Increase in cell-surface localization of parathyroid hormone receptor by cytoskeletal protein 4.1G

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The cell-surface localization of GPCRs (G-protein-coupled receptors) has emerged as one of critical factors of the GPCR-mediated signal transduction. It has been reported that the C-termini of GPCRs contain the sequences for sorting the receptors to cell surface. In the present study, we have searched for proteins that interact with the C-terminus of PTH (parathyroid hormone)/PTH-related protein receptor (PTHR) by using the yeast two-hybrid system, and identified a cytoskeletal protein 4.1G (general-type 4.1 protein) as an interactant with the C-terminus. Immunohistochemical study revealed that both PTHR and 4.1G were co-localized on plasma membranes, when they were transiently expressed in COS-7 cells. When 4.1G or the C-terminal domain of 4.1G (4.1G-CTD), a dominant-negative form of 4.1G, was co-expressed with PTHR in COS-7 cells, 4.1G, but not 4.1G-CTD, facilitated the cell-surface localization of PTHR, determined by

cell-surface biotinylation assay. PTH-(1–34) caused phosphorylation of ERK (extracellular-signal-regulated kinase) 1/2 in PTHR-expressed cells mainly mediated through EGF (epidermal growth factor) receptor. The phosphorylation was enhanced by the expression of 4.1G, but not 4.1G-CTD. PTH-(1–34) elevated $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration) independent of EGF receptor activation, and the elevation was enhanced by the expression of 4.1G, but not 4.1G-CTD. These data indicate that 4.1G facilitates the cell-surface localization of PTHR through its interaction with the C-terminus of the receptor, resulting in the potentiation of PTHR-mediated signal transduction.

Key words: 4.1G, cell-surface expression, extracellular-signal-regulated kinase 1/2 (ERK1/2), intracellular Ca^{2+} concentration, parathyroid hormone.

INTRODUCTION

It is widely known that GPCRs (G-protein-coupled receptors) transduce extracellular signals into cells through the activation of trimeric G-proteins. Recently, it has been shown that the proteins that interact with C-termini of GPCRs, such as GRKs (GPCR kinases) and β -arrestin, regulate the intracellular traffic of the GPCRs, including their cell-surface localization, internalization and recycling [1]. These proteins also regulate the GPCR-mediated signal transduction [1]. While agonist-activated GPCRs change their conformation and activate G-proteins, the activated GPCRs are then phosphorylated by GRKs. The phosphorylated GPCRs are bound to arrestins, which prevent their coupling to G-proteins, and terminate the signal transduction from GPCRs. The phosphorylated GPCRs are also translocated from the plasma membrane to inside the cells (internalization). Internalization reduces the number of cell-surface receptors, resulting in the reduction of the ligand binding. Internalized GPCRs, however, activate MAPK (mitogen-activated protein kinase) via arrestins in the cytosol [1,2]. Once GPCR has been desensitized and internalized, it may be either directed to lysosomes for degradation or recycled back to the plasma membrane in an active form [1].

PTH (parathyroid hormone) is involved in regulation of Ca^{2+} homeostasis, bone remodelling and maintaining blood phosphate concentration [3,4]. PTH-related protein is known to cause humoral hypercalcaemia of malignancy, which is caused by an increase in systemic bone resorption induced by bone-resorbing factors secreted from tumour cells [5]. The PTHR (PTH/PTH-related protein receptor) belongs to class II GPCRs, which include receptors for secretin, glucagons, calcitonin and several related peptides. PTHR is coupled to trimeric G-proteins G_q and G_s , which activate the phospholipase C pathway and adenylate cyclase pathway respectively [4]. It is generally accepted that many GPCRs transactivate RTKs (receptor tyrosine kinases). At present, the TMPS (triple-membrane-passing-signalling) mechanism of GPCR-induced EGF (epidermal growth factor) receptor activation is widely accepted as a model of RTK transactivation [6]. Also, phosphorylation and activation of the EGF receptor by Src in response to GPCR stimulation have been identified as an additional pathway of EGF receptor transactivation [7]. Recent reports demonstrate that the activation of PTHR results in the matrix-metalloprotease-dependent release of EGF-like peptides, such as heparin-binding EGF that transactivate EGF receptors [8].

