**Abstract:** Heterotrimeric G-proteins along with G-protein-coupled receptors (GPCRs) regulate many biochemical functions by relaying the information from the plasma membrane to the inside of the cell. The lipid modifications of Gα and Gγ subunits, together with the charged regions on the membrane interaction surface, provide a peculiar pattern for various heterotrimeric complexes. In a previous study, we found that Gαs and Gαi3 prefer different types of membrane-anchor and subclass-specific lipid domains. In the present report, we examine the role of distinct Gγ subunits in the membrane localization and spatiotemporal dynamics of Gαs and Gαi3 heterotrimers. We characterized lateral diffusion and G-protein subunit interactions in living cells using fluorescence recovery after photobleaching (FRAP) microscopy and fluorescence resonance energy transfer (FRET) detected by fluorescence lifetime imaging microscopy (FLIM), respectively. The interaction of Gγ subunits with specific lipids was confirmed, and thus the modulation of heterotrimeric G-protein localization. However, the Gα subunit also modulates trimer localization, and so the membrane distribution of heterotrimeric G-proteins is not dependent on Gγ only.

**Keywords:** protein-membrane interaction; spatiotemporal protein dynamics; G-proteins; FLIM-FRET; FRAP

1. Introduction

G-protein-coupled receptor (GPCR) stimulation results in the activation of a Gα subunit and a Gβγ complex of the heterotrimeric G-protein. Both the activated components of the heterotrimer modulate the function of downstream effector proteins located on the cytosolic surface of the cell membrane. The intracellular response effect is caused by second messenger molecules after the stimulation of effector proteins such as adenylyl cyclase, phospholipase C-β, and ion channels. There are 18 Gα, five Gβ, and 12 Gγ genes in the human genome [1]. The Gα subunit family is the most diverse among G-proteins and is responsible for the specificity of interactions with GPCRs [2]. There is evidence of distinct activity of the Gβγ dimer in terms of G-protein activation and modulation of effector proteins [3].

Several different lipids are covalently attached to Gα and Gγ subunits, and as hydrophobic anchors, lipids promote localization to cellular membranes. Apart from the subunits Gαi and Gα_gust, all Gα subunits are palmitoylated, and some are also myristylated. Palmitoylation is a post-translational modification that involves the addition of saturated 16-carbon palmitic acid to a specific cysteine in an amino acid sequence through a thioester bond (S-palmitoylation). This modification is unique among lipidations and can be quickly reversed under in vivo conditions [4,5]. It is also possible to attach...
palmitoleic acid by an amide bond to the glycine residue in the N-terminal fragment of the Gαs protein (N-palmitoylation) [6]. Myristylation is a co-translation or post-translation modification which consists of attaching the myristate (a 14-carbon saturated fatty acid) to the N-terminal glycine residue through a peptide bond [7]. The non-myristylated Gα subunits (Gαs, Gαq, and Gα12/13 families) contain the motif of basic residues, arginines and lysines, forming a charged surface on one side of the N-terminal helix [8–11]. Such a charged protein area is able to interact with negatively-charged lipid head groups. A positively charged surface is also present in the Gβ structure. Such a region, through electrostatic interactions favors binding to acidic membrane phospholipids [12].

All Gγ subunits undergo post-translational isoprenylation. Prenylation is an irreversible multistage modification that involves the transfer of a C15 farnesyl or C20 geranylgeranyl group to a cysteine residue within the C-terminal CaaX motif via a thioether bond. Farnesylation occurs in the Gγ1, Gγ9, and Gγ11 subunits; the other subunits of Gγ are geranylgeranylated [13]. According to the two-signal hypothesis or kinetic trapping [14–18] a single membrane binding signal might be insufficient for proper membrane docking. However, a combination of two or more signals such as lipid anchor, poly-basic sequence or other motifs can ensure high-affinity interaction with cellular membranes.

Apart from differences in the length of anchors, the literature distinguishes three groups of gamma subunits based on transient translocation to endomembranes. The Gγ1, Gγ11, Gγ9, and Gγ13 belong to a group characterized by rapid translocation, Gγ5 and Gγ10 translocate slowly, while the other Gγ subunits, previously identified as non-translocating, are currently described as the slowest translocating [19,20]. It is noteworthy, that the translocation process is acylation/deacylation cycle dependent and the activation of G-protein is not necessary [21]. It is currently accepted that the factors that influence the behavior of activated G-proteins are not only heterotrimer composition and lipid modification but also local membrane environment, receptor coupling, the presence or absence of effector proteins or accessibility of other interacting partners.

In previous reports, we showed a separate location of heterotrimers of Gαs and Gα13 subunits with Gβ1γ2 complex in HEK 293 cells [22]. Here we examined the effect of distinct Gγ subunits, Gγ2 and Gγ9, on heterotrimer behavior prior to receptor activation. The combined approach of two fluorescence microscopy techniques was used, fluorescence lifetime imaging microscopy–fluorescence resonance energy transfer (FLIM–FRET) technique to examine protein-protein interactions and the fluorescence recovery after photobleaching (FRAP) technique to monitor membrane dynamics of complexes. We confirmed the interaction of the Gγ subunit with specific lipids and thus the modulation of heterotrimeric G-protein localization. However, the membrane distribution of heterotrimeric G-proteins is not only Gγ dependent. Results presented herein indicate that the Gα subunit also modulates trimer localization and the nature of that impact is to some extent similar to that of Gγ. Therefore, the role of other subunits of G-protein trimer partitioning process appears to be significant.

