Intracellular protein traffic involves a tightly regulated series of events in which a membrane-bounded vesicles bud from one compartment and are specifically targeted to the next compartment, where they dock and fuse. A cell-free system that reconstitutes vesicle trafficking between the cis and medial Golgi cisternae has been used previously to identify several proteins involved in vesicular transport (N-ethylmaleimide-sensitive fusion protein, soluble N-ethylmaleimide-sensitive fusion protein attachment proteins, p115, and p16); however, these factors are insufficient to drive the transport reaction. We have used a modified version of this in vitro intra-Golgi transport assay to guide purification of a new transport-stimulating activity. The active component is a 13 S hetero-oligomeric complex consisting of at least five polypeptides (approximately 110, 109, 90, 82, and 71 kDa), which we term Golgi transport complex (GTC). Hydrodynamic properties suggest that GTC is approximately 800 kDa and nonglobular. We obtained peptide sequence information from the 90-kDa subunit (GTC-90) that allowed us to identify a number of GTC-90 cDNAs. Comparison of these cDNAs with one another and with the genomic sequence suggests that the GTC-90 mRNA is alternatively spliced. Anti-GTC-90 antibodies inhibit the in vitro Golgi transport assay, confirming the functionality of the purified complex. Subcellular fractionation indicates that GTC-90 exists in both membrane and cytosolic pools, with the cytosolic pool associated exclusively with the GTC complex. The membrane-associated pool of GTC-90 is localized to the Golgi apparatus.

Much of the intracellular transport of proteins between the various compartments of the secretory pathway is mediated by small membrane-bounded vesicles (1–4). These vesicles bud from one compartment and transport their cargo to the next compartment of the secretory pathway. A multitude of factors collaborate in this process: coat proteins (e.g. COPI (coatomer) or COPII) shape the membrane into a bud culminating in vesicle release (5); a number of integral membrane proteins of the p24 family function in cargo selection during budding (6); and a set of soluble and integral membrane proteins mediate target selection and membrane fusion (7).

Many of the proteins involved in vesicular transport have been identified and/or characterized through use of a well-characterized in vitro system that measures intra-Golgi protein traffic (8). The assay measures the glycosylation of a cargo protein (vesicular stomatitis virus (VSV) G protein) present in one population of Golgi stacks by a glycosyltransferase present in another population of Golgi. In this system, COPI-coated transport vesicles form under the direction of a small GTPase termed ADP-ribosylation factor and a cytosolic coat protein complex termed coatamer (9). The docking and fusion phases of the assay require a number of other proteins. For example, N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) are required at a time point just prior to fusion and thus have been thought for some time to be integral components of the membrane fusion apparatus (10, 11). Another protein identified with this system is p115 (12), which is also thought to act at the docking phase (13–15) and may act to tether the vesicle to the target membrane before assembly of the v/t-SNARE targeting complex (16, 17). Rab6 (18), which is a Golgi-associated member of a large family of low molecular weight GTP-binding proteins (19, 20), is also required in this cell-free system and most likely acts at the membrane docking step (21). Last, a protein termed p16 has recently been identified and was suggested to have a role in the docking and/or fusion phase of early Golgi transport (22).

The final fusion event in this assay system is likely to be mediated by members of a family of integral membrane proteins called SNAP receptors (SNAREs) (23–26). Specific members of the SNARE family can be found on distinct target membranes (t-SNAREs) or on vesicles (v-SNAREs). Cognate v-SNAREs and t-SNAREs interact to form stable complexes (23, 27–30), perhaps via coiled-coil domains (28, 31, 32). This cross-membrane v/t-SNARE complex has recently been termed a “SNAREpin,” and it has been shown that SNAREs in this topological configuration can induce fusion of the docked membranes in the absence of other protein factors (33). Recent studies of vacuole-vacuole docking and fusion in yeast, where the membranes involved in fusion possess both v- and t-SNAREs, have shown that NSF acts to disassemble v-t-SNARE complexes in the same membrane prior to, and as a prerequisite for, vesicle-target membrane docking and fusion (34–36). Thus, the requirement for NSF and SNAP in the intra-Golgi in vitro system at a point temporally close to membrane fusion may reflect the requirement for unassociated v-

