Identification of the Human Receptor Activity-modifying Protein 1 Domains Responsible for Agonist Binding Specificity*

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When co-expressed with receptor activity-modifying protein (RAMP) 1, calcitonin receptor-like receptor (CRLR) can function as a receptor for both calcitonin gene-related peptide (CGRP) and adrenomedullin (AM). To investigate the structural determinants of ligand binding specificity, we examined the extracellular domain of human (h) RAMP1 using various deletion mutants. Co-expression of the hRAMP1 mutants with hCRLR in HEK-293 cells revealed that deletion of residues 91–94, 96–100, or 101–103 blocked [125I]CGRP binding and completely abolished intracellular CAMP accumulation normally elicited by CGRP or AM. On the other hand, the deletion of residues 78–80 or 88–90 significantly attenuated only AM-evoked responses. In all of these cases, the receptor heterodimers were fully expressed at the cell surface. Substituting alanine for residues 91–103 one at a time had little effect on CGRP-induced responses, indicating that although this segment is essential for high affinity agonist binding to the receptors, none of the residues directly interacts with either CGRP or AM. This finding suggests that RAMPs probably determine ligand specificity by contributing to the structure of the ligand-binding pocket or by allosteric modulation of the conformation of the receptor. Interestingly, the L94A mutant up-regulated surface expression of the receptor heterodimer to a greater degree than wild-type hRAMP1, thereby increasing CGRP binding and signaling. L94A also significantly increased cell surface expression of the hRAMP1 deletion mutant D101–103 when co-transfected with hCRLR, and expression of a L94A/D101–103 double mutant markedly attenuated the activity of endogenous RAMP1 in HEK-293T cells.

CGRP and AM belong to the calcitonin family of regulatory peptides and are both highly potent vasodilators (1, 2). Although they share very little sequence identity, both contain ring structures comprised of six amino acids linked by a disulfide bridge and an amidated C terminus that are required for biological activity (3). Both of these peptides and their specific or common receptors are widely distributed among peripheral tissues and in the central nervous system, enabling them to exert a wide variety of biological effects (4, 5).

RAMPs are a recently identified group of single transmembrane domain accessory proteins that serve to transport CRLR to the cell surface where they form functional CGRP and AM receptors (6). The three RAMP isoforms (RAMP1, RAMP2, and RAMP3), which share only 30% sequence identity and differ in their tissue distributions, are all comprised of ~160 amino acids that make up a large extracellular N-terminal domain, a single membrane-spanning domain, and a very short cytoplasmic domain (6, 7). Co-expression of CRLR with RAMP1 leads to both proteins being presented at the plasma membrane as a heterodimeric CGRP receptor, whereas co-expression of CRLR with RAMP2 or RAMP3 enables the resultant heterodimer to function as an AM receptor (6, 8, 9). Upon binding their respective agonist, both receptors mediate a rise in intracellular CAMP levels as well as Ca2+ mobilization and undergo internalization and intracellular trafficking with similar kinetics (9). One recent study (10) shows that the CRLR/RAMP1 heterodimer functions fully as both an AM and a CGRP receptor, which may explain why many actions of AM are potently antagonized by the CGRP receptor antagonist CGRP-8–37.

An analysis of various RAMP1/2 chimeras showed the extra- cellular N-terminal domain to be crucial for defining CGRP and AM selectivity (11), which is consistent with earlier radioligand binding and functional assays (8). A more detailed analysis using various deletion mutants showed that a seven-residue segment situated between the residues (Trp-Cys and Tyr) conserved in human, rat, and mouse RAMP2 and RAMP3 is essential for high affinity agonist binding to AM receptors and that RAMP mutants in which these segments were deleted act as negative regulators of AM receptor function (12, 13). By contrast, there has been no detailed analysis of the extracellular domain(s) of RAMP1 that confer agonist specificity, although sequential truncation mutation of hRAMP1 showed the transmembrane domain to be critical for the functional expression of a CGRP receptor (14). To address this question, we analyzed the effects of co-expression in HEK-293 cells of CRLR with human (h) RAMP1 containing various deletion mutations and AM-selective RAMP1 containing various deletion mutations in its extracellular domain. Our results indicate that residues 91–103 of hRAMP1 are key determinants of high affinity agonist binding to the CRLR/RAMP1 heterodimer and that their deletion results in the formation of a dominant negative mutant capable of inhibiting endogenous hRAMP1 function.

