Structural Basis for Modulation of Gating Property of G Protein-gated Inwardly Rectifying Potassium Ion Channel (GIRK) by i/o-family G Protein α Subunit (Gα_{i/o})

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Background: Although Gβγ is known to activate GIRK, Gα_{i/o} also modulates GIRK gating.

Results: The α2/α3 helices of Gα_{i3} in the GTP-bound state directly bind to the αα helix of GIRK.

Conclusion: The complex model explains how Gα_{i/o} sequesters Gβγ efficiently from GIRK upon GTP hydrolysis.

Significance: The structural basis for the rapid closure of GIRK by Gα_{i/o} is provided.

G protein-gated inwardly rectifying potassium channel (GIRK) plays a crucial role in regulating heart rate and neuronal excitability. The gating of GIRK is regulated by the association and dissociation of G protein βγ subunits (Gβγ), which are released from pertussis toxin-sensitive G protein α subunit (Gα_{i/o}) upon GPCR activation in vivo. Several lines of evidence indicate that Gα_{i/o} also interacts directly with GIRK, playing functional roles in the signaling efficiency and the modulation of the channel activity. However, the underlying mechanism for GIRK regulation by Gα_{i/o} remains to be elucidated. Here, we performed NMR analyses of the interaction between the cytoplasmic region of GIRK1 and Gα_{i3} in the GTP-bound state. The NMR spectral changes of Gα upon the addition of GIRK as well as the transferred cross-saturation (TCS) results indicated their direct binding mode, where the Kd value was estimated as ~1 μM. The TCS experiments identified the direct binding sites on Gα and GIRK as the α2/α3 helices on the GTPase domain of Gα and the αα helix of GIRK. In addition, the TCS and paramagnetic relaxation enhancement results suggested that the helical domain of Gα transiently interacts with the αα helix of GIRK. Based on these results, we built a docking model of Gα and GIRK, suggesting the molecular basis for efficient GIRK deactivation by Gα_{i/o}.

G protein-gated inwardly rectifying potassium ion channel (GIRK) is a member of the inwardly rectifying potassium channel (Kir) family, regulating heart rate, neuronal excitability, and other physiological events. GIRK functions as a tetramer in which a long, ~88 Å K⁺ permeation pathway is formed by the transmembrane (TM) and cytoplasmic pore (CP) regions. A structural study of GIRK revealed two K⁺ ion gates: the helix bundle crossing on the cytoplasmic side of the TM region and the loops on the membrane side of the CP region, referred to as G-loops. Although the gating of Kir family proteins is generally regulated by phosphatidylinositol 4,5-bisphosphate, the opening of the GIRK gate is also triggered by the direct binding of the G protein βγ subunits (Gβγ). The stimulation of G protein-coupled receptors (GPCRs) causes the exchange of GDP with GTP on the α subunit (Gα), which decreases the affinity of Gα for Gβγ. Gβγ then dissociates from Gα in the GTP-bound state (Gα(GTP)) and associates with GIRK, resulting in the opening of the GIRK gate.

Although Gβγ directly binds to and activates GIRK, Gα also plays important roles in regulating GIRK. In native tissues, GIRK is activated only through the stimulation of GPCRs that couple to the G proteins belonging to the i/o-family.
family (G_{i/o}) (13). The activation and the deactivation of GIRK are also accelerated when the channels are co-expressed with G_{i/o} (14–16).

Efficient GIRK activation seems to be attributed to the preassociation of the heterotrimeric G protein in the i/o-family (G_{i/o}(GDP)βγ) with GIRK, in which G_{i/o} is directly associated with both GPCR and GIRK before GPCR activation (17, 18). Furthermore, the direct association of G_{i/o}(GDP)βγ with GIRK in the absence of GPCR-stimulation reportedly suppresses the basal activity of GIRK while maintaining the maximal evoked current by Gβγ in the presence of GPCR stimulation (14, 15, 19–22). This regulation mechanism is called “priming,” by which G_{i/o} reportedly plays an important role by facilitating a better response to GPCR activation.

On the other hand, the deactivation is accelerated by the direct interaction of G_{i/o}(GTP) with GIRK (18). The interaction enables G_{i/o} to rapidly bind to Gβγ upon GTP hydrolysis, which is presumably assisted by the regulator of G protein signaling (RGS) (23), resulting in the closure of the GIRK gate. Because the re-association of G_{i/o}(GDP) and Gβγ allows G_{i/o}(GDP)βγ to enter the next cycle of GIRK activation, the rapid reassociation would also accelerate the activation of GIRK (15). Thus, the direct interaction between GIRK and G_{i/o}(GTP) could contribute to the efficient activation and deactivation of the GIRK channel. In a FRET study investigating the conformational changes of the GIRK1/2 channel in Xenopus oocytes, the coexpression of G_{i/o}(GTP) with Gβγ conferred different FRET patterns, and the maximal GIRK current, which is presumably assisted by the regulator of G protein signaling, was modulated by G_{i/o} (24). Altogether, it is now evident that G_{i/o} not only functions as the donor and acceptor of Gβγ but also modulates the gating property of GIRK. To reveal the underlying molecular mechanism by which the G_{i/o} modulates GIRK, several studies have proved the interactions between G_{i/o} and the cytoplasmic region of GIRK. G_{i/o} binding to the N- and C-terminal fragments of GIRK was examined by pulldown assays in vitro (14, 15, 19, 20, 22, 24, 25), indicating that the C-terminal region of GIRK is essential for the i/o-family-specific binding to G_{i/o}. On the other hand, light is known about the GIRK binding site on G_{i/o}. Ga consists of a GTPase domain and a helical domain (26, 27) in which GDP or GTP is sandwiched by both domains and GPCRs bind to the GTPase domain (28). An electrophysiological study using a G_{i/o}/Ga chimeric, in which the helical domain was replaced, revealed that the helical domain of Gα is responsible for the specific activation of the GIRK channel (25). Thus, it remains unclear how G_{i/o} modulates the GIRK gating property, and therefore, the elucidation of the molecular recognition mode of G_{i/o} and GIRK is required.

In this study we performed the NMR analyses of the interaction between the cytoplasmic region of GIRK1 and G_{i/o}(GTP), which accelerates the deactivation of GIRK. The NMR spectral changes of Ga upon the addition of GIRK as well as the transferred cross-saturation (TCS) results indicated that Ga and the cytoplasmic region of GIRK1 directly bind to each other, with an estimated $K_D$ value of $\sim1$ mm. We identified the binding sites on Ga and GIRK, respectively, and examined the binding mode by paramagnetic relaxation enhancement (PRE) experiments. Based on these results, we built a docking model of Ga and the cytoplasmic region of GIRK, suggesting the molecular basis for the efficient deactivation of the channel by G_{i/o}.

**Experimental Procedures**

**Expression and Purification of Cytoplasmic Regions of Mouse GIRK1**—The N- and C-terminal cytoplasmic regions of mouse GIRK1 (residues 41–63 and 190–386) were fused into a single polypeptide, which is hereafter referred to as GIRK_{C-P,L}. The C-terminal region of GIRK_{C-P,L} is 15 residues longer than that of GIRK_{C,P} (residues 41–63 and 190–371). We previously analyzed the interaction of GIRK_{C-P} with Gβγ and confirmed its validity by the crystal structure (29). Preliminary TCS experiments using GIRK_{C-P} and Ga3 indicated that the C-terminal region of GIRK_{C-P} was involved in the interaction with G_{i/o}, suggesting that some interacting residues are missing in GIRK_{C-P}. Therefore, we extended the GIRK_{C-P} construct for 15 amino acids to obtain GIRK_{C-P,L}, which was used for the further analyses.