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1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive fusion protein; BBC, bovine brain cytosol; EST, expressed sequence tag; GTC, Golgi transport complex; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VSV, vesicular stomatitis virus; DTT, dithiothreitol; BSA, bovine serum albumin; MES, 4-morpholinoethanesulfonic acid; PCR, polymerase chain reaction; ASR, alternatively spliced region; TBS, Tris-buffered saline; MDBK, Madin-Darby bovine kidney; IP, immunoprecipitation; HPLC, high pressure liquid chromatography.
and t-SNAREs in both the donor and acceptor membranes for subsequent SNAREpin formation (33).

This cell-free system used to identify NSF, SNAP, p115, and p16 has been suggested to reconstitute the entire vesicular transport cycle, including vesicle budding, targeting, and fusion (7). Extensive electron microscopic and biochemical analyses have clearly indicated that vesicles form during the assay, and it has been suggested that they carry the VSV-G protein from the “donor” Golgi to the “acceptor” Golgi, where it is glycosylated, resulting in the signal obtained in the biochemical assay (3). However, several studies have shown that the assay signal is not affected by removal of ADP-ribosylation factor (13, 37, 38), which is required for vesicle formation (9), suggesting that the transfer of VSV-G protein from donor to acceptor Golgi occurs via a partial transport reaction encompassing only the membrane docking and fusion steps. In this regard, it is noteworthy that the four proteins purified based on their activity in this in vitro system (NSF, SNAPs, p115, and p16) impact on the docking or fusion phases of transport, as shown by both biochemical studies (10, 11, 13, 14, 22) and genetic analyses of their homologs in yeast (15, 39). In addition, removal of Rab proteins, which are generally thought to be involved in membrane docking and/or fusion and perhaps glycosylation of VSV-G.

Because the output of the intra-Golgi cell-free system is glycosylation of VSV-G, it is also possible that factors that affect glycosylation could exhibit activity. Indeed, it has been shown that the inclusion of uridine monophosphate kinase inhibition could exhibit activity. Indeed, it has been shown that the inclusion of uridine monophosphate kinase enhances the signal obtained by indirectly facilitating the uptake of radiolabeled UDP-sugar into the Golgi lumen, which is utilized by the glycosylation machinery (42). Thus, this cell-free system is a powerful tool for identification and analysis of transport factors and may be primarily dependent on those involved in membrane docking and/or fusion and perhaps glycosylation of VSV-G.

In this report, we describe the purification and initial characterization of a novel Golgi-associated protein complex containing at least five polypeptide chains, ranging from 71 to 110 kDa, that stimulates transport in this in vitro intra-Golgi transport system.

MATERIALS AND METHODS

General Procedures

pH measurements were done at room temperature; other manipulations were performed at 0–4 °C, unless otherwise noted. Dialysis membranes had a molecular mass cutoff of 12–14 kDa (BioDesign Inc.). Salt concentrations were determined by conversion from conductivity using a standard curve generated from 25 mM Tris-Cl, pH 8.0, 10 mM KCl, 10% glycerol (w/v) with 0–10 mM KCl or from 25 mM Tris-Cl, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol (w/v) with 0–500 mM Kp, (potassium phosphate). Protein concentrations were determined with the Bradford protein assay (43) (Bio-Rad) using a Beckman DU-64 spectrophotometer (Beckman Instruments). Unless otherwise noted, all fractions that were analyzed in the transport assay were first dialyzed into assay buffer (20 mM Hepes-KOH, pH 7.4, 100 mM KOAc, 1 mM DTT).

