Gγ identity dictates efficacy of Gβγ signaling and macrophage migration

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Running title: G protein γ subtype dictates Gβγ signaling

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ABSTRACT

G protein βγ subunit (Gβγ) is a major signal transducer and controls processes ranging from cell migration to gene transcription. Despite having significant subtype heterogeneity and exhibiting diverse cell and tissue specific expression levels, Gβγ is often considered a unified signaling entity with a defined functionality. However, the molecular and mechanistic basis of Gβγ’s signaling specificity is unknown. Here, we demonstrate that Gγ subunits, bearing the sole plasma membrane (PM)-anchoring motif, control the PM affinity of Gβγ and thereby differentially modulate Gβγ effector signaling in a Gγ-specific manner. Both Gβγ signaling activity and the migration rate of macrophages are strongly dependent on the PM affinity of Gγ. We also found that the type of C-terminal prenylation and 5–6 pre-CaaX motif residues at the PM-interacting region of Gγ control the PM affinity of Gβγ. We further show that the overall PM affinity of the Gβγ pool of a cell type is a strong predictor of its Gβγ signaling–activation efficacy. A kinetic model encompassing multiple Gγ types and parameterized for empirical Gβγ behaviors not only recapitulated experimentally observed signaling of Gβγ, but also suggested a Gγ-dependent, active–inactive conformational switch for the PM-bound Gβγ, regulating effector signaling. Overall, our results unveil crucial aspects of signaling and cell migration regulation by Gγ type–specific PM affinities of Gβγ.

Active G proteins GaGTP and Gβγ, interact, control a cohort of effectors and regulate the majority of metazoan signaling (1-3). Although Gα signaling has been the primary focus in the field, recent findings show that Gβγ subunits also regulate crucial signaling pathways and cellular functions. Some of the Gβγ effectors include phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3Kγ), adenylyl cyclase (AC) isoforms (activation of AC2, 4, 7, and inhibition of AC1, 5), inwardly rectifying potassium (GIRK) channels, PLC isoforms (PLCβ2, β3), Ca2+ channels (N, P/Q type), GPCR kinases (GRKs), and guanine nucleotide exchange factors (GEFs) such as Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division control protein 42 (Cdc42), guanine nucleotide exchange factor (FLJ00018), and p114-RhoGEF (4-13). These effectors coordinate a wide range of cellular and physiological functions such as cellular secretion, gene transcription, contractility, and cell migration, and are therefore involved in numerous pathological conditions including cancer and heart disease (1-3).

Among Gβγ controlled activities, chemokine GPCR activation governed cell migration plays a key role in many physiological functions, including embryonic development and immune responses. Altered cell motilities are implicated in pathological processes such as immune deficiencies, lack of wound healing, tissue repair and cancer metastasis (14-17). We have recently shown that Gβγ is a key regulator of inhibitory G protein (Gi) – coupled GPCR activation induced macrophage migration (18). In addition to PI3K-PIP3 signaling at the leading edge, we demonstrated that Gβγ mediated activation of

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PLCβ pathway is essential for macrophage migration.

Mammalian cells express twelve Gγ and five Gβ subunits, and form stable Gβγ dimers with the exception of Gβ5, giving rise to 48 possible combinations of Gβγ (19,20). It has been shown that most Gγ subtypes comparably interact with the two most predominant Gβ types in cells, Gβ1 and Gβ2, with the exception of Gγ11 for Gβ2 (21). Similar affinities of Ga11 for Gβγ types have also been demonstrated (20). Studies have suggested the possibility of specific Gβγ subtypes possessing higher affinities towards certain GPCRs or effectors. Using in vitro reconstituted heterotrimers and activated GPCRs, heterotrimers with certain Gγ subtypes exhibited higher affinities for specific GPCRs (20). In addition, specific structural motifs in GPCRs, preferring interactions with certain Gβγ isoforms also have been reported for adenosine family receptors (22,23). Assigned cellular functions to the availability of specific Gβ or Gγ subtypes have also been shown (24,25). For instance, modulation of Golgi vesiculation and cellular secretions by Gγ11 and differential ion channel control by Gγ9 and Gγ3 subunits have been demonstrated (24,25). Gγ3 and Gγ5 were shown to control predisposition of mice to seizures (26).

While these investigations have primarily assigned subunit identity of either Gβ or Gγ subtype to specific signaling activities and cellular functions, molecular and mechanistic basis of such a signaling specificity has not been provided. Gβ subunits have a conserved structure with a >80% identity among their isoforms. However, Gγ isoforms show a significant sequence diversity ranging from ~20-80% (19,27). Therefore, if the Gβγ diversity is a crucial modulator of its signaling and associated cell behaviors, the Gγ identity in these dimers is likely to be a primary regulator of Gβγ signaling. Although Gβγ is classically considered PM bound, recent work has shown that, upon GPCR activation, Gβγ translocates from the PM to internal membranes (IMs) until an equilibrium is reached (25). Interestingly, translocation half time (T1/2) and the extent [T] are governed by the type of accompanying Gγ subunit (28,29). These results further suggest that the PM-affinity of a Gβγ is Gγ subtype dependent. Since, Gγ provides the only PM anchor for Gβγ, the accompanying Gγ subtype-dependent translocation ability of Gβγ suggests that, Gγ subunit controls the PM-affinity of the accompanying Gβγ. Considering that the majority of Gβγ-effector interactions take place at the PM, Gγ-governed PM-affinity is likely to be crucial for Gβγ signaling. Thus, this study is focused on examining how cells employ a selected group of Gγ subunits to tune signal propagation from activated GPCRs to Gβγ effectors, controlling signaling and macrophage migration.

**RESULTS**

**Gγ subtype identity-specific control of PI3Kγ activation by Gβγ**

PIP3 is a major regulator of lamellipodia formation in the leading edge of migratory cells (30). Since Gβγ-PI3K interaction leads to the PIP3 generation (Fig. 1A), we examined whether PIP3 production is controlled in a Gγ subtype dependent manner. To interact with Gβγ and catalyze PIP3 production, PI3K subunit p110 should translocate to the PM upon activation (31,32). The signaling circuit that drives PIP3 production is composed of GPCRs, Gβγ and PI3Kγ subunits (Fig. 1A). PIP3 generation was measured using the translocation of a fluorescently tagged PIP3 sensor (Akt-PH-mCherry) from cytosol to the PM. We have previously shown that localized blue opsin activation results in a robust PIP3 production at the leading edge and directional motility of RAW264.7 cells (33). Since both blue opsin and chemokine receptor 4 (CXC4) activate G proteins with nearly similar efficiencies, blue opsin was employed to induce macrophage migration (Fig. S1, A-C). Activation of Gi-coupled GPCR, blue opsin, induced a robust PIP3 production in RAW cells (Fig. 1B-top). This suggests that the type/s of Gβγ in RAW cell supports PI3K activation. However, the same receptor activation in HeLa cells failed to produce an observable PIP3 response (Fig. 1B-lower). Interestingly, both cell types produced similar G protein activation upon blue opsin activation, when measured using translocation of YFP-Gγ9 and YFP-Gγ3 respectively, indicating equivalent G protein activations (Fig. S1, D-F). Therefore, either absence of proper type of Gβγ or low expression of PI3Kγ or both as the source of this lack PIP3 production. Similar to HeLa cells, PC12 cells also failed to show PIP3 production.
upon GPCR activation. However, unlike HeLa cells, PC12 cells exhibited augmented PIP3 generation upon expression of PI3Kγ, directing the study towards the type of Gβγ in HeLa cells (Fig. 1C). Real-time PCR data from RAW and HeLa cells revealed that they express substantially different Gγ subunit profiles (Fig. 1D). Compared to RAW cells, HeLa cells show a ~6 fold lower expression of Gγ3 while the expression of Gγ4 is also significantly lower in HeLa cells. Expression of Gγ3 in HeLa cells resulted in an elevated basal PIP3, even without GPCR activation (Fig. 1F). Blue opsin activation resulted in a robust PIP3 production in these cells. Although Gγ4 expression did not promote an elevation of basal PIP3, opsin activation exhibited a minor increase in PIP3 in HeLa cells (Fig. 1F-yellow arrows). Nevertheless, Gγ9 expressing HeLa cells failed to induce PIP3 production either at the basal state or upon opsin activation (Fig. 1F). Real-time PCR data indicated that overexpression of a Gγ subunit results in the reduction of the fractional contribution of endogenous Gγ subunits to the pool, making the introduced Gγ subunit dominant, creating nearly a mono-Gγ system (Fig. 1E).

