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Functions of the extracellular histidine residues of receptor activity-modifying proteins vary within adrenomedullin receptors

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ABSTRACT

Receptor activity-modifying protein (RAMP)-2 and -3 chaperone calcitonin receptor-like receptor (CRLR) to the plasma membrane, where together they form heterodimeric adrenomedullin (AM) receptors. We investigated the contributions made by His residues situated in the RAMP extracellular domain to AM receptor trafficking and receptor signaling by co-expressing hCRLR and V5-tagged-hRAMP2 or -3 mutants in which a His residue was substituted with Ala in HEK-293 cells. Flow cytometric analysis revealed that hRAMP2-H71A mediated normal hCRLR surface delivery, but the resultant heterodimers showed significantly diminished [^{125}I]AM binding and AM-evoked cAMP production. Expression of hRAMP2-H124A and -H127A impaired surface delivery of hCRLR, which impaired or abolishing AM binding and receptor signaling. Although hRAMP3-H97A mediated full surface delivery of hCRLR, the resultant heterodimers showed impaired AM binding and signaling. Other His residues appeared uninvolved in hCRLR-related functions. Thus, the His residues of hRAMP2 and -3 differentially govern AM receptor function.

Adrenomedullin (AM) is a potent vasodilator that has also been shown to exert powerful antioxidative and antiatherosclerotic effects [1,2]. Like calcitonin gene-related peptide (CGRP), AM belongs to the calcitonin superfamily of regulatory peptides [3,4]. It exerts its effects via two receptors, which along with a CGRP receptor were identified thanks to the discovery of a novel accessory protein, receptor activity-modifying protein (RAMP) [5]. Three RAMP isoforms (RAMP1, -2, and -3) have been identified in mammals [5,6]. All are comprised of ~160 amino acids and exhibit a common structure consisting of a large extracellular N-terminal domain, a single transmembrane-spanning domain and a very short cytoplasmic C-terminal tail. Despite these similarities, however, the three isoforms share less than 30% sequence identity [5,6]. One function of RAMP is to act as a chaperone, associating with calcitonin receptor-like receptor (CRLR) [7], probably within the endoplasmic reticulum (ER) [5,8], and mediating its transport to the cell surface, where the CRLR/RAMP heterodimer forms a functional receptor. Co-expression of CRLR and RAMP1 produces the CGRP-(8–37)-sensitive $CGRP_1$ receptor, while CRLR plus RAMP2 produces the highly AM-specific AM_1 receptor [9,10]. RAMP3 also enables CRLR to act as an AM receptor (AM_2 receptor), but its specificity for AM is not high [9,10]. All three heterodimeric receptors can mediate agonist-evoked cAMP production and Ca^{2+} mobilization [5,11].

The three RAMPs also differ with respect to their expression levels in tissues and are differentially affected by various pathological conditions [5,6,10], suggesting that each RAMP has a distinct function *in vivo*. Indeed, considerable attention has been paid to the structural and pharmacological differences among RAMPs. In particular, the functions of the extracellular domains of RAMPs have been extensively studied (for review, see Refs. [12–13]). These domains have been shown to not only assist in the folding and export of CRLR from intracellular compartments such as the ER and golgi, but also may define AM vs. CGRP specificity at the plasma membrane. For instance, we previously identified a region common to the extracellular domains of RAMP2 and -3 that is essential for formation of the AM binding pocket, though it is not part of the binding site itself [14–16]. That said, the residues within the RAMP extracellular domain that are directly involved in ER export and agonist binding to the two AM receptor subtypes are still not fully determined. Notably, hRAMP2 and -3 possess 4 and 2 His residues, respectively, all of which are present in their extracellular domains. Moreover, His124 of hRAMP2 and His97 of hRAMP3 are conserved in several mammal species tested [12]. These findings suggest that extracellular His residues may be key determinants of hCRLR function. To better understand their contribution to RAMP activity, we examined the effects of individual His → Ala

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substitutions in hRAMP2 and -3 on the function of hCRLR exogenously expressed in HEK-293 cells.

Materials and methods

Regents and antibodies. [^{125}I]hAM (specific activity 5 μCi/pmol) was produced in our laboratory [1]. Human AM was kindly donated by Shionogi & Co. (Osaka, Japan). Mouse anti-V5 antibody and FITC-conjugated mouse anti-V5 monoclonal antibody (anti-V5-FITC antibody) were purchased from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers.

