Probing thermostability of detergent-solubilized CB₂ receptor by parallel G protein–activation and ligand-binding assays

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G protein–coupled receptors (GPCRs) comprise a large class of integral membrane proteins involved in the regulation of a broad spectrum of physiological processes and are a major target for pharmaceutical drug development. Structural studies can help advance the rational design of novel specific pharmaceuticals that target GPCRs, but such studies require expression of significant quantities of these proteins in pure, homogenous, and sufficiently stable form. An essential precursor for these structural studies is an assessment of protein stability under experimental conditions. Here we report that solubilization of a GPCR, type II cannabinoid receptor CB₂, in a Façade detergent enables radioligand thermostability assessments of this receptor with low background from nonspecific interactions with lipophilic cannabinoid ligand. Furthermore, this detergent is compatible with a [35S]GTPγS radionucleotide exchange assay measuring guanine exchange factor activity that can be applied after heat treatment to further assess receptor thermostability. We demonstrate that both assays can be utilized to determine differences in CB₂ thermostability caused by mutations, detergent composition, and the presence of stabilizing ligands. We report that a constitutively active CB₂ variant has higher thermostability than the WT receptor, a result that differs from a previous thermostability assessment of the analogous CB₁ mutation. We conclude that both ligand-binding and activity-based assays under optimized detergent conditions can support selection of thermostable variants of experimentally demanding GPCRs.

CB₂ is a class A (rhodopsin-family) G protein–coupled receptor whose activity is critical to immunoregulatory and anti-inflammatory processes. Although it is highly expressed in lymphocytes, it is also found in many other tissues throughout the body. Similar to other class A GPCRs, agonist-bound CB₂ is a guanine exchange factor (GEF)³ that promotes the exchange of bound nucleotide from GDP to GTP within the α subunit of inhibitory heterotrimeric G protein (G_i). Nucleotide exchange induces a conformational change that enables Gα_i/o signaling activity. Gα_i-GTP inhibits adenylyl cyclase, resulting in deactivation of cAMP-dependent signaling cascades. As a result, CB₂ signaling ultimately produces changes in cell motility, cell proliferation, and cytokine secretion important to anti-inflammatory immune processes. Development of specific CB₂ therapeutics has been proposed as a method of selectively engaging the CB₂ function without any unwanted psychotropic or inflammatory effects from CB₁ stimulation or suppression (1). Although CB₂-specific ligands have been developed, none have yet progressed out of clinical trials as anti-inflammatory or immunomodulatory therapeutics (2).

Biophysical studies of ligand–receptor interaction may enable experimental validation of mechanisms of CB₂ functional selectivity and the development of effective cannabinoid therapeutics. Structural analysis of CB₂ has proven to be challenging because of its low thermostability and high conformational lability in detergent, common characteristics of class A GPCRs that impair experimental tractability (3). Although stabilized CB₁ and CB₂ mutants have been developed for X-ray crystallographic studies by computational methods and rational design (4, 5) and radioligand-binding thermostability assay have been described for CB₁ in membrane preparations (6, 7), no reliable radioligand-binding thermostability assay has yet been reported for detergent-solubilized cannabinoid receptors.

Cannabinoid ligands are challenging to adapt to conventional radioligand assays because of their intrinsic hydrophobicity and tendency to associate with lipids or detergent micelles. Here, we report a cannabinoid radioligand binding thermostability assay using the tritiated CB₂ agonist [³H]CP-55,940 conducted on CB₂ solubilized with Façade–TEG detergent that forms small micelles with excellent filtration characteristics (Fig. S1) (8, 9). In contrast to other commonly used mild detergents such as n-dodecyl-β-D-maltoside (DDM) or lauryl maltose-neopentyl glycol (LMNG) that contain long hydrophobic alkyl chains, Façade detergents consist of a rigid hydrophobic choyl group with multiple polyethylene-glycol-

³ The abbreviations used are: GEF, guanine exchange factor; GPCR, G protein–coupled receptor; DDM, n-dodecyl-β-D-maltoside; LMNG, lauryl maltose neopentyl glycol; CHS, cholesteryl hemisuccinate; 2-AG, 2-arachidonoyl glycerol; GTPγS, guanosine 5’-3-O-(thio)triphosphate.
tethered glycosides projecting out of only one plane. We hypothesized that the Façade detergents’ low micelle aggregation number, micelle mass, and hydrodynamic radius facilitate the removal of “free” ligand partitioned into the micelle, resulting in lower background radioactivity compared with conventional long-chain detergents (Fig. 1A). We further demonstrate that Façade–TEG improves CB2 thermostability relative to other detergents, promotes monodisperse CB2 reconstitution, and tolerates very high CB2 concentration (see “Results” section), additional characteristics that make it an attractive detergent for GPCR characterization by NMR, EPR, and other biophysical techniques.

