

Countercurrent Distribution of Two Distinct SNARE Complexes Mediating Transport within the Golgi Stack

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Genetic and biochemical evidence has established that a SNARE complex consisting of syntaxin 5 (Sed5)-mYkt6 (Ykt6)-GOS28 (Gos1)-GS15 (Sft1) is required for transport of proteins across the Golgi stack in animals (yeast). We have utilized quantitative immunogold labeling to establish the *cis-trans* distribution of the v-SNARE GS15 and the t-SNARE subunits GOS28 and syntaxin 5. Whereas the distribution of the t-SNARE is nearly even across the Golgi stack from the *cis* to the *trans* side, the v-SNARE GS15 is present in a gradient of increasing concentration toward the *trans* face of the stack. This contrasts with a second distinct SNARE complex, also required for intra-Golgi transport, consisting of syntaxin 5 (Sed5)-membrin (Bos1)-ERS24 (Sec22)-rBet1 (Bet1), whose v- (rBet1) and t-SNARE subunits (membrin and ERS24), progressively decrease in concentration toward the *trans* face. Transport within the stack therefore appears to utilize countercurrent gradients of two Golgi SNAREpins and may involve a mechanism akin to homotypic fusion.

INTRODUCTION

Intracellular membrane fusion results from the assembly of a target membrane t-SNARE, composed of one heavy chain (syntaxin) and two distinct light chains, with a cognate vesicle membrane v-SNARE (Söllner *et al.*, 1993; Weber *et al.*, 1998; Hu *et al.*, 2003). The resulting SNAREpin, held together by a bundle of four α -helices (Sutton *et al.*, 1998), forcefully perturbs the apposing lipid bilayers, thereby triggering fusion (McNew *et al.*, 2000b). Cognate SNARE pairing between bilayers is the immediate determinant of the specificity of membrane fusion (Fukuda *et al.*, 2000; McNew *et al.*, 2000a; Parlati *et al.*, 2000, 2002; Paumet *et al.*, 2001). The v-SNARE is functionally distinguished from the other subunits of the SNAREpin because fusion can only occur when the v-SNARE resides in the opposite bilayer from the remaining subunits, which comprise the t-SNARE (Fukuda *et al.*, 2000).

In particular, fusion of vesicles derived from the ER with the Golgi requires the pairing of the v-SNARE Bet1 (in yeast; rBet1 in animals) with the t-SNARE comprised of the syntaxin heavy chain Sed5 (syntaxin 5) and light chains Bos1

(membrin) and Sec22 (ERS24, also named Sec22b; Parlati *et al.*, 2000), abbreviated t-Sed5/Bos1, Sec22. The genes encoding these four “ER-Golgi” SNAREs are required for ER-to-Golgi transport in yeast *in vivo* (Newman and Ferro-Novick, 1987; Newman *et al.*, 1990; Shim *et al.*, 1991; Hardwick and Pelham, 1992; Cao and Barlowe, 2000). Liposomes bearing the v-SNARE Bet1 only fuse with liposomes bearing the cognate t-SNARE Sed5/Bos1, Sec22 (Parlati *et al.*, 2000, 2002; Paumet *et al.*, 2001).

The intracellular distribution of these SNAREs determined by immunoelectron microscopy fits well with their roles in ER-to-Golgi transport in animal cells (Paek *et al.*, 1997; Hay *et al.*, 1998; Orci *et al.*, 2000; Martinez-Menarguez *et al.*, 2001). The “short” Golgi-localized form of the t-SNARE heavy chain syntaxin 5 (Hui *et al.*, 1997), which lacks the ER-retrieval signal encoded in the additional exon of the “long” form, is located in every cisternae across the Golgi stack including the *trans*-Golgi network (TGN). By contrast, the t-SNARE light chains membrin, ERS24 and the v-SNARE rBet1 are located in the intermediate compartment (IC) and largely within the first two cisternae of the Golgi stack on the *cis* side. The presence of syntaxin 5 throughout the stack, well beyond its light chains membrin and ERS24 suggested that it could play a dual role, also serving as the heavy chain of a second, distinct intra-Golgi t-SNARE within later cisternae. Consistent with this, syntaxin 5/Sed5 coimmunoprecipitates with several additional SNAREs in extracts from animal and yeast cells (Sogaard *et al.*, 1994; Banfield *et al.*, 1995; Fischer von Mollard *et al.*, 1997; Hay *et al.*, 1997, 1998; Lupashin *et al.*, 1997; Xu *et al.*, 1998, 2002; Zhang and Hong, 2001; Parlati *et al.*, 2002; Shorter *et al.*, 2002).

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Abbreviations used: CHO, Chinese hamster ovary; CGN, *cis*-Golgi network; IC, intermediate compartment; GFP, green fluorescent protein; NRK, normal rat kidney cells; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TGN, *trans*-Golgi network; VSV, vesicular stomatitis virus; VSV-G, vesicular stomatitis virus glycoprotein.

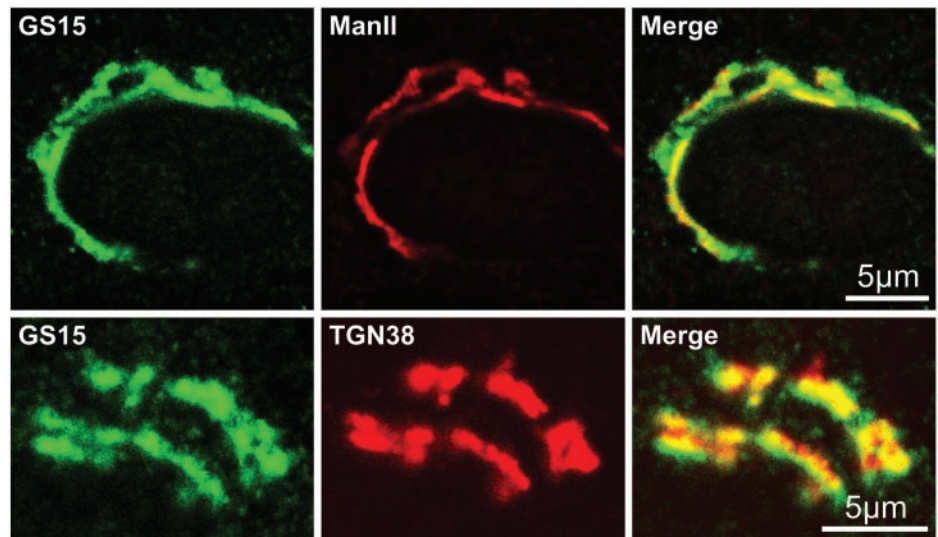


Figure 1. Localization of GS15 in NRK cells by immunofluorescence microscopy. Rat NRK cells were fixed and immunostained for GS15 and Mannosidase II (top panels) or GS15 and TGN38 (bottom panels) and analyzed by laser confocal microscopy as described in MATERIALS AND METHODS. Areas of colocalization of the two proteins in the merged images appear yellow.

Recently, this postulated second tetrameric fusogenic Sed5/syntaxin 5-based SNARE complex has been established (Parlati *et al.*, 2002). The t-SNARE consists of Sed5 (syntaxin 5) as the heavy chain together with Ykt6 (mYkt6) and Gos1 (GOS28) as the light chains. The cognate v-SNARE is Sft1 (GS15). The two Golgi SNAREpins are completely distinct functionally, as v-Sft1 will only fuse with t-Sed5/Ykt6, Gos1 and not with t-Sed5/Bos1, Sec22 and v-Bet1 will only fuse with t-Sed5/Bos1, Sec22 and not with t-Sed5/Ykt6, Gos1 (Parlati *et al.*, 2002).

Where within the Golgi stack does this novel t-SNARE (Sed5/Ykt6, Gos1) and its cognate v-SNARE (Sft1) reside? Which of these SNAREs are enriched in peri-Golgi vesicles compared with the cisternae? What are the relative molar abundances of Golgi SNAREs? Answering these questions is central to understanding how proteins flow in the Golgi stack because the pattern of localization and abundance of these SNARE proteins will pinpoint the fusion events that are required for transport of cargo within the Golgi stack.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies against GOS28, GS15, and GM130 were from Transduction Laboratories (Lexington, KY) and against membrin and rBet1 (clone 16G6) from StressGen Biotechnology (Victoria, British Columbia, Canada). Monoclonal anti-mannosidase II and anti-TGN38 antibodies were purchased from Covance Inc. (Richmond, CA) and BD Biosciences (San Diego, CA), respectively. Rabbit polyclonal antibodies against membrin and GS15 were obtained from Dr. F. Wieland (Biochemie-Zentrum, Heidelberg) and Dr. W. Hong (Institute of Molecular and Cell Biology, Singapore), respectively. A goat anti-Ykt6 antibody was generated commercially (Covance Inc.) to his6-tagged human Ykt6 according to the company's standard protocol. An anti-GS15 peptide antibody was generated commercially (SynPep Inc., Dublin, CA) by immunizing rabbits with a GS15 peptide (aa27–41) coupled through a C-terminal cysteine to KLH. These polyclonal antibodies were affinity-purified by Sulfolink (Pierce Inc., Rockford, IL) columns coupled with either GS15 peptide (aa27–41+cysteine) or his6-tagged human Ykt6. Purified antibodies were eluted with 0.1 M glycine, pH 2.8, and neutralized with 2 M Tris-Cl, pH 8.0. Rabbit polyclonal antibodies against GOS28, ERS24, and Syntaxin 5 were generated and affinity-purified as described previously (Nagahama *et al.*, 1996; Paek *et al.*, 1997; Orci *et al.*, 2000). Rabbit polyclonal antibodies against rBet1 and Vti1b were generated by immunizing rabbits with recombinant rBet1 (aa1–95) and Vti1b (aa1–206). These antibodies were affinity-purified as described previously for our other SNARE antibodies (Nagahama *et al.*, 1996; Paek *et al.*, 1997; Orci *et al.*, 2000).

