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A Heterotrimeric G Protein, $G\alpha_{i\text{-}3}$, on Golgi Membranes Regulates the Secretion of a Heparan Sulfate Proteoglycan in LLC-PK_1 Epithelial Cells

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Abstract. A heterotrimeric Gαi subunit, $\alpha_{i\text{-}3}$, is localized on Golgi membranes in LLC-PK_1 and NRK epithelial cells where it colocalizes with mannosidase II by immunofluorescence. The $\alpha_{i\text{-}3}$ was found to be localized on the cytoplasmic face of Golgi cisternae and it was distributed across the whole Golgi stack. The $\alpha_{i\text{-}3}$ subunit is found on isolated rat liver Golgi membranes by Western blotting and $G\alpha_{i\text{-}3}$ on the Golgi apparatus is ADP ribosylated by pertussis toxin. LLC-PK_1 cells were stably transfected with $G\alpha_{i\text{-}3}$ on an MT-1, inducible promoter in order to overexpress $\alpha_{i\text{-}3}$ on Golgi membranes. The intracellular processing and constitutive secretion of the basement membrane heparan sulfate proteoglycan (HSPG) was measured in LLC-PK_1 cells. Overexpression of $\alpha_{i\text{-}3}$ on Golgi membranes in transfected cells retarded the secretion of HSPG and accumulated precursors in the medial–*trans*-Golgi. This effect was reversed by treatment of cells with pertussis toxin which results in ADP-ribosylation and functional uncoupling of $G\alpha_{i\text{-}3}$ on Golgi membranes. These results provide evidence for a novel role for the pertussis toxin sensitive $G\alpha_{i\text{-}3}$ protein in Golgi trafficking of a constitutively secreted protein in epithelial cells.

MOVEMENT of newly synthesized proteins through the secretory pathway involves staged vesicular transport between sequential membrane compartments (14, 24, 27). Recently, the requirement for GTP binding proteins at many of these steps has been established (5) and an increasing number of monomeric G proteins, which are members of the *ras* supergene family, have been identified in the secretory pathway (10, 18, 22, 30). Yeast proteins such as Sec 4 and YPT1 are found on secretory vesicles and ER to Golgi vesicles respectively and are essential for vesicle fusion (29, 30). Related members of the mammalian rab G protein family have now also been identified on different vesicle populations and membrane compartments in the secretory pathway (10) including rab 6 which is on the Golgi cisternae (19).

In contrast to the monomeric G protein family, members of the heterotrimeric G protein family have not previously been implicated in vesicle trafficking. These larger G proteins, composed of α, β, and γ subunits, are classically involved in signal transduction events at the cell surface where they regulate disparate effectors such as adenylyl cyclase and ion channels (16, 37). Functionally, G proteins have been identified by their susceptibility to bacterial toxins, thus, the widely distributed Gs and Gi proteins may be activated by cholera toxin (Gs) or inhibited by pertussis toxin (Gi) respectively. Variability of the α subunits potentially allows specific G proteins to be assigned to different signal transduction functions in a cell or to couple different receptors to the same effector. There is topographical separation of different Gα subunits in polarized epithelial cells. In LLC-PK_1 cells there are only two pertussis toxin–sensitive αi subunits, $G\alpha_{i\text{-}2}$, which is localized on the basolateral cell membrane and $G\alpha_{i\text{-}3}$, which is found intracellularly in the region of the Golgi complex (13).

The present study was conducted to establish the association of $G\alpha_{i\text{-}3}$ with membranes in the Golgi complex and to investigate the potential involvement of the $\alpha_{i\text{-}3}$ subunit in Golgi trafficking. We have previously shown that $G\alpha_i$ genes can be stably transfected into LLC-PK_1 epithelial cells with chimeric metallothionein-I (MT-I)[1] genes, ensuring that controlled amounts of recombinant αi subunits are overexpressed and correctly targeted to their appropriate membrane domains (13); using this system we have produced transfected cells that overexpress $\alpha_{i\text{-}3}$ on the Golgi complex. In addition, we have established that basement membrane heparan sulfate proteoglycan (HSPG) is synthesized and secreted constitutively in a polarized fashion from the LLC-PK_1 cells (11) and MDCK cells (9). Quantitation of HSPG secretion and accumulation of Golgi-associated HSPG precursors can be used as an assay for the constitutive secretory pathway in these cells (11). Transfected cells were used to determine the effect of overexpression of $G\alpha_{i\text{-}3}$ on the intracellular pro-

1. *Abbreviations used in this paper*: HSPG, heparan sulfate proteoglycan; MT-1, metallothionein-1.

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cessing and secretion of HSPG. The results presented here demonstrate that $G\alpha_{i-3}$ on the Golgi cisternae is involved in the secretory pathway by regulating transport of HSPG precursors in the medial–*trans*-Golgi compartments.

Materials and Methods

Cell Culture

NRK cells were maintained as described (35) and LLC-PK_1 cells, a polarized epithelial cell line derived from pig kidney, were grown as previously described (13) in high glucose, Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, in 5% CO_2/95% air. Confluent monolayers of LLC-PK_1 cells were used at days 3–5 after plating when the expression of endogenous $G\alpha_i$ genes was maximal (20).

Antibodies

A polyclonal antibody (EC; kindly provided by Dr. A. Spiegel, National Institutes of Health, Bethesda, MD), raised against the carboxy-terminal decapeptide of $G\alpha_{i-3}$ subunit, was used for immunolocalization and immunoblotting of $G\alpha_{i-3}$. This antibody has been shown to primarily recognize the α_{i-3} (41 kD) subunit on Western blots, although it can also react more weakly with α_o and perhaps with other αi subunits (31). In LLC-PK_1 cells there is no expression of $G\alpha_o$ and we have shown that the EC antibody specifically recognizes only the α_{i-3} subunit in its native and denatured forms in these cells (13). The polyclonal antibody to HSPG was raised against rat glomerular HSPG and it specifically recognizes the core protein of the basement membrane HSPG, which is synthesized and secreted by a variety of epithelia and cell cultures (35, 36). The monoclonal antibody to mannosidase II (6) was kindly provided by Dr. B. Burke (Harvard Medical School, Boston, MA). Goat anti-rabbit IgG conjugated to FITC, biotin, or alkaline phosphatase were obtained from Vector Laboratories, Inc. (Burlingame, CA).

Transfection and Overexpression of $G\alpha_{i-3}$ in LLC-PK_1 Cells

The methods described previously (13) for the transfection and overexpression of the $G\alpha_{i-2}$ gene were used for the transfection of $G\alpha_{i-3}$ and its overexpression on a mouse MT-1 promoter in LLC-PK_1 cells. A full length rodent $G\alpha_{i-3}$ cDNA (a kind gift of Dr. R. Reed, Johns Hopkins University, Baltimore, MD) was ligated into the eukaryotic expression vector pMXX and cotransfected with a plasmid carrying the neomycin resistance gene (pSV2neo) into LLC-PK_1 cells by calcium phosphate precipitation (13). Clonal cells containing stably integrated plasmids were selected by resistance to geneticin (G418; Gibco Laboratories, Grand Island, NY) at 1 mg/ml. The stable cell clone (LLC-$PK_{1(3+5+6)}$ used for these experiments is referred to here as LLC-$PK_{1(\alpha i-3)}$ cells.

