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G_s and G_q signalings regulate hPEM-2-induced cell responses in Neuro-2a cells

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ABSTRACT

Rho family GTPase-specific guanine nucleotide exchange factors of the Dbl family regulate a variety of cellular events including cytoskeletal arrangement, signal transduction, and gene expression through activation of Rho family GTPases. In this study, we show that hPEM-2 is a downstream effector of G_s and G_q signaling in Neuro-2a neuroblastoma cells. Co-expression with hPEM-2 and GTPase-deficient (constitutively active) mutants of Gαs ($G\alpha_sQ213L$) or $G\alpha_q$ ($G\alpha_qQ209L$), but not other GTPase-deficient mutants of Gα subunit and Gβγ subunits, activated serum response element (SRE)-dependent gene transcription, which is known to be induced by Rho family activation. Although a dominant negative mutant of Rac1 strongly blocks $G\alpha_sQ213L$ or $G\alpha_qQ209L$/hPEM-2 activated SRE-dependent gene transcription, those of Cdc42 or RhoA are marginally affected. A PKA inhibitor, H-89, attenuated $G\alpha_s$/hPEM-2-activated SRE-dependent gene transcription. And a dominant negative mutant of c-Src and an Src inhibitor attenuated $G\alpha_qQ209L$/hPEM-2-activated SRE-dependent gene transcription. Experiments using hPEM-2 deletion mutants indicate that some regions of hPEM-2 play an important role in enhancing SRE activation by G_s and G_q signalings. These results reveal that G_s and G_q signalings regulate hPEM-2 functions through PKA and c-Src in Neuro-2a neuroblastoma cells, respectively.

1. Introduction

The Rho family of small GTPases, which comprises the key regulators of the actin cytoskeleton, has been shown to mediate the morphological changes that are observed during neural development and plasticity such as neurite outgrowth, axonal guidance and dendrite topology modifications. Members of the Rho family perform distinct roles in the regulation of the actin cytoskeleton [1]. RhoA is responsible for the formation of focal adhesions and the assembly of actin stress fibers, and it has been found to inhibit the formation of neurite outgrowths. Rac1 promotes the formation of membrane lamellae, whereas Cdc42 regulates the outgrowth of filopodia. Both Rac and Cdc42 positively regulate neurite outgrowth [2].

Like other small GTPases, activation of Rho GTPases, i.e., exchange of bound GDP by GTP, is catalyzed by guanine nucleotide exchange factors (GEFs) [3]. In particular, 69 different GEFs for Rho family members (RhoGEFs) are found in the human genome. A common feature of RhoGEFs is the Dbl homology (DH) domain responsible for exchange activity, followed by a pleckstrin homology (PH) domain supposed to be involved in the interaction of the RhoGEFs with cellular membranes. Besides this tandem motif, RhoGEFs often contain one or more additional signal transduction domains, such as PDZ, Src homology (SH) 2, SH3, and regulator of G protein signaling (RGS), which often function as molecular bridges between different signal transduction pathways. Many RhoGEFs are regulated by a variety of receptor signalings, including heterotrimeric GTP-binding protein (G-protein) signaling and growth factor receptor signaling [3,4].

Recently, it has been reported that morphological changes in neuroblastoma cells are regulated by a cAMP/protein kinase A (PKA)-dependent pathway [5]. However, a RhoGEF identified as β_1Pix has been shown to be phosphorylated and activated by the $G\alpha_s$/PKA-dependent pathway in mesangial cells [6]. In the present study, we investigated whether β_1Pix-related RhoGEFs were regulated by G_s signaling in neuroblastoma cells. We elucidated novel mechanisms by which G_s and G_q signaling regulated the human homologue of the Ascidian protein Posterior End Mark-2 (hPEM-2)-induced SRE-dependent gene transcription and cell morphology through PKA and c-Src activation in Neuro-2a neuroblastoma cells.

