The C-Terminus of the G Protein α Subunit Controls the Affinity of Nucleotides

ARTICLE *in* BIOCHEMISTRY · MARCH 2012

Impact Factor: 3.02 · DOI: 10.1021/bi201702d · Source: PubMed

5 AUTHORS, INCLUDING:

Takahiro Yamashita

Kyoto University

43 PUBLICATIONS **627** CITATIONS

SEE PROFILE

Takesi Matsuyama Hoyos

RIKEN

5 PUBLICATIONS **124** CITATIONS

SEE PROFILE

BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

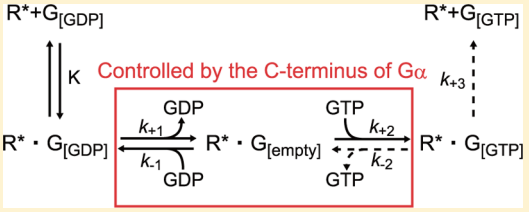
pubs.acs.org/biochemistry

The C-Terminus of the G Protein α Subunit Controls the Affinity of Nucleotides

Naoki Kimata, Takahiro Yamashita, Take Matsuyama, Yasushi Imamoto, and Yoshinori Shichida*

Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

ABSTRACT: The C-terminus of the G protein α subunit has a well-known role in determining the selective coupling with the cognate G protein-coupled receptor (GPCR). In fact, rhodopsin, a prototypical GPCR, exhibits active state [metarhodopsin II (MII)] stabilization by interacting with G protein [extra formation of MII (eMII)], and the extent of stabilization is affected by the C-terminal sequence of Gα. Here we examine the relationship between the amount of eMII and the activation efficiency of Gi mutants whose Giα forms have different lengths of the C-terminal sequence of Goα. The results show that both the activation efficiencies of Gi and the amounts of eMII were affected by mutations; however, there was no correlation between them. This finding suggested that the C-terminal region of Gα not only stabilizes MII (active state) but also affects the nucleotide-binding site of Gα. Therefore, we measured the activation efficiency of these mutants by MII at several concentrations of GDP and GTP and calculated the rate constants of GDP release, GDP uptake, and GTP uptake. These rate constants of the Gi mutants were substantially different from those of the wild type, indicating that the replacement of the amino acid residues in the C-terminus alters the affinity of nucleotides. The rate constants of GDP uptake and GTP uptake showed a strong correlation, suggesting that the C-terminus of Giα controls the accessibility of the nucleotide-binding site. Therefore, our results strongly suggest that there is a long-range interlink between the C-terminus of Giα and its nucleotide-binding site.



The G protein-mediated signal transduction cascade is one of the typical signaling systems widely present in organisms. The signal cascade is initiated when a G protein-coupled receptor (GPCR) responds to a stimulus and activates the G protein. The activated GPCR (R*) binds to the G protein and catalyzes its GDP−GTP exchange reaction. Then the G protein dissociates into active subunits, GTP-bound α subunit and βγ subunits. The G protein-mediated signaling is ubiquitously present in cells, and mammals possess thousands of GPCRs, which couple to a few dozen G proteins. Therefore, elucidating the activation mechanism of G protein by R* will have far-reaching implications. The activation mechanism of G protein has been extensively studied by means of various biochemical, molecular biological, spectroscopic, and crystallographic techniques.[1−5] These studies have focused on the binding of the G protein to R* or on the GDP−GTP exchange reaction that proceeds at the nucleotide-binding site. However, the receptor binding site and the nucleotide-binding site are separated by approximately 30 Å,[6] and little is known about interactions between the receptor binding site and the nucleotide-binding site, although it is reported that mutation of K341 of Giα alters the rates of GDP release and GTP uptake as well as the binding affinity for the G protein-activating state of rhodopsin [metarhodopsin II (MII)].[7]

Vertebrate rhodopsin, a prototypical GPCR, couples with Gt in the native photoreceptor cells, but it can also activate Gi and Go in vitro.[8,9] Because large amounts of the α subunit of Gi (Giα) can be expressed in *Escherichia coli* unlike Gtα,[10] the activation mechanism of G protein can be investigated in detail by using Gi mutants. Especially, the rhodopsin/Gi system has the advantage that the interaction of MII with the G protein can be studied by spectroscopy. Previous reports have shown that G protein selectively binds to R* with the C-terminal regions of its α and γ subunits, resulting in the GDP−GTP exchange reaction at the nucleotide-binding site.[11−17] In line with this, we have previously investigated the role of the C-terminus of Giα in the activation of the G protein by MII and found that the MII-induced activation efficiency of the Gi mutant having five C-terminal amino acids of Goα in its Giα [Gi/DGFY (see Figure 1)] is very similar to that of wild-type Gi (Giwt), whereas that of the Gi mutant having 11 C-terminal amino acids of Goα in its Giα [Gi/o11 (see Figure 1)] is significantly reduced.[9] It was spectroscopically shown that the amount of extra formation of MII (eMII), which correlates with the amount of the complex of MII and G protein, by Gi/o11 is greatly reduced as expected from the activation efficiency.[11,18] However, during the course of our study, we found that the amount of eMII by Gi/DGFY was also greatly reduced, which is inconsistent with the activation efficiency comparable to that of Giwt. These results suggested that the C-terminus of Gα has a role other than the modulation of the efficiency of formation of the complex, although the amount of eMII does not strictly correlate with the binding affinity between MII and Gi.

