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RGS-GAIP, a GTPase-activating Protein for $G\alpha_i$ Heterotrimeric G Proteins, Is Located on Clathrin-coated Vesicles

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RGS-GAIP (Gα-interacting protein) is a member of the RGS (regulator of G protein signaling) family of proteins that functions to down-regulate $G\alpha_i/G\alpha_q$-linked signaling. GAIP is a GAP or guanosine triphosphatase-activating protein that was initially discovered by virtue of its ability to bind to the heterotrimeric G protein $G\alpha_{i3}$, which is found on both the plasma membrane (PM) and Golgi membranes. Previously, we demonstrated that, in contrast to most other GAPs, GAIP is membrane anchored and palmitoylated. In this work we used cell fractionation and immunocytochemistry to determine with what particular membranes GAIP is associated. In pituitary cells we found that GAIP fractionated with intracellular membranes, not the PM; by immunogold labeling GAIP was found on clathrin-coated buds or vesicles (CCVs) in the Golgi region. In rat liver GAIP was concentrated in vesicular carrier fractions; it was not found in either Golgi- or PM-enriched fractions. By immunogold labeling it was detected on clathrin-coated pits or CCVs located near the sinusoidal PM. These results suggest that GAIP may be associated with both TGN-derived and PM-derived CCVs. GAIP represents the first GAP found on CCVs or any other intracellular membranes. The presence of GAIP on CCVs suggests a model whereby a GAP is separated in space from its target G protein with the two coming into contact at the time of vesicle fusion.

INTRODUCTION

Classical G protein-mediated signaling pathways are three-component systems consisting of serpentine (seven-transmembrane domain) plasma membrane (PM)[1] receptors, heterotrimeric G proteins composed of α, β, and γ subunits, and an effector, usually an enzyme or an ion channel (Gilman, 1987; Bourne *et al*., 1990; Neer, 1995; Hamm and Gilchrist, 1996). The newly discovered family of proteins known as RGS proteins (regulators of G protein signaling) constitute a fourth component of these systems (Dohlman and Thorner, 1997; Koelle, 1997; Neer, 1997; Berman and Gilman, 1998). RGS proteins serve as guanosine triphosphatase-activating proteins (GAPs) that accelerate the guanosine triphosphatase activity of Gαi/Gαq subunits by stabilizing the Gα subunit in its guanosine triphosphate (GTP)-to-guanosine diphosphate (GDP) transition state (Berman *et al*., 1996), returning them to their inactive GDP-bound form (Berman *et al*., 1996; Hunt *et al*., 1996; Watson *et al*., 1996), and thereby terminating the G protein signal. The RGS protein family has been implicated in desensitization and negative regulation of heterotrimeric G protein-signaling pathways in yeast, fungi, and nematodes (Dohlman *et al*., 1996; Koelle and Horvitz, 1996; Yu *et al*., 1996). In mammalian cells, RGS proteins have been

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[1] Abbreviations used: CCVs, clathrin-coated vesicles; GAIP, Gα interacting protein; HA, hemagglutinin; PFA, paraformaldehyde; PM, plasma membrane; PNS, postnuclear supernatant; PVDF, polyvinylidinedifluoride; RGS, regulator of G protein signaling; RM, residual microsomes.

implicated in the negative regulation of MAP kinase and phosphoinositide-phospholipase C activity and a loss of inhibition of adenylate cyclase activity by Gαi subunits (Druey *et al.*, 1996; Chatterjee *et al.*, 1997; Huang *et al.*, 1997; Yan *et al.*, 1997). RGS proteins may also regulate cell death as suggested by the finding that A28-RGS14 is transcriptionally activated by the tumor suppressor, p53 (Buckbinder *et al.*, 1997). The negative regulation of these cellular processes has been assumed to be due to the GAP activity of RGS proteins. However, RGS protein family members also have recently been suggested to function as effector antagonists that compete for effector binding to Gα (Hepler *et al.*, 1997; Berman and Gilman, 1998).

GAIP (Gα interacting protein) was the first RGS protein shown to interact directly with the heterotrimeric $G\alpha_{i3}$ subunit through the RGS domain common to all family members (De Vries *et al.*, 1995) and the first, along with RGS4, shown to have GAP activity (Berman *et al.*, 1996). Up to now, no RGS protein has been clearly localized within the cell. This is an important issue because there are already more mammalian RGS family members (>20) than Gαi/Gαq subunits, and multiple RGS proteins are expressed in the same tissue or cell type (De Vries *et al.*, 1995; Chen *et al.*, 1996; Druey *et al.*, 1996; Hunt *et al.*, 1996; Chen *et al.*, 1997; Faurobert and Hurley, 1997; Snow *et al.*, 1997).

Heterotrimeric G proteins have been localized on intracellular membranes as well as on the PM and appear to play a role in control of endocytic and secretory pathways (Bomsel and Mostov, 1992; Helms, 1995; Nürnberg and Ahnert-Hilger, 1996). $G\alpha_{i3}$ is found on Golgi membranes (Stow *et al.*, 1991; Wilson *et al.*, 1994; Denker *et al.*, 1996), and overexpression of $G\alpha_{i3}$ was found to slow transport of newly synthesized proteins along the exocytic pathway (Stow *et al.*, 1991). Previously we showed that in AtT-20 stably expressing GAIP, most of the GAIP (80–90%) is membrane associated and palmitoylated most likely via its cysteine string motif (De Vries *et al.*, 1996), but the nature of the membranes to which GAIP is anchored has not been established. Key unanswered questions are whether GAIP is associated with Golgi membranes or the PM and whether it is present on the same membrane domains as $G\alpha_{i3}$. Here we used cell fractionation and immunocytochemistry to determine where GAIP is located and report the unexpected finding that GAIP is not found on either the PM or Golgi membranes but is associated with clathrin-coated buds and vesicles (CCVs).

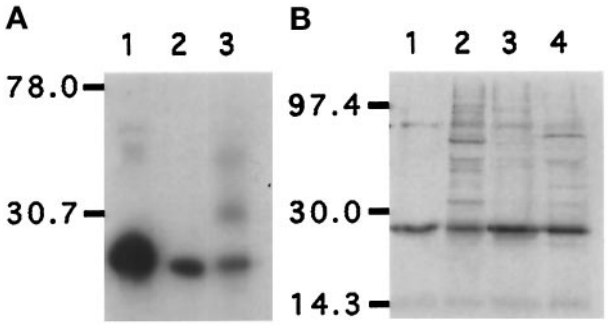

Figure 1. Characterization of anti-GAIP antibodies demonstrating that they recognize only GAIP, a ~25 kDa protein, by immunoblotting (A) or immunoprecipitation (B) of a lysate from AtT-20 pituitary cells stably expressing HA-GAIP. (A) Cells were homogenized, centrifuged at 600 × *g*, and the postnuclear supernatant was solubilized in Laemmli sample buffer. The proteins (50 μg/lane) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-HA (1 μg/ml; lane 1); anti-GAIP (C) antiserum (1:8000; lane 2), or anti-GAIP (N) antiserum (1:4000; lane 3) followed by appropriate goat anti-mouse or anti-rabbit IgG coupled to HRP and detection by ECL. (B) Cells were labeled for 4 h in 100 μCi/ml Easytag (Dupont, Boston, MA) after which they were homogenized, and a postnuclear supernatant was prepared and solubilized in RIPA buffer. The lysate was precleared with 20 μl Protein A/G-Sepharose (mAb 16B12) or protein A-Sepharose (rabbit sera), after which GAIP was precipitated with anti-HA (5 μg/ml; lane 1), anti-GAIP (N) (lane 2), anti-GAIP (C) (lane 3), or anti-$GAIP_{23–217}$ (lane 4) antisera diluted 1:333, and the immunoprecipitates were separated by SDS-PAGE and detected by autoradiography.