Secondary antibodies were from Molecular Probes (Eugene, OR; Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse), Jackson ImmunoResearch (West Grove, PA; FITC and Cy5 donkey anti-rabbit and

anti-mouse) and British BioCell International (Cardiff, United Kingdom; gold-conjugated goat anti-mouse and goat anti-rabbit IgG). Whole rabbit IgG was purchased from Sigma (St. Louis, MO). Fab fragments were prepared using an ImmunoPure Fab Kit (Pierce Inc.) according to the manufacturer's instructions. The Fab fragments were then dialyzed against (25 mM Tris-Cl, pH 7.4; 25 mM KCl, 10% glycerol) and concentrated using 20% PEG in the same buffer.

Immunofluorescence Microscopy

NRK or HeLa cells were seeded onto 12-mm glass coverslips in 12-well dishes and treated as described in the figure legends. Cell fixation, permeabilization, and subsequent antibody labeling was performed as described in reference (Volchuk *et al.*, 2000). For the labeling in Figure 1, rabbit anti-GS15 (1:50) and either mouse mannosidase II (1:2000) or mouse TGN38 (1:30) were used as the primary antibodies, followed by detection with Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 546-conjugated goat anti-mouse (both at 1:400 dilution) secondary antibodies. Rabbit polyclonal anti-GS15 antibodies from W. Hong (Xu *et al.*, 1997, 2002) and our anti-GS15 peptide antibody were used and both gave similar results. For the labeling in Figure 2A, goat anti-Ykt6 (1:20) and rabbit anti-GOS28 (1:100) were used as the primary antibodies followed by detection with FITC-conjugated donkey anti-goat and Cy5-conjugated donkey anti-rabbit (both at 1:1000 dilution) secondary antibodies. Immunofluorescence images were obtained with a Zeiss LSM 510 confocal microscope (Thornwood, NY).

Electron Microscopy and Quantitative Evaluation

Rat NRK cells were used for the immunolocalization of endogenous GS15, rBet1, membrin, ERS24, GOS28, syntaxin 5, and GM130. The cells were washed two times in PBS before fixation for 30 min at room temperature with 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The cells were washed three times in PBS, scraped gently with a plastic cell scraper, and pelleted by centrifugation in a microfuge (12,000 rpm, 2 min). Cell pellets were embedded in 12% gelatin and cooled on ice. Small blocs of gelatin-embedded cells were infused with 2.3 M sucrose, frozen in liquid nitrogen, and sectioned with a cryoultramicrotome. Thin sections were collected and immunogold labeled as described in Orci *et al.* (1997). Antibody dilutions were as follows: anti-GS15 from Dr. W. Hong (1:20), anti-membrin (1:70), anti-syntaxin 5 and anti-ERS24 (1:20), anti-rBet1 monoclonal (1:20) and anti-GOS28 (1:3). Double-label immunocytochemistry was performed by combined labeling of GM130 with ERS24, membrin, or GS15. GM130 antibody was revealed by goat anti-mouse IgG conjugated with 10-nm gold, and ERS24, membrin, or GS15 antibodies were revealed by goat anti-rabbit IgG conjugated to 15-nm gold particles.

The subcellular localization of the different antigens was analyzed quantitatively on thin cryosections incubated with the appropriate antibodies followed by protein A-gold. Mouse monoclonal anti-rBet1 was labeled with gold-conjugated goat anti-mouse IgG. Sections of immunolabeled Golgi areas with a clear *cis-trans* orientation were photographed and printed at a final magnification of 96,000 \times . For each antigen, 40 different Golgi areas were quantified (50 different Golgi areas were used for the rBet1 analysis). The compartments evaluated were as follows: a) intermediate compartment (IC), defined as the tubulo-vesicular membrane profiles located in the area limited by the *cis*-most Golgi cisternae and the transitional elements of the rough endoplasmic reticulum (RER); b) cisternae, defined as elongated stacked

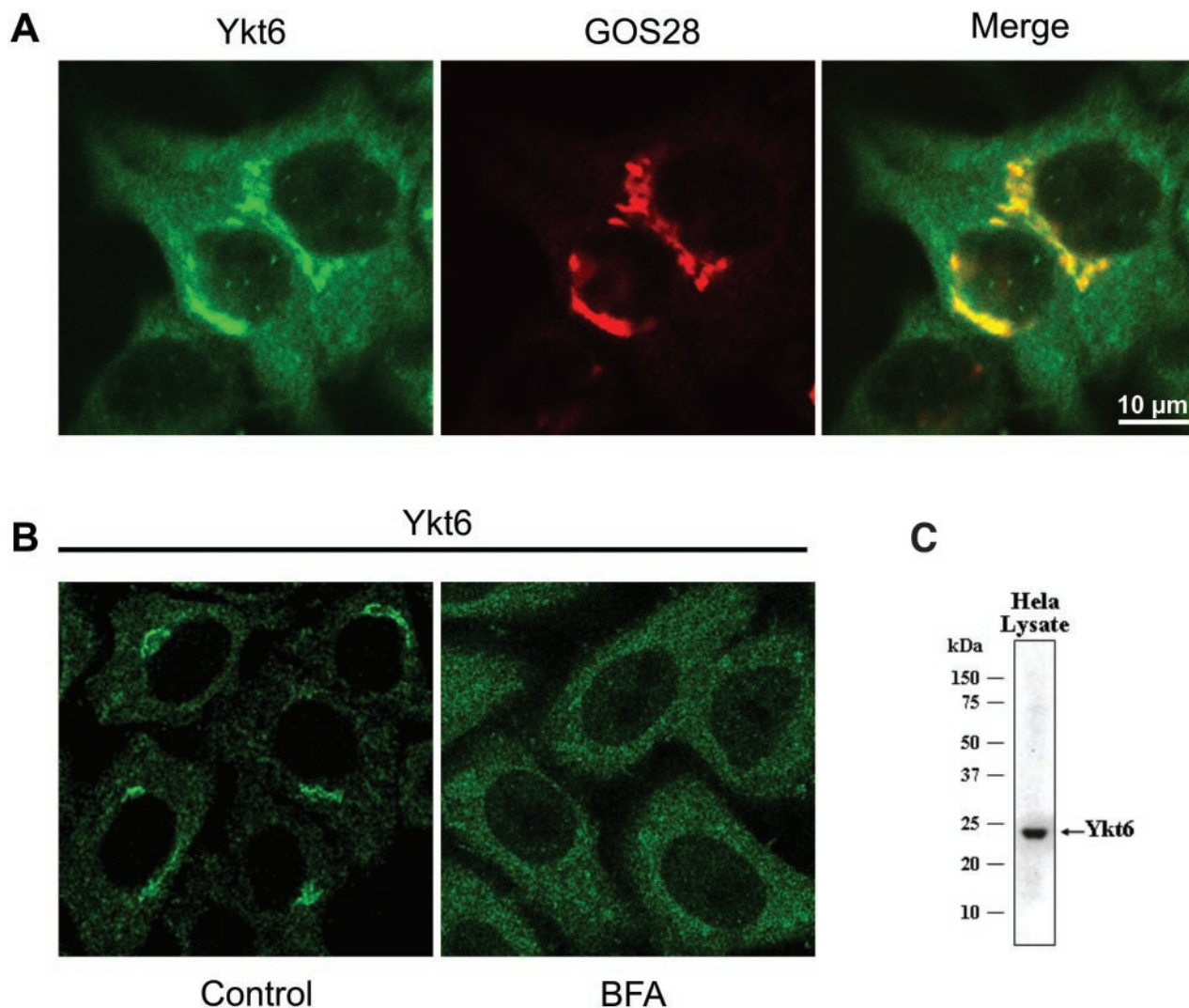


Figure 2. Localization of Ykt6 in HeLa cells by immunofluorescence microscopy. (A) Human HeLa cells were fixed and immunostained with a goat anti-Ykt6 antibody and a rabbit anti-GOS28 antibody as described in MATERIALS AND METHODS. (B) Human HeLa cells were stained for Ykt6 before (Control) and after treatment with brefeldin A (10 μ g/ml for 30 min; BFA). (C) HeLa cell total lysate (60 μ g) was resolved by SDS-PAGE and immunoblotted with a goat anti-Ykt6 antibody.

