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CLIC4 interacts with histamine H3 receptor and enhances the receptor cell surface expression

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Abstract

Histamine H3 receptor (H3R), one of G protein-coupled receptors (GPCRs), has been known to regulate neurotransmitter release negatively in central and peripheral nervous systems. Recently, a variety of intracellular proteins have been identified to interact with carboxy (C)-termini of GPCRs, and control their intracellular trafficking and signal transduction efficiencies. Screening for such proteins that interact with the C-terminus of H3R resulted in identification of one of the chloride intracellular channel (CLIC) proteins, CLIC4. The association of CLIC4 with H3R was confirmed in *in vitro* pull-down assays, coimmunoprecipitation from rat brain lysate, and immunofluorescence microscopy of rat cerebellar neurons. The data from flowcytometric analysis, radioligand receptor binding assay, and cell-based ELISA indicated that CLIC4 enhanced cell surface expression of wild-type H3R, but not a mutant form of the receptor that failed to interact with CLIC4. These results indicate that, by binding to the C-terminus of H3R, CLIC4 plays a critical role in regulation of the receptor cell surface expression.

Keywords: G protein-coupled receptor; Histamine H3 receptor; CLIC4; Trafficking

Histamine regulates numerous functions of central and peripheral nervous systems, including sleep-wake cycle, arousal, cognition, memory, and pain processing, through four receptor subtypes: H1, H2, H3 and H4 [1]. H3R has been known to inhibit synaptic release of a variety of neurotransmitters including histamine, acetylcholine, dopamine, noradrenaline, glutamate, γ-aminobutyric acid (GABA) [2]. Accordingly, H3R has attracted considerable interest from many pharmaceutical companies as a potential drug target for treatment of various pathological states including neuropathic pain and Alzheimer's disease [1].

In recent years, a growing number of cellular molecules have been identified to interact with C-terminal cytoplasmic domain of GPCR, and in most cases these interactions have been implicated in targeting, intracellular trafficking and subsequent signaling of GPCR [3]. Our previous work also showed that cell surface expression of PTH/PTH-

Abbreviations: CLIC4, chloride intracellular channel 4; ELISA, enzyme-linked immunosorbent assay; GPCR, G protein-coupled receptor; GST, glutathione-*S*-transferase; H3R, histamine H3 receptor; MBP, maltose binding protein.

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related protein receptor is regulated by interaction of its C-terminal domain with some cellular molecule [4]. With the advancement of the research, it is now becoming increasingly clear that intracellular trafficking and subcellular localization of GPCRs are regulated in elaborate detail through these interactions [5].

CLIC4 belongs to CLIC protein family that consists of seven highly homologous proteins: CLIC1-4, 5A, 5B (p64), and 6 (parchorin). These proteins are widely expressed in multicellular organisms and implicated in anion transport within various subcellular compartments. Among them, however, only CLIC1, CLIC4, and CLIC5 have been shown directly to display anion channel activities in reconstituted planar lipid bilayer systems, albeit with poor selectivity [6]. CLIC proteins share a core structural domain of approximate 240 amino acid residues with a single transmembrane domain. A crystal structure study on CLIC4 revealed that the core domain of this protein family has a feature as the omega class glutathione-*S*-transferase proteins, and pointed to the structural foundation of the ability of this domain to conformationally transform itself from a soluble globular protein to an integral membrane protein [7]: an unusual feature shared among CLIC family proteins to "autoinsert" themselves into membranes [8]. CLIC4 has been reported to locate in membrane systems including endoplasmic reticulum (ER), caveola and *trans*-Golgi network, mitochondrial inner membrane, dense core secretary vesicles, and plasma membrane [8]. A study on the CLIC-related protein EXC-4 in *Caenorhabditis elegans* indicated that the protein is indispensable for lumen formation of excretory tube of the nematode [9]. It is also reported that CLIC4 is essential for tubular morphogenesis of cultured human endothelial cells [10]. While other reports suggest that CLIC4 associates with a variety of cellular proteins including dynamin I, 14-3-3 proteins, and rhodopsin [11,12], biological roles of these interactions are largely unknown.

Adding to the reported functional diversity, we present here a new observation that CLIC4 exerts a critical influence on H3R cell surface expression through binding to the C-terminal cytoplasmic domain of the receptor.

Materials and methods

Cell culture. CHO and PC12 cells were grown in DMEM supplemented with 10% FCS, and with 10% horse serum and 5% FCS, respectively. These cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan).

Yeast two-hybrid screening. The screening was carried out using the ProQuest Two-Hybrid System (Invitrogen, CA, USA) according to the manufacturer's recommendation.