EXPERIMENTAL PROCEDURES

Reagents—[125I]hCGRP (specific activity 2 μCi/pmol) was produced in our laboratory using a modification of a method described previously (2). Human αCGRP was purchased from Peptide Institute (Osaka,
Japan). Human AM was kindly donated by Shionogi & Co. (Osaka, Japan). Rat FITC-conjugated monoclonal anti-HA antibody was from Roche Applied Sciences. All of the other reagents were of analytical grade and were obtained from various commercial suppliers.

**Expression Constructs**—Human CRLR and RAMP1 were modified to provide a consensus Kozak sequence as described previously (15). A HA epitope tag (YPYDVPDYA) was ligated in-frame to the 5’ end of the hRAMP1 cDNA, and the native signal sequences were removed and replaced with MKTLALSTYIFCLVFA, yielding HA-hRAMP1 (16). Human CRLR and HA-hRAMP1 were cloned into the mammalian expression vector pCAGGS/Neo (9) using the 5’-Xhol and 3’-Not sites. The sequences of the resultant constructs were all verified using an Applied Biosystems 310 genetic analyzer. HA-hRAMP1 was compared with the native sequence in the assays and was found to behave identically (data not shown).

Deletion mutations and single amino acid substitutions were carried out using a QuikChange kit (Stratagene, San Diego, CA) according to the manufacturer’s instructions with pRES-HA-hRAMP1, which was constructed by subcloning the coding sequence of HA-hRAMP1 into pRES1/Neo (Clontech, Palo Alto, CA). For each mutation, two complementary 30-mer oligonucleotides (sense and anti-sense) were designed to contain the desired mutation in their middle. To enable rapid screening of mutated clones, the primers carried an additional silent mutation introducing (or removing) a restriction site. The presence of each mutation of interest and the absence of undesired ones were confirmed by DNA sequencing. Individual HA-hRAMP1 mutants were then cloned into pCAGGS/Neo.

**Cell Culture and DNA Transfection**—HEK-293 and HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C under a humidified atmosphere of 95% air, 5% CO₂. For experimentation, cells were seeded into 24-well plates and, upon reaching 70–80% confluence, were transiently co-transfected with hCRLR plus HA-hRAMP1, one of the HA-tagged hRAMP1 mutants, or empty vector using LipofectAMINE transfection reagents (Invitrogen) according to the manufacturer’s instructions. The cells were incubated for 4 h in 250 μl of OptiMEM 1 medium (containing 2000 ng/well plasmid DNA, 2 μg/well LipofectAMINE, and 2 μg/well LipofectAMINE. As a control, some cells were transfected with empty vector (pCAGGS/Neo) (Mock). All of the experiments were performed 48 h after transfection.

**FACS Analysis**—Flow cytometry was carried out to assess the levels of whole cell and cell surface expression of HA-hRAMP1 or the HA-hRAMP1 mutant in HEK-293 cells. To evaluate cell surface expression, cells were harvested following transient transfection, washed twice with PBS, resuspended in ice-cold FACS buffer (9), and then incubated with anti-HA-FITC antibody (1:50 dilution) for 60 min at 4 °C in the dark. For evaluation of whole cell expression, cells were first permeabilized using IntraPrep™ reagents (Beckman Coulter, Fullerton, CA) as described previously (17, 18). Oligonucleotide primers and fluorescently labeled probes were prepared as described previously (10, 18). The levels of hCRLR, hRAMP1, and hAM mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA, which served as an internal control. DNA sequence analysis confirmed that the amplified products were identical to those of hCRLR and the three hRAMP isoforms (6, 19, 20).

**mRNA Expression Measured by Real-time Quantitative PCR**—Total RNA was extracted from transfected HEK-293 or HEK-293T cells using Total RNA isolation reagent (Invitrogen) and then reverse-transcribed using SuperScript reverse transcriptase (Invitrogen), yielding the respective cDNAs. The expression of mRNAs encoding hCRLR, the three hRAMP isoforms, and hAM was assessed using real-time quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA) as described previously (17, 18). Oligonucleotide primers and fluorescently labeled probes were prepared as described previously (10, 18). The levels of hCRLR, hRAMP1, and hAM mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA, which served as an internal control. DNA sequence analysis confirmed that the amplified products were identical to those of hCRLR and the three hRAMP isoforms (6, 19, 20).

