

A v-SNARE Implicated in Intra-Golgi Transport

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Abstract. We report the identification of a putative v-SNARE (GOS-28), localized primarily to transport vesicles at the terminal rims of Golgi stacks. In vitro, GOS-28, a Golgi SNARE of 28 kD, is efficiently packaged into Golgi-derived vesicles, which are most likely

COPI coated. Antibodies directed against GOS-28 block its ability to bind α -SNAP, partially inhibit transport from the *cis* to the medial cisternae, and do not inhibit budding of COP-coated vesicles, but do accumulate docked uncoated vesicles.

TRANSport vesicles shuttling cargo between intracellular organelles dock to their correct destination by the selective recognition of an address signal on vesicles by a receptor on the intended target membrane. According to the SNARE¹ hypothesis, this process is mediated at least in part by the unique pairing of SNAP receptors localized on vesicles (v-SNAREs) with their cognate t-SNARE on the target membrane (Söllner et al., 1993a). For example, in yeast, distinct compartment-specific SNARE complexes acting at the ER-Golgi (Søgaard et al., 1994) and at the Golgi-plasma membrane transport steps (Aalto et al., 1992, 1993; Protopopov et al., 1993) have been demonstrated and a t-SNARE at the vacuole has been postulated (Bennett et al., 1993). In mammalian cells, SNARE complexes involved in regulated exocytosis have been isolated (Söllner et al., 1993a,b). The only known t-SNARE acting early in the secretory pathway is syntaxin 5 (Bennett et al., 1993) and its yeast homologue, Sed5 (Hardwick and Pelham, 1992), which have been localized to the *cis*-Golgi network (Banfield et al., 1994). In both eukaryotes, the SNARE pattern within the Golgi apparatus is largely unknown.

In contrast to SNARE proteins, which have to be compartment specific to fulfill their proposed function, the machinery acting in the consumption of docked vesicles, in the fusion process, is a general one used at multiple transport steps. Indeed, general fusion proteins such as the

N-ethylmaleimide-sensitive fusion protein (NSF) (Block et al., 1988) and soluble NSF attachment proteins (SNAPs) (Clary and Rothman 1990; Clary et al., 1990) can be both found free in the cytoplasm and associated with the membranes of different organelles. The membrane interaction of NSF is mediated by the binding of SNAPs to SNAREs (Weidmann et al., 1989; Wilson et al., 1992). The assembled v-SNARE/t-SNARE complex provides high affinity sites for the binding of several SNAPs (Söllner et al., 1993b; Hayashi et al., 1995; Schiavo et al., 1995). A complex of SNAREs, SNAPs, and NSF can be isolated in vitro in the absence of hydrolyzable ATP, and migrates with 20 Svedberg (20S particle) in velocity centrifugation (Wilson et al., 1992). In the presence of ATP, the ATPase activity of NSF provides energy to dissociate the SNARE complex and to release SNAPs and NSF, thereby likely linking the process of vesicle docking to the initiation of vesicle fusion (Söllner et al., 1993b).

The secretory pathway is the major route for the delivery of newly synthesized molecules to their intracellular or extracellular destinations. This requires the maintenance of the compartments comprising the pathway (i.e., the constancy of their protein and lipid composition), despite a continuous flow of material passing through them. This appears to be accomplished by constant sorting events either excluding or enriching certain molecules in transport vesicles (Griffiths and Simons, 1986; Pelham and Munro, 1993). Transport vesicles enriched in certain cargo could either move in an anterograde or retrograde direction, either selecting molecules for their forward transport to a distinct organelle or retrieving escaped molecules backwards to their organelle of origin (Pelham, 1991). In addition to these sorting events, import into and export from a distinct compartment have to be balanced to maintain its size and volume. This implies that the transport containers, the vesicles, and the transport machinery have to be recycled. v-SNAREs that determine the address of a trans-

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1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; GOS-28, Golgi SNARE of 28 kD; GST, glutathione *S*-transferase; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VSV, vesicular stomatitis virus.

port vesicle are themselves part of this machinery and may play a major role in these processes. For example, a v-SNARE that has guided a vesicle to its destination becomes part of the target membrane after fusion has occurred. Most likely, this v-SNARE has to be cycled back to its compartment of origin, and it would seem only natural that the number of v-SNAREs available for forward transport is dependent on their recycling from their target membrane. This would couple and balance the vesicle flow between two compartments. To test such predictions, it is necessary to identify v-SNAREs and their interacting t-SNAREs in the central turntable for vesicular trafficking, the Golgi apparatus, and its *cis*- and *trans*-Golgi networks.

On one hand, the Golgi receives both anterograde transport vesicles from the ER and retrograde vesicles from downstream compartments; on the other hand, it produces retrograde vesicles moving back to the ER and anterograde vesicles moving toward the plasma membrane (Pelham, 1991; Miesenböck and Rothman, 1995). Therefore, the isolation and characterization of SNAREs from the Golgi will be essential for our understanding of how a cell maintains the integrity of its organelles by integrating vesicular flow patterns in a coordinated manner. Furthermore, the localization of SNAREs within the Golgi stack might provide us with information about the organization of the Golgi. For example, analysis of the distribution of SNAREs within a cisterna may help to define areas involved in vesicle budding and fusion.

In this paper, we describe the isolation of Golgi SNAREs and the identification, localization, and functional characterization of GOS-28, a v-SNARE, which can act in intra-Golgi transport.

Materials and Methods

Materials

The following materials were isolated as described elsewhere: Golgi membranes from CHO cells (Balch et al., 1984a), bovine brain cytosol (Malhotra et al., 1989), His₆-α-SNAP (Whiteheart et al., 1993), and recombinant His₆-NSF-myc (Söllner et al., 1993b).

Sequencing and Mass Analysis of Peptides

Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose, and the visualized bands were processed for internal amino acid analysis, as described (Erdjument-Bromage et al., 1994). Briefly, membrane-bound proteins were digested *in situ* with trypsin and the resulting peptides separated by narrowbore reverse-phase HPLC; the modular liquid chromatography system used in this study has been described elsewhere (Elicone et al., 1994). Selected peptides were then subjected to chemical microsequencing and matrix-assisted laser desorption (MALDI) mass spectrometry, also described previously (Söllner et al., 1993a; Erdjument-Bromage et al., 1994; Tempst et al., 1994).

