We previously identified a specific activation-dependent interaction between the α subunit of the heterotrimeric G protein, Gα, and a regulator of Rap1 signaling, Rap1GAP (Meng, J., Glick, J. L., Polakis, P., and Casey, P. J. (1999) J. Biol. Chem. 274, 36663–36669). We now demonstrate that activated forms of Gα are able to recruit Rap1GAP from a cytosolic location to the membrane. Using PC12 cells as a model for neuronal differentiation, the influence of Gα activation on Rap1-mediated cell differentiation was examined. Introduction of constitutively-activated Gαs into PC12 cells markedly attenuated the differentiation process of these cells induced by a cAMP analogue. Treatment of PC12 cells expressing wild type Gαs with a specific agonist to the α2-adrenergic receptor also attenuated cAMP-induced PC12 cell differentiation, demonstrating that receptor-mediated activation of Gαs was also effective in this regard. Furthermore, activation of Gαs decreased the ability of the cAMP analogue to trigger both Rap1 and extracellular-regulated kinase (ERK) activation. Differentiation of PC12 cells induced by nerve growth factor (NGF) is also thought to be a Rap1-mediated process, and Gα activation was found to attenuate this process as well. Rap1 activation, ERK phosphorylation, and PC12 cell differentiation induced by NGF treatment were all significantly attenuated by either transfection of constitutively activated Gαs or receptor-mediated Gα activation. Based on these findings, a model is proposed in which activation of Gαs results in recruitment of Rap1GAP to the membrane where it can effectively down-regulate Rap1 signaling. The implications of these findings in regard to a possible role for Gαs in neuronal development are discussed.

Guanine nucleotide-binding regulatory proteins (G proteins) serve as molecular switches in signaling cascades (1). GTP binding to the G protein α subunit, which is initiated by appropriately liganded G protein-coupled receptors (GPCRs) as well as other GTP exchange factors (GEFs), and dissociation of the tightly associated βγ complex from α-GTP produce the active forms of the G proteins that transmit signals to downstream targets, termed effectors. G protein α subunits possess intrinsic GTPase activity, and GTP hydrolysis returns the system to its resting state where the α subunit and βγ complex re-associate (2). This GTP hydrolysis step can be accelerated by GTPase-activating proteins (GAPs) such as the members of regulators of G protein signaling (RGS) family of proteins (3, 4).

Structural homology of the α subunits, and also functional differences, divides G proteins into Gαq, Gαi, Gαs, and Gα12 subgroups (2, 5). One particularly interesting member of the Gαi subfamily is Gαi6 (6, 7). Like other members of the Gαi family, Gα12 has the ability to inhibit some subtypes of adenyl cyclases (8) and is subject to both myristoylation and palmitoylation (9, 10). Mutation of either myristoylation site Gly2 or palmitoylation site Cys3 results in incorrect localization of Gα12 and loss of the ability to transduce signals to downstream effectors in the cell (11). Tissue distribution of Gα12 is quite restricted, being found primarily in brain, adrenal medulla, and platelets, while expression is virtually undetectable in other tissues (5, 12). Lack of a cysteine residue that is conserved in other members of the Gα family near the C terminus, Gα12 does not serve as a substrate for pertussis toxin (PTX)-catalyzed ADP-ribosylation (5, 6). Interestingly, the intrinsic rate of GTP hydrolysis by Gα12 is quite low compared with most other G protein α subunits, suggesting that RGSs such as RGSZ1 (one of the products of the RGS20 gene, Ref. 13) play important roles in the regulation of its signaling (14).

Monomeric G proteins consist of only one polypeptide and also cycle between two conformations induced by binding and hydrolysis of GTP (15). While Ras is probably the best known and best studied member in the family, the closely related Rap1 has attracted considerable attention recently (16). First identified as a molecule capable of reverting the oncogenic phenotype of Ras-transformed fibroblasts (17), Rap1 has been implicated as a key player in several cellular processes. In rat thyroid-like cells, thyrotropin activates Rap1 through a cAMP-mediated and PKA-independent mechanism and results in Akt phosphorylation (18, 19). In PC12 cells, Rap1 can be activated by elevation of cyclic AMP (cAMP) and contributes to prolonged activation of extracellular-regulated kinases (ERks) (20). Influx of Ca2+ in platelets also activates Rap1 and consequently promotes platelet aggregation (21). In HEK293 and NIH3T3 cells, Rap1 is required for cAMP-mediated inhibition of cell growth (22).