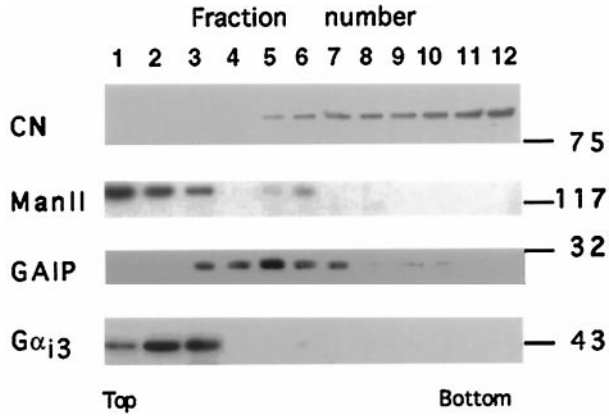

Figure 2. Distribution of GAIP in AtT-20 cell fractions prepared by sucrose density centrifugation as described in MATERIALS AND METHODS. The ER marker calnexin peaks in heavy fractions with the majority (71%) in fractions 9–12. The Golgi marker Man II and $G\alpha_{i3}$ are found in light fractions 1–3, which also contain PM. Fractions 1–3 contain 15, 44, and 40%, respectively, of the total $G\alpha_{i3}$ in the gradient. GAIP is associated with fractions 3–7 of intermediate density, which have 14, 17, 38, 17, and 13%, respectively, of the total. Thus, the overlap between the distribution of GAIP and that of $G\alpha_{i3}$ is limited to fraction 3 and is minimal (14%). Fractions were lysed in Laemmli buffer, and proteins from each fraction were separated by SDS-PAGE, transferred onto PVDF membranes, reacted with anti-GAIP (C) antiserum (diluted 1:2000) followed by goat anti-rabbit IgG coupled to HRP (1:3000), and immunoreactivity was detected by chemiluminescence (ECL).

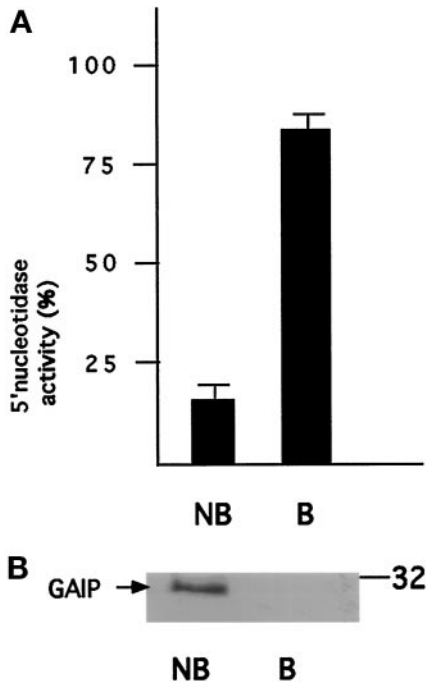

Figure 3. GAIP is associated with intracellular membranes in pituitary cells. GAIP-containing fractions 3–7 prepared from AtT-20 cells (see Figure 2) were pooled and diluted in PBS with protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin A), and 1.0-ml aliquots were incubated at 4 C overnight with WGA-agarose (400 μg/ml) to deplete them of PM. Bound (B) and nonbound (NB) membranes were assayed for 5′-nucleotidase activity (upper panel) and solubilized and immunoblotted for GAIP (lower panel). Most (80%) of the 5′-nucleotidase activity is found in the bound fraction. GAIP is found in the nonbound fraction. Data are means ± SD of three independent experiments.

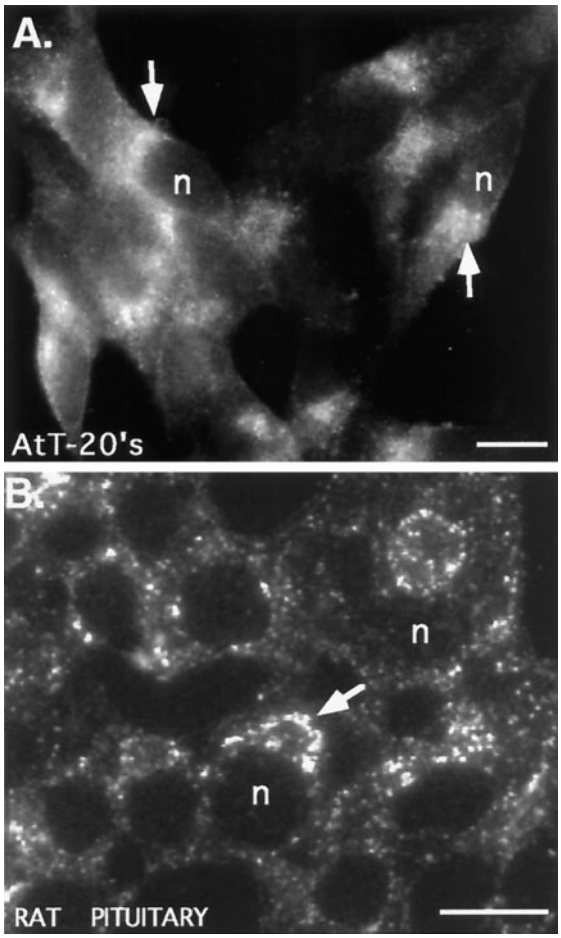

Figure 4. GAIP is concentrated in the Golgi region (arrows) in AtT-20 pituitary cells (A) and in rat pituitary tissue (B). No staining of the plasma membrane is seen. AtT-20 cells or semithin cryosections of rat pituitary tissue were fixed in 2% paraformaldehyde in 100 mM phosphate buffer, incubated with affinity-purified, anti-GAIP (C) IgG followed by donkey anti-rabbit F(ab′)$_2$-Texas Red, and examined by immunofluorescence. n, Nucleus. Scale bars, 10 μm.

