Development of an antibody fragment that stabilizes GPCR/G-protein complexes

Shoji Maeda1, Antoine Koehl2, Hugues Matile3, Hongli Hu1,2, Daniel Hilger1, Gebhard F.X. Schertler4, Aashish Manglik5,6, Georgios Skiniotis1,2, Roger J.P. Dawson3 & Brian K. Kobilka1

Single-particle cryo-electron microscopy (cryo-EM) has recently enabled high-resolution structure determination of numerous biological macromolecular complexes. Despite this progress, the application of high-resolution cryo-EM to G protein coupled receptors (GPCRs) in complex with heterotrimeric G proteins remains challenging, owing to both the relative small size and the limited stability of these assemblies. Here we describe the development of antibody fragments that bind and stabilize GPCR-G protein complexes for the application of high-resolution cryo-EM. One antibody in particular, mAb16, stabilizes GPCR/G-protein complexes by recognizing an interface between Gα and Gβγ subunits in the heterotrimer, and confers resistance to GTPγS-triggered dissociation. The unique recognition mode of this antibody makes it possible to transfer its binding and stabilizing effect to other G-protein subtypes through minimal protein engineering. This antibody fragment is thus a broadly applicable tool for structural studies of GPCR/G-protein complexes.
**G-protein coupled receptors (GPCRs)** make up the largest receptor family in the human genome, comprising around 800 members. GPCRs are expressed ubiquitously and play essential roles of signal transduction in response to a wide variety of extracellular stimuli such as photons, ions, neurotransmitters, hormones and proteins. Given their numerous physiological roles, GPCRs are implicated in numerous diseases and ~30% of marketed drugs are targeting this receptor family. Recent advances in GPCR crystallography have led to high-resolution structures of G-protein and arrestin complexes, which have enhanced our understanding of the structural details underlying ligand binding and signal transduction at the atomic level. The first crystal structure of a GPCR/G-protein complex was that of the β2 adrenergic receptor in complex with stimulatory G-protein, Gαs (G1α2R/G3γ). This was later followed by the crystal structure of A2A adenosine receptor in complex with miniGα (A2AR/miniGα) in which a highly engineered Gα, that consists of only the Gα ras-like domain was used in place of the full heterotrimer. The fact that such drastic protein engineering is needed to obtain a crystallizable quality complex reflects the difficulty inherent in GPCR–G-protein complex crystallography. Despite the technological advancement, crystallographic studies of these complexes remains extremely difficult. More recently, single-particle cryo-electron microscopy (cryo-EM) has emerged as an alternative technique with the ability to provide near-atomic resolution maps, as demonstrated for two class B GPCRs both in complex with Gαs: the glucagon-like peptide1 receptor/Gαs (Glp1R/G3γ) as well as the calcitonin receptor/Gαs (CTR/G3γ). These studies have highlighted the possibility of employing cryo-EM to obtain the structures of GPCR–G-protein complexes. Compared to class A GPCRs, class B receptors include a structured extracellular domain that may aid in particle alignment. Furthermore, for Gαs proteins, Nb35 stabilizes these complexes against GTPγS by stabilizing an interface between the Ras-like domain of the Gαs subunit and the Gβγ subunit. These factors make class B GPCR/Gα protein complexes more tractable targets for cryo-EM compared to class A or other G-protein subtype complexes.

Apart from the GPCR/Gα complex, the only structure available at high-resolution has been limited to the MetII rhodopsin/Gαi2 where the last 11-amino-acid fragment of Gαras-dsducin was co-crystallized with the activated rhodopsin. Although in silico analyses using this complex have provided insights into the conformational changes that allow Gαi coupling as well as general principles for G protein coupling specificity, experimental structures of other G-protein complexes are invaluable to understand how receptors selectively engage one G-protein subtype over others. G-protein mimetic nanobodies have been used as a surrogate to capture the active conformation of a receptor, but it may require an extensive effort to find such nanobodies and the trapped conformation may not necessarily represent the G-protein engaged state.

Here we describe the development of an antibody, termed mAb16, that recognizes the heterotrimeric Gαi/o type G protein and enhances the stability of GPCR-Gi/o complexes, while simultaneously adding an asymmetric feature that may aid with cryo-EM particle projection alignment. As antibodies typically bind to their targets in a rigid manner, such an antibody would be expected to enable structure determination of GPCR-G-protein complexes by cryo-EM. mAb16 recognizes a unique epitope, binding at the interface between the α and β subunits of heterotrimeric Gαi. While the antibody confers extra stability to GPCR/Gαi/o complex as well as increased resistance to GTPγS-triggered dissociation of the complex in a manner similar to Nb35 for Gαs, mAb16 and Nb35 bind to completely different epitopes. We have recently succeeded in obtaining a near-atomic resolution map of the mu-opioid receptor (μOR)/Gαi complex using this antibody fragment. Although this antibody is specific against Gαi/o-family G-proteins, its ability to bind and stabilize the heterotrimer can be transferred to other G-protein subtypes through a simple protein engineering strategy.