Abbreviations used: 4.1B, brain-type 4.1 protein; 4.1G, general-type 4.1 protein; 4.1N, neuron-type 4.1 protein; 4.1O, ovary-type 4.1 protein; 4.1R, red blood-cell type 4.1 protein; A₁AR, A₁ adenosine receptor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CTD, C-terminal domain; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMEM, Eagle's minimum essential medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FCS, foetal-calf serum; FERM, 4.1/ezrin/radixin/moesin; fura 2/AM, 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(amino-5-methylphenoxy) ethane-*N,N,N',N'*-tetra-acetic acid, penta-acetoxymethyl ester; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; HA, haemagglutinin; H+L, heavy and light chains; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; mGlu1 α , metabotropic glutamate receptor subtype 1 α ; PTH, parathyroid hormone; PTHR, PTH/PTH-related protein receptor; PTHR-C, C-terminus of PTHR; RTK, receptor tyrosine kinase; SABD, spectrin/actin-binding domain; sulpho-NHS-SS-biotin, sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate.

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Brain consists of neuronal cells and glial cells, and glial cells are composed of astrocytes, oligodendrocytes and microglia. Among these cell types, astrocytes play critical roles in the development and functional regulation of the central nervous system [9]. Furthermore, astrocytes contribute to brain homeostasis through regulating the concentrations of local ions and neuroactive substances and through releasing several neurotrophic factors [9]. Although astrocytes express PTHR [10,11], as well as many receptors or channels [12], the role of PTHR in function of astrocytes remains to be clarified.

In the present study, we have screened a human brain cDNA library to seek proteins that interact with the C-terminus of PTHR (PTH-C) using the yeast two-hybrid system, and we found 4.1G (general-type 4.1 protein) as an interactant with PTH-C via its CTD (C-terminal domain) (4.1G-CTD). The C-terminal amino acid sequence of PTHR (Gln⁴⁶⁷–Gly⁴⁹⁰) appears to be the important domain for the interaction with 4.1G-CTD. The amount of cell-surface PTHR was increased by the expression of 4.1G, accompanied by an enhancement of PTHR-mediated phosphorylation of ERK (extracellular-signal-regulated kinase) 1/2 and elevation of $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration). Thus it is suggested that 4.1G enhances PTHR-mediated signal transduction through enhancing the localization of PTHR to the plasma membrane.

EXPERIMENTAL

Materials

DMEM (Dulbecco's modified Eagle's medium) and EMEM (Eagle's minimum essential medium) were obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). FCS (foetal calf serum), anti-FLAG monoclonal antibody M2, monoclonal anti-GFP (green fluorescent protein) antibody and monoclonal anti- β -actin antibody were from Sigma (Tokyo, Japan). Anti-[phospho-p44/p42 MAPK (ERK)] antibody, anti-[p44/p42 MAPK (ERK)] antibody and anti-rabbit IgG conjugated with HRP (horseradish peroxidase) were from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-HA (haemagglutinin) 12CA5 monoclonal antibody was from Roche Diagnostics (Indianapolis, IN, USA). Alexa Fluor[®] 594-conjugated F(ab')₂ fragment of goat anti-mouse IgG [H + L (heavy and light chains)] was from Molecular Probes. ProQuest two-hybrid system, a human brain cDNA library, pcDNA3.1(+) vector, Platinum Taq DNA polymerase high fidelity and Lipofectamine[™] 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, U.S.A.). PfuTurbo DNA polymerase was from Stratagene (La Jolla, CA, U.S.A.). Sulpho-NHS-SS-biotin [sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] was from Pierce Biotechnology (Rockford, IL, U.S.A.). Fura 2/AM {1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(amino-5-methylphenoxy)ethane-*N,N,N'*, *N'*-tetra-acetic acid, penta-acetoxymethyl ester} was from Dojindo Laboratories (Kumamoto, Japan). Human PTH-(1–34) was from Peptide Institute (Osaka, Japan). Isogene was from Nippon Gene (Tokyo, Japan). Other chemicals or drugs were of reagent grade or the highest quality available.