We also developed a GEF assay to assess GPCR thermostability in detergent solution (Fig. 1B). The GEF assays are well-established for characterizing ligands or the influence of mutations on GPCR activity (10). Although radionucleotide exchange assays have long been used to characterize ligand properties of membrane or liposomal GPCR preparations, they are rarely applied to characterize detergent-reconstituted GPCRs (3, 11) Previous work by our laboratory demonstrated that GEF activity after heat treatment reveals differences in receptor stability (3). However, retention of GEF activity has not been utilized yet as a selection method to identify thermostabilized GPCR mutants.

As proof of principle, we investigated the thermostability of CB2 mutants relative to WT (Fig. 2). We use the Ballesteros–Weinstein numbering for identification of amino acids, which facilitates comparison of structural motifs, mutation effects, and ligand interaction between class A (rhodopsin-like) GPCR (12). This system is based on the presence of highly conserved residues in each of the seven transmembrane helices. It consists of two numbers where the first denotes the helix, and the second denotes the residue position relative to the most conserved residue in that helix, defined as number 50. Numbers decrease toward N terminus and increase toward C terminus. For example, the number 3.46 denotes a residue located in transmembrane helix 3, four residues before the most conserved residue, R3.50.

It was reported that the mutations T3.46I and T3.46A of CB1 produce constitutively active and inactive receptor variants, respectively. In addition, the CB1 T3.46I mutation reduces apparent thermostability, whereas the T3.46A mutation increased thermostability as measured by ligand-binding assays on heat-inactivated membrane preparations (6, 7). In both CB1 and CB2, the T3.46 position has been hypothesized to be involved in the regulation of the conserved DRY “ionic lock” motif bridging the cytoplasmic ends of helices 3 and 6 responsible for closing the G protein–binding interface in inactive conformations. The analogous T3.46I and T3.46A CB2 mutations were not extensively characterized, although it was reported that the T3.46A mutation was less responsive to agonist binding (4). Here we report that CB2 T3.46I and T3.46A mutants behave similarly to their CB1 counterparts, respectively representing constitutively active and inactive conformers. We also report a higher thermostability of the constitutively active T3.46I CB2 variant solubilized in detergent micelles relative to the WT receptor, a result that differs from a previous thermostability assessment of the analogous CB1 mutation.

Furthermore, we employed an additional CB2 construct as a useful control for the development of thermostability tests named “5-MUT” that contains five thermostabilizing mutations (Fig. 2). A similar construct containing these mutations in addition to N- and C-terminal truncations and replacement of the intracellular loop 3 was recently used for crystallization of CB2 receptor (4). Although the mutated receptor lost GEF activity, it is still capable of ligand binding, albeit at somewhat reduced affinity (4).

Results
Expression of CB2 variants

CB2 variants T3.46I and T3.46A were characterized as follows. The expression levels of these proteins in Escherichia coli
membranes were analyzed by Western blotting; ligand-binding properties were analyzed by a competition binding assay with radiolabeled [3H]CP-55,940; and the G protein–activation properties were analyzed by GEF assay on E. coli membranes containing recombinantly expressed receptor variants. The expression levels of the maltose-binding protein–CB2 fusion harboring the T3.46I mutation were slightly lower than for WT, possibly because of lower stability of this construct in bacterial membranes (Fig. S2). In contrast, levels of T3.46A expression appear to be higher compared with WT, suggesting improved stability.

Ligand-binding competition displacement assays with agonist CP-55,940 and inverse agonist SR-144,528 were conducted against [3H]CP-55,490 ligand to determine changes in affinity of mutants relative to WT (Table 1). The high-affinity synthetic cannabinoid ligand CP-55,940 is commonly used to assess the ligand-binding activity of the receptor and its activation behavior (13). The synthetic inverse agonist of CB2, SR-144,528, stabilizes CB2 in an inactive “basal” conformation (13). Similar to CP-55,940, it has a low-nanomolar affinity for the receptor and can be used for ligand-displacement experiments. As expected, the T3.46I mutation displayed 2-fold higher affinity for agonist over WT and a lower affinity for inverse agonist. In contrast, mutant T3.46A represents a constitutively inactive conformer, with low basal GEF activity as well as increased sensitivity to inactivating ligand relative to WT. Taken together, these results indicate that CB2 T3.46I and T3.46A mutants in membranes of E. coli cells behave similarly.