Abbreviations: DH, Dbl homology; hPEM-2, human homologue of the Ascidian protein Posterior End Mark-2; PH, pleckstrin homology; PKA, protein kinase A; RhoGEF, rho family small GTPase specific guanine nucleotide exchange factors; SH3, Src homology 3; SRE, serum response element.

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2. Materials and methods

2.1. Plasmids and antibodies

pcDNA3.1-RhoAT19N, pcDNA3.1-Rac1T17N, pcDNA3.1-Cdc42T17N, pcDNA3.1-Gα_sQ213L, pcDNA3.1-Gα_qQ209L, pcDNA3.1-Gα_{12}Q229L, pcDNA3.1-Gαi2Q205L, pcDNA3.1-Gβ_1, pcDNA3.1-Gγ2, and pcDNA3.1-m1 muscarinic acetylcholine receptor (M1R) were purchased from Missouri S&T cDNA Resource Center (Rolla, MO). These genes were subcloned with a pF4A-CMV vector (Promega, Madison, WI) by restriction enzyme digestion or polymerase chain reaction amplification. Complementary DNA clones for RhoGEF genes were isolated during the Kazusa human cDNA project, which aimed to accumulate information on the coding sequences of long cDNAs for unidentified human genes. All RhoGEF clones were subcloned into pFN21A-Myc or pFN21A-mAG vectors by restriction enzyme digestion or polymerase chain reaction amplification. pUSEamp-c-Src dominant negative was from Millipore (Billerica, MA). Humanized monomeric Azami-Green (mAG) expression plasmid (phmAG1-MCLinker) was purchased from MBL (Nagoya, Japan). To prepare the pFN21-mAG vector, the mAG gene was subcloned with a pFN21 vector (Promega, Madison, WI) by restriction enzyme digestion or polymerase chain reaction amplification. pSRE.L-luciferase reporter plasmid was purchased from Agilent Technologies, Inc. (Santa Clara, CA) and pRL-SV40 from NIPPON GENE Co. (Tokyo, Japan). DNA sequences of mutants were confirmed by an ABI Prism 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's protocol. Rabbit polyclonal antibodies against Gα_s, Gα_q and RhoA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against Myc epitope (9E10) was purchased from Roche Diagnostics (Indianapolis, IN) and mouse monoclonal antibodies against human Rac1 and Cdc42 from BD Biosciences (Franklin Lakes, NJ).

2.2. Cell culture, transfection and staining

Neuro-2a cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C. Transient transfection was performed using LipofectAMINE Plus according to the manufacturer's instructions (Invitrogen Co., Carlsbad, CA). To examine the effect of stimulus through M1R on SRE-dependent gene transcription, cells were co-transfected with M1AR and hPEM-2 or other plasmids and cultured for 16 h in DMEM supplemented with 1× Insulin–Tranferrin–Selenium-X (Invitrogen Co., Carlsbad, CA). Then cells were washed twice with DMEM, incubated for 2 h in DMEM, and stimulated with 1 mM carbachol for 6 h. The cells were starved in serum-free DMEM for 8 h and then fixed in 4% paraformaldehyde in phosphate-buffed saline. After applying coverslips, cells were visualized and photographed under a fluorescent microscope (BZ-9000; KEYENCE, Osaka, Japan). Cells of roughly equal and average fluorescence intensity were chosen as comparative examples.

2.3. Assay of SRE-dependent gene transcription

Neuro-2a cells seeded on 24-well plates were co-transfected with the indicated expression plasmids (400 ng of total DNA/well) together with the pSRE.L-luciferase reporter plasmid and the pRL-SV40 control reporter vector. After transfection, cells were washed once with phosphate-buffered saline and lysed with passive lysis buffer. Luciferase activities were determined with the dual-luciferase reporter assay system (Dual-Luciferase Reporter Assay System, Promega, Madison, WI) as described previously [7]. The activity of the experimental reporter was normalized against the activity of the control vector.

