Supporting Information

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SI Materials and Methods

Expression Constructs and Cells. Primary human hepatocytes (Celsis) were plated overnight on collagen-coated 96-well plates. Stable Flp-In (Invitrogen) 293 cell lines expressing WT glucagon receptor (GCGR), Y65A or R111A were generated using pcDNA5/FRT/V5-HisTOPO (Invitrogen). Stable 293 cells expressing WT GCGR, extracellular loop (ECL) 1, and ECL3 chimeric receptors were generated using pVITRO4 (Invivogen). Wt GCGR, Q113A/N/G, or ECL2 chimeric GCGR in pVITRO4 were transiently transfected into 293 cells. cDNAs corresponding to full-length GCGR and extracellular doman (ECD) residues Ala26 to Gln142 of human GCGR were subcloned into a modified pAcGP67 vector (BD) as a fusion to the gp67 signal sequence and a C-terminal, Tobacco Etch Virus (TEV) (Eton Bioscience) protease cleavable 6xHis tag. Some constructs also contained an AviTag biotinylation sequence (GLNDIFEAQKIEWHE) preceding the TEV protease cleavage site for in vivo biotinylation upon coexpression with BirA (Avidity). Single-site mutagenesis was performed using Quickchange II (Agilent). ECL chimeric receptors were generated by oligonucleotide-directed site mutagenesis.

Cell-Based Assays. PEPCK mRNA was measured using the Quantigene 2.0 bRNA assay system (Affymetrix) 6 h after addition of glucagon (2 nM) in insulin-free, BSA-free Krebs-Ringer buffer. Luciferase activity was measured in cells cotransfected with pGL4.29 (CRE-luciferase construct) and pRL-SV40 expressing renilla (Promega) 3–5 h after addition of glucagon or 100 nM 3-isobutyl-1-methylxanthine (IBMX). cAMP was measured using homogenous time resolved fluorescence (HTRF) (Cisbio). Antibodies were incubated with cells for 1 h before adding glucagon or IBMX.

Antibody Isolation and Expression. cDNAs encoding the mAb1 protein sequence (1) were synthesized. The mAb1 Fab was generated by LysC digestion (Wako) of IgG and purified on a SP-Sepharose column (GE Healthcare) using a linear gradient from 0 to 250 mM NaCl in 20 mM MES (pH 5.5). Fab fragments of mAb23 and mAb39 were obtained by selection of a phage display library (2) and affinity matured (2, 3). For mAb7, mice were immunized with cells expressing human GCGR. For all antibodies heavy chain (HC) and light chain (LC) Fab regions were subcloned into IgG1 expression vectors, which were cotransfected into 293T or CHO cells. Secreted IgG1 was collected and purified by protein A affinity chromatography.

Protein Expression. Full-length GCGR was expressed in *Sf9* cells as a fusion to an amino terminal gp67 signal sequence and C-terminal AviTag biotinylation sequence, as described for the isolated ECD. Viral particles were harvested by centrifugation at 20,000 × *g* for 6 h at 4 °C and then solubilized for 2 h at 4 °C in 30 mL of 200 mM NaCl, 10% (vol/vol) glycerol, 1% dodecyl maltoside (DDM), and 25 mM Hepes (pH 7.5). After centrifugation at 4 °C for 1 h at 100,000 × *g*, the supernatant was harvested. The presence of full-length GCGR in the viral particles and detergent solubilized fraction was confirmed by Western blotting. This material was used immediately in AlphaScreen assays (0.4 μL per 20-μL reaction), using buffer E [150 mM NaCl, 0.1% DDM (Anatrace), 0.1 mg/mL BSA, and 50 mM Tris (pH 7.5)].

Production of Recombinant GCGR ECD and Fab Complexes. Recombinant baculovirus stocks were generated with BaculoGold (BD). Expression was performed in suspension Sf9 cells for 48 h. Media was batch bound to Ni-Sepharose beads (GE Healthcare) in Tris-buffered Saline (TBS) (pH 8) and eluted with a gradient of 10–300 mM imidazole. ECD was cleaved with TEV protease, dialyzed into 200 mM NaCl and 20 mM Hepes (pH 7.2), and resolved by size exclusion chromatography (SEC). Fab/GCGR ECD complexes (1:1 molar ratio) were mixed overnight at 4 °C and then resolved by SEC. Peak Fab/GCGR ECD complex fractions were pooled and concentrated to 11 mg/mL.