GIRK_{C-P,L} was expressed in Escherichia coli cells. The uniformly $^2$H, $^{15}$N-labeled GIRK_{C-P,L} samples for the NMR analyses were prepared by growing E. coli in M9 minimal medium containing $^{14}$NH$_4$Cl, $[^3$H]glucose, and $[^3$H/$^{15}$N]Celtone base powder in 99% $^2$H$_2$O. GIRK_{C-P,L} was purified according to the same procedure as for GIRK_{C,P} (30). GIRK_{C-P,L} mainly eluted as a tetramer from the size exclusion chromatography column. The uniformly $^2$H, $^{15}$N-labeled and uniformly $^2$H-labeled GIRK_{C-P,L} samples for the TCS experiments were prepared without the denaturing and refolding procedure to preserve the amide hydrogen atoms as $^2$H in the core of the protein. The GIRK_{C-P,L} samples were incubated at 303 K for $>48$ h before the TCS experiments to prevent $^2$H/$^1$H exchange during the TCS measurements.

**Assignments of NMR Signals of GIRK_{C-P,L}**—The $^1$H,$^{15}$N transverse relaxation-optimized spectroscopy (TROSY) signals of GIRK_{C-P,L} were well dispersed and mostly overlapped with those of GIRK_{C,P}. The backbone assignments of residues 41–63 and 190–366 of GIRK_{C-P,L} were transferred from those of GIRK_{C,P} (BioMagResBank accession number 11067) (30). The assignments of residues 367–377 were established by triple resonance experiments (HNCACB and HNCA) (31, 32) using uniformly $^2$H,$^{13}$C,$^{15}$N-labeled GIRK_{C-P,L} as shown in supplemental Table 1. The backbone amide resonances for Asn-378–Val-386 could not be assigned because their signals were very weak or not observed, probably due to either the fast exchange of their amide hydrogen atoms with those of water molecules or the line-broadening due to conformational exchange.

**Expression and Purification of Ga3**—The Ga3 protein, with an N-terminal decahistidine tag followed by an HRV3C protease cleavage site, was expressed in E. coli cells. The uniformly $^2$H, $^{15}$N-labeled Ga3 samples for NMR analyses were prepared by growing E. coli in M9 minimal medium containing $^{14}$NH$_4$Cl, $[^3$H$]^{15}$N]Celtone base powder in 99% $^2$H$_2$O. Ga3 was purified as described (12, 33). Briefly, Ga3 was purified to homogeneity by chromatography on a HIS-Select Nickel Affinity Gel (Sigma) column, His-tag cleavage by Pre-
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Scission\textsuperscript{TM} Protease (GE Healthcare), and removal of the cleaved His tags and the PreScission\textsuperscript{TM} Protease (GE Healthcare) by HIS-Select\textsuperscript{TM} Nickel Affinity Gel (Sigma).

Spin Labeling of \( \alpha_{i3} \)—For PRE experiments, we first prepared the \( \alpha_{i3} \) mutant referred to as Hexa III, in which all six exposed Cys residues were substituted (\( \alpha_{i3} \)-C3S-C66A-C214S-C305S-C325A-C351I), according to the previous report (34). Using this construct as a template, cysteine substitutions were separately introduced to Ile-82 and Ser-153 by the QuikChange\textsuperscript{®} system (Stratagene). All mutations were confirmed by DNA sequencing.

Spin-labeling was performed in a buffer containing 10 mM Hepes-NaOH (pH 7.0), 50 mM KCl, 10 mM MgCl\(_2\), and 0.60 mM GTP\(_\gamma\)S. The \( \alpha_{i3} \) mutants were incubated with S-(1-oxo-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSL) at a molar ratio of 1:3 for each protein:MTSL at room temperature for 4 h. Under these conditions we confirmed that only the most reactive cysteine residue was modified, whereas the remaining buried native cysteine residues were not modified. The excess MTSL was removed by extensive washes with the buffer by ultrafiltration using an Amicon Ultra filter unit (Millipore). For the diamagnetic state experiments, ascorbate was then added to the MTSL-labeled \( \alpha_{i3} \) mutants at a molar ratio of 1:3 protein:ascorbate, and the solution was incubated at 4 °C for 12 h. The ascorbate was then removed by ultrafiltration. Thus, we prepared the MTSL-labeled Hexa III in the diamagnetic state.

**NMR Analyses**—All NMR experiments were performed on a Bruker Avance 600 spectrometer equipped with a cryogenic probe. The \(^1\)H,\(^15\)N TROSY spectra of \( \alpha_{i3} \) in the presence of various amounts of GIRK\(_{CP-1}\) were observed at 308K. TCS and PRE experiments were performed at 303 K. All spectra were processed by the Bruker TopSpin 2.1 software, and the data were analyzed by Sparky (T. D. Goddard and D. G. Kneller, Sparky 3, University of California, San Francisco, CA). The error bars are based on the signal-to-noise ratio calculated by the Sparky software. The backbone NMR signal assignments of \( \alpha_{i3} \) were reported previously (35).

To examine the spectral changes of \( \alpha_{i3} \) induced by the presence of GIRK\(_{CP-1}\), we prepared five samples, each containing the uniformly \(^2\)H,\(^15\)N-labeled \( \alpha_{i3} \) (0.28 mM) mixed with GIRK\(_{CP-1}\) at molar ratios (\( \alpha_{i3}:\text{GIRK}_{CP-1} \)) tetramer) of 1:0, 1:0.9, 1:1.8, 1:2.7, and 1:3.6 in the NMR sample buffer (10 mM HEPES-NaOH (pH 7.0), 50 mM KCl, 10 mM MgCl\(_2\), 0.6 mM GTP\(_\gamma\)S, 5 mM DTT), 1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), 10% \(^2\)H\(_2\)O, 90% \(^1\)H\(_2\)O). We then observed the \(^1\)H,\(^15\)N TROSY spectrum of each sample. The residues with overlapping resonances were omitted from the analyses.

The TCS experiments were performed as described with minor modifications (12, 36). In the TCS experiments observing \( \alpha_{i3} \), a solution containing uniformly \(^2\)H,\(^15\)N-labeled \( \alpha_{i3} \) (0.3 mM) and unlabeled GIRK\(_{CP-1}\) (0.25 mM as a tetramer) was prepared in buffer (10 mM HEPES-NaOH (pH 6.5), 50 mM KCl, 5 mM DTT, 1 mM DSS, 0.8 mM GTP\(_\gamma\)S, 20% \(^2\)H\(_2\)O, 80% \(^1\)H\(_2\)O). The saturation frequency was set at 0.83 ppm, and the maximum radiofrequency amplitude was 0.17 kHz for WURST-2 (adiabatic factor \( Q_0 = 1 \)). The saturation duration and the relaxation delay were set at 1.5 and 2.5 s, respectively. To evaluate the effect of the residual aliphatic protons within \( \alpha_{i3} \), TCS experiments were also performed under the same conditions as those mentioned above, with the sample containing uniformly \(^2\)H,\(^15\)N-labeled \( \alpha_{i3} \) (0.3 mM) and uniformly \(^1\)H-labeled GIRK\(_{CP-1}\) (0.25 mM as a tetramer). It should be noted that the residues with signal overlapping and/or signal-to-noise ratios less than 10 were excluded from the analyses.

