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The RhoA-specific guanine nucleotide exchange factor p63RhoGEF binds to activated $G\alpha_{16}$ and inhibits the canonical phospholipase Cβ pathway

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ABSTRACT

Heterotrimeric G proteins regulate diverse physiological processes by modulating the activities of intracellular effectors. Members of the $G\alpha_q$ family link G protein-coupled receptor activation to phospholipase Cβ (PLCβ) activity and intracellular calcium signaling cascades. However, they differ markedly in biochemical properties as well as tissue distribution. Recent findings have shown that some of the cellular activities of $G\alpha_q$ family members are independent of PLCβ activation. A guanine nucleotide exchange factor, p63RhoGEF, has been shown to interact with $G\alpha_q$ proteins and thus provides linkage to RhoA activation. However, it is not known if p63RhoGEF can associate with other $G\alpha_q$ family members such as $G\alpha_{16}$. In the present study, we employed co-immunoprecipitation studies in HEK293 cells to demonstrate that p63RhoGEF can form a stable complex with the constitutively active mutant of $G\alpha_{16}$ ($G\alpha_{16}$QL). Interestingly, overexpression of p63RhoGEF inhibited $G\alpha_{16}$QL-induced IP_3 production in a concentration-dependent manner. The binding of $PLC\beta_2$ to $G\alpha_{16}$QL could be displaced by p63RhoGEF. Similarly, p63RhoGEF inhibited the binding of tetratricopeptide repeat 1 to $G\alpha_{16}$QL, leading to a suppression of $G\alpha_{16}$QL-induced Ras activation. In the presence of p63RhoGEF, $G\alpha_{16}$QL-induced STAT3 phosphorylation was significantly reduced and $G\alpha_{16}$QL-mediated SRE transcriptional activation was attenuated. Taken together, these results suggest that p63RhoGEF binds to activated $G\alpha_{16}$ and inhibits its signaling pathways.

1. Introduction

G protein coupled receptors (GPCRs) receive extracellular stimuli and activate heterotrimeric (αβγ) guanine nucleotide binding proteins (G proteins) to regulate a host of effectors and channels in the cell. The activity of Gα subunits is regulated by the binding of guanine nucleotides. Agonist-bound GPCRs promote the nucleotide exchange of Gα subunits, thus favoring the formation of active GTP-bound Gα subunits to regulate downstream effectors. To some extent, GPCRs are analogous to the guanine nucleotide exchange factors (GEFs) that transduce signals from growth factor receptors to the monomeric GTPases such as the Ras and Rho proteins.

There are four members in the $G\alpha_q$ family, namely $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$. The involvement of $G\alpha_q$ family members in a signaling pathway can be determined by the measurement of the activity of phospholipase Cβ (PLCβ), the canonical effector of $G\alpha_q$ family members, in a pertussis toxin resistant manner. The activation of $G\alpha_q$ family members stimulates PLCβ which triggers the hydrolysis of phosphatidylinositol bisphosphate to form inositol trisphosphate (IP_3) and diacylglycerol (DAG) [1]. $G\alpha_{16}$ has been shown to activate PLCβ and stimulate a variety of downstream signaling pathways such as STAT3 [2–4], MAPKs [2,3] and NFκB [4–6]. In principle, G proteins can receive multiple signals and modulate diverse signaling pathways. Thus G proteins function as a molecular switch and have the potentials to interact with a number of proteins to stimulate a multitude of cascades. Increasing evidence have shown the interactions between G proteins and novel effectors or regulators, such as regulators of G protein signaling (RGS proteins) and activators of G protein signaling (AGS proteins). RGS2 was found to effectively inhibit all four $G\alpha_q$ family members [7]. It has been reported that $G\alpha_{16}$ proteins can interact with tetratricopeptide repeat 1 (TPR1) which serves as an adaptor protein for Ras signaling [8]. Recently, it has been discovered that myristoylated Ric-8A can bind to $G\alpha_{15}$ (the mouse homolog of human $G\alpha_{16}$) and enhances the olfactory receptor-induced calcium mobilization [9]. No further investigations have been reported on the binding proteins of $G\alpha_{16}$. G protein receptor kinase 2 (GRK2) was found to be selective for $G\alpha_q$ family members except $G\alpha_{16}$ [10]. Phosphoprotein 50 (EBP50) is a

