The Role of ICL1 and H8 in Class B1 GPCRs; Implications for Receptor Activation

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The first intracellular loop (ICL1) of G protein-coupled receptors (GPCRs) has received little attention, although there is evidence that, with the 8th helix (H8), it is involved in early conformational changes following receptor activation as well as contacting the G protein β subunit. In class B1 GPCRs, the distal part of ICL1 contains a conserved R12.48KLRCxR2.46b motif that extends into the base of the second transmembrane helix; this is weakly conserved as a [R/H]12.48KL[R/H] motif in class A GPCRs. In the current study, the role of ICL1 and H8 in signaling through cAMP, iCa2+ and ERK1/2 has been examined in two class B1 GPCRs, using mutagenesis and molecular dynamics. Mutations throughout ICL1 can either enhance or disrupt cAMP production by CGRP at the CGRP receptor. Alanine mutagenesis identified subtle differences with regard elevation of iCa2+, with the distal end of the loop being particularly sensitive. ERK1/2 activation displayed little sensitivity to ICL1 mutation. A broadly similar pattern was observed with the glucagon receptor, although there were differences in significance of individual residues. Extending the study revealed that at the CRF1 receptor, an insertion in ICL1 switched signaling bias between iCa2+ and cAMP. Molecular dynamics suggested that changes in ICL1 altered the conformation of ICL2 and the H8/TM7 junction (ICL4). For H8, alanine mutagenesis showed the importance of E3908.49b for all three signal transduction pathways, for the CGRP receptor, but mutations of other residues largely just altered ERK1/2 activation. Thus, ICL1 may modulate GPCR bias via interactions with ICL2, ICL4 and the Gβ subunit.

Keywords: GPCRs, signaling bias, CLR, RAMPs, mutagenesis

INTRODUCTION

GPCRs form the single largest protein family in the human genome, and they are also the largest single target for therapeutic agents (1). They all share a common architecture based around seven transmembrane helices (TM5), connected by three intracellular and three extracellular loops (ICLs and ECLs), often with an 8th helix (H8), immediately after TM7, lying parallel to the membrane. They
are divided into several families. The largest and best understood is the class A or rhodopsin-like family. The class B1, or secretin-like family is smaller but is made of receptors for physiologically important peptides such as glucagon, corticotrophin-releasing factor (CRF) and members of the calcitonin gene-related peptide (CGRP) family. There are cryo-electron microscope structures for most of these receptors, showing their structure with Gs or other G proteins (2) and crystal or cryo-EM structures are available for the transmembrane domains of the glucagon, glucagon-like peptide-1 (GLP-1), CRF type-1 (CRFR1), CGRP and parathyroid type-1 receptors (3–10).

The means by which class A GPCRs recognise G proteins is now understood in detail for a number of receptors (11). There is also growing understanding of GPCR binding to β-arrestins (12). It has been possible to trace how agonist binding leads to TMs 3 and 6 (and hence ICLs 2 and 3) moving apart, to allow an interaction with the C-terminus of the G protein. It remains unclear how different receptor-ligand combinations select between individual G proteins or β-arrestins, although this is key to understanding biased agonism - the phenomenon whereby agonists produce unique signals at receptors (10). The activation of class B1 GPCRs appears to be broadly similar although they have their own unique motifs; thus, residues at the base of TM2 and its junction with ICL1 function in combination with TM 3 to create a G protein-binding pocket (7–10). The individual microswitches used by class A and class B1 are distinct (13). For class B1 GPCRs, it seems that the key to receptor activation is the full engagement of the agonist peptide N-terminus with the TM domain of the GPCR, which initiates changes at the intracellular face of the receptor (14, 15). ICL1 has been little studied in either GPCR class. Mutations can disrupt cell surface expression (16, 17), indicating the loop is important for structural integrity of the receptor. It is adjacent to ICL2 and in the class A and B1 GPCR structures there is an interaction between it and the proximal region of H8. The junction between H8 and TM7 is sometimes known as ICL4. ICL1 has a role in receptor activation, as revealed by mutations in a range of GPCRs from class A and B1 (18–26). Hydrogen-deuterium exchange indicates that different agonists cause distinctive conformational changes in ICL1, similar to those seen at ICL4 (27). Changes in the ICL1–H8 unit precede movement of TMs 5 and 6 in the μ-opioid receptor, showing that they are an early event in GPCR activation (28, 29); this may be part of a conserved activation mechanism (30). There is also good evidence for ICL1 playing a role in G protein selectivity through splice variants in class B1 GPCRs (31). The mechanism behind the actions of ICL1 is unclear.

