Engineering a minimal G protein to facilitate crystallisation of G protein-coupled receptors in their active conformation

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Abstract

G protein-coupled receptors (GPCRs) modulate cytoplasmic signalling in response to extracellular stimuli, and are important therapeutic targets in a wide range of diseases. Structure determination of GPCRs in all activation states is important to elucidate the precise mechanism of signal transduction and to facilitate optimal drug design. However, due to their inherent instability, crystallisation of GPCRs in complex with cytoplasmic signalling proteins, such as heterotrimeric G proteins and β-arrestins, has proved challenging. Here, we describe the design of a minimal G protein, mini-Gs, which is composed solely of the GTPase domain from the adenylate cyclase stimulating G protein Gs. Mini-Gs is a small, soluble protein, which efficiently couples GPCRs in the absence of Gβγ subunits. We engineered mini-Gs, using rational design mutagenesis, to form a stable complex with detergent-solubilised β1-adrenergic receptor (β1AR). Mini G proteins induce similar pharmacological and structural changes in GPCRs as heterotrimeric G proteins, but eliminate many of the problems associated with crystallisation of these complexes, specifically their large size, conformational dynamics and instability in detergent. They are therefore novel tools, which will facilitate the biochemical and structural characterisation of GPCRs in their active conformation.

Key words: complex, G protein, G protein-coupled receptor, GPCR, Gα, mini G protein, mini-Gs

Introduction

G protein-coupled receptors (GPCRs) modulate cytoplasmic signalling, through heterotrimeric G proteins and β-arrestins, in response to extracellular stimuli, such as hormones and neurotransmitters (Rosenbaum et al., 2009). The central role of GPCRs in regulating cellular responses makes them an important therapeutic target (Lagerstrom and Schioth, 2008). GPCRs adopt different conformational states in response to binding different classes of ligand and coupling to cytoplasmic signalling proteins. Therefore, structure determination of GPCRs in all activation states is important to decipher the molecular mechanisms of signal transduction, and to facilitate optimal drug design.

Heterotrimeric G proteins are composed of α, β and γ subunits. Gα consists of a GTPase domain (GαGTPase), which is analogous to members of the small GTPase superfamily of proteins, and an α-helical domain (GαAH), which is unique to heterotrimeric G proteins (Sprang, 1997). In the inactive, GDP-bound state, Gα binds Gβγ, forming a heterotrimer with low basal nucleotide exchange activity (Fingar et al., 1987). The trimer is anchored to the cell membrane, through lipid modifications of both Gα and Gγ (Spiegel...
GPCRs catalyse rapid nucleotide exchange on heterotrimeric G proteins, but only weakly activate the isolated α subunit (Herrmann et al., 2006; Phillips et al., 1992).

Agonist binding to a GPCR promotes its transition to a structural state that can efficiently interact with heterotrimeric G proteins (Lebon et al., 2011b; Rasmussen et al., 2011b; Rosenbaum et al., 2011; Xu et al., 2011). The agonist-bound receptor engages the C-terminal region of Gα (Hamm et al., 1998), initiating a rotation and displacement of the α5 helix (Oldham et al., 2006). This ultimately destabilises the nucleotide-binding pocket and the GoGTase–GαAH domain interface, allowing GDP to dissociate (Alexander et al., 2014; Dör et al., 2015; Flock et al., 2015; Kaya et al., 2014; Sun et al., 2015; Van Eps et al., 2011). The resulting nucleotide-free ternary complex displays large, mutually induced structural changes in both the receptor and G protein (Rasmussen et al., 2011b), and is often characterised by increased agonist binding affinity of the receptor (Deleau et al., 1980). This complex can be trapped in the absence of guanine nucleotides (Bornancin et al., 1989), but is extremely short-lived in vivo due to rapid binding of GTP to Gα (Vuong et al., 1984). GTP binding triggers dissociation of the G protein from the receptor (Kuhn, 1981) and separation of Gα from Gβγ (Fung et al., 1981).

GPCR–G protein complexes are difficult targets for structural studies due to their large size, conformational dynamics, and instability in detergent. To date, only a single structure of a GPCR–G protein complex has been reported, namely the β2-adrenergic receptor (β2AR) bound to the adenylate cyclase stimulating G protein G1 (Rasmussen et al., 2011b). This structure provided the first atomic resolution insight into the organisation of the ternary complex, but further structures are required to fully decipher the molecular mechanisms of signal transduction and the specificity for G protein coupling. Given the difficulties in crystallising GPCR–G protein complexes, novel tools are needed to facilitate their high-throughput crystallisation.

Here, we report the design of a minimal G protein, termed mini-G1α, which is composed solely of the GoGTase domain from Gα1. Mini-Gα closely mimics the pharmacological and structural changes induced in GPCRs by heterotrimeric Gα. It is therefore a novel tool, which will facilitate the characterisation of GPCRs in their active conformation, and has allowed the structure determination of the adenosine A2A receptor in the fully active state (Carpenter et al., 2016).

**Materials and Methods**

**Cloning**

Details of G protein, β1AR and mini-G1 constructs used in this work are provided in Supplementary Tables SI-SIII, respectively. All G proteins used in this study were mutared to remove sites of lipid modification. G protein cDNAs were cloned into the transfer vector pBacPAK8 (Clontech), and baculoviruses were prepared using the flashBAC ULTRA system (Oxford Expression Technologies). Synthetic genes (Integrated DNA Technologies) for Nb80 (Rasmussen et al., 2011a) and Nb35 (Westfield et al., 2011) were cloned into pET26b (Novagen) for periplasmic expression in *Escherichia coli*. Mini-Gα constructs, which were derived from the long isoform of the human Gαα, gene, were cloned into the pET15b vector (Novagen) for expression in *E. coli*.

**Expression and purification of β1AR**

β1AR constructs were expressed in insect cells using the baculovirus expression system, and purified as described previously (Warne et al., 2003; Warne et al., 2011; Warne et al., 2009).

**Baculovirus expression of G proteins**

*Trichoplusia ni* cells (Expression Systems) were grown in ESF921 serum-free media (Expression Systems) in 5 L optimum growth flasks (Thompson Instrument Company). Immediately before infection, heat-inactivated foetal bovine serum (Sigma) was added to a final concentration of 5%. Cells were infected with third passage virus at a final concentration of 3%. In the case of co-infection with multiple viruses (for heterotrimeric G1, or Gβγ) each virus was added to a final concentration of 3%. The final volume of culture was 3 L per flask and the final cell density was 3 × 10^9 cells/ml. Cells were harvested 48 h post-infection by centrifugation at 5000 g for 5 mins, flash-frozen in liquid nitrogen and stored at −80°C.

