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Multicolor BiFC analysis of competition among G protein β and γ subunit interactions

Thomas R. Hynes, Evan Yost, Stacy Mervine, and Catherine H. Berlot*

Weis Center for Research, Geisinger Clinic, Danville, PA 17822-2623

Abstract

We have applied multicolor BiFC to study the association preferences of G protein β and γ subunits in living cells. Cells co-express multiple isoforms of β and γ subunits, most of which can form complexes. Although many $\beta\gamma$ complexes exhibit similar properties when assayed in reconstituted systems, knockout experiments *in vivo* suggest that individual isoforms have unique functions. BiFC makes it possible to correlate $\beta\gamma$ complex formation with functionality in intact cells by comparing the amounts of fluorescent $\beta\gamma$ complexes with their abilities to modulate effector proteins. The relative predominance of specific $\beta\gamma$ complexes *in vivo* is not known. To address this issue, multicolor BiFC can determine the association preferences of β and γ subunits by simultaneously visualizing the two fluorescent complexes formed when β or γ subunits fused to amino terminal fragments of yellow fluorescent protein (YFP-N) and cyan fluorescent protein (CFP-N) compete to interact with limiting amounts of a common γ or β subunit, respectively, fused to a carboxyl terminal fragment of CFP (CFP-C). Multicolor BiFC also makes it possible to determine the roles of interacting proteins in the subcellular targeting of complexes, study the formation of protein complexes that are unstable under isolation conditions, determine the roles of co-expressed proteins in regulating the association preferences of interacting proteins, and visualize dynamic events affecting multiple protein complexes. These approaches can be applied to studying the assembly and functions of a wide variety of protein complexes in the context of a living cell.

Keywords

multicolor bimolecular fluorescence complementation; heterotrimeric G protein; G protein $\beta\gamma$ complex; fluorescence microscopy; spectrofluorometer; CFP; YFP; subcellular targeting; live cell imaging

1. INTRODUCTION

Most combinations of the 5 G protein β subunits and 12 γ subunits that have been identified in mammals form dimers that exhibit similar abilities *in vitro* to modulate the activities of effectors such as adenylyl cyclase [1], phospholipase C [2], and GIRK channels [3]. However, there is evidence suggesting that specific $\alpha\beta\gamma$ combinations are required for particular receptor-G protein signaling pathways *in vivo* [4]. In most cases it is not known which $\alpha\beta\gamma$ heterotrimers

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*To whom correspondence should be addressed. Weis Center for Research, Geisinger Clinic, 100 North Academy Avenue, Danville, PA 17822-2623, Phone (570) 271-8661, FAX (570) 271-6701, E-mail: E-mail: chberlot@geisinger.edu.

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mediate particular GPCR signaling pathways, nor has it been possible to determine which $\beta\gamma$ combinations predominate in a particular cell type. The relative amounts of particular $\beta\gamma$ complexes in a cell will be determined both by the expression levels of each of the β and γ subunits as well as by their accessibilities to and relative affinities for each other. Multicolor BiFC makes it possible to quantify the association preferences of β and γ subunits for each other in intact cells.

Multicolor BiFC consists of the simultaneous visualization of the two fluorescent complexes formed when proteins fused to amino terminal fragments of YFP and CFP (YFP-N and CFP-N, respectively) interact with a common binding partner fused to a carboxyl terminal fragment of CFP (CFP-C). Complexes containing YFP-N and CFP-C fusion proteins are yellow, while those containing CFP-N and CFP-C fusion proteins are cyan, because the amino terminal fragment of the fluorescent protein, which contains the chromophore, determines the spectral properties of the complex [5]. In the experiments described here the fluorescent proteins are split at residue 158 such that the amino terminal fragment consists of residues 1–158 of the fluorescent protein and the carboxyl terminal fragment consists of residues 159–238 of the fluorescent protein. For multicolor BiFC, it is important to use CFP rather than YFP to produce the carboxyl terminal fragment, because BiFC is not obtained when the closely related fluorescent protein fragments CFP(1–154) and YFP(155–238) are used [5]. For competition analysis, we use Cerulean, a modified version of ECFP that is 2.5-fold brighter than ECFP [6], to produce Cer-N fusion proteins, because Cer-N fusions compete more effectively than do CFP-N fusions with YFP-N fusions. The reason for this difference is not clear, but could indicate greater stability of Cer-N-fusion/CFP-C-fusion complexes compared to CFP-N-fusion/CFP-C-fusion complexes.

The interaction preferences of β and γ subunits identified using BiFC probably reflect association preferences, because most available evidence indicates that BiFC is irreversible [7,8]. For $\beta\gamma$ complexes, this is not a concern, because they generally associate irreversibly. The only reported exceptions are $\beta_5\gamma_2$ [9] and $\beta_4\gamma_{11}$ [10], which are unstable *in vitro*. Efforts are underway in several laboratories to develop a reversible form of BiFC by means of splitting the fluorescent protein in different places and mutagenesis of the fluorescent protein fragments, which would enable dynamic analysis of reversible protein-protein interactions. Nevertheless, the relative association preferences of reversibly associating proteins can also be estimated using this approach.

