Conformational plasticity of the intracellular cavity of GPCR–G-protein complexes leads to G-protein promiscuity and selectivity

Manbir Sandhu, Anja M. Touma, Matthew Dysthé, Fredrik Sadler, Sivaraj Sivaramakrishnan, and Nagarajan Vaidehi

While the dynamics of the intracellular surface in agonist-stimulatedGPCRs is well studied, the impact of GPCR dynamics on G-protein selectivity remains unclear. Here, we combine molecular dynamics simulations with live-cell FRET and secondary messenger measurements, for 21 GPCR–G-protein combinations, to advance a dynamic model of the GPCR–G-protein interface. Our data show C terminus peptides of Gaα, Gaβ, and Gaq proteins assume a small ensemble of unique orientations when coupled to their cognate GPCRs, similar to the variations observed in 3D structures of GPCR–G-protein complexes. The noncognate G proteins interact with latent intracellular GPCR cavities but dissociate due to weak and unstable interactions. Three predicted mutations in β2-adrenergic receptor stabilize binding of noncognate Gaα protein in its latent cavity, allowing promiscuous signaling through both Gaα and Gaq in a dose-dependent manner. This demonstrates that latent GPCR cavities can be evolved, by design or nature, to tune G-protein selectivity, offering insights to pluridimensional GPCR signaling.

G-protein–coupled receptor | GPCR | functional selectivity | structural plasticity | dynamics

Significance

Structures of GPCR–G-protein complexes show how cognate G proteins interact with GPCRs. However, noncognate GPCR–G-protein interactions are poorly understood, despite their relevance in cells. The conceptual advancements in our study show 1) the C terminus of Gaα, Gaβ, and Gaq proteins assume a small dynamic ensemble of unique orientations when coupled to their cognate GPCRs, explaining the variations observed in the X-ray and cryo-EM structures of GPCR–G-protein complexes; and 2) the noncognate G proteins interact dynamically with latent, previously uncharacterized cavities within the GPCR cytosolic cavity. Engineering these latent cavities with hotspots to the noncognate G proteins tunes promiscuity in the GPCR. This study provides a framework for understanding how GPCR dynamics subtly modulate signaling in different pathways.

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1To whom correspondence may be addressed. Email: sivaraj@umn.edu or nvaidehi@coh.org.

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that tethering GPCRs to Go proteins with the length-adjustable α-helical ERK linker (36) allows scaling of the effective localized concentration of GPCR and Go protein to span various, plausible, cellular concentrations. SPASM is sensitive to measuring weak and dynamic protein–protein interactions in cellular conditions (29, 37, 38). This permits comparison between the binding affinities of cognate (canonical signaling partners) and noncognate (weak or uncharacterized partners) Go proteins at the same stoichiometric ratios with the GPCR, which is not feasible with other biophysical techniques used in live cells (1, 4, 39, 40). Recent findings with SPASM FRET sensors show a physiologic effect of noncognate G proteins to prime GPCR signaling within the cell (41), demonstrating the importance for probing these noncognate G-protein interactions within the cell.

The key findings from our study are as follows: 1) The Go peptides assume a small ensemble of unique orientations when coupled to a cognate GPCR. 2) The s-pep binds in a different IC cavity of its cognate GPCR and orients its C terminus toward TM helices 5 and 6 (TM5 and TM6) compared with i-pep and q-pep that orient toward TM2 and IC loop 1 (IC1). 3) MD simulations of β2AR complexed with the noncognate q-pep reveal formation of a transient cavity in the β2AR IC interface, resembling the stable IC cavity observed in the V1AR-qpep complex. Mutation of the hotspot residues identified for Go coupling in V1AR into β2AR stabilizes this transient cavity. We have generated a triple mutant, β2AR−Q142K,R567−R228L,Q229W, that displays dose-dependent, isoproterenol-induced, promiscuity toward Goα− and Goαε-coupled signaling pathways. 4) This promiscuous β2AR mutant demonstrates that GPCRs contain defined, latent IC receptor cavities showing weak interactions with noncognate G proteins. These latent cavities can couple to the noncognate G proteins if stabilized with the necessary hotspot residues, through mutagenesis or natural evolution. The promiscuous β2AR mutant thus serves as a model system to probe the dynamics of GPCRs exhibiting pluridimensional G-protein coupling. Our dynamics-based framework reveals the structural plasticity of the GPCR cytosolic pocket that underlies G-protein selectivity and the role of noncognate G-protein interactions in influencing GPCR dynamics. Furthermore, this study provides features of the GPCR–G-protein interaction that can be targeted by functionally selective drugs to tune therapeutic response to specific GPCR signaling pathways (42).