**Protein purification**

Details of G protein, nanobody and mini-Gα purifications are provided in the Supplementary material.

**Saturation binding assay**

Insect cell membranes containing β1AR were resuspended in assay buffer (20 mM HEPES pH 7.5, 100 mM NaCl). The sample was aliquoted and [3H]-dihydroalprenolol was added (to give final concentrations in the range of 0.25 nM to 256 nM), alprenolol was added to the negative control (1 mM final concentration). Samples were incubated at 20°C for 2 h, before filtering through 96-well glass fibre filter plates (Merck Millipore) and washing with ice-cold assay buffer. Radioactivity was quantified by scintillation counting and apparent *Kd* values were determined using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

**Competition binding assay**

Insect cell membranes containing β1AR were resuspended in assay buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1 mM ascorbate). The sample was aliquoted and binding partner (25 μM final concentration), isoprenaline (final concentrations in the range of 1 pM–100 nM), and apyrase (0.1 U/ml final concentration) were added. Alprenolol was added to the negative control (100 μM final concentration). Samples were incubated at 20°C for 1.5 h, before adding [3H]-dihydroalprenolol (5 or 20 nM final concentrations for β1ARNC or β1AR-84, respectively). Samples were incubated at 20°C for 1.5 h, before filtering through 96-well glass fibre filter plates and washing with ice-cold assay buffer. Radioactivity was quantified by scintillation counting and *Kd* values were determined using GraphPad Prism version 5.0.

**Competition binding assays using detergent-solubilised β1AR-84**

were performed using a similar protocol, except: all steps were performed at 4°C; membranes were solubilised with dodecyl maltoside (DDM; 0.1% final concentration) for 30 min, prior to addition of binding partner and ligands; separation of bound from free ligand (by gel filtration) was performed exactly as described in the thermo-stability assay protocol (below).

**Thermostability measurement of β1ARNC-mini-Gα complexes**

Thermostability assays were performed using a modified version of previously described methods (Lebon et al., 2011a; Serrano-Vega et al., 2008). Insect cell membranes containing β1ARNC were resuspended in assay buffer (25 mM HEPES pH 7.5, 400 mM NaCl,
Thermostability measurement of GDP-bound mini-Gs mutants by differential scanning fluorimetry

Differential scanning fluorimetry (DSF) was performed essentially as described previously (Niesen et al., 2007). Mini-G, mutants (30 μg) were diluted with assay buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1 mM GDP, 2 mM DTT). SYPRO-orange was added to give a final concentration of 2 μM. Thermostability measurements were performed using a Rotor-Gene Q (Qiagen). Samples were equilibrated for 90 s at 25°C before ramping from 25 to 99°C at 4°C/min. The apparent melting temperature (Tm) values were determined using GraphPad Prism version 5.0.

Gel filtration analysis of mini G protein complexes

The mini-G-βγ complex was prepared using mini-G399, a construct in which the N-terminal residues 6–25 were replaced and the L272D mutation was reversed (Supplementary Table SIII). Purified mini-G399 was mixed with non-lipidated Gi172 dimer in an equimolar ratio and incubated on ice for 4 h. The sample was loaded onto a Superdex-200 10/300 gel filtration column (GE healthcare), equilibrated with gel filtration buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1 μM GDP, 0.1 mM TCEP).

The β2AR-mini-G complex was prepared using β2ARANC purified in lauryl maltose neopentyl glycol (LMNG) detergent. Purified β2ARANC was mixed with a 1.2-fold molar excess of mini-G393 and incubated on ice for 4 h. The sample was loaded onto a Superdex-200 10/300 gel filtration column, equilibrated with gel filtration buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1 μM ascorbic acid, 1 μM isoproterenol, 0.002% LMNG). Peak fractions were analysed by SDS-PAGE on a 4–20% Tris-glycine gel (Thermo Fisher).

The gel filtration column was calibrated using molecular weight standards (Sigma), and the apparent molecular weight of samples was calculated using the calibration curve shown in Supplementary Fig. S9.

Statistical analysis

Unpaired, two-tailed t-tests were used to compare two data sets, and P values are quoted in the text.
there is only a small increase in agonist affinity upon binding a G protein (Carpenter et al., 2016).

To be most useful for the characterisation and structure determination of GPCRs in their active state, mini-Gs needed to fulfil a number of criteria. Mini-Gs should be stable enough in its basal conformation to allow high-yield expression and purification, promote the transition of β1AR to the high-affinity agonist-bound state, and form a stable complex with detergent-solubilised β1AR. The development of mini-Gs involved a number of key steps, some of which only became apparent as the work progressed: (i) development of a sensitive assay to detect G protein coupling to β1AR; (ii) isolation of the GαGTPase domain (mini-Gs) and demonstration of binding to β1AR; (iii) thermostabilisation of the β1AR-mini-Gs complex in detergent; (iv) thermostabilisation of the β1AR-mini-Gs complex in detergent; and (v) validation of the final mini-Gs construct. Details of each of these steps are given under the corresponding subheadings later.