2. DESCRIPTION OF METHOD

2.1 Producing and assessing functionality of BiFC $\beta\gamma$ complexes

The amino termini of the G protein β and γ subunits are associated as a coiled coil in $\beta\gamma$ complexes [11] (Fig. 1). Fusion of fluorescent protein fragments to the amino termini of each subunit (Fig. 1) results in BiFC [12–14]. As will be discussed at greater length in section 2.4, the abilities of different γ subunits to compete for a common β subunit are compared using fusions of γ subunits (red in Fig. 1A, green in Fig. 1B) to the carboxyl termini of YFP-N (yellow in Fig. 1A) and Cer-N (cyan in Fig. 1B). The β subunit to be competed for (magenta in Fig. 1, A and B) is fused to the carboxyl terminus of CFP-C (dark blue in Fig. 1, A and B). Conversely, the abilities of different β subunits to compete for a common γ subunit are compared using an alternative BiFC configuration; the β subunits (red in Fig. 1C, green in Fig. 1D) are fused to the carboxyl termini of YFP-N (yellow in Fig. 1C) and Cer-N (cyan in Fig. 1D). The γ subunit to be competed for (magenta in Fig. 1, C and D) is fused to the carboxyl terminus of CFP-C (dark blue in Fig. 1, C and D).

It is important to demonstrate that both of these configurations of BiFC $\beta\gamma$ complexes are functional in assay(s) relevant to the planned studies. We have demonstrated that YFP-N-

β_1 YFP-C- γ complexes potentiate α_s -mediated activation of adenylyl cyclase in COS-7 cells [12] and that CFP-C- β_5 Cer-N- γ complexes activate phospholipase C- β_2 expressed in HEK-293 cells [14]. Interestingly, co-expressed GFP- β_1 and untagged γ_2 , and co-expressed CFP- γ_2 and untagged β_1 , but not co-expressed GFP- β_1 and CFP- γ_2 , can potentiate activation of adenylyl cyclase [12]. This result suggests that although the $\beta\gamma$ structure can accommodate the equivalent of one fluorescent protein (produced by BiFC) at its amino terminus, two fluorescent proteins distort the structure enough to interfere with potentiation of α_s -mediated adenylyl cyclase activation. However, FRET has been reported between YFP- β_1 and CFP- γ_2 and YFP- β_1 /CFP- γ_2 , like untagged $\beta_1\gamma_2$, has been reported to increase basal N-type Ca^{2+} channel facilitation and attenuate noradrenaline-mediated inhibition of Ca^{2+} channels, as well as activate G-protein-gated inwardly rectifying K^+ (GIRK) channels [15]. Therefore, the functional consequences of fusing fluorescent proteins and their fragments to β and γ subunits may vary and should be tested for the effector of interest.

To apply multicolor BiFC to other proteins, it may be necessary to fuse the fluorescent protein fragments to regions other than the amino terminus. For instance, we have obtained BiFC with the β_2 -adrenergic receptor by fusing fluorescent protein fragments to the carboxyl terminus (data not shown). Moreover, sometimes, as with G protein α subunits, both the amino and carboxyl termini are important for function. We have identified internal regions of α_q [16] and α_s [17] in which insertion of full-length fluorescent proteins bordered by linkers consisting of Gly-Ser-Ser-Ser-Ser-Gly maintains full retention of function. Inserting fluorescent protein fragments at these sites also permits BiFC (data not shown).

2.2. Correlating complex formation with functionality

BiFC makes it possible to correlate $\beta\gamma$ function directly with the amount of $\beta\gamma$ complex formation in intact cells. For instance, we have compared the abilities of different CFP-C- β_5 Cer-N- γ complexes to activate phospholipase C- β_2 [14]. Phospholipase C- β_2 activity in cells expressing CFP-C- β_5 and different Cer-N- γ subunits was compared with the relative amounts of fluorescence produced by the CFP-C- β_5 Cer-N- γ complexes. Four of seven CFP-C- β_5 Cer-N- γ complexes (those containing Cer-N- γ_2 , Cer-N- γ_5 , Cer-N- γ_7 , or Cer-N- γ_{12}) activated co-expressed phospholipase C- β_2 , while the other 3 complexes (those containing Cer-N- γ_1 , Cer-N- γ_{10} , or Cer-N- γ_{11}) produced no activation above that seen in cells transfected with empty vector (Fig. 2A). No activity was obtained when CFP-C- β_5 was expressed without a Cer-N- γ subunit or when any of the Cer-N- γ subunits was expressed without CFP-C- β_5 (Fig. 2A). The three CFP-C- β_5 Cer-N- γ complexes that did not stimulate phospholipase C- β_2 exhibited only minimal fluorescence, indicating that lack of activity was due to the absence of complex formation (Fig. 2B). For the four CFP-C- β_5 Cer-N- γ complexes that activated phospholipase C- β_2 , the ratios of CFP-C- β_5 Cer-N- γ -stimulated activity (Fig. 2A) to amount of complex formation (Fig. 2B) were similar (Fig. 2C), indicating that the different efficacies of the CFP-C- β_5 Cer-N- γ combinations were due primarily to different amounts of complex formation. These results are in agreement with *in vitro* studies that found similar abilities of different $\beta\gamma$ complexes to modulate effectors [1–3]. In contrast, different $\beta\gamma$ complexes have been shown to exhibit a wider range of abilities to interact with G protein-coupled receptors [18–21].

