Compartmentalization of protein kinase A signaling by the heterotrimeric G protein G_o

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G_o, a member of the $G_o/_i$ family, is the most abundant heterotrimeric G protein in brain. Most functions of G_o are mediated by the $G_{\beta\gamma}$ dimer; effector(s) for its α-subunit have not been clearly defined. Here we report that $G_{o\alpha}$ interacts directly with cAMP-dependent protein kinase (PKA) through its GTPase domain. This interaction did not inhibit the kinase function of PKA but interfered with nuclear translocation of PKA while sparing its cytosolic function. This regulatory mechanism by which G_o bifurcates PKA signaling may provide insights into how G_o regulates complex processes such as neuritogenesis, synaptic plasticity, and cell transformation.

β-catenin | cAMP | Rap1 | somatostatin | cAMP response element binding protein

G_o is the most abundant heterotrimeric G proteins expressed in the brain (1) and is classified as a member of the G_i/G_o family. G_i and G_o proteins are activated by a common set of receptors that include α2 adrenergic, D2 dopamine, opioid, 5HT1, somatostatin (SST), and the muscarinic M2 and M4 receptors (2). To date, unlike G_i, which inhibits adenylyl cyclase, most functions of G_o can be interpreted through the actions of a common pool of $G_{\beta\gamma}$ dimers, and specific functions of $G_{o\alpha}$ have yet to be defined. Several indirect lines of evidence suggest that $G_{o\alpha}$ does function independent of $G_{\beta\gamma}$. The most compelling of these are that constitutively active $G_{o\alpha}$ promotes oncogenic transformation of NIH 3T3 cells (3) and that overexpression of $G_{o\alpha}$ is sufficient to promote neuritogenesis in neuroblastoma cell lines including PC12 (4), N1E-115 (4), Neuro2A (5), and, as we reported earlier, F11 cells (6). In this latter study we had found that both the wild-type $G_{o\alpha}$ and the Q205L mutant, which cannot interact with $G_{\beta\gamma}$, promote an increase in the number of cAMP-induced neurites at the expense of neurite extension. We had also found that this effect of $G_{o\alpha}$ is accompanied by a concomitant decrease in cAMP response element binding protein (CREB)-mediated gene expression, suggesting a cross-talk between G_o and cAMP-dependent PKA.

Functions of PKA isoforms are directly regulated by intracellular concentration of cAMP and expression of A kinase anchoring proteins (AKAPs). cAMP binds the regulatory (R) subunits and causes the release of catalytic (C) subunits (7). AKAPs interact with RII isoforms and direct the compartmentalization of PKA signaling (8). For example, PKAI isoforms with the RI regulatory subunits are soluble and widely expressed, whereas most PKAII isoforms with the RII subunits are associated with the particulate fractions of homogenates through interaction with various AKAPs (8). The predominant PKA isoform and principal mediator of cAMP action in the mammalian central nervous system is the RIIβ-containing PKAIIβ (9).

In the present study we report that $G_{o\alpha}$ has a previously unappreciated scaffolding role in the cytosolic compartment that prevents translocation of PKA into the nuclear compartment.

Results

To determine the interaction of G_o and PKA we incubated rat brain microsomal proteins with GST-$G_{o\alpha}$ fusion proteins and probed with antibodies against the predominant PKA isoform in the brain, PKAIIβ. As shown in Fig. 1*a*, both RIIβ and Cα of PKA were retained by GST-$G_{o\alpha}$. To confirm this interaction in the cell we coexpressed RIIβ and Cα with $G_{o\alpha}$ in 293T cells and immunoprecipitated for $G_{o\alpha}$ (Fig. 1*b*). Also in this case, both RIIβ and Cα were found associated to $G_{o\alpha}$, indicating that G_o::PKA interaction occurs in a cellular context. To determine the $G_{o\alpha}$-interacting subunit of PKA, we induced dissociation of R and C subunits by preincubating rat brain microsomal proteins in the presence of 10–500 μM cAMP for 20 min at 30°C. Addition of cAMP decreased the recovery of RIIβ from GST-$G_{o\alpha}$ in a concentration-dependent manner (Fig. 1*c*), indicating that $G_{o\alpha}$ interacts with C but not R subunits. We also noticed that cAMP treatment increased Cα binding to $G_{o\alpha}$ (Fig. 1*c*). The latter is likely due to more Cα being freed from the R_2C_2 holoenzymes, which tend to associate with scaffolding proteins through interaction with AKAPs (8). Ht31 is known to bind RII and compete effectively for RII binding to AKAP (10). Overexpression of Ht31, however, did not alter the interaction between $G_{o\alpha}$ and PKA (data not shown), eliminating the possibility that the $G_{o\alpha}$::PKA interaction is mediated by AKAP. Although less marked when compared with brain microsomes, the effect of cAMP to reduce the binding of RIIβ to $G_{o\alpha}$ was consistently evident also in 293T cells, where free Cα was present in excess (Fig. 1*d*). More directly, GST-$G_{o\alpha}$ was able to retain purified PKA-Cα but not PKA-RIIβ *in vitro* (Fig. 1*e*). Taken together, these results demonstrate that $G_{o\alpha}$ directly interacts with Cα within the intact cellular context.

