# Differential Distribution of α Subunits and βγ Subunits of Heterotrimeric G Proteins on Golgi Membranes of the Exocrine Pancreas

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***Abstract.*** Heterotrimeric G proteins are well known to be involved in signaling via plasma membrane (PM) receptors. Recent data indicate that heterotrimeric G proteins are also present on intracellular membranes and may regulate vesicular transport along the exocytic pathway. We have used subcellular fractionation and immunocytochemical localization to investigate the distribution of Gα and Gβγ subunits in the rat exocrine pancreas which is highly specialized for protein secretion. We show that $G\alpha_s$, $G\alpha_{i3}$ and $G\alpha_{q/11}$ are present in Golgi fractions which are >95% devoid of PM. Removal of residual PM by absorption on wheat germ agglutinin (WGA) did not deplete Gα subunits. $G\alpha_s$ was largely restricted to TGN-enriched fractions by immunoblotting, whereas $G\alpha_{i3}$ and $G\alpha_{q/11}$ were broadly distributed across Golgi fractions. $G\alpha_s$ did not colocalize with TGN38 or caveolin, suggesting that $G\alpha_s$ is associated with a distinct population of membranes. Gβ subunits were barely detectable in purified Golgi fractions. By immunofluorescence and immunogold labeling, Gβ subunits were detected on PM but not on Golgi membranes, whereas $G\alpha_s$ and $G\alpha_{i3}$ were readily detected on both Golgi and PM. Gα and Gβ subunits were not found on membranes of zymogen granules. These data indicate that $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i3}$ associate with Golgi membranes independent of Gβ subunits and have distinctive distributions within the Golgi stack. Gβ subunits are thought to lock Gα in the GDP-bound form, prevent it from activating its effector, and assist in anchoring it to the PM. Therefore the presence of free Gα subunits on Golgi membranes has several important functional implications: it suggests that Gα subunits associated with Golgi membranes are in the active, GTP-bound form or are bound to some other unidentified protein(s) which can substitute for Gβγ subunits. It further implies that Gα subunits are tethered to Golgi membranes by posttranslational modifications (e.g., palmitoylation) or by binding to another protein(s).

VESICULAR transport involves a series of highly specific budding, targeting, and fusion events in which proteins move between successive compartments along the exocytic pathway. Small GTP-binding proteins of the rab family (18, 36) have been identified as key regulators of this process. Recent evidence suggests that heterotrimeric G proteins (Gα)[1] also play a regulatory role in intracellular transport at multiple steps along the exocytic (5, 7, 9) as well as the endocytic (13) and transcytotic pathways (8).

The involvement of Gα subunits in vesicular transport was initially suggested by the finding that $AlF_4^-$, an activator of the heterotrimeric but not the small molecular weight G proteins (24, 26), inhibited intra-Golgi transport in a cell-free system (35). Gα proteins were implicated in another aspect of vesicular transport, coat formation, by experiments in which excess βγ subunits inhibited the association of ADP-ribosylation factor (ARF) and β-COP with Golgi membranes that occurs in the presence of GTPγS (15).

Direct evidence that Gα proteins regulate transport through the Golgi came from the demonstration that overexpression of $G\alpha_{i3}$ in LLC-PK1 cells retarded the secretion of heparan sulfate proteoglycan and accumulated precursors in the medial/*trans*-Golgi, and this effect was reversed by addition of pertussis toxin (which inactivates $G\alpha_i$ proteins) (52). However, in permeabilized NRK cells neither pertussis toxin nor cholera toxin (which constitutively activates $G\alpha_s$) had an effect on βγ-induced inhibition of VSV-G export from the ER (50). Thus, the available data do not point to a general mechanism by which Gα proteins regulate transport, but rather suggest there may be cell type– and compartment-specific variations. Localization studies support this assumption: $G\alpha_{i3}$ was localized to multiple Golgi cisternae in LLC-PK1 cells (52)

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1. *Abbreviations used in this paper*: AlkPDE, alkaline phosphodiesterase; ARF, ADP-ribosylation factor; DAG, diacylglycerol; Gα, heterotrimeric G protein alpha subunit; Man II, α-mannosidase II; PFA paraformaldehyde; PKC, protein kinase C; PM, plasma membrane; TfR, transferrin receptor.

and primarily to *cis*-Golgi elements in rat pituitary cells (60) by immunoelectron microscopy, whereas $G\alpha_s$ was localized to the nucleus and PM in S49 lymphoma cells (48) but partially overlapped with a *trans*-Golgi marker (TGN38) in PC12 cells by immunofluorescence (31). Similarly, based on subcellular fractionation, Gα subunits were found to be associated with a variety of intracellular membranes including chromaffin granules, synaptic vesicles (1), and constitutive and regulated secretory granules from PC12 cells (31).

To investigate the role of Gα subunits in vesicular transport, we turned to the exocrine pancreas, the classical model system in which the biosynthetic route of secretory proteins was defined (39). In the exocrine pancreas it is known that activation of PM receptors and Gα subunits of the s, q, and i families controls the final exocytic event, secretory granule release, via second messengers such as cAMP and diacylglycerol (DAG) (37, 38, 41, 49). However, there is as yet no data available on the role of Gα proteins in earlier transport events or in constitutive secretion. The highly organized architecture of intracellular compartments and the well-characterized transport machinery make the pancreas an ideal system for studying this question. Given the abundance of the intracellular machinery required for vesicular transport in the exocrine pancreas, Gα proteins should be expressed in amounts sufficient for detection by biochemical and immunocytochemical techniques if they constitute part of this machinery.

In the current study, we have determined the distribution of three Gα subunits ($G\alpha_s$, $G\alpha_{i3}$, and $G\alpha_{q/11}$) as well as Gβ subunits in intracellular compartments of exocrine pancreatic cells by subcellular fractionation and immunofluorescence and immunogold labeling. We show that all three Gα subunits are found in Golgi membranes independent of Gβ subunits and that $G\alpha_s$ has a different distribution among Golgi membranes than $G\alpha_{i3}$ and $G\alpha_{q/11}$.

## Materials and Methods

### Materials

Reagents and supplies were obtained from the following sources: male rats (100–150 g) were from Harlan Sprague Dawley (Indianapolis, IN), Ham's F12 medium from the UCSD Core Facility (La Jolla, CA), and FCS from GIBCO-BRL (Gaithersburg, MD). HL-1 was from Ventrex Labs (Portland, ME), Nutridoma from Boehringer Mannheim Corp. (Indianapolis, IN), and Nu-serum IV from Collaborative Research, Inc. (Bedford, MA). FITC-conjugated donkey anti-rabbit $F(ab')_2$ and Texas red-conjugated donkey anti-mouse $F(ab')_2$ cross-absorbed against human, mouse, rat, chicken, and goat were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Colloidal gold-conjugated goat anti-mouse and goat anti-rabbit IgG and the EnhancedChemiLuminescence (ECL) detection kit were from Amersham Life Sciences (Arlington Heights, IL). WGA-agarose and reagents for the α-mannosidase II (Man II) and alkaline phosphodiesterase (AlkPDE) assays were purchased from Sigma Chem. Co. (St. Louis, MO). All other chemicals were purchased from commercial sources and were of the highest grade.

### Antibodies

Affinity-purified rabbit IgG directed against the carboxy terminus of $G\alpha_s$ (RM) or $G\alpha_{i3}$ (EC) were kindly provided by Dr. A. Spiegel (NIDDK, Bethesda, MD). Mouse mAb 1D9 recognizing ARF 1, 3, 5, and 6 was a gift of R. Kahn (NCI, Bethesda, MD), mouse mAb H68.4 against the COOH terminus of the transferrin receptor (TfR) (59) was from I. Trowbridge (Salk Institute, La Jolla, CA), mouse mAb against Man II was prepared from ascites induced with 5CF3 hybridoma cells provided by Brian Burke (10), rabbit affinity-purified IgG against rab1b (44) was a gift of W. Balch (Scripps Research Institute, La Jolla, CA), mouse ascites against β-COP (2) were generated from M3A5 hybridoma cells provided by T. Kreis (University of Geneva). Rabbit antisera against the luminal domain of TGN38 (34) was a gift from K. Howell (University of Colorado, Denver, CO), and antisera directed against the amino terminus of cellubrevin (19) was from P. DeCamilli (Yale University School of Medicine, New Haven, CT). Monoclonal mouse anti-VIP21/caveolin IgG was purchased from Zymed Labs (South San Francisco, CA). Affinity-purified rabbit IgG recognizing the carboxy terminus of rab6 or the common carboxy terminus of the Gβ subunit (T-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit antisera to the common carboxy terminus (SW/1) and amino terminus (MS/1) of the Gβ subunit and to the common carboxy terminus of $G\alpha_{q/11}$ (QL) was purchased from New England Nuclear (Wilmington, DE).

### Pancreatic Lobule Preparation and Cell Fractionation

Rat pancreatic lobules were prepared as described (22, 23). Smooth membranes were separated from rough microsomes as in (23) except that the homogenization buffer consisted of 0.3 M sucrose, 10 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 5 mM benzamidine, and 5 mM EGTA. Total microsomes enriched in Golgi membranes were collected at the 0.3/1.3 M sucrose interface, adjusted to 1.35 M sucrose and loaded in a sandwich gradient as described (23). Fractions (1.0 ml) were collected from the bottom of the gradient and analyzed for rough ER, Golgi, and plasma membrane (PM) markers.

