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Interactions of GIPC with Dopamine D_2, D_3 but not D_4 Receptors Define a Novel Mode of Regulation of G Protein–coupled Receptors

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The C-terminus domain of G protein–coupled receptors confers a functional cytoplasmic interface involved in protein association. By screening a rat brain cDNA library using the yeast two-hybrid system with the C-terminus domain of the dopamine D_3 receptor (D_3R) as bait, we characterized a new interaction with the PDZ domain-containing protein, GIPC (GAIP interacting protein, C terminus). This interaction was specific for the dopamine D_2 receptor (D_2R) and D_3R, but not for the dopamine D_4 receptor (D_4R) subtype. Pull-down and affinity chromatography assays confirmed this interaction with recombinant and endogenous proteins. Both GIPC mRNA and protein are widely expressed in rat brain and together with the D_3R in neurons of the islands of Calleja at plasma membranes and in vesicles. GIPC reduced D_3R signaling, cointernalized with D_2R and D_3R, and sequestered receptors in sorting vesicles to prevent their lysosomal degradation. Through its dimerization, GIPC acts as a selective scaffold protein to assist receptor functions. Our results suggest a novel function for GIPC in the maintenance, trafficking, and signaling of GPCRs.

INTRODUCTION

A common regulatory mechanism of G protein–coupled receptors (GPCR) activity is their sequestration from the cell surface. Thus, whereas prolonged exposure to agonist leads receptors to internalization and degradation within lysosomes (Bohm *et al.*, 1997), brief stimulation involves internalization and trafficking to sorting compartments to recycle the receptors to the cell surface (Ferguson, 2001; Claing *et al.*, 2002). Desensitization initiates a cascade of events leading to signaling regulation starting from the uncoupling of the G protein in response to receptor phosphorylation by either second messenger–dependent protein kinases, like protein kinase A (Mason *et al.*, 2002) or G protein–coupled receptor kinases (GRK). In turn, receptor phosphorylation promotes arrestin binding, which targets many GPCR in intracellular clathrin-coated vesicles (Zhang *et al.*, 1997a, 1997b). Emerging alternative mechanisms have been proposed, involving β-arrestin–independent, clathrin-independent and dynamin-dependent internalization routes (Kohno *et al.*, 2002), suggesting that the complexity and specificity of GPCR-regulatory processes may reside in the combination of accessory protein functions to be unraveled yet.

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Abbreviations used: aa, amino acids; 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; GRK, G protein–coupled receptor kinase; GST, glutathione *S*-transferase; HEK293, embrionic kidney cells; PDZ, consensus sequence in PSD95/DLG/zo-1; RGS, regulator of G protein signaling.

Dopamine receptors are GPCRs mediating the pleiotropic actions of dopamine in the brain and peripheral tissues. Among them, D_2-like receptors (D_2R, D_3R, and D_4R), which are the major target for antipsychotic and anti-Parkinson's disease drugs (Missale *et al.*, 1998), differ in their brain distribution and pharmacological profiles. For instance, D_3R possesses a higher affinity for endogenous dopamine than D_2R and D_4R (Sokoloff and Schwartz., 1995). These receptors, however, share common signaling cascades, including inhibition of cAMP formation, regulation of ion channel activities, and stimulation of mitogenesis through the activation of mitogen-activated protein kinases (Sokoloff and Schwartz, 1995; Missale *et al.*, 1998). Despite the discrete distribution of the D_3R as compared with the D_2R, both receptors were reported to be coexpressed in neurons of striatum (Gurevich and Joyce, 1999). Whether members of the D_2-like receptor family fulfill distinct functional roles has not yet been satisfactorily addressed. Growing evidence suggests the involvement of various sets of signaling regulatory proteins like GRK and arrestins in D_1R (Jiang and Sibley, 1999) and D_2R desensitization (Itokawa *et al.*, 1996; Ito *et al.*, 1999; Iwata *et al.*, 1999), but a modest contribution of these proteins for the D_3R (Kim *et al.*, 2001; Kabbani *et al.*, 2002).

Identification of subtype-specific dopamine receptor-interacting proteins may provide important clues for the determination of the functional differences between dopamine receptors. To better understand the regulation of D_2-like receptor signaling, trafficking, and specificity, we sought to identify D_3R-interacting proteins by screening a rat brain cDNA library using the yeast two-hybrid system with the C-terminal tail of the D_3R as bait, bringing to light a novel interaction of D_3R with the PDZ (PSD95/Dig/ZO-1) domain-containing protein, GIPC (GAIP interacting protein, C terminus). This protein has previously been identified as an interacting protein for several transmembrane and membrane-associated proteins, including GAIP, a regulator of G protein signaling (De Vries *et al.*, 1998), β1-adrenergic recep-

tor (Hu *et al.*, 2003), semaphorine M-SemF (Cai and Reed, 1999), glucose transporter GLUT1 (Bunn *et al.*, 1999), tyrosine kinase receptors like the neurotrophin receptors Trk A and Trk B (Lou *et al.*, 2001), insulin-like growth factor 1 (IGF-1) receptor (Booth *et al.*, 2002), and transforming growth factor β (TGFβ) receptor type III (Blobe *et al.*, 2001). These studies suggest a possible role of GIPC in the regulation of vesicular trafficking (De Vries *et al.*, 1998; Lou *et al.*, 2002), receptor surface expression (Bunn *et al.*, 1999; Wang *et al.*, 1999; Blobe *et al.*, 2001), or G protein signaling (De Vries *et al.*, 1998; Lou *et al.*, 2001; Booth *et al.*, 2002; Hu *et al.*, 2003).

In this study, we report for the first time, a subtype-specific interaction between GIPC and the D_2R, D_3R but not D_4R. Evidence is provided that GIPC, when recruited by the D_2R or D_3R at the plasma membrane, reduced receptor signaling and increased receptor stability through their sequestration in sorting vesicles, away from degradation compartments. This mechanism may take part in the regulation of D_2R and D_3R activity, but not D_4R.

MATERIALS AND METHODS

Plasmids Constructs

C-terminus domains of the rat D_2R (aa 433–444), D_3R (aa 435–446), D_4R (aa 372–385), D_3R C-terminus mutants ($D_3\Delta C$; $D_3A_{442}K$; $D_3\Delta LKI$ and $D_3\Delta EFR$), human GAIP (aa 207–217), and human TrkB juxtamembrane domain (aa 458–544) were inserted in-frame downstream of the B42 activation domain into pEG202 vector (OriGene Technologies, Rockville, MD). GIPC mutants, i.e., the PDZ domain (aa 125–265), the ACP domain (aa 223–316), both domains (aa 125–316) or the N-terminus (aa 1–125) were constructed by PCR and subcloned into pJG4.5 downstream of the LexA domain (OriGene Technologies). The D_3R C-terminus coding sequence was subcloned into pGEX-2TK (Amersham Pharmacia Biotechnology, Piscataway, NJ). GIPC was tagged (Xpress/His-GIPC) at its N-terminus with the two epitopes (Xpress and poly-histidine) from pcDNA3.1HisC (Invitrogen Corp., San Diego, CA). Rat D_3R and mutant $D_3\Delta C$ were subcloned either into pcDNA3.1Hygro or pRc/CMV (Invitrogen Corp.). Other D_3R mutants were PCR-amplified and subcloned in pcDNA3.1Hygro. The human D_3R, D_2R short isoform and D_4R were fluorescently tagged at their N-terminus with the Enhanced Green Fluorescent protein fused to the nicotinic receptor α7 subunit signal peptide (Weill *et al.*, 1999) in the pCEP4 vector (Invitrogen Corp.) and pcDNA3.1hygro, respectively. $GFPD_3\Delta C$ was engineered by PCR using a specific mutant primer lacking the C-terminus cysteine residue. Human D_2R short isoform and rat D_3R were tagged at their N-terminus by PCR with the c-myc epitope (EQKLISEEDL). All constructions were verified by automated nucleotide sequencing (Licor, Lincoln, NE).

Yeast Two-hybrid Screening

Yeast two-hybrid screening was 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 upstream LexA binding sites. Transformants were grown upon selective medium and assayed for β-galactosidase to verify and quantify interactions between bait and prey by solid-support assay and liquid culture assay, respectively, with X-gal and *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrates, according to the Yeast Protocols Handbook from Clontech (Palo Alto, CA). Relative binding to GIPC is measured as follows: (βgal units $(\beta U)^{test} - \beta U^{crl}$)/($\beta U^{GAIP} - \beta U^{crl}$). One unit of βgal is defined as the amount that hydrolyzes 1 μmol of ONPG per min per cell. Sequences isolated from clones were compared with nucleotide sequence databases using BLAST searches.

