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| journal or publication title | Proceedings of the National Academy of Sciences |
| volume | 116 |
| number | 24 |
| page range | 12054-12059 |
| year | 2019-05-29 |
| URL | http://hdl.handle.net/10097/00128250 |

doi: 10.1073/pnas.1905993116



Variable G protein determinants of GPCR coupling selectivity

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Edited by Brian K. Kobilka, Stanford University School of Medicine, Stanford, CA, and approved May 8, 2019 (received for review April 9, 2019)

G protein-coupled receptors (GPCRs) activate four families of heterotrimeric G proteins, and individual receptors must select a subset of G proteins to produce appropriate cellular responses. Although the precise mechanisms of coupling selectivity are uncertain, the G α subunit C terminus is widely believed to be the primary determinant recognized by cognate receptors. Here, we directly assess coupling between 14 representative GPCRs and 16 G α subunits, including one wild-type G α subunit from each of the four families and 12 chimeras with exchanged C termini. We use a sensitive bioluminescence resonance energy transfer (BRET) assay that provides control over both ligand and nucleotide binding, and allows direct comparison across G protein families. We find that the G_s- and G_q-coupled receptors we studied are relatively promiscuous and always couple to some extent to G_{i1} heterotrimers. In contrast, G_i-coupled receptors are more selective. Our results with G α subunit chimeras show that the G α C terminus is important for coupling selectivity, but no more so than the G α subunit core. The relative importance of the G α subunit core and C terminus is highly variable and, for some receptors, the G α core is more important for selective coupling than the C terminus. Our results suggest general rules for GPCR-G protein coupling and demonstrate that the critical G protein determinants of selectivity vary widely, even for different receptors that couple to the same G protein.

GPCR | G protein-coupled receptor | G protein selectivity | ternary complex

G protein coupled receptors (GPCRs) exert many of their physiological effects by coupling to and activating heterotrimeric G proteins. The 16 human G α subunit genes are classified into four families (G_s, G_{i/o}, G_{q/11}, and G_{12/13}), and members of each family interact with different effector molecules to produce distinct cellular responses (1, 2). Individual cells generally express many different G proteins from multiple families, and, therefore, GPCRs must be able to selectively activate subsets of G proteins. Many receptors show a preference for G proteins from just one of the four families, although promiscuous coupling to G proteins from multiple families is also not uncommon (3, 4). The mechanisms of selective GPCR-G protein coupling are not completely understood, and the structural determinants of selectivity have not been determined with precision. Analysis of GPCR primary sequences has not revealed simple conserved motifs for coupling to different G protein families (5), and analysis of GPCR-G protein complex structures has only begun to uncover features that may be important for coupling selectivity (6–12). From the standpoint of the G protein, it has long been appreciated that the G α carboxy (C) terminus is critical for activation by receptors and also a key determinant of selectivity (13–17). The distal part of the C-terminal alpha helix (helix 5; H5) is enveloped by GPCR transmembrane (TM) domains during coupling (6), and several studies have shown that mutations within this region can promote coupling of receptors to noncognate heterotrimers (17–21). Although other G α regions have been shown to be important for recognition by individual GPCRs (22–25), the G α C terminus is widely considered to be the most important structural determinant of coupling selectivity.

Here, we test this idea by measuring coupling of GPCRs to four representative G α subunits (G α_s , G α_{i1} , G α_q , and G α_{12}) and 12 G α chimeras with the C terminus of one representative and the G α core region of another. We monitor coupling using a sensitive energy transfer assay that allows comparison of all GPCR-G protein combinations directly at the coupling step. Our results demonstrate unexpectedly promiscuous coupling of many GPCRs to wild-type G proteins and support the notion that the G α C terminus is one important determinant of coupling selectivity for many receptors. However, our results also show that the G α C terminus is a minor contributor to selective coupling of several other receptors. Taken together our findings suggest that GPCRs recognize widely distributed structural features of G proteins and are consistent with the suggestion that different GPCRs recognize different conserved selectivity determinants of a given G protein (5).

Results

Promiscuous Coupling to Wild-Type G Proteins. Coupling of a GPCR to a heterotrimeric G protein can be defined as the allosteric interaction between the ligand binding site of the receptor and the nucleotide binding site of the associated G α subunit, such that the agonist-bound state of the receptor promotes the nucleotide-free (empty) state of the G protein and vice versa (26, 27) (Fig. 1A). To quantify coupling we monitored agonist-dependent and nucleotide-sensitive association of GPCRs with

Significance

G protein-coupled receptors (GPCRs) regulate a wide variety of important cellular processes and are targeted by a large fraction of approved drugs. GPCRs signal by activating heterotrimeric G proteins and must couple to a select subset of G proteins to produce appropriate intracellular responses. It is not known how GPCRs select G proteins, but it is generally accepted that the G α subunit C terminus is the primary G protein determinant of coupling selectivity. We systematically studied coupling of GPCRs to four families of G proteins and chimeras with C terminal regions that were exchanged between families. We uncovered rules for coupling selectivity and found that different GPCRs can recognize different features of the same G protein for selective coupling.