**Statistical Analysis**—Results are expressed as means ± S.E. of at least three independent experiments. Differences between two groups were evaluated with Student’s t test. The differences among multiple groups were evaluated with one-way analysis of variance followed by Scheffe’s test. Values of p < 0.05 were considered significant.

**RESULTS**

**Construction and Characterization of hRAMP1 Deletion Mutants**—The extracellular domain of RAMP proteins is known to be crucially involved in defining CGRP and AM selectivity (8, 11). Therefore, to assess which regions of the CRLR/RAMP1 heterodimer are involved in binding hCGR and hAM and mediating cAMP accumulation, we constructed a group of hRAMP1 mutants with various deletions in their extracellular N-terminal domains (Fig. 1). Because all four cysteine residues in the extracellular domain of hRAMP2 (Cys₆⁸, Cys₈⁴, Cys₉⁹, and Cys₁₃¹) are known to be essential for proper delivery of the receptor proteins to the cell surface (21), the constructed hRAMP1 genes always encoded all six cysteine residues normally present in hRAMP1 (Fig. 1, asterisks). The resultant 17 mutant constructs were then transiently transfected into HEK-293 cells, which lack functional CGRP or AM receptors, even when transfected with hCRLR (10, 21, 22).

We initially analyzed the whole-cell expression of epitope-tagged hCRLR mutants in permeabilized cells using FACS (Fig. 2A). Immunoreactivity was detected in only 1.9% cells expressing the empty vector (Mock), which is well within the 2% limit of resolution characteristic of FACS analysis. When expressed alone or with hCRLR, HA-hRAMP1 was detected in 60.3 and 58.8% of cells, respectively. Similarly, co-expression of hCRLR with one of the hRAMP1 deletion mutants led to their full expression in 37–52% cells. Thus, the transfection efficiency
for the mutant receptor was comparable with that for hCRLR/HA-hRAMP1. Moreover, the staining pattern suggested that these proteins were probably in the endoplasmic reticulum, representing a pool of newly synthesized molecules not yet transported to the cell surface (data not shown).

We next analyzed the cell surface expression of the hRAMP1 mutants in nonpermeabilized cells (Fig. 2B). Empty vector and HA-hRAMP1 appeared at the surface of 1.7 ± 0.10% and 4.2 ± 0.28% of cells, respectively, which is consistent with earlier studies showing that most HA-hRAMP1 molecules are not transported to the cell surface when expressed alone (6, 23). When hCRLR was co-expressed with HA-hRAMP1, cell surface immunoreactivity was detected in 23.7 ± 2.3% cells. Among the 17 deletion mutants, D28–33, D34–39, D74–76, D78–80, D105–107, D109–112, and D113–118 were detected at the surface of 14–30% cells when co-transfected with hCRLR, which is comparable with the frequency seen with hCRLR/HA-hRAMP1. Cell surface immunoreactivity was detected in fewer cells co-transfected with hCRLR and each of the remaining 10 mutants (7–13%), but it still tended to be higher than was seen with transfection of HA-hRAMP1 alone.

The functionality of the hRAMP1 mutants was assessed by measuring agonist-induced intracellular cAMP accumulation (Fig. 3). Neither 10 nM hCGRP nor hAM elicited significant increases in cAMP in nontransfected HEK-293 cells or cells transfected with HA-hRAMP1 or hCRLR alone. On the other hand, the co-expression of hCRLR with HA-hRAMP1 enabled CGRP and AM to each elicit significant increases in cAMP, which is consistent with previous reports that CGRP and AM bind to the CRLR/RAMP1 heterodimer with similar affinities (8, 9, 12, 24, 25). Similar CGRP- and AM-evoked increases in cAMP levels were seen when hCRLR was co-expressed with D74–76, D105–107, or D109–112. By contrast, cells expressing hCRLR with D28–33, D34–39, D46–50, D51–55, D78–80, D83–86, D88–90, or D113–118 responded more selectively to CGRP. In particular, CGRP-induced cAMP production mediated by hCRLR/D78–80 or D88–90 was nearly 10-fold greater than that induced by AM, despite the fact that [125I]CGRP binding to the mutant receptors was much diminished (Fig. 4).

