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Short communication

THE GTPASE DOMAIN OF Galphao CONTRIBUTES TO THE FUNCTIONAL INTERACTION OF Galphao WITH THE PROMYELOCYTIC LEUKEMIA ZINC FINGER PROTEIN

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Abstract: Go, one of the most abundant heterotrimeric G proteins in the brain, is classified as a member of the Gi/Go family based on its homology to Gi proteins. Recently, we identified promyelocytic leukemia zinc finger protein (PLZF) as a candidate downstream effector for the alpha subunit of Go (Gαo). Activated Gαo interacts with PLZF and augments its function as a repressor of transcription and cell growth. G protein-coupled receptor-mediated Gαo activation also enhanced PLZF function. In this study, we determined that the GTPase domain of Gαo contributes to Gαo:PLZF interaction. We also showed that the Gαo GTPase domain is important in modulating the function of PLZF. This data indicates that the GTPase domain of Gαo may be necessary for the functional interaction of Gαo with PLZF.

Key words: Cell growth, Differentiation, Domain, G protein

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Abbreviations used: β-gal – β-galactosidase; BrdU – bromodeoxyuridine; Gαo – the alpha subunit of Go; G proteins – heterotrimeric GTP-binding proteins; GST – glutathione-*S*-transferase; PKA – cAMP-dependent protein kinase; PLZF – promyelocytic leukemia zinc finger protein

INTRODUCTION

Heterotrimeric GTP-binding proteins (G proteins) mediate signal transduction and amplification in a variety of cell-signaling pathways [1-3]. In its inactive state, the alpha subunit (Gα) of the heterotrimeric G protein contains a bound GDP and has a high affinity for the Gβγ heterodimeric subunit. When activated by an appropriate signal, membrane-bound G protein-coupled receptors bind the heterotrimer, forming a quaternary complex that catalyzes the exchange of the bound GDP for GTP in Gα. This exchange facilitates the sequential dissociation of Gα from the Gβγ dimer, and the release of these G protein subunits from the receptor. The dissociated Gα and/or Gβγ participate(s) in interactions with various downstream effectors.

Go, one of the most abundant G proteins expressed in the brain [4], is classified as a member of the Gi/Go family by virtue of its sequence homology to Gi proteins. However, unlike the α-subunits of Gi proteins, which inhibit adenylyl cyclase and decrease intracellular cyclic AMP levels, the α-subunit of Go (Gαo) has no effect on adenylyl cyclase; its role, other than to act as a reservoir of βγ subunits available for liberation upon receptor activation, is unclear at present. Recently, we identified PLZF as a Gαo-interacting partner [5]. Activated Gαo interacts directly with PLZF and enhances its cellular function as a repressor of transcription and cell growth. These findings indicate that Gαo can modulate cell growth-related gene expression via PLZF activation.

PLZF was initially identified as a fusion protein with the retinoid acid receptor resulting from a variant t(11;17) chromosomal translocation that occurs in a small subset of acute promyelocytic leukemia patients [6]. The PLZF protein, which is highly conserved among humans, mice, and chickens [7], is a nuclear protein that has at its C-terminus nine C_2-H_2 zinc finger motifs that bind directly to DNA [8]. The amino terminus of PLZF contains a BTB/POZ domain that mediates self-dimerization and transcriptional repression by binding to nuclear co-repressors, such as N-CoR, SMRT, Sin3A, and histone deacetylases [9-11]. PLZF functions as a transcriptional regulator of cell-cycle progression by binding to the promoter of target genes, such as those for cyclin A and the interleukin-3 receptor α chain [12-15]. Analysis of PLZF knock-out mice revealed that the protein is a growth inhibitor and pro-apoptotic factor in the limb bud *in vivo* [16]. PLZF is also involved in the differentiation of cells, including megakaryocytes [17], osteoblasts [18], and neurons [19]. In this study, we confirmed a direct interaction between Gαo and PLZF and characterized the functional domain of Gαo in modulating the function of PLZF.

MATERIALS AND METHODS

Plasmids

The plasmids pGEX2T-Gαo and pGEX2T-PLZF were respectively used to express the glutathione-*S*-transferase (GST) fusion proteins of Gαo and PLZF [5].

The plasmids pRC/CMV-Gαo and pRC/CMV-Gαo^{Q205L} were respectively used to express the wild-type and activated mutant forms (Gαo^{Q205L}) of Gαo [20]. The plasmids pcFLAG-PLZF and pcHA-PLZF were respectively used to express FLAG- and HA-tagged PLZF [5].

