Visualizing G protein signaling in living cells

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Citation for published version (APA):

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Chapter 3

Monitoring the activation of the heterotrimeric G protein $G_q$ in living cells by means of FRET ratio-imaging

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Summary

Activation of heterotrimeric G proteins is difficult to monitor in living cells. Here, we describe the development of a fluorescent G\textsubscript{α\text{q}} subunit with a CFP inserted into the helical domain. This fusion protein was able to productively interact with receptors, G\textsubscript{β\gamma} dimers and its effector PLC\textsubscript{β}. Several other insertion positions were tried, but these failed in providing functional fusion constructs. Importantly, we were able to develop a FRET pair, consisting of G\textsubscript{α\text{q}}-CFP and YFP-G\textsubscript{γ\text{2}}, that reports on the activation state of the heterotrimer upon activation of endogenous GPCRs with high sensitivity. Both ratio-imaging and FLIM data indicate that, contrary to the prevailing dogma, Gq changes conformation in living cells, rather than dissociating upon activation. Overexpression of a GPCR and a domain of PLC\textsubscript{β} were shown to increase the amount of active Gq without changing its conformation. Therefore, we conclude that Gq is not required to dissociate in order to trigger downstream signaling.
**Introduction**

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) are composed of Gα subunits and Gβγ dimers and can be activated by G protein coupled receptors (GPCRs). In mammalian cells, 17 genes encoding Gα subunits, 5 genes encoding Gβ subunits and 12 genes encoding Gγ subunits have been described, whereas the number of GPCRs is thought to amount up to about 1000 genes (Cabrera-Vera et al., 2003; Klein-Seetharaman, 2005). These receptors recognize ligands like hormones, neurotransmitters or paracrine factors. In addition, a large number of GPCRs is activated by stimuli present in the outside environment, e.g. pheromone molecules. Upon binding of a ligand the receptor changes in conformation and acts as a guanine nucleotide exchange factor (GEF). By inducing the exchange of GDP for GTP in the Gα subunit, the G protein becomes active. Both the Gα subunit and the Gβγ dimer relay information from the receptor to downstream signaling partners upon activation, a phenomenon that is thought to necessitate the physical separation of the two units (Clapham and Neer, 1997; Offermanns, 2003).

The family of Gα subunits consists of four classes; Gs, Gi, Gq and G12 (Simon et al., 1991). The subunits Gαq, Gα11, Gα14 and Gα16 belong to the Gq class. Gαq was cloned in 1990 (Strathmann and Simon, 1990), but it was not until 2005 that Tesmer et al. succeeded in crystallizing the protein, albeit without the N-terminus. Like all the other Gα subunits, Gαq consists of a Ras-like GTPase domain, an α-helical domain and an N-terminal segment (Figure 1). The GTPase domain combines the five conserved stretches needed for Mg²⁺ and nucleotide binding and exchange, whereas the α-helical domain has been proposed to function as an intrinsic GAP module and an effector recognition domain (Liu and Northup, 1998; Scheffzek et al., 1997; Sprang, 1997). The interactions between the GTPase domain and the α-helical domain keep the Gα subunit in its GDP-bound state by acting as a lid over the guanine nucleotide-binding pocket (Warner and Weinstein, 1999). In addition, the Gβγ dimer reduces the release of GDP upon interaction with the Gα subunit.

The Gβγ dimer is crucial for the interaction of a Gα subunit with a GPCR, since it is indispensable for induction of the high affinity agonist-binding conformation of the receptor and initiation of GDP/GTP exchange on the Gα subunit (Birnbaumer, 1990; Freissmuth et al., 1999). However, the mechanism by which the receptor causes guanine nucleotide exchange on the G protein is still a subject of debate. Two models have been proposed: the lever-arm theory and the gear-shift model (Cherfils and Chabre, 2003; Rondard et al., 2001). Recent data provide evidence for the gear-shift model (Gales et al., 2006). The principal receptor interaction sites on the Gα subunit are found in the N-terminus, the α2-helix, the α2-β4 loop, the α4-helix, the α4/β6 loop and the C-terminus.
Their relative importance differs among Gα subunits and GPCRs and also other regions have been reported to play a role (Figure 1) (Gilchrist et al., 2001; Slessareva et al., 2003). Gβγ interacts with Gα through two interfaces; the most extensive surface involves the switch region, the other site is formed by the N-terminus of the Gα subunit. Binding of GTP upon receptor activation disrupts the entire switch interface, which is thought to trigger the dissociation of Gβγ from Gα. The N-terminus of Gα subunits is only crystallized in the presence of GDP and Gβγ and it is postulated that Gβγ plays a role in the stabilization of this segment. Upon activation of the heterotrimer, the plasma membrane may fulfill this role in vivo (Chapter 1, Figure 10) (Lambright et al., 1996; Wall et al., 1995).

![Diagram](image)

**Figure 1.** A) Crystal structure of Gαq-GTP based on 2BCJ. The insertion sites described in this study are marked in yellow, except for N1 and N2. These sites are present in the N-terminus which has not been crystallized in case of Gαq. The names of the α helices and the β sheets are indicated. B) The amino acid sequence of Gαq. The insertion sites are indicated by black boxes. The residues implicated in interaction with other proteins are coloured. Blue= GPCR; green= Gαq; yellow= PLCβ. The regions needed for GTPase activity and GTP/Mg2+ binding are coloured in red (regions 1-5).

Heterotrimeric G proteins are peripheral membrane proteins by virtue of their lipid modifications. In general, plasma membrane targeting of heterotrimeric G proteins is essential for efficient interaction with receptors and effectors. Co- and posttranslational modifications have been reported for Gα and Gγ subunits. Many Gα subunits are dually
lipidated by a myristoyl and a palmitoyl tail. In contrast, Gαq is not myristoylated, but it contains two cysteines close to the N-terminus that can be decorated by palmitoyl moieties. For efficient fatty acid modification, both cysteines appear to be required (Smotrys and Linder, 2004; Wedegaertner et al., 1993). The Gγ subunit is a target for prenyltransferases that attach an isoprenoid group to the C-terminal cysteine in the CaaX box motif. However, prenylation is dispensable for the formation of the highly stable Gβγ dimer (Zhang and Casey, 1996). The prevailing hypothesis is that the heterotrimer assembles before reaching the plasma membrane, but the site of assembly is unknown. The Golgi was nominated for this role, but disruption of this system by brefeldin A does not prevent heterotrimeric G proteins from reaching the plasma membrane (Takida and Wedegaertner, 2004).

