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GIPC Recruits GAIP (RGS19) To Attenuate Dopamine D_2 Receptor Signaling[D]

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Pleiotropic G proteins are essential for the action of hormones and neurotransmitters and are activated by stimulation of G protein–coupled receptors (GPCR), which initiates heterotrimer dissociation of the G protein, exchange of GDP for GTP on its Gα subunit and activation of effector proteins. Regulator of G protein signaling (RGS) proteins regulate this cascade and can be recruited to the membrane upon GPCR activation. Direct functional interaction between RGS and GPCR has been hypothesized. We show that recruitment of GAIP (RGS19) by the dopamine D_2 receptor (D_2R), a GPCR, required the scaffold protein GIPC (GAIP-interacting protein, C terminus) and that all three were coexpressed in neurons and neuroendocrine cells. Dynamic translocation of GAIP to the plasma membrane and coassembly in a protein complex in which GIPC was a required component was dictated by D_2R activation and physical interactions. In addition, two different D_2R-mediated responses were regulated by the GTPase activity of GAIP at the level of the G protein coupling in a GIPC-dependent manner. Since GIPC exclusively interacted with GAIP and selectively with subsets of GPCR, this mechanism may serve to sort GPCR signaling in cells that usually express a large repertoire of GPCRs, G proteins, and RGS.

INTRODUCTION

A general concept of signal transduction establishes that distinct signaling pathways form through the combination of components from a common repertoire of enzymes to evoke distinct physiological responses. For instance, neurotransmitters can induce a wide range of direct effects on target cells through the activation of G protein–coupled receptors (GPCR), which in turn stimulate particular intracellular signaling components. Selective interactions between these components may serve to sort signaling pathways in cells that usually express a wide range of GPCRs, G proteins, and effectors. Regulator of G protein signaling (RGS) proteins exert their GTPase function through direct interactions on activated (GTP-bound) form of G proteins to limit their lifetime and terminate signaling (Berman and Gilman, 1998; Ross and Wilkie, 2000; Hollinger and Hepler, 2002). Although most RGS are promiscuous in their Gα subunit binding (De Vries *et al.,* 2000), recruitment of a particular RGS in G-mediated signaling cascades may not be dictated by the Gα subunit itself, but by the receptor that initiates G protein activation. Previous studies support this concept, showing that distinct GPCRs, although coupled to the same G protein, select different RGS to regulate their signaling (Wang *et al.,* 2002; Xu *et al.,* 1999). Because receptor-G protein complexes are membrane bound, cellular mechanisms must direct RGS, usually confined away from signaling components (Hollinger and Hepler, 2002), to target Gα subunits. Several RGS translocate to the plasma membrane (PM) when exposed to GTPase-deficient Gα subunits or through mechanisms initiated by G protein activation (Druey *et al.,* 1998; Saitoh *et al.,* 2001). How RGS assemble with the signaling machinery in living cells is a highly debated issue (Hepler, 2003; Roy *et al.,* 2003). Recently, the discovery of a direct functional interaction between RGS2 and the third intracellular loop of the M1 muscarinic acetylcholine receptor (Bernstein *et al.,* 2004) suggests the possibility of a new regulatory process dictated by the GPCR and not only the G protein.

Scaffolding proteins organize and assemble components of a machinery in local units of cells by spatially clustering proteins, like components of signal transduction pathways (Li and Montell, 2000; Hamazaki *et al.,* 2002). Several members of the RGS family display multiple protein interaction domains conferring scaffolding properties in addition to their GTPase activity. The "complex" RGS, including members of the RA, R7, and R12 subfamilies (reviewed by De Vries and Farquhar, 1999; Hollinger and Hepler, 2002) possess a highly ordered structure with multiple functional domains, in contrast with the "simple" RGS (members of the RZ and R4 subfamilies), which do not, suggesting that they may undertake different regulation mechanisms. Indeed, the

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Abbreviations used: CHO, Chinese hamster ovary cells; Ct, C-terminus; D_2R, D_3R, and D_4R, dopamine D_2, D_3, and D_4 receptors; GAIP, Gαi3-interacting protein; GIPC, GAIP-interacting protein C-terminus; GFP, green fluorescent protein; GPCR, G-protein–coupled receptor; GST, glutathione-*S*-transferase; HEK293, human embryonic kidney cells; ODN, oligodeoxynucleotide; PDZ, consensus sequence in PSD95/DLG/ZO-1; PM, plasma membrane; RGS, regulator of G-protein signaling.

complex RGS may assemble by itself to the signaling machinery (Snow *et al.*, 1998), whereas dynamic recruitment of the simple RGS may rely on accessory proteins.

The PDZ-domain–containing protein GIPC was identified by virtue of its interaction with GAIP, a member of the RZ RGS subfamily (De Vries *et al.*, 1998b). GIPC was recently demonstrated to interact with GPCRs, such as the dopamine D_2R and D_3R (Jeanneteau *et al.*, 2004) and β1-adrenergic receptors (Hu *et al.*, 2003), raising the possibility that GIPC may serve as a molecular adaptor between GPCR and RGS. In addition, the GTPase activity of GAIP targets Gαi/Gαo subunits (De Vries *et al.*, 1995; Berman *et al.*, 1996), which D_2-like receptors preferentially bind (Missale *et al.*, 1998). So, we carefully examined the role of GAIP and GIPC in the regulation of D_2R-mediated G signaling in living cells. In the present study, we describe how G signals elicited by dopamine agonists through the D_2R subtype are finely regulated by the elaboration of a highly ordered GIPC-dependent protein complex containing D_2R and GAIP.

MATERIALS AND METHODS

Plasmid Constructs

C-terminal domains of the human RGS2 (amino acids 201–211, accession number for the nucleotidic sequence: NM_002923), RGS9–2 (amino acids 661–671, accession number for the nucleotidic sequence: NM_003835), RGS12L (amino acids 1437–1447, accession number for the nucleotidic sequence: NM_198430), GAIP (amino acids 207–217, accession number for the nucleotidic sequence: AY585188), GAIPΔA216, a mutant lacking the C-terminus alanine, and RGSZ1 (amino acids 203–213, accession number for the nucleotidic sequence: NM_170587) were inserted in-frame downstream of the B42 activation domain in pEG202 (OriGene Technologies, Rockville, MD). Recombinant pEG202 plasmids encoding for the full-length (amino acids 1–333) or the last two-thirds (amino acids 119–333) of the open reading frame of GIPC (accession number for the nucleotidic sequence: AF089817) previously described (Jeanneteau *et al.*, 2004) were used in binary two-hybrid assays. Rat GIPC fused downstream of the LexA DNA-binding domain (OriGene Technologies) in pJG4.5 was obtained as described (Jeanneteau *et al.*, 2004). Rat GAIP coding sequence (accession number: NM_021661), amplified by RT-PCR from rat hippocampus total RNA with specific primers, was subcloned either in pGEX-2TK (Amersham Pharmacia Biotech, Piscataway, NJ) downstream of the glutathione-*S*-transferase (GST) or in pCMV-tag3B (BD Biosciences Clontech, Palo Alto, CA) downstream of the c-myc epitope or in pEGFPC1 (BD Biosciences Clontech) downstream of the Enhanced Green Fluorescent protein (EGFP). The GAIPΔA216 construct was obtained by hybridizing complementary primers lacking the C-terminal alanine (A216) codon at the *Apa*I restriction site in the open reading frame of GAIP, whereas GAIPS151A was obtained using the QuickChange Multi-Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The Xpress/His_6GIPC and GFP- or c-myc–tagged D_2R short isoform (accession number: NM_016574) and D_4R (accession number: U06925) were obtained as described (Jeanneteau *et al.*, 2004). Constructions were checked by nucleotide sequencing (Licor, Lincoln, NE).

Binary Two-hybrid Assays in Yeast

Yeast two-hybrid binary assays were performed using the DupLex-A Two-Hybrid system kit (OriGene Technologies) and supplied yeast strain EGY48 harboring the reporter genes LEU1 and β-galactosidase under the control of the upstream LexA binding sites. Transformants were grown on selective medium and assayed for β-galactosidase activity by using X-gal for solid-phase assays and *o*-nitrophenyl β-D-galactopyranoside (ONPG) for liquid-phase assays according to the Yeast Protocols Handbook from Clontech. Relative binding to GIPC is measured as follows: (βgal units (βU)^{test} − βU^{crl})/(βU^{D2R} − βU^{crl}). One unit of βgal is defined as the amount that hydrolyzes 1 μmol of ONPG per min per cell.

Cell Culture and Transfections

$CHOD_2R$ and HEK293 cells stably expressing His_6GIPC were obtained and maintained as described (Giros *et al.*, 1989; Jeanneteau *et al.*, 2004). Cell lines expressing GFPGAIP, GFPGAIPΔA216, or GFPGAIPS151A were obtained by transfection with Superfect (Qiagen, Santa Clarita, CA) using 10 μg of pEGFPC1GAIP, pEGFPC1GAIPΔA216, or pEGFPC1GAIPS151A, respectively, in $CHOD_2R$ cells. Clones were selected by resistance to neomycin and screened for GAIP expression by measuring GFP fluorescence and by Western blotting with the Living Colors Full-Length A.v. polyclonal anti-GFP antibody (BD Biosciences Clontech).