Isolation of GOS-28 cDNA

To isolate a cDNA encoding Golgi-SNARE of 28 kD (GOS-28), a two-stage PCR was performed with degenerate primers based on peptide sequence data obtained as described (Söllner et al., 1993a). For the first stage of PCR, the following primers were used: ATGTT(TC)GA(AG)-AC(AGTC)ATGGC (codons Met⁶⁶-Ala⁷¹, sense, 0.5 μM), TG(AG)AA(TC)TC(AG)TG(AGTC)GT(AG)TA (codons Tyr¹¹⁸-His¹²³, antisense, 0.5 μM) and a bovine liver cDNA (0.5 ng, Clontech) as template. The amplification reactions were performed for five cycles using an annealing temperature of 42°C (1 min at 94°C, 1 min at 42°C, and 2 min at 72°C) fol-

lowed by an additional 30 cycles at an annealing temperature of 55°C (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C). The last cycle was followed by an 8-min extension at 72°C. For the second stage, the PCR products from the first stage were diluted 1:1,000, and the same antisense primer as in stage 1 and the ATGGC(AGTC)AT(ATC)GA(AG)AT(ATC)GA (codons Met⁷⁰-Glu⁷⁵, sense) primer were added. The amplification conditions were the same as in stage 1. The products were fractionated by electrophoresis in 2% agarose gels. A major amplification product of ~180 bp was isolated, random labeled, and used as a probe for screening a CHO cDNA library constructed with the Uni-ZAP XR vector (Stratagene, La Jolla, CA). Hybridization was done at 50°C for 14 h in 5× SSPE (1× SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaH₂PO₄, pH 7.7), 5× Denhardt's solution, 0.5% SDS, in the presence of 20 μg/ml herring sperm DNA. The filters were washed twice in 2× SSPE, 0.1% SDS for 10 min at room temperature, followed by a 15-min wash at 50°C in 1× SSPE, 0.1% SDS. Positive clones were excised from the Uni-ZAP XR vector as inserts in pBluescript SK⁻ by coinfection with the helper phage, ExAssist, as described by the manufacturer (Stratagene), and were sequenced for both sense and antisense strands.

Preparation of Antibodies

To generate a polyclonal antibody to GOS-28, a fusion protein of GOS-28 with an amino-terminal extension containing six histidine residues (His₆-GOS-28) was prepared by insertion of the GOS-28 cDNA into a pQE11 vector (Qiagen, Chatsworth, CA). After expression in *Escherichia coli* (XL1-Blue; Stratagene), the protein was purified from inclusion bodies under denaturing conditions by affinity chromatography using Ni-nitrilotriacetic acid agarose as described by the manufacturer (Qiagen). His₆-GOS-28 was eluted in 8 M urea, 0.1 M NaH₂PO₄, and 0.01 M Tris (pH 4.5), and then injected into rabbits for immunization. Antibodies directed against GOS-28 were purified as described (Harlow and Lane, 1988), by affinity chromatography using His₆-GOS-28 conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and the eluate was dialyzed against PBS. Minor contaminations of antibodies cross-reacting with cellular antigens other than GOS-28 were affinity depleted. For this purpose, 500 μg of CHO protein was separated by SDS-PAGE, transferred onto nitrocellulose membrane, the region containing proteins with a 28-kD mol mass was cut out, and the remaining nitrocellulose membrane was incubated with the affinity-purified anti-GOS-28 antibodies in 0.1% BSA.

Fab fragments were prepared by incubating the affinity-purified anti-GOS-28 antibody with papain (Boehringer Mannheim Biochemicals, Indianapolis, IN), and any remaining intact antibodies and Fc fragments were removed by protein A-agarose chromatography (Mage, 1981). The Fab fragments were dialyzed against 10 mM MOPS-KOH (pH 7.5) and 150 mM KCl.

Western Blot Analysis

For Western blot analysis, proteins were fractionated by SDS-PAGE (Laemmli, 1970) and transferred onto nitrocellulose filters or polyvinylidene difluoride filters. Polyvinylidene difluoride membranes were used for the detection of the p24 antigen (Stamnes et al., 1995). Filters were blocked with 5% milk powder in TBS-Tween (0.1% Tween-20), and were incubated with primary antibodies as indicated (affinity-purified anti-GOS-28 antibody diluted 1:1,000, anti-p24 antiserum diluted 1:1,000, or anti-ζ-COP polyclonal antibody (Kuge et al., 1993) diluted 1:1,000). Immunoreactive bands were visualized with HRP-conjugated secondary antibody and chemiluminescence (ECL kit; Amersham Corp., Arlington Heights, IL).

Expression of Epitope-tagged GOS-28 in CHO Cells

To obtain CHO cells that stably express GOS-28 tagged with a myc epitope (EQKLISEEDL), the coding sequence for the myc epitope was added by PCR to the 5' end of the coding region of the GOS-28 cDNA, and the resulting construct was subcloned into the mammalian cell expression vector pcDNA3 (InVitrogen, San Diego, CA). The FLAG epitope (DYKDDDDK) was inserted after the initiation codon into the GOS-28 cDNA by loop-in mutagenesis using the unique site elimination method (Deng and Nickoloff, 1992) and the Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). CHO cells grown in MEM-α medium containing 10% FCS were transfected with the above-mentioned constructs using the Lipofectamine reagent (GIBCO-BRL,

Gaithersburg, MD), as described by the manufacturer. 48 h after the transfection, the cells were selected by the incubation with 1 mg/ml G418 sulfate (GIBCO-BRL). After 7 d, several resistant colonies were isolated by trypsinization in cloning cups, grown in a medium containing 0.5 mg/ml G418, and examined for myc-GOS-28 or FLAG-GOS-28 expression by Western blot analysis.

Immunolocalization

For immunofluorescence, CHO cells expressing FLAG- and myc-tagged GOS-28 fusion proteins were fixed with 4% paraformaldehyde, and were permeabilized by dehydration and rehydration with ethanol. Cells were subsequently exposed for 2 h at room temperature to affinity-purified anti-GOS-28 antibodies (1:5) followed by a 1-h exposure to goat anti-rabbit IgG coupled to FITC. Anti-FLAG (M2) (Brizzard et al., 1994) or anti-myc (9E10) (Evan et al., 1985) mAb revealed by FITC-coupled anti-mouse IgG were used to localize FLAG- or myc-tagged GOS-28 in the transfected CHO cell lines. Cells were counterstained with 0.03% Evans blue and examined with a confocal fluorescence microscope. For electron microscope immunolocalization, cells were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4), infiltrated with 2.3 M sucrose, and processed for cryoultramicrotomy according to the method of Tokuyasu (1986). Anti-GOS-28 affinity-purified antibodies (diluted 1:3) were localized with the protein A-gold method (Roth et al., 1978) or by goat anti-rabbit IgG-coupled gold. Anti-myc (9E10) antibodies (diluted 1:100 or 1:300) were localized with rabbit anti-mouse IgG followed by protein A-gold. After immunolabeling, sections were absorption stained with uranyl acetate (Tokuyasu, 1986) and examined in the electron microscope.

Cis- to Medial-Golgi Transport Assay

The assay measures transport from a donor Golgi, prepared from vesicular stomatitis virus (VSV)-infected 15B CHO cells (a mutant cell line that lacks UDP-[³H]-N-acetylglucosamine (UDP-GlcNAc) glycosyltransferase I) to an acceptor Golgi, prepared from uninfected wild-type cells, by incorporation of UDP-[³H]GlcNAc into the VSV G protein. The preparation of assay components and standard incubation conditions were as described previously (Balch et al., 1984a,b; Malhotra et al., 1989). Briefly, transport reactions contain bovine brain cytosol (5 μ l), donor Golgi (5 μ l), acceptor Golgi (5 μ l), and 0.4 μ M UDP-[³H]GlcNAc in transport buffer (25 mM Hepes/KOH [pH 7.0], 20 mM KCl, 2.5 mM Mg(OAc)₂, 5 mM creatine phosphate, 250 μ M UTP, 50 μ M ATP, 8 IU/ml creatine kinase) in a final volume of 50 μ l. Transport occurs during an incubation at 37°C for 1 h; then the membranes are lysed by detergent and the VSV G protein is immunoprecipitated.