In this study, we investigated the role of the C-terminal tail of Giα in the GDP−GTP exchange reaction as well as the interaction with MII. We constructed C-terminal mutants of Giα that have hybrid sequences of Giα and Goα (Figure 1) and compared the amount of eMII and the MII-induced activation efficiency of them. Estimation of the rate constants involved in

Received: November 11, 2011
Revised: February 6, 2012
Published: March 12, 2012

dx.doi.org/10.1021/bi201702d | *Biochemistry* 2012, 51, 2768−2774

C-terminal sequence of Giα

	340										350
Giwt	I	K	N	N	L	K	D	C	G	L	F
Gi/o11	I	**A**	N	N	L	**R**	**G**	C	G	L	**Y**
Gi/DGFY	I	K	N	N	L	K	**G**	C	G	L	**Y**
Gi/KAKR	I	**A**	N	N	L	**R**	D	C	G	L	F
Gi/K341A	I	**A**	N	N	L	K	D	C	G	L	F
Gi/K345R	I	K	N	N	L	**R**	D	C	G	L	F
Gi/D346G	I	K	N	N	L	K	**G**	C	G	L	F
Gi/F350Y	I	K	N	N	L	K	D	C	G	L	**Y**

Figure 1. Sequences of the 11 C-terminal amino acids of Giα mutants. Residue numbers are based on the numbering of Gtα; i.e., I340−F350 in Gtα correspond to I344−F354 in Giα. Amino acids that are different from those of Giwt are shown in bold.

the activation process of Gi revealed that the C-terminal sequence of Gα has a significant role in the GDP−GTP exchange reaction.

MATERIALS AND METHODS

Preparation of Rhodopsin. Bovine rod outer segments (ROSs) were isolated and purified from bovine retinas by the methods previously described.[19] All procedures were conducted under dim red lights (>650 nm) on ice. The ROSs were isolated with a standard discontinuous sucrose gradient (29/35.5%) method. The ROS membranes were then washed with 5 M urea and were repeatedly washed with a low-ionic strength buffer to remove membrane-associated proteins, such as Gt and phosphodiesterase, as previously described.[20] The washed membranes were stored at −80 °C until they were subjected to the experiments.

The membrane samples suspended in buffer A [30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 40 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0) at 4 °C] were sonicated at low power (dial was set to 3 of 10) on ice under a stream of nitrogen gas for 90 s with a sonicator to diminish turbidity (Ultrasonic W-220). Sonicated samples containing 7.5 μM rhodopsin in buffer A were subjected to spectroscopic measurements.

For the GTPγS binding assay, bovine rhodopsin was extracted with 1% *n*-dodecyl β-D-maltoside (DM) in buffer P {140 mM NaCl and 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 6.5)} and purified by rho1D4 antibovine rhodopsin antibody column chromatography, which had been equilibrated by buffer A supplemented with 0.02% DM. Rhodopsin extracted with DM was almost completely converted to MII when it was irradiated under our experimental conditions.

Preparation of Giα Mutants and Gtβγ. The rat Giα1 was expressed in *E. coli* strain BL21 using the Giα1 cDNA inserted into the pQE6 plasmid vector, and the expressed Giα1s were purified by three-step column chromatography using DEAE Sephacel (GE Healthcare), Phenyl Sepharose CL-4B (GE Healthcare), and Mono Q 5/50 GL (GE Healthcare) as previously described.[10] The concentrations of each Giα1 were determined by the Bradford method.

Bovine transducin β and γ subunits (Gtβγ) were prepared from isolated bovine ROSs by the method previously described.[21] First, isolated bovine ROSs were irradiated with a white fluorescent lamp and repeatedly washed with a low-ionic strength buffer (pH 7.2) to remove membrane-associated proteins except Gt. Next, Gt was liberated to the supernatant by the addition of GTP. Gtα and Gtβγ were then separately purified from the supernatant with concatenated liquid column chromatography [Blue Sepharose 6 Fast Flow (GE Healthcare) to trap Gtα and DEAE Toyopearl 650S (TOSOH) to trap Gtβγ]. The concentration of Gtβγ was determined by the Bradford method.

The purified wild-type and mutant Giα1s were mixed with equal molar amounts of Gtβγ. The amounts of these Gis were determined by [^{35}S]GTPγS uptake using a radiolabeled nucleotide filter binding assay. The heterotrimer of Giα and Gtβγ was used in all experiments.

Estimation of the Amount of the MII·G Complex. Absorption spectra after irradiation of rhodopsin in the sample were recorded with a CCD spectrophotometer as previously described.[19] Transient spectra (650−350 nm) with a wavelength resolution of 1.6 nm in time intervals of 9.7 ms were continuously recorded. The sample temperature was maintained at 4.0 ± 0.1 °C by a temperature controller (Neslab RTE-111) that was attached to the sample cell holder. The sample was irradiated with light from a xenon flash lamp (Nissin Electric) through a glass cutoff filter (Y-52, Toshiba). In this setup, ∼50% of the rhodopsin in the sample was bleached.

The transient spectra were analyzed as follows. Difference spectra of the ROS sample (7.5 μM rhodopsin) before and after irradiation in the absence ($Diff_{-G}$) or presence ($Diff_{+G}$) of 8 μM Gi were recorded without adding GTPγS and GDP. Then the $Diff_{+G}$ minus $Diff_{-G}$ spectrum was calculated for each mutant. The amount of eMII was estimated by the amplitudes of the $Diff_{+G}$ minus $Diff_{-G}$ spectra at 480 nm because there was large noise at <400 nm.

GTPγS Binding Assay. A radioisotope-labeled nucleotide filter binding assay, which measures the light-dependent GDP−GTPγS exchange by Gi, was conducted as described previously.[9] All procedures were conducted on ice. The mixture of rhodopsin and Gi (final concentrations of 6.25 and 600 nM, respectively) was irradiated with yellow light (through a Y52 cutoff filter, >500 nm) for 30 s or kept in the dark, and they were immediately mixed with a solution containing GDP and GTPγS. To estimate the GTPγS binding affinity of Gi, the concentrations of GTPγS in the assay mixture were varied from 0.01 to 10 μM, while no GDP was added to the mixture. To estimate the GDP binding affinity of Gi, the concentration of GTPγS in the assay mixture was fixed at 1 μM while the GDP concentration was varied from 0 to 16 μM. To measure the spontaneous activation efficiency of Gi, the concentration of GTPγS was fixed at 1 μM, and no rhodopsin or GDP was added to the assay mixture. After incubation for a selected time in the dark, the reaction was stopped by addition of the solution containing 10 μM GTPγS, and the reaction mixture was immediately filtered through a nitrocellulose membrane to trap [^{35}S]GTPγS bound to the Giαs. The amount of bound [^{35}S]-GTPγS was quantified by assaying the membrane with a liquid scintillation counter (LS 6000IC, Beckman).