Here we study the role of ICL1–H8 unit in the activation of class B1 GPCRs and in particular, the significance of a motif previously referred to as the [K/R]KHL motif (32) within ICL1 which mediates its interactions with H8. We have primarily concentrated on the calcitonin receptor-like receptor (CLR), a receptor we have studied (33, 34), in association with receptor activity-modifying protein 1 (RAMP1), this forms a receptor for CGRP which can also recognize the related peptide adrenomedullin (AM) (35). We have also examined the glucagon receptor (GCGR) and two splice variants of the CRFR1.

**METHODS**

**Materials**

Human CGRP and CRF were purchased from Bachem and human glucagon was purchased from Alta Biosciences. All peptides were made to 1 mM stocks in water containing 0.1% bovine serum albumin (BSA).

**Constructs and Site-Directed Mutagenesis of CLR and GCGR**

The CRFR1a and CRFR1b constructs were gifts from Dr. Simon Dowell (GSK, Stevenage, UK). ICL1 mutants of CLR were generated in a pIRE5-RAMP1-SNAP-CLR construct in-house by Sosei Heptares and confirmed through Sanger sequencing. ICL1 mutants of the GCGR were generated as for CLR except using a base vector of pcDNA3.1-GCGR-GFP. H8 mutants for CLR and GCGR were generated by using the QuikChange Lightening Site-Directed Mutagenesis Kit (Agilent Technologies) in accordance with the manufacturer’s instructions and sequenced in house at Cambridge University. All constructs were of human receptors and RAMP.

**Transfection and Cell Culture**

HEK 293T cells (a gift from Professor Colin Taylor) were cultured in DMEM/F12 GlutaMAX supplemented with 10% fetal bovine serum (FBS), and incubated at 37% in humidified 95% air, 5% CO2. Plasmids were transfected into HEK 293T cells using FuGENE HD according to the manufacturer’s instructions using a 1:3 w:v ratio of DNA : FuGENE and cultured for 48 hours prior to assaying.

**Quantification of Mutant GPCR Expression**

Cell surface expression of WT and mutant GPCRs was determined in HEK 293 cells via flow cytometry as previously described (36). Briefly, after 48 hours, cells were washed three times in fluorescence activated cell sorting (FACS) buffer (PBS supplemented with 1% bovine serum albumin (BSA) and 0.03% sodium azide) before and after 1 hour incubation at room temperature in the dark with appropriate primary antibody; rabbit anti-GGCR (AGR-024, diluted 1:50 (Alomone Labs)) or rabbit anti-SNAP (CAB4255, diluted 1:100, Invitrogen). Cells were then incubated for a further hour at room temperature in humidiﬁed 95% air, 5% CO2. Plasmids were transfected into HEK 293T cells using FuGENE HD according to the manufacturer’s instructions using a 1:3 w:v ratio of DNA : FuGENE and cultured for 48 hours prior to assay.

**Measurement of Intracellular cAMP Accumulation**

HEK 293 cells expressing WT or mutant GPCR were assayed for cAMP accumulation as previously described (33, 37). cAMP accumulation was measured after 30 minutes stimulation using LANCE® cAMP Detection Kit (Perkin Elmer Life Sciences) on a
Mithras LB 940 multimode microplate reader (Berthold Technologies). Data were normalized to the maximal level of cAMP accumulation from cells in response to 10 μM Forskolin (Sigma) stimulation.

**Measurement of Intracellular Calcium Mobilization**

Intracellular calcium mobilisation was measured in transfected HEK 293 cells as previously described using Fluo-4/AM and a Mithras LB 940 multimode microplate reader (33). Data were normalized to the maximal intracellular calcium release in response to 10 μM ionomycin.

**Measurement of Phospho-ERK1/2 (Thr202/Tyr204)**

HEK 293 cells expressing WT or mutant GPCR were serum starved overnight prior to assaying. Cells were then washed and resuspended in Ca^{2+} free HBSS and seeded at a density of 35000 cells per well in 384-well white Optiplates (Perkin Elmer). Cells were stimulated with ligand for 5 min, before lysis using the supplied lysis buffer and assayed for ERK1/2 phosphorylation using the phospho-ERK (Thr202/Tyr204) Cellular Assay Kit (Cisbio). Plates were read using a Mithras LB 940 multimode microplate reader and data normalized to the maximal response to 100 μM phorbol 12-myristate 13-acetate (PMA, Sigma) (34).

**Molecular Dynamics**

PDB structures for the inactive and active conformations of CLR complexed with RAMP1 (PDB codes 7KNT and 6E3Y, respectively) and the GCGR (PDB codes 5EE7 and 6WHC, respectively) were obtained from the PDB. Missing atoms were identified from the PDB header and are available in the original PDB file. They were replaced using MODELLER (38) prior to refinement and scoring using Rosetta (39). To reduce errors introduced by loop modelling, ICL2 was excluded from the MODELLER step. Furthermore, no attempt was made to refine existing regions of secondary structure as are as per the original crystallographic file.