A key negative regulator in Rap1 signaling pathway is Rap1 GTPase-activating protein (Rap1GAP) that is expressed most abundantly in brain and some tumor cell lines (23). Rap1GAP is a 663-residue protein, and the region from residue 75 to 416 comprises its “core” domain that has full GAP activity toward GDP-
bound inactive state, resulting in a down-regulation of Rap1 signaling.

In a previous study, we identified an interaction between the activated form of Goα and Rap1GAP, the aforementioned specific activator of Rap1 signaling. Characterization of the interaction revealed that the three proteins, i.e. Goα, Rap1GAP, and Rap1, could form a ternary complex, suggesting a role for Goα in Rap1-mediated signaling processes (26). However Rap1 single-turnover GTPase assays indicated that binding of Goα did not affect Rap1GAP’s ability to stimulate GTP hydrolysis by Rap1. In the present study, cell-based approaches are described to show that activated Goα can recruit Rap1GAP from a cytosolic location to the membrane. In PC12 cells, Goα-mediated recruitment of Rap1GAP was accompanied by a down-regulation of a Rap1-mediated signaling process, i.e. cell differentiation induced by either cAMP analogue or nerve growth factor (NGF). These studies shed light on the mechanism of how Rap1 signaling can be regulated by activation of Goα and provide insight into the role of heterotrimeric G proteins in PC12 cell differentiation.

EXPERIMENTAL PROCEDURES

Materials—HEK293 cells and S99 cells were obtained from the American Type Culture Collection. PC12 cells were generously provided by Pate Sleen (Duke University). His-tagged Rap1 was a gift from William Tschantsz (Duke University). RalGDS-RBD plasmid was provided by Johannes L. Bos (Utrecht University, The Netherlands). Goα was purified from Escherichia coli as described previously. Adrenal chromaffin cell membrane fractions were prepared as described (12).

The anti-Rap1GAP polyclonal antibody (25) and anti-Goα polyclonal antibody (5) were obtained from Santa Cruz Biotechnology, Inc. The anti-Rap1 monoclonal antibody, anti-ERK polyclonal antibody, and anti-phospho-ERK monoclonal antibody were purchased from Transduction Laboratories. Radioisotopic compounds ([γ-32P]GTP and [γ-35S]GTP-S) were purchased from PerkinElmer Life Sciences.

Plasmid Constructs—Plasmids containing the cDNA of rat Goα (both wild type and the Q205L mutant) containing the Glu-Glu epitope at residues 3–8 were the gift of Henry Bourne (University of California, San Francisco) and have been described (27). Plasmids containing full-length Rap1GAP (designated pCAN-Rap1GAP) and fragments thereof were also produced and described (25). The plasmid containing full-length Rap1GAP with an appended N-terminal His-tag (pRSET-Rap1GAP) was constructed by subcloning a KpnI- and EcoRI-digested fragment from pCAN-Rap1GAP that contained the Rap1 GAP coding sequence into the pRSET-C vector (Invitrogen). The plasmid containing full-length Rap1GAP with an appended N-terminal EGFP (N-GFP) Rap1GAP was constructed by subcloning a KpnI- and EcoRI-digested fragment from pCAN-Rap1GAP that contained the Rap1-GAP coding sequence into the EGF-P2 vector (Invitrogen).

Cell Culture and Transfection Conditions—HEK293 cells were cultured in Dulbecco’s Modified Eagle medium (Invitrogen) with 10% fetal bovine serum at 37 °C. PC12 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 5% heat-inactivated horse serum at 37 °C. Transfection was performed using Superfect® reagent (Qiagen) in 6-well Falcon plates. Standard protocols provided by the manufacturer were used. Briefly, cells were transfected at 70–80% confluency with 5.0 μg (the default level) or 15.0 μg of plasmid encoding Goα forms and/or 2.0 μg of the plasmid encoding N-GFP-Rap1GAP unless otherwise noted.