Optogenetic determination of PM affinities of 12 Gγ subunits using T\(_{1/2}\) of Gγ

Measurement of G protein activation upon ligand addition is prone to experimental artifacts due to inconsistencies associated with the agonist injection and variations in its diffusion through the culture media. This hinders calculation of precise T\(_{1/2}\) as well as extent of translocation Gβγ. Thus, to measure the dependency of Gγ type on translocation properties of Gβγ, optically controlled activation of GPCR-G protein signaling was utilized as follows. HeLa cells expressing blue opsin together with each of YFP tagged 1-13 Gγ types were examined for translocation upon activation of blue opsin (Fig. 2A-C). Cells were supplemented with 11-cis retinal for 5 min before opsin activation. Gβγ translocation was measured using YFP fluorescence dynamics in IMs (F\(_{IM}\) vs time curves), and the data were fitted to the logistic function (\(F_{IM} = \frac{T_{\text{max}} - T_{\text{base}}}{1 + (T_{1/2}/T_0)^n} + T_{\text{base}}\)), because GPCR activation results in an approximately sigmoidal increase in Gβγ in IMs, which reaches saturation over time. Using the fitted curves, T\(_{1/2}\) and the extent of translocation |T| = (\(T_{\text{max}} - T_{\text{base}}\)) of individual Gγ subtypes were calculated (Table S1). The plot of |T| vs T\(_{1/2}\) (Fig. 2D) exhibited a strong exponential decay correlation (adjusted R\(^2\)=0.94). This suggests that the Gγ types with moderate to slow translocation rates are translocation deficient (small |T|). These data also indicate that |T| and T\(_{1/2}\) of Gγ are linked and likely to be controlled by the ability of Gβγ to interact with the PM (Fig. 2E). Considering the link between the T\(_{1/2}\) of Gβγ dissociation from the PM and free energy of the associated transition state (\(\Delta G\)), the T\(_{1/2}\) of Gβγ was considered as an index of the PM residence time and the PM affinity of Gβγ because the T\(_{1/2}\) includes the effects of Gβγ shuttling between IMs and the PM.

T\(_{1/2}\) values of Gγ9 translocation were identical in HeLa, RAW and HEK cells (Fig. S1A-F). This demonstrates that translocation properties of Gγ types are conserved among cell types, suggesting conserved PM affinities of Gγ types. Although Gγ types only possess two types of lipid anchors (geranylgeranyl and farnesyl) at their carboxy terminal cysteine (in the CaaX motif), they exhibit a discrete series of T\(_{1/2}\) values (Table S1). Therefore, distinct regions of PM-interacting CaaX motifs of Gγ subunits appear to provide further control over their PM affinities, resulting in a discrete series of T\(_{1/2}\) values.

Gγ-dependent control of chemokine pathway mediated RAW cell migration

Since the subtype identity of Gγ controls PIP3 formation, we examined whether cell migration is also controlled in a Gγ type dependent manner. Real-time PCR data showed that ~30% of Gγ7 in WT RAW 264.7 cells is Gγ3, a high PM-affinity (HiAf) Gγ type (T\(_{1/2}\) = ~270 s) (Fig. 1D). In response to localized optical activation of blue opsin, RAW cells migrate efficiently with a leading edge velocity (vLE) of 0.82 µm/min and trailing edge velocity (vTE) of 0.51 µm/min (Fig. 3A, B). Knockdown of endogenous Gγ3 using the most effective shRNA identified by screening five constructs (Fig. S2) resulted in a complete cessation of cell migration (Fig. 3A, B). Non-specific shRNA did not affect WT RAW cell migration. Expression of HiAf-Gγ2 (T\(_{1/2}\) = ~181 s) in Gγ3 knocked down
cells resulted in rescuing the lost migration ability with νLE: 0.61 µm/min and νTE: 0.28 µm/min (Fig. 3A, B). Expression of low PM-affinity (LoAf) Gγ subtypes showed a marked reduction of migration, i.e. Gγ9 (T½ = ~5 s) νLE: 0.20 µm/min, νTE: 0.03 µm/min and Gγ1 (T½ = ~13 s) νLE: 0.24 µm/min, νTE: 0.04 µm/min (Fig. 3A, B). Further, expression of moderate PM-affinity (MoAf) Gγ4 (T½ = ~116 s) also reduced the migration ability of WT RAW cells substantially (νLE: 0.38 µm/min, νTE: 0.18 µm/min). Although, LoAf-Gγ expressing cells occasionally showed lamellipodia formation at the leading edge, trailing edge retraction was not observed. These data collectively suggest that the higher the PM affinity of Gγ, the greater the migration ability of RAW cell. To examine the universal nature of HiAf Gγ subunit requirement in chemokine pathways, we examined if the introduction of HiAf-Gγ3 helps non-migratory HeLa cells to migrate. Localized opsin activation in HeLa cells expressing Gγ3 showed a distinct trailing edge retraction with lamellipodia formation at the leading edge, resulting in a net movement of the cell. No such responses were observed in WT or Gγ9 expressing HeLa cells for similar signaling activation (Fig. S3).

Control of RAW cell migration by CaaX and pre-CaaX residues in the CT of Gγ

Since the CT of Gγ provides sites for Gbγ dimers to anchor and interact with the PM, which is required for Gbγ signaling, properties of their CT on RAW cell migration was examined. The CT sequences of Gγ exhibit a significant diversity (Fig. 2C) (19,27). Sequence alignment and structural data show that, after the conserved F59 residue in all Gγ subtypes (except Gγ13), CT region loops out from a conserved hydrophobic pocket on Gb (Fig. 2C, 4A), delineating its last contact point with Gb (Fig. 4A) (34). The pre-CaaX region of Gγ (between F59 and the CaaX) therefore appears to interact with the PM and partially modulates the PM-affinity of Gbγ. The lack of electron density for the CT of Gγ in Gbγ crystal structures indicates that, this region is unstructured and suggests dynamic interactions with the PM. We employed a group of Gγ mutants comprising the body of HiAf-Gγ with a substituted CaaX and or pre-CaaX motifs from LoAf-Gγ and vice-versa (Fig. 4B). Translocation properties of these mutants resembled properties of WT Gγ in which the introduced CT motifs were originated (Table S1). For instance, Gγ9 with pre-CaaX + CaaX of Gγ3 (Gγ9-γ3CT) exhibited similar translocation properties to Gγ3. On the contrary, Gγ3 with pre-CaaX + CaaX regions of Gγ9 (Gγ3-γ9CT) exhibited similar translocation properties to Gγ9 (Fig. 4C). The incorporation of an extra cysteine to Gγ3 CaaX moiety eliminated the translocation ability of Gbγ9 (Fig. S4A). This is likely due to the second geranylgeranyl lipid anchor attachment. Deletion of cysteine from the CaaX motif resulted in complete disruption of PM localization of Gbγ9, limiting it only to the cytosol (Fig. S4B), indicating the lipid anchor requirement for PM interaction of Gbγ. The cells expressing above-mentioned mutants were also examined for their ability to modulate RAW cell migration. For instance, Gγ9-γ3CT mutant induced cell migration. On the contrary, cells expressing Gγ3-γ9CT mutant failed to migrate, recapitulating migration behavior of RAW cells expressing Gγ9 (Fig. 4D, E). Collectively, these data suggest that CaaX and pre-CaaX residues of the CT of Gγ control the PM affinity and the signaling efficiency of Gbγ.