In the TCS experiments observing GIRK\(_{CP-1}\) a solution containing uniformly \(^2\)H,\(^15\)N-labeled GIRK\(_{CP-1}\) (0.25 mM as a tetramer) and unlabeled \( \alpha_{i3} \) (0.4 mM) was prepared in buffer (10 mM HEPES-NaOH (pH 6.5), 50 mM KCl, 5 mM DTT, 5 mM GSH, 0.8 mM GTP\(_\gamma\)S, 20% \(^2\)H\(_2\)O, 80% \(^1\)H\(_2\)O). The saturation frequency was set at 0.83 ppm, and the maximum radiofrequency amplitude was 0.17 kHz for WURST-2 (adiabatic factor \( Q_0 = 1 \)). The saturation duration and the relaxation delay were set at 3.0 and 2.0 s, respectively. To evaluate the effect of the residual aliphatic protons within \( \alpha_{i3} \), TCS experiments were also performed under the same conditions as those mentioned above, with the sample containing uniformly \(^2\)H,\(^15\)N-labeled GIRK\(_{CP-1}\) (0.25 mM as a tetramer) and uniformly \(^1\)H-labeled \( \alpha_{i3} \) (0.4 mM). It should be noted that the residues with signal overlapping and/or signal-to-noise ratios less than 10 were excluded from the analyses.

In the PRE experiments of the paramagnetic state, samples containing uniformly \(^3\)H,\(^15\)N-labeled GIRK\(_{CP-1}\) (0.075 mM as a tetramer) mixed with 0.2 mM oxidized Hexa III-Cys-MTSL(red) were prepared. In the experiments of diamagnetic state, samples containing uniformly \(^3\)H,\(^15\)N-labeled GIRK\(_{CP-1}\) (0.075 mM as a tetramer) mixed with reduced Hexa III-Cys-MTSL(red) in buffer (10 mM HEPES-NaOH (pH 7.0), 50 mM KCl, 1 mM DSS, 10% \(^2\)H\(_2\)O, 90% \(^1\)H\(_2\)O) were prepared. The \(^1\)H,\(^15\)N TROSY spectrum of each sample was recorded. The residues with overlapping resonances were omitted from the analyses. PRE was calculated as paramagnetic to diamagnetic signal intensity ratios (\( I_{\text{para}}/I_{\text{dia}} \)) (37).

Construction of Complex Models—The complex models of Ga-GIRK and Ga-GIRK-\( \gamma \) were obtained with the HADDOCK software (38).

First, we built a homology model of \( \alpha_{i3}(GTP) \) by the MODELLER software (39) using the crystal structure of \( \alpha_{i3}(GTP\gamma)S \) (PDB code 1GIA) as a template, whose amino acid sequence is 94% identical to that of \( \alpha_{i3} \). The crystal structure of GIRK\(_{CP} \) (PDB code 1N9P) and the structure of \( \gamma \) in the crystal structure of \( \alpha_{i3}(GDP)\gamma)S \) (PDB code 1GP2) were used, respectively, for dockings. The active residues used in the definition of the ambiguous interaction restraints for docking are listed in supplemental Table 2.

The Ga-GIRK-\( \gamma \) ternary complex model was built as follows. First, we built a docking model of the \( \gamma \)-GIRK complex with parameters listed in supplemental Table 2. Then, the Ga-GIRK and \( \gamma \)-GIRK complex models were superimposed by the GIRK structure.

**RESULTS**

**NMR Spectral Changes of \( \alpha_{i3} \) upon Binding to GIRK\(_{CP-1}\)**—To investigate the direct binding between \( \alpha_{i3} \) and GIRK\(_{CP-1}\), we observed a series of \(^1\)H,\(^15\)N TROSY spectra of 0.28 mM uni-
formally $^2$H, $^{15}$N-labeled $\Gamma\alpha_{i3}$ in the absence or presence of 0.25, 0.50, 0.75, and 1.0 mM GIRK$_{CP-L}$ as a tetramer. As the concentration of GIRK$_{CP-L}$ increased, most signals exhibited decreased intensity due to line-broadening without changing their chemical shifts, whereas a number of signals exhibited further intensity reductions and eventually disappeared in the presence of 0.75 mM GIRK$_{CP-L}$. In addition, several signals exhibited small but significant chemical shift changes (Fig. 1, A and B). Although the overall intensity reductions are caused by the slowing of the overall tumbling motion upon binding to GIRK$_{CP-L}$, the further intensity reductions and apparent chemical shift changes reflect the direct binding of GIRK$_{CP-L}$ to $\Gamma\alpha_{i3}$. The weighted averages of the chemical shift differences ($\Delta \delta$) were calculated using the equation ($\Delta \delta = [\sum \Delta \delta(i)/(\sum \Delta \delta(i)^2)]^{1/2}$). The titrations curves of $\Delta \delta$ were fit to the following theoretical formula to obtain the value of the dissociation constant ($K_d$).

$$
\Delta \delta = \frac{[\text{GIRK}_{CP-L} + [\Gamma\alpha_{i3}]] - K_d \cdot [\text{GIRK}_{CP-L}][\Gamma\alpha_{i3}]}{2[\text{GIRK}_{CP-L}]}
$$

(Eq. 1)

where $\Delta \delta_{sat}$ is the $\Delta \delta$ value when a saturating amount of GIRK$_{CP-L}$ is added (ppm), and $[\Gamma\alpha_{i3}]_{tot} = 0.28$ mM. The fitting of the titration curves of the chemical shift changes for the signals from Lys-209 and Trp-258 resulted in dissociation constant ($K_d$) values of 0.6 and 1.1 mM, respectively (Fig. 1C).

We evaluated the apparent chemical shift differences ($\Delta \delta$) of $\Gamma\alpha_{i3}$ in the absence of GIRK$_{CP-L}$ and the presence of 0.75 mM GIRK$_{CP-L}$, in which 35–50% of $\Gamma\alpha_{i3}$ was in the GIRK$_{CP-L}$-bound state, as estimated by the $K_d$ value of 0.6–1.1 mM (Fig. 1D). The residues with significant chemical shift changes are located in two regions; that is, the region from the $\alpha$2 helix and the following loop (Glu-207, Lys-209, Trp-211, His-213, Phe-215, and Glu-216) and the region from the $\alpha$3 helix and the following loop (Ser-246 and Trp-258). The other residues, Arg-312 and Thr-316, are located on the loop between the $\alpha$4 helix and the $\beta$6 strand. On the other hand, the residues with significant intensity reductions are located around the GTP binding site, in which Ser-44, Gly-45, Lys-46, Ser-47, and Gly-203 directly interact with the phosphate group of GTP$_{\gamma}$S.

As shown in Fig. 1F, the affected residues exhibited significant chemical shift changes, indicating that they are involved in the direct GIRK$_{CP-L}$ binding site and/or in the site(s) exhibiting a conformational change upon binding.

**GIRK$_{CP-L}$ Binding Site on $\Gamma\alpha_{i3}$ Revealed by Transferred Cross-saturation Experiments**—To identify the GIRK$_{CP-L}$ binding site on $\Gamma\alpha_{i3}$, TCS experiments were performed. The saturation of the GIRK$_{CP-L}$ resonances by the irradiation with radio frequency pulses caused the signal intensity reductions of the $^1$H, $^{15}$N TROSY signals of the $\Gamma\alpha_{i3}$ residues, which should be located in the GIRK$_{CP-L}$ binding site (Fig. 2A) (42, 43).

