The Second Intracellular Loop of the Calcitonin Gene-related Peptide Receptor Provides Molecular Determinants for Signal Transduction and Cell Surface Expression*

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The calcitonin gene-related peptide (CGRP) receptor is a heterodimer of a family B G-protein-coupled receptor, calcitonin receptor-like receptor (CLR), and the accessory protein receptor activity modifying protein 1 (RAMP1). It couples to Gs, but it is not known which intracellular loops mediate this. We have identified the boundaries of this loop based on the relative position and length of the juxtamembrane transmembrane regions 3 and 4. The loop has been analyzed by systematic mutagenesis of all residues to alanine, measuring cAMP accumulation, CGRP affinity, and receptor expression. Unlike rhodopsin, ICL2 of the CGRP receptor plays a part in the conformational switch after agonist interaction. His-216 and Lys-227 are essential for a functional CGRP-induced cAMP response. The effect of (H216A)CLR is due to a disruption to the cell surface transport or surface stability of the mutant receptor. In contrast, (K227A)CLR had wild-type expression and agonist affinity, suggesting a direct disruption to the downstream signal transduction mechanism of the CGRP receptor. Modeling suggests the loop undergoes a significant shift in position during receptor activation, exposing a potential G-protein binding pocket. Lys-227 changes position to point into the pocket, potentially allowing it to interact with bound G-proteins. His-216 occupies a position similar to that of Tyr-136 in bovine rhodopsin, part of the DRY motif of the latter receptor. This is the first comprehensive analysis of an entire intracellular loop within the calcitonin family of G-protein-coupled receptors. These data help to define the structural and functional characteristics of the CGRP-receptor and of family B G-protein-coupled receptors in general.

Several structural and functional motifs are well characterized within the intracellular domains of the largest known family (A) of GPCRs. These include the conserved (D/E)R(Y/H) motif on the boundary between transmembrane helix 3 and the second intracellular loop (ICL2) and which is well documented to have a significant role in the activation mechanism (2) and the NPXYY motif of TM7, which can have diverse roles in the constitutive phosphorylation, internalization, and signaling of many family A GPCRs (3, 4). In addition, residues within ICL2 juxtaposed to transmembrane helix 3 have been shown to be involved in receptor stability (5).

In contrast, much less is known about the important amino acids of family B receptors, which include the calcitonin family of receptors and which tend to bind larger peptide agonists. There are no obvious common motifs between family A and family B receptors; nevertheless, a shared mechanism involving a ligand-induced conformational distortion between the distal regions of TM3 and 6 has been proposed for the parathyroid hormone receptor (6) and the CGRP/adrenomedullin receptors (7). There are contrary reports arguing for the involvement of the second intracellular loop in receptor expression and activation within the family B GPCRs. A recent study on the VPAC1 receptor found that all residues of ICL2 could be mutated without altering receptor expression or adenyl cyclase response to vasoactive intestinal peptide (8). Conversely, a lysine residue in ICL2 of the parathyroid hormone (PTH) receptor was shown to be critical in activating the phospholipase C pathway while having no effect on the adenyl cyclase response (9).

The family B receptor CLR is unusual among GPCRs in that it requires the co-expression of one of a family of accessory proteins called RAMPs (receptor activity modifying proteins) for cell trafficking and ligand interaction (10). A functional CGRP receptor is formed by a CLR/RAMP1 heterodimer (11). In addition, an agonist-mediated signaling response requires the co-localization of a third membrane-associated accessory protein called the receptor component protein (12). Receptor component protein is thought to form an ionic interaction with the membrane adjacent to CLR (13). There are no studies exploring the role of intracellular regions of CLR involved in CGRP binding, cAMP signaling, or protein expression; indeed there are very few mutational analyses of the second intracellular loop for any family B GPCRs.