Cell Culture and Transfections

Chinese hamster ovary (CHO), COS7, and HEK293 cells were cultured in DMEM (Life Technologies, Rockville, MD) or alpha-modified Eagle's medium (αMEM, Life Technologies) for the latter, supplemented with 10% fetal calf serum and 100 μg/ml penicillin/streptomycin in a humidified atmosphere of 5% CO_2, 95% air. Cells were seeded in 10-cm dishes at 50–80% confluency and transiently transfected using calcium phosphate coprecipitation for HEK293 cells (Wigler *et al.*, 1977) or Superfect (Qiagen, Santa Clarita, CA) for CHO and COS7 cells. HEK/GIPC, HEK/$GFPD_2R$, and HEK/$GFPD_3R$ cell lines were obtained by transfection with 10 μg pcDNA3.1HisC-GIPC, pcDNA3.1hygro$GFPD_2R$, and pCEP4-$GFPD_3R$, respectively. HEK/$GFPD_2R$/GIPC and HEK/$GFPD_3R$/GIPC cell lines were obtained by transfection of the HEK/$GFPD_2R$ or HEK/$GFPD_3R$ cell lines with 10 μg pcDNA3.1HisC-GIPC. Clones were selected by resistance to hygromycin or neomycin and screened for receptor expression by measuring [^{125}I]iodosulpride binding or for GIPC expression by Western blotting with the anti-Xpress antibody (Invitrogen Corp.). D_3R mRNA expression was examined by Northern blot as described (Sokoloff *et al.*, 1990). GIPC expression was assessed by Northern blot and RT-PCR using a GIPC N-terminus (aa 1–366) PCR probe and specific primers, respectively.

GST Pull-down Assay

GST and GST-D_3R-Ct fusion proteins were produced in *Escherichia coli* BL21 after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h. Sonicated cells were incubated in 500 μl of 50 mM potassium phosphate buffer containing 150 mM NaCl (phosphate-buffered saline [PBS]) with 1% *n*-octyl β-D-glucoside for 1 h and centrifuged at 45,000 $\times$ *g* for 30 min. Supernatants were incubated with 100 μl of glutathione-Sepharose beads (50% slurry, Amersham Pharmacia Biotechnology) for 30 min and washed three times with 10 ml of ice-cold PBS. The immobilized fusion proteins were then incubated with 500 μl of cytosolic extracts (300 $\mu g \cdot ml^{-1}$) from Xpress/His-GIPC–transfected COS7 cells at 4°C for 1 h and washed three times with 10 ml of ice-cold PBS. Pellets were resuspended in SDS sample buffer and analyzed by Western blot using the anti-Xpress antibody (dil. 1:5000).

In Vitro Interaction

D_3R-expressing COS7 cells (Bmax = 1 pmol·mg protein^{-1}) were harvested in 500 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO), sonicated, and centrifuged at 50,000 $\times$ *g* for 30 min at 4°C. Membranes from three confluent 10-cm culture plates were solubilized by incubation for 45 min at 4°C in 500 μl of 50 mM Na/Na_2PO_4, pH 7.4, 300 mM NaCl, 40 mg/ml digitonin, 10 mg/ml deoxycholate, 10 mM dithiothreitol plus protein inhibitor cocktail, followed by centrifugation at 150,000 $\times$ *g* for 40 min at 4°C. Ni^{2+}-chelating Sepharose column (HisTrap, Amersham Pharmacia Biotechnology) was loaded with 1 ml of cytosolic extracts from COS7 cells overexpressing Xpress/His-GIPC (300 $\mu g \cdot ml^{-1}$), rinsed with 10 ml of 10 mM imidazole buffer (20 mM phosphate, 0.5 M NaCl, pH 7.4–7.6), and subsequently loaded with 1 ml of solubilized D_3R-expressing membranes from transfected COS7 cells (300 $\mu g \cdot ml^{-1}$). The flow-through was collected before extensive washes in 10 mM imidazole buffer. Bound proteins were eluted with 500 mM imidazole buffer and collected as 1-ml aliquots. Flow-through and elution fractions were analyzed by 10% SDS-PAGE and immunoblots revealed with anti-Xpress (dil 1:5000) and anti-D_3R antibody (dil 1:1000) as described (Diaz *et al.*, 2000). After stripping, membranes were probed with the D_3R antibody presaturated with 10 μg/ml its immunizing peptide (G15Y; Diaz *et al.*, 2000). In an independent experiment, the column was saturated with 900 μg of D_3R-Ct peptide (EFRKAFLKILSC) before loading solubilized D_3R membrane samples.

Immunoprecipitation of Endogenous Proteins

Membrane and soluble fractions from rat striatum were separated by centrifugation after sonication. Membranes were extensively washed and solubilized in the digitonin-cholate mixture previously described and receptors labeled with [^{125}I]iodosulpride (0.4 nM; Amersham Pharmacia Biotechnology). Endogenous GIPC was immunoprecipitated with the anti-GIPC N19 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein-A Sepharose (Amersham Pharmacia Biotechnology). GIPC-antibody complexes were extensively washed and incubated with iodo-labeled receptors for 2 h at 4°C. After centrifugation, the nonprecipitated receptors in the supernatant were assayed by filtration (Diaz *et al.*, 2000).

In Situ Hybridization

The N-terminus region of GIPC (nucleotides 1–366) was subcloned into pGEM-4Z (Promega, Madison, WI). The D_3R riboprobe corresponding to the sequence of the third intracellular loop of the receptor is described elsewhere (Sokoloff *et al.*, 1990). α-^{33}P labeling and in situ hybridization were performed as previously described (Diaz *et al.*, 1995).

Binding Assays

Binding experiments on cell membrane fraction were performed using [^{125}I]iodosulpride (0.1 nM) for D_2R and D_3R and [^{3}H]spiperone (0.4 nM) for D_4R, as previously described (Sokoloff *et al.*, 1992; Sautel *et al.*, 1995). Nonspecific binding was determined in the presence of 1 μM eticlopride. Binding data were analyzed by the nonlinear regression curve-fitting program PRISM (GraphPad, San Diego, CA). Protein concentration was estimated with the Coomassie protein assay reagent using bovine serum albumin (BSA) as a standard.

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]cAMP radioimmunoassay kit (DuPont/NEN, Boston, MA).

Immunofluorescence

Cells were grown on collagen-coated cover slips and fixed in 2% paraformaldehyde/PBS (pH 7.4) for 20 min at room temperature, washed twice in PBS/glycine buffer (0.1 M, pH 7.4) and permeated for 20 min with 0.05%

Table 1. Ligand binding properties of D_3R mutants

	D_3R	$D_3\Delta C$	$D_3\Delta LKI$	$D_3A_{442}K$
[^{125}I]iodosulpride binding (% of D_3R)	100 ± 2.3	29 ± 2.3	6 ± 2	34 ± 3.9
Dopamine Ki (nM)	18 ± 3	6.3 ± 2	388 ± 80	4.5 ± 2
Nafadotride Ki (nM)	1.4 ± 0.5	3.3 ± 0.7	nd	nd

Plasmids coding for D_3R and its mutants were transiently transfected in COS7 cells, and ligand binding was measured with 0.1 nM [^{125}I]iodosulpride. D_3R and its mutants were also transiently expressed in HEK293 cells, and varying concentrations (10^{-10}–10^{-6} M) of dopamine or nafadotride, a D_2-like antagonist, were used to inhibit [^{125}I]iodosulpride binding. Dissociation constants were obtained by the nonlinear regression curve-fitting program PRISM (GraphPad). (means ± SEM of data from three independent experiments). nd, not determined.

saponin while blocking in 10% fetal bovine serum/PBS. For the internalization assay, anti-myc antibody (Santa Cruz Biotechnology) was applied on living mycD_2R- and mycD_3R-transfected cells for 1 h before fixation, and detection of GIPC was performed after cell permeation. GIPC was labeled with the anti-Xpress antibody (dil 1:3000) in incubation buffer (0.1% BSA/PBS supplemented with 10% fetal bovine serum). Subsequent detection was performed using either an Alexa488-conjugated secondary antibody (Interchim, Lyon, France, dil 1:100) for mycD_2R, mycD_3R, or a biotin-coupled secondary antibody (Biogenex, San Ramon, CA, dil 1:70) and CY3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, dil 1:2000) for GIPC. To localize subcellular sequestration compartments, we used the following specific markers: an anti-CD71 antibody (Harlan Seralab, Loughborough, Leicestershire, United Kingdom; dil 1:10), an anti-P58K (Sigma, dil 1:50), an anticlathrin kindly provided by A. Schmidt (dil 1:50), an anti-EEA1 (Molecular Probes, Eugene, OR, dil 1:80), an anti-CD63 (Caltag Laboratories, Burlingame, CA, dil 1:200), which were revealed with a CY3-conjugated anti-mouse antibody (Molecular Probes, 1:200).

