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Rapid Mix Flow Cytometry Measurements of Subsecond Regulation of G Protein-Coupled Receptor Ternary Complex Dynamics by Guanine Nucleotides¹

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Abstract

We have used rapid mix flow cytometry to analyze the early subsecond dynamics of the disassembly of ternary complexes of G-protein coupled receptors (GPCRs) immobilized on beads to examine individual steps associated with guanine nucleotide activation. Our earlier studies suggested that the slow dissociation of G α and G $\beta\gamma$ subunits was unlikely to be an essential component of cell activation. However, these studies did not have adequate time resolution to define precisely the disassembly kinetics. Ternary complexes were assembled using three formyl peptide receptor constructs (wild type, FPR-G α_{i2} fusion, and FPR-GFP fusion) and two isotypes of the α subunit (α_{i2} and α_{i3}) and $\beta\gamma$ dimer ($\beta_1\gamma_2$ and $\beta_4\gamma_2$). At saturating nucleotide levels, the disassembly of a significant fraction of ternary complexes occurred on a subsecond time frame for α_{i2} complexes and $\tau_{1/2} \leq 4$ sec for α_{i3} complexes, time scales which are compatible with cell activation. $\beta_1\gamma_2$ isotype complexes were generally more stable than $\beta_4\gamma_2$ associated complexes. The comparison of the three constructs however proved that the fast step was associated with the separation of receptor and G protein and that the dissociation of the ligand or of the α and $\beta\gamma$ subunits was slower. These results are compatible with a cell activation model involving G protein conformational changes rather than disassembly of G $\alpha\beta\gamma$ heterotrimer.

Keywords

G protein-coupled receptor; guanine nucleotide; rapid mix flow cytometer; dissociation kinetics; detergent solubilized receptors

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Introduction

G protein-coupled receptors (GPCRs) represent the largest family of transmembrane signaling molecules in the human genome [1,2]. GPCRs are activated by interaction with specific ligands (hormones, neurotransmitters, growth factors, glycoproteins, cytokines, odorants and photons). These stimuli elicit conformational changes in the receptor, leading to binding and activation of intracellular G protein heterotrimers. G proteins are comprised of 27α , 5β , and 13γ subunit proteins [2]. The specific combination of α , β and γ subunits dictates not only receptor specificity, but also downstream effector specificity, providing the means of targeting specific physiological processes in response to specific external stimuli [1].

Currently, the consensus model of G protein activation involves the exchange of GDP for GTP catalyzed by ligand-activated GPCR results in the dissociation of the G protein heterotrimer into separate α and $\beta\gamma$ subunits which then interact independently with their cognate effectors [3,4]. However, more recent experimental evidence has led to questions about the absolute necessity of including subunit dissociation as an element of activation. For example, Klein, Levitzki and coworkers [5,6] used a $G\alpha$ subunit fused to its cognate $G\beta\gamma$ subunit, to show that both the fusion of subunits and therefore absence of dissociation of subunits did not impair signaling function. Other evidence has come from FRET assays using fluorescently tagged $G\alpha$ and $G\beta\gamma$ subunits (*vide infra*) [7–9].

The formyl peptide receptor (FPR) is a prototypical GPCR that binds to *N*-formyl methionine-containing peptides resulting from bacterial and mitochondrial protein synthesis, as well as other hydrophobic peptides [10]. The prototypical ligand, *N*-formyl-Met-Leu-Phe is capable of initiating leukocyte inflammatory responses such as adhesion, chemotaxis, superoxide generation and degranulation [11]. The receptor, cloned and overexpressed in tissue culture cells, has been solubilized and assembled with a formyl peptide ligand and G proteins to form a high agonist-affinity ternary complex, both in solution [12], or on beads, where it has been investigated by flow cytometry [13]. The detergent solubilized FPRs preserve native ligand binding affinity, and exhibit evidence of appropriate G protein assembly that discriminates full and partial agonists [14].

Bead-based studies have compared ternary complexes assembled with chimeric FPR fusion proteins [13]. The results, interpreted according to a ternary complex model [15], were consistent with the idea that the dissociation of the α and $\beta\gamma$ subunits in the presence of GTP γ S was slow compared to the typical kinetics of intracellular signals such as intracellular Ca^{2+} elevation and therefore could not likely account, kinetically, for cell activation. While fluorescent formyl peptides and FPR-GFP made it possible to study ternary complex dynamics in real-time, sample mixing and delivery times in flow cytometry limited kinetic resolution [16]. To minimize the dead time, online mixing schemes have been developed to achieve subsecond resolution [17]. However, for ternary complex reagents available only in limited quantities, we have recently described a small volume rapid mixing device [18] to measure subsecond disassembly kinetics in the presence of saturating concentrations of guanine nucleotides.

