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GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP

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ABSTRACT **We have identified a mammalian protein called GIPC (for GAIP interacting protein, C terminus), which has a central PDZ domain and a C-terminal acyl carrier protein (ACP) domain. The PDZ domain of GIPC specifically interacts with RGS-GAIP, a GTPase-activating protein (GAP) for $G\alpha_i$ subunits recently localized on clathrin-coated vesicles. Analysis of deletion mutants indicated that the PDZ domain of GIPC specifically interacts with the C terminus of GAIP (11 amino acids) in the yeast two-hybrid system and glutathione *S*-transferase (GST)-GIPC pull-down assays, but GIPC does not interact with other members of the RGS (regulators of G protein signaling) family tested. This finding is in keeping with the fact that the C terminus of GAIP is unique and possesses a modified C-terminal PDZ-binding motif (SEA). By immunoblotting of membrane fractions prepared from HeLa cells, we found that there are two pools of GIPC–a soluble or cytosolic pool (70%) and a membrane-associated pool (30%). By immunofluorescence, endogenous and GFP-tagged GIPC show both a diffuse and punctate cytoplasmic distribution in HeLa cells reflecting, respectively, the existence of soluble and membrane-associated pools. By immunoelectron microscopy the membrane pool of GIPC is associated with clusters of vesicles located near the plasma membrane. These data provide direct evidence that the C terminus of a RGS protein is involved in interactions specific for a given RGS protein and implicates GAIP in regulation of additional functions besides its GAP activity. The location of GIPC together with its binding to GAIP suggest that GAIP and GIPC may be components of a G protein-coupled signaling complex involved in the regulation of vesicular trafficking. The presence of an ACP domain suggests a putative function for GIPC in the acylation of vesicle-bound proteins.**

The recently discovered family of RGS proteins (for regulators of G protein signaling) act as GTPase-activating proteins (GAPs) that bind to α subunits of heterotrimeric G proteins of the $G\alpha_i/G\alpha_q$ subclass and enhance their GTPase activity (1–5), thus facilitating their deactivation. Moreover, some family members serve as effector antagonists that compete with effector for binding to $G\alpha$ subunits (4).

Today more than 20 different mammalian proteins are known to contain the diagnostic RGS domain, far more than there are $G\alpha_i/G\alpha_q$ subunits. Although their RGS domains are highly homologous (45–80%), the N and C termini of mammalian RGS proteins outside the common RGS domain share little sequence similarity, suggesting that they have specific interactions and functions.

Previously we have identified and characterized RGS-GAIP (6, 7), a RGS family member shown to directly interact with $G\alpha_{i3}$ through its RGS domain (6). We subsequently showed that GAIP is membrane-anchored (7) and, quite recently, that it is located on clathrin-coated vesicles and not on the plasma membrane (8). The localization of GAIP to clathrin-coated vesicles suggests GAIP may participate in the regulation of G protein-mediated pathways that control vesicular trafficking (8). These pathways are still poorly understood compared with G protein-linked pathways at the plasma membrane (9). Although the properties and interactions of the RGS domain of GAIP have been extensively studied, much less is known about the functions of the specific N and C termini of GAIP. The N terminus of GAIP, which includes a cysteine string motif, is palmitoylated and has been implicated in anchoring GAIP to membranes (7). The C terminus of GAIP, like many RGS proteins, is quite short (i.e., only 11 amino acids), and it is unique except for RET-RGS, the only RGS family member that has significant homology to GAIP outside of the RGS domain (10).

In this study, we used the yeast two-hybrid system to identify proteins that bind GAIP and therefore might be involved in its regulation or in signaling cascades that include GAIP. In this report we describe the isolation and characterization of a PDZ domain-containing protein that interacts specifically with the C terminus of GAIP, which we named GIPC (GAIP interacting protein, C terminus). GIPC interacts with GAIP and no other RGS family member and is the only protein described to date that binds to the C terminus of a RGS protein.

MATERIALS AND METHODS

Materials. Template cDNAs for bovine RET–RGS and rat RGS4 were provided by Eva Faurobert (Centre National de la Recherche Scientifique, Sophia-Antipolis, Nice, France) and Kirk Druey (National Institutes of Health), respectively. Polyclonal antisera to the C-terminal peptide sequence of GAIP (aa 208–217, QGPSQSSSEA) (9), to CALNUC (11) and to Man II (12) were prepared as described. A polyclonal anti-GFP antibody was a gift from Charles Zuker (University of California at San Diego, La Jolla). The complete mouse GIPC (mGIPC) cDNA as an expressed sequence tag (EST) (GenBank accession no. AA071924) and human ESTs for GIPC (hGIPC, GenBank accession nos. R12984, W06974, and T54433), were constructed and partially sequenced by the IMAGE Consortium (The Washington University–Merck EST Project, L. Hillier, unpublished data), and were purchased from Genome Systems (St. Louis) and sequenced by automated DNA sequencing.