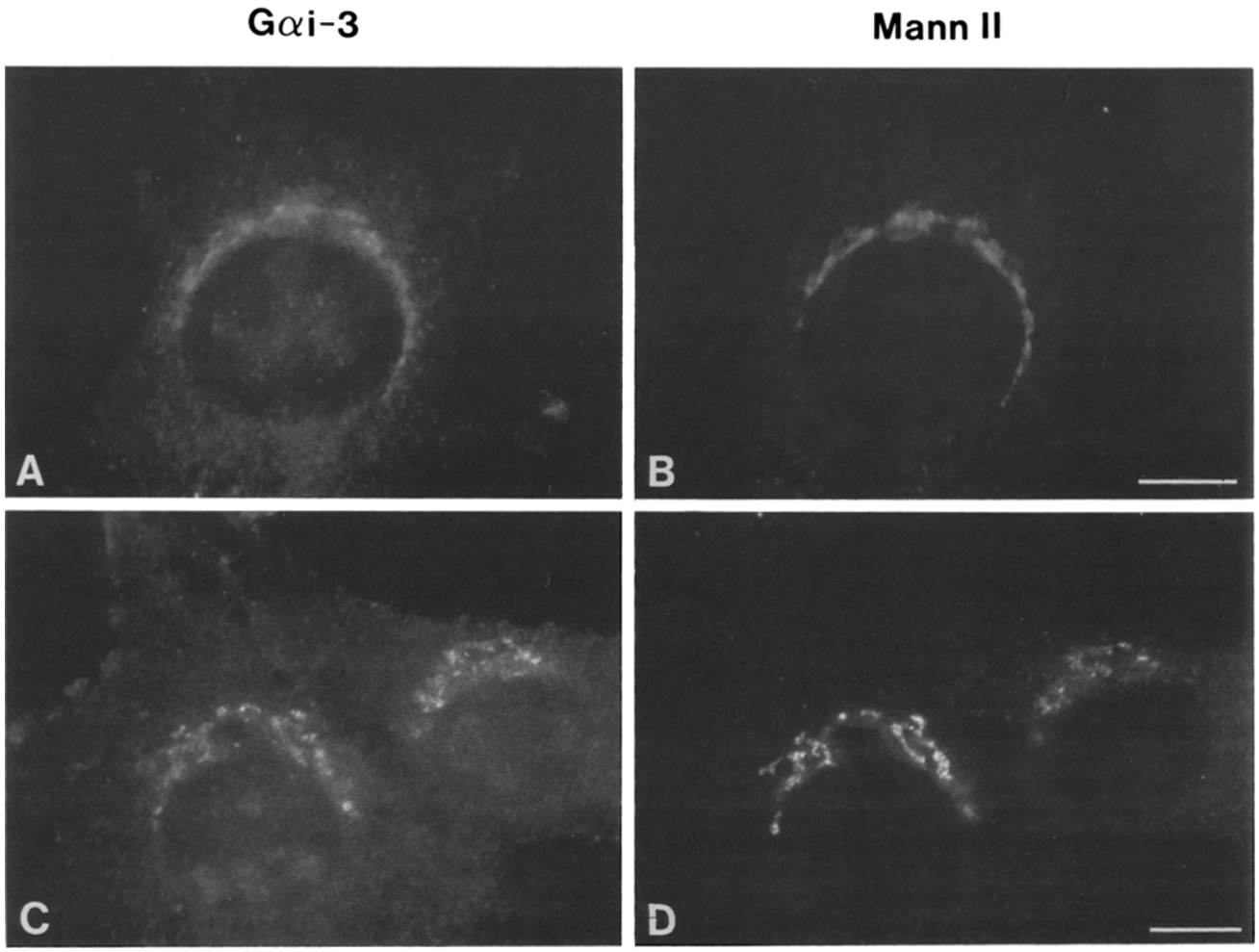

Figure 1. NRK cells were fixed and double-stained as described in the text to colocalize mannosidase II and $G\alpha_{i-3}$. The cells were photographed sequentially using the appropriate barrier filters to visualize Texas red–IgG labeling of $G\alpha_{i-3}$ (*A* and *C*) and FITC-IgG labeling of mannosidase (*B* and *D*). The field shown in *A* and *B* depicts $G\alpha_{i-3}$ and mannosidase staining over a condensed, perinuclear Golgi complex. Both antibodies stain almost exactly the same area. At lower magnification in *C* and *D*, there is staining of Golgi complexes in two adjacent cells. In these cells a reticular array of Golgi stacks can be seen within each complex and the individual stacks are stained by both antibodies. There is also diffuse cytoplasmic staining of $G\alpha_{i-3}$ in these cells. Bars, 10 μm.

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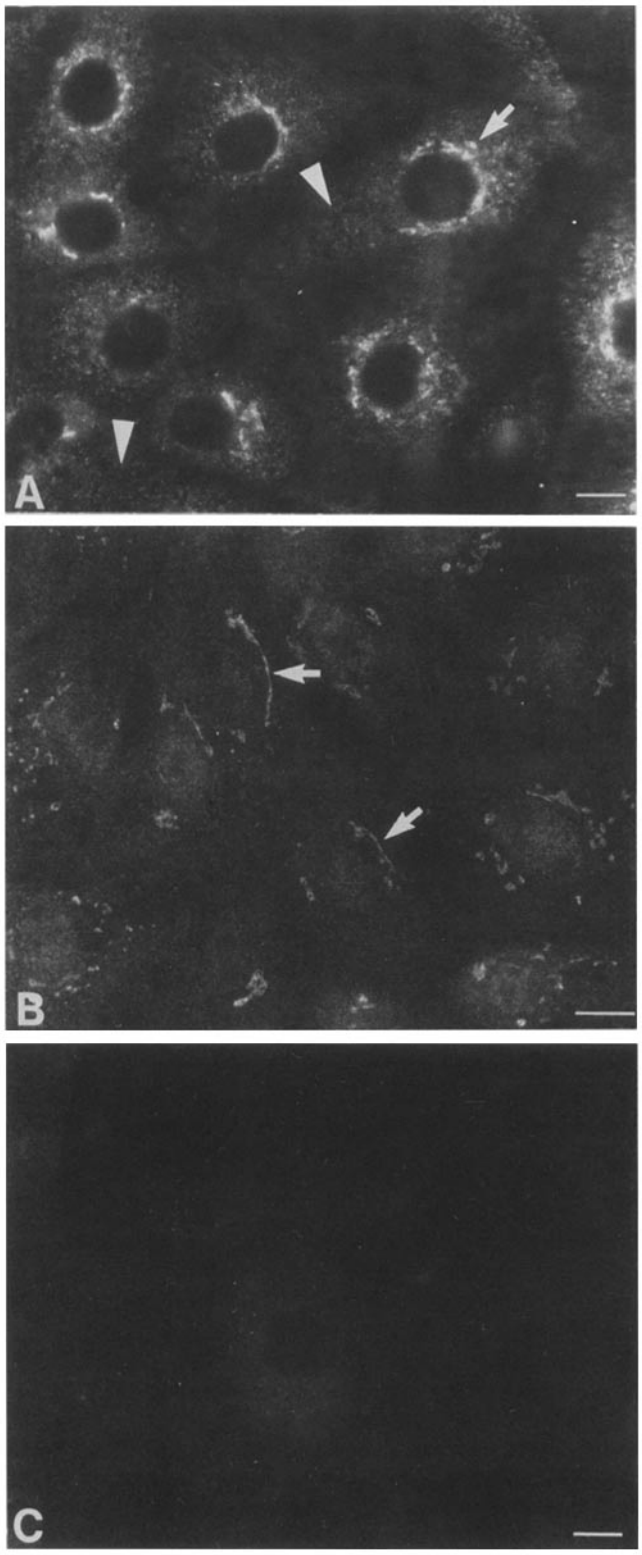

Figure 2. Monolayers of LLC-PK$_1$ cells stained by immunofluorescence show localization of G$\alpha_{i\text{-}3}$ in the perinuclear Golgi complex. Cells were either untreated (*A*) or pretreated with cycloheximide for 16 (*B*) or 32 h (*C*) to stop protein synthesis. The untreated cells have strong G$\alpha_{i\text{-}3}$ staining in the Golgi area (*arrows*) and weaker, diffuse cytoplasmic staining (*arrowheads*). After cycloheximide treatment (16 h; *B*) the cytoplasmic staining disappears leaving only staining of the Golgi membranes (*arrows*). Longer incubations of cells with cycloheximide (32 h; *C*) resulted in the eventual disappearance of Golgi staining. Bars, 10 μm.

Isolation of Rat Liver Golgi Membranes

Rat liver Golgi membranes were prepared by a modification of the method described by Ehrenreich et al. (12) in which total microsomes were separated on a discontinuous sucrose gradient. The Golgi membrane fraction collected from the 0.90/0.40 M interface was assessed morphologically and enzymatically and was found to contain >90% Golgi cisternal membranes. The purity of the Golgi fraction was shown by a significant (28-fold) enrichment in galactosyltransferase activity as measured by standard methods (21). This fraction was found to have no enrichment in marker enzymes from other, potentially contaminating membranes including canalicular membranes (alkaline phosphatase), sinusoidal membranes (Na^+/K^+ATPase) (4), or rough ER membranes (KCN-resistant NADH-oxireductase) (33).

Western Blotting

Aliquots of microsomal membranes prepared from LLC-PK$_1$ cells and rat liver Golgi membranes were loaded onto 10% gels for SDS-PAGE and transferred onto Immunobilon membrane (Millipore Continental Water Systems, Bedford, MA). Immunoblotting was performed with the EC antibody to detect G$\alpha_{i\text{-}3}$, followed by either alkaline phosphatase–labeled or ^{125}I-labeled second antibodies, as described (13). Autoradiographs were analyzed by densitometry to quantitate and compare the amount of $\alpha_{i\text{-}3}$ protein in uninduced and induced LLC-PK1($\alpha_{i\text{-}3}$) membranes.

ADP Ribosylation by Pertussis Toxin

Monolayers of LLC-PK$_1$ cells were incubated for 16 h in the presence of pertussis toxin (100 ng/ml) (List Biological Lab. Inc., Campbell, CA) for in vivo ADP ribosylation of Gα subunits in cells. Microsomal membranes (100,000 *g* pellet) from LLC-PK$_1$ cells and rat liver Golgi membranes were used for in vitro ADP ribosylation by pertussis toxin as described previously (23, 25). Aliquots of membrane proteins (20 μg) were incubated with 20 μl (2 μg) activated pertussis toxin or with an equivalent volume of buffer without toxin and 9 μCi of ^{32}P-NAD (800 Ci/mmol; New England Nuclear, Boston, MA) for 180 min at 4°C in 12.5 mM Tris-HCl (pH 7.5), 10 mM thymidine, 1 mM EDTA, 1 mM ATP, 0.1 mM GTP, and 0.1% Lubrol PX. The reaction was stopped by addition of 1 ml 20% ice-cold TCA. The TCA precipitates were washed with ether, solubilized in sample buffer, and separated on a 10% gel by SDS-PAGE, the gels were then dried and autoradiographed for 2–24 h at −70°C.

Immunocytochemistry

Cells were fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X 100, and then incubated sequentially in diluted EC (anti-αi-3) serum or anti-HSPG serum followed by goat anti-rabbit IgG-FITC. G$\alpha_{i\text{-}3}$ and mannosidase II were colocalized by double-labeling cells with sequential incubations in EC serum, anti-mannosidase culture supernatant, goat anti-rabbit IgG-Texas red and sheep anti-mouse IgG-FITC. Cells were viewed by conventional epifluorescence microscopy on a Nikon Microphot microscope or by scanning laser confocal imaging on a Biorad MRC-600 system.