In earlier studies, FPR ternary complexes reconstituted in detergent were analyzed in terms of the sensitivity of ligand dissociation kinetics to the presence of guanine nucleotides [12,13]. For clarity we will summarize the results of the two principal studies here. The first study [12] involved the measurement of the dissociation rate of the fMLFK-FITC from detergent solubilized FPR in the presence and absence of $G\alpha\beta\gamma$ heterotrimeric units. The results of that study showed that the dissociation kinetics of a fluorescent ligand from a G protein coupled FPR ($\tau_{1/2} \leq 100\text{sec}$) were much slower than the uncoupled FPR ($\tau_{1/2} \approx 20\text{sec}$). A follow up study involved the assembly of ternary complexes of detergent solubilized GPCRs on beads

designed to examine individual steps associated with GTP-mediated disassembly of ternary complexes [13]. These studies did not have adequate time resolution to define precisely the disassembly kinetics.

In the current study, ternary complexes were assembled using three receptor constructs (wild type, FPR-G α_{i2} fusion, and FPR-GFP fusion) and two isoforms of the α subunit (α_{i2} and α_{i3}) and $\beta\gamma$ dimer ($\beta_1\gamma_2$ and $\beta_4\gamma_2$). At saturating nucleotide levels, the disassembly of ternary complexes was biphasic, with a subsecond component that is compatible with cell activation. The comparison of the three constructs suggested that the fast step was associated with the separation of receptor and G protein and that the dissociation of the ligand or of the α and $\beta\gamma$ subunits was slower. GTP γ S induced separation of the receptor and G protein as did GDP (at half the rate), making it unlikely that RG separation is sufficient for activation. These results are compatible with a cell activation model regulated by the G protein conformational changes rather than disassembly of the G $\alpha\beta\gamma$ heterotrimer [7,9,19].

Materials and Methods

Materials

Plasticware was from VWR (West Chester, PA), and all chemicals and reagents were from Sigma (St. Louis, MO) except where otherwise noted. 6.7 μ m diameter streptavidin coated polystyrene beads (0.5% w/v) were purchased from Spherotech Inc., (Libertyville, IL). α_{i2} and α_{i3} G-protein subunits were purchased from Calbiochem (La Jolla, CA) and were used without further purification. FLAG-his tagged $\beta\gamma$ subunits ($\beta_1\gamma_2$ or $\beta_4\gamma_2$) were prepared and purified as described previously [13,20]. The subunits were stored at -80°C in 2–5 μ L volume aliquots depending on stock concentration (3–7 μ M). Membrane fractions from U937 cells expressing FPR (R), FPR-GFP (R^F), or FPR-G α_{i2} fusion protein (R-G α_{i2}) were prepared as described elsewhere [13] and used without further purification. The following buffers were used: HPSM (pH7.5, 30 mM HEPES, 100 mM KCl, 20 mM NaCl, 1 mM MgCl₂), DHPSM (HPSM with 0.1% n-dodecyl- β -D-maltoside, DOM), G-buffer (HPSM with 0.1% DOM and 1 mM dithiothreitol, DTT), and AMF solution (20 μ M AlCl₃, 10 mM MgCl₂ and 10 mM NaF).

Generation of FPR-G α_{i2} Fusion Constructs

FPR-G α_{i2} Fusion constructs were synthesized as previously described [21]. Appropriate clones were confirmed by dideoxy sequence analysis. The final fusion protein contained three alanine residues between the last amino acid of the FPR and the first amino acid of the G protein open reading frame [21].

Solubilization of FPR

The cloning of the FPR, its expression in U937 cells, generation of FPR-G α_{i2} and FPR-GFP fusion constructs and the membrane preparations have been described [13].

Fluorescent Labeling of α_{i3} Subunits

Proteins were labeled with the desired probe as described previously [22]. The samples were purified and concentrated by ultra filtration from phosphate buffered saline using YM-30 Microcon centrifugal filter devices (Millipore Corp. Bedford, MA). The fluorophore to protein (f/p) ratios were determined following standard procedures from the manufacturers. The f/p ratios were $\approx$ 2:1 for both Texas Red and fluorescein labeled proteins (hereafter G α_{i3} ^{fl}).

AMF Mediated Dissociation of G α •GDP from Heterotrimer on Beads

$\beta_1\gamma_2$ beads were incubated with 1 μ M G α_{i3} ^{fl} fl in G-buffer at 8°C for 1.5 hours with gentle mixing. The beads were then washed three times in G-buffer and were suspended to a final

concentration of 20,000 beads/ μ L. Subsequently, 40,000 beads were added to a 200 μ L aliquot of AMF in a 12 $\times$ 75mm Falcon tube (Becton Dickinson Labware, Lincoln Park, NJ).

Fluorescence intensity readings, that represented the dissociation of $G\alpha_{i3}^{\text{fl}}\cdot\text{GDP}\cdot\text{AlF}_4^-$ from the heterotrimer, were periodically recorded with the flow cytometer over a 20-hour time span at room temperature.