PM was separated from microsomes as follows: total microsomes were isolated as above, with the addition of protease inhibitors soy bean trypsin inhibitor (20 μg/ml), aprotinin (0.1 TIU/ml), chymostatin, leupeptin, antipain, and pepstatin A (all at 1 μg/ml). Microsomes were brought to 1.3 M sucrose, and 1.0 ml of sample was overlaid with 1.2 M (0.5 ml), 1.14 M (1.5 ml), 0.99 M (0.5 ml), and 0.9 M (0.5 ml) Tris-HCl-buffered sucrose for a total volume of 4.0 ml. The gradient was centrifuged to equilibrium at 170,000 *g* for 14 h, SW60 rotor, LM-80 Ultracentrifuge (Beckman Instrs., Inc., Palo Alto, CA). Fractions (0.5 ml) were collected from the bottom and analyzed as described below.

### Membrane Marker and Enzyme Assays

Protein and nucleic acid content were determined using the Nucleic Acid Soft-Pac module (Program 10) on a Beckman DU-64 Spectrophotometer. Man II activity was determined by hydrolysis of *p*-nitrophenyl-α-D-mannopyranoside (23, 55) and AlkPDE activity was determined by hydrolysis of thymidine 5′-monophosphate-*p*-nitrophenyl ester (45) with volumes reduced to facilitate the assays in 96-well plates. After incubation at 37°C for 15 min (AlkPDE activity) or 1 h followed by quenching with 100 mM NaOH (Man II activity) absorbances were measured at 405 nm using a Microplate Reader (Molecular Devices Corp., Sunnyvale, CA).

### Immunoblotting

Fractions were diluted by addition of 10 mM Tris-HCl buffer, pH 7.2, and membranes were pelleted at 100,000 *g* for 1 h, TLA45 rotor, TL-100 Ultracentrifuge. Pellets were resuspended in Laemmli sample buffer and boiled. Proteins were separated by 10% or 12% SDS-PAGE, transferred to polyvinylidine difluoride (PVDF) membranes and probed with primary antibodies. Antigen–antibody complexes were detected using HRP-conjugated anti-rabbit or anti-mouse IgG and the ECL kit. Quantitation of bands was determined using a LKB UltroScan XL Densitometer. To determine the enrichment of $G\alpha_s$ in subcellular membranes relative to the amount of PM in the fractions, densitometer values obtained from immunoblots for $G\alpha_s$ were divided by absorbance values obtained from the AlkPDE enzyme assay.

### Depletion of PM from Golgi-enriched Fractions by Absorption on WGA-Agarose

PM (fractions 6 and 7, pooled) or Golgi membranes (fractions 2 and 3, pooled) were diluted in PBS containing fresh protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin A, all at 1 μg/ml). 1.0-ml aliquots were incubated end-over-end at 4°C overnight with WGA-agarose (5–400 μg/ml). Control samples were incubated with agarose beads. After separation of bound from nonbound membranes by pelleting (1,000 *g* for 5 min), an aliquot (100 μl) of the nonbound material was removed for protein and AlkPDE determinations, while the remainder, representing PM-depleted

membranes, was pelleted (100,000 *g* for 1 h) and solubilized in Laemmli sample buffer. Membrane proteins were released from WGA-agarose by adding sample buffer, followed by boiling for 5 min. Samples were subject to 12% SDS-PAGE and immunoblotting.

AlkPDE assays were performed as described above, with the exception that for PM-depleted fractions, longer incubations (1 h) were required to obtain adequate activity to record a positive signal. Data are presented as percent remaining activity, and values were determined using the formula A = (absorbance after WGA/absorbance before WGA) × 100%. Because 7.9% corresponded to the sum of AlkPDE activity in fractions 2 and 3 of the flotation gradient, the remaining PM in Golgi fractions is .079A.

### *Immunolabeling*

Pancreatic lobules were fixed in 8% paraformaldehyde (PFA), 100 mM phosphate buffer, pH 7.4, (15 min) followed by 4% PFA in phosphate buffer (1 h). Samples were cryoprotected and frozen in liquid $N_2$ as described (23, 56). Semithin (0.5–1.0 μm) cryosections were incubated with primary antibodies (3 h at 4°C) followed by cross-absorbed FITC-conjugated donkey anti–rabbit $F(ab')_2$ or TRITC-conjugated donkey anti–mouse $F(ab')_2$ (1 h at 4°C). Ultrathin cryosections were incubated 2 h at 4°C with primary antibodies in 10% FCS/PBS, followed by 5- or 10-nm gold-conjugated goat anti–rabbit or goat anti–mouse IgG (2 h at 4°C). Grids were stained in 2% neutral uranyl acetate (10 min), adsorption-stained with 0.2% neutral uranyl acetate, 0.2% methyl cellulose, and 3.2% polyvinyl alcohol, and observed in a JEOL 1200 EX-II or Phillips CM-10 electron microscope. For quantitation of immunogold labeling of $G\alpha_s$ and $G\alpha_{i3}$, over 50 total micrographs were taken from seven experiments. From these, for each Gα subunit, seven micrographs in which *cis* and *trans* sides of the Golgi could be clearly identified were chosen for counting. A total of 250 gold particles in the Golgi region were counted for each Gα subunit. For TGN38, a total of 850 gold particles were counted from seven micrographs. The Golgi stack was divided down the middle and particles were scored as *cis* based on their presence on the side facing the nucleus and transitional elements of the ER, or as *trans* by the presence of condensing vacuoles.

## *Results*

### *Heterotrimeric α Subunits $G\alpha_s$ and $G\alpha_{i3}$ Are Found in the Golgi Region and PM by Immunofluorescence*

As a first approach to defining the role of Gα proteins in transport along the exocytic pathway, we investigated the localization of $G\alpha_s$ and $G\alpha_{i3}$ by immunofluorescence. Double-labeling experiments were performed on semithin cryosections of rat pancreas using affinity-purified anti-$G\alpha_s$ or $G\alpha_{i3}$ IgG and a mouse mAb to Man II, a *cis-trans*-Golgi marker in pancreatic exocrine cells (56). Both $G\alpha_s$ (Fig. 1 *B*) and $G\alpha_{i3}$ (Fig. 1 *E*) were detected on PM. In addition, both were detected in the Golgi region of the cell where they overlapped with Man II (Fig. 1, *C* and *F*). No signal for either Gα protein was detected on zymogen granule membranes. The partial overlap with Man II suggested that $G\alpha_s$ and $G\alpha_{i3}$ may be associated with Golgi membranes of the pancreatic exocrine cell. Since $G\alpha_{i3}$ had been previously localized to the Golgi in other cell types (53, 60) and information on the localization of $G\alpha_s$ is more limited, we focused on $G\alpha_s$ and compared its distribution in the Golgi to that of β-COP, ARF, and caveolin, proteins with unique Golgi localizations. Staining for the coatamer components (28, 51) β-COP (Fig. 2 *C*) and ARF (Fig. 2 *F*) was confined to the Golgi region and partially overlapped with $G\alpha_s$ (Fig. 2, *B* and *E*). With ARF there was also diffuse cytoplasmic staining attributed to the large cytosolic pool (~60% in the pancreas) of ARF. Caveolin was found both at the PM and in the Golgi region (Fig. 2 *I*) where it over-

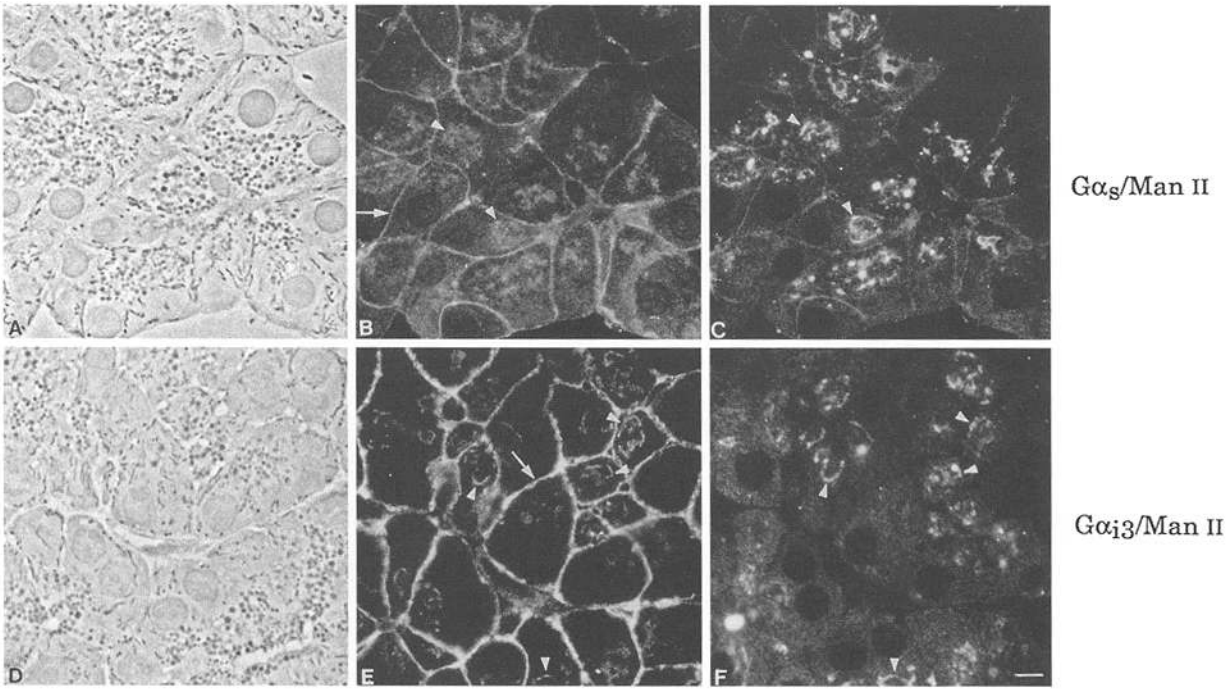


*Figure 1.* Localization of $G\alpha_s$ and $G\alpha_{i3}$ to the Golgi region and the PM of exocrine pancreatic cells. By immunofluorescence, both $G\alpha_s$ (*B*) and $G\alpha_{i3}$ (*E*) colocalize with Man II (*C* and *F*), a *cis-trans* Golgi marker in the pancreas (56). Arrowheads indicate regions of overlap between Gα proteins and Man II. The distribution of $G\alpha_s$ in the Golgi is more diffuse than $G\alpha_{i3}$. Arrows in *B* and *E* indicate PM labeling. Semithin cryosections were prepared from formaldehyde-fixed pancreas lobules as described in Materials and Methods. Sections were incubated with a mouse mAb to Man II (1:400) and affinity-purified rabbit IgG against either $G\alpha_s$ (1:400) or $G\alpha_{i3}$ (1:100), followed by Texas red–conjugated goat anti–mouse and FITC donkey anti–rabbit conjugates. Phase contrast images for each field are shown in *A* and *D*. Bar, 5 μm.

lapped extensively with $G\alpha_s$ (Fig. 2 *H*). In addition, labeling for both $G\alpha_s$ and caveolin was intense on endothelial cells of the capillaries surrounding the acini (data not shown).