Generation of Golgi-derived Coated Vesicles

Vesicle budding reactions contained CHO Golgi membranes (50 μ g/ml), bovine brain cytosol (2.4 mg/ml), 10 μ M palmitoyl Coenzyme A, 25 μ g/ml RNase A, and 20 μ M GTP γ S, in transport buffer. Control reactions were performed in the absence of GTP γ S. Reaction mixtures were assembled on ice and then incubated at 37°C for 15 min. After the incubation, the Golgi membranes were isolated by centrifugation for 20 min at 15,800 g. The Golgi membrane pellet was resuspended in 0.6 ml low salt stripping buffer (25 mM Hepes [pH 7.2], 2.5 mM Mg(OAc)₂, 0.2 M sucrose, and 50 mM KCl), reisolated by centrifugation, and subsequently washed with 0.6 ml high salt stripping buffer (25 mM Hepes [pH 7.2], 25 mM Mg(OAc)₂, 0.2 M sucrose, 250 mM KCl). After centrifugation, the high salt supernatant was adjusted to 52% (wt/wt) sucrose. The high salt supernatant containing the coated vesicles was overlaid with 0.5 ml of 45% sucrose and 1 ml of (each) 40, 35, and 30% sucrose, and centrifuged for 15 h at 287,000 g. 350- μ l fractions were collected from the gradient, diluted with 1 vol H₂O, and centrifuged at 356,000 g for 30 min to pellet the vesicles. The isolated vesicles were resuspended in Laemmli buffer for Western blot analysis.

Assembly of 20S Particles on Immobilized GST- α -SNAP

A glutathione S-transferase (GST) fusion protein of α -SNAP was prepared by inserting the α -SNAP cDNA into the pGEX-2T vector (Pharmacia). The GST α -SNAP fusion protein was expressed in *E. coli* and purified from the bacterial lysate on glutathione-agarose (Sigma Chemical Co.). CHO Golgi membranes were stripped of peripherally associated

proteins by incubating with 1 M KCl for 15 min on ice, and then recovered by centrifugation (15,800 g, 15 min, 4°C). The membrane pellet was resuspended in lysis buffer (20 mM Hepes-KOH [pH 7.2], 100 mM KCl, 1 mM DTT, 0.5% Triton X-100, 1% glycerol) and solubilized for 45 min at 4°C with gentle mixing, followed by centrifugation for 45 min at 100,000 g to remove detergent-insoluble material. The Golgi Triton X-100 extract (25 μ g of protein) was incubated with the indicated amounts of affinity-purified anti-GOS-28 antibody for 1 h at 4°C. Subsequently, the mixtures were incubated with GST- α -SNAP (1 μ g of protein) prebound to glutathione-agarose beads (15 μ l) without or with His₆-NSF-myc (5 μ g of protein), in the presence of 0.5 mM ATP and either 2 mM EDTA or 2 mM MgCl₂, for 1 h at 4°C in a final volume of 1 ml. Beads were then washed four times with 1 ml of the appropriate buffer, and proteins were eluted from the beads by incubation with 15 μ l 2 \times Laemmli buffer and then subjected to Western blot analysis.

Electron Microscopy to Examine the Effect of the Anti-GOS-28 Antibody on Vesicular Transport

Reactions (500 μ l) contained 10 μ M palmitoyl Coenzyme A, bovine brain cytosol (2.4 mg/ml), and CHO Golgi membranes (100 μ g/ml) in transport buffer. For the preparation of N-ethylmaleimide (NEM)-treated Golgi, CHO Golgi membranes were treated with 1 mM NEM for 15 min on ice, followed by an addition of 2 mM DTT and an incubation for 15 min on ice. Reactions were assembled on ice, preincubated on ice for 15 min with 5 μ g of affinity-purified anti-GOS-28 antibody and 20 μ M GTP γ S (see Table 1), and then incubated at 37°C for 15 min. The reactions were then treated with 1% glutaraldehyde, the membranes were isolated by centrifugation (15,800 g, 30 min, 4°C) and processed for electron microscopy, and the quantitative evaluation of vesicles and buds was performed as described previously (Orci et al., 1991).

Results

Isolation of SNAREs from a Golgi-enriched Preparation

To gain insight into the SNARE composition of the Golgi, we made use of the mammalian Golgi apparatus, which is morphologically well defined by the existence of several stacked cisternae. Therefore, a preparation enriched in Golgi was isolated from bovine liver (Tabas and Kornfeld, 1979). The membranes were extracted at pH 11.5 (Fujiki et al., 1982) to remove peripheral associated proteins and lysed by addition of Triton X-100. From this extract, potential SNARE proteins were isolated by their ability to assemble together with SNAPs and NSF in a 20S particle in the absence of hydrolysable ATP. The whole particle was immobilized on beads containing covalently coupled anti-myc antibodies that bind the myc epitope-tagged NSF in the 20S particle. In a control reaction, the beads were treated with Mg-ATP γ S to release proteins that were bound in an Mg²⁺ sensitive, ATP hydrolysis-independent manner (Fig. 1, lane 2). SNAPs and SNAREs were then eluted from the matrix by Mg-ATP treatment (Söllner et al., 1993a). In addition to several proteins in the molecular mass range of 70 kD, which are also visible in the control reaction, α -SNAP and six additional protein bands representing potential SNAREs were specifically eluted as shown in the Ponceau S-stained Western blot in Fig. 1, lane 1. These six proteins migrate with an apparent molecular mass of 45 (A), 31 (B), 26 (C), 24 (D), 21 (E), and 20 kD (F) in high-Tris urea SDS-PAGE. Microsequencing and mass spectrometric data obtained from several tryptic peptides derived from these six proteins were used to search protein sequence repositories and indicated that all bands corresponded to as yet unidentified proteins (data not shown). Further analy-

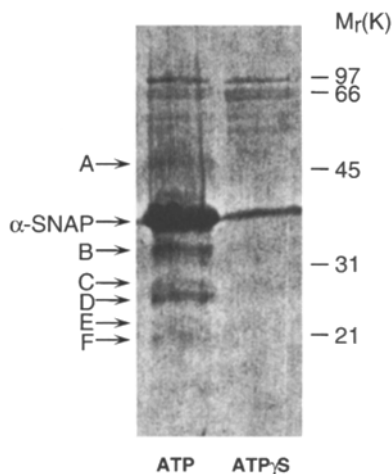


Figure 1. Purification of SNAREs from Golgi-enriched membranes. SNAREs were purified as described (Söllner et al., 1993a). Briefly, His₆-NSF-myc (500 μg of protein) and His₆-α-SNAP (500 μg of protein) were assembled into 20S particles by incubating them together with Triton X-100 extracts of enriched Golgi membranes (250 mg of protein) from bovine liver in the presence of ATPγS. The 20S particles were then isolated with anti-myc mAbs covalently coupled to protein G-Sepharose beads (2.5 ml of 2 mg 9E10 IgGs/mg protein G-Sepharose). The beads were washed, eluted with Mg-ATPγS, free Mg²⁺ was complexed by addition of EDTA, the ATPγS was exchanged against ATP, and then the bound 20S particles were disassembled by a specific elution step with Mg-ATP. Shown is a Ponceau S-stained Western blot of the specific (Mg-ATP) eluate and the control eluate (Mg-ATPγS). The following tryptic peptide sequences (one-letter code) of the protein band D were obtained by microsequencing as described under Materials and Methods: (1) YL(V/E)(N/Q)EL(D/Q)(X/Y), both residues observed during this cycle); (2) MFETMAIEIEQLAR; (3) MAEYTNAGVPSLNAALMHTL; (4) HRDILQDY-TEEFFK; (5) ERENLMGS; (6) RTELFLKEHDHLR. Two additional peptide sequences obtained from protein band D showed an identical match with α-SNAP, and they most likely represent contaminations from the dominant α-SNAP band.