Development of a sensitive assay to detect G protein coupling to β1AR
A sensitive competition binding assay was developed that could detect the interaction of different binding partners with β1AR, by measuring the affinity of agonist binding to the receptor. A heterologous competition format was used to determine the agonist binding affinity (Kᵢ) of β1AR by measuring binding of the antagonist 3H-dihydroalprenolol (3H-DHA; Supplementary Fig. S1) in the presence of increasing concentrations of the agonist isoprenaline. The concentration of binding proteins used in the assay was standardised to 25 μM, which was approximately 30-fold above the equilibrium dissociation constant (Kᵢ) for Nb80 binding to β1AR (Miller-Gallacher et al., 2014). Initially, we used a truncated form of turkey β1AR (Warne et al., 2003), which was designated β1AR<sup>ANC</sup> (Supplementary Table SII). This construct did not contain any thermostabilisation mutations, and behaved identically to full-length receptor in cell-signalling assays (Baker et al., 2011), despite containing truncations of disordered regions in the N-terminus and C-terminus. The Kᵢ for isoprenaline binding to β1AR<sup>ANC</sup> was 40 ± 0 nM in the absence of a binding partner, which shifted to 5.8 ± 0.8 nM, 17 ± 2 nM or 6.8 ± 0.6 nM in response to Nb80, Gs<sub>I</sub> or Gs<sub>II</sub>-Nb35, respectively (Supplementary Fig. S2). The shift in isoprenaline Kᵢ upon G protein binding to β1AR<sup>ANC</sup> was relatively small, which was unsuitable for detecting potentially small changes elicited upon binding of unstable G protein derivatives during the development of mini-Gs. We therefore used a minimally thermostabilised β1AR construct (β1AR-84; Supplementary Table SII), which contained four mutations that increased the stability preferentially of the inactive state of the receptor (Miller-Gallacher et al., 2014; Warne et al., 2011), in addition to the truncations at the N-terminus and C-terminus. β1AR-84 had a lower affinity for isoprenaline (Kᵢ of 2.6 ± 0.3 μM) in its uncoupled state than β1AR<sup>ANC</sup>, but showed a larger shift in agonist binding affinity when coupled to either Nb80, Gs<sub>I</sub> or Gs<sub>II</sub>-Nb35 (Kᵢ of 28 ± 1 nM, 271 ± 54 nM or 16 ± 4 nM, respectively; Table I and Supplementary Fig. S2). The competition binding data fitted best to single-site binding parameters. Therefore, the partial shift in isoprenaline Kᵢ observed for some binding partners most likely reflected incomplete stabilisation of the high-affinity agonist-bound state, rather than indicating partial coupling or mixed receptor populations. Although Gs<sub>I</sub> was able to couple β1AR, Nb35 was required to stabilise the Gs complex, resulting in β1AR-Gs<sub>II</sub>-Nb35 complexes with similar affinity for isoprenaline compared to the β1AR-Nb80 complexes (Table I and Supplementary Fig. S2).

The competition binding assay using β1AR-84 showed a 162-fold increase in isoprenaline affinity upon Gs<sub>I</sub>-Nb35 coupling to the receptor, which was far larger than the 6-fold shift in affinity induced by Gs<sub>II</sub>-Nb35 binding to β1AR<sup>ANC</sup>. We therefore used β1AR-84 in all subsequent competition binding assays during the development of mini-Gs.

Isolation of the Gα<sub>G</sub> GTPase domain and measuring binding to β1AR-84
The GαGTPase domain from Gα<sub>G</sub> had previously been expressed as an isolated protein, in order to determine its role in guanine nucleotide binding and hydrolysis (Markby et al., 1993), but its ability to couple to GPCRs had never been investigated. We isolated the Gα<sub>G</sub> domain by replacing the sequence corresponding to GoAH with a short glycine linker (Supplementary Table SIII). This construct, mini-G<sub>G</sub>,77 expressed poorly in E. coli and could not be purified to homogeneity, indicating that it was very unstable. Nevertheless, a small amount of partially pure protein could be prepared (Supplementary Fig. S3), and was tested for its ability to couple β1AR-84 (at 20°C) in either the presence or absence of Gβγ-Nb35. No significant shift in the isoprenaline Kᵢ of β1AR-84 (2.6 ± 0.3 μM) was observed in the presence of mini-G<sub>G</sub>,77 (1.9 ± 0.2 μM; P = 0.254), but mini-G<sub>G</sub>,77-Gβγ-Nb35 induced a large shift in isoprenaline affinity to 3.6 ± 0.8 nM (P = 0.004; Table I and Supplementary Fig. S2). Thus partially purified mini-G<sub>G</sub>,77 was functional, but the data also suggested that it was unable to couple β1AR-84 in the absence of Gβγ when assayed at 20°C. In contrast, when the assay was performed at 4°C mini-G<sub>G</sub>,77 induced a significant shift in isoprenaline Kᵢ from 2.1 ± 0.2 μM for uncoupled β1AR-84 (at 4°C) to 99 ± 12 nM (P < 0.001; Table I and Supplementary Fig. S2). This demonstrated that the isolated GαGTPase domain (mini-G<sub>G</sub>,77) could couple to β1AR-84 in the absence of Gβγ, but suggested that the thermostability of the GαGTPase domain was a limiting factor in its ability to stabilise the high-affinity agonist-bound state of the receptor.

Thermostabilisation of the β1AR-mini-Gs complex in membranes
Rational design mutagenesis was employed to thermostabilise mini-Gs in complex with membrane-embedded β1AR. Mutations were designed based on structural alignments (Fig. 2a and Supplementary Fig. S4) between Gα<sub>G</sub> (Sunahara et al., 1997) and Arl2 (Hanzal-Bayer et al., 2002); Arl2 is the small GTPase with the greatest structural similarity to Gα<sub>G</sub>. This initial mutagenic screen primarily targeted regions of Gα<sub>G</sub> that were close to the GoAH domain interface or that were known to be conformationally dynamic. Mutants were screened using the competition binding assay at both 4 and 20°C. Due to the low, and variable expression level of the mutants, it was not easy to standardise the concentration of mini-Gs mutants used in the assays. Therefore, the total mini-Gs purified from 1 L of E. coli culture was used per competition curve (Table I). Approximately 100 mutants were tested during this initial screen. Mutations that shifted the isoprenaline Kᵢ of β1AR-84 more than 2-fold compared to the parental mini-Gs construct (mini-G<sub>G</sub>,77) at either temperature were classed as positive. A total of 16 positive mutations, covering 12 unique positions were identified (Table I).

Some of the single mini-Gs mutants produced a near maximal shift in the agonist binding affinity of β1AR-84 in the competition binding assay, therefore, mutation combinations could not be
Table I. β1AR-84 competition binding data

| Binding partner | Mutation | CGN code | β1AR-84 isoprenaline Ki (nM) | Effect on expression |
|-----------------|----------|----------|-----------------------------|---------------------|
|                 |          |          | 4°C                         | 20°C               |
| None            | n.a.     | n.a.     | 2100 ± 180 (n = 12)         | 2600 ± 270 (n = 15) |
| Nb80            | n.a.     | n.a.     | n.d.*                      | 28 ± 1 (n = 2)      |
| Gs              | n.a.     | n.a.     | 420 ± 80 (n = 2)            | 271 ± 54 (n = 2)    |
| Gs–Nb35         | n.a.     | n.a.     | n.d.                       | 16 ± 4 (n = 3)      |
| Mini-Gs77       | Parental | n.a.     | 99 ± 12 (n = 4)             | 1900 ± 230 (n = 3)  |
|                 | H41I     | 41G.S3.3 | 32                          | 390                 |
|                 | H41V     | 41G.S3.3 | 51                          | 490                 |
|                 | A48L     | 48G.T3.1 | 43                          | 170                 |
|                 | G49D     | 49G.T3.1 | 25                          | 280                 |
|                 | E50N     | 50G.T3.1 | 37                          | 720                 |
|                 | R201A    | 201G.H3.2| 31                          | 1480                |
|                 | G226A    | 226G.H2.2| 86                          | 820                 |
|                 |          |          | 227–230 subd                |                    |
|                 | 227–230  | 227G.H2.2| 31                          | 1480                |
|                 | subd     |          | 86                          | 820                 |
|                 | A249D    | 249G.S4.7| 10                          | 35                  |
|                 | A249E    | 249G.S4.7| 70                          | 390                 |
|                 | S522D    | 522G.H3.3| 14                          | 94                  |
|                 | S522E    | 522G.H3.3| 38                          | 380                 |
|                 | S55–264  | 55G.H3.5 | 21                          | 20                  |
|                 | L72D     | 272G.H3.8| 7                           | 310                 |
|                 | L72E     | 272G.H3.8| 28                          | 750                 |

