Structure–function analyses reveal a triple β-turn receptor-bound conformation of adrenomedullin 2/intermedin and enable peptide antagonist design

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The cardioprotective vasodilator peptide adrenomedullin 2/intermedin (AM2/IMD) and the related adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) signal through three heterodimeric receptors comprising the calcitonin receptor–like class B G protein–coupled receptor (CLR) and a variable receptor activity–modifying protein (RAMP1, -2, or -3) that determines ligand selectivity. The CGRP receptor (RAMP1:CLR) favors CGRP binding, whereas the AM1 (RAMP2:CLR) and AM2 (RAMP3:CLR) receptors favor AM binding. How AM2/IMD binds the receptors and how RAMPs modulate its binding is unknown. Here, we show that AM2/IMD binds the three purified RAMP–CLR extracellular domain (ECD) complexes with a selectivity profile that is distinct from those of CGRP and AM. AM2/IMD bound all three ECD complexes but preferred the CGRP and AM2 receptor complexes. A 2.05 Å resolution crystal structure of an AM2/IMD antagonist fragment–bound RAMP1–CLR ECD complex revealed that AM2/IMD binds the complex through a unique triple β-turn conformation that was confirmed by peptide and receptor mutagenesis. Comparisons of the receptor-bound conformations of AM2/IMD, AM, and a high-affinity CGRP analog revealed differences that may have implications for biased signaling. Guided by the structure, enhanced-affinity AM2/IMD antagonist variants were developed, including one that discriminates the AM1 and AM2 receptors with ~40-fold difference in affinities and one stabilized by an intramolecular disulfide bond. These results reveal differences in how the three peptides engage the receptors, inform development of AM2/IMD-based pharmacological tools and therapeutics, and provide insights into RAMP modulation of receptor pharmacology.

Adrenomedullin 2/intermedin (AM2/IMD)2 is the newest member of the calcitonin peptide family, which also includes calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) (1, 2). It is a vasodilator like CGRP and AM, but it also has other effects in the central nervous system and cardiovascular, renal, respiratory, and endocrine systems (3–5). AM2/IMD is widely expressed, and its actions include hypothalamic-pituitary-adrenal axis activation (6); protective effects in hypertension, heart attack, heart failure, and ischemia reperfusion injury (3, 7–12); and promotion of adipocyte browning and reduction of insulin resistance and obesity (13, 14). An AM2/IMD knockout mouse revealed a role for the peptide in vascular lumen enlargement during both normal and pathological angiogenesis by promoting endothelial cell proliferation (15). In humans, plasma AM2/IMD is elevated in heart failure (16), and a gene polymorphism is associated with hypertension, kidney dysfunction, and cerebrovascular lesions (17). The diverse spectrum of AM2/IMD functions in multiple organ systems has generated considerable interest in exploiting AM2/IMD signaling to develop novel therapeutics for diabetes, obesity, and cardiovascular, cardiopulmonary, and renal diseases (3, 5).

Actions of AM2/IMD, AM, and CGRP are mediated by three heterodimeric cell surface receptors comprising a common class B G protein–coupled receptor (GPCR) subunit, the calcitonin receptor–like receptor (CLR), in association with a variable receptor activity–modifying protein subunit (RAMP1, -2, or -3) that determines peptide preference (18, 19). CGRP has highest affinity for the RAMP1:CLR complex, which is designated the CGRP receptor, whereas AM has the highest affinity for the RAMP2:CLR and RAMP3:CLR complexes, which are designated the AM1 and AM2 receptors, respectively. AM2/IMD was initially suggested to be a nonselective agonist (1), but it has since been shown to have a slight preference for the AM2 receptor (20, 21). This complex system in which three peptide ligands share a common receptor that is modulated by accessory membrane proteins is a model for accessory protein regulation of GPCR pharmacology. Notably, RAMPs also interact

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This article contains Tables S1–S4 and Figs. S1–S3.

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2The abbreviations used are: AM2/IMD, adrenomedullin 2/intermedin; CLR, calcitonin receptor–like receptor; RAMP, receptor activity–modifying protein; AM, adrenomedullin; CGRP, calcitonin gene-related peptide; ECD, extracellular domain; 7TM domain, 7-transmembrane domain; GPCR, G protein–coupled receptor; FP, fluorescence polarization/anisotropy; MBP, maltose-binding protein; ANOVA, analysis of variance; DMEM, Dulbecco’s modified Eagle’s medium; PEI, polyethyleneimine.
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with several other GPCRs (18), and a recent bioinformatics analysis suggested that RAMPs interact with more GPCRs than is currently appreciated (22).

Understanding how the three peptides bind the receptors is especially important considering the phenomenon of signaling bias in which different ligands stabilize distinct receptor conformations to yield different downstream signaling outcomes (23). RAMP:CLR complexes couple to G\textsubscript{q}, G\textsubscript{i}, G\textsubscript{s}, and β-arrestin. Although G\textsubscript{q}-mediated cAMP accumulation is commonly used as readout in structure–function studies of these receptors, activation of intracellular calcium mobilization and ERK1/2 also occurs, and these pathways and others have been implicated in the physiological actions of the peptides (3, 24). AM2/IMD, AM, and CGRP exhibit signaling bias at RAMP:CLR complexes with respect to cAMP and calcium signaling (25). Bias with respect to other pathways remains poorly studied. Understanding the structural basis of biased signaling may facilitate the design of drugs with desired bias profiles. Significant progress was recently made toward a structural understanding of signaling bias at the class B GLP-1 receptor (26–28), but we lack insight into bias mechanisms at the CGRP and AM receptors.