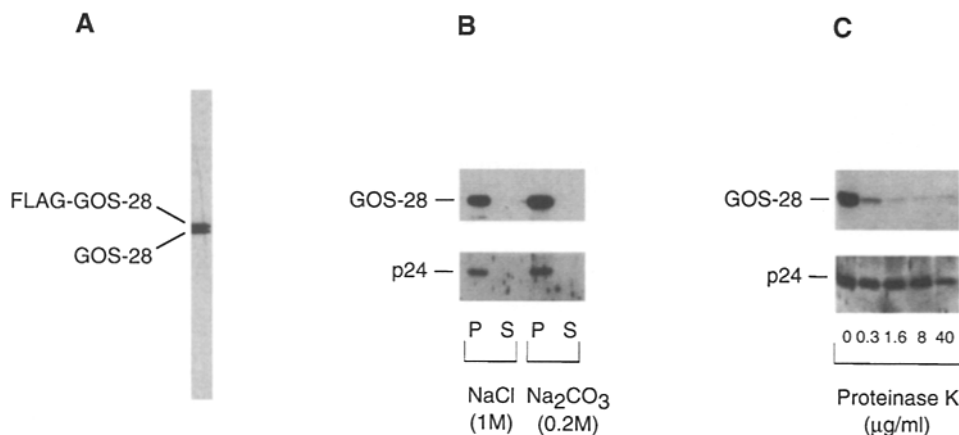


Figure 3. GOS-28 is a cytoplasmically oriented integral membrane protein. (A) Anti-GOS-28 antibodies specifically recognize GOS-28 in total cell extracts. CHO cells stably transfected with the FLAG epitope-tagged GOS-28 were lysed in Laemmli buffer, and the proteins were separated by SDS-PAGE, transferred on nitrocellulose, and immunodecorated with affinity-purified anti-GOS-28 antibodies as described in Materials and Methods. The upper band represents the FLAG epitope-tagged GOS-28; the lower band represents the endogenous GOS-28. These stably transfected cells were also used for the immunolocalization studies. (B) GOS-28 is an integral membrane protein. CHO Golgi membranes were incubated either with 1 M NaCl or 200 mM Na₂CO₃ (pH 11.3) for 30 min on ice. The membranes were isolated by centrifugation at 356,000 g for 45 min. The supernatants were precipitated by TCA, and the pellets were analyzed by immunodecoration of Western blots with anti-GOS-28 antibodies, as described in Materials and Methods. (C) The bulk of GOS-28 is exposed to the cytoplasm. CHO Golgi membranes were treated with indicated concentrations of proteinase K (Boehringer Mannheim) at 16°C for 30 min in 20 mM Hepes-KOH (pH 7.2), 25 mM KCl, and 200 mM sucrose. Reactions were stopped by the addition of 10 mM PMSF. Samples were precipitated by TCA and analyzed for GOS-28 and p24 as described in B.

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MAAGTSNYWEDLRKQARQLENELDLKLVSFSLKCTSYSHSSARDGGRDRY  50
SSDTPPLNGSSQDRMFETMAIEIEQLLARLTGVNDKMAEYTNAGVPSL  100
NAALMHTLQRHRDILQDYTHEFHKTANFMAIRERENLMGSVVRKDIESYK  150
SGSGVNNRRTELFLKEHDHLRNSDRLEETISIAMATKENMTSQRGMLKS  200
IHSKMNTLANRFPVNSLIQRINLRKRRSLILGGVIGICTILLLLYAFH  250

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Figure 2. Amino acid sequence of GOS-28. The amino acid sequence predicted from the cDNA sequence is shown. The peptide sequences determined by microsequencing are underlined. The carboxy-terminal hydrophobic domain is boxed. The heptad repeat showing the potential to form coiled coil structures is marked by dots. The sequence data are available from EMBL/GenBank/DBJ under the accession number U49841.

sis, including the isolation of the encoding cDNA, the topological characterization, the intracellular localization, and functional studies, was performed with GOS-28 (represented by protein band D in Fig. 1).

GOS-28 Is a Type II Membrane Protein with the Bulk of Its Mass Exposed to the Cytoplasm

A full-length clone encoding GOS-28 was isolated from a CHO cDNA library, and the predicted molecular mass (28.5 kD) of the protein encoded by the open reading frame was in close agreement with the apparent molecular size of 24 kD (Fig. 1, protein band D). GOS-28 migrates in a standard Laemmli SDS-PAGE with an apparent molecular mass of 31 kD (data not shown). All six peptide sequences obtained by microsequencing of the protein band D aligned perfectly with the translation product of the open reading frame (Fig. 2). GOS-28 has no obvious signal peptide, and contains a region (amino acids 63–95) with an inherently high probability to form coiled coil structures. The amino terminal region could also form coiled coil structures, but with lower probability. GOS-28 is further characterized by a carboxy-terminal hydrophobic region

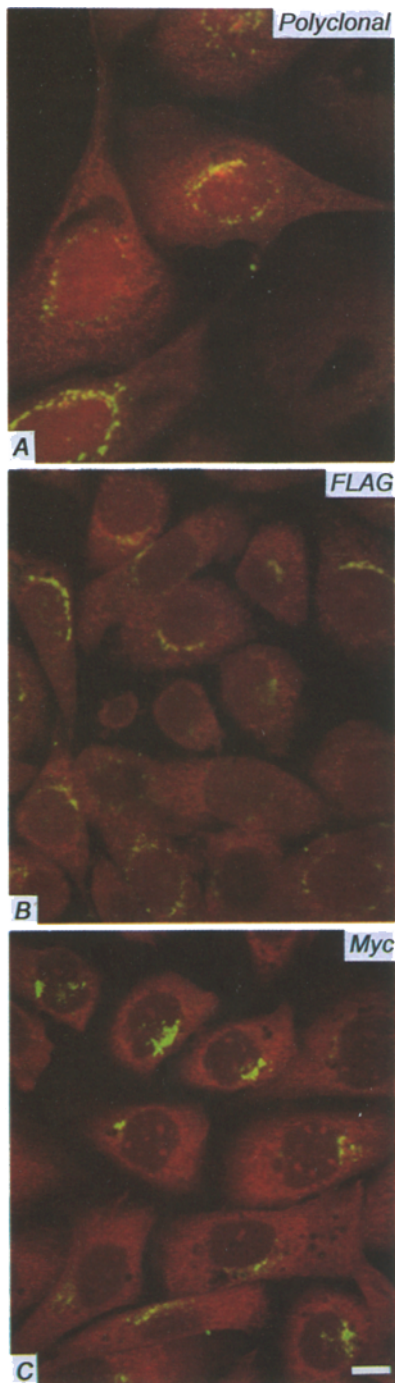


Figure 4. Immunofluorescence labeling of GOS-28 in CHO cells. Clusters of CHO cells viewed by confocal microscopy after immunofluorescent staining with affinity-purified anti-GOS-28 antibodies (A), FLAG mAb (B), or myc mAb 9E10 (C). In all three conditions, a distinct spotty juxtannuclear fluorescence is visible. $\times 490$. Bar, 10 μm .