In the case of a larger protein system, such as $\Gamma\alpha_{i3}$ and GIRK$_{CP-L}$, with molecular masses of 41 and 103 kDa, respectively, the enhanced $^1$H homonuclear dipolar-dipolar interactions might cause intramolecular saturation transfer from the residual protons in $\Gamma\alpha_{i3}$ (the exchangeable hydrogen atoms in the NH, OH, and SH groups and/or the hydrogen atoms, due to the incomplete $^2$H-labeling of $^2$H, $^{15}$N-labeled $\Gamma\alpha_{i3}$). To exclude these effects, we also performed a control experiment by using $^2$H-labeled GIRK$_{CP-L}$ instead of the unlabeled (i.e. $^1$H-labeled) protein to reflect only the artificial effects described above (Fig. 2B, supplemental Fig. 1, gray) and subtracted the intensity reduction ratios of this control experiment from those obtained by using unlabeled GIRK$_{CP-L}$ (supplemental Fig. 1, orange). The differences in the reduction ratios, $\Delta RR$, are shown in Fig. 2C, with the error bars calculated based on the signal-to-noise ratios. The minimum values of $\Delta RR$ within the error ranges ($\Delta RR_{min} = \Delta RR - error$) were utilized for the evaluation.

The residues with large intensity reductions ($\Delta RR_{min} > 0.08$, Fig. 2C) are located on the helical domain (Gly-89 in the $\alpha$A helix; Gly-112 and Ala-114 in the $\alpha$B helix) and the GTPase domain (Arg-208, Lys-209, Trp-211, Ile-212, His-213, Glu-216, and Gly-217 in the $\alpha$2 helix and the following loop; Met-240, Lys-248, Leu-249, Ile-253, Asn-256, and Trp-258 in the $\alpha$3 helix and the flanking loops; Glu-186 in the $\beta$2 strand). The mapping of these residues on the structure of $\Gamma\alpha$ revealed that the residues identified on the GTPase domain of $\Gamma\alpha_{i3}$ are clustered, indicating that this site (hereafter, referred to as the “$\alpha$2/$\alpha$3 site”) mainly contributes to the GIRK$_{CP-L}$ binding (Fig. 2D). This is also supported by the two alanine mutants of the identified residues (I212A and W258A) that exhibited impaired
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Binding affinity for GIRK CP-L (supplemental Fig. 2). It should be noted that Glu-186 seems separated from the cluster by the intervening residue, Phe-199 (supplemental Fig. 3). The amide group of Phe-199 is buried in the protein and is more than 6 Å away from the contiguous surface for GIRK CP-L binding, resulting in the lack of cross-saturation for Phe-199. Therefore, we conclude that the α2/α3 site is the major GIRK CP-L binding site.

The residues identified on the helical domain (Gly-89, Gly-112, and Ala-114), which are distant from the α2/α3 site, do not form a contiguous surface but are located on the same side of the protein surface as the α2/α3 site, suggesting that these residues transiently contact GIRK CP-L. We further investigated the interactions of the helical domain with GIRK CP-L (see below).

Gαs Binding Site on GIRK CP-L Revealed by TCS Experiments—Conversely, to identify the Gαs binding site on GIRK CP-L, TCS experiments observing GIRK CP-L were performed. In the same manner as the TCS experiments to identify the binding site on Gαs, we evaluated the cross-saturation from unlabeled Gαs (supplemental Fig. 4, orange) by subtracting the intensity reduction ratios of the control experiment in the presence of 2H-labeled Gαs (supplemental Fig. 4, gray). The differences in the reduction ratios, ΔRR, are shown in Fig. 3A, with the error bars calculated based on the signal-to-noise ratios. The minimum values of ΔRR within the error ranges (ΔRRmin) were utilized for the evaluation.

The residues with large intensity reductions (ΔRR > 0.02) are Glu-242, Val-358, Leu-365, Leu-366, Met-367, Ser-368, Ser-369, Leu-371, Ile-372, and Ala-373 as well as one of the C-terminal unassigned residues (hereafter, referred to as CT1). These residues were mapped on a single subunit (Fig. 3B) and two adjacent subunits (Fig. 3C) of GIRK CP (PDB code 1N9P).

All of these residues, except for Glu-242, are located on the eq of GIRK CP-L (0.75 mM as a tetramer). The signals with chemical shift differences larger than 0.008 ppm are labeled. The signal from Gly-45, which is one of the residues showing significant intensity reduction without a significant chemical shift change, is also labeled in parentheses. B, overlays of the spectra in the presence of 0–2.7 eq of GIRK CP-L (as a tetramer) are displayed for Gly-45 (left) and Trp-258 (right) as typical examples of the signals with significant intensity reductions and chemical shift changes, respectively. C, titration curves of the chemical shift differences for Lys-209 (left) and Trp-258 (right) are shown. D, shown are plots of the chemical shift differences of Gαs in the absence and presence of 2.7 eq of GIRK CP-L (as a tetramer). The error bars were calculated based on the digital resolutions of the spectra. The minimum values of Δω within the error ranges (Δωmin) were utilized for the evaluation. Bars corresponding to the residues with significant chemical shift differences, which have the minimum values of Δω within the error ranges (Δωmin) larger than 0.008 ppm, are colored red. Asterisks in D and E indicate the residues with no data due to signal overlapping, lack of assignments, or insufficient signal-to-noise ratio. The secondary structure elements of Gαs are depicted in gray below the sequence, and α2, α3, α4, and β6 are colored green. E, shown are plots of the normalized intensity ratios (R) of uniformly 2H,15N-labeled Gαs upon the addition of 1.8 eq of GIRK CP-L tetramer to GIRK CP. The intensities of the free Gαs were divided by a scaling factor of 1.78 (see supplemental Methods) and then were used for the calculation of the intensity ratios. The error bars were calculated based on the signal-to-noise ratios. Bars corresponding to the signals with the maximum value of R, within the error ranges (Rmax) lower than 0.42 are colored cyan. F, mapping of the affected residues on the GIRK structure in which 333 of 354 residues (94%) are identical to those of GIRK CP-L (PDB code 1GIA) is shown. The amide nitrogen atoms of the residues with apparent chemical shift differences and intensity reductions are shown as balls colored red and cyan, respectively. Proline residues and residues with no data are colored black. The α2, α3, α4, and β6 are colored green, and GTPγS is depicted by teal sticks.
C-terminal region around the αA helix of GIRK\textsubscript{CP-L} (Fig. 3). Therefore, we concluded that these residues are the $\text{G}_\alpha_i$ binding residues. Glu-242 would be involved in the direct binding to the $\text{G}_\alpha_i$, as explained under "Discussion."

It should be noted that the first two residues, Ile-372 and Ala-373, and CT1 were detected among the extended C-terminal 15 residues from GIRK\textsubscript{CP}. The other C-terminal residues showed no significant intensity reductions. Thus, we concluded
that the extension for 15 residues is sufficient for the interaction with Goα3.