Author contributions: N.O., N.V., and N.A.L. designed research; N.O., Q.W., S.G., and M.S. performed research; A.I. contributed new reagents/analytic tools; N.O., M.S., A.I., N.V., and N.A.L. analyzed data; and N.O., N.V., and N.A.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1905993116/-DCSupplemental.

Published online May 29, 2019.

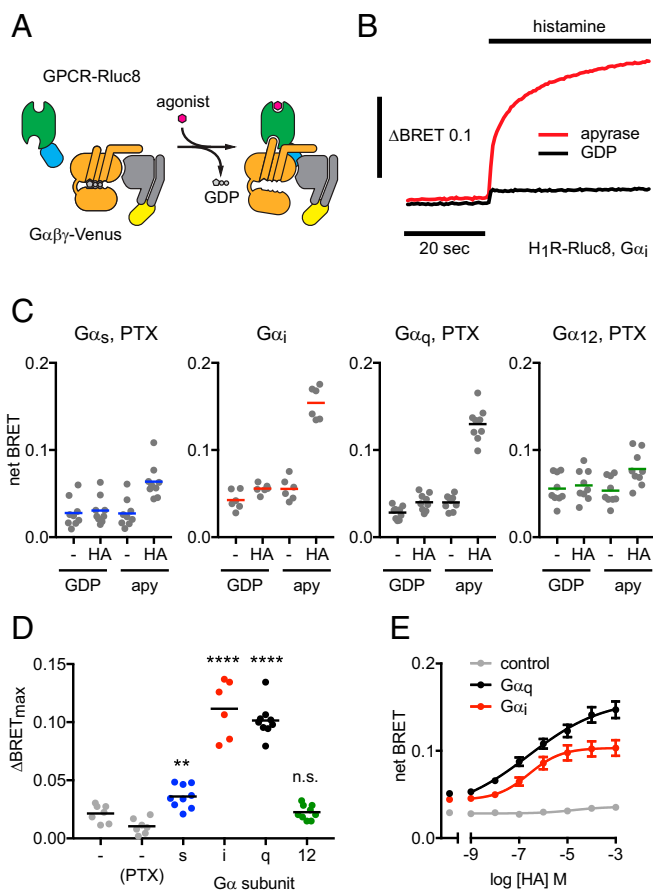


Fig. 1. Coupling to wild-type heterotrimers in permeabilized, nucleotide-depleted cells. (A) Receptors were fused to *Renilla* luciferase (Rluc8), and $G\beta\gamma$ dimers were fused to Venus. (B) Time course of BRET between H_1R -Rluc8 and $G\alpha_{i1}\beta\gamma$ -Venus in response to 100 μ M histamine (HA) in permeabilized cells in the presence (GDP) and absence (apyrase) of nucleotides. (C and D) H_1R couples to G_s , G_{i1} , and G_q heterotrimers; $**P = 0.0011$, $****P < 0.0001$, n.s. not significant; one-way ANOVA, Sidak's multiple comparisons. Pertussis toxin S1 subunit (PTX) was coexpressed in experiments with $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$. (E) Similar potency of histamine-induced coupling to G_{i1} ($EC_{50} = 258$ nM; $n = 4$) and G_q ($EC_{50} = 245$ nM; $n = 7$) heterotrimers.

different heterotrimers using bioluminescence resonance energy transfer (BRET) (28). To interfere as little as possible with receptor-G protein interactions, we positioned BRET donor and acceptor labels far from regions identified in functional and structural studies as important for coupling. A luciferase was fused to the GPCR C terminus, and complementary fragments of the fluorescent protein Venus were fused to $G\beta_1$ and $G\gamma_2$ subunits. These components were transfected together with unlabeled $G\alpha$ subunits into CRISPR/Cas9-edited HEK293 cells deficient in either $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ subunit families (three-family knockouts; 3GKO) or all four $G\alpha$ families (four-family knockouts; 4GKO) (29). When 3GKO cells were used, the S1 subunit of pertussis toxin (PTX) was coexpressed to prevent coupling to endogenous $G\alpha_{i/o}$ subunits, except when the overexpressed $G\alpha$ subunit was itself sensitive to PTX. Cells were permeabilized with digitonin and either supplemented with GDP or treated with apyrase to remove residual nucleotides.