Crystallization and Data Collection. Sitting drop vapor diffusion crystal trials were performed at 4 °C and 19 °C with drop volumes of 0.1 μL protein sample and 0.1 μL well solution. Hit optimization was by hanging drop vapor diffusion. The mAb1 Fab/GCGR ECD complex crystallized after 1–2 wk in 0.1 M Tris (pH 8.5), 0.2 M NaCl, 25% (wt/vol) PEG3350, and 0.1 M NaOAc (pH 4.6), 0.2 M (NH4)2SO4, 25% (wt/vol) PEG4000, as ~150 μm Å × 20-μm thin plates. Crystals were frozen in mother liquor with 25% (vol/vol) glycerol. Data were collected at the Advanced Light Source BL5.0.1 beamline.

Structure Determination and Refinement. The ECD/mAb1 Fab structure was solved by molecular replacement using Fc and Fv regions of Protein Data Bank ID codes 1FVC and 1FVD as search models. Clear F_o-F_c electron density was observed for the GCGR ECD and was rebuilt and refined using Coot (4) and PHENIX (5) (Table S2). The first and last residues of the GCGR ECD with interpretable electron density are V28 and M123, respectively. Ramachandran statistics for mAb1/ECD: Ramachandran outliers = 0.19%, 93.83% in the favored regions.

Computational Docking and Modeling. Modeling of the ECD–glucagon complex was achieved by first building a homology model of glucagon based on a GLP-1 structure (Protein Data Bank ID 3IOL, chain B) and then superimposing the GCGR ECD crystal structure on the GLP-1R ECD complex to generate a starting model for high-resolution docking using Rosetta v3.2 (6). Docking was achieved with the local refine flag selected. The structure with the lowest energy from a docking run was used for further analysis.

Binding of mAbs to Purified ECD and Full-Length GCGR in Alphascreen Format. The ability of WT GCGR ECD, mutants, or full-length receptor to compete with WT GCGR ECD for binding to antibodies was measured using a competition Alphascreen assay (PerkinElmer). Twenty-microliter reaction mixtures contained 20 μg/mL of streptavidin-coated donor beads, 20 μg/mL of protein A-coated acceptor beads, 10 nM biotinylated WT GCGR ECD, 1 nM of the tested antibodies, and nonbiotinylated competitor ECD at the indicated concentrations, in buffer E. Samples were incubated in a 384-well ProxiPlate (PerkinElmer) for 2–3 h at 22 °C. Signal was recorded using an EnVision 2103 plate reader using an AlphaScreen emission 570 filter.

Binding of mAbs to WT and Chimeric GCGR in ELISA Format. Baculovirus particles displaying full-length WT and ECL3 chimera GCGR were produced and purified as previously described (2). Baculovirus particles were mixed with PBS-0.5% BSA, DDM added to a concentration of 1%, and incubated on ice for 20 min. Debris and insoluble material in the lysates were removed by centrifugation at 18,000 × *g* for 20 min at 4 °C, and the supernatant was mixed with IgG serially diluted in PBS-0.5% BSA containing 0.1% DDM and incubated for 1 h at 4 °C. The amount of lysate used was twice the amount needed to saturate the ELISA wells

precoated with neutravidin (Pierce), determined in a preliminary experiment. After incubation, WT and ECL3 chimera GCGR bound to IgG was captured in ELISA plates coated with neutravidin for 10 min at room temperature. The plates were washed with PBS containing 0.1% DDM, and goat anti-human IgG HRP conjugate (Invitrogen) diluted in PBS-0.5% BSA with 0.1% DDM was added to the plates. After 30 min incubation the plates were washed as above, developed with a tetramethylbenzemidine (TMB) substrate for 3 min, and the reaction stopped with 1 M phosphoric acid.