Abbreviations: ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptors; GTPase, GTP hydrolyase; HEK, human embryonic kidney; MAPK, mitogen-activating protein kinase; NFkB, nuclear factor κB; PLCβ, phospholipase Cβ; SRE, serum response element; SRF, serum response factor; STAT3, signal transducer and activator of transcription 3; TPR1, tetratricopeptide repeat 1.

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$G\alpha_q$-binding protein, but it does not bind to $G\alpha_{16}$ [11]. However, it is not known if other $G\alpha_q$-binding proteins exhibit similar selectivity for the various members of the $G\alpha_q$ family.

Rho GTPases are important regulators of gene transcription and actin cytoskeletal remodeling in a variety of cellular events such as cell migration, proliferation and muscle contraction. Its involvement in regulating the hypertrophy of cardiomyocytes has been documented [12]. The activation of Rho GTPases is linked to an induction of hypertrophic cell growth and proliferation in vitro. In contrast, an in vivo study showed that an overexpression of RhoA resulted in heart failure [13]. Rho GTPases can be activated by a variety of GPCRs that are coupled to different G proteins. Upon hypertrophic stimuli, activation of G_q-coupled receptors leads to the stimulation of RhoA activity in cardiac myocytes [14]. The activity of Rho GTPases can be regulated by a family of Rho GTPase guanine nucleotide exchange factors (RhoGEFs). RhoGEFs are defined by the presence of a catalytic Dbl homology domain (DH domain) followed by a pleckstrin homology domain (PH domain) [15]. The guanine nucleotide exchange activity of RhoGEFs is catalyzed by the DH domain whereas the PH domain serves as a regulatory region to help this exchange activity. It has also been found that PH domain is important for phosphoinositides binding [16] and protein–protein interaction [17]. Several RhoA-specific GEFs, including PDZRhoGEF and p115RhoGEF, can interact with $G\alpha_{12/13}$ specifically. These proteins are important molecules in modulating the activation of RhoA mediated by GPCRs that are coupled to $G\alpha_{12/13}$. A Rho-specific guanine nucleotide exchange factor (GEF) known as p63RhoGEF appears to serve as a novel mediator of $G\alpha_{q/11}$ signaling via protein–protein interaction [18]. However, it is not clear if p63RhoGEF can modulate the signaling pathways of other $G\alpha_q$ family members such as $G\alpha_{16}$.

In this study, we demonstrate that p63RhoGEF forms signaling complexes with $G\alpha_{16}$ proteins with a higher affinity towards the constitutively active $G\alpha_{16}$QL. Expression of p63RhoGEF prevents the interaction between $G\alpha_{16}$ and its known binding proteins, including PLCβ and TPR1, thus leading to the suppression of $G\alpha_{16}$-driven STAT3 phosphorylation, Ras activation and SRE transcriptional activity. In contrast to the findings of $G\alpha_q$, $G\alpha_{16}$ failed to potentiate RhoA activation and SRF transcriptional activity by p63RhoGEF.