GST Pull-down Assay

GST and GSTGAIP fusion protein were produced in *Escherichia coli* BL21 after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h. Sonicated cells were agitated for 5 min in 300 μl B-PER Bacterial Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL), lysozyme (200 $\mu g \cdot ml^{-1}$, Research Organics, Cleveland, OH) plus protein inhibitors (Sigma, St. Louis, MO) and centrifuged at 12,000 × *g* for 5 min. Supernatants were incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h and washed three times with 10 ml of ice-cold phosphate-buffered saline (PBS). The same amount of immobilized fusion proteins was then incubated with solubilized D_2R-expressing membranes from transfected HEK293 cells (400 $\mu g \cdot ml^{-1}$) in the presence or absence of cytosolic extracts from HEK/GIPC cells (400 $\mu g \cdot ml^{-1}$) overnight at 4°C. Beads were washed three times with 10 ml of ice-cold PBS and resuspended in loading buffer. An equal amount of bound proteins was separated in each lane by SDS-PAGE (10%) and analyzed by Western blot using anti-GFP (1:7000), anti-Xpress (1:5000), or anti-GST antibodies (1:2000, Amersham Pharmacia Biotech).

Immunoprecipitation

$CHOD_2R$ cell lines coexpressing GFPGAIP or GFPGAIPΔA216 cells pretreated with 250 $\mu g \cdot ml^{-1}$ concanavalin A for 30 min were stimulated or not by 10 μM dopamine for 15 min. Cells were solubilized in the digitonin-cholate mixture previously described (Jeanneteau *et al.*, 2004) and receptors were labeled with [^{125}I]iodosulpride (0.1 nM; Amersham Pharmacia Biotech). GFPGAIP or GFPGAIPΔA216 were immunoprecipitated from the same amount of cell lysates (one 10-cm dish) with the anti-GFP antibody (1:5000, BD Biosciences Clontech) and protein A-Sepharose (Amersham Pharmacia Biotech). On one hand, bound proteins were centrifuged and the precipitated ^{125}I-labeled receptors were assayed by filtration (Diaz *et al.*, 2000). Relative efficiency of D_2R coprecipitation = (coprecipitated D_2R binding sites) − (nonspecific binding with 10 μM enomapride)/(total binding in the solubilized cell lysates). On the other hand, bound proteins were extensively washed in PBS and equal amounts were electrophoresed on 10% SDS-PAGE for further analysis by Western blot with anti-GFP (1:7000) and anti-His6 antibodies (1:3000).

In Situ Hybridization

Probes for rat GIPC and D_2R mRNAs were previously described (Sokoloff *et al.*, 1990; Jeanneteau *et al.*, 2004). ^{33}P-labeled riboprobes for D_2R and GIPC were synthesized with the Riboprobe Gemini System (Promega, Madison, WI), treated with RNAse-free DNAse (Roche, Basel, Switzerland), and recovered from Chroma spin-30 columns (BD Biosciences Clontech). Digoxigenin-labeled rat GAIP riboprobe (Grafstein-Dunn *et al.*, 2001), subcloned into pCRII (Invitrogen, San Diego, CA), was synthesized with the Ampliscribe transcription kit (Epicenter Technologies, Madison, WI). Cryostat sections (10 μm) of adult male Wistar rat brain and pituitary were prepared and hybridized as described (Grafstein-Dunn *et al.*, 2001). Slices were dipped in photographic emulsion (LMI; Amersham Pharmacia Biotech) and digoxigenin-UTP revealed with an alkaline phosphatase-conjugated secondary antibody (Roche). Relative signal levels were determined by visual inspection of autoradiographic and alkaline phosphatase emulsion-coated sections using a Zeiss Axiophot microscope under dark field conditions (Carl Zeiss, New York, NY). Coexpression reflected by colocalization of ^{33}P silver grains and alkaline phosphatase signal on counterstained dipped sections was quantified in >500 cells from each brain region in 3–4 sections from two different animals.

Binding Assays

Binding experiments were performed on cell membrane fraction from $CHOD_2R$ and cell lines stably expressing GFPGAIP or its mutants, using [^{3}H]spiperone (0.4 nM) as previously described (Jeanneteau *et al.*, 2004). To detect D_2R present at the surface of cells, binding experiments were performed in culture medium with [^{125}I]iodosulpride (0.1 nM). Competition binding studies were measured with increasing concentrations of dopamine in the presence of 0.05 mg ml^{-1} ascorbate. Nonspecific binding was determined in the presence of 1 μM enomapride. Data were analyzed by the nonlinear regression curve-fitting program PRISM (Graphpad, San Diego, CA).

Arachidonic Acid Release Assay

The release of [^{3}H]arachidonic acid (AA; Amersham Pharmacia Biotech; 200–220 Ci $mmol^{-1}$, 0.25 μCi ml^{-1}) in $CHOD_2R$ cells was measured as described (Piomelli *et al.*, 1991). Cells were incubated for 10 min at 37°C in 0.25 ml of DMEM with the appropriate dilution of quinpirole before stimulation by adding 0.25 ml of 2 mM ionophore (A23187, Sigma).

cAMP Accumulation Assay

Cells were preincubated with 10 μM 3-isobutyl-1-methylxanthine in αMEM for 25 min and treated with quinpirole in increasing concentrations for 10 min in the presence of 0.5 μM forskolin. The reaction was stopped by addition of 50 μl of ice-cold 0.1 M HCl. Cells were sonicated and cAMP accumulation was

assayed with the Rianen ^{125}I-labeled cAMP radioimmunoassay kit (DuPont/NEN, Boston, MA).

Immunofluorescence

Cells were grown on coverslips, fixed to perform immunofluorescence as described (Jeanneteau *et al.*, 2004). Tagged-receptors prominently localized to the PM of cells transfected with a low amount of cDNA (Jeanneteau *et al.*, 2004). MycGAIP was detected by a CY3-conjugated secondary anti-rabbit antibody (1:1000, Interchim, Lyon, France). The anti-P58K (1:50, Sigma), anticlathrin (1:50) kindly provided by A. Schmidt, anti-EEA1 (1:80, Molecular Probes, Eugene, OR), and anti-CD63 (1:200, Caltag Laboratories, Burlingame, CA) antibodies were used as described (Jeanneteau *et al.*, 2004). Cells were imaged as TIF files by a laser scanning confocal image system (Leica TCS SP II software, Deerfield, IL) coupled to a Leica DM R fluorescence microscope. Fluorescence from single- or double-labeled cells was quantified by transforming red, green, and yellow pixels in gray scale pixels using photoshop. PM and intracellular areas were traced and selected, and pixel intensity was measured using the NIH image 1.63 software (http://rsb.info.nih.gov/nih-image/). Colocalization data between GAIP and D_2R or D_4R were expressed as percentage of overlap in 10–15 cells each from three independent experiments. Translocation of GFPGAIP and mutants to the PM of CHO cells was quantified in terms of the PM/cytoplasm-specific fluorescence ratio in 15–50 cells each of three independent experiments.

Antisense Strategy

Synthetic phosphorothioate antisense oligodeoxynucleotides (ODNs) were prepared (Proligo Biochemie GmbH, Hamburg, Germany) to reduce the synthesis of GIPC. The following 20-base ODN sequence was used: ODN-AS 5′-gtggcatgagcagccagaag-3′ corresponding to nucleotides 60–80 of the rat GIPC gene sequence (AF089817). Antisense ODN control consisted of the sense sequence: the 20-base ODN-S 5′-cttctggctgctcatgccac-3′. Each ODN transfection using Lipofectamine 2000 (Invitrogen) was performed on distinct $CHOD_2R$ cell lines coexpressing GFPGAIP or GFPGAIPΔA216 according to the following 3-d schedule: 1 nmol on day 1, 2 nmol on day 2, and 3 nmol on day 3. At the end of the ODN treatment, cells were pretreated by 250 $\mu g \cdot ml^{-1}$ concanavalin A and subsequently stimulated by 10 μM dopamine. Translocation of GFPGAIP at the PM of cells was observed by confocal microscopy, and cells were thereafter harvested and sonicated. An equal amount of each cell lysate was separated on 10% SDS-PAGE for further analysis of specific protein expression levels by Western blot with anti-GIPC (1:200), anti-GFP (1:7000), and antiactin (1:1000, MP Biomedicals, Aurora, OH) antibodies. Experiments and blots are representative of two independent experiments.

Table 1.