Purification of Bovine Serum Albumin (BSA)

Six ml of 40 mg/ml BSA (fracion V, Sigma) in 25 mM Tris-Cl, pH 7.4, 100 mM KOAc, 1 mM DTT were loaded onto six 10.5-ml 10–25% glycerol gradients in the same buffer. The gradients were centrifuged in a Beckman SW41 rotor (Beckman Instruments Inc.) at 40,000 rpm (198,000 × grot) for 24 h. The gradients were then fractionated into 0–500 mM KCl, and the A280 peak was pooled, avoiding the leading edge of the peak that sedimented deeper into the gradient. The pool was loaded at 1.5 ml/min on an 8-ml Mono-Q column (HR 16/10; Amersham Pharmacia Biotech) equilibrated in 25 mM Tris-Cl, pH 7.4, 100 mM KCl, 1 mM DTT. The column was washed with 16 ml of equilibration buffer and eluted with a 60-ml gradient from 100 mM up to 1 M KCl in the same buffer. Fractions (1.5 ml) were collected, and those with a minimum of contaminating proteins, as determined by SDS-PAGE and Coomassie blue-staining, were pooled and was referred to as purified BSA.

Cis to Medial Intra-Golgi Transport Assay

The transport assay used in this purification is a modification of previously published assays (8, 12, 44), with the primary changes being inclusion of transport factors shown to be active in this assay, specifically NSF (45), α- and β-SNAP (44), and p115 (12), and the use of the N-acetylglucosaminyl transferase-deficient mutant Chinese hamster ovary cell line Lec1 (46), rather than 15B (8), for the preparation of VSV-G-bearing donor membranes (47). The 25-μl reactions, which were incubated at 37 °C for 1 h, contained 15 μl of chromatographic fractions to be assayed, 3 μl of Chinese hamster ovary (Lec1) VSV-infected donor Golgi and 2 μl of Chinese hamster ovary (wild type) acceptor Golgi membranes (–3 μg of protein). 25 ng of His6,NSF (48), 200 ng of His6-α-SNAP (48), 325 ng of p115 (12), 0.3 μl of UDP-[3H]-N-acetylglucosamine (American Radiochemicals), 200 ml Hepes-KOH, pH 7.4, 16 mM Tris-Cl, pH 7.4, 60 mM KCl, 2.5 mM Mg(OAc)2, 250 μM UTP, 100 μM ATP, 5 mM creatine phosphate, 12 IU/ml creatine kinase, 10 μM palmitoyl-coenzyme A (49), 40 μg/ml nucleotide monophospate kinase (42), 200 mM sucrose, 4.8% (w/v) glycerol, and 0.6 mM DTT. [3H]-N-acetylglucosamine incorporation was quantitated by immunoprecipitation of VSV-G protein and scintillation counting (8).

For the inhibition of in vitro transport with affinity-purified antibodies (Fig. 8), affinity-purified anti-GTC-90 (see below) was dialyzed extensively into assay buffer without DTT. The antibody was then serially diluted in assay buffer without DTT and added to a standard transport assay containing serial dilutions of either BSA, or the CHT2-I pool (see below). A preimmune IgG fraction was prepared on a protein A-Sepharose column according to the manufacturer’s instructions, dialyzed into assay buffer without DTT, and used as a control for the inhibition experiments.

Purification of GTC

Preparation of Bovine Brain Cytosol and Ammonium Sulfate Precipitation—Five cow brains were obtained immediately after slaughter, and bovine brain cytosol (BBC) was prepared and ammonium sulfate-precipitated as described previously (12), except that ammonium sulfate was added to a final concentration of 30% saturation at 0 °C, and the pellet was resuspended with and dialyzed against 25 mM MES-KOH, pH 6.8, 50 mM KCl, 1 mM DTT, 10% glycerol (w/v). The dialysate (228 ml) had a protein concentration of 36.7 mg/ml.