LLC-PK$_1$ cells grown on semipermeable filters were fixed in paraformaldehyde-lysine-periodate containing 0.01% glutaraldehyde for 4 h at 20°C, permeabilized in PBS with 0.5% ovalbumin with 0.005% saponin, and incubated in EC antibody overnight at 4°C. Cells were then incubated sequentially in biotinylated goat anti-rabbit IgG and avidin-HRP. The reaction with diaminobenzidine substrate, postfixation, and embedding for electron microscopy were carried out as previously described (35). Thin sections were cut and viewed on a Phillips 400 electron microscope.

Biosynthetic Labeling and Immunoprecipitation of HSPG

Confluent monolayers of LLC-PK$_{1(\alpha i\text{-}3)}$ cells on semipermeable filters (Transwell; Costar, Cambridge, MA) were preincubated in cysteine-free or sulfate-free medium and then incubated with 300 μCi/ml [^{35}S]cysteine (1,100 Ci/mmol; New England Nuclear, Boston, MA) or 300 μCi/ml [^{35}S]sulfate (carrier free; ICN Biomedicals, Inc., Costa Mesa, CA) which was added to the basal medium. After labeling, the cells were chased for various times in DMEM.

Quantitative immunoprecipitation of intracellular and secreted radiolabeled HSPG was carried out as previously described (11). After metabolic labeling, the basal and apical media were harvested separately and the cell layer was scraped and extracted in RIPA (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM TRIS, [pH 7.4]) buffer. These three compartments

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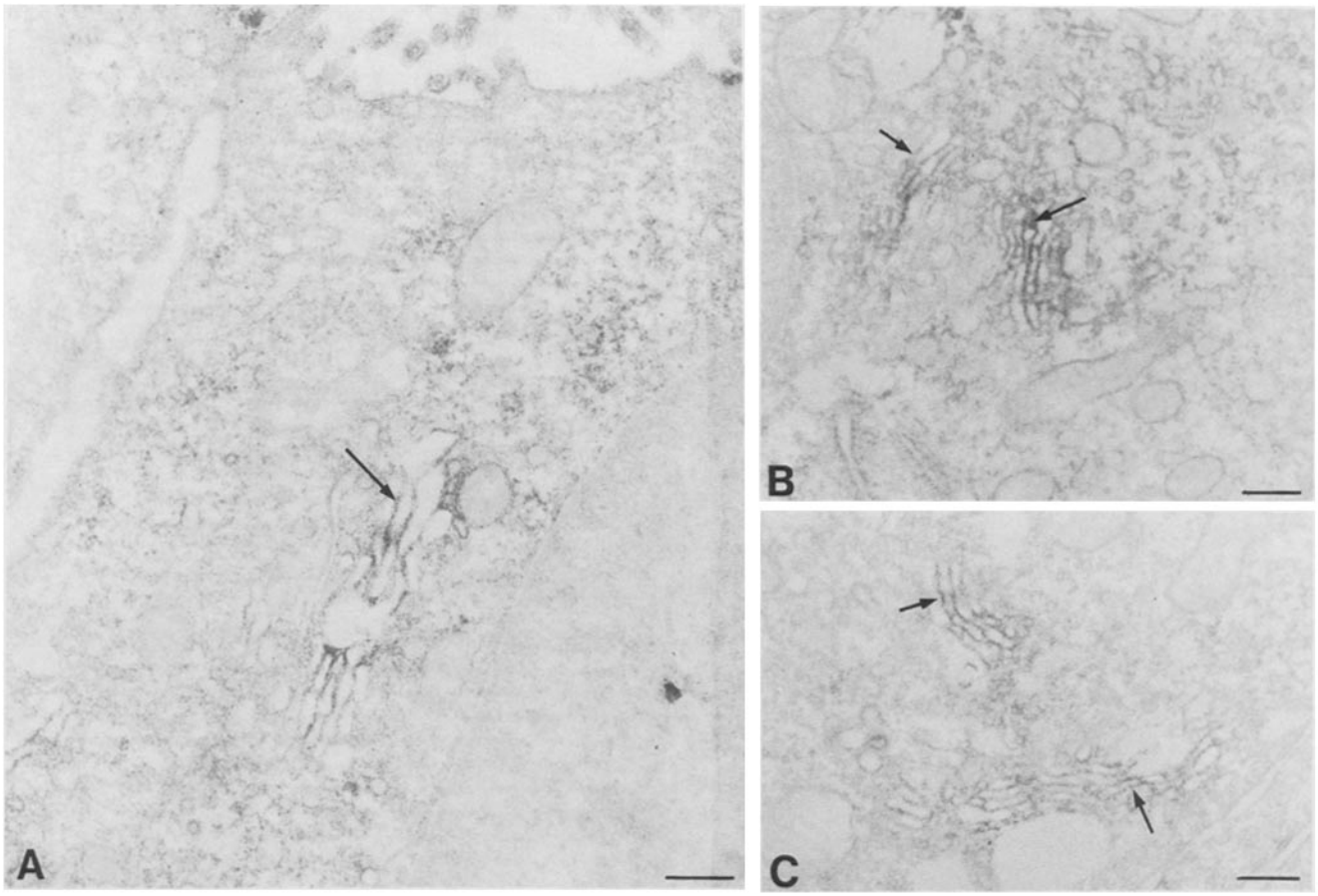

Figure 3. LLC-PK$_1$ cells grown on semi-permeable filters were prepared for immunoperoxidase staining and electron microscopy as described in the text. Immunoperoxidase staining of G$\alpha_{i\text{-}3}$ was found on membranes of the Golgi cisternae (*arrows*). Reaction product was deposited on the cytoplasmic face of the Golgi membranes and there was no staining in the lumens of the cisternae. The immunoperoxidase staining of the Golgi membranes was quite intense but it was necessary to titrate the peroxidase reaction to avoid excessive diffusion of the reaction product into the cytosol. G$\alpha_{i\text{-}3}$ was found in all Golgi stacks in the cells examined, this distribution is demonstrated in a number of Golgi stacks shown in three separate views of LLC-PK$_1$ cells in *A*, *B*, and *C*. In some stacks occasional cisternae were not stained as seen in *A*. There was no staining of $\alpha_{i\text{-}3}$ on plasma membranes or other intracellular membranes in these cells but diffuse patches of cytoplasmic staining were often seen, corresponding to the punctate cytoplasmic staining seen by immunofluorescence. Bar, 0.35 μm.

(apical and basal media and cells) were then used for immunoprecipitation using anti-HSPG serum and protein A-Sepharose beads. The final, RIPA-washed precipitates were boiled in SDS-PAGE sample buffer and electrophoresed on 5–10% SDS-PAGE gels. Gels were soaked in Autofluor, dried, and exposed to x-ray film at −70°C for 2–5 d. Radiolabeled bands on the films were quantitated by laser densitometry. To measure secretion of ^{35}S-labeled HSPG, aliquots of each immunoprecipitate from the medium samples were solubilized and counted in a liquid scintillation analyzer.

Results

G$\alpha_{i\text{-}3}$ Is Localized on Golgi Membranes in Epithelial Cells

The G$\alpha_{i\text{-}3}$ subunit was localized by immunofluorescence staining with a specific peptide antibody (EC) in two different cell types, NRK and LLC-PK$_1$ epithelial cells (Figs. 1 and 2). G$\alpha_{i\text{-}3}$ staining was most intense in the perinuclear region of the cells in a Golgi-like distribution, there was also faint punctate staining of $\alpha_{i\text{-}3}$ throughout the cytoplasm of these cells. The localization of $\alpha_{i\text{-}3}$ in the Golgi complex of NRK cells was confirmed by colocalization with a known Golgi marker, mannosidase II. The staining of mannosidase and $\alpha_{i\text{-}3}$ produced the same, overlapping pattern of reticular arrays of Golgi stacks in the perinuclear area in NRK cells (Fig. 1). G$\alpha_{i\text{-}3}$ staining was also concentrated in the perinuclear Golgi complex in LLC-PK$_1$ cells (Fig. 2 *a*), which have a more dispersed Golgi structure than NRK cells. When LLC-PK$_1$ cells were treated with cycloheximide to stop protein synthesis, the cytoplasmic staining disappeared after 16 h although there was still strong staining of the Golgi complex (Fig. 2 *B*); eventually, in the presence of cycloheximide for longer times (32 h), the Golgi staining also disappeared (Fig. 2 *C*). Immunofluorescence staining of NRK and LLC-PK$_1$ cells demonstrates that G$\alpha_{i\text{-}3}$ is concentrated in, but not restricted to, the Golgi complex in these cells. As a control for the specificity of the EC antibody, the antiserum was preabsorbed with the decapeptide before staining NRK or LLC-PK$_1$ cells, this resulted in the elimination of $\alpha_{i\text{-}3}$ staining in the Golgi and cytoplasm (data not shown).