Rap1 Single-turnover GTPase Assays—The procedure was essentially the same as previously described (26). Briefly, Rap1 was first incubated with [γ-35S]GTP at room temperature for 10 min and then isolated by gel filtration using a 1-ml spin column of G-25 Sephadex. GTP hydrolysis was carried out in the presence or absence of Rap1GAP protein or cell lysate. In experiments assessing the abilities of Goα forms to inhibit Rap1GAP-stimulated GTPase activity, the Rap1GAP protein was incubated with the Goα-GTP complex at 0 °C for 5 min before addition to the reaction. GTPase activity was measured by phosphate release as previously described (28).

PC12 Cell Differentiation Assays—PC12 cells were transfected with designated plasmids and allowed to recover 24 h before serum deprivation. Following 24 h of serum deprivation, cells were treated with either 10 μM 8-CPT, Calbiochem for an additional 24 h or 100 ng/ml NGF (Calbiochem) for 18 h and examined by microscopy. Where indicated, 10 μM UK14304 (Research Biochemicals Inc.) was applied 1 min before 8-CPT or NGF treatment. Differentiation of PC12 cells was assessed by measurement of neurite outgrowth; five random photographs were taken of more than 100 cells in each well, and only cells with neurite extensions longer than one cell body diameter were considered as differentiated cells.

ERK Activation Assays—PC12 cells were serum-starved for 24 h before 8-CPT or NGF treatment as above. In experiments using UK14304, the compound at a concentration of 10 μM was added 1 min prior to 8-CPT or NGF. Cells were harvested and disrupted, and extracts were centrifuged at 3,000 × g for 5 min to remove nuclei and cell debris. Rap1GDS-RBD (10 μg) coupled to Ni2+-nitrolotriacetic acid-agarose beads (Qiagen) was added to the supernatant and the mixture incubated at 4 °C for 60 min with gentle agitation. Beads were washed twice with 50 mM sodium HEPES, pH 7.7, 1 mM dithiothreitol, 0.1% Lubrol. Sample buffer was added and heated at 65 °C for 10 min to uncouple the proteins from the beads. Supernatant samples were subjected to SDS-PAGE followed by immunoblot with the Rap1-specific antibody.

PTX-catalyzed ADP-ribosylation Assays—The procedure used to ADP-ribosylate G proteins in PC12 cells was essentially the same as previously described. Briefly, cells were treated with or without PTX (100 ng/ml) for 2 h and membrane fractions prepared and suspended in 50 mM Tris-HCl, 1 mM EDTA, and 50 mM NaCl. The membrane suspension was then incubated with [32P]NAD and PTX (15 ng/ml) at 30 °C for 30 min to ribosylate any remaining PTX substrates and membranes subjected to SDS-PAGE and autoradiography.

Phase-contrast and Fluorescence Confocal Microscopy—PC12 cells were treated as described above in “PC12 cell differentiation assays,” washed with phosphate-buffered saline and viewed using a Nikon Eclipse TE300 inverted microscope (Nikon, Melville, N.Y.) under phase-contrast and 63-fold magnification. HEK293 cells were treated as described above, washed with phosphate-buffered saline, and viewed using a Zeiss LSM 410 invert confocal microscope (Carl Zeiss Microscopy Systems, Oberkochen, Germany) under green fluorescence detection mode and 63-fold magnification.

Miscellaneous—SDS-PAGE was performed on 10% polyacrylamide gels. For immunoblot, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) and processed by standard immunoblot procedures; bound antibodies were visualized by a secondary antibody conjugated to horseradish peroxidase (Promega). Intensity of the staining was analyzed by Scion Image for Windows (Scion Corporation).

RESULTS

In our previous study, Rap1GAP, a regulator of Rap1 signaling, was found to specifically interact with activated Goα. While binding to Rap1GAP blocked access of RGS proteins to the activated Goα, it did not affect the ability of Rap1GAP to act on Rap1 (26). These findings suggested to us another regulatory mechanism that Goα activation might employ, that of triggering recruitment of Rap1GAP to a specific cellular location to enhance its ability to act on, and thus down-regulate, Rap1 signaling. To address the question of the cellular localization of Rap1GAP, a GFP-Rap1GAP fusion protein was constructed and expressed in HEK293 cells. The GFP-Rap1GAP fusion protein retained functional activity, as cell extracts prepared after transfection exhibited 20-fold greater GAP activity toward Rap1 than extracts from mock-transfected cells (data not shown). Consistent with a previous report on the localization of Rap1GAP (23), GFP-Rap1GAP was localized predominantly in the cytosol (Fig. 1A).