### 2. Materials and Methods

#### 2.1. Chemicals and Protein Constructs

All chemicals were purchased from Sigma (Sigma Aldrich, Poznań, Poland) unless otherwise indicated. All DNA sequences of Gα, β, and γ subunits in pcDNA3.1+ used were purchased from UMR cDNA Resource Center (Bloomberg, PA, USA). Plasmids pEYFP–N1, pEGFP–N1, and pmCherry–N1 were purchased at Clontech (Mountain View, CA, USA). The fusion proteins of the Gα subunits with fluorescent proteins (FP) was performed as described earlier [22]. In the GαsIEK chimeric protein, the conserved region Ile26–Glu27–Lys28 (G.HN.43–45; (I/L)-(E/D)-(K/R) motif in Gα family) was modified into a sequence of three alanine residues. These mutations were carried out using the Quick Change method and resulted in the elimination of the interaction of the mutant GαsIEK protein with the Gβγ dimer [23]. The restriction-free cloning method [24] was used to modify the Gβ1 subunit. As a first step, the FP sequence was multiplied and cloned at the N-terminus of the Gβ1 subunit and this construct was used in the FLIM-FRET experiments. In the next PCR, a His-tag sequence was
added at the N-terminus of the FP. This modification enabled carrying out pull-down experiments. All constructs were checked by determining the nucleotide sequence.

2.2. Cell Culture

The HEK 293 cells (American Type Culture Collection, Manassas, VA, USA) were cultivated and transiently transfected as described earlier [22]. The cells were transfected with 0.15–0.6 µg of DNA/dish and the ratio of the DNA coding of the Gα, Gβ, and Gγ subunits was 2:3:3. For the pull-down assay, cells were cultured on a 100 mm diameter dish and the amount of DNA for transfection was increased accordingly.

2.3. Determination of Intercellular cAMP Concentration

Changes in cAMP concentration were determined by using the cAMP ELISA chemiluminescent kit (STA-500, Cell Biolabs Inc, San Diego, CA, USA). Cells were seeded onto six-well plates and 24 h later transfected with 1.85 µg plasmid DNA/well. HEK 293 cells were co-transfected with the D2R dopamine receptor in the case of Gαi or adenosine A2AR receptor in the case of Gαs and GαsIEK. Three days after transfection the cells were treated for 10 min with 1 µM sumanisole maleate in minimum essential medium (MEM) containing 0.5% fetal bovine serum (FBS) and phosphodiesterase inhibitors 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) or 0.1 mM 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro 20–1724) or for 15 min with 1 µM 2–phenylaminoadenosine in MEM medium containing 0.5% FBS. Non-transfected cells were used as controls. After stimulation, the concentration of cAMP in harvested cell lysates was determined according to the manufacturer’s instruction. The chemiluminescent signal was measured by using a Synergy H1 microplate reader (BioTek Instruments, Winooski, VT, USA). To minimize batch-to-batch variations, in each experiment the signals were normalized to the average signal in the control group (non-transfected cells), and the normalized data were summarized across all experiments.

2.4. Pull-Down Assay

Interaction of Gαs or GαsIEK mutants with the Gβ1 or Gβ1Gγ2 dimers was studied by pull-down on a nickel-charged affinity beads (NiNTA agarose). HEK 293 cells, transiently expressing bait His-tagged Gβ1-mCherry alone or full heterotrimeric GαsGβ1Gγ2 or GαsIEKGβ1Gγ2 complexes, were lysed in ice-cold lysis buffer (50 mM Tris, pH 8.0, 5 mM MgCl2, 10 mM β-mercaptoethanol, 100 mM NaCl, 20 µM GDP, benzonase, inhibitors). After sonication and centrifugation (50,000 g, 45 min, 4 °C), the supernatant (cytoplasmic fraction) containing His6-tagged mCherry-Gβ1 was loaded onto the NiNTA resin and washed with wash buffer (lysis buffer with 50 µM guanosine-5’-triphosphate, GTP, and without benzonase and inhibitors). After sonication and centrifugation (50,000 g, 45 min, 4 °C), the supernatant (cytoplasmic fraction) containing His6-tagged mCherry-Gβ1 was loaded onto the NiNTA resin and washed with wash buffer (lysis buffer with 50 µM guanosine-5’-triphosphate, GTP, and without benzonase and inhibitors) and utilized for studying interactions with Gαs. The membrane fractions containing membrane-bound GαsGβ1Gγ2 or GαsIEKGβ1Gγ2 heterotrimeres were suspended in wash buffer supplemented with 1% n-dodecyl-β-D-maltopyranoside, DDM (Anatrace, Maumee, OH, USA) and homogenized (Teflon–glass homogenizer, Sigma Aldrich, Poznań, Poland). After overnight solubilization (4 °C), clarified membrane fractions (50,000× g, 45 min, 4 °C) were incubated for 3 h at 4 °C with NiNTA resin.

In the next step, lysate from cells expressing Gαs or GαsIEK, after sonication and centrifugation (cytoplasmic fraction), were incubated for 3 h at 4 °C with His-Gβ1-trapped NiNTA. The membrane-bound Gαs or GαsIEK were isolated in the same way as full heterotrimers, followed by incubation with His-Gβ1-trapped NiNTA. Complexes bound to the beads were isolated by centrifugation, washed three times with ice-cold lysis buffer and eluted in wash buffer containing 0.5 M imidazole. As negative control, a cell lysate without protein expression (negative cell lysate) was used. The eluted complexes were separated on 15% polyacrylamide-SDS gel and visualized by Western blot using antibodies against Gαs (Novus, Centennial, CO, USA), His-tag (Proteintech, Rosemont, IL, USA), and mCherry (Proteintech).
2.5. Confocal Microscopy

HEK 293 cells producing Gαs, GαsIEK or Gαi3 or full heterotrimeric complexes (Gαsβ1γ2, Gαsβ1γ9, GαsIEKβ1γ2, GαsIEKβ1γ9, Gαi3β1γ2, Gαi3β1γ9) were imaged with the TCS SP5 laser-scanning confocal microscope (Leica Microsystems, Mannheim, Germany) with a 63 × 1.4 numerical aperture (NA) oil-immersion objective at 37 °C. Green or yellow fluorescence (mGFP or Citrine FP) was detected at 495–570 nm with 488 nm excitation (argon ion laser) and red (mCherry) at 610–700 nm with 594 nm excitation (laser diode). All measurements were taken on living cells at 37 °C in an air-stream cube incubator. Before imaging, the culture medium was replaced with fresh Dulbecco’s modified Eagle medium/nutrient mixture F-12 (DMEM-F12) containing 2% FBS without phenol red.