Estimations of the Affinities of Each Giα Mutant for GDP and GTPγS. The activation of the G protein by MII can be expressed as Figure 2. In this process, the dissociation constant of the MII·$G_{[GDP]}$ complex (K) is defined by

$$K = \frac{[\mathrm{MII}][\mathrm{G}_{[\mathrm{GDP}]}]}{[\mathrm{MII}\cdot\mathrm{G}_{[\mathrm{GDP}]}]} \quad (1)$$

dx.doi.org/10.1021/bi201702d | *Biochemistry* 2012, 51, 2768−2774

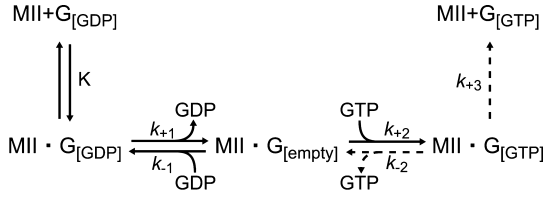


Figure 2. Reaction scheme of activation of the G protein by metarhodopsin II (MII). The scheme consists of four steps: (i) binding of MII and G protein in GDP-bound form, (ii) release of GDP from Gα in the complex, (iii) GTP uptake of Gα in the complex, and (iv) dissociation of the complex to release the activated G protein.

Because released $G_{[GTP]}$ and MII·$G_{[GTP]}$ complex are indistinguishable in our membrane binding assay using radiolabeled GTPγS, the sum of $G_{[GTP]}$ and MII·$G_{[GTP]}$ was written as

$$[G_{[GTP]}]_{total} = [G_{[GTP]}] + [MII{\cdot}G_{[GTP]}] \quad (2)$$

The rate of binding GTPγS to the G protein is then

$$v = \frac{d}{dt}[G_{[GTP]}]_{total} = k_{+2}[MII{\cdot}G_{[empty]}][GTP] \quad (3)$$

Because the apparent initial rate is observed at the pseudo-steady state, it follows that

$$\frac{d}{dt}[MII{\cdot}G_{[empty]}] = k_{+1}[MII{\cdot}G_{[GDP]}] - (k_{-1}[GDP] + k_{+2}[GTP]) \times [MII{\cdot}G_{[empty]}] = 0 \quad (4)$$

where the term $k_{-2}[MII{\cdot}G_{[GTP]}]$ is neglected because $[MII{\cdot}G_{[GTP]}]$ is negligible at approximately time zero. The total amount of MII is

$$[MII]_{total} = [MII] + [MII{\cdot}G_{[GDP]}] + [MII{\cdot}G_{[empty]}] \quad (5)$$

When these equations are solved, the initial rate of activation (v_0) is expressed by

$$v_0 = \frac{k_r k_{+2}[GTP]}{k_r + k_{-1}[GDP] + k_{+2}[GTP]}[MII]_{total} \quad (6)$$

where

$$k_r = k_{+1}\frac{[MII{\cdot}G_{[GDP]}]}{[MII] + [MII{\cdot}G_{[GDP]}]} = k_{+1}\frac{[G_{[GDP]}]}{K + [G_{[GDP]}]} \quad (7)$$

In the scheme in Figure 2, the rate constants of uptake of GDP (k_{-1}) and GTPγS (k_{+2}) by the MII·$G_{[empty]}$ complex represent the affinity of the G protein for GDP and GTPγS, respectively. To estimate the rate constants, dependency curves of v_0 on the concentrations of GDP and GTPγS were fitted with eq 6.

RESULTS

Several lines of evidence have demonstrated that the C-terminal region of Gα is essential for the selective interaction with the cognate GPCR.[13,18] We characterized roles of the amino acid residues in the C-terminus of Giα using Giwt and its mutants listed in Figure 1. Although these Giα mutants were not myristoylated at the N-terminal region as they were expressed in *E. coli*, Preininger et al. reported that myristoylated and unmyristoylated Gis show similar MII-induced activation efficiencies.[22] Moreover, because recombinant Giα was coupled with falnesylated native Gt$\beta\gamma$, the Gi heterotrimer we used was expected to hydrophobically interact with MII and/or ROS membranes.

Irradiation of rhodopsin yields a mixture of MII and its precursor, metarhodopsin I (MI), which is apparently biased toward MII in the presence of Gi because of the formation of the MII·Gi complex. This equilibrium shift was shown by the difference spectrum between $Diff_{+G}$ (difference spectrum before and after irradiation in the presence of Gi) and $Diff_{-G}$ (difference spectrum before and after irradiation in the absence of Gi) (see Materials and Methods). Figure 3A shows the $Diff_{+G}$ minus $Diff_{-G}$ spectra for Giwt (—) and its mutants (---). The absorbance increase of $Diff_{+G}$ minus $Diff_{-G}$ spectra at <400 nm and the decrease at 480 nm represent the extra formation of MII (eMII). The shapes of $Diff_{+G}$ minus $Diff_{-G}$ spectra of mutants were essentially identical to that of Giwt, while the amplitudes were varied, confirming that $Diff_{+G}$ minus $Diff_{-G}$ spectra represent eMII also for Gi mutants. The amounts of eMII by Gi were estimated by the absorbance decrease at 480 nm because of the large noise at <400 nm (Figure 3C). In these experiments, 7.5 μM rhodopsin in ROS was irradiated in the presence of 8.0 μM Gis, where ~50% of the rhodopsin was photoactivated. This ratio is a saturated level of eMII for Giwt[19] and Gi/o11 (this work), indicating that the amount of eMII represents the efficacy in shifting the equilibrium between MI and MII rather than the binding affinity for Gi.

Figure 3B shows the courses of uptake of GTPγS by Giwt and mutants induced by MII. The net increase in radioactivity due to the uptake of GTPγS by Gi was obtained by subtracting the nonspecific binding estimated by the measurements without irradiation. The GTPγS uptake occurred linearly in this region. The amount of GTPγS uptake indicated that only 7.5% of Gi in the sample was activated and 4.5% of GTPγS was taken up in the reaction time, showing that the depletion of Gi and GTPγS is negligible. Therefore, the initial velocity of GTPγS uptake (v_0) was obtained by fitting with a linear line. The activation efficiency of the Gi mutant was evaluated by v_0 (Figure 3C). The activation efficiency of Gi/o11 was lower but that of Gi/KAKR higher than that of Giwt in the presence of 1 μM GTPγS. Activation efficiencies of the other mutants were comparable to that of Giwt.

