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Characterization of the single transmembrane domain of human receptor activity-modifying protein 3 in adrenomedullin receptor internalization

Kenji Kuwasako [a,*], Kazuo Kitamura [b], Sayaka Nagata [b], Naomi Nozaki [a], Johji Kato [a]

[a] Frontier Science Research Center, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, Miyazaki 889-1692, Japan
[b] Division of Circulation and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, Miyazaki 889-1692, Japan

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ABSTRACT

Two receptor activity-modifying proteins (RAMP2 and RAMP3) enable calcitonin receptor-like receptor (CLR) to function as two heterodimeric receptors (CLR/RAMP2 and CLR/RAMP3) for adrenomedullin (AM), a potent cardiovascular protective peptide. Following AM stimulation, both receptors undergo rapid internalization through a clathrin-dependent pathway, after which CLR/RAMP3, but not CLR/RAMP2, can be recycled to the cell surface for resensitization. However, human (h)RAMP3 mediates CLR internalization much less efficiently than does hRAMP2. Therefore, the molecular basis of the single transmembrane domain (TMD) and the intracellular domain of hRAMP3 during AM receptor internalization was investigated by transiently transfecting various RAMP chimeras and mutants into HEK-293 cells stably expressing hCLR. Flow cytometric analysis revealed that substituting the RAMP3 TMD with that of RAMP2 markedly enhanced AM-induced internalization of CLR. However, this replacement did not enhance the cell surface expression of CLR, [^{125}I]AM binding affinity or AM-induced cAMP response. More detailed analyses showed that substituting the Thr^{130}–Val^{131} sequence in the RAMP3 TMD with the corresponding sequence (Ile^{157}–Pro^{158}) from RAMP2 significantly enhanced AM-mediated CLR internalization. In contrast, substituting the RAMP3 target sequence with Ala^{130}–Ala^{131} did not significantly affect CLR internalization. Thus, the RAMP3 TMD participates in the negative regulation of CLR/RAMP3 internalization, and the aforementioned introduction of the Ile–Pro sequence into the RAMP3 TMD may be a strategy for promoting receptor internalization/resensitization.

1. Introduction

Adrenomedullin (AM), like calcitonin (CT) gene-related peptide (CGRP), is a potent vasodilator that belongs to the CT family of six regulatory peptides [1]. AM can also powerfully inhibit oxidative stress, inflammation, apoptosis and atherosclerosis as well as promote angiogenesis and lymphangiogenesis [2,3]. Therefore, AM is expected to become a new drug for various cardiovascular diseases such as hypertension, heart failure, myocardial infarction, arteriosclerosis obliterans and secondary lymph edema [2,3].

The discovery of two receptor activity-modifying proteins (RAMP2 and RAMP3) led to the identification of two AM receptors [4]. Both accessory proteins transport CT receptor-like receptor (CLR) to the cell surface as a 1:1 heterodimer (CLR/RAMP2 or CLR/RAMP3), whereas RAMP1 enables CLR to function as a CGRP receptor [4]. All three RAMPs each comprise approximately 160 amino acids, and all exhibit a common structure: a large extracellular domain (ECD), a single transmembrane domain (TMD) and a short cytoplasmic C-terminal tail (C-tail). Despite these similarities, the three isoforms share less than 30% sequence identity, even within their TMDs (Fig. 1B) [4,5]. Interestingly, the expression levels of the three RAMPs vary among tissues [4,5] and are differentially affected by pathological conditions [3]. Although no selective AM receptor antagonist has been found, the two AM receptors have been suggested to cooperatively or differentially protect against various cardiovascular disease states [3].

