The Functional of Extracellular Cysteines in the Human Adrenomedullin Receptor

Kenji KUWASAKO, Kazuo KITAMURA, Tomohiko UEMURA, Yasuko NAGOSHI, Johji KATO, and Tanenao ETO

The function of the cysteine (C) residues in the extracellular loops of human (h)CRLR (C212, C225 and C282) and in the extracellular domain of hRAMP2 (C68, C84, C99 and C131) was examined. Using site-directed mutagenesis, the cysteine residues were substituted, one at a time, with alanine (A). Co-expression in HEK293 cells of hRAMP2 with the hCRLR C212A or C282A mutant significantly reduced the 50% of effective concentration (EC50) for AM-evoked cyclic adenosine monophosphate (cAMP) production, despite full cell surface expression of the receptor heterodimer. Co-expression of the C225A mutant had no effect on [125I]AM binding or receptor signaling. These results suggest that the cysteine residues in the first (C212) and the second (C282) extracellular loops form a disulfide bond that is important for stabilizing the receptor in the correct conformation for ligand binding and activation. Cells expressing hCRLR with the hRAMP2 mutant (C68A, C84A, C99A or C131A) showed no specific AM binding or AM-stimulated cAMP accumulation. Though abundant in the intracellular compartment, these receptors were not detected at the cell surface, suggesting that all four cysteine residues are essential for efficient transport to the plasma membrane. Cysteine residues in the extracellular loops of hCRLR and in the extracellular domain of hRAMP2 thus appear to play distinct roles in the cell surface expression and function of the receptor heterodimer.

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tion (11), suggests CRLR is a member of the GPCR superfamily. Consistent with that idea, the 461-amino acid human (h)CRLR contains seven transmembrane domains and three cysteine (C) residues in the first (C212 and C225) and the second (C282) extracellular loops. In addition, the 160-amino acid RAMPs possess a short cytoplasmic domain, a single transmembrane domain and a large extracellular domain containing 4–7 cysteine residues (12). In the present study, we used site-directed mutagenesis to substitute, one at a time, the cysteine residues in the extracellular loops of hCRLR or the extracellular domain of hRAMP2 with the aim of determining their respective roles in the structure and function of the AM receptor.

**Methods**

**Materials**

[^125I]hAM (specific activity 2 µCi/pmol) was produced in our laboratory (13). hAM was purchased from Peptide Institute (Osaka, Japan). Mouse fluorescein isothionate (FITC)-conjugated monoclonal anti-myc antibody (anti-myc-FITC antibody) was from Invitrogen (Carlsbad, USA). All other reagents were of analytical grade and obtained from various commercial suppliers.

**Expression Constructs**

hCRLR (14) and hRAMP2 (10) were modified to provide a consensus Kozak sequence as previously described (14). Initially, a myc epitope tag (EQKLISEEDL) was ligated, in-frame, to the 5’ end of the hCRLR and hRAMP2 cDNAs, and the native signal sequences were removed and replaced with MKTILALSTYIFCLVFA (15). The myc-hCRLR and -hRAMP2 were then cloned into pCAGGS/Neo expression vectors using the 5’ XhoI and 3’ NotI sites to generate pCAGGS-hCRLR and -hRAMP2 (11).

Single amino acid substitutions were carried out using a Quick Change kit (Stratagene, La Jolla, USA) according to the manufacturer’s instructions. pIRES-myc-hCRLR and -hRAMP2, which were constructed by subcloning the coding sequence of myc-hCRLR and -hRAMP2 into pIRES1/Neo (CLONTECH, Palo Alto, USA), served as the template. For each mutation, two complementary 30-mer oligonucleotides (sense and antisense) were designed to contain the desired mutation in their middle. To allow rapid screening of mutated clones, the primers carried an additional silent mutation that introduced or removed a restriction site. The presence of each mutation of interest and the absence of undesired ones was confirmed by DNA sequencing, myc-hCRLR and -hRAMP2 mutants were then cloned into pCAGGS/Neo.

The products from polymerase chain reaction were sequenced using an Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, Foster City, USA).