3. Results and discussion

3.1. hPEM-2 dependent gene transcription is enhanced by Gα_s and Gα_q signaling in Neuro-2a cells

Five RhoGEFs (β_1Pix, αPix, Asef, hPEM-2 and PDZ-RhoGEF) were used for a luciferase reporter gene assay in Neuro-2a cells to find out which RhoGEFs among these were activated by heterotrimeric G protein subunits. The first four RhoGEFs are structurally related each other [8,9] and PDZ-RhoGEF is known to mediate the activation of RhoA by heterotrimeric G protein Gα_{12} family that includes Gα_{12} and Gα_{13} [10]. We have previously reported that FLJ00018/PLEKHG2, a RhoGEF, induces Rac and Cdc42 activation through direct interaction of G$\beta\gamma$ with the N-terminal region of FLJ00018/PLEKHG2 [7]. As shown in Fig. 1A, overexpression of hPEM-2 and Asef led to increased luciferase expression, and co-expression with a GTPase-deficient mutant of Gα_s (Gα_sQ213L) greatly increased hPEM-2-induced gene transcriptional activity. In contrast, co-expression of β_1Pix with Gα_sQ213L had no effect on the gene transcriptional activity. Although this is in variance with findings showing that β_1Pix is phosphorylated and activated by a Gα_s/protein kinase A-dependent pathway in mesangial cells and is activated by the Gi pathway in RBL-2H3hm1 mast cells [6,11], the variations are probably caused by differences of the cellular signaling pathway in respective cells.

As the data obtained so far supported the activation of hPEM-2 by Gα_sQ213L, we studied the influence of various GTPase-deficient Gα subunit mutants and $\beta\gamma$ subunits on hPEM-2-induced luciferase expression (Fig. 1B). The transcriptional activities of several GTPase-deficient mutants of the G protein α subunit or G$\beta\gamma$ subunit-expressed cells were 1.5–2 times higher than mock (control) cells. Co-expression of hPEM-2 with Gα_sQ213L and a GTPase-deficient mutant of Gα_q (Gα_qQ209L) greatly increased the transcriptional activity in Neuro-2a cells.hPEM-2 has been demonstrated to function as a RhoGEF specific for Cdc42 in fibroblasts [9]. To clarify which subtype of Rho family proteins contributes to the increase of SRE-dependent gene transcription by hPEM-2 in Neuro-2a cells, we examined the effects of various Rho family dominant negative mutants (Fig. 1C). A dominant negative mutant of Rac1 completely inhibited SRE-dependent gene transcription induced by hPEM-2, whereas that of RhoA and Cdc42 inhibited it to a lesser extent. These results seem in variance with some of the previous reports [9,12]. This could be explained by differences of the intracellular signaling pathway, cell types and/or conditions of the experiments.

3.2. hPEM-2 dependent morphological change is regulated by Gα_s and Gα_q signaling in Neuro-2a cells

To examine whether the co-expression of hPEM-2 and Gα_sQ213L or Gα_qQ209L influences cell morphology, we co-transfected monomeric Azami-Green-tagged hPEM2 and Gα_sQ213L or Gα_qQ209L into Neuro-2a cells (Fig. 1D). Cells expressing Gα_sQ213L and Gα_qQ209L were circular, similar to control cells. In contrast, hPEM-2 expression led to cell flattening in Neuro-2a cells. In addition, co-expression of hPEM-2 and Gα_sQ213L or Gα_qQ209L enhanced cell spreading. These results suggest that G_s and G_q signalings regulate cell morphology through hPEM-2 in Neuro-2a cells.

3.3. PKA and c-Src were involved in Gα_s and Gα_q/hPEM-2-dependent gene transcription in Neuro-2a cells

To investigate whether hPEM-2 directly interacts with Gα_s and Gα_q, either Gα_sQ213L or Gα_qQ209L was co-expressed with Myc-