Modulation of RAW cell migration potential by Gγ subtype-dependent activation of PI3Kγ

Since PIP3 is a key regulator of chemokine induced cell migration, we examined if PIP3 production is Gγ-type dependent. RAW cells expressing the PIP3 sensor, Akt-PH-mCherry, showed a significant PIP3 accumulation at the leading edge upon localized optical activation of blue opsin (Fig. 5B, SI movie 1). Inhibition of Gbγ with gallein and PI3Kγ with wortmannin ceased PIP3 production and migration of RAW cells (Fig. 5A, B). A Gallein-like compound, fluorescein did not show any effect either PIP3 production or migration. Cells expressing Gγ3 showed a leading edge PIP3 production and a directional migration similar to the responses exhibited by WT RAW cells (Fig. 5C, SI movie 2). On the contrary, plots show that Gγ9 expressing RAW cells exhibit mild or no PIP3 production. These cells further failed to migrate as well. (Fig. 5C, SI movie 3). Gγ3 knocked down cells neither showed PIP3 production at the
leading edge nor cell migration upon opsin activation (Fig. 5C, D, SI movie 4). Additionally, RAW cells expressing Gγ3 mutants composed of either pre-CaaX or CaaX motifs or both from Gγ9 failed to produce PIP3 at the leading edge and subsequently migrate (Fig. 5F). Interestingly, cells expressing Gγ9-γ3CT mutant (both CaaX and pre-CaaX from Gγ3) showed both PIP3 production and cell migration. However, Gγ9 mutants with either pre-CaaX alone or CaaX alone from Gγ3 failed to show PIP3 production or cell migration. This can be understood by examining PM affinities (Tt1/2 values) of Gγ types and their mutants listed in Table S1. The order of Tt1/2 is: Gγ3 > γ9-γ3CT > γ3-γ9CaaX > γ9-γ3CaaX > γ3-γ9CT > γ9. PIP3 dynamics in RAW cells expressing Gγ types exhibited a reasonable fit to the logistic function with an adjusted R² > 0.93 (Fig. 5E). This comparative PIP3 response analysis illustrates that cells expressing only HiAf-Gγ subtype, including Gγ3, Gγ2, and Gγ9-γ3CT mutant elicited a significant PIP3 generation. Such a robust PIP3 production appears to be required for cell migration. Fitted curves also showed that both WT and HiAf-Gγ3 expressing RAW cells possess comparable mean rates of PIP3 production: 0.0022 sec⁻¹ and 0.0030 sec⁻¹, respectively. However, the mean rate of PIP3 generation in MoAf-Gγ4 expressing cells (0.0009 sec⁻¹) was more closer to Gγ9 (0.0007 sec⁻¹) and Gγ12 (0.0012 sec⁻¹) than to Gγ3. Collectively, these data indicate that, only Gγ types with the highest PM affinity support significant PIP3 production and RAW cell migration.

Gγ subtype - dependent control of Gβγ mediated PLCβ activation

Recently, we demonstrated that Gi-coupled GPCR activation induced RAW cell migration requires an increase in cytosolic calcium (Ca²⁺) which is governed by Gβγ mediated activation of PLCβ to induce trailing edge retraction (18). Thus, we examined if PLCβ activity in RAW cells is also controlled in a Gγ subtype dependent manner, in the same way it controlled PI3Kγ activation. Ca²⁺ mobilization upon endogenous Gi-coupled complement component 5a receptor (c5aR) activation in RAW cells with 10 μM c5a (35) was measured using a fluorescence probe for Ca²⁺, fluo-4-AM. WT and HiAf-Gγ3 expressing cells showed Ca²⁺ responses to a higher degree (Fig. 6A, B, D), while LoAf-Gγ9 expressing RAW cells showed minor or no Ca²⁺ response upon c5aR activation (Fig. 6C, D). Interestingly, MoAf-Gγ4 and Gγ12 expressing cells only exhibited a relatively weak response (Fig. 6E, F). Replacement of the entire CT or CaaX motif alone in Gγ3 with those of Gγ9 respectively, resulted in loss of Ca²⁺ mobilization ability of WT Gγ3 (Fig. 6G, H). Although, expression of Gγ9-γ3CaaX mutant failed to elicit Ca²⁺ mobilization, mutant Gγ9-γ3CT showed a Ca²⁺ response, which is equivalent to responses exhibited by WT as well as Gγ3 expressing RAW cells (Fig. 6G, H). In addition, we confirmed that the resultant Ca²⁺ responses are similar for Gγ with different fluorescent tags (Fig. 6A-D).

\[ T_{t1/2} \text{ of Gγ is a strong predictor of Gβγ effector activation ability} \]

The purpose was to examine the hypothesis that the extent of Gβγ effector responses elicited upon GPCR activation in a cell can be predicted using the averaged \( T_{t1/2} \) of endogenous Gγ pool. The experimental process to test this concept is given in Fig. 7A. PIP3 production in HeLa cells expressing each of the 12 Gγ subtypes upon blue opsin activation was measured and plotted against the \( T_{t1/2} \) of Gγ types (Fig. 7A-blue box). The extent of PIP3 production in each Gγ expressing cells was considered as the effector activation, \( [EF]_{exp}^{\gamma} \), and was measured using baseline-normalized increase of Akt-PH-mCherry fluorescence at the PM due to PIP3 production (Fig. 7B). \( T_{t1/2} \) values of each Gγ type translocation were also similarly calculated by measuring YFP-Gγ translocation (Fig. 2A-C). The fitted straight line on the resultant \( [EF]_{exp}^{\gamma} \) vs \( T_{t1/2} \) (HeLa effector plot-blue box) exhibited an R² value of 0.94 (Fig. 7C). This indicates a linear relationship between the Gγ effector responses and the PM affinities of Gβγ. Next, translocation properties of endogenous Gβγ pool in HeLa and RAW cells were measured using blue opsin activation induced YFP-Gβ1 translocation (Fig. 7D). Since Gβ translocates with endogenous Gγ, \( T_{t1/2} \) of Gβ was considered as an indicator of endogenous Gγ translocation and we termed it average \( T_{t1/2} (\text{avg}-T_{t1/2}) \). The fast \( T_{t1/2} \) of Gβ
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observed in Gγ9 expressing cells (T1/2 of Gβ1=7±2 s and Gβ2=6±1 s) confirms that Gβ represents translocation properties of endogenous or introduced Gγ (Fig. 7D, E, Table S2). The avg-T1/2 value observed for RAW cells (221±5 s) was greater than avg-T1/2 of HeLa cells (93±2 s) (Fig. 7D, Table S2). These results suggest that, compared to HeLa cells, RAW cells express more HiAf-Gγ types. This data is also in agreement with real-time PCR data of Gγ mRNA (Fig. 1D). To ensure that the type of Gβ does not influence endogenous Gγ translocation, similar experiments were performed in both HeLa and RAW cells, however expressing YFP-Gβ2 (Fig. 7E, Table S2). The observed T1/2 of Gβ1 and Gβ2 were comparable, suggesting that the type of Gβ does not alter the T1/2 of Gγ.

Next, blue opsin activation induced PIP3 production in WT HeLa and WT RAW cells were measured to obtain effector activity induced by endogenous Gβγ (|EF|exp or Δ[PIP3]). Data show that RAW cells possess a twofold higher effector activation ability than HeLa cells (Fig. 7F). The avg-T1/2 obtained above for both HeLa and RAW cells (Fig. 7E) were then extrapolated on the HeLa effector plot (Fig. 7C) to obtain predicted effector activities (|EF|calc). The ratio of experimental and calculated effector activities (|EF|exp : |EF|calc) for HeLa and RAW cells were found to be 0.82 and 0.99 respectively. This shows that |EF|exp : |EF|calc ratios for both HeLa and RAW cells are closer to one. Therefore, the avg-T1/2 of endogenous Gγ pool is a strong predictor of a cell’s Gβγ effector-activation ability.

PM-residing ability of Gβγ produced upon GPCR activation on its effector activation potential

Similar to HiAf-Gγ types, moderate affinity Gγ (MoAf-Gγ) types also maintain considerably high Gβγ concentrations on the PM after GPCR activation (Fig. 2B). However, it was unclear why MoAf-Gγ expression does not promote robust PIP3 productions, as seen in HiAf-Gγ expressing cells (Fig. 5E, 7B). To comprehend this, a model is proposed in which Gβγ on the PM stays in a transiently active conformation (Gβγ-PM*) which can activate effectors. We assume that, the lifetime (τ) of this transiently active conformation is dependent on the corresponding Gγ type or more specifically the PM affinity of Gγ type. Using Gγ12 as a model MoAf Gγ, we first examined if the observed lack of translocation in Gγ12 is controlled by factors other than its CT. We substituted CT of Gγ12 with CT of Gγ3 and Gγ9 respectively (Fig. S5). Compared to the moderate translocation observed in Gγ12 (T1/2 ~ 80 s, Table S1), Gγ12-Gγ9CT mutant showed a fast translocation with T1/2 ~ 8 s, resembling translocation properties of Gγ9. As expected, Gγ12-Gγ3CT mutant translocated slower than Gγ12 (T1/2 ~ 232 s) (Fig. S5). Similar changes were observed for Gγ3-Gγ9CT mutant (Fig. 4C). Since the CT of Gγ does not interact with the receptor, the fast translocating mutants of MoAf and HiAf Gγ suggest that, their heterotrimers are equally activated by the GPCR, as seen for heterotrimers with LoAf Gγ through their intense Gβγ translocation. These observations indicate that, though MoAf-Gβγ are liberated from the heterotrimer and reside on the PM, a fraction of them is not conformationally appropriate for Gβγ effector activation. These findings are consistent with recent reports suggesting that K-Ras possesses orientation dependent effector binding (36).