**Results**

**Selection of monoclonal antibodies.** Despite exhaustive attempts to crystalize a complex between rhodopsin and heterotrimeric Gαi1, we were unsuccessful in producing diffraction quality crystals. We presumed that this was due to the flexibility of the alpha-helical domain of Gα1, as this domain separates from Ras-like domain and becomes flexible upon receptor-mediated activation in the nucleotide-free state. We then set out to discover antibodies that could reduce this flexibility and facilitate crystallographic and cryo-EM structural studies of the complex. Mice were immunized with purified rhodopsin/Gαi1 complex and hybridoma cells were prepared from the isolated mice splenocytes. Clones that showed enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation positive reaction were screened further using an analytical size-exclusion chromatography (SEC) assay with purified monoclonal antibodies. Most of the SEC-positive clones were Gβγ-binders, reflecting that it is the most stable and rigid component of the complex. (Fig. 1a, c). Interestingly, we found a single clone that binds and confines GTPγS resistance to the rhodopsin/Gαi1 complex (Fig. 1a, b). Based on the clone identification number, we named its antibody mAb16. Notably, this clone does not show binding to any single component of Gαi1, but binds specifically to the intact heterotrimeric form of Gαi1, suggesting that it binds a composite epitope at the interface between Gαi1 and Gβγ subunits (Fig. 1c). We provide amino-acid sequence of mAb16 in the Supplementary Note 1.

**Crystal structure of Gαi1/scFv16.** In order to better understand the recognition mode of mAb16, we crystallized a fully soluble heterotrimeric Gαi1 in complex with mAb16 fragments. We tried both a Fab fragment (Fab16) and single-chain variable fragment (scFv16) derived from mAb16. Both Gα1/Fab16 and Gα1/scFv16 complexes formed crystals but only the scFv16 version diffraction to high resolution, presumably due to the intrinsic flexibility of the linker between the variable and the constant domain of the Fab. The crystal structure of the Gα1/scFv16 complex was solved at 2.0 Å by molecular replacement using Gα1 (PDB ID: 1GP2) and an scFv fragment (PDB ID: 4NKD) as search models (Table 1). The overall structure of Gα1 in complex with scFv16 is very similar to Gα1 alone (Fig. 2a). The relative position of Ras-like domain and alpha-helical domain of Gα1 moves closer to Gβ1 by a small rotation around the αn-B1 junction (Fig. 2b). This slight movement leads to two additional interactions between Thr182 of Gα1 and Asn119 of Gβ1, and Arg205 of Gα1 and Thr143 of Gα in Gα1, located in Switch I and Switch II region, respectively (Fig. 2b). This could be due to the tighter association between these two subunits mediated by scFv16, although it may be the consequence of different crystal contacts between Gα1 alone and Gα1/scFv16. The structure of the Gα1/scFv16 complex shows that scFv16 recognizes an epitope composed of the terminal part of the αn helix of Gα1 as well as part of the Gβ1 subunit (Fig. 2c, Supplementary Fig. 1). The complementarity-determining region 3 of the heavy chain (CDR-H3) extends to interact with Gβ1, with its tip and Gα1 with its side. CDR2-H2 and CDR1-H1 support the interaction with Gα1 and Gβ1, respectively by making hydrogen bonds and van der Waals contacts. CDR-L1 is exceptionally long and makes extensive contact with the edge of the αn helix together with CDR-L3 (Fig. 2c). There is no obvious interaction between scFv16 and Gβ2 subunit.
Fab16 would bind to all Gi/o family members, as it has poor sequence similarity to Gi\(\alpha\)o members at this epitope region (Fig. 3b).

