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A single mutation in helix 8 enhances the angiotensin II type 1a receptor transport and signaling

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ABSTRACT

The amphipathic helix 8 in the membrane-proximal C-terminus is a structurally conserved feature of class A seven transmembrane-spanning G protein-coupled receptors (GPCRs). Mutations of this helical motif often cause receptor misfolding, defective cell surface transport and dysfunction. Surprisingly, we demonstrated here that a single point mutation at Lys308 in helix 8 markedly enhanced the steady-state surface density of the angiotensin II type 1a receptor (AT1aR). Consistent with the enhanced cell surface expression, Lys308 mutation significantly augmented AT1aR-mediated mitogen-activated protein kinase ERK1/2 activation, inositol phosphate production, and vascular smooth muscle cell migration. This mutation also increased the overall expression of AT1aR without altering receptor degradation. More interestingly, Lys308 mutation abolished AT1aR interaction with β-COP, a component of COPI transport vesicles, and impaired AT1aR responsiveness to the inhibition of Rab6 GTPase involved in the Golgi-to-ER retrograde pathway. Furthermore, these functions of Lys308 were largely dependent on its positively charged property. These data reveal previously unappreciated functions of helix 8 and novel mechanisms governing the cell surface transport and function of AT1aR.

1. Introduction

G protein-coupled receptors (GPCRs) constitute a superfamily of cell surface receptors, which regulate a variety of cell functions. All GPCRs share common structural features characterized by a hydrophobic core of seven transmembrane-spanning α-helices, three intracellular loops, three extracellular loops, an extracellular N-terminus, and an intracellular C-terminus. Another important feature of the rhodopsin-like class A GPCRs is the formation of an additional amphipathic α-helix (helix 8) in the membrane-proximal C-termini. Helix 8 contains a high concentration of positively charged residues and mutation of this region often causes receptor misfolding, defective cell surface transport and dysfunction [1–5].

The precise function of GPCRs is regulated by their intracellular trafficking, including cell surface export, internalization, recycling and degradation. Among these trafficking processes, the molecular mechanisms underlying the cell surface export of newly synthesized receptors are not well understood. GPCRs are synthesized in the endoplasmic reticulum (ER). After being synthesized, properly folded receptors are transported to the cell surface *en route* through the ER-Golgi intermediate complex (ERGIC), the Golgi apparatus and the trans-Golgi network (TGN), during which the receptors undergo post-translational modifications (e.g. glycosylation) to attain mature status. At the same time, the receptors at each intermediate compartment may be transported back to the previous compartment. It has been well defined that COPI-, COPII- and clathrin-coated vesicles mediate the transport between these intracellular compartments. COPI vesicles are involved in both anterograde and retrograde transport between different Golgi stacks and from the Golgi to the ER. COPII vesicles exclusively transport nascent cargos from the ER to the Golgi. Clathrin-coated vesicles mediate vesicular transport between the plasma membrane, the TGN and the endosomal compartment. Therefore, the steady state cell surface receptor expression is a delicate balance of vesicle-mediated anterograde and retrograde transport through a series of intracellular compartments.

Angiotensin II (Ang II) plays an important role in the physiological function of virtually all organs and is involved in the development of many diseases, including diabetes, hypertension, myocardial infarction, congestive heart failure, stroke and cancer. There are two major Ang II receptors: type 1 receptor (AT1R) and type 2 receptor (AT2R), both are prototypic GPCRs. As demonstrated in many studies, AT1R mediates the most physiological actions of Ang II. AT1R mainly couples to the Gq

Abbreviations: GPCR, G protein-coupled receptor; Ang II, angiotensin II; AT1R, angiontensin II type 1 receptor; AT1aR, angiontensin II type 1a receptor; AT2R, angiontensin II type 2 receptor; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; WT, wild-type; IP, inositol phosphate; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; RASMC, rat aortic smooth muscle cell; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ER, endoplasmic reticulum..

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family G proteins and activates a variety of signal transduction pathways, including the activation of mitogen-activated protein kinases (MAPK) and phospholipase C [6–8].

The C-terminus is a very important domain in the regulation of AT1R function, including G protein coupling, signaling, trafficking, phosphorylation and interaction with cytosolic proteins [9–18]. Based on the newly published crystal structure, the helix 8 of AT1R runs away from the membrane [19] which is in contrast to other GPCRs in which helix 8 is parallel to the membrane bilayer. This helix 8 forms high affinity interaction with the negatively charged lipids of the plasma membrane [20–22]. A number of studies have demonstrated that small GTPases and interacting proteins are involved in the regulation of AT1R transport to the cell surface [23–30]. We have previously shown that AT1aR cell surface transport depends on Sar1, a crucial regulator in the formation of COPII vesicles, and on ARF1, which is required for the function of both COPI- and clathrin-coated vesicles, suggesting an important role of small coated vesicles in the cell surface targeting of AT1R. We have also demonstrated that the di-acidic motif and di-leucine motif in the C-terminus are essential for AT1aR cell surface transport [31–33]. Here, we report a surprising finding that a single point mutation at position Lys308 in helix 8 markedly enhances AT1aR cell surface export and function which is likely mediated via regulating overall expression and retrograde transport pathway of the receptor.