Preparation of G protein Coated Beads ($\beta\gamma$ Beads and $G\alpha\beta\gamma$ Beads) and LRG Assemblies

G protein $\beta\gamma$ dimers were prepared and purified as previously described [20,23]. Beads coated with ≈ 4 million M2 antiFLAG antibodies were prepared as previously described [22]. The M2 beads were resuspended in PBS buffer and stored in 25 μ L aliquots (40,000 beads/ μ L) at 4°C. A typical preparation of G protein coated beads, involved the incubation of a nominally equimolar amount of M2 beads (e.g. 15 pmol FLAG binding sites) and FLAG tagged $\beta\gamma$ subunits ($\beta_{1\gamma 2}$ or $\beta_{4\gamma 2}$) in 25 μ L of G-buffer for an hour at 4°C under mild vortex. The beads were then centrifuged and resuspended in G buffer at 40,000 beads/ μ L and stored at -80°C before use. The surface density of $\beta\gamma$ subunits on the beads was determined as previously described [24]. The functional activity of M2 antibodies drops over time, therefore over the course of more than 6 months, we noticed a reduction in the surface density of $\beta\gamma$ subunits of 25% [25]. The drop in activity of the M2 did not affect the integrity of the ternary complex assembly because it serves as a simple tether of the $\beta\gamma$ subunits to the beads. The change in surface coverage only affects the initial amplitude of the signal associated with the ternary complexes at disassembly. To assemble the heterotrimer, the $G\beta\gamma$ bearing-beads were incubated with an approximately equimolar amount of $G\alpha_i$ subunits at 4°C for an hour. The mixture was centrifuged, and resuspended in 25 μ L of G buffer to give the final bead density of 40,000beads/ μ L. The assembly of ligand and receptor (LR) complexes on beads displaying $G\alpha\beta\gamma$ was performed as previously described [13].

Kinetic Measurement of LRG Disassembly

Kinetic measurements of the disassembly of LRG complexes were carried out by mixing LRG beads as described above and 10^{-4} M guanine nucleotide in a small volume rapid mix device coupled to a flow cytometer. The flow cytometric analysis used a Becton-Dickinson FACScan flow cytometer (Sunnyvale, CA) interfaced to a Power PC Macintosh running the CellQuest software package. Instrumental details have been described elsewhere [18]. A 105 μ L aliquot of LRG beads in DHPSM (600 beads/ μ L) was loaded into one of the sample syringes of a small volume rapid mix device. An equal volume of DHPSM buffer or 0.2 mM guanine nucleotide was loaded into the other sample syringe. An experiment consisted of three consecutive runs, each used 35 μ L of the 105 μ L stock aliquot of sample in both syringes, and followed by the typical automated mixing sequence as described elsewhere [18]. Thus a single data set consisted of an average of three rapid mix runs. Rapid mix experiments were repeated several times for each LRG complex. The number of repetitions tended to be governed by the quality of data over different preparations of LRG beads. Manual-mix experiments using 50 μ L aliquots were also performed for comparison.

The time course data were converted to ASCII format using the FCSQuery program (Bruce Edwards, UNM HSC). Data were then analyzed and graphed using Prism software (GraphPad Software, San Diego, CA). To determine the dissociation characteristics, the fluorescence over time in blocked control samples was subtracted point by point from the fluorescence over time of ligand binding samples before normalization as described previously [13].

Results

Overview

The goal of the present work was to examine rapid steps associated with the disassembly of G protein and FPR ternary complexes on beads by flow cytometry so that the kinetics could be compared with the kinetics of cell activation. The disassembly of the LRG complex following guanine nucleotide activation can in principle occur at three junction points: *i.e.*, through the separation of the α subunit from $\beta\gamma$, through the separation of LR from $G\alpha$, or through the separation of L from RG. The identity of the break point can be delineated by kinetics (*cf.* Figure 2–Figure 5).

The Assembly of Heterotrimeric $G\alpha\beta\gamma$ and Ternary LRG on Beads

We used either $\beta_1\gamma_2$ or $\beta_4\gamma_2$ subunits tagged with a FLAG epitope to initiate the molecular assembly on beads. Trimeric $\alpha\beta\gamma$ complexes were formed by the addition of α_{i2} or α_{i3} subunits. Figure 1A shows the binding of fluorescein-labeled α_{i3} subunits to $\beta\gamma$ subunits on beads ($K_D \approx 32\text{nM}$). This value is within the range of affinity constants for the interaction between α and $\beta\gamma$ previously reported to vary from subnanomolar to tens of nM depending on experimental conditions and type of subunit [26, 27].

Figure 1B shows the comparative influence of α_{i2} and α_{i3} subunits with respect to the equilibrium binding of the ligand activated FPR. Because we are unable to produce sufficiently high concentrations of solubilized receptors (μM range) from our membrane preparations, it is not clear from Figure 1B how the subunit isotypes differ, in terms of the number of bound receptors at saturation the receptor-G protein affinity. However, the limited data set shown in Figure 1B suggests that the α_{i3} subunit favors a higher degree of ternary complex formation on *G beads*, than α_{i2} under similar experimental conditions. This is consistent with our previous finding in solution measurements [12]. The quantification of the ternary complexes on *G beads* was carried out using standard calibration beads (Quantum FITC MESF beads, www.Bangslabs.com).