Abbreviations: ACP, acyl carrier protein; EST, expressed sequence tag; GAIP, $G\alpha$ interacting protein; GAP, GTPase activating protein; GFP, green fluorescent protein; RGS, regulator of G protein signaling; GST, glutathione *S*-transferase; β-gal, β-galactosidase; r, rat; m, mouse; h, human.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. hGIPC, AF089816; rGIPC, AF089817; and mGIPC, AF089818).

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Library Screening. A rat RGS-GAIP cDNA sequence ($GAIP_{23–216}$), highly homologous to human GAIP (6), was obtained from a yeast two-hybrid screen of a rat GC cell cDNA library (11) in the pACT2 "prey" vector with rat $G\alpha_{i3}$ as a bait. Interaction screening in the yeast two-hybrid system was performed with $GAIP_{23–216}$ as bait in the same rat GC cell cDNA library. A total of 600,000 yeast transformants were plated on selective medium and scored for β-galactosidase (β-gal) activity by colony lift assay. Positive (blue) colonies were restreaked on selective medium, and prey plasmid DNA was rescued by electroporation into *Escherichia coli* HB101 (Bio-Rad). Purified pACT2 plasmids were retransformed into yeast strain SFY526 for one-to-one interactions with the original bait plasmid and various control plasmids. Inserts were submitted to restriction digestion, similar patterns were grouped, and one of each was sequenced (both strands) by automated sequencing.

BLAST Searches. Online BLAST searches were performed via the National Center for Biotechnology Information at the National Institute of Health, Bethesda, MD (13). PROSITE (Geneworks, IntelliGenetics) was used for protein analysis, and protein alignments were carried out with the CLUSTAL W program (14). We define homology as identity plus conserved amino acid substitutions.

Northern Blot Analysis. Multiple tissue blots of human, rat, and mouse poly(A^+) RNA (Clontech) were hybridized to probes labeled by random priming (Amersham) according to the manufacturer's instructions. Human, rat, and mouse GIPC probes (1,200, 1,030, and 1,000 bp, respectively) were ^{32}P-labeled to a specific activity of 10^9 cpm/μg. ExpressHyb solution (Clontech) was used under high-stringency conditions for hybridization (68°C) according to the manufacturer's guidelines, and high-stringency washes were performed in 0.1× SSC (SSC = 150 mM NaCl/15 mM sodium citrate, pH 7) plus 0.1% SDS at 65°C. Blots were exposed for autoradiography for 16 and 72 hr. Bands corresponding to GIPC were quantified by using SCANANALYSIS software (Biosoft, Cambridge, U.K.).

***In Vivo* Interactions in the Two-Hybrid System.** For analysis of the interaction between GIPC and GAIP, PCR fragments of the N-terminal (aa 1–79), RGS (aa 80–206), and RGS plus C-terminal (aa 80–217) domains of hGAIP were produced by PCR and cloned into pGBT9 vector as described (6). The C-terminal domains of hGAIP (aa 207–217 and aa 207–216) and rGAIP (aa 207–216) were produced via annealing of two oligonucleotides and cloned into pGBT9. The N-terminal (aa 1–130), C-terminal (aa 211–333), and PDZ domains (aa 124–226) of rGIPC were produced by PCR and cloned into pACT2. Full-length mouse RGS2 and RGSr/RGS16 were amplified by PCR. Full-length RET-RGS, RGS4, RGS2, and RGS-r/RGS16 were cloned into the pGBT9 vector. (Primer sequences for constructs made by PCR are available upon request.) All constructs were verified for in-frame cloning by automated DNA sequencing. For one-to-one interaction we used a colony lift assay with β-gal and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (15). We obtained the same qualitative results in our two hybrid assays regardless of whether we used hGAIP or rGAIP.

***In Vitro* Interactions.** hGAIP and RGS4 were cloned into pGEX-KG (Pharmacia), and glutathione *S*-transferase (GST)-fusion proteins were expressed in *E. coli* and purified as described (6). Coupled *in vitro* transcription-translation of mGIPC in pCMV-Sport2 (GIBCO/BRL) was performed by using the TnT rabbit reticulocyte lysate kit (Promega) in the presence of [^{35}S]methionine. To test for *in vitro* interactions between GAIP, RGS4, and GIPC, 10 μg of GST-GAIP, GST-RGS4, or GST was immobilized on glutathione-agarose beads (Sigma) and incubated with ^{35}S-labeled *in vitro*-translated GIPC for 2 hr at 4°C in 250 μl buffer A (50 mM Hepes, pH 7.5/2 mM $MgCl_2$/1 mM EDTA/100 mM NaCl) with gentle rocking. In some cases GST-GAIP was preincubated (1 hr at room temperature) with affinity-purified GAIP C-terminal antibody (30 or 300 μg). In others ^{35}S-labeled *in vitro*-translated GIPC was preincubated (1 hr at room temperature) with a peptide corresponding to the 10 C-terminal aa of hGAIP (10 μM or 1 mM) before incubation with GST–GAIP. Beads were washed three times in the same buffer, resuspended and boiled in 25 μl Laemmli buffer, and the supernatant was loaded on SDS/12% polyacrylamide gels. Gels were dried and exposed for autoradiography (16 to 72 hr) using Kodak X-Omat film. Bands corresponding to GIPC were quantified by using SCANANALYSIS software.