Specificity of interactions between GIPC and RGS or GPCRs

Candidate protein	C-ter sequence	Binding to GIPC[a]	Ref.[b]
RGS			
RGS 2	KPQITTEPHAT$_{COOH}$	−	1
RGS 9-2	EKEVICPWESL$_{COOH}$	−	1
RGS12L	PKTSAHHATFV$_{COOH}$	−	1
GAIP	LQGPSQSSSEA$_{COOH}$	+	1
RGS Z1	LQSLSEKSIEA$_{COOH}$	−	1
GAIPΔA216	LQGPSQSSSE-$_{COOH}$	−	1
RGS 4	ADCTSLVPQCA$_{COOH}$	−	2
RGS 16	PSGSPAEPSHT$_{COOH}$	−	2
Ret-RGS	LRSLSEKAVEA$_{COOH}$	−	2
GPCR			
D_2R	FRKAFMKILHC$_{COOH}$	+	3
D_3R	FRKAFLKILSC$_{COOH}$	+	3
D_4R	SVFRKTLRLRC$_{COOH}$	−	3
β_1AR	CRPGFASESKV$_{COOH}$	+	4
β_2AR	GRNCSTNDSLL$_{COOH}$	−	4
LHR	ALLDKTRYTEC$_{COOH}$	+	5

The C-terminus of RGS candidates were used in yeast binary two-hybrid assays to test for interactions with the full-length GIPC. Interactions previously reported are also indicated with references between brackets. PDZ-binding motifs are underlined.

[a] +, interaction; −, no interaction.

[b] References: 1, present study; 2, De Vries *et al.*, 1998; 3, Jeanneteau *et al.*, 2004; 4, Hu *et al.*, 2003; 5, Hirakawa *et al.*, 2003.

RESULTS

GIPC Selectively Interacted with GAIP and GPCRs

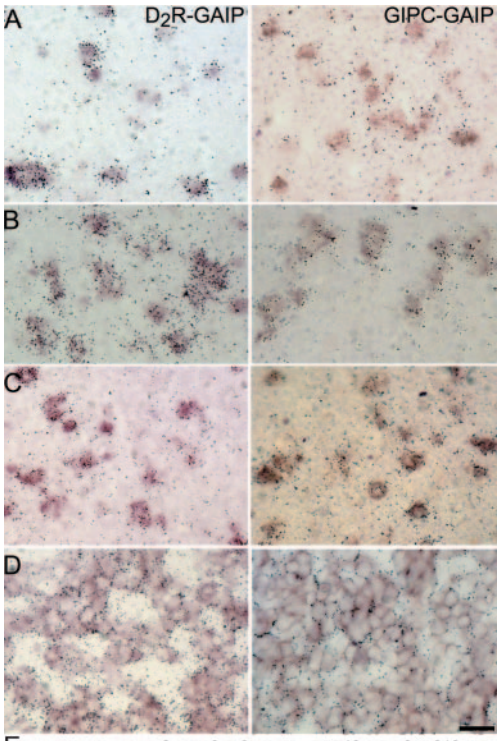
Because cells usually express a large repertoire of GPCR and RGS that could regulate the same signaling pathway (Hepler *et al.*, 1997), we investigated the selectivity of GIPC toward various RGS using binary yeast two-hybrid assays (Table 1). GIPC exclusively interacted with the C-terminal domain of GAIP among various RGS candidates for D_2R regulation, namely, RGS2, whose expression is up-regulated by D_2/D_3 antagonists (Robinet *et al.*, 2001); RGS9–2, which is mainly expressed in brain regions receiving dopamine innervations (Rahman *et al.*, 2003); RGS12, which has a type I PDZ-binding motif like GAIP (Table 1, underlined motifs); and RGSZ1, which shares 58% sequence identity with GAIP protein. A previous study (De Vries *et al.*, 1998b) also showed that full-length RGS2, RGS4, RGS16, and RET-RGS did not interact with GIPC. Interaction between GIPC and GAIP was based on a C-terminal PDZ-recognition motif (De Vries *et al.*, 1998b), in which the C-terminal alanine residue is critical because its deletion broke the interaction (Table 1). Moreover, GIPC binds to several GPCRs, including the dopamine D_2R, D_3R, β1-adrenergic (β_1AR) and LH (LHR) receptors, through different types of PDZ-binding motifs (Table 1, underlined motifs). However, GIPC did not interact with closely related PDZ-ligands such as that of RGS12, β_2AR, and D_4R. Hence, specificity of GIPC PDZ-recognition may rely on structural determinants inside and outside PDZ-binding motifs. To date, GIPC interacts with one unique RGS, GAIP (RGS19), but several specific GPCRs.

D_2R, GIPC, and GAIP Were Coexpressed in Neurons and Neuroendocrine Cells

The hypothesis that GIPC and GAIP associate with D_2R in brain was further examined by studying the expression pattern of all three mRNAs in cells of different brain regions. To this end, in situ hybridization with GAIP specific digoxigenin-stained and GIPC- or D_2R-specific ^{33}P-labeled riboprobes was performed on adjacent rat brain sections. Results show that D_2R, GIPC, and GAIP mRNAs were highly codistributed throughout rat brain regions. For instance, all three mRNAs were coexpressed in neurons of the striatum (Figure 1A), substantia nigra (Figure 1B) and ventral tegmental area (Figure 1C), which mainly contain dopaminoceptive and dopaminergic neurons as well as in neuroendocrine cells of the pituitary (Figure 1D). All the D_2R-positive neurons expressed GAIP, whereas almost all the GAIP-positive neurons expressed GIPC (Figure 1E). In agreement, both D_2R (Levey *et al.*, 1993) and GIPC (our unpublished results) proteins are broadly expressed in the striatum and pituitary. Nevertheless, although GAIP and GIPC mRNAs were coexpressed in the neocortex and hippocampus, D_2R mRNA was undetectable in these regions, a result that is consistent with the hypothesis that the GAIP-GIPC complex may bind to other receptors.

D_2R, GIPC, and GAIP Formed a Complex in which GIPC Was a Necessary Component

We investigated the formation of a protein complex between D_2R, GIPC, and GAIP, by using an in vitro pull-down assay (Figure 2A). Soluble GAIP immobilized as a GST fusion



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	GAIP	GIPC	D2R	D2R/GAIP	GIPC/GAIP
Striatum					
Ventro-lateral	+	+	++	>80 %	>91 %
Dorso-lateral	++	+	+++	>98 %	>98 %
SN	+	+	+	100 %	100 %
VTA	+	+	+	100 %	100 %
Pituitary					
Anterior	++	++	++	100 %	>95 %
Intermediate	++	++	+++	100 %	>95 %
Posterior	-	-	-	NA	NA
Neocortex	++	++	-	NA	>90 %
Hippocampus	++	++	-	NA	>90 %

Figure 1. GAIP, GIPC, and D_2R mRNAs were coexpressed in the rat brain and pituitary. In situ cohybridization of specific GAIP digoxigenin-stained (dark) and D_2R or GIPC ^{33}P-labeled (silver grains) riboprobes on adjacent rat brain sections. Codistribution of D_2R with GAIP mRNAs (left) and GIPC with GAIP mRNAs (right) in the dorsolateral striatum (A), substantia nigra (B), ventral tegmental area (C), and pituitary (D). Bar, 10 μm. (E) Quantification of D_2R, GAIP, and GIPC mRNAs cellular coexpression throughout rat brain tissues is expressed as percentage of double-labeled cells compared with the total number of cells counted ($n > 500$ cells) in each region. NA, not applicable.

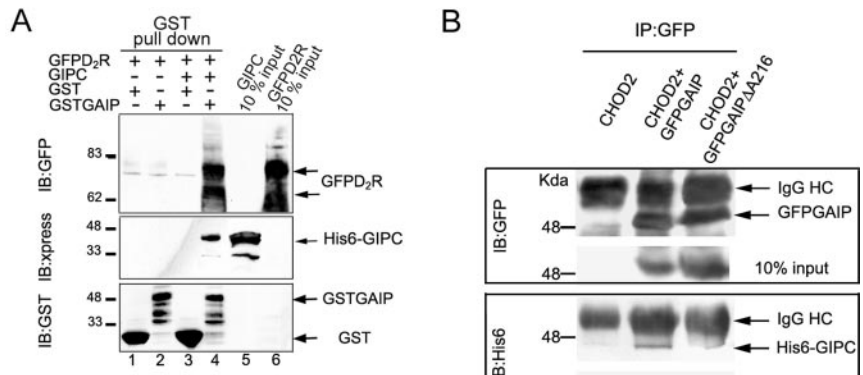
protein or GST alone were incubated with detergent extracts from HEK293 cells transfected with D_2R fused with GFP ($GFPD_2R$) in the presence or absence of cytosolic extracts from His_6GIPC-transfected HEK293 cells. $GFPD_2R$ did not coprecipitate with GST in the absence (Figure 2A, lane 1) or the presence (Figure 2A, lane 3) of recombinant His_6GIPC. In contrast, $GFPD_2R$ coprecipitated with GSTGAIP when His_6GIPC was added to the incubation assay (Figure 2A, lane 4), but not when His_6GIPC was omitted (Figure 2A, lane 2). The necessity of GIPC in the formation of a protein complex containing D_2R and GAIP was further assessed by immunoprecipitation with anti-GFP antibody (Figure 2B) of GFPGAIP or GFPGAIPΔA216, a mutant that cannot bind to GIPC (Table 1). The presence of His_6GIPC and D_2R was detected in the precipitates, respectively, by immunoblotting and receptor binding with a D_2-like selective radioligand, [I^{125}]iodosulpride. We found that both His_6GIPC and D_2R were coimmunoprecipitated with GFPGAIP in CHO cells transfected with all three proteins (Figure 2B, lane 2) but not in cells transfected with D_2R, His_6GIPC and GFPGAIPΔA216 (Figure 2B, lane 3). Therefore, coprecipitation of D_2R and His_6GIPC by GFPGAIP resulted, at least, from a direct interaction between GIPC and GAIP. Because both GAIP and D_2R interact with the PDZ-domain of GIPC and could compete for the same binding site (Lou *et al.*, 2002, Jeanneteau *et al.*, 2004), the formation of a complex between GAIP, GIPC, and D_2R should rely on either accessory proteins or GIPC dimerization. The latter hypothesis is supported by GIPC binding to itself in yeast binary two-hybrid assays (Figure 2C). GIPC may dimerize through its N-terminus, given that GIPC did not bind to a GIPC construct lacking its N-terminus (Figure 2C). In addition, the avidity of the GIPC-GIPC interaction measured by liquid-phase yeast two-hybrid assays was weaker than that of GIPC-GAIP and GIPC-D_2R (Figure 2C). This could account for the little efficiency of D_2R coprecipitation by GIPC-GAIP complexes. Indeed, the amount of D_2R coprecipitated with GFPGAIP-His_6GIPC complexes represented only 9.9 ± 2.3% of the total specific [^{125}I]iodosulpride binding sites measured in the solubilized cell lysates (Figure 2B, lane 2). Altogether, these results provide biochemical evidence for the occurrence of a protein complex, in which GIPC is a required component.