SP-Sepharose Chromatography—The protein concentration of the dialysate was adjusted to 10 mg/ml with 25 mM MES-KOH, pH 6.8, 50 mM KCl, 1 mM DTT, 10% glycerol (w/v) and was loaded at 7.0 ml/min onto a 450-ml SP-Sepharose Fast Flow (Amersham Pharmacia Biotech) column equilibrated in the same buffer. The column was washed with the same buffer, and the protein that flowed through the column was collected and dialyzed extensively against 25 mM Tris-Cl, pH 8.0, 1 mM DTT (T/D, pH 8.0) with 50 mM KCl. The material was then clarified in a Beckman SW41 rotor at 55,000 rpm for 15 min, resulting in a 1.2-liter pool with a protein concentration of 4.1 mg/ml.

DEAE-Sepharose Chromatography—The dialyzed SP-Sepharose flow-through was loaded at 4.0 ml/min onto a 250-ml DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech) column equilibrated in 25 mM Tris-Cl, pH 8.0, 1 mM DTT, 10% glycerol (w/v)/T/DG, pH 8.0 with 50 mM KCl. The column was washed with 300 ml of equilibration buffer, followed by elution with a 300-ml gradient from 50 to 350 mM KCl in T/D/G, pH 8.0, followed by a 150-ml gradient from 350 mM to 1 M KCl in T/D/G, pH 8.0. Fractions (15 ml) were collected, and an aliquot of each was assayed for transport activity after dialysis into assay buffer. The active fractions, usually from 55 to 170 mM KCl, were pooled (133 ml) and had a protein concentration of 2.4 mg/ml.

Cibacron Blue 3GA Chromatography—The salt concentration of the DEAE pool was adjusted to 100 mM KCl with the addition of T/DG, pH 8.0, 3.0 M KCl and loaded onto a 30-ml Cibacron Blue 3GA (Sigma) column equilibrated in T/D/G, pH 8.0, 100 mM KCl. The column was washed with 50 ml of equilibration buffer, followed by elution with 90 ml of T/DG, pH 8.0, 500 mM KCl. The eluted fractions were pooled (43 ml) and had a protein concentration of 2.4 mg/ml.

Butyl-Sepharose Chromatography—The salt concentration of the Blue 3GA pool was adjusted to 1.5 M KCl by the addition of T/DG, pH 8.0, 3 M KCl and loaded at 0.5 ml/min onto a 5-ml butyl-Sepharose Fast Flow (Amersham Pharmacia Biotech) column equilibrated in T/D/G, pH 8.0, 1.5 M KCl. The column was washed with 10 ml of equilibration buffer followed by elution with 25 ml of T/DG, pH 8.0, 3 mM KCl. The eluted material (9.0 ml) had a protein concentration of 4.3 mg/ml.
Ceramic Hydroxylapatite (CHT2-I) Chromatography—The salt concentration of the butyl pool was adjusted to 100 mM KCl by the addition of T/D/G, pH 7.4. Potassium phosphate (KP), pH 7.4, was then added to a final concentration of 2 mM, and the pool was loaded at 1.0 ml/min onto a 1 ml CHT2-I column (Bio-Rad) equilibrated in T/D/G, pH 7.4, 100 mM KP, 2 mM KP. The column was eluted with 4 ml of the buffer followed by the elution of the bound material with a 14-ml gradient from 2 to 125 mM KP, in the same buffer. Fractions (0.5 ml) were collected in “low adhesion” polypropylene microcentrifuge tubes (USA/Scientific Plastics) and immediately dialyzed in a microdialyzer (Life Technologies, Inc.) against assay buffer to remove the phosphate (extended exposure to phosphate seemed to leave the protein insoluble). The fractions were collected into low adhesion microcentrifuge tubes and stored at 20 °C.

Superox 6 Size Exclusion Chromatography—The CHT2-I pool was concentrated to a volume of 0.5 ml using an Ultrafree-4 centrifugal filter device (Millipore, Biomax-10K NMWL). The concentrated material was centrifuged for 10 min in a microcentrifuge to remove any insoluble material and then sized at 0.25 ml/min through a 24-ml Superose 6 column (Amersham Pharmacia Biotech) equilibrated in T/D, pH 7.4, 100 mM KOAc, 0.01% (v/v) Tween 20, 250 µg/ml purified BSA. Fractions (0.5 ml) were collected in low adhesion microcentrifuge tubes, and 15 µl of each fraction were assayed without prior dialysis. The active fractions, usually eluting between 0.25 and 0.45 ml of fractions, were collected into low adhesion microcentrifuge tubes (50). In addition, 7.5 µl of each fraction was then assayed for transport activity. The active fractions, usually eluting from 50–75 mM KCl, were pooled (3 ml) and had a protein concentration of 0.96 mg/ml.