The principal target (i.e. effector) of the Gq class is phospholipase C β (PLCβ) (Lee et al., 1992; Taylor et al., 1991). The Gα subunit interacts with the C-terminal domain of PLCβ and activates it upon stimulation of Gq-coupled GPCRs. In turn, PLCβ cleaves phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ opens up channels in the ER, which causes a rise in intracellular calcium and consequently the regulation of a large amount of proteins (Berridge, 1997; Bootman et al., 2001). Several other downstream partners of Gq have been described (Hubbard and Hepler, 2006). The Gq class of G proteins is involved in many processes, e.g. cell growth and proliferation, neuronal signaling, smooth muscle contraction and platelet aggregation (Wettschureck et al., 2004). A knockout of Gαq in mice causes cerebellar ataxia (Offermanns et al., 1997a) and increased bleeding time (Offermanns et al., 1997b), whereas a knockout of both Gαq and Gα₁₁ leads to embryonic lethality due to hypoplasia of the heart. Importantly, a cardiomyocyte-targeted knockout of both Gαq and Gα₁₁ indicates that myocardial hypertrophy is abolished. Normally, this phenomenon is triggered by pressure overload of the heart and increases the risk of cardiac failure (Offermanns et al., 1998; Wettschureck et al., 2001). Drugs to downregulate Gαq/₁₁ are therefore much sought after.

Recently, several sensors have been developed to monitor the activation state of specific heterotrimeric G proteins in living cells upon GPCR activation (Bunemann et al., 2003; Frank et al., 2005; Gibson and Gilman, 2006; Hein et al., 2006; Janetopoulos et al., 2001; Yi et al., 2003). Measuring the state of the G protein does not involve an amplification step and is therefore a more direct measure of GPCR activation than sampling second messenger production, a technique that is generally applied in pharmacology. Certainly, monitoring ligand-receptor dynamics shares this characteristic, but is not representative of the overall kinetics of G protein signaling. Here, we report on the development of a highly sensitive sensor based on CFP-tagged Gαq and YFP-tagged Gγ₂ that allows for real-time visualization of both the location and G protein activation
state of G\(\alpha_q\) in living cells. We describe the effects of stimulating endogenously as well as exogenously expressed receptors, while concurrently monitoring the influence of accessory proteins on the behavior of the sensor.

**Results**

**Subcellular location of G\(\alpha_q\)N-VFP fusion proteins**

In order to further our understanding on G\(_q\) signaling in living cells, we prepared visible fluorescent protein (VFP)-tagged human G\(\alpha_q\) subunits. Since neither N- nor C-terminal fusions of G\(_\alpha_5\) to VFP retained functionality (Sheridan et al., 2002; Yu and Rasenick, 2002), insertion of the fluorescent protein was opted for. The different insertion sites described in this study are highlighted in the 3D-structure of G\(\alpha_q\) (Figure 1). At first we tried to insert YFP in the N-terminus as described for the yeast G protein Gpa1 that was able to complement a Gpa1 deletion strain (Yi et al., 2003). This spectral variant of VFP was inserted between residues 16 and 17 of G\(\alpha_q\), a few amino acids downstream of the palmitoylated cysteines. This G\(\alpha_q\)-YFP, termed G\(\alpha_q\)N1-YFP, was found diffusely distributed in the cytosol and occasionally faintly at the plasma membrane when transiently expressed in HeLa cells. Fluorescence was also found in the nucleus of most cells (Figure 2A).

Since immunolabeling and cell fractionation studies demonstrated plasma membrane localization of G\(\alpha_q\) (Arthur et al., 1999; Evanko et al., 2000; Wedegaertner et al., 1993), G\(\alpha_q\)N1-YFP was judged to be malfunctioning. A lack of palmitoylation (S-acylation) could be the cause of this aberrant localization. Therefore, we incorporated a repeat of G\(\alpha_q\) residues 11-17 downstream of the YFP insertion to enable possible docking of the
putative palmitoyltransferase (PAT). Since the N- and C-termini of VFP variants are in close proximity to each other, the first and second part of $\alpha_q$ are thought to remain in close proximity as well. Unfortunately, this fusion protein, termed $\alpha_qN2$YFP did not tether to the membrane either (Figure 2B). Importantly, the N-terminus does not only provide a site for lipidation, it also plays a vital role in interaction with the $\beta\gamma$ dimer (Lambright et al., 1996; Rens-Domiano and Hamm, 1995; Wall et al., 1995). Since heterotrimerization has been shown to be essential for plasma membrane targeting, mislocalization of $\alpha_qN1/2$ may be caused by a defect in the interaction with the $\beta\gamma$ dimer (Evanko et al., 2001; Evanko et al., 2000; Fishburn et al., 1999; Michaelson et al., 2002; Rehm and Ploegh, 1997; Takida and Wedegaertner, 2003). Subsequently, a third insertion site was chosen between residues 37 and 38, which are situated in a loop separating the N-terminus from the $\beta1$ sheet. This loop was expected to provide enough flexibility and be favorably distal to the residues needed for lipidation and $\beta\gamma$ interaction in order to host a VFP protein. However, this fusion protein, named $\alpha_qN3$YFP, was not targeted to the plasma membrane (Figure 2C).

**The effect of $\beta\gamma$ expression on the location of $\alpha_qN$-VFP fusion proteins**

Expression of the $\alpha_qN$-VFP fusion proteins did not lead to plasma membrane localization. Since $\alpha_qN$ forms a heterotrimer, the effect of co-expression of $\beta1$ and $\gamma2$ subunits was studied. Remarkably, both $\alpha_qN1$YFP and $\alpha_qN2$YFP were predominantly found at the plasma membrane upon expression of wild-type or VFP-tagged $\beta1\gamma2$ subunits (Figure 2D and 2E), in contrast to $\alpha_qN3$YFP (Figure 2F). A similar phenomenon has been described by Evanko et al. (2001), who used $\alpha_t$ and $\alpha_q$ mutants deficient in high affinity interaction with $\beta\gamma$ dimers and showed that they only tether to the plasma membrane upon over-expression of $\beta\gamma$. These results show that, although $\alpha_qN1$YFP and $\alpha_qN2$YFP were not plasma membrane bound when expressed alone, they were able to localize to this compartment upon co-expression of $\beta\gamma$, indicative of the formation of heterotrimers. It is not clear why plasma membrane localization of $\alpha_qN3$YFP was not restored in the presence of high levels of $\beta\gamma$.

Interestingly, tubular structures were occasionally observed for $\alpha_q$ upon co-expression of $\beta\gamma$ as well as for VFP-tagged $\beta$ and $\gamma$ subunits (arrows, Figure 2E and data not shown). Association with the cytoskeleton has been described for several heterotrimeric G proteins among which $\alpha_t$ (Drmota et al., 1999; Ibarrondo et al., 1995).