**Cell Culture and DNA Transfection**

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B at 37ºC under a humidified atmosphere of 95% air/5% CO2. For experimentation, cells were seeded onto 24-well plates and, upon reaching 70% confluence, were transiently co-transfected with hRAMP2 and myc-hCRLR or one of its mutant expression constructs, or with hCRLR and myc-hRAMP2 or one of its mutants, using Lipofectamine transfection reagents (Invitrogen) according to the manufacturer’s instructions. Briefly, the cells were incubated for 3 h in 250 µl OptiMEM 1 medium containing 200 ng/well plasmid DNA and 2 µl/well Lipofectamine. As a control, some cells were transfected with an empty vector (pCAGGS/Neo). All experiments were performed 48 h after transfection.

**Fluorescence-Activated Cell-Sorting (FACS) Analysis**

Flow cytometry was performed to assess the levels of whole cell and cell surface expression of myc-hCRLR, myc-hRAMP2 and their respective point mutants in HEK293 cells. To evaluate cell surface expression, following transient transfection, cells were harvested, washed twice with phosphate-buffered saline (PBS), resuspended in ice-cold FACS buffer (11), and then incubated for 60 min at 4ºC in the dark with anti-myc-FITC antibody (1:1,000 dilution). For evaluation of whole cell expression, cells were permeabilized using IntraPrep™ reagents (Beckman Coulter, Fullerton, USA) according to the manufacturer’s instructions, after which they were incubated for 15 min at room temperature in the dark with anti-myc-FITC antibody (1:1,000 dilution). Following two successive washes with FACS buffer, both groups of cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter) and analyzed using EXPO 2 software (Beckman Coulter).

**Radioligand Binding Assays**

To assess whole-cell radioligand binding, transfected HEK293 cells in 24-well plates were washed twice with warmed PBS and incubated for 20 min at 37ºC with 0.1% bovine serum albumin (BSA) in PBS to reduce endogenous AM binding, after which the remaining adherent cells were washed with ice-cold PBS. The cells were then incubated for 6 h at 4ºC with 20 pmol/l[^125I]hAM in the presence (for nonspecific binding) or absence (for total binding) of 1 µmol/l unlabeled hAM in modified Krebs-Ringers-N-2-hydroxyethylpiperezine-N'-2-ethane sulfonic acid (HEPES) medium (11). Thereafter, the cells were washed twice with ice-cold PBS and harvested with 0.5 mol/l NaOH. The associated cellular radioactivity was measured in a γ-counter. Specific binding was defined as the difference between total binding and non-
specific binding.

**cAMP Measurements**

Cells were exposed to hAM in Hanks' buffer containing 20 mmol/l HEPES, 0.1% BSA and 0.5 mmol/l 3-isobutyl-1-methylxanthine (Sigma Chemical, St. Louis, USA) for 15 min at 37°C, after which the reactions were terminated by addition of lysis buffer (Amersham Biosciences, Buckinghamshire, UK). The resultant lysates were centrifuged at 2,000 rpm for 10 min at 4°C, and the cAMP content in samples of the supernatants was assayed using a commercial enzyme immunoassay kit according to the manufacturer’s (Amersham) instructions for a non-acetylation protocol.

**Statistical Analysis**

Results are expressed as the means ± SEM 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 to indicate statistical significance.

**Results**

**Whole Cell and Cell Surface Expression of hCRLR Mutants**

In this study, we used HEK293 cells for gene transfection because they express only low levels of hRAMP2 and hCRLR mRNA (expression ratio = 25:1), and they lack functional AM receptors (16).

We initially analyzed the whole cell expression of epitope-tagged hCRLR mutants in permeabilized cells using FACS (Fig. 1A). Surface and intracellular immunoreactivity was detected in only 1.7 ± 0.18% of cells expressing the empty vector (Mock), which was well within the 2% limit of resolution characteristic of FACS analysis. When expressed alone or with hRAMP2, myc-hCRLR was detected in 23.7 ± 0.18% and 19.8 ± 1.2% of cells, respectively. Likewise, co-expression of hRAMP2 with an hCRLR C212A, C225A or C282A mutant led to their full expression in 19–24% of cells. Thus, the transfection efficacy for the mutant receptor was comparable to that for myc-hCRLR/hRAMP2.

We next analyzed the cell surface expression of these hCRLR mutants in non-permeabilized cells (Fig. 1B). The empty vector and myc-hCRLR appeared at the surface of 1.5 ± 0.35% and 13.3 ± 0.79% of cells, respectively. When hRAMP2 was co-expressed with an hCRLR C212A, C225A or C282A mutant, cell surface immunoreactivity was detected in 9–19% of cells, which again was similar to myc-hCRLR/hRAMP2.