Data-guided computational modeling of Gγ subtypes driven signal propagation

As an extension to our previous model (37), reactions for Gβγ effector activation in a multi-Gγ containing cell were modeled, to decipher how a diverse group of Gγ types in a cell controls Gβγ effector activities. The optimized model (variation 3) encompasses reactions in Fig. 8A (see SI for Variation 1 and 2). The model includes novel circuits for Gβγ to (a) activate effectors, (b) translocate to IMs, and (c) be in fluctuating conformationally active and inactive states on the PM (GβγPM* ≡ GβγPM). Considering that many proteins fluctuate among multiple conformations, it is likely that Gβγ fluctuates among these structures while one particular conformation has a stronger affinity towards Gβγ effectors (Fig. 8A, B) (36,38,39). The kinetic curves show that Gβγ in the mono-Gγ3 system is primarily in the PM bound active state (Gβγ-PM*), and is available for effector activation (Fig. 8C). The simulations show low concentrations of Gβγ-PM* for mono-Gγ4 and mono-Gγ9 systems compared to mono-Gγ9. Simulations also demonstrate that, in mixed Gγ systems, Gγ3 is the primary contributor for the
active Gβγ on the PM (PM*) (Fig. 8D). Concentrations of activated effectors ([βγPM]E) were plotted as a function of time. The mono Gγ-systems exhibited effector responses (Fig. 8E), and are similar to the experimental PIP3 and Ca2+ responses observed in HeLa and RAW cells expressing specific Gγ subtypes, that we also defined as mono Gγ systems. Simulations show that mono-Gγ3 system rapidly activate 90% of the effectors in 195 s, while Gγ4 and Gγ9 systems exhibit minor effector activities.

Effector activation by mixed Gγ [βγ3+4+9]E system with equal compositions of HiAf-Gγ3, MoAf-Gγ4 and LoAf-Gγ9 (Fig. 8D) exhibited that Gγ3 interacts with effectors the most (Fig. 8E). In this system at 600 s, 99% of the effectors are activated and 82% of these effectors are bound to Gγ3 (i.e., [βγ3PM]E), 16% to Gγ4 ([βγ4PM]E), and 2% to Gγ9 ([βγ9PM]E). Here, effector activations by MoAf-Gγ4 and LoAf-Gγ9 are lower with respect to mono-Gγ7 systems. Thus, the model predicts that in mixed systems, physiological responses are primarily governed by HiAf-Gγ. This is also observed for 1:1 mixture of MoAf-Gγ4 and LoAf-Gγ9, where the activity is determined by the available higher affinity Gγ subtype (i.e., Gγ4, Fig. 8F). It is noteworthy that, if translocation rate constants are set equal for individual Gγ type (i.e.,kin = kout), this activity dominance of HiAf-Gγ is not observed. When fluctuation between active-inactive Gβγ conformations was not incorporated, equal effector activity from Gγ3 and Gγ4 is observed, contradicting experimental observations.

DISCUSSION

Considering diverse and unique tissue and cell type specific Gγ type distribution patterns, the Gγ identity specific regulation of Gβγ signaling can have a broader impact on the current understanding of GPCR-G protein signal transduction. If Gβγ were to be a unitary signaling entity, cells would have intense Gβγ signaling on all occasions of GPCR activation, which can be deleterious. For instance, RAW cells have a Gγ profile with HiAf-Gγ that supports PI3K activation and PIP3 production. However, for a usually immobile cell type like HeLa, intense PIP3 production may not serve a purpose and thus HiAf-Gγ expression is not required. Supporting this notion, Gγ3 expression allowed HeLa cells to produce PIP3 upon GPCR activation. The fundamental difference identified between the introduced Gγ3 over endogenous Gγ7 types in HeLa cells was the ability of Gγ3 to make Gβγ more available at the PM, where PIP3 production takes place. To catalyze PI2 to PI3, Gβγ recruits and activates PI3K subunits to the PM (40). Out of the 12 Gγ types, only Gγ3 and Gγ2 promoted PIP3 production. This is likely due to the weak translocation properties of Gγ3 that allows maintenance of a relatively higher concentration of free Gβγ on the PM. The Gγ dependent differential PIP3 generation in HeLa cells hints at a plausible mechanism of how Gβγ effectors are recruited to the PM and activated by PM bound fraction of HiAf-Gγ7. We recently showed that Gβγ controls PLCβ activation, induces Ca2+ mobilization, governing the trailing edge retraction during RAW cell migration (18). Similar to PI3Kγ, PLCβ1 and PLCβ2 are also cytosolic (41,42). Our data suggests that, PM targeting and-or activation of these Gγ effectors are likely to be governed by the PM-affinity of Gβγ. The extent of effector responses suggests that, the stronger the PM-affinity of Gβγ, the greater its potential to control signaling. Here we employed T1/2 as an index for the residence time on the PM or the PM affinity of Gβγ. The free energy of the translocation (ΔG) is considered as the energy required to dislodge Gβγ from the PM to the IM. Thus it is a direct measure of PM affinity of Gβγ to the PM. ΔG is related to a first order reaction equilibrium constant (Keq) by ΔG = -RT ln Keq. For the Gβγ translocation process, (GβγPM/GβγIM), Keq = kin/kout, and the half time, T1/2 can be expressed as k = 0.693/t1/2, thus ΔG = -RT ln (t1/2/41/2). This indicates that the longer the residence time on the PM, the greater the PM affinity. Translocation t1/2 of Gβγ is a complex measure which includes the shutting of Gβγ between the PM and IMs. However the initial reaction is dominated by Gβγ dislodging from the PM (kin), thus, as shown above, T1/2 is a fair approximation of the PM affinity of Gβγ.

Gγ subunits interact with the PM through the prenyl group. The type of prenylation is decided by the CaaX motif sequence of Gγ. The prenylation with 20-carbon geranylgeranyl lipid provides a
higher PM-affinity to Gβγ compared to the 15-carbon farnesyl lipid attachment. Except for farnesylated Gγ9, Gγ1 and Gγ11, all other Gγ types are geranylgeranylated. However, only Gγ3 and Gγ2 supported RAW cell migration, suggesting factors additional to the type of prenylation control the PM affinity of Gγ. Interestingly, pre-CaaX regions of Gγ3 and Gγ2 are composed of ~80% positively charged and hydrophobic residues, as opposed to ~50% in farnesylated-Gγ subunits. Extensive mutagenesis to the pre-CaaX region of Gγ suggested that this 5 or 6-residue region modulates Gβγ-PM interactions, in which positively charged and hydrophobic amino acids strengthen the PM-affinity. Previously reported translocation data of Gγ mutants with altered pre-CaaX residues further validate the role of this motif in controlling the PM-affinity (29). The complete loss of PM localization observed in Gγ9 upon cysteine removal from CaaX motif indicates that, pre-CaaX region only serves as a strong modulator of PM-affinity, while prenylation is essential for primary PM anchoring of Gβγ. By modulating properties of their pre-CaaX motifs, geranylgeranylated-Gγ subunits managed to possess a discrete series of PM affinities.

Heterotrimer with specific Gγ types have been shown to possess higher affinities towards certain GPCRs (20, 43). However, an exchange of the CT of slow translocating Gγ3 and moderate translocating Gγ12 with the CT of Gγ9 resulted in fast translocating mutants, comparable to Gγ9. This can suggest that either (a) heterotrimer activation process is controlled by the CT of Gγ through modulating Gaβγ-GPCR interactions, or (b) the PM-affinity of generated Gβγ is dependent on the CT of Gγ subunit. Regardless, the CT of Gγ should hold a crucial control over Gβγ function, although our data strongly support the possibility (b). We anticipate that, among the available Gβγ pool, LoAf and MoAf Gβγ types exist primarily to support GrGTP generation, while HiAf-Gβγ subunits activate Gβγ effectors. Our data also support that the PM bound Gβγ composed of HiAf-Gγ types stay a longer fractional time in the active conformation, compared to their LoAf and MoAf associates. Lack of migration ability in Gγ3-knocked down RAW cells strongly support this notion, since the remaining MoAf-Gγ types in RAW cells lack effector activation ability.