MATERIALS AND METHODS

Materials

Male rats (100–150 g) were from Harlan Sprague Dawley (Indianapolis, IN), DMEM medium from the UCSD Cell Core Facility (La Jolla, CA), FCS from GIBCO-BRL (Gaithersburg, MD), and horse serum from Hyclone Laboratories (Logan, UT). Wheat germ agglutinin (WGA)-agarose and the 5′-nucleotidase kit were purchased from Sigma Chemical (St. Louis, MO). The ECL detection kit was from Amersham Life Sciences (Arlington Heights, IL). FITC-conjugated donkey anti-rabbit F(ab′)$_2$ and Texas Red-conjugated donkey anti-mouse F(ab′)$_2$ cross-absorbed against human, mouse, rat, chicken, and goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-rabbit or anti-mouse IgG conjugates (5 or 10 nm gold) were purchased from Amersham.

Antibodies

Antiserum was prepared against human GAIP$_{23–217}$, which includes the RGS domain (amino acids 80 to 206), shared with other RGS family members. Antisera were also generated against the N terminus and C terminus of GAIP, which are unique. GAIP$_{23–217}$ was subcloned into pGEX-KG, expressed as a glutathione S-transferase (GST) fusion protein that was affinity purified on glutathione agarose beads, and injected into rabbits. For the N-terminal–specific antiserum, a PCR fragment of human GAIP DNA (coding for residues 1–79) was cloned into 5′ *Eco*RI and 3′ *Sal*I sites of the pET28a vector (Novagen, Madison, WI). His6-tagged N-terminal GAIP protein was produced in *Escherichia coli* (strain BL21(DE3)), purified by affinity chromatography, and injected into rabbits. For the C-terminal–specific antiserum, a peptide, QGPSQSSSEA, corresponding to the last 10 amino acids of GAIP (208–217), was coupled to keyhole limpet hemocyanin and injected into rabbits. The antiserum was affinity purified on the same peptide. The N-terminal antiserum, anti-GAIP (N), recognized 10 ng affinity-purified full-length GST-GAIP by immunoblotting at 1:4000, and the affinity-purified C-terminal IgG, anti-GAIP (C), detected 40 ng GST-GAIP at 1.2 μg/ml. All antisera recognized a single, 25-kDa band by immunoblotting (Figure 1A) or immunoprecipitation (Figure 1B) of a lysate prepared, respectively, from unlabeled or ^{35}S-methionine–labeled AtT-20 cells stably expressing HA-GAIP (De Vries *et al.*, 1996).

MAb 16B12 against the hemagglutinin (HA) epitope was purchased from BABCO (Berkeley, CA). Polyclonal antiserum to calnexin was a gift from J. Bergeron (McGill University, Montreal,

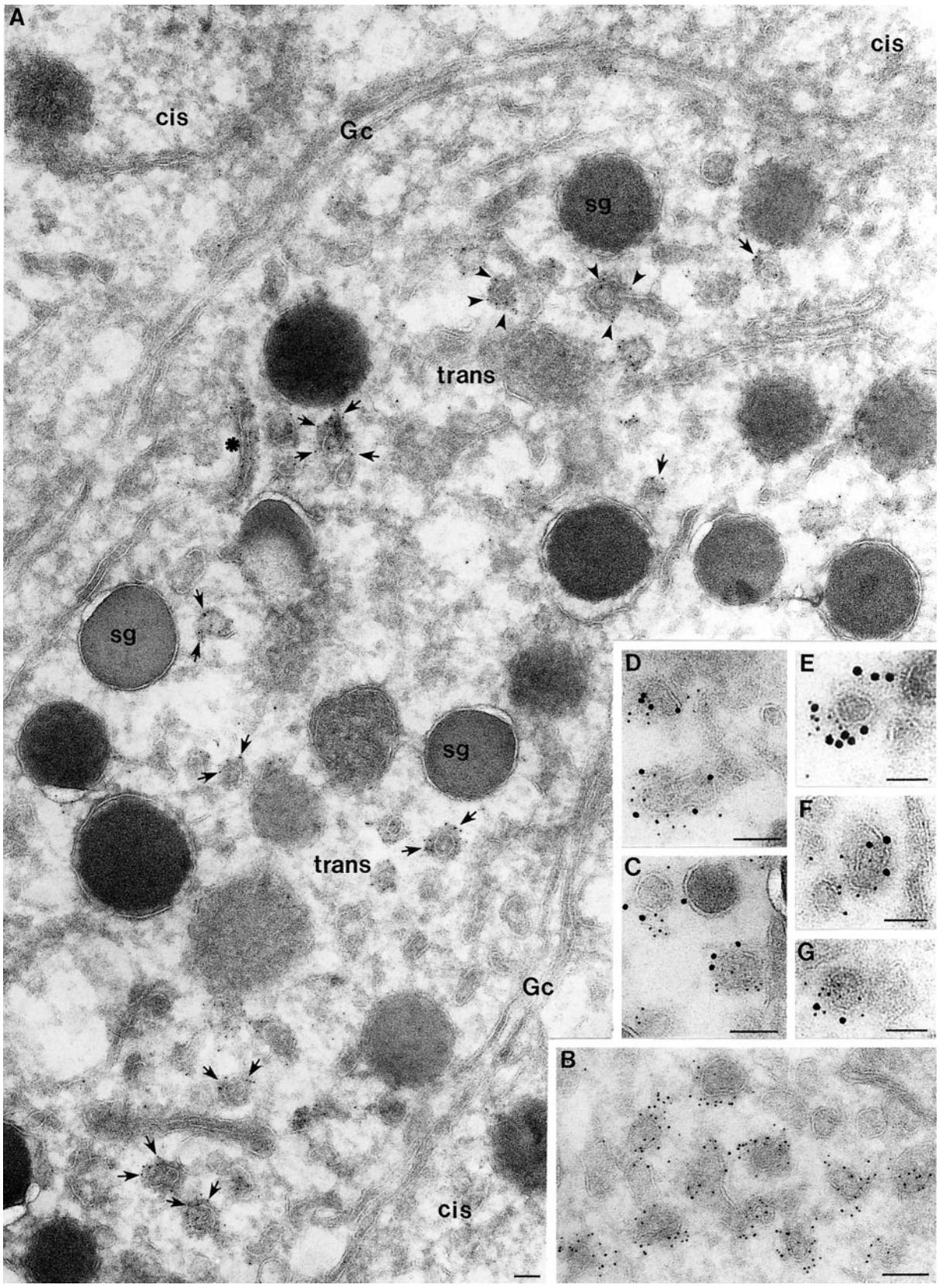

Figure 5.

Quebec, Canada). Mouse monoclonal antibodies against bovine and human clathrin (heavy chain) were obtained from Polysciences (Warrington, PA) and Dr. Francis Brodsky (University of California, San Francisco, CA), respectively. Polyclonal antiserum to caveolin was purchased from Transduction Laboratories (Lexington, KY). The EC polyclonal antibody against $G\alpha_{i3}$ was kindly provided by Dr. A. Spiegel (NIDDK, Bethesda, MD). Rabbit antiserum to α-mannosidase II (Man II) was prepared as described (Velasco *et al*., 1993).

