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TRIMERIC G PROTEIN α SUBUNITS OF THE Gs AND Gi FAMILIES LOCALIZED AT THE GOLGI MEMBRANE[1]

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SUMMARY: The occurrence of individual G proteins within Golgi membranes from liver and from adrenal medulla were analyzed by Western blotting. Two splice variants of Gαs and also Gαi-3 were found in both tissues. Additionally, Gαi-2, its 43-kDa splice variant and Gαo were strongly labeled in Golgi of adrenal medulla. Golgi preparations of liver contained comparable quantities of Gαi-2 and a second variant of 46 kDa, but no Gαo was detected. Immunoelectron microscopic studies of the above Golgi preparations showed that both Gαi-2 and Gαi-3 are localized at multivesicular structures identified as Golgi complexes. The occurrence of two stimulatory G proteins (Gαs-S and Gαs-L) and three or four α subunits of the Gi/Go type as components of the Golgi membrane support the notion that formation of different vesicle types might be regulated by individual stimulatory and inhibitory G proteins.

Trimeric G proteins play an important role by transducing signals from membrane receptors at the plasma membrane to intracellular effectors. Besides their function at the cytoplasmic phase of the plasma membrane, trimeric G proteins have also been found attached to intracellular compartments, such as endosomes and Golgi from rat liver (1), Golgi of rat kidney (2) and the endoplasmic reticulum of canine pancreas (3). The intracellularly localized trimeric G proteins are implicated in vesicle transport. Evidence for the involvement of G proteins in the vesicle formation at the trans-Golgi network (TGN) was obtained by several authors (4-8). In addition, protein transport between endoplasmic reticulum and Golgi in NRK cells (9) and endocytosis by epithelial cells is supported by G protein(s) (10,11).

The first step in the budding of a vesicle consists in the attachment of ARF, a small GTP-binding protein, and of coat proteins to the cytoplasmic phase of the donor membrane (12),

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The abbreviations used are: ARF, ADP-ribosylation factor; G protein, GTP-binding protein; HME, 10 mM Hepes/KOH, pH 7.5, 2 mM $MgCl_2$, 5 mM EDTA; PNS, post-nuclear supernatant; TBS, 10 mM Tris/HCl, pH 7.4, 100 mM NaCl; TGN, trans-Golgi network.

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followed by a second step, pinching off the coated vesicles. Both steps depend on trimeric G proteins: Binding of ARF is stimulated by aluminium fluoride (4), an activator of trimeric G proteins. The second step, budding of vesicles is inhibited by activated α subunits of the Gi/Go-type (5-8) and inactivation of these α subunits by pertussis toxin-catalyzed ADP-ribosylation supports vesicle formation (5,6,8).

Individual G proteins have been identified at the Golgi membrane by specific antibodies. Using this strategy, the α subunit of Gi-3 (Gαi-3) was detected in the Golgi of LLC-PK_1 cells (5,13) and in NIH 3T3 cells (14). Gαi-3 and Gαs were identified in the Golgi of PC12 cells (8). Gαo was localized in membrane fractions enriched in TGN (8) and in membranes of chromaffin granules and synaptic vesicles (15). Finally, a splice variant of Gαi-2 was localized in the Golgi of different tissues (16).

Except for the identification of the above trimeric G proteins and small G proteins, like ARF (17,18), Rab6 (17) and Rab8 (19) no further members of putative signal pathways for vesicle formation have been identified in the Golgi complex so far. The stimulation of trans-Golgi vesicle formation by activated PKC and PKA (20,21) provides the first evidence that kinase substrates might be involved. In this respect, inhibitory Gi proteins are preferential candidates, since the 40 kDa form of Gαi-2 was identified as a substrate for PKC in liver cells (22-25) and for PKA in hepatocytes (26). Therefore we investigated whether Gαi-2 might also be associated with Golgi membranes.

To analyze the association of Gi proteins with Golgi membranes and to compare secretory and non-secretory cells, Golgi preparations of liver and adrenal medulla were analyzed by Western blotting. Two splice variants of Gαs, two forms of Gαi-2 and Gαi-3, were found in preparations of both tissues. Additionally Gαo was identified as a component of Golgi membranes isolated from adrenal medulla.