Similar to most other heterotrimeric G proteins, Goα is localized primarily at the plasma membrane (11). When constitutively activated Goα (Goα-QL) was co-transfected with GFP-Rap1GAP, GFP-Rap1GAP translocated to the plasma membrane (Fig. 1B). Importantly, this translocation was not triggered by expression of either wild type Goα (Fig. 1C) or the QL variant of Goα lacking the N-terminal myristoylation site.
required for correct plasma membrane association (non-myristoylated QL G\(_{\alpha_2}\)) (Fig. 1D). We also tested the ability of G\(_{\alpha_2}\), another member in G\(_i\) subfamily, and G\(_{\alpha_{12}}\), a member of a distinct subfamily, for their abilities to recruit GFP-Rap1GAP to the plasma membrane of these cells. However, neither of these constitutively active forms of \(\alpha\) subunits influenced the cellular localization of GFP-Rap1GAP (Fig. 1, E and F), even though expression and membrane association of the two proteins were confirmed (results not shown).

In our previous study, deletion of N-terminal 74 amino acids of Rap1GAP was found to eliminate its ability to interact with G\(_{\alpha_2}\), even though its ability to act on Rap1 was completely preserved (26). As an additional test of the requirement for G\(_{\alpha_2}\) binding in the translocation of Rap1GAP, a construct encoding a GFP protein fused to this N-terminal deletion from of Rap1GAP (GFP-\(\Delta\)N-Rap1GAP) was created. Expression of GFP-\(\Delta\)N-Rap1GAP in HEK293 cells showed cytosolic localization of this protein similar to that seen with the full-length form (Fig. 1G). However, co-transfection of G\(_{\alpha_2}\) QL did not alter the cellular localization of GFP-\(\Delta\)N-Rap1GAP (Fig. 1H). Taken together, all these localization studies demonstrate that activated G\(_{\alpha_2}\) can specifically recruit Rap1GAP to the plasma membrane, and that the N-terminal 74 residues of Rap1GAP are required for this consequence of G\(_{\alpha_2}\) activation.

Having obtained evidence for a functional consequence of the G\(_{\alpha_2}\)-Rap1GAP interaction, i.e. a relocalization of Rap1GAP to the membrane where it could potentially act on Rap1 in that cellular location, we were keen to identify a Rap1-mediated biological process on which to test the influence of G\(_{\alpha_2}\) activation. Due to the emerging evidence of the importance of the Rap1/B-Raf/ERK signaling pathway in PC12 cell differentiation (20, 29), we chose this cell system for the studies of the functional consequences of G\(_{\alpha_2}\)-mediated Rap1GAP relocation to the membrane fraction. Cellular localization of Rap1 and Rap1GAP was examined in PC12 cells expressing the G-protein \(\alpha\) subunit. Endogenous Rap1 was detected only in the membrane fraction (Fig. 2), but not in the cytosol fraction (data not shown). In untransfected PC12 cells, cells expressing wild type G\(_{\alpha_2}\), or cells expressing constitutively active G\(_{\alpha_{2QL}}\), endogenous Rap1GAP was only detected in the cytosol fraction (Fig. 2). While the expression level of Rap1 and Rap1GAP was unchanged in transfected PC12 cells, expression of G\(_{\alpha_2}\) QL resulted in a redistribution of endogenous Rap1GAP to the membrane fraction (Fig. 2). This observation confirmed that active form of G\(_{\alpha_2}\) was able to recruit endogenous Rap1GAP to the cellular localization of Rap1, and this recruitment is specific to G\(_{\alpha_2}\).