Immunoperoxidase staining at the electron microscopy level was used to localize $\alpha_{i\text{-}3}$ on the membranes of the Golgi cisternae (Fig. 3, *A–C*). G$\alpha_{i\text{-}3}$ was localized by deposition of reaction product which was concentrated over all of the Golgi complexes in LLC-PK$_1$ cells. The $\alpha_{i\text{-}3}$ staining

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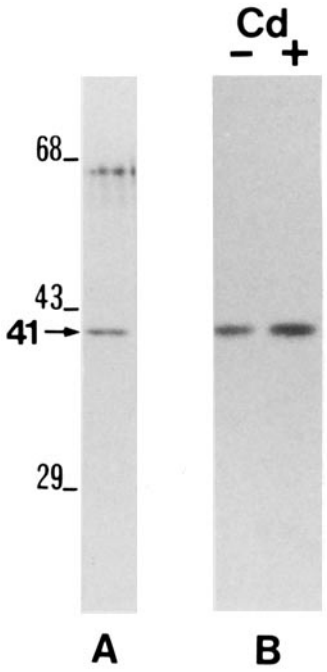

Figure 4. Western blot analysis of $G\alpha_{i\text{-}3}$ in Golgi membranes isolated from rat liver (*A*) and in microsomal membranes (100,000 *g* pellet, enriched in $\alpha_{i\text{-}3}$) from LLC-$PK_{1(\alpha i\text{-}3)}$ cells (*B*). Transfers were incubated with EC peptide antibody followed by an alkaline phosphatase-conjugated IgG (*A*) or an ^{125}I anti-rabbit IgG (*B*). The only protein band specifically recognized by the EC antibody in the rat liver Golgi membranes is the 41-kD, $\alpha_{i\text{-}3}$ subunit (*A*). In the Golgi membranes (*A*) there is also nonspecific sticking of antibody to denatured albumin at a higher molecular mass. Western blots were carried out to compare the amount of $G\alpha_{i\text{-}3}$ protein in microsomal membranes of uninduced (− *Cd*) and induced (+ *Cd*) LLC-$PK_{1(\alpha i\text{-}3)}$ cells (*B*). Total protein amounts in the microsomal membranes were measured by the Bradford assay and equal amounts of protein (20 μg) were loaded in each lane of the gel. LLC-$PK_{1(\alpha i\text{-}3)}$ cells transfected with the full-length cDNA of $\alpha_{i\text{-}3}$ on an MT-I promoter were induced with $CdCl_2$ to turn on expression of the $\alpha_{i\text{-}3}$ recombinant gene. The 41-kD band in the $CdCl_2$ induced cells (+ *Cd*) is more intensely labeled than in the uninduced cells (− *Cd*), corresponding to a 2.5-fold increase (measured by densitometry) in the amount of $\alpha_{i\text{-}3}$ protein in microsomal membranes after $CdCl_2$ induction.

was associated with the cytoplasmic face of the cisternae membranes since reaction product deposited on the membranes tended to diffuse into the cytoplasm immediately around the Golgi and there was no staining in the lumens of Golgi cisternae. In general, $\alpha_{i\text{-}3}$ was associated with all of the cisternae (*cis-trans*) of the Golgi stacks, although in some stacks there were occasional unstained cisternae (Fig. 3 *A*).

Western blotting with the EC antibody confirmed the presence of the $\alpha_{i\text{-}3}$, 41-kD subunit in Golgi membranes isolated from rat liver (Fig. 4 *A*). $G\alpha_{i\text{-}3}$ was found only on membranes and not in the cytosol of rat liver Golgi fractions and there was no recognition of other 40- or 41-kD proteins with peptide antibodies specific for other αi subunits ($\alpha_{i\text{-}1}$ and $\alpha_{i\text{-}2}$) on the Golgi membranes by Western blotting (data not shown).

$G\alpha_{i\text{-}3}$ Overexpressed in Transfected Cells Is Correctly Targeted to Golgi Membranes

LLC-$PK_{1(\alpha i\text{-}3)}$ cells, which were stably transfected with a chimeric MT-I-$\alpha_{i\text{-}3}$ gene, were used to overexpress $\alpha_{i\text{-}3}$ on Golgi membranes. Expression of the recombinant $\alpha_{i\text{-}3}$ gene could be induced in LLC-$PK_{1(\alpha i\text{-}3)}$ cells by exposure to $CdCl_2$ (1–10 μM), which produced a 4.4-kb mRNA of the recombinant $\alpha_{i\text{-}3}$ in addition to constant low amounts of the 3.2-kb mRNA of the endogenous $\alpha_{i\text{-}3}$ (data not shown). This resulted in increased expression of $\alpha_{i\text{-}3}$ protein in LLC-$PK_{1(\alpha i\text{-}3)}$ cells after induction with 5 μM $CdCl_2$ for 16 h (Figs. 4 *B* and 5). Western blots of microsomal membranes (100,000 *g* pellet) from untreated and induced LLC-$PK_{1(\alpha i\text{-}3)}$ cells showed a two- to threefold increase in the 41-kD, $\alpha_{i\text{-}3}$ protein in membranes of induced cells compared to untreated cells (Fig. 4 *B*); no $\alpha_{i\text{-}3}$ was found in the cytosol from these cells, either before or after induction.

Immunofluorescence localization and quantitative analysis of the fluorescent signal further showed that there was a threefold overexpression of $G\alpha_{i\text{-}3}$ in $CdCl_2$-induced, LLC-$PK_{1(\alpha i\text{-}3)}$ cells and that the overexpressed $\alpha_{i\text{-}3}$ was correctly targeted to the Golgi membranes. The immunofluorescent staining of $\alpha_{i\text{-}3}$ in $CdCl_2$-induced cells was increased in intensity, consistent with the presence of more $\alpha_{i\text{-}3}$ protein and more antibody binding (Fig. 5, *A* and *B*). The intensity of the fluorescent signal was quantitated using confocal image analysis which showed a threefold increase in staining intensity over the perinuclear Golgi complex in induced cells (Fig. 5 *C*). This result, together with the quantitation of $\alpha_{i\text{-}3}$ in microsomal membranes by Western blotting, confirms that a threefold increase in $\alpha_{i\text{-}3}$ protein synthesis leads directly to the association of three times more $\alpha_{i\text{-}3}$ with Golgi membranes.

$G\alpha_{i\text{-}3}$ on Golgi Membranes Is ADP Ribosylated by Pertussis Toxin

$G\alpha i$ subunits can be functionally uncoupled by pertussis toxin–induced ADP ribosylation. The $\alpha_{i\text{-}3}$ subunit on Golgi membranes is ADP ribosylated by pertussis toxin. Golgi membranes purified from rat liver were used for in vitro ADP ribosylation with pertussis toxin which showed that a 41-kD, $\alpha_{i\text{-}3}$ protein band was ADP ribosylated in the presence, but not in the absence, of pertussis toxin (Fig. 6, lanes *1* and *2*). Since the autoradiographs contained only a single band, $G\alpha_{i\text{-}3}$ appears to be the only pertussis toxin substrate found on Golgi membranes.

Previous work from this laboratory has shown that there are only two known pertussis toxin–sensitive G proteins expressed in LLC-PK_1 cells, i.e., $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$ (13). ADP ribosylation in vitro was performed on microsomal membranes (100,000 *g* pellet, enriched in $\alpha_{i\text{-}3}$) from LLC-PK_1 cells and the 41-kD, $\alpha_{i\text{-}3}$ subunit was ADP ribosylated in the presence, but not in the absence, of pertussis toxin (Fig. 6, lanes *3* and *4*). Pertussis toxin treatment of intact LLC-PK_1 cells results in the complete in vivo ADP ribosylation of all αi subunits present in these cells, such that no further ADP ribosylation can be detected by re-exposing membranes prepared from these cells to pertussis toxin in vitro (23). Using this method we confirmed that all of the available αi subunits in LLC-$PK_{1(\alpha i\text{-}3)}$ cells, both before and after induction of $\alpha_{i\text{-}3}$ overexpression, were ADP ribosylated in vivo by pertussis toxin (Fig. 6, lanes *5* and *6*).

Basement Membrane HSPG Is Secreted Constitutively by LLC-PK_1 Cells

To study the constitutive secretory pathway in LLC-PK_1 cells we followed the biosynthetic processing and constitutive secretion of the basement membrane HSPG. Intracellular and secreted forms of the HSPG were immunoprecipitated from the media and cell extracts of pulse-chase, [^{35}S]cysteine-labeled LLC-$PK_{1(\alpha i\text{-}3)}$ cells (Fig. 7). The 250-kD core protein in the rough ER is the most abundant intracellular

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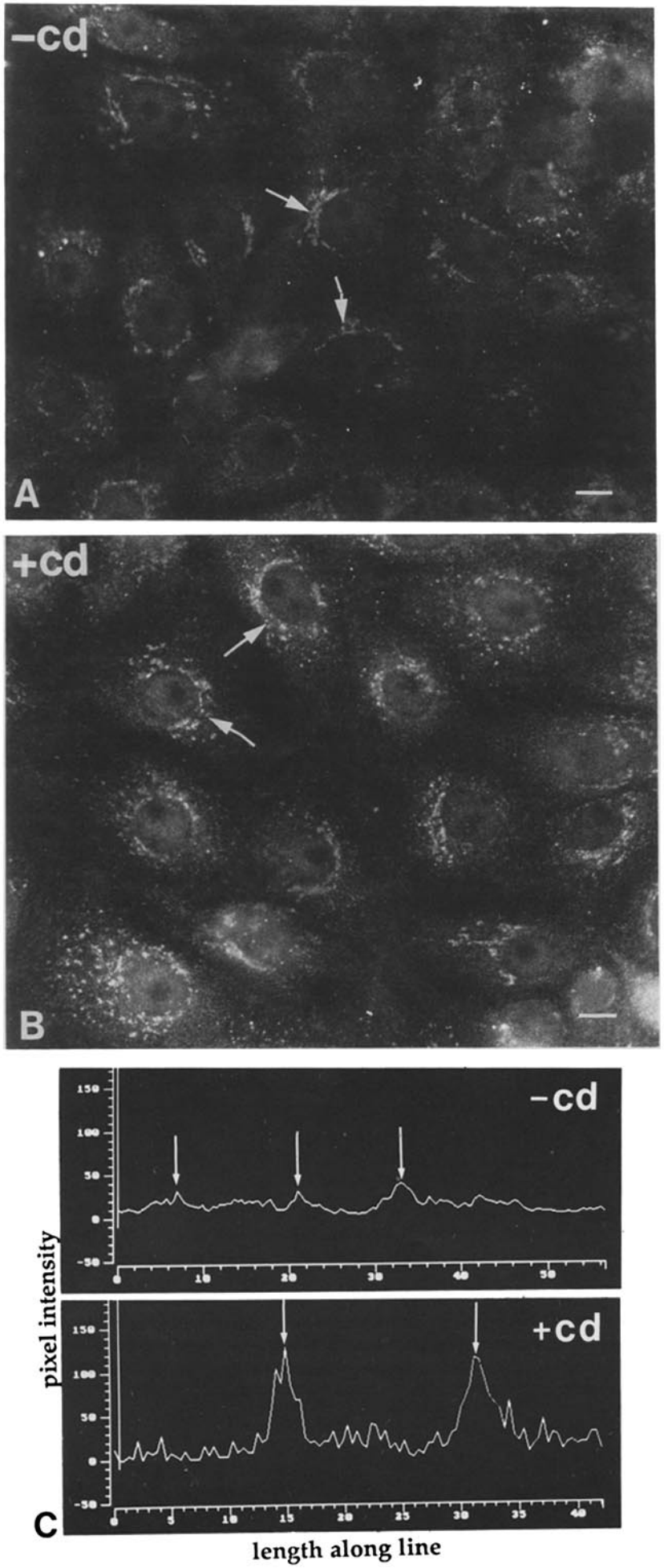