Application to other GPCR-Gi/o complexes. Since Fab16 was initially isolated as a stabilizing agent that confers resistance to GTP\(\gamma\)S triggered dissociation to a rhodopsin/Gi\(\alpha\) complex and was later found to bind to a panel of Gi\(\alpha\)o family members, we investigated whether it confers the same GTP\(\gamma\)S resistance to other Gi\(\alpha\)o type G-protein complexes. We chose a \(\mu\)-opioid receptor/Gi\(\alpha\) (\(\mu\)OR/Gi\(\alpha\)) and an M\(_2\) muscarinic acetylcholine receptor/G\(\alpha\)\(_{OA}\) (M\(_2\)R/G\(\alpha\)\(_{OA}\)) as representative family A Gi/o-coupling GPCR complexes. Purified \(\mu\)OR/Gi\(\alpha\) or M\(_2\)R/G\(\alpha\)\(_{OA}\) complex solubilized in detergent was incubated with GTP\(\gamma\)S in the presence or absence of Fab16, then analysed for dissociation by analytical SEC. Both \(\mu\)OR/Gi\(\alpha\) and M\(_2\)R/G\(\alpha\)\(_{OA}\) complexes showed a leftward peak shift upon incubating with Fab16, indicating its binding and also became GTP\(\gamma\)S resistant, as they showed much less dissociation in the presence of Fab16 than the complex alone (Fig. 3c). These data with GPCR/Gi\(\alpha\)\(_{OA}\) complexes are consistent with the binding experiments of Fab16 with G-protein alone, and indicate that it stabilizes Gi\(\alpha\)o type GPCR complexes in general. In order to show the applicability of scFv16 to structural analysis of GPCR-G protein complexes, we have recently solved a near-atomic resolution map of \(\mu\)OR/Gi\(\alpha\) complex using scFv16\(\alpha\). The presence of scFv16 enhanced complex stability towards specimen vitrification for cryo-EM, thereby enabling quality single-particle reconstructions.

Influence of Fab16 on nucleotide binding. In order to further investigate mAb16 for its protection mechanism against GTP\(\gamma\)S, we monitored the binding kinetics of GTP\(\gamma\)S to G-protein-free M\(_2\)R/G\(\alpha\)\(_{OA}\) complex. In the absence of Fab16, BODIPY-FL-GTP\(\gamma\)S, a fluorescent analogue of GTP\(\gamma\)S, binds to the complex with fast kinetics reflecting its ability to bind and trigger the dissociation of the complex. In contrast, BODIPY-FL-GTP\(\gamma\)S binds to M\(_2\)R/G\(\alpha\)\(_{OA}\)/Fab16 complex ~70 times slower and to a much lower extent.

Table 1 Data collection and refinement statistics

| Gi\(\alpha\)/scFv16\(\alpha\) |
|-------------------------------|
| **Data collection**           |
| Space group                   | P222, |
| Cell dimensions (\(\AA\))     | 58.51, 104.74, 211.82 |
| \(\alpha\), \(\beta\), \(\gamma\) (\(\circ\)) | 90.00, 90.00, 90.00 |
| Resolution (\(\AA\))          | 39.26–2.00(2.07–2.00)\(b\) |
| R\(_{wp}\) or R\(_{merge}\)    | 0.176(0.888) |
| \(\check{R}\)                  | 9.91(0.73) |
| Completeness (%)              | 99.16(99.47) |
| Redundancy                    | 4.6(4.8) |
| **Refinement**                |
| Resolution (\(\AA\))          | 39.26–2.00(2.07–2.00) |
| No. reflections               | 88,191(8710) |
| R\(_{wp}\)/R\(_{merge}\)      | 0.1746(0.2097/0.2682/0.2940) |
| No. atoms                     | 7567 |
| Protein                       | 41 |
| Ligand/ion                    | 628 |
| Water                         | 51.44 |
| B-factors (\(\AA^2\))        | 47.08 |
| Protein                       | 31.55 |
| Ligand/ion                    | 51.44 |
| Water                         | 1.19 |