Complexes between agonist-bound GPCRs and G proteins are short-lived when guanine nucleotides are present at concentrations similar to those found in the cytosol of intact cells and are greatly stabilized when guanine nucleotides are absent (30). Accordingly, nucleotide depletion significantly enhanced the magnitude of agonist-induced BRET between receptors and G

proteins (Fig. 1B), and maximal BRET was observed when agonist was present and GDP was absent (Fig. 1C). As a simple index of coupling, we defined $\Delta BRET_{max}$ as the difference between the BRET observed when receptor-G protein complexes are least stable (agonist absent, GDP present) and that observed when receptor-G protein complexes are most stable (agonist present, GDP absent). This experimental system allows direct comparison of receptor coupling to heterotrimers with $G\alpha$ subunits from different families and is sufficiently sensitive to detect secondary coupling to nonpreferred G proteins. Using this assay, we found that receptors generally coupled to the same G proteins as indicated by other methods and as annotated in the IUPHAR/BPS Guide to Pharmacology (3, 31). However, many receptors coupled to wild-type G proteins more promiscuously than expected. For example, we found that H_1 histamine receptors (H_1R), which are generally classified as G_q -coupled (3), couple strongly to both G_q and G_i heterotrimers, couple weakly to G_s heterotrimers, and do not couple significantly to G_{12} heterotrimers (Fig. 1D). The EC_{50} values for H_1R coupling to G_q and G_i were comparable (Fig. 1E), suggesting that activation of G_i heterotrimers by this receptor may have physiological significance, as some studies have indicated (32). We carried out similar experiments on a panel of 19 receptors (SI Appendix, Table S1), 17 of which are classified in the IUPHAR/BPS Guide to Pharmacology (3) as coupling primarily to G_s , G_i , or G_q proteins, and two of which are unclassified orphan receptors that transduce signals through $G_{12/13}$ proteins (33, 34). We found that promiscuous coupling to more than one wild-type G protein was a universal feature of the G_s -, G_q - and G_{12} -coupled receptors that we studied. In contrast, the G_i -coupled receptors that we studied were much more selective for G_i heterotrimers (Fig. 2 and SI Appendix, Fig. S1). From the perspective of the G protein, we found that G_i heterotrimers coupled to all of the receptors that we studied. It should be noted that the assay we used for these studies reflects allosteric coupling between agonist and nucleotide binding sites, but this is only a minimum requirement for agonist-dependent G protein signaling. Additional factors may be required for efficient G protein activation and signaling under physiological conditions in cells (see below).

Coupling to G Protein C-Terminal Chimeras. To assess the importance of the $G\alpha$ C terminus for coupling selectivity, we constructed 12 $G\alpha$ chimeras consisting of the main $G\alpha$ subunit core [HN.1-H5.16 using

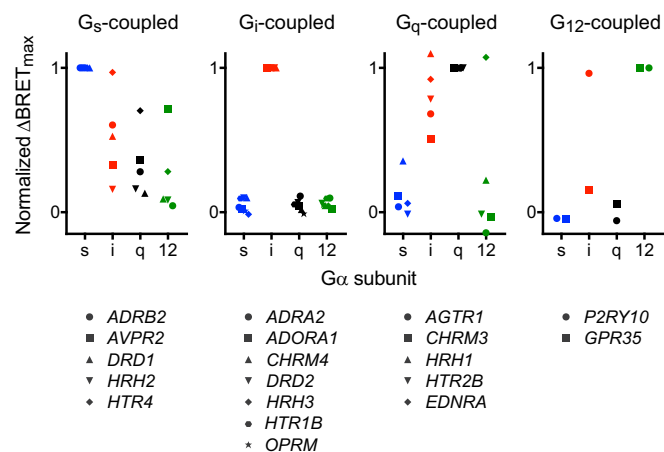


Fig. 2. G_i -coupled receptors are relatively selective. Normalized $\Delta BRET_{max}$ for a panel of G_s -, G_i -, G_q -, and G_{12} -coupled receptors. Each value represents the mean $\Delta BRET_{max}$ (normalized to the value obtained using the presumed cognate G protein) from $n = 3$ –6 independent experiments. Individual data points for each receptor are shown in SI Appendix, Fig. S1.