Nevertheless, we are aware that, in addition to Gγ diversity, there are converging and diverging pathways and signaling components including integrins, secretory proteins (i.e. matrix metalloproteinases), can influence the migration potential of a cell (44-46). Therefore, differences in cell migration potentials among cell types with diverse origins should be examined considering these potential inherent variables. While we are in concert with these reports, our findings demonstrate that Gβγ governed migration requires appropriate Gγ-types with higher PM-affinity. Supporting these findings, even a non-migratory cell type like HeLa, expressing HiAf-Gγ3 undergo directional migration upon blue opsin activation.

Avg-Tt1/2 of endogenous Gγ measured using Gβ translocation accurately predicted the ability of native Gβγ to control its effectors. Predicted effector activity using this method was similar to the PIP3 production observed in both RAW and HeLa cells. These observations suggest that Tt1/2 and therefore the PM-affinity of a Gγ type is a strong indicator of the ability of Gβγ to activate effectors. Thus, our observations collectively indicate that Gγ subunit diversity in a cell is a crucial factor in determining whether the cell has the ability to activate Gβγ effectors sufficient to orchestrate the intended behaviors, including migration.

In the kinetic model with multi-Gγ and embedded experimental observations that Tt1/2 ∝ PM-affinity and Tt1/2 ∝ |EF|; multiple mechanistic scenarios associated with G protein activation were attempted. The incorporation of an active-inactive conformation circuit to the PM-bound Gβγ was required to simultaneously capture all the experimental responses observed. These include the lack of effector activation by Gβγ associated with MoAf-Gγ. The incorporated circuits to the model indicated that (a) HiAf-Gβγ subtypes tend to readily activate effectors, initiating downstream signaling, (b) the majority of LoAf-Gβγ types translocate away from the PM to down regulate signaling, and (c) the fraction of MoAf-Gβγ that did not translocate tend to minimize signaling by oscillating between a PM bound active-inactive conformational states. While these conformational fluctuations are common for all types of Gβγ, the Gγ type and the PM-affinity decide the lifetime of their active state. The ability of this model to
recapitulate experimental responses indicates its reliability. Therefore, reactions and parameters embedded in our model are likely to closely reflect how PM affinities of Gγ subunits modulate information flow from the activated GPCRs to effectors. The model also allowed simulation of experimentally challenging in vivo conditions, including varying ratios of HiAf-Gγ: LoAf-Gγ and total Gβγ concentrations.

In summary, this study demonstrates that distinct translocation abilities of the 12 Gγ types provide Gβγ a diverse range of PM interaction and effector activation abilities. Since, most Gβγ-effector activities occur at the PM, data confirms that, the PM affinities of Gγ types expressed in a cell are deterministic to the potency of Gβγ effector as well as downstream signaling activation. Although we only show Gγ identity dependent control of PI3Kγ and PLCβ, and their regulation of cell migration, it is likely that a plethora of Gβγ mediated functions is similarly regulated. Since GPCR-G protein signaling is universally conserved and Gβγ signaling pathways are major drug targets, mechanisms we describe here can have a wide influence not only on cell migration but also in many areas of signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents**

The reagents; Gallein (TCI AMERICA), Fluo-4 AM (Molecular probes, Eugene, Oregon), Wortmannin 2APB (Cayman Chemical, Ann Arbor, MI), c5a (Eurogentec), U50488 hydrochloride (Tocris) were initially dissolved in DMSO and then diluted in HBSS (Gibco laboratories) before adding to cells. 11-cis retinal (National Eye Institute) was initially resuspended in absolute ethanol and 2 µl aliquots (50 µM) were further diluted (2 µl for each aliquot) with absolute ethanol before introducing (2 µl) to cells in dark. SDF-1α (PeproTech) was reconstituted in DI water to a concentration 100 µg/mL and further diluted with a buffer containing 0.1% BSA before adding to cells.

**DNA constructs and cell lines**

Engineering of DNA constructs used; blue opsin-mCherry, blue opsin-mTurquoise, Akt-PH-mCherry and YFP tagged Gγ1-Gy13, have been described previously (33,47,48). YFP-β1 and 2, kappa-opioid receptor, PI3K-CA-CFP and mCh-GPI were kind gifts from Professor N. Gautam’s lab, Washington University, St. Louis, MO. Gγ3, Gγ9 and Gγ12 mutants were generated using Gibson assembly (NEB) (49). Parent constructs; mCherry-Gγ3, mCherry-Gγ9, and YFP- Gγ12 were PCR amplified with overhangs containing expected nucleotide mutations. DpnI (NEB) digestion was performed on the PCR product to remove the parent construct. DpnI digested PCR product was then mixed with the Gibson assembly master mix (NEB) and incubated at 50 ºC for 45 min, which was followed by transformation of competent cells and plating on Ampicillin LB agar plates. All the constructs used in this study possess the Ampicillin resistant pcDNA 3.1 vector backbone. Cell lines (HeLa, RAW 264.7, PC12 and HEK cells) were originally purchased from the American Tissue Culture Collections (ATCC) and authenticated using a commercial kit to amplify 9 unique STR loci.

**Cell Culture and Transfections**

RAW 264.7 cells used in migration and PIP3 generation experiments were cultured in RPMI 1640 (10-041-CV; Corning, Manassas, VA) with 10% dialyzed fetal bovine serum (DFBS; from Atlanta Biologicals) and 1% Penicillin–Streptomycin (PS) in 60 mm tissue culture dishes. HeLa cells were maintained in minimum essential medium (MEM; from CellGro) supplemented with 10% DFBS and 1% PS. Around 80% cell confluency, the growth medium was aspirated, 2 mL versene (EDTA) (CellGro) was added, incubated for 3 minutes at 37 ºC, 5% CO2 incubator, and then cells were lifted and suspended in versene. The cell suspension was centrifuged at 1000 g for 3 minutes, versene(EDTA) was aspirated, and the cell pellet was resuspended in their normal growth medium (RPMI/ DFBS/ PS for RAW, and MEM/ DFBS/ PS for HeLa) at a cell density of 1 × 10⁶ /mL. For imaging experiments, cells were seeded on 35 mm glass-bottomed dishes (8 × 10⁴ cells on each) with 15 mm inner diameter, prepared using #1 German cover glasses.
cell seeding, dishes were washed with 2 N NaOH for 20 min, ethanol washed, and sterilized for one hour using UV irradiation. A day following cell seeding, cells were transfected with appropriate DNA combinations using the transfection reagent PolyJet (SignaGen), according to the manufacturer’s protocol and then incubated in a 37 °C, 5% CO₂ incubator. Cells were imaged after 16 hours of the transfection.

Knockdown of Gγ3 in RAW 264.7 cells

Five shRNAs (TRCN0000036794-98; Sigma-Aldrich) were screened in RAW cells by co-expressing with GFP-Gγ3. Cells were screened for GFP expression and the shRNA construct that induced the highest reduction in GFP-Gγ3 expression was selected as the most effective shRNA. The identified TRCN0000036795 shRNA (sequence: CCGGGCTTAAGATTGAAGCCAGCTTCTCGAGAAGCTGGCTTCAATCTTAAGCTTTTTG) was employed to knockdown Gγ3 in the subsequent experiments. A scrambled shRNA was used as the control.

Live cell imaging to monitor Gβγ translocation, PIP3 generation, and optogenetic control of cell migration

Imaging system: Spinning-disk XD confocal TIRF (total internal reflection) imaging system composed of a Nikon Ti-R/B inverted microscope, a Yokogawa CSU-X1 spinning disk unit (5000 rpm), an Andor FRAP-PA (fluorescence recovery after photo-bleaching and photo-activation) module, a laser combiner with 40–100 mW 445, 488, 515, and 594 nm solid-state lasers and iXon ULTRA 897BV back-illuminated deep-cooled EMCCD camera. Live cell imaging was performed using a 60X, 1.4 NA (numerical aperture) objective. In cell migration and PIP3 generation experiments, mCherry tagged receptor blue opsin and the PIP3 sensor Akt-PH were imaged for 10 min using 515 nm excitation–527 nm emission or 488 nm excitation 515 nm emission, respectively. Regular culture media or HBSS supplemented with 1g/mL glucose preincubated in a 37 °C, 5% CO₂ incubator for 30 minutes were used as the imaging medium. During imaging, cells were maintained at 37 °C. To prevent focal plane drifts, Nikon Perfect Focus System (PFS) was engaged.