Figure 5. Immunofluorescence staining was carried out to localize $G\alpha_{i\text{-}3}$ in LLC-$PK_{1(\alpha i\text{-}3)}$ cells before (*− cd*) and after induction (*+ cd*) with $CdCl_2$. Cells were either treated with 5 μM $CdCl_2$ or remained untreated for 16 h before fixation, permeabilization, and staining with the EC antibody. (*A*) In uninduced cells (*− cd*) there was strong Golgi staining of $\alpha_{i\text{-}3}$ in the perinuclear area and faint cytoplasmic staining. (*B*) In induced cells (*+ cd*) the overexpressed $\alpha_{i\text{-}3}$ was also localized on the Golgi in the perinuclear area but the intensity of this staining was brighter than in the uninduced cells. (*C*) Confocal imaging was used to quantitate the intensity of $\alpha_{i\text{-}3}$ staining in the induced and uninduced cells above. Images of immunostained cells were collected and the pixel intensity was measured along lines drawn through selected cells (*arrows*). In the two graphs the peaks indicated by the arrows are the points where the lines intersected areas of perinuclear Golgi staining. The top shows fluorescence readings for uninduced cells (*− cd*), there are small peaks (45 pixel units) of Golgi staining, while in the bottom, the fluorescence readings of induced cells (*+ cd*) show a threefold increase in pixel intensity (140 pixel units) in the Golgi areas (*arrows*), which is consistent with the brighter Golgi staining seen in the micrograph of induced cells in *B* above. Bars, 10 μm.

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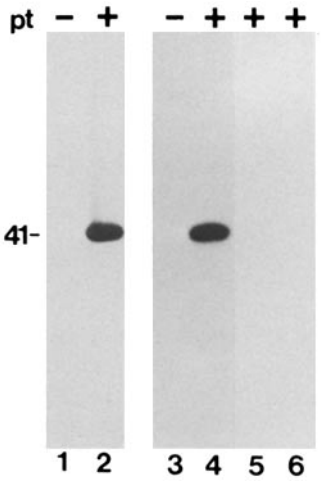

Figure 6. Pertussis toxin-induced ADP ribosylation of α_{i-3} in rat liver Golgi membranes and LLC-PK$_1$ microsomes. SDS-PAGE analysis of ADP ribosylation of membrane proteins incubated in vitro in the presence or absence of pertussis toxin (+ *pt* or − *pt*). In rat liver Golgi membranes, a single protein, at 41 kD, corresponding to α_{i-3}, was ADP ribosylated in the presence, but not in the absence, of pertussis toxin (lanes *1* and *2*). ADP ribosylation of the same protein, at 41 kD, was seen in LLC-PK$_1$ cell membranes (100,000 *g* pellet) incubated in vitro in the presence of pertussis toxin (lanes *3* and *4*). To show that all of the available αi subunits are ADP ribosylated when α_{i-3} is overexpressed, induced LLC-PK1$_{(\alpha i-3)}$ cells (+ Cd) (lane *6*) and uninduced LLC-PK1$_{(\alpha i-3)}$ cells (− Cd) (lane *5*), were exposed to pertussis toxin in vivo for 16 h. Membranes (100,000 *g* pellets) made from these cells, in both cases, were completely depleted of substrate and there was no ADP ribosylation when the membranes were re-exposed to pertussis toxin in vitro (lanes *5* and *6*).

Table I. Effect of $G\alpha_{i-3}$ on Secretion of ^{35}S-HSPG

Treatment	^{35}S-HSPG/30 min	^{35}S-HSPG/60 min
LLC-PK$_{1(\alpha i-3)}$ cells		
−Cd	399 ± 68	1,229 ± 86
+Cd	51 ± 35*	479 ± 88*
+Cd+PTX	729 ± 64*	1,684 ± 117‡
−Cd+PTX	701 ± 117§	1,547 ± 174‖
LLC-PK$_{1(\alpha i-2)}$ cells		
−Cd	277 ± 28	919 ± 29
+Cd	278 ± 24¶	923 ± 35¶
LLC-PK$_1$ cells		
−Cd	318 ± 36	1,045 ± 61
+Cd	330 ± 51¶	1,071 ± 96¶

Cells were pulse labeled with [^{35}S]cysteine, the basal medium was then collected and replaced each 30 min. ^{35}S-HSPG secreted into the medium was immunoprecipitated, solubilized, and the total cpm was counted. The cpm of ^{35}S-HSPG appearing in the basal medium in the first 30 min and first 60 min are shown above. Data are presented as mean cpm ± SE, $n = 4$. Statistical comparisons in LLC-PK$_{1(\alpha i-3)}$ cells were made to LLC-PK$_{1(\alpha i-3)}$ cells −Cd and in LLC-PK$_{1(\alpha i-2)}$ cells to LLC-PK$_{1(\alpha i-2)}$ cells −Cd and in LLC-PK$_1$ cells to LLC-PK$_1$ cells −Cd.
* $P < 0.0001$.
‡ $P < 0.001$.
§ $P < 0.004$.
‖ $P < 0.02$.
¶ No significant difference. *CD*, $CdCl_2$ (5 μM; 16 h) induction of cells to overexpress $G\alpha_{i-3}$ (LLC-PK$_{1(\alpha i-3)}$cells) or $G\alpha_{i-2}$ (LLC-PK$_{1(\alpha i-2)}$ cells) and treatment of wild type cells (LLC-PK$_1$ cells). *PTX*, treatment of intact cells with pertussis toxin (100 ng/ml; 16 h) to ADP ribosylate $G\alpha_{i-3}$.

precursor of the HSPG in these cells. This core protein is modified by the addition of xylose, O-linked oligosaccharides and sialic acid to a 450-kD precursor (O-glycanase and neuraminidase sensitive) in the medial–*trans*-Golgi. Then, upon addition of heparan sulfate glycosaminoglycan (GAG) chains and sulfation it is secreted in its mature form which appears as a high molecular mass smear at the top of SDS-PAGE gels (Fig. 7, lanes *4–6*). Sulfation occurs in the *trans*-Golgi (26) and is one of the last posttranslational modifications of the HSPG before it is secreted. Only the mature, sulfated form of the HSPG is secreted into the medium and only this mature form of the HSPG is seen in immunoprecipitates from [^{35}S]sulfate-labeled cells (Figs. 7 and 9). Immunoprecipitates of [^{35}S]cysteine-labeled HSPG also contain the A chain (400 kD) and B1,B2 chains (~200 kD) of laminin which is coprecipitated as a result of non-covalent complexing of the HSPG core protein and laminin at an early posttranslational stage (15).

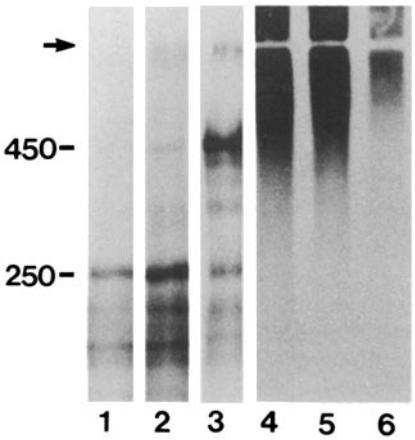

Figure 7. Biosynthesis of radiolabeled HSPG. Immunoprecipitates of radiolabeled HSPG in LLC-PK$_{1(\alpha i-3)}$ cell extracts (lanes *1–4*) or secreted into the medium (lanes *5* and *6*) were electrophoresed on 5–10% SDS-PAGE gels and exposed for fluorography. In lane *1*, after a 5-min pulse with [^{35}S]cysteine only the 250-kD precursor is immunoprecipitated; in lane *2*, after a 10-min pulse and a 10-min chase the 250-kD precursor is more heavily labeled and a band at 450-kD has appeared; in lane *3*, after a 10-min pulse and a 30-min chase the 250-kD precursor has been glycosylated to give the 450-kD precursor band. The other bands appearing in these lanes are the coprecipitated laminin. In lanes *4*, *5*, and *6* the sulfated, mature form of the HSPG is shown in immunoprecipitates from the cell layer (lane *4*), the basal medium (lane *5*), and the apical medium (lane *6*) of [^{35}S]sulfate-labeled cells. The mature HSPG is secreted in a polarized fashion, mostly into the basal medium.