Co-immunoprecipitation

GST, GST-Gαo, and GST-PLZF fusion proteins were purified according to a standard protocol [21]. Purified proteins (5 μg each) were mixed in PBTX buffer (PBS containing 5 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100 and protease inhibitors) containing 0.1% BSA, as indicated. The protein mixtures were pre-cleared by incubation with 20 μl of Protein A-Sepharose CL-4B beads (10% slurry; Amersham Bioscience) and then incubated with 1 μg of the indicated antibody with gentle rotation for 4 h at 37ºC, followed by incubation with 50 μl beads. After a 2-h room-temperature incubation, the beads were washed with PBTX buffer. The bound proteins were eluted with an SDS sample buffer, separated by SDS-PAGE and immunoblotted with antibodies against PLZF (1:100 dilution, Calbiochem) or Gαo (1:500 dilution, Santa Cruz Biotechnology).

Construction of the Gαo deletion mutants

Gαo deletion mutant expression plasmids (pGEX2T-GαoΔN, pGEX2T-GαoΔNH, pGEX2T-GαoΔG, and pGEX2T-GαoΔHL2G) encoding the GST-tagged version of the Gαo deletion mutants (respectively, GST-GαoΔN, GST-GαoΔNH, GST-GαoΔG, and GST-GαoΔHL2G) were constructed as follows. Gαo cDNA was amplified by PCR from a pRC/CMV-Gαo template using the following primers: for GαoΔN, 5'-CCA TGA TGA TGG CTT CTC T-3' and 5'-TCA GTA CAA GCC ACA GCC-3'; for GαoΔNH, 5'-CAA AAC AAC TGG CAT CGT A-3' and 5'-TCA GTA CAA GCC ACA GCC-3'; for GαoΔG, 5'-GGA ATT CGA TTC ATG GGA-3 and 5'-TAC GAT GCC AGT TGT TTT-3'; and for GαoΔHL2G, 5'-GGA ATT CGA TTC ATG GGA-3' and 5'-AGA GAA GCC ATC TTC ATG-3'. The amplified products were inserted into pGEX2T (Amersham Bioscience). The pGEX2T-GαoΔL2 deletion mutant plasmid was generated by the *BamHI* digestion and self-ligation of pGEX2T-Gαo. Expression plasmids (pcFLAG-GαoΔL2G) encoding FLAG-tagged versions of the Gαo deletion mutants (FLAG-GαoΔL2G) were created by the *BamHI* digestion and self-ligation of pcFLAG-Gαo.

GST pull-down assay

Bacterial cell lysates containing GST fusion proteins were incubated with glutathione-Sepharose 4B beads (Amersham Bioscience) for 1 h at 4ºC in PBTX buffer, and then washed extensively with PBXT. 293T cell extracts (500 μg) expressing the FLAG-PLZF protein were added to the beads and incubated for 1 h at 37ºC. After extensive washing with PBTX buffer, the bound proteins were

eluted with SDS sample buffer and subjected to immunoblot analysis using antibodies against FLAG (1:500 dilution, Sigma).

BrdU-incorporation assay

293T cells were plated on coverslips at a density of $1x10^3$ per coverslip and transfected with combinations of expression plasmids, as indicated in the text. The GFP expression plasmid, pEGFP (Clontech), was employed as a transfection marker under all conditions. After incubation for 24 to 48 h to allow expression by the transfected plasmids, the cells were labeled with 10 μM bromodeoxyuridine (BrdU) and incubated for 12 h, then washed with PBS and fixed in 4% paraformaldehyde for 15 min. After fixation, 2 N HCl was applied for 30 min followed by 0.1 M sodium borate (pH 8.5) for 10 min. The cells were subsequently incubated with antibodies against BrdU (1:250 dilution, Sigma) and GFP (1:500 dilution, Molecular Probe) at 4ºC overnight. Their immunoreactivity was visualized using Alexa Fluor 568-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probe). To visualize the nuclei, the cells were counterstained with DAPI (Vector Laboratories).

Luciferase reporter gene assay

293T cells were plated on 6-well tissue culture dishes at a density of $5x10^5$ cells per well and transfected with the indicated expression plasmids and CyclinA2-Luc reporter gene plasmid [22]. To normalize the transfection efficiency, the cells were transfected with 0.3 μg of a β-galactosidase (β-gal) expression plasmid (pCMV-β-gal). A constant total amount of plasmid DNA was maintained in the transfections by adding pcDNA3 (Invitrogen). After 24 h, the 293T cells were serum starved for 16 h and harvested. Cell lysates were assayed for luciferase and β-gal activity using a Luciferase assay system (Promega), as recommended by the manufacturer.