Calcitonin family peptides are ~40 amino acids in length, and they have an N-terminal disulfide bond ring structure important for receptor activation followed by a central α-helical region and an unstructured C-terminal region that ends with an amide modification (29). Receptor binding follows the class B GPCR two-domain model in which the C-terminal half of the peptide binds the GPCR extracellular domain (ECD), whereas the N-terminal half contacts the 7-transmembrane (7TM) domain and activates the receptor (30). N-terminally truncated peptides lacking the disulfide-bonded region are competitive antagonists (31). CGRP(8–37) and AM(22–52) have traditionally been used to distinguish the CGRP and AM\textsubscript{1,2} receptors, but we lack antagonists for discriminating the AM\textsubscript{1} and AM\textsubscript{2} receptors. Studies using the traditional antagonists provided evidence that AM2/IMD signaling in different cell types is mediated by the CGRP and/or the AM receptors depending on the effect (3). Additional tools for discriminating the receptors are needed to improve our ability to identify the receptor(s) responsible for a given peptide action.

Soluble fusion proteins in which the RAMP1 or RAMP2 ECD was tethered to the CLR ECD by a linker recapitulated the CGRP and AM binding preferences of the full-length CGRP and AM\textsubscript{1} receptors (32). Crystal structures of these constructs in complex with C-terminal antagonist fragments of a high-affinity CGRP analog and AM revealed that the two peptides bind a common site on CLR with different conformations and have minimal contact with the RAMPs (33). The structures and peptide mutagenesis studies supported a dual mechanism for RAMP function in which allosteric modulation of CLR and distinct RAMP contacts with the peptide C-terminal residue cooperate to determine CGRP and AM selectivity (34). Unfortunately, our understanding of how AM2/IMD binds the receptors and how RAMPs modulate its binding remains limited.

Here, we produced recombinant tethered ECD complexes for each of the three receptors and used them to show that AM2/IMD has a unique selectivity profile, preferring the CGRP and AM\textsubscript{2} ECD complexes. We determined a high-resolution crystal structure of an antagonist fragment of AM2/IMD bound to the CGRP receptor ECD complex, which revealed a triple β-turn receptor-bound conformation different from those of CGRP and AM. Guided by the structure, we designed novel AM2/IMD antagonists. Our results reveal differences in the receptor binding modes of the three endogenous ligands of the CGRP and AM receptors that have implications for biased signaling, inform development of AM2/IMD-based pharmacological tools and therapeutics, and provide mechanistic insights into RAMP accessory protein modulation of GPCR pharmacology.

Results

Expression and purification of soluble tethered RAMP–CLR ECD complexes of the human CGRP, AM\textsubscript{1}, and AM\textsubscript{2} receptors in their native N-glycosylated states

AM2/IMD has a unique selectivity profile for activation of cAMP signaling at the three RAMP:CLR complexes as compared with CGRP and AM (Fig. 1A), but the basis for this is unclear. We sought to characterize AM2/IMD binding affinity for soluble heterodimeric ECD complexes of the CGRP and AM receptors and compare it with AM and CGRP. In our previous studies examining CGRP and AM binding to tethered RAMP1–CLR and RAMP2–CLR ECD proteins, the fusion proteins were expressed in *Escherichia coli* and folded with a novel disulfide shuffling method (32–35). Maltose-binding protein (MBP) was included in the constructs to facilitate crystallization (33). Attempts to generate a properly folded MBP-RAMP3–CLR ECD fusion protein using this method failed (data not shown). As RAMP3 contains four N-linked glycosylation sites (36) as compared with one in RAMP2 and none in RAMP1 (Fig. 1A), we reasoned that the RAMP3 ECD might require N-glycans to fold. We therefore expressed N-glycosylated MBP-RAMP3–CLR ECD as a secreted protein from HEK293T cells using methods we previously used for the calcitonin receptor ECD (37–39). To assemble a complete set of the three tethered ECD complexes with each in their native glycosylation state (Fig. 1B), we similarly expressed the MBP-RAMP1–CLR ECD and MBP-RAMP2–CLR ECD proteins using HEK293T cells. Each of the fusion proteins was purified by immobilized metal-affinity and gel-filtration chromatography, which yielded highly purified proteins (Fig. S1, A and B). Each of the fusion proteins behaved as a monomer on gel filtration chromatography (Fig. S1, C–E).

AM2/IMD binds tethered ECD complexes of the CGRP, AM\textsubscript{1}, and AM\textsubscript{2} receptors with a unique selectivity profile

We previously developed a fluorescence polarization/anisotropy (FP) assay to assess peptide binding to tethered RAMP1- and RAMP2–CLR ECD constructs using an FITC-labeled high-affinity AM analogue AM(37–52) S45W/Q50W as probe (34). This assay was used to characterize peptide binding at the purified N-glycosylated MBP-RAMP–CLR ECD proteins. In saturation binding experiments, all three constructs exhibited nanomolar affinity for the probe with equilibrium dissociation constants (pK\textsubscript{d}) determined as 7.23 ± 0.02 (K\textsubscript{d} ~ 59 nM), 7.41 ±
0.04 $\left( K_D \sim \text{39 nM} \right)$, and 8.19 ± 0.04 $\left( K_D \sim \text{6.5 nM} \right)$ for the RAMP1, RAMP2, and RAMP3 constructs, respectively (Fig. 2, A–C). We then used a competition assay format to determine the equilibrium dissociation constants ($K_I$) for unlabeled AM2/IMD(16–47), AM(22–52), and CGRP(8–37) (Fig. 2, D–F) and Table 1). These traditional antagonist fragments include the C-terminal ECD-binding region and the central $\alpha$-helical region that contacts the CLR 7TM domain, but they lack the N-terminal disulfide-bonded portion required for 7TM domain activation (Fig. 1C). CGRP(8–37) bound the RAMP1 construct with an affinity ($K_I \sim \text{10 M}$) 4-fold stronger than the RAMP3 construct ($K_I \sim \text{40 M}$), and no binding was detected at the RAMP2 construct. AM(22–52) bound the RAMP2 and RAMP3 constructs equally well, with $K_I$ values ($\sim \text{5–6 M}$) 18-fold stronger than at the RAMP1 construct ($K_I \sim \text{100 M}$). AM2/IMD(16–47) had similar affinities for the RAMP1 and RAMP3 constructs ($K_I$ values $\sim \text{2–3 M}$) that were 5–8-fold stronger than at the RAMP2 construct ($K_I \sim \text{14 M}$).