2. Experimental procedures

2.1. Materials

Rat AT1aR in vector pCDM8 was kindly provided by Dr. Kenneth E. Bernstein (Department of Pathology, Emory University, Atlanta, GA). Antibodies against GFP and phospho-ERK1/2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against ERK1/2 were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-β-COP polyclonal antibody was from Thermo Scientific. High affinity anti-HA-fluorescein (3F10) was from Roche Molecular Biochemicals (Mannheim, Germany). Ang II was purchased from Calbiochem (San Diego, CA). Myo-[^{3}H]-inositol was from Perkin Elmer Life Sciences. Dowex AG1-X8 was from Bio-Rad (Hercules, CA). Rat aortic smooth muscle cells (RASMCs) were purchased from Cell Applications, Inc. (San Diego, CA). All other materials were obtained as described elsewhere [2,34].

2.2. Plasmid construction

To generate the pcDNA3.1(−) vector containing three HA at the *Xba*I and *Xho*I restriction sites, two primers (forward primer: 5′–CTAGAAT GTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTA CGCTTACCCATACGATGTTCCAGATTACGCTGATC-3′; reverse primer: 5′–TCGAGATCAGCGTA ATCTGGAACATCGTATGGGTAAGCGTAATCTGGAAC ATCGTATGGGTAAGCGTAATCTGGAACATCGTATGGGTACATT-3′) encoding three YPYDVPDYA and containing *Xba*I and *Xho*I restriction sites were synthesized, annealed and ligated into the pcDNA3.1(−) vector (Invitrogen Life Technologies, Carlsbad, CA), which was digested with *Xba*I and *Xho*I. To generate AT1aR tagged with three HA at its N-terminus (HA-AT1aR), the full-length AT1aR was amplified by polymerase chain reaction (PCR). The PCR product was digested with *Xho*I and *Hind*III, purified and ligated to HA-tagged pcDNA3.1(−) vector, which was digested with *Xho*I and *Hind*III. AT1aR tagged with GFP at its C-termini (AT1aR-GFP) was generated as described previously [23,34]. The GFP and HA epitopes have been used to label GPCRs including AT1aR, resulting in receptors with similar characteristics to the wild-type (WT) receptors. Glutathione S-transferase (GST) fusion protein constructs coding the C-terminus of AT1aR were generated in the pGEX-4 T-1 vector as described previously [3]. The constructs of small GTPases (Rab1N124I, Rab6Q72L, Sar1H79G and ARF1N126I) were generated as described previously [23–27]. All mutants were made with the Quick Change site-directed mutagenesis kit. The sequence of each construct used in this study was verified by restriction mapping and nucleotide sequence analysis.

2.3. Cell culture and transient transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin. RASMCs were cultured in Rat Smooth Muscle Cell Growth Medium (Cell Applications, Inc.). Transient transfection of the cells was carried out using Lipofectamine 2000 reagent (Invitrogen Carlsbad, CA).

2.4. Flow cytometric analysis of receptor expression

For measurement of the cell surface expression of AT1aR, HEK293 cells were cultured on 6-well dishes and transfected with 1 μg of HA-AT1aR for 36 h. The cells were collected, suspended in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) at a density of 1×10^7 cells/ml and incubated with high affinity anti-HA-fluorescein (3F10) at a final concentration of 2 μg/ml for 30 min at 4 °C. After washing twice with 0.5 ml of PBS/1% FCS, the cells were resuspended and the fluorescence was analyzed on a flow cytometer (Dickinson FACSCalibur) as described previously [23]. Since the staining with the HA antibodies was carried out in the unpermeabilized cells and only those receptors expressed at the cell surface were accessible to the HA antibodies, the fluorescence measurement reflected the amount of the receptors at the cell surface. For the measurement of total receptor expression, HEK293 cells were transfected with AT1aR-GFP for 36 h. The cells were collected, washed twice with PBS and resuspended in PBS containing 1% FCS at a density of 8×10^6 cells/ml. Total receptor expression was determined by measuring the GFP fluorescence. To determine the effect of drug treatment on receptor expression, HEK293 cells were incubated with MG132 (20 μM), chloroquine (100 μM) for 6 h at 37 °C. To determine the effect of small GTPases, HEK293 cells were cultured on 6-well dishes and transfected with 200 ng of AT1aR plasmid together with 800 ng of individual small GTPase plasmids.

2.5. Biotinylation of the cell surface AT1aR

In addition to flow cytometry the cell surface AT1aR expression was also measured by immunoblotting following biotinylation and isolation of cell surface proteins by using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). Briefly, HEK293 cells were cultured on 10-cm dishes and transfected with 4 μg of the pEGFP-N1 vector, AT1aR-GFP or K308E-GFP for 36–48 h. After washing, the cells were incubated with EZ-Link Sulfo-NHS-SS-Biotin for 30 min at 4 °C with gentle rotation. After the reaction was quenched, the cells were collected, lysed with lysis buffer containing protease inhibitors (Roche Molecular Biochemicals) and sonicated. The cell lysate was then centrifuged and the biotinylated proteins were isolated by using the NeutrAvidin Agarose column following the manufacturer's instructions. The biotinylated cell surface AT1aR was measured by immunoblotting using GFP antibodies.

2.6. Fluorescence microscopy

Subcellular distribution of AT1aR was analyzed by fluorescence microscopy as described previously [33,35]. Briefly, HEK293 cells were grown on coverslips pre-coated with poly-L-lysine in 6-well plates and transfected with 100 ng of AT1aR-GFP for 36 h. The cells were fixed with 4% paraformaldehyde-4% sucrose mixture in PBS for 15 min. The coverslips were mounted with prolong antifade reagent (Invitrogen) and images were captured using a Zeiss LSM780 confocal microscope equipped with a 63× objective. The cell surface expression of AT1aR was quantified by using ImageJ software.