presumably acting as a membrane anchor (see Fig. 2). Studies using a polyclonal antibody (Fig. 3 A) raised against recombinant GOS-28, which was expressed in and purified from *E. coli*, revealed that GOS-28 resists salt and sodium carbonate extraction of the Golgi (see Fig. 3 B). Both criteria are consistent with the properties expected for an

tegral membrane protein. The bulk of the protein seems to be exposed to the cytoplasm since GOS-28 was degraded by addition of low amounts of protease to isolated Golgi (see Fig. 3 C). A control protein, p24, an integral membrane protein, the bulk of whose mass is localized in the Golgi lumen was protease resistant (Stamnes et al., 1995). Protease treatment of detergent-solubilized Golgi showed that p24 is not inherently protease resistant (data not shown). These data, together with the existence of a single membrane-spanning region at the carboxy terminus of GOS-28, argue for a type II membrane protein. These results also confirm the identity of the isolated clone and GOS-28 obtained by its association with SNAPs and NSF.

GOS-28 Is Localized to the Golgi Region

Since the starting material used for the SNARE isolation was enriched for Golgi membranes, one would predict that the majority of the recovered SNAREs should be localized within the Golgi or representing SNAREs that mediate the docking of anterograde or retrograde vesicles to the Golgi. Immunofluorescence localization experiments were performed to confirm this for GOS-28. Polyclonal antibodies raised against GOS-28 and mAbs directed against the FLAG and myc epitope of amino terminal FLAG-GOS-28 and myc-GOS-28 fusion proteins stably expressed in CHO cells were used for these studies (see Fig. 4). In all three cases, GOS-28 exhibits a clear juxtannuclear staining pattern that is characteristic of Golgi proteins.

Electron microscope immunocytochemistry revealed that GOS-28 is primarily localized to vesicles and buds at the ends of the cisternae, with little labeling of the cisternae themselves both in situ and in cell-free assays (Figs. 5 and 6 A–C). The very low levels of labeling of the central regions of stacked cisternae precluded an assignment of GOS-28 to *cis* versus medial versus *trans* regions. Since this distribution of GOS-28 could be indicative of a functional specialization of the terminal rim of cisternae for vesicle budding and fusion, Golgi complexes were primed in vitro to accumulate coated buds and vesicles, and were further analyzed by electron microscopy (Fig. 6, B and C).

A few labeled vesicles were observed in relationship with the *cis*-Golgi pole, and occasionally buds on the transitional elements of the ER were also found labeled (data not shown). A comparable distribution of immunolabeling was observed in insulin cells of the rat pancreas (not shown).

GOS-28 Is Found in Coated Vesicles

The presence of GOS-28 in Golgi-associated buds and vesicles, as seen by electron microscopy, argues that GOS-28 is a v-SNARE. To confirm this, Golgi-derived transport vesicles were accumulated in vitro by incubating purified Golgi in the presence of cytosol, ATP, and a nonhydrolyzable GTP analogue, GTP γ S. GTP γ S binds to the ADP-ribosylation factor (ARF), thus keeping it in its active conformation, which recruits coatmer to membranes (Melancon et al., 1987; Serafini et al., 1991; Donaldson et al., 1992). The coat proteins deform the membrane, thereby shaping vesicles, which finally pinch off from their donor compartment (Ostermann et al., 1993). Since GTP γ S is, at best, only