AM2/IMD, AM, and CGRP vary considerably in their C-terminal half (Fig. 1C), so we determined the minimal ECD-binding region of AM2/IMD. AM2/IMD(26–47), AM2/IMD(29–40)
Table 1  Summary of FP competition binding of C-terminal peptide fragments to MBP-RAMP–CLR ECD fusion proteins

| Peptide            | R1 Selectivity R2 versus R1 | R2 Selectivity R1 versus R2 |
|--------------------|----------------------------|----------------------------|
| AM2/IMD(16–47)     | 5.58 (0.04)                | 0.76 (0.03)               |
| AM2/IMD(32–47)     | 5.50 (0.02)                | 0.77 (0.03)               |
| AM2/IMD(32–47) Y47F| 5.49 (0.04)                | 0.77 (0.03)               |

- Tukey’s multiple-comparison test. Significance is shown for each mutant as compared with the respective WT peptide of the same length (AM2/IMD(32–47) or AM2/IMD(16–47)).
- Statistical comparison of binding of WT AM2/IMD(16–47) and AM(22–52) to the R2–CLR ECD was done with an unpaired t test.
- Statistical comparison of binding of single peptides to the RAMP1–, RAMP2–, and RAMP3–CLR ECDs was done using one-way ANOVA with Tukey’s multiple-comparison test.
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We next sought to determine a crystal structure of AM2/IMD bound to one of the ECD complexes to reveal its ECD-binding mode. We used MBP-RAMP1–CLR ECD for crystallization because it strongly bound AM2/IMD and we could cost-effectively produce it in E. coli. We purified the bacterially produced protein to homogeneity as described previously (32, 33) (Fig. S1, A and B). AM2/IMD(16–47) bound the N-glycan-free MBP-RAMP1–CLR ECD with an affinity (K$_d$ ~5 μM) similar to that of the N-glycosylated version (Fig. S2A). Crystals of MBP-RAMP1–CLR ECD in complex with AM2/IMD(29–47) were obtained that diffracted to 2.05 Å resolution (Fig. S2B and C and Table S3). The structure was solved by molecular replacement and refined to good R factors with excellent geometry (Table S3). Three peptide–receptor complexes were present in the asymmetric unit. The Mol 2 complex exhibited the best peptide electron density and lowest peptide B factors. Clear, unambiguous electron density for AM2/IMD residues 32–47 was visible in the peptide-binding site (Fig. S2D). As there were no substantial differences in peptide binding between the three complexes, hereafter we describe the Mol 2 complex (chains B and E).

AM2/IMD primarily binds the CLR subunit (Fig. 3A). Its C-terminal Tyr-amide occupies the pocket over the CLR Trp-72 Trp shelf that is augmented by RAMP1 Trp-84 (chains B and C). The amide group hydrogen-bonds with the backbone of CLR Thr-122 at the base of the pocket. The AM2/IMD Tyr-47 phenyl ring contacts CLR Trp-72, the β1-β2 loop, and RAMP1 Trp-84. Tyr-47 is the only peptide residue that contacts the RAMP subunit (Fig. 3C). AM2/IMD adopts a striking conformation with a series of kinks resulting from residues Pro-38, Pro-41, and Pro-44 in a triple β-turn sequence in which the last residue of each turn is the first residue in the next turn (Fig. 3, A and B). Turn 1, 2, and 3 comprise residues 37–40 (APVD), 40–43 (DPSS), and 43–46 (SPHS), respectively (Fig. 3, D–F). Turn 3 contacts the CLR turret loop and helps position the C-terminal Tyr-amide in the pocket. Ser-46 of turn 3 hydrogen bonds with CLR Trp-121, and Pro-41 of turn 2 and Ala-37 of turn 1 contact the CLR hydrophobic patch (Fig. 3, B and C). Before the triple β-turn, the peptide–receptor contacts primarily involve hydrogen bonds between the AM2/IMD backbone and CLR (Fig. 3C). AM2/IMD–receptor contacts are summarized in Table S4.

AM2/IMD turn 1 is a type VIII β-turn, and turns 2 and 3 are type I β-turns (40). In addition, three-residue Asx- and ST-turns (41) are present within turns 2 and 3, respectively. The Asx-turn within turn 2 involves a hydrogen bond between the Asp-40 side chain and the backbone amide nitrogen of Ser-42 (Fig. 3E). The Asp-40 side chain is also within hydrogen-bond-
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Figure 3. Crystal structure of AM2/IMD antagonist fragment bound to MBP-RAMP1–CLR ECD. A, orthogonal views of the AM2/IMD-bound RAMP1–CLR ECD complex shown in a cartoon representation with selected side chains as sticks. The dotted line indicates the disordered tether connecting the ECDs. MBP is omitted for clarity. B, RAMP1–CLR ECD shown as a surface representation with AM2/IMD shown as a cartoon backbone with side-chain sticks. C, AM2/IMD shown as sticks and the RAMP1–CLR ECD shown as a cartoon with select residues shown as sticks highlighting peptide–receptor contacts. Dotted lines, intermolecular hydrogen bonds. D–F, close-up views of the three AM2/IMD β-turns with intramolecular hydrogen bonds shown as dotted lines. G–I, single-point FP competition binding assay with AM2/IMD alanine scan mutants at each of the three purified N-glycosylated MBP-RAMP–CLR ECD proteins. G–I are representative of two independent experiments. Error bars, S.D. for the duplicate technical replicates within the representative experiment.