MATERIALS AND METHODS

Materials—Bovine suprarenal glands were obtained from a local slaughter house and Wistar rats were from Moellegard (Schönwalde, Berlin).

Antibodies—C-terminal peptides of Gαs, Gαi-3 and Gαo - extended at the N-terminus by a chloroacetylglycyl group - were obtained from Dr. R. Dölling (Biotez, Berlin). To distinguish Gαi-1 and Gαi-2, the internal peptides 111-124 and 112-126, respectively, were synthesized according to (27) with chloroacetylglycyl groups extending the N-termini. Keyhole limpet hemocyanin or bovine serum albumin was treated with iminothiolane, dialyzed against TBS, and conjugated with the chloroacetyl-modified peptides for 24 h at 4°C. The conjugates (200 μg) were used for injecting rabbits subcutaneously and for boosting two or three times. Peptide-specific antibodies were affinity purified from sera by chromatography on peptide-modified, iminothiolane-activated aminohexyl-Sepharose and eluted with 0.1 M glycine-HCl, pH 2.5, according to (28). The purified antibodies against Gαi-1 (AB-i-1), Gαi-2 (AB-i-2), Gαs (AB-s), and Gαo (AB-o) are monospecific as tested by Western blotting using a G protein mixture extracted from total membranes of bovine brain. AB-i-3 recognizes also Gαi-2 (see Fig. 2a and b, lanes 3). Antibodies raised against the identical C-terminal peptide of Gαi-1 and Gαi-2 (AB-i-1/2) recognize both G proteins. Due to the absence of Gαi-1 in Golgi preparations, only Gαi-2 is visualized. Antibodies (AB-st) against an N-terminal peptide of 15 amino acids were used for detection of sialyltransferase (29).

Preparation of Golgi membranes from rat liver—Golgi membranes were isolated according to a modification of the procedures described before (30,31). All sucrose solutions were in 10 mM Hepes-KOH, pH 7.5, 2 mM $MgCl_2$ and 5 mM EDTA (HME). Four male rats (150 - 200 g) were killed by decapitation and the livers were excised, minced and immediately placed in 0.25 M sucrose-HME on ice. The tissue was homogenized in the same buffer (4 ml per g tissue) using a loosely fitted teflon-glass Potter-Elvehjem homogenizer at 350 rpm with 8 strokes. Nuclei and intact cells were removed by centrifugation at 200 x g for 10 min. The post-nuclear supernatant (PNS) was layered in 65 ml portions on top of 30 ml 1.3 M sucrose-HME and centrifuged at 110 000 x g for 30 min. The crude membrane fraction, that appeared as a white band on top of the 1.3 M sucrose layer, was removed by aspiration, adjusted to 1.2 M sucrose-HME and overlaid with 1.1 M, 1.0 M and 0.5 M sucrose-HME. After centrifugation at 110 000 x g for 90 min, the light Golgi fraction (G1) banding at the 0.5/1.0 M sucrose interphase and the heavy Golgi fraction (G2) banding between 1.0 and 1.1 M sucrose was collected separately.

Preparation of Golgi membranes from adrenal medulla—Suprarenal glands were cut and adrenal medullas were collected by scraping with a scalpel. Tissue obtained from 50 glands was homogenized in 0.25 M sucrose-HME at 900 rpm and processed as above. Preparation of Golgi vesicles of PC12 and HepG2 cells is described elsewhere[3].

Enzyme assays—Na^+/K^+ ATPase (EC 3.6.1.3.) was determined according to (32,33) and sialyltransferase (EC 2.4.99.1) according to (34) modified in (35).

Western blotting—Proteins were separated on 10 % acrylamide / 0.27 % bisacrylamide gels according to (36) in the presence of 4 M urea (8). After blotting for 90 min at 10 V, nitrocellulose membranes were blocked in 5 % skimmed milk for at least 1 h. Blots were incubated with anti-G protein antibodies (1 - 5 µg/ml) in 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1 % Tween 20 for 2 h and with anti-rabbit IgG conjugated with horseradish peroxidase (Boehringer, Mannheim) for 1 h and developed with Fast stain (Sigma, Deisenhofen).