Animal and tissue preparations for immunohistochemistry were performed as previously described (Diaz *et al.*, 2000). Rat brain frontal sections (10 μm) were rinsed in 0.1 M glycine/PBS after fixation with paraformaldehyde 2%, immersed in blocking solution (5% donkey serum, 0.4% BSA, 0.1% gelatin, and 0.1% Tween in Tris-buffered saline (TBS) for 1 h and incubated for 48 h with the anti-D_3R (dil. 1 :5000) and anti-GIPC (dil. 1 :500) antibodies in 5% donkey serum, and 0.05% Tween in TBS. The sections were alternatively incubated for 1 h with an anti-donkey–AlexA488 (dil. 1:500, Interchim) and an anti-rabbit biotin-conjugated (dil. 1:300, Amersham Pharmacia Biotechnology) that was further coupled to Streptavidin-CY3 (Dil. 1:4000, Jackson ImmunoResearch). The sections were rinsed several times for 40 min in TBS containing 0.1% gelatin and 0.05% Tween-20 after each incubation and, as for cell cultures, mounted on glass slides using Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence was captured using a laser scanning confocal image system (Leica, Deerfield, IL).

RESULTS

Critical D_3R C-terminal Residues for Ligand Binding

Structural determinants in the C-terminal domain of various GPCR are critical to regulate receptor trafficking and function, like D_1R through its interaction with the accessory protein DRiP78 (Bermak *et al.*, 2001). To assess the role of D_3R C-terminus domain in its receptor function, we transfected D_3R mutants ($D_3\Delta C$, $D_3A_{442}K$, and $D_3\Delta LKI$) in COS7 cells and performed ligand-binding analysis with [^{125}I]iodosulpride, a D_2R/D_3R radioligand (Table 1). Mutations of the D_3R C-terminus sequence profoundly affected ligand-binding properties because D_3R mutants showed reduced [^{125}I]iodosulpride binding (up to 94%). This may reflect receptor instability or an expression defect. We further examined the pharmacological profile of D_3R mutants. A mutant lacking the LKI motif ($D_3\Delta LKI$) displayed a 20-fold lower affinity for dopamine. In contrast the mutant devoid of its C-terminal cysteine residue ($D_3\Delta C$) and the mutant that has its lysine$_{442}$ substituted by an alanine residue ($D_3A_{442}K$) displayed threefold higher affinities for dopamine (Table 1). To investigate the influence of the deletion of the C-terminus cysteine on receptor expression, we compared mRNAs expression levels of D_3R and its $D_3\Delta C$ mutant in transiently transfected COS7 cells as assayed by Northern blot with a D_3R-specific probe. The expression level of $D_3\Delta C$ mRNA was higher than wild-type D_3R (unpublished data), suggesting that this mutation did not induce an expression defect. Thus, because the deletion of the D_3R C-terminus cysteine decreased neither receptor mRNA expression nor its affinity for dopamine, this mutation might interfere with protein–protein interactions, suggesting a critical role for the D_3R C-terminus in D_3R function.

GIPC Interacted with D_2R, D_3R, But Not D_4R

To identify proteins that interact with the D_3R, we performed a yeast two-hybrid screening of a rat brain cDNA library using the D_3R C-terminus cytoplasmic tail as bait. From 4.5×10^6 primary transformants screened, we isolated 50 colonies. Two of these clones coded for regions of rat GIPC. Sequence analysis revealed two conservative discrepancies ($V_{252}I$ and $D_{270}E$) between these sequences and the published sequence of rat GIPC (De Vries *et al.*, 1998). GIPC, mostly known as a PDZ (PSD95/Dlg/ZO-1) domain-containing protein (aa 125–225), displays two putative functional domains (Figure 1A), an acyl carrier protein domain (ACP) within the C-terminal region (aa 264–315), and a proline-rich N-terminal region (aa 1–56). The larger clone encoded the full-length open reading frame (ORF) of GIPC (aa 1–333), whereas the shorter contained the last two-thirds of the ORF (aa 119–333; Figure 1A). This suggests that the GIPC N-terminal domain did not take part in the interaction with the D_3R C-terminus.

To examine whether GIPC interacts with other dopamine D_2-like receptors that are conserved at their C-termini (Figure 1B), we tested the D_2R and D_4R C-terminal domains in binary two-hybrid assays. GIPC interacted with D_2R and D_3R C-termini but not with that of the D_4R (Figure 1B). This is in agreement with the fact that C-termini of D_2R and D_3R share a higher sequence identity than with the D_4R. To compare the relative strength of interactions between GIPC and its various partners, we used a liquid phase assay of the two-hybrid system. Side-by-side comparisons of relative binding of several proteins to GIPC revealed a stronger interaction with dopamine receptor subtypes D_2R and D_3R than with the regulator of G protein signaling, GAIP, and the neurotrophic tyrosine kinase receptor type 2, TrkB (Figure 1C, $p < 0.01$). Taken together, our results indicate that the GIPC C-terminal region strongly interacts with D_2R and D_3R, but not with the D_4R.

Characterization of the Mutual Interaction Domains

To localize the binding site of D_3R on GIPC, each domain of GIPC was individually tested for its capacity to bind to the D_3R C-terminus in binary two-hybrid assays. Whereas neither the PDZ nor the ACP domains alone were able to bind to the D_3R C-terminus, the combination of both domains restored the interaction (Figure 1A). These observations suggest that the PDZ and ACP domains act together to carry out such an interaction. GIPC was also able to bind to itself through its N-terminal region (Figure 1A), suggesting a possible dimerization.

To delineate the GIPC target motif within the D_3R C-terminus, D_3R C-terminal mutants, constructed through site-directed mutagenesis, were used in binary two-hybrid assays with the full-length GIPC (Figure 1B, lower part). D_2-like C-terminal sequences display a carboxy terminus type III PDZ-binding motif (X-X-C_{COOH}). Although interaction with GIPC