It has been long believed that each RAMP ECD mediates agonist binding to the CLR/RAMP heterodimer [6–8], which in turn mediates intracellular cAMP production and Ca^{2+} mobilization [4,9]. Very recently, crystal structural analysis revealed that human (h)RAMP1 ECD or hRAMP2 ECD forms an agonist-binding pocket together with hCLR ECD [10]. Upon binding of AM, recombinant or endogenous human CLR/RAMP2 and CLR/RAMP3 undergo rapid internalization via clathrin-coated vesicles [9,11,12], as is observed for many G protein-coupled receptors (GPCRs) [13]. Additionally, like many GPCRs [13], CLR/RAMP3 can be recycled back to the plasma membrane to promote the functional restoration of signal transduction, so-called "resensitization" [14], whereas CLR/RAMP2

Abbreviations: AM, adrenomedullin; CLR, calcitonin receptor-like receptor; eGFP, enhanced green fluorescent protein; RAMP, receptor activity-modifying protein; TMD, transmembrane domain; WT, wild type.

* Corresponding author. Fax: +81 985 85 9718.
E-mail address: kuwasako@fc.miyazaki-u.ac.jp (K. Kuwasako).

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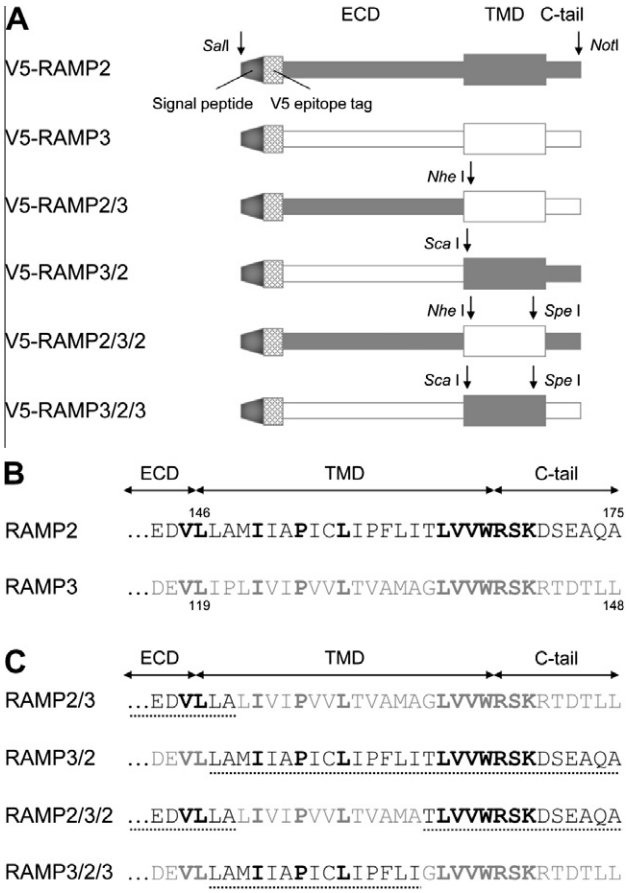

Fig. 1. (A) Schematic representation of the human RAMP domain swap constructs. *Gray boxes* are from RAMP2; *open boxes* are from RAMP3. V5 epitope-tagged RAMP chimeras were constructed using three restriction sites: *Nhe*I, *Sca*I and *Spe*I (see Section 2). ECD, extracellular domain or ectodomain; TMD, transmembrane domain; C-tail, cytoplasmic C-terminal tail. (B) Amino acid sequence alignment of the TMDs and C-tails of human RAMP2 and -3. The sequences are aligned for maximum homology; an alignment of all the sequences was presented by MacLatchie et al. [4]. The numbers indicate the amino acid positions in accordance with Sexton et al. [5]. Conserved amino acids are in bold. (C) Alignment of the four RAMP chimeras. The dotted underline indicates amino acid sequence of human RAMP2.

is targeted to lysosomes for degradation [9,11,12]. That is, receptor internalization is the primary mechanism of acute signal termination or desensitization of G protein signaling and is required for receptor resensitization [13]. It is noteworthy that hRAMP3 mediates hCLR internalization much less efficiently than does hRAMP2 [15]. Therefore, it is important to develop strategies to enhance the internalization of CLR/RAMP3 to promote receptor resensitization. However, little is known about the molecular basis of RAMP3 domains during CLR internalization. To address this issue, we constructed various chimeras targeting the TMDs and C-tails of hRAMP2 and hRAMP3 and characterized hRAMP3 domains during hCLR internalization by transiently transfecting their chimeric constructs into HEK-293 cells stably expressing hCLR.

