GAIP, a Gαi-3-binding protein, is associated with Golgi-derived vesicles and protein trafficking

FIONA WYLIE, KIRSTEN HEIMANN, TAM LUAN LE, DARREN BROWN, GLENN RABNOTT, AND JENNIFER L. STOW
Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, Queensland 4072, Australia

Wylie, Fiona, Kirsten Heimann, Tam Luan Le, Darren Brown, Glenn Rabnott, and Jennifer L. Stow. GAIP, a Gαi-3-binding protein, is associated with Golgi-derived vesicles and protein trafficking. Am. J. Physiol. 276 (Cell Physiol. 45): C497–C506, 1999.—Proteins of the regulators of G protein signaling (RGS) family bind to Gα subunits to downregulate their signaling in a variety of systems. Gα-interacting protein (GAIP) is a mammalian RGS protein that shows high affinity for the activated state of Gαi-3, a protein known to regulate post-Golgi trafficking of secreted proteins in kidney epithelial cells. This study aimed to localize GAIP in epithelial cells and to investigate its potential role in the regulation of membrane trafficking. LLC-PK1 cells were stably transfected with a c-myc-tagged GAIP cDNA. In the transfected and untransfected cells, GAIP was found in the cytosol and on cell membranes. Immunogold labeling showed that membrane-bound GAIP was localized on budding vesicles around Golgi stacks. When an in vitro assay was used to generate vesicles from isolated rat liver and Madin-Darby canine kidney cell Golgi membranes, GAIP was found to be concentrated in fractions of newly budded Golgi vesicles. Finally, the constitutive trafficking and secretion of sulfated proteoglycans was measured in cell lines overexpressing GAIP. We show evidence for GAIP regulation of secretory trafficking before the level of the trans-Golgi network but not in post-Golgi secretion. The location and functional effects of GAIP overlap only partially with those of Gαi-3 and suggest multiple roles for GAIP in epithelial cells.

HETERO TrIMERIC G PROTEINS regulate a variety of signal transduction pathways in eukaryotic cells, such as the photoreception mechanism, hormone receptor signaling, ion channel regulation, and membrane trafficking (4, 21, 48). In recent years, G protein subunits have been localized on a variety of organelle membranes, including the endoplasmic reticulum (1), the Golgi complex (20), and endosomal structures (8). In addition, it has been demonstrated that vesicle trafficking in the secretory pathway is regulated at multiple steps by heterotrimeric G proteins (33, 42, 44, 49, 51). The signal transduction pathways regulating vesicular transport are not well defined. Effectors, receptors, and other regulators of heterotrimeric G proteins in these pathways remain to be characterized.

GAIP-interacting protein (GAIP) is a member of the recently described regulators of G protein signaling (RGS) family (17, 30, 46). These proteins are able to negatively regulate heterotrimeric G protein activity through specific binding to Gα subunits. The RGS functional prototype is Sst2 in yeast, a phoromone-sensitive agent that negatively regulates the activity of Gpa1 (6, 16, 18). There are at least 21 RGS proteins already cloned and, with the exception of the cholera-sensitive subunit Gαs, all other Gα subunits bind one or more of the RGS proteins with varying affinities (38). GAIP was identified in a yeast two-hybrid screen using Gαi-3 as the bait (14). In vitro, GAIP binds activated forms of Gαi-3 with high affinity and other members of the Gαi subfamily with varying but lower affinities. GAIP and other RGS proteins have been shown to act as GTPase-activating proteins (GAPs) for heterotrimeric G proteins; the kinetics of this activity differ according to the binding affinity for the interacting Gα subunit (3, 7, 52). RGS proteins are characterized by a stretch of 125 amino acids (19, 46) (the RGS domain) that interacts with the Gα subunit (14) and mediates the GTPase activity of the RGS protein (43). In addition to in vitro experiments, this GTPase activity has been demonstrated in defined G protein pathways in intact cells or microsomal fractions (19, 26, 39). Recently, RGS9 was identified as a highly efficient GAP for Gαt (transducin) in the retina, where, in conjunction with the cGMP phosphodiesterase effector, it regulates recovery of visual transduction (24). GAIP thus has the potential to act as a powerful regulator of Gαi-3 signaling pathways. Due to the localization of heterotrimeric G proteins on Golgi membranes and the demonstrated involvement of Gαi-3 in vesicular transport, the RGS protein GAIP was investigated as a possible player in the regulation of protein trafficking. Data in two recent studies indicate that GAIP is involved in vesicular trafficking. In colonic epithelial cells, GAIP was demonstrated to regulate trafficking in the autophagic sequestration pathway, and the effects of overexpressing GAIP were consistent with its function as a GAP for Gαi-3 in this pathway (41). Another recent report shows that, in pituitary and liver cells, membrane-bound GAIP is localized on clathrin-coated vesicles (13). The full inventory of trafficking pathways involving GAIP now stands to be elucidated. The locations and roles of GAIP may vary between cell types, as do those of Gαi-3. The specific membrane locations of GAIP and other RGS proteins might therefore serve as a preliminary indication of their links with specific Gα subunits and their potential roles in different steps in vesicle trafficking. In this paper, we have...
investigated the distribution of GAIP on intracellular membranes in kidney epithelial cells, its interactions with membrane-bound G\textsubscript{O\textalpha,3}, and its involvement in G\textsubscript{O\textalpha,3}-regulated trafficking in the secretory pathway.