The best scoring inactive and active conformations of each receptor type were used for an essential dynamics simulation (40). Each protein was embedded in an equilibrated solvated membrane consisting of 280 POPC lipids. NaCl was added at a concentration of 150 mM, with extra Cl ions added to the solvent to neutralize the system. Protonation states of charged residues were determined using Propka (41) prior to the simulation start. Initial equilibration simulations (100 ns) were performed using Gromacs (42) at 310 K for both the active and inactive receptor states for each receptor family. The equilibrated structures were then used for an essential dynamics simulation. During the essential dynamics simulations, each inactive conformation had a fixed potential applied to the first eight non rotational/translations eigenvectors that increased in fixed increments per step to drive the system from the inactive to the active state. Simulations were performed with fixed increments of 1.2 × 10^{-6} nm per each simulation step (2 fs). Each simulation was performed 10 times. The amber-ILDN forcefield was used in all simulations. The simulations were combined into an inactive to active trajectory using the best scoring snapshot, using the Rosetta scoring function at each timestep. The snapshots were not chosen, but are equally spaced conformations that describe the trajectory from inactive to active state. The RMSD between snapshots was approximately 0.01 Angstroms.

**Generation of Weblogo’s**

GPCR sequences were obtained for 289 human GPCRs in Class A and Class B1 from GPCRdb (https://gpcrdb.org) (43). Sequences were aligned for their ICL1 and H8 using GPCRDB outputs. Weblogo’s were then created using free software from https://weblogo.berkeley.edu using the frequency method where height of each letter represents the frequency of each amino acid.

**RESULTS**

**Sequence Conservation Within ICL1 and H8**

There is a high level of conservation in both ICL1 and H8 in all GPCRs (Figures 1A, B) and especially Class B1 GPCRs (Figures 1C, D). In Class B1 GPCRs only, ICL1 contains an R12.48bKLRCxR2.46b motif that extends into TM2 (using the Wootten Class B1 numbering, 46); the C and R in bold are absolutely conserved. In H8 there is an N7.61bG motif that extends into TM1 and TM2 (using the Wootten Class B1 numbering, 46); the C and R in bold are absolutely conserved. In H8 there is an N7.61bG motif that extends into TM1 and TM2 (using the Wootten Class B1 numbering, 46); the C and R in bold are absolutely conserved.

**Mutational Analysis of the ICL1-H8 Regions of CLR**

To understand the function of ICL1 and H8, we first performed an alanine scan centred on the R12.48bKLRCxR and E8.49bXxxX motifs in CLR. The ICL1-H8 motifs (underlined) are represented in the sequences Y165FSLSCQR173/2.46b and Y165FSLSCQR173/2.46b.
Almost all mutants were expressed at the cell surface at a third or greater than that of WT (Tables S1, S2 – except I394A), a level which we have previously established that causes little change in agonist potency (47). It should however be noted that we had not checked for effects on efficacy/Emax. L169A expression was reduced by 72% and there were also sizeable decreases in expression for E390A, I394A, I397A and L398A and R399A; the expression of L395A and K167A were reduced by 50%.

When cAMP production was measured, mutation of F166, L169, C171 and Q172 (Table S3A and Figure 2) all reduced CGRP potency or Emax while S170A slightly increased it. For H8 and the base of TM7 there were significant reductions in pEC50 for N388A, E390A (also decreasing Emax), V391A and W399A (Table S3A).

To provide a comparison of how the ICL1 mutants might influence downstream signaling activity, we renormalised the cAMP accumulations data shown in Figure 2 to now account for the maximal level of cAMP accumulation achievable for HEK 293T cells (stimulations with the non-selective adenylyl cyclase activator forskolin), (Figure 3A and Table S3B). Beyond cAMP accumulation, CLR can also couple to Gaq/11 to increase intracellular calcium (33, 48). When this response is measured, the ICL1 mutants show a somewhat different pattern of activity; whilst L169A and C171A again reduce potency, with S168A there was a decrease in apparent affinity calculated from the operational model (Figure 3B and Table S4). No mutants enhanced coupling. Alanine mutation of the ICL1 appeared to show little overall effect upon CGRP-induced ERK1/2 activation [which was largely independent of Protein Kinase A activation, (34)], with only K167A showing any significant changes; an increase in potency but decrease in Emax (Figure 3C and Table S5).