GAIP Colocalized with the Activated D_2R in a GIPC-dependent Manner

The requirement of GIPC in the formation of GAIP-GIPC-D_2R complexes was further investigated in HEK293 cells by comparing the colocalization of mycGAIP and $GFPD_2R$ in the presence or absence of overexpressed His_6GIPC. $GFPD_4R$, which does not bind to GIPC (Jeanneteau *et al.*, 2004), was used as a negative control. Colocalization between mycGAIP and $GFPD_2R$ or $GFPD_4R$ was quantified as the percentage of overlapping fluorescent signals. In wild-type HEK293 cells that express similar native GIPC level than in rat brain sample tissues but low endogenous levels compared with other cell lines as observed by Western blot (Supplementary Information 1), distribution of mycGAIP appeared as punctate and distinct from that of $GFPD_2R$. Treatment with 3 μM quinpirole, a dopamine agonist, did not significantly affect the GAIP-D_2R colocalization background from 24.7 ± 7.5 to 27.2 ± 9.0% (Figure 3, A, B, and G). However, the GAIP-D_2R colocalization rate increased from 27.2 ± 9 to 52.6 ± 14.5% ($p < 0.05$; Figure 3, D, E, and G) in cells transfected to stably overexpress His_6GIPC (Supplementary Information 1B). Interestingly, mycGAIP colocalized with $GFPD_2R$ at the PM and in vesicles (Figure 3E, see arrows). In contrast, colocalization between mycGAIP and $GFPD_2R$ was not apparent when the receptor was not activated (Figure 3, A and D) or after a longer exposure to the agonist (>10 min, our unpublished results), indicating that the formation of a complex in a cell context could be transient. In addition, $GFPD_4R$ was unable to participate in such a process (Figure 3, C, F, and G), suggesting that the formation of the protein complex containing GAIP, GIPC, and D_2R is specific.

Dynamic Subcellular Localization of GFPGAIP

In CHO cells that express endogenous GIPC, a GFPGAIP fusion protein displayed a vesicular endosomal-based localization (Figure 4A), as assessed with specific cellular biomarkers (Supplementary Information 2). Nevertheless, GFPGAIP also localized faintly to the PM of a few cells (1–5%), but also to the cytoplasm and the nucleus as a result of its overexpression (our unpublished results). The coexpression of D_2R did not affect the subcellular distri-



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Interactions tested	Yeast 2-hybrid assays	
	Solid phase	Liquid phase
GIPC:D_2	+	1 ± 0.1
GIPC:GAIP	+	0.72 ± 0.07
GIPC:GIPC	+	0.3 ± 0.08
GIPC:GIPCΔNter	-	n.d.
GIPC:Vector	-	0.017 ± 0.001
Vector:D_2	-	n.d.

Figure 2. GAIP, GIPC, and D_2R formed a coprecipitable complex. (A) Pull-down assays with GST or GSTGAIP fusion protein were performed in the presence (lanes 3 and 4) or absence of GIPC (lanes 1 and 2). Bound proteins (lanes 1–4) were separated on 10% SDS-PAGE and analyzed by Western blot using anti-GFP, anti-Xpress, or anti-GST antibodies. The amount of proteins loaded in each well was adjusted to 50 μg, and the expression levels in 10% of cell lysates are shown in lanes 5 and 6. (B) Coimmunoprecipitation assays using $CHOD_2R$ cells transfected with His_6GIPC and GFPGAIP, or GFPGAIPΔA216. Because RGS main substrates are active G proteins, $CHOD_2R$ cells were stimulated with 10 μM dopamine for 15 min and extensively rinsed with ice-cold PBS before harvesting. Cells were solubilized, and lysates were incubated with anti-GFP antibody, protein A-Sepharose, and 0.1 nM [^{125}I]iodosulpride in the presence or absence of 1 μM enomapride to measure nonspecific binding. Epitope tags of GAIP or GAIPΔA216 and GIPC were detected by immunoblots (representative of 4 independent experiments) in precipitates rinsed with PBS. Equal amounts of proteins were resolved by 10% SDS-PAGE, and expression levels are shown in cell lysates corresponding to 50 μg protein. The efficiency of D_2R coprecipitation by the anti-GFP antibody was estimated by measuring the amount of D_2R-specific binding sites in the precipitates compared with in the whole solubilized cell lysate. [^{125}I]iodosulpride binding was expressed as mean ± SEM of data from four independent experiments. Paired *t* test: *$p < 0.05$ vs. $CHOD_2R$ cells; #$p < 0.05$ vs. +GFPGAIP. (C) Solid and liquid phase yeast two-hybrid assays. The yeast strain EGY48 was cotransformed with the full-length GIPC cDNA and D_2R, GAIP, or GIPC. The relative strength of protein-protein interactions observed in transformants was monitored by β-galactosidase liquid-phase assay using ONPG as substrate. Controls were performed by using pEG202 or pJG4.5 empty vectors. Binding to GIPC was compared with D_2R-GIPC interaction defined arbitrary as 1. Values are means ± SEM of data from four experiments and two different transformations. n.d., not determined.

bution of GFPGAIP (Figure 4B), whereas the receptor activation with 3 μM quinpirole, elicited the translocation of GFPGAIP to the PM (Figure 4C, see arrows), an effect that was blocked in the presence of 50 μM haloperidol, a dopamine antagonist (Figure 4D). These results were quantified and expressed as a PM/cytoplasm-specific fluorescence ratio that represents the relative proportion of GFPGAIP localized at the PM (Figure 4G). The translocation rate of GFPGAIP to the PM was significantly increased upon D_2R activation from 7.7 ± 5 to 49.6 ± 18%

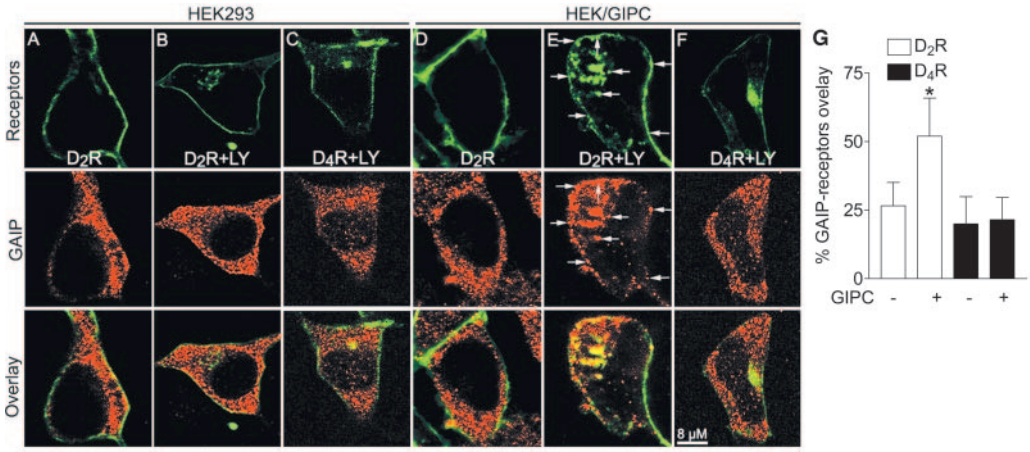

Figure 3. GIPC-dependent clustering of GAIP with the activated D_2R. Double-staining experiments of myc-GAIP and $GFPD_2R$ in transfected HEK293 cells (A–C) and GIPC-stably overexpressing HEK293 cells (D–F). $GFPD_4R$ that does not interact with GIPC was also tested as a negative control (C and F). Transfected cells were either untreated (A and D) or stimulated (B, C, E, and F) for 5 min by 3 μM quinpirole (LY). The antimyc antibody, revealed by a CY3-conjugated secondary antibody, and GFP were visualized and captured by confocal fluorescent microscopy. Arrowheads indicate examples of colocalized clusters. (G) Colocalization between myc-GAIP and GFP-D_2R or -D_4R was compared in the presence or absence of His_6GIPC overexpression by recording the intensity of yellow pixels in 10–15 cells using the NIH image 1.63 software. Colocalization was expressed as the percentage of total receptor pixel intensity. Paired *t* test *$p < 0.05$ vs. -GIPC.