Taken together, the immunofluorescence results strongly suggested that $G\alpha_s$ and $G\alpha_{i3}$ are associated with Golgi membranes where they partially overlap with ARF, β-COP, and caveolin.

### *$G\alpha_s$ Is Restricted to Smooth Membrane Fractions of Rat Pancreas*

We next turned to subcellular fractionation in an attempt to isolate the compartment(s) associated with these Gα subunits. Using an established protocol (23) to separate smooth membranes (endosomes, Golgi, PM) from rough microsomes (rough ER, transitional ER elements), we found that $G\alpha_s$ fractionated exclusively with smooth membranes. Fig. 3 shows that the bulk of the rough microsomes were present in fractions 1-3 (based on measurement of nucleic acid; Fig. 3 *A*), Golgi membranes were enriched in fractions 9 and 10 (based on Man II activity; Fig. 3 *B*), and PM was enriched in fractions 10-11 (based on AlkPDE activity; Fig. 3 *B*). Thus, there was a significant overlap of Golgi and PM markers in fraction 10 (Fig. 3 *B*).

By immunoblotting, $G\alpha_s$ (Fig. 3 *C*) was restricted to those fractions enriched in Golgi membranes (fractions

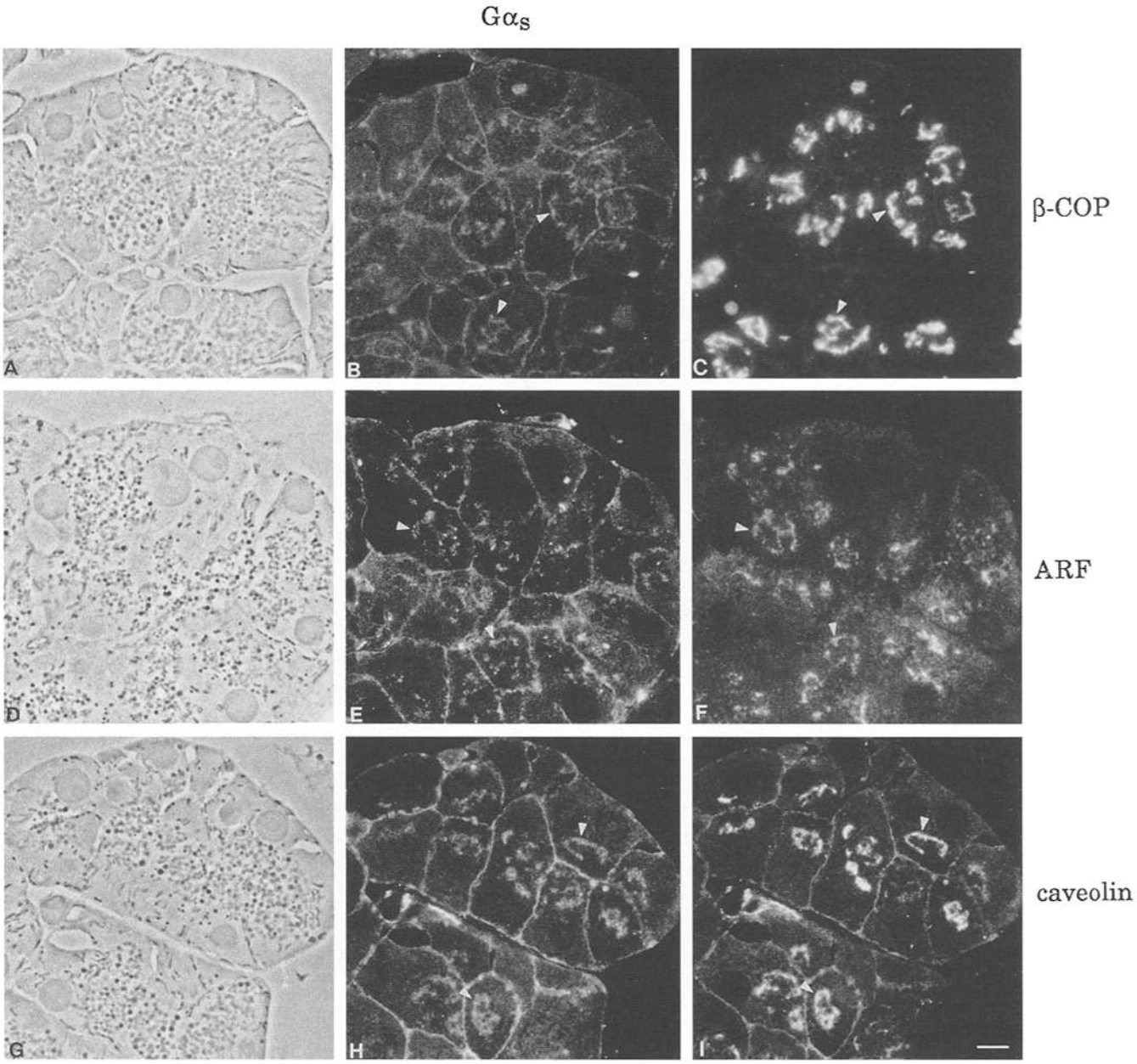


*Figure 2.* $G\alpha_s$ staining overlaps with that of β-COP, ARF, and caveolin. Areas of partial overlap of $G\alpha_s$ with these three proteins in the Golgi region are indicated by arrowheads. $G\alpha_s$ staining most closely resembles that of caveolin. Semithin cryosections were incubated with rabbit antibody to $G\alpha_s$ (*B*, *E*, and *H*) and the following mouse mAb: (*C*) β-COP (1:200), (*F*) ARF (1:100), and (*I*) caveolin (1:200), followed by the appropriate conjugates (as in Fig. 1). Phase contrast images for each field are shown in *A*, *D*, and *G*. Bar, 5 μm.

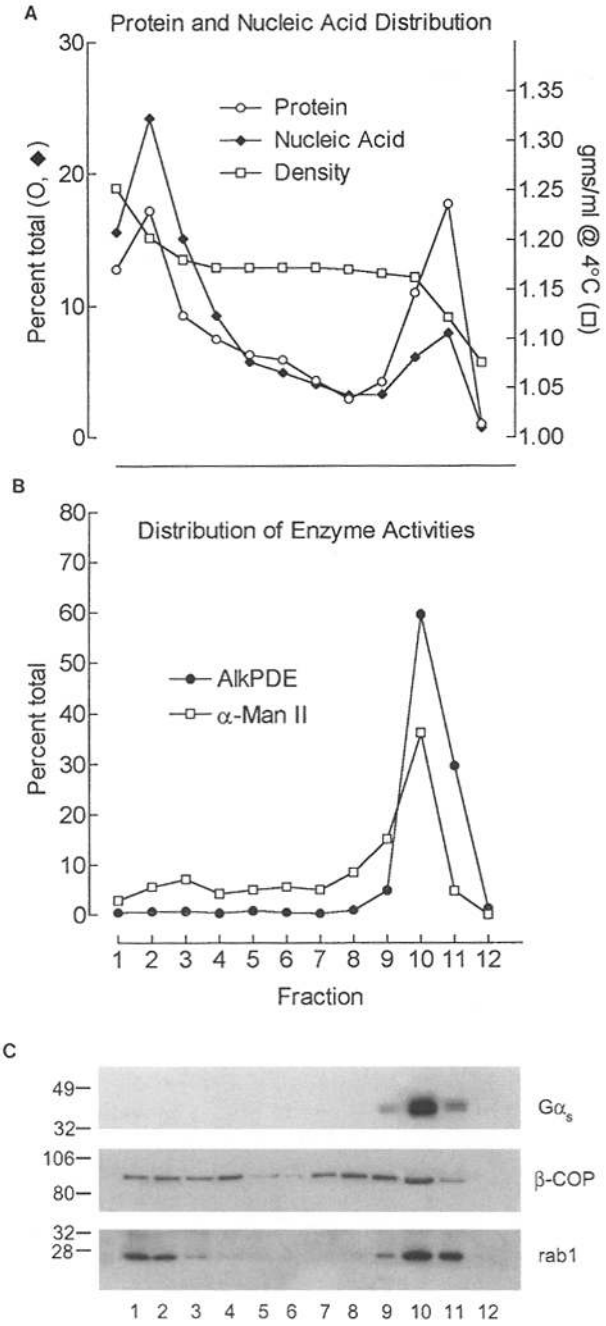


*Figure 3.* $G\alpha_s$ is restricted to smooth membrane fractions. Total microsomes were subfractionated by centrifugation and fractions (1.0 ml) were analyzed for rough ER, Golgi, and PM markers. 55% of rough microsomes, based on nucleic acid content (*A*), are found in fractions 1-3 ($d$ = 1.25–1.18 g/ml), whereas 90% of PM, monitored by AlkPDE activity (*B*), and 41% of Golgi membranes, monitored by Man II activity (*B*), sediment together in fractions 10-11 ($d$ = 1.16–1.12 g/ml). Immunoblot analysis of these fractions (*C*) shows $G\alpha_s$ is restricted to smooth membrane fractions (fractions 9-11), while β-COP and rab1 are associated with both smooth membranes (fractions 9-11) and rough microsomal fractions (fractions 1-3). Molecular weights are indicated on the left. Data shown are representative of one of four experiments. Fraction 1 corresponds to the bottom of the gradient.