**Table 1**

Assessment of ligand binding on CB2 receptor expressed in E. coli membranes

| Competing ligand | $K_i$ vs. [3H]CP-55,490 |
|------------------|------------------------|
| CP-55,940        | WT T3.46I T3.46A       |
|                  | 3.49 ± 0.54 1.95 ± 0.12 6.5 ± 0.14 |
| SR-144,528       | 3.67 ± 0.67 4.45 ± 0.49 3.1 ± 0.28 |

**Figure 3.** G-protein activation by CB2 WT and T3.46A and T3.46I mutants. The membranes were prepared, and the assay was performed as described under “Experimental procedures.” The results are averages of two independent measurements with the data points indicated by the dots.
to their CB₁ counterparts, representing constitutively active and inactive conformers, respectively.

Additionally, we expressed the 5-MUT along with truncated variants of CB₂ (truncated at residue 20 to the N terminus and residue 326 to the C terminus) in *E. coli* cells. The Western blotting (Fig. S3A) demonstrates a high expression level of the MUT-5 construct, suggesting improved stability in *E. coli* membranes. The combination of five thermostabilizing mutations increased the expression level of both the full-length CB₂ protein, as well as its truncated variant N-20/C-326 (Fig. S3A).

The GEF activity of 5-MUT was assessed (Fig. S3B). As expected, even in the presence of saturating concentrations of high-affinity agonist CP-55,940, the GEF activity of this construct is less than 5% of activity compared with WT receptor (Fig. S3B).

**Thermostability assessment workflow**

A typical protein purification protocol is described under “Experimental procedures” and involves solubilization of the receptor expressed in cell membranes in a mix of three detergents, DDM, CHAPS, and the cholesterol derivative cholesteryl hemisuccinate (CHS). The latter has been shown to enhance thermal stability of GPCR (3). The micelles formed by this detergent mix are large (14), which complicates spectroscopic characterization of GPCR by methods like solution-state NMR that require small micelles that reorient more rapidly. Furthermore, WT CB₂ receptor requires a temperature of 4 °C or lower in DDM/CHAPS/CHS micelles and denatures rapidly at higher temperature (3). Therefore, it is desirable to improve thermostability of GPCR by introducing thermostabilizing mutations as well as selecting a detergent that better protects the receptor while keeping micelle size low. Here we describe a workflow that allows direct characterization of recombinant detergent-solubilized receptor by two complementary assays that assess fundamental properties of GPCR, namely, their ability to (i) bind specific ligands(s) and (ii) activate cognate G protein in response to agonist binding (Fig. 1).

The radioligand retention test requires efficient separation of the ligand–receptor complex from the unbound ligand (Fig. 1A). The complementary G protein activation assay can be performed on the same sample, provided that detergents used for solubilization of GPCR do not affect interaction between the receptor and its cognate G protein and do not inhibit the nucleotide exchange on the G₁₅ subunit of the G protein (Fig. 1B).

**Detergent optimization**

Our early attempts to assay CB₂–radioligand interaction in detergent micelles suitable for extraction and purification of the recombinant receptor expressed in *E. coli* cells were hampered by very high nonspecific partitioning of radioligand into both “empty” and CB₂-containing detergent micelles (3, 15). To reduce partitioning of the hydrophobic cannabinoid ligands into micelles, a series of detergent conditions were screened. Briefly, radioligand was preincubated with detergent at 4 °C, and the sample was applied to a size-exclusion gel-filtration spin column equilibrated with the same detergent (see “Experimental procedures”). The amount of radioligand in the eluent was measured and compared with total concentration of radioligand originally added to the sample. Lower detergent concentration reduces the amount of radioligand partitioned into micelles (Fig. 4). Among several detergent formulations analyzed, Façade–TEG at concentrations of 0.1–0.3 mM had the lowest nonspecific partitioning of ligand into micelles (3, 15). To reduce partitioning of the hydrophobic cannabinoid ligands into micelles, a series of detergent conditions were screened. Briefly, radioligand was preincubated with detergent at 4 °C, and the sample was applied to a size-exclusion gel-filtration spin column equilibrated with the same detergent (see “Experimental procedures”). The amount of radioligand in the eluent was measured and compared with total concentration of radioligand originally added to the sample. Lower detergent concentration reduces the amount of radioligand partitioned into micelles (Fig. 4). Among several detergent formulations analyzed, Façade–TEG at concentrations of 0.1–0.3 mM had the lowest nonspecific partitioning of ligand into micelles, yielding a radioactive background of only 0.1% of total radioligand added. This is significantly less than the values observed for other commonly used detergents such as LMNG and DDM.