Cytosolic Ca²⁺ measurements

For intracellular Ca²⁺ measurements, RAW cells seeded on glass-bottom dishes and maintained in a 37°C with 5% CO₂ were transfected with a Gγ subtype on the following day of cell seeding. After 12 to 16 h of transfection, cells were washed twice with Ca²⁺ containing HBSS (pH 7.2) and incubated for 30 min at room temperature with the fluorescent Ca²⁺ indicator, fluo-4 AM in the dark. After incubation, cells were washed twice with HBSS and 500 µl of HBSS was then used as imaging medium. The fluorescence intensity of fluo-4 AM was continuously imaged at 1 s intervals using 488 nm excitation–515 nm emission to capture signal before activation for 50 s. Endogenous c5aRs in RAW cells were activated with 10 µM c5a. Observed fluo-4 AM fluorescence increase due to Ca²⁺ release was base line normalized.

Real-time PCR, transcriptome and RNAseq data analysis

In order to obtain the Gγ profile of WT HeLa and WT RAW 264.7 cells, RNA was extracted from cells grown in 100 mm tissue culture dishes after reaching 90%-100% cell confluency. RNA extraction was performed using the GeneJet RNA purification kit following their given protocol. Extracted RNA was used as the template for cDNA synthesis with Radiant cDNA synthesis kit. cDNA product was quantified using the nanodrop and used for real-time PCR (BioRad CFX96Real-Time qPCR system) in 96 well plates to obtain the Gγ profile. Radiant Green Lo-ROX...
qPCR kit (Alkali Scientific) was used in real-time PCR experiments and β actin gene was used as the housekeeping gene. To screen the Gγ profile alteration with Gγ3 and Gγ9 overexpression, HeLa cells were seeded in 100 mm tissue culture dishes, transfected with GFP-Gγ3 and GFP-Gγ9 respectively at 70%-80% cell confluency, and RNA was extracted after confirming greater than 70% transfection efficiency by observing under the microscope. This was followed by cDNA preparation and real-time PCR.

**Statistics and reproducibility**

Results of all quantitative assays (Gβγ translocation, cell migration, and PIP3 generation) are expressed as standard error of mean (SEM) from n numbers of cells (indicated in the figure legends) from multiple independent experiments. Statistical analysis of cell migration data of WT and mutant Gγ subtypes was performed using two-tailed unpaired t-test. P value < 0.05 was considered as statistically significant.

**Computational modeling**

The dynamic nature of the GPCR signal transduction has been modeled by a series of ordinary differential equations (SI Equations 2) which encompasses the series of reactions in Fig. 8A. Computations were performed in a custom Python 2.7 script with odeint module to numerically integrate the ordinary differential equations. The equations are an extension of our previous model for a ligand activated signal transduction and is extended to allow for effector activation by multiple types of Gβγ subunits (HiAf, MoAf, and LoAf). The reaction mechanism is similar to our previous publication (37) with the classical GPCR activation cycle, and includes novel circuits for Gβγ to (1) activate effectors, (2) translocate to IMs, and (3) be in a conformationally inactive structure on the PM. The equations describe the rates of heterotrimer dissociation, heterotrimer association (SI Equations 1), Gα(GTP) hydrolysis, Gβγ translocation to IMs, Gβγ oscillation to an inactive configuration, and Gβγ effector activation. The rates for heterotrimer dissociation and Gα(GTP) hydrolysis assume Michaelis-Menten kinetics, while all others are assumed as first or second order reactions. The ordinary differential equations define the rates of formation or depletion of the important species in the signaling network (i.e., Gα(GDP)βγ, Gα(GDP), Gα(GTP), GβγPM, GβγPM*, GβγIM, and GβγEF). To incorporate multiple Gβγ subunits and consequently the Gγ diversity, there is an ordinary differential equation for each Gβγ type except the Gα(GTP) and Gα(GDP) concentrations. Numerically integrating these functions, the concentration of the species over time was determined.
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Author contributions: Kanishka Senarath conducted most of the experiments (Gγ translocation, cell migration, real time PCR, PIP3 generation, Ca2+ generation) and formal analysis of results. John L. Payton performed the computational modeling based on the experimental results. Dinesh Kankanamge generated Gγ3 mutant with extra Cysteine and Gγ9 mutant with Cysteine to Alanine mutation in the CaaX motif and conducted experiments. Praneeth Siripurapu performed PIP3 generation experiments in RAW cells with Gβγ and PI3K inhibitors, Gallein and Wortmannin respectively. In addition, he conducted PIP3 generation experiments in HeLa cells with multiple Gγ subtypes. Mithila Tennakoon generated Gγ12 mutants, conducted translocation experiments and analyzed data. John L. Payton critically reviewed the manuscript and wrote the computational modeling section. Ajith Karunarathne and Kanishka Senarath conceptualized the project and wrote the manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
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Figure 1.

A. 

\[
\begin{align*}
\text{GPCR} & \quad \Downarrow \quad \text{G}_\beta_G \\
\text{PI3K}_\gamma & \quad \Downarrow \quad \text{PIP3}
\end{align*}
\]

B. 

Akt-PH-mCh

C. 

PI3K-CFP

D. 

Relative expression

E. 

Relative expression

F. 

Akt-PH-mCh

16
Fig. 1. Gγ identity-controlled PIP3 generation. (A) Pathway for Gβγ mediated PI3K activation. (B) Wild type (WT) HeLa and RAW 264.7 cells expressing the PIP3 sensor; Akt-PH-mCherry and blue opsin-mTurquoise. Cells supplemented with 50 µM 11-cis-retinal were imaged every 5 s for mCherry (with 594 nm). Blue opsin activation with 445 nm blue light induced translocation of cytosolic PIP3 sensor to the PM only in RAW cells but not in HeLa cells. The plot shows the accumulation Akt-PH-mCherry on the PM. Blue arrow points initiation of optical activation (at 30 s). Intensities are baseline normalized. (C) PI3Kγ expression in a HeLa cell failed to induce PIP3 generation on blue opsin activation (black trace). PC12 cells that showed no PIP3 response, elicited a robust repose upon expression of PI3Kγ (green trace). Blue arrow indicates optical activation. (D) Comparison of real time PCR Gγ profiles of HeLa and RAW cells. HeLa cells express mRNA for Gγ12 and Gγ5 in abundance, while Gγ4 and Gγ3 are prominent in RAW cells. (E) Gγ9 (red) and Gγ3 (green) overexpression induced changes to the Gγ profile in HeLa cells. The overexpressed Gγ type appears to dominate native Gγ. (F) HeLa cells expressing Gγ3, blue opsin-mTurquoise, and Akt-PH-mCherry showed an intense PIP3 generation compared to the WT cells upon blue opsin activation. Images and the plot show Gγ4 expression showed a minor (blue trace), while Gγ9 showed no PIP3 generation (green trace), compared to WT (black trace) and Gγ3 (red trace) on the PM. The plot shows the corresponding PIP3. Intensity values are baseline normalized, blue arrow indicates optical activation (Scale bar: 5 µm, Error bars:SEM).
Figure 2.

A.  

| Gy1 | Gy2 | Gy3 | Gy4 | Gy5 | Gy7 | Gy8 | Gy9 | Gy10 | Gy11 | Gy12 | Gy13 |
|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|
| Basal | | | | | | | | | | | |
| 50 s | | | | | | | | | | | |
| 600 s | | | | | | | | | | | |

B.  

C.  

| LoAf | Tt1/2 (s) | MoAf | Tt1/2 (s) | HiAf | Tt1/2 (s) |
|------|----------|------|----------|------|----------|
| Gy9--NPFE-KGGC-far | 5±1 | Gy5--NPFRPQKV-C-far | 71±3 | Gy4--NPFRERKFFC-gerger | 116±2 |
| Gy1--NPFKELKGGC-far | 13±2 | Gy12--NPFKDKKT-C-gerger | 80±2 | Gy8--NPFRDKRLFC-gerger | 124±1 |
| Gy11--NPFE-KGSC-far | 38±2 | Gy10--NPFRERPS-C-gerger | 97±4 | Gy2--NPFRKFFC-gerger | 181±4 |
| Gy7--NPFKDDKP-C-gerger | 41±2 | Gy13--NPWVE-KGKC-gerger | 100±3 | Gy3--NPFRKFFC-gerger | 270±4 |

D.  