2.3. Quantifying the amount of complex formation under non-competing conditions

Single color BiFC is used to quantify the formation of $\beta\gamma$ complexes in the absence of competition. For instance, to compare the abilities of different γ subunits to form complexes with the same β subunit, each Cer-N- γ subunit is co-expressed in limiting amounts with an excess of the CFP-C- β subunit. The amounts of plasmid that will produce a linear dose-response of fluorescence versus amount of transfected plasmid need to be determined empirically. For HEK-293 cells (1.6×10^6 per 60-mm dish) transiently transfected with 6 μl of Lipofectamine 2000 (Invitrogen) and analyzed 48 hours after transfection, 2.4 μg of CFP-C- β_1 plasmid co-

transfected with 0.0033 to 0.03 μg of Cer-N- γ plasmid yielded a linear relationship [13]. In the case of $\beta_5\gamma$ complexes, the fluorescence intensities of the complexes spanned a larger range and for the less intense ones, 0.1 to 0.3 μg of Cer-N- γ plasmid was necessary to produce detectable signals and yielded linear relationships between amounts of fluorescence and amounts of transfected plasmid (Fig. 3A) [14]. The relative amount of complex formation is calculated as the slope of fluorescence versus amount of transfected plasmid (Fig. 3A). It is important to keep the total amount of plasmid constant using empty vector to control for promoter competition. We have less experience with Cer-N- β subunits and CFP-C- γ subunits, but in a study using Cer-N- β_1 , Cer-N- β_5 , and CFP-C- γ_2 , we found that 2.4 μg of CFP-C- γ_2 co-expressed with 0.00033 to 0.03 μg of either Cer-N- β_1 or Cer-N- β_5 plasmid yielded a linear relationship of fluorescence to Cer-N- β dose [14].

2.3.1. Measurement of BiFC in cell populations—Fluorescence can be quantified in individual cells using fluorescence microscopy [5,13,22], but we have found that it is easier and less time-consuming to obtain quantitative results by measuring the fluorescence in cell populations using a spectrofluorometer. Two days after transient transfection of HEK-293 cells (1.6×10^6 per 60-mm dish) the cells are washed once in 4 mL of HBSS+ CaCl_2 media (20 mM Hepes, pH 7.2, 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 1 mM CaCl_2). 2 mL of HBSS+EDTA media (20 mM Hepes, pH 7.2, 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 0.5 mM EDTA) are then added to the dish, cells are scraped off the dishes with a rubber policeman, passed through a pipet several times to break up clumps, and suspended in a 1 cm square glass cuvette with a magnetic stir bar.

When measuring cells in suspension it is important to minimize the signal due to light scattering, and to subtract out the signal due to autofluorescence. The monochrometers used in most spectrofluorometers transmit enough light at wavelengths outside the band-pass region to generate a large background signal in highly scattering samples. This scattering signal can be eliminated through the use of band-pass and long-pass filters, which are generally much better than the monochromator at blocking light outside the band-pass region. Autofluorescence cannot be eliminated, but varies as a function of cell density, which can be determined with a light scattering measurement that is made at a wavelength outside that of the fluorescent signal emission.

We collect our data on a PC1 photon-counting spectrofluorometer (ISS, Champaign, IL) configured with motorized filter wheels on both the excitation path between the excitation monochromator and the sample, and on the emission path between the sample and the emission monochromator as described [13]. The slits on the excitation and emission monochromators are set to 16 nm. To eliminate the light scattering signal from the cell suspensions during measurements of CFP and YFP intensity, band-pass filters are used in the excitation filterwheel in combination with long-pass filters in the emission filterwheel. For the CFP channel measurement the excitation monochromator is set to 430 nm with a 430/25 band-pass filter, and the emission monochromator is set to 480 nm with a 455 long-pass filter. For YFP channel measurements the excitation is set to 492 nm with a 492/18 band-pass filter, and emission is set to 530 nm with a 515 long-pass filter. A dilute solution of glass milk, which scatters significantly, but is not fluorescent, should produce little if any signal in the CFP and YFP channels. For the light scattering channel measurement, the excitation monochromator is set to 650 nm with a 1.3 OD neutral density filter in combination with a 590 long-pass filter, and the emission monochromator is also set to 650 nm with no filter. All filters are from Chroma. Control of the monochrometers, motorized filterwheels and data acquisition is done using the Vinci software program (ISS) and allows the measurements of CFP, YFP and light scattering to be made in quick succession without repositioning the sample.