Competition binding data showing the isoprenaline Ki of β1AR-84 in the presence of different binding partners. Data are from a single experiment performed in duplicate unless otherwise stated; where two or more independent experiments were performed, data represent mean ± SEM, from the number (n) of independent experiments performed in duplicate. The effect of mutations on the expression level of mini-Gs was estimated from SDS-PAGE gels. Expression levels are described as being equal to (=), more than 2-fold lower than (−), or more than 2-fold higher than (+) the parental construct.

*Common Gs numbering (CGN) system (Flock et al., 2015).

bNot applicable.

dSubstitution of switch II residues 227–230 with two glycine residues.

Reliably tested using this assay, as further small increases in agonist affinity would be difficult to accurately measure. Instead, the mutation combinations were tested in complex with detergent-solubilised β1ARNC using a thermostability (Tm) assay (Table II). The agonist [3H]-norepinephrine ([3H]-NE) was used in the Tm assay, however due to the high background signal associated with this ligand, a maximum concentration of 200 nM could be used. This was approximately equal to the high background signal associated with this ligand, a maximum concentration of 200 nM could be used. This was approximately (β1AR-84 isoprenaline (nM) Effect on expression)

### Table II. β1AR-84 competition binding data

| Binding partner | Mutation | CGN code | β1AR-84 isoprenaline Ki (nM) | Effect on expression |
|-----------------|----------|----------|-----------------------------|---------------------|
|                 |          |          | 4°C                         | 20°C               |
| None            | n.a.     | n.a.     | 2100 ± 180 (n = 12)         | 2600 ± 270 (n = 15) |
| Nb80            | n.a.     | n.a.     | n.d.*                      | 28 ± 1 (n = 2)      |
| Gs              | n.a.     | n.a.     | 420 ± 80 (n = 2)            | 271 ± 54 (n = 2)    |
| Gs–Nb35         | n.a.     | n.a.     | n.d.                       | 16 ± 4 (n = 3)      |
| Mini-Gs77       | Parental | n.a.     | 99 ± 12 (n = 4)             | 1900 ± 230 (n = 3)  |
|                 | H41I     | 41G.S3.3 | 32                          | 390                 |
|                 | H41V     | 41G.S3.3 | 51                          | 490                 |
|                 | A48L     | 48G.T3.1 | 43                          | 170                 |
|                 | G49D     | 49G.T3.1 | 25                          | 280                 |
|                 | E50N     | 50G.T3.1 | 37                          | 720                 |
|                 | R201A    | 201G.H3.2| 31                          | 1480                |
|                 | G226A    | 226G.H2.2| 86                          | 820                 |
|                 |          |          | 227–230 subd                |                    |
|                 | 227–230  | 227G.H2.2| 31                          | 1480                |
|                 | subd     |          | 86                          | 820                 |
|                 | A249D    | 249G.S4.7| 10                          | 35                  |
|                 | A249E    | 249G.S4.7| 70                          | 390                 |
|                 | S522D    | 522G.H3.3| 14                          | 94                  |
|                 | S522E    | 522G.H3.3| 38                          | 380                 |
|                 | S55–264  | 55G.H3.5 | 21                          | 20                  |
|                 | L72D     | 272G.H3.8| 7                           | 310                 |
|                 | L72E     | 272G.H3.8| 28                          | 750                 |

Competition binding data showing the isoprenaline Ki of β1AR-84 in the presence of different binding partners. Data are from a single experiment performed in duplicate unless otherwise stated; where two or more independent experiments were performed, data represent mean ± SEM, from the number (n) of independent experiments performed in duplicate. The effect of mutations on the expression level of mini-Gs was estimated from SDS-PAGE gels. Expression levels are described as being equal to (=), more than 2-fold lower than (−), or more than 2-fold higher than (+) the parental construct.

Common Gs numbering (CGN) system (Flock et al., 2015).

bNot applicable.

dSubstitution of switch II residues 227–230 with two glycine residues.

Deletion of switch III residues 255–264.
Table II. β1AR\textsubscript{ANC} thermostability data