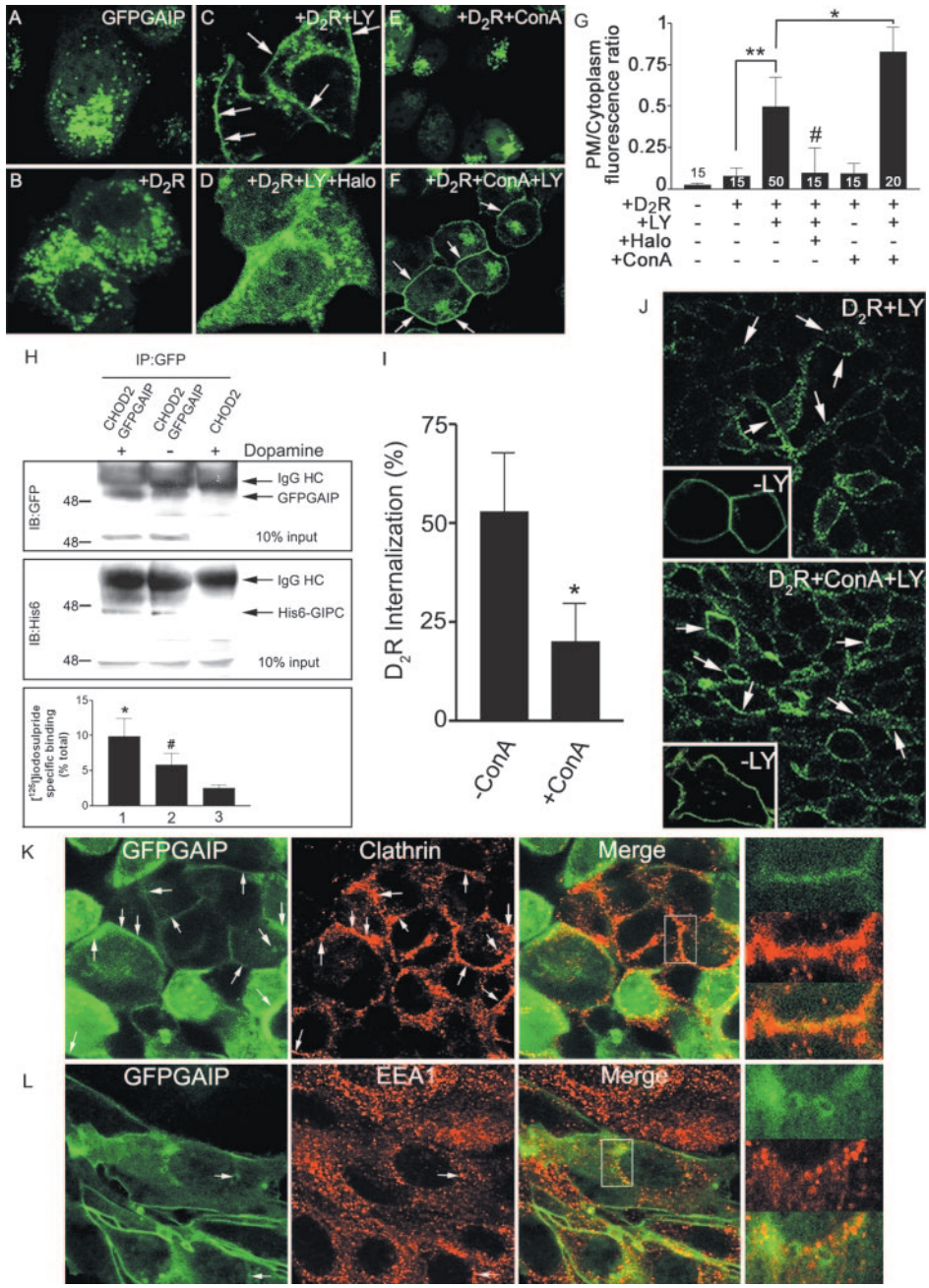

Figure 4. Subcellular distribution of GFPGAIP was dynamically regulated by D_2R activation. Subcellular distribution of GFPGAIP in transfected CHO (A) and $CHOD_2R$ cells (B–F) treated for 10 min with 3 μM quinpirole (LY), a dopamine agonist (C), plus 50 μM haloperidol (Halo), a dopamine antagonist (D) or with 250 $\mu g \cdot ml^{-1}$ concanavalin A (ConA) for 30 min (E) before D_2R activation with 3 μM LY (F). GFP fluorescence was visualized by confocal microscopy and arrowheads indicate translocation of GFPGAIP to the PM. (G) Translocation of GFPGAIP to the PM was quantified as means ± SEM of data from three independent experiments in 15–50 cells using the NIH Image 1.63 software and was expressed as a PM/cytoplasm specific fluorescence ratio. Paired *t* test: *$p < 0.05$ D_2R+LY vs. D_2R+LY+conA; **$p < 0.01$ D_2R+LY vs. untreated cells, #$p < 0.05$ vs. D_2R+LY; n, represents the number of cells that were quantified. (H) Effect of D_2R activation by dopamine (10 μM, 15 min) on the relative content of GFPGAIP, His_6GIPC, and D_2R in the protein complex immunoprecipitated by anti-GFP antibody. The amount of proteins resolved by 10% SDS-PAGE was adjusted and the expression levels shown in cell lysates corresponding to 50 μg protein. Paired *t* test *$p < 0.05$ vs. -GFPGAIP, #$p < 0.05$ vs. +dopamine. (I) Effect of ConA upon D_2R internalization. The extracellular epitope tag of $mycD_2R$ was labeled by adding the antimyc antibody on unpermeated $mycD_2R$-transfected CHO cells pretreated or not with 250 $\mu g \cdot ml^{-1}$ concanavalin A for 30 min and subsequently by 3 μM dopamine for 30 min. Values are expressed as the percentage of total $mycD_2R$ fluorescence in 50 cells using the NIH Image 1.63 software and are mean ± SEM of data from five independent experiments. Paired *t* test: *$p < 0.01$ vs. -conA. (J) Effect of ConA upon D_2R distribution at the PM. $MycD_2R$ was detected at the surface of unpermeated cells upon ConA treatment in the presence or absence (insets) of 3 μM LY. Arrows show clusters containing $mycD_2R$. Double labelings with anticlathrin (K) and anti-EEA1 (L) were performed on GFPGAIP-stably expressing $CHOD_2R$ cells treated with 250 $\mu g \cdot ml^{-1}$ concanavalin A and 3 μM LY for 3–5 min before fixation. Arrows indicate colocalization captured by confocal fluorescent microscopy.

($p < 0.01$ vs. untreated cells). To test the possibility that translocation of GFPGAIP at the PM may have resulted in the formation of complexes containing GAIP, GIPC, and D_2R, we performed immunoprecipitation with anti-GFP antibody (Figure 4H) whether CHO cells, transfected by all three proteins, were stimulated or not by 10 μM dopamine for 15 min. We found that both His_6GIPC- and D_2R-binding sites coimmunoprecipitated with GFPGAIP, more efficiently upon D_2R activation (9.9 ± 2.3%, $p < 0.05$; Figure 4H, lane 1) than when D_2R remained inactive (5.7 ± 1.6%; Figure 4H, lane 2).

Moreover, the translocation rate of GFPGAIP to the PM was accentuated to 82.3 ± 15% ($p < 0.05$ vs. LY-treated cells) when the clathrin-dependent endocytotic pathway that is used by active D_2R (Kim *et al.*, 2001), was inhibited by the addition of 250 $\mu g \cdot ml^{-1}$ concanavalin A (Figure 4F), a treatment that did not affect GFPGAIP subcellular localization in unstimulated cells (Figure 4E). So, we hypothesized that GFPGAIP and active D_2R may have been trapped in clathrin-coated pits at the PM. Indeed, treatment with concanavalin A blocked D_2R internalization by up to 50% ($p < 0.01$; Figure 4I) and exacerbated D_2R localization within microdomains of the PM as suggested by its punctate distribution (Figure 4J, see arrows). In addition, GFPGAIP colocalized with clathrin mostly at the PM (Figure 4K, see arrows) and faintly with EEA1 upon D_2R activation (Figure 4L), despite