To subtract the signal due to autofluorescence, measurements using the CFP, YFP, and light scattering channels are made each day on a dilution series of cells transfected with vector alone. The linear relationships between the autofluorescence signals in the CFP and YFP channels versus the light scattering signal are calculated. CFP, YFP and light scattering measurements are then made for samples containing fluorescent proteins, and the scatter value is used to calculate the amount of autofluorescence to subtract from the CFP and YFP measurements.

2.3.2. Controlling for the expression levels of Cer-N-fusion proteins—

Immunoblots are used to quantify expression under the same transfection conditions as the fluorescence measurements are made. We have found that polyclonal antibodies to residues 3 to 17 of GFP (Anti-GFP, N-terminal; Sigma-Aldrich, St. Louis, MO) and to full-length GFP (Rockland Immunochemicals, Gilbertsville, PA) work well for detecting and comparing expression levels of Cer-N fusion proteins. Fig. 4A shows an immunoblot of Cer-N- γ subunits using the polyclonal antibody to residues 3 to 17 of GFP and Fig. 4B shows an immunoblot of Cer-N- β subunits using the polyclonal antibody to full-length GFP. The antigen-antibody complexes were detected using the ECL Western blotting protocol (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) (Fig. 4A) or with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) (Fig. 4B) and were imaged using a Lumi-Imager (Roche Applied Science, Indianapolis, IN) (Fig. 4A) or with a FluorChem SP Imaging System (Alpha Innotech, San Leandro, CA) (Fig. 4B). Bands are quantified using IPLab software (BD Biosciences) (Fig. 3B and Table 1). We have found that under the conditions in which BiFC signals vary linearly as a function of the amount of transfected plasmid (Fig. 3A), the expression levels as determined by immunoblots also vary linearly (Fig. 3B) [14]. The relative amount of expression is calculated as the slope of band intensity versus amount of transfected plasmid (Fig. 3B and Table 1). The relative amounts of complex formation are calculated as the ratio of BiFC intensity to expression level (Fig. 3C) [14].

2.4. Using multicolor BiFC to compare the abilities of subunits to compete for a common binding partner that is expressed in limiting amounts

To compare the abilities of different γ subunits to compete for a common β subunit, one of the γ subunits (red in Fig. 1A) is fused to the carboxyl terminus of YFP-N (yellow in Fig. 1A) and each of the γ subunits (green in Fig. 1B) to be compared is fused to the carboxyl terminus of Cer-N (cyan in Fig. 1B). The β subunit to be competed for (magenta in Fig. 1, A and B) is fused to the carboxyl terminus of CFP-C (dark blue in Fig. 1, A and B). We quantify competition as the loss of yellow fluorescence of the CFPC- β /YFP-N- γ complex when Cer-N- γ subunits are co-expressed (Fig. 3, D and E). Conversely, to compare the abilities of different β subunits to compete for a common γ subunit, one of the β subunits (red in Fig. 1C) is fused to the carboxyl terminus of YFP-N (yellow in Fig. 1C) and each of the β subunits to be compared (green in Fig. 1D) is fused to the carboxyl terminus of Cer-N (cyan in Fig. 1D). The γ subunit that is competed for (magenta in Fig. 1, C and D) is fused to the carboxyl terminus of CFP-C (dark blue in Fig. 1, C and D). Competition is quantified as the loss of yellow fluorescence of the CFP-C- γ /YFP-N- β complex when Cer-N- β subunits are co-expressed.

The relative preferences of the limiting subunit for the competing subunits are expressed as the IC_{50} for inhibition of yellow fluorescence produced by the CFP-C-subunit/ YFP-N-subunit complex. For example, for inhibition of association of YFP-N- γ_2 with CFP-C- β_5 by Cer-N- γ subunits, the IC_{50} is defined as μg of Cer-N- γ subunit plasmid that produces a 50% decrease in the intensity of CFP-C- β_5 YFP-N- γ_2 . To determine IC_{50} values, the data are fit to:

$$Y = (100)/(1 + X/a)^b \quad \text{Eq. (1)}$$

where X is μg of transfected Cer-N- γ plasmid, Y is the % of maximal fluorescence produced by CFP-C- β_5 YFP-N- γ_2 , a is the half-maximal inhibitory concentration (IC_{50}) of the Cer-N- γ subunit, and b is the slope factor. These IC_{50} values are normalized by multiplying by the relative expression levels of the Cer-N- γ subunits (Fig. 3E and Table I).