Expression of GIPC-GFP in Mammalian Cells. To express GIPC with a C-terminal green fluorescent protein (GFP) tag in mammalian cells, the complete coding sequence of rGIPC was subcloned into pEGFP-N1 (Clontech). CsCl-purified plasmid was transfected (4 μl Lipofectamine plus 1 μg plasmid; GIBCO/BRL) into HeLa cells (70% confluency) plated on coverslips in 35-mm wells. Twenty hours posttransfection cells were fixed with 2% paraformaldehyde (30 min) in phosphate buffer, and the GFP-fluorescent signal was observed with a Zeiss Axiophot (FITC excitation and emission filters). In some cases fixed cells were permeabilized with 0.1% Triton X-100 in PBS and incubated with polyclonal antiserum against Man II (diluted 1:400) or affinity-purified anti-GAIP IgG (8) followed by a cross-absorbed Texas red donkey anti-rabbit $F(ab')_2$ conjugate (Jackson ImmunoResearch).

Preparation of Anti-GIPC Antiserum. rGIPC was subcloned into pET28a(+) vector (Novagen), expressed as a His-tagged protein, affinity purified on Ni-NTA agarose beads (Qiagen, Chatsworth, CA), and injected into rabbits. The antiserum recognized 10-pg affinity-purified His-tagged rGIPC by immunoblotting (1:5000 dilution). It also recognized a endogenous GIPC (40 kDa) and GIPC-GFP (65 kDa) by immunoblotting of a lysate prepared from HeLa cells expressing GIPC-GFP. The same 65-kDa GIPC-GFP band was also detected using anti-GFP antibody.

Immunocytochemical Localization of Endogenous GIPC. For immunofluorescence, HeLa cells were fixed, permeabilized, and incubated in anti-GIPC IgG (1 hr) and Texas red donkey anti-rabbit $F(ab')_2$ as above. For immunogold labeling, HeLa cells were fixed in 8% formaldehyde (30 min), 4% formaldehyde (1 hr), and processed for ultrathin cryosectioning as described (8). Ultrathin cryosections were incubated with anti-GIPC IgG followed by incubation with a 5-nm gold, goat anti-rabbit IgG conjugate (Jackson ImmunoResearch). Sections were stained for 10 min in 2% neutral uranyl acetate followed by absorption staining and embedment in 3.2% uranyl acetate, 0.2% methyl-cellulose, 0.1% polyvinyl alcohol.

Preparation and Analysis of Membrane Fractions. HeLa cell fractions were prepared as described for AtT-20 cells (8). Postnuclear supernatant, 100,000 × *g* supernatants, and 100,000 × *g* pellets were normalized by volume, proteins were separated by SDS/PAGE (16) and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using anti-GIPC antiserum (1 hr, 1:5000 dilution) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) (1 hr, 1:3000 dilution). Detection was by enhanced chemiluminescence (Amersham), and GIPC bands were quantified using SCANANALYSIS software.

RESULTS

Identification of GIPC. Screening a rat GC cell pituitary library using the yeast two-hybrid system with RGS–GAIP as bait yielded 22 positive clones. One clone (present 3 times out of 22) contained a 1,400-bp insert and was later named GIPC. BLAST searches in the dBEST database with the cDNA sequence revealed the presence of several expressed mouse and human ESTs. One mouse EST (GenBank accession no.

AA071924) and three human ESTs (GenBank accession nos. R12984, W06974, and T54433) were sequenced. The presence of an in-frame upstream stop codon in human EST T25830 suggested it included the full length ORF. The full-length rat GIPC (rGIPC) clone isolated from our two hybrid screen contained an ORF of 333 aa but no poly(A) tail. The mouse and human ORFs were the same length as rGIPC, and these cDNAs also had poly(A) signals and poly(A) tails.