This report describes the systematic substitution of all residues of ICL2 of human CLR. Mutations were constructed either individually or in pairs replacing the side chains with a single methyl group (alanine). The naturally occurring alanine residues at position 221 and 224 were replaced with glycine. These mutants were transfected into mammalian cells in combination with a RAMP1 construct and analyzed using a tritiated cAMP radio receptor assay. The assay compared the CGRP-stimulated cAMP accumulation of mutant with wild-type CLR. Mutants with impaired function were further characterized for cellular...
expression, ligand affinity, and surface localization. We identify His-216 and Lys-227 as key residues for normal receptor function and utilize molecular modeling to reveal the likely spatial positioning of the second intracellular loop in the predicted active and inactive states of the receptor. Our findings are discussed in the context of previously proposed motifs within this region in other family B receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human αCGRP was from Calbiochem. Peptides were dissolved in distilled water and stored as aliquots at −20 °C in non-stick microcentrifuge tubes (Thermo Life Sciences, Basingstoke, UK). Unless otherwise specified, chemicals were from Sigma or Fisher. Cell culture reagents were from Invitrogen or Sigma. [125I]iodohistidyl-αCGRP (2000 Ci/mmol) was from Amersham Biosciences.

**Expression Constructs and Mutagenesis**—Human CLR with an N-terminal hemagglutinin (HA) epitope tag (YPYDVPDYA) was provided by Dr. S. M. Foord (GlaxoSmithKline) and was subcloned into pcDNA3 (−) (Invitrogen) before mutagenesis. Introduction of the mutations was confirmed by sequencing (Functional Genomics, Birmingham, UK).

**Cell Culture and Transfection**—Cells in 12-well plates were transiently transfected with wild type (WT) or mutant HA epitope-tagged human CLR and RAMP 1. The transfected cells were treated with 3.7% formaldehyde for 15 min after aspiration of growth medium. The cells were then washed 3 times with 0.5 ml of Tris-buffered saline (TBS). Nonspecific binding of the antibody was blocked with 1% BSA in TBS for 45 min. The cells were treated with 250 μl of primary antibody (mouse, anti-HA antibody 12CA5 (Sigma) diluted 1:1000 in TBS with 1% BSA) for 1 h, and the cells were washed again 3 times with 0.5 ml of TBS. A further block step was performed for 15 min before the cells were incubated with 250 μl of secondary antibody (anti-mouse, horseradish peroxidase-conjugated (Sigma) diluted 1:1000 in TBS) for 1 h. The cells were washed a further three times before development with 0.05 M sodium citrate and 0.05% H2O2. The absorbance measured by the ELISA showed a linear dependence on the DNA concentration used in the transfection.

**Immunohistochemistry**—Cos-7 cells were seeded in 6-well plates containing nitric acid-washed glass coverslips (12 mm) and transfected using polyethyleneimine as described above. Cells were fixed and washed with phosphate-buffered saline (PBS) as described previously for the ELISA. Cells were blocked with 1% BSA in PBS for 45 min followed by incubation with an anti-HA primary antibody (diluted to 1:3000 in 3% (w/v) for 60 min. Cells were washed 3 times with PBS before reblocking with 1% BSA in PBS for 15 min at room temperature. Cells were labeled with a staining mixture including goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Sigma) (diluted to 1:500 in 10% (v/v) goat serum in PBS), Alexa 546 phalloidin (Molecular Probes, Leiden, The Netherlands), and Hoescht 33342 (Sigma) for 60 min at room temperature in the dark. After a further three washes, coverslips were mounted on glass slides before confocal microscopy.

**Confocal Microscopy**—Confocal microscopy was performed using a Nikon Optiphot II laser scanning microscope at 60× magnification under immersion oil. The HA-tagged receptors were visualized by exciting the secondary antibody, actin filaments were visualized by exciting the Alexa 546 phalloidin, and nuclei were visualized by exciting the Hoescht 33342. The appropriate wavelength was used according to manufacturer’s instructions in each case. Images were captured at 10 random sites for each slide from three separate experiments.