Yeast two-hybrid screening and assay of protein–protein interaction

A yeast two-hybrid system was used to search the proteins that interacted with PTH-C, as described previously [13]. We also used the yeast two-hybrid system to determine the interacting domain of PTH-C with 4.1G-CTD. Wild-type PTH-C and its various segments were subcloned into the pDBLeu vector, and 4.1G-CTD was subcloned into the pPC86 vector. The positive clones were selected by their *HIS3*, *URA3* and *lacZ* reporter gene expressions.

Cell culture and transfection

COS-7 cells were cultured in a humidified atmosphere at 37 °C under 5% CO₂ in DMEM supplemented with 10% FCS, 100 units/ml penicillin G and 100 µg/ml streptomycin. Human astrocytoma (1321N1) cells were cultured in the same medium with 5% FCS. Transfections of the vectors were performed using Lipofectamine[™] 2000 reagent according to the manufacturer's protocol. The vector-transfected cells were used for experiments 2 days after the transfection.

Plasmid construction

HA–PTH and PTH–GFP were prepared as described previously [13]. C-terminally HA-tagged 4.1G-CTD (4.1G-CTD–HA) in pcDNA3.1(+) vector was obtained by PCR from h4.1G/pcDNA3 (kindly provided by Dr John G. Conboy, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA, U.S.A.) using primers of 5'-CCCCGCTAGCACCATGTGTAACAATTTCTGA-3' (sense) and 5'-AACTAGAAGGCACAGTCGAG-3' (antisense), and amplified with 26 cycles (94 °C for 1 min, 61 °C for 30 s and 70 °C for 50 s) using PfuTurbo DNA polymerase. Then the PCR product was digested by *NheI* and *EcoRI*, and the fragment was subcloned into pcDNA3.1(+) vector. After removal of the HA-tag by *Apal* and *SacI*, annealed two oligonucleotides, 5'-CGACTACAAGGACGACGATGACAAAT-AAGGGCC-3' (sense) and 5'-CTTATTTGTCATCGTCGTCC-TTGTAGTCGAGCA-3' (antisense), which included FLAG tag, were inserted into the digested vector. C-terminal FLAG-tagged human full-length 4.1G (FLAG–4.1G) was obtained from human astrocytoma (1321N1) cells by the reverse transcription–PCR technique as described previously [14,15]. First, total RNA of 1321N1 cells was extracted using Isogene, and the cDNA was reverse-transcribed from the RNA. Then 4.1G cDNA was PCR-amplified 35 cycles (94 °C for 1 min, 61 °C for 30 s and 68 °C for 4 min) using Platinum Taq DNA polymerase high fidelity with primers of 5'-GGGCATAAGCTTTGGCCATGACTA-3' (sense) and 5'-GCATTAGGGCCCCGAATAGTGGTGTGGCATT-3' (antisense). We obtained two cDNAs, full-length 4.1G (3137 bp) and its splicing variant (2927 bp). The shorter form lacks the codons for 70 amino acids from Lys⁶¹² to Gly⁶⁸¹. We chose to use the longer cDNA, which corresponds to the human 4.1G sequence (GenBank[®] accession number NM_001431). The cDNA was digested by *HindIII* and *NheI*, and was subcloned into FLAG–4.1G-CTD/pcDNA3.1(+) to prepare the full-length 4.1G construct.

Immunohistochemistry

COS-7 cells were co-expressed with PTH–GFP and FLAG–4.1G (4.1G and 4.1G-CTD). On the next day, the cells were seeded on poly(L-lysine)-coated cover glasses in a 12-well plate, and they were cultured for 24 h. Then the cells were rinsed with PBS containing 1 mM Mg²⁺ and 0.1 mM Ca²⁺ twice, and were fixed with PBS containing 4% (w/v) paraformaldehyde for 20 min at room temperature (23 °C). Next, the cells were incubated with PBS containing 0.5% (v/v) Triton X-100 for 10 min at room temperature. After three washes with TBS (Tris-buffered saline: 20 mM Tris/HCl and 150 mM NaCl), pH 7.5, containing 5% (w/v) non-fat dried milk, the preparation was incubated for 1 h with the same buffer for blocking. After cells were incubated with anti-FLAG antibody M2 in the buffer for 30 min, they were treated with Alexa Fluor[®] 594-conjugated F(ab')₂ fragment of goat anti-mouse IgG (H + L) in the buffer for an additional 30 min at room temperature. Then the cells were washed three times with PBS, and mounted on to glass slides. The cells were observed under a confocal laser-scanning microscope (Leica TCS NT, Wetzlar, Germany).