the common G protein numbering (CGN) system] of $G\alpha_s$ (long isoform), $G\alpha_{i1}$, $G\alpha_q$, or $G\alpha_{12}$ and the final 10 amino acids (H5.17–26) of each of the others. This portion of the $G\alpha$ C terminus is deeply embedded in the TM domains of GPCRs in all receptor–G protein complex structures and has been called the interface module of the C-terminal alpha helix (helix 5 or H5) (35). Previous mutagenesis studies have primarily targeted residues within this region to alter coupling selectivity (36). This region is also unstructured in G protein crystal structures (35), suggesting that it is not involved in folding or stability of the $G\alpha$ subunit core and, therefore, can be exchanged without compromising the structural integrity of heterotrimers. We confirmed the ability of $G\alpha$ chimeras to form heterotrimers by monitoring sequestration of overexpressed $G\beta\gamma$ dimers (37), and all expressed at comparable levels as indicated by immunoprecipitation (SI Appendix, Fig. S2). Conditions were optimized to provide roughly equivalent stoichiometry for each receptor–G protein pair, with the latter in excess. For simplicity, we refer to heterotrimers that incorporate these chimeras as G_{si} , G_{sq} , G_{s12} . We generated full coupling profiles (4 wild-type $G\alpha$ subunits and 12 chimeras) for 14 GPCRs and plotted the results as $\Delta BRET_{max}$ heat maps (Fig. 3). Similar heat maps were constructed for GDP-sensitive BRET in the absence of agonist ($\Delta BRET_{GDP}$; SI Appendix, Fig. S3) and agonist-induced BRET in the presence of GDP ($\Delta BRET_{ag}$; SI Appendix, Fig. S4).

In agreement with our results using wild-type $G\alpha$ subunits, we found that G_s -, G_q - and G_{12} -coupled receptors coupled more promiscuously to G protein chimeras than G_i -coupled receptors.

The G_s -, G_q - and G_{12} -coupled receptors that we studied always coupled well to several chimeras in addition to wild-type G proteins, sometimes with $\Delta BRET_{max}$ values that approached or even exceeded that of the cognate wild-type G protein (Fig. 3). The G_i -coupled receptors that we studied were more selective. For example, the D_2 dopamine receptor (D_2R) failed to couple to any $G\alpha$ chimera with a $\Delta BRET_{max}$ value that reached half of that observed with wild-type $G\alpha_{i1}$. We also observed that the $G\alpha_{12}$ core was often nonpermissive for coupling, particularly for G_i - and G_q -coupled receptors. This was sometimes partially overcome when a cognate C terminus was present, e.g., for A_1R and H_1R . By comparison, the $G\alpha_{12}$ C terminus was more accommodating to most receptors (Fig. 3). This suggests that many receptors reject $G\alpha_{12}$ core regions rather than the C terminus to select against G_{12} heterotrimers. The most promiscuous receptor that we studied was the endothelin A (ET_A) receptor, which coupled well to $G\alpha_{i1}$, $G\alpha_q$, $G\alpha_{12}$, and eight of the nine chimeras bearing the C termini of these subunits. This receptor coupled poorly to $G\alpha_s$ and chimeras bearing the $G\alpha_s$ C terminus, suggesting that selection against G_s coupling for this receptor is determined primarily by the C terminus.

We anticipated that GPCRs would couple poorly to chimeras bearing noncognate C termini, i.e., adding a noncognate C terminus to a cognate $G\alpha$ core would impair coupling. Indeed, this was observed for all 42 receptor-chimera pairs that we studied. However, the extent of impairment was highly variable, ranging from a near complete loss of coupling to almost no loss (SI Appendix, Fig. S5A). G_i -coupled receptors, in particular the M_4 acetylcholine receptor (M_4R) and D_2R , were the most sensitive to noncognate C termini. Conversely, we expected that GPCRs would generally couple well to chimeras bearing cognate C termini, i.e., noncognate $G\alpha$ core regions would only modestly impair coupling. However, we found that a noncognate $G\alpha$ core was often as detrimental to coupling as a noncognate C terminus (SI Appendix, Fig. S5B). Although adding a cognate C terminus to a noncognate $G\alpha$ core usually enhanced coupling, the gain of coupling was again highly variable and rarely a complete “switch.” For most of the receptors that we studied, coupling selectivity was determined both by the $G\alpha$ subunit core and the C terminus. A clear C terminus-dominant coupling pattern was observed for only a few receptors, the most obvious examples being H_1R , $P2RY10$, and $GPR35$ (Fig. 3). Other receptors, such as H_2R and A_1R , showed a more balanced influence of the cognate C terminus and G protein core. These receptors coupled equally well to chimeras bearing either the cognate C terminus or the cognate G protein core, although coupling to these chimeras was never as efficient as coupling to cognate wild-type G proteins. Unexpectedly, D_1R and M_3R showed coupling that was determined almost entirely by the G protein core and was virtually insensitive to the $G\alpha$ C terminus (Fig. 3). Full concentration–response curves for these receptors confirmed the patterns observed with single saturating concentrations of agonists. Specifically, for the D_1R and M_3R agonist potency was greater for chimeras with cognate G protein cores, whereas for the H_1R agonist potency was greater for chimeras with cognate C termini (SI Appendix, Fig. S6 and Table S2).

