When co-expressed with a receptor activity-modifying protein (RAMP) accessory protein, calcitonin receptor-like receptor (CRLR) can function as a calcitonin gene-related peptide receptor (CRLR-RAMP1) or an adrenomedullin (AM) receptor (CRLR-RAMP2/3). Here we report on the structural domain(s) involved in selective AM binding that were examined using various RAMP chimeras and deletion mutants. Co-expression of chimeric RAMPs and CRLR in HEK293 cells revealed that residues 77–101, situated in the extracellular N-terminal domain of human RAMP2 (hRAMP2), were crucial for selective AM-evoked cAMP production. More detailed analysis showed that deletion of hRAMP2 residues 86–92 significantly attenuated high-affinity 125I-AM binding and AM-evoked cAMP production despite full cell surface expression of the receptor heterodimer and that deletion of hRAMP3 residues 59–65 had a similar effect. There is little sequence identity between hRAMP3 residues 59–65 and hRAMP2 residues 86–92; moreover, substituting alanine for Trp86 (Ala87), Met88, Ile90, Ser91, Arg92, or Pro92 of hRAMP2 had no effect on AM-evoked cAMP production. It thus seems unlikely that any one amino acid residue is responsible for determining selective AM binding or that AM binds directly to these peptide segments. Instead these findings suggest that the respective seven-amino acid sequences confer selectivity either by directly contributing to the structure of ligand binding pocket or by allosteric modulation of the conformation of CRLR.

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 proteins contain ring structures comprised of six or seven amino acids linked by a disulfide bridge and an amidated C terminus required for biological activity (3). Both peptides and their specific or common binding sites are widely distributed among peripheral tissues and in the central nervous system and mediate a wide variety of biological effects (4–6).

In 1998, McLatchie et al. (7) identified and cloned the RAMP family of accessory proteins that includes three isoforms (RAMP1, RAMP2, and RAMP3) and serves to transport the CRLR to the cell surface, thereby forming functional CGRP and AM receptors. RAMPs consist of ~160 amino acids and possess a large extracellular N-terminal domain, a single transmembrane-spanning domain, and a short cytoplasmic domain. Co-expression of RAMP1 with CRLR leads to both proteins being presented at the plasma membrane as a CGRP receptor, while co-expression of RAMP2 or -3 allows CRLR to function as an AM receptor (7–10). Interestingly, although RAMP2 and -3 share only 30% sequence identity and differ in their tissue distributions, they generate essentially equivalent AM receptors when co-expressed with CRLR in HEK293T cells (9).

It was initially believed that mature glycosylation of CRLR in the presence of RAMP1 determined CGRP specificity, while core glycosylation in the presence of RAMP2 or -3 determined AM specificity (7, 9). However, unlike in mammalian cells, co-expression of CRLR with RAMP1 or RAMP2 in Drosophila Schneider 2 cells led to RAMP-independent, uniformly mature glycosylation of CRLR; moreover, the functional properties of the CGRP and AM receptors formed were indistinguishable from those in mammalian cells (11). This is consistent with a more recent study directly demonstrating that the distinct pharmacology conferred by each RAMP is independent of the CRLR glycosylation state (12). In fact, although core-glycosylated CRLR predominates when co-expressed with RAMP2 or -3, cross-linking experiments revealed that 125I-AM binds only to the mature forms. On the other hand, analysis of various RAMP1/2 chimeras showed the extracellular N-terminal domain to be crucial for defining CGRP or AM selectivity (12), which is consistent with earlier radioligand binding and functional assays (9).