We examined whether the interaction is specific to the G_i/G_o family. For this we coexpressed Cα and the FLAG-epitope-tagged full-length α-subunit of G_i and assessed $G_{i\alpha}$ in the immunoprecipitated complex with Cα (Fig. 2*a*). Strikingly, $G_{i\alpha}$ did not bind to Cα. A chimeric protein, $G_{i\alpha}/G_{o\alpha}$, containing $G_{i\alpha}$[1–212] and $G_{o\alpha}$[214–354], could bind to Cα, whereas $G_{o\alpha}/G_{i\alpha}$ containing $G_{o\alpha}$[1–213] and $G_{i\alpha}$[213–354] could not (Fig. 2*b*). The results indicate that the specific determinant(s) of the $G_{o\alpha}$::Cα interaction resides in the C-terminal half of $G_{o\alpha}$. This region encompasses only amino acids contributing to the GTPase domain of Gα-subunits and excludes their helical domain.

We examined the effect of the G_o::PKA interaction on the kinase function of PKA. Increasing amounts of purified $G_{o\alpha}$ (up

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Abbreviations: SST, somatostatin; AKAP, A kinase anchoring protein; CCh, carbachol; 8Br-cAMP, 8-bromo-cAMP; CREB, cAMP response element binding protein.

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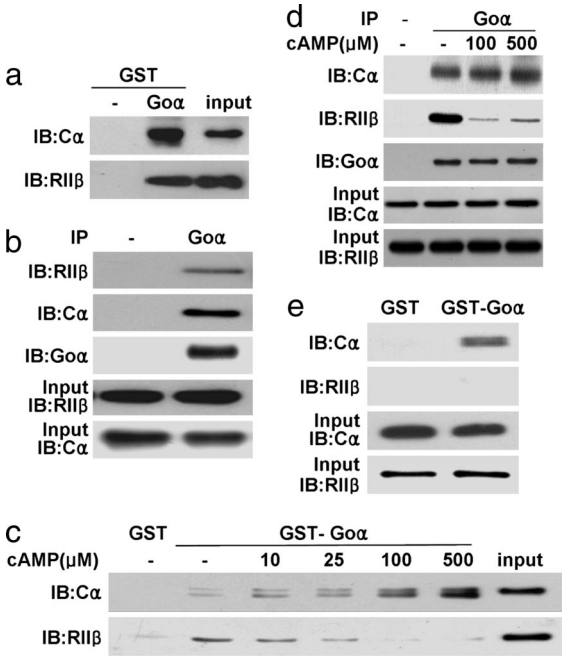

Fig. 1. $G_{o\alpha}$ directly interacts with PKA-Cα. (*a*) Brain microsomal proteins were incubated with GST-$G_{o\alpha}$ and immunoblotted against Cα and RIIβ. (*b*) 293T cell lysates overexpressing $G_{o\alpha}$, Cα, and RIIβ were immunoprecipitated with anti-$G_{o\alpha}$ antibody and immunoblotted against Cα and RIIβ. (*c*) Brain microsomal proteins were preincubated with cAMP and then with GST-$G_{o\alpha}$. Retention of PKA subunits was visualized by immunoblotting. (*d*) 293T cell extracts overexpressing $G_{o\alpha}$, Cα, and RIIβ were preincubated with cAMP and immunoprecipitated with anti-$G_{o\alpha}$ antibody. Association of PKA with $G_{o\alpha}$ was determined by immunoblotting. (*e*) Purified Cα or RIIβ was incubated with GST-$G_{o\alpha}$. Retention of Cα or RIIβ was examined with immunoblotting. All of the experiments were carried out at least three times, and the most representative results are presented.