2.6. Fluorescence Lifetime Imaging

A time-domain fluorescence lifetime imaging FLIM was performed with a confocal laser-scanning microscope (TCS SP5; Leica Microsystems, Mannheim) additionally equipped with a single-photon counting device with picosecond time resolution (PicoHarp 300, PicoQuant, Berlin, Germany). Details of the instrumentation are as described previously [22,25]. Images were recorded using the following settings: 63× oil-immersion objective with numerical aperture (NA) 1.4 at 37 °C with a frame size of 512 × 512 pixels and 470 nm laser in pulse mode at 40 MHz. Fluorescence was detected by a single-photon avalanche photodiode (τ-SPAD, PicoQuant) in a narrow range of 500–550 nm (band-pass filter). Citrine and mCherry fluorophores were used as FRET pairs. SPAD signals were analyzed with the SymPhoTime software (PicoQuant). The decay of Citrine intensity distribution was approximated in the subsequent fluorescence lifetime analysis by a bi-exponential decay model wherein we estimated four parameters—fluorescence lifetimes (τ) and relative abundances of the components of the donor molecules in the sample. FLIM images were generated using the SymPhoTime software (PicoQuant) by displaying pixel-wise average lifetimes in pseudo-colors. During the analysis, the instrument response function (IRF) was applied to obtain short lifetime components with a high accuracy.

Reduction of fluorescence lifetime between donor-only and FRET samples were calculated from the means of donor-only and FRET samples, with inclusion of fractional standard errors. The FRET efficiency (E) was calculated based on the following equation:

\[ E = 1 - \frac{\tau_{da}}{\tau_d} \]  

where: \( \tau_{da} \) is the lifetime of donor in the presence of acceptor molecules, and \( \tau_d \) is the lifetime of the donor only [26]. The energy transfer was analyzed only at the plasma membrane.

2.7. FRAP Measurements

All experiments using the FRAP microscopy technique were performed and analyzed as described earlier [22]. Briefly, FRAP images were collected by using the Leica TCS SP5 confocal scanning microscope with LAS AF software and an immersion lens 63 × 1.4 NA. Experiments were performed on transiently transfected live cells of the HEK 293 line at 37 °C. Just before imaging, the culture medium was replaced with fresh DMEM-F12 medium without phenol red and enriched with 2% FBS serum. Data was collected for at least 100 s after the photobleaching impulse.

2.8. Statistical Analyses

The FRAP and FLIM-FRET data was collected for at least five independent experiments. The distribution of data was determined (normality by Shapiro–Wilks’ W test; additionally, shape of the distribution by skewness and kurtosis analysis). Data were presented as mean ± standard error of the mean (S.E.M.) and the unpaired t-test was performed when the data were normally distributed. The assumption of equality of variances was verified by Levene’s test. Otherwise, data were represented as median ± median absolute deviation (MAD) and the Mann–Whitney U test was executed. Outliers, whose presence was evaluated by the box plot method or Grubbs’s test, were
excluded from statistical analysis. The number of samples in each experiment \( n \) and \( p \)-values are presented in figure legends and tables. Statistical analysis was performed with Statistica (data analysis software system), version 13 (TIBCO Software Inc., Palo Alto, CA, USA, 2017; http://statistica.io).

3. Results

3.1. Functionality of Fluorescently-Tagged G-Proteins

We have shown previously that stimulatory \( \mathrm{G}_s \) and inhibitory \( \mathrm{G}_{\alpha_3} \) subunits are located in distinct types of the membrane domains, depending on their specific activation state \([22,25]\). In previous experiments we reported that co-transfection of \( \mathrm{G}_s \) or \( \mathrm{G}_{\alpha_3} \) with the \( \mathrm{G}_\beta_1\gamma_2 \) dimer and the dopamine \( \mathrm{D}_1 \) receptor influences the membrane location of \( \mathrm{G}_s \) and, to a lesser extent, of the \( \mathrm{G}_{\alpha_3} \), respectively \([25]\). In their presence, \( \mathrm{G}_s \), complexed with \( \mathrm{G}_\beta_1\gamma_2 \), relocates outside the liquid-ordered membrane domains. Because of the possibility that this result arose from the concurrent actions of the \( \mathrm{G}_\beta_1\gamma_2 \) dimer and the \( \mathrm{D}_1 \) receptor, in the present study we focused solely on the effect of \( \mathrm{G}_\beta\gamma \). More specifically, we addressed the question whether the localization of the heterotrimer at the plasma membrane was controlled only by the \( \mathrm{G}_\beta\gamma \) dimer.

Tagging proteins of interest with FP might reduce their biological function due to undesired conformational changes or steric hindrance introduced by the tags. In order to avoid this, we selected loop-tagged \( \mathrm{G}_s \) and \( \mathrm{G}_{\alpha_3} \) and amino-terminal-tagged \( \mathrm{G}_\beta_1 \) for further optimization and analyses. The FP sequence was inserted into the L1 loop of the \( \mathrm{G}_s \) subunit (the loop from Glu71 to Ser82 residue (G.h1ha.7-G.h1ha.18)) was exchanged), and into the second loop (\( \alpha \)BC loop) within the helical domain of the \( \mathrm{G}_{\alpha_3} \) subunit (FP sequence cloned after Ala114 residue (H.hbhc.2)). All investigated proteins displayed plasma membrane localization as shown by the fluorescence images, confirming that the presence of tags does not disturb their membrane-binding ability (Figure 1). The loop fusion \( \mathrm{G}_s \) has been reported to be functional \([27]\). Additionally, intracellular cAMP level was measured in order to check the functional activity of all designed fusion proteins.