Guanine Nucleotide Activation of the Heterotrimeric $G\alpha\beta\gamma$ Complex

We examined the stability of *G bead* heterotrimeric complexes (from Figure 1) in the presence of 0.1 mM GTP γ S, excess soluble α_{i3} subunits, and AlF_4^- . Figure 2A shows the effects of adding 0.1 mM GTP γ S to *G beads* bearing fluorescein labeled α_{i3}^{fl} subunits. The results show no appreciable loss of intensity within the time frame of the 40 second experiment. A reasonable explanation for the result is that the nucleotide binding pocket of the fluorescein labeled α_{i3}^{fl} subunit was already occupied by a tightly bound GDP ($K_d \approx 10^{-11}\text{ M}$) [28], and could not be displaced by GTP γ S without the catalytic assistance of a ligand activated receptor. AlF_4^- can emulate the γ -phosphate of GTP in the nucleotide-binding pocket [29,30] and can activate the GDP-bound G protein in purified or native systems [31,32].

We therefore incubated a sample of *G beads* with 20 μM AlF_4^- and analyzed the sample over a 20 hour period. The results are shown in curve *a* in Figure 2B. The data show a fast ($\approx 35\%$) drop in intensity during the first minute ($\tau_{1/2} < 30\text{seconds}$). This is followed by a slow phase decay ($\tau_{1/2} \approx 100\text{min}$). The loss in intensity is consistent with the release of $G\alpha_{i3}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (curve *a* in Figure 2B). Interestingly the fast component decay is comparable to the result obtained by Sarvazyan *et al* [26], while the slow dissociation was comparable to the measurements of Zelent *et al* [27]. Our data appear to suggest two distinct populations of G proteins that display different levels of reactivity. At present we don't have an explanation for this behavior except to point out that the biphasic form of dissociation is reproduced in the disassembly of LRG (Figure 2C *vide infra*). Curve *b* in Figure 2B shows the same biphasic behavior of the “self-exchange” reaction between the fluorescein labeled α_{i3} and excess

unlabelled α_{i3} . Here the rate of exchange between the soluble and bound subunits is slower ($\tau_{1/2} \approx 13\text{min}$) compared to the fast decay component of fluoroaluminate-mediated dissociation of α_{i3} . Figure 2C shows the dissociation of FPR-GFP from *G-beads* after the manual addition of GTP γ S. The data shown in Figure 2C is clearly missing important early kinetic data, and therefore gives a clear rationale for the need of rapid mix cytometry. It is important to note that in the presence of LR, the nucleotide-binding pocket is empty. The LRG complex on beads is only stable when the pocket is empty and rapidly disassembles when either GDP or GTP are added. Thus the ternary complex formed in live cells survives for the short duration of the dissociative interchange between GDP and GTP [3, 4].

Rapid Mix Measurement of Guanine Nucleotide-Induced Disassembly of Ternary Complexes

In these experiments we used either GTP or GDP to activate LRG complexes on *G beads*. We also examined the comparative effects of subunit isotypes (α_{i2} vs. α_{i3} and $\beta_{1\gamma 2}$ vs. $\beta_{1\gamma 4}$) on the kinetics of disassembly of ternary complexes. The results are summarized in Table 1. An individual data set is comprised of an average of a minimum of three runs of the rapid mix flow cytometer. In some cases unique data sets were collected on the same day using the same stock of LRG beads, all the data sets were averaged before kinetic analysis (e.g. rows 1 and 2 are comprised of and average of 6 runs). The kinetic data shown with standard deviations represent an average of at least 3 unique kinetic analysis data sets collected over different days using unique LRG bead preparations. Representative experimental data are presented below. Figure 3 shows the dissociation kinetics of the ligand, fMLFK-FITC (L^F) from an intact complex $L^F R-\alpha_{i2}\beta_{1\gamma 2}$. The potential break points for this complex as shown by arrows in Figure 3B involve L^F and α_i subunit. However, the experimental results shown in Figure 3 are consistent with the sole breaking of L^F from R and leaving $\alpha-\beta\gamma$ junction intact. The rationale is as follows: In Figure 3A, the traces show (a) the fluorescence time course of the complex after dilution with buffer; (b) dissociation of L^F from beads after rapidly mixing with 0.2mM GTP γ S; and (c) background fluorescence of $L^F R-\alpha_{i2}\beta_{1\gamma 2}$ ternary complex prepared in the presence of GTP γ S. Figure 3B shows the dissociation of L^F corrected for dilution effects and background. The trace shown in Figure 3B corresponds to the dissociation caused by GTP γ S ($\tau_{1/2} = 18\text{sec}$). The GDP results are listed in Table 1 ($\tau_{1/2} = 34\text{sec}$). The fluorescence decay is similar to the ligand dissociation rate measured from a G protein uncoupled receptor [12]. Figure 3C shows corroborating evidence in favor of the breaking of the L^F -R junction while leaving the heterotrimer intact. When GTP γ S was added to the ternary complex in the presence of excess L^F (curve *b* in Figure 3C), the putative dissociation of L^F was inhibited due to rebinding of excess soluble L^F , whereas one would not expect excess L^F to affect the decay in any manner if the break point involved the heterotrimer.