Cell Culture

Murine AtT-20/D-16v pituitary cells, obtained from Richard Mains (Johns Hopkins University, Baltimore, MD), were grown in DME-high glucose, supplemented with 10% horse serum, 2.5% FCS, penicillin G, and streptomycin sulfate. AtT-20 cells stably expressing HA-tagged GAIP were prepared and grown as described previously (De Vries *et al*., 1996).

Subcellular Fractionation

AtT-20 cells were fractionated as described by Wendland and Scheller (1994). Briefly, cells of two confluent 100-mm plates were combined by scraping into 1 ml ice-cold PBS containing PMSF (1 mM) and aprotinin (100 U/ml). All the following steps were performed at 4°C. The cells were homogenized by ten passages through a 30.5 gauge needle, and a postnuclear supernatant (PNS) was prepared by centrifugation for 10 min at 5000 × *g*. Five hundred microliters were layered on a discontinuous sucrose gradient (0.2, 0.4, 0.6, 1.0, 1.4, and 1.8 M, 750 μl each) and centrifuged for 2 h at 24,000 rpm (Beckman SW50.1 rotor). Twelve fractions (400 μl) were collected from the top and centrifuged for 1 h at 100,000 × *g*. The resultant pellets were solubilized in Laemmli sample buffer, and the proteins were separated by SDS/PAGE. Quantitation of the amount of GAIP in cell fractions was obtained by densitometry using the ScanAnalysis program (Biosoft, Cambridge, United Kingdom).

Rat liver homogenization and preparation of cytosol and total microsomal membranes were as described previously (Jin *et al*., 1996). Briefly, total microsomal membranes were adjusted to 1.24 M sucrose and loaded at the bottom of a 32-ml discontinuous sucrose gradient composed of 8 ml each of 1.18, 1.15, 0.86, and 0.25 M sucrose and centrifuged at 82,000 × *g* (25,000 rpm, SW28 rotor) for 3 h. Bands at the interface between 0.25 M/0.86 M and 0.86 M/1.15 M sucrose, enriched in Golgi elements, were collected and designated Golgi light and Golgi heavy fractions (Saucan and Palade, 1994). Fractions 1.15 and 1.18 were defined as carrier vesicle fraction 1 and 2 (CV1 and CV2), and fraction 1.24 was defined as the residual microsome fraction (RM) (Jin *et al*., 1996). The protein concentration of each fraction was determined by BCA assay (Pierce Chemical Co., Rockford, IL), and 50 μg of protein of each fraction were solubilized in Laemmli sample buffer and separated by SDS/PAGE.

SDS-PAGE and Immunoblotting

Proteins were separated on 10% or 12% SDS gels using a Bio-Rad minigel apparatus. After electrophoresis, the separated proteins were transferred to polyvinylidinedifluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were incubated with primary antibodies followed by secondary antibodies (anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase, Bio-Rad) in 5% calf serum/PBS, 0.1% Tween 20, for 1 h each, washed three times for 15 min each between each incubation, and detected by ECL according to the manufacturer's instructions.

Depletion of Plasma Membranes from Subcellular Fractions by WGA-Agarose Absorption

WGA-agarose absorption was performed as described previously (Denker *et al*., 1996). Briefly, GAIP-containing fractions were diluted in PBS with protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin A). Aliquots (1.0 ml) were incubated at 4°C overnight with WGA-agarose (400 μg/ml). The bound fraction was collected by sedimentation (1,000 × *g*, 5 min), and the nonbound fraction (PM-depleted membranes), was pelleted (100,000 × *g*, 1 h) and solubilized in Laemmli sample buffer. Membrane proteins were released from WGA-agarose by adding sample buffer, followed by boiling for 5 min, and separated by SDS/PAGE and immunoblotting.

5′-Nucleotidase Assay

GAIP-containing fractions (as detected by immunoblotting) from AtT-20 cells and rat liver were pooled, and 5′-nucleotidase assays were performed on the pooled fractions as described by the manufacturer. WGA-bound and nonbound fractions were prepared as described above, and the beads (bound fraction) were resuspended in the same volume as the nonbound fraction. 5′-Nucleotidase activity was measured on the total volume of both fractions and on non-WGA–treated controls.

Immunocytochemistry

For immunofluorescence, AtT-20 cells were fixed in 2% paraformaldehyde (PFA) in 100 mM phosphate buffer, pH 7.4, for 1 h, followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min. Cells were then incubated 1 h at room temperature with primary antibody, followed by a 1-h incubation in cross-absorbed FITC-conjugated donkey anti-rabbit $F(ab')_2$. Cells were washed and mounted in 25% PBS, 75% glycerol with 1 mg/ml *p*-phenylenediamine.

For immunogold or immunofluorescence labeling of cryosections, rat pituitary and liver were perfusion fixed in 8% PFA, 100 mM phosphate buffer, pH 7.4 (15 min), followed by 4% PFA in phosphate buffer (1 h). Samples were cryoprotected and frozen in liquid nitrogen as described (Hobman *et al*., 1992; McCaffery and Farquhar, 1995). Semithin (0.5–1.0 μm) cryosections were prepared and incubated with primary rabbit polyclonal or mouse monoclonal antibodies (3 h at 4°C) followed by incubation in cross-absorbed FITC-conjugated donkey anti-rabbit or TRITC-conjugated donkey anti-mouse $F(ab')_2$ (1 h at 4°C). Ultrathin cryosections were prepared and incubated 2 h at 4°C with primary antibodies in 10% FCS/PBS, followed by 5- or 10-nm gold-conjugated goat anti-rabbit or anti-mouse IgG (2 h at 4°C). Grids were stained in 2% neutral uranyl acetate (10 min), adsorption-stained with 0.2% neutral uranyl acetate, 0.2% methyl cellulose, and 3.2% polyvinyl alcohol, and observed in a JEOL 1200 EX-II (JEOL USA, Peabody, MA) or Philips CM-10 electron microscope (Philips Electronic Instruments, Mahwah, NJ).

Figure 5 (facing page). GAIP is found on CCVs located in the Golgi region of rat pituitary cells by immunoelectron microscopy. (A–B) Ultrathin cryosections of pituitary sections labeled with affinity-purified anti-GAIP (C) IgG detected with 5 nm gold. GAIP is localized predominantly on the outer (cytoplasmic) surface of coated buds (arrowheads) or vesicles (arrows) found on the trans side (trans) of the Golgi cisternae (Gc) in a somatotrope (growth hormone-producing cell). Only rarely is GAIP seen on tubular elements with buds (asterisk), which usually are not labeled. Secretory granules (sg), also concentrated on the trans side of the stack, are found in the vicinity of the labeled vesicles. (C–G) Similar preparations of sections double labeled for GAIP (5 nm gold) and clathrin (10 nm gold) showing their presence on the same vesicles. Rat pituitary was fixed in 8% paraformaldehyde (PFA), 100 μM phosphate buffer, pH 7.4 (15 min), followed by 4% PFA in phosphate buffer (1 h), and ultrathin cryosections were incubated as described in MATERIALS AND METHODS. Scale bars, 0.1 μm.