*The data set was collected from one single crystal

*Values in parentheses are for highest-resolution shell

As the \(\alpha\) subunits of all Gi\(\alpha\) family members have high sequence similarity at the epitope residues in the aN helix (Fig. 3a) and can form a complex with G\(_{i\alpha}\), we expected that Fab16 would bind to all Gi\(\alpha\) family proteins. Using analytical fluorescent SEC, we show that Fab16 can bind to five different Gi\(\alpha\)
on alignment of G
in cyan, G
subunits in order to generalize scFv16 binding to all G-protein
next, we sought to engineer the
provide GTP
transfer Fab16 binding ability to G11, a G
family member, and
affected when the complex is bound to Fab16 (Fig.4a). Next we
bind Fab16 and form stable complexes with respective GPCRs
(Supplementary Fig. 2b, c.) The same minimal region when
bind to Fab16 in the absence of Nb35 but becomes extremely slow or
which the
gamm subunit and forms a stable complex with the
Because mAb16
originally raised against and indeed binds to Rhodopsin/Gi1 that
The
G
11iN complex bound to Fab16 showed GTPγS resistance (Fig. 5c) consistent with G11i and G11N complexes. Negative stain EM visualization of the M1/R/G11iN complex reveals a
discrete sample (Fig. 5d). These results demonstrate that Fab16 (or scFv16) can be used as a tool with broad variety of GPCR/G-protein complexes by substituting the αN helix of other G-subunit with equivalent region of Ga1. Scanning the chimera junction between Ga11 and Ga1, shows that a smaller substitution in the middle of the αN helix is still tolerated for the expression and the heterotrimer formation (Supplementary Fig. 2a). When replaced with the equivalent residues with this minimal chimeric region (residues 1–18 of Ga11), both G11 and Ga11 are enabled to
binding interface of
β
δ
γ
S, whereas the M1R/
G11iN complex formed with Fab16 and form stable complexes with respective GPCRs (Supplementary Fig. 2b, c.) The same minimal region when transferred to G12i also enables Fab16 binding (Supplementary Fig. 2d). The binding interface of Gβ1 subunit to scFv16 is limited compared to Ga subunit in the crystal structure. These residues are mostly conserved among Gβ family members except Gβ5 (Supplementary Fig. 3). There is no direct interaction between
γ
2 and scFv16 in the crystal structure. The fact that mAb16 was originally raised against and indeed binds to Rhodopsin/Gi1 that is composed of Gy1 from the native bovine retina and still binds G-proteins or GPCR/G-protein complexes composed of Gy2 indicates that Fab/scFv16 binds to the heterotrimeric G-protein regardless of the composition of the γ-subunit. Therefore, the binding ability to Fab16 is transferable to broad range of G-protein family members with minimal chimeric constructs. We provide amino-acid sequences of G-protein chimera constructs in the Supplementary Note 2 as well as the primers used for the construction in the Supplementary Table.

Discussion
In this work, we have developed a unique antibody fragment that recognizes an interface on heterotrimeric Gi1. The antibody confers the GPCR–Gai10 complexes the resistance to GTPγS-induced dissociation. This property is also observed with formerly

Fig. 2 Crystal structure of Gαi/scFv16 and characterization of Fab16. a Overall structure of Gi1/scFv16 complex. Cartoon representation with Ga1, in gold, Gβ in magenta, scFv-heavy chain in light grey and scFv-light chain in light blue. b Superposition of Gαi/scFv16 structure onto Gi1 (PDB: 1GP2) based on alignment of Gβγ subunits. Ga1, (1GP2) in grey and Gαi/scFv16 in the same colour code as in a. For clarity, Gβγ subunits and scFv16 is shown as transparent cartoon. Arrows show a slight rotational displacement of Ga1 towards Gβ compared to Gi1 alone. Additional interactions are formed between switch I and switch II of Ga1 and Gβγ. c Interaction between Ga1 and scFv16. The residues participating in the interactions are depicted with stick models in the expanded panels. Residue numbers are shown with Common Gα Numbering (CGN) code for Ga1.
identified Nb35 for Gs complexes. Nb35 and mAb16 engage distinct epitopes at the G-protein interface: Nb35 binds at the switch II and αIII helix of Ga1, and Gβ, while mAb16 engages the αN helix of Ga1 and Gβ. The switch II region adopts a distinct conformation upon binding of GTPγS compared to the nucleotide-free or GDP-bound state observed in the crystal structures as well as in the EPR spectroscopic measurement. Nb35 is reported to suppress nucleotide exchange turnover of the CTR/Gi complex and indeed it prevents GTPγS binding to the β2AR/Gs complex (Fig. 4c), which we presume due to the fixed conformation of the switch II in the nucleotide-free state and steric clash of the switch III with Nb35. On the other hand, mAb16 binds 40 Å away from the nucleotide-binding pocket with no direct contact with this region yet prevents the binding of mAb16 binds 40 Å away from the nucleotide-binding pocket with no direct contact with this region yet prevents the binding of mAb16.
other family members or to evolve the nanobody itself. On the other hand, the binding surface of mAb16 to mAb16 is located in a small stretch of the αN helix. Since the αN helix apparently serves as a separate module from the Ras-like domain to interact with Gβγ, it is more amenable to generating functional chimeras. Previous studies have shown that the entire αN helix of Gαα, Gα12 and Gα13 can be substituted with the corresponding region of Gαi1 to produce functional chimeric Gαi1, Gαi12 and Gαi13. These engineered G proteins retained the biochemical properties of their wild-type counterparts. In fact, our G protein chimeras were transferred to the equivalent residues of Gαα, Gα12 and Gα13, respectively. The usefulness of the antibody fragment in generating functional heterotrimers with co-expressed Gαα, Gα12 and Gα13 is reportedly important for the receptor selectivity. Chimeric Gα11 protein has also been made with various lengths of αN from Gαα, Gα12 and Gα13 to investigate the functional role of this region. 