2. Materials and methods

2.1. Materials

[^{125}I]hAM (specific activity 2 μCi/pmol) was produced in our laboratory [16]. Human AM was kindly donated by Shionogi & Co. (Osaka, Japan). Mouse anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA). Fluorescein phycoerythrin (PE)-conjugated rabbit anti-mouse secondary antibody was from Exalpha Biologicals, Inc.

2.2. Expression constructs

Double V5 epitope-tagged RAMP2 and RAMP3 (V5-RAMP2 and V5-RAMP3, respectively) were prepared as described previously [17] and cloned into the mammalian expression pIRES1/Neo (Clontech, CA) using the 5′-*Sal*I and 3′-*Not*I sites (Fig. 1A), yielding pIRES-V5-RAMP2 and pIRES-V5-RAMP3.

Four hRAMP chimeras were constructed according to our previously described procedure [8,15,17,18]. To construct the TMD plus C-tail swap chimeras (V5-RAMP2/3 and V5-RAMP3/2) and TMD swap chimeras (V5-RAMP2/3/2 and V5-RAMP3/2/3) (Fig. 1A, C), *Nhe*I and *Sca*I restriction sites were introduced at the 5′ end of the TMDs of hRAMP2 and hRAMP3, respectively, and a *Spe*I site was introduced at the 3′ end of both RAMP ECDs. The individual DNA fragments were amplified by PCR using primers containing the restriction sites. The separate RAMP fragments were then ligated into the pIRES1/Neo expression vector.

Sequential substitution of the hRAMP3 TMD sequences with the corresponding sequence from hRAMP2 (Fig. 4A) to single and double amino acid substitutions (Fig. 4A) were carried out using a QuickChange kit (Stratagene, USA) according to the manufacturer's instructions, with pIRES-V5-RAMPs serving as the templates. For each mutation, two complementary 30- to 40-mer oligonucleotides (sense and antisense) were designed with the mutation in the middle.

The resulting chimeric and mutant constructs were all sequenced using an Applied Biosystems 310 Genetic Analyzer.

2.3. Cell culture and DNA transfection

HEK-293 cells stably expressing an hCLR–eGFP fusion protein [9] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 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. Transient transfection of the cells was accomplished using Lipofectamine™ with Plus™ reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, the cells were seeded into 12- or 24-well plates and, upon reaching 70–80% confluence, were transiently transfected with the empty vector (pIRES1/Neo (*Mock*) or V5-tagged wild-type (WT)), pIRES-chimeric or pIRES-mutant construct; V5-RAMP2 and/or V5-RAMP3 were included in each transfection set. The DNA complex with transfection reagents was formed by incubating the cells for 4 h in OptiMEM 1 medium containing plasmid DNAs, Plus regent, and Lipofectamine reagent. All of the experiments were performed 36–48 h after transfection.

2.4. Flow cytometric analysis

Flow cytometry was used to assess the cell surface expression levels of V5-tagged receptor proteins. Following transient transfection of the indicated cDNAs for V5-RAMP WT, chimeras or mutants into CLR-eGFP-expressing HEK-293 cells in 12-well plates, the cells were washed once with ice-cold PBS and then non-enzymatically harvested with ice-cold FACS buffer [9]. After washing with the buffer, the cells were incubated for 60 min at 4 °C in the dark with anti-V5 monoclonal antibody (1:1000 dilution). Thereafter, the cells were incubated for 60 min at 4 °C in the dark with fluorescein PE-conjugated rabbit anti-mouse secondary antibody (1:400 dilution) and then washed twice with FACS buffer. The cells were then subjected to flow cytometry and analyzed as described previously [15].