Cyclin A expression

293T cells ($1.5x10^6$ cells/dish) were plated on 100-mm tissue culture dishes. The cells were transfected with appropriate combinations of expression plasmids, as indicated in the text, then lysed with RIPA buffer (50 mM Tris-Cl, pH.7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors). Extracts were subjected to immunoblot analysis using antibodies against cyclin A (1:500 dilution, Santa Cruz Biotechnology) or α-tubulin (1:1000 dilution, Upstate Biotechnology).

RESULTS AND DISCUSSION

Gαo interacts directly with PLZF

In our previous study, we identified PLZF as a Gαo-interacting protein and showed that Gαo binds directly to PLZF and promotes its cellular functions [5]. To confirm that these proteins directly interact, we purified the GST fusion

proteins of Gαo and PLZF from bacterial lysates for use in co-immunoprecipitation assays. As shown in Fig. 1, after the indicated incubation, the purified proteins were co-immunoprecipitated, showing that Gαo interacts with PLZF without the assistance of other proteins.

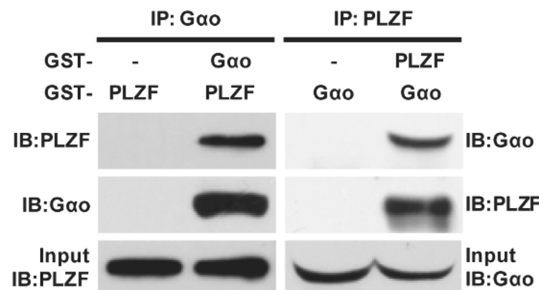

Fig. 1. Direct interaction between Gαo and PLZF. Purified GST, GST-Gαo and GST-PLZF were incubated and subjected to a co-immunoprecipitation assay, as described in the Materials and Methods section. The input indicates lanes loaded with the 10% purified protein mixtures used for immunoprecipitation.

The GTPase domain of Gαo contributes to PLZF interaction

G proteins consist of at least three domains: an N-terminal domain; an α-helical domain unique to the highly homologous family of heterotrimeric G-proteins; and a GTPase domain common to members of the GTPase superfamily. These domains are interconnected by two linker sequences (L1 and L2). To identify the regions of Gαo that bind to PLZF, we constructed a series of deletion mutants and expressed them as GST fusion proteins with the GST epitope at the N-termini (Fig. 2A). GST pull-down assays were performed by incubating purified GST-tagged deletion mutants with 293T cell extracts containing exogenously expressed FLAG-PLZF and examining for PLZF retention by Gαo mutant proteins. Deleting the N-terminus (GST-GαoΔN) alone or together with the helical domain (GST-GαoΔNH) did not affect Gαo interactions with PLZF (Fig. 2B, lanes 3 and 4). The mutant lacking the L2 domain (GST-GαoΔL2) likewise retained the ability to interact with PLZF (Fig. 2B, lane 7). Importantly, deleting the GTPase domain alone (GST-GαoΔG) or together with the helical domain (GST-GαoΔHL2G) abolished PLZF binding (Fig. 2B, lanes 5 and 6). These results indicate that the specific determinant of Gαo:PLZF interaction resides in the C-terminal half of Gαo (residues 213 to 354). This region includes only amino acids that contribute to the formation of the GTPase domain of Gαo, and excludes the helical domain. We previously found that Gαo interacts directly through its GTPase domain with cAMP-dependent protein kinase (PKA), thereby compartmentalizing PKA signaling [21]. Thus, the GTPase domain of Gαo may be necessary for its interaction with related proteins.