For quantitation, the number of gold particles/μm PM length was determined by counting the number of gold particles and tracing the membrane contour with a cartographer's wheel. The number of gold particles/μm membrane of coated buds and coated vesicles was determined as follows: gold particles on coated buds or CCVs were counted in 16 fields each from pituitary and liver containing a total of 65 vesicles or buds. The diameter of the coated buds or CCVs was measured, and the mean diameter (~90 nm) and average circumference (2πr X 45 = 0.28 μm) were calculated. The results were expressed as gold particles/μm membrane.

RESULTS

While the function of GAIP as a GAP and the fact that it is anchored to membranes have been established, its localization in the cell has not been determined. To localize endogenous GAIP, we prepared GAIP-specific antibodies against the C and N termini of GAIP (outside the 120-amino acid RGS domain) and determined the distribution of GAIP by cell fractionation and by immunofluorescence and immunogold labeling on cultured AtT-20 pituitary cells and rat liver and pituitary tissue because they express high levels of endogenous GAIP (De Vries *et al.*, 1995).

GAIP Fractionates with Intracellular Membranes in Pituitary Cells

Initially we prepared mouse pituitary AtT-20 cell fractions on sucrose density gradients using a procedure designed to separate light membranes (PM, Golgi, and other smooth membranes) from heavy membranes (ER, dense-core vesicles, and secretory granules) (Wendland and Scheller, 1994). When the fractions were solubilized and immunoblotted for marker proteins, calnexin, a resident ER protein (Wada *et al.*, 1991), sedimented with heavy fractions and peaked in fractions 10–12, whereas the Golgi marker Man II (Velasco *et al.*, 1993) was found only in light fractions (1–3) (Figure 2). GAIP was found in fractions 3–7 of intermediate density (Figure 2), whereas $G\alpha_{i3}$ codistributed with Man II in light fractions (1–3). Thus the distribution of GAIP and $G\alpha_{i3}$ overlapped only in fraction 3, which contained 14% of the total GAIP in the gradient, with the remainder (86%) found in fractions 4–7. To eliminate the possibility that the presence of GAIP was due to contaminating PM, we treated fractions 3–7 with WGA-Agarose to selectively remove PM (Denker *et al.*, 1996). As anticipated, WGA-bound fractions were enriched in the PM marker 5′-nucleotidase (Figure 3A) (Drummond and Masanobu, 1971); however, GAIP remained in the nonbound fraction and was not detected in the bound fraction (Figure 3B). These results indicate that, in AtT-20 pituitary cells, GAIP is associated with intracellular membranes, not the PM, and has a distinct distribution from ER, Golgi, or PM markers.

GAIP Is Localized on Clathrin-coated Buds or Vesicles Located Near the TGN in Pituitary Cells

When GAIP was localized by immunofluorescence in AtT-20 cells (Figure 4A) and semithin cryosections of rat pituitary (Figure 4B), punctate staining for GAIP was distributed throughout the cytoplasm but was concentrated in the Golgi region. The punctate pattern was most evident in semithin cryosections (arrow, Figure 4B). No PM staining was observed.

To determine the nature of the compartment in which GAIP is located, we carried out immunogold labeling at the EM level on ultrathin cryosections of rat pituitary. We detected GAIP on coated vesicles, 70–100 nm diameter, located mainly on the trans side of the Golgi stack (Figure 5, A and B, and Figure 6A) or on coated buds in continuity with tubular cisternae (Figure 6,B–D). When sections were double labeled for clathrin, the major coat protein of CCVs (Brodsky, 1997; Robinson, 1997), clathrin, and GAIP were localized on the same vesicles (Figure 5, C–G). No staining of the PM was evident (Figure 6A).

The abundant GAIP labeling of coated buds contrasted markedly with the sparse labeling of the membranes of the tubular cisternae with which they were in continuity. Counts of gold particles/μm membrane revealed that the concentration of gold particles on coated buds and vesicles (10.4 gold particles/vesicle or 37/μm membrane) was 93× that of the membranes of the tubular cisternae with which they were in continuity (0.4/μm membrane). We conclude that GAIP is concentrated on clathrin-coated buds and CCVs located on the trans side of the Golgi stack in rat pituitary cells.

GAIP Is Concentrated in Vesicular Carrier-enriched Fractions from Rat Liver

Next we determined the distribution of GAIP in rat liver—a well characterized system in which fractions enriched in carrier vesicles can be separated from Golgi and other membranes by sucrose gradient centrifugation (Saucan and Palade, 1994; Jin *et al.*, 1996). Golgi light (GL), Golgi heavy (GH), carrier vesicle 1 and 2 (CV1 and CV2), and RM fractions were prepared, solubilized, and immunoblotted for GAIP and marker proteins. GAIP was most abundant in CV1, CV2, and RM fractions, with the peak in CV2 (Figure 7). No GAIP signal was detected under these conditions in the GL fraction, and only trace amounts of GAIP were found in the GH fraction. Densitometric analysis established that GAIP is 18× more abundant in CV1 and 26× more abundant in CV2 than in GH. CV1 and CV2 fractions typically contain a mixture of transcytotic vesicles, vesicles derived from early and late endosomes, TGN-derived vesicles, and ER-to-Golgi transport vesicles (Saucan and Palade, 1994; Jin *et al.*, 1996). $G\alpha_{i3}$