**Contribution of Helical Domain of Goα3 to Interaction with GIRKCP-L, as Investigated by PRE Experiments—**The interaction of the helical domain of Goα3 with GIRKCP-L was investigated by PRE experiments. PRE arises from magnetic dipolar interactions between the nuclear spin and the unpaired electron spin of the paramagnetic center, which enhances the relaxation of the nuclear spins, leading to the line-broadening and thus the intensity reduction of the NMR signals of the residues within about 20 Å of the spin label (37, 44). In addition, PRE can detect a transient interaction, because the PRE effect occurs within about 20 Å of the spin label (37, 44). In addition, PRE can thus the intensity reduction of the NMR signals of the residues associated with transient interactions between the nuclear spin and the unpaired electron spin of the label, which is a potential approach to identifying transient interactions.

PRE arises from magnetic dipolar interactions between the nuclear spin and the unpaired electron spin of the paramagnetic center, which enhances the relaxation of the nuclear spins, leading to the line-broadening and thus the intensity reduction of the NMR signals of the residues within about 20 Å of the spin label (37, 44). In addition, PRE can detect a transient interaction, because the PRE effect occurs within about 20 Å of the spin label (37, 44). In addition, PRE can thus the intensity reduction of the NMR signals of the residues associated with transient interactions between the nuclear spin and the unpaired electron spin of the label, which is a potential approach to identifying transient interactions.

**DISCUSSION**

**Direct Interaction between Goα3 and Cytoplasmic Region of GIRK—**NMR analyses were performed to probe the interaction between GIRKCP-L and Goα3. Because cross-saturation is a phenomenon depending on the intermolecular 1H-1H distances, the TCS results from GIRKCP-L to Goα3 (Fig. 2) and vice versa (Fig. 3) indicated that the α2/α3 site of Goα3 and the αA helix of GIRKCP-L directly interact with each other. Our relaxation matrix calculations (43, 46) in which the on and off rates of the GIRKCP-L-Goα3 interaction are also considered, suggested that under the current experimental conditions the cross-saturation effect should be observed for the amide hydrogen atoms of GIRKCP-L within 6 Å from Goα3 and for those of Goα3 within 5 Å from GIRKCP-L. Furthermore, the direct interaction was verified by the mutations of the interacting residues on Goα3 (which impaired the affinity for GIRKCP-L (I212A and W258A, supplemental Fig. 2).

Most of the residues with apparent chemical shift changes are located in the GIRKCP-L binding site consisting of the α2 and α3 helices of Goα3, whereas the other residues, Arg-312 and Thr-316, exist on the α4/β6-loop that is adjacent to the α3 helix, reflecting the conformational changes of these residues upon GIRKCP-L binding (Fig. 1). The fitting of the titration curves of the chemical shift changes of Lys-209 and Trp-258, the GIRKCP-L binding residues, resulted in the $K_d$ values of 0.6 and 1.1 mM, respectively, which are within the range of the fitting error (Fig. 1C). Thus, the $K_d$ value for the binding of Goα3 and GIRKCP-L was estimated as 1 mM. Although this $K_d$ value is quite large as a value for a protein-protein interaction, it would fall in the nanomolar to micromolar range on the cell membrane, considering the reduced dimensionality effects (12, 47, 48). The $K_d$ value on the order of 1 mM is 4 times of the $K_d$ value of 0.25 mM that we previously reported for the GIRKCP-L-Gβγ interaction (12), which is consistent with the report that the affinity of the cytoplasmic region of GIRK1 for Gβγ was 4–5-fold stronger than that for Goα3 (15).

As shown in Fig. 1, E and F, accelerated signal intensity reductions were observed for the Goα3 residues at the GTP binding region, which reflect the larger chemical shift changes for the residues in the intermediate exchange regime. These residues are distant from the α2/α3 site, which is the direct GIRKCP-L binding interface, suggesting that the conformational change around the GTP binding site is induced by GIRKCP-L binding to the α2/α3 site. In particular, Ser-44, Gly-

![FIGURE 2. TCS from GIRKCP-L to Goα3, A, shown is a selected portion of the 1H,15N TROSY spectra of the uniformly 2H,15N-labeled Goα3 (0.30 mM) in the presence of GIRKCP-L (0.25 mM as a tetramer), which was recorded without (left) and with (right) radio frequency irradiation. Cross-sections are also shown for the signals from Ala-99 and Trp-258. B, procedures were the same as A, except that 2H-labeled GIRKCP-L, was used instead of unlabeled (1H-labeled) GIRKCP-L, as a negative control. C, shown is a plot of the difference in the reduction ratios (ΔRR) originating from the backbone amide groups with and without irradiation in the presence of unlabeled GIRKCP-L, and 2H-labeled GIRKCP-L (see also supplemental Fig. 1). The residues indicated by asterisks are those with no data mostly due to overlapping of the resonances or insufficient signal-to-noise ratio. The error bars were calculated based on the signal-to-noise ratios. Bars corresponding to the residues with significant intensity reductions (minimum values of ΔRR (ΔRRmin) > 0.08) are colored red. The secondary structure elements of Goα3 are depicted in gray below the sequence, and αA, αB, αC, and αD are colored green. D, mapping of the affected residues in the TCS experiment on the Goα3 structure (PDB code 1GIA) is shown. The backbone nitrogen atoms of the affected residues are shown as red balls in the ribbon diagram of the Goα3 structure (left). The affected residues are colored red on the surface representations of the Goα3 structure, whereas the residues with no data, including proline residues, are colored black (center and right). The αA, αB, αC, and αD are colored green.
45, Lys-46, Ser-47, and Gly-203 are the residues directly interacting with the β- or γ-phosphate groups of GTP. This might be related to the report that the GTPase activity of Gαi is enhanced upon binding to its effector, adenylate cyclase (49), which also binds to the α2/α3 site of Gαq (50). However, the significance of the conformational change at the GTP binding
site in terms of GIRK regulation remains unclear and is beyond the scope of this paper.

**Binding Mode of Gα and Cytoplasmic Region of GIRK**—Although the TCS results revealed that the αA helix of GIRKCP-L directly binds to the α2/α3 site of Gαi3, the PRE results indicated the αA helix of GIRKCP-L is within 20 Å of Ile-82 of Gαi3. However, the αA helix of GIRKCP-L, which binds to the α2/α3 site, should be more than 20 Å away from the spin-labeled site, suggesting that another αA helix in a neighboring subunit of the GIRKCP-L tetramer must come close to the spin-labeled site (Fig. 5).

To build a model of the Gαi3-GIRKCP-L complex satisfying the NMR-derived structural information, rigid body docking was performed by using the HADDOCK program (38). Because the structure of Gαi3(GTP) is not available, we built a homology model by the MODELLER software of Gαi3(GTP) from the crystal structure of Gαi3(GTP·γS) (PDB code 1GIA) in which 94% of the 354 residues are identical to Gαi3 (39) and used it for the construction of the complex model. The residues in the α2/α3 site of Gα and in the αA helix of GIRKCP-L, identified by the TCS experiments, were specified as the “active residues” in the program so that they formed the interface of the complex. Although the information about the residues on the helical domain derived from the TCS and PRE experiments was not used for the calculation, the helical domain of Gα was calculated to be proximal to the αA helix in a neighboring subunit of GIRKCP-L, probably due to the restraint of Glu-242 on the subunit interface of GIRKCP-L, as determined from the TCS experiments (Fig. 5). In this binding mode, Glu-242 of GIRKCP-L at a distance from the αA helix, approaches the side chain of Glu-186 of Gαi3, which accounts for its intensity reduction in the TCS experiment.