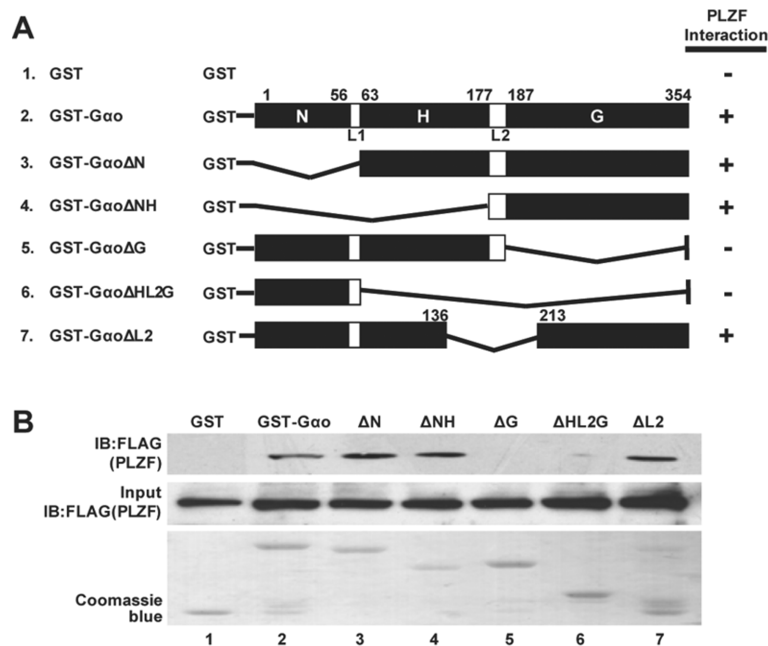

Fig. 2. The GTPase domain of Gαo contributes to PLZF interactions. A – A schematic diagram of the structures of Gαo GST fusion proteins (full-length and deletion mutants). N, N-terminus; L1, linker 1; L2, linker 2; H, helical domain; G, GTPase domain. B – Beads charged with bacterially expressed GST fusion proteins were incubated with 293T cell extracts expressing 10 μg of the FLAG-PLZF plasmid. Bound proteins were immunoblotted with anti-FLAG antibodies. The input indicates lanes loaded with the 10% 293T cell extracts used in the GST pull-down assays. Coomassie blue staining was used to show GST fusion protein levels.

The Gαo GTPase domain-deletion mutant does not modulate PLZF activity

Gαo activation augments PLZF's function as a cell growth suppressor and transcriptional repressor [5]. To establish the effects of the Gαo GTPase deletion mutant on cell growth regulation by PLZF, we performed BrdU incorporation assays on 293T cells co-expressing $G\alpha o^{Q205L}$ or FLAG-GαoΔL2G, a mutant lacking residues 137 to 354 of Gαo, together with HA-PLZF. The GFP expression plasmid, pEGFP, was employed as a transfection marker under all conditions. Co-expression of PLZF and $G\alpha o^{Q205L}$ reduced the percentage of BrdU-positive S-phase cells among the GFP-positive cells (Fig. 3A and B). Importantly, this marked repression was completely abolished by co-expression of GαoΔL2G and PLZF. Next, to determine the effects of the Gαo GTPase deletion mutant on the transcriptional repressor function of PLZF, we evaluated the expression of cyclin A2, a target gene of PLZF, employing a luciferase reporter gene assay. Co-expression of PLZF and $G\alpha o^{Q205L}$ decreased cyclin A2

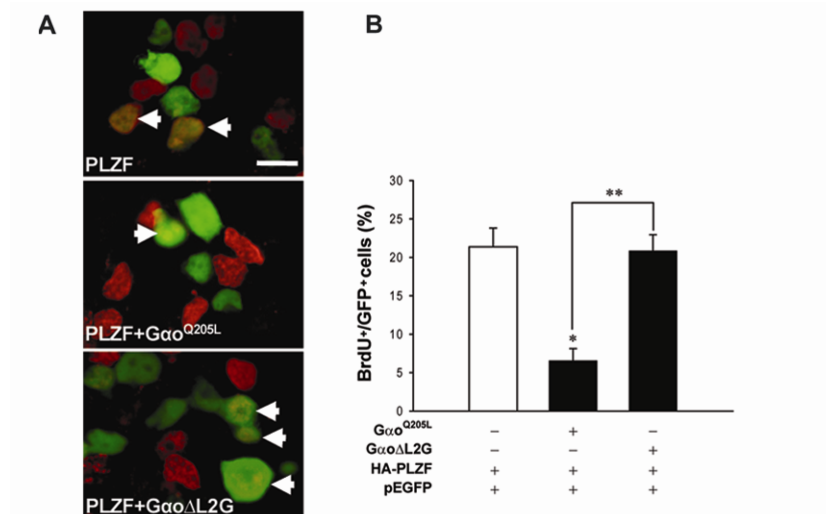

Fig. 3. The effect of Gαo GTPase-domain deletion on PLZF-mediated cell growth. 293T cells were transfected with expression plasmids for $G\alpha o^{Q205L}$ (1 μg), FLAG-GαoΔL2G (1 μg), HA-PLZF (3 μg) and pEGFP (0.5 μg), as indicated. After 24 h, cells were pulsed with BrdU and then labeled with anti-BrdU and -GFP antibodies. A – The $BrdU^{+}/GFP^{+}$ cells are indicated by arrows. Scale bar = 20 μm. B – The extent of BrdU incorporation was assessed in GFP-positive cells. The data is presented as the average ± S.E. of at least three independent experiments. *$p < 0.01$, compared to control. **$p < 0.01$.