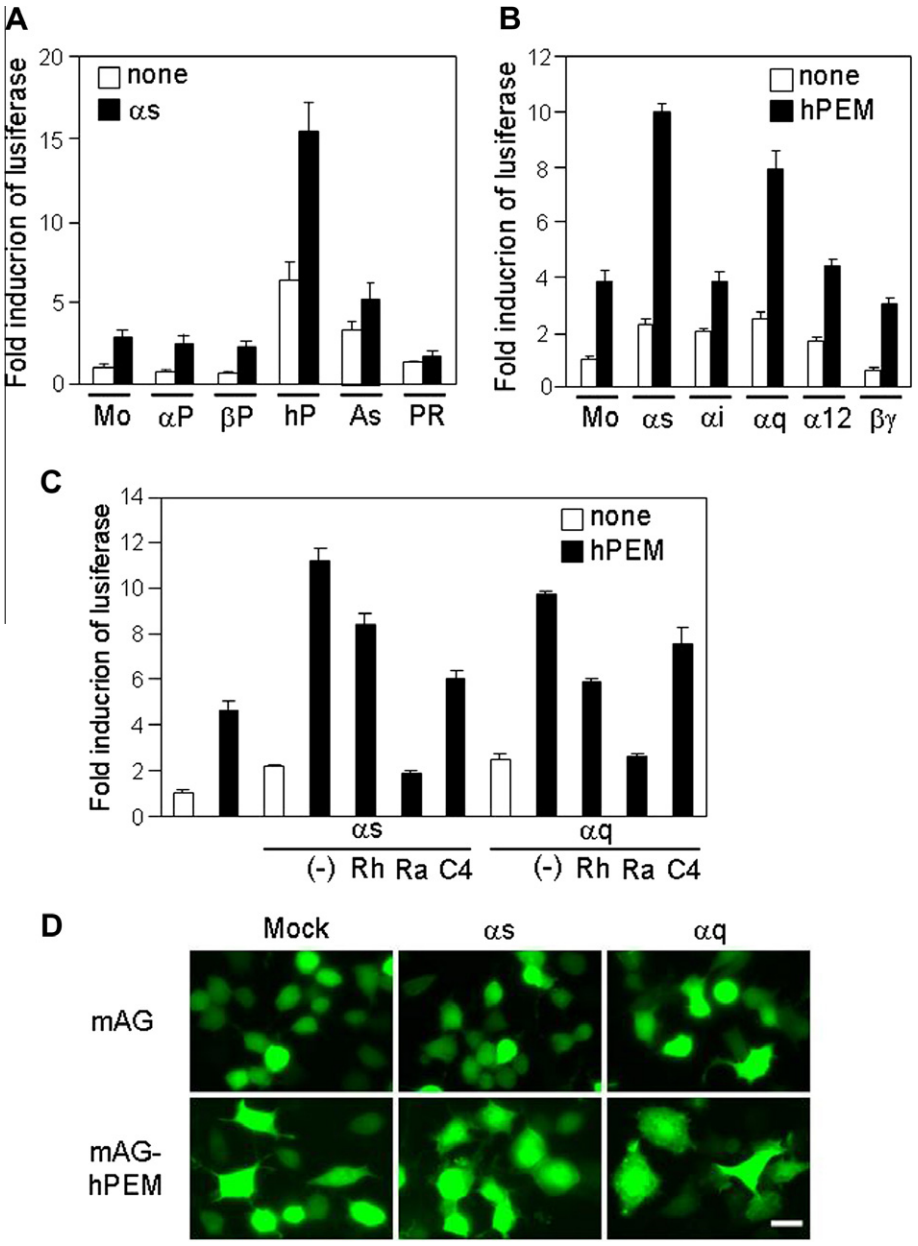

Fig. 1. hPEM-2-induced SRE-dependent gene transcription and cell morphological changes through $G\alpha_s$ and $G\alpha_q$ in Neuro-2a. (A) Effects of RhoGEFs on SRE-dependent expression were analyzed by luciferase reporter gene assay. Cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, pFN4A-CMV control vector (Mo), $G\alpha_sQ213L$ (α_s) and expression vectors for various RhoGEF clones that includes αPix (αP), β_1Pix (βP), hPEM-2(hP), Asef(As), and PDZ-RhoGEF (PR) as indicated. (B) GTPase-deficient mutants of heterotrimeric G protein were co-expressed with hPEM-2 to identify which were related to hPEM-2-induced SRE-dependent expression. Cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, pFN4A-CMV control vector (Mo), expression vectors for hPEM-2 (hPEM), $G\alpha_sQ213L$ (α_s), $G\alpha i2Q205L$ (αi), $G\alpha_qQ209L$ (α_q), $G\alpha_{12}Q2$ 26L (α_{12}), $G\beta_1$ and $G\gamma_2$ (βγ) as indicated. (C) Dominant negative mutants of Rho family small GTPases were co-expressed with hPEM-2 to find out those related to hPEM-2-induced SRE-dependent expression. Cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, expression vectors for hPEM-2 (hPEM), $G\alpha_sQ213L$ (α_s) or $G\alpha_qQ209L$ (α_q) and dominant negative mutants of RhoA (Rh), Rac1 (Ra) or Cdc42 (C4), as indicated. In all experiments, the average of luciferase activities obtained with the control was taken as 1.0, and relative activities are shown as a fold induction of luciferase expression. Results show means ± SD from three separate experiments. (D) Neuro-2a cells were transfected with mAG or mAG-tagged hPEM2 (hPEM) with or without $G\alpha_sQ213L$ (α_s) and $G\alpha_qQ209L$ (α_q). The results shown are representative of three independent experiments. Scale bar, 10 μm.

fused hPEM-2 in Neuro-2a cells, and cell lysates were immunoprecipitated with anti-Myc antibody and analyzed for co-precipitated $G\alpha_s$ or $G\alpha_q$ protein. However, the $G\alpha_s$ and $G\alpha_q$ proteins did not co-precipitate with hPEM-2 (data not shown).