| Binding partner | Mutation | CGN code\textsuperscript{a} | Apparent $T_m$ of β1AR\textsubscript{ANC} in DDM, measured by $^3$H-NE binding (°C) | Stability of GDP-bound mini-Gs, measured by DSF (°C) |
|-----------------|----------|-----------------------------|---------------------------------------------------------------------------------|---------------------------------------------|
| None            | n.a.     | n.a.                        | 25.9 ± 0.0 (n = 3)                                                              | n.a.                                        |
| Nh80            | n.a.     | n.a.                        | 32.0 ± 0.0 (n = 3)                                                              | n.a.                                        |
| Gt–Nh80         | n.a.     | n.a.                        | 35.8 ± 0.1 (n = 3)                                                              | n.a.                                        |
| Gt             | n.a.     | n.d.\textsuperscript{c}     | 50.1 ± 0.1 (n = 3)                                                              | n.a.                                        |
| Mini-G\textsubscript{162} | A249D |                           | 25.1 (n = 1)                                                                    | 60.6 ± 0.1 (n = 3)                          |
| Mini-G\textsubscript{164} | A249D, SII\textsuperscript{d} |                           | 28.6 (n = 1)                                                                    | 66.5 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{165} | A249D, S252D, SII |                           | 28.5 ± 0.2 (n = 2)                                                              | 68.7 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{169} | A249D, S252D, SII, L272D |                           | 28.8 (n = 1)                                                                    | 67.1 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{183} | G49D, E50N, A249D, S252D, SII, L272D |                           | 28.7 ± 0.2 (n = 4)                                                              | 72.5 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{199}\textsuperscript{e} | G49D, E50N, A249D, S252D, SII, L272D |                           | 29.2 ± 0.2 (n = 17)                                                             | 72.5 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{254} | Mini-G\textsubscript{199} + M60A | 60\textsuperscript{G.H.1} | 31.5 ± 0.3 (n = 5)                                                              | 70.3 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{350} | Mini-G\textsubscript{199} + L63Y | 63\textsuperscript{G.H.11} | 30.9 ± 0.4 (n = 2)                                                              | 70.7 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{340} | Mini-G\textsubscript{199} + I372A | 372\textsuperscript{G.H.4} | 34.0 (n = 1)                                                                    | 66.6 ± 0.1 (n = 3)                          |
| Mini-G\textsubscript{303} | Mini-G\textsubscript{199} + V373I | 375\textsuperscript{G.H.7} | 31.5 ± 0.6 (n = 3)                                                              | 70.3 ± 0.0 (n = 3)                          |
| Mini-G\textsubscript{352} | Mini-G\textsubscript{199} + L63Y, I372A |                           | 34.5 (n = 1)                                                                    | 64.7 ± 0.1 (n = 3)                          |
| Mini-G\textsubscript{345} | Mini-G\textsubscript{199} + I372A, V373I |                           | 35.0 (n = 1)                                                                    | 65.4 ± 0.1 (n = 3)                          |
| Mini-G\textsubscript{393}\textsuperscript{f} | Final mini-Gs, construct |                           | 34.1 ± 0.5 (n = 3)                                                              | 65.3 ± 0.0 (n = 3)                          |

Thermostability data for either detergent-solubilised β1AR\textsubscript{ANC} complexes or mini-Gs mutants in the GDP-bound state. Apparent $T_m$ values represent the mean ± SEM from the number (n) of independent experiments performed in duplicate. Some apparent $T_m$ values were determined from a single experiment, with an assumed error of ±0.5°C. Apparent $T_m$ values for mini-Gs in the GDP-bound state were determined by differential scanning fluorimetry (Supplementary Fig. S6).

\textsuperscript{a}Common Gs numbering (CGN) system (Flock et al., 2015).
\textsuperscript{b}Not applicable.
\textsuperscript{c}Not determined.
\textsuperscript{d}Deletion of switch III residues 255–264 is referred to as SIII.
\textsuperscript{e}Mini-G\textsubscript{199} contains the same mutations as mini-G\textsubscript{183}, but has a redesigned linker region (Supplementary Table SIII), and was used as the parental construct for screening detergent-stabilising mutations.
\textsuperscript{f}Mini-G\textsubscript{393} contains the same mutations as mini-G\textsubscript{345}, but has an additional truncation of the N-terminus and redesigned linker region (Supplementary Table SIII), and was used as the starting construct for crystallisation trials.

designed to stabilise the region around switch III, through a potential interaction with Arg265. The G49D and E50N mutations, which are located in the P-loop, were designed to reduce flexibility and conformationally constrain this region, through potential interactions with Arg265 and Lys293, respectively. The sixth mutation (L272D) was designed to conformationally constrain switch II, through potential interactions with a cluster of charged and polar residues (227–233) within its N-terminal region (Fig. 2b).

Thermostabilisation of the β1AR\textsubscript{ANC–mini-Gs} complex in detergent

The majority of mutations from the first mutagenic screen did not stabilise the β1AR–mini-Gs complex in detergent. We hypothesised that this was because they did not specifically stabilise mini-Gs in its receptor-bound conformation, therefore, a second panel of approximately 150 mutants were designed with the intention of stabilising the receptor-bound conformation of mini-Gs. This mutagenic screen was based on the structure of the βAR-Gs complex (Rasmussen et al., 2011b), and focused on regions of Gs, that undergo large conformational changes upon receptor binding. Many of the mutations tested during this second screen were destabilising to mini-Gs in its basal GDP-bound state, therefore they could not be tested individually (i.e. in the mini-G\textsubscript{161} parental construct). Instead, they were added to mini-G\textsubscript{199}, which was identical to mini-G\textsubscript{183}, except that it contained a modified linker region (Supplementary Table SIII). This construct was very stable in its basal GDP-bound state (72.5°C) and could thus negate the destabilising effects of the additional mutations. Mini-Gs mutants were screened in complex with detergent-solubilised β1AR\textsubscript{ANC} using the $^3$H-norepinephrine $T_m$ assay. Four stabilising mutations were identified (Table II), the best of which (I372A) increased the apparent $T_m$ of the complex from 29.2 to 34.0°C and it combined additively with V373I, to give an apparent $T_m$ of 35.0°C. This was 3.0°C higher than the β1AR\textsubscript{ANC–Nh80} complex and only 0.8°C lower than the β1AR\textsubscript{ANC–Gs–Nh80} complex. The other mutations did not combine additively with I372A and V373I and were rejected (results not shown).

Both of the positive detergent-stabilising mutations were located within the α1 or α5 helices. Alignment of Gs, in its receptor-bound conformation (Rasmussen et al., 2011b) with the GTP-bound structure (Fig. 2c; Sunahara et al., 1997) identified an unfavourable steric clash across the α1–α5 helix interface, involving residues Met60, His64 (from the α1 helix) and Ile372 (from the α5 helix). This clash was predicted to prevent close packing of the C-terminal region of the α1 helix against the α5 helix and the core of the GtGTPase domain, thus exposing the core of the protein to the solvent. The I372A mutation was designed to eliminate this clash and facilitate better packing in this region. Similarly, the V373I mutation was designed to improve packing between the α5 helix and the core of the protein in its receptor-bound conformation (Fig. 2d).