Six of the mutants (D41–45, D59–65, D67–71, D91–94, D96–100, and D101–103) did not support either [125I]CGRP binding (Fig. 4) or agonist-evoked cAMP accumulation (Fig. 3), even though their cell surface expression was similar to that of hCRLR/D78–80 and D88–90, which did support receptor function. To better understand what accounts for this difference, we carried out a detailed FACS analysis in which the behavior of D41–45, D59–65, D67–71, D91–94, D96–100, and D101–103 was compared with that of D46–50, D51–55, and D88–90. When whole-cell expression of the epitope-tagged hRAMP1 mutants was analyzed, we found that all were expressed in 42–53% cells and these levels changed little when the mutants were co-transfected with hCRLR (Fig. 5A). An analysis of their cell surface expression showed that when the mutants were expressed alone, cell surface immunoreactivity was detected in 3.3–4.7% cells. This is comparable with the levels seen with transfection of HA-hRAMP1 alone (compare Figs. 2B and 5B) and suggests that similar to HA-hRAMP1, these mutants interact with the small amounts of endogenous CRLR or calcitonin receptor expressed in HEK-293 cells (22, 26).

RAMP1 Domains Involved in Agonist Specificity

FIG. 2. FACS analysis of HEK-293 cells expressing hCRLR and hRAMP1 or one of its deletion mutants. A, whole cell expression of the indicated HA-tagged proteins in permeabilized cells estimated by flow cytometry. Forty-eight hours after transfection, cells were permeabilized and then incubated for 15 min at room temperature with monoclonal anti-HA-FITC antibody. Mock incubation with the antibody served as the control. Bars represent the average of two experiments. B, cell surface expression of the indicated HA-tagged proteins in nonpermeabilized cells estimated by flow cytometry. Following transient transfection, cells were incubated for 1 h at 4°C with monoclonal anti-HA-FITC antibody. Mock incubation with the antibody served as the control. Bars represent means ± S.E. of three independent experiments (*, p < 0.05 versus Mock).

Cell surface expression of all of the mutants was significantly increased when they were co-transfected with hCRLR. In particular, cell surface immunoreactivity was detected in 9.4 ± 0.12% cells co-expressing D51–55 and hCRLR. This is 2.7-fold higher than that observed in the absence of hCRLR and led to significant increases in both [125I]CGRP binding and CGRP-

FIG. 3. Agonist-evoked cAMP production in HEK-293 cells co-expressing hCRLR and hRAMP1 or one of its deletion mutants. Cells were transiently transfected with empty vector, HA-RAMP1, hCRLR, or hCRLR plus HA-hRAMP1 or one of its deletion mutants, after which they were incubated for 15 min at 37°C with 10 nM hCGRP or hAM and then lysed. The resultant lysates were analyzed for cAMP content. Symbols represents means ± S.E. of three independent experiments (*, p < 0.005 versus Mock).
incubated for 4 h at 4°C with 100 pmol/liter [125I]hCGRP in the presence (for nonspecific binding) or absence (for total binding) of 1 μmol/liter unlabeled hCGRP. Bars represent means ± S.E. of three experiments (*, p < 0.002 versus corresponding nonspecific binding).

Fig. 4. [125I]CGRP binding to HEK-293 cells co-expressing hCRLR plus hRAMP1 or one of its deletion mutants. Shown is the total (open bars) and nonspecific (filled bars) binding. Cells were transiently transfected with the indicated HA-tagged proteins and then incubated for 4 h at 4°C with 100 pmol/liter [125I]hCGRP in the presence (for nonspecific binding) or absence (for total binding) of 1 μmol/liter unlabeled hCGRP. Bars represent means ± S.E. of three experiments (*, p < 0.002 versus corresponding nonspecific binding).

A

B

Fig. 5. FACS analysis of HEK-293 cells expressing mutant hRAMP1 with or without hCRLR. A, whole-cell expression of the indicated HA-tagged proteins in permeabilized cells. Cells were analyzed as in Fig. 2A, B, cell surface expression of the indicated HA-tagged proteins in nonpermeabilized cells. Cells were analyzed as in Fig. 2B. In both preparations, cells transfected with empty vector or hCRLR alone served as controls. Bars represent means ± S.E. of three experiments (*, p < 0.0001 versus empty vector; #, p < 0.0001 versus hCRLR alone; †, p < 0.05 versus corresponding CRLR(−)).

induced cAMP accumulation (Figs. 3 and 4). Similar levels of cell surface immunoreactivity (8.8–9.3%) were detected in cells expressing hCRLR and D91–94, D96–100, or D101–103. However, although their cell surface expression was 2.0- to 2.4-fold greater than in cells not expressing hCRLR, there was a complete absence of [125I]CGRP binding and signaling.