Immunoelectron microscopy—Golgi-enriched fractions were bound to formvar-coated grids that had been activated by pentylamine treatment. Membrane proteins were fixed with 4 % formaldehyde and 0.25 % glutaraldehyde in 10 mM Hepes-KOH, pH 7.5. Aldehydes were quenched with 0.12 % glycine, 1 % bovine serum albumin in 10 mM Hepes-KOH. Proteins were incubated with specific antibodies in TBS containing 0.1% Tween 20 for 1 h and rinsed, thereafter, with buffer three times. Bound antibodies were labeled by protein A bound to 10 nm gold particles (Utrecht Univ., School of Med., Dept.Cell Biol.) for 30 min. After washing with TBS/0.1 % Tween and distilled water, membrane structures were stained with 2 % aqueous uranyl acetate.

The content of stacked Golgi membranes was quantitated according to Weibel (37). Briefly, a lattice of points was laid onto photographs of negatively stained Golgi preparations (magnification 10,000 x). Points superimposed with Golgi structures were counted and compared with the number of points overlapping with any membrane structure that were set to 100 %. Since multivesicular Golgi stacks are only analyzed in two dimensions, the actual amount of membranes is certainly higher than the value measured by this method.

RESULTS

Isolation of Golgi-enriched fractions from rat liver and bovine adrenal medulla—Light and heavy Golgi fractions from rat liver isolated as described in Material and Methods comprised 0.15% and 0.26 %, respectively, of the protein in the post-nuclear supernatant (PNS). Fraction G1 contained 67 ± 3 % and fraction G2 72 ± 2% of the membranes in the form of multivesicular structures shown in Fig. 1, a-d, that have been identified as Golgi complexes before (30). Besides

[3] Westermann, P. et al., manuscript in preparation.