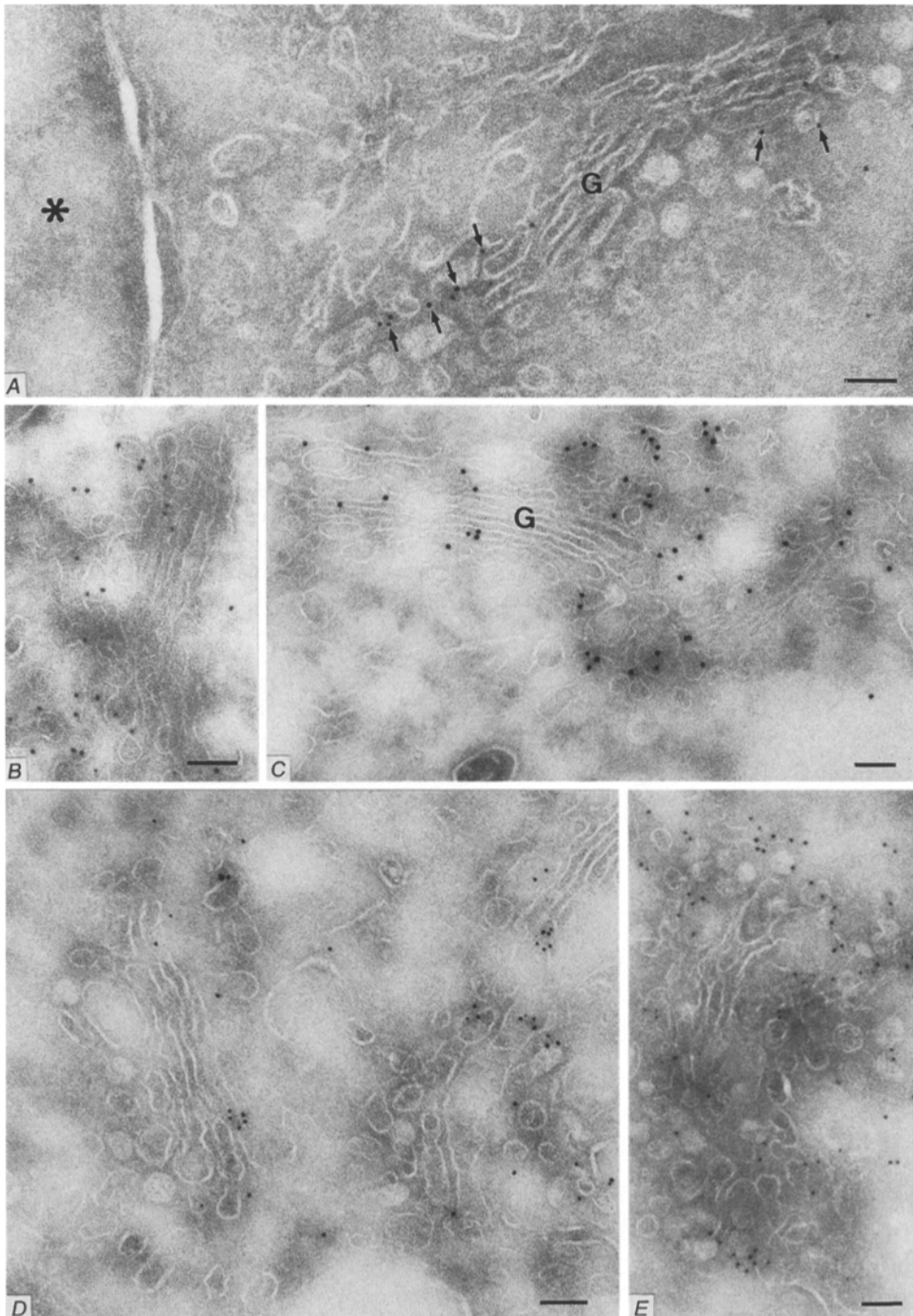


Figure 5. Immunolocalization of GOS-28 to the Golgi region of CHO cells. (A–C) Thin cryosections of the Golgi area of non-transfected CHO cells labeled with affinity-purified anti-GOS-28 antibodies, showing a predominance of labeling of endogenous GOS-28 on the vesicle/bud elements situated on the lateral parts of the cisternae (A, arrows) forming the Golgi stack (G). Approximately 30% of the Golgi-associated vesicles were labeled. A comparable pattern of labeling of myc-tagged, over-expressed GOS-28 is obtained with the monoclonal anti-myc antibody on another set of cryosections (D and E) from stably transfected CHO cells. The predominant distribution of immunogold particles over the lateral bud/vesicular elements of the Golgi stack is particularly evident in E. Incubation with preimmune serum did not elicit labeling. The asterisk in A indicates the nucleus. In A, B, D, and E, antibodies were detected with protein A-gold (size of particles = 10 nm); in C, antibodies were detected with goat anti-rabbit IgG-gold (size = 15 nm). A, $\times 79,000$; B, $\times 75,000$; C, $\times 56,000$; D, $\times 67,000$; E, $\times 62,000$. Bar, 0.1 μm .

slowly hydrolyzed, ARF and coatamer are locked on the vesicles, thereby preventing fusion and accumulating COPI-coated vesicles, which differ in their density from the originating compartments. Density equilibrium centrifugation thus separates COPI-coated vesicles produced in this manner from parental Golgi membranes.

Indeed, GOS-28 is shifted into the COPI-coated vesicle peak (marked by ζ -COP) only when $\text{GTP}\gamma\text{S}$ is added (Fig. 7), implying that GOS-28 is a v-SNARE because it is efficiently packed into vesicles. Interestingly, the distribution of GOS-28 only coincides with about half of the ζ -COP, suggesting that some COPI-coated vesicles contain GOS-28

while others do not. This is consistent with the finding that only 30% of vesicles label for GOS-28 (Fig. 5).

The presence of GOS-28 in COPI-coated vesicles was also shown by electron microscope immunocytochemical analysis of vesicles produced from isolated Golgi stacks by purified ARF and coatamer (Fig. 6 B), and in complete transport reactions inhibited by aluminum fluoride (Fig. 6 C). The cisternal areas labeled with anti-GOS-28 antibodies coincide with those labeled by anti- ζ -COP antibody (Fig. 6 D), indicating that the terminal rims of the Golgi cisternae are likely to be the active zones of vesicle budding. Although all these data strongly suggest the presence

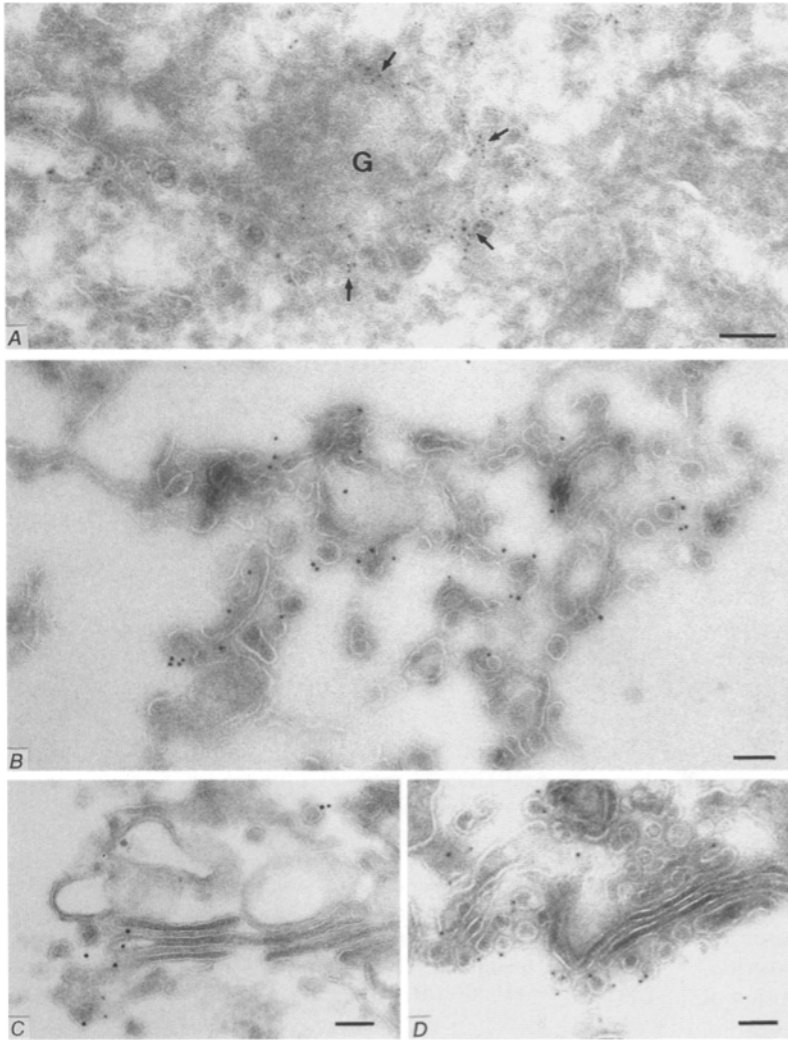


Figure 6. GOS-28 is concentrated in vesicles and buds at the terminal rims of Golgi cisternae. (A) Tangential view of a CHO cell Golgi complex (G) in situ showing the distribution of GOS-28 on peripherally located vesicular elements (arrows) in CHO cells stably transfected with overexpressed FLAG-tagged GOS-28. Immunolabeling was done with affinity-purified anti-GOS-28 antibodies. (B) Cell-free transport reactions. An isolated Golgi complex with cisternal and coated vesicular elements: only the latter shows a distinct GOS-28 immunolabeling on the outer aspect of the vesicle membrane. Golgi fractions were incubated with purified ARF and coatamer in presence of GTP, and were labeled with affinity-purified anti-GOS-28 antibodies. (C) Lateral part of an isolated Golgi complex. The GOS-28 immunolabeling appears restricted to the cisternal tips and associated vesicles. Golgi fractions were incubated in the presence of cytosol and aluminum fluoride, as described by Orci et al. (1989). Samples were labeled with affinity-purified anti-GOS-28 antibodies. (D) Section comparable to that in C, but immunolabeled with anti- ζ -COP antibodies. The labeling pattern is essentially similar to that with anti-GOS-28 antibodies. The Golgi fractions were incubated in presence of cytosol and GTP γ S, as described by Orci et al. (1989). In all experiments, the protein A-gold labeling technique described in Materials and Methods was used; size of gold particles = 10 nm. A, $\times 90000$; B, $\times 65000$; C and D, $\times 64000$. Bar, 0.1 μ m.

of GOS-28 in COPI-coated vesicles, we can not exclude that GOS-28 might be also found in a different class of Golgi-derived vesicles.