Cell Binding Assay. Cells (5×10^5) expressing GCGR variants were plated into 96-well high-bind plates [Meso Scale Discovery (MSD)]. After 1 h cells were blocked with 30% FBS/PBS for 30 min. The mAbs were added to cells in 3% FBS/PBS and incubated for 1 h at room temperature. After three washes with PBS, 2 μg/mL sulfo-labeled secondary antibody (MSD) in 3% FBS/PBS was added for 1 h. Binding was measured using Read Buffer T (MSD) on a MSD6000 plate reader.

Shotgun Alanine Scanning of GCGR ECD. *Escherichia coli* were co-infected with a phagemid (pS2202b) (7) that was modified to contain human GCGR ECD (Ala26 to Gln142) and M13-KO7 helper phage to generate M13 bacteriophage particles displaying the maltose binding protein secretion signal, followed by an epitope tag (amino acid sequence: SMADPNRFRGKDLGS), followed by GCGR ECD and ending with the mature M13 gene-8 major coat protein on the surface. Libraries, containing $\sim 10^{10}$ unique members, were constructed using previously described methods (8). For each mutated position, the codon was designed to encode either WT or alanine. For some residues, two other extra mutations might be introduced (9). Phages from the libraries were propagated in *E. coli* XL1-blue as described previously (8). Phage solutions (10^{12} phage/mL) were added to BSA-blocked, 96-well Maxisorp immunoplates that had been coated with capture mAb. For the display selection an antibody that recognized the epitope tag fused to the N terminus of GCGR ECD was used, whereas for the functional selection, mAb1 or mAb23 were used. Individual clones from the fourth round of selection were screened with spot phage ELISA. Clones exhibiting signals at least twofold greater than signals on control plates coated with BSA were considered positive. These positive clones were subjected to DNA sequence analysis. Approximately 100 positive clones were sequenced for each library, and *F* values for each position were calculated as described previously (9).

Glucagon Scanning Mutagenesis. cDNA encoding for the full-length glucagon peptide was fused to the N terminus of M13 major coat protein g8 by Kunkel mutagenesis (10) using pS2202b1 as template. The mutated scanning library was constructed by Kunkel mutagenesis according to the standard protocol (11). Residues S16-T29 were "soft randomized" using a doping codon in which each base position was a mixture of 70% WT base and 10% of the other three bases that would result in a ~50% mutation rate at the amino acid level. A stop template was used to construct a library that contained $\sim 2 \times 10^{10}$ unique members. The library was cycled against recombinant, biotinylated GCGR-ECD for four rounds in solution as previously described (11).

Limited Proteolysis of WT and Chimeric GCGR. Stable 293 cells expressing the various full-length GCGR constructs were resuspended in buffer containing 120 mM NaCl, 10% glycerol, and 50 mM Tris (pH 8.0). Endoproteinase Lys-C was added at 5, 10, 20, and 40 μg/mL and the samples incubated at 22 °C for 90 min. Digestions were stopped by the addition of SDS sample buffer lacking DTT. Samples were immediately resolved by SDS/PAGE, followed by transfer to nitrocellulose for Western blotting.

Western Blotting. Cell lysates were prepared by solubilizing cells in lysis buffer M (Roche) with protease inhibitors. Purified ECD protein was added to the lysate at 0.05 mg/mL. SDS sample buffer with or without DTT (as indicated) was added to lysates, and samples were resolved by SDS/PAGE followed by transfer to nitrocellulose for Western blotting. Blots were incubated with 10 μg/mL anti-GCGR antibodies in TBST + 5% nonfat milk, followed by probing with a goat anti-human secondary antibody conjugated to HRP or alkaline phosphatase (AP). HRP was detected using an ECL detection kit (Pierce). AP was detected using SigmaFast tablets (Sigma).

Data Analysis. EC_{50} and IC_{50} values were determined from nonlinear regression models using Prism software (GraphPad Software).