9-10) and PM (fractions 10-11). Fraction 10, which contained the highest content of both Golgi and PM, also contained the greatest amount of $G\alpha_s$.

The distribution of $G\alpha_s$ was much more restricted than that of β-COP and rab1b, markers for ER to Golgi and intra-Golgi carrier vesicles and the intermediate compartment (44, 46). Both β-COP and rab1b were broadly distributed in rough microsomes as well as smooth membrane fractions (Fig. 3 *C*). The restricted distribution of $G\alpha_s$ did not merely reflect protein levels, because Golgi fractions enriched in $G\alpha_s$ typically contained only 15% of the total protein distributed across the gradient. In separate experiments pancreatic zymogen granules were isolated, and their membranes were prepared and analyzed for the presence of $G\alpha_s$ by immunoblotting. $G\alpha_s$ was not enriched in comparison to total membranes (data not shown).

### *Separation of Golgi Membranes from PM Reveals Two Distinct Pools of $G\alpha_s$ and $G\alpha_{i3}$*

To further characterize the Golgi and PM pools of Gα proteins, we devised a fractionation method for separation of PM from Golgi membranes. After equilibrium centrifugation of microsomes in a flotation gradient, PM floated to the top ($d$ = 1.15–1.12 g/ml) whereas rough microsomes and most of the Golgi membranes remained at the load ($d$ = 1.17). Representative data from one of seven fractionation experiments are presented in Fig. 4: ~85% of the PM (AlkPDE activity) was recovered in fractions 5-8 (Fig. 4 *B*), and ~58% of the Golgi membranes (Man II activity) in fractions 1-3 (Fig. 4 *B*). Fractions 1-2 (load fractions) and fraction 3 were enriched in total protein and rough microsomes (Fig. 4 *A*). Significant amounts of Golgi membranes (typically 14–15%) were isolated in fraction 3 ($d$ = 1.16 g/ml). Therefore, fractions 2 and 3 were enriched in Golgi membranes but not PM, and fraction 4, which was not enriched in either population of membranes, separated Golgi membranes from PM (fractions 5-8).

Immunoblotting analysis of pelleted membranes from gradient fractions demonstrated that Man II (data not shown), β-COP, and rab1b (Fig. 4 *C*) were restricted to fractions 1-3. $G\alpha_s$ was resolved into two pools, one peaked with the Golgi-enriched fractions (fractions 2-3) and the other with PM-enriched fractions (fractions 5-8) (Fig. 4 *C*). No difference in the relative amounts of the long (52 kD) and short (45 kD) forms of $G\alpha_s$ was seen among the different membrane populations. The recently cloned extra long form of $G\alpha_s$, XLαs (27), was not detected. By dividing densitometry values from $G\alpha_s$ immunoblots by absorbance readings from AlkPDE assays for each fraction, we determined that $G\alpha_s$ was enriched threefold over AlkPDE in the light Golgi fraction (fraction 3) when compared with fraction 4, which had an equivalent amount of AlkPDE activity. This further indicated that $G\alpha_s$ found in fraction 3 was associated with membranes other than contaminating PM.

$G\alpha_{i3}$ was also found in both Golgi and PM-enriched fractions (5-8) but had a broader distribution across the gradient: it was found in heavy (fraction 1) as well as light (fractions 2 and 3) Golgi fractions. We also investigated the distribution of $G\alpha_{q/11}$, a G protein involved in signaling through phospholipase Cγ. By immunoblotting the distribution of $G\alpha_{q/11}$ was similar to that of $G\alpha_{i3}$, i.e., it was

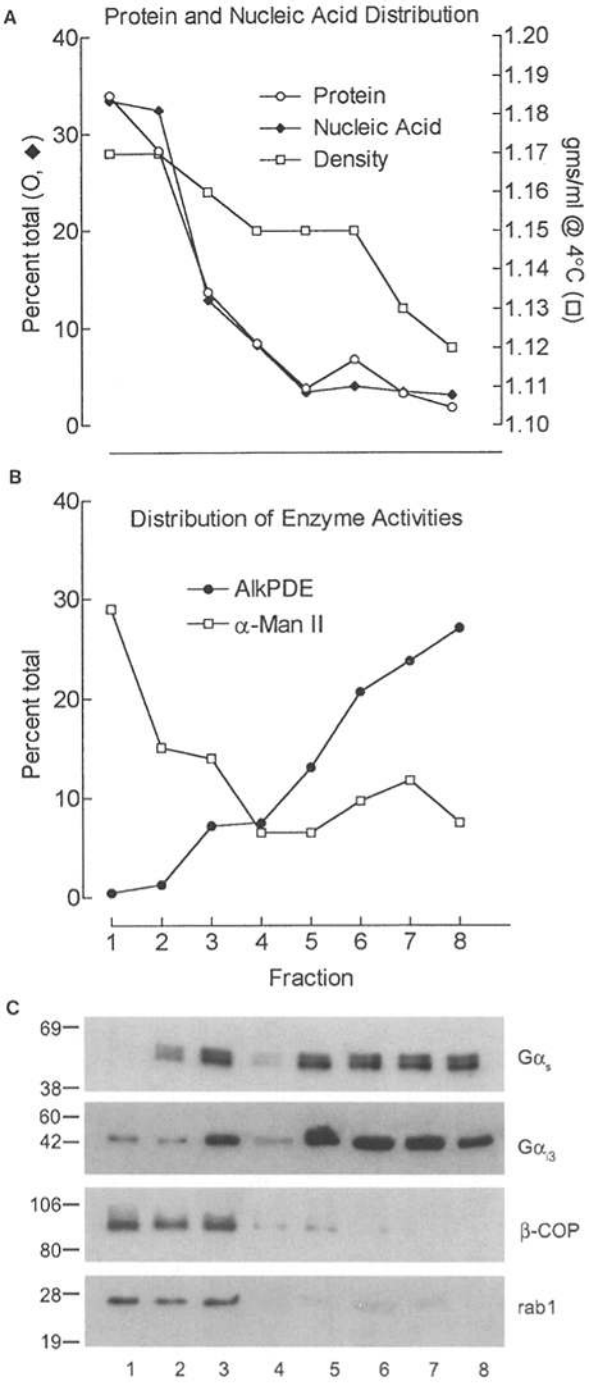


*Figure 4.* Separation of PM from Golgi membranes reveals two pools each of $G\alpha_s$ and $G\alpha_{i3}$, one associated with Man II activity and another associated with AlkPDE activity. Total microsomes were subfractionated by centrifugation and fractions (0.5 ml) were analyzed as in Fig. 3. The majority (85%) of PM based on AlkPDE activity (*B*) is recovered in fractions 5-8, whereas 76% of the protein (*A*), 79% of the rough microsomes (nucleic acid) (*A*), and 58% of the Golgi membranes (Man II activity) (*B*) is recovered in fractions 1-3 ($d$ = 1.17–1.16 g/ml). By immunoblotting (*C*), $G\alpha_s$ and $G\alpha_{i3}$ are detected in fractions enriched in both Golgi (fractions 1-3) and PM (fractions 5-8). The amounts of long (52 kD) and short (45 kD) forms of $G\alpha_s$ in each fraction are similar. After longer exposures, $G\alpha_s$ was still undetectable in fraction 1. β-COP and rab1 are restricted to Golgi fractions. Data are representative of one of seven experiments.

present in Golgi fractions 1-3 and all PM fractions (data not shown). These biochemical data demonstrate that $G\alpha_s$, $G\alpha_{i3}$, and possibly $G\alpha_{q/11}$ are associated with Golgi membranes.

### *Depletion of Residual PM from Golgi Fractions Does Not Deplete Gα Subunits*

Although the pooled Golgi fractions (fractions 2 and 3) contained only 7.9% (maximum) of the total AlkPDE activity distributed across the gradient, we were concerned that contamination by PM could explain the signal for $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{i3}$ associated with these fractions. To deplete Golgi-enriched fractions of PM we carried out lectin absorption on WGA-agarose beads. The depletion step relied on the fact that most PM vesicles fractionate outside out and therefore vesicles derived from PM will bind WGA via exposed sialic acid residues whereas vesicles derived from TGN membranes and transport vesicles (which fractionate outside-in) will not bind.