Table 1. Distribution of SNAREs in the Golgi area of NRK cells

	Percentage of total gold particles				Percentage of total Golgi stack gold particles over <i>cis</i> to <i>trans</i> cisternae				
	IC	Golgi stack	Peri-Golgi vesicles	TGN	Cisterna 1	Cisterna 2	Cisterna 3	Cisterna 4	Cisterna 5
GS15	5 \pm 1	71 \pm 2	6 \pm 1	18 \pm 2	4 \pm 1	10 \pm 2	18 \pm 2	33 \pm 2	35 \pm 3
GOS28	8 \pm 2	66 \pm 4	16 \pm 3	10 \pm 2	16 \pm 3	25 \pm 3	27 \pm 3	18 \pm 3	14 \pm 2
rBet1	72 \pm 3	23 \pm 3	3 \pm 1	2 \pm 1	60 \pm 6	20 \pm 5	6 \pm 3	10 \pm 4	3 \pm 2
Membrin	20 \pm 3	68 \pm 4	9 \pm 2	3 \pm 1	70 \pm 4	23 \pm 4	7 \pm 2	0.2 \pm 0.2	0.5 \pm 0.4
ERS24	35 \pm 4	47 \pm 3	6 \pm 1	12 \pm 2	64 \pm 4	14 \pm 3	10 \pm 3	4 \pm 1	8 \pm 2
Syntaxin 5	11.5 \pm 2	73 \pm 2	3 \pm 1	12 \pm 2	23 \pm 2	20 \pm 2	20 \pm 2	24 \pm 3	13 \pm 2

Refer to MATERIALS AND METHODS in the Electron Microscopy and Quantitative Evaluation section for the details of the quantitations. Data are the mean \pm SEM.

membrane profiles enclosing a lumen (lateral cisternal rims and buds were included in this compartment); cisternae were numbered C1 to C5 from *cis* to *trans*; c) peri-Golgi lateral vesicles, defined as circular ~70-nm membrane profiles not connected to cisternae on the sections and located within 200 nm of the lateral rim of the cisternae; d) TGN, defined as the tubulo-vesicular membrane profiles at the *trans*-side of the Golgi stack. The distribution of gold particles over the compartments defined above was expressed as a percentage of the total number of gold particles. For the cisternal *cis-trans* distribution of the labeling (Table 1, right side), the number of gold on each cisterna was expressed as the percentage of the total gold present over the stack of cisternae. The linear density was quantified as follows: the length of linear profiles (such as cisternae or tubulovesicles) was measured with an electronic pen. The length of peri-Golgi vesicles was estimated by multiplying the circumference of a 70-nm circle times the total number of vesicles counted. The number of gold particles on each compartment was recorded with the same pen and the labeling densities were expressed as the number of gold per μm of membrane. The data represent the mean of the values obtained on 40 different Golgi areas (or 50 different Golgi areas for rBet1), \pm SEM. For unknown reasons, the rBet1 antibody also elicited a gold labeling over the nucleoplasm.

³⁵S-Methionine Cell Labeling, SNARE Immunoprecipitation, and Molar Ratio Calculation

Rat NRK cells were labeled for 24 h with 100 $\mu\text{Ci}/\text{ml}$ ³⁵S-methionine (in vivo cell labeling grade, Amersham, Piscataway, NJ) in methionine-free DME containing 10% dialyzed FCS and 100 μM cold methionine. The cells were subsequently washed, lysed in Triton-X 100 lysis buffer (1% TX-100, 100 mM KCl, 20 mM HEPES, pH 7.3, 2 mM EDTA, 1 mM dithiothreitol (DTT), 230 μM PMSF, supplemented with protease inhibitor tablets; Roche, Nutley, NJ), and incubated for 1 h at 4°C. The lysate was clarified by centrifugation (14,000 rpm, 10 min, 4°C). The protein concentration of the lysate was determined using the BCA Protein Assay (Pierce Inc). SNARE proteins were quantitatively immunoprecipitated from 100 μg of lysate by adding 5–10 μg of antibodies specific for each SNARE for 1 h at 4°C under constant rotation. A protein A-Agarose slurry (20 μl ; Roche) was added to the mixture and incubated for an additional 2 h at 4°C. The beads were subsequently washed three times 5 min each with lysis buffer and once for 5 min with lysis buffer containing 0.1% TX-100. The washed beads were boiled (5 min) in 2 \times NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) β -mercaptoethanol. The radiolabeled SNARE immunoprecipitates were resolved by SDS-PAGE, and the gel was dried and exposed to a Molecular Dynamics (Sunnyvale, CA) Storage Phosphor screen and processed by a PhosphorImager. The molar ratios of the various SNARE proteins were determined as follows: Band intensities in arbitrary units of the individual SNAREs were quantified by ImageQuant software. To account for the fact that different SNAREs have different amounts of methionine, which causes the band intensity, the intensity values for each SNARE were normalized to the number of methionines in GOS28. GOS28 with 11 methionines based on its cDNA sequence (the highest number of methionines for the various SNAREs) was assigned a normalization factor of 1 (i.e., ERS24 with 7 methionines has a normalization factor of 1.57, etc.). In this way the relative levels of each SNARE in arbitrary units present in 100 μg of starting protein was determined.

Similarly, the collective band intensities of 1% (1 μg) of all ³⁵S-labeled proteins in the starting material was quantified using a PhosphorImager and ImageQuant software. This value was multiplied by 100 to give the relative intensity of proteins in arbitrary units in 100 μg total protein. This value was then normalized for the average methionine content of human proteins (2.29%, determined from analysis of the Human Genome Project). The individual SNARE values (in arbitrary units) were divided by this number to obtain the amount of each SNARE (in grams) in the starting material. By taking into account the true molecular weights of each SNARE (g/mol) the moles/mg cell protein of each SNARE was obtained yielding the molar ratios of the SNARE proteins within cells (see values presented in the Figure 7 legend).

Cell-free Transport Assay

Wild-type CHO cells and a mutant clone 15B (defective in UDP-GlcNAc glycosyltransferase I) were cultured as described (Balch *et al.*, 1984a). The 15B cells were infected with VSV and Golgi membranes from the infected 15B cells (donor Golgi) and wild-type CHO cells (acceptor Golgi) were prepared as described previously (Balch *et al.*, 1984a). Cytosol was prepared from wild-type CHO cells by a modified protocol originally used for bovine brain cytosol preparation (Malhotra *et al.*, 1989). Briefly, 3 L of CHO cells were grown to a density of $3\text{--}5 \times 10^5$ cells/ml. The cells were washed with PBS, resuspended in homogenization buffer (250 mM sucrose, 10 mM Tris-Cl, 50 mM KCl, pH 7.4) and homogenized with a ball bearing homogenizer. The homogenate was centrifuged in a SW55 rotor for 1 h at 55K rpm at 4°C. The supernatant was removed and dialyzed against 25 mM Tris-Cl, 50 mM KCl, 0.5 mM DTT, pH 7.4. The dialyzed supernatant was incubated at 37°C for 30 min and centrifuged as above. The supernatant (cytosol) was decanted, the

protein concentration was assayed (Bio-Rad, Hercules, CA), and aliquots were frozen in liquid nitrogen and stored at -80°C .

Transport assays were conducted according to a previously published protocol (Balch *et al.*, 1984a). Briefly, 50 μl reactions contained the following (final concentrations): distilled water (20 μl), buffer (25 mM KCl, 25 mM HEPES, pH 7.0, 2.5 mM magnesium acetate; 5 μl), 0.5 μCi UDP-GlcNAc (New England Nuclear, Boston, MA) dried before resuspension, ATP-regenerating system (0.2 mM ATP, 1.0 mM UTP, 5.0 mM creatine phosphate, 18.2 IU/ml creatine kinase; 5 μl), and 75 μg cytosol (10 μl) and ~1.5 μg donor (5 μl) and acceptor Golgi (5 μl). Fab fragments (dialyzed in 25 mM Tris-Cl, pH 7.4, 25 mM KCl, 10% glycerol) or dialysis buffer alone were substituted for the water in reactions containing antibodies. Premixes of all ingredients except the ATP-regenerating system were incubated on ice for 10 min, before the ATP-regenerating system was added, and samples were further incubated at 4°C for 15 min. The reactions were then transferred to a 37°C water bath for 60 min. The samples were then placed on ice for 5 min and lysed by buffer containing 1% Triton X-100, and VSV-G was immunoprecipitated as described (Balch *et al.*, 1984a). The immune precipitate was recovered on glass filters (Whatman, Clifton, NJ) and washed, and incorporated radioactivity was determined by scintillation counting. A sample without cytosol addition and/or a complete reaction incubated on ice and processed as the other samples was used to determine the background signal. This value was subtracted from all samples. For each antibody the assay was performed at least twice on different days with triplicate samples for each condition.