Overexpression of $G\alpha_{i-3}$ on Golgi Membranes Retards Constitutive Secretion in a Pertussis Toxin–reversible Manner

LLC-PK$_{1(\alpha i-3)}$ cells grown on filters in Transwell chambers were pulse-labeled with [^{35}S]cysteine, chased in "cold" medium and then radiolabeled HSPG was immunoprecipitated from the basal medium which was collected at 30 min intervals, to determine the rate and amount of HSPG secretion, as previously described (11). The results shown in Table I and Fig. 8 are representative of four experiments carried out to measure secretion of HSPG under different manipulations of $G\alpha_{i-3}$. In LLC-PK$_{1(\alpha i-3)}$ cells induced with $CdCl_2$ to overexpress α_{i-3} on Golgi membranes there was a significant reduction in the amount of HSPG secreted compared to uninduced cells. Quantitation of the ^{35}S-HSPG immunoprecipitated from the basal medium during the first 30- and 60-min periods showed that there was a highly significant decrease (>60% decrease) in the amount of ^{35}S-HSPG secreted during both of these periods following overexpression of α_{i-3} (Table I,

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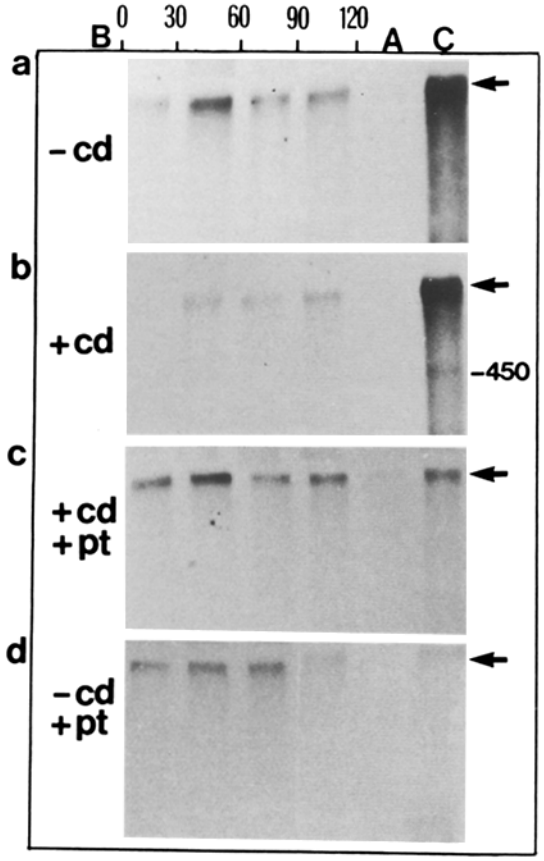

Figure 8. Rate of secretion of HSPG. LLC-PK$_{1(\alpha i\text{-}3)}$ cells were pulse labeled with [^{35}S]cysteine for 15 min and chased in cold medium for 30 min. Then, the apical and basal media were replaced and a collection period was started (time *0*) where the basal medium was collected at 30-min intervals (*30*, *60*, *90*, and *120*); after this, the total apical medium (*A*) and cell extracts (*C*) were collected and each of these samples was used for immunoprecipitation of the HSPG. Filters of cells used for each condition were matched for incorporation of isotope into total protein. (*a*) In control cells (− *cd*), labeled HSPG was first detected in the basal medium during the first 30 min period (lane *1*) and continued to be secreted throughout each of the following collections (lanes *2–4*). (*b*) After induction of LLC-PK$_{1(\alpha i\text{-}3)}$ cells (+ *cd*) there was a 30-min lag before the first appearance of HSPG in the basal medium (lane 2) compared to the − cd cells in *a*. There was no HSPG secreted in the first 30 min (lane 1) and much smaller amounts of HSPG were secreted throughout the rest of the collection (lanes 2–4). There was also retention of the 450-kD form of the HSPG in the cell extracts (lane 6) following induction. (*c*) LLC-PK$_{1(\alpha i\text{-}3)}$ cells were induced with $CdCl_2$ (+ *cd*) and treated with pertussis toxin (*pt*) for 16 h before the experiment. In spite of the retardation of secretion caused by + cd alone (*b*), in the presence of pertussis toxin there was a significant amount of HSPG secreted earlier, in the first 30-min collection (lane 1) and there was more HSPG secreted in each of the following times also (lanes 2–4). Some of the HSPG was secreted even earlier than the beginning of the collection period and was thus not accounted for. There was concomitantly less HSPG remaining in the cell layer. (*d*) Treatment of − cd cells with pertussis toxin also caused faster secretion of HSPG; there was again more HSPG secreted in, and before, the first collection time (lane 1) than in the control cells (*a*).

LLC-PK$_{1(\alpha i\text{-}3)}$ cells). The decrease in HSPG secretion was not seen following $CdCl_2$ treatment of either wild-type LLC-PK$_1$ cells or LLC-PK$_{1(\alpha i\text{-}2)}$ cells, which are stably transfected with the closely homologous $\alpha_{i\text{-}2}$ gene (13) (Table I, LLC-PK$_1$(αi-2) cells and LLC-PK$_1$ cells). These data indicate that the retardation of HSPG secretion is a specific action of the $\alpha_{i\text{-}3}$ subunit since it is not produced by overexpression of another αi subunit and is also not due to the effects of $CdCl_2$ alone.

The retardation of HSPG secretion caused by overexpression of G$\alpha_{i\text{-}3}$ was reversed by treatment of the cells with pertussis toxin (Table I). Pertussis toxin ADP ribosylates the $\alpha_{i\text{-}3}$ subunit on the Golgi membrane (see Fig. 6), resulting in functional uncoupling of the $\alpha_{i\text{-}3}$ protein. LLC-PK$_{1(\alpha i\text{-}3)}$ cells, either uninduced or induced with $CdCl_2$, were treated with pertussis toxin for 16 h before the experiment. The retardation of secretion caused by overexpressed $\alpha_{i\text{-}3}$ in induced cells was completely overcome by pertussis toxin treatment, which caused faster secretion of HSPG and resulted in a significant increase in the amount of ^{35}S-HSPG secreted in the initial periods. Notably, treatment of uninduced LLC-PK$_{1(\alpha i\text{-}3)}$ cells with pertussis toxin also caused faster secretion of HSPG since the pertussis toxin completely ADP ribosylates both the endogenous and the overexpressed $\alpha_{i\text{-}3}$ subunits (Table I).

The effects of overexpression of G$\alpha_{i\text{-}3}$ and pertussis toxin on HSPG secretion are shown also by SDS-PAGE analysis of immunoprecipitates (Fig. 8). A qualitative comparison of these gels supports the data in Table I and demonstrates the differences in the amount of ^{35}S-HSPG secreted during the initial collections of basal medium following induction of $\alpha_{i\text{-}3}$ expression and pertussis toxin treatment. Thus the amount of HSPG secreted by cells overexpressing $\alpha_{i\text{-}3}$ (+ Cd) is greatly reduced compared to uninduced cells. The decreased secretion of HSPG in turn resulted in an increased amount of HSPG remaining in the cells at the end of the collection. In particular, there was retention of the 450-kD form of the HSPG in the cell extracts which was not seen in control cells (Fig. 8 b, lane *6*). In both induced and uninduced cells the rate of HSPG secretion is increased by pertussis toxin treatment as reflected by the increased amounts of HSPG in the basal media at early time points. In addition, these gels show that neither increased $\alpha_{i\text{-}3}$ on Golgi membranes nor pertussis toxin affected the polarity of HSPG secretion, since there was no increase in the amount of HSPG precipitated from the apical medium during the experiment (Fig. 8, lane *5*).

HSPG Is Retained in the Golgi Membrane in Response to Overexpressed G$\alpha_{i\text{-}3}$

The retardation of HSPG secretion in induced LLC-PK$_{1(\alpha i\text{-}3)}$ cells was concomitant with an accumulation of the 450-kD form of the HSPG in the medial–*trans*-Golgi. Small amounts of the 450-kD precursor form are normally seen in labeled cells (Fig. 9 *a*, lane 1) but in the presence of overexpressed $\alpha_{i\text{-}3}$, there was an increase (280 ± 25%, n = 5) in the amount of this Golgi precursor immunoprecipitated from cell extracts (Fig. 9 *a*, lane *2*). This accumulation of the 450-kD form of HSPG following LLC-PK$_{1(\alpha i\text{-}3)}$ induction was reproducible in five separate experiments. Earlier biosynthetic precursors (250 kD) of the HSPG, or the mature form of the HSPG, did not show significant increases after $\alpha_{i\text{-}3}$ overex-