**TM Prediction**—Individual TM helix prediction of 58 diverse Family A GPCR sequences was performed using the web-based versions of TMHMM2 (17) and HMMTOP2 (18). A consensus prediction for the boundaries of TMIII and TMIV was generated by visual inspection, and from this initial survey, a ClustalW (19) profile alignment (using the Blosum matrix) was created against 58 peptide hormone family A GPCRs. The resulting alignment was used to generate an initial homology based model using the high resolution X-ray crystal structure of bovine rhodopsin as a template. Further refinement of the homology model was achieved through molecular dynamics (MD) simulations of the receptor embedded in a dipalmitoylphosphatidylcholine bilayer. A series (n = 5) of 5-ns MD simulations were carried out using the GROMOS96 force field parameter set, with minor modifications, as implemented in GROMACS (20). The resulting trajectories were conurate-

**Assay of cAMP Production**—Growth medium was removed from the cells and replaced with Dulbecco’s modified Eagle’s medium containing 500 μM isobutylmethylxanthine for 30 min. αCGRP in the range 10 pm to 1 μM was added for a further 15 min. Ice-cold ethanol (95–100% v/v) was used to extract cAMP, which was subsequently measured by radio-receptor assay as previously described (16).
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TABLE 1
Conservation of residues within ICL2 of CLR
ICL2 boundaries are assumed from the model presented in this study. The table compares CLR with 57 diverse GPCR examples from family B taken from www.expasy.ch.

|   | CLR % Identity | CLR % Identity | CLR % Identity |
|---|---------------|---------------|---------------|
| Tyr-214 | 100 | Val-221 | 15 |
| Leu-215 | 100 | Ala-222 | 27 |
| His-216 | 56 | Val-223 | 21 |
| Thr-217 | 47 | Phe-224 | 42 |
| Leu-218 | 71 | Ala-225 | 29 |
| Ile-219 | 48 | Glu-226 | 50 |
| Val-220 | 36 | Lys-227 | 28 |

![Amino acid sequence of ICL2 from human CL showing the positions of the mutated residues.](image)

FIGURE 1. Amino acid sequence of ICL2 from human CL showing the positions of the mutated residues. Residues in black showed reductions in the CGRP-stimulated adenylylate cyclase activation in this study. Residue numbering is from the start of the predicted mature transcript of CLR (15).

RESULTS

Identification of the Boundaries of ICL2—According to the Swiss-Prot data base entry Q16602 (www.ExPasy.org), ICL2 of the CLR would be predicted to encompass residues from (and including) Leu-215 to Met-230. An alignment of this region of 57 family B GPCRs in Table 1 shows the most conserved amino acids. The TM/loop boundaries were more precisely defined by using a consensus of previously validated TM prediction methods and remote sequence alignment to a known structure. Visual inspection of the TM prediction results revealed that, although the boundaries for TM4 were well defined, those for TM3 exhibited variability. Essentially, the predicted boundaries for TM3 were clustered into two populations. The first population, which included the majority of sequences studied (40 of 58), exhibited a TM3 boundary at the equivalent position to residues 214 and 243 (including the signal peptide) of the CLR. Significantly, this would position the conserved cysteine residue located at the extracellular end of TM3 in the same positions as that observed in family A receptors. The boundaries for TM3 of the second, smaller population was between the equivalent residues to 227 and 247 (CLR). We have, therefore, used the boundaries exhibited by the first, larger population for subsequent loop modeling studies. As previously mentioned, the predicted boundaries for TM4 were well defined and included residues 250—270 (CLR). Furthermore, sequence alignment of the predicted TM4 segment against the residues corresponding to the high resolution x-ray crystal structure of rhodopsin positioned a conserved tryptophan residue found in both family A and B GPCRs in the same position.

Effect of ICL2 Substitution Mutants on cAMP Accumulation—To identify the contribution to CGRP-stimulated signaling provided by residues within this ICL2 and adjoining segments, residues between Tyr-214 and Tyr-233 inclusive (see Fig. 1) were mutated to alanine individually or in pairs to generate the constructs (Y214A/L215A)CLR, (H216A)CLR, (T217A)CLR, (I218A/I219A)CLR, (V220A/V221A)CLR, (A222G)CLR, (Y223A/F224A)CLR, (A225G)CLR, (E226A)CLR, (K227A)CLR, (Q228A/H229A)CLR, (L230A/M231A)CLR, (W232A)CLR, and (Y233A)CLR. Each mutant receptor construct was co-expressed with a RAP1 vector in Cos-7 cells, and the CGRP-stimulated cAMP accumulation was compared with wild-type CLR. In each
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FIGURE 2. CGRP-stimulated cAMP response of the ICL2 mutants. Cos-7 cells were transfected with WT/RAMP1 or mutant/RAMP1 and assayed for CGRP-stimulated cAMP production. Open circles, WT type receptors. Mutant receptors are as indicated. Data are representative of three to four similar experiments. Points are the mean ± S.E. of triplicate points. a, mutations with wild-type cAMP signaling; b, (V223A/F224A)CLR; c, (W232A)CLR; d, (H216A)CLR; e, (K227A)CLR.