Efficient coupling to G proteins with noncognate $G\alpha$ C termini was unexpected, and suggested either that the C terminus contributes little to the stability of some receptor–G protein complexes or, alternatively, that some receptors can interact strongly with both cognate and noncognate C termini. To better understand the basis of efficient coupling to $G\alpha$ subunits with noncognate C termini, we performed atomistic molecular dynamics (MD) simulations of D_1R complexes with G_s , G_{si} , G_{sq} , and G_{s12} . These simulations revealed that in all cases, the C terminus contributed a substantial portion (at least one-third) of the total G_s protein interaction energy with D_1R (SI Appendix, Fig. S7B) and, therefore, was critical for the overall stability of

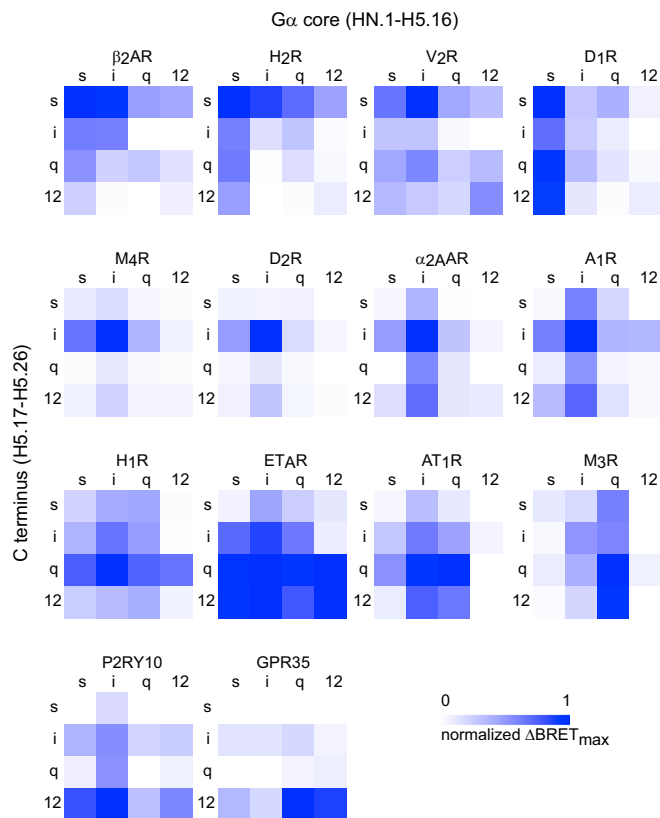


Fig. 3. Coupling to heterotrimers with C-terminal $G\alpha$ subunit chimeras. Heat maps of $\Delta BRET_{max}$ (normalized to the largest value) for G_s -coupled (Top), G_i -coupled (second row), G_q -coupled (third row), and G_{12} -coupled (Bottom) receptors. Maps are arranged from left to right depending on coupling that is determined more (e.g., H_1R) or less (e.g., D_1R) by the $G\alpha$ C terminus (H5.17–H5.26). Each value represents the mean normalized $\Delta BRET_{max}$ from $n = 3$ –6 independent experiments.

the complex. However, this receptor accommodated noncognate C termini with small energetic penalties (*SI Appendix, Fig. S7*), which corresponds to the observation that the C terminus was not critical for D₁R selectivity. These simulations also identified several regions of the G α_s core that contribute to the interaction and may also contribute to coupling selectivity for this receptor, including HN, the hns1, s2s3, and h4s6 linkers and N-terminal residues of H5 (*SI Appendix, Fig. S7A*).

We then asked if receptors that interacted well with nucleotide-free G proteins bearing noncognate G α C termini in our direct BRET coupling assay and MD simulations could also efficiently activate these G proteins and produce downstream signals. We found that wild-type G_s, G_{si}, G_{sq}, and G_{s12} all effectively restored potent activation of adenylate cyclase in response to D₁R activation in cells lacking endogenous G α_s subunits (Fig. 4A and *SI Appendix, Fig. S8*), whereas our assays did not detect signals mediated by wild-type G_{i1}, G_q, or G_{i2} in these cells (*SI Appendix, Fig. S9*). Likewise, we found that wild-type G_q, G_{qi}, G_{qs}, and G_{q12} all fully restored calcium release from intracellular stores in response to M₃R activation in cells lacking endogenous G α_q subunits (Fig. 4B and *SI Appendix, Fig. S8*), whereas our assays did not detect signals mediated by wild-type G_s, G_{i1}, or G_{i2} in these cells (*SI Appendix, Fig. S9*). Notably, both D₁R and M₃R coupled significantly to G_{i1} heterotrimers in our direct BRET assay, with Δ BRET_{max} values that were approximately half that of the respective cognate heterotrimers (Fig. 2) but failed to inhibit cAMP accumulation through G_{i1} (*SI Appendix, Fig. S9*). This suggests that receptors may couple to heterotrimers, as defined by formation of an agonist- and nucleotide-sensitive complex, and yet fail to activate a given signaling pathway even when overexpressed. In contrast to what we observed with D₁R and M₃R, G protein chimeras with noncognate C termini only partially restored calcium release from intracellular stores in response to H₁R activation. The rank order of restoration by chimeras was G_{qi} > G_{qs} > G_{q12} (Fig. 4C), which was consistent with the rank order of H₁R coupling to

