Cellular Signalling 25 (2013) 690–697

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Suppression of adenylyl cyclase-mediated cAMP production by plasma membrane associated cytoskeletal protein 4.1G

Toshihiro Goto [a], Ayano Chiba [a], Jun Sukegawa [c], Teruyuki Yanagisawa [c], Masaki Saito [a,b,d,e,*], Norimichi Nakahata [a,b,1]

[a] Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan
[b] Tohoku University International Advanced Research and Education Organization, Institute for International Advanced Interdisciplinary Research, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan
[c] Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai 980-8575, Japan
[d] Department of Biological Informatics and Experimental Therapeutics, Akita University Graduate School of Medicine, 1-1-1, Hondo, Akita City, Akita 010-8543 Japan
[e] Department of Biochemistry, Tokyo Women's Medical University, 8-1, Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

ARTICLE INFO

Article history:
Received 5 August 2012
Received in revised form 22 November 2012
Accepted 25 November 2012
Available online 29 November 2012

Keywords:
Protein 4.1
Adenylyl cyclases
Cyclic AMP
Parathyroid hormone receptor
FERM (4.1, ezrin, radixin, moesin) domain

ABSTRACT

It has been shown lately that activity of G protein-coupled receptors (GPCRs) is regulated by an array of proteins binding to carboxy (C)-terminus of GPCRs. Proteins of 4.1 family are subsets of subcortical cytoskeletal proteins and are known to stabilize cellular structures and proteins at the plasma membrane. One of the 4.1 family proteins, 4.1G has been shown to interact with the C-terminus of GPCRs and regulate intracellular distribution of the receptors, including parathyroid hormone (PTH)/PTH-related protein receptor (PTHR). PTHR is coupled to trimeric G proteins G_s and G_q, which activate the adenylyl cyclase/cyclic AMP (cAMP) pathway and phospholipase C pathway, respectively. During the course of investigation of the role of 4.1G on adenylyl cyclase/cAMP signaling pathway, we found that 4.1G suppressed forskolin-induced cAMP production in cells. The cAMP accumulation induced by forskolin was decreased in HEK293 cells overexpressing 4.1G or increased in 4.1G-knockdown cells. Furthermore, PTH -(1-34)-stimulated cAMP production was also suppressed in the presence of exogenously expressed 4.1G despite its activity to increase the distribution of PTHR to the cell surface. In cells overexpressing FERM domain-deleted 4.1G, a mutant form of the protein deficient in plasma membrane distribution, neither forskolin-induced nor PTH -(1-34)-stimulated cAMP production was not altered. The suppression of the forskolin-induced cAMP production was observed even in membrane preparations of 4.1G-overexpressing cells. In 4.1G-knockdown HEK293 cells, plasma membrane distribution of adenylyl cyclase 6, one of the major subtypes of the enzyme in the cells, showed a slight decrease, in spite of the increased production of cAMP in those cells when stimulated by forskolin. Also, cytochalasin D treatment did not cause any influence on forskolin-induced cAMP production in HEK293 cells. These data indicate that plasma membrane-associated 4.1G regulates GPCR-mediated G_s signaling by suppressing adenylyl cyclase-mediated cAMP production.

1. Introduction

It is widely known that GPCRs transduce extracellular signals into cells through activation of trimeric G proteins. Activation of signaling pathway mediated through G_s, G_q and G_{12} family proteins induces adenylyl cyclase/cyclic AMP (cAMP) pathway, phospholipase C/intracellular Ca^{2+}/diacylglycerol pathway, and ROCK/Rho pathway, respectively. These GPCR-mediated signal transductions are regulated by various mechanisms including the receptor localization at microdomains termed lipid rafts [1] or oligomerization of GPCRs [2], and also controlled by activator of G protein-signaling (AGS) or regulator of G protein-signaling (RGS) independent of G protein activity [3].

It has also been shown that the proteins interacting with C-termini of GPCRs regulate physiological functions of GPCR. For example, GPCR kinases (GRKs) and β-arrestins control the intracellular distribution of

Abbreviations: AGS, activator of G protein-signaling; cAMP, cyclic adenosine monophosphate; CTD, carboxy-terminal domain; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; Epac, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; FERM, 4.1-ezrin-radixin-moesin; HA, hemagglutinin; HRP, horseradish peroxidase; GPCR, G protein-coupled receptor; GRK, GPCR kinase; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PKA, protein kinase A; PTH, parathyroid hormone; PTHR, PTH/PTH-related protein receptor; RGS, regulator of G protein-signaling; SABD, spectrin/actin-binding domain; siRNA, small interfering RNA.