**Results**

**Cognate GPCR–Go-Protein C Terminus Complexes Reveal Distinct Conformations for Goα−, Goε, and Goαε Signaling Pairs.** We performed atomistic MD simulations and generated a minimum of 1-μs ensembles for seven different class A GPCRs bound to full agonists and complexes with each of three Go peptides (SI Appendix, Table S1). From the MD data, we detect that s-pep, i-pep, and q-pep insert in distinct cavities within the IC interface of their respective cognate GPCRs (Fig. 1A and B and SI Appendix, Fig. S1B). The N terminus of the Go peptides, which protrude out of the GPCR IC cavity, are highly flexible during the MD simulations and normally engaged in intramolecular interactions with the “Ras” domain of the Go protein (43, 44). Therefore, we omitted the N-terminal region for analysis of receptor–G-protein contacts. The C terminus of the Go peptides (indicated by “**”, Fig. 1B) insert into the GPCR IC cavity and retain helicity. We have used the axis defined by this helical region of the Go peptide [Common G-protein Numbering, residues H5.12 to H5.26 (17)] for our analyses of Go-peptide orientation.

The GPCR conformations shown (Fig. L4 and SI Appendix, Fig. S1B) are the centroid of the most populated conformation cluster from the ensemble of MD trajectories with the cognate Go peptide bound. The Go-peptide conformations shown in Fig. 1B, are centroids from the top three populated clusters of these simulations. The central region of all of the three Go peptides are anchored to TM5 and ICL2 of their given GPCRs. The extreme C termini of i-pep and q-pep orient toward TM2, ICL1, and ICL2 in their cognate GPCRs, while the C terminus of s-pep orients toward an interface between TM6 and TM7 (Fig. 1B and SI Appendix, Fig. S1B). We calculated the insertion of the Go peptides in their cognate GPCRs as the angle between the principal axis of the GPCR TM core bundle and principal axis of the Go-protein α5 helix for each cognate GPCR–Go-protein simulation. We did the same for the X-ray and cryo-Electron Microscopy (cryo-EM) structures (Fig. 1C and SI Appendix, Fig. S1B and Table S2). The three Go-protein subtypes show different angles of insertion in the GPCR IC cavity. There is also variation in the insertion angles even among the three Go-coupled receptors studied here. Our previous FRET sensor studies (29, 37) have shown differences in coupling strengths of Goα to β2AR, β3AR, and D1R in the order β2AR > β3AR > D1R. As shown in SI Appendix, Fig. S1C,
the calculated average interaction energy from MD simulations of s-pep with β2AR, β3AR, and D1R showed the same trend as observed in the FRET sensor experiments. We speculate that the differences in the α5-helix insertion may modulate the strength of interaction between GPCR and Gα peptide.

Fig. 2. Dynamic properties of the cognate and noncognate agonist–GPCR–Gα-peptide interfaces that stabilize a signaling complex. (A) First-order torsional entropy values calculated at 300 K, using torsion angles distribution for the GPI residues of each GPCR in the presence of cognate and noncognate Gα peptides. Values are shown as means from five replicate simulations ± SEM for s-pep (red), i-pep (green), and q-pep (blue). Significance was calculated using two-sided ANOVA; ****P < 0.0001. (B) Visual model of the sampled rotamer conformations for the GPI residues of the β2AR with the highest entropy values when bound to noncognate Gα peptides. The spread of sampled rotamer angles is shown in transparent sticks. (C) Population distribution of the MD simulation snapshots for β2AR, CB1R, and V1A R when bound to their respective agonists and cognate Gα peptides, with respect to interresidue distances between TM3 and TM6, and TM3 and TM7, shown in SI Appendix, Fig. S2A; *** denotes the interresidues distances for X-ray and cryo-EM structures, colored as in A, based on G-protein preference: 3SN6-Gαs−bound β2AR (red); 5G53-mini-Gαs−bound A2AR (red); 6DDE-Gαi−bound μOR (green); 6G79-Gαi−bound A1R (green); 6G79-Gαo−bound 5HT-1BR (green); and 6CMO-Gαi−bound Rhodopsin (green). (D) Representation of centroids from conformational clusters of the cognate and noncognate Gα peptides bound to β2AR (shown as pink cylinders). The top clusters making up 85% of the conformational ensemble are shown. The noncognate i-pep (nine clusters, green) and q-pep (three clusters, blue) in β2AR show greater flexibility as multiple conformation clusters compared with s-pep (one cluster, red). (E) Model derived from the data in this figure: Both receptor and (cognate and noncognate) Gα peptides are highly dynamic upon interaction (Left). Thermodynamically favorable interactions allow the GPCR IC cavity to clamp onto the Gα peptide and stabilize the dynamics of the complex (Right). See also SI Appendix, Fig. S2.
Only Cognate GPCR–Gα-Peptide Pairs Stabilize Clamping of GPCR IC Cavity on α5 Helix. GPCRs and G proteins cluster in plasma membrane domains (45), inflating the relative concentration of cognate and noncognate G proteins compared with GPCRs (5). Gupta et al. (41) showed that noncognate G proteins can synergize the signaling efficacy of cognate G proteins. To assess how cognate and noncognate G-protein interactions affect the GPCR IC cavity, we calculated the first-order torsional entropy of the GPCR residues which interface the G protein (GPI) (Fig. 2A and SI Appendix, Table S4). A schematic of side-chain conformations of the βAR residues with highest entropy is shown (Fig. 2B). The GPI residues show lower entropy when coupled to their cognate G proteins compared with noncognate G proteins. We also observed increased flexibility in the GPCR IC cavity measured as the distance between residues 3.50 and 6.30 (SI Appendix, Fig. S2A) (46) when bound to noncognate G proteins (SI Appendix, Fig. S2B and Table S3). The residue notations shown are the Ballesteros–Weinstein GPCR numbering system (47). The reduced entropy and flexibility of cognate interactions allows the GPCR residues in the IC cavity to form strong enthalpic interactions with the G protein, except in the Gqα-coupled α1AR. We and others have shown, through live-cell coupling data, that α1AR interacts promiscuously with all three Gα peptides (29, 48).