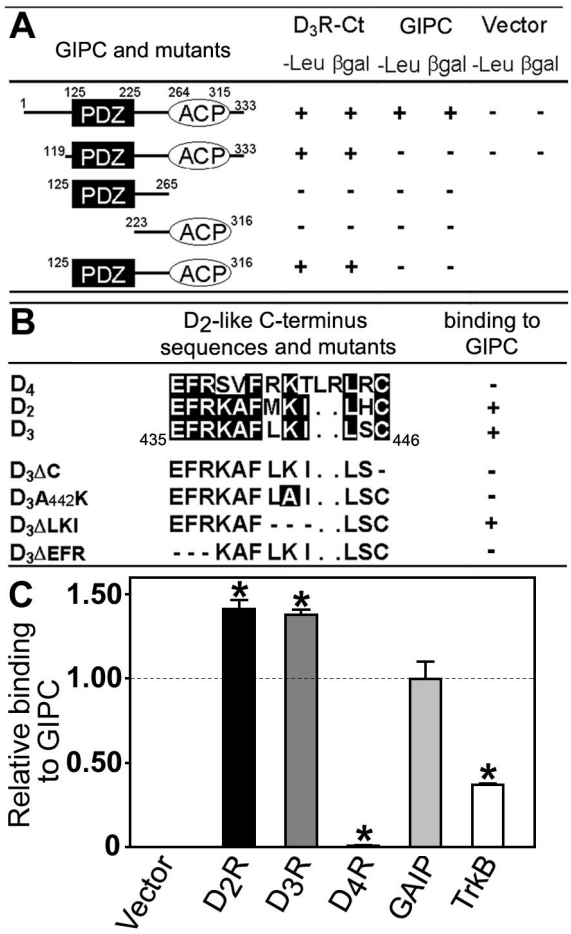

Figure 1. Characterization of the interactions in the yeast two-hybrid system. (A) Cotransformants of the full-length GIPC cDNA (aa 1–333) or GIPC deletion mutants (including aa 119–333, aa 125–265, aa 223–316, aa 125–316) and the C-terminus of the D_3R (D_3R-Ct) or vector alone were screened for growth on leucine-deprived medium (−Leu) and β-galactosidase activity (βgal). Dimerization of GIPC was assessed in binary two-hybrid assays using GIPC cDNA as bait and prey. (B) For mapping of the GIPC binding site, D_2R, D_3R, and D_4R C-termini and D_3R C-terminus mutants were used in binary two-hybrid assays. +, interaction; −, no interaction. (C) The yeast strain EGY48 was cotransformed with the full-length GIPC cDNA and the putative interacting domains of the D_2R, D_3R, D_4R, GAIP, and TrkB. The relative strength of protein–protein interactions observed in transformants was monitored by β-galactosidase liquid assay using ONPG as substrate. Binding to GIPC was compared with GAIP-GIPC interaction defined as 1. Values are means ± SEM of data from four experiments and two different transformations. *$p < 0.01$ versus GAIP-GIPC interaction.

was not only based on its PDZ domain as it also required the association of its ACP domain, removal of the D_3R C-terminal cysteine residue ($D_3\Delta C$) abolished this interaction. However, this could not explain the subtype specificity of such an interaction, because the D_4R also displays a C-terminal cysteine residue. Therefore, we next focused on residues outside the PDZ-binding motif. Removal of the EFR motif ($D_3\Delta EFR$) also abolished the interaction with GIPC (Figure 1B). In addition, the $D_3A_{442}K$ mutant did not bind to GIPC contrary to the $D_3\Delta LKI$ mutant, in which the loss of the LKI residues resulted in a shift of the K_{438} toward the PDZ-binding motif where it is in a position to compensate for the loss of the K_{442} (Figure 1B). This suggests that critical residues outside the PDZ-binding motif may carry out the interaction with GIPC together with the C-terminal cysteine residue. Moreover, the D_4R C-terminal sequence contains two additional amino acids adjacent to its type III PDZ-binding motif compared with the D_2R and D_3R C-termini and may possess a different secondary structure of importance for receptor subtype GIPC-binding specificity. Taken together, these results indicate that specific interaction with GIPC required the C-terminal cysteine residue, which is part of the PDZ-binding motif, while other residues outside this motif likely ensure proper secondary structure of the receptor C-terminus.

D_3R and GIPC Interacted In Vitro

To verify the interaction between the D_3R C-terminus and GIPC, we performed a pull-down assay (Figure 2A). GST and GST-D_3R-Ct fusion protein expressed in *E. coli* were isolated on glutathione-Sepharose beads and incubated with lysates from COS7 cells transiently expressing Xpress/His-GIPC. Xpress/His-GIPC bound to GST-D_3R-Ct, but not to GST.

To confirm the interaction with the full-length recombinant D_3R, we performed affinity chromatography using an Xpress/His-GIPC construct bound onto a (poly)His-Trap column. Unbound proteins from D_3R-transfected COS7 cells were collected in the flow-through, and bound proteins were eluted and analyzed by subsequent immunoblotting with an anti-Xpress antibody and a specific anti-D_3R antibody (Diaz *et al.*, 2000). D_3R appeared on blot as typical 60–80-kDa species (Figure 2B), probably representing different glycosylation forms of the receptor (Diaz *et al.*, 2000). D_3R was retained on the column and coeluted with Xpress/His-GIPC (Figure 2B, left). In an independent experiment, the addition of an excess of a synthetic D_3R C-terminal peptide, inhibited by 75% the interaction between Xpress/His-GIPC and D_3R, of which immunoreactivity was mostly detected in the flow-through (Figure 2B, right). Moreover, the D_3R antibody presaturated with the immunizing peptide (G15Y, 10 mg/ml, Diaz *et al.*, 2000) failed to produce any signal (unpublished data). These results provide biochemical evidence for an interaction between GIPC and mature and immature D_3R.

Endogenous GIPC Interacted with D_2R/D_3R from Rat Striatum

To validate an interaction between endogenous proteins, an anti-GIPC antibody and [^{125}I]iodosulpride to detect D_2R/D_3R binding sites from extracts of the rat striatum, were used. Solubilized D_2R/D_3R receptors were incubated with [^{125}I]iodosulpride and the immunopurified GIPC-bound protein-A-Sepharose. Bound versus unbound proteins were separated by centrifugation, and the nonprecipitated receptors were assayed in the supernatant. Immunoprecipitation of GIPC with the anti-GIPC antibody was monitored by Western blot (Figure 2C, inset). GIPC from solubilized rat striatum was maximally immunoprecipitated with a 1:100 dilution of anti-GIPC antibody (lane 3), faintly with a 1:500 dilution (lane 2) but not without the use of the anti-GIPC antibody (lane 1). As shown in Figure 2C, solubilized D_2R/D_3R binding sites in the supernatant were depleted by immunopurified GIPC by up to 20%, depending on the dilution of the anti-GIPC antibody. This effect was almost abolished by presaturation of the anti-GIPC with an excess of its immunizing peptide, when the antibody was tested at the lowest dilution (1:100). Assaying the radioactivity retained on the GIPC-absorbed beads also showed, less reproducibly, that ~19% of [^{125}I]iodosulpride binding sites were immunoprecipitated with the lowest antibody dilution tested (1:100, unpublished data). These results support a physiological interaction between endogenous GIPC and D_2R/D_3R dopamine receptors from striatum.

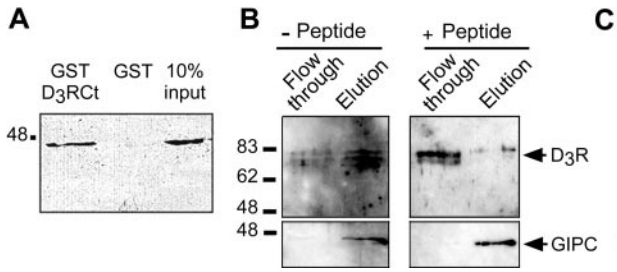

Figure 2. Interaction of GIPC with D_2R and D_3R. (A) GST and GST-D_3R C-terminus fusion protein ($GSTD_3RCt$) bound to glutathione-Sepharose beads, were incubated with extracts from COS7 cells overexpressing Xpress/His-GIPC. Bound proteins were analyzed by Western blot with an anti-Xpress antibody. (B) Binding of GIPC to the full-length D_3R. Xpress/His-GIPC immobilized on an Ni^{2+}-chelating column was incubated with solubilized D_3R-containing membrane extracts from COS7 cells before extensive washes and elution with imidazole. Flow-through and elution fractions were analyzed by Western blot with anti-Xpress and anti-D_3R antibodies. The experiment was performed without (left) or with (right) addition of an excess of the peptide EFRKAFLKILSC identical to D_3R-Ct. (C) Endogenous GIPC interacts with D_2R/D_3R from rat striatum. Receptors from striatum were solubilized and labeled with [^{125}I]iodosulpride, whereas endogenous GIPC from rat striatum was separately immunoprecipitated with anti-GIPC antibody in increasing concentrations, in the presence or absence of its immunizing peptide. Labeled receptors and immunopurified GIPC were incubated together and the nonprecipitated receptors were assayed (mean ± SEM of 5 determinations from 2 independent experiments and values are expressed as the percentage of immunoprecipitation with no anti-GIPC antibody). Inset: Western blot of immunoprecipitated GIPC from rat striatum.1, no antibody; 2 and 3, 1:500 and 1:100 dilutions, respectively.

GIPC and D_3R Colocalized in Rat Brain

To compare the localization patterns of GIPC and D_3R mRNAs in the brain, we performed in situ hybridization with GIPC- and D_3R-specific ^{33}P-labeled riboprobes on adjacent rat brain sections. GIPC displayed a widespread expression throughout the adult rat brain, yet with different hybridization signal intensities in various brain regions. Indeed, the expression level was higher in the granular layer of the cerebellum, pontine nuclei, granular layer of the dentate gyrus, olfactory tract, and cortex than in the striatum (Figure 3A). The addition of an excess of unlabeled GIPC riboprobe prevented signal detection in an adjacent section (unpublished data). Both D_3R and GIPC mRNAs were observed in the nucleus accumbens and the islands of Calleja (Figure 3A), and coexpressed in the granular cells of the islands of Calleja wherein all cells expressed D_3R mRNAs (Diaz et al., 1995).