The data shown in Figure 3 indicate that under our experimental conditions, the potential separation of R- α from $\beta\gamma$ can be no faster than the dissociation of L^F from the receptor and can only match that rate. It is tempting to allude to the dynamics of the fluoroaluminate-mediated disassembly of the heterotrimer (Figure 2) where the dissociation of the α_{i3} subunit is shown to occur along a timeframe comparable to the dissociation of the ligand. By elimination, we have ascribed the subsecond disassembly of the ternary complex in Figure 4 to be a measure of the dissociation of FPR-GFP from *G beads* (Table 1 rows 5 and 6). Finally, the kinetic behavior of wild type FPR complexes as shown in Figure 3, generally matched that of the FPR-GFP system, in that the apparent dissociation of the L^F from the G beads occurred at much faster rates, $1 < \tau_{1/2} \leq 5\text{sec}$ (Table 1), than the putative L^F dissociation rate of $\tau_{1/2} < 20\text{sec}$ from the uncoupled receptor, indicating that the receptor dissociates from the G protein prior to the departure of the fluorescent ligand from the receptor. Figure 5B and 5C show the relative influence of α_{i2} and α_{i3} subunits on the dissociation of the receptor from the *G beads*. As shown in Table 1, α_{i2} bearing systems tended to break at a faster rate than the α_{i3} systems (*cf.* Figure 5B vs 5C).

Discussion

The intent of this study was to evaluate in a particle-based detergent solubilized receptor system the steps analogous to those which define how transmembrane GPCRs transduce ligand-initiated activation into intracellular responses. In particular, we measured the time scale of disassembly of the ternary complex components initiated by the addition of either GTP γ S or GDP. The most significant aspects of our present data show that: a) the fastest step associated with ternary complex disassembly is the departure of the receptor from the G α subunit. b) On the biologically relevant time scale, the heterotrimeric subunits remain intact, in contrast to the paradigm in which the activation of GPCRs by nucleotides involves the dissociation of G α subunits from G $\beta\gamma$ [4,33] c) In this system GDP can initiate the disassembly of ternary complexes, although GDP bound G proteins are generally believed to be “inactive” in cells [33].

The prevailing model for G protein-mediated signal transduction is that the dissociation of the G α subunit from G $\beta\gamma$ subunits is an essential component of activation. A crucial argument in favor of subunit dissociation originates from cumulative X-ray structural data, which suggest that the domains that are necessary for interaction with effectors are found at the interface of the α and $\beta\gamma$ subunits [34]. It has therefore been logically argued that subunit dissociation is necessary to remove the steric blockade to effector interactions. A recent paper by Tesmer *et al* [35] has reported on an X-ray crystallographic study that provided structurally correlated “snapshots” of inactive and activated heterotrimeric G $\alpha_q\beta\gamma$ protein. A most compelling structural feature of the data is that a fully dissociated G α_q subunit was shown to be bound to a G protein coupled receptor kinase 2 (GRK2) reorientated away from its erstwhile resting state partner, the $\beta\gamma$ subunit. The authors suggest that these snapshots represent the initial and final frame of a “signaling movie” where the intervening details are at present ill defined or unknown.

An interesting point of detail is that much of the strongest structural evidence, which favors subunit dissociation involves the specific isoforms such as α_s and α_q subunits and may not necessarily be an essential element of signaling processes that involve α_i subunits as recent studies have shown [8]. Furthermore, other experimental evidence has led to questions about the absolute necessity of including subunit dissociation as an element of activation [5–9,19].

The main points of open discussion have been delineated as follows: 1) How ligand activated GPCRs effect the guanine nucleotide exchange at G α [36,37]; 2) Whether G α , G $\beta\gamma$, and GPCRs remain associated after activation [4,8,38,39]; 3) How the G protein subunits and their effectors are spatially arranged during signaling [6,40].

In the present study we have used modular molecular assemblies of ternary complexes on beads, and rapid mix flow cytometry to examine the kinetics of disassembly at specific junction points of the complex (Table 1). We will, in part, discuss our data in the context of the first two open questions stated above. Because model systems, rather than live cells, were used in our study, the results can be viewed in the circumstances where it is possible to bring experimental context to hypothetical steps that have so far remained ill-defined or controversial in the current paradigm of G protein activation.

How Ligand Activated GPCRs Effect the Guanine Nucleotide Exchange at G α

In cells, the ternary complex of ligand, GPCR, and heterotrimeric G protein complexes, has a very short lifetime, which is regulated by the kinetics of nucleotide exchange between the inactive GDP-bound form and the active GTP-bound form [3,33]. The exchange is initiated by the contact between the heterotrimeric G protein entity and a ligand activated receptor. The ternary complex thus formed survives for the short duration of the dissociative interchange

between GDP and GTP under rapid mass transfer considerations. The mechanism by which ligand activated GPCRs catalyze the exchange of GDP for GTP is not clear [3]. It is however believed to involve three flexible regions designated switch I, switch II, and switch III on the α subunit that have been shown to adopt different conformations in the presence of GDP, GTP γ S, and other nucleotide adducts and analogs [41,42]. Because the switch regions are at least 30Å away from the cytoplasmic peptide loops of the receptor, it has been proposed that the receptor must achieve the catalytic feat by means of “action at a distance” [43]. It has been suggested that the GPCR manages the nucleotide exchange through “lever-arm” and “gear-shift” mechanisms [36,43]. The suggested mechanism predicts that the ligand activated receptor effects the release of GDP by using the G $\beta\gamma$ -subunit to directly (“the lever arm”) [43] or indirectly (“the gear shift”) [36] distort the α -subunit switch regions (I and II) and allow nucleotide release and recognition [34]. Another hypothesis suggests that the activated receptor may use a “latch” on the surface of the G α -subunit and thus prompt GDP release [37,42].