* Corresponding author at: Department of Biochemistry, Tokyo Women's Medical University, 8-1, Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. Tel./fax: +81 3 5269 7416.
E-mail address: saimasa@research.twmu.ac.jp (M. Saito).

[1] We dedicate this work to Dr. Norimichi Nakahata.

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http://dx.doi.org/10.1016/j.cellsig.2012.11.020

the receptor immediately after ligand binding and modify the signal transduction through the receptor [4]. On the other hand, Tctex-1 (*t*-complex testis expressed-1) interacts with C-terminus of parathyroid hormone (PTH)/PTH-related protein receptor (PTHR), and is involved in agonist-induced PTHR internalization [5]. 4.1G, one of the other PTHR-interactants, facilitates plasma membrane localization of PTHR, and promotes PTHR-mediated G_q-dependent $[Ca^{2+}]_i$ elevation and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation [6].

PTHR is coupled to either G_s or G_q depending on cell types and their environments [7]. Cyclic AMP is the prototypical second messenger, and is widely involved in cell fate determination, from differentiation and development to cell death [8–10]. This soluble second messenger further activates effector proteins such as protein kinase A (PKA), exchange protein activated by cAMP (Epac) and cAMP phosphodiesterases (PDEs) [11,12]. Recently, it has been reported that the dynamic intracellular distribution of PTHR is important in sustained cAMP production mediated by the receptor [13,14].

Protein 4.1 family provides mechanical stability to plasma membrane and plays essential roles in maintaining cell shape [15]. The family consists of five members, namely 4.1R (red blood cell-type), 4.1G (general-type), 4.1N (neuron-type), 4.1B (brain-type) and 4.1O (ovary-type), and they are mainly composed of three domains: FERM (4.1-ezrin-radixin-moesin) domain, SABD (spectrin/actin-binding domain), and CTD (carboxy-terminal domain) [16]. FERM domain is commonly found in a family of peripheral membrane proteins that mediate the linkage of cytoskeletal proteins to plasma membrane [17,18]. SABD participates in maintenance of cell shape by being associated with cytoskeletal proteins such as spectrin and actin [15,19]. There is a report of a splicing variant of 4.1G, which lacks K612-G681 [6]. The lacked amino acid sequence in the splicing variant corresponds to the most part of SABD. CTD associates with some of plasma membrane receptors such as PTHR [6], metabotropic glutamate receptors [20,21], A_1 adenosine receptor [22] and dopamine D_2 and D_3 receptors [23]. 4.1G-CTD mutant, which lacks FERM and SABD, distributes in the cytoplasm. While the full-length 4.1G protein increases the amount of cell surface PTHR, CTD mutant of 4.1G does not affect the amount of PTHR on cell surface [6].

In the present study, we have examined the physiological roles of 4.1G on G_s/adenylyl cyclase-mediated cAMP accumulation, along with intracellular distribution of 4.1G. We demonstrate here that membrane-associated 4.1G regulates adenylyl cyclase-mediated cAMP production, and thereby modulates PTHR-mediated G_s signaling.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimum essential medium (EMEM) were obtained from Nissui pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) was from JRH Biosciences (Lenexa, KS, USA). Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-mouse IgG (H + L), rhodamine-phalloidin, and Lipofectamine 2000 transfection reagent were from Life Technologies (Carlsbad, CA, USA). ECL Western blotting detection reagents and anti-mouse IgG conjugated with horseradish peroxidase (HRP) were from GE Healthcare (Buckinghamshire, England). Phusion High-Fidelity DNA Polymerase was from New England Biolabs (Ipswich, MA, USA). PTH-(1–34) was from Peptide Institute (Osaka, Japan). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), avidin-agarose, anti-FLAG M2 antibody, anti-β-tubulin antibody and anti-β-actin antibody were from Sigma (St. Louis, LO, USA). Anti-hemagglutinin (HA) antibody (16B12) was from Covance (Princeton, NJ, USA). Anti-integrin-α_V antibody was from BD Biosciences (San Jose, CA, USA). Human anti-chicken IgG conjugated with HRP was from Jackson ImmunoResearch (West Grove, PA, USA). Anti-human 4.1G antibody was kindly given by Dr. Sharon Krauss (University of California, Berkeley, CA, USA). Cyclic AMP assay kit "Yamasa" was from Yamasa (Tokyo, Japan). siRNA cocktail (siTrio Full Set) was from B-Bridge International (Mountain View, CA, USA). Sulfo-NHS-SS-biotin was from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals or drugs were of reagent grade or the highest quality available.