Validation of mini-Gs

The detergent-stabilised construct (mini-G\textsubscript{s,345}) was modified for crystallographic applications by changing the linker and shortening...
Fig. 2 Rational design of mutations to stabilise mini-Gα. (a) Structural alignment of Gαs (PDB code 1AZT; Sunahara et al., 1997), coloured magenta and grey, and Arl2 (PDB code 1KSH; Hanzal-Bayer et al., 2002), coloured green. The Gαs GTPase domain aligns to Arl2 with an RMSD of 1.9 Å, despite sharing sequence identity of only 25%, determined using the DALI server (Holm and Rosenstrom, 2010). See Supplementary Fig. S4 for a sequence alignment between Gαs and Arl2. The inset shows an expanded view of mini-Gα residues (shown as sticks and underlined name) that were mutated (G49D, E50N, A249D, and S252D) to match the corresponding residue in Arl2. Residues with which the mutations potentially interact are shown as sticks. (b) Mutation of Leu272, which is located within the α3 helix of Gαs (PDB code 1AZT; Sunahara et al., 1997), to aspartic acid allows potential interactions with a cluster of charged and polar residues (227–233) in the N-terminal region of switch II. (c) Alignment of Gαs in its GTP-bound conformation (PDB code 1AZT; Sunahara et al., 1997), coloured magenta, and GPCR-bound conformation (PDB code 3SN6; Rasmussen et al., 2011b), coloured cyan. In the GPCR-bound conformation Ile372 (α5 helix) sterically clashes with Met60 and His64 (α1 helix), preventing close packing of the α1 helix against the core of the GαTPase domain. (d) The V375I mutation (modelled using PyMOL) was designed to increase hydrophobic contacts between the core of the GαTPase domain and the α5 helix in its GPCR-bound conformation (PDB code 3SN6; Rasmussen et al., 2011b). Residues that interact with Val375 are shown as sticks, additional contacts (less than 4.2 Å), which are predicted to be formed by the α-carbon (*) of the isoleucine mutation are displayed as dashed lines.

β struct, mini-Gα393 (Supplementary Fig. S7 and S8), was able to elicit the N-terminus (Supplementary Table SIII). The final stabilised construct, mini-Gα393 (Supplementary Fig. S7 and S8), was able to elicit an equal or greater shift in isoprenaline affinity compared to either Nb80 or Gα–Nb35 (Fig. 3a–c) whether the experiments were performed using membrane-embedded βiARANCE (Kᵢ of 4.1 ± 1.1 nM compared to 5.8 ± 0.8 nM and 6.8 ± 0.6 nM, respectively), membrane-embedded βiAR-84 (Kᵢ of 3.6 ± 0.0 nM compared to 28 ± 1 nM and 16 ± 4 nM, respectively) or detergent-solubilised βiAR-84 (Kᵢ of 4.7 ± 0.4 nM compared to 83 ± 2 nM and 23 ± 7 nM, respectively). Crucially, for the mini-Gα393 complex, high-affinity isoprenaline binding was maintained when βiAR-84 was solubilised in detergent, and was 17-fold higher than that of the Nb80 complex and 5-fold higher than that of the Gα–Nb35 complex. Thus mini-Gα393 is an ideal protein for the formation of stable GPCR complexes in detergent solution.

The biochemical properties of mini-Gα393 also make it ideal for structural studies of GPCR complexes. Mini-Gα393 was readily purified to homogeneity with a yield of 100 mg of purified protein per litre of E. coli culture, and it could be concentrated to over 100 mg/ml (Supplementary Fig. S9). Analytical gel filtration showed that mini-Gα393 bound to purified βiARANCE in LMNG (Fig. 3d and e), demonstrating that the complex could be purified in detergent. Recent innovations in electron cryo-microscopy (cryo-EM; Bai et al., 2015) suggest that large GPCR–G protein complexes could be amenable for structure determination by single particle imaging. It is therefore useful to note that mini-Gα399, a construct in which the N-terminal
residues 6–25 were replaced and the L272D mutation was reverted to wild type (Supplementary Table SIII), retained its ability to form a heterotrimer with G\_\beta\gamma, and thus better suited to cryo-EM applications.

Crystallisation of GPCRs often requires the use of short chain detergents, so the thermostability of the \(\beta_1\) AR\_\DeltaNC–mini-G\_\beta\gamma complex compared to Nb35 complexes in short chain detergents, measured by competition binding assay. The response in isoprenaline \(K_i\) induced by G\_\beta\gamma–\DeltaNC alone or in complex with Nb80, G\_\beta\gamma–\DeltaNC, or mini-G\_\beta\gamma, in different detergents (Supplementary Fig. S10). Uncoupled \(\beta_1\) AR\_\DeltaNC did not survive solubilisation in NG or OG. Colours correspond to those used in (a). (h–i) GTP-mediated dissociation of \(\beta_1\) AR–84 complexes, measured by competition binding assay. The response in isoprenaline \(K_i\) induced by G\_\beta\gamma, mini-G\_\beta\gamma, or mini-G\_\beta\gamma–\DeltaNC coupled to \(\beta_1\) AR–84 was measured in the presence or absence of GTP/\(\gamma\_S\) (250 \(\mu\)M). (a–c,h,i) Data are representative of at least two independent experiments, each performed in duplicate, with error bars \(\pm\) SEM. (g) Data represent mean \(\pm\) SEM of at least two independent experiments, each performed in duplicate.

and compared to the analogous complexes with Nb80 and G\_\beta\gamma–\DeltaNC (Fig. 3g and Supplementary Fig. S10). Under all conditions tested uncoupled \(\beta_1\) AR\_\DeltaNC was significantly less stable than when bound to either mini-G\_\beta\gamma, Nb80, or G\_\beta\gamma–\DeltaNC. Similarly, \(\beta_1\) AR\_\DeltaNC was always more stable bound to mini-G\_\beta\gamma compared to Nb80. \(\beta_1\) AR\_\DeltaNC–mini-G\_\beta\gamma complexes were also considerably more stable than \(\beta_1\) AR\_\DeltaNC–\DeltaNC complexes in short chain detergents, although the later was marginally more stable in DDM. The striking improvement in the thermostability by 5°C–8°C of the \(\beta_1\) AR\_\DeltaNC–mini-G\_\beta\gamma complex in NG and OG in comparison to other complexes suggests it has significant advantages for the structure determination of receptors in the active state.