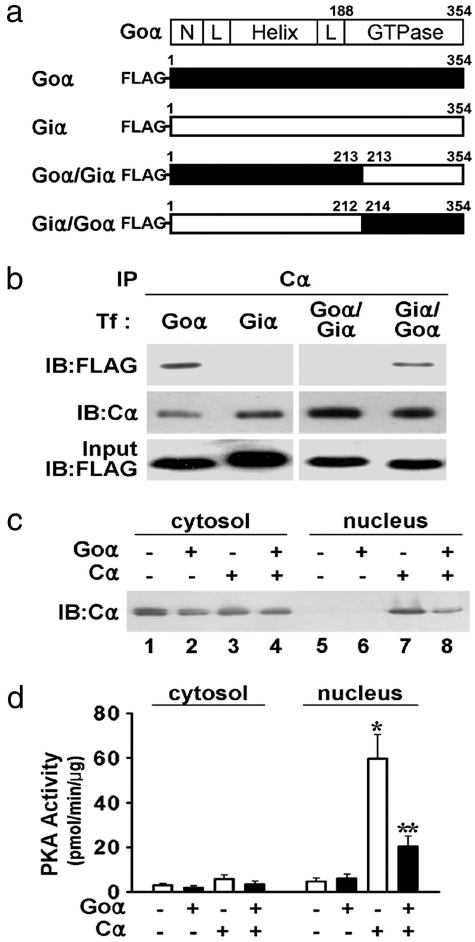

Fig. 2. GTPase domain of $G_{o\alpha}$ contributes to Cα interaction. (*a*) Schematic presentations of $G_{o\alpha}$ and $G_{i\alpha}$ and chimeric proteins with a FLAG-tag at the N terminus. The GTPase domain of $G_{o\alpha}$ (214–354 aa) was substituted by the corresponding region of $G_{i\alpha}$ (213–354 aa) and vice versa. (*b*) Immunoprecipitation was carried out with cell lysates from 293T cells expressing Cα with FLAG-tagged $G_{o\alpha}$, $G_{i\alpha}$, or chimeric proteins. Twenty percent from each of the precipitates was immunoblotted with anti-Cα antibody to verify successful immunoprecipitation. (*c*) COS7 cells were transfected with expression vectors for Cα and $G_{o\alpha}$. Nuclear and cytosolic fractions were immunoblotted for the presence of Cα. (*d*) Kinase activity of the free Cα-subunit is shown as an average ± SE from four independent experiments. Note that catalytic activity of nuclear Cα was increased with respect to the values of untransfected cells (*, $P < 0.005$) and Cα-transfected cells (**, $P < 0.05$).

to 1 μg) did not inhibit the catalytic activity of purified Cα under the condition in which 10 units of RIIβ completely suppressed its kinase activity (Fig. 3*a*). To further confirm the lack of effect of G_o on PKA we developed a method that allowed us to assess the kinase activity in the $G_{o\alpha}$::Cα complex. We incubated His-tagged Cα with $G_{o\alpha}$ or RIIβ in the form of purified or GST fusion proteins. We captured His-Cα and associated $G_{o\alpha}$ or RIIβ using magnetic beads that recognize the His-epitope (Fig. 3 *b* and *c*). As expected, GST-RIIβ and purified RIIβ bound to His-Cα on magnetic beads (Fig. 3*b*, lanes 5–7 and 14–16) and reduced the kinase activity of Cα (Fig. 3*c*). In contrast, neither GST-$G_{o\alpha}$ nor purified $G_{o\alpha}$ inhibited the kinase activity of Cα (Fig. 3*c*), even though they bound to His-Cα (Fig. 3*b*, lanes 2–4 and 11–13). The data clearly showed that the interaction with $G_{o\alpha}$ does not interfere with the kinase activity of PKA and suggested a scaffolding function of G_o.

To examine the subcellular location of PKA-Cα, we overexpressed $G_{o\alpha}$ and Cα in COS7 cells and determined Cα levels in cytosolic and nuclear fractions by Western blot analysis (Fig. 2*c*). In untransfected COS7 cells, most Cα was found in the cytosolic fraction. After overexpression of Cα, a substantial portion of Cα was translocated to the nucleus. Interestingly, upon coexpression with $G_{o\alpha}$, the amount of Cα in the nuclear fraction decreased (Fig. 2*c*, compare lanes 7 and 8). The decreased protein level of Cα in nuclei correlated with a corresponding decrease in the catalytic activity in the nuclear compartment (from 59.8 to 20.5 pmol/min per μg) (Fig. 2*d*), supporting the finding that $G_{o\alpha}$ interferes with nuclear translocation of the free Cα.