While the changes in potency for cAMP and Ca2+ mobilisation appear to suggest changes in signaling bias profiles, to formally confirm this, and to remove potential confounding issues of system bias, we refitted the data in Figure 3 using the operational model of receptor agonism (44). Since the ERK1/2 activation seemed to tolerate mutation at any positions in ICL1, all data was normalised to this parameter (Figure 3D and Tables S3B, S4, S5). This analysis reconfirmed that mutations of residues L169 to Q172 all resulted in a signaling response that was biased away from Ca2+ mobilisation and towards cAMP. Moreover, mutations of C171 and Q172 also displayed bias away from cAMP accumulation to a lesser extent than for mobilisation of Ca2+.

We next performed the same array of assays as described for the ICL1 mutants on the CLR H8 mutants. Normalisation of the cAMP accumulation data showed that N388A, E390A, V391A, L395A and W399A all displayed reduced potency (Figure 4A and Table S3B). For Ca2+ mobilisation, only E390A showed a dramatic reduction in potency (Figure 4B and Table S4). Unlike the ICL1 mutants, mutations at every position within the H8 motif changed either potency (with increases for E390A, V391A, and Q392A) or Emax when ERK1/2 activation was quantified (Figure 4C and Table S5). Analysis using operational parameters confirmed these observations (Figure 4D and Tables S3B, S4, S5). I394A displayed reduced cell surface expression and failed to generate responses for Ca2+ mobilisation or ERK1/2 activation so was excluded from any analysis although it did show a WT cAMP response, for reasons that are not obvious. Thus, the effects of mutation on ICL1 and

FIGURE 1 | Conservation of the [K/R][K/R]xx[T/R] and EFxxxL motifs. Alignment of the amino acids from 298 human GPCRs (both Class A and B1) for (A) ICL1 and (B) Helix 8 depicted as a weblogo’s. Position marked using the Ballesteros-Weinstein numbering systems combined. Alignment of the amino acids from human Class B1 GPCR alone for (C) ICL1 and (D) Helix 8 depicted as a weblogo’s (45). Position marked using the Wootten Class B1 numbering, (46). The height of each letter represents the frequency of each amino acid. Amino acids are colour coded to their chemical properties: polar amino acids (G,S,T,Y,C) are green, neutral (Q,N) are magenta, basic (R,K,H) are blue, acidic (D,E) are red, and hydrophobic (A,V,L,I,P,W,F,M) are black. Figures generated using https://weblogo.berkeley.edu..
H8 differ depending on which coupling pathway is examined and ERK1/2 activation is much more sensitive to mutations in H8 than cAMP or Ca2+ elevation. These differences are highlighted in the bias plots for ICL1 and H8.

After preliminary experiments using saturation mutagenesis of ICL1 on cAMP production, the effect of glutamate, arginine, histidine, glycine and isoleucine were explored in detail, to examine the effect of charge and size at each position. This showed that substitutions at every position can alter receptor activation and/or receptor expression. Several interesting features appear from this extended data. Substitution by R within the motif is well tolerated apart from at L169; S168R increases CGRP potency by almost 100-fold and there was a small increase in Emax with Q172R. Substitution by H was best tolerated at C171 and replacement by E gives particularly large decreases in activity at every position apart from F165. Replacement of L169 by any residue apart from I reduces expression by at least 2/3 compared to WT. Glycine substitution, generally deleterious, increases potency at L169 (albeit with a reduced Emax) and C171.

Extension of Alanine Scan of ICL1 to the GCGR

In the GCGR, the sequence of ICL1 is C165/1.66L12.47b SKLHCTR and the corresponding H8 sequence is NE406/8.49b VQSEL (underlined residues are the motifs, as shown for CLR in section 3.1 and 3.2). This is a more typical H8 sequence as 8.53b is E in 12/15 human class B GPCRs. To probe the differences/conservation of regions of activity, we focussed upon mutations of the ICL1 region of the GCGR. C171A failed to express and so was without activity in all signaling assays; K168A, L169A and H170A showed a 50% reduction in expression. For the other mutants, cell-surface expression was not changed (Figure 6A). Alanine mutation of L16912.50 and T17212.52 reduced the extent of cAMP production; the other mutants that expressed were without effect on this pathway (Figure 6B). By contrast, both elevation of Ca2+ mobilisation and (in contrast to the CLR) ERK1/2 signaling were far more sensitive to alanine substitution throughout ICL1 (Figures 6B–E and Table S6).