**MATERIALS AND METHODS**

Antibodies. Polyclonal antibodies were raised in both chickens and rabbits against human GAIP expressed as a glutathione S-transferase (GST) fusion protein. Animals were injected with glutathione-purified GST-GAIP protein. IgG fractions were purified from chicken egg yolks (Eggstract, Promega, Madison, WI), and rabbit sera were affinity purified over cyanogen bromide-activated Sepharose conjugated to the GST-GAIP fusion protein, and then antibodies were additionally affinity purified on thrombin-cleaved GAIP peptide columns. Antisera were tested for reactivity and specificity by immunoblotting on GST-GAIP and on cell extracts (see Fig. 1). An affinity-purified goat polyclonal antibody (C-20) raised against a 20-residue GAIP peptide (197–216) was additionally affinity purified on thrombin-cleaved GAIP peptide columns. Antisera were tested for reactivity and specificity by immunoblotting on GST-GAIP and on cell extracts (see Fig. 1).

Cell culture and transfection. LLC-PK\textsubscript{1} cells (pig kidney epithelial cells) were grown, passaged as confluent monolayers in DMEM containing 2% L-glutamine and 10% FCS, and transfected as previously described (5, 49). Cells were transfected with cDNA encoding human GAIP, either ligated to a c-myc expression tag (MEQKLISEEDLN) at the COOH-terminal end and cloned into a pCB7 vector (Pharmacia, Uppsala, Sweden) or directly into a pcDNA3.1 expression vector (Invitrogen, San Diego, CA). Transfections were carried out using 10 µg cDNA and pcDNA3.1 expression vector (Invitrogen, San Diego, CA).

Cell culture and transfection. LLC-PK\textsubscript{1} cells (pig kidney epithelial cells) were grown, passaged as confluent monolayers in DMEM containing 2% L-glutamine and 10% FCS, and transfected as previously described (5, 49). Cells were transfected with cDNA encoding human GAIP, either ligated to a c-myc expression tag (MEQKLISEEDLN) at the COOH-terminal end and cloned into a pCB7 vector (Pharmacia, Uppsala, Sweden) or directly into a pcDNA3.1 expression vector (Invitrogen, San Diego, CA). Transfections were carried out using 10 µg cDNA and pcDNA3.1 expression vector (Invitrogen, San Diego, CA).

Transfections were carried out using 10 µg cDNA and pcDNA3.1 expression vector (Invitrogen, San Diego, CA). Transfections were carried out using 10 µg cDNA and pcDNA3.1 expression vector (Invitrogen, San Diego, CA).

Golgi vesicle budding assay. A Golgi membrane fraction was prepared from homogenates of rat liver or Madin-Darby canine kidney (MDCK) cells by density gradient centrifugation, based on the method of Leelavathi et al. (31). Golgi membranes were incubated in vitro with cytosol (1 mg/ml protein), 100 µM guanosine 5'-(3-thiotriphosphate) (GTP\textsubscript{\gamma}S), and a modified HKM buffer [in mM: 25 HEPES (pH 7.2), 20 KCl, and 2.5 Mg(CH\textsubscript{3}COO)\textsubscript{2}] containing 0.5 mM dithiothreitol and an ATP-regenerating system (1.25 U/ml creatine phosphokinase, 81 mM creatine phosphate, and 28.6 mM ATP) to generate budded vesicles (10). Budded vesicles remaining Golgi cisternae were separated by centrifugation at 17,500 g for 10 min. The vesicles in the supernatant were then separated from the remaining cytosol by ultracentrifugation at 100,000 g for 90 min. Fractions were analyzed by SDS-PAGE and immunoblotting using antibodies to vesicle-associated proteins (10) and to GAIP.