Because of the high cost of Façade–TEG, this detergent was used only in the last step of sample preparation. The purification procedure included solubilization of CB₂ from cell membranes and a chromatographic purification on StrepTactin resin in DDM/CHAPS detergents as described earlier (16). The purified protein was then immobilized on nickel-affinity resin, taking advantage of the His₁₀-affinity tag at the C terminus of recombinant CB₂ protein. Detergents were exchanged to 0.3
mm Façade–TEG/CHS (10:1 mol/mol) as described under “Experimental procedures.” Additionally, a high-affinity ligand (either agonist CP-55,940 or inverse agonist SR-144,528) was present during detergent solubilization and chromatographic purification to stabilize the receptor (3). The purified protein was monodisperse as assessed by size-exclusion chromatography in a buffer supplemented with Façade–TEG (Fig. 5).

As described in Fig. 1B, we sought to assess CB2 thermostability by the ability of the GPCR to activate cognate G protein in detergent. Unfortunately, commonly used detergents such as DDM and CHAPS are not readily compatible with GEF assays because they interfere with interaction between the GPCR and G protein or inhibit the nucleotide exchange on the Ga subunit (3). This does not apply to the small micelles of the Façade–TEG detergent, which have properties that are better suited to study interaction of CB2 with G protein. At concentrations of Façade–TEG of 0.1–0.3 mM, close to the critical micelle concentration of 0.2 mM, (8) activation of G protein on agonist-bound CB2 was measured reliably (Fig. S4). However, higher concentrations of Façade–TEG resulted in either partial or total loss of activation, presumably as a result of receptor destabilization and aggregation. Therefore, all subsequent experiments were conducted at Façade–TEG concentrations in the range of 0.1–0.3 mM.

Importantly, the CB2 protein in Façade–TEG micelles can be concentrated to very high levels without concomitant co-concentration of solubilizing detergents. For example, 2.5 mg of purified CB2 were concentrated to 1.75 mM using a spin concentrator with a molecular weight cutoff of 30 kDa. The concentration of Façade–TEG in the sample was determined to be 77 mM as measured by 1H-NMR (see “Experimental procedures”), suggesting an efficient separation of the protein-containing micelles from protein-free micelles by the membrane of the concentrator. If an aggregation number of Façade–TEG of 20–25 is assumed (8), this corresponds to an approximate concentration ratio between the protein-containing and “empty” micelles of 1:1. Low content of empty micelles is of potential benefit for downstream applications requiring high concentrations of protein and reasonably low concentrations of solubilizing detergents. Methods like solution- and solid-state NMR spectroscopy, X-ray crystallography, and cryo-EM may potentially benefit from such conditions.

Solution-state NMR analysis also revealed that highly concentrated CB2 preparations in Façade–TEG contain lipids carried over from the plasma membrane of E. coli from which the receptor was extracted (Fig. S5). Samples yielded NMR resonances that are characteristic of hydrocarbon chains (Fig. S5A, see peaks labeled \(-CH_2\)n and \(CH_3\)). The presence of significant amounts of the detergent DDM that shows the same resonances was excluded: addition of a small aliquot of DDM did not yield evidence for the presence of other characteristic DDM resonances. The ligand CP-55,940 contributes to those resonances as well, but the expected concentrations are lower. Therefore, we assume that these are mostly hydrocarbon chain resonances of trace amounts of lipids that are co-concentrated with CB2 during sample purification. Experiments are underway to determine lipid species and their concentrations. The presence of lipids deserves further attention because they are likely to improve thermal stability of GPCR in Façade–TEG micelles.

CHS has been previously shown to significantly stabilize CB2 in DDM/CHS detergent micelles (3). To explore whether CHS has a similar stabilizing effect on CB2 in Façade–TEG detergent, we performed thermostability assessment by the GEF assay. Aliquots of 20 nM CB2 in 0.3 mM Façade–TEG in the presence of saturating concentrations of CP-55,940 were subjected to a temperature ramp of 1 °C/min, and the residual activity was measured by the GEF assay as described under “Experimental procedures.” The background radionucleotide signal was determined by a filtered sample containing the assay reaction mixture without CB2. The data represent averages of two measurements and were normalized to the highest value for each curve. See Table S1 for T_m values and confidence intervals.
CB_2 receptor thermostability in Façade–TEG detergent