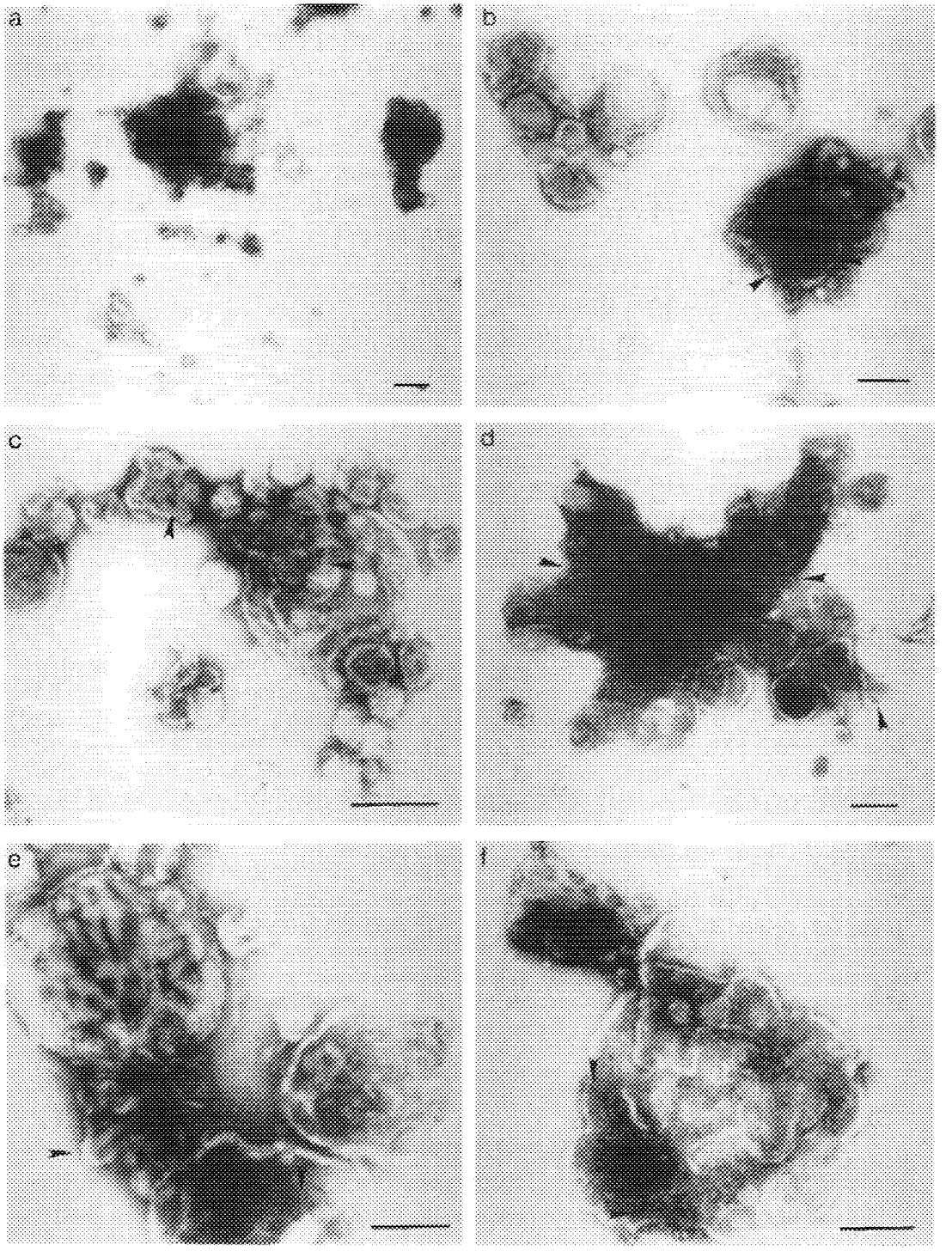

Fig. 1. Electron microscopic immunochemistry of Gαi-2 (c,e), Gαi-3 (d,f), and sialyltransferase (b) in Golgi fractions G1 of rat liver (a-d) or bovine adrenal medulla (e-f). Antibodies used are AB-i-1/2 (c,e), AB-i-3 (d,f) and AB-st (b). Arrowheads are used for indicating membrane-bound protein A-gold particles. Bars: 0.2 μm.

morphological criteria, binding of antibodies against sialyltransferase (Fig. 1b) and determination of sialyltransferase as a marker enzyme supports this notion (Tab. 1). The absence of plasma membranes as contamination was confirmed by measuring the ouabain-sensitive Na^+/K^+ ATPase (Tab. 1).

Golgi fraction G1 and G2 of adrenal medulla contain 0.9 % and 1.0 % of total PNS proteins. Multivesicular structures (Fig. 1 e and f) comprised 74 ± 8% of the G1 fraction and 82 ± 5% of the G2 fraction. Sialyltransferase as a marker for trans-Golgi was assayed by Western blotting (Fig. 2b, lane 6). Activities of sialyltransferase and of ouabain-sensitive Na^+/K^+ ATPase were similar to liver preparations. Enzyme concentrations of isolated Golgi fractions correspond to values published for Golgi membranes of liver (30,31). Due to the lower ATPase content in G1 fraction of adrenal medulla, further experiments were performed with G1 fractions from both tissues. In addition to Golgi structures, small vesicles of a diameter between 50 and 100 nm and larger vesicles of 100 to 200 nm were observed in fractions G1 (Fig. 1a and b).

Immunogold labeling of α subunits of Gi-2 and Gi-3 localized at isolated Golgi membranes—G1 fractions from liver bound to membrane-coated grids were fixed and thereafter incubated with antibodies that specifically recognize the C-terminus of Gαi-2 (Fig. 1c) and Gαi-3 (Fig. 1d). Bound antibodies were visualized by incubation with protein A bound to gold particles. AB-i-1/2 labeled 85 ± 5 % of all Golgi structures, whereas AB-i-3 decorated 40 ± 10 % of Golgi structures. Smaller vesicles were not labeled and background labeling was also very low. Only one subtype of large vesicles was labeled by both antibodies AB-i-1/2 (32 ± 7 %) and AB-i-3 (12 ± 4 %).