wild-type G α subunits (Fig. 1). These results confirm that some GPCRs can efficiently couple to and activate heterotrimers with a variety of noncognate G α C termini. Taken together with our direct coupling BRET results, these findings indicate a highly variable influence of the G α subunit C terminus on coupling selectivity for different GPCRs.

Discussion

In this study, we used energy transfer between GPCRs and heterotrimeric G proteins to monitor allosteric coupling between agonist and nucleotide binding. This approach allowed us to directly compare coupling of several receptors to multiple G protein families without relying on downstream signals such as second messenger production. The use of G protein-deficient cell lines restricted coupling to defined wild-type and chimeric G α subunits, and permeabilization allowed us to control occupancy of both agonist and nucleotide binding sites.

Our results revealed that many receptors couple more promiscuously than expected. For example, every GPCR that we tested coupled to G_{i1} to some extent, and every G_s-coupled receptor that we tested also coupled somewhat to G_q. As a general rule, it appears that receptors that couple primarily to G_i heterotrimers are more selective than receptors that couple primarily to G_s and G_q heterotrimers. These trends are not readily apparent in annotated databases of coupling selectivity (3, 4). We suspect that the sensitivity of our assay, which detects stable complexes between agonist-occupied receptors and nucleotide-free G proteins, allowed us to detect secondary coupling (particularly to G_{i1}) that might easily be overlooked in functional studies. Indeed, our results with D₁R and M₃R coupling to G_{i1} suggest that this method can detect interactions that are too inefficient to lead to physiologically significant G protein activation in cells. However, it is also possible that weak secondary coupling interactions have physiological significance under certain circumstances, as previously demonstrated for β_2 AR activation of G_i heterotrimers (38, 39). We also suspect that a single assay that reports coupling to G proteins directly from all four families is likely to produce a more accurate profile of subtype selectivity than comparison across families with multiple assays based on second messenger accumulation and/or gene expression.

Our results with wild-type G α subunits are consistent with recent computational and structural studies, which have suggested that the outward displacement of transmembrane domain 6 (TM6) is restricted in active G_i-coupled receptors (8, 10–12, 40, 41). This produces a relatively small pocket in the cytoplasmic surface of the receptor that can only accommodate the relatively small C termini of G_{i/o} family G α subunits. This mechanism predicts stringent rejection of G_s and G_q heterotrimers by G_i-coupled receptors, but no similar barrier to promiscuous G_i activation, as we observed. However, our results with G α chimeras also show that some G_i-coupled receptors (e.g., α_2 AR and A₁R) can tolerate noncognate C termini to some extent and, therefore, suggest that the G α_i core region also partly determines selectivity for these receptors.

Overall our results support the well-established role of the G α subunit C terminus as a key determinant of receptor-G protein coupling selectivity. However, our findings also emphasize that other selectivity determinants exist that are equally important or more important for G protein recognition by many receptors. This is consistent with several previous reports of selectivity determinants that lie outside of the distal C terminus (22–25) and suggests that recognition of several spatially distributed regions of G α subunits is likely to be a general property of GPCRs. Remarkably, a few receptors virtually ignored the distal C terminus for the purposes of coupling selectivity. This demonstrates that the broad functional diversity of GPCRs extends to the mechanism of receptor-G protein coupling selectivity and