RESULTS

Subcellular Localization of Golgi SNAREs in Mammalian Cells

In this study we examine in detail the distribution of Golgi SNAREs. All localization studies were performed exclusively on endogenous SNAREs to avoid potential artifacts, which can result from the use of tagged or overexpressed proteins. Double-label immunofluorescence localization of GS15 and the medial Golgi enzyme mannosidase II in rat NRK cells revealed a partial colocalization, in agreement with previous reports (Figure 1, top panels; Xu *et al.*, 1997, 2002). In addition, we observed significant colocalization with a TGN marker TGN38 (Figure 1, bottom panels), indicating that this SNARE distributes across the Golgi stack and is also present in the *trans*-Golgi face.

Ykt6 lacks a transmembrane domain and consequently only a fraction (<50%) of the endogenous protein is associated with membranes (McNew *et al.*, 1997; Zhang and Hong, 2001). A recent study however has reported that Ykt6 is not present in the Golgi of mammalian cells, but in an unidentified specialized compartment primarily in neuronal cells (Hasegawa *et al.*, 2003). This contrasts with the results of Zhang and Hong (2001), who showed that endogenous Ykt6 is present in Golgi-enriched membranes from liver by Western blotting and in the Golgi area of NRK cells by immunofluorescence microscopy. To test whether Ykt6 is indeed a Golgi-localized SNARE, we generated a goat antibody to the human Ykt6 protein. The antibody recognizes a single band by Western blotting of HeLa cell total lysate (Figure 2C). By immunofluorescence microscopy in HeLa cells (Figure 2A), this antibody gave a perinuclear Golgi pattern that colocalized to a large extent with GOS28, a Golgi SNARE localized throughout the Golgi stack (Nagahama *et al.*, 1996; Orci *et al.*, 2000; and Figure 6). As a control, the immunofluorescence signal was completely blocked by preincubation of the Ykt6 antibody with recombinant Ykt6 protein (unpublished data). This is consistent with the observation that immunoprecipitation of GOS28 from rat liver Golgi extracts coimmunoprecipitates Ykt6 (Xu *et al.*, 2002, and our unpublished observations). In addition, treating the cells with brefeldin A caused a complete dispersal of the Golgi staining for Ykt6 (Figure 2B), indicating that the majority of the Ykt6 is localized in the Golgi stack (Klausner *et al.*, 1992). Unfortunately, despite testing several antibodies to

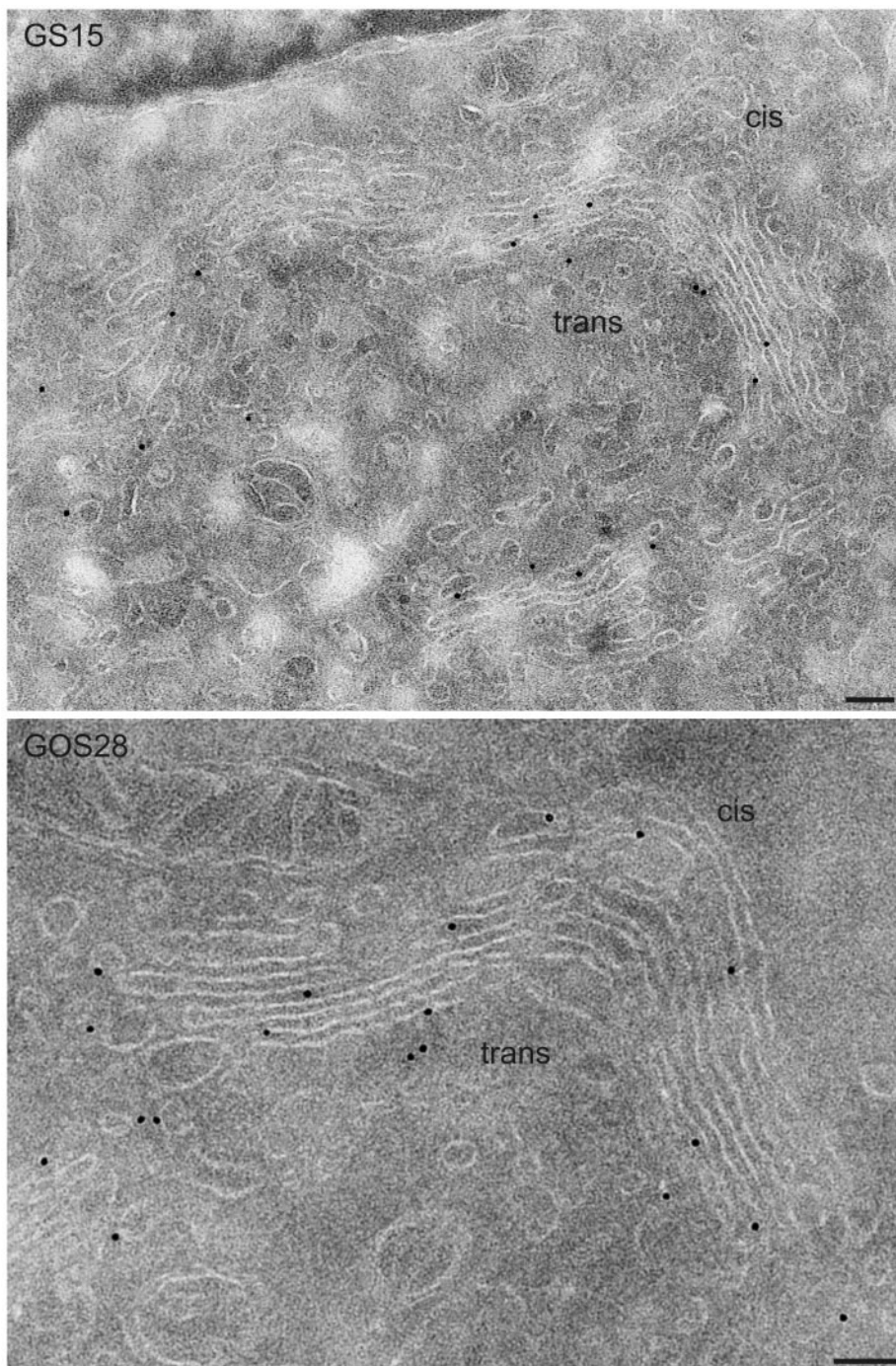


Figure 3. Ultrathin cryosections of NRK cells showing immunogold labeling of GS15 and GOS28. GS15 is preferentially localized over the *medial* and *trans* cisternae of the Golgi stack, whereas GOS28 is distributed across the stack. The quantitative evaluation of the labeling is shown in Tables 1 and 2. Bar, 100 nm.

Ykt6, none have proven to be useful for immunogold localization by EM, precluding a more precise localization at the cisternae level. Although Ykt6 is indeed a bona fide Golgi SNARE protein, it is not exclusively localized there (Hasegawa *et al.*, 2003). The yeast Ykt6 protein has been implicated in post-Golgi transport steps (Ungermann *et al.*, 1999), and we did observe significant punctate staining that could be of endosomal origin or other organelles (Figure 2A and 2B).

Representative examples of the ultrastructural localization of GS15 as well as several other Golgi SNAREs are shown in Figures 3–5. GS15 is most prominent in the *medial* and *trans*

portion of the stack (Figure 3). Figure 5C shows double labeling with the *cis* Golgi marker GM130 (Nakamura *et al.*, 1995). This result agrees well with the immunofluorescence localization observed with *medial* and *trans*-Golgi markers (Figure 1). It is only in partial agreement with the electron microscopic distribution reported by Xu *et al.* (2002), who observed a primarily medial distribution of endogenous GS15 in CHO and 3T3-L1 cells. However, no quantitative data for the distribution were presented in this study. Syntaxin 5 and GOS28 are localized throughout the Golgi stack, whereas ERS24, rBet1, and membrin reside primarily in the *cis* face (Figures 3–5; Tables 1 and 2).