The elongation of the inter residue distance between residues 3.50 and 6.30 and the contraction of the residue distance between 3.50 and 7.53 are characteristics of GPCR activation (46, 49, 50) (SI Appendix, Fig. S2A). MD trajectories of β2AR, β3AR, and D1R complexed with s-pep projected on these two distances show ensembles of states close to the conformation in the crystal structure of β2AR with nucleotide-free Gα [Protein Data Bank (PDB) ID code 3SN6] and adenosine 2A Receptor (A2AR) bound to mini-Gα protein (PDB ID code 5G53; Fig. 2C and SI Appendix, Fig. S2C). Both CB2R and V1aAR, with i-pep and q-pep, respectively (Fig. 2C), show ensembles representing the active state identified in the cryo-EM structures of μ Opioid Receptor (μOR) and Rhodopsin with nucleotide-free trimeric Gα (PDB ID codes 6DDE and 6CMO). We also observe that the α2AR and α1AR both sample active states similar to the Lα2AR bound to mini-Gα, Serotonin 1B Receptor (5HT1B) bound Gα protein, and β2AR bound to nucleotide-free Gαq (PDB ID codes 5G53, 6G79, and 3SN6). These distances in the X-ray and cryo-EM structures of G-protein–bound class A GPCRs are also shown in SI Appendix, Table S2.

We analyzed the Gα-peptide conformational dynamics by clustering the Gα-peptide MD simulation trajectories using root-mean-square deviation (RMSD) in coordinates. The cognate Gα peptide is stabilized in the majority of the seven GPCRs, revealed by fewer conformational clusters compared with the number of clusters sampled by noncognate Gα peptides (SI Appendix, Table S5). In the cognate interaction of β2AR with the s-pep, >85% of the MD snapshots are located within the top cluster (Fig. 2 D, Left), whereas the noncognate i-pep (green) and q-pep (blue) sample only 30% (top nine clusters to reach >85% population) and 67% (top three clusters to reach >85% population), respectively, of the population within the top cluster (Fig. 2 D, Center and Right). Taken together, these results show that the GPCR clamps tighter on the cognate Gα C terminus, lowering the flexibility, and improves the enthalpic interaction leading to productive signaling (Fig. 2E). The noncognate Gα peptides show high flexibility, show weaker interactions in the GPCR IC cavity, and eventually fall out of the cavity.

Identifying Amino Acid Hotspots in the C Terminus α5 Helix That Confer Selectivity to GPCRs. We used an iterative combination of MD simulation analysis and SPASM experiments to identify the amino acid residues in each Gα peptide which confer selectivity to their cognate receptors among the seven studied (SI Appendix, Fig. S3). We identified residues on the Gα peptide that remain in helical conformation, and show above-average favorable interaction energies and sustained contacts (with >50% frequency) during the dynamics with the GPCR (shown in yellow boxes and bold, colored font, Fig. 3A and Materials and Methods). We observe these predicted selectivity hotspot residues to be both conserved and mutated across the Gα peptides. Where applicable, the hotspot residues were swapped with homologous positions from another Gα peptide, and binding was tested with the cognate GPCRs for both the cognate and mutated noncognate Gα peptides. For the hotspots conserved in both position and sequence across Gα peptides, the residue was mutated to alter amino acid physical characteristics and test disruption in the cognate complex.

We hypothesized that the swapping mutations would enable GPCRs to couple to noncognate Gα peptides with appropriate “cognate-like” swapping mutations. We tested this swapping between s-pep and q-pep and also between i-pep and q-pep using β2AR for Gs coupling, V1aAR for Gq coupling, and CB2R for Gαi coupling. The mutations were made in SPASM FRET sensor constructs and transiently transfected into HEK-293T cells. FRET ratio is measured as agonist-stimulated minus unstimulated FRET, and comparisons to the wild type (WT) were calculated (SI Appendix, Table S6). The swapping mutations in the cognate Gα peptides led to significant reduction in FRET intensity changes upon treatment with agonist as shown in Fig. 3 B, i for β2AR with the q-like mutations in s-pep, Fig. 3 B, ii for V1aAR with s-like mutations in q-pep, Fig. 3 B, vii for CB2R with q-like mutations in the i-pep, and Fig. 3 B, viii for V1aR with i-like mutations in q-pep. These results affirm the conclusion that Gαi residue E392H5.24, Gαq residues L349I15.16, E355H5.22, and N357H5.24, and Gαs residue G352H5.25 are some of the selectivity hotspot residues. The details of the FRET data are discussed in SI Appendix, Table S6.