Immediately outside ICL1, R1732.46bA reduced the potency of glucagon, almost 100-fold, on cAMP accumulation (pEC50 WT, 9.09 ± 0.1; pEC50 R173A, 7.14 ± 0.1, p>0.01, n= 5). A limited characterisation of H8 was carried out, focussing on the two charged residues E4068.49b and E4108.543b. The only effect on cAMP production was a very small increase in basal activity seen with E406A (WT 7.6 ± 1.1% of response to forskolin, E406A, 11.1 ± 1.5%, p>0.05, n=5). With the double mutant E406AE410A, the basal was further increased to 19.6 ± 1.4% (p<0.01), again with no change in potency. However, for E406A, expression was 53.8 ± 5.8% of WT and this was further reduced to 21.5 ± 1.3% with E406AE410A, suggesting an increase in the effectiveness of coupling to Gs given the increase in basal and the maintenance of potency.

ICL1 in the CRF-Type 1 Receptor (CRFR1)

Our data suggested that ICL1 might have a role in determining G protein preference of Class B1 GPCRs. The CRFR1 exists in two
splice forms (1a and 1b) which differ due to the presence of a 29 amino acid insert in the RKLR motif which does not influence cell surface expression (49). Analysis of the signaling profiles suggests that the CRF1a receptor, which lacks the insert, is biased towards Ca\textsuperscript{2+} signaling, which is consistent with the motif being important for G protein specificity (Figure 7 and Table S7).

**Molecular Dynamics Simulation of ICL1 During Activation of the CGRP Receptor**

A comparison between the inactive and active CGRP and GluR structures (the latter in complex with both Gs and Gi) indicated that ICL1 only underwent subtle changes during the process of receptor activation. To further understand why mutations throughout this region can change activation, a molecular dynamic simulation was used to study the inactive-active transition in the CGRP receptor (Figure 8 and Videos S1A, B). Early during the simulation, the proximal region of ICL1 underwent a flexing movement centred on S168\textsuperscript{12.49}. This subtly changes the orientation of ICL1 with respect to both ICL2 and H8. This was followed by movements of ICL2. Around the middle of the simulation, N388\textsuperscript{7.61}b and E390\textsuperscript{8.49} in ICL4 move towards L169\textsuperscript{12.50}. There is then a bending of the distal part of TM1 but ICL1 with H8 and ICL2 move largely as a rigid body as a consequence of this. At the very end of the simulation there are further movements of N388 and E390, coupled eventually to a change in the side-chain orientation of R173. At this stage, the G protein binding pocket is now fully open. The distance between L169 in ICL1 and E390 in H8 reflects these changes (Figure 8B). Broadly similar movements were seen during a molecular dynamics simulation of the glucagon receptor, with both early and late movements in ICL1 (Video S2).

**DISCUSSION**

The role of ICL1 in mediating signal transduction is poorly explored. In this paper, we confirm evidence (32) evidence for a
structural motif in the loop which is an important player in determining the specificity of G protein-coupling. It is possible to interpret some of the data using the current cryo-EM and crystal structures with further insight coming from molecular dynamics simulations. We have also used mutagenesis to explore the role of individual residues. Mutagenesis data needs to be interpreted with care as the mutants themselves can change the structure of a protein; however, it can be useful to explore interactions predicted from structural and modelling studies.

At first sight, the claim that ICL1 is important in modulating G protein signaling may seem surprising. The loop is usually short and could be considered simply as a linker between TMs 1 and 2. It makes no direct contact with the α subunits of G proteins. Furthermore, its conformation in G protein associated GPCRs is very similar to that of the apo-receptors. However, it has several interfaces in Class B1 GPCRs, as seen from structures (3–5, 8, 9, 50–55) (Figure 9). The distal end of the loop faces towards TM3/4 and ICL2. It can indirectly influence ICL3 via changing the position of H8. In the inactive receptor, it can contact a string of bound water molecules that fit between TMs 2 and 7, at least in the high resolution GCGR structure, 5EE7 (2.5Å). The H8-TM7 junction (ICL4) makes important contacts with the G protein when it binds to the receptor. ICL1 contacts the Gβ subunit of the bound G protein (Figure 9E) and R²-helix faces the Gα subunit (Figures 9B, F). The contacts between ICL2 and, especially, ICL4 are of considerable potential significance as it is likely that these are key determinants of G protein selectivity (2, 56). ICL4 (between TM7 and H4) directly contacts the H5 helix of Gα subunits, which enters the cavity that opens on the cytosolic surface of GPCRs on agonist activation. ICL2 interacts with “rim” residues that flank H5 when it associates with GPCRs. The subtle changes observed in ICL4, depending on the nature of the interacting G protein, are powerful drivers of G protein association and specificity. In our molecular dynamics simulation of CLR, a very early event was a subtle movement of the proximal end of ICL1 followed by rearrangements of the TM2/ICL2 interface and then ICL4. We suggest that these underlie the actions of ICL1 in G protein-coupling. Some caution is needed in extrapolating between the molecular dynamics simulation as structural data suggests large scale movements of the cytoplasmic face of CLR require a G protein to stabilise them (14); however, the simulations may have value in reflecting the conformational changes that are required for the G protein interaction.
It is possible to suggest functions for some individual amino acids within ICL1. The majority of crystal structures show that the side chain of L169 (residue 169 in both CGRP and GCGR) in the middle of the loop points towards H8 (Figure 9). Thus L169 seems to be required to stabilise the H8 interaction, consistent with our observations that almost any mutation here reduces receptor expression. Residue 12.49 is usually basic and its sidechain points beneath the receptor; where a G protein is present it interacts with D312 of Gβ (Figures 9B, E). In the CGRP receptor, 12.49 is serine (perhaps connected to D312 and H8 by water molecules) and, interestingly, its mutation to arginine increases CGRP potency. This may work by