Expression construct. Human RAMP2 and -3 were modified to provide a consensus Kozak sequence, and their native signal sequences were replaced with the influenza hemagglutinin signal sequence (MKTILALSTYIFCLVFA) [11]. To facilitate cell surface detection of hRAMP2, a double V5 epitope tag (GKPIPNPLLGLDST) was inserted between amino acids 55 and 56 in the hRAMP2 N-terminus, downstream of the signal sequence [17], yielding V5-tagged-hRAMP2 (V5-hRAMP2). Double V5-tagged hRAMP3 (V5-hRAMP3) has been described previously [18]. V5-hRAMP2 and -3 were cloned into the mammalian expression vector pCAGGS/Neo [11] using the 5′ XhoI and 3′ NotI sites. The sequences of the resultant constructs were all verified using an Applied Biosystems 310 Genetic Analyzer. V5-hRAMP2 and -3 were compared to native sequences in the assays and found to behave identically (data not shown).

Each extracellular His residue of hRAMP2 and -3 (Fig. 1) was substituted with an Ala using a site-directed mutagenesis kit (Quik Change®, Stratagene) according to the manufacturer's instructions, with pIRES-V5-hRAMPs serving as the template. All constructs were confirmed by DNA sequencing. Individual V5-hRAMP mutants were then cloned into pCAGGS/Neo.

Cell culture and DNA transfection. Human embryonic kidney (HEK)-293 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 0.25 mg/ml G 418 at 37 °C under a humidified atmosphere of 95% air/5% CO_2. For experimentation, cells were seeded into 12- or 24-well plates and, upon reaching 70–80% confluence, were transiently transfected with the indicated cDNAs using LipofectAMINE transfection reagents (Invitrogen) according to the manufacturer's instructions. As a control, some cells were transfected with empty vector (pCAGGS/Neo) (*Mock*). All of the experiments were performed 48 h after transfection.

Fluorescence-activated cell-sorting (FACS) analysis. To evaluate cell surface expression of the indicated V5-hRAMP along with hCRLR, cells grown in 12-well plates were harvested following transient transfection, washed twice with PBS, resuspended in ice-cold FACS buffer [11], and then incubated for 60 min at 4 °C in the dark with FITC-conjugated anti-V5 antibody (1:500 dilution). For evaluation of intracellular and/or surface expression of the indicated V5-hRAMP, HEK-293 cells were first permeabilized using IntraPrep™ reagents (Beckman Coulter) according to the manufacturer's instructions and then incubated with anti-V5-FITC antibody (1:500 dilution) for 15 min at room temperature in the dark. Following two successive washes, both groups of cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter) [11].

Whole-cell radioligand binding assay. HEK-293 cells transfected in 24-well plates were washed twice with prewarmed PBS and then incubated for 4 h at 4 °C with 20 pM [^{125}I]hAM in the presence (for nonspecific binding) or absence (for total binding) of 1 μM unlabeled hAM in modified Krebs–Ringers–Hepes medium [11]. The cells were then washed twice more with ice-cold PBS and solubilized with 0.5 M NaOH. The resultant cell lysate was collected, and the associated cellular radioactivity was measured in a γ-counter.

Measurement of intracellular cAMP. In Hanks' buffer containing 20 mM Hepes and 0.2% bovine serum albumin, transfected cells were exposed to the indicated concentrations of hAM for 15 min at 37 °C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). The reactions were terminated by addition of lysis buffer (Amersham Biosciences), after which the cAMP content was determined using a commercial enzyme immunoassay kit according to the manufacturer's instructions (Amersham) for the non-acetylation protocol.

Statistical analysis. Results are expressed as means ± SEM of at least three independent experiments. Differences between two groups were evaluated using Student's *t* tests; differences among multiple groups were evaluated using one-way analysis of variance followed by Scheffe's tests. Values of $p < 0.05$ were considered significant.