To find out what kind of signaling molecules function in the $G\alpha_s$ or $G\alpha_q$/hPEM-2-induced SRE-dependent gene transcription, we examined the effects of some inhibitors on the $G\alpha_s$ or $G\alpha_q$/hPEM-2-induced SRE-dependent gene transcription. It is well known that $G\alpha_s$ stimulates the cAMP/PKA signaling pathway. A recent study showed that the EP2 prostaglandin E_2 receptor, which preferentially couples to the $G\alpha_s$ family, was expressed in Neuro-2a [15]. To test whether a G_s-coupled receptor system enhances SRE-dependent gene transcription through hPEM-2, we examined the effects of butaprost, a specific agonist for EP2 receptor in hPEM-2 expressing cells. The treatment of butaprost significantly enhanced SRE-dependent gene transcription in hPEM-2 expressing cells, but

not in control cells. The enhancement of gene transcription by butaprost treatment was reduced by the treatment of a PKA inhibitor, H-89 (Fig. 2A). Furthermore, we clarified the effect of H-89 on $G\alpha_s$/hPEM-2-induced SRE-dependent gene transcription. As a result, the gene transcription was significantly reduced by H-89 (Fig. 2B). These results suggest that hPEM-2-induced SRE-dependent gene transcription involves the $G\alpha_s$/PKA pathway.

As it is well known that $G\alpha_q$ stimulates the c-Src signaling pathway [13,14], we examined the effect of a selective Src family kinase inhibitor, SU6656, on $G\alpha_q$/hPEM-2-induced SRE-dependent gene transcription. The transcription was attenuated by SU6656 (Fig. 3A). To check whether activation of c-Src was required for $G\alpha_q$/hPEM-2-induced SRE-dependent gene transcription, the cells were co-expressed with the dominant negative mutant of c-Src (SrcDN). $G\alpha_q$/hPEM-2-induced SRE-dependent gene transcription was also reduced by co-expression of the dominant negative mutant of c-Src (Fig. 3B). These results suggest that the $G\alpha_s$/hPEM-2-induced SRE-dependent gene transcription involved PKA activation, and the $G\alpha_q$/hPEM-2-induced SRE-dependent gene transcription involved c-Src activation. To examine whether a G_q-coupled receptor system would enhance the SRE-dependent gene transcription through hPEM-2, we co-expressed hPEM-2 together with