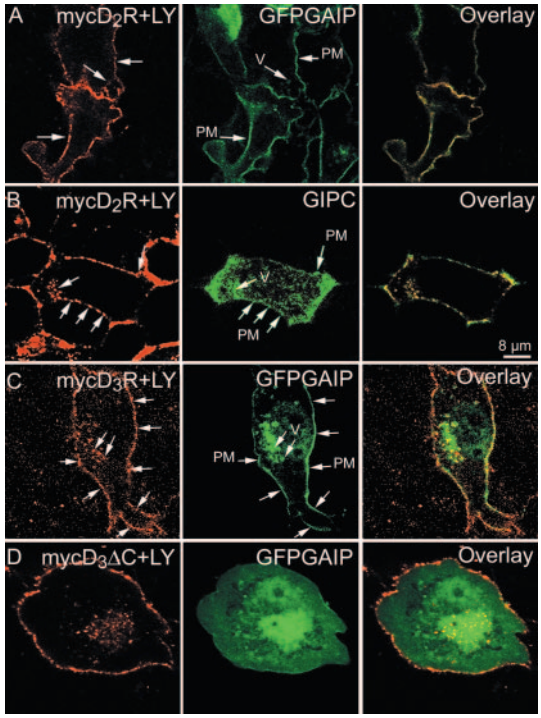

Figure 5. D_2R colocalized with GAIP and GIPC. Double-staining experiments in $CHOD_2R$ cells transfected with either $mycD_2R$ and GFPGAIP (A) or $mycD_2R$ and His_6GIPC (B). Cells were treated with 3 μM quinpirole (LY) for 10 min, and clathrin-dependent internalization was blocked by pretreatment with 0.45 M sucrose for 30 min. Cells were sequentially stained for $mycD_2R$ and GIPC under nonpermeant and permeant conditions, respectively; GAIP was detected by the GFP fluorescence. $MycD_3R$ (C) that interacted with GIPC (Jeanneteau *et al.*, 2004) contrary to $mycD_3\Delta C$ (D) was also tested. The activated $mycD_2R$ and $mycD_3R$ formed clusters that colocalized with GFPGAIP at the PM and with GIPC at the PM and in vesicles (V). Arrowheads indicate examples of colocalized clusters.

the endosomal-based localization displayed by GFPGAIP at steady state (Supplementary Information 2). This suggests that GFPGAIP moved to clathrin-coated pits to meet with GIPC and active D_2R in agreement with previous data reporting the codistribution of GIPC and GAIP within clathrin-coated pits, where GAIP interacted with Gαi3 (Lou *et al.*, 2002; Elenko *et al.*, 2003). Collectively, these results indicate that D_2R activation regulates the spatial distribution of GFPGAIP.

D_2R, GIPC, and GAIP Colocalized at the PM

The above results raised the question of where GAIP, GIPC, and D_2R assemble. Because the formation of complexes containing GAIP, GIPC, and D_2R was thought to be transient, double-labeling experiments were conducted when internalization by the clathrin-coated endocytotic pathway was inhibited by hypertonic sucrose (0.45 M). To detect the active D_2R that are expected to recruit GAIP, the antitag antibody was applied on living $mycD_2R$-transfected cells under nonpermeant conditions before the addition of quinpirole, because this antibody did not induce, by itself, receptor internalization (Jeanneteau *et al.*, 2004). We observed that GFPGAIP strictly colocalized with active D_2R prominently at the PM and in rare endocytotic vesicles (Figure 5A, arrows). In addition, His_6GIPC colocalized with the activated $mycD_2R$ at the PM and also in endocytotic vesicles (Figure 5B, arrows), confirming the close subcellular association of the three proteins. Colocalization was not complete, likely as a result of interactions between His_6GIPC and undetected $mycD_2R$ or other ligands. Similarly, GFPGAIP colocalized with active $mycD_3R$ at the PM and in endocytotic vesicles (Figure 5C), which is consistent with the fact that GIPC cointernalizes with D_2R and D_3R (Jeanneteau *et al.*, 2004). However, $D_3\Delta C$, which does not interact with GIPC (Jeanneteau *et al.*, 2004), was unable to undertake such a process (Figure 5D). Therefore, complexes containing GAIP, GIPC, and D_2R may form predominantly at the PM.

GAIP Controlled D_2R Signaling in a GIPC-dependent Manner

To characterize the functional role of GAIP upon D_2R signaling, $CHOD_2R$ cells that express endogenous GIPC were used to stably express GFPGAIP. In these cells upon D_2R activation, 49.6 $\pm$ 18% of GFPGAIP translocated to the PM (Figure 6, A and B), where G proteins are presumably activated. GFPGAIP attenuated quinpirole-induced [^{3}H]AA release, a typical D_2R-mediated response (Piomelli *et al.*, 1991), by shifting the EC_{50} of quinpirole by about one order of magnitude (EC_{50} = 144 $\pm$ 30 vs. 18 $\pm$ 6 nM, $p < 0.05$), without affecting the maximal response (Figure 6D). To confirm that such an effect resulted from the GTPase activity of GAIP, we used a mutant (GAIPS151A) that has less RGS activity, because it lacks its Erk1/2-dependent phosphorylation site responsible for the stimulation of its GTPase activity (Ogier-Denis *et al.*, 2000). When stably expressed in $CHOD_2R$ cells, 31.4 $\pm$ 8% of GFPGAIPS151A translocated to the PM of cells stimulated by quinpirole, an effect that was blocked by haloperidol (Figure 6, A and B; $p < 0.05$ vs. untreated cells), implying that the mutation did not alter the formation of the protein complex. GFPGAIPS151A produced an intermediate rightward shift of the quinpirole dose-response (EC_{50} = 48 $\pm$ 7 nM), without affecting the maximal response (Figure 6D), confirming that regulation was mediated by the GTPase activity of GAIP. In contrast, the GAIPΔA216 mutant that did not interact with GIPC (Figure 2B), still displayed the same vesicular-based distribution as GFPGAIP and GFPGAIPS151A in unstimulated cells, as verified using specific cellular biomarkers (compare Supplementary Information 2, 3, and 4), but neither translocated to the PM upon D_2R activation (Figure 6, A and B; $p < 0.05$ vs. GFPGAIP) nor attenuated the D_2R-mediated response (EC_{50} = 11 $\pm$ 5 nM; Figure 6D). Indeed, GFPGAIPΔA216 did not colocalize with $mycD_2R$ and His_6GIPC in these cells (our unpublished results). Expression levels of native GIPC and heterologous D_2R, GFPGAIP, or its mutants in these various cell lines were similar, as assessed by radioligand binding for the D_2R and immunoblotting using anti-GIPC and anti-GFP antibodies (Figure 6C).

Furthermore, GFPGAIP also attenuated the quinpirole-induced inhibition of cAMP accumulation triggered by forskolin (0.5 μM), with an EC_{50} increasing from 3.1 $\pm$ 0.67 to 10 $\pm$ 1.46 nM ($p < 0.05$ $CHOD_2R$ vs. $CHOD_2R$/GFPGAIP), without change in the maximal response (Figure 6E). GFPGAIPS151A moderately regulated the potency of quinpirole with an EC_{50} of 4.5 $\pm$ 0.7 nM, whereas GFPGAIPΔA216 was inefficacious (EC_{50} = 2.8 $\pm$ 0.41 nM).

Using the same cells, complete inhibition curves of D_2R binding (Figure 6F) show that dopamine competed with surface D_2R binding in a biphasic manner, a feature common to GPCRs (Castro and Strange, 1993); the two sites corresponded to the high-affinity state, coupled to the Gα protein and to the low-affinity, uncoupled state, respectively. The high-affinity state was affected by the overexpres-