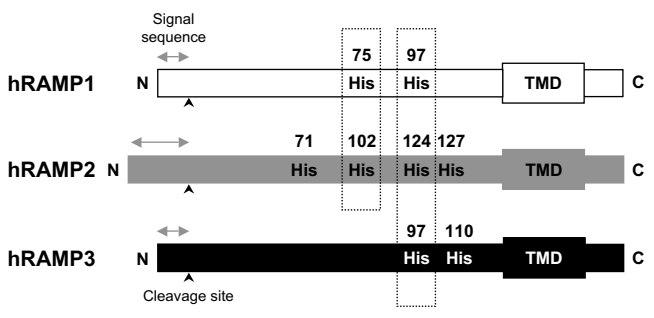

Fig. 1. Positions of extracellular His residues of the three hRAMPs. Each hRAMP possesses a large extracellular N-terminal domain containing signal sequence and cleavage site, a single transmembrane-spanning domain and a very short cytoplasmic C-terminal tail. Each contains 2 or 4 histidine (His) residues located in its extracellular domain. *Hatched boxes* indicate conserved His residues: His97 of hRAMP1, His124 of hRAMP2, and His97 of hRAMP3.

Results

Total and cell surface expression of wild-type or mutant hRAMP in the absence and presence of hCRLR

We previously showed that HEK-293 cells express only low levels of endogenous hCRLR, hRAMP1, and -2 [14]. Moreover, AM elicits no increase in intracellular cAMP in these cells, even when they are transfected with hCRLR (Fig. 4A) [19] or hRAMP2 [19], indicating they lack functional hRAMPs and hCRLR. We therefore used HEK-293 cells as a model with which to study the effects of transfecting AM receptor component genes.

We initially used FACS to analyze the total expression of V5 epitope-tagged hRAMP mutants in permeabilized cells (Fig. 2A). Surface and intracellular immunoreactivity were detected in only 1.44% of cells transfected with empty vector (control), which was well within the 2% limit of resolution characteristic of FACS analysis. This immunoreactivity was not affected by co-transfection of hCRLR. By contrast, when expressed alone or together with hCRLR, FITC-labeled hRAMP2 was detected in 19.2% or 35.1% of cells, respectively. Very similar results were observed when cells were transfected with hRAMP2-H71A or -H102A, with or without hCRLR i.e., substituting His71 or His102 with Ala had little or no effect on hRAMP2 expression, with or without co-transfection of hCRLR. Similarly, hRAMP3 and its H97A and H110A mutants were detected in 37–40% of cells when expressed alone, and their total expression increased to 48–52% when they were co-expressed with hCRLR. Thus, the transfection efficacies of all the mutants were comparable to those for the corresponding wild-type

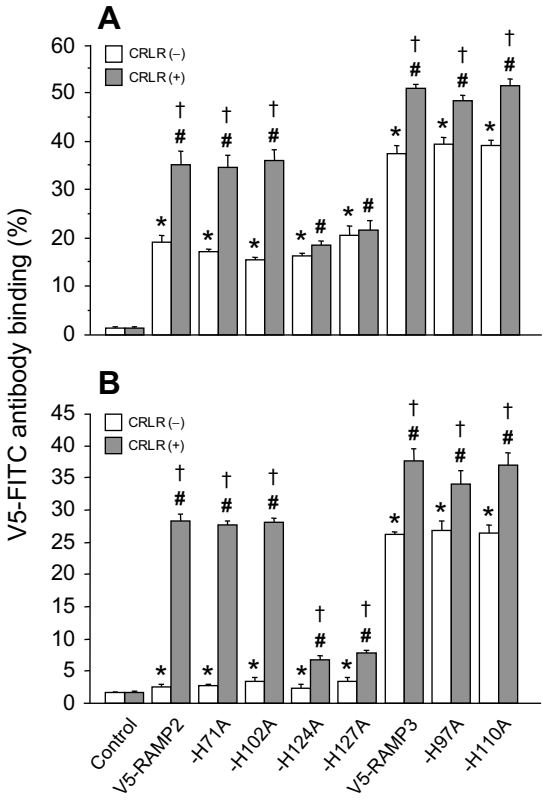

Fig. 2. FACS analysis of total and cell surface expression of V5-hRAMP following transfection into HEK-293 cells, with or without hCRLR. (A) Total expression of the indicated V5-tagged proteins. Forty-eight hours after transfection, cells were permeabilized with IntraPrep™ reagents and then incubated for 15 min at room temperature with monoclonal anti-V5-FITC antibody; Mock incubation with the antibody served as the control. Levels of each FITC-labeled V5-hRAMP protein expressed in the cytoplasm and/or at the cell surface were estimated by flow cytometry. (B) Cell surface expression of the indicated V5-tagged proteins in intact cells. Following transfection, cells were incubated for 1 h at 4 °C with monoclonal anti-V5-FITC antibody; Mock incubation with the antibody served as the control. Conventions are the same as in panel A. In both panels bars represent means ± SEM of three independent experiments. $^{*}p < 0.0001$ vs. empty vector; $^{\#}p < 0.0001$ vs. hCRLR alone; $^{\dagger}p < 0.004$ vs. corresponding CRLR (−).