The three residues that TCS identified on the helical domain of Gαi3 (Gly-89, Gly-112, and Ala-114) showed relatively weak cross-saturation, and they do not form a continuous binding surface, presumably because the αA helix of GIRKCP-L transiently accesses the Gαi3 helical domain. This can be accounted for by the conformational flexibility of the αA helix. In the crystal structure, the αA helix of GIRKCP-L is stabilized by the crystal contacts with the αA helix of another tetramer. In addition, the C-terminal part of the helix (residues 367–368) exhibited very weak or no NOEs that are typical for an α helix (data not shown), and the chemical shift index calculated by the 13C chemical shifts indicated that the C-terminal residues after Met-367 are unstructured. Altogether, we concluded that the α2/α3 site of Gαi3 mainly recognizes the αA helix of GIRKCP-L in one subunit of the tetramer, whereas the helical domain of Gαi3 transiently approaches the αA helix in another neighboring subunit.

The N terminus of Gα is modified by a lipid moiety in vivo and anchored to the cell membrane (51), which can be accounted for by this binding mode. Although the most N-ter-
Structural Basis for Modulation of GIRK-gating by G\textsubscript{ai/o}(GTP)

FIGURE 5. The proposed model of G\textsubscript{ai/o}(GTP) and the cytoplasmic region of the GIRK complex. Left, two adjacent subunits of a GIRK\textsubscript{CP} tetramer (PDB code 1N9P), viewed from the inside of the tetramer, are depicted by a ribbon diagram, and a homology model of G\textsubscript{ai/o} built from the crystal structure of G\textsubscript{ai/o}(GTP\textsubscript{S}) (PDB code 1GIA) by the MODELLER software (39) is shown in a surface representation. Residues 371–386 of GIRK1 are depicted schematically. The nitrogen atoms of the GIRK\textsubscript{CP-L} residues identified in the TCS experiments and the residues with PRE effects from MTSL, modified to I82C on the helical domain of G\textsubscript{ai/o}, are shown as red and magenta balls, respectively. The a2/a3 residues of G\textsubscript{ai/o} identified in the TCS experiments are colored blue. Center, shown is the binding model of G\textsubscript{ai/o} in the GTP-bound state and the cytoplasmic region of GIRK1 (PDB code 1N9P), obtained with HADDOCK software (38). The residues on GIRK\textsubscript{CP-L} and the a2/a3 residues on G\textsubscript{ai/o}, identified by TCS were defined as the active residues in the program so as to form the interface of the complex. The residues 358–370 on G\textsubscript{ai/o} were defined as “semi-flexible segments” to allow them to move during the simulated annealing. Only the two adjacent subunits of the GIRK tetramer are shown from the inside of the tetramer. Right, the model displayed at the center is rotated by 180 degrees, and all of the subunits of the GIRK tetramer are shown.

The helical domain of G\textsubscript{ai/o} is reported to be important for the i/o-family-specific activation of the GIRK channel, as determined with a G\textsubscript{ai/o}/G\textsubscript{ao} chimera (25). However, the main GIRK binding site for the G\textsubscript{ai/o}(GTP) revealed in this study was the a2/a3 site on the GTase domain, not on the helical domain. The activation specificity should be accomplished by the binding of G\textsubscript{ai/o}(GDP)\textsubscript{βγ} to GIRK, which seems to occur at a different site from that for G\textsubscript{ai/o}(GTP).

Previously, the G\textsubscript{ai/o} binding regions in GIRK1 were investigated by pulldown assays using the cytoplasmic fragments of GIRK (14, 15, 20, 22, 24). The cytoplasmic C-terminal residues 320–369 of GIRK1 were indicated to be important for strong binding to G\textsubscript{ai/o} (14), whereas the N-terminal fragment of GIRK1 (residues 1–84) also bound to G\textsubscript{ai/o}(GTP) (15, 20). The a2/a3 binding regions of GIRK1 revealed in this study are involved in the previously defined C-terminal region, and we identified three additional residues, Leu-371, Ile-372, and Ala-373. These residues vary among the GIRK subtypes, and GIRK1 possesses more hydrophobic and fewer charged residues than the other subtypes (supplemental Fig. 6), suggesting that the G\textsubscript{ai/o} binding affinity might differ among the subtypes. Unfortunately, the N-terminal binding region was not identified here, as the construct used in our study lacks residues 1–40 and 64–84.

Modulation of GIRK by G\textsubscript{ai/o}—GIRK is activated by the G\textsubscript{βγ} binding, and G\textsubscript{ai/o} modulates the gating property of GIRK. G\textsubscript{ai/o}(GDP)\textsubscript{βγ} is assumed to be precoupled with GPCRs and GIRK, which facilitates the efficient gate opening of GIRK, with the high specificity of GPCR signaling to GPCRs and GIRK, which facilitates the efficient gate opening of GIRK while maintaining the maximal current evoked by the GPCR stimulation, which is referred to as priming (14, 15, 19–22). On the other hand, G\textsubscript{ai/o}(GTP) binding to GIRK accelerates its deactivation (15).

Recently, a two-site model was proposed for the G protein binding sites on GIRK (“anchoring site” and “activation site”) (19, 22). The anchoring site is relevant to precoupling and priming for GIRK when the site accommodates G\textsubscript{ai/o}(GDP)\textsubscript{βγ} and to the binding of G\textsubscript{ai/o}(GTP) when G\textsubscript{βγ} shifts to the activation site, which was revealed by our NMR analyses as the border of the two neighboring subunits of the GIRK tetramer (12). In this
In this study we have identified the anchoring site for G\(_{\alpha_i/o}(GTP)\) on GIRK (Figs. 5 and 7). Notably, it is unknown whether the site is identical to the one for G\(_{\alpha_i/o}(GDP)\)\(_{\gamma}\). Upon ligand stimulation of the precoupled GPCR, the GDP-GTP exchange on G\(_{\alpha_i/o}\) in the complex with G\(_{\beta\gamma}\) at the anchoring site for G\(_{\alpha_i/o}(GDP)\)\(_{\gamma}\) decreases the affinity of G\(_{\alpha_i/o}\) for G\(_{\gamma}\), allowing G\(_{\beta\gamma}\) to activate GIRK by binding to the activation site. When the GPCR stimulation ends, GTP on G\(_{\alpha_i/o}\) at the anchoring site for G\(_{\alpha_i/o}(GTP)\) is rapidly hydrolyzed to GDP with assistance from RGS (23). Thus, G\(_{\alpha_i/o}\) at the anchoring site for G\(_{\alpha_i/o}(GTP)\) immediately re-associates with G\(_{\beta\gamma}\), leading to the closure of the GIRK gate.

In this study we revealed that the cytoplasmic region of GIRK1 and the GTP-bound G\(_{\alpha_i}\) directly bind to each other, and the \(\alpha\) helix of GIRK1 corresponds to the anchoring site for G\(_{\alpha_i/o}(GTP)\). The mapping of the G\(_{\beta\gamma}\) binding residues (12) on the structure of the G\(_{\alpha_i}\)-GIRK complex indicated that the binding sites of G\(_{\alpha_i}\)(GTP) and G\(_{\beta\gamma}\) on GIRK do not overlap with each other, and therefore, G\(_{\alpha_i}\)(GTP) and G\(_{\beta\gamma}\) can simultaneously bind to GIRK (Fig. 7). Furthermore, the mapping of the RGS binding site on the structure of G\(_{\alpha_i}\) suggested that RGS can bind to G\(_{\alpha_i}\) in the complex with GIRK (supplemental Fig. 7). Therefore, the G\(_{\alpha_i}\)-GIRK-G\(_{\beta\gamma}\) ternary complex model obtained here provides the structural basis for the rapid closure of the GIRK channel upon signal termination through the efficient removal of the proximal G\(_{\beta\gamma}\) from GIRK.