Methods

Protein expression and purification. Rhodopsin/Gαi1 complex was purified as described previously. Briefly, bovine rhodopsin with three mutations, N2C, M257Y and N282C, was stably expressed and purified from HEK293S GnTi cells using ID4 immunosilicon chromatography. Purified rhodopsin was incubated with Gαi1 reconstituted from recombinant Gαi1 subunit from Escherichia coli BL21 (DE3) cells (Novagen) and Gβγ subunit purified from bovine retina (W L Lawson Company). Rhodopsin/Gαi1 complex formation was triggered by the irradiation through 495 nm long-pass filter in the presence of apyrase (Sigma-Aldrich). Rhodopsin/Gαi1 complex was separated from the free rhodopsin using Gαi1, by SEC on a Tricorn 10/600 column packed with Superdex 200 (GE healthcare) in a buffer containing 100 mM NaCl, 20 mM Hepes pH 7.5, 0.01% lauryl maltoside neopentyl glycol (MNG), 2 mM 2-mercaptoethanol.

μOR with a cleavable amino and carboxy-terminal FLAG- and His-tag was expressed in Spodoptera frugiperda Sf9 insect cells using baculovirus infection system (Expression Systems). Cells were solubilized in 1% n-dodecyl-β-D-maltoside (DDM) (Anasatrce), 0.2% 5-cholesterol hemisuccinate (CHS) (Steraloids) and the soluble fraction was purified by Ni-chelating sepharose chromatography. The eluted protein was supplemented with 2 mM CaCl2, loaded onto M1 anti-FLAG immunosilicon column (prepared in house) and washed with progressively lower concentrations of the antagonist naloxone (Sigma-Aldrich). Receptor was eluted in a buffer consisting of 100 mM NaCl, 20 mM Hepes pH 7.5, 0.1% DDM, 0.01% CHS with 50 mM naloxone, and further purified by SEC on a Superdex 200 10/300 column in a buffer containing 1 mM lofentanil (Tronto Research Chemicals) to exchange the ligand. Monomeric fractions were pooled, further supplemented with a twofold molar excess of lofentanil and concentrated to ~100 μM for complex formation.
and 345–354 were added to TM5 and TM6, respectively. The IC50 of M1R was extended by 15 amino-acid residues from TM6. Primer sequences for these modifications are provided in the Supplementary Table. The amino-acid sequences for these receptors are provided in the Supplementary Note 2. These receptors were purified essentially in the same way as mOR with detergent exchange to MNG during M1 FLAG chromatography and using aprotinin (Sigma-Aldrich) and iperox (Sigma-Aldrich) in place of naloxone and lofentanil, respectively.

Heterotrimeric G-proteins were expressed and purified as previously described. Briefly, Trichoplusia ni (Hi5) insect cells (Expression Systems) were co-infected with two viruses, one encoding the wild-type human Ga subunit and another encoding the wild-type human β1y2 subunits with a decahistidine tag inserted at the amino terminus of the β1 subunit with HRV-3C protease cleavable site. In the case of Gi1α complex, additional virus encoding Ric8A was also co-infected. Cells were harvested 48 h post infection, lysed in hypotonic buffer and lipid-modified heterotrimeric G-protein was extracted in a buffer containing 1% sodium cholate (Sigma-Aldrich) and 0.05% DDM. The soluble fraction was purified using Ni-chelating sepharose chromatography, and the detergent was exchanged from cholate/DDM mixture to DDM alone. Following elution, HRV-3C protease (in-house prepared) was added and the protein was dialyzed against a buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM MgCl2, 0.05% DDM, 100 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma-Aldrich), 10 μM GDP (Sigma-Aldrich). Cleaved heterotrimeric G-protein was further purified by reloading over Ni-sepharose resin. The flow through was collected and purified over a size-exclusion chromatography using a Superdex 200 10/300 column.