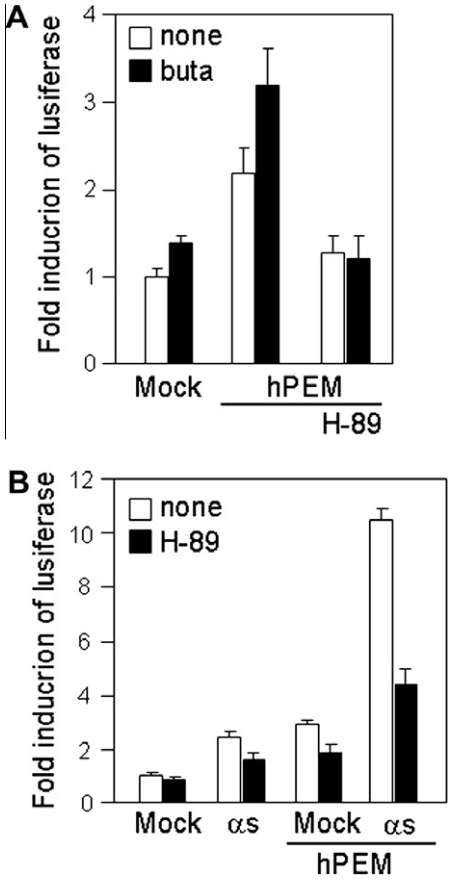

Fig. 2. Involvement of PKA and $G\alpha_s$-coupled receptor in hPEM-2-induced SRE-dependent gene transcription. (A) Effects of $G\alpha_s$-coupled receptor on SRE-dependent expression were analyzed by luciferase reporter gene assay. Neuro-2a cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs and expression vectors for hPEM-2 (hPEM) as indicated. Then cells were starved, incubated in the presence or absence of 10 μM H-89 for 1 h, and stimulated for 6 h with or without 10 μM butaprost (buta). In all experiments, the average luciferase activity obtained with the control was taken as 1.0, and relative activities are shown as a fold induction of luciferase expression. Results are means ± SD from three separate experiments. (B) Effects of a PKA inhibitor, H-89, on $G\alpha_s$ /hPEM-2-induced SRE-dependent expression were analyzed by luciferase reporter gene assay. Neuro-2a cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, expression vectors for hPEM-2 and $G\alpha_sQ213L$ (α_s) as indicated. Then cells were starved and incubated for 6 h with or without H-89 before cell lysis.