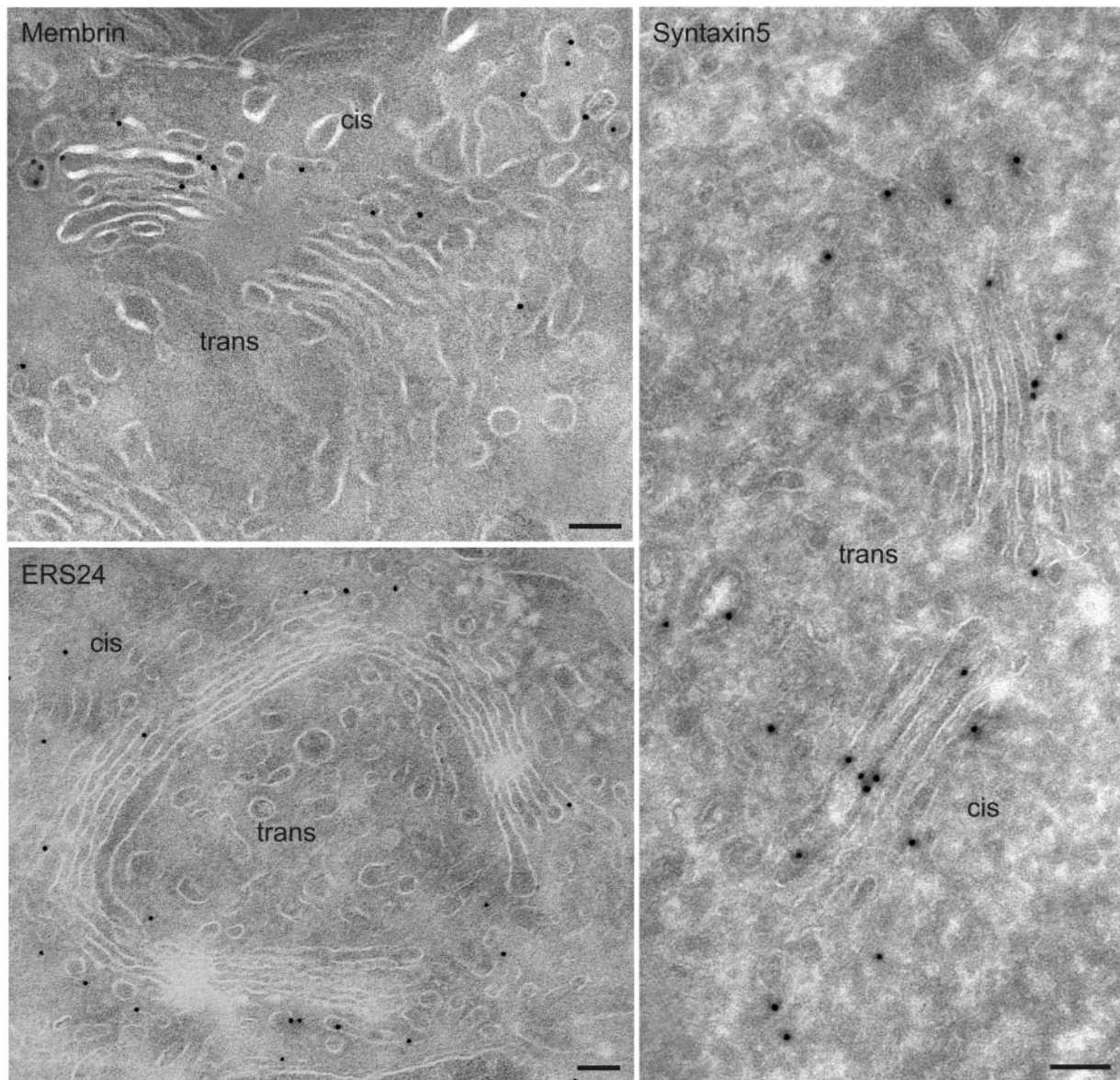


Figure 4. Ultrathin cryosections of NRK cells showing immunogold labeling of membrin, ERS24, and syntaxin 5. Membrin and ERS24 distribute mostly to the *cis* side of the Golgi, including elements of the intermediate compartment, whereas syntaxin 5 is present throughout the entire Golgi stack. The quantitative evaluation of the labeling is shown in Tables 1 and 2. Bar, 100 nm.

Tables 1 and 2 provide quantitative results regarding the intra-Golgi distribution of GS15, syntaxin 5, ERS24, membrin, rBet1, and GOS28 in NRK cells. The data presented here for these SNAREs are in good agreement with previously published results in other cell types (Paek *et al.*, 1997; Hay *et al.*, 1998; Orci *et al.*, 2000; Martinez-Menarguez *et al.*, 2001). We also provide extensive data on the cisterna-by-cisterna concentrations of these SNAREs based on the linear density of labeling (Figure 6) and on the cisternal vs. peri-Golgi vesicle abundance and concentration of each SNARE (Table 1 and 2; see also Figure 8). ERS24, membrin, and rBet1 are concentrated most in the IC/*cis*-Golgi network (CGN) and the *cis*-Golgi (cisternae 1 and 2) and are greatly reduced in concentration at the *trans* end of the stack (Figure 6A). Syntaxin 5 is most abundant in the *cis*-most cisterna and is evenly distributed

at ~40% of its peak concentration in the Golgi stack (Figure 6B). The high level of syntaxin 5 observed in cisterna 1 compared with the rest of the stack may likely be due to the fact that the polyclonal antibody used for localization recognizes both the 34-kDa Golgi form and the 41-kDa ER/IC form of syntaxin 5 (Hui *et al.*, 1997). The 41-kDa form is also likely present in cisterna 1 (Hui *et al.*, 1997); thus, the signal observed is a combination of the two splice forms. GOS28, like syntaxin 5, also distributes approximately equally throughout the stack, declining to ~50% of its peak concentration in the TGN. GS15 exhibits the opposite distribution, with progressively higher concentrations toward the *trans* side of the stack, resulting in a gradient of a factor of ~9 in concentration from IC/CGN to the last Golgi cisternae (Figure 6B).

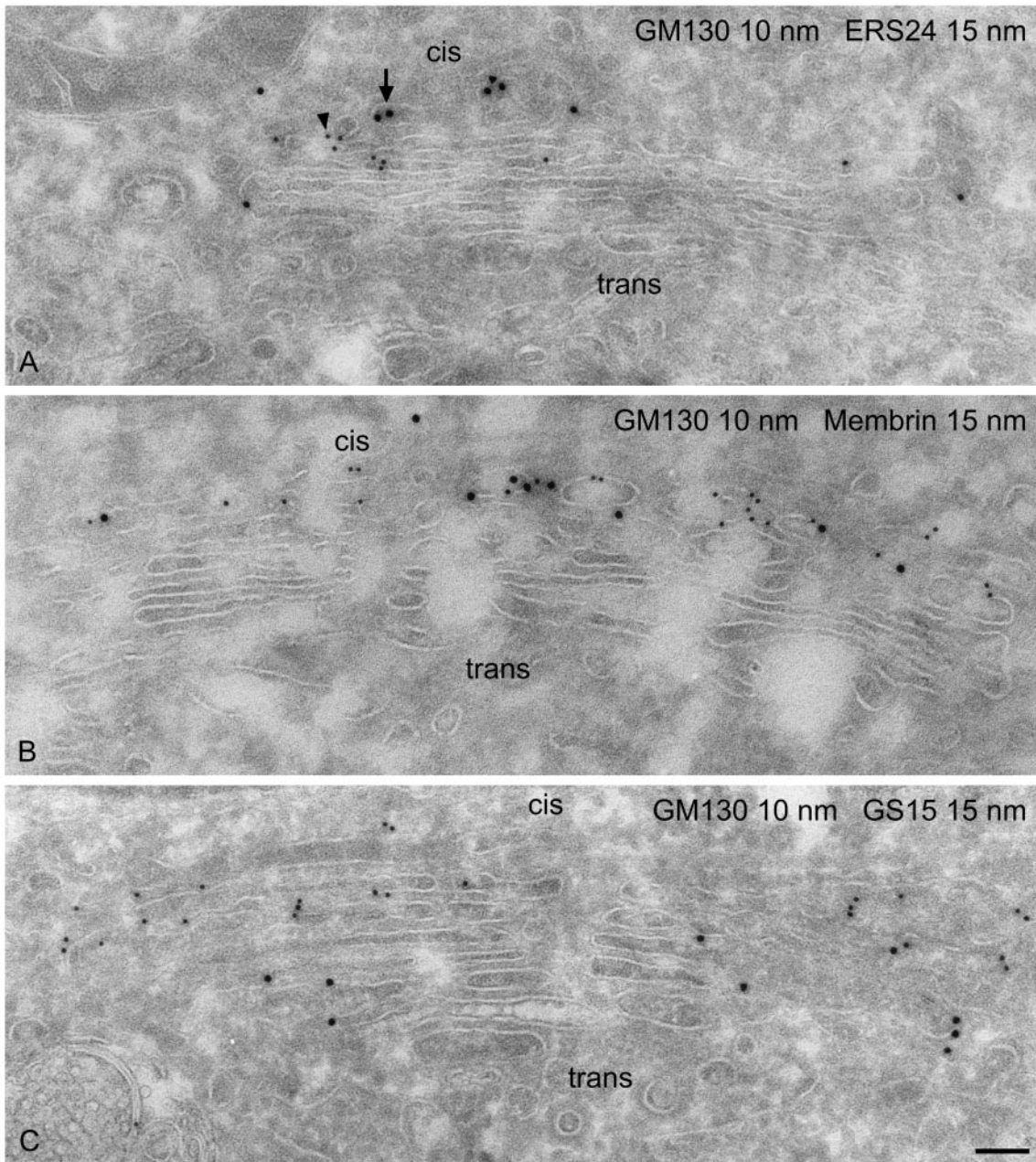


Figure 5. Assessment of the *cis-trans* polarity of the Golgi stack. Double labeling of GM130 and ERS24 (A), GM130 and membrin (B), GM130 and GS15 (C). GM130, a *cis*-Golgi marker, colocalizes with ERS24 and membrin but not with GS15, a SNARE localized to the *medial/trans*-Golgi. GM130 was visualized with small (10 nm) gold particles (arrowhead), whereas ERS24, membrin, and GS15 were revealed by large (15 nm) gold particles (arrow). Bar, 100 nm.