TABLE 2

Functional parameters of WT/RAMP1 and mutant receptors

| Mutant                   | pEC50<sub>WT</sub> | pEC50<sub>pEC50</sub> |
|--------------------------|--------------------|------------------------|
| Y214A/L215A/GLR         | 9.02 ± 0.21 (3)    | 8.91 ± 0.36 (3)        |
| H216A/GLR               | 9.41 ± 0.13 (3)    | 8.37 ± 0.37 (3)<sup>a</sup>|
| T217A/GLR               | 9.36 ± 0.21 (3)    | 9.33 ± 0.35 (3)        |
| L218A/I219A/GLR         | 9.66 ± 1.01 (3)    | 9.37 ± 1.04 (3)        |
| V220A/V221A/GLR         | 9.75 ± 0.36 (3)    | 9.64 ± 0.11 (3)        |
| A222G/GLR               | 9.54 ± 0.12 (3)    | 9.54 ± 0.34 (3)        |
| A225G/GLR               | 9.62 ± 0.05 (3)    | 9.02 ± 0.13 (3)<sup>b</sup>|
| Q228A/H229A/GLR         | 9.63 ± 0.21 (3)    | 9.34 ± 0.19 (3)        |
| E226A/GLR               | 10.07 ± 0.09 (3)   | 9.87 ± 0.20 (3)        |
| (K227A)CLR              | 9.97 ± 0.37 (3)    | 8.91 ± 0.23 (3)<sup>c</sup>|
| (Q228A/H229A)CLR        | 9.60 ± 0.75 (3)    | 9.55 ± 0.52 (3)        |
| (L230A/M231A)CLR        | 9.44 ± 0.21 (3)    | 9.65 ± 0.39 (3)        |
| (W232A)CLR              | 9.27 ± 0.10 (3)    | 8.85 ± 0.32 (3)<sup>d</sup>|
| (Y233A)CLR              | 8.84 ± 0.06 (3)    | 8.50 ± 0.10 (3)        |

<sup>a</sup> Significantly different from WT, p < 0.01, as assessed by paired Student’s t test.
<sup>b</sup> Significantly different from WT, p < 0.05, as assessed by paired Student’s t test.

As shown in Fig. 2a and Table 2, the pEC50 for cAMP production in response to CGRP was not significantly different for the seven alanine substitution mutants (Y214A/L215A)CLR, (T217A)CLR, (L218A/I219A)CLR, (V220A/V221A)CLR, (Q228A/H229A)CLR, (L230A/M231A)CLR, and (Y233A)CLR or the two glycine substitution mutants (A222G)CLR and (A225G)CLR when compared with WT CLR co-transfected with RAMP1. Additionally, none of the point mutations in this study had basal levels of stimulation in excess of that seen for the wild-type receptor (data not shown). This suggests none of these substitution mutations forms a constitutively active receptor.

In contrast to the mutants described above, two constructs including a double alanine substitution of the valine and phenylalanine residues at positions 223 and 224 (V223A/F224A)CLR as well as a single mutation of tryptophan to alanine at position 232 (W232A)CLR, both resulted in a small loss in the ability of CLR to elevate cAMP in response to CGRP (Table 2 and Fig. 2, b and c). The disruption in signaling resulted in a significant reduction in the pEC50 value of 4- and 2.6-fold for mutants (V223A/F224A)CLR and (W232A)CLR, respectively. However, when the His-216 or Lys-227 were mutated to alanine, the perturbation of stability. To investigate this possibility, we constructed and analyzed a double mutant, (E226A/K227A)CLR. The signaling profile of this mutant was not significantly different to the effects seen by the (K227A)CLR mutant alone (pEC50 values for wild-type and (E226A/K227A)CLR were 8.79 ± 0.08 and 8.28 ± 0.09, respectively, n = 3). These data suggest a direct role for the basic lysine side chain in the activation mechanism of the receptor.