The \( \mathrm{G}_s \) and \( \mathrm{G}_{\alpha_3} \) subunits are distinct and provide the specificity for activation and inhibition, respectively, of adenylyl cyclase. Depending on the \( \mathrm{G}_s \) subclass, HEK 293 cells were co-transfected with adenosine \( \mathrm{A}_{2A} \) or the dopamine \( \mathrm{D}_2 \) receptor. In response to extracellular stimuli with a suitable agonist (2-phenylaminoadenosine or sumanirole), a rapid increase or reduction in the production of cAMP was detected for \( \mathrm{G}_s \) or \( \mathrm{G}_{\alpha_3} \), respectively (Figure 1A,B). These results indicate that the tagged G-protein heterotrimers are signaling-active. The \( \mathrm{G}_s \) heterotrimers were capable of more than eight-fold induction in cAMP over the basal level. In turn, \( \mathrm{G}_{\alpha_3} \) heterotrimers showed pronounced inhibitory effect. \( \mathrm{G}_{\alpha_3} \), tagged with Citrine or mGFP, was examined and no significant differences in cAMP production between these fusion proteins were found. Intriguingly, trimers composed of \( \mathrm{G}_\beta_1\gamma_2 \) changed the basal cAMP level in HEK 293 cells less efficiently than trimers consisting of \( \mathrm{G}_\beta_1\gamma_2 \). Especially \( \mathrm{G}_{\alpha_3}\beta_1\gamma_9 \). This finding may be explained by a potentially less effective formation of the full heterotrimeric \( \mathrm{G}_\alpha\beta_1\gamma_9 \) complex than \( \mathrm{G}_\alpha\beta_1\gamma_2 \). It was previously reported that some combinations of \( \mathrm{G}_s \) and \( \mathrm{G}_\beta\gamma \) were less stable and dissociated over longer periods of time \([28]\). On the other hand, the coupling of the \( \mathrm{G}_\alpha\beta_1\gamma_2 \) protein to dopamine \( \mathrm{D}_2 \)R or adenosine \( \mathrm{A}_{2A} \)R, in response to agonist activation, may be more productive than interaction with \( \mathrm{G}_\alpha\beta_1\gamma_9 \). In cryogenic electron microscopy (cryo-EM) structures, there are no visible interactions between the \( \mathrm{G}_y \) subunit and \( \mathrm{A}_{2A} \)R \([29]\) or with other receptors like 5-HT1B \([29]\), \( \mathrm{A}_1 \)R \([30]\) or \( \mu \)OR \([31]\). Nonetheless, the subclass of the \( \mathrm{G}_y \) subunit is essential in governing the formation of a GPCR–G-protein complex, as it was reported for NTR1, adenosine A1 and muscarinic M2 receptors \([28,32,33]\). It has been shown that, especially, the C-terminal amino acid sequence of the \( \mathrm{G}_y \) subunit and its native acyl group are important determinants for the interaction between GPCR and the G-protein heterotrimer \([33]\).
Figure 1. Cellular localization of Gαsβ1γ2, Gαsβ1γ9, Gαi3β1γ2, and Gαi3β1γ9 heterotrimers. Representative confocal images of fluorescent protein (FP)-tagged Citrine–Gα subunits and mCherry–Gβ1γ dimers in transiently co-transfected HEK 293 cells. Localization of heterotrimers: (A) Gαsβ1γ2, (B) Gαsβ1γ9, (C) Gαi3β1γ2, (D) Gαi3β1γ9, (E) Gαi3β1γ2, (F) Gαi3β1γ9. Scale bar, 10 µm.
3.2. Analysis of G-Protein Interactions in Live Cells Using FLIM–FRET Microscopy

FLIM–FRET was employed to assess whether the Gγ subunits played a role in plasma membrane localization and formation of the G-protein heterotrimer. For these reasons, we performed experiments to evaluate the molecular interactions of two dimers, Gβ1γ2 and Gβ1γ9, with Gαs and Gαi3. As we have reported, these two different Ga subclasses show different lipid preferences and, consequently, are localized in distinct types of membrane domains [25]. Thus, we examined here if heterotrimer formation was also controlled in a Gα-subclass dependent manner.

We monitored FRET by measuring the lifetime of the donor fluorophore (Citrine) in the absence and presence of the acceptor (mCherry), as described in the Materials and Methods section. In our experimental system, Citrine (Citrine–Gαi3β1γ2, Citrine–Gαsβ1γ2) exhibited a double exponential decay with lifetimes of 2.7 ± 0.1 ns (τ1) and 3.4 ± 0.15 ns (τ2), implying the existence of two donor species. The amplitude of each of these lifetimes was approximately 50%. The presence of multi-exponential fluorescence decays in various intrinsically FP such as Citrine, cyan fluorescent protein (CFP), enhanced cyan fluorescent protein (ECFP), monomeric green fluorescent protein (mGFP), and Discosoma red fluorescent protein (DsRed) has been reported in several articles [34–36]. In the FRET system (the cells expressing Citrine–Gα were additionally transfected with mCherry–Gβ1 and the appropriate Gγ), the donor emission curves were also fitted to a double exponential decay model. However, a shortening of the fluorescence lifetime due to FRET was observed only for the short component τ1, while τ2 remained almost unchanged (compared to the donor alone). This indicates that only one donor species (characterized by lifetime τ1) underwent FRET (FRETing donor fluorophore state) and that the other species with the longer lifetime (τ2) was not engaged in FRET (non-FRETing donor fluorophore state). Therefore, only the FRETing component was used to calculate FRET efficiency (Figure 2C).

Lifetime shortening due to FRET was also observed in the FLIM images as a uniform change in color toward the blue hues across all pixels (Figure 2A).