We further tested regulation of subcellular compartmentalization of Cα by $G_{o\alpha}$ using immunocytochemistry (Fig. 4). COS7 cells were transfected with expression vectors for FLAG-tagged $G_{o\alpha}$, $G_{i\alpha}$, or chimeric proteins and then stimulated with forskolin, an adenylyl cyclase activator, for 20 min. In the absence of $G_{o\alpha}$, most of endogenous Cα (green) moved into the nucleus (blue). Importantly, coexpression of $G_{o\alpha}$ (red) reduced nuclear Cα whereas $G_{i\alpha}$ did not. Consistently, expression of $G_{i\alpha}/G_{o\alpha}$ containing the Cα-interacting domain of $G_{o\alpha}$ also inhibited nuclear translocation of Cα whereas in contrast $G_{o\alpha}/G_{i\alpha}$ lacking binding potential to Cα as shown in Fig. 2*b* did not.

We next investigated whether this interaction occurs in nontransfected cells expressing normal complements of G_o and PKA. For this we used GH4C1 rat pituitary tumor cells, wherein G_i/G_o can be activated by SST or carbachol (CCh) receptors (11, 12). We used 8-bromo-cAMP (8Br-cAMP), a cell-permeable cAMP analogue, to directly activate PKA and thereby bypassed the effects G_o's $\beta\gamma$ dimers and/or coactivated $G_{i\alpha}$ had on cAMP formation. Pretreatment with 100 nM SST or 100 μM CCh for 5–15 min strongly attenuated cAMP-induced phosphorylation of CREB (Fig. 5*b*).

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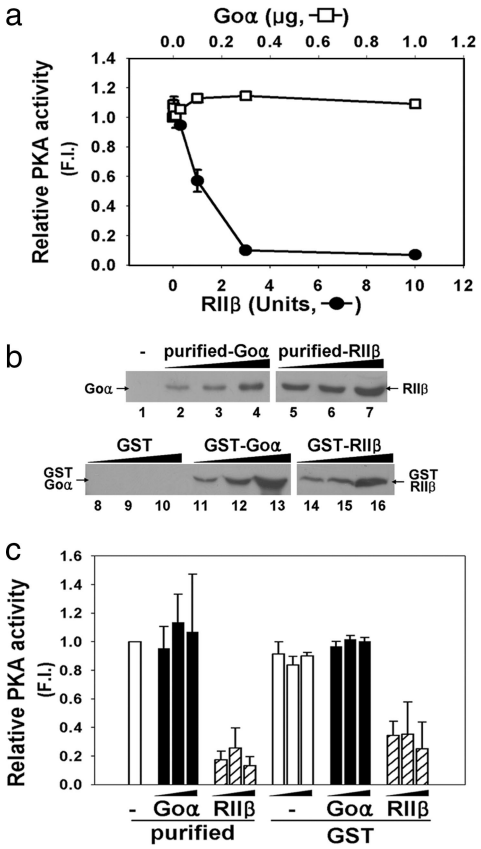

Fig. 3. Effects of G_o::PKA interaction on kinase activity of Cα. (*a*) Purified Cα was incubated with increasing amounts of purified-$G_{o\alpha}$ or RIIβ and subjected to PKA assay. $G_{o\alpha}$ could not inhibit the kinase function of Cα. (*b*) $G_{o\alpha}$ or RIIβ (5, 15, and 50 μg each) was added to His-Cα in the form of purified or GST fusion protein. Association of $G_{o\alpha}$ and RIIβ to His-Cα on magnetic beads was visualized by immunoblotting. (*c*) Twenty percent of the Cα-containing complex in *b* was subject to kinase assays using Kemptide as a substrate. Results are presented as means ± SEM from three independent experiments.

The inhibition of CREB phosphorylation upon activation of G_o by SST or CCh was accompanied by reduced translocation of the Cα into the nuclei (shown for SST in Fig. 5*c*). These results strongly support our previous finding that activation of G_o inhibits PKA-dependent CREB activation in F11 cells (6).