Soluble Gβγ subunit for crystallography was expressed and purified from Trichoplusia ni (Hi5) insect cells. Hi5 cells were infected with baculovirus encoding the human β1y2 subunits with a cysteine 68 of y2 subunit mutated to thiol (Gβ1γ2G88S) to remove the geranylation modification. A decahistidine tag was attached at the amino terminus of the β1 subunit with HRV-3C protease cleavable site. Cultures were harvested 48 h post infection. Cells were lysed in the lysis buffer (10 mM Tris pH 7.4, 5 mM 2-mercaptoethanol, 160 μg/ml benzamidine and 2.5 μg/ml leupeptin). Following centrifugation, the supernatant was incubated with Ni-chelating sepharose resin. The resin was washed with a high salt buffer (20 mM Hepes pH 7.5, 500 mM NaCl, 20 mM imidazole and 2 mM 2-mercaptoethanol) then a low salt buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 20 mM imidazole and 100 μM TCEP). The protein was eluted with an elution buffer (2 mM Hepes pH 7.5, 100 mM NaCl, 250 mM imidazole and 100 μM TCEP) and dialysed against 20 mM Hepes pH 7.5, 100 mM NaCl and 100 μM TCEP after adding HRC-3C protease to cleave amino-terminal His-tag. Gβ1γ2G88S was further purified by re-loading over Ni-sepharose resin. The flow through was collected and purified over a size-exclusion chromatography using a Superdex 200 10/300 column.

The human Ga11 subunit with HRV-3C protease cleavable site. The amino-acid sequences described previously using agonists lofentanil, iperox, Bi-167107 for heterotrimeric G-protein was extracted in a buffer containing 1% sodium cholate (Sigma-Aldrich) and 0.05% DDM. The soluble fraction was purified using Ni-chelating sepharose chromatography, and the detergent was exchanged from cholate/DDM mixture to DDM alone. Following elution, HRV-3C protease (in-house prepared) was added and the protein was dialyzed against a buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM MgCl2, 250 mM imidazole, 10 μM GDP and 100 μM TCEP and dialysed against 20 mM Hepes pH 7.5, 100 mM NaCl and 100 μM TCEP after adding HRC-3C protease to cleave amino-terminal His-tag. Gβ1γ2G88S was further purified by reloading over Ni-sepharose resin. The flow through was collected and purified over a size-exclusion chromatography using a Superdex 200 10/300 column.

GPCR/G-protein complex was prepared essentially in the same way as described previously using agonists lofentanil, iperox, Bi-167107 for mOR, M1R and M2R, B2AR, respectively. Briefly, receptor was mixed with 1.5 molar excess G-protein. Following the incubation at room temperature for 1 h, apyrase was added and the reaction mixture was transferred to 4 °C and further incubated for 4 h to overnight. Prior to loading M1 FLAG column, 1% MNG and 0.1% CHS was added. The MNG concentration was progressively lowered during M1 FLAG wash. FLAG eluted protein was further purified by size-exclusion chromatography on a Superdex 200 10/300 column.

Monoclonal antibody production and characterization. For the antigen, rhodopsin–cGα complex was stabilized by crosslink using BS-3 (ThermoFisher). Naval Medical Research Institute (NMRI) mice were immunized intraperitoneally with the emulsified antigen. This study was carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzver- ordnung) of the Swiss Bundesamt für Veterinärwesen. The protocol was ethically approved by the Ethikkommission der Stadt Basel (Permit Number: 2375/2013). Mice with strong ELISA reaction to the antigen were killed and the spleen was removed. Isolated splenocytes were fused with the myeloma cell partner (PAI

**Fig. 5** | Generation of chimeric G-proteins. a Alignment of the αN helix of the G-protein subfamilies and the sequence of the chimeric Ga subunits. Transferred region from Gaα11 in Gaβ2AR is shown in orange. b, c Analytical SEC of β2AR/Gaα11 and M1R/Gaα11N complexes incubated with GTPγS in the presence or absence of Fab16. Protein elution profiles were monitored by the intrinsic tryptophan fluorescence. d Negative stain electron microscopy image of purified the M1R/Gaα11N/ScFvFab16 complex. β2AR was purified in the same way as described previously. Briefly, Sf9 insect cells were lysed by osmotic shock prior to solubilization of the membrane fraction by DDM. Solubilized receptor was first purified by M1 anti-FLAG immunoaffinity chromatography, followed by alpenrol-sepharose chromatography (alpenrol-sepharose resin prepared in-house) to isolate only functional receptor. Alpenrol-sepharose eluate was concentrated on M1 FLAG affinity resin, and then washed with ligand-free buffer for 1 h at room temperature to eliminate bound alpenrol. Receptor was eluted in a buffer consisting of 20 mM Hepes pH 7.5, 350 mM NaCl, 0.1% DDM and 0.01% CHS and further purified by size-exclusion chromatography on a Superdex 200 10/300 column (GE Healthcare) in buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 0.05% DDM, 0.005% CHS and 1 μM BI-167107 (Boehringer Ingelheim). The eluted receptor was concentrated to ~100 μM for complexing.