Figure 7. Thermostability of WT, T3.46I, and 5-MUT variants of CB_2. A, thermostability of WT, T3.46I, and 5-MUT CB_2, assessed by a [^3H]CP-55,490 radioligand-binding thermostability assay in Façade–TEG. Aliquots of 20 nM CV exchanged into 0.3 mM Façade–TEG buffer were equilibrated with [^3H]CP-55,490 and subjected to a 1 °C/min temperature gradient (dotted line). The samples were then processed with size-exclusion chromatography columns, and the content of bound radioligand was determined as described under “Experimental procedures.” Data points with error bars represent combined measurements from two separate experiments. The data were normalized to the highest and lowest values in each curve. The T_m values are 33.68 °C for WT, 38.42 °C for T3.46I, and 60.96 °C for 5-MUT (Table S2). The [^3H]CP-55,490 associated with CB_2 in 0.3 mM Façade–TEG were equilibrated with of [^3H]CP-55,490 and subjected to heat treatment at 42 °C for the indicated time intervals. The amount of [^3H]CP-55,490 associated with CB_2 protein was then determined as described under “Experimental procedures.” Each curve represents the result of one experiment. The background radioactivity from nonspecific binding of ligand to CB_2 micelle was estimated from radioactivity retention at long incubation times and subtracted from all data points. The data were normalized to the largest value for each curve. The data were fitted to a single exponential decay, and apparent half-lives are for 3.9 (2.3–6.6, 95% confidence interval) min for WT and 14.8 (6.5–49.6, 95% confidence interval) min for T3.46I. The large 95% confidence interval for T3.46I reflects the lack of data points representing a plateau of radioactivity retention within the incubation interval.

Thermostability of T3.46I, 5-MUT, and WT was assessed using a radioligand thermostability assay described above. Aliquots of 20 nM CB_2 were incubated at a fixed temperature (42 °C) for variable amounts of time to measure thermostability at elevated temperature over time. The data were fitted to an exponential decay curve. The T3.46I mutant displayed a longer half-life (14.8 min) at 42 °C compared with the WT construct (3.9 min), further demonstrating that the T3.46I constitutively active mutation improves CB_2 thermostability under conditions of a detergent screen (Fig. 7B). The 5-MUT construct did not show any decrease in ligand retention during the entire length of temperature incubation, as expected (Fig. 7B).

The radioligand thermostability assay described above differs from similar assays in literature, because of both the application of a 1 °C ramp rather than incubating separate samples at different temperatures for a set amount of time and the use of relatively low equimolar-to-receptor concentration of radioligand. There was no background radioactivity from nonspecific binding of ligand to empty micelles (Fig. 5). The nonspecific interaction with protein was determined by separate measurement of a data point on fully heat-denatured protein or by asymptotic extrapolation of the temperature curve corresponding to a data point of entirely heat-denatured protein. Nonspecific association of the hydrophobic ligand with such denatured
CB$_2$ receptor thermostability in Façade–TEG detergent

Figure 8. Thermostability of CB$_2$, WT and T3.46 mutants measured by GEF assay. Aliquots of 20 nM CB$_2$ in 0.1 mM Façade–TEG in the presence of saturating concentrations of CP-55,940 were subjected to a temperature ramp of 1 °C/min, and the residual activity was measured by the GEF assay as described under “Experimental procedures.” The background radionucleotide signal was determined by a filtered sample containing the assay reaction mixture without CB$_2$. The data were normalized to the highest value for each curve. The $T_m$ values were 48.96 °C for WT, 55.66 °C for T3.46I, and 54.05 °C for T3.46A (see Table S3 for confidence interval values).

CB$_2$-containing micelles constituted up to 20–25% of total radioactivity.

Finally, results of the radioligand-binding thermostability assay were compared with the GEF thermostability assay (Fig. 8). Samples of detergent-solubilized WT, T3.46I, and a constitutively inactive T3.46A mutant were heated with a temperature ramp of 1 °C/min in the presence of saturating concentrations of CP-55,940 and then analyzed by measurement of [$^{35}$S]GTP-$\gamma$-S binding to Go$_{i1}$. As with radioligand thermostability assessments, both mutations at position 3.46 yielded higher apparent $T_m$ values than WT CB$_2$ (Table S3).

Thermostability of the 5-MUT construct was challenging to assess by GEF activity (Fig. S3). Its already low activity was lost within 30 min of incubation (Fig. S7). Therefore, although this thermostabilized mutant does fully retain the ability to bind CP-55,940 at 42 °C for at least 1 h (Fig. S6), its structural features related to activation of G protein appear to be more susceptible to thermal inactivation.

Ligands affect the thermostability of CB$_2$ in Façade–TEG measured by GEF assay

The nucleotide exchange assay was used to assess the stabilizing effects of various ligands of CB$_2$ in Façade–TEG micelles (Fig. 9 and Table S4). The protein was dispersed in Façade–TEG buffer to a concentration of 20 nM, exposed to a series of cannabinoid ligands at a concentration of 10 μM (20 μM for 2-arachidonoyl glycerol (2-AG)), and subjected to a temperature ramp of 1 °C/min. The aliquots were withdrawn at the indicated time intervals, and residual CB$_2$ activity was measured by the GEF assay as described under “Experimental procedures.” The $T_m$ values were: 44.5 °C for CP-55,940, 43.8 °C for WIN-55,212-2, 31.3 °C for WIN-55,212-3, 34.8 °C for 2-AG, and 33.7 °C without ligand (see Table S4 for 95% confidence interval values).