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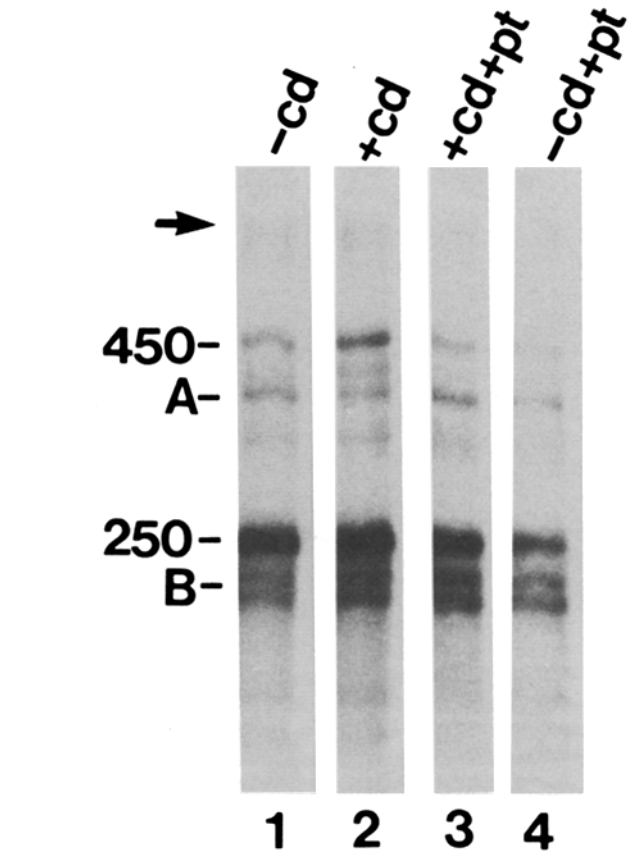
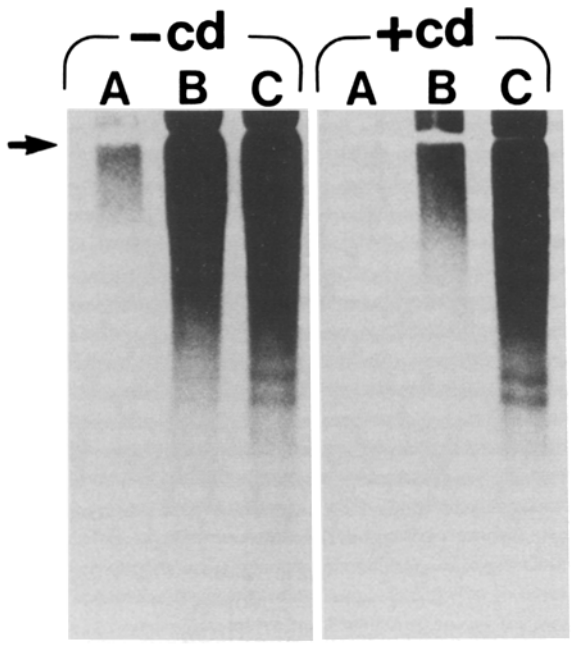


A



B

pression. The amount of the 450-kD Golgi precursor, in both uninduced and induced cells, was decreased by pertussis toxin. Pertussis toxin–induced ADP-ribosylation of both endogenous and overexpressed $\alpha_{i\text{-}3}$ resulted in a decreased amount of the 450-kD precursor which appeared to be cleared from the Golgi membrane faster than in control cells (Fig. 9 *a*, lanes *3* and *4*).

The effect of $\alpha_{i\text{-}3}$ overexpression on the passage of the HSPG through the *trans*-Golgi compartment was further observed in [^{35}S]sulfate-labeled uninduced and induced LLC-PK$_{1(\alpha i\text{-}3)}$ cells (Fig. 9 *b*). In control, uninduced cells, approximately equal amounts of sulfated HSPG were recovered from the basal medium and the cell extract following a 3-h labeling period (Fig. 9 *b*). After overexpression of $\alpha_{i\text{-}3}$, there was less incorporation of [^{35}S]sulfate into HSPG and relatively less of this sulfated HSPG was secreted. This is consistent with the effect of overexpressed $\alpha_{i\text{-}3}$, which is to accumulate the 450-kD (presulfation) precursor in the medial–*trans*-Golgi, retarding its delivery into the *trans*-Golgi where there is less HSPG available for sulfation and secretion.

The accumulation of HSPG in the secretory pathway caused by overexpression of $\alpha_{i\text{-}3}$ could also be seen by immunofluorescence staining. Normally, HSPG moving constitutively through the secretory pathway is present in intracellular compartments in relatively small concentrations, producing faint, diffuse intracellular staining in most cells (Fig. 10 *A*). At steady state, the majority of the HSPG is secreted and is localized extracellularly in clumps of matrix at the base of the cell monolayer. After overexpression of $\alpha_{i\text{-}3}$ in LLC-PK$_{1(\alpha i\text{-}3)}$ cells, many cells showed an increased amount of intracellular HSPG staining which was found in a perinuclear, Golgi-like distribution. This increased intracellular

Figure 9. (*A*) LLC-PK$_{1(\alpha i\text{-}3)}$ cells were treated with $CdCl_2$ (*cd*) and pertussis toxin (*pt*) as indicated on the lanes. Cells were pulsed for 20 min with [^{35}S]cysteine and then chased for 2 h; cell extracts with comparable amounts of isotope incorporation were used for immunoprecipitation of HSPG. The amounts of the 250- and 450-kD precursors and the mature HSPG (at the *arrow*) found in the cell extracts were quantitated by densitometry in five experiments, a representative experiment is shown. There was an increased amount (280 ± 25%) of the 450-kD precursor in the cell extracts after $CdCl_2$ treatment of cells (lane *2*) compared with uninduced cells (lane *1*). This accumulation was reversed, and the basal amount of 450-kD HSPG was further depleted, in cells (+ *cd* or − *cd*) treated with pertussis toxin (lanes *3* and *4*). Although in lane *4* there was apparent depletion of the 250-kD band, the general finding was that the amount of the 250 kD band was unchanged, after $CdCl_2$ or pertussin toxin. The induction of $\alpha_{i\text{-}3}$ in + cd cells causes an accumulation of the 450-kD precursor in the Golgi and this is reversed by pertussis toxin. (*B*) Induced cells (+ *cd*) or uninduced cells (− *cd*) were labeled with [^{35}S]sulfate and the amounts of sulfated, immunoprecipitated HSPG in the cell extracts (*C*), the basal medium (*B*), and apical medium (*A*) were compared after 3 h. In the control cells (− *cd*) there was an equal amount of sulfated HSPG recovered from the cell layer and basal medium. After induction (+ *cd*) the amount of sulfated HSPG recovered from the total sample was significantly reduced. There was significantly less secretion of newly sulfated HSPG into the medium and less sulfated HSPG in the cell layer.

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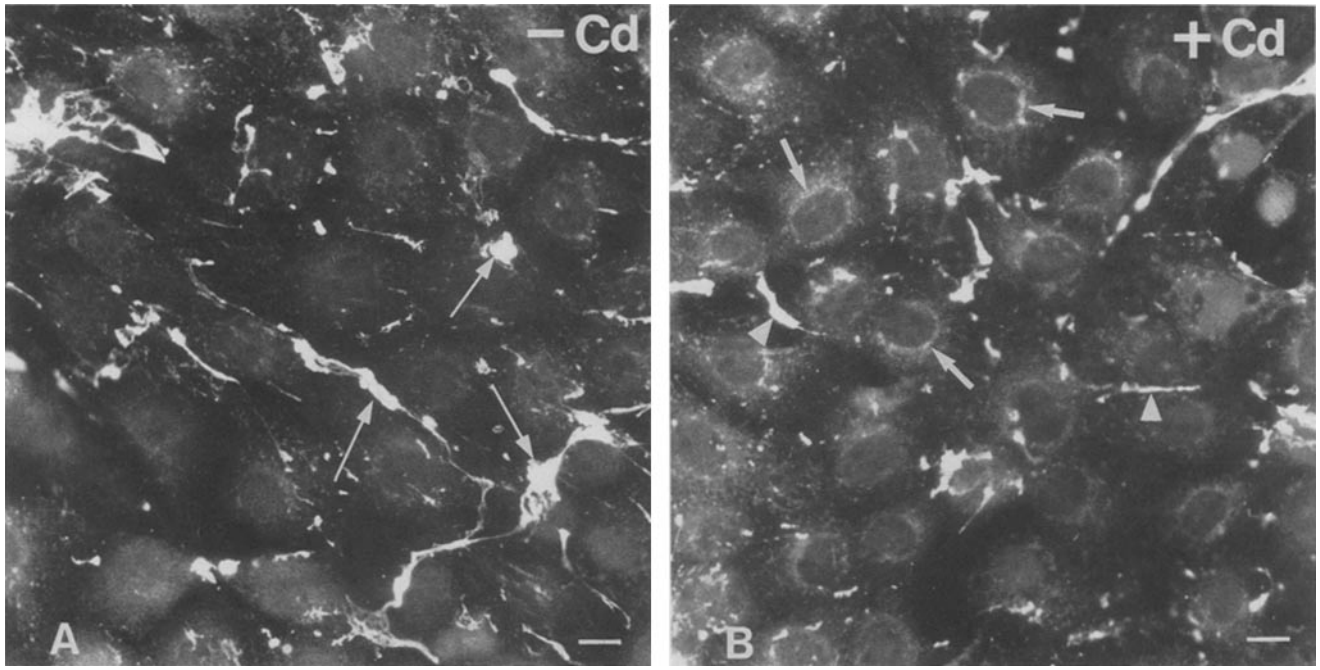

Figure 10. Immunofluorescence staining of HSPG in fixed, permeabilized LLC-PK$_{1(\alpha i\text{-}3)}$ cells. (*A*) In control (− *cd*) cultures the HSPG was found predominantly in clumps of extracellular matrix (*arrows*), there is only faint intracellular staining of HSPG in most of the cells representing HSPG precursors moving constitutively through the secretory pathway. (*B*) In induced cells (+ *cd*) the secreted HSPG is still found in the extracellular matrix (*arrowheads*) but there is also now brighter intracellular staining of HSPG, which is concentrated in the perinuclear region (*arrows*) in many cells. This increased staining of HSPG in the Golgi area is consistent with the retention of the radiolabeled HSPG Golgi precursors seen after induction of G$\alpha_{i\text{-}3}$. Bars, 10 μm.

staining is consistent with the reduced secretion and increased accumulation of HSPG precursors in the Golgi (Fig. 10 *B*).