For M1R and M2R, we used constructs in previously published studies with some modifications. Briefly, T4L in the ICL3 of M1R was removed, and residues 219–232
mouse myeloma cells, derived from P3-x63-AG8) using polyethylene glycol 1500 (Roche Diagnostics). The fusion mixture was plated into multi-well plates (Thermo Scientific Nun MicroWell Cell Culture High Flange 96-Well Microplates) and clones hybridomas were selected by growing in HAT medium supplemented with culture supernatant of mouse macrophages P388. IgG positive clones were screened by ELISA for reactivity against Rhodopsin/Gi complex. Clones that showed a positive reaction in an ELISA assay and by immunoprecipitation were further characterized with the specific antibodies or Fab fragments. Initial SEF analysis using rhodopsin/Gi or each component was carried out in 20 mM Hepes pH 7.5, 100 mM NaCl, 2 mM mercaptoethanol and 0.01% MNG using Superdex 200 210/200 column.

Conclusions of the heavy-chain (VH-CH1) and light-chain (VL-CL) of mAb16 were cloned into the modified pVL1392 vector containing a GP67 secretion signal immediately prior to the amino terminus of the scFv16. Octahistidine-tag with HRV-3C protease cleavage site was attached to the carboxy-terminus of VH-CH1 for the expression System, and purified by Ni-sepharose chromatography. Supernatant from baculovirus infected cells was pH balanced by addition of Tris pH 7.5. Chelating agents were added with a G protein. The single-chain variable fragment of mAb16 (scFv16) was cloned into a modified pVL1392 vector containing a GP67 secretion signal immediately prior to the amino terminus of the scFv16. Octahistidine-tag with HRV-3C protease cleavage site was attached to the carboxy-terminus.

Both Fab16 and scFv16 were expressed in secreted form from Trichoplusia ni Hi5 insect cells using the baculovirus infection method (Expression Systems), and purified by Ni-sepharose chromatography. Supernatant from baculovirus infected cells was pH balanced by addition of Tris pH 7.5. Chelating agents were added with a G protein. The single-chain variable fragment of mAb16 (scFv16) was cloned into a modified pVL1392 vector containing a GP67 secretion signal immediately prior to the amino terminus of the scFv16. Octahistidine-tag with HRV-3C protease cleavage site was attached to the carboxy-terminus.

The binding assay of Fab16 with heterotrimeric-G protein subtypes, Fab16 was first labelled with Alexa Fluor 488 NHS Ester (ThermoFisher Scientific) in 20 mM MES pH 6.5. Free dye was removed by G-50 desalting column (GE healthcare). Monomeric fractions were pooled, concentrated and flash frozen in liquid nitrogen until use.

The fusion mixture was plated into multi-well plates (Thermo Scientific Nun MicroWell Cell Culture High Flange 96-Well Microplates) and clones hybridomas were selected by growing in HAT medium supplemented with culture supernatant of mouse macrophages P388. IgG positive clones were screened by ELISA for reactivity against Rhodopsin/Gi complex. Clones that showed a positive reaction in an ELISA assay and by immunoprecipitation were further characterized with the specific antibodies or Fab fragments. Initial SEF analysis using rhodopsin/Gi or each component was carried out in 20 mM Hepes pH 7.5, 100 mM NaCl, 2 mM mercaptoethanol and 0.01% MNG using Superdex 200 210/200 column.

Conclusions of the heavy-chain (VH-CH1) and light-chain (VL-CL) of mAb16 were cloned into the modified pVL1392 vector containing a GP67 secretion signal immediately prior to the amino terminus of the scFv16. Octahistidine-tag with HRV-3C protease cleavage site was attached to the carboxy-terminus.