![Figure 5](image-url)
promoting G protein interaction. K12.48 of CLR also H-bonds to D52 and T50 of Gb. In all class B1 GPCR structures, the absolutely conserved C2.44b faces towards H8 and R2.46b, where its side chain is close to E8.49b, perhaps helping to stabilise the ionic interaction between the arginine and glutamic acid. The polarizability of the sulphur atom in cysteine may be significant here and explain the absolute conservation of the residue. There are also receptor-specific contacts for this residue. For the CGRP and active CRF1 receptors, its sidechain is orientated to G8s, although still close to R2.46b. In the inactive GCCR (5EE7) and GLP-1R (5VEW), it also H-bonds to a water molecule in the cleft between H8 and ICL1. Alanine mutation of C2.44b in the PTH1 receptor results in loss of receptor expression, further suggestive of a role in all class B1 GPCRs. In the GCGR where the 2.45b is the shorter threonine a bridging water molecule allows a hydrogen bond between the backbone amides of 2.45b, 2.46b and the phenolic hydroxyl of Y248.35b (Figure 9H). In the CRF1R, L150.245b mediates hydrophobic interactions.

For all class B1 GPCRs, there are a complex set of interactions between the absolutely conserved R2.46b at the ICL1/TM2 junction and E8.49b and N7.61b at the H8/TM7 junction in the inactive receptors, probably involving water molecules (52) (Figures 9B, F). In the active receptors, these contacts undergo rearrangement to include Q390 and E392 at the C-terminus of H5 of G8s (Figure 8). This pattern of interaction is broadly similar in the PTH1, GLP-1, CGRP and calcitonin receptors (6NIY) (8, 53, 54), although the structures show differences in the orientation of the side chains.

Whilst it is possible explain some of the mutagenesis data in this study, it is hard to convincingly rationalise every result. Perhaps the most economical explanation is to suggest that mutations anywhere along ICL1 have the potential to change its conformation and so indirectly alter its interactions with ICL2 and ICL4. For this to be plausible, ICL1 must have a degree of flexibility. The molecular dynamics simulation suggests this is possible. However, the GPCR structures also speak to this issue. Perhaps not surprisingly, in the crystal structures ICL1 has a high B-factor, consistent with mobility. However, for the GCGR, two distinct conformations are seen, differing in their orientation of L169.12.50 (Figures 9D, E). For the CRFR1 structures, in the inactive 4Z9G, H8 cannot be resolved and the sidechain of L12.50 also points between TM1 and 2; it is in a
single turn of a helix at the proximal end of ICL1. However, in the active structures (6PB0, 6P9X), where H8 is seen, the helix is lost and L12.50 now faces H8. Thus, ICL1 is not rigid and mutations could have unpredictable effects on its structure. Hydrogen-deuterium exchange has demonstrated that even in the absence of CGRP, ICL1 of CLR/RAMP1, with the other intracellular loops, has a high rate exchange, consistent with flexibility (14).

It is worth considering how H8 is adapted as a partner to ICL1. E8.49b of H8 faces centrally into ICL1 (Figures 9A–E); its presence may partially explain why the introduction of glutamate anywhere in ICL1 is deleterious as this is likely to result in ionic repulsion. The small increase in basal activity seen in the GCGR when it is substituted by alanine may be explained by the presence of an interaction between it and R346 at the base of TM6 seen in the inactive receptor (5EE7), which helps constrain the base of the receptor in a closed form. E8.49b is probably important in maintaining the orientation of H8 as it can interact directly or indirectly to Q/N7.61b in both active and inactive receptors (Figures 9A, B, D, F), hence explaining why alanine mutagenesis reduces receptor expression. Apart from the junction with TM7, the rest of H8 appears to be of little importance for G protein coupling but not for ERK1/2 activation. This presumably reflects the role that H8 plays in β-arrestin recruitment and subsequent ERK1/2 phosphorylation (58–61).