Cell-surface biotinylation assay

Cell-surface biotinylation was carried out by a modification of the method described previously [13]. In brief, COS-7 cells co-expressed with PTHR-HA and FLAG-4.1G were rinsed with ice-cold PBS twice, and they were incubated for 20 min twice with 0.5 mg/ml sulfo-NHS-SS-biotin in PBS. Then the cells were rinsed with PBS twice, and they were lysed with ice-cold RIPA buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P40, 0.5% (w/v) deoxycholic acid and 0.1% (w/v) SDS] containing 1 mM PMSF. Next, the lysates were centrifuged at 15 000 *g* for 5 min at 4°C, and HA-tagged protein in supernatants was immunoprecipitated by incubation with anti-HA antibody 12CA5 overnight at 4°C. The immunoprecipitated proteins were solubilized in the sample buffer without DTT (dithiothreitol) [125 mM Tris/HCl, pH 7.4, 4% (w/v) SDS, 10% (v/v) glycerol and 0.2 mg/ml Bromophenol Blue], separated by SDS/PAGE, and transferred on to nitrocellulose membranes. The blots were probed with streptavidin conjugated to HRP and visualized using a chemiluminescence detection system.

Detection of PTHR-GFP protein, FLAG-4.1G protein and β -actin

To detect PTHR-GFP, FLAG-4.1G, FLAG-4.1G-CTD and β -actin, transfected cells were rinsed with PBS twice, and lysed in sample buffer with 1 mM DTT. Equal amounts of protein were separated by SDS/PAGE and transferred on to PVDF membranes. The blots were probed with anti-GFP antibody, anti-FLAG antibody M2 or anti- β -actin antibody for 1 h at room temperature, and then they were blotted using anti-mouse IgG conjugated with HRP for 1 h at room temperature. The signals were visualized using a chemiluminescence detection system.

Detection of phosphorylation of ERK1/2

PTHR-HA and FLAG-4.1G were co-expressed in COS-7 cells. The cells were rinsed twice with the incubation buffer (EMEM buffered with 20 mM Hepes, pH 7.35, containing 0.5% BSA), and they were pre-incubated for 1 h at 37°C. Then the medium was replaced with the incubation buffer containing 100 nM PTH, and the cells were incubated for indicated time. The reactions were terminated by the removal of the medium and an addition of the sample buffer described above with 1 mM DTT. The samples were separated by SDS/PAGE and transferred on to PVDF membranes. The blots were probed with anti-[phospho-p44/p42 MAPK (ERK)] antibody or anti-[p44/p42 MAPK (ERK)] antibody for 1 h at room temperature, and then they were blotted using anti-rabbit IgG conjugated to HRP for 1 h at room temperature. The signals were visualized using a chemiluminescence detection system.

Measurement of $[Ca^{2+}]_i$ using a fluorescent indicator

The change in the $[Ca^{2+}]_i$ was monitored by the fura 2 method described previously [16]. In brief, COS-7 cells co-expressed with PTHR-HA and FLAG-4.1G were incubated with 1 μ M fura 2/AM for 15 min. After centrifugation of the cells at 250 *g* for 2 min to remove extracellular fura 2/AM, the cells were suspended in modified Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.18 mM $CaCl_2$, 5.6 mM glucose and 10 mM Hepes, pH 7.4). Fluorescence at 510 nm by excitation at 340 nm and 380 nm was observed with a spectrofluorimeter (Hitachi, F2000). Relative $[Ca^{2+}]_i$ was calculated as the fluorescence ratio excited at 340 nm and 380 nm.

Statistical analysis

Data were expressed as means $\pm$ S.E.M., and the significant difference was analysed with unpaired Student's *t* test.