The overall distribution of GIPC protein, as assessed by immunohistochemistry, perfectly matched that of its mRNAs and was observed as a diffuse cellular and punctate labeling that was totally displaced by the addition of an excess of the immunizing peptide and absent when the anti-GIPC antibody was omitted (unpublished data). The D_3R mainly localized to the plasma membrane and in vesicles, as suggested by the punctate distribution previously described (Diaz *et al.*, 2000). The GIPC and D_3R proteins partially colocalized in clusters at the plasma membrane (P) and in vesicles (V) in the granular cells of the islands of Calleja (Figure 3B, see arrows). These results support a physiological interaction between the two proteins in neurons in vivo.

D_2R, D_3R, But Not D_4R, Caused Translocation of GIPC to the Plasma Membrane

To further determine the subcellular site of the interaction between GIPC and dopamine receptors, we performed immunofluorescence studies. Wild-type HEK293 cells or stably expressing Xpress/His-GIPC (HEK/GIPC) were transiently transfected with $GFPD_2R$, $GFPD_3R$, or $GFPD_4R$ (Figure 4), which possess ligand-binding characteristics similar to that of wild-type receptors (unpublished data). In HEK/GIPC cells devoid of detectable dopamine receptor subtypes expression, GIPC was diffusely distributed in the cytoplasm (Figure 4, arrows). On single transfection in HEK293 and HEK/GIPC cells and under determined conditions, i.e., low amount of transfected DNA (<0.1 μg/well of a 12-well plate) and examination shortly after transfection (24 h), GFP-tagged receptors were prominently expressed at the plasma membrane (Figure 4, A and B, insets). GIPC translocated to the plasma membrane and colocalized with D_2R or D_3R

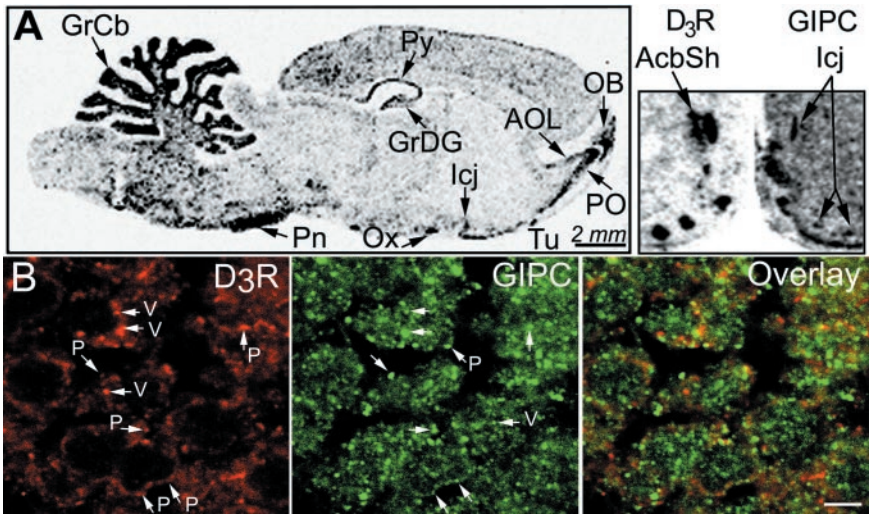

Figure 3. Distribution of GIPC and D_3R in the rat brain. (A) In situ hybridization on a sagittal section with a ^{33}P-labeled GIPC riboprobe and frontal adjacent sections with ^{33}P-labeled D_3R and GIPC riboprobes shown in mirror-image orientation. AOL, anterior olfactory nucleus, Acbsh, accumbens shell; lateral part; GrCb, granular layer of cerebellum; GrDG, granular layer of dentate gyrus; Icj, islands of Calleja; OB, olfactory bulb, PO, primary olfactory cortex; Py, pyramidal cell layer of the hippocampus; PN, pontine nuclei; Ox, optic chiasm; Tu, olfactory tubercle. (B) Immunofluorescence on a 10-μm rat brain section with the anti-GIPC and anti-D_3R antibodies revealed with an anti-donkey-AlexA488 and an anti-rabbit biotin-conjugated antibody that was further coupled to streptavidin-CY3. Arrows point to colocalization found in the granular cells in the islands of Calleja. P, plasma membrane; V, vesicles; Scale, 4 μm.

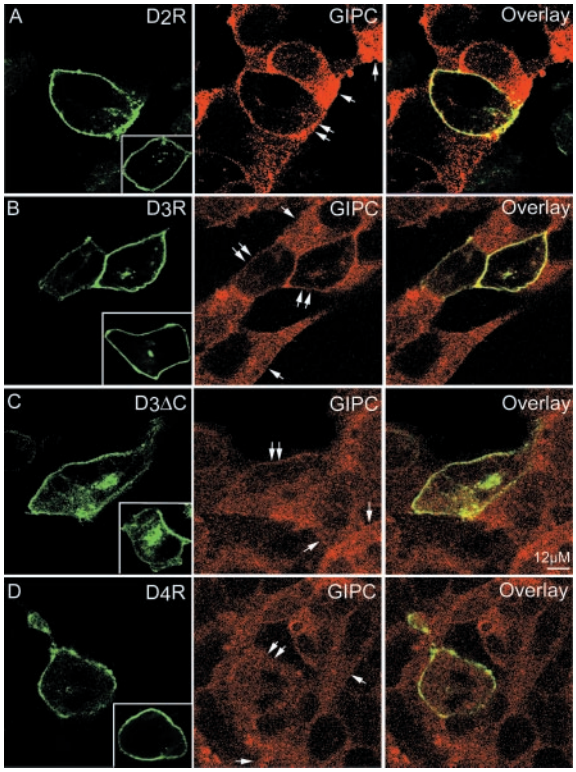

Figure 4. D_2R-, D_3R-dependent translocation of GIPC to the plasma membrane. HEK293 and HEK/GIPC cell lines were transiently transfected with cDNAs encoding for $GFPD_2R$ (A), $GFPD_3R$ (B), $GFPD_3\Delta C$ (C), or $GFPD_4R$ (D). CY3-labeled Xpress antibody for GIPC and GFP fluorescence were observed by confocal microscopy. For comparison, cells expressing receptors but not GIPC are shown in insets. Arrows show cells expressing GIPC alone and double arrows show cells coexpressing GIPC and a dopamine receptor subtype.

in cells transiently expressing these receptors (Figures 4, A and B, double arrows). However, GIPC translocation to the plasma membrane was less efficient when transiently overexpressed in HEK293 cells stably expressing dopamine receptors (unpublished data). To confirm that this effect resulted from a direct interaction between GIPC and the D_2R or D_3R, we used $GFPD_4R$ and the $GFPD_3\Delta C$ mutant, which does not to interact with GIPC. In HEK293 and HEK/GIPC cells, $GFPD_3\Delta C$ was expressed at the plasma membrane, but also more readily in intracellular compartments than $GFPD_3R$ (Figure 4C, inset), which may explain the reduction of $D_3\Delta C$ ligand binding (Table 1). Moreover, $GFPD_4R$ and $GFPD_3\Delta C$ did not cause any GIPC translocation (Figures 4, C and D, double arrows). In addition, mutation of GIPC in its D_3R-binding site by deletion of its ACP domain resulted in a distribution similar to that of the wild-type GIPC, but a complete loss of its translocation and colocalization with the D_3R upon cotransfection (unpublished data). These data show that, through a direct interaction, the D_2R and D_3R are able to recruit GIPC at the plasma membrane.