2.4.1. Optimization of amounts of transfected Cer-N-subunit, YFP-N-subunit, and CFP-C-subunit plasmids—In competition experiments, the subunit that is competed for is expressed in a limiting amount compared to the competing subunits. The optimum amounts of plasmids to be transfected are determined empirically by measuring competition between fusions of the same subunit to Cer-N and YFP-N. Loss of YFP fluorescence from CFP-C- β /YFP-N- γ or YFP-N- β /CFP-C- γ complexes is measured in the presence of a range of amounts of Cer-N- γ or Cer-N- β subunits, respectively. The initial fluorescence of the CFP-C- β /YFP-N- γ or YFP-N- β /CFP-C- γ complex needs to be high enough to provide a workable dynamic range of fluorescent intensities in the presence of the competing Cer-N- γ or Cer-N- β subunits. The β subunit fusions generally express at lower levels than do the γ subunit fusions, so the optimal conditions for competition of γ subunits for β subunits may be different from those for competition of β subunits for γ subunits. For example, to compare γ subunits competing for β_1 or β_5 , we transfected HEK-293 cells (1.6×10^6 per 60-mm dish) with 0.6 μg each of plasmids expressing CFP-C- β_1 or CFP-C- β_5 and YFP-N- γ_2 and 0, 0.01, 0.03, 0.09, 0.27, 0.81, or 2.43 μg of each Cer-N- γ subunit using 6 μl of Lipofectamine 2000 [13,14] (Fig. 3D). The total amount of plasmid was maintained at 3.63 μg by making up the difference with empty vector (pcDNA1/Amp). To compare β_1 and β_5 competing for γ_2 , we transfected HEK-293 cells with 0.3 μg of CFP-C- γ_2 plasmid, 0.6 μg of YFP-N- β_1 plasmid, and 0.033, 0.1, 0.3, 0.9, 2.7, or 8.1 μg of plasmids encoding either Cer-N- β_1 or Cer-N- β_5 . The total amount of plasmid was maintained at 9 μg using pcDNA1/Amp (see Fig. 8 of [14]). The expression levels of the competitors should be determined under the conditions of competition so that IC_{50} values can be normalized to the amounts of the expressed competitors (Fig. 3E and Table 1).

2.5. Determining the roles of interacting proteins in the subcellular targeting of complexes

Multicolor BiFC makes it possible to simultaneously image the subcellular localization patterns of two complexes that share a common subunit. In this way, the roles of alternative binding partners in regulating the localization patterns of complexes can be determined. These studies have demonstrated that the binding partners of both β and γ subunits play roles in subcellular targeting. For example, dual imaging of $\beta_5\gamma_2$ and $\beta_1\gamma_2$ in HEK-293 cells demonstrated a role for β subunits in targeting γ subunits (Fig. 5, A–C). CFP-C- γ_2 Cer-N- β_5 localizes to discrete cytoplasmic structures (Fig. 5, A and C) that exhibit co-localization with markers for the ER and Golgi apparatus [14], while CFP-C- γ_2 YFP-N- β_1 associates with the plasma membrane (Fig. 5, B and C), confirming previous studies using single color BiFC [12]. Conversely, dual imaging of $\beta_5\gamma_2$ and β_5 complexed with RGS7, a member of the R7 family of regulators of G protein signaling (RGS) proteins, revealed that β_5 localization can be determined by its binding partner. In contrast to the localization of CFP-C- β_5 YFP-N- γ_2 on discrete cytoplasmic membranes (Fig. 5, D, F, G, and I), CFP-C- β_5 Cer-N-RGS7 produces a diffuse cytoplasmic signal (Fig. 5, E and F), and CFP-C- β_5 Cer-N-RGS7-t, containing a truncated form of RGS7 lacking the DEP domain, localizes preferentially in the nucleus (Fig. 5, H and I). The roles of co-expressed proteins in regulating the targeting of these complexes can also be determined. For instance, we observed that Cer-C- β_5 Cer-N- γ_2 and Cer-C- β_5 Cer-N-RGS7 were targeted preferentially to the plasma membrane by wild-type α_o and a constitutively activated α_o mutant, respectively [14].

2.6. Studying the formation of protein complexes that are unstable *in vitro*

The G protein β_5 subunit differs from other β subunits in having divergent sequence and subcellular localization patterns. Although $\beta_5\gamma_2$ can modulate effector proteins [3,23–27] (Fig.

2), β_5 associates with R7 family regulators of G protein signaling (RGS) proteins when purified from tissues [28]. The fact that $\beta_5\gamma_2$ dimers are unstable under non-denaturing buffer conditions [9,29] may explain why they have yet to be isolated. Using multicolor BiFC, we found that Cer-N- γ_2 competed three times more effectively than did Cer-N-RGS7 for CFP-C- β_5 [14] (Fig. 6A), illustrating the advantage of using BiFC in intact cells to identify protein interaction partners.

2.7. Determining the roles of co-expressed proteins in regulating the association preferences of interacting proteins

Using multicolor BiFC, the potential roles of co-expressed proteins in determining the association preferences of alternative binding partners can be investigated. R7 family binding protein (R7BP), which, like β_5 and RGS7, is highly expressed in the nervous system, can target β_5 RGS7 complexes to the plasma membrane [30]. We found that in the presence of co-expressed R7BP, the preference of CFP-C- β_5 for Cer-N- γ_2 over Cer-N-RGS7 was lost. Cer-N- γ_2 and Cer-N-RGS7 now competed similarly with YFP-N- γ_2 for CFP-C- β_5 [14] (Fig. 6B). The formation of β_5 complexes will also be influenced by the association preferences of its alternative binding partners. For instance, γ_2 can also form complexes with other β subunits. Using multicolor BiFC, we found that Cer-N- β_1 competed four times more effectively than did Cer-N- β_5 for CFP-C- γ_2 [14] (Fig. 6B). This preferential interaction of γ_2 with β_1 rather than β_5 could decrease formation of $\beta_5\gamma_2$ complexes by sequestering some of the available γ_2 away from β_5 .