RESULTS

Screening for the proteins which interact with PTHR

In order to identify proteins interacting with the CTD (amino acid residues from Gly⁴⁶⁷ to Met⁵⁹³) of PTHR (PTHR-C), yeast two-hybrid screening was performed using PTHR-C as a bait and human brain cDNA library as a prey. Analysis of approx. 10⁶ yeast clones transformed with human brain cDNA library yielded 15 positive clones determined by their *HIS3*, *URA3* and *lacZ* reporter gene expressions. Nucleotide sequencing of these cDNA clones revealed five clones of 4.1G-CTD (Figure 1A).

Interaction between PTHR-C and 4.1G-CTD using the yeast two-hybrid system

To define the interacting domain of PTHR-C with 4.1G-CTD, PTHR-C was divided into 12 small segments, and subjected again to yeast two-hybrid analysis using *HIS3* and *lacZ* reporter gene expressions (Figure 1B). As a result, 4.1G-CTD interacted strongly with CN-1, -2, -3 and -8 (Figure 1B), suggesting that the amino acid sequence of CN-8 (24 amino acids between Gln⁴⁶⁷ and Gly⁴⁹⁰ in PTHR-C) is important for the interaction with 4.1G (Figure 1C).

Intracellular localization of PTHR and 4.1G

We examined the intracellular localization of PTHR and 4.1G immunohistochemically using confocal microscopy (Figure 2). After PTHR-GFP and FLAG-4.1G were co-expressed in COS-7 cells, the cells were immunolabelled with anti-FLAG antibody M2 (mouse) and Alexa Fluor[®] 594-conjugated F(ab')₂ fragment of goat anti-mouse IgG (H + L) to visualize 4.1G. PTHR-GFP was localized on the plasma membrane, cytosol and the perinuclear region. On the other hand, FLAG-4.1G was localized on plasma membrane and cytosol. A merged photograph indicated that PTHR and 4.1G co-localized on plasma membranes and cytosol, but not the perinuclear region.

Augmentation of PTHR localization on the plasma membrane by 4.1G

We investigated the effect of 4.1G on the amount of PTHR on the cell surface. COS-7 cells were transfected with HA-PTHR, and PTHR on the cell surface was measured by a combined method of immunoprecipitation and cell-surface biotinylation [13] (Figure 3). PTHR on the cell surface increased significantly when COS-7 cells were co-expressed with FLAG-4.1G, compared with control cells (Figure 3, upper panel). Protein 4.1 families are mainly composed of three domains: FERM (4.1/ezrin/radixin/moesin) domain, SABD (spectrin/actin-binding domain) and CTD [17]. We used 4.1G-CTD as a dominant-negative form, which is able to bind to PTHR-C, but does not exert its function, because 4.1G-CTD lacks the FERM domain and the SABD. In the cells that expressed FLAG-4.1G-CTD, there was no increase in PTHR localization on the plasma membrane (Figure 3, upper panel). These results indicate that 4.1G binds to PTHR-C, and facilitates the targeting of PTHR to the cell surface, with the aid of the FERM domain and the SABD.

Although the level of PTHR on the plasma membrane is increased by expression of 4.1G, there is a possibility that 4.1G expression would affect the total amount of PTHR protein in cells through transcriptional regulation and/or stabilization of 4.1G mRNA. Therefore we examined whether 4.1G expression affects the amount of PTHR in cells. PTHR-GFP and FLAG-4.1G (4.1G or 4.1G-CTD) were co-expressed in COS-7 cells, and the expression levels of PTHR-GFP in the cells were determined by Western blotting. As shown in Figure 4, the level of PTHR-GFP

Figure 1 4.1G is a binding protein of PTHR-C

(A) Identified 4.1G cDNA fragments interacting with PTHR-C. Reported human 4.1G mRNA (GenBank® accession number NM_001431), coding region of full-length 4.1G, and coding region of 4.1G-CTD are shown as grey bars. One of the representative cDNA clones of 4.1G as a result of yeast two-hybrid screening is shown by a black line. (B) Determination of peptide fragments bound to 4.1G in PTHR-C. A yeast two-hybrid system was used between PTHR-C and 4.1G-CTD. Wild-type PTHR-C and its various subsegments (CC- and CN- series) were tested. +, positive interaction; -, negative interaction. One-letter amino acid codes are used. (C) Alignment of amino acid sequences of CN-8.