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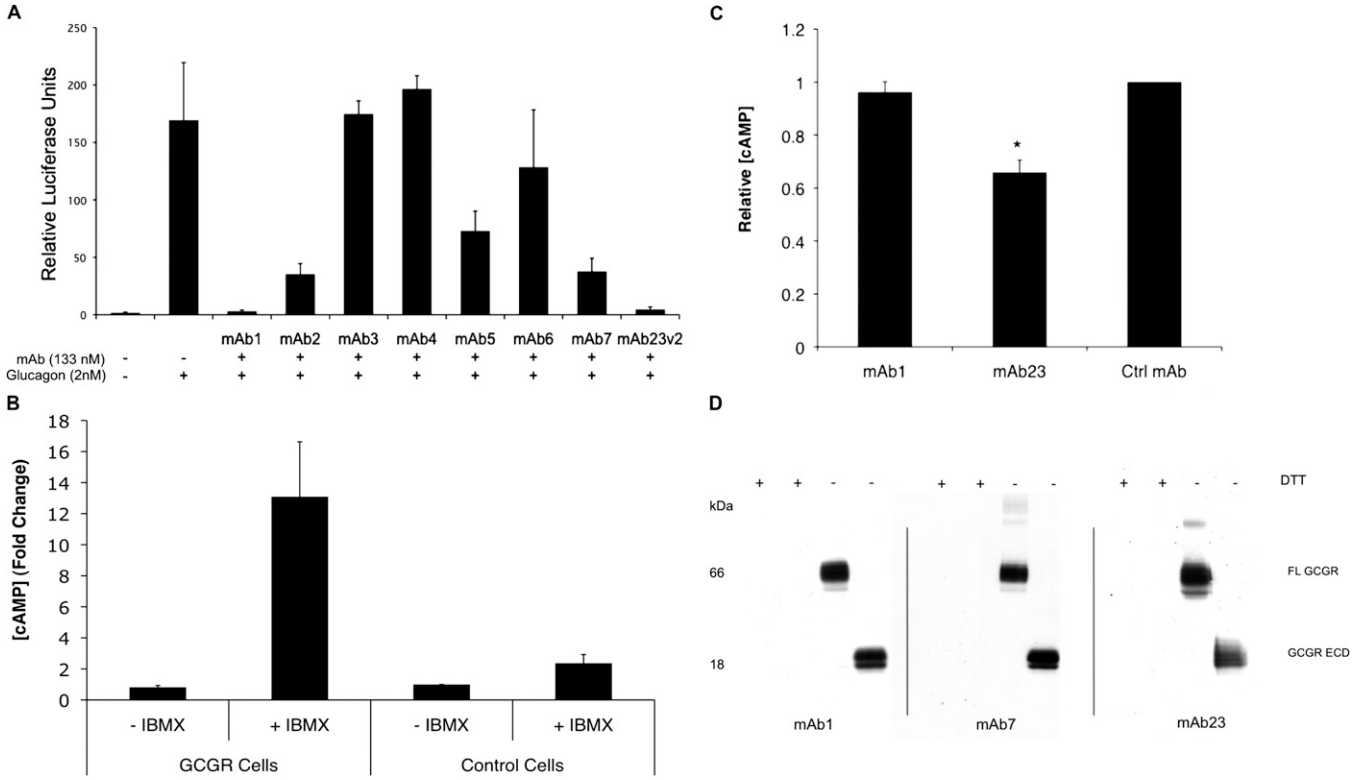

Fig. S1. Inhibitory anti-GCGR mAbs recognize conformational epitopes. (*A*) Identification of mAb7 and mAb23 as antibodies that block glucagon-induced activation of CRE-luciferase expression in 293 cells overexpressing human GCGR. Data shown are mean ± SD, $n = 3$. (*B*) Constitutive GCGR activity in 293 cells overexpressing human GCGR. Data shown are mean ± SD, $n = 3$. (*C*) Basal cAMP levels are reduced by mAb23 in 293 cells overexpressing human GCGR. Control antibody does not recognize GCGR. Mean ± SD, $n = 5$. $^*P < 0.05$. (*D*) mAb1, mAb7, and mAb23 recognize conformational epitopes on the GCGR ECD. Western blots of lysates from cells expressing full-length human GCGR or with purified recombinant ECD added were probed with mAb1, mAb7, and mAb23. DTT was either added to or omitted from the SDS sample buffer, and gels were run under nonreducing, denaturing conditions.