Relative Molar Concentrations of SNAREs across the Stack and in Vesicles

It is evident from the localization data that each cisterna contains a mixture of all seven of the subunits comprising the two Golgi-localized SNAREpins, v-rBet1: t-syntaxin 5/membrin, ERS24 and v-GS15: t-syntaxin 5/mYkt6, GOS28, but their ratios in the mixture vary across the stack. In particular every Golgi cisterna contains varying ratios of two alternative sets of t-SNARE light chains (membrin+ERS24 vs. mYkt6+GOS28) that will compete for binding to a common heavy chain (syntaxin 5), to form one or the other t-SNARE. The data in Tables

1 and 2 and Figure 6 speak to how each SNARE (with the exception of Ykt6) distributes across the stack, but not to the actual number of copies of each SNARE in each compartment, the more fundamental quantity in establishing which t-SNARE prevails in this competition. Therefore, we wished to assess the relative molar abundance of the various SNAREs throughout the cisternae and in the transport vesicles.

To measure the molar abundance of SNAREs in whole cells, rat NRK cells were labeled with ³⁵S-methionine to a steady-state (24 h) and SNARE proteins were quantitatively immunoprecipitated from the lysate. In each case the pre-

Table 2. Distribution of SNAREs in the Golgi area of NRK cells

	IC	Golgi stack	Peri-Golgi vesicles	TGN
GS15	0.04 ± 0.01	0.65 ± 0.04	0.60 ± 0.14	0.55 ± 0.06
GOS28	0.79 ± 0.21	0.52 ± 0.04	1.15 ± 0.18	0.38 ± 0.08
rBet1	4.45 ± 0.44	0.29 ± 0.04	0.15 ± 0.07	0.08 ± 0.03
Membrin	1.08 ± 0.18	0.51 ± 0.03	0.91 ± 0.19	0.11 ± 0.05
ERS24	3.02 ± 0.39	0.54 ± 0.07	0.85 ± 0.22	0.64 ± 0.10
Syntaxin 5	1.10 ± 0.19	1.01 ± 0.09	0.51 ± 0.17	0.69 ± 0.10

Refer to the MATERIALS AND METHODS section in the Electron Microscopy and Quantitative Evaluation section for the details of the quantitations. Data are the mean ± SEM. Values are concentration (gold particles/μm membrane).

cipitated SNARE was evident as a major band in SDS-PAGE of the expected molecular weight (unpublished data). The bands were quantified and normalized according to the known methionine content of each SNARE to yield data on the relative molar abundance of the SNAREs, which are

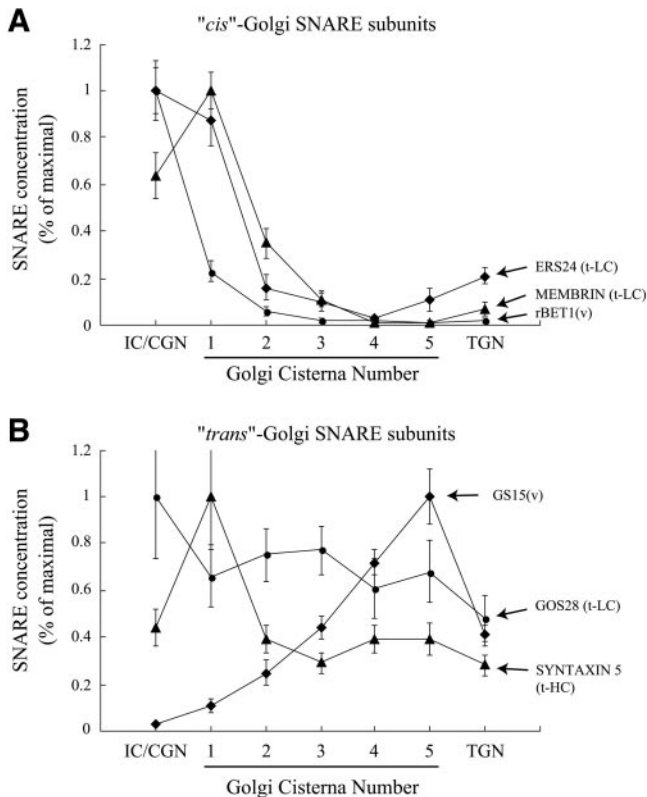


Figure 6. Distribution of SNAREs within the IC, Golgi stack, and TGN. The distribution of the indicated SNAREs within the IC, Golgi stack (cisterna 1 (*cis*) to cisterna 5 (*trans*)), and TGN, were obtained as outlined in MATERIALS AND METHODS. Shown is the linear density of labeling (gold particles/μm membrane) within these compartments. For each SNARE its distribution was normalized to the value in the compartment having the highest labeling density. (A) Data for the subunits of the *cis*-SNAREpin, v-rBet1, and light chains membrin and ERS24. t-LC, t-SNARE light chain; v, v-SNARE. (B) Data for the subunits of the *trans*-SNAREpin, v-GS15, and t-SNARE heavy chain syntaxin 5 and t-light chain GOS28 (t-HC, t-SNARE heavy chain). Error bars, SEM

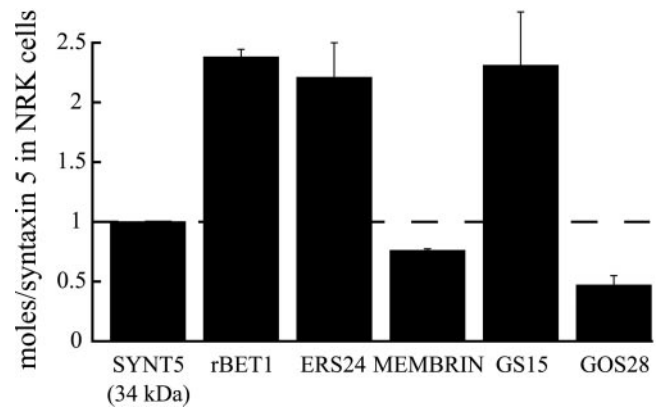


Figure 7. Relative molar amounts of Golgi SNAREs in cells. Rat NRK cells were uniformly labeled with ^{35}S methionine as described in MATERIALS AND METHODS. Individual SNARE proteins were immunoprecipitated and resolved by SDS-PAGE. The amount of each ^{35}S -labeled SNARE in arbitrary units was determined using a Molecular Dynamics PhosphorImager. The absolute abundance (pmol/mg cell protein) of the individual SNAREs were calculated as stated in MATERIALS AND METHODS and are as follows: rBet1, 317 ± 9; GS15, 308 ± 61; ERS24, 295 ± 39; syntaxin 5 (34 kDa), 133 ± 28; membrin, 101 ± 2; GOS28, 63 ± 10. Values were obtained from three independent experiments and shown are the mean ± SD normalized to syntaxin 5. SYNT5, syntaxin 5 (34-kDa Golgi form).

presented in Figure 7, normalized to the common heavy chain syntaxin 5 (34-kDa Golgi-localized form). Because only a small fraction of these SNAREs reside outside the IC/Golgi (12% of rBet1, 11% of ERS24, 9% of membrin, and 7% of syntaxin 5 are in the ER; Hay *et al.*, 1998), the total cellular ratios are, within experimental error, representative of the Golgi area. Lack of a suitable antibody precluded results for mYkt6.

Several notable features emerge from this analysis. First, the various light chains are present in broadly similar molar amounts relative to each other and to their common heavy chain, syntaxin 5, consistent with a dynamic competition to form one or the other t-SNARE throughout the stack.

Second, and surprisingly, the two v-SNAREs rBet1 and GS15 are present in molar excess (~2.3-fold) over syntaxin 5. The actual molar ratio of each of these v-SNAREs to its cognate t-SNARE will be even greater than this for two reasons. First, the syntaxin 5 will be partitioned between two t-SNAREs, only one of which can bind rBet1 or GS15. Second, the v-SNAREs are heavily concentrated toward the *cis* (rBet1) or *trans* (GS15) face, whereas syntaxin 5 evenly distributes in the stack. Therefore, for both SNAREpins the concentration of v-SNARE is much greater than the concentration of t-SNARE in the Golgi complex.

Does this same condition hold in the peri-Golgi transport vesicles? Figure 8A shows the concentration of each SNARE in the vesicles relative to its concentration in cisternae, calculated from the data in Table 2 by dividing (for each SNARE) the linear density of labeling (gold particles/μm) in the peri-Golgi vesicles by the linear density of labeling in the Golgi stack. Evidently, all of the SNAREs (data lacking for Ykt6) are also present in the vesicles, where they are neither dramatically concentrated or excluded. In the case of GOS28, this SNARE is about twofold concentrated in vesicles compared with cisternae, consistent with an earlier study (Nagahama *et al.*, 1996).