Features of GIPC. The predicted molecular weight of GIPC is 36,300 with a theoretical pI of 5.7. The deduced amino acid sequence (Fig. 1*A*) predicts a rather hydrophilic protein lacking a transmembrane domain or N-terminal signal sequence. PROSITE analysis shows seven potential protein kinase C and six potential casein kinase II phosphorylation sites. Sequence alignment shows a very high degree of homology between the three mammalian GIPC proteins and two *Caenorhabditis elegans* proteins (Fig. 1*A*). No homologs were found in the *Saccharomyces cerevisiae* genome database. The human and rat proteins are 96% identical, and the mouse and rat proteins are 99% identical. The mammalian GIPC proteins are 32% identical (50% homologous) to the *C. elegans* proteins (C35 and F-44). A BLAST search revealed the presence of a PDZ domain between aa 125 and 225 (Fig. 1*A*). PDZ domains contain 80–100 aa and are found in many signaling molecules where they are involved in protein–protein interactions (17–19). Sequence alignments with other PDZ domains revealed that the PDZ domain of GIPC shows closest homology to the third PDZ domain of atrophin-1 interacting protein 1 (AIP), which contains five PDZ domains (20), and to the second PDZ domain of glutamate receptor interacting protein (GRIP), which contains seven PDZ domains (21) (Fig. 1*B*). One *S. cerevisiae* ORF (YJB7) showed homology to the PDZ domain of GIPC. Two ORFs of unknown function (F44D12.4 on chromosome III and C35D10.2) from the *C. elegans* genome sequencing project also showed significant homology to GIPC outside the PDZ domain. Interestingly, the PDZ domains of the mammalian and *C. elegans* proteins show a higher degree of homology (68%) than their overall homology. Another putative functional motif in GIPC revealed by PROFILESCAN analysis (PROSITE program) is an acyl carrier protein (ACP) domain, aa 264 and 320.

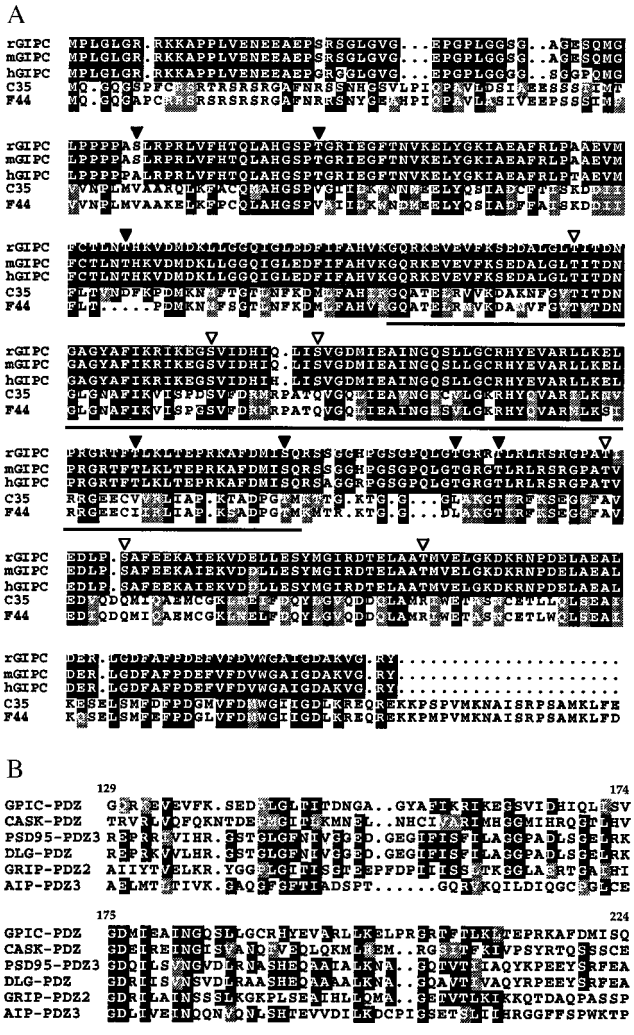

FIG. 1. (*A*) Alignment of human, rat, mouse and two *C. elegans* GIPC proteins (C35 and F44). The PDZ domain is underlined, and seven putative protein kinase C (▼) and six casein kinase II (▽) sites are indicated. (*B*) Sequence alignment of selected PDZ domains. Identical residues are highlighted in deep gray, conserved residues in pale gray. Amino acid numbering corresponds to that of GIPC. The sequences are as follows: CASK (residues 486–518, AF032119), PSD95–PDZ3 (residues 308–401, P78352), DLG–PDZ3 (residues 462–555, Q12959), GRIP–PDZ2 (residues 668–764, U88572), AIP–PDZ3 (residues 601–691, AF038563). The numbers in parenthesis refer to the positions of amino acids and the GenBank accession numbers, respectively.

GIPC Interacts Specifically with GAIP. To determine the specificity of GIPC for GAIP, we tested several other members of the RGS family, RGS2, RGS4, RGS-r/RGS16, and RET-RGS, for their ability to interact with GIPC in the two hybrid system. None of those tested interacted with GIPC (Fig. 2*A*), including RET–RGS, the family member that shows the highest homology to GAIP. We conclude that the interaction of GIPC with GAIP is highly specific for GAIP.