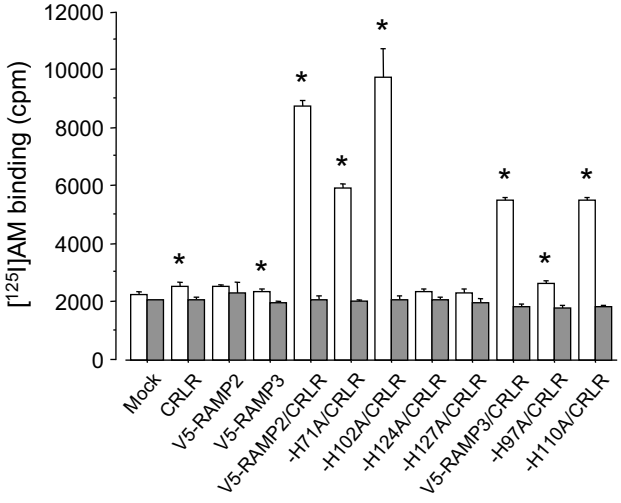

Fig. 3. [^{125}I]AM binding to HEK-293 cells co-expressing hCRLR with wild-type or mutant hRAMP. Total (open bars) and nonspecific (gray bars) binding are shown. Cells were transfected with the indicated V5-tagged genes and then incubated for 4 h at 4 °C with [^{125}I]AM (20 pM) in the presence or absence of 1 μM unlabeled AM. Bars represent means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. corresponding nonspecific binding.

hRAMPs. Consistent with an earlier report [18], immunocytochemical analysis revealed that almost all the mutants were located in the ER (not shown).

We next analyzed the cell surface expression of these mutants in intact cells (Fig. 2B). Surface immunoreactivity was detected in only 1.60% of cells transfected with empty vector, and that was unaffected by co-transfection of hCRLR. Upon transfection of hRAMP2 alone, surface expression of the protein was detected in 2.49% of cells. Likewise, its mutants appeared at the surface of 2.4–3.4% of cells in the absence of hCRLR. Co-transfection of hCRLR led to marked increases in surface expression of hRAMP2 and its H71A and H102A mutants (~28% of cells). By contrast, hRAMP2-H124A and -H127A appeared at the surface of only 6.81% and 7.74% of cells, respectively, when co-transfected with hCRLR. Surface expression of hRAMP3, hRAMP3-H97A and -H110A was detected in ~26% of total cells, even when expressed alone; co-transfection of hCRLR significantly increased their surface expression to 34–38% of cells.

[^{125}I]AM binding to receptors comprised of hCRLR and hRAMP point mutants

We next evaluated the binding of [^{125}I]AM to cells co-expressing hCRLR with wild-type or mutant hRAMP (Fig. 3). There were no remarkable differences among cells transfected with empty vector, hCRLR or hRAMP2 or -3. In cells co-expressing hCRLR with hRAMP2, the specific AM binding was ~35-fold higher than in control cells (Mock) (IC_{50} = 3.6 nM). The greatest AM binding was observed in cells co-expressing hCRLR with hRAMP2-H102A (IC_{50} = 7.4 nM). Unexpectedly, the maximum specific binding of AM to cells co-transfected with hCRLR and hRAMP2-H71A (IC_{50} = 15.1 nM) was ~40% lower than that seen with hRAMP2, though there was no difference in the level of surface expression of the two heterodimeric receptors (Fig. 2B). By contrast, specific AM binding in cells co-expressing hRAMP2-H124A or -H127A did not differ from control. Co-transfection of hRAMP3 or hRAMP-H110A significantly increased the specific binding of AM to hCRLR-transfected cells (IC_{50} = 36.7 nM and 58.3 nM, respectively). However, co-transfection of hRAMP3-H97A led to a marked reduction in the specific [^{125}I]AM binding, despite full surface expression of the receptors (Fig. 2B).