**Subcellular location of $\alpha_{H/G}$-VFP fusion proteins**

Next, four additional insertion sites outside of the N-terminus were made of which one, termed $\alpha_qH1$YFP, localized to the plasma membrane upon transient expression in
various cell lines (MDCK, N1E-115 neuroblastoma, HEK293 and NIH-3T3) (HeLa: Figure 3A). The VFP insertion site of this fusion protein was based on a functional Giq HA-tagged in the α helical domain (residues 125-ENPYVD-130 replaced by DVPDYA, Wedegaertner et al., 1993; Wilson and Bourne, 1995) and on Giq-VFP, as described by Hughes et al. (2001). The other fusion proteins, GiqH2-YFP, GiqH3-YFP, GiqG1-YFP and GiqG2-YFP, did not localize to the plasma membrane by themselves (Figure 3B-E) nor in the presence of Gβ1γ2 (data not shown) and showed varying degrees of fluorescence in the nucleus.

![Figure 3. HeLa cells expressing A) GiqH1-YFP; B) GiqH2-YFP; C) GiqH3-CFP; D) GiqG1-CFP and E) GiqG2-CFP. Bars, 10 μm.](image)

**Activation of PLCβ by Giq-VFP fusion proteins**

PLCβ is activated by Giq upon stimulation of certain GPCRs and catalyzes the hydrolysis of PtdInsP2 into DAG and IP3. The latter molecule is soluble and diffuses into the cytosol where it opens calcium channels in the ER, as mentioned above. In order to study the ability of Giq-VFP fusion proteins to activate PLCβ, the Q209L mutation was applied. Wild-type Giq that contains this mutation is constitutively active, because its GTPase activity is abrogated (Kalinec et al., 1992). The mutant was coexpressed with the PH domain from PLCβ1, which is a sensor of PtdInsP2 levels and is found at the plasma membrane in resting cells (Lemmon et al., 1995; Stauffer et al., 1998). Application of this mutation in the Giq-VFP fusion proteins rendered GiqH1-YFP constitutively active, as was concluded from its capacity to cause translocation of PHδ1 to the cytosol in a similar way as non-tagged Giq-QL (Figure 4A and 4B and data not shown). The other Giq-VFP fusion proteins described in this study were not able to cause translocation of the PHδ1 domain, as shown for GiqG2-QL-CFP (Figure 4C and 4D). Interestingly, GiqH1-QL-YFP was severely compromised in plasma membrane localization and showed considerable fluorescence in the nucleus.
Effect of lipidation/N-terminal cysteines on PLCβ activity

The effect of palmitoylation of the two cysteines in the N-terminus of $\alpha_q$ on localization and interaction with PLCβ is controversial. In order to get insight into this matter we exchanged the N-terminus of $\alpha_q$H1-YFP for that of $\alpha_t$. Instead of being palmitoylated, $\alpha_t$ is co-translationally myristoylated and does not contain cysteines that can receive a palmitoyl moiety (Yang and Wensel, 1992). Upon expression of this chimera in HeLa cells fluorescence was detected diffusely throughout the cytosol (data not shown). Co-expression of $\alpha_{2}G2$ did not lead to an increase in fluorescence at the plasma membrane. Notwithstanding this lack of a visible localization to the plasma membrane, rendering the protein constitutively active did cause PHβ1 to translocate to the cytoplasm, indicating that PLCβ is activated by the chimera (Figure 4E-F).

Receptor-coupling of $\alpha_q$H1-VFP and downstream signaling

The method described above indicates that $\alpha_q$H1-YFP is able to activate PLCβ and provides a quick assay, but it does not provide information on the potential of $\alpha_q$H1-YFP to interact with a GPCR and be activated by it. Therefore, the last 5 amino acids of

Figure 4. HeLa cells co-expressing A) $\alpha_q$H1(Q209L)-YFP and B) RFP-PHβ1. HeLa cells co-expressing C) $\alpha_q$G2(Q209L)-CFP and D) YFP-PHβ1. HeLa cells co-expressing E) $\alpha_q$H1(Q209L)-YFP and F) CFP-PHβ1. Bars, 10 μm.
$\text{G}_{\alpha q}$H1-YFP (EYNLV) were exchanged for those of $\text{G}_{\alpha q1}$ (DCGLF), leading to $\text{G}_{\alpha q15}$H1-YFP. Conklin et al. (1993) showed that this small stretch was enough to change the preference of $\text{G}_{\alpha q}$ towards $\text{G}_{\gamma}$-coupled receptors. Activation of the $\text{G}_{\alpha q}$-coupled serotonin 5HT1A receptor in HeLa cells co-transfected with $\text{G}_{\alpha q15}$H1-YFP led to an increase in cytosolic calcium, as observed using the fluorescent calcium indicator Fura-2 (11 out of 16 cells expressing $\text{G}_{\alpha q15}$H1-YFP, Figure 5A). These results suggest that $\text{G}_{\alpha q15}$H1-YFP can functionally interact with the 5HT1A receptor and in turn activate PLC$_{\beta}$. Remarkably, cells that were co-transfected with 5HT1AR and wild-type $\text{G}_{\alpha q}$ also showed an increase in cytosolic calcium upon addition of serotonin (7 cells out of 47, Figure 5B). Therefore, we opted for another system in which to investigate the functionality of $\text{G}_{\alpha q}$H1-YFP. Offermanns et al. (1998) described the development of $\text{G}_{\alpha q11}$ deficient mice and prepared mouse embryonic fibroblasts (MEF$_{q11}$(-/-)) from these mice (Zywietz et al., 2001). This cell line, as well as a MEF cell line prepared from wild-type mice, expresses the B2 receptor that couples to $\text{G}_{\alpha q}$ (Vogt et al., 2003). As expected, addition of bradykinin (BK, 1$\mu$M) led to an increase in cytosolic calcium in the wild-type MEF cell line, but not in the MEF$_{q11}$(-/-) cell line. Expression of $\text{G}_{\alpha q}$H1-YFP (Figure 5C) or $\text{G}_{\alpha q}$ in MEF$_{q11}$(-/-) caused an increase in cytosolic calcium upon addition of BK (Figure 5D). These results indicate that $\text{G}_{\alpha q}$H1-YFP can substitute for the wild-type $\text{G}_{\alpha q}$ in living cells devoid of endogenous $\text{G}_{\alpha q11}$ proteins.