The activation of the G protein by MII can be expressed by the scheme shown in Figure 2, where at least four steps of interaction between MII and G protein occur. First, MII forms a complex with G protein in a GDP-bound form ($G_{[GDP]}$). Then, GDP is released from Gα in the complex ($G_{[empty]}$), and GTP binds to Gα in the complex ($G_{[GTP]}$). Finally, the complex is dissociated to release the activated Gα.[23] If the C-terminus of Giα affects only the equilibrium between MI and MII (i.e., k_{+2} in eq 3 is not changed), we should expect that a smaller amount of eMII, which is the active (catalytic) complex, results in a lower Gi activation efficiency. However, our results show that, while that for active complex for all Gi mutants was smaller than that for Giwt, most mutants have the same or an even larger activation efficiency (Figure 3C). Thus, the activation efficiency is not solely correlated with the amount of active complex, but the C-terminus of Giα has an additional role in the activation process.

We then measured the initial rate of activation of Gi at various GTPγS concentrations to characterize the GTP uptake reaction (Figure 4A). Typical results were obtained in Gi/o11. The initial rate of uptake of GTPγS by Gi/o11 was smaller than that by Giwt at high concentrations of GTPγS (1 and 10 μM),

dx.doi.org/10.1021/bi201702d | *Biochemistry* 2012, 51, 2768–2774

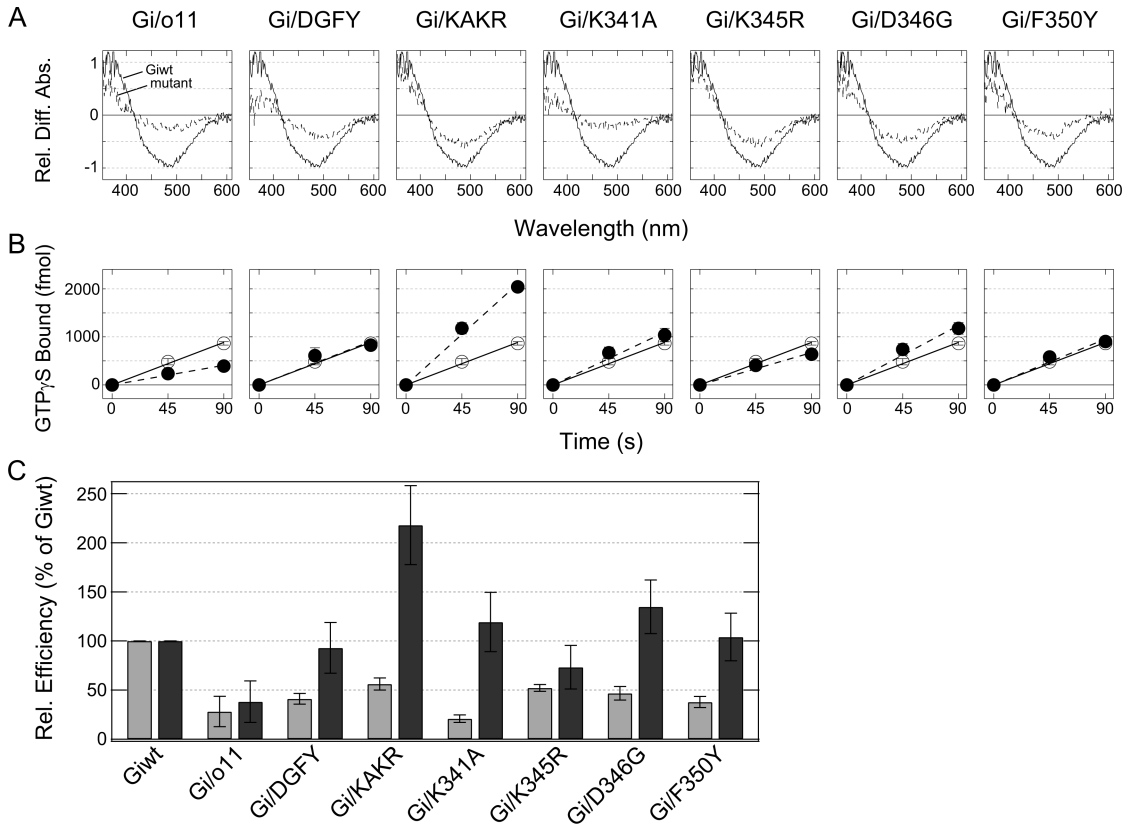


Figure 3. Amount of extra formation of MII (eMII) and activation efficiencies of Gis. (A) Spectroscopic assay of the formation of the complex of MII and Gi (MII·Gi). The $Diff_{+G}$ minus $Diff_{-G}$ spectrum (see Materials and Methods) was calculated for each mutant (---). The $Diff_{+G}$ minus $Diff_{-G}$ spectrum for Giwt is shown for comparison (—). These spectra were the averages of three or four measurements. The amount of eMII was estimated by the absorbance decrease of the $Diff_{+G}$ minus $Diff_{-G}$ spectra at 480 nm. (B) MII-induced GTPγS uptake of Giwt (○) and each mutant (●) with 1 μM GTPγS and no GDP. Radioactivities without light irradiation at respective times were subtracted from those after light irradiation. They were fit with linear lines, and the activation efficiency was estimated by the initial rate of GTPγS uptake (solid and dashed lines). Each point represents the average of at least three independent experiments, and the error bars represent standard deviations. (C) Relative amounts of eMII by Gis estimated by spectroscopy (gray bars) and efficiencies of activation of Gis by MII estimated by GTPγS uptake (black bars). They are normalized to the value of Giwt. Each bar represents the average of at least three independent experiments, and the error bars represent standard deviations.

while it was higher than that by Giwt at low concentrations of GTPγS (0.01 and 0.1 μM) (Figure 4A). This reversal was also observed in other mutants (e.g., Gi/K341A). At low concentrations of GTP, the rate-limiting step of the activation process is likely to be GTP uptake. Because Giwt has a smaller activation efficiency than Gi/o11 at low GTP concentrations, Giwt would have a lower efficiency of GTP uptake than Gi/o11. On the other hand, at high GTP concentrations, the rate-limiting step is likely to be GDP release. Thus, Giwt would release GDP more readily than Gi/o11. Therefore, these results suggest that the C-terminus of Giα affects the binding affinity of GDP and GTP.