**Radioligand Binding to hCRLR Mutant Receptors**

Analysis of the binding of 20 pmol/l [125I]AM to receptors comprised of hRAMP2 complexed with the indicated mutant revealed no remarkable differences among cells expressing the empty vector or myc-hCRLR (Fig. 2). On the other hand, in cells co-expressing hRAMP2 and myc-hCRLR, C212A, C225A or C282A, the specific AM binding was 85–95-fold higher than in control cells (Mock).
The EC<sub>50</sub> for AM-induced cAMP production in intact HEK293 cells was 2.0 × 10⁻⁷ mol/l (Fig. 3A), which is indicative of the absence of functional AM receptors in this cell type. Virtually identical responses were obtained with cells expressing myc-hCRLR alone (Fig. 3A). This is in contrast to our earlier finding that AM-induced cAMP production was 2-fold higher in HEK293 cell transfectants stably expressing CRLR than in intact cells (11). Most likely this difference is largely explained by differences in transfection efficacy between transient and stable transfectants.

In cells co-expressing hRAMP2 and myc-hCRLR or

**cAMP Production Mediated via hCRLR Mutant Receptors**

The EC<sub>50</sub> for AM-induced cAMP production in intact HEK293 cells was > 2.0 × 10⁻⁷ mol/l (Fig. 3A), which is indicative of the absence of functional AM receptors in this cell type. Virtually identical responses were obtained with cells expressing myc-hCRLR alone (Fig. 3A). This is in contrast to our earlier finding that AM-induced cAMP production was 2-fold higher in HEK293 cell transfectants stably expressing CRLR than in intact cells (11). Most likely this difference is largely explained by differences in transfection efficacy between transient and stable transfectants.

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C225A, AM elicited concentration-dependent increases in cAMP that reached levels nearly 80-fold greater than in cells expressing the empty vector, and there was no apparent difference in potency: the EC50 for AM was $6.2 \times 10^{-10} \text{mol/l}$ with both cell types (Fig. 3B). Cells expressing hRAMP2 and C212A or C282A responded comparatively weakly to AM in the concentration range tested: the EC50 values were $6.8 \times 10^{-8} \text{mol/l}$ and $2.2 \times 10^{-8} \text{mol/l}$, respectively (Fig. 3B).

### Whole Cell and Cell Surface Expression of hRAMP2 Mutants

Figure 4A shows the whole cell expression of epitope-tagged hRAMP2 mutants in permeabilized cells detected using FACS. The frequency of immunoreactivity in cells expressing the empty vector, myc-hRAMP2 or myc-hRAMP2/hCRLR was 1.5 $\pm$ 0.32%, 27.7 $\pm$ 1.4% and 40.6 $\pm$ 1.9%, respectively. When co-expressed with hCRLR, the hRAMP2 C68A, C84A, C99A or C131A mutant was detected in 24–29% of cells. By contrast, cell surface immunoreactivity was detected in <10% of cells (Fig. 4B), suggesting that these four cysteine residues in the extracellular domain of hRAMP2 are all involved in the cell surface expression of the hRAMP2/hCRLR heterodimer. The empty vector, myc-hRAMP2 and myc-hRAMP2/hCRLR appeared at the surface of 1.9 $\pm$ 0.08%, 13.1 $\pm$ 0.64% and 30.2 $\pm$ 1.4% of cells, respectively.

hRAMP2 also contains a cysteine residue in its one transmembrane domain (C155). When we analyzed the immunoreactivity of non-permeabilized and permeabilized cells co-expressing hCRLR and the hRAMP2 C155A mutant, we found that the mutant receptor was fully expressed at the cell surface and in the intracellular compartment at a level comparable with myc-hRAMP2/hCRLR (Fig. 4A and B).

### Radioligand Binding to hRAMP2 Mutant Receptors

Analysis of [$^{125}$I]AM binding revealed no remarkable differences among cells expressing the empty vector or myc-hRAMP2 alone (Fig. 5). The specific AM binding in cells expressing hCRLR and myc-hRAMP2 or C155A was 50–60-fold greater than that in Mock, while binding in cells expressing the other mutants (C68A, C84A, C99A and C131A) was $\leq 5$-fold greater than in Mock.