**Figure 5.** A) HeLa cells were co-transfected with $\text{G}_{\alpha q15}$H1-YFP and 5HT1AR and loaded with Fura 2-AM. The Fura intensity was monitored upon addition of 100 $\mu$M serotonin (Sigma). The two traces (diamonds and triangles) are from non-transfected cells, the other traces (filled and open circles) are from cells expressing $\text{G}_{\alpha q15}$H1-YFP. B) HeLa cells were co-transfected with $\text{G}_{\alpha q}$ and 5HT1AR and loaded with Fura 2-AM. The Fura intensity was monitored upon addition of 100 $\mu$M serotonin (Sigma). The traces of two cells in which calcium was mobilized are shown. C) MEF cells deficient in $\text{G}_{\alpha q11}$, transduced with $\text{G}_{\alpha q}$H1-YFP. Bar, 10 $\mu$m. D) $\text{G}_{\alpha q11}$ deficient MEF cells were transduced with $\text{G}_{\alpha q}$H1-YFP and loaded with Fura 2-AM and its intensity was measured upon addition of bradykinin (1$\mu$M) ($n=12$). The traces of two cells (filled and open circles) are shown.
Monitoring the dynamic interaction between GαqH1-VFP and Gβγ by means of FRET

Conformational changes in the heterotrimer upon activation have been described for the human Gα1,2,3, Gαz, Gαo and Gαs proteins, the yeast Gα Gpa1 and the Dictyostelium Gα2 using FRET (Bunemann et al., 2003; Frank et al., 2005; Hein et al., 2006; Janetopoulos et al., 2001; Yi et al., 2003). However, the interaction between Gαq and Gβγ upon activation of its GPCRs has not yet been monitored. Fluorescence resonance energy transfer (FRET) is a distance- and conformation-dependent process and therefore it is very suitable in studies on the dynamic properties of protein interactions in living cells. With the purpose of assessing the interaction between Gαq-CFP and the Gβγ dimer, the N-terminus of Gγ2 was fused to YFP and dual emission ratiometric FRET-imaging experiments were performed. However, addition of histamine in order to stimulate the endogenous H1 receptor in HeLa cells did not result in a change in intensities of donor or acceptor (Figure 6A). To assure that the CFP and YFP fluorophores are in close enough proximity to allow efficient FRET in the heterotrimer, we assessed the FRET efficiency by measuring the fluorescence lifetime of the donor fluorophore (Gαq-CFP) in the presence and in the absence of the acceptor YFP-Gγ2. In the presence of the acceptor, the lifetime of the donor decreased about 23%, reflecting efficient FRET in the intact heterotrimer (Figures 6B-F, Table 1).

Figure 6. A) Representative time course of the change in FRET ratio upon addition of histamine (100μM) in cells expressing GαqH1-CFP (left image) and YFP-Gγ2 (right image), inset: the CFP and YFP intensity traces that belong to the ratio. GαqH1-CFP intensity (B) and phase lifetime image (D) in the absence of acceptor YFP-Gγ2. GαqH1-CFP intensity (C) and CFP phase lifetime image (E) in the presence of acceptor YFP-Gγ2. (F) CFP phase lifetime histogram; lifetime distribution in the presence (left population) and in the absence of the acceptor (right population). Scale 1-5 ns. Bars, 10 μm.
To investigate whether the orientation of the fluorescent proteins is not optimal for detecting subtle conformational changes and/or distance-related alterations, we removed 8 amino acids from the C-terminus of CFP, thereby forcing the CFP fluorophore (and its dipole moment) in another orientation. This construct proved functional in MEF_{Gq11}(-/-) cells (Figure 7A).

### Table 1. Apparent FRET efficiencies

<table>
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<th>Constructs</th>
<th>Apparent FRET efficiencies</th>
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<th>based on εM, ± s.d.</th>
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<tr>
<td>G_{αq1}CFP (n=14)</td>
<td>0.0 ± 4.8%</td>
<td>0.0 ± 0.8%</td>
<td></td>
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<tr>
<td>G_{αq1}CFP+YFP-G_{Y2} (n=8)</td>
<td>22.9 ± 4.3%</td>
<td>16.3 ± 2.6%</td>
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<tr>
<td>G_{αq1}CFPΔ8 (n=15)</td>
<td>0.0 ± 0.1%</td>
<td>0.0 ± 1.0%</td>
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<tr>
<td>G_{αq1}CFPΔ8+YFP-G_{Y2} (n=12)</td>
<td>25.5 ± 0.5%</td>
<td>17.2 ± 4.7%</td>
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Figure 7. A) G_{αq1}H1-CFPΔ8 similarly caused an increase in [Ca^{2+}] upon addition of BK (n=6). B) Representative time course trace of the change in FRET ratio upon addition of histamine (100μM) in cells expressing G_{αq1}H1-CFPΔ8 (left image) and YFP-G_{Y2} (right image) (Ratio change: 0.05 ± 0.04 (n=54)). G_{αq1}H1-CFPΔ8 intensity (C) and phase lifetime image (E) in the absence of acceptor YFP-G_{Y2}. G_{αq1}H1-CFPΔ8 intensity (D) and phase lifetime image (F) in the presence of acceptor YFP-G_{Y2}. (G) Phase lifetime histogram; lifetime distribution in the presence (left population) and in the absence of the acceptor (right population). Scale 1-5 ns. Bars, 10 μm.
The intensity traces that belong to these cells unequivocally show that the intensity of CFP increased, whereas the sensitized emission of YFP decreased (Figure 7B), indicating a loss of FRET. Fluorescence lifetimes measured by means of FLIM showed that GαqH1-CFPΔ8 has a similar low fluorescence lifetime in the presence of the acceptor YFP-Gγ2, as GαqH1-CFP (Figure 7C-G, Table 1). Apparently, judging from the lack of a clear ratio-change in GαqH1-CFP, Gq does not predominantly dissociate upon activation but merely undergoes a conformational change.

**Proteins that influence the interaction between GαqH1-VFP and Gβγ**

Over-expression of the H1 receptor in HeLa cells led to intensity changes with significantly larger amplitudes (Figure 8A). Similarly large ratio changes were obtained upon activation of an over-expressed B2 receptor (data not shown). In addition, recovery was not observed at the timescale that led to almost complete recovery observed for endogenous receptor levels, suggesting that the high amount of receptors cannot be rapidly desensitized by the endogenous machinery, comprising e.g. GRKs and PKCs (Bohm et al., 1997). Upon activation of Gq in the presence of over-expressed H1R, the lifetime of Gαq-CFPΔ8 increased about 7%, but did not return to the control CFP-lifetime observed in the absence of YFP-Gγ2 (Figures 8D-H, Table 2). Again, this finding suggests that, even in the presence of an over-expressed activating receptor, Gαq and Gβγ do not completely dissociate upon activation. Thus, judging from the FLIM data, the larger amplitude of the response represents a larger amount of activated Gq proteins.

A plethora of proteins regulates the activation state of heterotrimeric G proteins. One of the proteins that regulates Gq is PLCβ1Δ2. Whereas this protein is activated by Gαq, it turns itself off by acting as a GTPase accelerating protein (GAP) for Gαq. The C-terminal domain (CT domain) of PLCβ is responsible for interaction with Gαq and GAP activity. Co-expression of this module led to ratio-changes with amplitudes similar to those observed upon over-expression of the H1 receptor (Figure 8B, Table 2). Interestingly, the recovery rates were much faster than those observed in the presence of over-expressed receptors, while they were slower than those observed in the absence of the C-terminal domain. CFP lifetimes increased about 6% upon addition of histamine in the presence of the CT domain (Figures 8I-M, Table 2). This change in lifetime is similar to the one observed for over-expressed receptors, suggesting that the interaction with the CT domain does not induce overall dissociation nor a larger conformational change, which is in line with a previous report (Evanko et al., 2005). Apparently, the CT domain retains Gq in its active state by shielding it from e.g. PLCβ/RGS proteins, thereby accumulating Gαq-GTP. Over-expression of both H1R (representing an increase in koff/GEF activity) and the CT domain (representing a decrease in kon/GAP activity) led to even larger FRET ratio amplitudes, whereas the lifetimes measured increased about 6% (Figures 8C, 8N-R, Table 2).
Table 2. Changes in FRET upon addition of agonist.