Peptide and receptor mutagenesis validates the AM2/IMD antagonist conformation and suggests that AM2/IMD agonist binds the intact receptor in the same manner

To estimate the relative contribution of each AM2/IMD side chain to ECD binding, we performed an alanine scan of residues 35–47 in the context of the 32–47 fragment. Ala-37 was substituted with Gly. Binding of the mutant peptides to the three N-glycosylated MBP-RAMP–CLR ECD fusion proteins was assessed in a single point FP competition assay (Fig. 3, G–I). In agreement with the structure, reduction of binding affinity began with A37G, and all subsequent side chains other than Ser-42 contributed to binding at each of the three complexes. Tyr-47 was the most important side chain, consistent with its role in occupying the CLR pocket (Fig. 3, B and C). Ser-43 was also quite important, presumably reflecting its role in stabilizing H9252-turn 3 (Fig. 3F).

To ensure that our structural findings apply to the AM2/IMD agonist binding the intact CGRP receptor in cells, we co-expressed full-length RAMP1 and CLR with single alanine substitutions within the CLR or RAMP ECDs in COS-7 cells and determined the effects of the substitutions on the ability of AM2/IMD(1–47) agonist to activate cAMP signaling. Nine mutations in the CLR ECD and one in the RAMP1 ECD were selected based on the proximity of their side chains to AM2/IMD in the structure. The mutant CLR receptors were previously shown to express at cell surface levels not significantly different from WT (33). The RAMP1 W84A mutation moderately reduced cell surface expression, but not enough to substantially alter signaling response (42). Representative concentration–response curves for each mutant are presented in Fig. 4 (A and B), and the data are summarized in Table 2 and
Effects of alanine substitutions in RAMP1:CLR on AM2/IMD activation of cAMP signaling

| Receptor mutant | WT pEC_{50} ± S.E. (n) | Mutant pEC_{50} ± S.E. (n) | -Fold change | Mutant E_{max} (% of WT) ± S.E. (n) |
|----------------|--------------------------|-----------------------------|--------------|-------------------------------------|
| CLR T37A       | 8.43 ± 0.10 (3)          | 6.91 ± 0.06* (3)            | 33           | 98.9 ± 3.08 (3)                     |
| CLR W72A       | 8.33 ± 0.08 (3)          | 6.51 ± 0.23* (3)            | 146          | 121.1 ± 17.1 (3)                    |
| CLR F92A       | 8.50 ± 0.08 (3)          | 6.22 ± 0.14* (3)            | 191          | 55.9 ± 19.2 (3)                     |
| CLR D94A       | 8.47 ± 0.03 (3)          | 7.35 ± 0.11* (3)            | 12           | 131.9 ± 8.52 (3)                    |
| CLR F95A       | 8.36 ± 0.05 (3)          | 6.21 ± 0.16* (3)            | 143          | 108.1 ± 15.2 (3)                    |
| CLR H114A      | 8.27 ± 0.09 (3)          | 6.89 ± 0.07* (3)            | 24           | 98.2 ± 21.2 (3)                     |
| CLR R119A      | 8.52 ± 0.14 (5)          | 6.61 ± 0.19* (5)            | 83           | 109.9 ± 22.25 (3)                   |
| CLR W121A      | 8.29 ± 0.10 (3)          | <5.3 (3)                    | >1,000       | Undeterminable                      |
| CLR Y124A      | 8.41 ± 0.04 (3)          | 6.63 ± 0.09* (3)            | 60           | 37.1 ± 7.49* (3)                    |
| RAMP1 W84A     | 8.38 ± 0.07 (4)          | 7.32 ± 0.15* (3)            | 12           | 81.3 ± 9.52 (3)                     |

a Statistical significance of pEC_{50} values was determined by a paired t test comparing the mutant pEC_{50} with the WT pEC_{50}.

b Statistical significance of E_{max} values was determined by a ratio paired t test comparing the mutant E_{max} with the WT E_{max} before normalization.

The AM2/IMD receptor-bound conformation differs from those of AM and a high-affinity CGRP analog

Superimpositions of the AM2/IMD-bound RAMP1–CLR ECD structure with our previous structures of the high-affinity CGRP analog (CGRPmut)-bound RAMP1–CLR ECD and AM-bound RAMP2–CLR ECD (33) revealed that the three peptides occupy the same site on CLR, but with distinct conformations (Fig. 5, A and B). Shared features include occupancy of the pocket by their amidated C-terminal residue (Tyr or Phe) and a type I β-turn before this residue that contacts the CLR turret loop. The phenyl rings of CGRPmut Phe-37 and AM2/IMD Tyr-47 make similar hydrophobic contact with RAMP1 Trp-84 (Fig. 5A). AM Tyr-52 hydrogen-bonds with RAMP2 Glu-101, and presumably AM2/IMD Tyr-47 would also make this contact when binding the AM1 receptor (Fig. 5B). The peptides diverge before their common β-turn with CGRPmut lacking secondary structure, AM containing a single α-helical turn, and AM2/IMD having two additional β-turns (Fig. 5, A and B). AM2/IMD β-turns 1 and 2 create unique bulges in the path of the backbone as compared with CGRPmut and AM. Before the AM2/IMD β-turn 1, the AM2/IMD and AM backbones follow a similar path along the CLR β3-β4 loop that differs significantly from that of CGRPmut.