E.  

\[ y = y_0 + Ae^{-t} \]

\[ R^2 = 0.9359 \]
Fig. 2. Gγ-identity driven differential translocation of Gβγ. (A) HeLa cells expressing blue opsin-mTurquoise and each of the 12 Gγ subunit with a YFP fluorescent tag. Cells were supplemented with 50 µM retinal and were imaged for YFP (515 nm) and activated with 445 nm light at 2 s intervals in a time-lapse series. This process was continued for 10 mins where the YFP fluorescence changes reached the equilibrium. (B) Plots show baseline normalized YFP fluorescence increase in IMs over time (Error bars: SEM, n= 10; Scale bar: 5 µm). (C) Alignment of carboxy termini (CT) sequences of 12 Gγ, indicating the properties of amino acids (red: acidic, blue: basic, green: hydrophobic uncharged, Black: other residues) and their translocation half time values (T1/2). Here, Gγ types are grouped, based on their PM affinities. (D) Plot of T1/2 vs. |T| shows an exponential decay relationship. (E) Schematics of GPCR activation induced G protein heterotrimer activation and dissociation. LoAf-Gβγ translocate from the PM to IMs faster compared to HiAf-Gβγ, while HiAf-Gβγ interact with effectors to initiate signaling pathways leading to cellular responses efficiently compared to LoAf-Gβγ.
Fig. 3. Subtype specific control of macrophage migration by Gγ. (A) RAW 264.7 cells expressing blue opsin-mCherry and a selected Gγ subunit, supplemented with 50 µM 11-cis-retinal. Blue opsin was activated in confined regions of cells using a 445 nm laser with 0.22 µW/µm² power in every 2 s interval (white boxes). The images show cells before and after 20 mins of blue opsin activation. Note the difference in cell movement towards the optical input with respect to the Gγ type the cell possesses. Gγ3 expressing cell shows an almost identical cell migration as the WT, and Gγ2 also supports migration. Note the inhibition of cell migration in Gγ3 knocked down cells. This migration loss was rescued by expressing HiAf-Gγ2, but none other. (B) Bar graph shows the relative displacement of cells’ leading and trailing edges, with blue opsin activation (Error bars: SEM, n= 12, * P= 0.021, ** P< 0.0001, *** P< 0.0001; Scale bar: 5 µm).
Figure 4.

A. Gγ3 with Gγ9 CaaX
   Gγ3 with Gγ9 CT
   Gγ9 with Gγ3 CaaX

B. Gγ3 WT    ----- NPFREKKFFCALL
   Gγ3 with Gγ9 CaaX ---- NPFREKKFFCLIS
   Gγ3 with Gγ9 CT     ---- NPFRE-KGGCLIS
   Gγ9 WT            ---- NPFKE-KGGCLIS
   Gγ9 with Gγ3 CaaX   ---- NPFKE-KGGLL
   Gγ9 with Gγ3 CT    ---- NPFKEKKFFCALL

C. Gγ3 with Gγ9 CaaX
   Gγ3 with Gγ9 CT
   Gγ9 with Gγ3 CaaX

D. Gγ3 with γ9 CaaX   Gγ3 with γ9 CT   Gγ9 with γ3 CaaX  Gγ9-γ3 CT

E. 

Distance migrated (μm)

- Leading edge
- Trailing edge

- WT
- γ3
- γ3-γ9
- γ9
- γ9-γ3 CaaX
- CaaX CT

Tt1/2 (s)

- γ9
- γ3 with γ9 CT
- γ9 with γ3 CaaX
- γ9-γ3 CT

- 5±2
- 14±2
- 21±3
- 37±3
- 186±5
- 270±4

F/M

- γ9
- γ3 with γ9 CT
- γ9 with γ3 CaaX
- γ9-γ3 CT

- 0.00
- 1.00
- 1.40
- 2.00
- 2.20

Time (s)

- 0
- 100
- 200
- 300
- 400
- 500
- 600

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Fig. 4. Carboxy terminus of Gγ governs rates of Gβγ translocation and the extent of cell migration.  
(A) Crystal structure of the CT region of Gγ in complex with Gβ (~F59 of Gγ-the last Gβ contact point) exposing the hydrophobic binding pocket in Gβ. (B) Sequence alignment of CT mutants of Gγ3 and Gγ9. 
(C) HeLa cells expressing GFP-Gγ mutants and blue opsin-mCherry, supplemented with 11-cis retinal. The cells were imaged for GFP (488 nm) to capture blue opsin activation induced translocation. Note the significant difference in mutant translocation compared to WT counterparts. (Error bars: SEM, n=10; Scale bar: 5 µm). (D) RAW 264.7 cells expressing each of the mutant Gγ and blue opsin-mCherry, supplemented 11-cis-retinal. Blue opsin in cells were activated locally (white box) in 2 s intervals for 20 min to induce migration. (E) The histogram shows the movement of leading and trailing edges. Permutations to the CT sequences clearly altered the cell migration ability (Error bars: SEM, n=12, * P= 0.0009 for the leading edge and 0.5714 for the trailing edge, ** P< 0.0001, *** P< 0.0001, **** P< 0.0001; Scale bar: 5 µm).
Figure 5.

A. 

```
    GPCR
     ↓
Gallein  Gβγ
     ↓
    PI3K  Wortmannin
     ↓
    PIP3
```

B. 

```
t=0   10 mins

WT

Gallein

Wortmannin

Gallein

Wortmannin
```

C. 

```
t=0   10       0      10      0       10  0           10 mins

WT

Gγ3

Gγ3 shRNA

Gγ4

Gγ12

Gγ9
```

D. 

```

Time (s)

PI3 generation

γ3

γ3 shRNA

γ4

γ12

γ9
```

E. 

```

Time (s)

PI3 generation

WT

γ3

γ4

γ12

γ9
```

F. 

```
t=0   10       0      10      0       10  0           10 mins

Gγ9 with γ3 CT

Gγ3 with γ9 CaaX

Gγ9 with γ3 CaaX

Gγ3 with γ9 CT
```

G. 

```

Time (s)

PI3 generation

γ9 with γ3 CT

γ3 with γ9 CaaX

γ9 with γ3 CaaX

γ3 with γ9 CT
```
Fig. 5. Gγ type dependent activation of PI3Kγ during macrophage migration. (A) GPCR mediated PIP3 generation pathway and its selected inhibitory points. (B) RAW 264.7 cells expressing Akt-PH-mCherry and blue opsin, supplemented with 50 µM 11-cis-retinal. On localized blue opsin activation with 445 nm (white box), WT cells showed the PIP3 production at the activated leading edge. Cells treated with PI3K inhibitor; wortmannin and Gβγ inhibitor gallein inhibited both PIP3 production and cell migration, confirming that PIP3 is required for directional cell migration. (C) RAW 264.7 cells expressing Akt-PH-mCherry, blue opsin-mTurquoise, a Gγ subunit (Gγ3, Gγ9, Gγ4, Gγ12) and supplemented with 50 µM 11-cis-retinal. On localized blue opsin activation with 445 nm (white box), Gγ3 expressing cells showed PIP3 generation at the leading edge. However, Gγ4, Gγ12 and Gγ9 expressing cells showed minor/no PIP3 accumulation. Gγ3 knocked down cells also showed no PIP3 generation. (D) Plots show the PIP3 generation with Gγ3, Gγ9, Gγ4, Gγ12 overexpression and Gγ3 knockdown compared to the WT (E) Smoothed curves fitted to logistic function show Gγ type dependent differential PIP3 responses. (F) RAW 264.7 cells expressing Akt-PH-mCherry, blue opsin-mTurquoise, each of the mutant Gγ types and supplemented with 50 µM 11-cis-retinal. Upon migration induction, cells expressing the mutant Gγ9-Gγ3CT showed both PIP3 as well as migration. Failure to exhibit migration in Gγ9-Gγ3CaaX cells shows the significance of the pre-CaaX motif of Gγ in Gβγ signaling. Gγ3-Gγ9CT mutant cells exhibited neither PIP3 production nor migration. (G) The plot shows PIP3 generation in RAW cells expressing CT mutants of Gγ (Error bars: SEM, n=15; Scale bar: 5 µm).
Figure 6.