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<th>FRET change based on $\tau_M$ ± s.d.</th>
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<td>G$<em>{q\alpha}$-CFPΔ8+YFP-G$</em>{Y2}$</td>
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<tr>
<td>+H1R (n=6)</td>
<td>5.6 ± 3.2%</td>
<td>4.1 ± 1.3%</td>
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<tr>
<td>+CT (n=6)</td>
<td>4.5 ± 2%</td>
<td>1.5 ± 0.8%</td>
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<td>+H1R+CT (n=4)</td>
<td>5 ± 1.2%</td>
<td>3.9 ± 2.9%</td>
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FRET remaining after addition of agonist

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<th>FRET change</th>
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<tr>
<td>+H1R</td>
<td>19.9 ± 3.2%</td>
</tr>
<tr>
<td>+CT</td>
<td>21.0 ± 2.0%</td>
</tr>
<tr>
<td>+H1R+CT</td>
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</tbody>
</table>

Figure 8. (A) Representative time courses of the change in FRET ratio upon addition of histamine in cells expressing G$_{q\alpha}$-H1-CFPΔ8 and YFP-G$_{Y2}$ in the presence of over-expressed H$_1$R (Ratio change: 0.12 ± 0.04, n=8); (B) in the presence of over-expressed CT domain (Ratio change: 0.23 ± 0.09, n=10); (C) in the presence of over-expressed H$_1$R and over-expressed CT domain (Ratio change: 0.29 ± 0.05, n=12). Insets: the CFP and YFP intensity traces that belong to the ratios.

The effects of the C-terminal domains of PLC$\beta_3$ and PLC$\beta_{4a}$ were examined as well and the average amplitude of the ratio-changes differed as follows: CT$\beta_{4a}$>CT$\beta_3$>CT$\beta_{4a}$ (Table 3). The results indicate that CT$\beta_{4a}$ is a less potent amplifier of the amount of G$_q$ present in the active state, suggesting that it shows a lower affinity for G$_{q\alpha}$. These findings support the results presented in Chapter 4.
Table 3. Ratio-changes for FRET between Gαq-CFPΔ8 and YFP-Gγ2 upon addition of histamine in the presence of the various CT domains.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Ratio change ± s.d.</th>
<th>Number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gαq-CFPΔ8+YFP-Gγ2+CTβ1a</td>
<td>0.23 ± 0.09</td>
<td>10</td>
</tr>
<tr>
<td>Gαq-CFPΔ8+YFP-Gγ2+CTβ1b</td>
<td>0.21 ± 0.01</td>
<td>3</td>
</tr>
<tr>
<td>Gαq-CFPΔ8+YFP-Gγ2+CTβ1c</td>
<td>0.10 ± 0.03</td>
<td>8</td>
</tr>
</tbody>
</table>

Additionally, FRET measurements were done in HEK293 cells, which endogenously express muscarinic acetylcholine receptors (Lin et al., 1999). However, stimulation with carbachol did not lead to a change in FRET, although an increase in intracellular calcium was observed (Figures 9A-B and data not shown). Importantly, co-expression of the C-terminal domain of PLCβ1a did cause a large ratio-change upon addition of carbachol (Figures 9C-D).

Discussion

Location, lipidation and Gβγ interaction of Gαq-VFP fusion proteins

Most of the VFP-tagged Gαq proteins that were screened were found to be non-functional. The fact that fluorescence of both GαqN1-YFP and GαqN2-YFP was found in the cytosol and the nucleus of most cells suggests that these proteins are either broken down or not lipidated. Co-expression of Gβγ rescued plasma membrane localization and eliminated fluorescence in the nucleus, which is indicative of stabilization of the proteins. Probably,

Figure 9. HEK293 cells were transfected with GαqH1-CFPΔ8, Gβ1, and cpV6-Gγ2. FRET ratiometric images were recorded upon addition of 100μM carbachol (Sigma). The YFP/CFP ratio is shown in graph A, while the intensity traces belonging to this ratio are depicted in B. HEK293 cells were transfected with GαqH1-CFPΔ8, Gβ1, cpV6-Gγ2, and RFP-CTβ1a. FRET ratiometric images were recorded upon addition of 100μM carbachol (Sigma). The YFP/CFP ratio is shown in graph C, while the intensity traces belonging to this ratio are depicted in D.
these proteins display decreased affinity for $G_{\beta\gamma}$ and are therefore unable to attain the plasma membrane in the presence of endogenous levels of $G_{\beta\gamma}$ dimers. The N-terminus is one of the two interfaces necessary for interaction with $G_{\beta\gamma}$. Apparently, insertion of a fluorescent protein in this region weakens interaction with $G_{\beta\gamma}$. Additional defects may also contribute to the lack of plasma membrane localization of these proteins. The N-terminus is involved in receptor interaction as well and, most importantly, dictates lipidation. It may be the lack in palmitoylation that makes the proteins instable rather than compromised $G_{\beta\gamma}$ binding, since a specific $G_{\beta\gamma}$ interaction mutant of $G_{\alpha_q}$ (I25A, E26A) did not display a shorter half-life than the wild-type protein (Johansson et al., 2005).

It is not clear why plasma membrane localization of $G_{\alpha_q}N3$-YFP was not restored in the presence of high levels of $G_{\beta\gamma}$. It might be that the protein is completely misfolded. Alternatively, the insertion site of the fluorescent protein may be too close to the plasma membrane for the G protein to correctly orient itself towards the $G_{\beta\gamma}$ dimer and the membrane. Indeed, a NMR study on $G_{\alpha_H}$ in the presence of membrane tubules indicates that the site of insertion is near the membrane and situated at the end of the N-terminal helix, close to a loop reaching into the cytoplasm (Zhang et al., 2004). Apparently, the folding of GFP is thermodynamically favourable at all of these sites, since fluorescence is consistently detected (Sheridan et al., 2002).