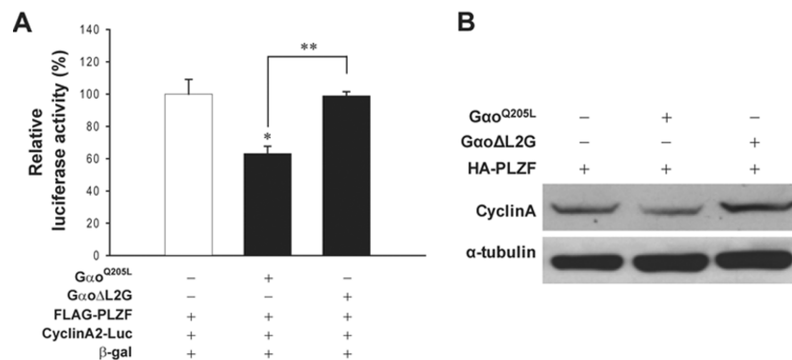

Fig. 4. The effect of Gαo GTPase-domain deletion on PLZF-mediated cyclin A expression. A – 293T cells were transfected with expression plasmids for $G\alpha o^{Q205L}$ (0.3 μg), FLAG-GαoΔL2G (0.3 μg), FLAG-PLZF (0.01 μg), CyclinA2-Luc (0.3 μg), and β-gal (0.3 μg), as indicated. Cell extracts were subsequently assayed for luciferase reporter activity. The data is presented as the average ± S.E. of at least three independent experiments. *$p < 0.05$, compared to control. **$p < 0.01$. B – Extracts of 293T cells transfected with expression plasmids for $G\alpha o^{Q205L}$ (3 μg), FLAG-GαoΔL2G (3 μg), and HA-PLZF (20 μg) were subjected to immunoblot analysis using an antibodies against cyclin A. α-tubulin was used as an internal control.

gene expression compared with the control level (Fig. 4A). Co-expression of PLZF and GαoΔL2G did not alter cyclin A2 promoter-dependent expression compared with PLZF alone. Changes in the cyclin A protein expression levels were consistent with the data obtained from luciferase reporter assays (Fig. 4B). These results collectively suggest that the GTPase domain of Gαo serves a functional role as a regulator of PLZF activity.

The GTPase domain of G proteins plays a pivotal role in the functional interaction between G protein and several other proteins, including PKA, tubulin, and adenylyl cyclase. The GTPase domain of Gαo specifically interacts with PKA and contributes to its subcellular localization [21]. The part of the GTPase domain (α2−β4 and α3−β5 domain) in Gαs protein contributes to interaction with tubulin, the building block of microtubules, and their interaction modulates microtubule polymerization [23]. The analysis of the crystal structure of adenylyl cyclase and Gαs reveal that the GTPase domain of Gαs plays an important role in the functional interaction [24]. In this study, the Gαo GTPase domain contributes to the specific interaction with PLZF and modulates its functions. Therefore, the GTPase domain of G proteins is an important region in signal transduction of heterotrimeric G proteins.

It was recently reported that PLZF expression is downregulated in some cancers, and there is evidence to suggest that PLZF may act as a tumor suppressor [25, 26]. Ectopic expression of PLZF in several cell lines induced growth arrest and apoptosis [27-29]. In our previous study, activation of the Gi/Go-coupled cannabinoid receptor significantly increased PLZF-mediated cell growth arrest in the human acute promyelocytic leukemia cell line, HL60. Thus, if these functional properties of PLZF and the signaling pathways for PLZF activation are supported by additional *in vitro* and *in vivo* experiments, it may be possible to utilize PLZF as a molecular target in cancer management.

Gαo is the most abundant heterotrimeric G protein expressed in the brain; its expression in neuroblastoma cell lines, including PC12, N1E-115, Neuro2a, and F11 cells, is sufficient to promote neuritogenesis [30-32]. In addition, as noted above, PLZF has been shown to induce cell differentiation in a neuronal cell line [19]. It is thus tempting to speculate that GTPase domain-mediated interactions of Gαo with PLZF play an important role in neuronal differentiation.

In this study, we confirmed a direct interaction between Gαo and PLZF, and determined that the GTPase domain of Gαo contributes to its functional interaction with PLZF. Collectively, these findings suggest that upon activation, Gαo acts via its GTPase domain to promote the function of PLZF.

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