We performed reciprocal, gain-of-coupling experiments by introducing cognate hotspot residue mutations into homologous structural positions in noncognate Gα peptides. We performed FRET assays for β2AR with s-like mutations in q-pep (Fig. 3 B, ii), V1aAR with q-like mutations in s-pep and also q-like mutations in i-pep (Fig. 3 B, iv and vi), and CB2R with i-like mutations in q-pep (Fig. 3 B, viii). These data show that the following residue positions mediate significant increase in G protein coupling to the noncognate GPCR: Gqα residues E355Q15.22, E355Q15.22/L490Q16.16 with β2AR (Fig. 3 B, ii); Gαq residues Q384E15.12/E392N16.14 with V1aAR (Fig. 3 B, iv); and Gαs residue T340K15.15 with V1aAR (Fig. 3 B, vi). Gαqα residues Q350K15.17, N357G15.24 with CB2R (Fig. 3 B, viii). Taken together, these results show that positions H5.12 and H5.17 are involved in ancillary roles within the Gαi and Gαqα interactions. These experiments suggest that the IC cavity of a given GPCR recognizes a small number of critical structural features in the α5 helix of the Gα protein, and, if these minimal features are present in the correct orientation, the Gα protein can complex with the GPCR. This is exemplified in MD simulations of the β2AR with the noncognate q-pep becoming stabilized in the GPCR IC cavity, similar to the cognate s-pep, with the addition of the s-pep H5.16 and H5.22 hotspots (L349Q/E355Q) (Movie S1).

Rational Design of a Promiscuous β2AR Gαi-Coupled Receptor. Fig. 4 shows the contribution from residues in each TM and ICL region in the Gαi-Cα, Gαqα-Cα, or Gαs-Cα-coupled receptors toward binding their cognate Gα peptides. The relative sizes of the circles reflect the percentage of total contacts (SI Appendix, Table S7) contributed from the TM or ICL region of the given GPCR. Specifically, Gαi-coupled receptors interact with the s-pep primarily through contacts on TM3, TM5, and TM6. The i-pep contacts the residues in TM3, TM5, TM6, and ICL2 in the Gαqα-coupled receptors. Most contacts in Gαs-coupled receptors are from...
**Fig. 3.** Hotspots in the G-protein α5 helix identified in cognate GPCR-Gα-peptide pairs. (A) Sequence alignment of the α5 helix of Gαs, Gαi, and Gαq C termini. The residues on the Gα peptides that make up the GPCR-interfacing residues (based on frequency of interaction) with their cognate receptors are shown in yellow boxes. The significant energetically favorable residue hotspots are marked in bold and colored font in the respective sequences and shown in stick representation in the cartoon of the Gα peptides shown below the alignment. The C termini of the peptides are marked with an asterisk for visual orientation. (B) Selectivity “hotspot” residues predicted from MD simulations were validated in SPASM FRET sensors, by mutating the Gα-peptide residue to a homologous residue of another Gα protein, and testing the interaction of the mutant Gα peptide with the original cognate GPCR (i, iii, v, vii) or the cognate GPCR of the homologous “donor” Gα peptide (ii, iv, vi, viii). Republished with permission of American Society for Biochemistry and Molecular Biology, from ref. 29; permission conveyed through Copyright Clearance Center, Inc. Mean FRET values were compared by one-way ANOVA and Tukey’s comparison of means. Significance is denoted as *P < 0.05, **P < 0.01, ***P < 0.001. (C) The schematic model depicts that mutations to the selectivity hotspots in the α5 helix orient noncognate Gα peptides into a cognate-like orientation within a given GPCR, by making the Gα peptide amenable to the GPCR cavity available for binding. See also SI Appendix, Figs. S3 and S4.
TM2, TM3, TM5, TM6, and ICL2. Both Gαq- and Gαq-coupled receptors, but not Gαi-coupled receptors, contact the C terminus of their respective peptides through ICL1 residues. The predicted pairwise interactions between the Gα peptides and their respective cognate GPCRs are given in SI Appendix, Table S8.

Similar to the swapping mutations we tested in Gα-peptide hotspots, we predicted GPCR hotspot swapping mutations to allow promiscuous coupling of β2AR to Gαq. We observed that the residues Q384(s-pep)/L349(q-pep) (H5.16) make sustained interactions with W245Q, H5.24 (β2AR)/W244S, H5.22 (V1aAR), respectively (Fig. 4 B, i and ii). Although Q384(s-pep)/L349(q-pep) interact with residues on TM5 in both β2AR and V1aAR, the hydrophilic interaction pair in β2AR-s-pep is swapped to a hydrophobic interaction pair in V1aAR-q-pep (Fig. 4 B, i and ii), suggesting that this interaction pair could be a selectivity filter. To further strengthen the binding and coupling of Gαq to β2AR in the TM5 region, we proposed the double mutant R228I (β2AR)/Q142L (V1aAR). From the MD simulation analysis, we observe that the dynamics of noncognate β2AR-Qq-pep complex samples a finite but small population of the conformation similar to that of the cognate V1aAR-Qq-pep interaction. This guides our hypothesis that GPCRs may couple to different Gα proteins with different interfaces, but the interfaces for the noncognate Gα proteins could be latent cavities with weak interactions. We predicted that mutations of the β2AR TM5 interface that mimic V1aAR may stabilize the short-lived V1aAR-Qq-pep–like orientation observed in β2AR-Qq-pep. We expressed and tested a β2AR–R228Iβ2AR-Q142K construct which produced an IP-1 signal about fivefold greater than WT β2AR (Fig. 5 A, Left). We also measured cAMP activity from the double-mutant construct, which showed a nonsignificant reduction in the Gαq pathway activity (Fig. 5 A, Right). This result suggests that the β2AR-DM does complex with Gαq protein, and also with Gαi, but with less coupling strength.