Consistent with a previous finding (29), treatment of PC12 cells with a cAMP analogue, 8-CPT, promoted cell differentiation (Fig. 3). Expression of G\(_{\alpha_2}\) QL in PC12 cells greatly decreased the 8-CPT-induced differentiation of the cells, while no significant effect on differentiation was observed when wild type G\(_{\alpha_2}\) was expressed (Fig. 3, C and D). Furthermore, when the non-myristoylated G\(_{\alpha_2}\) QL variant was used in the experiment, there was no significant decrease in the ratio of differentiated PC12 cells (Fig. 3E), indicating that correct localization of G\(_{\alpha_2}\) was required for the effect on the differentiation process. These experiments were performed several times and the results, summarized in Fig. 3F, clearly indicate a specific effect of activated G\(_{\alpha_2}\) on PC12 cell differentiation. We also performed an additional set of experiments in which constitu-
tively active forms of \( \text{G}_{\alpha_9} \) or \( \text{G}_{\alpha_12} \) were introduced into the PC12 cells; neither of these \( \alpha \) subunits affected 8-CPT-induced differentiation of PC12 cells even though they were clearly expressed in the PC12 cells (data not shown). The results of all these studies support the conclusion that the \( \text{G}_{\alpha_9} \)-Rap1GAP interaction is specific and that correct localization of activated \( \text{G}_{\alpha_9} \) is required to influence differentiation of PC12 cells induced by the cAMP analogue.

Differentiation of PC12 cells through the cAMP-Epac-Rap1 axis leads to activation of B-Raf, phosphorylation of ERKs, and accompanying downstream events (29). If the effect of activated \( \text{G}_{\alpha_9} \) on the differentiation of PC12 cells was mediated through a \( \text{G}_{\alpha_9} \)-Rap1GAP interaction, we reasoned that activation of Rap1 and phosphorylation of ERKs should be reduced when
activated $G_\alpha_i$ was present in the cells. Activation of Rap1 in PC12 cells was assessed through the use of pull-down experiments using GST-RalGDS, which specifically binds and stabilizes the GTP-form of Rap1 (30). When treated with 8-CPT, activation of endogenous Rap1 was observed within 1 min, and the effect persisted for at least 30 min (Fig. 4 and data not shown). When PC12 cells were transfected with the constitutively active form of $G_\alpha_z$, Rap1 activation stimulated by 8-CPT treatment was almost completely blocked, while expression of neither wild type $G_\alpha_z$ nor the non-myristoylated $G_\alpha_z$ QL variant had any significant effect on 8-CPT-induced Rap1 activation (Fig. 4, upper panel).

Recent studies on aggregation of platelets obtained from $G_\alpha_i$-null mice showed decreased susceptibility to epinephrine, which has been interpreted as indication that $\alpha_2A$-adrenergic receptors preferentially couple to $G_\alpha_i$ in platelets (31, 32). Using a specific agonist for $\alpha_2A$-adrenergic receptors, UK14304, we examined the effects of activating the $\alpha_2A$-adrenergic receptor system on Rap1 activation in PC12 cells. Treatment with UK14304 alone did not affect Rap1 activation in PC12 cells, nor did it affect PC12 cell differentiation induced by 8-CPT (data not shown). However, if PC12 cells were transfected with wild type $G_\alpha_z$ and then treated with UK14304 prior to the addition of 8-CPT, Rap1 activation by the cAMP analogue was markedly attenuated (Fig. 4, upper panel). This observation supports the hypothesis that $\alpha_2A$-adrenergic receptors couple to $G_\alpha_z$ in PC12 cells, and activation of these receptors leads to the activation of $G_\alpha_z$, which results in a down-regulation of Rap1 signaling.

To ensure that the above results were not due to overexpression of $G_\alpha_z$, we compared the expression levels of $G_\alpha_z$ in transfected PC12 cells with adrenal chromaffin cells, the cell type in which the pheochromocytoma tumors from which PC12 cells are derived (36). Either during tumor development or establishment of the PC12 line, the cells seem to have down-regulated $G_\alpha_z$ expression as $G_\alpha_z$ was virtually undetectable in untransfected PC12 cells (data not shown). Transfection of the PC12 cells the amounts of plasmid used in these studies resulted in increased expression of $G_\alpha_z$ in the membrane fraction of the cells, and the expression levels obtained were in the range of that observed in the parental adrenal chromaffin cells (Fig. 4, lower panel), indicating that the level of $G_\alpha_z$ in the transfected PC12 cells was similar to its physiological level in the adrenal medulla. We next examined whether 8-CPT-induced phosphorylation of ERKs was attenuated by activation of $G_\alpha_z$ in PC12 cells, which would be the predicted consequence of reducing Rap1 activation. While the total amounts of ERK p42 and p44 did not change upon treatment with 8-CPT, phosphorylation of ERKs detected with a phosphospecific antibody was markedly increased (Fig. 5). This ERK activation was completely blocked by expression of $G_\alpha_z$ QL, but not by wild type $G_\alpha_z$ or the non-myristoylated $G_\alpha_z$ QL variant (Fig. 5). In addition, treatment of cells expressing wild type $G_\alpha_z$ with the $\alpha_2A$-adrenergic receptor agonist, UK14304, again mimicked the effect of expressing $G_\alpha_z$ QL in the cell.