$G_{\alpha_q}H1$-VFP was the only protein that localized at the plasma membrane independently of the overexpression of other proteins, suggesting that it can form heterotrimers with endogenous $G_{\beta\gamma}$ dimers and become lipidated. $G_{\alpha_q}H2$, H3, G1 and G2 were not able to attain the plasma membrane whether or not $G_{\beta\gamma}$ dimers were overexpressed. Additionally, they were found in the nucleus, suggesting that these proteins are not being (efficiently) lipidated. Likely these protein fusions are not correctly folded, prohibiting their interaction with $G_{\beta\gamma}$ dimers. Although the insertion sites of both $G_{\alpha_q}H1$ and $G_{\alpha_q}H3$ are based on epitope-tagged $G_{\alpha_q}$ proteins, the insertion site of $G_{\alpha_q}H3$ is deleterious. Apparently, insertion of a large tag (e.g. VFP) at this locale is not tolerated. The fact that the $\alpha_B$-$\alpha_C$ loop (i.e. the H1 insertion site) is able to accommodate such a large insert is consistent with the notion that the yeast $G_{\alpha}$ subunit Gpa1 harbors a 100-residue insertion at this site, as compared to mammalian $G_{\alpha}$ subunits (Nakafuku et al., 1987).

**Functionality of $G_{\alpha_q}H1$-VFP**

Certainly, the fact that $G_{\alpha_q}H1$-VFP localizes to the plasma membrane does not provide adequate proof of functionality. As an initial assay we introduced a mutation that renders wild type $G_{\alpha_q}$ constitutively active. In accordance with the results on subcellular location, only $G_{\alpha_q}H1$-VFP was able to activate PLC$\beta$. Strictly, this does not mean that the other protein fusions are completely unable to activate PLC$\beta$, since a slight activation cannot be
excluded using this approach. In addition, spontaneous GDP exchange in G\textsubscript{\alpha q} is almost negligible and in need of a GEF (e.g. a receptor). The introduced mutation merely decreases the GTP hydrolysis rate without increasing the rate of GDP exchange (Coleman and Sprang, 1999), suggesting that the amount of GTP-bound, constitutively active G\textsubscript{\alpha q} must be low. In combination with other debilitating mutation(s), for instance those that lead to compromised plasma membrane targeting, the amount of active G\textsubscript{\alpha q} may be too low to hydrolyze an appropriate amount of PtdInsP\textsubscript{2} for the PH domain to translocate to the cytoplasm.

Subsequently, receptor-coupling was examined. Since the Gq class of G\alpha subunits is pertussis toxin (PTX)-resistant, it is impossible to inactivate the endogenous Gq proteins with this toxin (Fields and Casey, 1997). Therefore, we prepared the G\textsubscript{\alpha q/\gamma} chimera that no longer couples to Gq-GPCRs, introduced the G\textsubscript{\alpha q}-coupled 5HT\textsubscript{1A} receptor into HeLa cells and monitored calcium upon addition of serotonin. Unfortunately, both G\textsubscript{\alpha q} wild-type and G\textsubscript{\alpha q}\textsubscript{H1-YFP} gave a similar response, which might be explained by promiscuity of the 5HT\textsubscript{1A} receptor. Indeed, over-expression in heterologous systems has been shown to result in a reduction in G protein coupling specificity (Selbie and Hill, 1998). As a consequence, we transduced G\textsubscript{\alpha q}\textsubscript{H1-YFP} into MEF cells derived from G\textsubscript{\alpha q/\gamma} knockout mice and were able to monitor increases in intracellular calcium upon addition of bradykinin. These results show that G\textsubscript{\alpha q}\textsubscript{H1-YFP} effectively interacts with GPCRs and PLC\beta. In addition, we can state that it can also form heterotrimers with G\beta\gamma, because receptors bind G\alpha subunits poorly and fail to trigger GDP exchange in the absence of G\beta\gamma (Freissmuth et al., 1999).

It remains to be established whether G\textsubscript{\alpha q}\textsubscript{H1-VFP} is able to productively interact with its other partners (Chapter 1, Section 3.11). Among the most structurally heterogeneous regions of Gq is the\alpha B-\alpha C loop (Kimple et al., 2002), suggesting that highly specific functions are regulated through this loop and the insertion of an VFP may therefore inhibit interaction with as yet unidentified or non-characterized partners.

**Lipidation and PLC\beta activation**

The role of palmitoylation in PLC\beta activation by G\textsubscript{\alpha q} remains controversial. Studying lipidation by means of mutagenesis of residues that are assumed to be lipided \textit{in vivo} cannot differentiate between the effect of a lack of lipidation and the effect of the lack of the residue \textit{per se}. Hepler et al. (1996) described non-lipidated G\textsubscript{\alpha q} subunits capable of activating PLC\beta at levels comparable to the wild-type protein. Therefore, they ascribe the abrogation of PLC\beta signaling using G\textsubscript{\alpha q} cysteine mutants (C9/10S) to the lack of the cysteine residues, not the lipid tails. Yet, their results are derived from \textit{in vitro} studies and localization was not examined in living cells. In addition, inhibitory peptides encoding
the N-terminus of \( \alpha_q \) have not been found and exchanging the N-terminus for that of \( \alpha_t \) leads to activation of PLC\( \beta \) (Arkinstall et al., 1995; Wedegaertner et al., 1993). Moreover, a portion of the C9/10S double mutants were shown to be resistant to solubilization by detergents, suggesting that these proteins are misfolded (Edgerton et al., 1994). We were able to confirm the results published by Wedegaertner et al. (1993), indicating that the two cysteine residues in the N-terminus of \( \alpha_q \) are not mandatory for PLC\( \beta \) activation in living cells. \( \alpha_q \)-H1-VFP was not detected at the plasma membrane consistent with the fact that myristate provides barely enough hydrophobicity to anchor a protein to membranes. These results suggest that \( \alpha_q \) does not require a high residence time at the plasma membrane to activate PLC\( \beta \). However, a comparison between Figures 4A-B and 4E-F indicates that \( \alpha_q \)-H1-VFP is a much more potent activator.

**Monitoring Gq activation**

The activation of heterotrimeric G proteins is difficult to monitor, since direct measurement of GTP binding and hydrolysis is not feasible in living cells (Milligan, 2003). Therefore, intracellular calcium is often used as a measure of Gq activity. Unfortunately, other heterotrimeric G proteins can cause increases in intracellular calcium by means of calcium entry from the extracellular milieu and by activation of PLC\( \beta \) via G\( \beta\gamma \) dimers. In addition, increases in intracellular calcium triggered by Gq-coupled receptors are amplified signals that are modulated by downstream factors (e.g. calcium store depletion and regulation of effector function by Ca\(^{2+}\)-activated kinases). Hence they are not ideal to quantify the total extent of G protein activation. The FRET pair described here enables the measurement of G protein activation before amplification of the response occurs. The actual quantification of the amount of Gq that becomes active upon addition of agonist remains difficult, because our experiments are done in the presence of endogenous G protein subunits. VFP-tagged subunits can therefore interact with endogenous subunits, which abolishes the ability to measure FRET or changes in FRET upon activation. It will be difficult to circumvent these problems since this entails knocking down at most 5 G\( \beta \) subunits and 12 G\( \gamma \) subunits, depending on the complement of heterotrimeric G proteins present in the MEF \( \alpha_{q/11} \) deficient cells. Still, the effects of accessory proteins can be tested with the help of this sensor, as shown for the CT domain, and therefore it constitutes an important advance in the field of Gq research.