Discussion

The data presented here provide the first evidence that a heterotrimeric G protein is involved in the constitutive secretory pathway. The heterotrimeric, pertussis toxin–sensitive G$\alpha_{i\text{-}3}$ subunit was localized on the Golgi membranes of epithelial cells and was shown to influence the rate of HSPG trafficking through the Golgi complex in LLC-PK$_1$ cells. The G$\alpha_{i\text{-}3}$ subunit (41 kD) has previously been assigned more traditional roles in signal transduction and ion channel regulation at the cell surface (7, 8), and the association of $\alpha_{i\text{-}3}$ with Golgi trafficking now represents a novel function for this Gαi subunit.

In LLC-PK$_1$ cells and NRK cells, G$\alpha_{i\text{-}3}$ was found predominantly on Golgi membranes and was also found diffusely through the cytoplasm. The localization of the G$\alpha_{i\text{-}3}$ subunits on the cytoplasmic face of the Golgi cisternae in LLC-PK$_1$ cells is consistent with their cytosolic origin and also shows that the $\alpha_{i\text{-}3}$ is not merely present in the Golgi as a biosynthetic transient. The relative longevity of $\alpha_{i\text{-}3}$ on these membranes in cycloheximide-treated cells is further evidence that it is a resident Golgi protein. The $\alpha_{i\text{-}3}$ subunit was also identified by Western blotting and by ADP ribosylation on isolated Golgi membranes from rat liver where it was found to be the only pertussis toxin substrate. Although G$\alpha_{i\text{-}3}$ is found on the Golgi complex in all of the cell types we have examined, in some cells, such as A6 toad kidney cells, the $\alpha_{i\text{-}3}$ subunit is also concentrated near the apical cell surface where it regulates Na^+ channel activity (8) (Ausiello, D., J. Stow, H. Cantiello, and D. Benos, manuscript in preparation). Thus, G$\alpha_{i\text{-}3}$ may also have other roles in some cell types.

Previous studies have shown evidence for the presence of pertussis toxin–sensitive heterotrimeric G proteins on intracellular membranes in the secretory pathway, although their function has not previously been determined. A pertussis toxin-sensitive 41-kD protein, named G_{RER}, was found on isolated rough ER membrane fractions from dog pancreas (2). A pertussis toxin sensitive 41-kD protein was identified on plasma membranes and endosomal membranes isolated from rat liver and a Gα subunit antibody which recognized this protein also recognized a 41-kD protein in the "heavy" Golgi membrane fraction (1). A 46-kD membrane-associated protein which was ADP ribosylated by pertussis toxin has also been found on rat liver Golgi membranes (38) although in this study the specific Gα subunit was not identified. The relationship of these other pertussis toxin sensitive subunits on Golgi membranes is unclear and could be addressed by direct comparison of the subunits with specific peptide antibodies. Since, in the current study, we found only a single pertussis toxin substrate on the Golgi membranes, which we identified as G$\alpha_{i\text{-}3}$ with specific antibodies, the evidence suggests that the subunits identified in these previous studies may indeed also be G$\alpha_{i\text{-}3}$.

Ultrastructural immunolocalization revealed that $\alpha_{i\text{-}3}$ is present on all, or most, of the cisternae in Golgi stacks although there was no evidence of $\alpha_{i\text{-}3}$ staining on vesicles associated with the Golgi membrane. The $\alpha_{i\text{-}3}$ subunit therefore resides on the Golgi cisternae in the company of monomeric G proteins, such as rab 6, which has also been localized by immunoperoxidase on Golgi membranes (19). The distribution of these G proteins throughout the Golgi

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stack varies. While $G\alpha_{i\text{-}3}$ is found on all cisternae, rab 6 is more concentrated on the medial and *trans*-Golgi cisternae and the ARF (ADP ribosylation factor) protein was found to be concentrated on the *cis*-Golgi cisternae (34).

The mechanisms for targeting α subunits to different membrane domains are unknown. In LLC-PK_1 cells the targeting of $\alpha_{i\text{-}3}$ to the Golgi is highly specific since we have shown that a homologous $\alpha_{i\text{-}2}$ subunit is not found on Golgi membranes, but is instead directed to the basolateral membrane (13). In transfected LLC-$PK_{1(\alpha i\text{-}3)}$ cells, the overexpressed $\alpha_{i\text{-}3}$ was correctly targeted to the Golgi membranes under the conditions of induction of the MT-I promoter used in these experiments that resulted in a threefold increase in $G\alpha_{i\text{-}3}$ protein. However, when $\alpha_{i\text{-}3}$ was overexpressed in large amounts in transiently transfected cells with a Rous sarcoma viral promoter, it was overexpressed on the Golgi membrane but in addition, excess $\alpha_{i\text{-}3}$ was distributed all over the cell, suggesting that the targeting of αi subunits may be a saturable process (Stow, J., E. Heltzman, and L. Ercolari, unpublished observations). The transfected LLC-$PK_{1(\alpha i\text{-}3)}$ cells provide a system in which to study the function of $\alpha_{i\text{-}3}$ in the secretory pathway in intact cells. A threefold increase in the amount of $\alpha_{i\text{-}3}$ on Golgi membranes perturbed Golgi trafficking and resulted in significant retardation of HSPG secretion. The biological function of the heterotrimeric $G\alpha$ subunits is to serve as signal transducers, with the paradigm being their ability to link ligand receptor complexes to effector systems, such as enzymes or ion channels. In the most extensively studied model, G protein coupling to adenylyl cyclase, it has been shown that the steady-state interactions among receptors, $G\alpha$ subunits, and the catalytic subunit of adenylyl cyclase, result in a significant amplification of the signal transduction response (32). Due to differences in the stoichiometry of the various subunits, and the half-lives of the activated species, a twofold change in α subunit concentration can lead to a 50–100% change in enzyme activity (32), thus the observed two- to threefold change in $\alpha_{i\text{-}3}$ on Golgi membranes that results in a 60% change in HSPG secretion is consistent with the amplification response for heterotrimeric G protein pathways.

If the overexpressed $\alpha_{i\text{-}3}$ is exacerbating the effect of the endogenous $\alpha_{i\text{-}3}$, it suggests that the normal action of $\alpha_{i\text{-}3}$ is a rate-limiting step, or regulatory step, in transport through the later stages of Golgi transport. Our results do not rule out the potential involvement of $\alpha_{i\text{-}3}$ in earlier stages of Golgi transport, since it is localized throughout the Golgi stack. However, only the medial-late Golgi HSPG precursors, the 450-kD form and sulfated forms in the *trans*-Golgi, can be easily measured in these cells. The rate-limiting step imposed on Golgi traffic by either endogenous or overexpressed $\alpha_{i\text{-}3}$ was overcome by the functional uncoupling of the α subunit by ADP ribosylation with pertussis toxin. Our current results, and previous studies (23), show that $\alpha_{i\text{-}3}$ on Golgi membranes is ADP ribosylated by pertussis toxin both in vitro and in intact cells. The effect of pertussis toxin suggests that $G\alpha_{i\text{-}3}$ on Golgi membranes is bound to its $\beta\gamma$ subunits, since only α subunits bound to $\beta\gamma$ are targets for pertussis toxin induced ADP ribosylation (16, 37). In addition, since the monomeric G proteins are not ribosylated by this toxin, the effect of pertussis toxin shows that the retardation of secretory transport is a specific effect of the heterotrimeric $\alpha_{i\text{-}3}$ subunit, the only pertussis toxin–sensitive α subunit that is a resident Golgi protein in these cells.

Previous studies have shown that GTPγS inhibits vesicular transport from the rough ER to the Golgi in yeast and in mammalian cells (3, 28, 30), as well as between Golgi compartments (22). The effects of GTPγS in these studies have been ascribed to the monomeric G proteins which have been identified on different vesicle populations. GTPγS has also been used to implicate GTP-binding proteins at later stages of both the constitutive and regulated secretory pathways (17, 39). In a cell-free system, GTPγS has been shown to block the budding of vesicles carrying sulfated HSPG out of the *trans*-Golgi (39). Interestingly, while the inhibition of GTP hydrolysis by the use of the analogue, GTPγS, has universally been shown to "activate" heterotrimeric $G\alpha$ subunits, it results in the "inhibition" of monomeric G proteins that are involved in vesicle trafficking between the rough ER and Golgi (3, 30). Interpretation of the effects of GTPγS on the secretory pathway must take into account the actions of both monomeric and heterotrimeric G proteins.

The actual role of monomeric and heterotrimeric G proteins on the dynamics of the secretory pathway must await the identification of the receptor and effector elements with which these G proteins presumably interact. Using the G protein signal transducer paradigm, it is likely that the $\alpha_{i\text{-}3}$ on the Golgi is coupled to an effector which directly, or indirectly, modifies Golgi trafficking. This effector system is unknown at this time. However, given that α subunits can couple to multiple enzymes and ion channels, including phospholipases (8) and Na^+ (7) and K^+ channels for $\alpha_{i\text{-}3}$, it is possible that changes in phospholipid metabolism, ionic gradients or pH, could serve as the effector systems for induction or inhibition of vesicle movement. The elucidation of this pathway is the focus of our current work.

The studies presented here were conducted in the polarized, epithelia cell line, (LLC-PK_1), which has been used extensively in the last decade as a model for epithelial function, including HSPG secretion (11). It remains to be shown, however, whether $\alpha_{i\text{-}3}$ plays a prominent role in regulation of secretion in other epithelial or nonpolar cells. It can be said that $\alpha_{i\text{-}3}$ has been localized by immunocytochemistry on the Golgi membranes of many other epithelial cells, including NRK cells, MDCK, and A6 cells (data not shown), and that it is present in rat liver Golgi membranes, consistent with the hypothesis that the novel signal transduction role for $G\alpha_{i\text{-}3}$ proposed in this manuscript is a general phenomenon.

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