MD simulations of the q-pep bound to β2AR-DM-q-pep were started from a Gαq-like and Gαi-like orientation. Results show favorable interaction in the Gαq-like orientation (Fig. 4 B, iii), with E355H of Gαq stably binding to Q142L in β2AR-DM. We predicted that a third mutation of Q142K to lysine in β2AR-DM would further strengthen the q-pep interaction with β2AR in Gαq-like orientation. As predicted, the triple mutant β2AR-Q142Lβ2AR-Q142Kβ2AR-Q142L (V1aAR-Q142K–Q142L construct) produced an IP-1 signal about threefold greater than WT β2AR (Fig. 5 A, Left). Measurement of agonist-induced cAMP showed a significant increase in the triple mutant compared with WT β2AR (Fig. 5 A, Left). Measurement of agonist-induced cAMP showed a significant increase in the triple mutant compared with WT β2AR (Fig. 5 A, Right). We
tested whether G_q signaling played a role in this decreased cAMP activity, but assays suggest this effect is insensitive to pertussis toxin (SI Appendix, Fig. S3C). Additionally, the dynamics of the triple mutant β3AR:s-pep does not show lowering in s-pep binding (SI Appendix, Fig. S4D). We observe E225S/G322S in the triple-mutant β2AR complementing the Q38H/R16E and the E392H/L24E hotspot spots shows orientation to the TM6/TM7 region where it maintains contact with K270E/328E, R328S/55, and R333S/55. Dose–response curves reveal how the triple mutation in β2AR affects the potency and efficacy for the Gα_i and Gq_i interactions. For the Gq_i pathway, we observe a reduction in the EC_{50} of isoproterenol from 2.97 μM to 17.00 nM in the production of IP-1 by the β2AR triple mutant, and approximately fourfold increase in overall efficacy (Fig. 5, B, Left). In the Gα_i pathway, the EC_{50} of isoproterenol for cAMP production increased from 0.28 nM in the WT to 55.13 nM in the triple mutant, with approximately threefold reduction in overall efficacy (Fig. 5, B, Right).

We summarize the results from these data in a model (Fig. 5C). We propose that GPCRs have latent cavities within their IC surface to bind different G proteins. The cavity in which the cognate G protein binds is attractive, with enthalpically favorable hotspots that lower the entropy of the complex and stabilize the agonist-bound GPCR interaction with the cognate G protein. Although GPCRs possess latent cavities for noncognate G proteins, the cavities are dynamic and unable to stabilize the noncognate G proteins, since they lack affinity. Promiscuous GPCRs have hotspot residues in the respective G-protein binding cavities that make them attractive to multiple G proteins. We note that possible selectivity hotspots outside of the Gα_i-protein C terminus have not been probed in this study.

**Dynamic Reshaping of the IC Cavity in β2AR.** MD results suggest that the triple-mutant undergoes dynamic reshaping of the IC cavity to bind both Gα_i and Gq_i proteins. MD simulations of the triple-mutant β2AR coupled to s-pep and q-pep started from both Gα_i-like and Gq_i-like orientations revealed s-pep only binds in the Gα_i-like cavity, and q-pep only binds in the Gq_i-like cavity (Fig. 6A, i and ii). The Gα_i-interacting hotspots are shown as salmon-colored surface and span TM5, TM6, and TM7 and helix 8, with strongest interacting residues shown as spheres (Fig. 6A, ii). The residues that make contact with the q-pep are shown in blue surface, with the strongest interacting positions shown as blue spheres (Fig. 6A, ii). The Gq_i-interacting residues projected on the IC surface of s-pep–bound WT β2AR show that Gα_q-interacting residues are spread out and form a dispersed cavity when Gα_i is bound (Fig. 6A, iii). In the triple-mutant, the IC surface reshapes and positions the Gα_i-interacting residues into a trident-like pattern spanning the IC portions of TM3, TM5, and TM6 and ICL1 and ICL2 (Fig. 6A, iv). We projected this dynamic cavity on the interresidue distances between TM3 and TM6 and between TM3 and TM7, and we observe that WT β2AR:q-pep samples a β2AR cavity similar to the Gα_i-bound crystal structure. This suggests that the lack of q-pep stabilizing hotspots prevents the stabilization of the β2AR conformation observed in the triple-mutant complex with q-pep (Fig. 6B, Left). The q-pep interaction in the triple mutant shows a very distinct conformation, with a narrower cavity between TM3 and TM6 and a slightly wider cavity between TM3 and TM7. This shrinking of the TM3 to TM6 distance is similar to that observed in the interaction of WT β2AR with the Goi protein in previous MD simulations (S1). The β2AR conformation sampled by the triple mutant with s-pep is similar to that sampled by WT β2AR:s-pep (Fig. 2B), but the most populated conformational cluster shifts to a smaller TM3 to TM7 distance compared with WT β2AR.