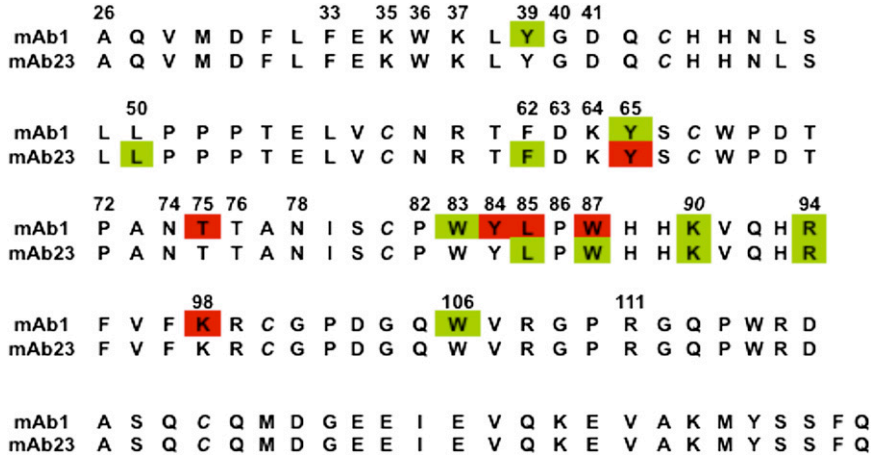

Fig. S2. A linear epitope map for mAb1 and mAb23 interactions with the ECD. Residues that have a calculated *F* value (9) between 3 and 5 or >5 are labeled in green or red, respectively.

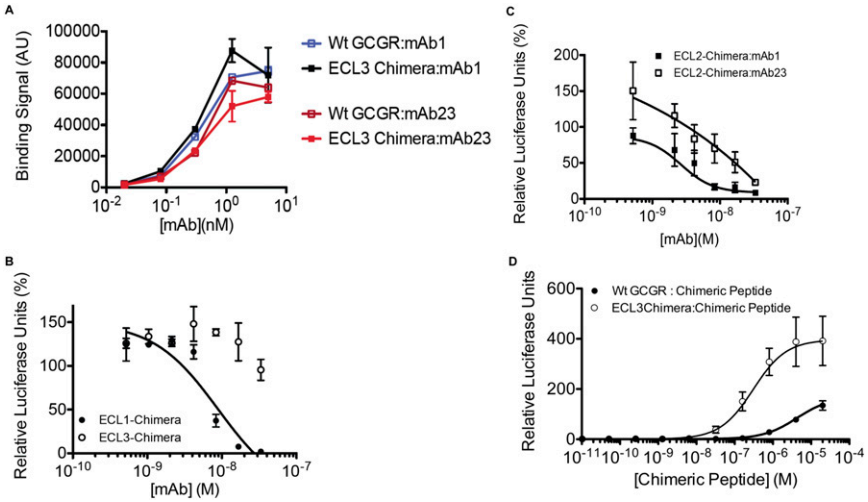

Fig. S3. (*A*) mAb1 and mAb23 bind to cells expressing WT GCGR or ECL3 chimeric GCGR with comparable affinities. (*B*) mAb1 fails to inhibit activation of ECL3 chimeric receptor. (*C*) The activity of the ECL2 chimeric receptor can still be blocked by mAb1 and mAb23. (*D*) A glucagon:GLP-1 chimeric peptide (HSQGTFTSDYSKYLEGQAAKEFIAWLVKGRG) that bypasses the ECD activates ECL3 chimeric receptor with greater potency than WT GCGR.