Remarkably, about 40% of today’s medicines target GPCRs (Klein-Seetharaman, 2005). However, side-effects of these drugs are frequently encountered. Many of these adverse effects are probably caused by the fact that most GPCRs do not trigger a single downstream signaling pathway. Their biological response often results from the concerted stimulation of several G proteins, leading to the activation of multiple downstream pathways (Scheer et al., 1996). A drug directed towards a GPCR would therefore inhibit
all of these pathways, whereas they are normally not all implicated in the disease that needs to be treated. For that reason, inhibitors of G proteins would be attractive drug candidates as opposed to antagonists of GPCRs (Freissmuth et al., 1999). The FRET pair we developed may aid in the development of new drugs by facilitating activity measurements in living cells.

Initial \textit{in vitro} experiments suggested that activation leads to dissociation of the heterotrimer into the G\textsubscript{\textalpha} subunit and the G\beta\gamma dimer (Katada et al., 1984). These experiments have evolved into a dogma that was recently challenged by several findings that indicate that the intact heterotrimer can play a role in signaling (Bunemann et al., 2003; Digby et al., 2006). In the process of developing the Gq activity FRET sensor, we obtained a G\textalpha\textsubscript{q}-CFP/YFP-G\gamma\textsubscript{2} pair that showed clear FRET as analyzed by FLIM measurements, but did not show a decrease in FRET ratio upon activation. This finding suggests that the majority of the G\alpha\textsubscript{q}/G\beta\gamma\textsubscript{1}/G\gamma\textsubscript{2} heterotrimers does not dissociate upon activation. The fact that the removal of 8 amino acids from the C-terminus of CFP does lead to detectable changes in FRET suggests that the initial orientation of the CFP fluorophore (and its dipole moment) inserted in G\alpha\textsubscript{q} was not optimal for the detection of more subtle conformational changes. As explained in Box 2, Chapter 1, the orientation of the chromophores plays an important role in the outcome of FRET efficiency by influencing the Förster radius for energy transfer and gives rise to the \( \kappa^2 \) orientation factor. A similar deletion of the C-terminal end of CFP has been shown to have a profound effect on the FRET efficiency in yellow cameleon (Truong et al., 2001).

Moreover, even in the presence of overexpressed H1R (increase in GEF activity) and the CT\beta1 domain (decrease in GAP activity) the CFP fluorescence lifetime did not increase to control levels. This again underscores that dissociation of the Gq heterotrimer is not the conventional mechanism of activation, but is rather accomplished by a conformational change, implying that it is the intact heterotrimer that signals to downstream partners. In addition, it suggests that the larger ratio changes obtained in the presence of overexpressed H1R/CT\beta1 domain are caused by the accumulation of GTP-bound Gq, as opposed to an increase in distance between G\alpha\textsubscript{q} and G\beta\gamma. Importantly, G\beta\gamma dimers have been shown to activate PLC\beta and it is tempting to speculate that they might play a role in the activation mechanism of PLC\beta by G\alpha\textsubscript{q}, especially now that Evanko et al. (2005) found G\beta\gamma dimers to be essential for G\alpha\textsubscript{q}-mediated PLC\beta activation. Certainly, the insertion of the VFP at another position in G\alpha\textsubscript{q} could give a different read-out and elucidate conformational rearrangements occurring upon activation of the heterotrimer in living cells. Unfortunately, we were not able to find other functional insertion sites for G\alpha\textsubscript{q}, as described above.

The remaining question is: how does a trimer signal? The observed FRET changes indicate that the helical domain rotates away from the N-terminus of G\gamma, but that...
complete dissociation is not a general mechanism. Gβ is most severely affected in binding to Gα when it contains mutations in the region that interacts with the N-terminus of Gα, indicating that the N-terminus is crucial in mediating the interaction with Gβ (Li et al., 1998). Therefore we hypothesize that, upon activation, the contact of Gβ with the switch regions is lost, whereas its contact with the N-terminus remains. This would enable an opening-up of the trimer, allowing Gα and Gβγ to interact with their downstream signalling partners. However, a lack of dissociation might also imply that interaction with certain effectors is impossible. Accessory proteins may cause dissociation of Gα and Gβγ by forcing them apart. The recently published crystal structure of the receptor kinase GRK2 in complex with Gαq-GTP and Gβγ exemplifies this idea (Tesmer et al., 2005).

Materials and Methods

Constructs

The pcDNA3.1+ plasmids containing the cDNA encoding human Gαq, Gαq-Q209L, Gβ1, Gβ2, SHTαR and H1R were obtained from the UMR cDNA Resource Center (www.cdna.org). GαεN1-VFP was prepared by inserting spectral variants of GFP in between residues 17 and 18. GαεN2-VFP was prepared by inserting spectral variants of GFP in between residues 17 and 18. In addition, the residues LSEEAK were repeated after the insertion position of the FP. GαεN3-VFP was prepared by inserting spectral variants of GFP in between residues 37 and 38. GαεH2-VFP was prepared by inserting spectral variants of GFP after residue 139, thereby changing residue N137 to K137. The linkers are identical to the ones described below for GαεH1-VFP. GαεH3-VFP was prepared by inserting spectral variants of GFP after residue 170, thereby changing residue A171 to G171. GαεG1-VFP was prepared by inserting spectral variants of GFP after residue 170, thereby adding two additional residues (EL). GαεG2-VFP was prepared by inserting spectral variants of GFP in between residues 307 and 308. The linkers present in the GαεH3-VFP, GαεG1-VFP and GαεG2-VFP are SG and GS.

In analogy with Hughes et al. (2001), Wedegaertner et al. (1993) and Wilson and Bourne (1995), we inserted spectral variants of GFP in the βC loop of Gαq in between residues 127 and 128, thereby changing residue N126 into D126. The linkers present in this construct are SGGGGS at the N-terminus of the FP and SGSGGD at the C-terminus of the FP. In order to make the Gαε-CFPΔ8 construct, the last 8 amino acids (i.e. HGMDELYK) of the CFP were removed. The linkers present in this construct are SGGGGS at the N-terminus of the FP and LGI at the C-terminus of the FP.

In order to make GαεH1-CFP, the N-terminus of Gαq (MTLESIMACCLSEEA) was swapped for that of Gαε (MGAGASAE). In order to make Gαεβ1h1-CFP, the C-terminal four amino acids of Gαq were swapped for those of Gαε (see text).

Gαε-VFP was amplified by means of PCR and inserted into the retroviral vector pBABE-puro (providing puromycin resistance), obtained from Addgene Inc. (Cambridge, MA, USA).