To further examine the structural domains of hAM receptors that confer agonist specificity, we analyzed the effects of co-expressing CRLR and various chimeras and deletion mutants in HEK293 cells. Our results indicate that seven-residue segments situated between three residues conserved in both hRAMP2 and hRAMP3 (amino acids 86–92 and 59–65, respectively) are key determinants of high affinity agonist binding to the hAM receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**

A plasmid hCRLR cDNA (13) was a kind gift from Dr. Kenji Kangawa (National Cardiovascular Research Institute, Osaka, Japan). 125I-hAM (specific activity, 2 μCi/μmol) was produced in our laboratory (2). Human αCGRP and AM were purchased from Peptide Institute (Osaka,
Expression Constructs—Human CRLR and RAMPs were modified to provide a consensus Kozak sequence as described previously (14). Expression vector pCAGGS-hCRLR was constructed by cloning hCRLR cDNA into the mammalian expression vector pCAGGS/Neo (10) using the 5′ XhoI and 3′ NotI sites. In addition, a Myc epitope tag (EQKLISEEDL) was ligated in-frame to the 5′-end of all hRAMP cDNAs and the corresponding sequence was removed and replaced with Mkt1-LALSTYIFCFLVFA (15). A cDNA encoding green fluorescent protein, not conjugated with hCRLR, was also inserted into the vector to confirm hCRLR expression levels. myc-hRAMPs were then cloned into pCAGGS/Neo without green fluorescent protein cDNA.

myc-hRAMP1/2 chimeras were constructed by digestion at three unique restriction sites (BamHI, Bsp126I, and MflI) located in the extracellular N-terminal domain of hRAMP2. As these sites are absent in hRAMP1, corresponding DNA fragments containing them were prepared by PCR using primers containing the sites. The separate fragments of myc-hRAMP1 and -2 were then ligated and cloned into the same expression vector. Chimeric RAMPs were all sequenced using an Applied Biosystems 310 Genetic Analyzer.

Deletion mutations and single amino acid substitutions were carried out using a Quick Change kit (Stratagene) according to the instructions of the manufacturer with pIRE5-myc-hRAMPs serving as the template, which was constructed by subcloning the coding sequence of each myc-hRAMP into pIRE51/Neo (CLONTECH). For each mutation, two complementary 30-mer oligonucleotides (sense and antisense) were designed to contain the desired mutation in their middle. To allow a 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 was confirmed by DNA sequencing. Individual myc-hRAMP mutants were then cloned into pCAGGS/Neo.

Cell Culture and Transfections—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G, 100 units/ml streptomycin, and 50 μg/ml amphotericin B at 37 °C under a humidified atmosphere of 95% air, 5% CO2. To obtain cells stably expressing hCRLR, HEK293 cells were grown to 70% confluence in 35-mm culture dishes and then transfected with 1 μg of linearized pCAGGS-hCRLR using LipofectAMINE (Life Technologies, Inc.) according to the instructions of the manufacturer. Two days after transfection, cells were selected in medium containing 1 mg/ml G418 (Life Technologies, Inc.), after which a pool of G418-resistant colonies was trypsinized and diluted, and single cells were seeded into 96-well plates. Stable transfectants were then isolated ~3 weeks later, and a single cell line in which almost 100% of cells expressed green fluorescent protein (assayed by flow cytometry) and functionally responded to CRLR agonists when co-transfected with hRAMPs (assayed by cAMP assay) was selected for further analysis. The transfectant cell line was maintained in the same Dulbecco’s modified Eagle’s medium containing 0.25 mg/ml G418.

Transient transfection of HEK293 cells stably expressing hCRLR was also performed using LipofectAMINE. The cells were seeded into 24-well culture plates, and at 70% confluence cells were transfected with empty expression vector (pCAGGS/Neo) or with wild-type, chimeric, or mutant expression constructs. This was accomplished by incubating the cells for 3 h in 250 μl of OptiMEM 1 medium containing 100 ng/well plasmid DNA and 2 μl/well LipofectAMINE. All experiments were performed 48 h after transfection.