ELISA Analysis of Cell-surface Expression—The mutants with disrupted cAMP signaling were subsequently analyzed for their comparative ability to traffic to the cell surface using a whole-cell enzyme-linked immunosorbent assay to detect the surface expression of the engineered
HA-tag epitope. The ELISA data are shown graphically in Fig. 3a, and maximum absorbance values ($AB_{\text{max}}$) are summarized with their S.E. in Table 3. The alanine substitutions to the hydrophobic residues Val-223/Phe-224 and Trp-232 approximately halved cell-surface expression to 54.2/61.8% of wild type, respectively. (H216A)CLR was barely detected at the cell surface, with an expression level just 10.2/3.2% of wild type. (K227A)CLR shows cell-surface expression levels comparable with wild type (90.9/9.16%).

To validate that the ELISA analysis presents a valuable measure of cell surface expression, we investigated the cell surface localization of HA-tagged CLR using immunofluorescence confocal microscopy (Fig. 3b). As expected, no signal was visible in cells transfected with untagged RAMP1 alone (Fig. 3b, i). When HA-CLR was co-expressed with RAMP1, fluorescence was seen both inside the cell and on the plasma membrane (Fig. 3b, ii). In cells transfected with HA-CLR alone, little fluorescence was seen on the plasma membrane, although there was intense intracellular staining (Fig. 3b, iii). Although the microscopy experiments are a qualitative measure and cannot be a sound basis for estimates of total cellular expression, they confirmed the cell surface expression of these receptors, thus demonstrating the ELISA represents genuine surface expression.

**RADIOLIGAND ANALYSIS OF TOTAL CELL RECEPTOR EXPRESSION ($B_{\text{max}}$)**—The total cellular expression of transfected receptors was compared with wild type using radioligand binding analyses. Total expression ($B_{\text{max}}$) values were estimated from $^{125}$I-labeled CGRP radioligand binding. $AB_{\text{max}}$, relative cell surface expression of receptors as measured by detection of HA tags in an ELISA. Data are normalized to WT as 100%.

| Mutant        | $B_{\text{max}}$ | $AB_{\text{max}}$ | $K_d$ |
|---------------|-----------------|-----------------|--------|
|               | pmol/mg         | %              | nM     |
| WT            | 1.60 ± 0.48 (12)| 100 (3)        | 8.71 ± 0.07 (9) |
| H216A         | 1.45 ± 0.50 (3) | 10.2 ± 3.2 (3) | 8.47 ± 0.09 (3) |
| V223A/F224A   | 2.91 ± 0.96 (3) | 54.2 ± 6.2 (3) | 8.60 ± 0.19 (3) |
| K227A         | 1.62 ± 0.16 (3) | 90.9 ± 10.8 (3) | 9.16 ± 0.43 (3) |
| W232A         | 1.02 ± 0.36 (3) | 61.8 ± 3.8 (3) | 8.62 ± 0.16 (3) |

*Significantly different from WT; $p < 0.05$ compared to WT using one-way analysis of variance followed by Dunnett’s test.

**TABLE 3**

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FIGURE 3. a, ELISA for H216A, V223A/F224A, K227A, and W232A. Surface expression of the four mutants with effects on adenylate cyclase activation is shown. **, significantly different from control; $p < 0.01$, 1-way analysis of variance followed by Dunnett’s test. b, confocal microscopy. Cos-7 cells were transiently transfected with HA-tagged CLR and RAMP1. Cells were fixed, and immunohistochemistry was processed as shown under “Experimental Procedures.” Images are representative from three separate experiments. i, cell transfected with RAMP1 alone; ii, cell transfected with HA-CLR and RAMP1; iii, cell transfected with HA-CLR alone.
CLR, and (H216A)CLR was reflective of a reduced localization or surface stability and not reduced protein expression or gross misfolding of the receptor.