In order to make YFP-GαT, we amplified GαT by means of PCR and cloned it behind circular-permutated Venus derived from YCam3.60 (Nagai et al., 2004), a kind gift of Dr. A. Miyawaki (RIKEN, Wako, Japan). A similar fusion to the N-terminus of GαT has been shown to retain functionality (Ruiz-Velasco and Ikeda, 2001).

pcDNA6V5HisB containing rat PLCβ1A and pcDNA3 containing rat PLCβ3 were kind gifts of Dr. L. Runnels (University of Medicine and Dentistry of New Jersey, NJ, USA). Full-length rat PLCβ4 in pCMV2-FLAG was a generous gift of Dr. Suh (Pohang University of Science and Technology, Pohang, South Korea). The CT domain of PLCβ1A (795-1216) was amplified by means of PCR and fused to the C-terminus of mStrawberry (Shaner et al., 2004), a kind gift of Dr. R.Y. Tsien (Howard Hughes Medical Institute, CA, USA). Similarly, CTβ3 (845-1236) and CTβ4 (822-1175) were prepared.
The pcDNA3 vector containing human B2R was a gift from Dr. C. Liebmann (Friedrich-Schiller-University, Jena, Germany).

Phø was a gift from Dr. T. Meyer (Stanford University Medical Center, Stanford, CA, USA) and was fused to the C-terminus of mRFP1. All constructs were verified by means of sequencing.

Structure representation
The ribbon diagram of Gq\(\gamma\)-GTP was generated using coordinates from Protein Data Bank file 2BCJ (Tesmer et al., 2005) and visualized with PyMol.

Cell culture & transfection/transduction
MEF cells devoid of G\(\alpha_q/11\) subunits (Zywietz et al., 2001), derived from G\(\alpha_q/11\) knockout mice (Offermanns et al., 1998) were cultured in DMEM (#21969-035) supplied with 1x non-essential amino acids, 10% FBS, 1x L-glutamine, Penicilline (100U/ml) and Streptomycine (100\(\mu\)g/ml) (Invitrogen, Breda, The Netherlands). For transduction of these cells at the Dutch Cancer Institute, the retroviral vector pBABE-G\(\alpha_q\)-YFP was transfected into Phoenix-Eco package cells and the supernatant containing viral particles was harvested 48h after infection. Cells were incubated with 1ml of this supernatant in the presence of 10\(\mu\)l Dotap (1mg/ml) and after 48h, cells were selected on 2\(\mu\)g/ml puromycin for the duration of two weeks. For imaging, these cells were cultured on glass cover slips and loaded with the calcium indicator Fura-2 AM (2\(\mu\)g/ml final concentration) (Sigma). For non-transduced MEF cells were cultured on glass cover slips and transfected with plasmids encoding G\(\alpha_q\)-CFP8\& by means of lipofectamine (Invitrogen, Breda, The Netherlands). After over-night incubation at 37°C, 5% CO\(2\), the cells were loaded with Fura and mounted in an Attofluor cell chamber (Invitrogen, Breda, The Netherlands), submerged in medium (140mM NaCl; 5mM KCl; 1mM MgCl\(2\); 1mM CaCl\(2\); 10mM glucose; 20mM HEPES, pH 7.4) and observed on a confocal microscope.

HeLa cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM+Glutamax (#61965-059), 10% FBS, Penicilline (100U/ml) and Streptomycine (100\(\mu\)g/ml) (Invitrogen, Breda, The Netherlands). HEK293 cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM (#21969-035), 10% FBS, Penicilline (100U/ml) and Streptomycine (100\(\mu\)g/ml), L-glutamine (2mM) (Invitrogen, Breda, The Netherlands). Transfection of HeLa and HEK293 cells and preparation for imaging purposes were similar as described above for MEF cells.

Confocal microscopy
Mammalian cells were imaged using a Zeiss LSM 510 confocal laser scanning microscope (Carl-Zeiss GmbH, Germany). A Zeiss Plan-A 63x/1.4 oil-immersion objective was used. Samples were excited with a 488 nm argon laser line controlled by an acousto-optical tunable filter. For YFP/Fura the following settings were used: as primary dichroic mirror, HFT 488; as secondary dichroic mirror, NFT 570, thereby splitting the Fura fluorescence from the YFP fluorescence. For CFP/YFP/RFP the following settings were used: first a 80/20 mirror, subsequently the dichroic mirror, NFT 490, was followed by the 505-550 bandpass filter to yield the YFP image. The longpass 650 filter was used to obtain the Fura image. The MEF cells, which endogenously express the bradykinin B\(2\) receptor that couples to G\(\alpha_q\) (Vogt et al., 2003), were stimulated with 1\(\mu\)M (final concentration) bradykinin (Sigma) and the Fura intensity was followed in time as a measure of intracellular calcium levels. For CFP/YFP/RFP the following settings were used: first a 80/20 mirror, subsequently the dichroic mirror NFT 570, thereby splitting the RFP fluorescence from the CFP/YFP fluorescence. Finally, the dichroic mirror, NFT 515, was used to discriminate between CFP and YFP fluorescence. To yield the RFP image the LP 585 filter was applied, for CFP the BP 470-500 nm filter was used and for YFP the BP 540/20 nm filter. In order to abolish crosstalk the images were acquired in the multi-tracking mode, where the 514 nm laser line was coupled to the YFP detection channel, the 458 nm laser line to the CFP detection channel and the 568 nm laser line to the RFP detection channel.
Wide-field fluorescence microscopy

Ratiometric FRET measurements in HeLa and HEK293 cells were done on a Zeiss Axiosvert 200M wide field fluorescence microscope kept at 37°C, equipped with a Zeiss Plan-Neofluar 40x/1.30 oil-immersion objective (Carl-Zeiss GmbH, Germany) and a Cairn Xenon Arc lamp with monochromator (Cairn research, Kent, UK). Images were recorded with a cooled CCD camera (Coolsnap HQ, Roper Scientific). Fluorophores were excited with 420 nm light (slit width 30 nm), CFP emission was detected with the bandpass 470/30 filter and YFP (sensitized) emission was detected with the bandpass BP535/30 filter by turning the filter wheel. The exposure time for each image was 200 ms. HeLa cells, which endogenously express the histamine H1 receptor (Tilly et al., 1990), were stimulated with 100 μM (final concentration) histamine (Sigma) and CFP/YFP emission was followed in time.

Fluorescence lifetime imaging microscopy

Frequency-domain FLIM measurements were performed using the set-up described by van Munster and Gadella (2004a). The objective used was a Zeiss Plan Apochromat 63x/1.4 oil-immersion objective. Samples were excited by means of a 442 nm Helium-cadmium laser modulated at 75.1 MHz and a BP 480/40 nm emission filter was used to detect CFP fluorescence. FLIM stacks of 24 phase images permuted in recording order (van Munster and Gadella, 2004b) were acquired with an exposure time of about 0.1-0.5 seconds each.

Acknowledgments

We thank Dr N. Divecha for enabling us to do retroviral transductions in his lab at the NKI (Amsterdam) and stimulating discussions.

References