GIPC Interacts with the C Terminus of GAIP. To identify the site of interaction of GIPC with GAIP, we used the two-hybrid, β-gal filter assay. GIPC did not interact with the N terminus of GAIP (aa 1–79) and showed an extremely weak interaction with its RGS domain (aa 80–206) (Fig. 2*B*). When the C terminus of GAIP (aa 206–217) was included, we observed strong interaction with GIPC. Next we tested directly the C-terminal 11 aa of GAIP and found it interacted with GIPC, demonstrating that this region of GAIP is responsible for GIPC binding (Fig. 2*B*). Deleting the last amino acid (alanine) of the C terminus of GAIP abolished the interaction. These results (*i*) demonstrate that the C terminus of GAIP is responsible for its binding to GIPC, and (*ii*) define the most C-terminal alanine residue of GAIP as a major determinant in the binding.

GAIP Interacts with the PDZ Domain of GIPC. To determine what region of GIPC interacts with GAIP, we divided the molecule into three parts: N-terminal (aa 1–130), PDZ (aa 124–226), and C-terminal (aa 210–333) domains and tested all three for interaction with GAIP in the two-hybrid assay. Fig. 2*C* shows that the PDZ but not the N- and C-terminal domains of GIPC interacts with GAIP.

Interaction Between GST–GAIP and *in Vitro*-Translated GIPC. We also performed an *in vitro* interaction assay with GST–GAIP bound to glutathione-agarose beads and *in vitro*-translated GIPC. GIPC bound weakly but specifically to GST-GAIP (Fig. 3, lane 3) but not to GST alone (Fig. 3, lane 2). By contrast no interaction was detected between GIPC and RGS4 (Fig. 3, lane 4). As expected, the RGS domain, $GAIP_{80–206}$ (Fig. 3, lane 5), and the N terminus, $GAIP_{1–79}$ (Fig. 3, lane 6), did not bind to GIPC. When GST–GAIP was preincubated with affinity-purified IgG (300 μg) raised against the C terminus of hGAIP (aa 208–217), binding to GIPC was abolished (Fig. 4, lane 5), whereas the same amount of irrelevant antibody did not perturb binding (Fig. 4, lane 6). Also, when GAIP C-terminal peptide was added to the binding mixture, it reduced interaction (Fig. 4, lane 8), whereas an unrelated peptide did not (Fig. 4, lane 9). Collectively, these results confirm that GIPC binds specifically to the C terminus of GAIP.

A

BAIT	Interaction	PREY
GAIP	+++	GIPC
RET-RGS	---	GIPC
RGS4	---	GIPC
RGS2	---	GIPC
RGS-r/RGS16	---	GIPC

B

BAIT		Interaction	PREY
N RGS C (1 79 206 217)	GAIP	+++	GIPC
	GAIP-N	---	GIPC
	GAIP-RGS	+/-	GIPC
	GAIP-RGS+C	+++	GIPC
LQGPSQSSSEA	GAIP-C11	+++	GIPC
LQGPSQSSSE	GAIP-C10	---	GIPC

C

BAIT	Interaction	PREY	
GAIP	+++	GIPC	N PDZ C (1 125 225 333)
GAIP	---	GIPC-N	(1 130)
GAIP	---	GIPC-C	(210 333)
GAIP	+++	GIPC-PDZ	(124 226)

FIG. 2. (A) GIPC interacts specifically with GAIP. (*B*) GIPC interacts with the C terminus of GAIP. (*C*) GAIP interacts with the PDZ domain of GIPC. The β-gal filter assay was performed on Leu^-, Trp^- plates, and intensity of color was scored as the following: ---, no color (background) after 16 hr; +/−, very faint color (but above background after 16 hr); +++, strong color after 2 hr. Yeast cotransformed with void bait and prey vectors were taken as background.

GIPC mRNA Is Widely Expressed in Mammalian Tissues. In a multiple human tissue Northern blot, an mRNA of ≈1.8 kb was detected: pancreas > skeletal muscle > brain, kidney, placenta > lung, liver ≫ heart (Fig. 5). Several striking differences were noted between expression of GIPC and GAIP mRNA in human tissues: In brain, kidney and skeletal muscle expression of GAIP is much lower than GIPC, whereas in lung and liver GAIP is higher (6). The same variability of GIPC mRNA expression among different tissues was found in the rat and mouse. Human and rat showed the same size mRNA, but the mouse mRNA was slightly larger (≈2.0 kb) (data not shown).

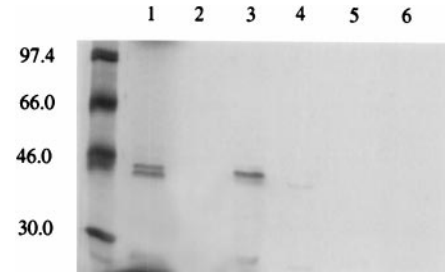

FIG. 3. GIPC interacts specifically with GAIP *in vitro*. GST-fusion proteins bound to glutathione-agarose beads were incubated with *in vitro*-translated, radiolabeled GIPC as described. GIPC bound specifically to GAIP (lane 3) but not to GST alone (lane 2). GIPC did not bind to RGS4 (lane 4), to the RGS domain of GAIP ($GAIP_{80–206}$, lane 5), or the N-terminal domain of GAIP ($GAIP_{1–79}$, lane 6). GIPC appears as a 40-kDa doublet (lane 1) in ^{35}S-labeled *in vitro*-translated GIPC product.