**CGRP Affinity-measured Using Radioligand Displacement Studies**—In contrast to the reduced signaling efficacy of the above four mutants, CGRP-affinity measured using $^{125}$I-labeled CGRP radioligand binding experiments was unaffected. Binding curves are shown in Fig. 4 with $pK_d$ values presented in Table 3. This supports the suggestion that the effects seen by (H216A)CLR, (V223A/F224A)CLR, and [W232]CLR are not indirect effects on agonist binding. Interestingly, the wild-type affinity shown by (K227A)CLR suggests an effect on the activation mechanism of the CGRP receptor that is structurally downstream of conformational switch from ground state ($R$) to the high affinity activated state ($R^*$). The affinity of CGRP to the ground state of CLR/RAMP1 heterodimer has previously been shown to be reduced (7).

Loop Modeling—To further understand the effects of (K227A)CLR and (H216A)CLR, we set about to construct models of ICL2 in the probable inactive and active conformations of CLR. To assess the effectiveness of a loop modeling protocol, it must first be able to predict the conformation of loops from a protein whose structure has been solved to high resolution. Rhodopsin is the only GPCR whose structure is known in atomic detail, and as such, we have used the protocol of de Bakker et al. (29) to predict the conformation of ICL2.

The ICL2 of bovine rhodopsin is defined by the sequence $^{140}$CKPM-SNFRFG$^{149}$, where C$^{140}$ and G$^{149}$ represent the intracellular ends of TM3 and TM4, respectively. Fig. 5, a and b, show the backbone conformation of the native structure, and superimposed on this is the highest-scored predicted conformation of ICL2 that was selected from an initial ensemble of 1000 random conformations. It is clear that the use of the AMBER/GBSA energy function resulted with the selection of a conformation with a fold that was very close to the native loop. Structural deviation between the native and predicted conformation of ICL2 was measured, which resulted with a heavy atom root mean-squared deviation of less than 1.0 Å.

The Predicted Structure of ICL2 of CLR—It is reasonable to assume that the conformation of the framework on which the loops are predicted would have an impact on the final loop orientation. With this in mind we have performed the same method as discussed above for bovine rhodopsin, on ICL2 from the CLR receptor under two different starting conditions to explore the effects of conformational change on the orientation of the loop. The conditions for the loop predictions were 1) homology modeling and MD refinement based on the ground state x-ray crystal structure coordinates of bovine rhodopsin and 2) molecular dynamics.
lar modeling, which utilized a refined TM helical bundle based experimentally derived distance constraints, which are consistent with an active conformation of bovine rhodopsin.

Comparison of the loop predictions for each of the starting conditions revealed distinct loop conformations for the inactive and a proposed active state of the receptor (Fig. 5c). It is clear that in the predicted inactive conformation, ICL2, acts to occlude the intracellular portion of the receptor. In contrast, the proposed active state revealed a conformational change that would allow access of G-proteins with the intracellular portion of the receptor. This is in contrast to the x-ray crystal structure of bovine rhodopsin where ICL2 does not act as a steric constraint in the inactive state of the receptor. However, several molecular dynamic studies (30) have demonstrated an increased root mean-squared fluctuation of the intracellular loops compared with the remainder of the protein, which is consistent with the B-factors found in the structural Protein Data Bank file. This suggests that ICL2 is dynamic and may adopt other conformations that are not observed in the rhodopsin x-ray crystal structures. In the model, the residues that alter cAMP production all undergo distinct conformational changes. The side chain of Lys-227 moves so that it is oriented toward the putative G-protein binding pocket in the active state of the receptor (Fig. 5c). Val-233 and Phe-234 undergo significant translations, but the relative orientations of their side chains relative to each in the active and inactive other remain constant; no other pair of side chains in ICL2 shows this property (Fig. 5d). His-216 shows virtually no change in position; it is located between a cavity between TMs 3, 4, and 5 and is lipid-facing (Fig. 5e). However, it is in a very similar position to Tyr-136 of bovine rhodopsin, part of the DRY motif of that receptor.