**Discussion**

The 3D structures of GPCRs–G-protein complexes predominately inform us on how cognate G proteins interact with GPCRs in the nucleotide-free state. The dynamics of the agonist-bound GPCR has been well characterized by spectroscopic and computational studies (30–32, 52–55). However, the dynamics of the GPCRs with their cognate and, especially, noncognate G proteins is poorly understood, despite their relevance in cellular mechanisms. 2) The C terminus of Gα_i affects the potency and efficacy for the Gα_i–bound GPCR has been well characterized by spectroscopic and computational studies (30–32, 52–55). However, the dynamics of the GPCRs with their cognate and, especially, noncognate G proteins is poorly understood, despite their relevance in cellular mechanisms. 3) Promiscuity of GPCRs is dynamic and unable to stabilize the noncognate G proteins, since they lack affinity. Promiscuous GPCRs have hotspot residues in the respective G-protein binding cavities that make them attractive to multiple G proteins. We note that possible selectivity hotspots outside of the Gα_i-protein C terminus have not been probed in this study.
a high degree of structural plasticity, and the cognate G protein reduces the entropy of residues in the GPCR cavity (Fig. 2A), stabilizing the receptor and enabling it to clamp down on the Gα C terminus (SI Appendix, Fig. S2B). The presence of enthalpically favorable intermolecular contacts between the cognate G protein and its preferred cavity leads to full complexity and productive signaling (Fig. 7A, iii and Fig. 7B). In contrast, the weak interactions between the GPCR cavity and noncognate G proteins result in the dissociation of Gα C terminus without productive complexation and signaling (Fig. 7A, iv). Incorporation of single G-protein–selective residues in the latent cavities, whether by evolution or engineering, is sufficient to reshape the GPCR IC surface for productive coupling with the noncognate G proteins. We hypothesize that promiscuously coupling GPCRs evolved to make these latent cavities highly attractive, while selective GPCRs are under evolutionary pressure to optimize the affinity between one cognate G protein and cavity.

One of the caveats of this study is our focus on only the C terminus of the G protein. The Gα C terminus is a known determinant of G-protein selectivity, and it has long been established that swapping the last three amino acids between the Gαi and Gαq isoforms is sufficient to confer promiscuous signaling from chimeric G proteins in HEK293 cells (28). Our focus on GPCR–Gα C terminus interactions prevents confounding effects from the integration of signaling downstream of endogenous and chimeric G proteins. Nonetheless, we acknowledge that other regions are likely involved in G-protein selectivity. Another caveat is that many of our MD simulations were started from a homology model of the receptor–Gα-peptide complex. The accuracy of our dynamics and hotspot predictions will be enhanced as more structures of the GPCR–G-protein complexes emerge in literature.

This study fills the knowledge gap in linking the dynamics of ligand–GPCR complexes to the dynamics of G-protein coupling and provides a framework to interpret variance in the strength of interaction between different GPCRs and G proteins.

Materials and Methods

Modeling of the GPCRs and Agonists. The summarized modeling details are given in SI Appendix, Table S1. Structures of the GPCRs studied were modeled based on homology to either β2AR, µOR, or CB1R templates. The models were then aligned to the active states of β2AR or Rhodopsin in the 3SN6 and 4J4Q PDB structures. Gα peptides were modeled using the α5 helix of Gαi in 3SN6, and then aligned to the α5 helix of Gαq or transducin in 3SN6 and 4J4Q. Structures were minimized using the MacroModel [Schrodinger Release 2015-4: MacroModel (2015); Schrödinger, LLC] application before simulation with Groningen Machine for Chemical Simulations (GROMACS). For more details, please see SI Appendix.

Details of MD Simulations. MD simulations were performed in explicit POPC lipid bilayer and water using gromos 53a6 force field and following a standard protocol for GPCRs used in our laboratory (29). Details are in SI Appendix.