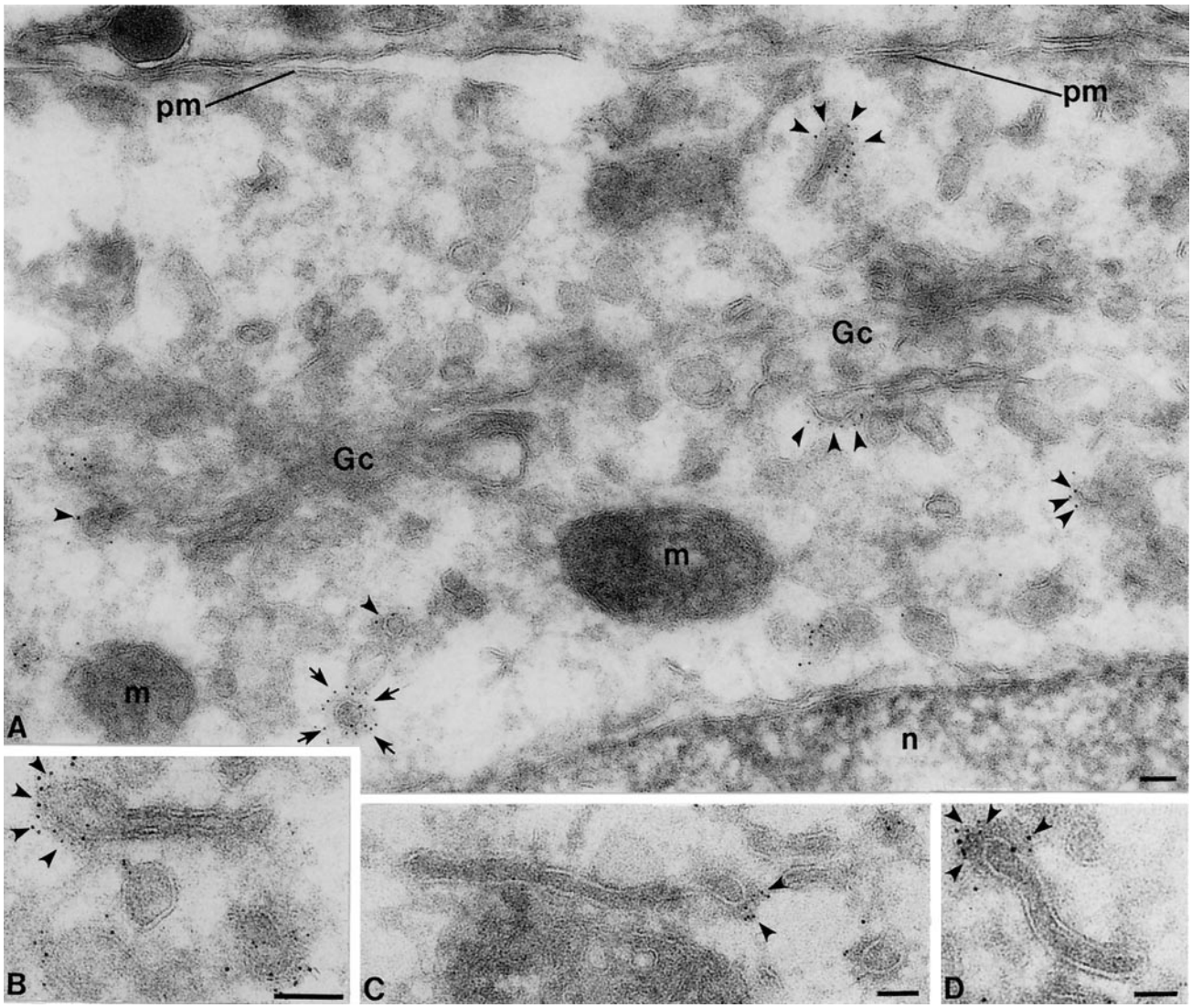

Figure 6. GAIP is present on clathrin-coated buds (arrowheads) and CCVs (arrows) but is not detected on the PM (pm) or tubular cisternae with coated buds. (A) Numerous coated buds located in the Golgi region are heavily labeled for GAIP. Counts of gold particles indicate that labeling of buds is ~90× higher than that on the tubular cisternae with which they are in continuity. The plasma membrane (pm) and Golgi membranes (Gc) are not labeled above background. (B–D) Enlargement of tubular cisternae with coated buds heavily labeled for GAIP. Note the lack of labeling of the tubular cisternae with which the buds are in continuity. Preparation same as Figure 5. Scale bars, 0.1 μm.

showed a much broader distribution across the gradient than GAIP (Figure 7) but was most abundant in the GH and CV1 fractions. The Golgi marker Man II was found mainly in the Golgi fractions (GL and GH) as expected (Figure 7). Interestingly, GAIP did not cofractionate with caveolin (Figure 7), a protein said to be associated with G protein-signaling complexes (Li *et al*., 1995). To prove that the GAIP signal was not due to PM contamination of the CV1, CV2, and RM fractions, we pooled these fractions and treated them with WGA-agarose to remove PM. We obtained similar results as with AtT-20 cells: the PM marker 5′-nucleotidase was highly enriched in the WGA-bound fraction (Figure 8A), but GAIP was found exclusively in the nonbound fraction (Figure 8B). From these results we conclude that 1) GAIP is associated with intracellular membranes rather than the PM in rat liver; and 2) GAIP is most abundant in fractions enriched in transport vesicles.

GAIP Is Localized on Clathrin-coated Pits and Vesicles Located Near the PM in Rat Liver

To determine the nature of the transport vesicles with which GAIP is associated, we carried out immunofluorescence on semithin cryosections of rat liver. With

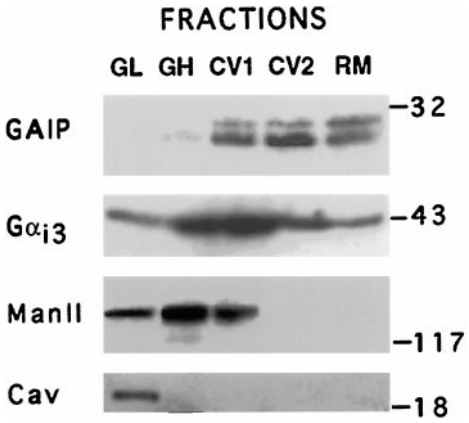

Figure 7. Distribution of GAIP in fractions prepared from rat liver. GAIP is concentrated in fractions enriched in carrier vesicles (CV1 and CV2). It is not detected in Golgi light (GL) fractions and is barely detectable in Golgi heavy (GH) fractions. Rat liver was homogenized and fractions were prepared by sucrose gradient centrigugation as described in MATERIALS AND METHODS. Fifty micrograms each of Golgi light (GL), Golgi heavy (GH), carrier vesicles 1 and 2 (CV1 and CV2), and residual microsome (RM) fractions were solubilized in Laemmli buffer and immunoblotted for GAIP (N-terminal antibody, dilution 1:4000), $G\alpha_{i3}$ (EC antibody, dilution 1:1000), Man II (dilution 1:2000), and caveolin (dilution 1:4000) as in Figure 1. The presence of two bands for GAIP is likely due to posttranslational modification (palmitoylation or phosphorylation).