Fig. 5 *A* shows that with increasing concentrations of lectin, increasing amounts of PM (based on AlkPDE activity) could be absorbed from PM fractions with a corresponding reduction in the amount remaining in the nonbound fraction (Fig. 5 *A*). At higher WGA concentrations (200 μg/ml), 85% of PM was absorbed (data not shown). The decrease in AlkPDE was accompanied by parallel and even greater decreases in $G\alpha_s$, $G\alpha_{i3}$, and Gq proteins from PM fractions (Fig. 5 *B*): 62% of PM was absorbed at 100 μg/ml WGA, while >90% of $G\alpha_s$ and ~70–80% of $G\alpha_{i3}$ was removed from the fractions. These data indicate that PM can be efficiently depleted from PM fractions by lectin absorption.

When Golgi-enriched fractions were subjected to absorption on WGA-agarose, 42% of the residual PM (based on AlkPDE activity) was removed at 100 μg/ml WGA (Fig. 5 *C*). Therefore, only a small fraction (4.6%) of the total PM in the original gradient was present in this pooled Golgi fraction after lectin absorption. Assays for AlkPDE activity in Golgi membranes required extension of incubation times (from 15 min to 1 h) to obtain a positive signal. Significantly, absorption of PM did not lead to detectable decreases in either the long or the short forms of $G\alpha_s$ or of $G\alpha_{q/11}$ or $G\alpha_{i3}$ (Fig. 5 *D*). Gα subunits recovered in the WGA pellets presumably represent residual PM-associated Gα. Densitometry on bound and nonbound fractions showed that for all three Gα subunits ~10, 20, and 30%, respectively, of the total protein was recovered in the bound fractions at 5, 50, and 100 μg/ml WGA. No additional Gα subunits were recovered in the WGA pellets at 200 μg/ml WGA, indicating that 30% represents the maximum amount of contaminating PM Gα present in the Golgi fractions.

This experiment demonstrated that (*a*) lectin absorption is a powerful tool for removal of PM, and (*b*) $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i3}$ are present in a Golgi fraction >95% depleted of PM. The minor amount of residual AlkPDE activity (not removed by increased amounts of WGA) remaining in the pooled Golgi fractions was assumed to be due to the residual PM which reseals outside-in after homogenization.

### *Gα Subunits Partially Cofractionate with Trans-Golgi Markers and ARF*

To further investigate the nature of the intracellular membranes associated with $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i3}$, we per-

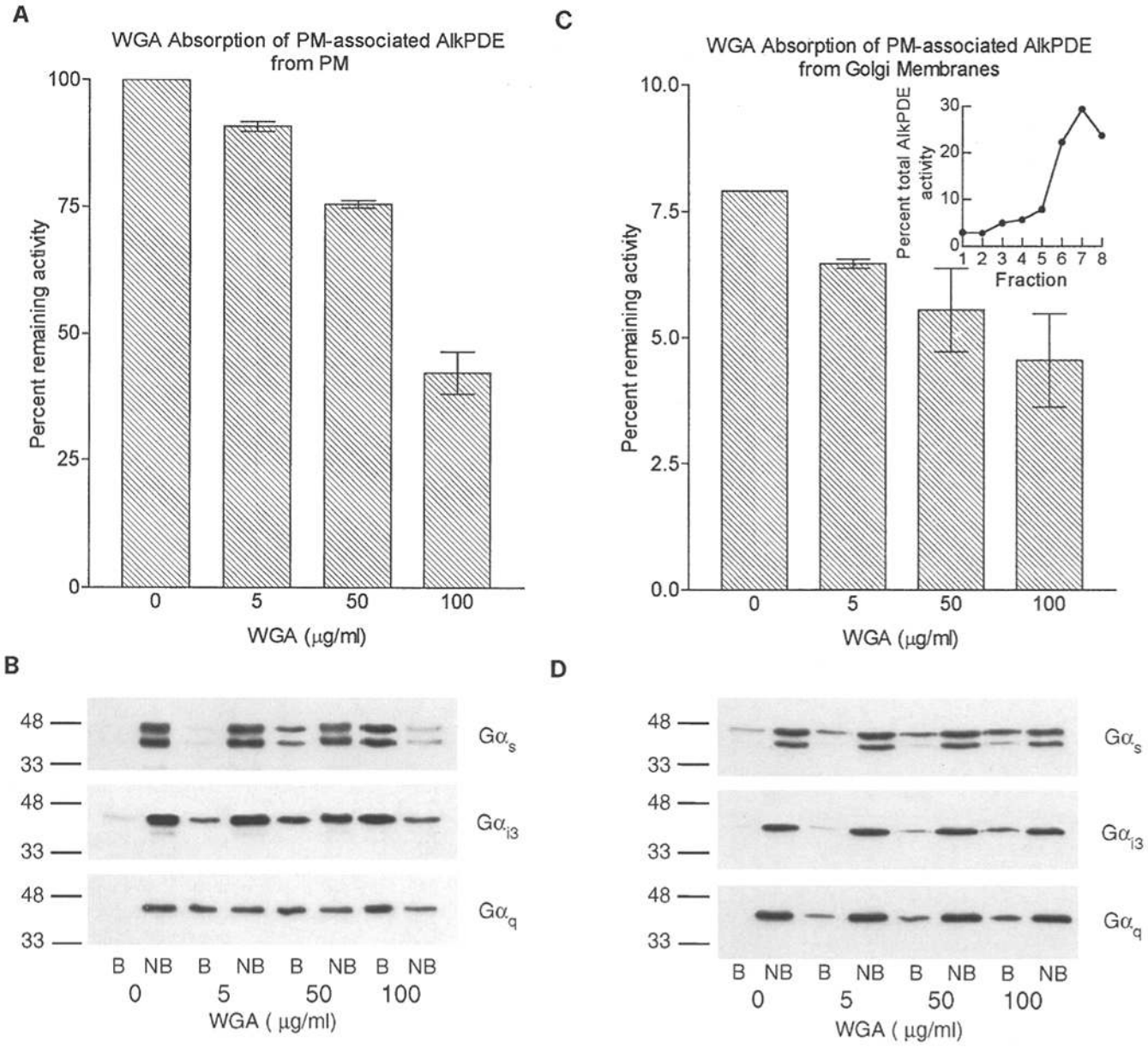


*Figure 5.* Depletion of PM from Golgi fractions by absorption on WGA-agarose does not deplete Gα subunits. Fractions were prepared as in Fig. 4. Golgi (fractions 2 and 3, pooled) and PM (fractions 6 and 7, pooled) were incubated with increasing amounts of WGA-agarose, and bound (*B*) and nonbound (*NB*) fractions were quantitated as described in Materials and Methods. *A* and *B* show experiments performed on PM. (*A*) WGA-agarose at 100 μg/ml depletes >60% of the AlkPDE activity from PM. (*B*) Immunoblot analysis shows that at 100 μg/ml WGA, >90% of the $G\alpha_s$ is bound and appears in the WGA pellet (*B*), whereas roughly 70–80% of the $G\alpha_{i3}$ and $G\alpha_{q/11}$ is depleted. *C* and *D* show experiments performed on Golgi membranes. (*C*) WGA-agarose removes >40% of residual PM from Golgi fractions based on AlkPDE activity (*inset*). (*D*) By immunoblotting, Gα subunits remain associated with Golgi membranes in the nonbound fraction (*NB*). At 100 μg/ml and greater concentrations of WGA, 30% of each Gα subunit is recovered in the bound fraction. (*A* and *C*) Results of enzyme assays are presented as percent of control activity remaining in the nonbound fraction (avg. ±SEM, $n = 3$). The inset in *C* shows the distribution of AlkPDE activity across the gradient. (*B* and *D*) Nonbound (*NB*) and bound (*B*) membranes were separated by SDS-PAGE and probed with the indicated antibodies.

formed immunoblot analysis (Fig. 6) with ARF and with the *trans*-Golgi markers, TGN38 (25), rab6 (3, 20), and caveolin (16). ARF (Fig. 6) was present in all Golgi fractions, particularly in fraction 1, along with the bulk of the rough microsomes and heavy Golgi membranes but was not enriched in the TGN-enriched fraction (fraction 3). Based on the absence of ARF labeling of PM (by immunofluorescence) (Fig. 2 *C*) and on the presence of TfR in fractions 5-7 (by immunoblotting) (see below), we concluded that endosomes (rather than PM) are responsible for the ARF signal observed in fractions 5-7. Thus, the distribution of heterotrimeric G proteins partially overlapped with that of the small GTPase ARF but had a distinctive distribution. TGN38 was largely confined to Golgi-enriched fractions (fractions 1-3) and was most concentrated in the lightest Golgi fraction (fraction 3). Rab6 was more broadly

distributed, with distinct peaks in both Golgi and PM-enriched fractions (fractions 3 and 6). Caveolin was heavily concentrated in PM-containing fractions 5-7 but was also found in Golgi fraction 3 (Fig. 6). This was consistent with the extensive overlap between caveolin and $G\alpha_s$ observed by immunofluorescence (Fig. 2, *H* and *I*). The strong signal for caveolin in PM fractions was attributed in part to the presence of PM derived from endothelial cells. TfR, an early endosomal marker, was broadly distributed (fractions 3-8) but was not enriched in the light Golgi fraction (fraction 3). This indicates that the $G\alpha_s$ detected was not due to contaminating endosomes (13). Overall, the immunoblotting results demonstrated that all three Gα subunits cofractionate with Golgi membranes and the light Golgi fraction is enriched in all three subunits. The primary difference in the distribution of the Gα proteins was that $G\alpha_{i3}$ and $G\alpha_{q/11}$ were present in the heaviest Golgi fraction (fraction 1) whereas $G\alpha_s$ was not.