Fraction G1 isolated from adrenal medulla was studied by the same method: Golgi complexes were labeled to 60 ± 10 % by antibody AB-i-3 and to 50 ± 10 % by antibody AB-i-1/2

TABLE 1. Distribution of sialyltransferase and ouabain-sensitive Na+/K+ ATPase in subcellular fractions of rat liver and bovine adrenal medulla

	Sialyltransferase Specific activity (pmoles sialic acid transferred per 90 min and mg protein)	Ouabain-sensitive Na+/K+ ATPase Specific activity (nmoles Pi released per min and mg protein)
Rat liver:		
PNS	3.5	200
Golgi fraction G1	32.5	14
Golgi fraction G2	64.3	12
Bovine adrenal medulla:		
PNS	4.6	240
Golgi fraction G1	35.8	30
Golgi fraction G2	42.8	90

Each figure represents the mean of 3 independent preparations. Enzymes were assayed in triplicate as described in Material and methods.

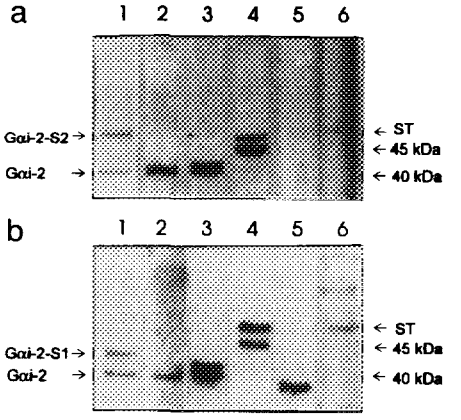

Fig. 2. Immunoblot analysis of G protein α subunits of Golgi fraction G1 from rat liver (a) or from bovine adrenal medulla (b). Gαi-2 and its splice variants, Gαi-2-S1 and Gαi-2-S2, decorated with AB-i-2, lane 1; Gαi-2 decorated with AB-i-1/2, lane 2; Gαi-3 (upper band) decorated by AB-i-3, lane 3; Gαs-S and Gαs-L decorated with AB-s, lane 4; Gαo decorated with AB-o, lane 5; sialyltransferase (ST) decorated with AB-st, lane 6. Molecular weight standards are indicated: ovalbumin,45 kDa; alcohol dehydrogenase, 40 kDa.

(Fig. 1e and f). The subfraction of large vesicles was labeled to 35 ± 5 % by AB-i-3 and to 25 ± 5 % by AB-i-1/2. Probing with AB-o stained a small fraction of Golgi complexes isolated from adrenal medulla, whereas Golgis obtained from liver were not decorated (data not shown).

G proteins of Golgi fractions analyzed by Western blotting—Detection of native, membrane-bound G proteins by antibodies might be incomplete due to shielding of the reactive C-terminus by interactions with putative receptors or other membrane proteins. Therefore, Golgi membrane proteins were denaturated by SDS treatment, separated on polyacrylamide gels and blotted. Western blots of proteins of fractions G1 from rat liver were decorated with G protein subtype-specific antibodies: distinct signals of Gαi-2 (Fig. 2a, lane 1/lower band and lane 2), of Gαi-3 (lane 3, upper band) and of both variants of Gαs (lane 4) were observed. A second signal obtained with AB-i-2 (Fig. 2a, lane 1/upper band) with an apparent molecular weight of 46 kDa was larger than the splice variant described by (16) and may therefore represent a third form of Gαi-2, characteristic for liver. The antibody against Gαi-3 cross-reacts with Gαi-2 (Fig. 2a, lane 3, lower band). There were no signals observed for Gαo (Fig. 2a, lane 5) and for Gαi-1 (data not shown).

Analysis of Golgi from bovine adrenal medulla with the same antibodies resulted in a pattern similar to that of rat liver (Fig. 2b, lanes 1 - 4). Three differences have been observed: 1. The splice variant of Gαi-2 was visualized with an apparent molecular weight of 43 kDa (Fig. 2b, lane 1) in accordance with (16). 2. The relative intensities of both forms of Gαs differ in the two tissues: Gαs-L produced a more intensive signal in rat liver whereas in Golgi of adrenal medulla the smaller form (Gαs-S) was more prominent. 3. Gαo was detected in Golgi fractions of adrenal medulla (Fig. 2b, lane 5).