Receptor function of dimers comprised of hCRLR plus hRAMP point mutants

When we further characterized the receptors by assessing their capacity to mediate AM-evoked cAMP production, we found that AM elicited little or no cAMP production in HEK-293 cells expressing hCRLR alone (Fig. 4A), confirming that the cells used in this study endogenously express no functional hRAMP proteins. By contrast, AM elicited marked accumulation of cAMP in cells co-transfected with hCRLR plus hRAMP2 (EC_{50} = 0.32 nM) or hRAMP2-H102A (EC_{50} = 0.37 nM) (Fig. 4B). The concentration–response relationship for cells expressing hCRLR/hRAMP2-H71A was shifted significantly rightward, as compared to curve for cells expressing CRLR/hRAMP2 (EC_{50} > 100 nM) (Fig. 4B), but there was no significant difference in their maximal responses. hRAMP2-H124A and -H127A did not mediate increases in cAMP accumulation beyond that seen with hCRLR alone (Fig. 4B). AM also elicited marked increases in cAMP (EC_{50} = 0.28 nM) in cells co-expressing hCRLR with hRAMP3 (Fig. 4C). Very similar responses were also seen with hRAMP3-H110A (EC_{50} = 0.31 nM) (Fig. 4C). In cells co-transfected with hRAMP3-H97A, the EC_{50} value for AM (1.68 nM) was about

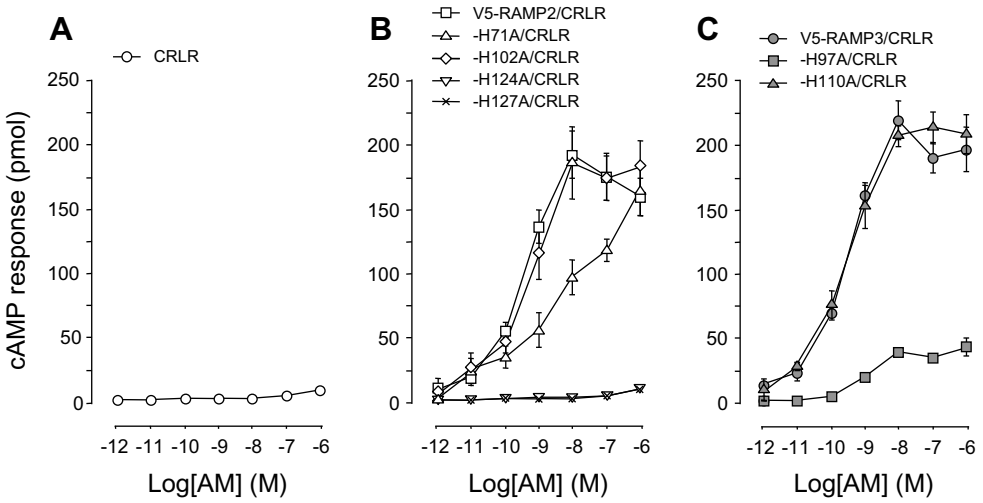

Fig. 4. AM-evoked cAMP production in HEK-293 cells co-expressing hCRLR with wild-type or mutant hRAMP. Cells were transfected with hCRLR plus empty vector (A), hCRLR plus V5-hRAMP2 or its point mutant (B) or hCRLR plus V5-hRAMP3 or its point mutant (C), stimulated with the indicated concentrations of AM for 15 min at 37 °C and then lysed. The resultant lysates were analyzed for cAMP content. Bars represent means ± SEM of four independent experiments.

6-fold higher than that for wild-type hRAMP3, and the maximal responses were diminished by ~80% (Fig. 4C).

Discussion

Attachment of various short epitope-tags to the N-terminus of hRAMPs enables one to assess not only their intracellular and cell surface expression but also their interaction with hCRLR. In previously reported immunocytochemical and FACS analyses, co-transfection of CRLR led to significant increases in the cell surface expression of all epitope-tagged RAMPs [14,16,17,19,20], which is consistent with the targeting of CRLR/RAMP heterodimers to the cell surface. The present FACS analysis revealed that surface delivery of all the hRAMP2 and -3 mutants was also significantly increased by co-transfection with hCRLR, and when expressed alone, the point mutants appeared at the cell surface to the same degree as the corresponding wild-type hRAMP2 and -3 (hRAMP3 > hRAMP2). Surface expression of RAMP2 and -3 without co-transfection of CRLR is also observed in *Xenopus* oocytes [21], COS-7 cells [17,20] and other group's HEK-293 cells [17], despite the fact that they all possess no functional endogenous CRLR. Our previous study showed that AM elicited little or no cAMP production in HEK-293 cells transfected with hRAMP2 alone [19], most likely because these cells lack functionally relevant endogenous hCRLR. The surface expression of wild-type and mutant hRAMPs in the absence of hCRLR may reflect their self-transport to the cell surface [21], or their interaction with other G protein-coupled receptors (e.g., receptors able to interact particularly with hRAMP3) [12,17].