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in a humidified atmosphere at 37 °C under 5% CO_2 in DMEM supplemented with 10% FCS, 50 U/ml penicillin G, and 50 μg/ml streptomycin. For transfection, HEK293 cells were seeded into 6-well plates. After 24 h, transfection of the cells with plasmid DNA (2 μg) or siRNA cocktail (100 nM) was performed using Lipofectamine 2000 reagent according to the manufacturer's protocol. The plasmid DNA- or the siRNA-transfected cells were used for experiments 2 or 3 days after the transfection, respectively. The siRNA cocktail of human 4.1G was a mixture of three duplexes; (GGG AAA GAC GAA AGA GUA ATT)/(UUA CUC UUU CGU CUU UCC CTT), (GGA AAA UGU AGG UGC CCA ATT)/(UUG GGC ACC UAC AUU UUC CTT), and (GGG AAG AUU AAG UAA GAA ATT)/(UUU CUU ACU UAA UCU UCC CTT).

2.3. Plasmid construction

HA-tagged PTHR (HA-PTHR) and FLAG-tagged full length of 4.1G (4.1G-FL-FLAG) were prepared as previously described [6]. C-terminally FLAG-tagged 4.1G-ΔFERM (4.1G-ΔFERM-FLAG), which lacked FERM domain, was obtained by PCR from 4.1G-FL-FLAG/pcDNA3.1(+). Coding region of 4.1G-ΔFERM-FLAG was amplified by Phusion High-Fidelity DNA polymerase using primers of 5′-CCC AAA GCT TGC CAT GTT GGA GGA ACT GGA TAA GGC C-3′ (sense) and 5′-AAC TAG AAG GCA CAG TCG AG-3′ (antisense) with 30 cycles (98 °C for 10 s, 58 °C for 10 s and 72 °C for 1 min 30 s). Then, the PCR product was digested by HindIII and ApaI, and the fragment was subcloned into pcDNA3.1(+) vector. Preparation of a FLAG-tagged 4.1G-SV (FLAG-4.1G-SV) plasmid was carried out by a slight modification of a method described previously [6]. First, cDNA from human astrocytoma 1321N1 cells amplified by Phusion High-Fidelity DNA polymerase using primes of 5′-GGG CAT AAG CTT TGG CCA TGA CTA-3′ (sense) and 5′-GCA TTA GGG CCC GAA TAG TGG TGT GGC ATT-3′ (antisense). We obtained two cDNAs, full-length 4.1G (3137 bp) and its splicing variant (2927 bp). We chose to use the shorter cDNA, which corresponds to the 4.1G-SV sequence. The cDNA was digested by HindIII and NheI, and was subcloned into FLAG-4.1G-CTD/pcDNA3.1(+) to prepare the 4.1G-SV construct.

2.4. Immunohistochemistry

HEK293 cells were transfected with the expression plasmid of 4.1G-FL-FLAG or 4.1G-ΔFERM-FLAG, or with siRNA cocktail of 4.1G. On the next day, the cells were seeded on poly-L-lysine coated cover glasses in a 24-well plate, and they were cultured for 24 h or 48 h. Then, the cells were rinsed with phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) containing 1 mM Mg^{2+} and 0.1 mM Ca^{2+} (PBS(+)) twice, and were fixed with PBS containing 4% (w/v) paraformaldehyde for 10 min at room temperature. The cells were incubated with PBS containing 0.1% (w/v) Triton X-100 for 10 min at room temperature. After washing three times with TBS (20 mM Tris–HCl and 150 mM NaCl, pH 7.4) containing 5% skim milk, the preparation was incubated for 1 h with the same buffer for blocking. The cells were incubated with blocking buffer containing anti-FLAG M2 antibody for 1 h, and they were treated with blocking buffer containing Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-mouse IgG (H + L) and rhodamine-phalloidin (5 U/ml) for additional 1 h at room temperature. Then, the cells were washed three times with PBS, and mounted onto slide glasses. The cells were observed under a confocal laser scanning microscope FV1000 (Olympus, Tokyo, Japan).