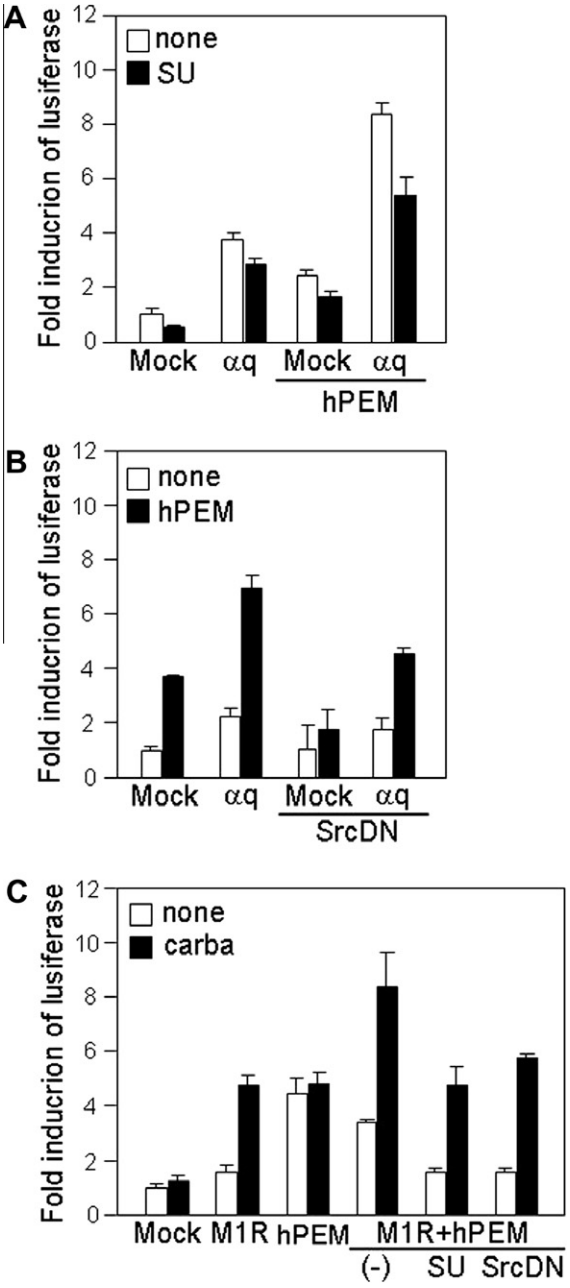

Fig. 3. Involvement of c-Src and $G\alpha_q$-coupled receptor in hPEM-2-induced SRE-dependent gene transcription. (A) Effects of c-Src inhibitor, SU6656 on $G\alpha_q$ /hPEM-2-induced SRE-dependent expression were analyzed by luciferase reporter gene assay. Cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, expression vectors for hPEM-2 and $G\alpha_qQ213L$ (α_q) as indicated. Then cells were starved, incubated in the presence or absence of 10 μM SU6656 for 12 h before lysis. (B) Effects of the c-Src dominant negative mutant on $G\alpha_q$/hPEM-2-induced SRE-dependent expression were analyzed by luciferase reporter gene assay. Cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, expression vectors for hPEM-2, $G\alpha_qQ213L$ (α_q), and a dominant negative mutant of c-Src (SrcDN) as indicated. (C) Effects of $G\alpha_q$-coupled receptor on hPEM-2-induced SRE-dependent expression were analyzed by luciferase reporter gene assay. Neuro-2a cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, expression vectors for hPEM-2 (hPEM) and M1 muscarinic acetylcholine receptor (M1R) as indicated. Then cells were starved, incubated in the presence or absence of 10 μM SU6656 (SU) for 1 h, and stimulated for 6 h with or without 100 μM carbachol. In all experiments, the average luciferase activity obtained with the control was taken as 1.0, and relative activities are shown as a fold induction of luciferase expression. Results are means ± SD from three separate experiments.

the m1 muscarinic acetylcholine receptor (M1R) in Neuro-2a cells. The M1R preferentially coupled to the $G\alpha_q$ family, but could additionally activate other G proteins. The combined expression of hPEM-2 and M1R led to a significant enhancement of luciferase expression by carbachol treatment, whereas stimulation was not observed in cells expressing hPEM-2 alone. The expression of SrcDN and the treatment with SU6656 strongly inhibited carbachol-induced luciferase production (Fig. 3C). These results suggest that hPEM-2-induced SRE-dependent gene transcription involves the $G\alpha_q$/c-Src pathway.