### cAMP Production Mediated via hRAMP2 Mutant Receptors

We found no remarkable differences in AM-evoked cAMP production among cells expressing empty vector (Mock), myc-hRAMP2 alone, or one of the four hRAMP2 mutants (C68A, C84A, C99A and C131A) together with hCRLR (Fig. 6A). By contrast, cells expressing C155A and hCRLR readily responded to AM, and the EC50 of $5.4 \times 10^{-10} \text{mol/l}$ was comparable to that obtained with cells expressing myc-hRAMP2/hCRLR (EC50 $= 1.6 \times 10^{-9} \text{mol/l}$) (Fig. 6B). Apparently, C155, situated in the transmembrane domain of hRAMP2, is not involved in the cell surface expression or function of the AM receptor.
Discussion

Previous pharmacological and biochemical analyses of mutant GPCRs in which the cysteine residues in the first and second extracellular loops were replaced by serine or alanine indicated that the substituting amino acid had little effect on the expression and function of platelet-activating factor receptor (2) and vasopressin V2 receptor (5). On the other hand, with rhodopsin, another seven-transmembrane-domain GPCR, replacement of the two cysteine residues in the first and the second extracellular loops with serine disrupted the tertiary structure and reduced the stability of the receptor protein (17). This was probably due to the hydrophilic nature of the serine side chain; indeed, when the two cysteine residues were replaced with alanines, the tertiary structure of the resultant rhodopsin mutant appeared to be almost normal (18). Therefore, in order to minimize the effects of the substitution on proper protein folding, we decided to replace the extracellular cysteine residues of hCRLR and hRAMP2 with alanine rather than with serine.

Most seven-transmembrane-domain GPCRs contain a pair of cysteine residues in the first and second extracellular loops that are linked via a disulfide bond (1–9). With respect to platelet-activating factor receptor (2), µ-opioid receptor (3), M3 muscarinic acetylcholine receptor (4), and vasopressin V2 receptor (5), the extracellular disulfide bonds appear necessary for proper transport of the receptor to the plasma membrane i.e., the mutant receptors failed to appear at the cell surface. In the present study, by contrast, co-expression of hRAMP2 with an hCRLR mutant in which the cysteine residues in the first (C212) and the second (C282) extracellular loops were substituted with alanine resulted in full expression of the receptor heterodimer at the cell surface (Fig. 1B) and high-affinity AM binding comparable to that seen with unmutated CRLR/RAMP2 (Fig. 2); nevertheless, AM-induced cAMP production was significantly diminished (Fig. 3B). This finding of diminished receptor function is similar to that obtained with thyrotropin receptor (5), β-adrenergic receptor (7), corticotropin-releasing factor receptor (8) and thromboxane A2 receptor (9). The fact that mutation of C225 in the first extracellular loop of hCRLR had no effect on [125]IAM binding or receptor function (Figs. 2 and 3B) suggests that C212 and C282 form a disulfide bond that is crucial for maintaining proper receptor functionality. Still, we cannot rule out the possibility that C212 and C282 link to a cysteine in the large extracellular domain of hCRLR, hRAMP2 or both.

When co-transfected with hCRLR, none of the four hRAMP2 mutants tested (C68A, C84A, C99A and C131A) appeared at the cell surface (Fig. 4B). The abundantly produced mutant receptors remained in the intracellular compartment (Fig. 4A) and showed no specific ligand binding or signaling capability (Figs. 5 and 6A). Apparently, all four of the hRAMP2 cysteine residues tested are essential for the proper delivery of the receptor proteins to the cell surface membrane, which is consistent with previous findings that mutation of the participating cysteine residues drastically reduced the maturation and transport of the proteins from the endoplasmic reticulum due to misfolding and aggregation (19). We recently reported that the seven-amino acid segment between the second and third cysteine residues in human and rat RAMP2 most likely contributes to the structure of the ligand-binding pocket, but not to the ligand-binding sites (20, 21). This means that C84 and C99 are probably the residues involved in forming a disulfide bond. Additional experiments will be needed to clarify whether disulfide bonds are also formed within the RAMP2 molecule or between RAMP2 and CRLR.

In summary, we have shown that C212 and C282 likely form a disulfide bond critical to the structure and function of the hCRLR. We have also shown that substituting any one of four cysteine residues (C68, C84, C99 and C131) in hRAMP2 yields an apparently normal receptor protein that is not transported to the cell surface. Cysteine residues in the extracellular loops of hCRLR and in the extracellular domain of hRAMP2 thus appear to play distinct roles in the cell surface expression and function of AM receptors.

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