Under our experimental conditions, *stable* LRG assemblies on beads are created in circumstances where the contact between the GPCR and *G beads* causes the ejection of GDP from the α_i nucleotide pocket with little to no chance of rebinding, due to the negligible concentrations of soluble nucleotides (GDP or GTP γ S), in solution. The nucleotide pocket remains empty until the addition of excess nucleotides during the rapid mix experiments. Relative to transient cellular processes, the lifetimes of these ternary complexes on our beads are long.

Whether G α , G $\beta\gamma$, and GPCRs Remain Associated after Activation

The most unequivocal result from our experiments is the fate of the GPCR following nucleotide activation. We observe that the receptor typically dissociates from the heterotrimeric G protein during a subsecond timeframe. The data also strongly suggest that the G protein heterotrimer appears to remain intact within the time frame of signal initiation (Table 1). Interestingly our kinetics for the dissociation of FPR-GFP or wild type FPR from G α_i in the detergent solubilized system are much faster than was reported in cells from experiments involving FRET between β_2 AR and G α_s [19]. We do not know whether this reflects differences between the intact cells and detergent, the difference between specific receptors and G proteins, the possibility that their measurement has contributions from a higher order topographical redistribution, or the possibility that ligand dissociation and rebinding [44–46] is rate-limiting in their measurement of RG dissociation.

One argument in favor of a fast dissociation rate constant of the GPCR from heterotrimeric G proteins is that it allows for the incorporation of several elements of GPCR ternary complex models that have been postulated to account for the rapid signaling kinetics by hypothesizing that receptors, their G proteins and effectors are compartmentalized in microdomains [47,48] where they diffuse freely and couple with specificity upon agonist stimulation. The locally high concentrations of signaling components would permit subsecond kinetics observed with GPCR signaling [19,49]. One can envision a case where a ligand activated receptor can hop from one G protein heterotrimer to the next, thereby activating several G proteins during the lifetime of a bound ligand.

Our data also suggest that the heterotrimer remains intact during the early events of activation. Although there is much recent evidence that concurs with our results [6–9], it is difficult to arrive at a definitive conclusion because of the model nature of our experiments. One might infer from structural data that the interface domains of the components of the ternary complex involves an intricate system of ON and OFF allosteric switches that trigger their stepwise disassembly from erstwhile partners in exchange for new ones [50]. The reason why we did not observe subunit dissociation could be due to the absence of effectors that could potentially be responsible for turning ON a switch mechanism that may cause the rapid dissociation of the

α subunit. As a case in point, under basal conditions, GDP, which has a reported affinity of 10^{-11} M [28] for the α subunit, is readily ejected in a millisecond time frame [19] from the binding pocket upon a transient contact between the ligand activated receptor during the GDP/GTP nucleotide exchange step of GPCR activation. This point is made in recognition of the incomplete nature of our model system. Nevertheless, our data fall in the column, which militates against subunit dissociation as an essential element of the early events in GPCR activation. It also worth noting that it has recently emerged from FRET studies that subunit dissociation might be governed by a region that is found in $G\alpha_i$ subunits and not $G\alpha_s$ subunits [8]. Thus the lack of subunit dissociation in some systems might be representative of different modes of activation that are a function of G protein isotype.

A variety of subunit isotypes can induce the receptor to achieve conformational states which display a higher affinity for the ligand [51]. In Table 1, our data show that the substitution of β_1 for β_4 in the $G\beta\gamma$ dimer has a small but measurable effect ($\tau_{1/2} = 18.3$ sec vs. 12.4 sec) on the dissociation rate of the ligand, after the receptor uncouples from the G protein ($\tau_{1/2} < 1$ sec). It is conceivable that the memory of the conformational state induced by the G protein lingers after the G protein interaction is disconnected. Interestingly, our equilibrium binding [13] and G protein activation data (Table 1, rows 1–2) show that the stability of the complexes and dissociation rates of receptor or ligand depend on the isotype of the G proteins. Other data have shown that the stability of the LRG complexes improved according to the rank $\alpha_{i1} < \alpha_{i2} < \alpha_{i3}$ [12]. In Table 1, the rate of nucleotide mediated separation of receptor and G protein appears to be similar with either a β_1 or β_4 component. However, the same process appears to be sensitive to the isotype of the α subunit, where α_{i2} containing ternary complexes separate at rates that are several times faster than α_{i3} containing complexes.