2.8. Visualizing dynamic events affecting multiple protein complexes

Previously, we visualized the β_2 -adrenergic receptor (β_2 AR)-stimulated trafficking patterns of α_s and $\beta_1\gamma_7$ in HEK-293 cells and found that both G_s subunits internalized and co-localized on vesicles upon stimulation of the β_2 AR [17]. α_s and $\beta_1\gamma_7$ internalized more rapidly than did the β_2 AR and, in contrast to the β_2 AR, their internalization was insensitive to a dominant negative mutant of dynamin 1 [17]. Multicolor BiFC makes it possible to monitor the β_2 AR-stimulated internalization of pairs of $\beta\gamma$ complexes simultaneously. In this way, differences in the kinetics and localization patterns of $\beta\gamma$ complexes can be visualized and quantified. For example, we have found that $\beta_1\gamma_{11}$ internalizes more rapidly than does $\beta_1\gamma_7$ and appears to localize on different subcellular structures (Fig. 7). Further experiments will reveal if these differences result from the different prenyl groups on the γ subunits (geranylgeranyl on γ_7 , farnesyl on γ_{11}) [31].

3. CONCLUDING REMARKS

The formation of $\beta\gamma$ complexes *in vivo* is likely to reflect the affinities of β and γ subunits for each other as well as their accessibilities to each other and their associations with other co-expressed proteins. By estimating the relative association preferences of potential binding partners in the context of a living cell, multicolor BiFC takes all of these factors into account. This approach quantifies the interaction preferences of β and γ subunits expressed heterologously in intact cells. In the particular cell of interest, it is important to determine which β and γ subunits are expressed endogenously at the protein level and to quantify these expression levels. Combining this information with the relative association preferences determined with multicolor BiFC should provide a reasonable estimate of the predominance of specific endogenous $\beta\gamma$ complexes. In addition, a recently described proximity ligation *in situ* assay that enables visualization of endogenous protein complexes in fixed samples [32] may provide a complementary means of corroborating the results of multicolor BiFC.

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FIG. 1.

Models of fluorescent $\beta\gamma$ complexes produced with multicolor BiFC. The split fluorescent protein at the top of each model is joined by linkers (orange) to the $\beta\gamma$ dimer at the bottom. The CFP-C fragment (dark blue) is combined with either the YFP-N fragment (yellow) to produce yellow fluorescence or the Cer-N fragment (cyan) to produce cyan fluorescence. (A and B) YFP-N- γ and Cer-N- γ compete for CFP-C- β . β is magenta and γ is red (A) or green (B). (C and D) YFP-N- β and Cer-N- β compete for CFP-C- γ . γ is magenta and β is red (C) or green (D). The structures of the fluorescent protein fragments are adapted from the structure of GFP [33]. The structures of β and γ are from the structure of an α_t/α_{i1} chimera complexed with $\beta_t\gamma_t$ [34].

FIG. 2.

Comparisons of the abilities of different β₅ and γ combinations to activate phospholipase C-β₂ and to form complexes. (A) Activation of phospholipase C-β₂ in cells expressing CFP-C-β₅ and Cer-N-γ subunits. HEK-293 cells were transfected with 3 μg of phospholipase C-β₂ plasmid and where indicated, 2.4 μg of CFP-C-β₅ plasmid, and 0.3 μg of Cer-N-γ plasmids. The total amount of plasmid in each transfection was maintained at 5.7 μg by making up the difference with pcDNAI/Amp. Inositol phosphate (IP) formation in cells labeled with [³H] inositol was measured as described [35]. Values represent the means ± S.E. from 6 experiments performed in triplicate. (B) Fluorescence intensities of CFP-C-β₅Cer-N-γ complexes expressed under the same transfection conditions as in (A). Values represent the means ± S.E. from 3

experiments performed in duplicate. (C) Normalization of phospholipase C- β 2 activation by coexpressed CFP-C- β 5 and Cer-N- γ subunits to the fluorescence intensities of the CFP-C- β 5Cer-N- γ complexes. IP levels obtained in cells transfected with pcDNAI/Amp (background) were subtracted from the levels in cells expressing CFP-C- β 5 and either Cer-N- γ 2, Cer-N- γ 5, Cer-N- γ 7, or Cer-N- γ 12. These background-subtracted activities were divided by the corresponding CFP-C- β 5Cer-N- γ intensities. Reproduced with permission from [14].

FIG. 3.