To confirm this idea, we examined whether the C-terminus of Giα in the MII·Gi complex also affects the affinity for GDP by measuring the initial rate of activation of Gi at various GDP concentrations (Figure 4B). The profile was strikingly altered by the mutation of the C-terminus of Giα. The initial rates of uptake of GTPγS by Giwt and Gi/K345R were almost independent of the concentration of GDP in this range, whereas those of the other Gi mutants were effectively reduced by GDP, implying that these mutants have an affinity for GDP higher than that of Giwt or Gi/K345R. These findings indicated that the structure and/or the dynamics of the C-terminus of Giα are interlinked with the structure of the nucleotide-binding site.

To examine whether the effect of the C-terminus of Giα on its affinity of GDP and GTP is mediated by the formation of a complex with MII, we measured the rate of spontaneous nucleotide exchange of Gi mutants (Figure 5). They were affected by the C-terminal mutation of Giα, indicating that the C-terminus of Giα affects the affinity of nucleotides without formation of a complex with MII. However, the efficiencies of spontaneous nucleotide exchange of Gi mutants were not correlated with MII-induced ones. Therefore, facilitation of nucleotide exchange by MII depends on the C-terminal sequence of Giα.

We then conducted kinetic analyses based on the scheme in Figure 2. The dependencies of the initial rate of GTPγS uptake on the GTPγS and GDP concentrations shown in Figure 4 were simultaneously fit by eq 6 to estimate k_r, k_{-1}, and k_{+2} (Table 1). As shown in the G protein activation scheme (Figure 2), k_{-1} and k_{+2} are the rate constants for the binding of GDP and GTPγS to the MII·$G_{[empty]}$ complex, respectively, and k_r represents the total efficiency of the formation of the MII·$G_{[GDP]}$ complex and GDP release. Replacement of amino acids in the C-terminus of Giα significantly changes these rate constants, indicating that the C-terminus affects the affinities for both GTP and GDP. It should be noted that Giwt has smaller k_{-1} and k_{+2} values and a larger k_r than most of the mutants. Because the ratio of k_r and k_{-1} (k_r/k_{-1}) represents the efficiency of GDP release, the affinity of Giwt for GDP is significantly lower than those of most Gi mutants.

dx.doi.org/10.1021/bi201702d | Biochemistry 2012, 51, 2768−2774

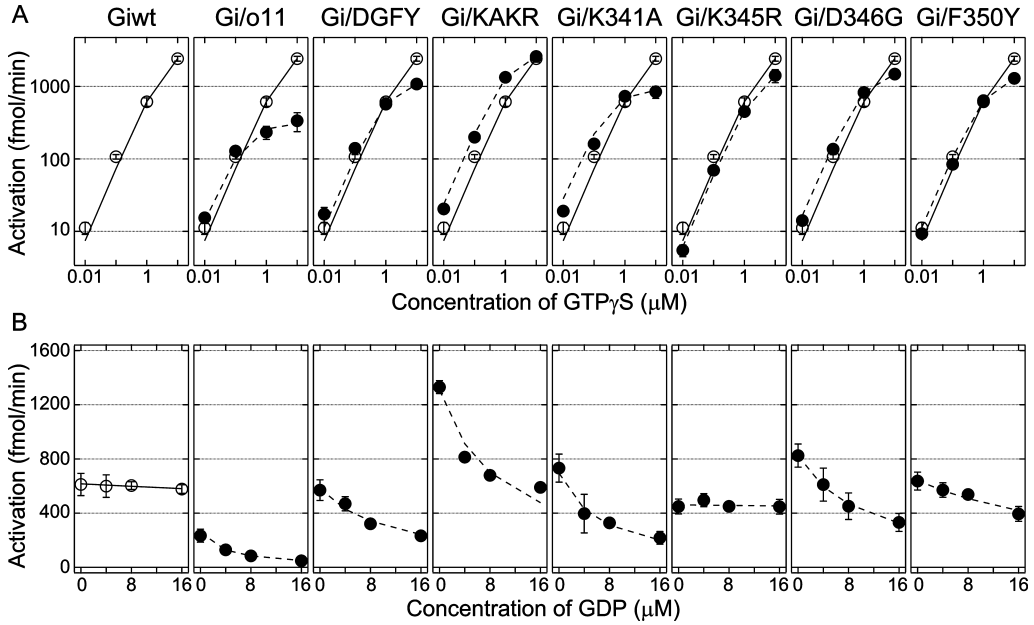


Figure 4. Dependence of the MII-induced activation efficiencies of Gis on the concentration of nucleotides. (A and B) Activation efficiencies of Giwt (○) and mutants (●) at various concentrations of GTPγS (A) or GDP (B). No GDP was added to varying concentrations of GTPγS (A) and a concentration of GTPγS fixed at 1 μM with varying concentrations of GDP (B). The dependencies on GTPγS and GDP concentrations for each mutant were simultaneously fit by eq 6 to estimate k_r, k_{-1}, and k_{+2} (solid lines for Giwt and dashed lines for mutants). Each point represents the average of at least three independent experiments, and the error bars represent standard deviations.

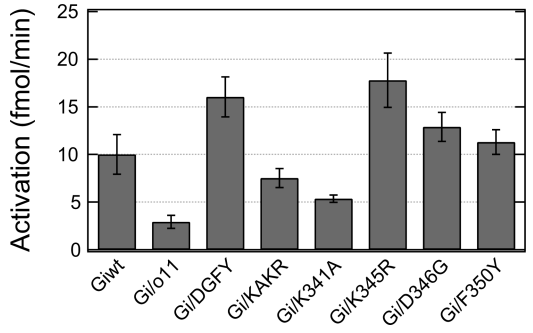


Figure 5. Spontaneous activation efficiencies of Gis. Rates of GTPγS uptake were estimated in the presence of 1 μM GTPγS. No GDP or rhodopsin was added. Each bar represents the average of at least three independent experiments, and the error bars represent standard deviations.