Response to GDP

Our data show that GDP is capable of inducing separation of ternary complex components, and is consistent with a number of literature reports where GDP was shown to induce effector activation [52–54]. In one study, GDP was reported to be capable of activating $G\alpha_s$ to the degree that subsequent interaction with adenylyl cyclase was measured [54]. It has been suggested that the affinity of a GTP-bound $G\alpha_s$ for adenylyl cyclase is ten times higher than its GDP bound counterpart [55]. The reduced affinity of the GDP-bound form for adenylyl cyclase was proposed to be part of a timing mechanism to limit cyclase activation, after the hydrolysis of GTP to GDP, thus allowing the GDP-bearing $G\alpha_s$ subunit to dissociate from the cyclase and rejoin the $\beta\gamma$ subunits. While our kinetic results demonstrate that both GDP and GTP have comparable effects on the ternary complex (*cf.* Table 1, Figure 4B), we do not know if downstream effector activity such as inhibiting cyclase activity by $G\alpha_i$ would ensue at a reduced efficacy.

Summary

The modular assembly of G proteins, detergent solubilized receptors and ligands on beads has the potential to offer insight into the thermodynamic and kinetics of GPCR complexes. Much of the advantage of this platform is the enabling of the practitioner to define reagent stoichiometry and identity, e.g., isotypes of promiscuous G protein subunits can be clearly defined. The assembly of ternary complexes on beads has the potential of meeting the challenge of a systematic study that could lead to the elucidation of differences in dynamic and equilibrium roles played by different isotypes of α and $\beta\gamma$ subunits [13,23,26].

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Glossary

Abbreviations

GPCR

G protein-coupled receptor

FPR

formyl peptide receptor

α

G α subunit

β

G β subunit

γ

G γ subunit

$\beta\gamma$

G $\beta\gamma$ dimer

$\alpha\beta\gamma$

G $\alpha\beta\gamma$ heterotrimer

GFP

green fluorescent protein

GTP γ S

guanosine 5'-O-(3-thio)triphosphate

FITC	fluorescein isothiocyanate
L	ligand
fMLFF	formyl-Met-Leu-Phe-Phe
L^F	fluorescent ligand
fMLFK-FITC	FITC conjugated formyl-Met-Leu-Phe-Lys
R	receptor
R^F	fluorescent receptor
FPR-GFP	FPR-GFP fusion protein
R-Gai2	FPR-Gai2 fusion protein
G	G protein or heterotrimeric regulatory of G protein
LR	ligand-receptor complex
RG	receptor-G protein complex
LRG	ligand-receptor-G protein complex
DOM	n-dodecyl- β -D-maltoside
DTT	dithiothreitol
BSA	bovine serum albumin
FLAG	FLAG peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys.

Figure 1. Assembly of G protein and ternary complex

A. Binding curve of fluorescein labeled $G\alpha_{i3}^{fl}$ subunits to $\beta\gamma$ subunits on beads. Total binding (filled circles), specific binding (open squares), and non-specific binding (filled squares) are shown in curves a, b, and c, respectively. The FLAG epitope-tagged $\beta\gamma$ subunits are attached to the beads via M2 antibodies. Beads displaying M2 without $\beta\gamma$ subunits were used as controls for nonspecific binding. The K_d was 32 nM (insert). B. Comparison of ternary complex formation with $G\alpha_{i3}$ and $G\alpha_{i2}$ subunits. Titration of ligand (fMLFF) activated FPR-GFP on *G* beads of the type $\beta_1\gamma_2\cdot\alpha_{i2}$ or $\beta_1\gamma_2\cdot\alpha_{i3}$.

Figure 2. A. Stability of bead-based assemblies

Plot of mean channel fluorescence (MCF) of *G-beads* versus time. The arrow indicates the addition of 0.1mM GTP γ S to G α_{13}^{fl} bearing G beads. Data show that GTP γ S does not cause subunit dissociation, when the nucleotide-binding pocket is blocked by high affinity binding GDP. B. (a) Dissociation of G α_{13}^{fl} •GDP•AlF $_4^-$ from the $\beta\gamma$ subunit on G beads in the presence of AlF $_4^-$. The G beads appear to bear two distinct populations of G proteins corresponding to the biphasic dissociation of G α_{13}^{fl} •GDP•AlF $_4^-$. These characteristics have been previously described separately in the literature. (b) Self-exchange of G α_{13}^{fl} with unlabeled G α_{13} subunits. C. Dissassembly of L•FPR^{GFP}-G $\alpha\beta\gamma$ after manual addition of GTP γ S to G beads on a flow cytometer. Data show (a) the fluorescence time course of the complex after dilution with buffer,

(b) dissociation from beads of LFPR^{GFP} after rapid mixing with 0.2mM GTP γ S, and (c) background fluorescence of L•FPR^{GFP}-Ga $\beta\gamma$ prepared in the presence of GTP γ S. The data corresponding to the rapid kinetics of disassembly are lost during the 10 second deadtime.