FIGURE 6. Properties of the binding interfaces on G\(_{\alpha_i}\) and GIRK1. An “open-book” display of the G\(_{\alpha_i}\)-GIRK complex model (Fig. 5) is shown in surface representations. Residues 371–386 of GIRKCP-L are depicted schematically. The residues on GIRKCP-L and the \(\alpha_2/\alpha_3\) residues on G\(_{\alpha_i}\) identified by TCS (labeled by bold letters) and their surrounding residues with C\(_{\alpha_i}\) atoms within 6.0 Å, are colored according to their side-chain properties: acidic, basic, hydrophobic, and hydrophilic residues are colored red, blue, green, and yellow, respectively.

FIGURE 7. A model of the G\(_{\alpha_i}\)-GIRK-G\(_{\beta\gamma}\) ternary complex. Left, the nitrogen atoms of the G\(_{\beta\gamma}\) binding residues on GIRK1 (12) are mapped as magenta balls on the complex model of G\(_{\alpha_i}\) and the GIRK tetramer (Fig. 5). Right, shown is a ternary complex model of G\(_{\alpha_i}\)-GIRK-G\(_{\beta\gamma}\). First, the binding model of G\(_{\beta\gamma}\)-GIRK was built by using HADDOCK with parameters listed in supplemental Table 2. The G\(_{\beta\gamma}\) binding residues on GIRKCP-L, identified in our previous study (12) and the residues on G\(_{\beta\gamma}\) reported to be important for GIRK binding (54, 55) were defined as the active residues in the software. Then the G\(_{\alpha_i}\)-GIRK complex model shown in Fig. 5 and the G\(_{\beta\gamma}\)-GIRK complex model were superimposed by the GIRK structure, rendering the G\(_{\alpha_i}\)-GIRK-G\(_{\beta\gamma}\) ternary complex model. G\(_{\beta}\) and G\(_{\gamma}\) are colored magenta and violet, respectively. The residues involved in the intermolecular interactions between G\(_{\alpha_i}\) and G\(_{\beta\gamma}\), within a distance of 4 Å in the G\(_{\alpha_i}\)(GDP)/\(\beta_1\gamma_2\) (PDB code 1GP2) structure, are colored orange.
It should be noted that the α2 helix of Gαia in the GIRK binding site (the α2/α3 site) is located in the switch II region, which is known to alter its conformation upon GDP-GTP exchange and is included in the Gβγ binding site. Thus, the anchoring site of Gαia(GDP)/βγ is different from that of Gαia(GTP) revealed here. Structural analyses of the interaction between Gαia(GDP)/βγ and GIRK will provide a complete understanding of the modulation mechanism of the GIRK-gating by Gαi/β proteins.

REFERENCES

1. He, C., Zhang, H., Mirshahi, T., and Logothetis, D. E. (1999) Identification of a potassium channel site that interacts with G protein βγ subunits to mediate agonist-induced signaling. J. Biol. Chem. 274, 12517–12524
2. Bichet, D., Haass, F. A., and Jan, L. Y. (2003) Merging functional studies with structures of inward-rectifier K+ channels. Nat. Rev. Neurosci. 4, 957–967
3. Hibino, H., Inanobe, A., Furutani, K., Murakami, S., Findlay, I., and Kurauchi, Y. (2010) Inwardly rectifying potassium channels. Their structure, function, and physiological roles. Physiol. Rev. 90, 291–366
4. Kuo, A., Gulbis, J. M., Antcliff, J. F., Rahman, T., Lowe, E. D., Zimmer, J., Kosaza, T., Casey, P. J., and Slesinger, P. A. (2005) Pertussis-toxin-sensitive G protein subunits selectively bind to C-terminal domain of neuronal GIRK channels. Evidence for a heterotrimeric G-protein-channel complex. Mol. Cell Neurosci. 28, 375–389
5. Rubinstein, M., Peleg, S., Berlin, S., Brass, D., and Dascal, N. (2007) Gαi3 primes the G protein-activated K+ channels for activation by coexpressed Gβγ in intact Xenopus oocytes. J. Physiol. 581, 17–32
6. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1995) G protein regulation of cardiac muscarinic potassium channel. Am. J. Physiol. 269, C821–C830
7. Kurachi, Y. (1995) G protein regulation of cardiac muscarinic potassium channel. Am. J. Physiol. 269, C821–C830
8. Ito, H., Tung, R. T., Sugimoto, T., Kobayashi, I., Takahashi, K., Katada, T., Ui, M., and Kurachi, Y. (1992) On the mechanism of G protein βγ subunit activation of the muscarinic K+ channel in guinea pig atrial cell membrane. Comparison with the ATP-sensitive K+ channel. J. Gen. Physiol. 99, 961–983
9. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) The βγ subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature 325, 321–326
10. Reuveny, E., Slesinger, P. A., Inglese, J., Morales, I. M., Iniguez-Llhuil, J. A., Lefkowitz, R. J., Bourne, H. R., Jan, L. Y., and Jan, L. Y. (1994) Activation of the cloned muscarinic potassium channel by G protein βγ subunits. Nature 370, 140–146
11. Wickmann, K. D., Iniguez-Llhuil, J. A., Davenport, P. A., Taussig, R., Kravivinsky, G. B., Linder, M. E., Gilman, A. G., and Clapham, D. E. (1994) Recombinant G protein βγ subunits activate the muscarinic-gated atrial potassium channel. Nature 366, 255–257
12. Yokogawa, M., Osawa, M., Takeuchi, K., Mase, Y., and Shimada, I. (2011) NMR analyses of the Gγβ binding and conformational rearrangements of the cytoplasmic pore of the G protein-activated inward rectifying potassium channel 1 (GIRK1). J. Biol. Chem. 286, 2215–2223
13. Hille, B. (1992) G protein-coupled mechanisms and nervous signaling. Neuron 9, 187–195
14. Ivanina, T., Varon, D., Peleg, S., Rishal, I., Porozov, Y., Dessauer, C. W., Keren-Raifman, T., and Dascal, N. (2004) Gαia and Gαiai3 differentially interact with, and regulate, the G protein-activated K+ channel. J. Biol. Chem. 279, 17260–17268
15. Berlin, S., Tsemakhov, V. A., Castel, R., Ivanina, T., Dessauer, C. W., Keren-Raifman, T., and Dascal, N. (2011) Two distinct aspects of coupling between Gαia and G protein-activated K+ channel (GIRK) revealed by fluorescently labeled Gαia subunits. J. Biol. Chem. 286, 33223–33235
16. Benians, A., Leaney, J. L., Milligan, G., and Tinker, A. (2003) The dynamics of formation and action of the ternary complex revealed in living cells using a G-protein-gated K+ channel as a biosensor. J. Biol. Chem. 278, 10851–10858
17. Kovoor, A., and Lester, H. A. (2002) GI Irks GIRKs. Neuron 33, 6–8
18. Slesinger, P. A., Reuveny, E., Jan, Y. N., and Jan, L. Y. (1995) Identification of structural elements involved in G protein gating of the GIRK1 potassium channel. Neuron 15, 1145–1156
19. Berlin, S., Keren-Raifman, T., Castel, R., Rubinstein, C. W., Ivanina, T., and Dascal, N. (2010) Gαia and Gαiai3 jointly regulate the conformations of a Gγβ effector, the neuronal G protein-activated K+ channel (GIRK). J. Biol. Chem. 285, 6179–6185
20. Peleg, S., Varon, D., Ivanina, T., Dessauer, C. W., and Dascal, N. (2002) Gαi3 controls the gating of the G protein-activated K+ channel. J. Biol. Chem. 277, 87–99
21. Rubinstein, M., Peleg, S., Berlin, S., Brass, D., and Dascal, N. (2007) Gαi3 primes the G protein-activated K+ channels for activation by coexpressed Gβγ in intact Xenopus oocytes. J. Physiol. 581, 17–32
22. Rasmussen, S. G., DeBree, 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., Sgouros, K., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the β2 adrenergic receptor G protein complex. Nature 477, 549–555
23. Nishida, M., and Mackinnon, R. (2002) Structural basis of inward rectification. Cytoplasmic pore of the G protein-gated inward rectifier GIRK1 at 1.8 Å resolution. Cell 111, 957–965
24. Yokogawa, M., Muramatsu, T., Takeuchi, K., Osawa, M., and Shimada, I. (2009) Backbone resonance assignments for the cytoplasmic regions of G protein-activated inward rectifying potassium channel 1 (GIRK1). Biochim. Biophys. Acta 1786, 125–128
25. Salzmann, M., Vervushkin, K., Wider, G., Senn, H., and Wuthrich, K. (1998) TROSY in triple-resonance experiments. New perspectives for sequential NMR assignment of large proteins. Proc. Natl. Acad. Sci. U.S.A. 95, 13585–13590
26. Salzmann, M., Wider, G., Vervushkin, K., Senn, H., and Wuthrich, K. (1999) TROSY-type triple-resonance experiments for sequential NMR assignments of large proteins. J. Am. Chem. Soc. 121, 844–848
27. Yoshida, K., Kofuku, Y., Ueda, T., Mase, Y., Yokogawa, M., Osawa, M., Terashima, Y., Matsushima, K., and Shimada, I. (2010) NMR analyses of the interaction between CCR5 and its ligand using functional reconstitution of CCR5 in lipid bilayers. J. Am. Chem. Soc. 132, 6768–6777
28. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) Conformational changes in the amino-terminal helix of the G protein αi3, following dissociation from Gβγ subunit and activation. Biochemistry 41, 9962–9972
29. Mase, Y., Yokogawa, M., Osawa, M., and Shimada, I. Backbone resonance assignments for G protein αi3 subunit in the GTP-bound state. Biochim. Biophys. Acta 1806, in press
30. Nakashima, T., Miyazawa, M., Sakakura, M., Terasawa, H., Takahashi, H., and Shimada, I. (2002) Determination of the interface of a large protein complex by transferred cross-saturation measurements. J. Mol. Biol. 318, 245–249
37. Battiste, J. L., and Wagner, G. (2000) Utilization of site-directed spin labeling and high resolution heteronuclear nuclear magnetic resonance for global fold determination of large proteins with limited nuclear Overhauser effect data. *Biochemistry* **39**, 5355–5365