Immunogold labeling. Cells for cryosectioning were fixed in 4% paraformaldehyde-0.1% glutaraldehyde (pH 7.4) for 1 h, scraped off the culture dish, and pelleted in 2% gelatin. Sections (80 µm) collected on Formvar/carbon-coated copper grids were blocked in 1% BSA in PBS and then incubated in primary antibody for 1 h. Bound antibody was visualized using protein A conjugated to 10-nm colloidal gold particles (Dr. J. W. Slot, Dept. of Cell Biology, Utrecht, The Netherlands). Sections were contrasted and embedded in 1% uranyl acetate-2% methylcellulose on ice for 10 min and viewed in a JEOL 1010 microscope at 80 kV. Perforated MDCK cells were prepared from monolayers plated at confluence on 24-mm Transwell filters (Corning Costar, Cambridge, MA) (28). Briefly, the filters were incubated at 20°C for 20 min, washed, and partially dried, and then the apical membranes were perforated by application and removal of a nitrocellulose filter. The filters were incubated at 37°C for 15 min in wash buffer [in mM: 25 HEPES-KOH (pH 7.2), 2.5 Mg(CH\textsubscript{3}COO)\textsubscript{2}, 50 KCH\textsubscript{3}COO, 5 EGTA, and 1.8 CaCl\textsubscript{2}] containing cytosol to 1 mg/ml, aluminum fluoride (AlF\textsubscript{3}: 50 µM AlCl\textsubscript{3}, 30 mM NaF),
and an ATP-regenerating system (1 mM creatine phosphate, 8 U/ml creatine phosphokinase, and 50 µM ATP-Na). The filters were then fixed in 0.1% glutaraldehyde-4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature; strips were excised from the filter and immunogold labeled as described above. The filter strips were then postfixed for 1 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate containing 0.1 g/ml sucrose, 12.5 mM CaCl₂, 70 mM KCl, and 12.5 mM MgCl₂, followed by 1% osmium tetroxide in potassium ferrocyanide for 30 min. The samples were then stained en bloc in 0.5% uranyl acetate in 50% ethanol, dehydrated through a graded series of ethanols, and embedded in Epon 812 resin. Ultrathin sections were cut (Reichardt Ultracut S), contrasted in lead acetate and uranyl acetate, and viewed as described above.

Secretion of sulfated proteoglycan. GMLLC-PK1 (clones 1 and 7), untransfected LLC-PK1 cells, and transiently transfected LLC-PK1 cells were plated at confluence on 24-mm Transwell filters (Corning Costar) and used to measure the sulfation and secretion of [35S]sulfate-labeled basement membrane heparan sulfate proteoglycan (bmHSPG), as described previously (11, 49). Transiently transfected cells with equivalent levels of transfection were plated in triplicate wells for each experiment. Cells were washed and preincubated in modified sulfate-free Fischer’s medium (GIBCO BRL, Grand Island, NY) and then metabolically labeled for 3 h at 37°C using 500 µCi/ml [35S]sulfate (carrier free; ICN Biomedical) added to the basal medium. Duplicate filters were also labeled with [35S]sulfate (carrier free; ICN Biomedical) added to the basal medium. Duplicate filters were also labeled with [35S]sulfate (carrier free; ICN Biomedical) added to the basal medium. Duplicate filters were also labeled with [35S]sulfate (carrier free; ICN Biomedical) added to the basal medium.

Whole cell homogenates or total membrane fractions from GMLLC-PK1 cells were extracted by incubation for 10 min at room temperature in HKM buffer (30 mM HEPES, pH 7.4, with 20 mM KCl and 5 mM magnesium acetate) containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Extracts were then centrifuged at 14,000 g for 10 min at 4°C to remove unextracted material. Incubations and washes were carried out in HKM buffer with 1% CHAPS. The supernatant was incubated overnight at 4°C with EC antibody, raised against Gαi3. Protein A-Trysacryl beads (Pierce) resuspended in 10 mM Tris-HCl (pH 7.4) were added to the supernatant and incubated for 1 h at room temperature with constant mixing. After brief centrifugation, the supernatant was collected as the depleted starting material and the beads were collected as the immunoprecipitate. The beads were washed several times in excess 10 mM Tris-HCl (pH 7.4), and finally immuno precipitated proteins were solubilized in SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and immunoblotted using both EC and GAIP-specific antibodies.