GIPC Affected a D_3R-mediated Response

Because D_3R provokes a massive translocation of GIPC to the plasma membrane, the question arose as to whether GIPC could affect agonist-induced D_3R signaling. To address this question, we first developed a cell line stably

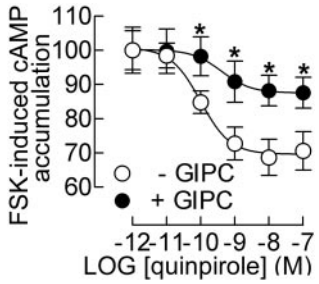

Figure 5. GIPC reduces D_3R-mediated response. Inhibition of cAMP accumulation by quinpirole (LY) in increasing concentrations in HEK/$GFPD_3R$ and HEK/$GFPD_3R$/GIPC cell lines in the presence of 0.5 μM forskolin (FSK). Results are expressed as a percentage of forskolin-stimulated cAMP accumulation and are means ± SEM of data from seven independent experiments. *$p < 0.05$ versus + GIPC.

expressing the human D_3R ($GFPD_3R$), which was subsequently transfected with GIPC. In HEK293 cells, D_3R stimulation by the D_2-like agonist, quinpirole (LY), inhibited cAMP accumulation triggered by forskolin (0.5 μM) in a concentration-dependent manner, with an EC_{50} of 0.11 ± 0.02 nM (mean ± SEM) and a maximal inhibition of 31 ± 0.6% (Figure 5). The overexpression of GIPC reduced the maximal inhibition of cAMP accumulation by forskolin after D_3R stimulation to 12 ± 0.3% ($p < 0.01$) with a significant rightward shift in the dose–response curve (EC_{50} = 0.42 ± 0.06 nM, $p < 0.01$; Figure 5). This result suggests a role of GIPC in the negative regulation of the D_3R signaling.

D_2R, D_3R, But Not D_4R, Cointernalized with GIPC

PDZ proteins have previously been shown to regulate receptor internalization, so we investigated the effect of GIPC on such a process in HEK/GIPC cells transiently transfected with extracellular epitope-tagged receptors ($mycD_2R$, $mycD_3R$, and $GFPD_4R$). We used $GFPD_4R$ instead of $mycD_4R$ in these experiments, because $mycD_4R$ did not readily internalize upon activation. To detect receptors at the cell surface and those internalized upon quinpirole (LY) stimulation, we applied the anti-myc antibody on living $mycD_2R$- and $mycD_3R$-transfected cells, as this antibody did not induce, by itself, receptor internalization (unpublished data). To properly distinguish internalized from newly synthesized D_4R, we used the previously determined experimental conditions, in which the receptors are prominently expressed at the plasma membrane. On stimulation with quinpirole, D_2R, D_3R, and D_4R receptors internalized with different kinetics, rapid and strong for D_2R and D_4R after a 30 min-exposure to 2 μM and 5 μM quinpirole (LY), respectively (unpublished data). In contrast, exposure to 2 μM quinpirole for 1 h induced a low extent of internalization of the D_3R with a slower kinetic rate than that of the D_2R (unpublished data). GIPC partially colocalized with D_2R-labeled vesicles upon stimulation with quinpirole for 1 h (Figure 6A) and strictly cotrafficked with D_3R from the plasma membrane to endocytotic vesicles (Figure 6B), an effect blocked in the presence haloperidol (50 μM), a D_2-like antagonist (unpublished data). Unlike D_2R and D_3R, D_4R-labeled endocytotic vesicles were never labeled with the anti-GIPC antibody (Figure 6C). Thus, GIPC cointernalized with D_2R, D_3R but not D_4R. The nonexhaustive labeling of D_2R-labeled vesicles with GIPC may suggest that it is subsequently released during vesicular sorting.

GIPC Altered Plasma Membrane Localization of the D_2R

To investigate the function associated to the persistent interaction between GIPC and the internalized D_2R and D_3R,

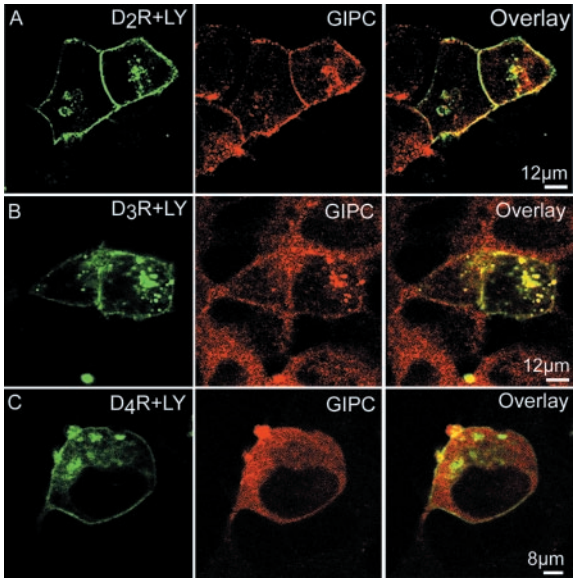

Figure 6. Cointernalization of GIPC with the D_2R, D_3R but not the D_4R. HEK/GIPC cells were transiently transfected with mycD_2R (A), mycD_3R (B), and GFPD_4R (C) cDNAs and stimulated by quinpirole (LY), a D_2-like agonist (2 μM for D_2R and D_3R, and 5 μM for D_4R) for 1 h. The anti-myc antibody was added on living cells, which were subsequently fixed, permeabilized, and incubated with the anti-Xpress antibody. Anti-myc and Xpress antibodies were revealed with secondary antibodies coupled to Alexa488 and CY3, respectively, the D_4R was detected by the GFP fluorescence and visualized by confocal microscopy.

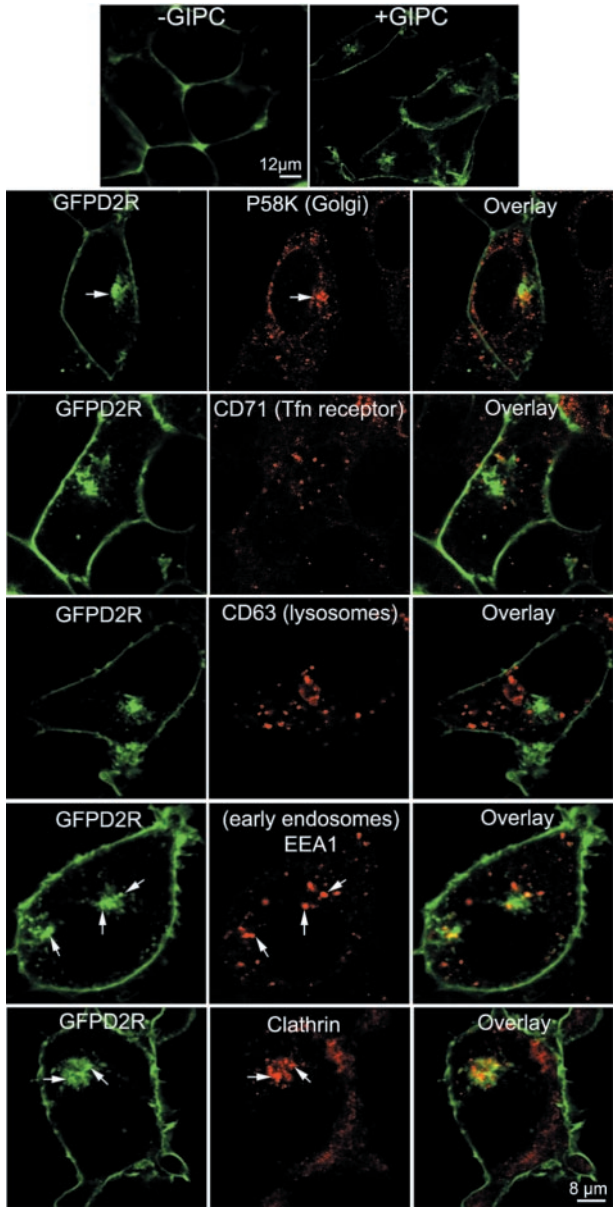

Figure 7. GIPC induces D_2R sequestration in endosomes. HEK/GFPD_2R (top panel, left) and HEK/GFPD_2R/GIPC (top panel, right) cells were seeded on glass cover slips, fixed, and permeabilized before subsequent detection of the different subcellular markers. Markers were all revealed with a CY3-conjugated anti-mouse antibody and GFP fluorescence visualized by confocal microscopy. The following markers, CD71, CD63, P58K, EEA1, labeled the transferrin receptor, lysosomes, the Golgi apparatus, and early endosomes, respectively.

we focused on receptor trafficking and sorting. GFPD_2R localized strictly to the plasma membrane, when stably expressed in HEK293 cells (Figure 7, top panel, left), although its transient expression resulted in faint labeling in the Golgi apparatus (Figure 4). In contrast, when stably coexpressed with GIPC, GFPD_2R partially localized in vesicles (Figure 7, top panel, right), as observed with GFPD_3R (unpublished data). To identify the nature of these vesicles, we performed immunocytochemistry with several biomarkers. Receptor-bearing vesicles were different from recycling vesicles where the transferrin receptor CD71 constitutively traffics, and lysosomes as revealed by the marker CD63 (Figure 7). However, vesicles partially colocalized with the Golgi apparatus marker P58K, the early endosome marker EEA1 and clathrin-coated vesicles (Figure 7). These results suggest that GIPC altered D_2R and D_3R subcellular localization independently of receptor activation by either redistributing or sequestrating receptors in sorting vesicles.