In addition to cAMP, several lines of evidence indicate that NGF-triggered differentiation of PC12 cells is also mediated by signaling through the Rap1/B-Raf/ERK pathway (20, 29). Given the observations noted above that activated $G_\alpha_i$ down-regulates Rap1-dependent signaling in PC12 cells, we reasoned that the $G_\alpha_i$-Rap1GAP interaction should also impact on NGF action on these cells. To test this hypothesis, PC12 cells were treated with NGF and differentiation of the cells determined. As expected, nearly 100% PC12 cells developed neurite extensions when treated with NGF (data not shown). The ability of NGF to trigger this differentiation pathway was not affected by introduction of wild type $G_\alpha_i$ into the cells, but expression of $G_\alpha_i$ QL in PC12 cells markedly attenuated cell differentiation. As with the 8-CPT studies, we also tested whether correct localization of $G_\alpha_i$ was important in this process by expressing the non-myristoylated $G_\alpha_i$ QL in the cells; again, the non-myristoylated $G_\alpha_i$ subunit was largely ineffective in blocking cell differentiation. Additionally, treatment of PC12 cells expressing wild type $G_\alpha_i$ with UK14304 resulted in a substantial reduction of the number of cells exhibiting the differentiated phenotype in response to NGF, again demonstrating the effectiveness of receptor-mediated activation of $G_\alpha_i$ in this regard.

The $\alpha_2A$-adrenergic receptor couples to several members of the $G$ subfamily $G$ proteins (33–35). However, given that we had to express wild type $G_\alpha_i$ in the PC12 cells to see the effect by the $\alpha_2A$-adrenergic receptor agonist, UK14304, it seemed unlikely that any of the endogenous $G$ proteins were responsible for the effect. Nonetheless, we felt that it was important to rule out this possibility. $G_\alpha_z$ can be distinguished from other members in $G$ subfamily in which $G_\alpha$ signaling is PTX-resist-
The binding of the unactivated (Gz), which exhibit Rap1GAP transfection, was either left untreated or treated with 8-CPT, NGF, or UK14304, results in the presence of membrane-associated Rap1GAP determined by immunoblot analysis.

DISCUSSION

PC12 cells have been used as a model system to study neuronal differentiation for over 20 years. Classically, differentiation of these cells has been studied in response to treatment with NGF, although cAMP is also known to trigger the differentiation process (29). Recent studies in these cells have implicated Rap1 signaling in this process (36, 37). Recent findings that activated forms of Gz, a link between receptor-mediated activation of this heterotrimeric G protein and Rap1-mediated cellular processes such as PC12 cell differentiation. The data presented in this study confirm this link and point to a potentially significant role for Gz in the neuronal differentiation process.

Based on the findings of the current studies, we propose a recruitment model to explain how activation of Gz is able to negatively impact a cellular differentiation process mediated through Rap1. Upon binding of NGF to its receptors in PC12 cells, Rap1 is activated to its GDP-bound form, leading to downstream events including phosphorylation of ERKs and PC12 cell differentiation. Ligation of an appropriate GPCR, e.g., the α2A-adrenergic receptor and the agonist UK14304, results in the activation of Gz, by the receptor and subsequent recruitment of Rap1GAP from the cytosol to the membrane. This recruitment event brings Rap1GAP to the sites where activated Rap1 is located, and the subsequent acceleration of GTP hydrolysis by Rap1 transforms Rap1 to its inactive GDP-bound state (Fig. 10). This model is in contrast to one previously proposed for the modulation of Rap1 activity by binding of Gz to Rap1GAP (38). In that study, data was reported indicating that the binding of the unactivated (i.e., GDP-ribosylated) Gz to Rap1GAP resulted in an increased, rather than decreased, activation of Rap1 in PC12 cells (38). However, despite numerous attempts we have been unable to demonstrate binding of Gz to the mammalian Rap1GAP used in our study (data not shown); it is possible that the difference in results is species-specific.