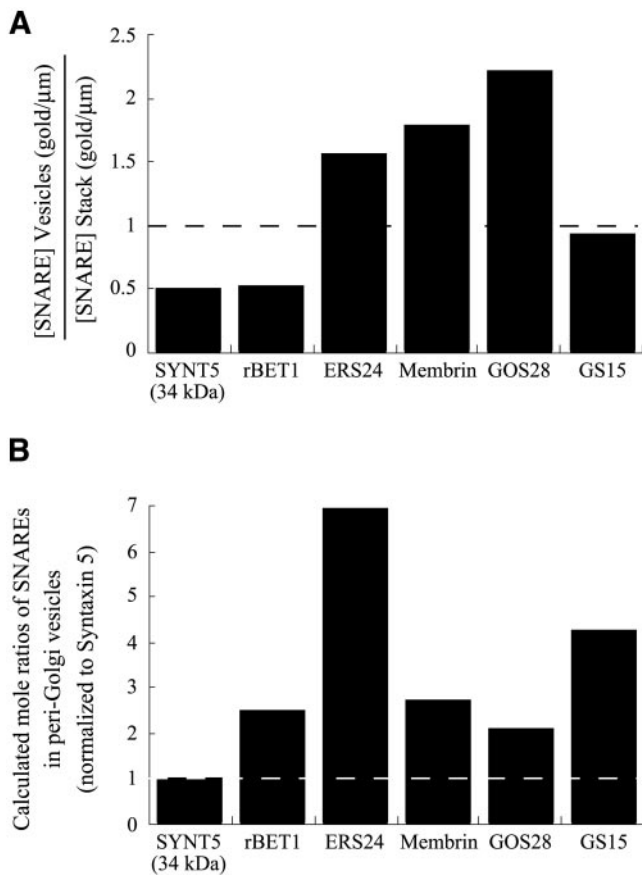


Figure 8. Concentration of SNAREs in peri-Golgi vesicles relative to the common Golgi t-SNARE heavy chain, syntaxin 5. (A) Shown for each SNARE is the ratio of the linear density (gold particles/ μm) within lateral peri-Golgi vesicles (from Table 2) relative to the linear density in the Golgi stack (from Table 2). SYNT5, syntaxin 5 (34-kDa Golgi form). (B) Shown is the calculated mole ratio of the various SNAREs to syntaxin 5 in peri-Golgi vesicles. The values were obtained by multiplying the values in Figure 7 (mole ratios of the SNAREs in cells) by the values in panel A (linear density of gold particles in peri-Golgi vesicles/Golgi stack), and renormalizing to syntaxin 5.

Combining the data in Figure 8A with the data in Figure 7 (molar abundance of SNAREs in the cell), it is evident that the concentration of the v-SNAREs GS15 and rBet1 are also in excess of syntaxin 5 in Golgi-associated vesicles (Figure 8B). Therefore, the morphological and biochemical analysis implies that $[\text{v-SNARE}] \gg [\text{t-SNARE}]$ both in peri-Golgi vesicles and in the Golgi cisternae. This in turn suggests a fusion mechanism akin to homotypic fusion, as will be explored in the DISCUSSION.

Both Golgi SNAREpins Are Required for Cell-free Golgi Transport

GOS28 has previously been shown to be functionally required for intra-Golgi transport in the cell-free system (Nagahama *et al.*, 1996). GOS28 and other SNAREs are also required in a more complex permeabilized cell assay measuring the transport of VSV-G protein starting in the ER to the medial Golgi (endoglycosidase H-resistance). Unfortunately, this assay does not distinguish between requirements

for ER-to-Golgi from intra-Golgi transport, because both must occur for the assay to register (endoglycosidase H resistance is not conferred until the removal of mannose from the N-linked oligosaccharide chain by mannosidase II, after the activity of NAGTI, which is concentrated in the medial Golgi; Dunphy *et al.*, 1985). Essentially complete inhibition was observed in this assay with antibodies to syntaxin 5 (Rowe *et al.*, 1998), ERS24 (Zhang *et al.*, 1999), rBet1 (Zhang *et al.*, 1997), mYkt6 (Zhang and Hong, 2001), and marked inhibition was reported for GOS28 (Subramaniam *et al.*, 1996).

To directly establish requirements for intra-Golgi transport, we have tested the effects of Fab fragments targeted to the subunits of the two alternative Golgi SNAREpins on reconstituted transport using isolated Golgi membranes (Balch *et al.*, 1984a). Polyclonal antibodies to SNARE proteins were affinity purified and Fab fragments were generated. The specificity of each Fab was confirmed as only a single expected band was observed in Western blots of whole cell lysates and/or Golgi membranes (unpublished data).

The Fab directed to syntaxin 5, the common Golgi t-SNARE heavy chain, completely inhibited transport (Figure 9). Fab fragments against ERS24, rBet1, GOS28, and GS15 also profoundly inhibited transport in a dose-dependent manner. The inhibition was specific based on the following data: 1) control rabbit Fab fragments had no effect, nor did Fab fragments against another SNARE, Vti1b (Figure 9A), 2) the inhibitory effects were completely neutralized by prior denaturation of the antibodies by boiling, and 3) preincubating the antibodies with recombinant soluble SNAREs lacking transmembrane domains (rBet1 and GOS28) or the GS15 immunizing peptide, neutralized the inhibitory effects (unpublished data). Unfortunately, suitable antibodies directed to membrin and mYkt6 were not available for these experiments. In conclusion, subunits of both Golgi SNAREpins, v-rBet1: t-syntaxin 5/membrin, ERS24 and v-GS15: t-syntaxin 5/mYkt6, GOS28 are functionally required for intra-Golgi transport, which must therefore require fusion processes mediated by each SNAREpin.

DISCUSSION

Opposing cis-trans Gradients of the Two SNAREpins within the Golgi Stack

That transport in the Golgi stack relies upon membrane fusion has long been suggested by its morphology in the cell (Palade, 1975) and its intrinsic capacity to bud and fuse vesicles (Balch *et al.*, 1984b) and most recently is evident from the specific requirement for individual SNARE proteins found within this organelle. The localization of these SNAREs along the cis-trans axis within the stack can therefore provide answers to important questions concerning where fusion occurs and the pattern of membrane flow, motivating this comprehensive study.

The nearly even distribution of the heavy chain (syntaxin 5) and one light chain (GOS28; and likely the other light chain mYkt6 from Figure 2 at the immunofluorescence level) suggest that the combination of the three, i.e., the functional t-SNARE syntaxin 5/mYkt6, GOS28, is present throughout the Golgi stack. On the other hand the cognate v-SNARE GS15 is localized in a cis < trans gradient (Figure 6B), suggesting on the face of it that the concentration of this Golgi SNAREpin and accordingly its ability to function in fusion,

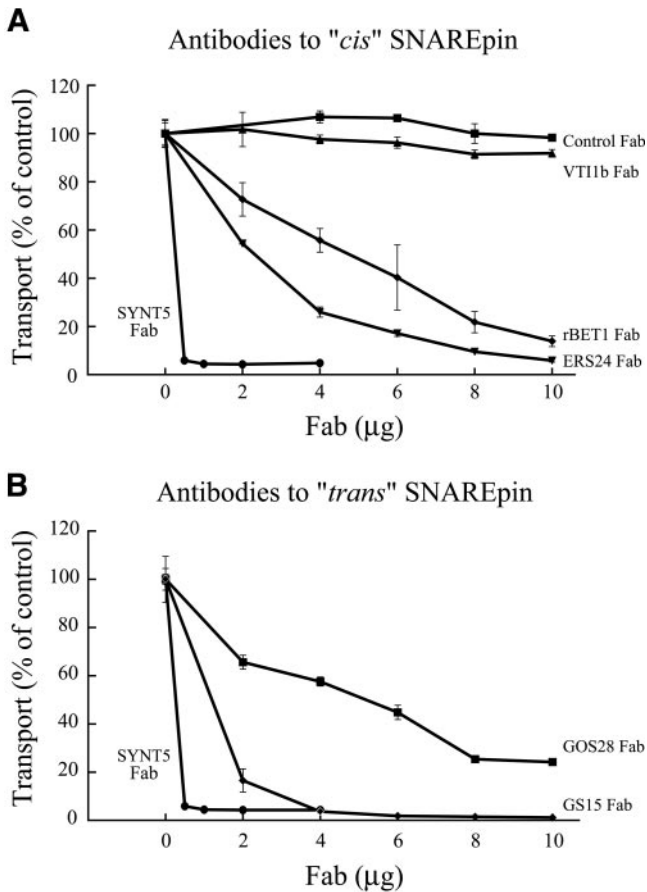


Figure 9. Requirement for SNAREs in cell-free reconstituted Golgi transport. Transport-coupled glycosylation of VSV-G protein was measured. Purified Golgi membranes from VSV-infected 15B cells (donor) and wild-type CHO cells (acceptor), were incubated with cytosol and ATP as described in MATERIALS AND METHODS. The assay was performed with increasing concentrations of the indicated Fab fragments or (as controls) nonspecific rabbit Fab or a Fab directed against Vti1b, as indicated. Triplicate samples were used for each condition generating the SDs shown. SYNT5, syntaxin 5.

may progressively increase toward the *trans* face of the Golgi stack. This contrasts with the *cis*-Golgi SNAREpin whose t-SNARE (a compound of the distributions of syntaxin 5 [*cis* = *trans*], membrin [*cis* > *trans*], and ERS24 [*cis* > *trans*]) and whose v-SNARE rBet1 (*cis* > *trans*), each progressively decrease toward the *trans* face of the stack (Figure 6A). The simultaneous requirement for both SNAREpins for reconstituted Golgi transport (Figure 9) implies that they function coordinately in membrane fusion processes that maintain the ongoing transport activity of the Golgi apparatus.