Single Amino Acid Substitutions of Residues 91–103 of hRAMP1—The aforementioned findings suggest that CGRP selectivity is determined within a region of hRAMP1 spanning amino acids 91–103. To assess the extent to which any single amino acid residue within that segment affects the capacity of CGRP to induce cAMP production, residues 91–103 were substituted at one time with alanine. We initially analyzed the cell surface expression of epitope-tagged mutants using FACS (Fig. 6). Cell surface immunoreactivity was detected in 24.0 ± 1.0% cells co-transfected with HA-hRAMP1 and hCRLR. Co-transfection of hCRLR with F93A, Y100A, or F101A led to their expression at the cell surface in only 8–11% cells, although the proteins were observed to be diffusely distributed throughout the cytoplasm of most cells (data not shown). The other eight mutants (R91A, F92A, V96A, H97A, G98A, R99A, F102A, and S103A) appeared at the surface of 18–24% cells, a level comparable to hCRLR/HA-hRAMP1. Apparently, the cell surface expression of D91–94, D96–100, and D101–103 is specifically inhibited by the substitution of Phe93, Tyr100, and Phe101, respectively. Surprisingly, cell surface expression of hCRLR/L94A was 1.6-fold higher than that of hCRLR/HA-hRAMP1.

As a negative control, we also constructed a C104A mutant because this cysteine residue is conserved among all three RAMPs and is essential for the cell surface expression of the receptor (21). As expected, hCRLR/C104A was detected at the surface of only 4% cells.

To confirm their ligand selectivity, we examined the binding of [125I]CGRP to receptor heterodimers comprised of hCRLR complexed with the indicated point mutant (Fig. 7). The specific CGRP binding to cells co-expressing hCRLR and HA-hRAMP1 was 7300 cpm/well, and the nonspecific/total binding ratio was 0.26. Similar levels of specific binding (5300–7400 cpm/well) were seen in cells co-expressing hCRLR with R91A, V96A, R99A, R102A, S103A, D76–78, or D79–82, whereas levels of specific binding in cells co-expressing hCRLR with F92A, F93A, H97A, or Y100A were somewhat lower (1700–2400 cpm/well). Consistent with their level of presentation at the cell surface (Fig. 6), CGRP binding to cells expressing hCRLR with L94A or G98A was 42 and 27%, respectively, higher than in cells expressing hCRLR/HA-hRAMP1. Conversely, CGRP binding to cells co-expressing hCRLR with F101A was 94% lower than in cells expressing hCRLR/HA-hRAMP1.

The functionality of the receptors comprised of hCRLR and each of the point mutants was then evaluated by measuring CGRP-evoked cAMP production. Consistent with the binding assays (Fig. 7), the C104A substitution markedly impaired CGRP-evoked cAMP production. In all of the other cases, however, the EC50 values obtained with the mutant receptors were not significantly different from that obtained with hCRLR/HA-hRAMP1 (Table I). Cells expressing hCRLR/L94A exhibited the strongest responses, which were 2.5-fold greater than in cells expressing hCRLR/HA-hRAMP1, whereas cells expressing hCRLR/F101A exhibited the smallest response, which was 42% smaller than that seen with hCRLR/HA-hRAMP1. Taken together, these results indicate that among residues 91–103 of hRAMP1, no single amino acid contributes significantly to the ligand selectivity of the CRLR/RAMP1 heterodimer.

