylation (%Max) for different conditions: EGFP, PhLP, PhLP (M1-G149), and PhLP (Q94-E283).
Figure 7

A

C S1P HGF

EGFP

M1 E283

M1 G149

B

Relative Migration Cell (%Max)

|          | C     | S1P   | HGF   |
|----------|-------|-------|-------|
| EGFP     | 50    | 100   | 22    |
| PhLP     |       |       |       |
| PhLP-M1-G149 |   |       |       |
Figure 8

A

| C    | S1P  | HGF |
|------|------|-----|
|      |      |     |

EGFP

M1

E283

M1

G149

B

Capillary Tube Length (%Max)

|         | C    | S1P  | HGF |
|---------|------|------|-----|
| EGFP    |      |      |     |
| PhLP    |      |      |     |
| PhLP-M1-G149 |      |      |     |
Figure 10: Western blot analysis showing the expression levels of Gβ1, Gβ2, Gβ3, Gβ4, and Gβ5 in untransfected, control, PhLP, PhLP-M1-G149, and PhLP-Q94-E283 cells. AKT phosphorylation is also observed.
Figure 11
Differential inhibitor of Gbetagamma-signaling to AKT and ERK derived from Phosducin-like protein: Effect on sphingosine-1-phosphate-induced endothelial cell migration and in vitro angiogenesis

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J. Biol. Chem. published online April 29, 2009 originally published online April 29, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.008839

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