Figure 9. Ligand effect on thermostability of CB$_2$ measured by the GEF assay. The CB$_2$ protein was dispersed at a concentration of 20 nM in 0.2 mM Façade–TEG–SUPPLEMENTED with 10–20 μM of indicated cannabinoid ligands (20 μM for 2-AG) and subjected to a temperature ramp of 1 °C/min. The aliquots were withdrawn at the indicated time intervals, and residual CB$_2$ activity was measured by the GEF assay as described under “Experimental procedures.” The $T_m$ values were: 44.5 °C for CP-55,940, 43.8 °C for WIN-55,212-2, 31.3 °C for WIN-55,212-3, 34.8 °C for 2-AG, and 33.7 °C without ligand (see Table S4 for 95% confidence interval values).

Discussion

Utilization of Façade–TEG as the primary component yields reduced nonspecific partitioning of hydrophobic ligands into the membrane mimetic environment surrounding the GPCR with greatly improved signal-to-noise ratio of the radioligand assay. Nonspecific interaction of hydrophobic or amphipathic ligands with membranes and micelles is a common problem that limits application to GPCR including the selection of protein variants with higher thermal stability. Also, the detergent system permits selection of stabilized mutants via a GEF activity thermostability assay, which reports protein stability in terms of signaling activity and is less prone to interference from nonspecific interaction of ligands with protein micelles.

As demonstrated in Fig. 9, the radionucleotide (GEF) thermostability assay can be conducted either with or without pre-bound ligand. As such, this assay allows for selection of thermostable mutants with preserved ligand-binding and G protein–activation features (6).

CB$_2$ T3.46A and T3.46I are constitutively active and inactive mutants, respectively

The 3.46 position and associated helix 3–helix 6 interface region has previously been demonstrated to play an important role in controlling GPCR conformational transitions via regulation of internal allostERIC microswitches (17). As expected, CB$_2$ T3.46I and T3.46A display similar ligand-binding properties and GEF activity relative to their CB$_1$ counterparts and are constitutively active and inactive mutants of CB$_2$, respectively. In CB$_2$, T3.46I displays enhanced affinity for agonist (Table 1)
but has a reduced sensitivity of signaling in response to agonist concentration (Fig. 3). Because T3.46 is distant from the ligand-binding site (4), the pattern of T3.46l activity changes suggest a global, preferential stabilization of an active R* conformation of CB2. This T3.46A mutant displays reduced agonist affinity (Fig. S3E) while dramatically lowering constitutive activity of the receptor (Fig. 3), suggesting preferential stabilization of the R (basal) state. Notably, saturating concentrations of agonist increase GEF activity of T3.46A to a level similar to that of the WT.

We observed a higher thermostability of the constitutively active T3.46l mutant protein solubilized in Façade–TEG micelles compared with WT CB2 receptor. This result conflicts with previously reported thermostability of the analogous CB1 mutation assessed by radioligand binding on receptor preparations in HEK293 cell membranes (6). A possible reason for this discrepancy is the difference in conditions at which the thermostability studies were performed. Although the stability of CB2 receptor and its variants was measured in cell membranes without the addition of high-affinity ligands (6), our studies were performed on purified proteins solubilized in Façade–TEG in the presence of agonist CP-55,940. It is likely that the higher affinity of T3.46l mutant for CP-55,940 contributes to a more rigid receptor–ligand complex that better withstands temperature treatment, compared with the WT CB2.

**GPCR facilitated nucleotide exchange on G protein presents a useful adjunct to radioligand thermostability assays**

Recent advances in GPCR structural biology enable the biophysical characterization of WT receptors utilizing tools such as stabilizing nanobodies, ultra-high-affinity ligands, and miniaturized G proteins (18, 19). Although GPCR constructs thermostabilized via measurement of radioligand binding preserve biologically relevant ligand-binding features, these proteins often have significantly reduced or even abolished GEF activity. This may be the result of stabilizing mutation bias toward inactive conformations of the receptor or because of the relationship between increased conformational rigidity and thermostability. Loss of such signaling activity may limit their use as a model system for the study of dynamic aspects of GPCR signal transduction. Addition of radioligand during heating has been utilized to select for receptor variants stabilized in conformations promoted by ligand. Selection via GEF assay may enable selection of conformations specific to ligands with no available radiolabeled ligand.