As shown in Figure 2B, different combinations of Ga subunits and Gβ1γ dimers gave varying levels of donor lifetime changes (box chart), and thus unequal FRET efficiencies. The highest FRET efficiency of 50% was obtained in cells expressing Gαi3β1γ2 and the efficiency was only 5% lower for the Gαi3β1γ9 heterotrimer. In contrast to Citrine–Gαi3, Citrine–Gαs exhibited a lower FRET signal when paired with either mCherry–Gβ1γ2 or mCherry–Gβ1γ9. The lifetime of Citrine–Gαs in cells co-expressing the mCherry–Gβ1γ2 dimer was found to be 2.12 ± 0.21 ns, amounting to 19.7% energy transfer efficiency, and a similar value was obtained for the complex with the mCherry–Gβ1γ9 dimer, giving a FRET signal of 19.5%.

An important question is whether the differences in FRET efficiency we observed are a measure of the efficiency of association between the interacting proteins or a consequence of structural differences between the heterotrimers. In the case of two proteins labeled with donor and acceptor molecules, FRET is highly dependent not only on the distance between the donor and acceptor but also on the stoichiometry of macromolecular interactions as well as on the donor fraction taking part in complex formation with acceptors. Therefore, an increase in FRET efficiency in our system can be interpreted as a quantitatively higher percentage of complexes formed between a particular Ga and Gβγ, especially when we are comparing trimers with the same Ga. Since Citrine in fusion proteins is integrated into other loops in the Ga and Gα3 structure (Figure 3), the structural differences between their heterotrimeric subunits should also be considered.
which cannot be uniquely attributed to only a single factor. Most probably the FRET signal reflects a mixture of energy donors (Citrine–Gα) and energy acceptors (mCherry–Gβγ and Citrine–Gα).

Thus, the energy transfer efficiency for these complexes may be related to their content subunits and dimers. Gαi3β1γ9 heterotrimers exhibited a double exponential decay model. FRET (FRETing donor Citrine–Gα) and donor in the presence of acceptor (mCherry–Gβγ). The median is shown as a line in the box, while the bottom and top boundaries represent the lower and upper quartile, respectively. Statistical significance of the difference in the donor fluorescence lifetimes τ1 detected in the absence and presence of energy acceptor using Mann–Whitney U test (* p < 0.02, ** p < 0.002, *** p < 0.0002). Gαs n = 42; Gαs and Gβ1γ2 n = 71; Gαs, Gβ1γ9 n = 46; Gαi3, and Gβ1γ2 n = 64; Gαi3 and Gβ1γ9 n = 72; GαsIEK and Gβ1γ2 n = 42; GαsIEK and Gβ1γ9 n = 38. (C) Plot of calculated fluorescence resonance energy transfer (FRET) efficiency percentage E derived from τ1, error bars represent standard errors.

Considering the results of intracellular cAMP concentrations obtained for Gαi3 heterotrimers, it seems reasonable to conclude that FRET efficiency for these complexes may be related to their content at the plasma membrane. The smaller FRET efficiency between Citrine–Gαi3 and mCherry–Gβγ is reflected in a smaller decrease in cAMP concentration when compared to Gαi3β1γ2. No differences were found in FRET efficiency between the investigated Gαs heterotrimers, indicating a similar level of Gαsβ1γ2 and Gαsβ1γ9 complexes at the plasma membrane. However, as shown in Figure 4B, a slight difference in the cAMP concentrations of Gαsβ1γ2 and Gαsβ1γ9 can be observed, although it is not statistically significant and is smaller than between Gαi3 heterotrimers. Thus, even if there is a difference in the concentrations of these complexes at the plasma membrane, it is not substantial. On the contrary, there are significant differences in FRET signals between Gαs and Gαi3 heterotrimers which cannot be uniquely attributed to only a single factor. Most probably the FRET signal reflects a mixture of effects, i.e., a potentially lower percentage of trimers composed of Gαs and Gβ1γ2 or Gβ1γ9 dimers than Gαi3 heterotrimers, and structural differences between these heterotrimers due to different localizations of fluorescence donors (Figure 3). The estimated difference in the distance separating mCherry–Gβ1 and Citrine–Gαs is, on average, 6.5 Å longer than in the Gαi3 heterotrimer. Thus, the energy transfer efficiency in the Gαs heterotrimers should be lower by about 7% than in Gαi3 heterotrimers, assuming Förster distance R0 of 56.6 Å for this pair of fluorophores [38,39].
As shown in Figure 1, Gα previously shown to have a strong propensity to disrupt membrane localization and palmitoylation of permeabilization of cell membranes, leading to a disruption of the native membrane. The residue 2019 cAMP production was observed as compared to the wild-type Gα or Gβ. We also examined cAMP production to ensure that the mutation introduced in Gα fulfilled its role. As shown in Figure 4B, a clear reduction in cAMP production was observed as compared to the wild-type Gα and the cAMP concentration was expected that the formation of the heterotrimer would be impaired. Similar mutation constructs were suitable mutation is believed to impair the assembly of a functional heterotrimer [40]. We engineered a construct a mutant of Gα heterotrimer was not a ff 26 −Glu27−Lys28 of Gα has long been recognized as being essential for its interaction with Gβ, and a suitable mutation is believed to impair the assembly of a functional heterotrimer [40]. We engineered a GαIEK mutant in which each of the interacting amino acid residues was substituted with alanine and expected that the formation of the heterotrimer would be impaired. Similar mutation constructs were previously shown to have a strong propensity to disrupt membrane localization and palmitoylation of the Gα subunit as well as deficient binding to Gβγ [23]. To examine the effect of the IEK mutation on the ability of Gα to interact with Gβγ, GαIEK was co-expressed with Gβ1γ2 or Gβ1γ9 in HEK 293 cells. As shown in Figure 1, GαIEK exhibited plasma membrane localization and co-localization with Gβ1γ2 or Gβ1γ9, but to a lower extent than the wild-type Gα. We also examined cAMP production to ensure that the mutation introduced in Gα fulfilled its role. As shown in Figure 4B, a clear reduction in cAMP production was observed as compared to the wild-type Gα and the cAMP concentration was...
approximately three times lower than for $G_{\alpha s}$. These findings suggest a lower $G_{\text{asIEK}}$ heterotrimer content than that of $G_{\alpha s}$ at the plasma membrane, which led to a reduced activation of adenylyl cyclase.