A. mCherry-GPI  
No γ transfection

B. mCherry-γ3  
GFP-γ3

C. mCherry-γ9  
GFP-γ9

D. 

E. YFP-γ4  
YFP-γ12

F. c5a

c5a

c5a

c5a

c5a

G. GFP-γ3 with γ9 CaaX  
GFP-γ3 with γ9 CT

H. GFP-γ9 with γ3 CaaX  
GFP-γ9 with γ3 CT
Figure 6. PLCβ activation induced differential Ca\textsuperscript{2+} response with different Gγs. RAW 264.7 cells expressing different WT Gγs and Gγ mutants were stimulated with 10 µM c5a addition to activate endogenous c5a receptors (c5aRs) after 30 min fluo-4 incubation. Cells were imaged at 40X magnification to capture the Ca\textsuperscript{2+} response. (A) Control (mCherry-GPI and untransfected) and, (B) Gγ3 expressing cells showed greater Ca\textsuperscript{2+} response compared to, (C) Gγ9 expressing cells, which showed almost no Ca\textsuperscript{2+}. Scale bar, 10 µm. (D) Plot shows the difference in fluo-4 signal (GFP fluorescence) increase in cells, indicating differential Ca\textsuperscript{2+} release to the cytoplasm depending on the Gγ subtype they overexpress. Also, it shows that the fluorescent tag of the Gγ subtype is not affecting the Ca\textsuperscript{2+} response (n=8). (E, F) MoAf-Gγ4 and Gγ12 expressing cells showed minor Ca\textsuperscript{2+} response with c5aR activation(n=8). (G, H) Gγ9 mutants with Gγ3 CaaX and Gγ3 CT showed an increased Ca\textsuperscript{2+} response compared to WT Gγ9, while Gγ3 with Gγ9 CaaX and Gγ9 CT showed a reduced Ca\textsuperscript{2+} response compared to WT Gγ3, confirming differential Gβγ-effector interactions with respect to the difference in the PM affinity thus different PM residence times of Gβγ (Error bars: SEM(; Scale bar: 10 µm).
Figure 7.

A. 
- Expression of a Gγ
- Determine \( T_{1/2, ITI} \)
  Repeat for all Gγs

Determine avg-\( T_{1/2} \) of cells using Gβ translocation

\[ \Delta \] \( \beta_1 \)-YFP \( \beta_2 \)-YFP 
mCh-γ9

Before After Before After

B. 
Gγ1-4
Gγ5-9

\[ \gamma_1, \gamma_4, \gamma_7, \gamma_8 \]

\[ \gamma_2, \gamma_3, \gamma_6, \gamma_9 \]

\[ \gamma_{10-12} \]

Fitted PIP3 responses

C. 
\[ y = a + bx \]
\[ R^2 = 0.94 \]

| \( \gamma_i \) | \( T_{1/2} \) |
|--------------|
| \( \gamma_1 \) | 6 ± 2 |
| \( \gamma_2 \) | 7 ± 2 |
| \( \gamma_9 \) | 6 ± 1 |
| \( \gamma_{10} \) | 6 ± 1 |

D. 
β1-YFP
β2-YFP
mCh-γ9

Before After Before After

E. 
HeLa
RAW

β1-YFP
β2-YFP

Before After Before After

F. 

[Graph showing fitted PIP3 responses for different cell types]
Fig. 7. Testing $T_{1/2}$ of $G_\gamma$ as a predictor of a cell’s ability to control $G_{\beta\gamma}$ effectors. (A) Experimental process of predicting $G_{\beta\gamma}$ effectors activity using $G_\gamma$ type dependent PM affinity ($T_{1/2}$). (B) Plots showing the extent of blue opsin activation induced PIP3 generation in HeLa cells expressing each of the 12 $G_\gamma$ types. Smoothed and logistic function fitted curves of PIP3 generation with all $G_\gamma$s. (C) Plot of $|EF|$ vs $T_{1/2}$ of all 12 $G_\gamma$ types. The $|EF|$ was measured using PIP3 production on the PM in HeLa cells expressing each of the 12 $G_\gamma$ types and Akt-PH-mCherry. (D) HeLa cells expressing blue opsin-mTurquoise, mCherry-$G_\gamma$9, either YFP-$G_\beta$1 or YFP-$G_\beta$2 respectively, supplemented with 50 µM 11-cis-retinal. On blue opsin activation, both $G_\beta$1 and $G_\beta$2 exhibited $T_{1/2}$ closer to that of $G_\gamma$9, further confirming that the translocation properties of $G_\beta$ represent the prominent $G_\gamma$ subtype expressed in the cell. (E) HeLa and RAW cells expressing blue opsin-mTurquoise and either YFP-$G_\beta$1 or YFP-$G_\beta$2 respectively, were supplemented with 50 µM 11-cis-retinal. Cell was imaged for YFP and blue opsin was activated with 445 nm light every 3 s. $G_\beta$ translocation exhibited the average translocation properties of the entire pool of endogenous $G_\gamma$. $G_\beta$ type does not influence translocation properties endogenous $G_\gamma$ in HeLa cells. The plot shows that $T_{1/2}$ of $G_\beta$1 and $G_\beta$2 translocation was closer to the $T_{1/2}$ of the most abundant $G_\gamma$ of each cell type. (F) Blue opsin activation induced experimental $|EF|$ (PIP3 response) measured in WT HeLa and RAW cells expressing blue opsin and the PIP3 sensor (Error bars: SEM, n=10; Scale bar: 5 µm).
Figure 8.

A. 

\[ R + L \xrightarrow{k_1} RL \xrightarrow{k_2} RL-\alpha G \gamma \xrightarrow{\alpha GTP} \alpha G \gamma + \beta Y_{PM} \xrightarrow{k_{PM}} \beta Y^n_{PM} \]

\[ \beta Y^n_{PM} \xrightarrow{k_{out}} \beta Y^n_{IM} \]

\[ \beta Y^n_{IM} \xrightarrow{k_{in}} \beta Y^n_{PM} \]

\[ n=1-5, 7-13 \]

B. 

Active G\beta\gamma on the PM

Inactive G\beta\gamma on the PM

G\beta\gamma on in IMs

C. 

Mono-G\gamma3 system

Mono-G\gamma4 system

Mono-G\gamma9 system

D. 

G\gamma3:G\gamma4:G\gamma9 – 1:1:1 system

Active G\beta\gamma on the PM

Inactive G\beta\gamma on the PM

G\beta\gamma on in IMs

E. 

Mono-G\gamma systems

G\gamma3:G\gamma4:G\gamma9 (1:1:1)

F. 

\[ \% \text{ of active EF at 600 s} \]

\[ \% \text{ of active EF at 600 s} \]
Fig. 8. Data guided computational modeling of signal transduction from GPCRs to the cell interior in multi-$\gamma$ systems. (A) The reactions representing the proposed mechanism of GPCR-G protein activation used in the model. (B) $G\beta\gamma$ fluctuation between active-inactive conformations ($G\beta\gamma_{PM}^n \leftrightarrow G\beta\gamma_{PM}^n$), which is assumed in the optimized model. (C-E) Concentrations of signaling entities ($G\alpha(GDP)\beta\gamma$, $G\alpha(GDP)$, $G\alpha(GTP)$, $G\beta\gamma_{PM}$, $G\beta\gamma_{PM*}$, $G\beta\gamma_{IM}$, and $G\beta\gamma_{EF}$) as a function of time for the four cases considered in the model ((1) mono-HiAf-$\gamma_3$, (2) mono-MoAf-$\gamma_4$, (3) mono-LoAf-$\gamma_9$, and (4) equal mix of $\gamma_3$, $\gamma_4$ and $\gamma_9$). (F) $G\beta\gamma$ effector responses in a multi-$\gamma$ system. The model predicts the responses are primarily dominated by the HiAf-$\gamma$. This indicates that the highest affinity $\gamma$ of the pool dictates the signaling activity in general.
Gγ identity dictates efficacy of Gβγ signaling and macrophage migration
Kanishka Senarath, John L Payton, Dinesh Kankanamge, Praneeth Siripurapu, Mithila Tenakoon and Ajith Karunarathne

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