Characterization of hRAMP1 Double Mutants—To further examine the effects of the L94A point mutation on the cell surface expression of D91–93, D96–100, and D101–103, we constructed three double mutants, L94A/D91–93, L94A/D96–100, and L94A/D101–103, and analyzed their expression using FACS (Fig. 8A). Whole-cell expression of the double mutants was similar to that seen with the various deletion mutants (compare Figs. 2A and 8A). An analysis of the cell surface expression showed that when expressed alone, L94A/D91–93, L94A/D96–100, and L94A/D101–103 appeared at
the surface of $4.0 \pm 0.44, 3.8 \pm 0.56, \text{and } 6.8 \pm 0.68\%$ cells, respectively, which was similar to HA-hRAMP1 alone (compare Figs. 2B and 8B). Their cell surface expression increased significantly to $10.6 \pm 1.1, 9.3 \pm 0.68, \text{and } 18.9 \pm 1.4\%$ cells when co-expressed with hCRLR. Notably, cell surface expression of hCRLR/L94A/D101–103 was comparable to that of hCRLR/HA-hRAMP1.

We then examined the effects of L94A/D101–103 on CGRP-evoked cAMP production via hCRLR/hRAMP1. As mentioned above, HEK-293 cells lack functional CGRP or AM receptors, even when transfected with hCRLR, which was consistent with our finding that they endogenously express no hRAMP1 and only low levels of hRAMP2 (Fig. 9). On the other hand, HEK-293T cells abundantly express endogenous hRAMP1 as well as a smaller amount of hRAMP2, which is consistent with earlier findings (6). Both CGRP and AM had a small effect on the cAMP content of HEK-293T cells expressing empty vector (Mock). Maximal cAMP levels reached ~6-fold over base line (EC$_{50}$ = 0.82 or 52 nM, respectively) (Figs. 10, A and B). In cells transfected with hCRLR alone, CGRP and AM elicited cAMP accumulation that were 6- and 10-fold, respectively, greater than that in mock-transfected cells (EC$_{50}$ = 0.41 or 32 nM, respectively). Co-transfection of hCRLR with L94A/D101–103 reduced CGRP-evoked cAMP accumulation by 38–51% (Fig. 10A) and AM-evoked accumulation by 23–42% (Fig. 10B). Thus, L94A/D101–103 attenuated the functional effect of both endogenous hRAMP1 and hRAMP2, presumably because of competition between the mutant and the endogenous proteins.

**DISCUSSION**

Since the discovery of RAMPs, various RAMP chimeras have been used to investigate the structural determinants of CGRP and AM receptor specificity (8, 11, 12, 27). When RAMP chimeras were co-transfected into cells together with CRLR, their binding characteristics and functionality revealed that the determinants of CGRP and AM selectivity reside in the extracellular domains of RAMP. Moreover, Hilairet et al. (11) showed that when complexed with CRLR, RAMPs are situated close to the agonist binding pocket because $[^{125}\text{I}]$CGRP and $[^{125}\text{I}]$AM could be cross-linked with RAMP1 and RAMP2 or RAMP3, respectively. This suggests that RAMPs determine ligand speci-
RAMP1 Domains Involved in Agonist Specificity

Specificity by contributing to the structure of the ligand-binding pocket or by allosteric modulation of the conformation of the receptor. Consistent with that idea, we recently used various RAMP chimeras and deletion mutants to show that a seven-residue segment situated between the residues (Trp-Cys and Tyr) in hRAMP2 (amino acids 86–92) and hRAMP3 (amino acids 59–65) is essential for high affinity agonist binding to hAM receptors but not for the interaction of RAMP with CRLR (12). We also found that the same seven-residue segment situated between Trp-Cys and Tyr was conserved in rat RAMP2 and rRAMP3 (amino acids 93–99 and 58–64, respectively) was also involved in agonist binding specificity (13). However, these findings did not enable us to draw any conclusions as to which structural domain(s) of human RAMP1 confer agonist specificity despite the use of the eight RAMP chimeras (RAMP1/2 and RAMP2/1) (12). Therefore, we decided to construct a group of deletion mutants that would enable a more detailed analysis of the extracellular domain of hRAMP1.