Flow cytometry was performed to assess levels of cell surface expression of various myc-hRAMP2 and -3 deletion mutants in HEK293 cells stably expressing hCRLR. Following transient transfection, the cells were harvested, washed twice with PBS, resuspended in ice-cold FACS buffer (20 mM PBS, pH 7.4, 1% fetal bovine serum, 1 mM EDTA, 0.02% sodium azide), and incubated for 45 min at 4 °C with monoclonal c-Myc antibody (1:1000 dilution). Following two additional washes with FACS buffer, the cells were incubated for 30 min at 4 °C in the dark with fluorescein PE-conjugated rabbit anti-mouse secondary antibody (1:400 dilution) in ice-cold FACS buffer. Then after two successive washes with FACS buffer, the cells were adjusted to a density of 2 × 106 cells/tube in FACS buffer containing 5 μg/ml propidium iodide, subjected to flow cytometry on an EPICS XL flow cytometer (Beckman Coulter), and analyzed using the CellQuest software program (BD Biosciences, San Jose, CA). The fraction of cells expressing myc-hCRLR (green fluorescence) was determined by addition of lysis buffer (Amersham Biosciences, Inc.).

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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 tests; differences among multiple groups were evaluated with a one-way analysis of variance followed by Scheffe’s tests. Values of \( p < 0.05 \) were considered significant.

RESULTS

Construction and Characterization of Chimeric RAMPs—To roughly assess which structural domains of the hAM receptor mediate cAMP accumulation upon binding AM, we constructed a group of hRAMP chimeras by using three unique restriction sites (BamHI, Bsp1286I, and MfiI), which enabled us to generate six hybrid genes by swapping DNA restriction fragments (Fig. 1). These RAMP chimeras were then transiently transfected into HEK293 cells stably expressing hCRLR and characterized by measuring agonist-induced intracellular cAMP accumulation (Fig. 2). We found that AM but not CGRP increased the cAMP content of untransfected cells slightly; maximal cAMP levels reached ~2.5-fold over baseline, which was consistent with the finding that intact HEK293 cells endogenously express hRAMP2 (data not shown). When co-expressed with myc-hRAMP1 or -2, hCRLR mediated marked, concentration-dependent increases in cAMP in response to CGRP or AM (Fig. 2, a and b). In cells co-expressing myc-hRAMP1 and hCRLR, both CGRP and AM elicited accumulations of cAMP that were nearly 20-fold greater than in cells expressing hCRLR alone (EC\(_{50}\) = 0.34 or 1.39 nM, respectively). On the other hand, cells expressing hCRLR and myc-hRAMP2 responded more selectively to AM (EC\(_{50}\) = 0.46 nM). Note that these EC\(_{50}\) values are compatible with those previously obtained in HEK293 cells stably expressing hCRLR and hRAMPs (10).

Among the six chimeric constructs, replacement of residues 36–76 of myc-hRAMP2 with the corresponding myc-hRAMP1 sequence had little effect on myc-hRAMP2-mediated cAMP production (Fig. 2c). Likewise, replacement of residues 102–175 or 128–175 of myc-hRAMP2 with the corresponding myc-hRAMP1 sequence had little effect on myc-hRAMP2-mediated responses to AM (EC\(_{50}\) = 0.43 and 0.40 nM, respectively (Fig. 2, g and h)). When myc-hRAMP2 residues 36–124 were replaced by the corresponding sequence from myc-hRAMP1, however, expression of the resultant chimera induced a leftward shift in the CGRP concentration-response curve, yielding a response comparable to those mediated by myc-hRAMP1 (Fig. 2e).

Two of the chimeras were incapable of mediating cAMP accumulation in response to either AM or CGRP (Fig. 2, d and f). This is explained by the observation that neither was detectable in the cells unless the cells were permeabilized (as judged...
by PE fluorescence of permeabilized cells). 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).

The above findings suggest that AM selectivity is determined within a region between amino acids 77 and 101 of hRAMP2. We therefore constructed two chimeras in which residues 77–101 were replaced with the corresponding sequence from hRAMP1 to produce an hRAMP2/1/2 chimera, and conversely, an hRAMP1/2/1 chimera was generated by inserting the residues 77–101 of hRAMP2 into the corresponding sequence of hRAMP1 (Fig. 3A). In cells co-expressing myc-hRAMP1/2/1 with CRLR, AM produced a greater increase in cAMP than did CGRP (EC50/H11005 = 0.58 nM) (Fig. 3B), and the responses were very similar to those observed in cells expressing hRAMP2/1 (Fig. 2g). By contrast, myc-hRAMP2/1/2 failed to mediate cAMP production in response to AM or CGRP (Fig. 3B). These results confirm that the amino acid sequence determining the selectivity of AM-evoked cAMP production via the CRLR/hRAMP2 complex is situated among residues 77–101 of hRAMP2.