In the calcitonin and CRF1 receptors, splice variants exist in ICL1 (31). In this study, we show that the insertion changes receptor bias of the CRF1 receptor. For the calcitonin receptor there is reduced coupling to both cAMP and Ca2+ signaling, although it is not clear if there is any change in bias. It
is not possible to provide any detailed mechanistic explanation with excessive speculation; however, it is significant that endogenous variants of ICL1 show differences in bias.

The equivalent of the RKLK motif can be recognized in class A GPCRs, where the ICL1 residues form a loosely conserved [R/H]14-16K[R/H] motif; the H8 motif is E8.49-Fxxx(F Figure 1) (32). ICL1 has been identified as being important for constraining class B1 A and C GPCRs in inactive states (13), perhaps hinting at a wider role the motif may play. ICL1 is in close proximity to the binding sites for a number of allosteric antagonists that bind to the intracellular face of GPCRs (50, 62, 63) and also the probable site of action of pepducins based on ICL1 sequences (64, 65). Given the number of interactions it can make, it may be a useful area for drug targeting.

In conclusion, ICL1 can influence the architecture of the G protein-binding pocket to favour either Gs or Gq/11 coupling.
making this an important regulator of G protein specificity. Residues at the distal end of the loop are particularly important and we suggest that they work largely by influencing the interplay between ICL4 and H5 of the G protein Gα subunit.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusion of this article will be made available by the authors, on reasonable request.

**AUTHOR CONTRIBUTIONS**

IW, KB, SR, NJR, MH, and JS performed the experiments and modelling. CAR, AJ, DRP, and GL conceived the idea and analysed data. DRP, GL, and CAR wrote the manuscript.

**REFERENCES**

1. Katritch V, Cherezov V, Stevens RC. Structure-Function of the G Protein-Coupled Receptor Superfamily. *Annu Rev Pharmacol Toxicol* (2013) 53:531–56. doi: 10.1146/annurev-pharmtox-032112-135923

2. García-Nafria J, Tate CG. Cryo-EM Structures of GPCRs Coupled to Gα, Gβ, and Gδ. *Mol Cell Endocrinol* (2019) 488:1–13. doi: 10.1016/j.mce.2019.02.006

3. Ehrenmann J, Schoppe J, Klenk C, Rappas M, Kummer L, Dore AS, et al. High-Resolution Crystal Structure of Parathyroid Hormone 1 Receptor in Complex With a Peptide Agonist. *Nat Struct Mol Biol* (2018) 25:1086–92. doi: 10.1038/s41594-018-0151-1

4. Hollenstein K, Kean J, Bortolato A, Cheng RK, Dore AS, Jazayeri A, et al. Structure of Class B GPCR Corticotropin-Releasing Factor 1 Receptor. *Nature* (2013) 499:438–43. doi: 10.1038/nature12357

5. Jazayeri A, Rappas M, Brown AJH, Kean J, Erey JC, Robertson NJ, et al. Crystal Structure of the GLP-1 Receptor Bound to a Peptide Agonist. *Nature* (2017) 546:254–8. doi: 10.1038/nature22800

6. Karageorgos V, Venihaki M, Sakellaris S, Pardalos M, Kontakis G, Matsoukas MT, et al. Current Understanding of the Structure and Function of Family B GPCRs to Design Novel Drugs. *Hormones (Athens)* (2017) 18:45–59. doi: 10.1007/s42000-018-0009-5

7. Liang YL, Khoshouei M, Radjainia M, Zhang Y, Glukhova A, Tarrasch J, et al. Phase-Plate Cryo-EM Structure of a Class B GPCR-G-Protein Complex. *Nature* (2017) 546:118–23. doi: 10.1038/nature22327

8. Liang YL, Khoshouei M, Glukhova A, Furness SGB, Zhao P, Clydesdale L, et al. Phase-Plate Cryo-EM Structure of a Biased Agonist-Bound Human GLP-1 Receptor-Gs Complex. *Nature* (2018) 555:121–5. doi: 10.1038/nature25773

9. Liang YL, Khoshouei M, Deganutti G, Glukhova A, Koole C, Peat TS, et al. Cryo-EM Structure of the Active, Gs-Protein Complexed, Human CGRP Receptor. *Nature* (2018) 561:492–7. doi: 10.1038/s41586-018-0553-y

10. Wootten D, Christopoulos A, Marti-Solano M, Babu MM, Sexton PM. Mechanisms of Signalling and Bias Agonism in G Protein-Coupled Receptors. *Nat Rev Mol Cell Biol* (2018) 19:638–53. doi: 10.1038/s41580-018-0049-3