We found that specific [^{125}I]AM binding and maximal AM-evoked cAMP production were markedly diminished in cells expressing hCRLR/hRAMP3-H97A, despite full surface expression of the mutant heterodimers. This His residue is conserved in chimpanzees, monkeys, pigs, dogs, rats, and mice [12], which suggests that His97 participates in the shared function of RAMP3 and that its side chain is crucially involved in efficient binding of AM to AM_2 receptors. We also found that the specific binding of AM to cells expressing hCRLR/hRAMP2-H71A was significantly impaired, though the maximal evoked accumulation of cAMP was unaffected, and there was no difference in the cell surface expression of the wild-type and mutant hCRLR/hRAMP2 heterodimers. Unlike His97 of hRAMP3, His71 of hRAMP2 is not conserved among the aforementioned mammals [12], so it is likely the respective side chains of the two residues are differently situated. Our findings suggest that as a result the contribution made by His71 of hRAMP2 to efficient AM binding to AM_1 receptors is smaller than that made by His97 of hRAMP3 to AM_2 receptors.

CRLR is not transported to the cell surface in the absence of RAMP [2,5,6], and in the present study cell surface expression of hCRLR was markedly reduced when it was co-transfected with hRAMP2-H124A or -H127. That total expression of these mutants was comparable to that of wild-type hRAMP2 in the absence of exogenous hCRLR suggests that the reduction in their cell surface expression was not due to diminished synthesis of the mutants or to enhanced degradation (e.g., due to misfolding). It should be noted that, in the absence of hCRLR, both mutants appeared at the cell surface at levels similar to those of wild-type hRAMP2, which suggests there was little or no impairment of their surface delivery, despite the absence of the receptor. These two mutations also impaired or abolished cAMP accumulation mediated via hCRLR. That both His124 and His127 are conserved among mammals [12] suggests that within hRAMP2 the side chains of both residues are involved in efficient surface expression of hCRLR (probably through interaction with hCRLR), as well as in AM binding and signaling in hCRLR/hRAMP2 heterodimers.

The assumption that conserved amino acid motifs contribute to functions of GPCRs implies that the side chains of those amino acids have similar functions in different GPCRs [22,23]. In the present study, however, the conserved His residues of hRAMP2 and -3 (His124 and His97, respectively) exerted differing effects on hCRLR function. His97 is also conserved at position 97 in the hRAMP1 extracellular domain (Fig. 1). Cells co-expressing hRAMP1-H97A with hCRLR showed marked increases in CGRP-stimulated cAMP production comparable to those seen in hCRLR/hRAMP1, though the cell surface expression of hCRLR/hRAMP1-H97A was only about 70% of that seen with the wild-type heterodimers [14]. Similarly, the highly conserved Ser-Lys sequence within the cytoplasmic C-terminal tails of hRAMP2 and -3 played distinct roles in the surface delivery and internalization of hCRLR, whereas that of hRAMP1 had no effect on hCRLR trafficking and function [18]. The mechanism underlying the distinct effects of the conserved His residues, as well as those of the Ser-Lys sequence, on the cellular trafficking and function of these two AM receptors remains to be determined.

hRAMP1 contains another His residue at position 75 in its extracellular domain (Fig. 1). Deleting residues 74–76 of hRAMP1 led to an approximately 30% reduction in surface expression of hCRLR and its activation [14]. Apparently, the His residues of hRAMP1, unlike those of hRAMP2 and -3, do not participate in hCRLR trafficking and signaling.

In conclusion, we found that His124 and His127 of hRAMP2 are both crucial for transport of AM_1 receptors to the plasma membrane, and that His97 of hRAMP3 is important for AM binding and receptor signaling, but not for surface delivery of AM_2 receptors. Thus, the His residues of hRAMP2 and -3, even those that are conserved, differentially govern the function of their respective AM receptors.

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