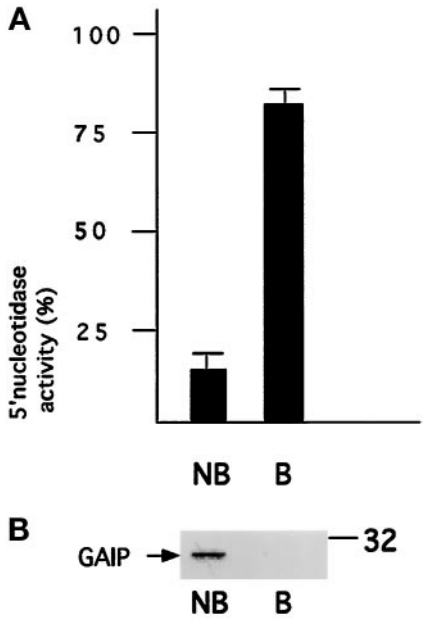

Figure 8. GAIP is associated with intracellular membranes in rat liver. Fractions CV1, CV2, and RM (see Figure 7) were pooled and treated with WGA to remove PM. Bound (PM) and nonbound (intracellular membranes) fractions were assayed for 5′-nucleotidase activity (A) or solubilized and immunoblotted for GAIP (B). Most (85%) of the 5′-nucleotidase activity is found in the bound fraction (B), whereas GAIP is found only in the nonbound fraction (NB). Data are means ± SD from three independent WGA treatments.

anti-GAIP antibodies, punctate staining was seen at the periphery of hepatocytes, particularly along the sinusoidal domain of the PM (Figure 9B). Little or no staining for GAIP was seen in the Golgi region. This suggested that GAIP is located at or near the PM. Double labeling for GAIP and clathrin showed a striking overlap in their distribution at the cell periphery, suggesting these proteins may reside in the same structures (Figure 9C). To determine whether this is the case, we carried out immunogold labeling on rat liver sections with affinity-purified anti-GAIP (C) antibodies. We found GAIP associated with coated vesicles located near the PM (Figure 10, A–E). Occasionally, labeling for GAIP was found on coated pits distributed along the sinusoidal PM (Figure 10F), suggesting GAIP's association with endocytic vesicles. By contrast, the PM adjacent to the coated pits showed little staining. Counts of gold particles indicated that GAIP is 60× more concentrated on coated buds or vesicles (7.6 gold particles/vesicle or 27/μm membrane) as on the noncoated regions in continuity with the coated buds (0.45 gold particles/μm membrane). By double immunogold labeling for GAIP and clathrin, GAIP colocalized with clathrin on the same vesicles (Figure 10, G–I). We also found variable mitochondrial staining with anti-GAIP antibodies, an observation that is currently under investigation. We conclude that in rat liver, GAIP is localized on coated pits and CCVs, very likely of endocytic origin.

DISCUSSION

We showed previously that GAIP is anchored to membranes by palmitoylation, most likely via its cysteine string motif (De Vries *et al*., 1996). Palmitoylation is a reversible lipid modification and, in the case of $G\alpha_i$ proteins, is considered to regulate their association with specific subdomains of the PM (Wedegaertner *et al*., 1995; Mumby, 1997). Here we show that GAIP cosediments with intracellular membranes by cell fractionation and is not detected in WGA-purified PM, indicating that GAIP is located on intracellular membranes rather than the PM. By immunofluorescence and immunoelectron microscopy, we clearly established that GAIP is found on clathrin-coated buds and CCVs in several cell types. Two general subclasses of CCVs are distinguished based on their origin: 1) CCVs derived from the TGN involved in the transport of lysosomal enzymes and membrane proteins to late endosomes and/or lysosomes, and 2) CCVs derived from the PM or from endosomes involved in receptor-mediated endocytosis (Brodsky, 1997; Robinson, 1997). GAIP appears to be present on both general subclasses of CCVs, with the TGN-derived CCVs predominating in pituitary cells and PM-derived CCVs predominating in hepatocytes and several cultured cell lines (NRK, HeLa). TGN-derived CCVs are known

to carry lysosomal enzymes bound to mannose 6-phosphate receptors destined for delivery to lysosomes via early or late endosomes. PM (coated pit)-derived CCVs are involved in the internalization of a number of receptors and their cargo including G protein-coupled receptors (Zhang *et al*., 1996). Recently, additional less well characterized populations of CCVs have been described whose functions are unclear (Robinson, 1997). GAIP is widely expressed in many tissues (pituitary, liver, lung, placenta, heart), but its expression is low in brain (De Vries *et al*., 1995), which is a rich source of CCVs. Whether GAIP is present on all CCVs or only on specific subpopulations of CCVs remains to be established.

GAIP is the first GAP to be localized on CCVs. Except for ras-GAP whose putative relocation to the PM has been documented (Clark *et al*., 1991), all other GAPs are cytosolic proteins. GAIP is the first RGS protein to be located within the cell, and with the exception of RGS3 and SST2, there is little information on whether or not other family members are membrane associated. RGS3 has been shown to sediment in membrane fractions (Neill *et al*., 1997), and Sst2, the yeast RGS, codistributes on sucrose density gradients with PM and Golgi markers and with Gpa1, the yeast Gα subunit with which it interacts (Dohlman *et al*., 1996). The structure of RET-RGS, found in retina, suggests it may be a membrane protein because it has a predicted transmembrane domain and a cysteine string motif similar to that found in GAIP (Faurobert and Hurley, 1997).

Our cell fractionation results indicate little overlap in the distribution of GAIP and $G\alpha_{i3}$ in AtT-20 pituitary cells and only a partial overlap in rat liver. $G\alpha_{i3}$ has been localized to Golgi membranes and to caveolae-enriched fractions (Sargiacomo *et al*., 1993) but not to CCVs in the cell models and tissues previously studied, including rat pituitary (Wilson *et al*., 1994) and pancreas (Denker *et al*., 1996). Interestingly, it has recently been reported that GAIP regulates a $G\alpha_{i3}$-dependent autophagy pathway in intestinal cells (Ogier-Denis *et al*., 1997).

It is still not clear whether $G\alpha_{i3}$ acts through classical or alternative G protein cycles on intracellular membranes, since no G protein-coupled receptors, effectors, or $\beta\gamma$ subunits have been detected to date on intracellular membranes (Helms, 1995). The localization of GAIP on clathrin-coated buds and CCVs suggests a model for regulation of $G\alpha_{i3}$ in which GAIP and $G\alpha_{i3}$ are located on different membrane domains, and GAIP is transported via vesicular transport to its target Gα subunit. According to this model, only upon fusion of the vesicle with its target would the GAIP on vesicles and the Gα subunit on membranes come into transient contact. Alternatively, GAIP might function to turn off G protein signaling during budding. However, its presence on CCVs suggests GAIP's involvement in downstream events. In either model the possibility should be considered that GAIP could act, not only as a GAP to turn off G protein signaling, but also as an effector antagonist (Berman and Gilman, 1998). Spatial separation may provide an additional layer of regulation to assure that the GAP and its target are only transiently brought into contact via vesicular transport