Figure 2 Intracellular distribution of PTHR and 4.1G in COS-7 cells

PTHR-GFP and FLAG-4.1G were co-expressed in COS-7 cells. FLAG-4.1G was immunostained using anti-FLAG antibody M2 (mouse) and Alexa Fluor® 594-conjugated F(ab')₂ fragment of anti-mouse IgG (H + L). Confocal micrographs are from one representative experiment of three.

was slightly increased in the cells that were co-expressed with 4.1G (Figure 4, top panel). This increase, however, was much less than that shown for the localization of PTHR at the plasma membrane. β -Actin was used as an internal control (Figure 4, middle panel). Expressions of FLAG-4.1G and FLAG-4.1G-CTD were also detected (Figure 4, bottom panel).

Augmentation of PTHR-mediated phosphorylation of ERK1/2 by 4.1G

Next, we examined the effect of 4.1G on PTH-(1–34)-induced ERK1/2 phosphorylation. To determine the levels of ERK1/2 phosphorylation in PTHR-HA-expressed COS-7 cells, the cells

Figure 3 Effects of 4.1G on the amount of cell-surface PTHR

HA-PTHR and FLAG-4.1G (4.1G or 4.1G-CTD) were co-expressed in COS-7 cells. Upper panel: cell-surface biotinylation assay. The amount of PTHR on the cell surface was increased by FLAG-4.1G. Lower panel: detection of 4.1G proteins in COS-7 cells. Expressions of both FLAG-4.1G and FLAG-4.1G-CTD proteins were confirmed. Results are representative of three experiments. IB, immunoblot; IP, immunoprecipitation. Molecular-mass sizes are given in kDa.

Figure 4 Effects of 4.1G on expression of PTHR in COS-7 cells

PTHR-GFP and FLAG-4.1G (mock, 4.1G or 4.1G-CTD) were co-transfected into COS-7 cells. The expression level of PTHR-GFP protein was compared among the cells co-transfected with mock, FLAG-4.1G and FLAG-4.1G-CTD (top panel). Note that FLAG-4.1G only slightly increased the expression of PTHR-GFP protein. β -Actin was used as an internal control (middle panel). Expression of FLAG-4.1G (4.1G or 4.1G-CTD) is shown in the bottom panel. Results are representative of three experiments. IB, immunoblot; IP, immunoprecipitation. Molecular-mass sizes are given in kDa.

were incubated with 100 nM PTH-(1–34) for various periods. PTH-(1–34) caused the phosphorylation of ERK1/2 in a time-dependent manner, with a peak at 5–20 min (Figure 5A). It is widely known that activation of GPCRs causes transactivation of the EGF receptor, and then facilitates the phosphorylation of MAPK cascades mediated via Ras [18]. To investigate whether PTHR-mediated ERK1/2 phosphorylation is caused through transactivation of EGR receptor, PTHR-HA-expressed COS-7 cells were incubated with AG1478 (100 nM), an inhibitor of the EGF receptor, for 10 min before the treatment of PTH-(1–34). As a result, PTH-(1–34)-induced phosphorylation of ERK1/2 was clearly inhibited by AG1478 (Figure 5B). EGF (10 ng/ml)-induced phosphorylation of ERK1/2 was also suppressed by AG1478. These results demonstrate that PTH-(1–34) caused the phosphorylation of ERK1/2 through transactivation of the EGF receptor in HA-PTHR-expressed COS-7 cells. Next, we examined

Figure 5 Effects of 4.1G on PTH-(1–34)-induced phosphorylation of ERK

(A) Time-dependency of PTH-(1–34)-induced phosphorylation of ERK. HA-PTHR-expressed COS-7 cells were treated with 100 nM PTH-(1–34) for the indicated times. Phosphorylated ERK1/2 and total ERK1/2 were detected as described in detail in the Experimental section. (B) PTH-(1–34)-induced phosphorylation of ERK1/2 via transactivation of the EGF receptor. HA-PTHR was expressed in COS-7 cells. Cells were treated with 10 ng/ml EGF or 100 nM PTH-(1–34) for 10 min in the absence or presence of 100 nM AG1478. PTH-(1–34)-stimulated ERK1/2 phosphorylation was inhibited by AG1478. Results are representative of three experiments. (C) Effects of 4.1G on PTH-(1–34)-induced ERK1/2 phosphorylation. HA-PTHR and FLAG-4.1G (4.1G or 4.1G-CTD) were co-expressed in COS-7 cells. The cells were treated with 100 nM PTH-(1–34) for 10 min. PTH-(1–34)-stimulated phosphorylation of ERK1/2 was augmented by FLAG-4.1G. Results are means $\pm$ S.E.M. for three experiments. (* P < 0.05 compared with mock).