38. de Vries, S. J., van Dijk, M., and Bonvin, A. M. (2010) The HADDOCK web server for data-driven biomolecular docking. *Nat. Protoc.* **5**, 883–897

39. Sali, A., Potterton, L., Yuan, F., van Vlijmen, H., and Karplus, M. (1995) Evaluation of comparative protein modeling by MODELLER. *Proteins* **23**, 318–326

40. Matsuo, H., Walters, J., Teruya, K., Tanaka, T., Gasser, G., Lippard, S., Kyogoku, Y., and Wagner, G. (1999) Identification by NMR spectroscopy of residues at contact surfaces in large, slowly exchanging macromolecular complexes. *J. Am. Chem. Soc.* **121**, 9903–9904

41. Mal, T. K., Masutomi, Y., Zheng, L., Nakata, Y., Ohta, H., Nakatani, Y., Kokubo, T., and Ikura, M. (2004) Structural and functional characterization on the interaction of yeast TFIID subunit TAF1 with TATA-binding protein. *J. Mol. Biol.* **339**, 681–693

42. Shimada, I., Ueda, T., Matsumoto, M., Osawa, M., Takeuchi, K., Nishida, N., and Takahashi, H. (2009) Cross-saturation and transferred cross-saturation experiments. *Prog. Nucl. Magn. Reson. Spectrosc.* **54**, 123–140

43. Matsumoto, M., Ueda, T., and Shimada, I. (2010) Theoretical analyses of the transferred cross-saturation method. *J. Magn. Reson.* **205**, 114–124

44. Clore, G. M., and Iwahara, J. (2009) Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes. *Chem. Rev.* **109**, 4108–4139

45. Clore, G. M. (2011) Exploring sparsely populated states of macromolecules by diagnostically and paramagnetic NMR relaxation. *Protein Sci.** 20**, 229–246

46. Takahashi, H., Miyazawa, M., Ina, Y., Fukunishi, Y., Mizukoshi, Y., Nakamura, H., and Shimada, I. (2006) Utilization of methyl proton resonances in cross-saturation measurement for determining the interfaces of large protein–protein complexes. *J. Biol. NMR* **34**, 167–177

47. Ozcan, F., Klein, P., Lemmon, M. A., Iwahara, J., and Schlessinger, J. (2006) On the nature of low and high affinity EGF receptors on living cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 5735–5740

48. Stratikos, E., Mosyak, L., Zaller, D. M., and Wiley, D. C. (2002) Identification of the lateral interaction surfaces of human histocompatibility leukocyte antigen (HLA)-DM with HLA-DR1 by formation of tethered complexes that present enhanced HLA-DM catalysis. *J. Exp. Med.* **196**, 173–183

49. Scholich, K., Mullenix, J. B., Wittpoth, C., Poppleton, H. M., Pierre, S. C., Lindorfer, M. A., Garrison, J. C., and Patel, T. B. (1999) Facilitation of signal onset and termination by adenylyl cyclase. *Science* **283**, 1328–1331

50. Tesmer, J. J., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Crystal structure of the catalytic domains of adenylyl cyclase in a complex with GαtGTPγS. *Science* **278**, 1907–1916

51. Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995) Lipid modifications of trimeric G proteins. *J. Biol. Chem.* **270**, 503–506

52. Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T., and Tesmer, J. J. (2005) Snapshot of activated G proteins at the membrane: the Gα1q-GRK2-Gβγ complex. *Science* **310**, 1686–1690

53. Johnston, C. A., Loboanov, E. S., Shankaranarayanan, A. S., Low, J., Ramer, J. K., Blaesius, R., Fredericks, Z., Willard, F. S., Kuhlman, B., Arshavsky, V. Y., and Siderovski, D. P. (2006) Minimal determinants for binding activated Gαt from the structure of a Gαt1-peptide dimer. *Biochemistry* **45**, 11390–11400

54. Albsoul-Younes, A. M., Sternweis, P. M., Zhao, P., Nakata, H., Nakajima, S., Nakajima, Y., and Kozasa, T. (2001) Interaction sites of the Gαt subunit with brain G protein-coupled inward rectifier K+ channel. *J. Biol. Chem.* **276**, 12712–12717

55. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shekter, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R., Miller, R. J., Jan, L. Y., Lefkowitz, R. J., and Hamm, H. E. (1998) Molecular basis for interactions of G protein βγ subunits with effectors. *Science* **280**, 1271–1274