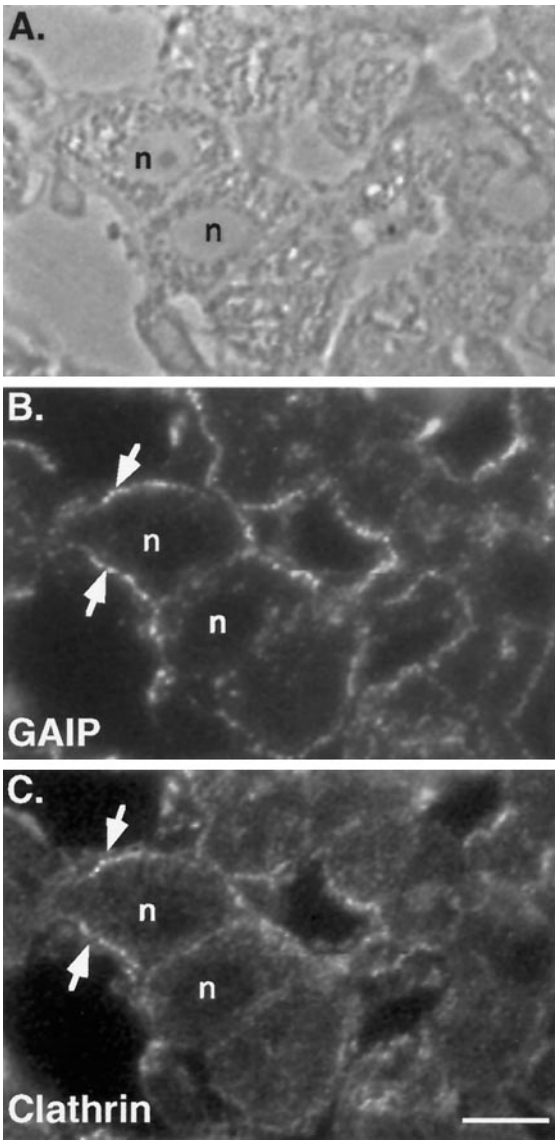

Figure 9. GAIP is distributed near the PM in rat liver. Staining for both GAIP (B) and clathrin (C) is found at the periphery of hepatocytes concentrated along the sinusoidal domain of the PM (arrows). The corresponding phase contrast image is shown in panel A. Semithin cryosections of rat liver were fixed in 2% paraformaldehyde in 100 mM phosphate buffer, pH 7.4, for 1 h, and incubated with affinity-purified, anti-GAIP (C) IgG followed by donkey anti-rabbit $F(ab')_2$-Texas Red, and examined by immunofluorescence. n, Nucleus. Scale bars, 10 μm.

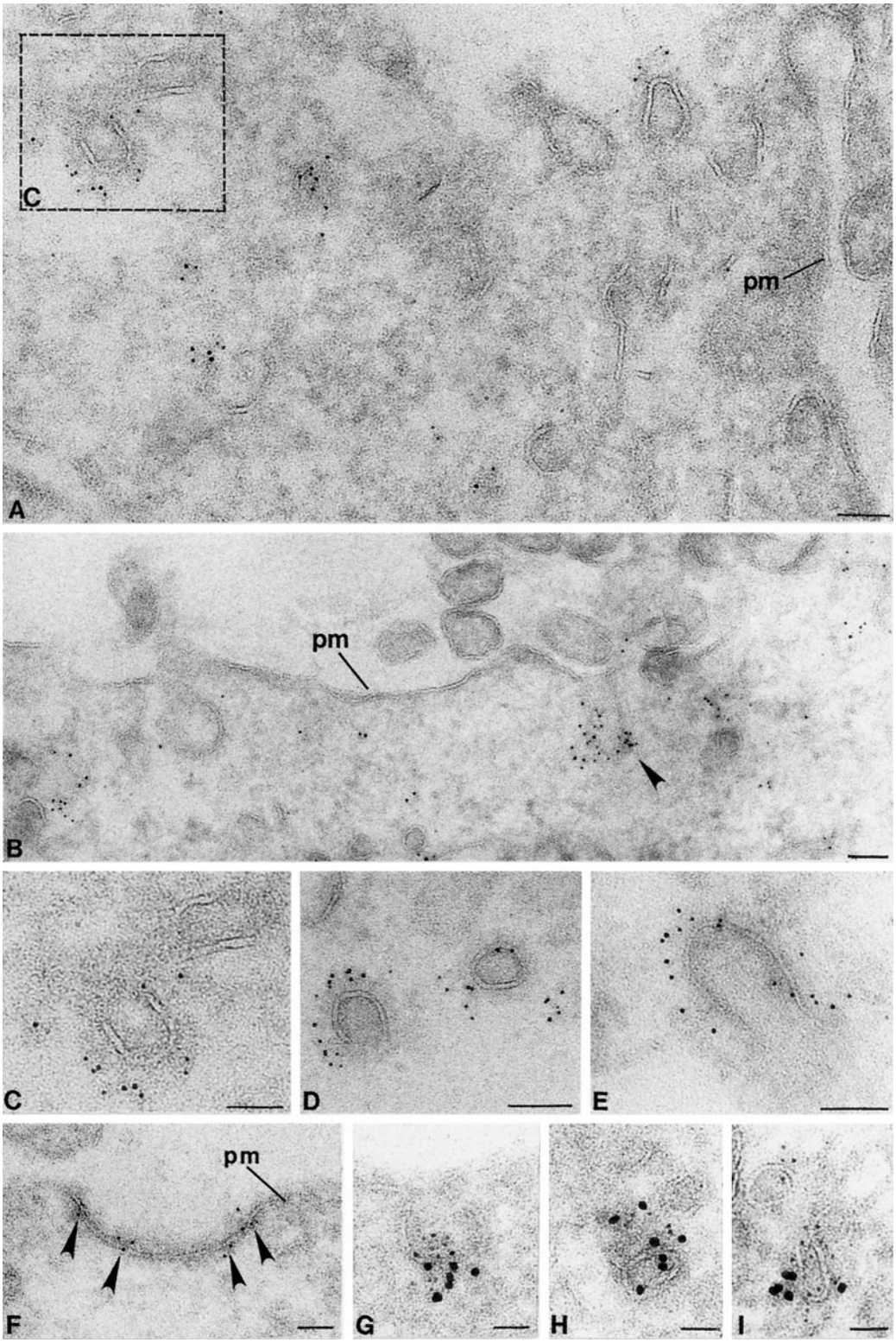

Figure 10. Immunogold labeling demonstrating GAIP's association with clathrin-coated pits and vesicles located near the sinusoidal PM in rat liver. (A and B) Numerous vesicles are labeled for GAIP. Note that the PM (pm) itself with which the coated pits are sometimes in continuity is not labeled. (G–I) Double labeling for GAIP (5 nm gold) and clathrin (10 nm gold) demonstrating their presence on the same vesicles. Rat liver was fixed in 8% paraformaldehyde (PFA), 100 mM phosphate buffer, pH 7.4 (15 min), followed by 4% PFA in phosphate buffer (1 h), and incubated as in Figure 4. Scale bars, 50 nm.

when a particular signal transduction cascade is activated.

Our work raises several intriguing questions: 1) How is GAIP targeted to CCVs, i.e., are there specific targeting signals encoded in its sequence? Or, does GAIP's interaction with other proteins determine its localization? 2) Are other RGS proteins found on transport vesicles or do they have compartment-specific or domain-specific localizations? 3) What purpose does the concentration of GAIP on CCVs serve?

Our findings suggest that the cell has evolved a system for regulation of intracellular G protein signaling that keeps the inactivators (GAPs) separated from the activators (G proteins). Thus, GAIP's localization on CCVs suggests a new paradigm for G protein signaling during vesicular transport.

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