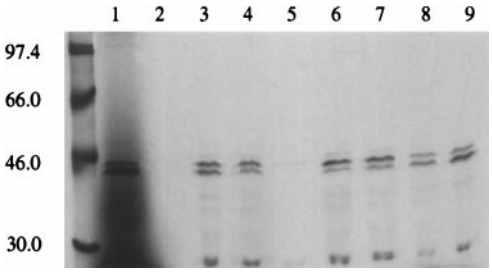

FIG. 4. GIPC interacts with the C terminus of GAIP. GST–GAIP fusion protein bound to glutathione-agarose beads was incubated with *in vitro*-translated GIPC (lane 3) as described. Addition of 30 μg (lane 4) or 300 μg (lane 5) of anti-GAIP C-terminal IgG reduced the binding of GIPC to GAIP, whereas 300 μg anti-CALNUC antibody (lane 6) had minimal effect. When 10 μM (lane 7) or 1 mM (lane 8) of GAIP C-terminal peptide were added the binding of GIPC to GAIP was also reduced 16% and 43%, respectively. A control peptide (1 mM, lane 9) had little effect on binding. Binding to GST alone (lane 2) was taken as background, and the signal obtained after binding of GIPC to GST–GAIP (lane 3) (with background subtracted) was defined as 100% in arbitrary units. Lane 1, ^{35}S-labeled *in vitro*-translated GIPC product.

GIPC Is Associated with Both Membrane and Cytosolic Fractions. Next we investigated whether GIPC is a soluble (cytosolic) or membrane-associated protein. Immunoblotting analysis of crude membrane (100,000 × *g* pellet) and cytosolic (100,000 × *g* supernatant) fractions prepared from HeLa cells demonstrated the presence of GIPC in both cytosolic (70%) and membrane (30%) fractions (Fig. 6). The level of expression of GAIP and its distribution remained unchanged in HeLa cells transfected with and overexpressing GIPC–GFP compared with mock-transfected cells (data not shown).

Distribution of GIPC–GFP in HeLa Cells. To determine the localization of GIPC, we initially expressed the protein with a GFP tag fused to its C terminus. HeLa cells were transfected with GIPC–GFP and analyzed by fluorescence 20–24 hr after transfection to avoid extensive overexpression. Three different patterns of GIPC–GFP signal were observed in different cells: diffuse cytoplasmic staining (63% of transfected cells) (Figs. 7*C*), bright punctate cytoplasmic staining (23% of transfected cells) (Fig. 7 *A* and *B*), and concentrated juxtanuclear signal (14% of transfected cells), which partially overlapped with that of the Golgi marker Man II. The signals for GIPC–GFP and GAIP did not appear to overlap significantly.

Localization of Endogenous GAIP. When endogenous GIPC was localized by immunofluorescence with anti-GIPC

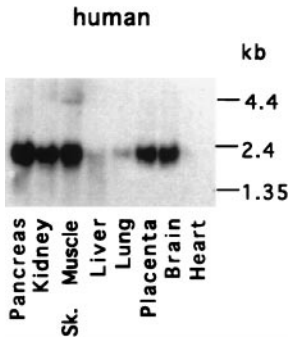

FIG. 5. Expression and tissue distribution of human GIPC mRNA. The autoradiograph was exposed for 16 hr at −70°C with intensifying screens. hGIPC showed an ubiquitous 1.8-kb transcript with highest expression levels in pancreas, skeletal muscle, kidney, placenta, and brain.

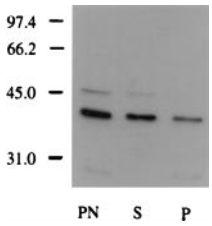

FIG. 6. GIPC is present in both membrane and cytosolic fractions. In HeLa cells most of the endogenous GIPC (70%) is found in the soluble (S) or cytosolic fraction, prepared from a postnuclear supernatant (PN), but the remainder (30%) sediments with the membrane pellet. Fractions were immunoblotted with anti-GIPC, antiserum detected by enhanced chemiluminescence, and quantified as described.

IgG, its distribution resembled that in the transfected cells as it was distributed in both a diffuse and a punctate staining pattern throughout the cytoplasm (Fig. 7*D*). The concentrated juxtanuclear pattern seen in transfected HeLa cells was not present. By immunogold labeling on ultrathin cryosections of HeLa cells, GIPC was often detected on the cytoplasmic surface of the membranes of small vesicles typically located in clusters near the cell membrane (Fig. 8). Scattered gold particles were also detected in the cytoplasm. We assume that the diffuse cytoplasmic staining and the vesicular labeling correspond, respectively, to the soluble and membrane-associated pools of GIPC.