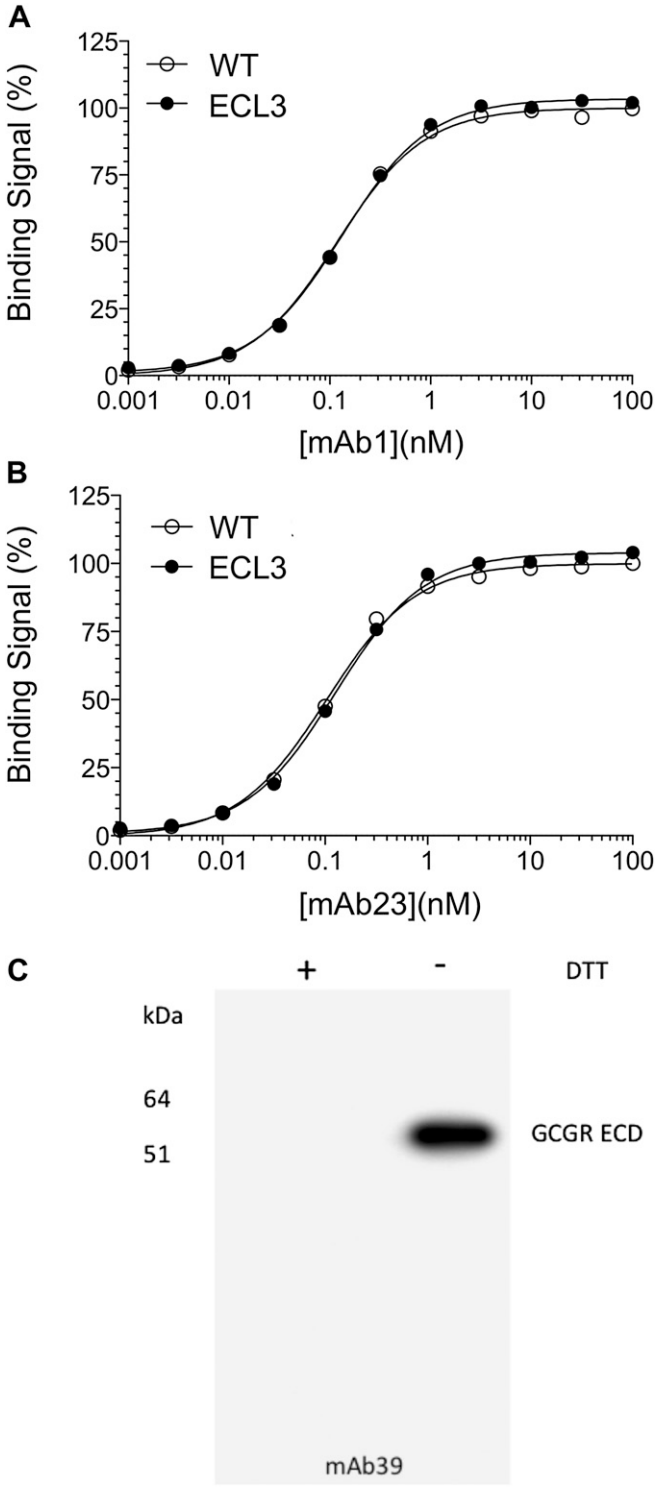

Fig. S4. Antibodies mAb1 (*A*) and mAb23 (*B*) bind similarly to detergent-solubilized WT GCGR and ECL3 chimeric GCGR. Data shown are mean ± SD from two independent experiments, normalized to binding of each antibody to WT GCGR. The data shown are from experiments performed in parallel with those in Fig. 5*E*. (*C*) mAb39 recognizes a conformational epitope on the GCGR ECD. DTT was either added to or omitted from the SDS sample buffer and the samples resolved under denaturing conditions. Westerns of recombinant ECD were probed with mAb39.