It is useful to compare the C-terminal portion (Fig. 5C), the middle region (Fig. 5D), and the N-terminal portion (Fig. 5E) of the antagonist fragments. RAMP-dependent changes in the

Figure 4. Validation of the structure through mutagenesis of the full-length CGRP receptor and cAMP signaling assays in COS-7 cells. A and B, concentration–response curves comparing stimulation of cAMP accumulation by AM2/IMD(1–47) agonist at the indicated CGRP receptors with substitutions in the CLR (A) or RAMP1 (B) subunits. Each panel is representative of three or more independent experiments. Error bars, S.D. for the duplicate technical replicates within the representative experiment. V, vehicle control lacking agonist. C, structure of the AM2/IMD-bound complex colored according to the effect of the substitutions on AM2/IMD agonist cAMP signaling potency.
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**Structure-guided design of AM2/IMD antagonists with enhanced affinities and altered selectivity profiles**

We reasoned that stabilizing the AM2/IMD β-turns or providing it with the ability to make additional receptor contacts should allow the development of high-affinity variants. Modeling predicted that a Trp at AM2/IMD position 45 (H45W) could adopt two possible rotamers in which the indole either intramolecularly contacts Pro-44 to stabilize β-turn 3 or provides additional contact to CLR Ser-117 in the turret loop (Fig. 6A). As Val-39 in β-turn 1 contacts Ser-43 and Ser-46 in β-turn 3, we predicted that mimicking these contacts with a covalent linkage should enhance affinity by enforcing the receptor-bound conformation. A left-handed disulfide bond with reasonable geometry (43) could be modeled connecting positions 39 and 46 (Fig. 6B). We also sought to improve upon the natural preference of AM2/IMD for the RAMP1- and RAMP3–CLR ECD complexes to develop an antagonist that discriminates the AM1 and AM2 receptors. We predicted that substituting the C-terminal Tyr with Phe (Y47F) would reduce affinity for the RAMP2–CLR ECD because of the lost ability to hydrogen-bond with RAMP2 Glu-101 and the inability of RAMP2 Phe-111 to contact the remaining phenyl ring. The Y47F substitution should not substantially alter binding to the RAMP1 and RAMP3 complexes because they each have Trp-84 to contact the phenyl ring (Fig. 6C).

We first assessed the effects of these substitutions on peptide binding to the purified tethered ECD complexes. Selected competition binding curves are shown in Fig. 6 (D–F), and the pK_I values and receptor selectivities are summarized in Table 1 and in scatter plot format in Fig. 6G. In the short 32–47 fragment, H45W enhanced affinity ~20–30-fold at each complex, the V39C/S46C/Y47F disulfide slightly enhanced affinity at the RAMP1 and RAMP3 constructs, and Y47F decreased binding to the RAMP2 construct while having only minor effects on binding to the RAMP1 and RAMP3 constructs. The H45W/Y47F double mutant exhibited equal affinities for the RAMP1 and RAMP3 constructs (K_I ~390 nM) and ~75-fold weaker affinity for the RAMP2 construct (K_I ~30 µM) (Fig. 6G and Table 1). Adding the disulfide bond to make the quadruple V39C/H45W/S46C/Y47F mutant slightly enhanced affinity at the RAMP1 and RAMP3 constructs (K_I ~160 nM) but reduced selectivity, as it bound the RAMP2 construct with only ~20-fold weaker affinity (K_I ~3.2 µM). In the longer 16–47 fragment, the double H45W/Y47F mutations had similar effects, but there was a reduced ability to discriminate the RAMP2 and RAMP3 constructs (~30-fold difference in K_I values) (Fig. 6G and Table 1).

Last, we assessed the abilities of AM2/IMD variants to antagonize cAMP signaling at the full-length CGRP, AM1, and AM2 receptors transiently expressed in COS-7 cells. We derived apparent pK_a values from the dextral shifts in the agonist concentration–response curves caused by a fixed concentration of antagonist. This value estimates the binding affinity of the antagonist for the full-length receptor. The H45W/Y47F double mutant results are shown for the 32–47 (Fig. 7, A–C) and 16–47 fragments (Fig. 7, D–F), and representative curves for the other antagonists are shown in Fig. S3. The pK_a values...
values and receptor selectivities are summarized in Table 3 and in scatter plot format in Fig. 7G. The 32–47-based antagonists bound the intact receptors with pK_B(app) values similar to their pK_I values for tethered ECD binding except that they all bound the AM1 receptor a bit better than MBP-RAMP2–CLR ECD. The 32–47 H45W/Y47F double mutant exhibited equal affinities for the CGRP and AM2 receptors (K_B(app) ~250 nM) and ~40-fold weaker affinity for the AM1 receptor (K_B(app) ~10 μM) (Fig. 7G and Table 3). The disulfide-containing quadruple mutant bound the CGRP and AM2 receptors with affinities similar to those of the double mutant, but it exhibited reduced selectivity due to enhanced binding of the AM1 receptor. The 16–47-based antagonists were more potent because of their additional contacts with the CLR 7TM domain, and like the shorter fragments, they exhibited decreased selectivity at the intact receptors as compared with the ECD complexes. The 16–47 H45W/Y47F double mutant exhibited very strong picomolar potency at the AM2 receptor, but it retained single-digit nanomolar potency at the CGRP and AM1 receptors and was thus only ~15-fold selective for AM2 over AM1.