Co-transfection of hCRLR and hRAMP1 deletion mutants D41–45, D59–65, D67–71, D91–94, D96–100, or D101–103 eliminated CGRP and AM binding as well as evoked cAMP accumulation. When expressed alone, the six deletion mutants were detected at the cell surface of only 4%, which is similar to HA-hRAMP1. That cell surface expression of the deletion mutants was significantly increased by co-transfection of hCRLR is consistent with recent FACS and immunohistochemical findings that the appearance of epitope-tagged RAMP molecules at the cell surface is significantly increased by CRLR (6, 13, 21) or other Class II G protein-coupled receptors (28). In addition, Flahaut et al. (23) recently showed that in a Xenopus...
oocyte expression system, RAMP1 is neither N-glycosylated nor transported to the plasma membrane when expressed alone but that introduction of N-glycosylation sites into its sequence (D58N/G60S, Y71N, and K103N/P105S) allowed its cell surface expression at levels similar to those seen when wild-type RAMP1 is co-expressed with CRLR. Still, CRLR-induced increases in the cell surface expression of the deletion mutants were 2.5-fold lower than what was seen with HA-hRAMP1. In the context of earlier studies (22, 26), this finding suggests that these mutants probably interact more potently with calcitonin receptor than with CRLR, both of which are endogenously expressed in HEK-293 cells.

The CRLR-induced increases in the cell surface expression of D91–94, D96–100, and D101–103 were very similar to that seen with D51–55. Our finding that hCRLR/D51–55 mediated full CGRP binding and signaling, whereas the others did not, suggests that residues 91–103 of hRAMP1 are especially important for high affinity agonist binding and signaling. Residues 59–65 of hRAMP1 correspond to residues 86–92 of hRAMP2 and 59–65 of hRAMP3, which as mentioned above we found to be crucial for high affinity agonist binding to hAM receptors and to be situated between the conserved residues (Try-Cys and Tyr) common to humans, rats, and mice (7). Notably, hRAMP1 residues 91–103 are also situated between the conserved residues (Pro-Asn and Cys).

When alanines were substituted one at a time for residues 91–103 of hRAMP1, co-expression of F101A with hCRLR reduced the magnitude of these responses by 42% as compared with those observed with hCRLR/HA-hRAMP1 and [125I]CGRP binding to the mutant receptor was also markedly diminished. In addition, this amino acid residue is conserved among all three RAMPs, although its precise role in determining agonist specificity is still not completely clear. The remaining amino acid residues show little sequence identity among RAMPs, suggesting that the three segments (residues 91–94, 96–100, and 101–103) do not directly interact with the binding agonist but confer selectivity by contributing to the structure of the ligand binding pocket or through allosteric modulation of the conformation of CRLR.

The expression of L94A up-regulated surface expression of the receptor heterodimer more potently than did wild-type RAMP1, leading to increased CGRP binding and signaling. To our knowledge, there has been only one other study showing up-regulation. In the C terminus of the β2-adrenergic receptor, L339A substitution caused a 1.8-fold increase in the number of binding sites as compared with the wild-type receptor with no changes in ligand affinity (29). The mechanism by which the substitution of the hydrophilic leucine residue with hydrophobic alanine increased the number of binding sites is not clear. However, we did find that whereas L94A substitution significantly increased cell surface expression of hCRLR/D101–103, there were no differences in the cell surface expression of heterodimers comprised of CRLR and the L94A/D91–93, L94A/D96–100, D91–94, or D96–104. This finding suggests that residues 91–93 and 96–100 are necessary for the increase in cell surface expression of L94A.

Cys21) and amidation of the C-terminal residue of the CGRP and AM molecules are both essential for agonist binding and receptor activation (3), deleted segments that strongly inhibited both CGRP- and AM-evoked cAMP production may be involved in the binding of those structures. Deletions that selectively reduced only AM-evoked responses may contribute to the interaction with agonist residues other than those making up the ring and amidated C terminus.

We also tested the hypothesis that the L94A/D101–103 double mutant can act as a negative regulator of CGRP or AM receptor function. To address that question, we used HEK-293T cells transfected with hCRLR, which form functional CGRP and AM receptors with endogenous hRAMP1 and hRAMP2. Consistent with our hypothesis, co-expression of L94A/D101–103 with hCRLR significantly diminished CGRP- and AM-evoked cAMP accumulation. Previous studies have shown that the dominant negative activity of truncated V2 vasopressin receptors resulted from the reduction of cell surface expression of the full-length receptor protein and the formation of heterodimeric complexes involving the truncated and full-length forms (30). Although several studies (31–34) have investigated the relative affinities of various RAMP forms for CRLR, it remains unclear whether the inhibition of receptor function by dominant negative RAMP mutants is attributed to competitive inhibition, formation of heterodimeric complexes, or both. A detailed analysis of dominant negative effects of L94A/D101–103 is currently ongoing.

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