DISCUSSION

Defining the precise molecular roles of residues within the intracellular loops of G-protein-coupled receptors is of fundamental importance. These loops are the key to dissecting many aspects of pharmacological specificity, and individual residues within ICL2 have been shown to distinguish between different α-subunits of the G-protein complex (9). There are few functional studies published for the analysis of the transmembrane helix 3 proximal region of ICL2 for the family B receptors, although recent observations have suggested an involvement and possible movement of the bottom of TMs 3 and 6 in the activation of glucagon-like peptide 1 and CL receptors (7, 31), in line with current models of activation for the family A. Unlike family A, there is no crystal structure of a family B GPCR to use for modeling studies. This is a particular problem in the region of transmembrane helix 3 for CLR, where two different proposals for the loop-helix boundary have been published (10, 32). Our re-evaluation of the likely TM-loop boundary suggests that the prediction of Chang et al. (3) is closer to the likely structure. Having established the likely loop-helix boundaries for ICL2, we have been able to model this region. The data suggest that movement of ICL2 is likely to play a significant role in CLR activation by exposing a binding pocket for G-proteins. This prediction is consistent with our findings from the mutagenesis which demonstrate that residues in the loop are implicated in activation of adenylyl cyclase.

Alanine-scanning mutagenesis of the entire second intracellular loop of CLR highlighted four residues or sets of residues that are important for signaling via cAMP. (V223A/F224A)CLR and (W232A)CLR showed small effects on surface expression and signaling. A previous study on the prostaglandin receptors suggested that a cluster of hydrophobic aromatic amino acids in ICL2 played a role in Gαi coupling (33), and it is possible that a similar factor is at work here. (K227A)CLR had a direct negative effect on cAMP signaling without disrupting surface expression. The wild-type agonist affinities of all these mutants imply that, although the residues are important for G-protein interaction (either directly or indirectly), they are not important in stabilizing the active conformation of the receptor around the CGRP binding state. The native uncoupled, ground state of the receptor has a significantly lower affinity for CGRP than the active form (7). The role for Lys-227 may involve downstream interaction with the Gαs, although it may also be a candidate for receptor component protein interaction, which is thought to involve an ionic combination centered around the second intracellular loop of CLR (13). An effect specific for CL is consistent with the observation that interactions between the second intracellular loop and the Gαs subunit have been specifically dismissed for other members of both family B (9) and family A GPCRs (34). A lysine in a similar position in the family B parathyroid hormone receptor was shown to be required for signal transduction (albeit Gαt and not Gαs-coupled) (9). The modeling suggests a mechanism for the effects of Lys-227, with the side chain pointed into a newly created cavity. It would now be able to interact with any G-protein that occupied the pocket. Interestingly, visual inspection of the active and inactive conformations of ICL2 reveals that Lys-227 is the only residue to be orientated closer to the G-protein binding pocket in the active state than in the inactive state (Fig. 5c). Furthermore, the side-chain length and charged state of Lys may make it able to form direct interactions at longer distances, as compared with other charged residues located in the ICL2. The modeling also sheds light on the roles of Val-233 and Phe-234. The unique preservation of the orientation of their side chains suggest that these residues may help maintain the loop integrity in this region of the receptor.

A surprising result was the identification of His-216 as important for cell-surface expression. The receptor can bind CGRP normally in cell homogenates, suggesting that the receptor folds correctly and associates with RAMPs but fails to be translocated to the cell surface. It may be part of a transport motif, or alternatively, it may act to mask an endoplasmic reticulum retention signal. The extreme C terminus of RAMP1 acts as an endoplasmic reticulum retention signal that must be overridden after association with CLR (35); His-216 may be involved in this process. The corresponding tyrosine to Tyr-214 in the glucagon-like peptide 1 receptor has been proposed to be the DRY-equivalent tyrosine residue in family B GPCRs (7, 31). Conversely, our data show that Tyr-214 has no functionally important role in the CGRP receptor, and therefore, this is not part of a family B motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family. It is not out of the question that His-216, with a role in surface expression, may be the third member of a DRY-like motif conserved across the entire family.
receptor. It will be interesting to discover if ICL2 plays similar roles in other family B GPCRs.

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