Discussion

AM2/IMD, AM, CGRP, and their receptor complexes provide a model system for accessory membrane protein modulation of GPCR pharmacology. Defining how each of the three peptides binds the receptors and how RAMPs alter CLR peptide selectivity is thus of fundamental interest for understanding GPCR signaling. With the biochemical and pharmacological data reported here and the new AM2/IMD-bound RAMP1–CLR ECD crystal structure to complement our previous structures of RAMP1–CLR ECD with bound CGRPmut and AM–bound RAMP2–CLR ECD (33), we now have a comprehensive view of how each of the endogenous peptide ligands binds the ECD complexes of the CGRP and AM receptors and a better understanding of how the RAMP subunits determine ligand selectivity.

An important contribution of this work is production of all three recombinant RAMP–CLR ECD complexes in their N-glycosylated forms. To our knowledge, this is the first report of a purified AM1 receptor ECD complex. Previous studies were limited to the CGRP and AM1 receptor ECD complexes or CLR
ECD alone produced in bacteria and therefore lacking N-glycans (32–35, 44–47). The three glycosylated tethered ECD fusions generally recapitulated the peptide selectivity profiles observed for the intact receptors (Table 1 and Fig. 1A), albeit with reduced binding affinities. The latter is expected because the ECD complexes only contact the C-terminal half of the peptides. The ECD complexes thus appear to contain the primary peptide selectivity determinants, although there is evidence that the membrane-embedded domains of the receptors also contribute to selectivity (48, 49). Interestingly, among the three traditional antagonist fragments, AM2/IMD(16–47) was actually the highest-affinity ligand for the CGRP and AM receptors (50–52) warrant future studies on the roles of CGRP and AM receptor N-glycans.

The AM2/IMD-bound crystal structure revealed a striking triple β-turn receptor-bound conformation at the CGRP receptor ECD complex. The three Pro residues, Pro-38, Pro-41, and Pro-44 presumably favor the kinked conformation. The AM2/IMD alanine scan results supported the conformation observed in the crystal structure, and the similar pattern observed for the effects of the mutants at each of the three ECD complexes (Fig. 3, G–I) suggests that AM2/IMD binds each receptor in a similar manner. One discrepancy is the lack of an effect of the S42A substitution despite its apparent intramolecular hydrogen bond with Asp-40 (Fig. 3E). This suggests that the importance of AM2/IMD Asp-40 for receptor binding stems from its role in the Asx-turn within β-turn 2 and/or its intermolecular hydrogen bond with CLR Asn-128. The most important residue for receptor binding was the C-terminal residue Tyr-47, which was also observed for the C-terminal residues of CGRP (Phe-37) and AM (Tyr-52) (32, 47). This is explained by its role in occupying the CLR ECD pocket, which appears to be a
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Table 3

| RAMP3-CLR vs | RAMP2-CLR vs | RAMP1-CLR vs |
|--------------|--------------|--------------|
| Selectivity R1 | Selectivity R2 | Selectivity R3 |
| AM2/IMD peptide | AM2/IMD peptide | AM2/IMD peptide |

AM2/IMD, AM, and CGRPmut share some features in their receptor-bound conformations, but they also have striking differences (Fig. 5). Occupancy of the pocket by the peptide C-terminal Tyr/Phe-amide is important for each, as is the common β-turn before this residue that contacts the CLR turret loop. They also share hydrophobic residues or residues with hydrophobic groups at two positions (AM2/IMD Ala-37/Pro-41, AM Ala-42/Ile-47, CGRPmut Thr-30/Val-32) that contact the CLR hydrophobic patch. Significant differences include the two additional β-turns unique to AM2/IMD, the presence of an α-helical turn in AM, and the difference in the N-terminal portion of the antagonist fragment of CGRP as compared with those of AM2/IMD and AM. How do these features contribute to receptor selectivity? Prior studies suggested that the RAMPs determine ligand selectivity of CLR through both a direct peptide contact component and an allosteric component (33, 34). The AM2/IMD structure and variant design studies provide additional support for this dual mechanism. RAMP1 can contact peptides having either a C-terminal Phe or Tyr via Trp-84 contact with their phenyl ring, RAMP2 can only contact peptides with a C-terminal Tyr via a Glu-101 hydrogen bond with the hydroxyl group, and RAMP3 is a hybrid with Glu-74 to hydrogen-bond with a Tyr hydroxyl and Trp-84 to contact a Phe/Tyr phenyl ring. These contacts explain the decreased AM1 receptor affinity of AM2/IMD variants with Y47F (Figs. 6 and 7) and AM variants with Y52F (34) and the increased AM1 receptor affinity of CGRP variants with F37Y (34). Other subtleties of the selectivity profiles, such as AM preferring the AM1 and AM2 ECD complexes and AM2/IMD preferring the CGRP receptor and AM1 ECD complexes despite both peptides sharing a C-terminal Tyr, likely arise at least in part from different effects of the RAMPs on CLR conformation and how the different peptide conformations accommodate these changes.

The different ECD complex-bound conformations of the peptides may provide a structural basis for biased signaling, at least in part. Although the available structures are antagonist-bound ECD complexes, these are still relevant for understanding how the C-terminal half of the agonists binds the full-length receptors. Indeed, CLR Asp-94 was absolutely critical for receptor antagonist simply by blocking access to the pocket (33, 53).

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IMD shown in Fig. 5E may similarly give rise to signaling bias. The structures may thus help guide future studies of bias mechanisms.