Anti-GOS-28 Antibodies Inhibit Intra-Golgi Transport by Blocking the Interaction of GOS-28 and α -SNAP

Isolated Golgi membranes were incubated with increasing amounts of affinity-purified IgGs directed against GOS-28. The samples were then incubated under conditions reconstituting transport. Transport was measured by glycosylation of the cargo protein VSV G (incorporation of radiolabeled GlcNAc). Transport was blocked by the antibody in a dose-dependent manner. The maximum inhibition was 40–50%, and was reached by the addition of 0.5 μ g IgG to 5 μ g Golgi protein in a 50- μ l reaction (see Fig. 8A). Addition of 1 μ g purified cytoplasmic domain of GOS-28 to a reaction had no effect on transport, but completely prevented the inhibitory effect of 1 μ g anti-GOS-28 IgG (data not shown), thus excluding unspecific effects caused by the addition of IgG. In addition, control IgG prepared from preimmune serum had no significant effect on transport. To avoid potential aggregation of Golgi stacks or potential clustering of GOS-28 on the membranes caused by

the bivalent IgGs, Fab fragments were prepared. The addition of Fab fragments also resulted in a partial inhibition of transport, similar to that obtained with the IgG, but the Fab had to be added at three- to fourfold higher concentrations to observe the maximal effect (see Fig. 8B). This could be explained by an inactivation of the Fabs during their preparation procedure or by the lower avidity of Fabs.

To establish at which particular stage the antibody blocks transport, the reactions were analyzed by electron microscopy. Therefore, transport reactions were either incubated with GTP γ S, known to accumulate coated vesicles (Melancon et al., 1987), or with the anti-GOS-28 antibody to test for any morphological effects caused by the antibody. A combination of anti-GOS-28 antibody and GTP γ S was used to test if the antibody blocks the formation of coated vesicles or acts at a later step. In other reactions, the membranes were treated with NEM to block the NSF activity, thus accumulating docked uncoated vesicles (Malhotra et al., 1988). The control reaction consisted of standard transport conditions. As shown in Table I, NEM caused, as expected, a fourfold increase (relative to the control) in uncoated circular profiles associated with the Golgi, and did not result in accumulation of coated vesicles. The anti-GOS-28 antibodies increased the number of uncoated cir-

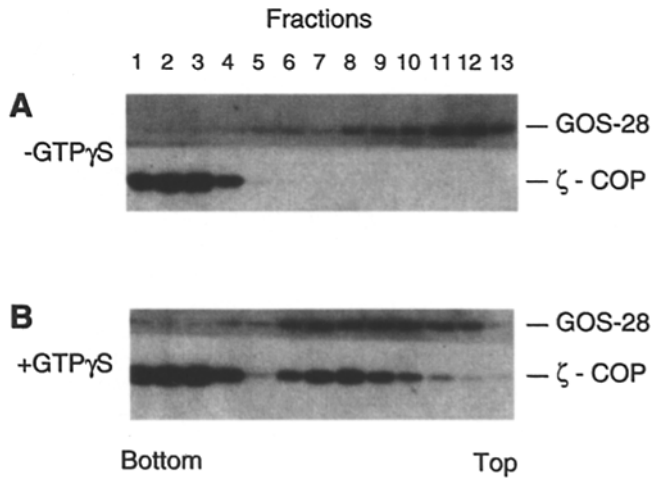


Figure 7. GOS-28 is present in Golgi-derived coated vesicles. Golgi membranes were incubated under transport conditions in the presence or absence of GTP γ S, as described in Materials and Methods. Briefly, after the GTP γ S treatment, the membranes were isolated and incubated with buffer containing 250 mM KCl to remove associated vesicles. After centrifugation, the supernatant containing the vesicles was adjusted to 52% sucrose and overlaid with a 30–52% sucrose gradient. Gradient fractions were subjected to Western blot analysis with anti- ζ -COP polyclonal antibodies and anti-GOS-28 polyclonal antibodies.

cular profiles associated with the Golgi by 3.2-fold, similar to the effect of NSF inactivation. The antibody did not inhibit the accumulation of coated vesicles by GTP γ S, demonstrating that the anti-GOS-28 antibody inhibits transport at some stage after coated vesicle budding. Therefore, while the anti-GOS-28 antibody does not block the formation of coated vesicles, it clearly blocks the consumption of docked uncoated vesicles before fusion can occur.

A putative SNARE protein such as GOS-28 should bind SNAP, particularly when it is assembled in v-t-SNARE complexes. To test this, detergent extracts of Golgi membranes were incubated with an affinity matrix containing immobilized α -SNAP (GST- α -SNAP fusion protein bound to glutathione-agarose). GOS-28 associated with α -SNAP (Fig. 9, lane 1), implying that GOS-28 can interact directly with α -SNAP or, more likely, that GOS-28 assembled in a v-t-SNARE complex provides high affinity sites for the binding of α -SNAP. This interaction is not a dead-end process because NSF releases the bound GOS-28 from the immobilized α -SNAP in presence of Mg-ATP, a hallmark property of SNAREs (Söllner et al., 1993b) (Fig. 9, lane 9).

Table I. Anti-Gos-28 Antibodies Cause the Accumulation of Docked Uncoated Vesicles

Sample	Density of total circular profiles	Density of uncoated circular profiles	Density of coated circular profiles
		(number per μm^2)	
Control	8.73 \pm 0.48	2.01 \pm 0.29	6.73 \pm 0.37
+GTP γ S	18.75 \pm 0.79	1.92 \pm 0.36	16.83 \pm 0.78
+NEM	13.23 \pm 0.66	8.10 \pm 0.55	5.13 \pm 0.36
+anti-GOS-28 IgG	11.42 \pm 0.60	6.51 \pm 0.55	4.90 \pm 0.39
+anti-GOS-28 IgG and GTP γ S	20.76 \pm 0.83	0.95 \pm 0.20	19.81 \pm 0.84

Untreated or NEM-treated CHO Golgi membranes were incubated under transport conditions in the presence or absence of affinity-purified anti-GOS-28 antibodies and GTP γ S, as described in Materials and Methods. For electron microscopy, membranes were fixed, collected by centrifugation, and processed as described (Orci et al., 1991). For each of the samples the density of coated vesicles/buds and uncoated circular profiles associated with the Golgi were analyzed in 20–30 separate Golgi areas in each of the three independent experiments. Numbers are mean values \pm SEM.