Computational Data Analysis. One-microsecond ensemble trajectories were used for analyzing intermolecular contacts and interaction energies for GPCR–peptide pairs. Individual energies were calculated for each amino acid of the Gα peptides with the entire GPCR using the GROMACS “energy” application. The total nonbond energy from short-range (within 12 Å) coulombic and van der Waals forces was extracted from an energy log file and summed for the total nonbond interaction energy. Gα-peptide residues showing above-average interaction energy (Fig. 3A) are considered critical residues and potential “hotspots.” Intermolecular contacts were calculated in Visual Molecular Dynamics (VMD) using Tcl scripts to identify the frequency of pairwise interactions within 5 Å between peptides and receptors. Contacts made with greater than or equal to 50% frequency were deemed critical contacts. Peptide residues deemed “critical” from both interaction energy and intermolecular contact analysis were strongly considered for their role as “hotspots” for G-protein selectivity.
between the residues in this pair is used as a standard indicator of receptor activation state (46). We also measured the distance between the Cx atoms of the residues 3.50 and 7.53 as another indication of receptor activation state. Conformational clustering method. RMSD clustering in coordinates was used to determine the number of conformational clusters sampled by Gx peptides within the 1-μs ensemble of simulations. Cx atoms from the GPCR TM5s were aligned for the least-squares fit to serve as a frame of reference for comparing peptide orientations based on the backbone atoms of Gx-peptide positions H5.12 to H5.26. The aligned Gx-peptide conformations were clustered using the "gromos" method in the GROMACS "cluster" application, with a cutoff of 2 Å (58). This procedure identifies the centroid with the largest sample size of neighboring structures within the cutoff distance and sorts them to a unique cluster, repeating the same procedure with the remaining, unsorted structures.

Statistical analysis. The mean ± SEM was determined from each of five 200-ns replicates of the 1-μs ensemble trajectory. Means were compared using one-way ANOVA followed by Tukey’s posttest to assess significance for multiple comparisons, using GraphPad Prism version 7.00 for Windows (GraphPad Software, https://www.graphpad.com) (Fig. 2A and SI Appendix, Fig. S4A). The Kolmogorov–Smirnov test statistic was calculated for each distribution of GPCR IC cavity width (TM3 to TM6 distances), to compare the variance of each distribution (59). The sample size of the distribution was calculated as 50,000 frames. Each comparison rejected the null hypothesis and P values were too small to be calculated, due to limitations of machine precision (limited to 2.2 × 10⁻¹⁶), but all P values for each comparison were significantly less than 1.0 × 10⁻⁴ (SI Appendix, Fig. S5B and Table S3).

Experimental Methods
Experiments were conducted similarly to procedures outlined in our previous study (29). Details of “Reagents and buffers,” “Molecular cloning,” and “Mammalian cell preparation and sensor expression” are found in SI Appendix.

cAMP Assays. HEK293T-Flp-in cells were transiently transfected (XtremeGENE HP) according to manufacturer's instructions. Where indicated, 10 h after transfection, cells were incubated with 100 ng·mL⁻¹ pertussis toxin (PTX) for 20 h. Between 28 h and 32 h posttransfection, (XtremeGENE HP) HEK293T cells expressing indicated sensor were harvested to assess cAMP levels using the bioluminescent cAMP Glo assay (Promega). Cells were gently suspended in their original media, were counted using a hemocytometer, and were spun down (350 × g, 3 min). Cells were resuspended in an appropriate volume of PBS (pH 7.4; Gibco) supplemented with 800 μM ascorbic acid and 0.2% dextrose (wt/vol) to reach 4 × 10⁶ cells/mL density. Cell suspensions were aliquoted into 384-well opaque plates. To assess Emax for cAMP production, cells were incubated with 100 μM of isoproterenol for 15 min at 37 °C. For dose-response curves, cells were incubated under the same conditions with a range of isoproterenol concentrations from 100 fM to 100 μM. Subsequently, cells were lysed and the protocol was followed according to the manufacturer's recommendation (Promega). Luminescence was measured using a microplate luminometer reader (SpectraMax M5e; Molecular Devices). The cAMP levels (relative luminescence unit) were evaluated by subtracting the isoproterenol concentrations from 100 fM to 100 μM. Subsequently, cells were lysed and the protocol was followed according to the manufacturer's recommendation (Promega). Luminescence was measured using a microplate reader (SpectraMax M5e; Molecular Devices). The cAMP levels (relative luminescence unit) were evaluated by subtracting the isoproterenol concentrations from 100 fM to 100 μM. Subsequently, cells were lysed and the protocol was followed according to the manufacturer's recommendation (Promega).

IP-1 Assays. At 28 h to 32 h posttransfection, (XtremeGENE HP) HEK293T cells expressing the indicated sensor were harvested to assess IP-1 levels using the IP-One HTRF assay kit (Cisbio). Cells were gently suspended in their original media, were counted using a hemocytometer, and were spun down (350 × g, 3 min). An appropriate volume of Stibm buffer (Cisbio: 10 mM Heps, 1 mM CaCl2, 0.5 mM MgCl2, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4) was added to reach 3 × 10⁶ cells/mL density. Cells were incubated with 100 μM isoproterenol at 37 °C for 120 min. For dose–response curves, cells were incubated under the same conditions with a range of isoproterenol concentrations from 100 fM to 100 μM. Following the manufacturer's protocol, each reaction suspension was then incubated for 1 h shaking (500 rpm) at room temperature with 15 μL of IP-1 conjugated to d2 dye and 15 μL of terbium cryptate-labeled anti–IP-1 monoclonal antibody prepared and stored as recommended by the manufacturer. IP-1 FRET spectra were collected by exciting samples at 340 nm (band-pass 15 nm). Emission counts were recorded from 600 nm to 700 nm (band-pass 10 nm) using a long-pass 475-nm filter. Raw IP-1 signal was calculated from the 665 nm to 620 nm ratio. Data are presented as a change in raw IP-1 ratio following drug treatment. Each experiment had four repeats per condition and was independently repeated at least three times (n > 3). To obtain EC₅₀ and Emax, dose–response data were fit to a sigmoidal dose–response equation using nonlinear least-squares regression.