β_5 interacts preferentially with γ_2 rather than γ_1 , γ_5 , γ_7 , γ_{10} , γ_{11} , or γ_{12} . (A) Fluorescence intensities of CFP-C- β_5 Cer-N- γ complexes when CFP-C- β_5 is not limiting. HEK-293 cells were transfected with 2.4 μ g of plasmid expressing CFP-C- β_5 and the indicated μ g of Cer-N- γ plasmids. The total amount of plasmid in each transfection was maintained at 2.7 μ g by making up the difference with pcDNA1/Amp. Values represent the means $\pm$ S.E. from 3 experiments performed in duplicate. CFP-C- β_5 Cer-N- γ intensities/ μ g Cer-N- γ plasmid, determined from linear fits to the data were as follows (means $\pm$ S.E., $\times 10^{-4}$): $\beta_5\gamma_1$, 3.93 ± 0.27 ; $\beta_5\gamma_2$, 408 ± 44.8 ; $\beta_5\gamma_5$, 21.8 ± 2.36 ; $\beta_5\gamma_7$, 106 ± 4.34 ; $\beta_5\gamma_{10}$, 6.72 ± 0.94 ; $\beta_5\gamma_{11}$, 11.3 ± 1.40 ; $\beta_5\gamma_{12}$, 22.0 ± 0.93 . (B) Expression levels of Cer-N- γ subunits when co-expressed with an excess of CFP-C- β_5 . HEK-293 cells were transfected as in (A). Values represent the means $\pm$ S.E. from 3 experiments. Cer-N- γ expression/ μ g plasmid, determined from linear fits to the data were as follows (means $\pm$ S.E., $\times 10^{-6}$): γ_1 , 1.82 ± 0.29 ; γ_2 , 16.87 ± 1.50 ; γ_5 , 4.77 ± 1.00 ; γ_7 , 10.51 ± 2.13 ; γ_{10} , 2.78 ± 0.05 ; γ_{11} , 8.48 ± 0.28 ; γ_{12} , 5.54 ± 0.59 . (C) Normalization of CFP-C- β_5 Cer-N- γ intensities to Cer-N- γ expression levels. The intensity/ μ g Cer-N- γ plasmid of each CFP-C- β_5 Cer-N- γ complex was divided by the corresponding Cer-N- γ expression/ μ g plasmid. (D) Competition between Cer-N- γ subunits and YFP-N- γ_2 for limiting amounts of CFP-C- β_5 . The intensity of CFP-C- β_5 YFP-N- γ_2 was measured in the presence of each Cer-N- γ subunit or empty vector. HEK-293 cells were transfected with 0.6 μ g each of plasmids expressing CFP-

C- β_5 and YFP-N- γ_2 , and the indicated μg of each Cer-N- γ plasmid. The total amount of plasmid in each transfection was maintained at 3.63 μg using pcDNAI/Amp. Values represent the means $\pm$ S.E. from 3 experiments performed in duplicate. (E) CFP-C- β_5 YFP-N- γ_2 intensity is expressed as a function of the relative amounts of co-expressed Cer-N- γ . Expression levels were determined in HEK-293 cells transfected with 0.6 μg each of plasmids expressing CFP-C- β_5 and pcDNAI/Amp, and 0.03, 0.09, 0.27, or 2.43 μg of each Cer-N- γ plasmid. The total amount of plasmid in each transfection was maintained at 3.63 μg using pcDNAI/Amp. The expression levels of the Cer-N- γ subunits varied linearly and the data were fit by linear regressions. The plasmid amounts used in (D) were multiplied by Cer-N- γ expression/ μg plasmid to yield the normalized amount of each Cer-N- γ subunit. CC indicates CFP-C and YN indicates YFP-N. Reproduced with permission from [14].

FIG. 4.

Expression levels of Cer-N-subunits are controlled for using immunoblots. (A) Expression levels of Cer-N- γ subunits co-expressed with CFP-C- β_5 . HEK-293 cells were transfected as in Figs. 3A and 3B. μg indicate the amount of transfected Cer-N- γ plasmid or vector. The vector control lane was taken from a gel run, transferred, and immunoblotted in parallel with the one containing CFP-C- β_5 and Cer-N- γ subunits. (B) Expression levels of Cer-N- β_1 and Cer-N- β_5 co-expressed with CFP-C- γ_2 . HEK-293 cells were transfected with 2.4 μg of CFP-C- γ_2 plasmid and the indicated amounts (in μg) of plasmid expressing Cer-N- β_1 or Cer-N- β_5 . The total amount of plasmid in each transfection was maintained at 2.43 μg by making up the difference with pcDNAI/Amp. For each lane, 15 μg of total cell lysates were resolved on NuPage Bis-Tris 4–12% gels (Invitrogen), transferred to nitrocellulose, and processed as described in the text.

FIG. 5.