DISCUSSION

Within the cell several pathways for the transport of newly synthesized proteins exist and may be regulated independently. Evidence for this notion has been obtained, (i) by characterization of different transport pathways for membrane proteins and secreted proteins (38), (ii) by isolation of heparan sulfate proteoglycan-transporting vesicles (39) and (iii) by analysis of basolateral and apical transport pathways in polarized epithelial cells (40). Therefore, different signaling pathways might exist, using stimulatory and inhibitory G proteins (8). In addition to the stimulatory G protein subunit, Gαs (8), several inhibitory G proteins function on Golgi membranes, from which Gαi-3 (5,8,13,14) and a splice variant of Gαi-2 (16) have been characterized so far. The splice variant of Gαi-2 could also be recognized in our Golgi preparation from adrenal medulla as a weak signal (Fig. 2b, lane 1) by antibody AB-i-2 raised against the internal peptide 112-126.

The stimulation of protein transport by PKC (20) and/or PKA (21) led us to analyze whether Gαi-2, a G protein α subunit that can be phosphorylated by PKC (22-24,41), is found in Golgi preparations. Indeed, Gαi-2 of 40 kDa was identified both, in liver and in adrenal medulla by two independent methods: Western blotting and immunoelectron microscopy. Additional to the 40 kDa form of Gαi-2, two larger forms were detected: (i) a 43 kDa form (Gαi-2-S1) corresponding to the splice variant described by (16) was visualized in adrenal medulla and (ii) a second species of 46 kDa (Gαi-2-S2) was recognized in Golgi preparations from liver. Presently we are studying whether Golgi-bound Gαi-2 variants become phosphorylated and if this phosphorylation influences vesicle formation at the TGN.

Gαo is expressed in neuronal tissues (42-45), testis (45) and adipocytes (45). This G protein subunit has been localized in the cytoplasmic matrix, between endoplasmic reticulum and Golgi of neurons (44) and was found attached to membranes of synaptic vesicles (15) and of secretory granules (15,46). Its attachment to secretory granules (15) and a stimulation of regulated secretion by antibodies against Gαo indicates that this G protein may be involved in maturation of secretory granules or their fusion with the cell membrane. The localization of Gαo at the Golgi of adrenal medulla, described here, is an indication that Gαo may additionally be involved in the formation of immature secretory granules at the TGN.

The localization of Gαi-3 on Golgi membranes that has been described before (5,8,13,14) was confirmed by immunoelectron microscopic studies with antibody AB-i-3. The antibodies raised against the C-terminal peptide of Gαi-3 (AB-i-3) cross-react with Gαi-2, as has been observed also by others (47). In order to check whether this cross-reactivity had an impact on immunodetection of Gαi-3, the C-terminal peptide of Gαi-3 was added: More than 70 % of immunogold labeling of Golgi structures could be blocked. No significant reduction in labeling was obtained with the C-terminal peptide of Gαi-2 (data not shown). Both results confirm the specificity of Gαi-3 detection.

Gαs, a G protein that stimulates apical transport, both, in vivo and in vitro (7) and post-Golgi transport in vitro (8) was identified in two forms at the Golgi membranes both in liver and adrenal medulla. In liver, the small form (Gαs-S) is found at about twice the level of the larger

one (Gαs-L), whereas in adrenal medulla, the larger form is the predominant one (Fig. 2a and b, lanes 4).

The results presented here implicate, that two stimulatory G protein α subunits (Gαs-S and Gαs-L) and three inhibitory G protein α subunits (Gαi-2, a splice variant of Gαi-2, and Gαi-3) are needed for the constitutive secretion in liver cells. The individual stimulatory or inhibitory G proteins may serve in controlling the formation of vesicles for the different constitutive signaling pathways.

Gαo found additionally in neural and neuroendocrine cells is probably linked to the regulated secretory pathway. Its association with Golgi membranes is indicative of a function during the formation of immature secretory granules.

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