Figure 4. Effect of different $G_{\gamma}$ subunits on the production of cAMP. HEK 293 cells were transiently transfected with Citrine–$G_{\alpha i3}$, dopamine receptor $D_2R$, mCherry–$G_{\beta_1i}G_{\gamma_2}G_{\gamma_9}$, or Citrine–$G_{\alpha sIEK}$, adenosine receptor $A_2AR$ and mCherry–$G_{\beta_3}G_{\gamma_9}$. The activity of the investigated proteins after stimulation of the $D_2$ receptor with sumaninole maleate or the $A_2A$ receptor with 2-phenylaminoadenosine was determined by measurements of cAMP levels. Data are presented as percentage of mean cAMP level in control (non-transfected cells) which are considered as intrinsic cAMP levels after stimulation of the $D_2$ or $A_2A$ receptor. Differences in cAMP levels between samples were evaluated by the unpaired t-test. (A) Citrine–$G_{\alpha i3}$: comparison with the control: **$p < 0.01$ ***$p < 0.001$, Citrine–$G_{\alpha i3}$ mCherry–$G_{\beta_1i}G_{\gamma_2}$ vs. Citrine–$G_{\alpha i3}$ mCherry–$G_{\beta_1i}G_{\gamma_2}$ **$p < 0.01$ and mGFP–$G_{\alpha i3}$ mCherry–$G_{\beta_1i}G_{\gamma_2}$ vs. mGFP–$G_{\alpha i3}$ mCherry–$G_{\beta_1i}G_{\gamma_2}$ ***$p < 0.001$ (n = 8). (B) Citrine–$G_{\alpha sIEK}$: comparison with the control: **$p < 0.01$ ***$p < 0.005$, Citrine–$G_{\alpha sIEK}$ mCherry–$G_{\beta_1i}G_{\gamma_2}$ vs. Citrine–$G_{\alpha sIEK}$ mCherry–$G_{\beta_1i}G_{\gamma_2}$ **$p < 0.01$ and Citrine–$G_{\alpha sIEK}$ mCherry–$G_{\beta_1i}G_{\gamma_9}$ vs. Citrine–$G_{\alpha sIEK}$ mCherry–$G_{\beta_1i}G_{\gamma_9}$ ***$p < 0.005$ (n = 8). (C) In a pull-down assay, $G_{\alpha sIEK}$ was found to interact with $G_{\beta_1i}G_{\gamma_9}$. Recombinant His-tagged mCherry–$G_{\beta_1}$ bound to NiNTA beads were incubated with Citrine–$G_{\alpha s}$ or Citrine–$G_{\alpha sIEK}$ cell lysates. Beads were precipitated, and the amount of $G_{\alpha s}$ was detected by Western blotting, using an antibody specific to $G_{\alpha s}$. The anti-His-tag antibody was used for visualization of His-tagged mCherry–$G_{\beta_1}$. The figure is representative of three independent experiments.

To further confirm that the observed increase in cAMP concentration over the basal level resulted from the direct interaction of $G_{\beta_1}$ with $G_{\alpha sIEK}$, we performed a pull-down assay. Cell lysates containing
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Citrine–GasIEK or Citrine–Gas were loaded onto NiNTA agarose baited with His6-tagged forms of mCherry–Gβ1 and then the complexes were eluted. Furthermore, purified proteins were detected by immunoblotting with an antibody against the N-terminal epitope of Gas and against the His6-tag of Gβ1. Figure 4C summarizes the results of all pull-down experiments, showing the binding of GasIEK to His6-tagged Gβ1. No clear signal was observed for the negative cell lysate (Figure 4C), thus confirming that the band detected by the anti-Gas antibody is GasIEK associated with Gβ1 and not endogenous Gas present in the cell lysate. Incubation of the membrane fraction of cells overexpressing GasIEK and Gβ1γ2 with the NiNTA resin resulted in a signal from GasIEK, once again indicating the presence of the full GasIEKβ1γ2 heterotrimer. Since these pull-down experiments are qualitative or at best semi-quantitative in nature, we did not attempt to quantify the relative amounts of the GasIEKβ1γ2 and Gasβ1γ2 heterotrimers.

Our data strongly suggest that the IEK mutation in Gas is Gβγ binding-deficient, the heterotrimer is formed less efficiently and has impaired activity but is still able to activate adenyl cyclase. The examined Gβ-binding surface on the Gas subunit may not be the only essential interacting region of the heterotrimer assembly. The mutation did not prevent the heterotrimer formation but confirmed the specificity and sensitivity of the measured FRET signals. The FRET efficiency between Citrine–GasIEK and mCherry–Gβγ dimers was reduced to 12.9% and 9.4% as compared to wild-type Gas in the presence of Gβ1γ2 and Gβ1γ9 dimers, respectively (Figure 2C). These results demonstrate that differences in the heterotrimer levels of the mutant GasIEK and the wild-type protein are reflected in the detection of a lower FRET signal and are also consistent with the results of intracellular cAMP concentration measurements. Overall, this study confirmed the sensitivity and accuracy of FLIM–FRET as a suitable tool for studying the interactions of signaling proteins.

3.3. Effect of Gβγ Dimer on Gas Diffusion

As stated earlier, the apparent diffusion coefficients of full heterotrimers differ significantly from those of monomers of Gas subunits. The formation of the complex of the Gas subunit with the Gβγ dimer modulates the mobility of the full heterotrimer, while the presence of a specific receptor is significant as well. Here, the presence of the Gβ1γ9 dimer caused an increase in apparent diffusion coefficients for all observed Gas subunits but in a different manner (Table 1). In the case of co-expression of the Gas subunit with the Gβ1γ9 dimer, the diffusion coefficient was 0.475 μm²/s, and was significantly higher compared to the value of 0.424 μm²/s obtained with the Gβ1γ2 dimer (Table 1). Conversely, for the Gas subunit, the measured value of apparent diffusion coefficient in the presence of the Gβ1γ9 dimer was 0.202 μm²/s and was higher than for the Gas subunit itself (0.130 μm²/s, Table 1), but significantly lower than the value measured in the presence of the Gβ1γ2 dimer–0.246 μm²/s (Table 1).