11. Thal DM, Vuckovic Z, Draper-Joyce CJ, Liang YL, Glukhova A, Christopoulos A, et al. Recent Advances in the Determination of G Protein-Coupled Receptor Structures. *Curr Opin Struct Biol* (2018) 51:28–34. doi: 10.1016/j.sbi.2018.03.002

12. Gurevich VV, Gurevich EV. The Structural Basis of the Arrestin Binding to GPCRs. *Mol Cell Endocrinol* (2019) 484:34–41. doi: 10.1016/j.mce.2019.01.019

13. Hauser AS, Kooistra AJ, Munk C, Heydenreich FM, Veprinatsev DB, Bouvier M, et al. GPCR Activation Mechanisms Across Classes and Macro/Microscale. *Nat Struct Mol Biol* (2021) 28:879–88. doi: 10.1038/s41594-021-00674-7

14. Josephs TM, Belousoff MJ, Liang YL, Piper SJ, Cao J, Garama DJ, et al. Structure and Dynamics of the CGRP Receptor in Apo and Peptide-Bound Forms. *Science* (2021) 372(6538):eaab7258. doi: 10.1126/science.abc2758

15. Wang X, Cheng X, Zhao L, Wang Y, Ye C, Zou X, et al. Molecular Insights Into Differentiated Ligand Recognition of the Human Parathyroid Hormone Receptor 2. *Proc Natl Acad Sci U S A* (2021) 118:e2101279118. doi: 10.1073/pnas.2101279118

16. Bentrup J, Schwab K, Pak WL, Paulsen R. Site-Directed Mutagenesis of Highly Conserved Amino Acids in the First Cytoplasmic Loop of Drosophila Rh1 Opsin Blocks Rhodopsin Synthesis in the Nascent State. *EMBO J* (1997) 16:1600–9. doi: 10.1093/emboj/16.7.1600

17. Wess J. Molecular Basis of Receptor/G-Protein-Coupling Selectivity. *Pharmacol Ther* (1998) 80:231–64. doi: 10.1016/S0163-7258(98)00030-8

18. Amatruda TT3rd, Dragas-Graonic S, Holmes R, Perez HD. Signal Transduction by the Formyl Peptide Receptor. Studies Using Chimeric Receptors and Site-Directed Mutagenesis Define a Novel Domain for Interaction With G-Proteins. *J Biol Chem* (1995) 270:28010–3. doi: 10.1074/jbc.270.47.28010

19. Hirata T, Kakiuzuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S, Arg60 to Leu Mutation of the Human Tromboxane A2 Receptor in a Dominantly Inherited Bleeding Disorder. *J Clin Invest* (1994) 94:1662–7. doi: 10.1172/JCI8070

20. Kleinuz G, Jaeschke H, Worth CL, Mueller S, Gonzalez J, Paschke R, et al. Principles and Determinants of G-Protein Coupling by the Rhodopsin-Like Thromtinopin Receptor. *PloS One* (2010) 5:e9745. doi: 10.1371/journal.pone.0009745

21. Liu B, Wu D. The First Inner Loop of Endothelin Receptor Type B is Necessary for Specific Coupling to Galphal 13. *J Biol Chem* (2003) 278:2384–7. doi: 10.1074/jbc.M208683200

22. Mathi SK, Chan Y, Li X, Wheeler MB. Scanning of the Glucagon-Like Peptide-1 Receptor Localizes G Protein-Activating Determinants Primarily to the N Terminus of the Third Intracellular Loop. *Mol Endocrinol* (1997) 11:424–32. doi: 10.1210/mend.11.4.9913

23. Wu V, Yang M, McRoberts JA, Ren J, Seensalu R, Zeng N, et al. First Intracellular Loop of the Human Cholecystokinin-A Receptor is Essential for Cyclic AMP Signaling in Transfected HEK-293 Cells. *J Biol Chem* (1997) 272:9037–42. doi: 10.1074/jbc.272.14.9037

24. Yu J, Polgar P, Lubinsky D, Gupta M, Wang L, Mierke D, et al. Coulombic and Hydrophobic Interactions in the First Intracellular Loop Are Vital for Bradykinin B2 Receptor Ligand Binding and Consequent Signal Transduction. *Biochemistry* (2005) 44:3295–306. doi: 10.1021/bi048288i

25. Zhang B, Yang X, Tiberi M. Functional Importance of Two Conserved Residues in Intracellular Loop 1 and Transmembrane Region 2 of Family A...
Conflict of Interest: AJ and NJR were employees of Sosei Heptares at the time the work was performed. Neither are now employed by Sosei Heptares.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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