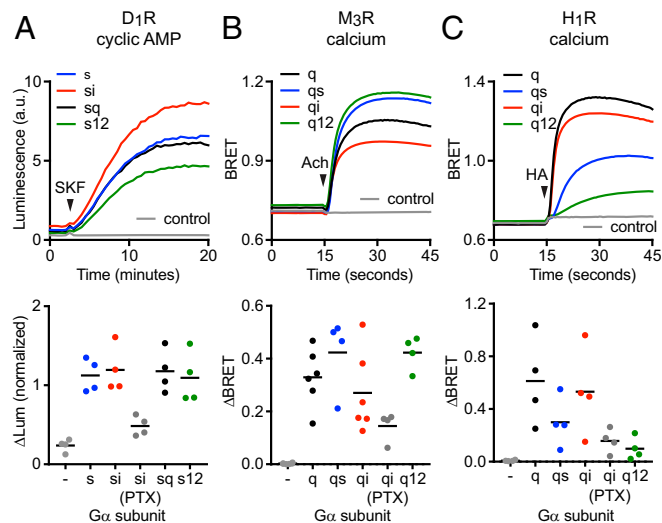


Fig. 4. Activation of G α subunit chimeras with noncognate C termini. (A) G_{si}, G_{sq}, and G_{s12} chimeras fully restored cAMP accumulation in response to D₁R stimulation (10 μ M SKF 81297) in cells lacking endogenous G α_s family subunits ($n = 4$). (B) G_{qs}, G_{qi}, and G_{q12} chimeras fully restored calcium release in response to M₃R stimulation (100 μ M acetylcholine) in cells lacking endogenous G α_q family subunits ($n = 6$). (C) G_{qi} but not G_{qs} and G_{q12} chimeras fully restored calcium release in response to H₁R stimulation (100 μ M histamine) in cells lacking endogenous G α_q subunits ($n = 4$). Traces are averages of several experiments, and data points represent independent experiments. Pertussis toxin S1 subunit (PTX) was coexpressed as indicated.

provides direct evidence to support the prediction that different receptors will recognize different conserved features of a particular G protein family (5).

Materials and Methods

Materials. Trypsin, Dulbecco's phosphate-buffered saline (DPBS), phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), Minimal Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, and L-glutamine were from GIBCO (ThermoFisher Scientific). Some receptor ligands, D-luciferin, and forskolin were purchased from Cayman Chemical. The remaining receptor ligands, digitonin, apyrase, protease inhibitor, and GDP were purchased from MilliporeSigma. All detergents were purchased from Anaspec. PEI MAX was purchased from Polysciences Inc.

Plasmids. Several different GPCR-luciferase constructs were made by appending either the *Renilla* luciferase variant Rluc8 or NanoLuc (Nluc) directly to the receptor C terminus either by QuikChange PCR or by subcloning into pRluc8-N1 or pNluc-N1 vectors. The V2R-Rluc8 plasmid was received as a gift from Kevin Pflieger (Harry Perkins Institute of Medical Research, Nedlands, WA, Australia). In some cases, a short GGSG linker was inserted between the GPCR and luciferase. Untagged GPCR sequences were obtained either from the cDNA Resource Center, <https://www.cdna.org/home.php?cat=0> (Bloomsburg University), as a gift from Jonathan Javitch (Columbia University, New York, NY) or as a gift from Bryan Roth (University of North Carolina, Chapel Hill, NC; PRESTO-Tango Kit: no. 1000000068, Addgene, Watertown, MA). Plasmids encoding $G\alpha$ subunits were purchased from the cDNA Resource Center. To generate chimeric $G\alpha$ subunits and $G\alpha_{Q227L10delctv}$ we used the PCR, reverse primers incorporating alternative C-terminal sequences, and wild-type $G\alpha$ templates to amplify full-length $G\alpha$ subunit sequences that were ligated into pcDNA3.1(+) using KpnI and XhoI. To generate p115RhoGEF-Rluc8, the sequence encoding p115RhoGEF was amplified from p115RhoGEF-GFP and ligated into a Rluc8-N1 vector with BglII and AgeI. All plasmid constructs were verified by Sanger sequencing. A plasmid encoding the S1 subunit of pertussis toxin (PTX-S1) was kindly provided by Stephen R. Ikeda (National Institute on Alcohol Abuse and Alcoholism, Rockville, MD). Plasmids encoding masGRKct-Rluc8, Venus-Kras, Venus-1-155-G γ_2 , and Venus-155-239-G β_1 have been described previously (37, 42). The Glosensor-22F cAMP plasmid (E2301) was obtained from Promega, p115RhoGEF-GFP was received as a gift from Phil Wedegaertner (Thomas Jefferson College, Philadelphia, PA), and the pT7-CalfluxVTN plasmid (43) was a gift from Carl Johnson (Vanderbilt University, Nashville, TN; Addgene plasmid 83926).

Cell Culture and Transfection. HEK 293 cells (American Type Culture Collection) were propagated in plastic flasks and on six-well plates according to the supplier's protocol. HEK 293 cells with targeted deletion of *GNAS* and *GNAL* (GSKO), HEK 293 cells with additional targeted deletion of *GNAS*, *GNAL*, *GNAQ*, *GNA11*, *GNA12*, and *GNA13* that are G protein three family knockouts (3GKO), and HEK 293 cells with additional targeted deletions to the 3GKO cells of *GNAI1*, *GNAI2*, *GNAI3*, *GNAT1*, *GNAT2*, *GNAZ*, and *GNAO1* that are G protein four family knockouts (4GKO) were derived, authenticated, and propagated as previously described (29, 44). Cells were transiently transfected in growth medium using linear polyethylenimine MAX (PEI MAX; MW 40,000) at an N/P ratio of 20 and were used for experiments 12–48

h later. Up to 3.0 μ g of plasmid DNA was transfected in each well of a six-well plate.