### *Distinctive Distribution of Gα and βγ Subunits*

We next compared the distribution of Gα to that of Gβγ subunits. βγ subunits interact with α subunits of G proteins and have recently been shown to have distinct functions in signal transduction (11, 40) and coatamer binding to Golgi membranes (15). By immunoblotting, Gβ subunits (which are tightly associated with γ) were equally distributed in all PM fractions (fractions 5-8) and were also detectable in the TGN-enriched fraction (fraction 3); however, they were not detectable in fractions enriched in rough microsomes and heavy Golgi membranes (fractions 1-2). This was surprising, as all three Gα subunits, $\alpha_s$, $\alpha_{q/11}$, and $\alpha_{i3}$ were present in fraction 2, and $\alpha_{i3}$ and $\alpha_{q/11}$ were present in fraction 1 (see Figs. 4–5, $G\alpha_{q/11}$ data not shown). In this experiment and all subsequent immunoblotting experiments for Gβ, antibodies against the common carboxy and common amino terminus of β-subunit isoforms 1-4 were mixed together to increase the sensitivity of detection for Gβ by immunoblot.

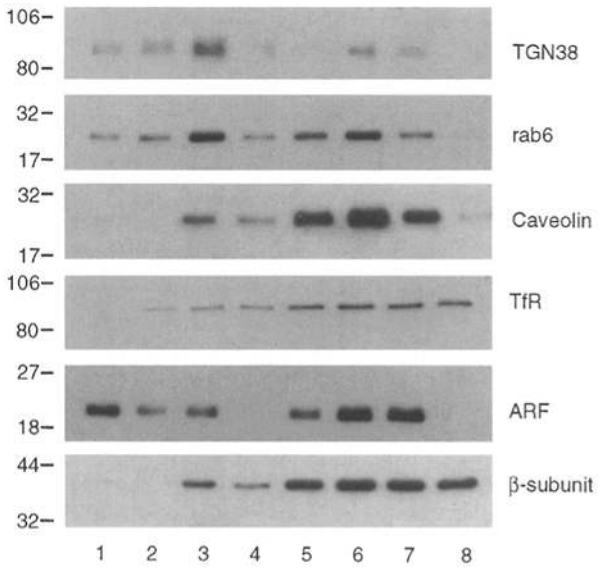


*Figure 6.* Distribution of Gβ subunits and other marker proteins in gradient fractions. Gβ subunits are equally distributed in all PM fractions (fractions 5-8) and are also detected at lower concentration in fraction 4 and the TGN-enriched fraction (fraction 3). They are not detectable in rough microsomes and heavy Golgi membranes (fractions 1-2). The distribution of TGN38, rab6, caveolin and ARF differs across the gradient; however, all four are present in the lightest Golgi fraction 3. At shorter exposures, TGN38 could be detected only in this fraction. Golgi fractions are not enriched in endosomes (*TfR*). After transfer and blocking, PVDF membranes were cut horizontally to yield strips representing high, medium, or low molecular weight proteins. Membranes were then incubated with the primary antibodies indicated on the right. Molecular weights are indicated on the left.

To further analyze the distribution of Gα and Gβ subunits, immunoblots across the flotation gradient for $G\alpha_{q/11}$ (data not shown), $G\alpha_s$ (Fig. 4 *C*), $G\alpha_{i3}$ (Fig. 4 *C*), and Gβ (Fig. 6) were scanned and the percent of the total of each subunit in each fraction was determined. 70–79% of all Gα subunits were associated with PM (fractions 5-8), whereas >90% of Gβ was distributed in these fractions. However, only 7% of Gβ partitioned into Golgi fractions 1-3, whereas between 16–26% of all Gα subunits were distributed in these fractions.

The distribution of Gβ between WGA bound and nonbound fractions was determined (one experiment). Using PM, Gβ showed a distribution profile similar to $G\alpha_{i3}$, in that ~70% was recovered in the bound fraction at 100 μg/ml WGA. However, using Golgi membranes, we could not detect a signal for Gβ at equivalent exposure times, a result of the limiting amount of PM in the starting material. In fact, exposure times of several hours were required to see a signal for Gβ (as opposed to several minutes for all Gα subunits). This indicated to us that Gβ was present at significantly lower levels in the Golgi when compared to PM.

To follow up this finding, increasing amounts (5–40 μg) of WGA-purified Golgi membranes and mock-treated (agarose) PM were loaded on the same gel, separated by SDS-PAGE, transferred and immunoblotted with the Gβ antibody mix. At 5 μg and exposure times as short as 30 s, Gβ subunits were easily detected in PM fractions, but were undetectable in Golgi fractions. Even at 20 μg Golgi membranes, Gβ was barely detectable. To obtain a signal for Gβ in Golgi fractions comparable to that obtained with 5 μg PM at 30-s exposures, 40 μg of protein and exposure times of 2 min were required. Although semiquantitative, this indicates a 30-fold greater level of Gβ in PM than Golgi membranes. This faint signal observed for Gβ could be accounted for by the small (<5%) amount of PM which reseals outside-in after fractionation (see Fig. 5). From these results we conclude that at least some Gα subunits present on Golgi membranes are not associated with Gβ subunits.

### *Immunogold Localization of $G\alpha_s$ and $G\alpha_{i3}$ to Golgi Membranes*

To localize $G\alpha_s$ and $G\alpha_{i3}$ to specific populations of Golgi membranes, we carried out immunogold labeling with affinity-purified anti-$G\alpha_s$ (RM) or anti-$G\alpha_{i3}$ (EC) IgG on ultrathin cryosections of rat pancreas. $G\alpha_s$ (Fig. 7 *A*) was detected on all Golgi membranes, but labeling was heavier on membranes of the *trans*-Golgi, including those of the *trans* cisternae, the TGN, and associated vesicles. Representative micrographs in which the percent of gold parti-

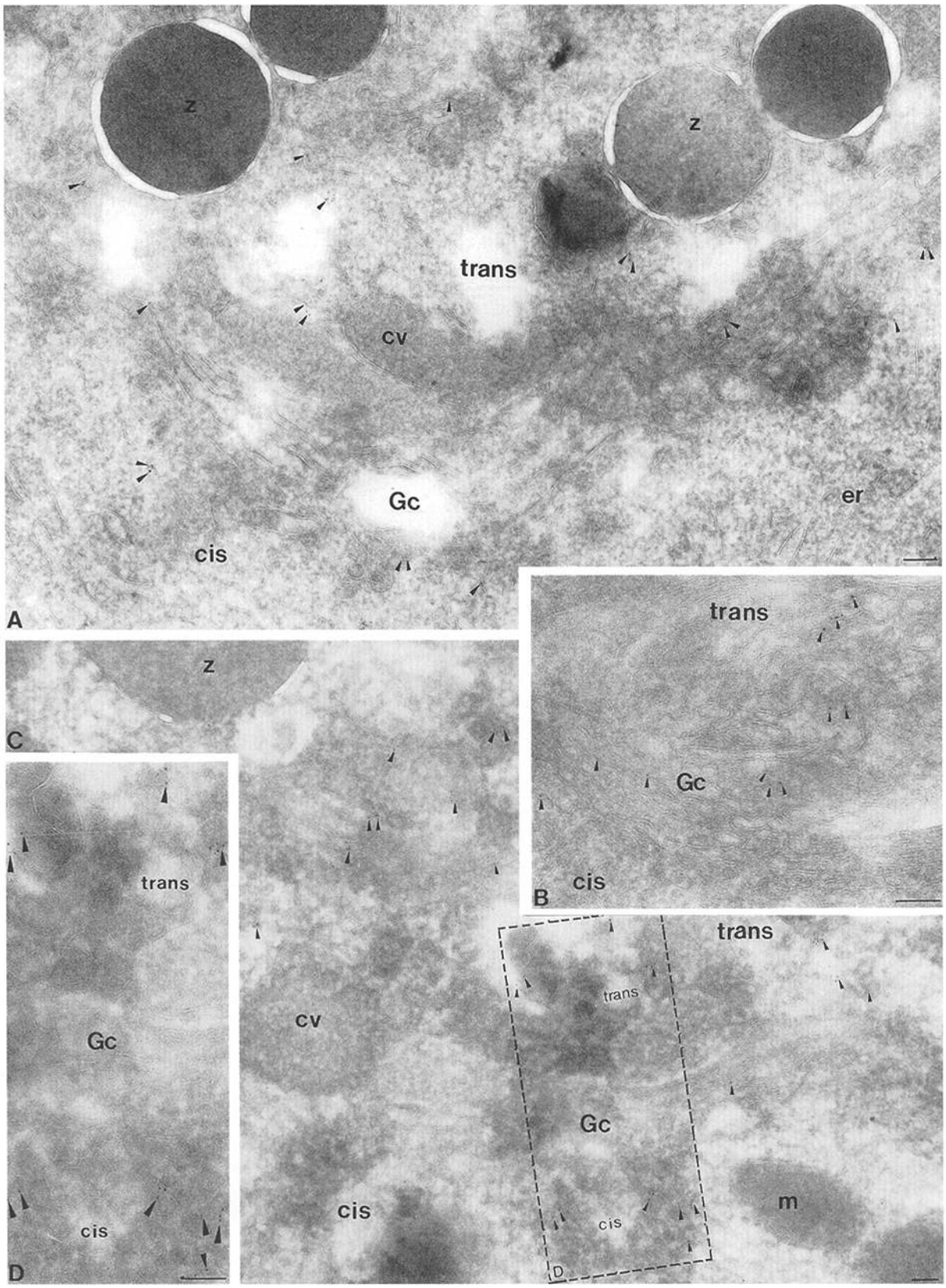


*Figure 7.* Immunogold localization of $G\alpha_s$ (*A* and *B*) and $G\alpha_{i3}$ (*C* and *D*) to Golgi membranes. (*A*) $G\alpha_s$ (*arrowheads*) is present primarily on vesicles and membranes on the *trans* side (*trans*) of the Golgi stack. Labeling is not detected on ER (*er*) membranes. (*B*) High magnification inset showing $G\alpha_s$ on Golgi membranes. (*C*) $G\alpha_{i3}$ is found on vesicles on both the *cis* and *trans* side of the Golgi stack (*arrowheads*) as well as on the cisternae (*Gc*). (*D*) Enlargement of the boxed area shown in *C*. Control experiments in which specimens were incubated with secondary antibody alone showed negligible labeling. Ultrathin cryosections prepared from formaldehyde-fixed pancreas lobules as described in Materials and Methods were incubated with affinity-purified anti-$G\alpha_s$ (1:50) or $G\alpha_{i3}$ (1:100) IgG, followed by 5-nm gold conjugated goat anti–rabbit IgG. *cv*, condensing vacuole; *m*, mitochondria. Bar, 0.1 μm.

cles on *cis* vs *trans* membranes was counted revealed that 38% of the gold was over *cis* cisternae whereas 62% was associated with *trans* (identified by the presence of condensing vacuoles). Label was not detected on granule membranes, nuclei, or ER. Therefore, immunogold labeling provided direct evidence that the majority of $G\alpha_s$ in the Golgi is present on *trans*-Golgi elements including the TGN and associated vesicles.