It is important to note that the $T_m$ values of CB2 variants obtained by the GEF assay (Figs. 6 and 8) differ slightly from the values obtained with the radioligand thermostability assays (Fig. 7A). The likely explanation is that the radioligand assay is performed at relatively low (usually equimolar to receptor) concentration of ligand CP-55,940, whereas the GEF thermostability curves are obtained in the presence of saturating concentrations of high-affinity ligands that are known to stabilize the CB2 receptor. This is further supported by the similarity between the $T_m$ value given by WT GEF assessment without ligand (33.7 °C; Fig. 9) and the $T_m$ value given by WT radioligand assessment (33.7 °C; Fig. 7A). Regardless, care must be taken when directly comparing $T_m$ values obtained with different types of thermostability assays.

**Façade detergent has favorable properties for GPCR characterization and downstream biophysical applications**

Façade detergents present a promising platform for biophysical characterization of GPCR. The small micelle size may promote fast tumbling of GPCR in micelles that improves spectral resolution in solution-state NMR experiments. The ability to concentrate protein to high levels without loss of functionality improves sensitivity of spectroscopic studies. The Façade–TEG detergent is equal or even superior to other detergents in its ability to stabilize the functional structure of CB2. Preliminary results from solution-state NMR experiments on isotopically labeled CB2 in Façade–TEG show improved thermal stability and spectral resolution. The reduced nonspecific interactions of ligands with the membrane mimetic environment are beneficial for structural studies on ligand–receptor interaction. Additionally, CB2 retains high GEF activity in Façade–TEG, making it an attractive system to study GPCR features related to signal transduction.

In summary, we present a framework for generation of thermostable GPCR mutants that selects for features required for functional activity. We envision the GEF-based thermostability assessment to be utilized as an adjunct to radioligand thermostability assessment for development of GPCR constructs that stabilize signaling-active conformers. We propose its use as tool to expand the repertoire of stabilized GPCR constructs available for structural and biophysical characterization. The highly sensitive radionucleotide-exchange thermostability assay may provide a path to circumvent issues with nonspecific interaction of lipophilic ligands with membranes/detergent micelles into which the GPCR is embedded.

**Experimental procedures**

**Materials**

Chromatographic resin nickel–nitroltriacetic acid was purchased from Qiagen. Streptactin XT was from IBA Life Sciences (Goettingen, Germany). The detergents CHAPS, LMNG, and DDM were from Anatrace (Maumwee, OH). CHS–Tris salt was from AnaTrace. The detergent Façade–TEG (3a,7a,12a-tri[(O-β-D-glucopyranosyl)methyloxy]-cholane) was from Avanti Polar Lipids (Alabaster, AL).

The potent nonselective CB2 agonist CP-55,940 ((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol, the high affinity selective CB2 inverse agonist SR-144,528 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]−H-pyrazole-3-carboxamide, a potent CB1 and CB2 agonist WIN-55,212–2, [(3S)-2,3-dihydroxy-5-methyl-3-(4-morpholinylnethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate, and a competitive neutral antagonist of CB2 WIN-55,212–3, [(3S)-2,3-dihydroxy-5-methyl-3-(4-morpholinylnethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, methanesulfonate were from Cayman Chemical (Ann Arbor, MI).
3H-Labeled CP-55,940 was from PerkinElmer Life Sciences. All other reagents were from Sigma–Aldrich.

Molecular graphics and analyses

Molecular graphics and analyses were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (20).

Cloning

CB2 point mutants were generated with a QuikChange XL site-directed mutagenesis kit (Agilent, Santa Clara, CA) following standard protocols. CB2 constructs were hosted on an isopropyl β-D-thiogalactopyranoside–inducible plasmid backbone with ampicillin resistance reported previously (16, 21). CB2 constructs were expressed as a maltose-binding protein fusion with tags for immobilized-metal affinity chromatography (His10 tag) and Streptactin XT affinity medium (Twin-Streptag). Primers for mutagenesis were ordered from Eurofin Genomics (Louisville, KY).

CB2 expression and purification

CB2 variants were expressed in BL21 (DE3) E. coli culture and purified on milligram scale as previously described (16). In brief, 10 liters of 2YT medium containing 0.2% glucose supplemented with ampicillin was inoculated with an overnight culture of E. coli. After reaching an optical density of 0.4, CB2 expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside and 2.5 μM CB2 agonist CP-55,490. Expression was conducted for an additional 42 h at 20 °C. After expression, the cells were harvested by centrifugation, washed with cold PBS, and lysed in a cell homogenizer (Avestin). The receptor was solubilized for 1 h at 4 °C under continuous stirring by addition of concentrated detergent to final concentrations of (0.1% CHS, 1.0% DDM, 0.5% CHAPS, all w/v). The insoluble material was removed by centrifugation at 170,000 × g for 1 h, and the solubilized receptor was then purified by two successive rounds of column affinity chromatography on nickel–nitrilotriacetic acid (Qiagen) and StrepTactin XT affinity medium (Twin-Streptag). Primers for mutagenesis were ordered from Eurofin Genomics (Louisville, KY).