DISCUSSION

In this study, we showed that replacement of amino acid residues in the C-terminus of Giα changes rate constants of MII-induced GDP release, GDP uptake, and GTP uptake, clearly indicating that the C-terminus of Giα affects not only the interaction with R* but also the GDP−GTP exchange reaction. The finding that the spontaneous nucleotide exchange of Gi was also affected by the C-terminal mutations of Giα also supports this. It has been reported that the activation efficiency of the G protein is affected by mutations distant from the nucleotide-binding site.[7,24] Characterization of Gi/K341L showed that this mutation enhances complex formation, GDP release, and GTP uptake.[7] This work further showed that mutations of other amino acid residues in the C-terminal tail of Giα, even the C-terminal amino acid at position 350, also affect the rate constants of nucleotide binding and release, suggesting that this correlation is not specific for Lys341. Therefore, the overall structure and/or the dynamics of C-terminal tail of Giα would regulate the affinity of nucleotides.

Table 1. Rate Constants of GDP binding (k_{-1}) and GTP Binding (k_{+2}) and Apparent Rate Constants of the Formation of the MII·$G_{[empty]}$ Complex from MII and $G_{[GDP]}$ (k_r) of Giwt and Its Mutants Calculated by Global Fitting Analysis

	k_{-1} ($\times 10^{-3}$ μM^{-1} s^{-1})	k_{+2} ($\times 10^{-3}$ μM^{-1} s^{-1})	k_r ($\times 10^{-3}$ s^{-1})
Giwt	2.18 ± 1.24[a]	99.2 ± 2.2	479 ± 7
Gi/o11	60.9 ± 23.6	196 ± 61	41.7 ± 2.9
Gi/DGFY	27.3 ± 4.4	152 ± 11	158 ± 5
Gi/KAKR	76.8 ± 13.3	318 ± 25	388 ± 13
Gi/K341A	78.5 ± 19.8	388 ± 75	122 ± 6
Gi/K345R	0.36 ± 1.03	82.0 ± 3.1	246 ± 5
Gi/D346G	41.8 ± 2.1	223 ± 5	217 ± 2
Gi/F350Y	11.4 ± 1.7	153 ± 7	194 ± 4

[a]Standard deviation calculated by curve fitting analysis.

Previous reports showed that the 11-mer peptide derived from the C-terminal sequence of Gtα (Gt-CT) binds to MII, mimicking Gt binding.[12] It was also reported that the affinities of Gt-CT for MII depend on the amino acid residue corresponding to Lys341.[25] Although Gt-CT having the K341A mutation can bind to MII as efficiently as the wild-type peptide,[25] our spectroscopic experiments indicate that the amount of eMII is reduced (Figure 3A) and the affinity for nucleotides is affected (Table 1) by the K341A mutation in Giα. This indicates that K341 affects both the local structure of the C-terminal region and the internal structure of Giα, which in turn regulates the binding to nucleotides as well as the interaction with MII.

The amount of eMII induced by the Gi mutant (Figure 3C) negatively correlates with the rate constant of uptake of GDP by MII·$G_{[empty]}$ (k_{-1}) (correlation coefficient of −0.64) (Figure 6). Under our experimental condition, the concentration of Gi is at saturation level for eMII. Therefore, this tendency suggests that binding of $G_{[GDP]}$ is less effective for eMII than $G_{[empty]}$, which is

dx.doi.org/10.1021/bi201702d | Biochemistry 2012, 51, 2768−2774

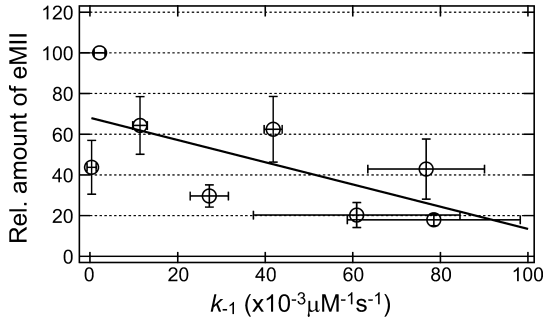


Figure 6. Correlation between the amount of eMII and the rate constant of GDP uptake of MII·$G_{[empty]}$. The relative amount of eMII (Figure 3A) was plotted vs the rate constant of GDP uptake of MII·$G_{[empty]}$ (k_{-1}), which was fitted with a line (correlation coefficient of −0.64). The error bars for the vertical axis and horizontal axis represent the standard deviations of experimental values (Figure 3C) and global fitting analyses (Table 1), respectively.

consistent with previous report that the binding affinity of $G_{[empty]}$ and MII is much higher than that of $G_{[GDP]}$ and MII.[26] If the conformational change in Giα induced by the interaction with MII decreases k_{-1}, it in turn contributes to the shift of the equilibrium between MI and MII. Our results suggest that the formation of the active complex is regulated by the affinity for GDP as well as the structure of the binding interface in the C-terminal region of Giα.

The rate constants of MII-induced binding of GDP to Gi mutants (k_{-1}) strongly correlate with those of GTPγS binding (k_2) (correlation coefficient of 0.93) (Figure 7), indicating that

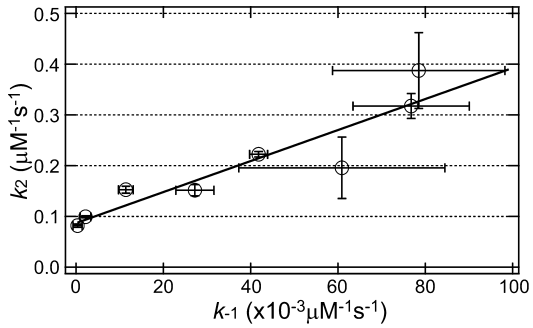


Figure 7. Correlation between the affinities for GDP and GTPγS. Rate constants of MII-induced GTPγS uptake (k_2) were plotted vs those of GDP uptake (k_{-1}), which was fitted with a line (correlation coefficient of 0.93). The error bars represent standard deviations of global fit analyses (Table 1).

the structural change of the C-terminus of Giα by the mutations alters the accessibility to the nucleotide-binding site. However, it should be noted that the regression line did not pass through the origin of the coordinates. This indicates that Gi with small k_2 and k_{-1} values such as Giwt almost exclusively binds to GTP, while Gi with large k_2 and k_{-1} values readily binds to GDP and GTP and shows poor selectivity of nucleotides. The poor accessibility to the nucleotide-binding site results in slow GTPγS uptake, but it is likely to be essential to avoid the reuptake of GDP.