the effect of 4.1G on PTHR-mediated phosphorylation of ERK1/2 (Figure 5C). Although PTH-(1–34) induced phosphorylation of ERK1/2 much more potently in the cells that expressed FLAG-4.1G than in control cells, it did not show increased phosphorylation of ERK1/2 in the cells that expressed FLAG-4.1G-CTD.

Non-involvement of the EGR receptor in 4.1G-augmented PTHR-mediated $[Ca^{2+}]_i$ elevation

To test the effect of 4.1G on other signalling pathways, PTH-(1–34)-induced $[Ca^{2+}]_i$ elevation was examined. Similar to ERK1/2 phosphorylation, PTH-(1–34)-induced $[Ca^{2+}]_i$ elevation was potentiated by the expression of FLAG-4.1G, but not FLAG-4.1G-CTD (Figure 6A). We investigated the involvement of EGF receptor in the augmentation of PTHR-mediated $[Ca^{2+}]_i$ elevation by 4.1G. Although PTH-(1–34) elevated $[Ca^{2+}]_i$, AG1478 did

Figure 6 4.1G facilitated PTH-(1-34)-induced $[Ca^{2+}]_i$ elevation without transactivation of the EGF receptor

(A) Effects of 4.1G on PTH-(1-34)-induced $[Ca^{2+}]_i$ elevation. COS-7 cells expressing HA-PTHR and FLAG-4.1G (4.1G or 4.1G-CTD) were loaded with fura 2/AM. PTH-(1-34) (100 nM)-induced $[Ca^{2+}]_i$ elevation was enhanced by the expression with FLAG-4.1G, but not with FLAG-4.1G-CTD (left-hand panel). The quantified data are shown (right-hand panel). The results are means $\pm$ S.E.M. for six individual experiments. (* $P < 0.05$ compared with mock). (B) Effects of EGF on $[Ca^{2+}]_i$ elevation. HA-PTHR-expressing COS-7 cells were treated with 10 ng/ml EGF (top panel) or 100 nM PTH-(1-34) (middle and bottom panels) in the absence (top and middle panels) or presence (bottom panel) of 100 nM AG1478. EGF did not cause $[Ca^{2+}]_i$ elevation, and PTH-(1-34)-induced $[Ca^{2+}]_i$ elevation was not influenced by AG1478. Results are representative of three experiments.

not reduce the increase at all (Figure 6B, middle and bottom panels). Moreover, EGF treatment of the cells did not cause $[Ca^{2+}]_i$ elevation (Figure 6B, top panel). These results indicate that 4.1G augmented PTHR-mediated $[Ca^{2+}]_i$ elevation independently of the transactivation of EGF receptor.

DISCUSSION

A few intracellular proteins have been found to interact with PTHR-C, such as β -arrestin [19,20] and Tctex-1 [13]. While β -

arrestin is involved in internalization and recycling of PTHR [19,20], cytoplasmic dynein light chain Tctex-1 regulates the internalization of PTHR, as shown previously by us [13]. In the present study, we identified cytoskeletal protein 4.1G as a binding partner of PTHR-C, using a yeast two-hybrid system. 4.1G belongs to the protein 4.1 family, which play critical roles in plasma membrane stability, morphology and deformation [21]. The first member of protein 4.1 was originally identified as an abundant protein of human erythrocytes, and is called band 4.1 or 4.1R (red-blood-cell-type). To date, several protein 4.1 homologues have been identified, such as 4.1B (brain-type) [22], 4.1G [23], 4.1N (neuron-type) [24] and 4.1O (ovary-type) [25], in addition to 4.1R.