Table 1. Lateral diffusion coefficients of investigated Gas subunits fusion protein constructs in the presence of Gβ1γ2 or Gβ1γ9 dimers.

|                         | D_{app} (μm²/s) | Mf (%) | n  |
|-------------------------|----------------|--------|----|
| Gas                     | 0.130 ± 0.004  | 84.5 ± 1.5 | 49 |
| Gas Gβ1γ2               | 0.246 ± 0.009  | 92.4 ± 0.8 | 143|
| Gas Gβ1γ9               | 0.202 ± 0.007  | 89.5 ± 1.1 | 92 |
| Gasγ3                   | 0.338 ± 0.022  | 94.2 ± 1.7 | 34 |
| Gasγ3 Gβ1γ2             | 0.424 ± 0.014  | 93.5 ± 0.9 | 66 |
| Gasγ3 Gβ1γ9             | 0.475 ± 0.021  | 92.8 ± 1.2 | 60 |
| GasIEK Gβ1γ2            | 0.214 ± 0.010  | 87.8 ± 1.5 | 61 |
| GasIEK Gβ1γ9            | 0.214 ± 0.005  | 89.7 ± 0.9 | 108|

In the experiments where the co-expression took place, the diffusion of Gas subunits was measured. Values represent the mean ± S.E.M., D_{app} — apparent diffusion coefficient, Mf — mobile fraction, † — data from Reference [22].

For the modified GasIEK subunit, the apparent diffusion coefficient obtained in the presence of the Gβ1γ2 or Gβ1γ9 dimers was the same (0.214 μm²/s). The modification of the GasIEK subunit did
not affect the diffusion of this trimer subunit with the Gβ1γ9 dimer and the difference was statistically insignificant. However, the diffusion rate was reduced significantly for the subunit Gasβγ in the presence of the Gβ1γ2 dimer in comparison to the Gasβ1γ2 heterotrimer (0.246 μm²/s, Table 1). This may indicate a selective character of mutations within the region of interaction of the Gα subunit with the Gβγ dimer. On the other hand, the modified Gasβγ subunit was less effective in creating complexes with a Gβγ dimer (lower FRET efficiency). The diffusion coefficient observed may, therefore, be reduced by the presence of a monomeric subunit at the cell membrane.

4. Discussion

In this study, we have combined two live-cell imaging microscopic methods, FLIM–FRET and FRAP, to investigate the molecular-level assembly properties and the trafficking dynamics of G-protein heterotrimers within the cell membrane. The membrane dynamics of heterotrimer complexes were monitored by FRAP. The studied Gas and Gαi3 subunits are expressed in most types of cells. Similarly, the Gβ1 and Gγ2 subunit expression is ubiquitous, whereas Gγ9 is mostly present in olfactory epithelium [1].

Although much progress has been made in understanding the molecular details of how G-proteins interact with GPCRs and regulate the activity of their downstream targets, it is less clear how activated GPCRs initiate this process and what the trafficking pathway of the heterotrimeric G-proteins is within the plasma membrane. The role of the plasma membrane lateral organization in the spatiotemporal distribution of GPCRs and G-proteins appears to be essential in the process of extracellular signal transduction. However, the molecular basis for the interaction of signaling molecules with lipids is still not fully understood but seems to be of key importance in understanding the functional selectivity and activation speed of cellular responses to G-protein activation. Previous evidence supports the direct role of the Gy subunit in G-protein activation by a receptor [41–43], and also suggests Gy diversity as a crucial modulator of G-protein membrane localization behaviors as well as trimer assembly [44–46]. Using the same FLIM–FRET and FRAP approach, we previously reported that preferences in localization within the membrane of the stimulatory and inhibitory Ga subunits, Gas and Gαi3, are different and further modulated by the dopamine D1 receptor, as well as by the Gβ1γ2 dimer [22,25]. The association of Gβ1γ2 with the GDP-bound Ga subunit translocates G-proteins outside the liquid-ordered membrane domains, which is particularly evident for Gas [25]. Here we confirmed the ability of the Gy subunit to bind specific lipids, and consequently, to influence the membrane localization of the full heterotrimeric complex of the G-protein; but our results further showed that its membrane distribution was not only Gy subclass dependent.

In this study, we examined the impact of two distinct Gy subunits, Gγ2, and Gγ9, on the membrane localization of G-proteins. These Gy differ, among others, in membrane anchors at carboxyl-terminal cysteine in the CaaX motif. The prenyl group promotes tethering of Gβγ complexes to membranes, sorting of particular lipid domains, as well as playing a role in the translocation properties of the Gβγ dimer and effector activation [47,48]. The Gγ2 belongs to the group of slowest translocating Gy subunits (t1/2 ~130 s) whereas Gγ9 belongs to the fastest one (t1/2 ~ 10 s) [19,20]. The prenylation of Gγ2 with a 20-carbon geranylgeranyl lipid, together with positively charged residues in the C-terminal domain, provides it with a higher affinity for the plasma membrane than Gγ9 with the 15-carbon farnesyl lipid attachment and fewer positively charged residues. The presence of a five-residue or six-residue cluster of positively charged amino acids in the pre-CaaX region modulates Gy–membrane interactions, strengthening the plasma membrane affinity [20], and also governs the membrane-interacting ability of Gβγ [42]. Considering how many possible Gβγ and Ga combinations exist, a key question in G-protein signaling is whether the plasma membrane location of heterotrimers composed of distinct subunits is only Gβγ-dependent. Indeed, our FRAP data for Gy subunits correlated well with translocation rates in a Gy-dependent manner only for heterotrimers composed of Gαi3. The mobility of Gαi3β1γ9 is much higher than that of Gasβ1γ2 and its population in the membrane is also lower. On the contrary, Gβ1γ2 associated with Gas diffuses faster than Gasβ1γ9. In fact, both Gasβγ heterotrimers
diffused significantly slower when compared with their Goi3 associates. At the same time, if the concentrations of both the Goi3 heterotrimer at the plasma membrane are comparable, the reduced mobility of Goi3,Goi1,Goi0 cannot be considered to be the result of a higher proportion of uncomplexed Goi3. The diffusion data thus indicated that the membrane localization of G-proteins was dependent not only on Goi2 but also on the Goi subtype since distinct heterotrimeric combinations showed different mobility characteristics.