3.4. Some regions of hPEM-2 related with G protein signalings in Neuro-2a cells

Next, to determine the region in hPEM-2 essential for enhancement of SRE-dependent gene transcription by G_s and G_q, we performed the reportor gene experiment with four truncated mutants of hPEM-2; WT (amino acids (aa) 1–516), SH3D (aa 89–516), DP1 (aa 89–434), DP2 (aa 107–434). The levels of luciferase production in SH3D- and DP1-expressing cells were higher than the levels in WT-expressing cells. The levels of luciferase production induced by co-expression of SH3D and $G\alpha_sQ213L$ were higher than the levels in SH3D-expressing cells. In contrast, the levels of luciferase production induced by co-expression of SH3D and $G\alpha_qQ209L$ were similar to the levels in SH3D-expressing cells (Fig. 4B). These results suggest that the region including the SH3 domain of hPEM-2 contains the negative regulatory region for the ability of SRE-dependent luciferase production and plays an important role in interaction with the G_q signaling. However, the levels of luciferase production induced by co-expression of DP1 and $G\alpha_sQ213L$ or $G\alpha_qQ209L$ were higher than the levels in DP1-expressing cells (Fig. 4B). In addition, the level of luciferase production by DP1 was almost the same as that by WT. These results suggest the possibility that DP1 also contains a region associated with interaction with G protein signalings. In addition, these results also suggest the possibility that the C terminal region of hPEM-2 contributes to the increase of luciferase production by hPEM-2. On the other hand, although the expression level of DP2 was the same as the levels of other mutants (data not shown), DP2 was inactive (Fig. 4B). This result suggests that the deletion of amino acid sequences near the DH domain influences the structure and function of the DH domain that interacts with Rho GTPase and constitutes the minimal unit required for nucleotide exchange. A previous report shows that the region near the DH domain of hPEM-2 is critical for interaction with gephyrin, the receptor-anchoring protein [16]. This issue requires further, more detailed examination in the future.

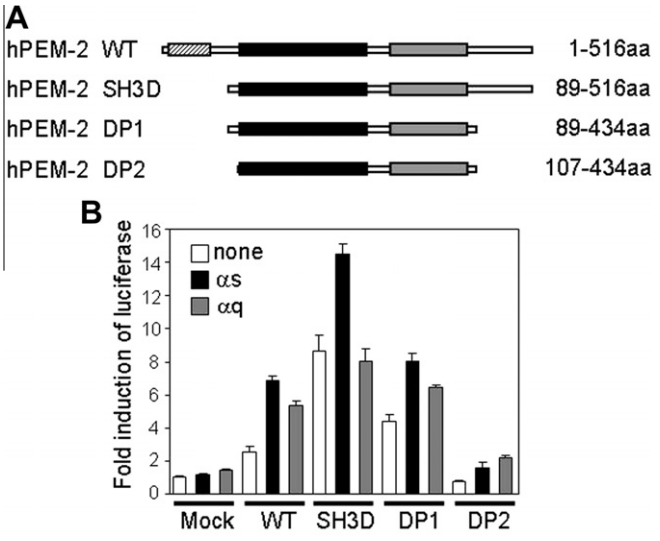

Fig. 4. hPEM-2 transmits two G protein signals by using a different region. (A) A schematic diagram to illustrate various hPEM-2 deletion mutants is shown. WT, SH3D, DP1 and DP2 constructs code for amino acid residues 1–516, 89–516, 89–434, 107–434 of hPEM-2, respectively. (B) hPEM-2-induced SRE-dependent gene transcription was analyzed using hPEM-2 deletion mutants. Cells were co-transfected with pSRE.L-luciferase, pRL-SV40 plasmid DNAs, expression vectors for various hPEM-2 deletion mutants, and $G\alpha_sQ213L$ (α_s) or $G\alpha_qQ209L$ (α_q) as indicated. The average luciferase activity obtained with the control was taken as 1.0, and relative activities are shown. Results are means ± SD from three separate experiments.

In this present study, we report for first time that G_s and G_q signalings enhance hPEM-2-stimulated SRE-dependent gene transcription by PKA- and c-Src-dependent pathways, respectively. These findings raise the possibility that other signaling pathways regulate hPEM-2-induced SRE-dependent gene transcription, because two inhibitors partially inhibited it, respectively. These issues call for clarification in future studies. hPEM-2 also binds to gephyrin, a protein component of the postsynaptic protein network of inhibitory synapses [17,18]. Thus, although we are aware of the protein–protein interaction, the activation mechanisms of hPEM-2, a binding partner of gephyrin, are still unknown. Gephyrin is known to interact with glycine receptors or several proteins controlling actin cytoskeletal dynamics including profilin and Mena/VASP [18,19]. It is feasible that G_s or G_q/hPEM-2 signaling systems probably carry out a certain function in glycine receptor signaling in the nervous system. Further studies may unravel the physiological function of this signaling system of hPEM-2.

Acknowledgments

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