The interacting domain of PTHR-C to bind to 4.1G was Gln⁴⁶⁷–Gly⁴⁹⁰ (24 amino acids), which is the proximal region of the seventh transmembrane domain. On the other hand, the interacting domain of 4.1G with PTHR-C was the CTD of 4.1G, because 4.1G-CTD was identified from the results of yeast two-hybrid screening.

We obtained full-length cDNA of 4.1G from 1321N1 cells. Although Sun et al. [26] reported that 4.1G is distributed in heart, brain, placenta, lung, skeletal muscle, kidney, pancreas and gonads, it has not been reported previously that astrocytes in brain or astrocytoma cell lines express 4.1G. This is the first report of 4.1G expression in astrocytoma 1321N1 cells.

Several receptors have been shown to interact with protein 4.1 families, such as one of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor complex GluR1 with 4.1G and 4.1N [27], the other complex GluRD (GluR4) with 4.1R, 4.1G, 4.1N and 4.1B [28], dopamine D₂ and D₃ receptors with 4.1R, 4.1G, 4.1N and 4.1B [29], and A₁AR (A₁ adenosine receptor) with 4.1G [30]. It has been suggested that protein 4.1 family members contribute to stabilization of these receptors on plasma membrane. Ins(1,4,5) P_3 receptor, an intracellular Ca^{2+} channel, was also reported to bind to 4.1N [31–33]. In the present study, using a cell-surface biotinylation assay, we show for the first time that 4.1G enhanced the localization of PTHR on plasma membrane in COS-7 cells.

We examined the effect of 4.1G on PTH-(1-34)-induced signal transduction. Our data and the previous reports have shown that PTH-(1-34) transactivates the EGF receptor through PTHR, resulting in the activation of the ERK cascade [8,34]. When we measured ERK1/2 phosphorylation in COS-7 cells co-expressed with PTHR and 4.1G, PTH-(1-34)-induced ERK1/2 phosphorylation was augmented in the cells that expressed 4.1G, but not 4.1G-CTD. The augmentation may be due to the increased expression of cell-surface PTHR by 4.1G. However, there is a possibility that 4.1G facilitates the localization of EGF receptor on the plasma membrane and augments the PTHR-mediated cellular response through the EGF receptor. However, 4.1G did not enhance EGF-induced ERK1/2 phosphorylation (M. Saito and N. Nakahata, unpublished work). In addition, PTH-(1-34)-induced $[Ca^{2+}]_i$ elevation was augmented by the expression of 4.1G, but EGF had no effect on $[Ca^{2+}]_i$ level in COS-7 cells. These results suggest that the augmentation of PTHR-mediated signal transduction by 4.1G is mainly mediated through an increase in cell-surface localization of PTHR.

There are a few reports of the role of the protein 4.1 family in other receptor-mediated signal transduction. A₁AR-mediated inhibition of cAMP accumulation was reduced by 4.1G, and A₁AR-mediated intracellular Ca^{2+} release was also reduced by 4.1G [30]. mGlu1 α (metabotropic glutamate receptor subtype 1 α)-mediated cAMP accumulation was reduced by 4.1G [35]. These observations are not consistent with our present data that 4.1G augmented PTHR-mediated ERK1/2 phosphorylation and

[Ca²⁺]_i elevation through the augmented localization of PTHR to cell membrane. These observations raise the possibility that 4.1G might change the conformations of A₁AR and mGlu α , and therefore reduce the signal transductions. Actually, radioligand-binding affinity of A₁AR was reduced by expression of 4.1G [30]. It is uncertain, however, why there is such a difference in 4.1G action between A₁AR/mGlu α and PTHR so far. Thus it is necessary to clarify the detail of mechanisms of 4.1G to modify GPCR signalling in future experiments.

In conclusion, the present study demonstrates that 4.1G, one of the protein 4.1 family, is involved in the increase in the localization of PTHR on to the plasma membrane through the binding to PTHR-C. Furthermore, 4.1G augmented PTH-(1–34)-induced ERK1/2 phosphorylation and [Ca²⁺]_i elevation. This report on 4.1G interaction with PTHR provides the evidence for one regulation of cell-surface expression of GPCRs.

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