We found that $G_{o\alpha}$-mediated inhibition of PKA function was specific to the nuclear compartment by using two approaches. First, we took advantage of the fact that PKA is a priming kinase for glycogen synthase kinase 3β. PKA phosphorylates β-catenin on both Ser-45 and Ser-675 (13, 14) in the extranuclear compartment where glycogen synthase kinase 3β forms a complex with axin and adenomatous polyposis coli, or presenilin. Addition of 8Br-cAMP increased phosphorylation of β-catenin on Ser-45, which was unaffected by 100 nM SST (Fig. 5*d*) under conditions where SST inhibited CREB activation in the nuclear compartment (Fig. 5 *b* and *c*). Second, we tested the effect of $G_{o\alpha}$ on Rap1 activation by cAMP in the extranuclear compartment. Rap1 is activated by cAMP through two paths: nucleotide exchange mediated by EPAC, a Rap1GEF (15), and PKA-dependent phosphorylation in the cytosolic compartment (16). To bypass the EPAC-dependent activation of Rap1 we coexpressed PKA-Cα together with $G_{o\alpha}$ and Rap1 and measured the level of active Rap1-GTP using RalGDS (17). Rap1-GTP was moderately increased after expression of Cα (Fig. 5*e*, compare lanes 1 with 2). Importantly, coexpression of $G_{o\alpha}$ with Cα did not inhibit the PKA-mediated Rap1 activation (Fig. 5*e*, compare lanes 2 and 4). Instead $G_{o\alpha}$ seemed to increase Rap1-GTP (Fig. 5*f*), which is likely because of $G_{o\alpha}$-induced promotion of proteosomal degradation of Rap1-GapII through a ubiquitin-dependent pathway (5). The data clearly showed that only the nuclear function of PKA is specifically inhibited by $G_{o\alpha}$ whereas the cytosolic functions of PKA are not only saved but rather facilitated by $G_{o\alpha}$.

Discussion

cAMP is a soluble second messenger that regulates various cellular functions including cell motility, growth, metabolism, ion channel conductivity, and synaptic plasticity (18). Most effects of cAMP are mediated through PKA-dependent phosphorylation, and the specificity of the PKA signaling pathway is in turn directed by anchoring proteins, AKAPs. AKAPs contain a conserved amphipathic helix that binds the R subunit of PKA and a unique targeting motif that directs the AKAP complex to a specific subcellular location (8). Our study shows that G_o resembles AKAPs in as much as it serves as an anchoring protein for PKA and directs its subcellular location in the cytosol/membrane compartment. However, G_o is distinct from traditional AKAPs in several aspects. First, the PKA-C subunits, not the R subunits, interact with G_o. Second, given that the PKA-C associated with G_o still retains its catalytic activity, G_o recruits free, active Cαs and reinforces the PKA action at locations where G_o is located. Finally, through its interaction with $G_{o\alpha}$, functions of PKA can be dynamically regulated by G_o-coupled receptor agonists, in addition to merely responding to changes in the level of cAMP.

The present study shows that only the cytosolic functions of PKA such as activation Rap1 are not only maintained but rather facilitated by $G_{o\alpha}$. Rap1 plays a key role in the activity-dependent regulation of dendritic growth and remodeling in the nervous system (19) and neuronal differentiation in PC12 cells (20). Therefore, our previous finding that $G_{o\alpha}$ increases the number of neurites in F11 cells may be partly ascribed to the G_o::PKA interaction, which may contribute to fine-tuning the Rap1 function in the plasma membranes.

G_o constitutes as much as 2% of membrane proteins, which is the highest for any G proteins in nonsensory cells (1). In addition, G_o is the most abundant heterotrimeric G protein found in growth cone membranes (21) and postsynaptic densities (22). In striatum, G_o is the major G protein transmitting dopamine signals through D2R (23), and disturbance of dopamine signals through type 2 receptors (D2R) is related to Parkinson's disease (24).

In conclusion, G_o only attenuates nuclear functions of PKA such as CREB activation while sparing cytosolic functions of PKA such as glycogen synthase kinase 3β phosphorylation and Rap1 activation. The bifurcating functions of G_o in PKA signaling are derived from its scaffolding function, which maintains PKA-Cα in association with G_o in the membrane/cytosol even when the intracellular level of cAMP increases. The scaffolding function is specific to G_o and resides in its GTPase domain. Because a given ligand such as dopamine can either increase or decrease intracellular cAMP concentration depending on the receptor types, multiple occupancy of various G protein-coupled receptors by ligands at the same time may lead to diverse effects in regulating generation of cAMP, which in turn indicates the need for compartmentalization of the downstream cAMP signaling. Our finding may provide insights into understanding diverse roles of G_o/G_i-coupled receptors as well as G_o-specific functions in the nervous system.