RESULTS

GAIP is found in membrane and cytosol cell fractions. Affinity-purified chicken and rabbit antibodies raised against a GST-GAIP fusion protein were shown to recognize the fusion protein and a single, 29-kDa band, corresponding to GAIP, in cell extracts (Fig. 1); this same band was also recognized by an antibody (C-20) raised against a COOH-terminal peptide of GAIP (Fig. 1A). The chicken and rabbit antibodies did not recognize bands corresponding to other proteins or other known RGS proteins in these extracts and were determined to be specific for GAIP. The antibodies were used to probe for endogenous GAIP in a number of cell lines. Cell extracts were fractionated into total microsomal membranes and cytosol and immunoblotted for GAIP using the chicken antibody; four of these are shown in Fig. 1B. Endogenous GAIP was expressed at roughly equivalent, relatively low levels in all cells tested. In cultured epithelial cell lines, GAIP was detected pre-
dominantly (90%) in the cytosol fractions with much smaller amounts in the membrane fractions. In macrophages (RAW 264.7), a band of slightly faster mobility was recognized by the GAIP antibody in membrane fractions. This shift in electrophoretic mobility could reflect conformational differences between cytosolic and membrane-bound forms of GAIP due to posttranslational modifications such as palmitoylation.

LLC-PK1 cells were stably transfected with a c-myc-tagged GAIP cDNA (Fig. 1C) or transiently transfected with an untagged GAIP construct (Fig. 1C). Stably transfected clonal cell lines expressing different levels of c-myc-GAIP were used in subsequent experiments and are referred to as GMLLC-PK1 cells. Immunoblots of cell extracts labeled with antibodies against either GAIP or the c-myc tag showed the presence of recombinant, c-myc-tagged GAIP in both the cytosol and membrane fractions of stably transfected GMLLC-PK1 cells. Transient transfection also resulted in expression of recombinant GAIP in both cytosol and membrane fractions. As in untransfected cells, most of the overexpressed GAIP was found in the cytosol (60–70%), although a proportion (30–40%) of recombinant GAIP was found also in membrane fractions. Both untagged GAIP (Fig. 1Cii) and c-myc-GAIP (Fig. 1Ci) showed the same distribution in cytosol and membrane fractions.

Localization of GAIP in cells. Immunofluorescence staining, carried out on a variety of cell lines and transfected cells, showed distributions of GAIP similar to that found by immunoblotting. GMLLC-PK1 cells overexpressing c-myc-tagged GAIP showed predominantly cytoplasmic staining (Fig. 2). Although a heavier concentration of GAIP staining was often observed in the perinuclear region (Fig. 2A), no specific staining of any intracellular membranes or organelles, or any staining of the cell surface, was detected at this level. The more sensitive method of immunogold labeling at the electron microscopy level also was used to localize GAIP. Ultrathin cryosections of GMLLC-PK1 cells showed c-myc-GAIP labeling throughout the cytoplasm (Fig. 3A). Again, no concentrated immunogold labeling of plasma membrane or of other organelles was obvious in the cryosectioned cells, perhaps due to the abundant cytoplasmic staining. Immunogold labeling was therefore also carried out on perforated MDCK cells from which the bulk of the cytosolic proteins had been removed, leaving only intracellular organelles and the proteins associated specifically with them (Fig. 3, B–D). Specific gold labeling of endogenous GAIP was found predominantly adjacent to membranes, showing the effective removal of cytosolic GAIP. Membrane labeling of GAIP was seen on a proportion of vesicles in the vicinity of Golgi stacks and not on any other membranes or organelles in MDCK cells. GAIP labeling was commonly on newly budded vesicles and on the budding ends of Golgi cisternae, with only occasional labeling on Golgi cisternal membranes. The specific population of GAIP-labeled vesicles could not be identified in these samples. Some, but not all, of the vesicles had electron-dense coats, and yet other vesicles, with identifiable electron-dense, clathrin coats, were not labeled (Fig. 3).

In double-labeling experiments, GAIP-labeled vesicles were not colabeled for p200/myosin II, which is found on a population of trans-Golgi network (TGN)-derived vesicles (27) (data not shown). The low level of GAIP labeling in these sections was consistent with the relatively small amount of membrane-bound endogenous GAIP detected by immunoblotting in MDCK cell fractions. In all, immunostaining showed a diffuse distribution of GAIP throughout the cytoplasm and specific labeling of membrane-bound GAIP on a population of Golgi-derived vesicles.