$G\alpha_{i3}$ was detected throughout the Golgi stack as well as on clusters of vesicles on both the *cis* and *trans* side of the stacked cisternae. Using the same approach as for $G\alpha_s$, the distribution of $G\alpha_{i3}$ in representative micrographs of *cis* vs *trans* cisternae was determined. $G\alpha_{i3}$ was found to be uniformly distributed across the stack (48% *cis*, 52% *trans*). This result is in agreement with the biochemical data (Fig. 4) demonstrating association of $G\alpha_{i3}$ with heavy as well as lighter Golgi membranes. By contrast, TGN38 was found to be largely restricted to *trans*-cisternae and the TGN (9% *cis*, 91% *trans*).

### *β Subunits Are Not Detected on Golgi Membranes by Immunofluorescence and Immunogold Labeling*

The immunoblotting data indicated that $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i3}$ may be associated with heavy Golgi membranes in the absence of Gβ. To check this finding we localized Gβ in rat pancreas using an antibody (SW/1) to the common carboxy terminus of Gβ subunits 1-4. Gβ subunits were detected largely on the PM; no signal was seen in the Golgi region either by immunofluorescence (Fig. 8 *B*) or by immunogold (Fig. 8 *C*) labeling. These results indicate that under these conditions, Gβ is not detectable on Golgi membranes. Taken together, our localization data and biochemical data suggest that Gα subunits are present in

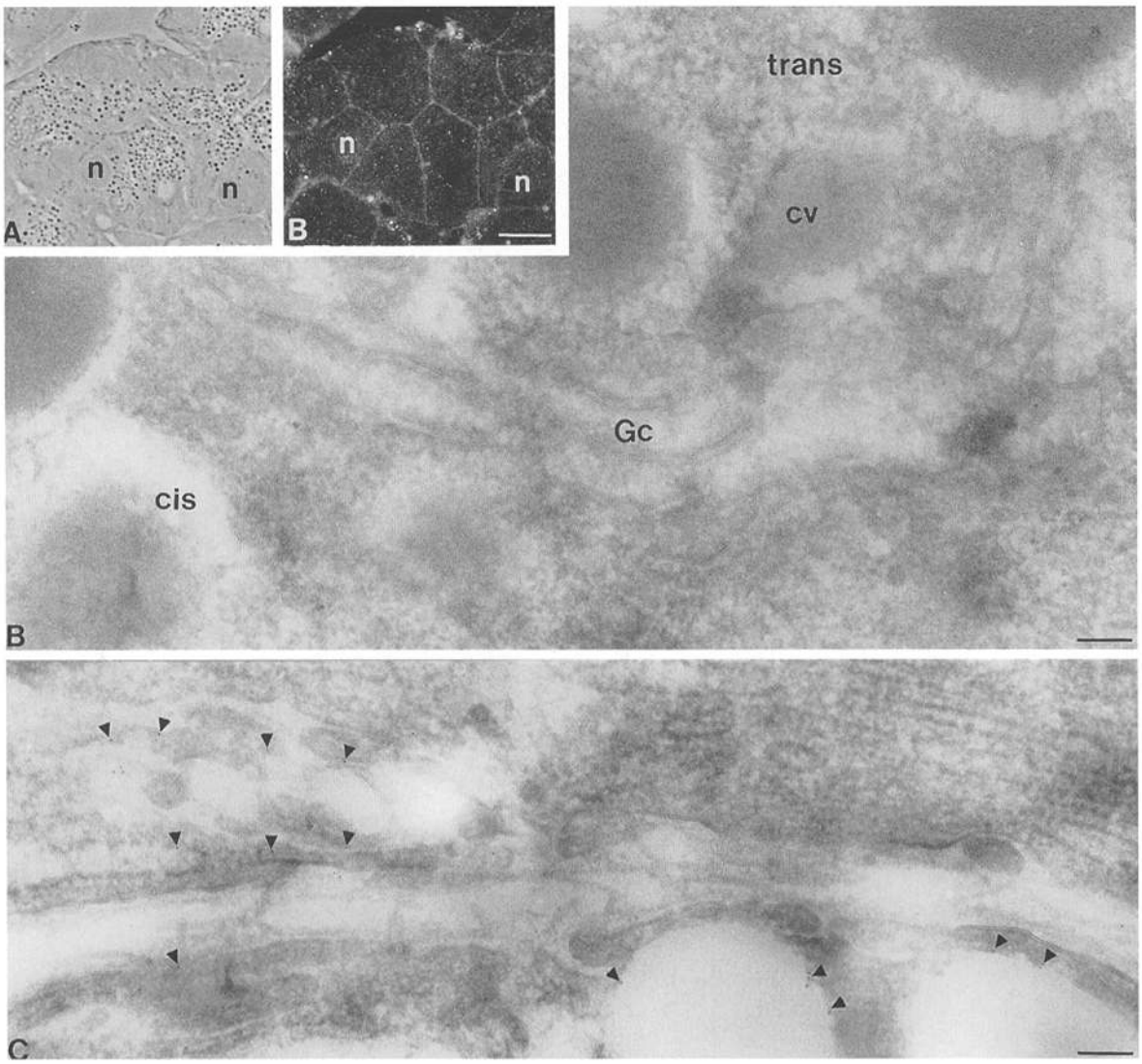


*Figure 8.* Gβ subunits are detected on the PM but not on Golgi membranes. (*B*) By immunofluorescence Gβ subunits are easily detected on the PM of the acinar cell, but no staining is seen in the Golgi region. (*C*) Gβ subunits are not detected on Golgi membranes by immunogold labeling. (*D*) The PM is clearly labeled (*arrowheads*) for Gβ. (*A*) Phase contrast microscopy of the field shown in *B*. Semithin (*A* and *B*) or ultrathin (*C* and *D*) cryosections were incubated with affinity-purified anti-Gβ (1:50) IgG, followed by FITC (*A* and *B*) or 5-nm gold (*C* and *D*)-conjugated goat anti–rabbit IgG.