Mono-Q Chromatography—The Superose 6 pool was loaded at 0.3 ml/min onto a 1-ml Mono-Q column (HR55, Amersham Pharmacia Biotech) equilibrated in T/D, pH 7.4, 100 mM KCl. The column was washed with 2.5 ml of the same buffer followed by elution with a 7.5-ml gradient from 0.1 to 1.0 M KCl in the same buffer. Fractions (0.25 ml) were collected in low adhesion tubes, and 3 µl of each fraction were assayed without prior dialysis. The active fractions, usually eluting between 250–300 mM KCl, were pooled (0.7 ml). This column acted primarily as a concentration step.

Glycerol Gradient Sedimentation—The Mono-Q pool was layered onto a 4.7-ml linear 10–25% glycerol (w/v) gradient in T/D, pH 7.4, 100 mM KOAc. The gradient was centrifuged at 60,000 rpm in a Beckman SW55 rotor at 55,000 rpm for 6.5 h with slow acceleration and deceleration. Fractions (0.6 ml) were collected into low adhesion microcentrifuge tubes and analyzed by electrophoresis and silver staining (50). In addition, 7.5 µl of each fraction were assayed directly for transport activity. The active fractions, sedimenting at approximately 15 S, were pooled. The activity co-purified with a hetero-oligomeric complex composed of at least five subunits (see below), which we have termed the GTC.

Sequence Determination of Tryptic Peptides of the 90-kDa GTC Subunit—Pools from three GTC purifications (150 µg of total protein, estimated) were prepared for peptide sequencing by 6% trichloroacetic acid precipitation in the presence of 0.02% deoxycholate to concentrate them for SDS-8% polyacrylamide gel electrophoresis. After electrophoresis, the gel was washed for 1 h in HPLC grade water and copper-stained (Bio-Rad) according to the manufacturer’s instructions. Each of the bands of the GTC complex was excised along with control gel slices of the same volume and relative molecular weight range from an adjacent lane in which no protein had been loaded. The gel slices were washed twice in 50% acetonitrile (Pierce) for 10 min, gently vortexing every 2 min. The supernatant was discarded, and the wet gel slices were frozen and stored at −80 °C.

Sequences of three tryptic peptides derived from the 90-kDa subunit (GTC-90) were obtained at the Harvard Microchemistry Facility (Harvard University, Cambridge, MA), where trypsin digestion, HPLC purification, and sequencing of the peptides by either Edman degradation (one peptide) or mass spectroscopy (two peptides) were performed.

Cloning and Sequencing of a GTC-90 cDNA—Sequence of Human Expressed Sequence Tags (ESTs) Corresponding to GTC—The three peptide sequences obtained from GTC-90 were used to search the dbEST data base with the BLAST algorithm (51). Three overlapping I.M.A.G.E. Consortium (LLNL) cDNA clones (52) were found that coded for the three peptides. Two of these, I.M.A.G.E. Consortium clone numbers 53376 and 626427 were obtained from Genome Systems (St. Louis, MO), and the complete nucleotide sequences of the two cDNAs were determined.

PCR Amplification of the 5′-End of the GTC-90 cDNA—To obtain the 5′-end of the GTC-90 cDNA, a HeLa cDNA library (Stratagene) in BlueScript SK−(53) was PCR-amplified in two rounds using a pair of primers nested in the 5′-end of clone 626427 and one primer in the BlueScript polylinker (M13-2) (53). In the first round of PCR, the outside GTC-90-specific primer and M13-2 were used to amplify an approximately 700-bp product which had been lyzed by heating at 98 °C in water. The second round of PCR was performed with the product from the first round using M13-2 and the inside GTC-90-specific primer. The product of round 2 was digested with XbaI and BglII and subcloned into a vector containing the 5′-end of EST-626427 linearized with the same enzymes. Plasmids containing the largest inserts were sequenced. One of the inserts contained an additional 492 base pairs of the open reading frame, which we termed 626427-A.