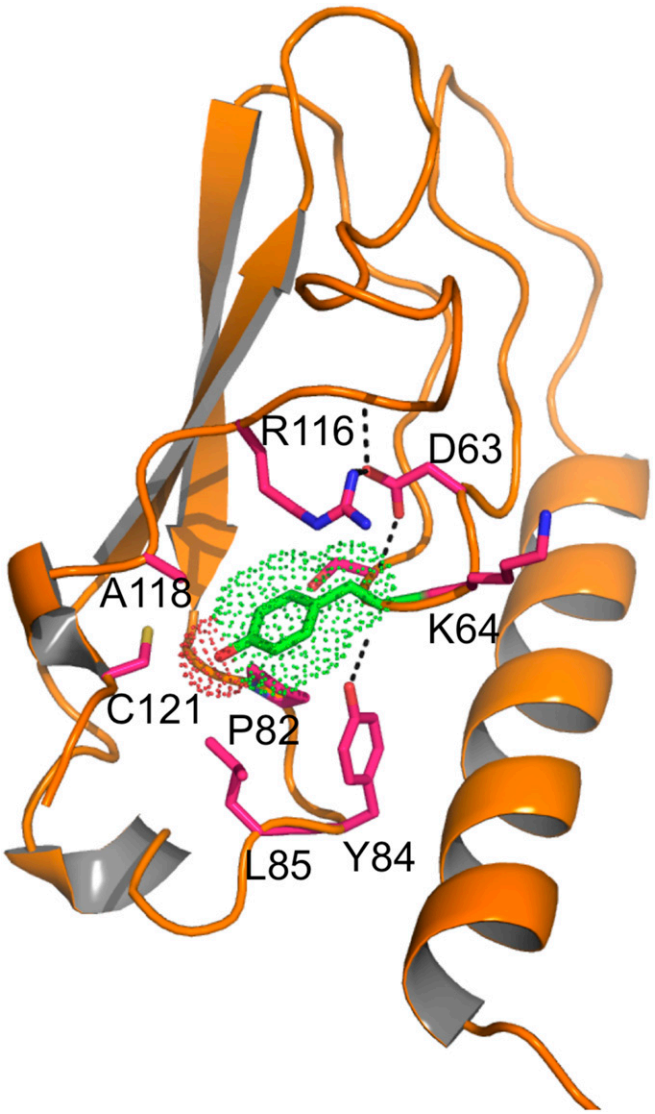

Fig. S5. Residues within van der Waals contact distance of Tyr65 are highlighted, with their sidechains displayed as sticks. The sidechain area of Tyr65 is highlighted as red and green dots.

Table S1. Kinetic binding data from surface plasmon resonance (SPR) binding analysis of Fab fragments to GCGR ECD

Fab	K_{on} ($M^{-1}s^{-1}$)	K_{off} ($M^{-1}s^{-1}$)	K_D (nM)
mAb1	1.8×10^5	4.6×10^{-4}	2.5
mAb7	4.3×10^5	9.7×10^{-4}	2.3
mAb23v3	2.5×10^5	2.1×10^{-4}	0.8

SPR was performed as previously described (1).

1. Hötzel I, et al. (2011) Efficient production of antibodies against a mammalian integral membrane protein by phage display. *Protein Eng Des Sel* 24:679–689.

Table S2. Data collection and refinement statistics for the mAb1/GCGR ECD complex

Parameter	mAb1/GCGR ECD
Data collection	
Space group	$P12_11$
Cell dimensions	
a, *b*, *c* (Å)	46.4, 62.2, 105
α, β, γ (°)	90.0, 93.9, 90.0
Resolution (Å)	43.47–2.64 (2.79–2.64)
R_{sym} or R_{merge}	0.108 (0.39)
$I/\sigma I$	14.4 (2.4)
Completeness (%)	99.3 (97.3)
Redundancy	3.9 (3.8)
Refinement	
Resolution (Å)	43.47–2.64
No. reflections	17,586
R_{work}/R_{free}	0.231/0.279
No. atoms	4,291
Protein	4,181
Ligand/ion	28
Water	112
B-factors	
Protein	44
Ligand/ion	30
Water	31
rmsd	
Bond lengths (Å)	0.006
Bond angles (°)	0.91

Values in parentheses are for highest-resolution shell.

Table S3. K_d values for mAb1 and mAb23 binding to cells expressing WT GCGR or the ECL chimeras

	mAb1				mAb23			
Variable	WT GCGR	ECL1 chimera	ECL2 chimera	ECL3 chimera	WT GCGR	ECL1 chimera	ECL2 chimera	ECL3 chimera
K_d (nM)	0.44	0.44	0.18	0.45	0.7	0.76	0.48	0.44