Fractionation of Golgi vesicles from rat liver and MDCK cells. Density gradient centrifugation was used to further study the association of GAIP with Golgi-derived vesicles. Rat liver Golgi membranes and cytosol from rat liver homogenates and MDCK cells were used
to reconstitute in vitro vesicle budding in the presence of GTP\(_\gamma\)S (10). The distribution of GAIP was compared with other peripheral, vesicle-associated proteins, p200/myosin II and β-COP, which are known to bind to distinct populations of Golgi-derived vesicles under these conditions (10, 35). In this assay, β-COP and p200/myosin II derived from the cytosol bind to vesicles in a GTP-dependent fashion (Fig. 4, lane 3). Most of the GAIP was also initially found in the cytosol; it was not detected in the freshly isolated stacked-Golgi membrane fraction (Fig. 4, lane 2). After incubation, GAIP was enriched in budded vesicle fractions (lanes 3 and 6) but was not present in significant amounts on remnant Golgi cisternae (lane 4). This pattern suggests that GAIP binds to vesicles during budding and is not retained on cisternal membranes. These results are consistent with the immunogold labeling in showing that GAIP is found primarily on Golgi-derived vesicles and that little, if any, GAIP appears to reside on Golgi cisternae. These data also highlight the temporal segregation of GAIP and G\(_{\alpha_i-3}\). As can be seen in Fig. 4, all of the G\(_{\alpha_i-3}\) is membrane bound, and it resides on the Golgi cisternal membranes (lanes 2 and 4). After budding, some of the G\(_{\alpha_i-3}\) fractionates with the vesicles (lane 6), and this is the only site where GAIP and G\(_{\alpha_i-3}\) may be colocalized.

Membrane-bound GAIP associates with activated G\(_{\alpha_i-3}\). The partial overlap of G\(_{\alpha_i-3}\) (on Golgi membranes and vesicles) and GAIP (in the cytosol and on vesicles) raises the question of whether or not these two proteins do interact in cells. In assays using purified components, RGS proteins have been shown to bind specifically to G\(_{\alpha}\) subunits and with highest affinity to a transitionally activated state of G\(_{\alpha}\), induced by the addition of AlF\(_4^-\) and GDP in the presence of Mg\(^{2+}\) (2). We tested the ability of c-myc-GAIP expressed in GMLC-PK1 cells to bind to G\(_{\alpha_i-3}\), which is found on the Golgi membranes in these cells (49). G\(_{\alpha_i-3}\) was immunoprecipitated from both whole cell extracts and mem-