Calculation of first-order torsional entropy. The first-order torsional entropy of the G-protein interacting residues shown in Fig. 2 (limited to 2.2 × 10⁻¹⁶), but all P values for each comparison were significantly less than 1.0 × 10⁻⁴ (SI Appendix, Fig. S5B and Table S3).

Calculation of the insertion angles of the G protein. Calculation of the insertion angles of the G protein. The procedure identifies the centroid with the largest sample size of neighboring structures within the cutoff distance and sorts them to a unique cluster, repeating the same procedure with the remaining, unsorted structures.

Statistical analysis. The mean ± SEM was determined from each of five 200-ns replicates of the 1-μs ensemble trajectory. Means were compared using one-way ANOVA followed by Tukey’s posttest to assess significance for multiple comparisons, using GraphPad Prism version 7.00 for Windows (GraphPad Software, https://www.graphpad.com) (Fig. 2A and SI Appendix, Fig. S4A). The Kolmogorov–Smirnov test statistic was calculated for each distribution of GPCR IC cavity width (TM3 to TM6 distances), to compare the variance of each distribution (59). The sample size of the distribution was calculated as 50,000 frames. Each comparison rejected the null hypothesis and P values were too small to be calculated, due to limitations of machine precision (limited to 2.2 × 10⁻¹⁶), but all P values for each comparison were significantly less than 1.0 × 10⁻⁴ (SI Appendix, Fig. S5B and Table S3).

Experimental Methods
Experiments were conducted similarly to procedures outlined in our previous study (29). Details of “Reagents and buffers,” “Molecular cloning,” and “Mammalian cell preparation and sensor expression” are found in SI Appendix.

Calculation of first-order torsional entropy. The first-order torsional entropy of the G-protein interacting residues shown in Fig. 2A was calculated using methods developed in-house (30). Further details of this analysis can be found in SI Appendix.

Calculation of the insertion angles of the G protein. We measured the angle between the principal axis of the helical portion of the Gx peptide and the C terminus of the Gx proteins, cognate and noncognate, complexed with an agonist-bound GPCR. Double-sided curved arrows are drawn to show (i) balanced dynamic movement of GPCR TM bundle and Gx C terminus between the dominant and latent cavities in the IC interface during the apo state, (ii) or skewed toward the dominant cavity upon initial complexation with cognate G protein, and (iii) skewed to the latent cavity upon complexing with a noncognate G protein. (iii) Strong, unidirectional arrows reveal stabilization of the dominant cavity during G-protein activation. (b) Schematic free-energy landscape describes the relative stability of the GPCR during agonist binding, transient interaction with G proteins, and full complexation with a cognate G protein.

Fig. 7. The Goldlocks Effect: Cognate peptides fit “just right” for productive activation and signaling. (A) A model of the dynamics of the GPCR IC cavity and the C terminus of the Gx proteins, cognate and noncognate, complexed with an agonist-bound GPCR. Double-sided curved arrows are drawn to show (i) balanced dynamic movement of GPCR TM bundle and Gx C terminus between the dominant and latent cavities in the IC interface during the apo state, (ii) or skewed toward the dominant cavity upon initial complexation with cognate G protein, and (iii) skewed to the latent cavity upon complexing with a noncognate G protein. (iii) Strong, unidirectional arrows reveal stabilization of the dominant cavity during G-protein activation. (b) Schematic free-energy landscape describes the relative stability of the GPCR during agonist binding, transient interaction with G proteins, and full complexation with a cognate G protein.
data were fit to a sigmoidal dose–response equation using nonlinear least-squares regression. Compared with the cAMP data, the IP-1 data were better fitted by fitting to a linear model (\( \text{Residual}^2 = 0.05 \)) than by fitting to a linear model (\( \text{Residual}^2 = 0.01 \)).

**Statistical Analysis.** Data are expressed as mean values ± SEM. Experiments were independently conducted at least three times, with three to six technical repeats per condition (\( n > 3 \)). Statistical analysis was performed using GraphPad Prism 7.0c (GraphPad Software, Inc.). Statistical significance was performed for individual experiments using paired Student’s t test. To assess how the data varied across experimental repeats, data were pooled, and paired or unpaired Student’s t tests were conducted to evaluate significance.

One-way ANOVA with a Tukey’s posttest was performed to assess significance when evaluating comparisons between multiple conditions (Figs. 3B and 5A) with P values \( P < 0.05 \); \( \ast P < 0.01 \); \( \ast\ast P < 0.001 \); and \( \ast\ast\ast P < 0.0001 \).

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