Fig. 3 Validation of mini-G\_\beta\gamma (a–c) A competition binding assay was used to measure the change in affinity \(K_i\) of isoprenaline induced by Nb80, G\_\beta\gamma–Nb35, or mini-G\_\beta\gamma coupling to: (a) membrane-embedded \(\beta_1\) AR\_\DeltaNC, (b) membrane-embedded \(\beta_1\) AR–84 and (c) DDM-solubilised \(\beta_1\) AR–84. (d) Analytical gel filtration analysis of \(\beta_1\) AR\_\DeltaNC binding to mini-G\_\beta\gamma. The apparent molecular weight of mini-G\_\beta\gamma was 23 kDa (17.1 ml), which compares well with the theoretical value of 27 kDa. The apparent molecular weight of \(\beta_1\) AR\_\DeltaNC was 139 kDa (13.2 ml), which is consistent with the 45 kDa receptor being associated with a large detergent micelle; the shoulder at 11 ml (> 300 kDa) probably represents aggregated receptor. A mixture of \(\beta_1\) AR\_\DeltaNC and mini-G\_\beta\gamma (1:2-fold molar excess) resolved as a predominant peak with an apparent molecular weight of 160 kDa (12.9 ml). The 21 kDa increase in the apparent molecular weight of the mini-G\_\beta\gamma complex compared to uncoupled \(\beta_1\) AR\_\DeltaNC is consistent with mini-G\_\beta\gamma binding with 1:1 stoichiometry. (e) SDS-PAGE analysis of the gel filtration eluate confirmed the presence of both \(\beta_1\) AR\_\DeltaNC and mini-G\_\beta\gamma in the peak fractions. (f) Analytical gel filtration analysis of \(\gamma\_S\) binding to mini-G\_\beta\gamma. The apparent molecular weights of mini-G\_\beta\gamma (Supplementary Table SIII) and \(\gamma\_S\) were 32 kDa (16.4 ml) and 42 kDa (15.8 ml), respectively, which is in close agreement with the theoretical values of 29 kDa and 46 kDa, respectively. An equimolar mixture of mini-G\_\beta\gamma and \(\gamma\_S\) resolved as a single peak with an apparent molecular weight of 73 kDa (14.6 ml). The 31 kDa increase in the apparent molecular weight of the mini-G\_\beta\gamma–\(\gamma\_S\) complex compared to \(\gamma\_S\) is consistent with mini-G\_\beta\gamma binding with 1:1 stoichiometry. (g) Thermostability of detergent-solubilised \(\beta_1\) AR\_\DeltaNC alone or in complex with Nb80, G\_\beta\gamma–\DeltaNC, or mini-G\_\beta\gamma, in different detergents (Supplementary Fig. S10). Uncoupled \(\beta_1\) AR\_\DeltaNC did not survive solubilisation in NG or OG. Colours correspond to those used in (a). (h–i) GTP-mediated dissociation of \(\beta_1\) AR–84 complexes, measured by competition binding assay. The response in isoprenaline \(K_i\) induced by G\_\beta\gamma, mini-G\_\beta\gamma, or mini-G\_\beta\gamma–\DeltaNC coupled to \(\beta_1\) AR–84 was measured in the presence or absence of GTP/\(\gamma\_S\) (250 \(\mu\)M). (a–c,h,i) Data are representative of at least two independent experiments, each performed in duplicate, with error bars \(\pm\) SEM. (g) Data represent mean \(\pm\) SEM of at least two independent experiments, each performed in duplicate.
The nucleotide-binding properties of the mutants were not extensively studied in this work, but one interesting observation was that the β1AR-84-mini-Gs393 complex was resistant to dissociation by physiological concentrations of GTP (Fig. 3h and i). GTPγS fully reversed the shift in Kᵢ induced by Gₛ binding to β₁AR-84 from 271 ± 44 nM to 2.7 ± 0.1 μM. The shift in isoprenaline Kᵢ induced by mini-Gₛ404 (an identical construct to mini-Gₛ393, except that the I372A and V375I mutations were reverted to wild type; Supplementary Table III) binding to β₁AR-84 was almost fully reversed by GTPγS (from 18 ± 2 nM to 700 ± 60 nM). However, there was no significant difference in the isoprenaline Kᵢ of the β₁AR-84-mini-Gₛ393 complex in either the presence or absence of GTPγS (3.6 ± 0.0 nM compared to 5.2 ± 0.7 nM; P = 0.137). This unresponsiveness to GTPγS was caused by the I372A mutation (see Discussion), because mini-Gₛ391 (an identical construct to mini-Gₛ393, except that only the V375I mutation was reverted to wild type; Supplementary Table III), behaved in a similar fashion to mini-Gₛ393 (Supplementary Fig. S11). Unresponsiveness to GTP is a useful property that should allow the formation of stable GPCR-mini-Gₛ complexes in vivo, which may be a novel method to improve expression and purification of unstable GPCRs.

Discussion

Several novel approaches have been developed to stabilise and crystallise GPCRs in their active conformation, including complexation with G protein–derived peptides (Scheerer et al., 2008), G protein-mimicking nanobodies (Huang et al., 2015; Kruse et al., 2013; Rasmussen et al., 2011a; Ring et al., 2013) and a nanobody-stabilised heterotrimeric G protein (Rasmussen et al., 2011b). All of these complexes appear to stabilize the receptor in its active state, which is characterised by an outward movement of helix 6 and conserved conformational changes of residues within the core of the receptor, particularly R₃₅₀, Y₅₅₈ and Y₇₅₃ (Huang et al., 2015). The β₁AR-Gₛ complex provided the first insight into the organisation of the native GPCR–Gₛ protein interface, which is something that other binding proteins cannot recreate, but frustratingly, complexes involving heterotrimeric Gₛ are also the most difficult to crystallise, due to their large size and dynamic nature. Therefore, we designed a minimal G protein that offers significant advantages to the crystallisation of native-like GPCR–Gₛ protein complexes, specifically mini-Gₛ is a small, soluble, highly expressed protein, which readily forms a detergent-stable complex with GPCRs.

Our lab has previously determined the structures of both β₁AR and the adenosine A₂₃ receptor (A₂₃R) bound to agonists (Lebon et al., 2011b; Warne et al., 2011), and therefore crystallisation trials were conducted for both β₁AR and A₂₃R in complex with mini-Gₛ, employing a parallel approach of lipidic cubic phase (LCP) and vapour diffusion. Crystals were obtained quickly for wild type A₂₃R in complex with mini-Gₛ by vapour diffusion in the detergent octylthioglucoside (OTG), and we were able to solve the structure to 3.4 Å resolution (Carpenter et al., 2016). β₁AR crystallisation trials are still at an early stage, and have not yet yielded crystals that diffract to sufficient resolution for structure determination. The molecular organisation of A₂₃R–mini-Gₛ is remarkably similar to that of the β₁AR–Gₛ complex (Rasmussen et al., 2011b), with A₂₃R adopting a conformation that closely resembles Gₛ-bound β₁AR. In addition, mini-Gₛ bound to A₂₃R is very similar to the analogous region in Gₛ bound to β₁AR (Rmsd 0.9 Å; Carpenter et al., 2016). Thus mini Gₛ proteins are useful surrogates for heterotrimeric Gₛ proteins to stabilise and determine structures of GPCRs in their active conformation. However, it must be appreciated that GPCRs have not evolved to be stable in the activated state bound to a Gₛ protein, but instead have evolved to be unstable so that signalling lasts for short, defined, periods of time before being terminated. Therefore, additional approaches, such as thermostabilisation of the GPCR (Tate, 2012) bound to mini-Gₛ, may be required before the structures of many complexes can be determined. The high-level expression and stability of mini-Gₛ makes this a trivial undertaking compared to using the wild type heterotrimeric Gₛ protein. It is also important to note that further modifications of mini-Gₛ, such as deletions and mutations, may be required to facilitate crystallisation of different GPCRs. The crystal structure of the A₂₃R–mini-Gₛ complex was solved using mini-Gₛ414, which is identical to mini-Gₛ393 except that it contains the additional mutation L63Y (Table II). Well diffracting crystals of the A₂₃R–mini-Gₛ complex were grown using either mini-Gₛ393 or mini-Gₛ414, however, the A₂₃R–mini-Gₛ414 complex produced a different crystal form that diffracted to slightly better resolution, and was thus used for structure determination. There was no discernible difference between these two complexes in either the competition binding assay or thermostability assay (results not shown), suggesting that the main effect of the L63Y mutation was on crystallogenesis.