---

**Fig. 3. Ultrastructural localization of GAIP.** A: Cryosections of GMLLC-PK1 (clone 1) cells were labeled with anti-c-myc (1:10) and 10-nm protein A-gold; labeling was distributed throughout cytoplasm, with some gold labeling in areas of tubular vesicular membranes, although there was no specific gold labeling on any organelles. B–D: Ultra-thin resin sections of perforated MDCK cells, labeled before embedding with rabbit anti-GAIP (1:10) and protein A-gold. Although cytosol has been removed from these cells, individual organelles are still intact. These fields depict perinuclear areas, including Golgi stacks. Gold labeling (arrowheads) was seen on vesicles and some tubular structures in the vicinity of Golgi stacks (G) and sometimes on budding ends of cisternae (arrow, micrograph C). Scale bars, 0.2 µm.
brane fractions of GMLLC-PK1 cells in buffers containing combinations of Mg$^{2+}$, AlF$_4^-$, and GDP (Fig. 5). The 41-kDa $\alpha_{i3}$ was immunoprecipitated under all conditions from these samples. Immunoprecipitates were analyzed by immunoblotting to detect coprecipitated GAIP. Only small amounts of c-myc-GAIP were coprecipitated with $\alpha_{i3}$ from untreated cell extracts or in the presence of added Mg$^{2+}$ and AlF$_4^-$ alone. The coprecipitation of GAIP with $\alpha_{i3}$ was substantially enhanced in the presence of both AlF$_4^-$ and GDP, suggesting a specific and conformation-dependent interaction between these two proteins (Fig. 5A). To decipher whether this bound GAIP was derived primarily from the cytosol or from the smaller membrane-associated GAIP pool, $\alpha_{i3}$ was immunoprecipitated from separate extracts of membranes or whole cell lysates (Fig. 5B). Quantitation of coprecipitated proteins revealed that $\alpha_{i3}$ bound a higher proportion of GAIP from its membrane-bound pool (~30%) than of GAIP from the whole cell extract (~5%), which is mostly cytosolic. These results suggest that, in cells, membrane-bound GAIP can bind to the transitional activation state of $\alpha_{i3}$ and that GAIP and $\alpha_{i3}$ are unlikely to sustain this interaction throughout the whole GTP-GDP cycle.
Overexpression of GAIP retards constitutive secretory trafficking. We and others have previously measured the constitutive trafficking and secretion of \(^{35}\text{S}\) sulfate-labeled bmHSPG to assay regulatory effects of \(G\alpha\) subunits on vesicular trafficking (5, 33, 49). Proteoglycans, such as bmHSPG, are heavily sulfated at the level of the TGN. In LLC-PK\(_1\) cells, we have shown that the intracellular trafficking of bmHSPG can be retarded at pre- and post-TGN steps by overexpression of \(G_{\alpha_i3}\) (5, 49). A similar assay was used in this study to investigate a functional role for GAIP by measuring the effects of GAIP overexpression on constitutive trafficking in GMLLC-PK\(_1\) cells. Untransfected LLC-PK\(_1\), cells, GMLLC-PK\(_1\), cells, and LLC-PK\(_1\), cells transiently transfected with vector only were labeled with \(^{35}\text{S}\) sulfate; radiolabeled bmHSPG precursors in cell extracts and secreted bmHSPG, collected from the apical and basal media, were analyzed by SDS-PAGE, fluorography, and densitometry (Fig. 6). Compared with control LLC-PK\(_1\) cells and those transfected with vector alone (pcDNA3.1), all GMLLC-PK\(_1\) cells produced significantly lower amounts of sulfated bmHSPG (Fig. 6), although equivalent levels of early bmHSPG precursors were produced in all cell lines, as determined by \(^{35}\text{S}\) cysteine labeling of bmHSPG (data not shown). Overexpression of GAIP in stably transfected GMLLC-PK\(_1\) cells reduced \(^{35}\text{S}\) sulfate incorporation into the constitutive trafficking of bmHSPG (data not shown). The proportion of \(^{35}\text{S}\) labeled bmHSPG in cell extracts are represented as % of total \(^{35}\text{S}\) bmHSPG found in transfected cells than in the controls. Because \(^{35}\text{S}\) sulfate is added to the bmHSPG at the level of the TGN, these results imply that overexpression of GAIP significantly retards delivery of bmHSPG precursors to the TGN, i.e., that trafficking in earlier steps of the secretory pathway is retarded. Sulfation function in GMLLC-PK\(_1\) cells was tested in the presence of \(\beta\)-o-xylloside acceptor and was found not to be compromised by GAIP overexpression (data not shown). GAIP had little or no detectable effect on post-TGN secretion of \(^{35}\text{S}\) sulfate-labeled bmHSPG into the basal medium of GMLLC-PK\(_1\) cells. The proportion of \(^{35}\text{S}\) bmHSPG in cell layers and in medium was the same in GMLLC-PK\(_1\) cells and control cells. Also, the polarity of bmHSPG secretion (90% basal; 10% apical) was unchanged in GMLLC-PK\(_1\) cells. Together these data suggest that overexpression of GAIP does not affect \(G_{\alpha_i3}\)-regulated post-TGN constitutive secretory function.