Materials and Methods

GST Pull-Down Assay. Full-length cDNA of $G_{o1\alpha}$ (GenBank accession no. M17526) was inserted to pGEX-2T and expressed in *Escherichia coli* BL21 cells by using a standard protocol. Bac-

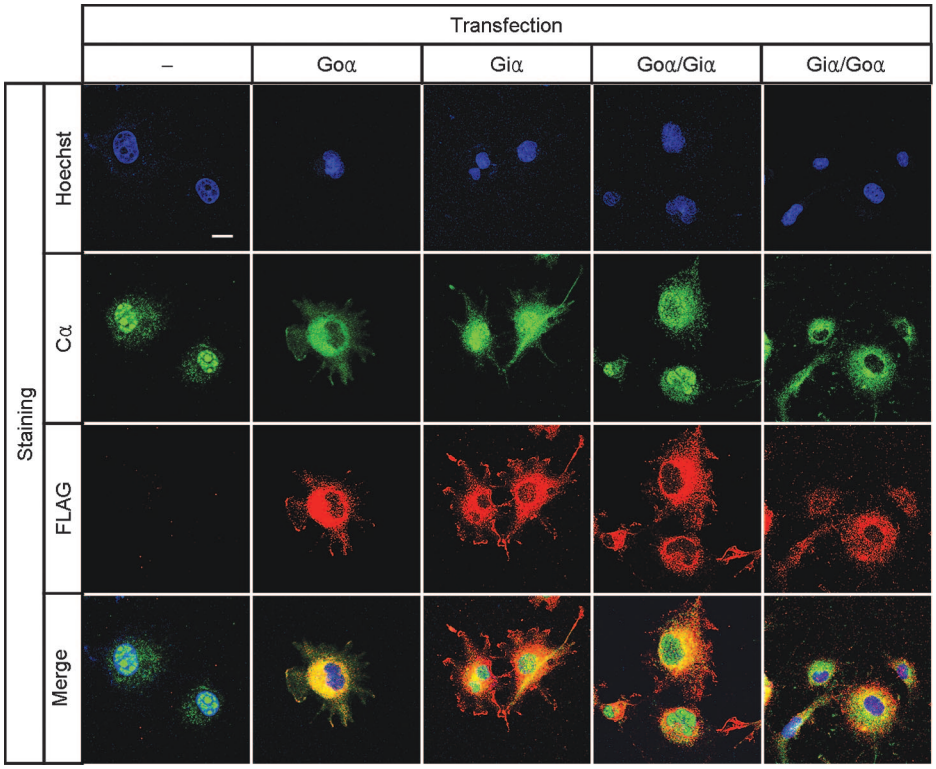

Fig. 4. Effects of G_o::PKA interaction on nuclear translocation of Cα. COS7 cells were transfected with various FLAG-tagged Gα constructs and stimulated with 30 μM forskolin for 20 min. To clearly demonstrate the nuclear area, confocal images at a focal plane around the nucleus are presented. Red, FLAG-Gα; green, Cα; blue, nucleus. The images are representative of results obtained in three independent experiments.

terial cell lysates containing GST fusion proteins were incubated with glutathione Sepharose 4B beads for 1 h at 4°C in PBTX buffer (PBS containing 1% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 mM phenylmethylsulfonyl fluoride) and then washed extensively with the PBTX. Rat forebrain microsomes were prepared as reported (25) and solubilized with PBTX. Either 500 μg of microsomal proteins or purified Cα and RIIβ proteins (Sigma-Aldrich, St. Louis, MO) was added to the beads and incubated for 1 h at 37°C. After washing the beads extensively with PBTX, the bound proteins were eluted with SDS sample buffer and subjected to immunoblot analysis by using antibodies against Cα (diluted 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) or RIIβ (diluted 1:500; BD Biosciences, Palo Alto, CA). Input lanes contained 10% of the extracts from GST pull-down assay.

Coimmunoprecipitation. 293T cells were transiently transfected with appropriate combinations of expression plasmids of the full-length $G_{o1\alpha}$ (pRC/CMV-$G_{o\alpha}$) (26); FLAG-tagged $G_{o\alpha}$ and $G_{i1\alpha}$ (GenBank accession no. AF493905); chimeric proteins of $G_{o\alpha}$ and $G_{i\alpha}$; RIIβ (GenBank accession no. NM_002736) in pcDNA3 (pcDNA3-RIIβ); and Cα (GenBank accession no. NM_002730) in pcDNA3 (pcDNA3-Cα) as indicated. Forty-eight hours after transfection, cells were lysed in PBTX, and 500 μg of soluble proteins was precleared by incubating 20 μl of protein A-Sepharose CL-4B beads (50% slurry). Five hundred micrograms of protein dissolved in 500 μl of PBTX was incubated with 1 μg of antibody against $G_{o\alpha}$ or Cα (Santa Cruz Biotechnology) with gentle rotation for 4 h at 37°C, and then with 50 μl of beads. After a 2-h incubation, beads were washed with PBTX and the bound proteins were eluted with SDS sample buffer and subjected to immunoblot analysis by using indicated antibodies. Input lanes contain 10% of the extracts used for immunoprecipitation.