Figure 3. Rapid mix disassembly of FPR-Gα_{i2} ternary complex

Red arrows in the scheme show junction points of disassembly, with the larger arrow indicating the weaker link that actually is shown to break in the experiment. A. Data show (a) filled squares: the fluorescence time course of the complex after dilution with buffer, (b) open squares: dissociation from beads of fMLFK-FITC (L^F) after rapid mixing with 0.2 mM GTPγS, and (c) filled circles: background fluorescence of $L^FR-\alpha_{i2}\beta_1\gamma_2$ prepared in the presence of GTPγS. B. Dilution and background corrected data showing measurements of L^F dissociation kinetics from Gα_{i2}-FPR complexed to $\beta_1\gamma_2$ subunits on beads after the addition of 0.1 mM GTPγS. The data were fit to a single exponential model; the half time $\tau_{1/2}$ of GDP is ≈ 34 sec (data not shown), compared to that of GTPγS (≈ 18 sec). C. Manual mix flow

cytometry measurements of the putative dissociation of L^F from $G\alpha_{i2}$ -FPR on G beads on beads after the addition of GTP γ S. Data sets show (a) open squares: effect of dilution on L^F - $\alpha_{i2}\beta_1\gamma_2$ in DHPSM buffer, (b) dotted line: represents the apparent inhibition of L^F dissociation by 20 nM L^F from the ternary complex following addition of 0.1 mM GTP γ S which uncouples the receptor ligand high affinity interaction from the G protein. (c) crosses: 0.1 mM GTP γ S added to ternary complex. (d) filled circles: background fluorescence of L^F - $\alpha_{i2}\beta_1\gamma_2$ prepared in the presence of GTP γ S.

Figure 4. Rapid mix disassembly of FPR-GFP (R^F) ternary complex

Red arrows in the scheme show junction points of disassembly, with the larger arrow indicating the weaker link that actually is shown to break in the experiment. A. Representative data show (a) the fluorescence time course of the complex after dilution with buffer, (b) dissociation from beads of R^F after rapid mixing with 0.2 mM GTP γ S, and (c) background fluorescence of $LR^F\alpha_{12}\beta_{1\gamma_2}$ prepared in the presence of GTP γ S. B. Dilution and background corrected data showing measurements of R^F dissociation kinetics from *G beads* following the addition of 0.1 mM GDP (a) or 0.1 mM GTP γ S (b). The data were fit to a two-phase exponential model. The effect of the two nucleotides is distinguished by the half time $\tau_{1/2}$ of the fast components: for

GDP the half time is ≈ 1.2 sec, compared to ≈ 0.7 sec for $\text{GTP}\gamma\text{S}$. The halftime values for the subsecond decay are estimates, constrained by the time resolution of the data.

Figure 5. Representative disassembly of wildtype FPR ternary complex

Red arrows in the scheme show junction points of disassembly, with the large arrow indicating the weaker link that actually is shown to break in the experiment. A. Data show (a) the fluorescence time course of the complex after dilution with buffer, (b) dissociation from beads of L^F after rapid mixing with 0.2 mM GTP γ S, and (c) background fluorescence of $L^F R \alpha_{i2} \beta_1 \gamma_2$ prepared in the presence of GTP γ S. B. Dilution and background corrected data showing measurements of $L^F R$ dissociation kinetics from $G \alpha_{i2} \beta_1 \gamma_2$ beads after the addition of 0.1 mM GTP γ S. C. Dilution and background corrected data showing measurements of $L^F R$ dissociation kinetics from $G \alpha_{i3} \beta_1 \gamma_2$ beads after the addition of 0.1 mM GTP γ S.

Table 1

Summary of experimental results of LRG dissociation kinetics measured by small volume rapid mix flow cytometry. A single data set represents an average of at least 3 runs of successive rapid mix runs (see text for details). The standard deviations represent the variance of at least 2–3 unique data sets collected over different days.

	Ligand	Receptor	Gα	βγ	τ _{fast} (sec)	Junction ^c
1 ^a	L ^F	R-Gα ₁₂		β ₁ γ ₂	18.3 (GTPγS) 33.8 (GDP)	L-RG
2 ^a	L ^F	R-Gα ₁₂		β ₄ γ ₂	12.4 (GTPγS)	L-RG
3 ^b	L ^F	R	Gα ₁₂	β ₁ γ ₂	0.8 ± 0.2 (GTPγS) 1.5 ± 0.4 (GDP)	LR-G
4 ^b	L ^F	R	Gα ₁₂	β ₄ γ ₂	0.7 ± 0.1 (GTPγS)	LR-G
5 ^b	L	R ^F	Gα ₁₂	β ₁ γ ₂	0.8 ± 0.1 (GTPγS) 1.1 (GDP)	LR-G
6 ^b	L	R ^F	Gα ₁₂	β ₄ γ ₂	0.6 ± 0.05 (GTPγS)	LR-G
7 ^b	L ^F	R	Gα ₁₃	β ₁ γ ₂	3.1 ± 0.2 (GTPγS)	LR-G
8 ^b	L	R ^F	Gα ₁₃	β ₁ γ ₂	5.8 (GDP) 2.4 ± 0.2 (GTPγS) 5.2 ± 0.6 (GDP)	LR-G

^a Kinetic data analyzed using a single-phase exponential model.

^b Kinetic data analyzed using a two-phase exponential model. The data only shows the results of the analysis of the fast component. The slower component was typically two orders of magnitude or more than the fast component (cf. Figure 3). We have attributed the slow component to receptor misfolding (see text for details). Dissociation produced by GTPγS was always faster and to a greater extent than GDP.

^c Experimentally measured point of dissociation in the ternary complex.