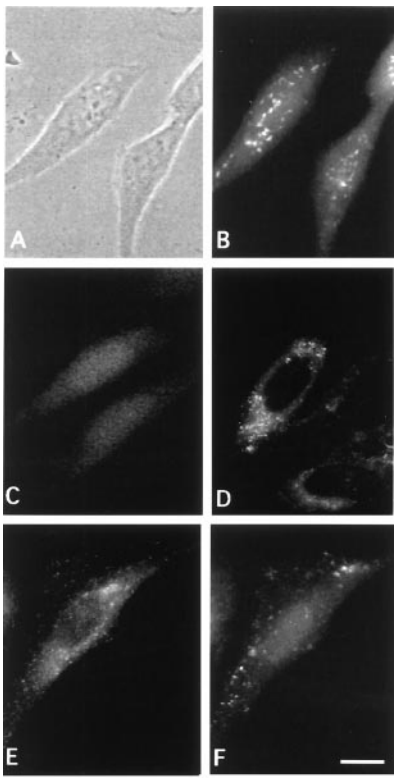

FIG. 7. Localization of GIPC–GFP and endogenous GIPC in HeLa cells. Phase contrast (*A*) and fluorescence (*B*, *C*, *E,* and *F*) micrographs of HeLa cells transiently transfected with GIPC–GFP. Two patterns of staining are seen 20 h after transfection: a punctate (*B*) and a diffuse (*C*) cytosolic staining pattern. (*D*) Endogenous GIPC shows a similar diffuse and punctate vesicular staining pattern in nontransfected HeLa cells by immunofluorescence. (*E*) When cells expressing GIPC–GFP were stained with affinity purified GAIP IgG, little overlap was seen in the distribution of GIPC-GFP (*E*) and GAIP (*F*). In *D*, nontransfected HeLa cells were aldehyde-fixed and incubated with rabbit polyclonal anti-GIPC antibody followed by Texas Red-conjugated donkey anti-rabbit F(ab′)$_2$ as described. (×600.)

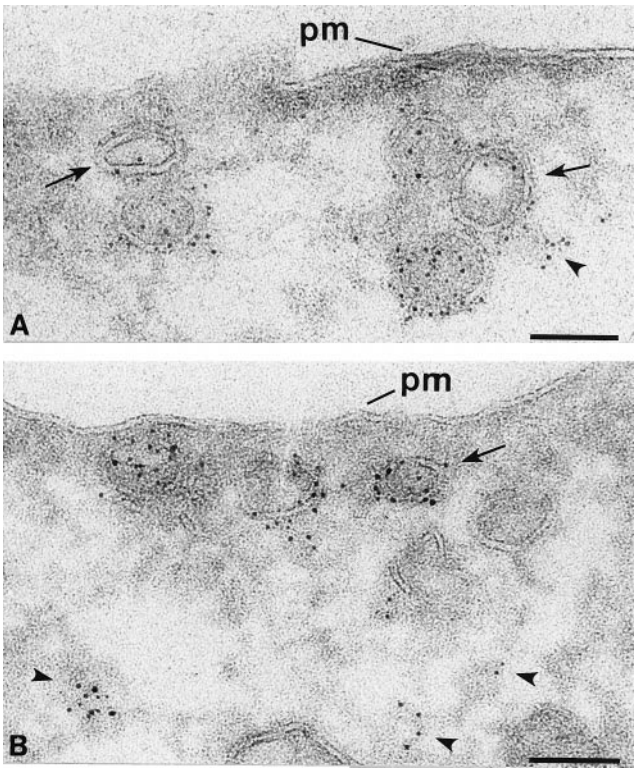

FIG. 8. GIPC is associated with vesicles in HeLa cells. (*A* and *B*) Gold particles are found on vesicles (arrows), which are typically arranged in clusters located near the plasma membrane (PM). Some gold particles are also seen scattered throughout the cytoplasm (arrowheads). Cells were processed as described. Ultrathin cryosections were incubated sequentially with rabbit polyclonal anti-GIPC IgG and 5-nm gold, goat anti-rabbit IgG conjugate. (×120,000.)

DISCUSSION

More than 20 different RGS proteins have been identified up to now (1–3). Aside from their RGS domains, which have high homology and have been shown to bind to G protein α subunits, these proteins share little sequence similarity, which suggests diversity in interactions and function among different family members.

A few functions have already been ascribed to the N termini of individual RGS proteins (e.g., membrane anchoring). We previously provided evidence that GAIP has an N-terminal cysteine string motif (7) that probably anchors it to membranes, and the same was also shown for RET–RGS (10). The N-terminal 33 aa of RGS4 was also found to be necessary for its localization to the plasma membrane, and deletion of this domain impaired RGS4 function (22). Four other RGS family members, SST2 from yeast, FLBA from *Aspergillus*, EGL-10 from *C. elegans*, and mammalian RGS7, have N-terminal DEP domains that may play a role in the regulation of GDP–GTP exchange by small GTP-binding proteins.