Fig. 7. Effect of activation of Gz on NGF-induced Rap1 activation. Rap1 activation was evaluated by standard GST-RalGDS pull-down experiments as described in the legend to Fig. 4. Precipitated activated Rap1 was analyzed by SDS-PAGE and immunoblot. The density of each band is reported relative to NGF treatment alone. WT, wild type; QL, constitutively active form; non-myr QL, non-myristoylated QL variant. Plus sign (+) indicates the usage of the indicated compound, while the minus sign (−) indicates the absence of the compound. The forms and amounts of plasmids used in each transfection are specified for each condition, and cell lysates were subjected to immunoblot with anti-Rap1 antibody.

Fig. 8. Effect of activation of Gz on NGF-induced ERK activation. Activation of ERKs in PC12 cells was estimated as described in the legend to Fig. 5. The density of the lower band (p42 ERK) for each condition is reported relative to NGF treatment alone. The upper panel shows phosphorylated ERKs and the lower panel shows total ERKs detected with anti-phospho-ERK and anti-ERK antibodies, respectively.

Fig. 9. Rap1GAP translocates to the membrane fraction upon UK14304 treatment of PC12 cells expressing wild type Gαz. PC12 cells expressing wild type (WT) Gαz were either left untreated or treated with 8-CPT, NGF, or UK14304 as described under “Experimental Procedures.” Cells were lysed after 48 h and membrane fractions isolated by centrifugation at 100,000 × g. Samples containing 40 μg of protein from each membrane fraction were resolved by SDS-PAGE and immunoblot.
related as chicken Rap1GAP was used in the Goα study (38). Regardless of the source of the discrepancy, we feel our results are quite clear and point to a specific consequence of Goα activation on Rap1 signaling in neuronal cells.

Recently, Goα knockout mice were generated and the impact of loss of Goα tested in both platelets and the central nervous system (32, 39). When mice were challenged in a model for testing their responses to antidepressant drugs, complete loss of responses to two selective norepinephrine reuptake inhibitors was observed in Goα knockout mice (32). This finding suggests the signaling pathways invoked by these antidepressant drugs are mediated primarily through Goα. Interestingly, recent studies of the effects of antidepressant drugs on adult rat hippocampus revealed that chronic treatment of antidepressant drugs increased neurogenesis at the expense of cell differentiation (40, 41). Our findings that activation of Goα attenuates PC12 cell differentiation seem to be a link between the results discussed above. It is tempting to speculate that Goα signaling plays an important role in cell fate determination of neuronal cells. In this scenario, treatment with antidepressant drugs leads to increases in circulating norepinephrine that binds to Gβγ-coupled receptors, and the subsequent activation of Goα leads to suppression of signaling pathways that promote cell differentiation and thereby allow the cell proliferation associated with neurogenesis. In Goα knockout mice, treatment with this class of antidepressant drugs cannot lead to activation of Gβγ and its downstream effectors, resulting in maintenance of cell differentiation without further neurogenesis.

The tissue distribution and expression pattern of Goα, Rap1GAP, and Rap1 provide another hint for their biological functions. Goα and Rap1GAP are found predominantly in brain. While Rap1 is expressed ubiquitously, it is especially abundant in brain. The developmental expression pattern of Goα has been determined at the mRNA level. Interestingly, this analysis showed that Goα expression reaches its peak at the time when target tissue innervation is occurring, which is a process involving neurogenesis (42). Together with the data presented in this paper demonstrating the involvement of Goα in the process of neurite development and in coupling to α2A-adrenergic receptors, we postulate that Goα signaling pathways may participate the regulation of neuronal development in vivo. Further evaluation of this regulatory network should lead to an increased understanding of the cellular and molecular basis of how neurogenesis is controlled.

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