Construction and Characterization of hRAMP2 Deletion Mutants—To gain a more precise understanding of how residues 77–101 of hRAMP2 confer agonist selectivity, we constructed the 10 hRAMP2 deletion mutants shown in Fig. 4 and carried out a detailed pharmacological characterization following their transient transfection into HEK293 cells stably expressing hCRLR. We initially analyzed the cell surface expression of epitope-tagged mutants using FACS (Fig. 5). Cell surface immunoreactivity was detected in only 1.82 ± 0.02% of cells expressing hCRLR alone. This markedly increased to 53.0 ± 1.0% of cells when myc-hRAMP2 was co-expressed with hCRLR. Co-expression of deletion mutant D76–78, D79–82, D86–89, or D90–92 with hCRLR led to their full expression at the cell surface in 47.2 ± 0.03, 21.7 ± 1.9, 16.8 ± 0.5, and 29.9 ± 1.3% of cells, respectively. The other mutants appeared at the surface of only <6% of cells, although the proteins were diffusely distributed throughout the cytoplasm (data not shown).

To confirm the ligand selectivity of the various deletion mutants, we examined the binding of 125I-AM to the receptors comprised of hCRLR complexed with the indicated mutants (Fig. 6A). As mentioned above, transfection of hCRLR into

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**FIG. 4.** Schematic representation of the N-terminal domain of wild-type and mutant hRAMP2. Residues 64–105 of wild-type RAMP2 are shown in single-letter amino acid code. The asterisks represent cysteine residues; the numbers indicate the amino acid positions in accordance with Husmann et al. (17). Solid lines denote the amino acid sequences deleted from the wild-type hRAMP2.

**FIG. 5.** FACS analysis of HEK293 cells co-expressing hCRLR with hRAMP2 or its deletion mutants. A, cell surface expression of myc-tagged hRAMP2 and its deletion mutants. HEK293 cells stably expressing hCRLR were transiently transfected with empty vector (Mock), myc-hRAMP2, or the indicated mutants. The cells were then incubated with monoclonal c-Myc antibody for 45 min at 4 °C followed by fluorescein PE-conjugated rabbit anti-mouse secondary antibody for 30 min at 4 °C. Samples incubated with secondary antibody but not c-Myc antibody served as the control. Cell surface expression of each construct was estimated by flow cytometry. The results represent the means ± S.E. of three independent experiments. *, p < 0.001 versus control.

B, representative results in cells co-expressing hCRLR with myc-hRAMP2 or one of four deletion mutants (D76–78, D79–82, D86–89, and D90–92). The number of cells was estimated by flow cytometry.
HEK293 cells, which endogenously express hRAMP2, led to production of an AM receptor, enabling AM-specific binding (199 ± 14 Bq/μg of protein). In cells co-expressing myc-hRAMP2 and hCRLR, the specific AM binding was 4.4-fold higher than in cells expressing hCRLR alone. Two of the deletion mutants, D76–78 and D79–82, were capable of supporting 125I-AM binding that was 94 and 32% of that seen with myc-hRAMP2, indicating that expression of hRAMP2, which was consistent with their level of presentation of hAM receptor.

We then assessed AM-evoked cAMP production in HEK293 cells co-expressing hCRLR and one of the 10 deletion mutants (Fig. 6B). As with myc-hRAMP2, co-expression of D76–78 with hCRLR mediated significant cAMP production in response to 100 nM AM. Similarly, D79–82 evoked a smaller but significant increase in cAMP. Consistent with the binding assays (Fig. 6A), however, the remaining mutants markedly impaired AM-evoked cAMP production, indicating residues 86–92 of hRAMP2 to be essential for agonist binding and functionality of the hAM receptor.