We designed novel AM2/IMD antagonist variants guided by the structure and the thesis that stabilizing the turns should enhance affinity (H45W and V39C/S46C disulfide substitutions) and altering RAMP contacts should alter selectivity (Y47F substitution). For the short ECD-binding 32–47 variants, there was good agreement between their pKi values for tethered ECD complex binding and their apparent pKi values for antagonizing the full-length receptors with the exception that the variants all bound the intact AM receptors better than MBP-RAMP2–CLR ECD. This discrepancy may be due to some aspect of our MBP-RAMP2–CLR ECD fusion protein that does not fully recapitulate the AM ECD complex, or alternatively in the AM receptor the membrane-embedded domains of the receptor may provide additional contact to the 32–47 fragment. The variants were thus less selective in the cell-based assay than at the purified ECD complexes. Nonetheless, the AM2/IMD(32–47) H45W/Y47F variant discriminated the AM1 and AM2 receptors with ~40-fold difference in affinities. To our knowledge, this antagonist distinguishes these receptors better than any available peptide antagonist, albeit with moderate binding affinities. Incorporating these two substitutions into the 16–47 fragment generated a very potent antagonist, but the receptor selectivity was diminished. These results are consistent with the membrane-embedded portions of the receptors affecting selectivity. The V39C/S46C disulfide enhanced affinity only slightly as compared with WT, but because this substitution eliminates the Ser-46 side chain–mediated network of intra- and intermolecular hydrogen bonds (Fig. 3, C and F), it seems likely that the disulfide does in fact impart a significant affinity increase. The ability to incorporate a disulfide bond into AM2/IMD could be valuable for therapeutic development because it might increase peptide stability in plasma, which is an important consideration for these peptides (54, 55).

In conclusion, the new structure revealed a unique triple β-turn ECD complex–binding mode of AM2/IMD, and comparing this with the receptor-bound CGRPmut and AM conformations revealed differences among the peptides that have implications for biased signaling. Novel AM2/IMD antagonist variants were designed, and the crystal structure and the peptide design studies support a dual allostery and direct peptide contact mechanism for RAMP-altered peptide selectivity. These results significantly expand our understanding of accessory protein modulation of GPCR pharmacology and will facilitate development of peptide therapeutics targeting the CGRP and AM receptors.

Experimental procedures

Reagents

General chemicals were from Sigma-Aldrich. Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/liter glucose and L-glutamine was from Lonza (Basel, Switzerland). Fetal bovine serum was from Life Technologies, Inc. COS-7 cells (CRL-1651) and HEK293T cells (HCL-4517) were from ATCC and Thermo Fisher Scientific, respectively. Restriction enzymes and Gibson assembly master mix were from New England BioLabs. Crystal screens were from Hampton Research.

Plasmids

All plasmids used human RAMP and CLR sequences. The plasmid for bacterial expression of the MBP-RAMP1.24–111-(GSA)3–CLR.29–144-H6 crystallization construct was described previously (33). Mammalian expression constructs were generated using the pHLsec vector for secreted protein expression (37). MBP-RAMP1.24–111-(GSA)3–CLR.29–144-H6 and MBP-RAMP2.55–140[L106R]-{(GS)5}–CLR.29–144-H6 construct sequences were PCR-amplified from previously described bacterial expression plasmids (32, 34) and inserted into pHLsec using the Agel and KpnI sites. The MBP-RAMP3.25–111-(GSA)3–CLR.29–144-H6 mammalian expression construct was constructed by Gibson assembly with overlapping fragments of MBP, CLR ECD, and RAMP3 ECD assembled into the Agel and KpnI sites. Numbers in the construct names indicate the amino acid residues used for the ECDs. The L106R substitution in RAMP2 prevented dimerization of the fusion construct and had no effect on the function of the intact RAMP2:CLR complex in cells (33). MBP ended with an NAAAFL linker sequence, as described previously (56). Primer sequences are available from the authors upon request. Coding sequences were confirmed by automated DNA sequencing by the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research core facility. Full-length WT and mutant Myc-tagged RAMP1 and HA-tagged CLR mammalian expression plasmids used for the structure validation cAMP-signaling assays were described previously (33). Mammalian expression plasmids encoding full-length untagged RAMP1, RAMP2, RAMP3, and CLR used for the antagonism of cAMP signaling assays were described previously (34). Mammalian expression plasmids were purified using a Midi Kit (Macherey-Nagel) according to the manufacturer’s directions.

Protein expression and purification

For mammalian cell expression of the N-glycosylated fusion proteins, HEK293T cells were grown to 80–90% confluence in DMEM supplemented with 10% fetal bovine serum at 5% CO2 and 37 °C in preparation for transient transfection. For each protein construct, six T175 flasks were transfected with 50 μg of plasmid DNA and 75 μg of polyethyleneimine (PEI) per flask as described previously (39). PEI Max was used for the MBP-RAMP3–CLR ECD construct. Optimized conditions for expression of each construct were as follows: 3 days at 37 °C with 5% CO2 for MBP-RAMP1.24–111-(GSA)3–CLR.29–144-H6, 5 days at 37 °C and 5% CO2 with 4 mM valproic acid added to the medium for MBP-RAMP2.55–140[L106R]–{(GS)5}–CLR.29–144-H6, and 5 days at 30 °C with 5% CO2 for the MBP-RAMP3.25–111–{(GS)5}–CLR.29–144-H6 construct. Culture media were harvested, and the proteins were purified by immobilized metal affinity chromatography followed by size exclusion chromatography and stored at ~80 °C as described (39). Yields were ~1–3 mg of purified protein. The N-glycan–free MBP-RAMP1.24–111–{(GSA)3}–CLR.29–144-H6 fusion protein used
for crystallization was expressed in E. coli Origami B (DE3) cells and purified as described previously (32, 33). Protein concentrations were determined by UV absorbance at 280 nm using extinction coefficients calculated from Tyr, Trp, and cystine residues and were further confirmed by Bradford assay with a BSA standard curve. Concentrations are stated in terms of the monomeric fusion proteins.