GIPC Increased the Number of D_2R and D_3R Binding Sites

To further test the above mentioned possibilities, we studied the effect of GIPC on the expression of the D_2-like receptors by assaying [^{125}I]iodosulpride binding to the wild-type recombinant D_2R and D_3R, and [^{3}H]spiperone binding to the wild-type recombinant D_4R using HEK293 and HEK/GIPC cells that were transiently transfected with the D_2R, D_3R, $D_3\Delta C$, or D_4R. Receptor overexpression was allowed for 48 h posttransfection and followed by ligand-binding studies on total cell membrane preparations to include receptors at the cell surface and in vesicles. [^{125}I]iodosulpride binding was 75–100% higher when D_2R or D_3R was transfected in HEK/GIPC cells than in HEK293 cells, whereas no effect of GIPC on either $D_3\Delta C$ that has reduced ligand binding (Table 1) or D_4R binding could be noted (Figure 8A). A similar but reduced effect (+30%) was found when Xpress/His-GIPC was transiently transfected in HEK293 cells stably expressing either $D_3\Delta C$ or D_3R and CHO cells stably expressing either D_2R, D_3R, or D_4R (unpublished data). In fact, GIPC increased the maximal number of D_3R expressed (Bmax = 721 ± 35 vs. 589 ± 39 fmol·mg protein^{-1}, $p < 0.05$), without any change in [^{125}I]iodosulpride binding affinity (K_d = 1.2 ± 0.015 vs. 1.197 ± 0.06 nM; unpublished data). Moreover, GIPC overexpression did not affect mRNA levels of heterologous D_3R as assessed by Northern blot anal-

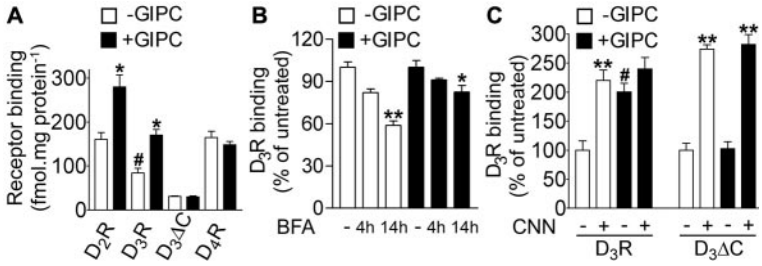

Figure 8. GIPC affects D_2R, D_3R ligand binding and prevents D_3R vacuolar degradation. (A) [^{125}I]iodosulpride (0.1 nM) binding for the D_2R and D_3R and [^{3}H]spiperone (0.4 nM) for the D_4R in HEK293 and HEK/GIPC cell lines transiently transfected with pCMV-mycD_2R, pCMV-D_3R, pCMV-$D_3\Delta C$, or pCMV-D_4R. Values are the mean ± SEM of data from four experiments. $^{\#}p < 0.05$ versus $D_3\Delta C$; $^{**}p < 0.05$ versus -GIPC. (B) Effect of brefeldin A (BFA), an ER-to-Golgi protein translocation inhibitor, on D_3R binding in HEK293 (open columns) or HEK/GIPC (filled columns) transiently transfected with pCMV-D_3R. Values are expressed as percentage of untreated cells and are means ± SEM of data from four experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ versus untreated cells. (C) Effect of concanamicyn A (CNN), a vacuolar protein degradation inhibitor, incubated for 14 h, on D_3R and $D_3\Delta C$ binding in HEK293 (open columns) or HEK/GIPC (filled columns) transiently transfected with pCMV-D_3R and pCMV-$D_3\Delta C$. Values are expressed as percentage of untreated cells and are means + SEM of data from three experiments, $^{**}p < 0.01$ versus untreated cells, $^{\#}p < 0.01$ versus untreated cells-GIPC.

ysis (unpublished data), suggesting that elevated receptor expression was a consequence of protein stabilization. These results establish that GIPC specifically increased D_2R and D_3R protein expression in virtue of its direct interaction with the two receptors by their sequestration during biosynthesis, processing, or degradation.

GIPC Did Not Interfere with D_3R Maturation

Previous data (Liu *et al.*, 2001) showed that only newly synthesized tyrosinase-related protein-1 (TRP-1) associates with GIPC, primarily in the juxtanuclear Golgi region to help its biosynthetic sorting. Having found that GIPC interacted with the different D_3R maturation forms (Figure 2B) together with the fact that stably expressed GIPC translocated better at the plasma membrane with D_2R and D_3R (Figure 4) than transiently expressed GIPC (unpublished data), we hypothesized that interaction with GIPC establishes during receptor maturation. To block the trafficking of newly synthesized D_3R and thus their maturation, we used brefeldin A (BFA), an endoplasmic reticulum-to-Golgi apparatus protein translocation inhibitor. Indeed, in HEK293 cells transiently transfected with D_3R, treatment with BFA resulted in a time-dependent decrease of [^{125}I]iodosulpride binding, reaching 40% after 14 h (Figure 8B) as the cellular pool of receptors is depressed by degradation (Fukuchi *et al.*, 1986). However, when measured in HEK/GIPC cells transiently transfected with D_3R, the decrease of [^{125}I]iodosulpride binding observed after BFA treatment was limited to <15% (Figure 8B), suggesting that GIPC still increased receptor binding despite treatment by stabilizing receptors on postmaturation.

GIPC Prevented D_3R Degradation

To examine whether GIPC prevented D_3R degradation as a possible explanation for GIPC-induced receptor sequestration, we assayed the effect of GIPC on receptor ligand binding in the presence of a set of degradation inhibitors containing the proteasome inhibitor I, a blocker of chymotrypsin-like activity; MG132, a potent reversible inhibitor of the 26-S proteasome; lactacystin, a specific irreversible inhibitor of the 20-S proteasome; and concanamicyn A (CNN), which inhibits acidification of organelles as in lysosomes and the Golgi apparatus (Woo *et al.*, 1992). In HEK293 cells transiently expressing the D_3R, a 14-h incubation with the proteasome inhibitors had no effect on D_3R degradation (unpublished data), indicating that this pathway was not required. On the contrary, CNN treatment increased by 130% the number of D_3R and $D_3\Delta C$ binding sites (Figure 8C), suggesting that D_3R degradation, as that of many GPCRs, required the vacuolar proteolytic pathway. In HEK/GIPC cells transiently transfected with the D_3R, [^{125}I]iodosulpride binding was twice as high as in wild-type HEK293 cells and CNN did not significantly increase D_3R expression (Figure 8C). In contrast, CNN increased $D_3\Delta C$ binding in HEK/GIPC cells. Thus the effect GIPC on D_3R expression is not synergistic with CNN because GIPC mimicked the effect of CNN by inhibiting receptor degradation. These results support the role of the endosomal or lysosomal compartments in regulating D_3R expression and suggest that GIPC, via its direct interaction with D_3R but not with $D_3\Delta C$, protects receptors from degradation, probably preventing vesicular sorting to lysosomes.