Detergent exchange

An amount of 200 μl of purified CB2 was bound to 200 μl of HisPur Co2+ (ThermoFisher) resin in buffer A and incubated under shaking for 2 h at 4 °C. The protein sample was then applied to a 1.0-ml spin column and washed on resin five times with 0.5 ml of 0.3 mM Façade–TEG buffer (20 mM HEPES, pH 7.5, 100 mM NaCl) with 10 μM CP-55,490 and an additional 5 × 0.5 ml of Façade–TEG buffer. The protein was then eluted in three 200-μl fractions of 0.3 mM Façade–TEG buffer containing 250 mM imidazole with 15% glycerol without additional ligand. Eluent fractions were combined and concentrated to 200 μl with a gel-filtration column. The preparation was divided into 10–20-μl aliquots and then flash-frozen and stored for later assessment. Protein concentration was measured with a Bio-Rad DC assay.

CB2 receptor thermostability in Façade–TEG detergent

Thermostability assays

Ligand binding—1 ml of 0.3 mM Façade–TEG buffer was added to detergent-exchanged CB2 and mixed by pipetting to a final concentration of CB2 estimated at 12 nM. A volume of 10 μl of a 10× diluted [3H]CP-55,490 stock (PerkinElmer Life Sciences) in ethanol was added to each 1-ml sample. The samples were mixed by pipetting and incubated on ice for 2 h to equilibrate. The samples were then split into eight 120-μl fractions and kept at either a constant temperature or heated at a rate of 1 °C/min from 4 to 74 °C under shaking at 350 rpm. After the desired incubation time or temperature point was reached, aliquots of samples were removed and placed on ice. After 5 min on ice, the aliquots were briefly centrifuged and loaded on a 0.5-ml Zeba Spin column (ThermoFisher) equilibrated with 0.3 mM Façade–TEG buffer. The columns were spun for 2 min at 1,500 × g to elute the receptor–ligand complex. The eluent was transferred to a scintillation vial, and 4 ml of CytoScint scintillation fluid (MP Biomedicals, Solon, OH) was added to samples. The samples were then vortexed, and radioactivity was measured on a scintillation counter.

For temperature ramping curves, the Tm values were calculated by fitting background-subtracted data to specific binding using GraphPad Prism. For constant temperature curves, half-lives were calculated by fitting background-subtracted data to a single exponential decay curve in GraphPad Prism. The level of nonspecific interaction of ligand with CB2–containing micelles (radiation background) was determined using heat-reactivated CB2 (exposure to 80 °C for 20 min).

[35S]GTP nucleotide exchange assays—The subunits of G protein were expressed and purified as described previously (22). The nucleotide exchange assay was performed as previously described (3). Aliquots of CB2, in Façade–TEG–buffered tubes were dispersed in 0.1–0.3 mM Façade–TEG buffer supplemented with a saturating concentration (40 μM) of ligand, mixed by pipette aspiration, and incubated on ice for 1 h. Heat inactivation of aliquots was conducted as described above for ligand binding. After heat treatment, the samples were placed on ice until application of the nucleotide exchange assay.

Competition ligand-binding assay—Cell membranes expressing CB2 were prepared from 30 ml of E. coli BL21 (DE3) cell cultures. The cells were collected, centrifuged, washed with PBS buffer, and lysed in a French press. Membranes were prepared according to a previously published procedure (21). Ligand-binding experiments were performed as described previously (3, 21).

Size-exclusion chromatography

Size-exclusion chromatography of purified CB2 protein was performed on a Superose 6 Increase 10/300 GL column (GE Healthcare) equilibrated with two column volumes of 50 mM Tris, pH 7.5, 150 mM NaCl, 0.25 mM Façade–TEG, 0.025 mM CHS, and 5 μM CP-55,940 on an AKTA Purifier 100 automated chromatography system (GE Healthcare). The chromatography was performed at 4 °C at a solvent flow rate of 0.25 ml/min using detection at 280 nm. Fractions of 0.5 ml were collected.

NMR analysis of Façade–TEG

Sample composition, including concentrations of Façade detergent, CHS stabilizing ligand, and lipids was measured by
dissolving small aliquots (5–20 μl) of concentrated CB2 in Façade–TEG in 550 μl of deuterated methanol/chloroform (1:1, v/v) and recording 1H NMR solution-state spectra. Absolute concentrations were determined by comparison with test samples containing known concentrations of individual components.

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