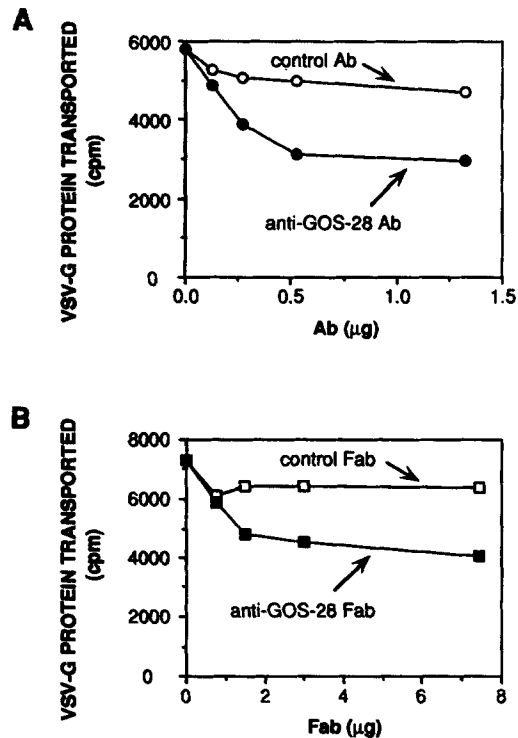


Figure 8. Anti-GOS-28 antibodies inhibit *cis*- to medial-Golgi transport in vitro. Enriched Golgi fractions were assayed for transport of VSV G protein between *cis*- and medial-Golgi compartments, as described (Balch et al., 1984a,b; see also Materials and Methods). The reactions (50 μ l) were preincubated on ice for 1 h with the indicated amounts of preimmune IgG (A), affinity purified anti-GOS-28 antibody (A), control Fab fragments prepared from preimmune IgG (B), or anti-GOS-28 Fab fragments (B) before incubation at 37°C for 1 h.

The accumulation of docked uncoated vesicles revealed by electron microscopy would imply that anti-GOS-28 antibody should block fusion, but not docking. Consistent with this, anti-GOS-28 antibody blocked the binding of GOS-28 to α -SNAP in a dose-dependent manner until a maximum inhibition of \sim 80% was reached by adding 2.5 μ g IgG to 25 μ g Golgi protein (see Fig. 9, lanes 2–4). Preimmune antibodies had no effect on the interaction of GOS-28 with α -SNAP (Fig. 9, lane 5). The anti-GOS-28 IgG/Golgi ratio needed to obtain maximum inhibition of the GOS-28/ α -SNAP interaction is the same as that is needed for maximum inhibition of transport, implying that the in-

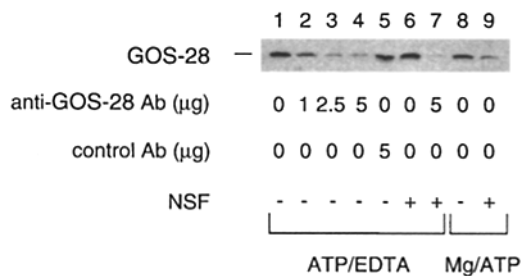


Figure 9. Anti-GOS-28 antibodies inhibit the assembly of the 20S fusion particle. Triton X-100 extract of CHO Golgi membranes (25 μg protein) was preincubated with the indicated amounts of anti-GOS-28 antibody for 1 h on ice, as described in Materials and Methods. Then GST-α-SNAP (1 μg) prebound to glutathione beads was added, and the incubation continued for another hour at 4°C in the absence or presence of 5 μg His₆-NSF-myc and either ATP/EDTA or ATP/Mg²⁺ as indicated. Proteins bound to the beads were isolated, the samples were washed, and the proteins were further analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by labeling with anti-GOS-28 antibodies, as described in Materials and Methods.

teraction measured in the detergent extract faithfully represents the effects seen in the *in vitro* transport assay.

Discussion

Here, we report the isolation and characterization of a 28-kD Golgi membrane protein, GOS-28, fulfilling all of the criteria currently available to define a v-SNARE. GOS-28 has a single membrane-spanning region at its carboxy terminus, and the bulk of the protein (the amino terminal part) is exposed to the cytoplasm and has the inherent probability to form a coiled coil structure. Furthermore, GOS-28 binds directly or indirectly to α-SNAP, and ATP hydrolysis by NSF results in dissociation of this interaction. Morphological studies revealed that GOS-28 is localized to the Golgi apparatus with an enrichment on vesicular components at the terminal rims of the Golgi. Electron microscopy analysis and fractionation show the efficient inclusion of GOS-28 in COP-coated vesicles, budding *in vitro*. Antibodies directed against GOS-28 partially inhibit transport from *cis*- to medial-Golgi, accumulating uncoated docked vesicles, and also inhibit the binding of GOS-28 to α-SNAP, implying that the binding of α-SNAP to complexes containing GOS-28 is needed for fusion after docking.

A possible discrepancy in our findings is that while anti-GOS-28 antibodies block transport by only 40–50%, they inhibit GOS-28 binding to α-SNAP by at least 80%. Among other possibilities, this difference could reflect an essential difference between the two assays. The membranes used in the transport assays contain sufficient endogenous α-SNAP to fulfill transport (Clary and Rothman, 1990), implying that two populations of SNAREs might exist in them: one free of endogenous α-SNAP and another associated with endogenous α-SNAP. The former would be inhibited by GOS-28 antibody, but the latter would not, resulting in the partial inhibition of transport. On the other hand, in the binding assay, the GST-α-SNAP matrix would only bind the free GOS-28 pool, which will also be accessible to the anti-GOS-28 antibodies. The an-

tibody would then efficiently block the GOS-28-α-SNAP association, but only partially inhibit transport.

What is the role of GOS-28 as a v-SNARE in Golgi transport processes? What routes within the stack are specified by this v-SNARE? Answering these important questions will require identifying the cisternae of origin of GOS-28, as well as the location of its partnering t-SNAREs in (or outside of) the stack. Unfortunately, because of the extremely efficient packing of GOS-28 into vesicles, it has not been possible to pinpoint the origin of vesicles containing GOS-28 in favor of *cis* versus *trans* cisternae, and the partnering t-SNAREs are still unknown. Attempts to test for the interaction with one potential partner, syntaxin 5 (Sed5), have revealed binding (data not shown), but its specificity could not be conclusively demonstrated.

The inhibition of reconstituted *cis*-to-medial transport by anti-GOS-28 antibody would suggest that GOS-28 is a v-SNARE for this step. The need for GOS-28, however, could be indirect; e.g., GOS-28 could be necessary for retrograde transport from medial- to *cis*-Golgi, or for both anterograde and retrograde steps connecting these compartments. For example, Pelham and colleagues have reported that a v-SNARE, Sft1p, is localized to the medial-Golgi in yeast (Banfield et al., 1995) and partners the *cis*-Golgi t-SNARE Sed5p (Sogaard et al., 1994). Yet, Sft1p is required for *cis*-to-medial transport *in vivo*. As proposed by Pelham, Sft1p could be used for retrograde transport from later cisternae to the *cis* compartment, and the block in anterograde transport could be a secondary consequence of the lack of recycling of critical anterograde machinery (perhaps Sft1p itself) back to the *cis*-Golgi. *In vivo* (Banfield et al., 1995), the anterograde block is complete; *in vitro*, it would be partial if multiple rounds were required. This could be another explanation (see above) of why antibodies to GOS-28 partially inhibit *cis*-to-medial transport *in vitro*, reflecting the fraction of anterograde transport that is dependent on recycling *in vitro* under the conditions we used.

Although the precise function of GOS-28 has not been completely established, GOS-28 is clearly a new member of the SNARE family, and the first mammalian v-SNARE localized to the Golgi and implicated in intra-Golgi transport. The predominant localization of GOS-28 to vesicular structures at the terminal rims of Golgi cisternae and its low abundance at the central cisternal region indicate that the terminal rims of Golgi cisternae are functionally specialized sites for the entry and exit of transport vesicles. Further understanding of the functional organization of the Golgi stack and its vesicular flow patterns will require the discovery of additional SNAREs and possibly other targeting molecules, as well as understanding their interactions.

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