**Synthetic peptides**

Table S1 lists the sequences of all peptides used in this study. αCGRP(1–37), AM(13–52), and AM2/IMD(1–47) agonists were purchased from Bachem (Torrance, CA). All other peptides were custom-synthesized and HPLC-purified by RS Synthesis (Louisville, KY). The lyophilized powders were dissolved at −10 mg/ml in sterile ultrapure water, and multiple aliquots were stored at −80 °C. Concentrations of stock solutions (diluted in 10 mM Tris-HCl, 1 mM EDTA (TE), pH 8.0) were determined by UV absorbance at 280 nm with extinction coefficients calculated from Tyr, Trp, and cysteine residues. For the CGRP(8–37) and AM2/IMD(32–47) Y47A peptides lacking Tyr and Trp residues, peptide concentration was calculated based on assumed 80% peptide content. For CGRP(1–37), peptide concentration was calculated based on the 77.9% peptide content reported by Bachem. The concentration of FITC-aminoheptanoic acid-hAM(37–52) S45W/Q50W was determined by absorbance at 495 nm using the FITC extinction coefficient (72,000 M⁻¹ cm⁻¹ at pH 8.0).

**FP peptide binding assay**

Saturation and competition equilibrium binding assays were performed as described previously using FITC-AM(37–52) S45W/Q50W as probe (34, 38). The reaction buffer conditions were 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mg/ml fatty acid–free BSA, 0.5 mM maltose, 0.5 mM EDTA, 0.1% Tween 20. The probe was used at a concentration of 7 nM in all experiments, and in the competition assays, the purified ECD fusion protein constructs were used at a concentration corresponding to their $K_D$ determined from the saturation experiments (60, 40, and 7 nM for the RAMP1-, RAMP2-, and RAMP3-containing fusion proteins, respectively). Fluorescence anisotropy readings were obtained using a PolarStar Omega plate reader (BMG Labtech). Data analysis and nonlinear regression fitting of the binding curves to exact analytical equations expressed in terms of the total ligand and receptor concentrations were performed with PRISM version 7 as described previously (34, 38, 57). In some competition assays with low-affinity peptides, a high-affinity peptide was included in the assay, and the bottom of the curves were constrained to be the same to enable more accurate affinity determination.

**cAMP signaling assays in COS-7 cells**

For assays assessing the effects of alanine substitutions in receptor components, the cells were transiently transfected in 96-well plates with WT or mutant receptor complexes (125 ng of each plasmid per well) using PEI as described previously (33, 34). Two days after transfection, cells were serum-starved for 30 min at 37 °C in DMEM containing 0.1% (w/v) fatty acid–free BSA and 1 mM 3-isobutyl-1-methylxanthine, following which they were stimulated with AM2/IMD(1–47) agonist for 30 min at 37 °C. After stimulation, cells were lysed as described (34), and cAMP in the lysates was measured with a LANCE CAMP kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions. To account for day-to-day variation in absolute cAMP levels, the data were normalized to percentage of WT receptor control. The concentration–response curves were fit by nonlinear regression to a fixed-slope dose–response stimulation equation in PRISM 7 (GraphPad Software, Inc., La Jolla, CA) to determine the pEC$_{50}$ and $E_{\text{max}}$ values. The assays assessing antagonism of cAMP signaling by truncated peptide variants were performed as described (34). The concentration–response curves in the absence and presence of a fixed antagonist concentration were fit by nonlinear regression to the Gaddum/Schild EC$_{50}$ shift model in PRISM 7 with the Hill and Schild slopes constrained to 1, assuming competitive binding to derive an apparent $pK_B$.

**Crystallization, diffraction data collection, structure solution, and refinement**

Bacterially produced MBP-RAMP1.24–111-(GSA)$_3$-CLR.29-144-H$_6$ was incubated with AM2/IMD(29–47) and concentrated to 30 mg/ml as described (33). Crystals were grown by hanging-drop vapor diffusion with a reservoir solution of 16% (w/v) PEG3350, 0.1 M sodium cacodylate, pH 6.5, and 0.1 M DL-malic acid, pH 7.0. Microseeding was used to improve crystals for data collection. Crystals were dialyzed to reservoir solution supplemented with 10% (v/v) PEG400 for cryoprotection and flash-frozen in liquid nitrogen. Diffraction data were collected remotely at the Advanced Photon Source (Argonne, IL) LS-CAT beamline 21-ID-G. Data from a single crystal were processed with HKL2000 (58) and the CCP4 suite (59). Molecular replacement was completed with Phaser (60) using ligand-free MBP and RAMP1–CLR ECD search models. The structure was rebuilt with COOT (61), and REFMAC5 (62) was used for TLS (translation/libration/screw) and restrained refinement with automatic NCS (non-crystallographic symmetry) restraints.

**Molecular modeling and structure figures**

PyMOL (Schrodinger LLC) was used for structure figure preparation and modeling of AM2/IMD variants using the mutagenesis wizard. Structural superimpositions used the align command to overlay structures based on the common CLR ECD subunit.

**Statistical analysis**

The binding and signaling assays were performed with a minimum of three independent replicates (on different days). Each individual experiment was performed with duplicate technical replicates. The CAMP signaling assays comparing WT and mutant receptors were done in a paired manner with each mutant compared with WT within each of the independent experiments. The $pK_B$ values resulting from the FP binding assays and the $pEC_{50}$, $E_{\text{max}}$, and $pK_B\text{(app)}$ values resulting from the signaling assays are reported as means of the independent replicates ± S.E. Statistical comparisons of the $pK_B$ or $pK_B\text{(app)}$ values for different peptides binding a single receptor complex...
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or for a single peptide binding the three receptor complexes were done using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc multiple-comparison test. Statistical comparisons of the pEC_{50} and E_{max} values for WT versus mutant receptors were done using a paired two-tailed t test and ratio t test, respectively. These paired t tests were done before normalization. Significance was determined as p < 0.05. Statistical tests were performed with PRISM 7.

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