### DISCUSSION

The intracellular distribution of GAIP and its potential participation in protein trafficking were investigated in kidney epithelial cells. Our current studies on endogenous GAIP in a variety of cells and on recombinant GAIP in transfected LLC-PK\(_1\) cells indicate that GAIP exists in both membrane-bound and cytosolic pools. The majority of endogenous GAIP was found as a soluble pool in the cytoplasm, and relatively small amounts were found on membranes. In our hands, transfected cells expressed proportionately more (10-30% more) membrane-bound GAIP than untransfected cells, suggesting that membrane binding sites for GAIP are not saturated at steady state. This may lead to, and be reflected in, differences in the relative levels of membrane-bound and cytosolic GAIP measured at different times. De Vries et al. (12) showed variable amounts of membrane-bound GAIP in different cell lines; only 30% of overexpressed GAIP was in membrane fractions in transfected COS cells, whereas >80% of GAIP in ATT20 cells was in the membrane fraction, which coincided with heavy and predominant labeling of GAIP on membranes in pituitary cells (13). The differences observed, between this and previous studies (12, 13), in the relative distribution of GAIP between membrane and cytosol might reflect differences in the cell types used or be due to cycling of GAIP between the two pools. The relationship between pools of membrane-bound and cytosolic GAIP remains unclear at this stage. GAIP has been shown to be modified for membrane attachment through palmitoylation at a number of cysteine residues near the NH\(_2\) terminus (12), and palmitoylation in other proteins, such as \(G_{\alpha}\) subunits, provides a basis for their transient and regulated attachment to membranes (32). GAIP may move dynamically between the two sites, as is suggested by the GTP-dependent attachment of GAIP to Golgi membranes in the vesicle budding assay.
In kidney epithelial cell lines, immunolocalization and cell fractionation studies revealed that GAIP is attached to vesicles associated with the Golgi complex. Labeling of the relatively small pool of membrane-bound GAIP cannot be distinguished at the immunofluorescence level or by immunogold labeling on transfected cells, where staining of the abundant cytosolic GAIP dominated. Membrane-bound GAIP was localized by immunogold labeling in perforated MDCK cells. The use of perforated MDCK cells has proven useful, and sometimes uniquely successful, for immunogold labeling of peripheral proteins bound to the cytoplasmic face of Golgi membranes or vesicle membranes (22, 27, 28). Labeling of membrane-bound GAIP was restricted to a subpopulation of vesicles or budding membrane structures around the Golgi stack. This distribution indicates that GAIP associates selectively with only some of the budding vesicles; similar distributions (on selected subpopulations of vesicles) are seen for other vesicle-associated proteins, such as β200/myosin II, β-COP, and γ-adaptin, in these preparations. Further characterization of these vesicle populations is needed to identify the particular vesicles binding GAIP in MDCK cells. DeVries et al. (13) have shown localization of GAIP on cisternal-coated vesicles, both those budding from the Golgi and from the plasma membrane, in pituitary cells and liver cells. The vesicles labeled here in MDCK cells could include Golgi-derived cisternal-coated vesicles, although not all labeled vesicles had recognizable coats and no plasma membrane-derived vesicles were labeled in perforated cells. The paucity of labeling on Golgi cisternae, together with the very small amount of GAIP detected in rat liver Golgi cisternae fractions (Fig. 4), suggests that GAIP associates with the vesicle membranes only during or after budding and that it does not reside on Golgi membranes.

Just how the localization of GAIP on these Golgi vesicles relates to its interaction with Goi-3 is not clear. The presence of both GAIP and Goi-3 on Golgi-associated membranes hints at a Golgi-specific functional link between the two proteins. Goi-3 is found on the Golgi membranes of many cell types by immunofluorescence labeling (reviewed in Ref. 50); in LLC-PK1 cells, Goi-3 is exclusively located on Golgi membranes (20, 49). At an ultrastructural level, Goi-3 appears across the Golgi stack in LLC-PK1 cells (49), and in excocrine pancreatic cells Goi-3 is also seen from cis- to trans-Golgi and on vesicles at both sides of the Golgi stack by immunogold labeling (15). Studies in several cell types show that Goi-3 regulates multiple pre- and post-Golgi trafficking steps, suggesting that it may function at multiple and variable sites across the Golgi stacks of different cells (5, 33, 49, 53). Current and previous data (13) showing that membrane-bound GAIP is restricted to budded vesicles seemingly limit the overlapping locations of Goi-3 and GAIP, and their potential to interact, within the Golgi milieu. Coimmunoprecipitation of GAIP with Goi-3 confirmed that indeed the two proteins do, at some time, interact. The binding of GAIP to Golgi vesicle membranes does not apparently depend on Goi-3 interaction, since vesicles are produced in the presence of GTPγS and GAIP does not bind with high affinity to Goi-3-GTPγS (Fig. 5) (2). Any interaction of Goi-3 on Golgi membranes with GAIP on vesicle membranes would be most likely to occur during the process of vesicle budding.

Studies using fusion proteins (3, 14, 52) have previously demonstrated high-affinity binding of GAIP to the Goi-3 subunit, and one very recent study has now confirmed that recombinant GAIP binds to Goi-3 in intestinal cells (41). We also showed that c-myc-GAIP in extracts of overexpressing GM LLC-PK1 cells is coprecipitated with Goi-3. Interaction occurred with highest affinity in the presence of AlF4− and GDP, which emulates the transitional state on the Gsα subunit. The GAP function of RGS proteins has been attributed to the ability of these proteins to interact with, and stabilize, this transitional form (2). Our experiments additionally show that the membrane-bound form of GAIP, and not that in the cytosol, accounts for the majority of binding to Goi-3. Soluble forms of GAIP and Goi-3 must be able to interact, since their binding in solution has been demonstrated and GAIP was originally identified as a soluble binding partner for Goi-3 in the yeast two-hybrid system (14). It is likely that in intact cells GAIP-Goi-3 interactions do occur at the membrane, since Goi-3 in cells is always tightly bound to membranes (5). In the context of intact cells, it is also possible that intervening protein interactions might either enhance or prevent Goi-3 interactions with membrane-bound or cytosolic pools of GAIP.