DISCUSSION

An important functional role for the cytoplasmic C-terminus of D_3R, which served as bait in the yeast two-hybrid screening, was initially suggested by the high conservation of this domain within the D_2-like receptors. The crystal structure of rhodopsin, a prototypical GPCR, predicts that its C-terminal domain exists as an amphipathic helix anchoring this domain at the intracellular face of the membrane. These results show that several mutations in the D_3R C-terminal domain produced a dramatic reduction of ligand binding, suggesting that it confers a proper conformation to the ligand-binding pocket or binds the receptor to regulatory cytoplasmic partners (Bermak *et al.*, 2001; Xiang *et al.*, 2002). For instance, a D_3R mutant that lacks its C-terminal cysteine, $D_3\Delta C$, displayed reduced ligand binding, but quite an intact pharmacological profile, suggesting that the mutation may have impaired receptor trafficking and/or maturation. In agreement with a role for GIPC in these latter function, $D_3\Delta C$ did not interact with GIPC. The interaction of GIPC with both D_2R and D_3R seems to involve an unusual mode of PDZ recognition (Harris *et al.*, 2001), because both receptors have a PDZ type III consensus motif X-X-C (Harris and Lim, 2001) at their C-terminus, whereas most GIPC-interacting proteins binding sites match the PDZ type I consensus motif S/T-X-V/A/L/I (De Vries *et al.*, 1998; Hu *et al.*, 2003). Moreover, both the PDZ and ACP domains of GIPC were required to interact with D_3R. Although the necessity of the ACP domain could have resulted from instability or improper folding of the PDZ domain fusion protein in yeast, previous studies have shown that the ACP domain of Kermit, the GIPC *Xenopus* homologue, is also required to interact with frizzled 3, a receptor for the Wnts, and with neuropilin 1, a receptor for Semaphorin III (Cai and Reed, 1999; Tan *et al.*, 2001). In addition, GIPC binds to an internal motif of the TrkB receptor although interaction is less potent with an internal than a C-terminal PDZ-binding consensus. The interactions of GAIP and TrkA are based on two distinct sites in the PDZ domain of GIPC (Lou *et al.*, 2001), highlighting the plasticity of the PDZ-based recognition by GIPC. Thus, the conformation of the whole cytoplasmic tail might also be of crucial importance because the D_4R C-terminus, which displays the X-X-C motif, but is two amino acids longer than that of D_2R and D_3R, did not bind to

GIPC. Hence, whereas PDZ domain specificity is primarily determined by the chemical nature of the last and third from last residues of the PDZ-binding consensus sequence (Harris *et al.*, 2001), amino acids that are crucial for the structural integrity of the hairpin required for interaction with the PDZ domain are at least as important as residues making direct contacts.

Scaffolding proteins usually have multiple PDZ domains, contrary to GIPC, which has a single PDZ domain. Nevertheless, GIPC is able to interact with itself through its N-terminus region to form homo-oligomers, thus containing multiple PDZ-binding sites permitting the formation of macro-molecular multicomplexes. Hence, GIPC possesses structural and functional characteristics favorable to its role as a scaffold protein, believed to be involved in organizing and assembling protein complexes by spatially clustering cytosolic proteins, which are usually components of signal transduction pathways of transmembrane receptors or channels (Li and Montell, 2000; Hamazaki *et al.*, 2002).

Although GIPC mRNA is known to be highly expressed in the brain, its distribution among brain regions at the cellular level was previously unknown (De Vries *et al.*, 1998; Bunn *et al.*, 1999). Here GIPC mRNAs were found to be enriched in several brain regions, including the granular layer of cerebellum and dentate gyrus, pontine nuclei, olfactory tract, and cortex. Such an ubiquitous distribution for GIPC is consistent with the variety of its ascribed binding targets. Furthermore, expression of GIPC mRNAs in the rat brain strictly matched that of its protein counterpart, and the GIPC and D_3R mRNAs and proteins colocalized in neurons of the islands of Calleja. Not only do these anatomical studies support the formation of a GIPC/D_3R complex in vivo, but an antibody directed toward GIPC immunoprecipitated D_2R/D_3R from the striatum. Collectively, these results show that GIPC and dopamine receptors are found in close association in neurons, which supports the physiological relevance of their interaction.

In neurons and cell cultures, GIPC protein was detected as a diffuse cellular and punctate staining typical of vesicular localization, which is consistent with the identification of distinct soluble and membrane-based pools (Lou *et al.*, 2002). However, when coexpressed with D_2R or D_3R independently of receptor activation, GIPC massively translocated from the cytoplasm to the plasma membrane as previously described for the *Xenopus* GIPC homologue (Tan *et al.*, 2001). This effect likely resulted from a direct interaction, because a D_3R mutant or the wild-type D_4R, neither of which bound to GIPC in the yeast two-hybrid system, were unable to recruit GIPC at the plasma membrane. Such an effect may reflect clustering of cytosolic proteins coupled to signal transduction. Accordingly, GIPC reduced both the maximal inhibition of forskolin-induced cAMP accumulation, a typical D_3R-mediated response (Griffon *et al.*, 1997) and quinpirole potency to evoke this response. Our results suggest a role of GIPC in D_3R signaling, by reducing its transduction via $G_i\alpha/G_o\alpha$, which are preferentially coupled to D_2-like receptors. In agreement, it was shown that GIPC regulates the β1-adrenergic receptor-mediated, $G_i\alpha$-dependent ERK activation (Hu *et al.*, 2003). Because GIPC binds to GAIP, a regulator of G protein signaling that serves as GTPase-activator for $G_i\alpha$ and $G_q\alpha$ subunits of heterotrimeric G proteins (De Vries *et al.*, 1995), we hypothesize that GIPC, GAIP, and the D_3R form a multimeric complex. According to this hypothesis, GIPC might constitute a physical link between the two other components, to turn off $G_i\alpha$ and then act negatively on D_3R signaling. The function of GIPC would be to promote GAIP-mediated $G_i\alpha$-GTP hydrolysis that terminates the G protein signal and recycles the resulting $G_i\alpha$-GDP for another round of G protein activation. Such an effect would result in termination of the receptor transduction cascade. Further studies are needed to directly demonstrate the formation of a GPCR/GIPC/GAIP multimeric complex. At this stage, it can be hypothesized that the subtype specificity of interacting proteins, as the suggested interaction of GIPC with D_2R, D_3R, but not D_4R, may be relevant to receptors sharing a common signaling pathways, such as the D_2-like receptors.

Agonist-induced GPCR signaling is rapidly downregulated through receptor phosphorylation by GRK, followed by arrestin binding leading to receptor internalization (Claing *et al.*, 2002). GIPC could take part in such a multiprotein scaffolding complex. However, little effect of GIPC on agonist-induced D_3R internalization was found (unpublished data), which is consistent with previous observations concerning the β1-adrenergic receptor (Hu *et al.*, 2003). Although GIPC had no effect on receptor endocytosis, it cointernalized with D_2R or D_3R, which is consistent with the association of GIPC in clathrin-coated pits and clathrin-coated vesicles (Lou *et al.*, 2002). Colocalization of GIPC with receptors in endocytotic vesicles, after a prolonged agonist stimulation, was complete with D_3R and partial with D_2R, suggesting the possible dissociation of GIPC-receptor complexes in sorting vesicles. Such discrepancies probably reflect different internalization kinetics, rapid for the D_2R and slow for the D_3R (unpublished data). Such a persistent interaction with D_2R and D_3R could reflect other functional roles for GIPC, such as in assisting receptor trafficking after its internalization. Indeed, GIPC promoted agonist-independent sequestration of D_2R in early endosomes and clathrin-coated vesicles, thus providing protection of receptors against degradation. As expected for a GPCR, the vacuolar route is mainly involved in D_3R degradation. Nevertheless, GIPC may exert its protective effect independently of the degradation route, because it also protects the TGFβ type III receptor from ubiquitin/proteasome-mediated degradation (Blobe *et al.*, 2001). It is noteworthy that overexpression of the GIPC-binding partner, GAIP, by stimulating GTPase activity of $G_{i3}\alpha$, regulates autophagic sequestration and thus degradation (Ogier-Denis *et al.*, 1997). The GIPC-mediated receptor stabilization may reflect GIPC interaction with cytoskeletal-binding proteins, such as α-actinin1, or cytoskeletal motors, such as kinesin KIF1B and myosin VI (Bunn *et al.*, 1999). Recent data demonstrated that myosin VI is recruited on GIPC-coated vesicles to promote vesicular trafficking of nascent endocytotic vesicles to early endosomes (Aschenbrenner *et al.*, 2003).

In summary, depending on the cytoplasmic effectors recruited, the stable and selective interaction with scaffold protein GIPC may exert distinct roles to assist receptor functions, i.e., signaling, trafficking, and sorting. Indeed, through dimerization, GIPC may alternatively recruit either RGS or cytoskeleton-associated proteins (Bunn *et al.*, 1999) to uncouple the D_2R and D_3R from their signaling cascade and to subsequently link the receptor to the cytoskeleton, leading to receptor sequestration in vesicles and protection against degradation. The mechanism described here could represent a previously unrecognized process of regulation of the D_2R and D_3R, and possibly other GPCRs.

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