Further, in cells expressing D86–89, D90–92, or D86–92, the total 125I-AM binding was significantly reduced to 18, 24, and 21% of that obtained with empty expression vector, respectively (Fig. 8A), and AM-induced cAMP production was not different from that seen in cells expressing hCRLR alone (Fig. 8B). When an alanine was substituted for Trp65 (Ala65), Met88, Ile89, Ser90, Arg91, or Pro92 of myc-hRAMP2 to assess which of these residues might affect the capacity of AM to induce cAMP production, no significant change in EC50 value or response magnitude was seen (Table I), indicating that no single amino acid residue within this segment is responsible for the ligand selectivity of the AM receptor.

Characterization of hRAMP3 Deletion Mutant D59–65—We next attempted to define the structural domains of the CRLR-RAMP3 receptor, which also selectively binds AM. Note that although the sequence alignment in Fig. 9 shows residues Trp83, Cys84, and Tyr93 of hRAMP2 to be conserved in hRAMP3, the corresponding seven-amino acid segment in between shows little sequence identity. Foord and Marshall (16) hypothesized that RAMPs might directly affect ligand binding by contributing or masking binding sites, or they might enable different binding states of CRLR by allosterically modifying its conformation. With that hypothesis in mind, a myc-hRAMP3 deletion mutant, D59–65, was co-expressed with hCRLR in HEK293 cells, after which FACs analysis showed it to be present at the surface of 53.8 ± 2.4% of cells (Fig. 10). The IC50 for AM binding in cells expressing D59–65, D86–92, or empty expression vector was >1 μM under conditions where the IC50 was 54 nM in cells expressing myc-hRAMP2 (Fig. 11A). In addition, total AM binding in cells expressing D59–65 was reduced by 14–24% as compared with cells expressing empty vector, which is similar to the reduction seen in cells expressing D68–92. The specific binding of 125I-AM to cells expressing empty vector, D86–92, and D59–65 was 157, 126, and 111 Bq/μg of protein, respectively.

The levels of cAMP accumulation induced by 1 μM AM in cells expressing D59–65 was ~37% lower than that seen in expressing empty vector (Fig. 11B), indicating that hRAMP3
**TABLE I**

AM-mediated cAMP response in cells stably expressing hCRLR with co-expression of myc-hRAMP2 or mutant hRAMP2

| Plasmid DNA | cAMP production | Maximal response |
|-------------|-----------------|-----------------|
|             | EC₅₀ pmol/mg protein |                      |
| myc-hRAMP2  | 1.51 ± 0.26      | 1173 ± 48       |
| W86A        | 1.40 ± 0.50      | 1083 ± 83       |
| M88A        | 1.73 ± 0.52      | 1221 ± 133      |
| I89A        | 1.46 ± 0.42      | 1123 ± 61       |
| S90A        | 1.97 ± 0.48      | 1195 ± 83       |
| R91A        | 0.82 ± 0.17      | 1260 ± 60       |
| P92A        | 0.92 ± 0.26      | 1120 ± 10       |

**FIG. 8.** Effects of D86–89, D90–92, and D86–92 on radioligand binding and functional responses in hCRLR-expressing cells. A, total 125I-AM binding. HEK293 cells expressing hCRLR were transiently transfected with empty vector (Mock) or the indicated deletion mutant, after which they were incubated for 3 h at 4 °C with 125I-hAM (20 μM). Bars represent means ± S.E. of at least three experiments. *, p < 0.001 versus Mock. B, evoked production of cAMP. Cells co-expressing hCRLR and empty vector (Mock), D86–89, D90–92, or D86–92 were treated with the indicated concentrations of AM for 15 min at 37 °C. Values are the means ± S.E. of three experiments.