The Goi subunits are palmitoylated and mostly myristoylated, depending on the specific Goi-subclass. The dual, N-palmitoylation and S-palmitoylation, of Goi is similar to the N-myristoylation and S-palmitoylation motif of the Goi class, but they differ in the number of positive charge residues at the N-terminus [6,49]. The membrane binding area of Goi or Goi3 is limited to two sites on the surface of the protein and the membrane [30]. However, most of the membrane binding area of Goi is formed by the N-terminus with covalently attached lipids. Consequently, since the IEK mutation reduces palmitoylation, apart from disrupting Gβ1 coupling, it also affects proper membrane localization of Goi. Since this mutation reduces the palmitoylation, it affects the specific binding of Goi to the plasma membrane [23]. Indeed, our diffusion data suggest that the N-termini residues of Goi function as an essential signal to ensure the correct localization of the subunit at the plasma membrane. Unlike for the heterotrimer formed by the wild-type Goi and Goi3, the diffusion coefficient of distinct GoiIEKβ1γ2 and GoiIEKβ1γ9 heterotrimers is equal, as is the intracellular cAMP concentration, indicating that the mutation eliminates the specificity of the GoiIEK heterotrimers. However, it is difficult to clearly indicate whether the substitution of charged residues in the Gβγ interacting surface or the reduced palmitoylation impairs the specificity of heterotrimer membrane targeting. Nevertheless, the presented results support the notion of the presence of membrane attachment signals in the N-termini of Goi subunits.

As we previously reported, preferences in localization within the membrane of the stimulatory and inhibitory Goi subunits are different [25]. Goi prefers solid-like domains (insensitive to cholesterol and structure or composition of lipid rafts), while Goi3 prefers the more fluid regions of the membrane and also detergent-resistant lipid rafts. The membrane mobility of Goi is relatively slow, while the Goi3 diffusion is much faster. When Gβγ dimer binds to the Goi, despite the increase in the molecular weight of the complex, it accelerates the lateral diffusion of Goi in all tested heterotrimers. Thus, this finding again strengthens the hypothesis that the Gβγ dimer not only affects the diffusion of Goi but also relocates complexed Goi within the plasma membrane. Notably, it is evident in the case of Goi heterotrimer because the apparent diffusion coefficient is more than 1.5 times greater than for the uncomplexed subunit. As demonstrated previously, the Gβγ dimer is responsible for the rapid relocation of Goi from the lamellar membrane region where it resides as a monomer [25,51]. The Gβγ complex remains associated with the non-lamellar regions which may explain the acceleration of the diffusion rate of heterotrimer compared to monomeric Goi [51]. Interestingly, as mentioned above, the lateral mobility of the G-proteins composed of the Gβ1γ2 dimer differs significantly from those containing Gβ1γ9 dimer; the diffusion rates of distinct heterotrimers are characterized by different values of diffusion coefficients. Hence, while the Gβγ dimer defines the affinity of the complete heterotrimer for the lipid phase, the differences in the prenyl moieties are not sufficient to explain the differential diffusion of the full heterotrimers. Our data correspond with the FRET-clustering analysis of fluorescently-tagged heterotrimeric G-protein-derived membrane anchors [52]. The authors have shown that the N-terminal sequences of Goi/o and Gao and their heterotrimers (the N-terminal part of Goi2 or Gao merging with the C-terminal part of Gγ2) were clustered together in different domains. Moreover, postulated membrane domains partially share their area causing overlapping domains, thus strengthening co-clustering. Upon activation the heterotrimers dissociate and the Goi subunits displace into a subclass-specific domains. Our FRAP data do not support the pre-activation co-clustering of the distinct heterotrimeric G-proteins. A possible explanation for this discrepancy in conclusions correlates with the use of membrane anchors of Goi and Gγ subunits but not, as in the present study, with full-length G-proteins.
In addition to those previously identified, both components, Gβγ dimer and Ga, determine final membrane localization of the full heterotrimer. These findings imply that the dissociation of the G-protein on activation and subsequent re-association on deactivation, are also influenced by the subclass of the Ga subunit. Yet, the FRAP measurements could not precisely resolve membrane distribution of G-protein but strongly suggested the possibility of different localizations at the plasma membrane of particular heterotrimers (membrane domains/regions differing in lipid composition and properties). The results presented here indicate that the diffusion rates of heterotrimers composed of different Gβγ and Ga subunits were not directly related to the membrane dissociation Gγ-pattern that were determined by the nature of the prenyl group and by basic residues in the C termini of Gγ. The role of the Ga subunit in determining the membrane localization by interacting with lipids and Gβγ dimers shown in the present study suggests that the Gγ translocation rate can, consequently, be also affected by the Ga subunit.

The mobility of the G-proteins and dissociated subunits is heterogeneous, suggesting non-random distribution within the cell membranes, which may strongly reflect the natural functions of these proteins. Many studies have emphasized the importance of the clustering of membrane proteins in a manner dependent on their functional state [53,54]. Results obtained in the present study show that divergent heterotrimers localize to distinct membrane locations due to the combined lipid modifications on Ga and Gγ, together with a different number and distribution of adjacent positively charged residues (i.e., various classes of G-proteins located in distinct domains relocate upon activation and dissociation into subunits). Since the cell membrane is currently considered as a highly complex structure, the role of distinct types of membrane domains in the spatiotemporal organization of GPCRs and G-proteins in the process of signal transduction needs further studies.

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