Simultaneous imaging of two complexes sharing a common subunit demonstrates the roles of alternative binding partners in subcellular localization. CFP (A) and YFP (B) images from the same cell expressing Cer-N- β_5 CFP-C γ_2 and YFP-N- β_1 CFP-C- γ_2 . In the merge image (C), Cer-N- β_5 CFP-C- γ_2 is green and YFP-N- β_1 CFP-C- γ_2 is red. HEK-293 cells were transfected with the following amounts (in μ g) of plasmids: CFP-C- γ_2 , 0.4; Cer-N- β_5 , 0.1; YFP-N- β_1 , 0.2; and mCherry-Mem, 0.0025 (a plasma membrane marker [14]). YFP (D) and CFP (E) images from the same cell expressing CFP-C- β_5 YFP-N- γ_2 and CFP-C- β_5 Cer-N-RGS7. In the merge image (F), CFP-C- β_5 YFP-N- γ_2 is green and CFP-C- β_5 Cer-N-RGS7 is red. HEK-293 cells were transfected with the following amounts (in μ g) of plasmids: CFP-C- β_5 , 0.3; YFP-N- γ_2 and Cer-N-RGS7, 0.15; and mCherry-Mem, 0.0025. (G) YFP and (H) CFP images from the same cell expressing CFP-C- β_5 YFP-N- γ_2 and CFP-C- β_5 CFP-N-RGS7-t, which contains a truncated form of RGS7 lacking the DEP domain. HEK-293 cells were transfected with the following amounts (in μ g) of plasmids: CFP-C- β_5 , 0.3; YFP-N- γ_2 and CFP-N-RGS7-t, 0.15; and mCherry-Mem, 0.0025. In the merge image (I), CFP-C- β_5 YFP-N- γ_2 is green and CFP-C- β_5 Cer-N-RGS7-t is red. Scale bar = 10 μ m. Figures (D), (E), (G), and (H) are reproduced with permission from [14].

FIG. 6.

Multicolor BiFC demonstrates that multiple factors influence formation of β_5 complexes. (A) In HEK-293 cells co-expressing CFP-C- β_5 , YFP-N- γ_2 , and either Cer-N- γ_2 or Cer-N-RGS7, Cer-N- γ_2 competes 3-fold more effectively for CFP-C- β_5 than does Cer-N-RGS7. (B) Co-expression of R7BP influences competition between γ_2 and RGS7 for β_5 . In the presence of R7BP, Cer-N-RGS7 and Cer-N- γ_2 compete with similar efficacies for CFP-C- β_5 . In addition, other β subunits may affect the relative amounts of β_5 complexes. Cer-N- β_1 competes 4-fold more effectively for CFP-C- γ_2 than does Cer-N- β_5 . Data for these results are shown in [14].

FIG. 7.

The stimulus-induced localization pattern of $\beta_1\gamma_{11}$ differs from that of $\beta_1\gamma_7$. YFP (top), CFP (middle), and merge (bottom) images from the same cell expressing CFP-C- β_1 YFP-N- γ_7 and CFP-C- β_1 Cer-N- γ_{11} acquired at the indicated times before and after stimulation with 10 μ M isoproterenol. In the merge image, colocalization of CFP-C- β_1 YFP-N- γ_7 (red) and CFP-C- β_1 Cer-N- γ_{11} (green) is indicated in yellow. HEK-293 cells were transfected with the following amounts (in μ g) of plasmids: α_s , 0.3; CFP-C- β_1 , 0.3; YFP-N- γ_7 , 0.1125; Cer-N- γ_{11} , 0.0375; β_2 AR, 0.1; and mCherry-Mem, 0.0025. More YFP-N- γ_7 than Cer-N- γ_{11} plasmid was used to normalize the fluorescence intensities of the two $\beta\gamma$ complexes. The same result was obtained in cells expressing CFP-C- β_1 YFPN- γ_{11} and CFP-C- β_1 Cer-N- γ_7 .

TABLE 1

Competition of Cer-N- γ subunits with YN- γ_2 for dimerization with CC- β_5 in live HEK-293 cells Values represent the mean $\pm$ S.E. from 3 experiments.

γ subunit	IC ₅₀ (μ g CerN- γ) ^a	Cer-N- γ expression/ μ g ^b (X 10 ⁻⁵)	Normalized IC ₅₀ ^c (X 10 ⁻⁵)
γ_1	3.10 $\pm$ 1.08	1.38 $\pm$ 0.23	4.27 $\pm$ 1.65
γ_2	0.15 $\pm$ 0.02	4.44 $\pm$ 0.77	0.66 $\pm$ 0.14
γ_5	2.15 $\pm$ 0.59	4.39 $\pm$ 0.17	9.43 $\pm$ 2.64
γ_7	0.40 $\pm$ 0.06	6.78 $\pm$ 0.58	2.68 $\pm$ 0.49
γ_{10}	2.66 $\pm$ 0.48	3.27 $\pm$ 0.07	8.71 $\pm$ 1.57
γ_{11}	1.94 $\pm$ 0.54	7.88 $\pm$ 0.58	15.27 $\pm$ 4.41
γ_{12}	1.60 $\pm$ 0.31	5.62 $\pm$ 0.46	9.01 $\pm$ 1.88

^aDefined as μ g of Cer-N- γ plasmid that produced a 50% decrease in the intensity of CC- β_5 YN- γ_2 , calculated from the data in Fig. 3D.

^bCalculated from linear fits of Cer-N- γ expression/ μ g plasmid. Expression levels were determined as described in the legend to Fig. 3E.

^cDefined as IC₅₀ X Cer-N- γ expression/ μ g plasmid.

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