Among all Gi mutants, those having the K341A mutation (Gi/o11, Gi/KAKR, and Gi/K341A) have larger k_{-1} and k_{+2} values than Giwt and other mutants (Table 1), indicating that K341 largely reduces the accessibility of GDP and GTP. Conversely, Giwt and mutants in which K341 is conserved have larger apparent constants for dissociation of GDP from Giα (k_r/k_{-1}) than those with the K341A mutation. Therefore, K341 is a key residue for efficient GDP release in the GDP−GTP exchange reaction. Noting that a high concentration of GTP is present inside cells (>100 μM[27]), the small k_2 would not be a disadvantage for the whole activation efficiency of the G protein. On the other hand, because of the significant concentration of GDP inside cells (>10 μM[27]), a large k_r and a small k_{-1} are essential for efficient activation of the G protein. Therefore, it is likely that Gi has been optimized to release GDP by suppressing the reuptake of GDP at the cost of slow GTP uptake, and K341 is essential for this nature of Gi.

We showed that the rate of spontaneous nucleotide exchange of Gi is affected by its C-terminal sequence (Figure 5). Although it has been reported that the spontaneous nucleotide exchange of Go is much faster than that of Gi, that of Gi/o11 is much slower than that of Giwt.[28] Therefore, it is likely that the nucleotide exchange involves multiple regions of Gi. In fact, deletion of the N-terminus of Giα changes the efficiency of nucleotide exchange, meaning that the N-terminus also affects the efficiency of GDP−GTP exchange.[29]

Recently, the crystal structures of the complexes of MII and the Gt-CT analogue and the active β-adrenergic receptor and Gs were determined.[5,30] In both structures, the C-terminus of Gα forms a characteristic structure called the "C-cap helical structure", and the amino acid residues studied here (K341, K345, D346, and F350) are oriented toward the receptor rather than the center of Gα. This is largely different from that of receptor-free Gi in which the C-terminal structure was not resolved.[31,32] The results presented here indicate that the C-terminus of Gi also controls GDP−GTP exchange, implying that the formation of the C-cap structure by R* induces structural changes in the nucleotide-binding site. Upon observation of these structures, helix α5 of Gs bound to the adrenergic receptor[30] is elongated compared to that of GDP-bound Gi.[31] In the structure of Gt-CT with MII, the side chain of K341 is close to that of F350, both of which interact with intracellular loops of MII by hydrophobic interaction.[5] The K341A mutation would affect the formation of the C-cap structure because alanine has a shorter alkyl chain than lysine, resulting in the changes in the affinity for nucleotides being larger than those of other mutations.

We have capitalized on the advantage of rhodopsin in the amount of the complex of the active state and Gi is estimated by spectroscopic measurements, which is independent of the biochemical assay of Gi activation efficiency. Crystal structures of MII and the β-adrenergic receptor[5,30] suggest that the helical arrangements of MII and the active β-adrenergic receptor are quite similar, indicating that these GPCRs have a shared activation mechanism. Thus, our findings that the C-terminus of Gα regulates the affinity of the nucleotide might be applicable for the other GPCRs.

■ CONCLUSION

We showed a direct interlink between the C-terminus and the nucleotide-binding site in Gα. The C-terminus regulates the affinities of Giα for GDP and GTP in addition to the interaction with R*. Given the physiological conditions of the cell, it is likely that the G protein was optimized to enhance release of GDP, rather than uptake of GTP, for which K341 was identified as a key residue.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan. Telephone: +81-75-753-4213. Fax: +81-75-753-4210. E-mail: shichida@rh.biophys.kyoto-u.ac.jp.

Funding

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan [Grants-in-Aid for Scientific Research to Y.S. (20227002, 60127090), Y.I. (23370070, 23107715), and T.Y. (23770074)]. N.K. and Y.S. were supported by Grants-in-Aid for Scientific Research and the Global Center of Excellence Program "Formation of a Strategic Base for Biodiversity and Evolutionary Research: From Genome to Ecosystem", and T.M. was supported by Grant-in-Aid for JSPS Fellows 10J01416.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

GPCR, G protein-coupled receptor; R*, active state of GPCR; MII, metarhodopsin II; MI, metarhodopsin I; eMII, extra formation of MII; Giwt, wild-type Gi; Gα, α subunit of G protein; ROS, rod outer segment(s); GTPγS, guanosine 5′-*O*-(γ-thio)triphosphate; $G_{[GDP]}$, $G_{[empty]}$, and $G_{[GTP]}$, GDP-bound, nucleotide-unbound, and GTP-bound G protein, respectively; DM, *n*-dodecyl β-D-maltoside.