The *cis* > *trans* distribution of the *cis*-Golgi SNAREpin suggests that it functions in membrane fusion events that are progressively directed toward the *cis* face within the stack, i.e., retrograde vesicle transport within the Golgi. This suggests, of course, that the *cis*-SNAREpin does double duty, because it also fuses ER-derived COPII vesicles with the Golgi at its *cis* face (Newman and Ferro-Novick, 1987; Newman *et al.*, 1990; Shim *et al.*, 1991; Hardwick and Pelham, 1992; Cao and Barlowe, 2000).

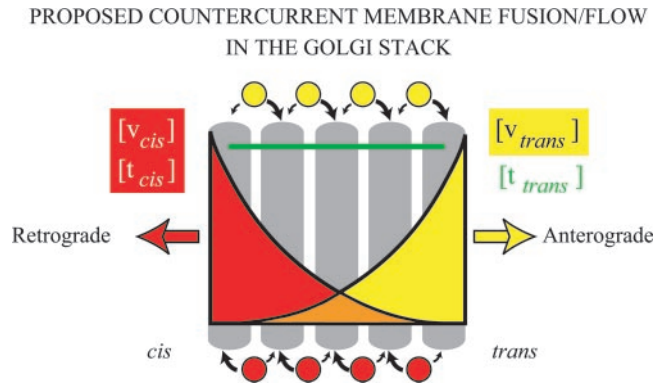


Figure 10. Countercurrent distribution of SNAREpins and its implication for vesicle transport patterns in the Golgi stack. Distinct *cis*- and *trans*-Golgi SNAREpins are needed for the operation of the Golgi stack. They distribute in countercurrent concentration gradients across the stack. Two types of COPI vesicles bud at every level of the Golgi stack (Orci *et al.*, 1997). One contains retrograde-directed cargo and the *cis*-Golgi v- and t-SNAREs (v_{cis} and t_{cis}). The other contains anterograde-directed cargo and the *trans*-Golgi v- and t-SNAREs (v_{trans} and t_{trans}). Both vesicles fuse homotypically with cognate SNAREs. They are restrained from dissociating by a carpet of tethers (Orci *et al.*, 1998; Sonnichsen *et al.*, 1998; Seemann *et al.*, 2000) and therefore are restricted to fusion with adjacent cisternae on either side. With these conditions, at each level of the stack a vesicle will have a higher probability of fusing to the neighboring cisternae that contains the higher—rather than the lower—concentration of its cognate (homotypic) SNAREs. The opposing *cis*-*trans* distribution of the two cognate SNARE pairs would mean that the “retrograde” vesicles carrying *cis*-Golgi SNAREs should have a systematically higher probability of fusing up their gradient toward the *cis* face, whereas the “anterograde” vesicles carrying *trans*-Golgi SNAREs would, to the contrary, fuse preferentially up their gradient toward the *trans* face. In other words, the two types of vesicles would engage in systematically biased random walks toward the opposite ends of the stack, as individual vesicles essentially chromatograph up their gradients. This mechanism would naturally create a net flow of the cargo contained in the “anterograde” vesicles across the stack in the *cis*-to-*trans* direction, and a net flow of the cargo contained in the “retrograde” vesicles in the opposite *trans*-to-*cis* direction (this model is a refinement of the “percolating vesicle” model which envisioned an unbiased random walk; Pelham and Rothman, 2000). The countercurrent of cargo could of course be fine-tuned by additional layers of specificity involving the distributions of regulatory proteins like rabs and tethers. This model for vesicular transport in the stack does not speak to the cisternal progression/maturation process that likely occurs concurrently (Pelham and Rothman, 2000).

By the same reasoning, the present work suggests as the simplest possibility that the oppositely oriented *trans*-SNAREpin should support anterograde flow, i.e., transport across the Golgi that depends on membrane fusion within the stack that is progressively directed toward the *trans* face, presumably involving transport vesicles. The alternative, that membrane fusion supports transport by direct fusion of adjacent cisternae without an intervening transfer vesicle, seems remote because three-dimensional reconstruction fails to reveal continuities between nonequivalent cisternae (Rambourg *et al.*, 1987; Ladinsky *et al.*, 1999).

Having said this, the *trans* > *cis* distribution of the *trans*-Golgi SNAREpin does not speak against the possibility of a concurrent cisternal progression/maturation process, which likely occurs in parallel with vesicular transport (Pelham and Rothman, 2000).

Homotypic Fusion between Vesicles and Cisternae in the Golgi?

The concentration of the v-SNAREs (rBet1 and GS15) exceeds that of their cognate t-SNAREs (limited by the concentration of their common syntaxin heavy chain, syntaxin 5) in Golgi cisternae and in the vesicles forming from them. This condition is reminiscent of what occurs during homotypic fusion between closely related or identical membranes in which both the v-SNARE and its cognate t-SNARE reside in both partners (Mellman and Warren, 2000). For example, in the yeast vacuole almost all of the cognate SNAREs exist in v-t SNARE complexes residing within each membrane partner wherein the v-SNARE and the t-SNARE are bound to each other and therefore unavailable to pair between membranes to form the SNAREpins that bridge the two membranes and are required for fusion (Nichols *et al.*, 1997; Ungermann *et al.*, 1998). For homotypic fusion to occur, the v- and t-SNAREs bound up within each partner first need to be separated by the ATPase of NSF so they are then free to partner between membranes (Ungermann *et al.*, 1998). In the case of Golgi cisternae and vesicles, because $[v] \gg [t]$, separation of SNAREs by NSF/SNAP need only occur in one of the partners to free up t-SNARE there; excess free v-SNARE will always be available in the other partner.

The activation of v-t SNAREs for fusion is ordinarily linked to the assembly of the COPI coat preventing the direct fusion of cisternae, but cisternal fusion can be triggered by “uncoupling” membrane fusion from transport vesicle budding, either by brefeldin A (Klausner *et al.*, 1992) or by removing ARF or coatomer (Orci *et al.*, 1993; Elazar *et al.*, 1994). Molecular structure studies are now revealing the subtle mechanisms used by coats to regulate fusion and couple it to budding (Miller *et al.*, 2003; Mossessova *et al.*, 2003).

How may the *cis*-Golgi and *trans*-Golgi SNAREpins distribute between the two compositionally distinct populations of COPI vesicles that have previously been identified (Orci *et al.*, 1997)? The “retrograde” population of COPI vesicles contain retrograde-directed KDEL salvage receptor and the *cis*-Golgi v-SNARE rBet1 (Martinez-Menarguez *et al.*, 2001). The “anterograde” population of COPI peri-Golgi vesicles contain the *trans*-Golgi t-SNARE light-chain GOS28 and anterograde-directed cargo (VSV-G protein).¹ Although the data are limited, so far they are consistent with the simple idea that the vesicles carrying anterograde-directed cargo have the *trans*-Golgi v- and t-SNAREs, whereas the vesicles carrying retrograde-directed cargo have the *cis*-Golgi SNAREs. With this in mind, it is our current hypothesis that the oppositely distributing *cis*- and *trans*-Golgi SNARE complexes are separately packaged into the two distinct populations of COPI vesicles. The needed segregation could occur during budding from a single bilayer phase,

¹While Klumperman and colleagues (Martinez-Menarguez *et al.*, 2001) have confirmed the two types of COPI vesicles identified by Orci *et al.* (1997), i.e. VSV-G-positive and KDEL receptor-positive vesicle populations, the VSV-G population is less abundant in their studies than in ours (Orci *et al.*, 1997; Orci *et al.*, 2000). It should be noted that, whereas Orci and colleagues studied VSV-viral infected cells, Martinez-Menarguez and colleagues employed cells transfected with plasmid encoding GFP-tagged VSV-G. VSV-G is much more abundantly expressed in infected cells (where all cell protein synthesis is replaced by only five major viral proteins) than in transfected cells. Therefore, the load of protein needing transport in the Golgi is much larger in our studies.

perhaps using isotypes of coatomer (Blagitko *et al.*, 1999; Futatsumori *et al.*, 2000; Hahn *et al.*, 2000; Lee *et al.*, 2000; Wegmann *et al.*, 2003) or before budding by segregation into distinct lateral membrane domains, as is the case for different rab proteins in the same endosomes (Sonnichsen *et al.*, 2000; Barbero *et al.*, 2002).

In summary, the countercurrent distribution of SNAREs (Figure 10) naturally suggests opposing streams of vesicles whose cargo would be subject to repeated rounds of purification in successive cisternae (the countercurrent distribution; Craig, 1948; Rothman, 1981).

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