Fractionation and PKA Activity Assay. Cytosolic and nuclear fractions from COS7 cells were prepared as described (27). PKA assays were performed with 3–10 μg of soluble proteins of cytosolic, nuclear, or membrane fractions to a 50-μl reaction mixture containing 50 mM Tris·HCl (pH 7.4), 1 mM DTT, 10 mM $MgCl_2$, 30 μM kemptide (Sigma-Aldrich), 5 μM ATP, 10 μCi (1 Ci = 37 GBq) [γ-^{32}P]ATP, and 40 mM β-glycerophosphate, with or without 30 μM protein kinase inhibitor (Sigma-Aldrich). After incubation at 30°C for 10 min, the mixture was transferred to a phosphocellulose membrane and washed with 1% phosphoric acid, and the remaining radioactivity was determined by using a liquid scintillation counter. The specific PKA activity was defined as the difference between radioactivities with and without PKI. Specific activity was presented as an average ± SEM from four experiments.

Immunofluorescence Staining. Expression vectors corresponding to 1 μg of FLAG-tagged α-subunits of G protein were transfected into COS7 cells by using DEAE-dextran in six-well tissue culture dishes. Forty-eight hours after transfection, forskolin (30 μM) was added for 20 min at 37°C, and the cells were fixed with 4% paraformaldehyde in PBS for 5 min and incubated for 1 h at room temperature with antibodies against FLAG (Sigma-Aldrich) and Cα. Cells were then washed with PBS and incubated for an additional 30 min at room temperature with Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Willow, OR) and counterstained with Hoechst (Sigma-Aldrich). Images were acquired with a LSM510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