BRET and luminescence assays. Validation of $G\alpha$ subunit ability to form heterotrimers: HEK 293 cells were transiently transfected with masGRKct-Rluc8, Venus-1-155-G γ_2 , Venus-155-239-G β_1 , pcDNA3.1(+), and a $G\alpha$ subunit in a (1:1:1:5:0) ratio or a (1:1:1:4:1) ratio or a (1:1:1:0:5) ratio. After a 24-h incubation, cells were washed twice with 1 \times DPBS, harvested by trituration, and transferred to opaque black 96-well plates.

Measurement of coupling between receptor and G protein in nucleotide-depleted cells. 3GKO or 4GKO cells were transiently transfected with a GPCR-Rluc8 and $G\alpha$ subunit pair, Venus-1-155-G γ_2 , Venus-155-239-G β_1 , and pcDNA3.1(+) or PTX-S1 in a (1:3:1:1:1) ratio. Experiments with $G\alpha_i$ C termini were conducted in 4GKO cells for $G\alpha_i$ cognate receptors and in 3GKO cells for all other receptors. Experiments with $G\alpha_i$ C termini were conducted without PTX-S1, all other $G\alpha$ subunits were cotransfected with PTX-S1. After a 48-h incubation, cells were washed twice with permeabilization buffer (potassium permeabilization solution; KPS) containing 140 mM KCl, 10 mM NaCl, 1 mM MgCl $_2$, 0.1 mM KEGTA, 20 mM NaHEPES (pH 7.2), harvested by trituration, permeabilized in KPS buffer containing 10 μ g \cdot mL $^{-1}$ high purity digitonin, and transferred to opaque black 96-well plate. Measurements were made from permeabilized cells supplemented either with 0.5 mM GDP or 2 U \cdot mL $^{-1}$ apyrase, in both cases with or without agonist (SI Appendix, Table S1).

Data processing. Net BRET was calculated as the raw BRET ratio minus the same ratio measured from cells expressing only the BRET donor. Heatmaps represent Δ BRET $_{max}$ (Fig. 3; BRET in the presence of agonist and apyrase minus BRET in the presence of GDP alone), Δ BRET $_{GDP}$ (SI Appendix, Fig. S2; BRET in the presence of apyrase alone minus BRET in the presence of GDP alone), and Δ BRET $_{ag}$ (SI Appendix, Fig. S3; BRET in the presence of agonist and GDP minus BRET in the presence of GDP alone). Background Δ BRET (presumably due to endogenous $G\alpha$ subunits remaining in 3GKO and 4GKO cells) measured from control cells not expressing exogenous $G\alpha$ subunits was routinely subtracted. Control cells expressed PTX-S1 for experiments with non- $G\alpha_i$ C termini. Normalized Δ BRET values were obtained by dividing the Δ BRET observed for each chimera in a heatmap by the highest value observed for each receptor. For the Glosensor assays with G_{s_r} , $G_{s_{ir}}$, $G_{s_{sq}}$, and $G_{s_{12}}$, vehicle-subtracted luminescence changes in response to agonist were normalized to vehicle-subtracted luminescence changes in response to forskolin. For the Glosensor assay with G_{i_1} the percent change in luminescence was given as agonist-induced change in luminescence over baseline luminescence. For the Calflux assay Δ BRET $_{HA}$ and Δ BRET $_{Ach}$ were calculated from the kinetic experiments by subtracting the average of the time points before agonist addition from the average of the final five time points after agonist addition.

Detailed methods related to second messenger assays, BRET and luminescence measurements, $G\alpha$ subunit immunoprecipitation and structural modeling and molecular dynamics simulations can be found in SI Appendix.

ACKNOWLEDGMENTS. We thank Steve Ikeda, Jonathan Javitch, Kevin Pflieger, Philip Wedegaertner, Carl Johnson, and Bryan Roth for providing plasmid DNA used in this study. This work was supported by National Institutes of Health Grants GM130142 (to N.A.L.) and GM117923 (to N.V.) and Ruth L. Kirschstein National Research Service Award Individual Fellowship GM131672 (to N.O.). A.I. was funded by the Advanced Research & Development programs PRIME JP17gm5910013 and LEAP JP17gm0010004 from Japan Agency for Medical Research and Development, and a grant-in-aid for scientific research KAKENHI 17K08264 from the Japan Society for the Promotion of Science.

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