2. Materials and methods

2.1. Materials

HEK293 cells were purchased from American Type Culture Collection (ATCC; Rockville, MD). The cDNAs encoding the full-length p63RhoGEF, p63RhoGEFΔN and p63RhoGEFΔC were kindly provided by Dr. Thomas Wieland (Institut für Pharmakologie und Toxikologie, Universität Heidelberg, Germany). Plasmid DNA of FLAG-tagged TPR1 was donated by Dr. Richard D. Ye (University of Illinois, USA). The plasmid of HA-tagged H-Ras was purchased from Missouri S&T cDNA Resource Center. Anti-c-myc and Anti-$G\alpha_{16}$ were obtained from Roche Applied Science and Torrey Pines Biolabs respectively. Ras assay reagent and Anti-Ras were obtained from Upstate Biotechnology. Other antibodies were from Cell Signaling (Beverly, MA). Anti-c-Myc EZview Affinity Gel was from Sigma Aldrich (St. Louis, MO, USA). Cell culture reagents, including LipofectAMINE PLUS, were purchased from Invitrogen. The luciferase reporter gene, pSRE-Luc and pSRF-Luc, were from Clontech laboratories (Palo Alto, CA, USA) and Stratagene (La Jolla, CA, USA) respectively. The luciferase substrate and lysis buffer were obtained from Roche Diagnostics (Mannheim, Germany).

2.2. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured in Minimum Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin, and grown at 37 °C in an environment of 5% CO_2. Renewal of growth medium was performed every 2 to 3 days. HEK293 cells were transferred to 12-well plates at 3×10^5 cells/well (for phospholipase C assay, SRE Luciferase assay) or to 10-cm plates at 3×10^6 cells (for co-immunoprecipitation). Transfection was performed by means of LipofectAMINE PLUS reagents following the supplier's instructions.

2.3. Co-immunoprecipitation

HEK293 cells were seeded on 10-cm plates at 3×10^6 cells and grown for 24 h before transfection. Cells were transfected with various cDNAs for 2 days using LipofectAMINE PLUS reagents. Cells were rinsed with PBS and scrapped off from the dish and transferred to a centrifuge tube. Then, the cells were crosslinked with 1 μM dithiobis (succinimidyl)propionate (DSP) in 1 ml of PBS for 45 min at room temperature. The reaction was terminated by incubating the cells with 20 mM Tris HCl for 15 min and cells were lysed with 1 ml of RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin). The cell lysates were incubated at 4 °C for 30 min. The cell lysates were centrifuged at 14,000 $\times g$ in a precooled centrifuge and the pellets were cleared and discarded. The immunoprecipitating antibodies were then added to the supernatant and incubated overnight at 4 °C. The immunocomplexes were captured by the addition of 20 μl of 50% protein A-agarose bead and incubated for 1 h at 4 °C. The samples were then centrifuged for 1 min in a microcentrifuge and the pellets were washed three times with 1 ml of RIPA lysis buffer. The immunoprecipitated complexes were eluted by adding 60 μl of SDS sample buffer. The total cell lysates and immunoprecipitates were separated by SDS-PAGE and analyzed by Western blot.

2.4. Ras and Rho activation assays

The cellular level of activated Ras was measured using a Ras activation assay kit following the manufacturer's instructions. In brief, the transfected HEK293 cells were serum starved after 24 h transfection. The cells were washed with PBS and lysed with Mg^{2+} Lysis Buffer (MLB, 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The cell lysates were scrapped off from the dish and transferred to a new centrifuge tube and incubated for 15 min at 4 °C with agitation. 1 mg of cell lysates was then incubated for 1 h at 4 °C with 20 μg of Ras assay reagent. After washing the beads with MLB, the bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE separation and detected by Western blot with an antibody against Ras. Similarly, the activity of Rho was determined by the Rho activation assay kit.

2.5. SRE-meditated luciferase reporter gene assay

HEK293 cells were seeded onto 12-well plates at 3×10^5 cells/well and grown for 24 h prior to transfection. Cells were transiently co-transfected with various cDNAs including pSRE-Luc using LipofectAMINE PLUS reagents. After 24 h transfection, cells were treated with serum-free MEM medium overnight. The cells were lysed with 150 μl of lysis buffer from Roche Diagnostics luciferase assay kit and then gently shaken on ice for 30 min. 25 μl of cell lysates were transferred into 96-well microtiter plates. To detect the luciferase activity, 25 μl lysis buffer and 25 μl luciferase substrate were added and the resulting luminescence was measured by a micotiter plate luminometer MicroLumatPlus LB96 V from EG&G Berthold.