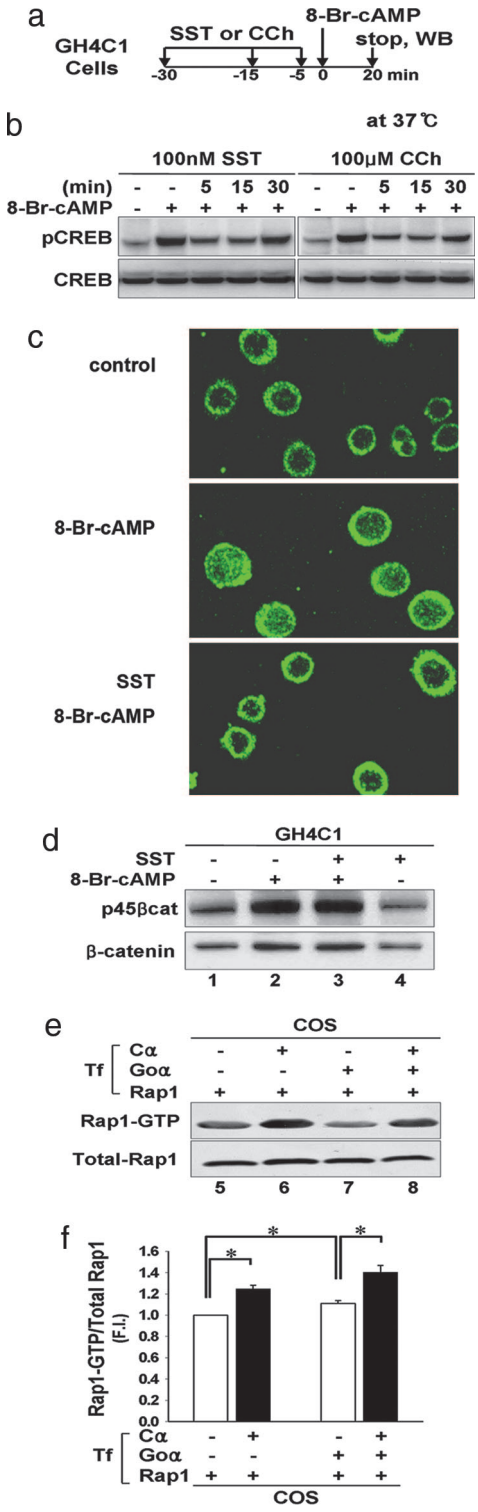

Fig. 5. Differential effects of $G_{o\alpha}$ on nuclear and cytosolic function of Cα. (*a*) GH4C1 cells were first incubated in the presence of 100 nM SST or 100 μM CCh for 5–30 min and then treated with 8Br-cAMP. Twenty minutes later immunoblotting was carried out. (*b*) Activation of CREB as visualized with anti-phospho-CREB antibody was blocked by pretreatment with SST or CCh. The total CREB was not altered during this period. (*c*) Confocal images show the nuclear translocation of PKA-Cα after treatment with 8Br-cAMP for 20 min (*Middle*), which was blocked by pretreatment with SST for 15 min (*Bottom*). The images are representative of results obtained in four independent experiments. (*d*) Phosphorylation of β-catenin at Ser-45 induced by 8Br-cAMP was attenuated by preincubation of GH4C1 cells in the presence of 100 nM SST. The total amount of β-catenin did not alter significantly. The results are representative of four independent experiments. (*e*) COS7 cells were transfected with expression vectors for Rap1, Cα, and $G_{o\alpha}$. Activated Rap1 (Rap1-GTP) was determined by using GST-RalGDS-RBD. Ten percent of the input was immunoblotted to detect total Rap1. (*f*) The results from three independent experiments are presented as averages ± SEM (*, $P < 0.05$).

Phosphorylation of CREB and β-Catenin. GH4C1 cells were grown as described (28). Media were replaced with fresh F10 medium supplemented with 0.5% FBS 12 h before the experiment. SST and CCh were added for the indicated times to final concentrations of 100 nM and 100 μM, respectively. Then 8Br-cAMP was added to give a final concentration of 1 mM, and the incubation continued for a final 20 min. After rinsing with ice-cold PBS, the cells were lysed with SDS sample buffer. The lysates were subject to immunoblot analysis by using anti-Phospho-CREB antibody (Ser-133; Upstate Biotechnology, Charlottesville, VA). The total CREB was visualized by using anti-CREB antibody to verify the equal loading (Upstate Biotechnology). Alternatively, immunoblotting was carried out by using an antibody that recognizes phosphorylation of β-catenin by PKA at Ser-45 (Cell Signaling Technology, Danvers, MA). The total amount of β-catenin was visualized with anti-β-catenin antibody (Cell Signaling Technology).

Magnetic Bead Capture Assay. pET-28-Cα was generated by inserting cDNA of PKA-Ca into pET-28(a) (Novagen, Darmstadt, Germany) and used for transformation of *E. coli* BL21. After being induced with 0.5 mM isopropyl β-D-thiogalactoside, cells were harvested and lysed by ultrasonication in lysis buffer (50 mM NaH_2PO4, pH 8.0/300 mM NaCl/10 mM imidazole/0.05% Tween 20/1 mM DTT/protease inhibitors). Soluble fractions were obtained and mixed with 5, 15, or 50 μg of purified or GST fusion $G_{o\alpha}$ and RIIβ proteins for 1 h at room temperature. Then Ni-NTA magnetic agarose beads (Qiagen, Valencia, CA) were added to the mixture and incubated for 30 min at room temperature according to the manufacturer's instructions. The beads were separated to the side walls by using a magnetic stand. After the beads were washed with lysis buffer by using the magnetic stand, the Cα::$G_{o\alpha}$ or Cα::RIIβ complexes were eluted in 25 μl of lysis buffer containing 250 mM imidazole. Five microliters each of the eluted proteins was used for PKA assay in duplicates with and without PKI or for Western blot analysis as described above. Results obtained from three independent experiments are presented as averages ± SEM.

Rap1 Activation Assay. COS cells were transfected with expression vectors for 8 μg of $G_{o\alpha}$, 5 μg of PKA-Cα, and 5 μg of Rap1 (HA-tagged) by using polyetyleneimine as DNA carriers. Total amount of DNA was adjusted to 18 μg by using pcDNA3. After 24 h, the medium was replaced with DMEM with 0.5% FBS for 16 h. Cells were treated with 30 μM forskolin and 100 μM IBMX for 10 min and then lysed in lysis buffer (25 mM Tris·HCl, pH 7.5/150 mM NaCl/5 mM $MgCl_2$/1% Nonidet P-40/1 mM DTT/5% glycerol and protease inhibitors), and levels of activated Rap were measured by use of the RalGDS binding domain as described (17). Briefly, RalGDS-RBD (RalGDS-Rap1 binding domain) was expressed as a GST fusion protein in bacteria, extracted in bacterial lysis buffer containing DTT and protease inhibitors, and then incubated with glutathione beads. The beads containing RalGDS-RBD were incubated with 450 μg of proteins of total cell lysates in the presence of DTT and protease inhibitors for 1 h at 4°C. After washing, GTP-bound Rap1 was determined by immunoblotting by using a mouse monoclonal anti-HA antibody (Roche). In parallel, 50 μg of proteins of total cell lysates was used for immunoblotting to verify similar expression of Rap1 in various transfection conditions.

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