■ REFERENCES

(1) Shichida, Y., and Imai, H. (1998) Visual pigment: G-protein-coupled receptor for light signals. *Cell. Mol. Life Sci. 54*, 1299−1315.
(2) Bockaert, J., and Pin, J. P. (1999) Molecular tinkering of G protein-coupled receptors: An evolutionary success. *EMBO J. 18*, 1723−1729.
(3) Gether, U. (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr. Rev. 21*, 90−113.
(4) Scheerer, P., Park, J. H., Hildebrand, P. W., Kim, Y. J., Krauss, N., Choe, H. W., Hofmann, K. P., and Ernst, O. P. (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature 455*, 497−502.
(5) Choe, H. W., Kim, Y. J., Park, J. H., Morizumi, T., Pai, E. F., Krauss, N., Hofmann, K. P., Scheerer, P., and Ernst, O. P. (2011) Crystal structure of metarhodopsin II. *Nature 471*, 651−655.
(6) Oldham, W. M., and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat. Rev. Mol. Cell Biol. 9*, 60−71.
(7) Herrmann, R., Heck, M., Henklein, P., Kleuss, C., Hofmann, K. P., and Ernst, O. P. (2004) Sequence of interactions in receptor-G protein coupling. *J. Biol. Chem. 279*, 24283−24290.
(8) Kanaho, Y., Tsai, S. C., Adamik, R., Hewlett, E. L., Moss, J., and Vaughan, M. (1984) Rhodopsin-enhanced GTPase activity of the inhibitory GTP-binding protein of adenylate cyclase. *J. Biol. Chem. 259*, 7378−7381.
(9) Terakita, A., Yamashita, T., Nimbari, N., Kojima, D., and Shichida, Y. (2002) Functional interaction between bovine rhodopsin and G protein transducin. *J. Biol. Chem. 277*, 40−46.
(10) Lee, E., Linder, M. E., and Gilman, A. G. (1994) Expression of G-protein α subunits in *Escherichia coli*. *Methods Enzymol. 237*, 146−164.
(11) Emeis, D., Kuhn, H., Reichert, J., and Hofmann, K. P. (1982) Complex formation between metarhodopsin II and GTP-binding protein in bovine photoreceptor membranes leads to a shift of the photoproduct equilibrium. *FEBS Lett. 143*, 29−34.
(12) Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988) Site of G protein binding to rhodopsin mapped with synthetic peptides from the α subunit. *Science 241*, 832−835.
(13) Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. (1993) Substitution of three amino acids switches receptor specificity of $G_q\alpha$ to that of $G_i\alpha$. *Nature 363*, 274−276.
(14) Kisselev, O. G., Ermolaeva, M. V., and Gautam, N. (1994) A farnesylated domain in the G protein γ subunit is a specific determinant of receptor coupling. *J. Biol. Chem. 269*, 21399−21402.
(15) Kisselev, O. G., Meyer, C. K., Heck, M., Ernst, O. P., and Hofmann, K. P. (1999) Signal transfer from rhodopsin to the G-protein: Evidence for a two-site sequential fit mechanism. *Proc. Natl. Acad. Sci. U.S.A. 96*, 4898−4903.
(16) Natochin, M., Muradov, K. G., McEntaffer, R. L., and Artemyev, N. O. (2000) Rhodopsin recognition by mutant $G_s\alpha$ containing C-terminal residues of transducin. *J. Biol. Chem. 275*, 2669−2675.
(17) Slessareva, J. E., Ma, H., Depree, K. M., Flood, L. A., Bae, H., Cabrera-Vera, T. M., Hamm, H. E., and Graber, S. G. (2003) Closely related G-protein-coupled receptors use multiple and distinct domains on G-protein α-subunits for selective coupling. *J. Biol. Chem. 278*, 50530−50536.
(18) Morizumi, T., Kimata, N., Terakita, A., Imamoto, Y., Yamashita, T., and Shichida, Y. (2009) G protein subtype specificity of rhodopsin intermediates metarhodopsin Ib and metarhodopsin II. *Photochem. Photobiol. 85*, 57−62.
(19) Morizumi, T., Imai, H., and Shichida, Y. (2005) Direct observation of the complex formation of GDP-bound transducin with the rhodopsin intermediate having a visible absorption maximum in rod outer segment membranes. *Biochemistry 44*, 9936−9943.
(20) Shichi, H., and Somers, R. L. (1978) Light-dependent phosphorylation of rhodopsin. Purification and properties of rhodopsin kinase. *J. Biol. Chem. 253*, 7040−7046.
(21) Fukada, Y., Matsuda, T., Kokame, K., Takao, T., Shimonishi, Y., Akino, T., and Yoshizawa, T. (1994) Effects of carboxyl methylation of photoreceptor G protein γ-subunit in visual transduction. *J. Biol. Chem. 269*, 5163−5170.
(22) Preininger, A. M., Parello, J., Meier, S. M., Liao, G., and Hamm, H. E. (2008) Receptor-mediated changes at the myristoylated amino terminus of $G\alpha_{i1}$ proteins. *Biochemistry 47*, 10281−10293.
(23) Heck, M., and Hofmann, K. P. (2001) Maximal rate and nucleotide dependence of rhodopsin-catalyzed transducin activation: Initial rate analysis based on a double displacement mechanism. *J. Biol. Chem. 276*, 10000−10009.
(24) Marin, E. P., Krishna, A. G., and Sakmar, T. P. (2001) Rapid activation of transducin by mutations distant from the nucleotide-binding site: Evidence for a mechanistic model of receptor-catalyzed nucleotide exchange by G proteins. *J. Biol. Chem. 276*, 27400−27405.
(25) Herrmann, R., Heck, M., Henklein, P., Kleuss, C., Wray, V., Hofmann, K. P., and Ernst, O. P. (2006) Rhodopsin-transducin coupling: Role of the Gα C-terminus in nucleotide exchange catalysis. *Vision Res. 46*, 4582−4593.
(26) Bennett, N., and Dupont, Y. (1985) The G-protein of retinal rod outer segments (transducin). Mechanism of interaction with rhodopsin and nucleotides. *J. Biol. Chem. 260*, 4156−4168.
(27) Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) The GTPase superfamily: Conserved structure and molecular mechanism. *Nature 349*, 117−127.
(28) Linder, M. E., Ewald, D. A., Miller, R. J., and Gilman, A. G. (1990) Purification and characterization of $G_{o\alpha}$ and three types of $G_{i\alpha}$ after expression in *Escherichia coli*. *J. Biol. Chem. 265*, 8243−8251.
(29) Herrmann, R., Heck, M., Henklein, P., Hofmann, K. P., and Ernst, O. P. (2006) Signal transfer from GPCRs to G proteins: Role of the Gα N-terminal region in rhodopsin-transducin coupling. *J. Biol. Chem. 281*, 30234−30241.
(30) Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature 477*, 549−555.
(31) Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) The structure of the G protein heterotrimer $G_{i\alpha1}\beta_1\gamma_2$. *Cell 83*, 1047−1058.
(32) Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature 379*, 311−319.