**DISCUSSION**

We have shown that seven amino acids located between three conserved residues in the extracellular N-terminal domains of hRAMP2 (86–92) and hRAMP3 (59–65) are essential for high affinity agonist binding to hAM receptors as well as for the functionality of the receptor. Previous studies have shown that RAMPs serve as accessory proteins influencing CRLR glycosylation and determining ligand specificity (7, 9), and it was proposed that mature glycosylation of CRLR in the presence of RAMP1 and core glycosylation in the presence of RAMP2 or -3 determines whether CRLR binds CGRP or AM. More recent evidence suggests that the distinct pharmacology conferred by individual RAMPs does not depend on the CRLR glycosylation state, however. For instance, unlike in mammalian cells, co-expression of CRLR with RAMP1 in Drosophila Schneider 2 cells does not affect CRLR glycosylation, although the CRLR-RAMP1 complex acted as a CGRP receptor with pharmacological properties indistinguishable from those in mammalian cells (11). Moreover, Hilariet et al. (12) showed that 125I-AM binds only to the mature glycosylated CRLR, even though the core-glycosylated forms of the receptor predominate when CRLR is co-expressed in mammalian cells with RAMP2 or -3. Those investigators also showed that, when complexed with CRLR, RAMPs are situated close to the agonist binding pocket as 125I-CGRP and 125I-AM could be cross-linked in RAMP1 and RAMP2 or -3, respectively (12), which supports the idea that the distinct pharmacology acquired by CRLR reflects the direct participation of RAMPs in ligand binding. Interestingly, the CRLR-RAMP1 complex is sensitive to peptide N-glycosidase F, while CRLR-RAMP2 and -3 are resistant to the enzyme (12). This indicates that distinct conformations of the receptor are induced by the different RAMPs, irrespective of whether an agonist is bound.

In the present study, 125I-AM binding was significantly diminished by expression of two hRAMP2 mutants, D86–89 and D90–92 (Fig. 8). However, as substituting an alanine for Trp^{86} (Ala^{87}), Met^{88}, Ile^{89}, Ser^{90}, Arg^{91}, or Pro^{92} caused no significant change on AM-evoked cAMP production and as amino acids 86–92 of hRAMP2 and 59–65 of hRAMP3 show little sequence identity, it seems unlikely that any single amino acid residue is responsible for determining selective AM binding or that AM binds directly to these sequences. More likely, these seven amino acid sequences confer selectivity by contributing directly...
to the structure of the ligand binding pocket or perhaps indirectly through allosteric modulation of the conformation of CRLR. Furthermore, as the ring structure (Cys16–Cys71) and amidation of the C-terminal residue of the AM molecule are both essential for agonist binding and receptor activation (18), the finding that expression of D86–92 both segments (residues 79–92) contributes to the cell surface, will require further experiments with endogenous hRAMP2. Consistent with our hypothesis, co-expression of hRAMP2 D86–92 or hRAMP3 D59–65 with hCRLR significantly diminished specific 125I-hAM binding and AM-evoked cAMP production. Previous studies have shown that the dominant negative activity of truncated V2 vasopressin receptors resulted from reduction of cell surface expression of the full-length receptor protein and formation of heterodimeric complexes involving the truncated and full-length forms (19). Although several studies have investigated the relative affinities of various RAMP forms for CRLR (8, 16, 17, 20), it remains unclear whether the inhibition of receptor function by dominant negative RAMP mutants is due to competitive inhibition, formation of heterodimeric complexes, or both. A detailed analysis of dominant negative effects of D86–92 and D59–65 is currently ongoing.

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**FIG. 11. Effects of hRAMP3 mutant D59–65 on competitive binding and functional responses in cells stably expressing hCRLR.** A, competitive binding of 125I-AM to HEK293 cells co-expressing hCRLR with empty vector (Mock), myc-hRAMP2, myc-hRAMP2 mutant D86–92, or myc-hRAMP3 mutant D59–65. All experiments were carried out using 20 pM 125I-hAM. Symbols represent the average of two experiments. B, evoked production of cAMP. Cells co-expressing hCRLR and empty vector (Mock), D86–89, D90–92, or D86–92 were treated with the indicated concentrations of AM for 15 min at 37 °C. Symbols are means ± S.E. of three experiments.
The Seven Amino Acids of Human RAMP2 (86) and RAMP3 (59) Are Critical for Agonist Binding to Human Adrenomedullin Receptors
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