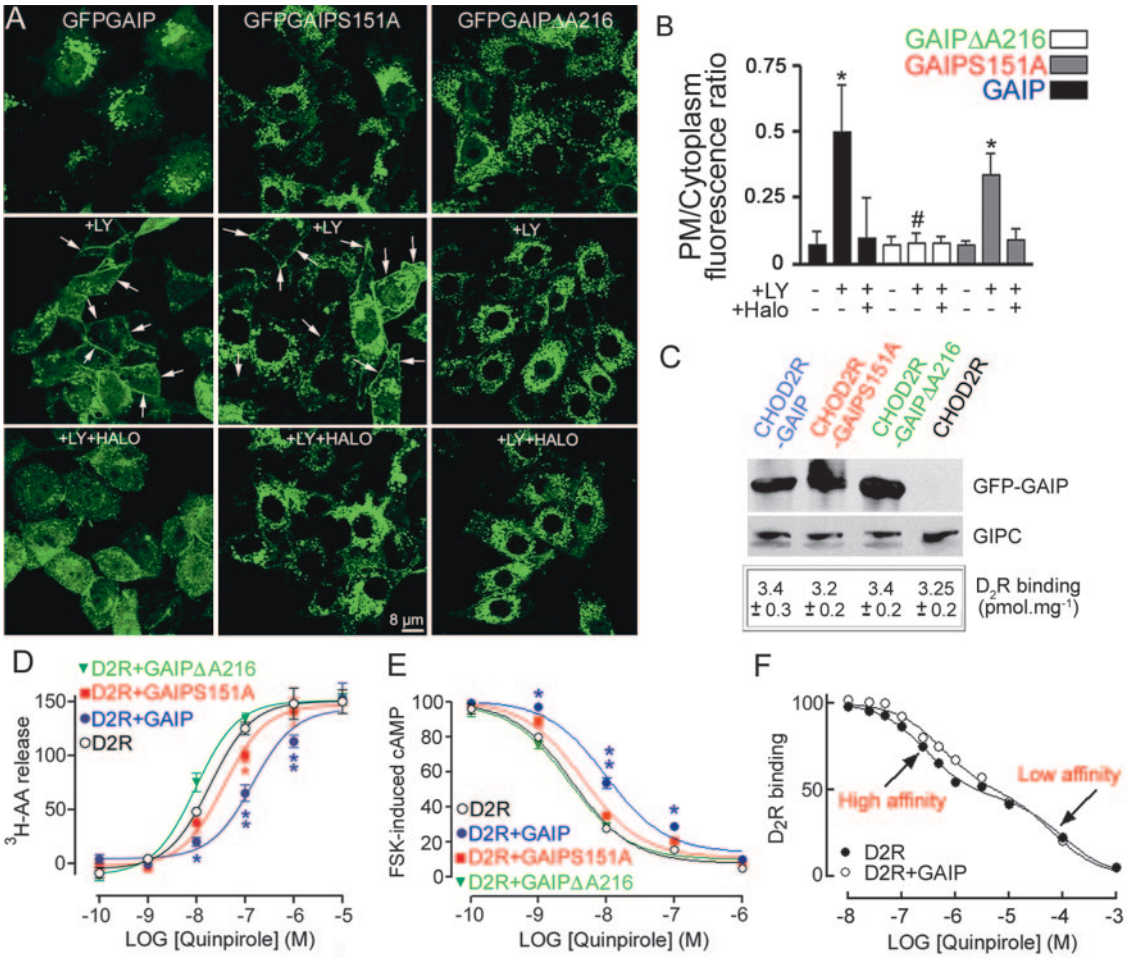

Figure 6. GAIP attenuated D_2R signaling in a GIPC-dependent manner. (A) Effect of D_2R activation on the localization of GAIP and its mutants. GFPGAIP or its mutants were stably expressed in $CHOD_2R$ cells that were either untreated (top) or treated for 10 min with 3 μM quinpirole (LY, middle), or with 3 μM LY plus 50 μM haloperidol (Halo, bottom). Arrows indicate translocation at the PM and quantification of PM/cytoplasm specific fluorescence ratio is shown in). Means ± SEM of 45 representative cells from 3–5 independent experiments. Paired *t* test: $^{\#}p < 0.01$ vs. GAIP+LY; $^{*}p < 0.01$ vs. untreated control cells. (C) Expression levels of GFPGAIP and mutants, GIPC and D_2R in the cell lines developed were determined respectively by immunoblot using anti-GFP and anti-GIPC antibodies and [^{3}H]spiperone specific binding. (D) [^{3}H]arachidonic acid (AA) release was stimulated by LY in increasing concentrations in $CHOD_2R$ cell lines stably expressing GFPGAIP or its mutants and triggered by addition of 4 μM calcium ionophore. Results are expressed as percentage of ionophore induction and are means ± SEM of data from 4–6 independent experiments. (E) Inhibition of cAMP accumulation by LY in increasing concentrations in $CHOD_2R$ cell lines stably expressing GFPGAIP or its mutants in the presence of 0.5 μM forskolin (FSK). Results are expressed as percentage of forskolin-stimulated cAMP accumulation and are means ± SEM of data from three independent experiments. Paired *t* test: $^{*}p < 0.05$ and $^{**}p < 0.03$ vs. $CHOD_2R$ cells. (F) Dopamine competition for [^{125}I]iodosulpride (0.2 nM) binding to living $CHOD_2R$ cells in the absence and the presence of overexpressed GAIP. Data fit better for a two-site model in which the high- and low-affinity sites represent the receptor state, respectively, coupled and uncoupled to the G proteins. Values are expressed as the percentage of total receptor binding and are mean ± SEM of four determinations from two independent experiments.

sion of GFPGAIP, which reduced dopamine affinity by twice (EC_{50} = 281 ± 61 vs. 550 ± 85 nM), but the low-affinity state did not change. These results suggest that GAIP reduced receptor coupling to the G proteins. Collectively, we showed that D_2R dynamically recruited GFPGAIP through GIPC to attenuate receptor signaling at the level of the G protein coupling.

Knockdown of GIPC Reduced D_2R-mediated Translocation of GAIP to the PM

To further demonstrate the participation of GIPC in D_2R-mediated translocation of GAIP, we studied the effects of GIPC knockdown by antisense ODNs in $CHOD_2R$ cells expressing GFPGAIP or GFPGAIPΔA216. The efficacy and selectivity of the ODN treatments were assessed through the expression levels of GIPC, GFP, and actin. The active ODN-AS reduced GIPC immunoreactivity by more than 50%, compared with inactive ODN-S or vehicle (Figure 7A). GIPC knockdown did not alter actin expression but tended to decrease GFPGAIP and GFPGAIPΔA216 immunoreactivities by an unknown mechanism.

In GIPC knockdown cells, the vesicular-based distribution of GFPGAIP or GFPGAIPΔA216 was unchanged compared with that observed in cells treated with inactive ODN-S or vehicle. However, the D_2R-mediated translocation of GFPGAIP to the PM was reduced in GIPC knockdown cells compared with control cells treated with inactive ODN-S or

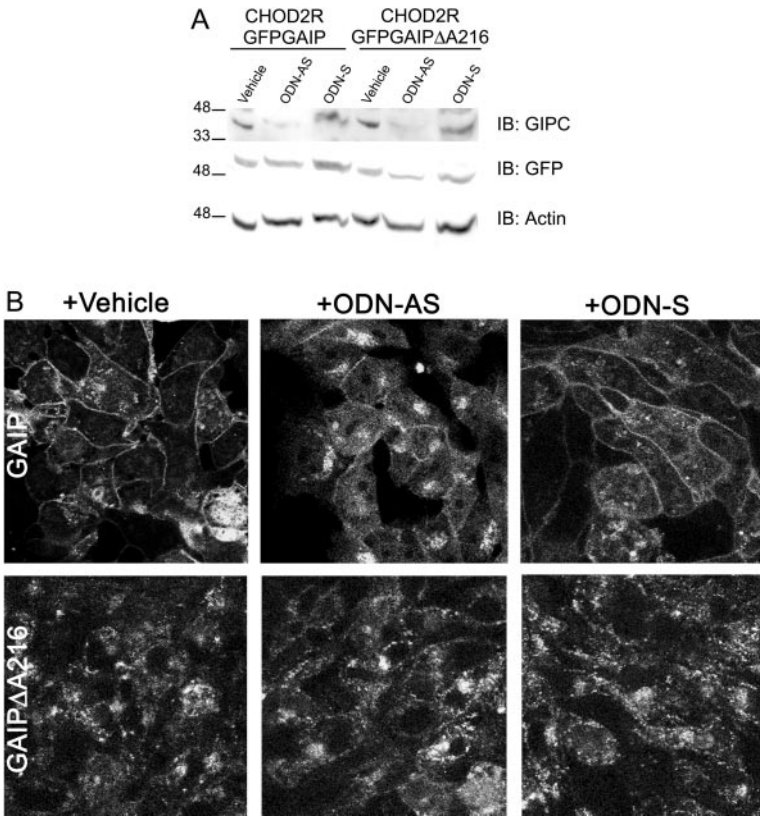

Figure 7. Knockdown of GIPC expression reduced translocation of GFPGAIP to the PM. (A) The efficacy and selectivity of the active ODN-AS treatments were verified by Western blot with antibodies directed to GIPC and actin. The expression levels of GFPGAIP and GFPGAIPΔA216 in response to ODNs treatments were also tested with anti-GFP antibody. Details of the 3-d antisense procedure are outlined in *Materials and Methods.* (B) At the end of the ODNs treatments, cells were treated with 250 $\mu g \cdot ml^{-1}$ concanavalin A and 10 μM dopamine for 15 min before monitoring the translocation of GFPGAIP or GFPGAIPΔA216 at the PM by confocal microscopy. Vehicle corresponds to the transfection reagent deprived of ODN.

vehicle (Figure 7B). In addition, GFPGAIPΔA216, which does not bind to GIPC, was unable to translocate to the PM upon D_2R activation in either GIPC knockdown or control cells treated with inactive ODN-S or vehicle. Therefore, recruitment of GFPGAIP by active D_2R depended on endogenous GIPC.

DISCUSSION

It is known that RGS acts as GTPase for many Gα subunits, but cellular mechanisms underlying RGS and Gα pairing remained unclear. The aim of this study was to examine the contribution of GIPC, a scaffold protein, in the assembly of a specific RGS-GPCR complex in living cells and the functional role of this assembly on GPCR-mediated G protein signaling. Specifically, we have studied the complex formed with GAIP (RGS19) and D_2R, which both selectively interact with GIPC (De Vries *et al.*, 1998b; Jeanneteau *et al.*, 2004). The PDZ-domain of GIPC that mediates interactions with GAIP and GPCR has the particularity to bind to either class of PDZ-binding motifs, but to discriminate closely related PDZ-binding consensus. This feature seems to confer to GIPC a high degree of selectivity toward its binding partners. Nevertheless, it cannot be excluded that other interactions with GIPC could involve an atypical internal PDZ-binding motif distinct from the classical C-terminal PDZ-recognition consensus (Lou *et al.*, 2002).