Goi-3 has been demonstrated to regulate protein trafficking in a variety of pathways (5, 33, 40, 41, 49, 53). We have previously shown that in LLC-PK1 cells overexpression of Goi-3 subunits downregulates polarized (basolateral) constitutive secretion (49). The assay used for these studies measures the trafficking of precursors and the secretion of terminally sulfated bmHSPG from polarized LLC-PK1 cells. This same assay was applied here to cells overexpressing GAIP. GAIP overexpression reduced the amount of [35S]sulfate-labeled bmHSPG in cells, although levels of [35S]cysteine-labeled bmHSPG precursors were unchanged, suggesting that trafficking of bmHSPG before the level of the TGN, where sulfation occurs, was retarded. GAIP overexpression, even at high levels, did not alter the kinetics of post-TGN secretion of bmHSPG. GAIP is thus implicated in regulating constitutive trafficking early in the secretory pathway. This correlates with one of the stages of trafficking affected by overexpression of Goi-3, which retards trafficking of bmHSPG precursors in the pre-TGN Golgi (by ~3-fold). Overexpression of Goi-3 also significantly reduces post-TGN secretion of [35S]sulfate-labeled bmHSPG (5, 49), a step that is not affected by GAIP. Thus GAIP appears to function in only one of the steps of constitutive trafficking similarly regulated by Goi-3. The known G protein-coupled functions for GAIP, to date, are as a GAP and as a potential effector antagonist (25, 54). In the current assay, GAIP did not downregulate the Goi-3 response, as would be expected for GAP activity. The retardation of secretary
trapping caused by GAIP in LLC-PK1 cells either masks a $\mathrm{G}_{\alpha_i-3}$-GAP activity of GAIP or reflects an additional or alternative function for GAIP in this trafficking pathway. Recently, GAIP functioning as a GAP was demonstrated in another trafficking pathway. GAIP overexpression downregulated the $\mathrm{G}_{\alpha_i-3}$ response in an autophagic sequestration pathway in colonic epithelial cells (41). This study also found that GAIP mRNA expression levels were altered during differentiation and were regulated by $\mathrm{G}_{\alpha_i-3}$ activity, suggesting that, in this pathway, GAIP is a highly reactive modulator of G protein signaling (41). The precise requirements for GTPases and other modulating proteins to downregulate $\mathrm{G}_{\alpha_i}$ signaling in the constitutive secretory pathway has not yet been well established, since the roles of receptor activation and G protein cycling in this system are not known.

It is possible that GAIP has more than one function in vesicular trafficking. This is supported by our finding that GAIP binds only to the transitionally active state of $\mathrm{G}_{\alpha_i-3}$ and that only a small proportion of the total GAIP (membrane-bound pool only) interacts with $\mathrm{G}_{\alpha_i-3}$ in these cells. This leaves open the possibility that the vast majority of overexpressed, cytosolic GAIP performs additional functions. RGS proteins are known to have diverse structural features. The presence of a COOH-terminal cysteine string motif (12) potentially links GAIP to the family of cysteine string proteins that are found on secretory vesicles and granules, although the function of this protein family is not yet known (9, 23, 29). RGS3 has an NH2-terminal coiled-coil domain (19), whereas RGS12 has a COOH-terminal coiled-coil domain that has been proposed as a potential site for cytoskeletal interactions (47). Binding of RGS proteins to Switch I/II regions of $\mathrm{G}_{\alpha}$ supports their function as effector antagonists for some, but not all, effectors of $\mathrm{G}_{\alpha}$ subunits (36, 37) The data reported here demonstrate the localization of GAIP on Golgi-derived vesicles in epithelial cells. Functional data are consistent with the regulatory participation of GAIP in early stages of the constitutive secretory pathway, but GAIP does not appear to modulate $\mathrm{G}_{\alpha_i-3}$-regulated post-Golgi secretion. Future mutational analyses of functional domains in GAIP will ultimately reveal the extent and diversity of its regulatory roles in protein trafficking pathways.

We thank Brandon Sullivan and Dennis Ausiello (Massachusetts General Hospital, Harvard Medical School) for providing the GAIP cDNA constructs and for helpful discussions and comments on the manuscript. This work was supported by a grant from the National Health and Medical Research Council of Australia (to J. L. Stow). J. L. Stow is a Wellcome Trust Senior Research Fellow. Address for reprint requests: J. L. Stow, Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, QLD 4072, Australia. Received 16 July 1998; accepted in final form 2 November 1998.

REFERENCES


ROLE OF GAIP ON GOLGI VESICLES