Both Fab16 and scFv16 were expressed in secreted form from Trichoplusia ni Hi5 insect cells using the baculovirus infection method (Expression Systems), and purified by Ni-sepharose chromatography. Supernatant from baculovirus infected cells was pH balanced by addition of Tris pH 7.5. Chelating agents were added with a G protein. The single-chain variable fragment of mAb16 (scFv16) was cloned into a modified pVL1392 vector containing a GP67 secretion signal immediately prior to the amino terminus of the scFv16. Octahistidine-tag with HRV-3C protease cleavage site was attached to the carboxy-terminus.
21. Mixon, M. B. et al. Tertiary and quaternary structural changes in galpha induced by GTP hydrolysis. Science 270, 954–960 (1995).
22. Coleman, D. et al. Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. Science 265, 1405–1412 (1994).
23. Van Eps, N., Oldham, W. M., Hamm, H. E. & Hubbell, W. L. Structural and dynamical changes in an subunit of a heterotrimeric G protein along the activation pathway. Proc. Natl Acad. Sci. USA 103, 16194–16199 (2006).
24. Brandt, D. R. & Ross, E. M. GTPase activity of the stimulatory GTP-binding regulatory protein of adenylate cyclase, Gs. Accumulation and turnover of enzyme-nucleotide intermediates. J. Biol. Chem. 260, 266–272 (1985).
25. Chung, K. Y. et al. Conformational changes in the G protein Gs induced by the [brg]2 adrenergic receptor. Nature 477, 611–615 (2011).
26. Medkova, M., Preininger, A. M., Yu, N.-J., Hubbell, W. L. & Hamm, H. E. Conformational changes in the amino-terminal helix of the G protein alpha (ii) following dissociation from Gbetagamma subunit and activation. Biochemistry 41, 9962–9972 (2002).
27. Downes, G. B. & Gautam, N. The G protein subunit gene families. Genomics 62, 544–552 (1999).
28. Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T. & Tesmer, J. J. Snapshot of activated G proteins at the membrane- the Galphaq-GGR2-Gbetagamma complex. Science 310, 1686–1690 (2005).
29. Kreutz, B. et al. A new approach to producing functional ga subunits yields the activated and deactivated structures of Ga 12/13proteins. Biochemistry 45, 167–174 (2006).
30. Nishimura, A. et al. Structural basis for the specific inhibition of heterotrimeric Gq protein by a small molecule. Proc. Natl Acad. Sci. USA 107, 13666–13671 (2010).
31. Yamaguchi, Y., Katoh, H. & Negishi, M. N-terminal short sequences of alpha subunits of the G12 family determine selective coupling to receptors. J. Biol. Chem. 278, 14936–14939 (2003).
32. Osawa, S., Dhanasekaran, N., Woon, C. W. & Johnson, G. L. G alpha i-G alpha s chimeras define the function of alpha chain domains in control of G protein activation and beta gamma subunit complex interactions. Cell 63, 697–706 (1990).
33. Russell, M. & Johnson, G. L. G protein amino-terminal alpha i2/alpha s chimeras reveal amino acids important in regulating alpha s activity. Mol. Pharmacol. 44, 255–263 (1993).
34. Gregorio, G. G. et al. Single-molecule analysis of ligand efficacy in beta2AR-G-protein activation. Nature 547, 68–73 (2017).
35. Thal, D. M. et al. Crystal structures of the M1 and M4 muscarinic acetylcholine receptors. Nature 531, 7282–7287 (2016).
36. Kabsch, W. XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132 (2010).
37. Evans, P. Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82 (2006).
38. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
39. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501 (2010).
40. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 (2010).
41. Afonine, P. V. et al. Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr. D Biol. Crystallogr. 68, 352–367 (2012).
42. Flock, T. et al. Universal allosteric mechanism for Ga activation by GPCRs. Nature 524, 173–179 (2015).

Acknowledgements

We thank Jean-Philippe Carralot (F. Hoffmann-La Roche Ltd) for help in antibody generation, Martin Siegrist, Georg Schmid, Bernard Ruten, Doris Zulauf, Stephanie Kueng (Roche Non-Clinical Biorepository) and Ralf Thoma for technical assistance for biomass and cell line generation. Shoji Maeda was supported by the Roche Postdoctoral Fellowship (RPF ID: 113). This work was supported by NIH grant R01GM083118 to B.K. K. B.K.K. is a Chan Zuckerberg Biohub investigator.

Author contributions

S.M. prepared rhodopsin/Gi1 complex for immunization and selection, performed mAb characterization, Fab and scFv cloning, expression and purification with assistance from A.M., prepared M1RGi1, M1RGi2, J2AR/Gi1, performed Gi1/scFv16 crystallization, structure determination and characterization. A.K. prepared mOR/Gs. H.M. performed immunization and antibody selection with assist from R.J.P.D. H.H. performed negative stain EM visualization. D.H. prepared β2AR/Gi, and provided advice for G-protein experiments. G.S. provided advice on EM analysis and interpretation. G.F.X.S. initiated the project. R.J.P.D., and B.K.K. supervised the project.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-06002-w.

Competing interests: B.K.K. is a co-founder of and consultant for ConfometRx. R.J.P.D. is employed by Roche Pharmaceuticals. The remaining authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2018