Even though we showed that D_2R activation initiated the translocation of GAIP to the PM, it was unclear to what degree G protein or receptor activation state influences the recruitment of GAIP. The necessity of GIPC in this process is supported by the absence of GAIP translocation in cells expressing $D_3\Delta C$ or D_4R, two proteins that do not bind to GIPC; it is confirmed by the attenuation of this process upon GIPC knockdown by antisense oligolucleotides. Furthermore, the formation of a coprecipitable protein complex containing D_2R, GIPC, and GAIP critically depended on a direct interaction between GIPC and GAIP because GAIPΔA216, a mutant deprived of its GIPC-binding motif, did not. Thus, clustering of GAIP to the PM was not dictated by G protein activation itself, but by active receptors paired with GIPC. The formation of a GPCR-GAIP complex through GIPC could favor targeting to the correct Gα substrate generated by receptor activation. For instance, GAIP interacts with its cognate $G_i\alpha 3$ subunit upon δ-opioid receptor (DOR) activation (Elenko *et al.*, 2003). Whether GIPC links GAIP to DOR as it clustered GAIP with D_2R, despite the absence of apparent PDZ-binding motif in DOR C-terminus, is currently unknown. If the functions of GAIP are dictated by the receptor and not the Gα subunit alone, then this would explain why GAIP is so promiscuous with regard to its interactions with Gα subunits in vitro (De Vries and Farquhar, 1999). In a cellular environment, GAIP may have not been free to pair up with any available Gα activated by D_2R in GIPC knockdown cells or when clustering of D_2R-, GIPC-, and GAIP-containing complexes was prevented by the use of mutant proteins. Therefore, D_2R could selectively sort GAIP at the PM to orient toward the linked Gα proteins and optimize its GTPase activity. This is corroborated by the high specificity of the PDZ domain of GIPC, which binds to GAIP, but not to other RGS.

The cooccurrence of D_2R, GIPC, and GAIP in neurons and neuroendocrine cells further supports the physiological relevance of this concept. GIPC could take part in an endogenous mechanism to regulate the availability of GAIP in signaling microdomains of the cell. Because the GTPase activity of GAIP is constitutive at least in purified solution-based assay (Berman *et al.*, 1996; Hepler *et al.*, 1997), cellular mechanisms like compartmentalization of signaling compo-

nents would help the regulation of its functions. Nevertheless, the recruitment to the PM of RGS2 and RGS4, two other simple RGS like GAIP, occurred when coexpressed with GPCRs or G proteins and was independent of their activation state (Roy *et al.,* 2003). Because RGS2 and RGS4 do not bind to GIPC, the dynamic and spatial regulation of these RGS may rely on different cellular mechanisms. Indeed, other "simple" RGS bind to common components of G protein signaling like Gβ5 subunit (Dowal *et al.,* 2001), which could ensure their recruitment or stabilization to membranes. Palmitoylation and amphipathic helices were also demonstrated to dictate membrane attachment of GAIP (De Vries *et al.,* 1996) as well as other RGS devoid of physical link with GIPC (De Vries *et al.,* 1998b) like RGS4 (Tu *et al.,* 2001) and RGS16 (Druey *et al.,* 1999). To what extent GIPC compared with palmitoylation causes recruitment of GAIP to the PM upon D_2R activation requires further investigations. Palmitoylation has been involved in the regulation of protein interactions (Mumby, 1997), dynamic membrane anchoring of signaling proteins, targeting within specialized microdomains of the PM as well as endocytosis (Qanbar and Bouvier, 2003). Whether GIPC, which has a putative acyl carrier protein domain (ACP) likely acting as an acylation cofactor, regulates palmitoylation has to be further examined.

We propose that GIPC acts as a scaffold protein, organizing and assembling protein complexes resulting in a spatial clustering of GAIP with D_2R and associated signaling components. The orchestration of these events and their localization at the cellular level characterized by the use of physical interaction defective mutant proteins and GIPC gene expression knockdown are summarized as follows: When activated, D_2R couples to the heterotrimeric G protein to catalyze GTP/GDP exchange on its α subunit, which carries the signal toward the tertiary effector protein to produce receptor-mediated responses. Receptor activation would also timely recruits signaling regulatory elements, like GAIP, by virtue of its interaction with GIPC. In turn, the GTPase activity of GAIP would promote $G_i\alpha$-GTP hydrolysis that terminates the G protein signal and recycles the resulting $G_i\alpha$-GDP for another round of G protein activation (Berman *et al.,* 1996), leading to the reduction of the subsequent receptor-mediated signaling cascade. This was evidenced at the level of G protein coupling by the observed decreased dopamine affinity at the D_2R high-affinity state upon GAIP overexpression and also at the level of downstream effectors by the observed down-regulation of two different D_2R-mediated responses.

It cannot be excluded at this point that recruitment of GAIP is a result of GIPC dimerization, although the affinity of GIPC for itself is weak as indicated by yeast two-hybrid. These findings raise questions as to the possibility that additional yet unidentified accessory proteins, like G proteins, may strengthen the coclustering of D_2R, GIPC, and GAIP or that preexisting steady state GIPC-GAIP and GIPC-D_2R complexes pair up after activation through conformational change of the receptor, the G protein, or GIPC. To date, interaction between GIPC and GPCR was shown to be independent of receptor activation state (Hu *et al.,* 2003; Jeanneteau *et al.,* 2004). Receptor activation in a coordinate effort with its cognate linked G proteins could catalyze their combination with GAIP through GIPC via posttranslation modifications. To support this hypothesis, both D_2R and GAIP have been shown to be phosphorylated, glycosylated, and palmitoylated (De Vries *et al.,* 1996; Missale *et al.,* 1998; Fischer *et al.,* 2000; Garzon *et al.,* 2004), but the dynamics of these processes remain unclear.

A previous study (Rahman *et al.,* 2003) demonstrated that RGS9–2, the C-terminus of which does not interact with GIPC (Table 1), also attenuates D_2R signaling in the basal ganglia, an effect that was reversed in RGS9 knockout mice. The reason why D_2R signaling would use two distinct RGS is unclear particularly because the GTPase activity of GAIP and RGS9–2 both target $G_i\alpha/G_o\alpha$ (Rahman *et al.,* 1999). Because GPCRs are believed to couple simultaneously or successively to multiple G proteins to trigger various intracellular signals (Selbie and Hill, 1998; Hermans, 2003), it is conceivable that both RGS9–2 and GAIP regulate distinct D_2R-signaling pathways. Indeed, RGS9–2 accelerates the off-kinetics of D_2R-induced GIRK currents (Rahman *et al.,* 2003), whereas GAIP was here found to participate in the regulation of adenylate cyclase and phospholipase A2 signaling cascades as well as in vesicular trafficking in accordance with previous studies (Lou *et al.,* 2002; Wylie *et al.,* 2003). In agreement with this latter observation, GAIP and GIPC were closely associated with clathrin as assessed by electron microscopy (De Vries *et al.,* 1998a; Fischer *et al.,* 1999). Additionally, we showed that D_2R, GIPC and GAIP colocalized within microdomains of the PM, probably clathrin-coated pits, where GFPGAIP codistributed upon D_2R activation. Vesicle budding requires GTPase activity (Wylie *et al.,* 2003) and such a process could recruit GAIP and GIPC in clathrin-coated pits to initiate receptor endocytosis. In support of this hypothesis, GIPC cointernalized with D_2R (Jeanneteau *et al.,* 2004) and myosin VI (Aschenbrenner *et al.,* 2003), a cytoskeleton motor, physically linked to GIPC to facilitate the translocation of GIPC-bearing endocytotic vesicles from cell peripheries.

Another related function of GIPC has been suggested in MAP-kinase signaling cascade regulation as its overexpression was found to reduce receptor-induced Erk1/2 activation (Lou *et al.,* 2002; Hu *et al.,* 2003). In turn, activated Erk1/2 increases the GTPase activity of GAIP through phosphorylation on its Ser151 residue (Ogier-Denis *et al.,* 2000), the mutation that reduced the effect of GAIP on D_2R signaling. Hence, GIPC may participate in a feedback regulation loop to limit the activity of GAIP on G protein signal. Clathrin-coated pits, where receptors, GIPC, GAIP, and G proteins could all meet, are important sites for the assembly of endocytosis and MAP-kinase signaling machineries (Luttrell *et al.,* 1999). Indeed, β-arrestin2, which serves as a scaffold protein for GPCR endocytosis (Goodman *et al.,* 1996) and GPCR-induced Erk activation (Tohgo *et al.,* 2002), colocalized with GIPC in D_2R-expressing cells stimulated by quinpirole (Supplementary Information; Figure 5). Thus, the GIPC and GAIP could take part in the GPCR-associated scaffold that connects both tightly related machineries.

GIPC is the first protein identified so far to functionally link a GPCR to an RGS. GIPC interacts with a large array of other transmembrane proteins, such as tyrosine kinase receptors (Lou *et al.,* 2001) and a transporter (Bunn *et al.,* 1999), and several GIPC family members have been described (Katoh, 2002). Therefore, the mechanism described here may be more general and serve to sort signaling proteins among a large repertoire within the interconnected signaling network.

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