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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.

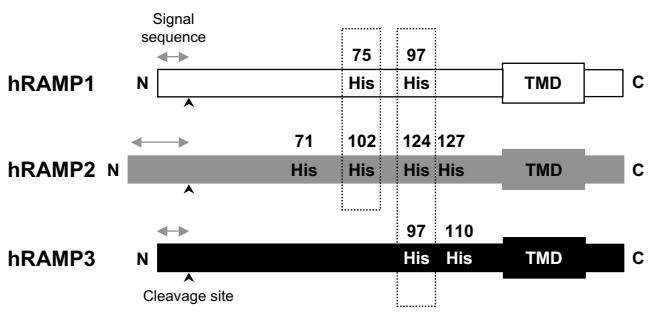

Fig. 1. Positions of extracellular His residues of the three hRAMPs. Each hRAMP possesses a large extracellular N-terminal domain containing signal sequence and cleavage site, a single transmembrane-spanning domain and a very short cytoplasmic C-terminal tail. Each contains 2 or 4 histidine (His) residues 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