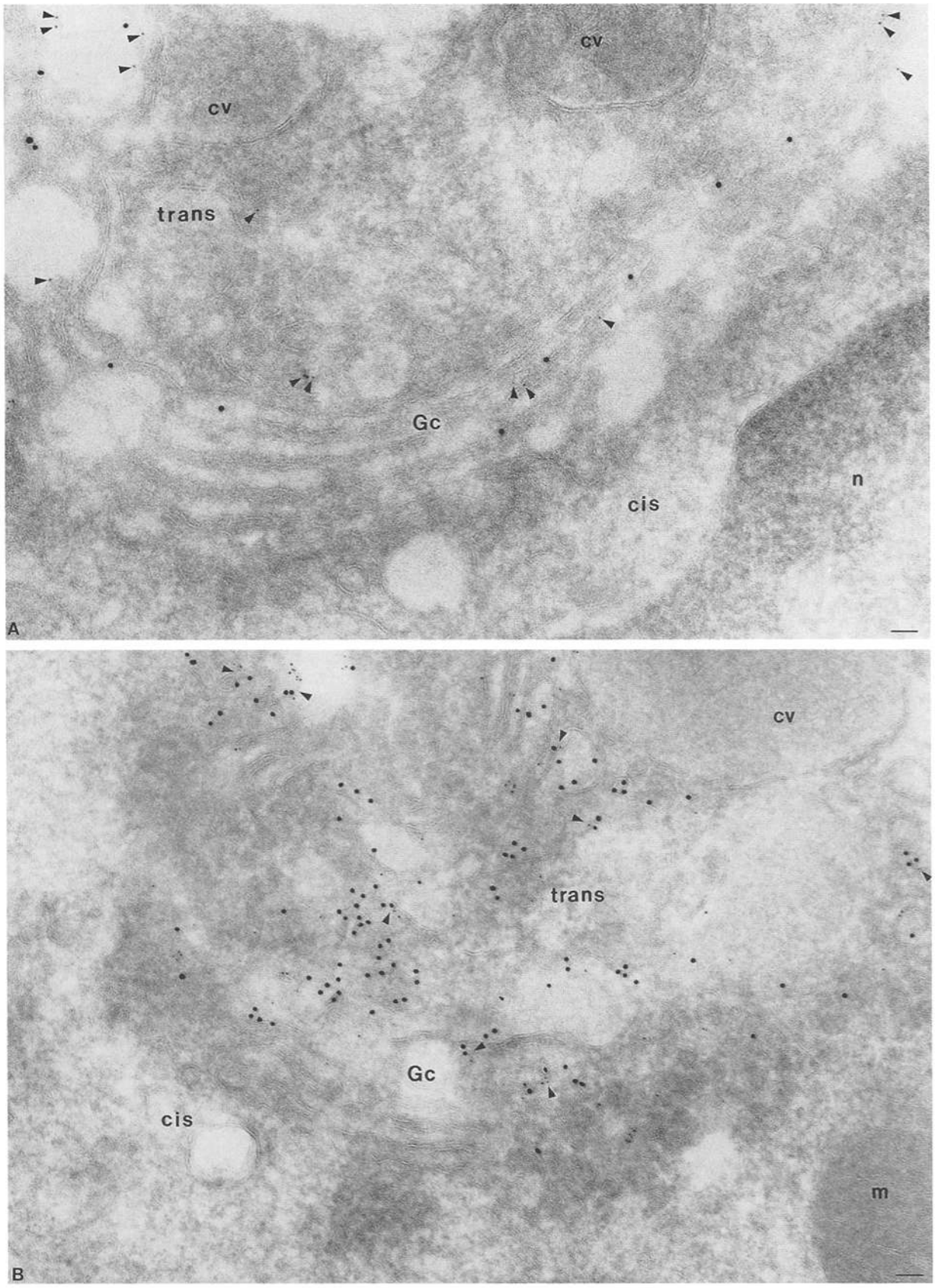


*Figure 9.* Caveolin colocalizes with TGN38 but not with $G\alpha_s$ in the *trans*-Golgi. (*A*) Caveolin (10 nm gold) is found primarily on *trans* and (to a lesser extent) medial cisternae of the Golgi stack. $G\alpha_s$ (5 nm gold, *arrowheads*) and caveolin are in close proximity but for the most part their distribution on *trans*-Golgi membranes does not coincide. (*B*) TGN38 (10 nm gold) is associated with several *trans* cisternae in the Golgi stack, as well as with vesicles on the *trans* side of the Golgi. Areas of colocalization (*arrowheads*) of TGN38 with caveolin (5 nm gold) can be observed. Sections were incubated with a mouse monoclonal IgG against caveolin (1:100) and rabbit polyclonal IgG against either $G\alpha_s$ (*A*, 1:50) or TGN38 (*B*, 1:100), followed by gold-conjugated goat anti–mouse and anti–rabbit IgG. *Gc*, Golgi complex; *cv*, condensing vacuole; *n*, nucleus. Bar, 0.1 μm.

the Golgi independent of their interaction with Gβ subunits.

### *$G\alpha_s$ Does Not Colocalize with Caveolin in the TGN*

$G\alpha_s$ and caveolin have been shown to copurify in PM caveolae (12, 32). To determine whether there is overlap between $G\alpha_s$ and caveolin in the Golgi, we performed double immunogold labeling experiments using polyclonal anti-$G\alpha_s$ and monoclonal anti-caveolin IgG. Caveolin (Fig. 9, 10-nm gold) was associated primarily with *trans* cisternae and vesicles clustered near the dilated rims of the cisternae and to a lesser extent with medial and *cis* cisterna. As indicated earlier, $G\alpha_s$ (5 nm gold, *arrowheads*) was also associated with *trans*-Golgi membranes and the TGN but for the most part its distribution did not directly coincide with that of caveolin.

As $G\alpha_s$ and TGN38 were enriched in the same Golgi fractions, we were interested in determining if they colocalized on the same Golgi elements. We were unable to localize $G\alpha_s$ and TGN38 in the same sections due to the unavailability of antibodies suitable for double labeling. We therefore approached the problem indirectly and investigated whether the distribution of caveolin and TGN38 coincided. We found that the distribution of the two proteins did coincide on *trans* cisternae and associated vesicles (Fig. 9, *arrowheads*) in some areas. The fact that the distribution of TGN38 and caveolin overlapped, but $G\alpha_s$ and caveolin did not, indicated that $G\alpha_s$ is on a separate domain of *trans*-Golgi membranes than either caveolin or TGN38.

## *Discussion*

By immunoblot analysis of Golgi fractions and immunofluorescence and immunogold labeling of pancreatic lobules, we have obtained new information on the association of heterotrimeric G proteins with Golgi membranes in the exocrine pancreatic cell. First, we showed that three Gα subunits, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i3}$, are present on both Golgi membranes and PM, and that these subunits differ in their distribution among pre-Golgi and Golgi membranes: $G\alpha_s$ is found preferentially in light Golgi membrane fractions enriched in TGN38, a *trans*-Golgi/TGN marker. By contrast $G\alpha_{i3}$ and $G\alpha_{q/11}$ are more broadly distributed across the gradient being found in heavy membrane fractions along with β-COP, rab1b, and ARF as well as in the light Golgi fraction overlapping with $G\alpha_s$. We were able to rule out that the presence of Gα subunits in Golgi fractions results from contamination by PM because the depletion of >95% of PM by WGA lectin absorption of Golgi fractions did not deplete $G\alpha_s$, $G\alpha_{q/11}$, or $G\alpha_{i3}$. $G\alpha_{i3}$ has previously been localized to Golgi cisternae by immunoelectron microscopy (54, 60), but the distribution of different families of Gα proteins among Golgi elements has not been investigated up to now.

We have also made the novel and intriguing observation that Gβ subunits of heterotrimeric G proteins are not present or are present in very low concentrations in Golgi membranes, they could not be detected in the Golgi by immunofluorescence or immunogold labeling but were readily detected on the PM. Moreover, Gβ subunits were not detected in heavy Golgi fractions by immunoblotting and were barely detectable in lighter Golgi fractions enriched in TGN38. Thus, many of the Gα subunits present in the Golgi are not associated with Gβ. This raises the question, What is the role of Gα in Golgi function if Gβγ subunits are absent? If the paradigm in the Golgi is the same as that in the PM, the presence of free Gα subunits in the Golgi implies that they are mainly in the active, GTP bound form. βγ subunits are known to lock Gα in the GDP bound conformation, preventing Gα from activating effectors until an activated PM receptor, acting as a guanine nucleotide exchange factor, is formed. Constitutive vesicular transport and membrane recycling are continuous processes, and if Gα subunits play a regulatory role in either of these events, it might be advantageous to remove the negative regulation imposed on Gα by Gβ. Regulation could still be imposed by the interaction of Gα with its effector, such as a vSNARE, coat protein, or another Golgi protein. There is already a precedent for such regulation, as phospholipase $C\beta_1$ can increase the GTPase activity of $G\alpha_{q/11}$ by acting as a GAP (6).

Since βγ subunits also assist in anchoring Gα subunits to the PM, our findings suggest that Gα subunits are tethered to the Golgi by other mechanisms than binding to Gβ subunits, such as association with as yet unidentified proteins or by posttranslational modifications. In this regard it is noteworthy that all three Gα subunits that were found in the Golgi in the pancreas are known to be palmitoylated (14, 33, 58), and palmitoylation appears to be both requisite and sufficient for membrane attachment of $G\alpha_s$ and $G\alpha_{q/11}$ (58). In fact, cleavage of the thio-ester bond linking the palmitate group to the Gα subunit is thought to be one mechanism by which Gα subunits, once activated, are released from the PM (57). The fact that the half-life of the palmitate group is shorter than the half-life of the protein (30), suggests that Gα subunits undergo cycles of de- and repalmitoylation. The possibility exists that the Golgi may represent a site for repalmitoylation once activated subunits are released from the PM and serve as a reservoir for recruitment of Gα subunits to the PM. The subcellular compartment in which palmitoylation of Gα subunits occurs has not been identified, but an acyltransferase which palmitoylated H-ras was found to copurify with Golgi and not PM markers (21).

Another novel observation of this study was that $G\alpha_{q/11}$, which acts upstream in the diacylglycerol/protein kinase C (PKC) signaling pathway, is found on Golgi membranes. The finding of $G\alpha_{q/11}$ on Golgi membranes is intriguing in view of recent findings in NRK cells indicating that (*1*) a diacylglycerol/phorbol ester–binding protein is involved in exit from the ER (17), and (*2*) a phorbol ester–binding isoform of PKC, PKCε, is present on Golgi membranes of transfected 3T3 cells where it is believed to modulate Golgi function (29).

Several mechanisms have been proposed by which Gα subunits may regulate vesicular transport along early stages of the exocytic pathway: (*1*) binding of coat proteins β-COP and ARF to Golgi membranes (15), (*2*) vesicle budding (4, 5), or (*3*) differential sorting or delivery of TGN-derived transport vesicles to different domains of the PM (42, 43). A fourth possibility is that Gα subunits interact with SNARE proteins, providing a molecular trigger for docking or targeting. Since SNAREs exist on many

membranes along the biosynthetic route (47), the specificity of targeting and fusion must depend on other factors, such as the small GTPases, rab-specific GDIs, GDSs, and GAPs (18). The heterotrimeric G proteins could provide an additional level of regulation or specificity to this process.

It remains to be seen whether Golgi membranes serve as a reservoir of Gα subunits and at which stage of vesicular trafficking, i.e., coat assembly, budding, targeting, or fusion, heterotrimeric G proteins are involved. The approaches presented in this comprehensive study of the pancreas establish a unique system in which Golgi Gα subunits may be examined distinct from their PM counterparts. Using this system, we hope to elucidate further biochemical differences between the two populations of Gα proteins and to identify putative Golgi-specific receptors or effectors.

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