In vitro pull-down experiment. The plasmid encoding GST-fused H3R (amino acids 414–445) was constructed from pGEX-5X-2 plasmid (GE Healthcare, UK). The coding sequence for CLIC4 (amino acids 56–253) obtained in the screening was inserted into pMAL-c2 vector (New England BioLabs, MA, USA) to generate MBP-CLIC4 fusion protein. The point mutations of H3R were generated with the aid of QuickChange site-directed mutagenesis kit (Stratagene, CA, USA). Cleared lysates of *Escherichia coli* containing GST-H3R or MBP-CLIC4 fusion protein were mixed and incubated for 1 h. After further incubated with glutathione-Sepharose 4B (GE Healthcare) for 1 h, proteins associated with the beads were separated on SDS–PAGE and probed with anti-MBP antibody (Cell Signaling Technology, MA, USA). Signals were visualized by HRP-conjugated secondary antibody and ImmunStar HRP substrate (Bio-Rad Laboratories, CA, USA).

Antibody production and immunoprecipitation. A recombinant CLIC4 protein (amino acids 56–253) was used to raise mouse anti-CLIC4 antibody. The antibody was affinity purified by using MicroLink protein coupling gel (Pierce, IL, USA). For production of H3R specific antibody, rabbits were immunized with GST-fused N-terminal domain of H3R (amino acids 1–30), and affinity purified by using MBP-H3R(1–30). Immunoprecipitation was performed as described [13].

Generation of recombinant adenoviruses. The recombinant adenoviruses (rAd-CLIC4, rAd-H3R, rAd-H3RF419A, and rAd-βgal) were generated from pAxCAwtit cosmid vector (Nippon Gene, Japan) according to manufacturer's recommendation. In all experiments, total number of viruses was adjusted by adding rAd-βgal expressing β-galactosidase.

Primary culture. Cerebella of E17 Wistar rat embryos were dissected according to protocols provided by Sumitomo Bakelite Co., Ltd. (Tokyo, Japan). Cerebellar cells cultured on poly-D-lysine/laminin-coated coverslips were subjected to immunofluorescence analysis on 22 days *in vitro*. All experimental procedures were complied with the Animals (Scientific Procedures) Act 1986 and the guidelines of the Ministry of Health, Labour and Welfare of Japan, and approved by Institute for Animal Experimentation Tohoku University Graduate School of Medicine.

Immunofluorescence microscopy. PC12 cells plated on coverslips precoated with collagen type IV were coinfected with rAd-H3R (MOI = 2) and rAd-CLIC4 (MOI = 100). Forty-eight hours after infection, cells were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA). After blocking in PBS containing 20% normal goat serum, the cells were incubated with anti-H3R antibody. To locate intracellular CLIC4, cells were permeabilized with 0.1% Triton X-100 in PBS, and incubated with anti-CLIC4 antibody. Cells were then incubated with Alexa Fluor 546-conjugated anti-rabbit and Alexa Fluor 488-conjugated anti-mouse antibodies (Invitrogen), and inspected by Digital Eclipse C1 confocal microscope (Nikon, Japan). Cerebellar cells were fixed in 4% PFA in PBS, and then in cold methanol. Cells were permeabilized with 0.2% Triton X-100 in PBS, and blocked in 1% blocking reagent (TSA kit, Invitrogen). Blocked cells were incubated with anti-H3R and anti-CLIC4 antibodies, and then with Alexa Fluor 546-conjugated anti-rabbit antibody, HRP-conjugated anti-mouse antibody (Cell Signaling Technology), Alexa Fluor 488 tyramide (TSA kit), and Alexa Fluor 647-conjugated anti-calbindin D-28K antibody.

Flow cytometry. Cell surface H3R of PC12 cells coinfected with rAd-H3R (MOI = 2) and rAd-CLIC4 was labeled with anti-H3R antibody, followed by Alexa Fluor 488-conjugated anti-rabbit antibody. Cell-associated fluorescence was detected by a BD FACSCalibur flow cytometer (BD Biosciences, CA, USA). To measure whole cell H3R expression, the cells were permeabilized with 0.1% Triton X-100 in PBS. All data were analyzed with CellQuest software (BD Biosciences).

Radioligand receptor binding assay. PC12 cells infected with rAd-H3R (MOI = 2) and rAd-CLIC4 were incubated for 1 h in binding buffer (50 mM Tris–HCl, pH 7.4, and 5 mM EDTA) with 2 nM [^{3}H]-(R)-(−)-α-methylhistamine ([^{3}H]-RaMH), a selective H3R agonist. After the cells were separated and washed on glass fiber filters, the cell-associated radioactivity was measured. Nonspecific binding was defined as ligand binding in the presence of 10 μM unlabeled RaMH. Saturation experiments were conducted at five concentrations of [^{3}H]-RaMH from 5 to 500 nM by using PC12 cells infected with rAd-H3R (MOI = 2) and rAd-CLIC4 (MOI = 0 or 30). Curve fitting was made with GraphPad Prism version 4.00 (GraphPad Software Inc., USA).

Cell-based ELISA. CHO cells infected with rAd-H3R (MOI = 2) or rAd-H3RF419A (MOI = 2) and rAd-CLIC4 were seeded on collagen I-coated 24-well plates. Forty-eight hours after infection, cells were fixed in 4% PFA in PBS and blocked in PBS containing 5% fat-free milk. Cell surface H3R was detected with anti-H3R antibody, and HRP-conjugated anti-rabbit secondary antibody, followed by OPD colorimetric assay.