The development of mini-Gₛ involved extensive screening to identify key deletions and point mutations that improved both the conformational homogeneity and thermostability of the β₁AR–mini-Gₛ complex (Fig. 4). Deletion of the GaAH domain, which is the most dynamic region of Gₛ in its GPCR-bound conformation, significantly reduced the conformational heterogeneity in the β₁AR–mini-Gₛ complex. This deletion also eliminated the requirement of Gₛβγ subunits for GPCR coupling, removing the need for exogenous components, such as Nb35, to stabilise the Ga–Gₛβγ interface. In the absence of Gₛβγ subunits the N-terminus of mini-Gₛ could be partially deleted, resulting in a more compact protein. The switch III

![Fig. 4 A model of heterotrimeric Gₛ highlighting the region that corresponds to mini-Gₛ (magenta). The model of heterotrimeric Gₛ was constructed by superposition of the crystal structures of Gₛ41 (PDB code 1AZT; Sunahara et al., 1997) and heterotrimeric Gₛ591 (PDB code 1GOT; Lambright et al., 1996). Residues that were mutated in mini-Gₛ (shown as spheres) were clustered in three regions of the protein: the nucleotide-binding pocket (green), switch II (blue), and the α5 helix (yellow). Regions of Gₛ that were deleted in mini-Gₛ (GaAH, switch III and half of the N-terminal helix) are coloured grey. The Gₛβγ subunits, which are not required for mini-Gₛ coupling to GPCRs are shown as ribbons and coloured grey. GDP is shown as sticks and coloured orange.](https://academic.oup.com/peds/article-abstract/29/12/583/2607331)
deletion removed a dynamic region of mini-Gs, replacing it with defined secondary structure components, and resulted in a significant improvement in the thermostability of the complex. A total of seven point mutations were required to fully stabilise mini-Gs in complex with β1AR (Fig. 4). Extensive mutagenic screens were employed that targeted most regions of mini-Gs, however, all of the mutations utilised in the final construct were clustered around three regions of the protein (nucleotide-binding pocket, switch II, and α5 helix). The G49D, E50N, A249D and S252D mutations were designed to stabilise the nucleotide-binding pocket and P-loop (Fig. 2a), and all of these mutations improved the thermostability of both the basal GDP-bound state of mini-Gs and the β1AR–mini-Gs complex. The L272D mutation, which was located adjacent to switch II, was designed to conformationally constrain this flexible region (Fig. 2b), and resulted in improved thermostability of the β1AR–mini-Gs complex. The I372A mutation, which was located in the α5 helix, were designed to improve packing between the core of protein and the α1 or α5 helices, respectively (Fig. 2c and d). These two mutations specifically improved the thermostability of the β1AR–mini-Gs complex in detergent. None of these mutations were located within the receptor-binding site, ensuring that the native GPCR–G protein interface was maintained, and allowing mini-Gs to be co-crystallised with other Gs-coupled receptors. During the course of this work the I372A mutation was also independently reported to stabilise a complex between rhodopsin and the adenylylate cyclase inhibiting G protein G3 (Sun et al., 2015).

The G protein engineering work has also provided insight into the mechanism of G protein activation. Binding of Go to β1AR triggers displacement of the G protein α5 helix to a position that is predicted to sterically clash with the α1 helix. This unfavourable clash appears to prevent close packing of the C-terminal region of the α1 helix against the α5 helix and the core of the GTPase domain (Fig. 2e), making this region a disordered in the β1AR–G protein complex (Rasmussen et al., 2011b). The α1 helix forms part of the nucleotide-binding pocket and directly connects to the P-loop (Fig. 2c), which is the main determinant of nucleotide binding affinity in G proteins (John et al., 1990). Destabilisation of the α1 helix has previously been suggested to be a key event in receptor-mediated nucleotide exchange (Flock et al., 2015; Kaya et al., 2014; Sun et al., 2015), however the mechanism of this destabilisation was unclear. Here, we identified a potential steric clash between Ile372 from the α5 helix and residues from the α1 helix (in particular Met60), which appears to play an important role in this nucleotide exchange. Mutation of Ile372 to alanine, which was predicted to eliminate the steric clash, was shown to inhibit GTP-mediated dissociation of the β1AR–mini-Gs complex. These data indicate that, in Gs, Ile372 acts as a relay between the GPCR-binding site (α5 helix) and the key regions of the nucleotide-binding pocket (α1 helix and P-loop), allowing the receptor to allosterically destabilise the nucleotide-binding pocket and modulate nucleotide exchange. We suggest that the I372A mutation uncouples GPCR binding from occupancy of the nucleotide-binding pocket, a hypothesis that was supported by the presence of GDP in one of the two copies of mini-Gs, in the asymmetric unit of the A2AR–mini-Gs complex (Carpenter et al., 2016).

Mini G proteins are novel tools that have many potential applications, including characterisation of receptor pharmacology in response to different classes of agonists (full, partial and weak), binding affinity and kinetic studies, thermostabilisation of GPCRs in their active conformation, drug discovery, and structure determination of native-like GPCR–G protein complexes. Furthermore, all of the mutations reported here are located within conserved regions of the Gs subunit. Therefore, the concept is potentially transferable to all classes of heterotrimeric G proteins, which would allow the production of a panel of mini G proteins capable of coupling any GPCR.

**Supplementary data**

Supplementary data are available at PEDS online.

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