Up to now information on the interactions of the C termini of RGS proteins has been virtually nonexistent. Using the yeast two-hybrid system, we identified a PDZ domain-containing protein, GIPC, that interacts specifically with the C terminus of GAIP. The 80–100 aa PDZ domain (named after PSD-95, mammalian postsynaptic density protein, Dlg, *Drosophila* disc-large protein, and ZO-1, a mammalian tight junction protein), is found in >50 proteins that are often components of signaling networks. The PDZ domain has no clearly defined function but typically is involved in protein–protein interactions and the formation of protein networks (17–19). Some PDZ domain containing proteins are involved in G protein-mediated signaling pathways. Best studied is the *Drosophila* Ina D protein,

which via five PDZ domains serves as a scaffold to assemble different components of the phototransduction cascade—i.e., the G protein effector phospholipase C-β, the light-activated calcium channel, and protein kinase C (23). Some PDZ domains bind to a specific motif [(S/T)XV] in the extreme C termini of other proteins (24–26). GAIP contains a similar but distinct motif (SEA) at its extreme C terminus, in keeping with our finding that its C terminus interacts with the PDZ domain of GIPC. The importance of the very last amino acid (position 0) of GAIP for binding was demonstrated by our finding that deletion of this residue abolished interaction with GIPC. Our finding that RET–RGS, whose last three amino acids are VEA, does not interact with GIPC indicates that the serine residue in position −2 is essential for binding of GAIP to the PDZ domain of GIPC.

Recently Rousset *et al.* (27) have isolated an incomplete GIPC cDNA via a two-hybrid screen by using the viral Tax transactivator protein from HTLV-1 as a bait. In their report, GIPC (which they named TIP-2 for Tax interacting protein, clone *2*) is one of six cellular proteins of unknown function which interact via their PDZ domains with the C terminus of Tax. Changing the last valine of Tax to alanine (TEV to TEA) abolishes interaction of Tax with all of the six above proteins except GIPC. Thus both GAIP and Tax can interact with the PDZ domain of GIPC. This finding indicates that the PDZ domain of GIPC can interact when either alanine or valine are present in position 0 of the C terminus. Interestingly, CXC chemokine receptor 5, a coreceptor for HTLV-1/HIV, is linked to a $G\alpha_i$ signaling pathway (28). Very recently Snow *et al.* (29) showed that an alternatively spliced form of RGS12 contains both an N-terminal PDZ domain and a C-terminal PDZ-binding motif (ATFV). The PDZ domain of RGS12 is capable of interacting with the C terminus of the interleukin 8 receptor B (also known as the CXC chemokine receptor 2). These authors also suggest that the C terminus of the CXC chemokine receptor 5 (STGL) has a potential RGS12 PDZ-binding motif.

It is intriguing that in addition to a PDZ domain, GIPC contains an ACP domain at its C terminus. ACP domains are found in animal fatty acid synthases and function as acceptors for acyl moieties (30). This feature suggests a possible role for GIPC as a carrier molecule of palmitoyl moieties for the palmitoylation of GAIP, $G\alpha$ subunits, or other signaling molecules.

Although GIPC and GAIP show a rather ubiquitous tissue distribution based on Northern blot analysis, the expression levels of their mRNAs in most tissues examined are quite different. We did not detect a significant change by immunoblotting in the level of expression of GAIP in HeLa cells as a result of transient overexpression ($\approx$25% transfection efficiency) of GIPC. Analysis of the endogenous expression levels of both proteins and of their intrinsic stabilities in different tissues is required to shed light on the relationship between GAIP and GIPC expression.

We have recently shown that GAIP is associated with membranes (7) and specifically is localized on clathrin-coated vesicles involved in membrane trafficking (8). PDZ domain proteins have been found in the cytosol, associated with membranes, usually plasma membranes, or in the nucleus (17–19). Our biochemical results and immunocytochemical findings indicate that in HeLa cells there are two pools of GIPC: one membrane associated and one soluble. By immunofluorescence, the membrane-associated pool of GIPC has a punctate, cytoplasmic distribution, and immunogold labeling at the electron microscope level revealed that it is associated with small vesicles frequently located near the cell membrane. Interestingly, when the distribution of GFP–GIPC and GAIP was analyzed by immunofluoresence, little overlap was evident. It was not possible so far to colocalize GAIP and GIPC at the electron microscope level due to lack of suitable antibodies (both are rabbit polyclonal antibodies). Further studies are necessary to establish whether GAIP and GIPC are located on the same or different vesicles. The ability of GAIP to bind to both GIPC and $G\alpha_{i3}$ (6, 7) together with the finding that both GAIP and GIPC are associated with vesicles suggests that these proteins may be components of a G protein-linked signaling network involved in control of vesicular trafficking.

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