According to splice site prediction analysis of the genomic DNA (54), the remaining GTC-90 sequence was contained within a single exon. Thus, the remaining 5′ sequence was obtained by PCR using a human bacterial artificial chromosome encoding the 5′-end of GTC-90 as a template (bacterial artificial chromosome RG020D02 from chromosome seven (55)). The amplified material was subcloned into 626427-A after digestion of both with BamHI and ScaI. Because this construct was missing the putative alternatively spliced region, it was termed GTC-90 (−ASR).

Construction of a Putative Full-length GTC-90 cDNA—The GTC-90 (−ASR) construct does not contain the region coding for amino acids 557–593, because this region was absent from the original EST (number 626427) derived from HeLa cells. Because this region contains one of the peptides found in our purified brain protein, we chose to insert the exon encoding this region to make the putative full-length brain construct. To accomplish this, EST 53376, which contains the region, was subcloned into BlueScript II SK−(53) after digesting both with BstII and XbaI. This construct, which represents the 3′-end of the GTC-90 cDNA, was termed 53376-BSSK. The 5′-end of the GTC-90 cDNA was then subcloned onto 53376-BSSK by digestion of GTC-90 (−ASR) with BamHI and XbaI and ligating it to 53376-BSSK, which was digested with the same enzymes. This putative cDNA construct was termed full-length GTC-90.

Production of Affinity-purified Anti-GTC-90 Antibodies—A construct encoding an N-terminal hexahistidine partial-GTC-90 fusion protein (His6-53376) was constructed using an I.M.A.G.E. cDNA clone. Clone 53376 was digested with HindIII and ligated into linearized pQE-30 (Qiagen) to yield the plasmid QE-53376. M15 cells (Qiagen) were transformed with the plasmid QE-53376, and expression was induced for 18 h with 0.2 mM isopropyl-β-d-thiogalactoside. Cell lysates were made in TBS (20 mM Tris-Cl, pH 7.5, 500 mM NaCl) in the presence of 5 mM DTT and 1.5% sarcosyl (56). After sonication and clarification by centrifugation, Triton X-100 was added to 4%, and the detergent lysate was incubated for 45 min with 2 ml of glutathione-Sepharose (Amersham Pharmacia Biotech) equilibrated in TBS. The mixture was poured into a Millipore filter and then washed by which had been followed by 10 ml of 100 mM HEPES-NaOH, pH 7.4, 150 mM NaCl. The column was eluted with 100 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.1% SDS. The eluate was gel-filtered on a PD-10 column (Amersham Pharmacia Biotech) and cross-linked to 0.5 ml of Affi-Gel-15 (Bio-Rad) according to the manufacturer’s instructions using 100 µl HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.1% SDS as a cross-linking buffer. The antibody was affinity-purified according to previously published protocols (55) except that the
low pH elution used 100 mM glycine, pH 2.5, 10% ethylene glycol (v/v). Briefly, after passing serum over the affinity column and washing the column, the antibodies were eluted sequentially with pH 2.5 buffer followed by elution with 100 mM triethylamine, pH 11.5. The affinity-purified antibodies used in the experiments described here are anti-His53 antibodies eluted at pH 11.5, and are referred to as anti-GTC-90.

**Immunoblotting, Immunofluorescence Microscopy, and Immunoprecipitation**

For detection of GTC-90 by immunoblots, proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (NEN Life Science Products). The blots were then processed according to previously published protocols (55) using 5% nonfat dry milk in phosphate-buffered saline, 0.1% Tween 20 as a blocking agent. Blots were incubated overnight at 4°C with primary antibody at the indicated dilutions and with secondary antibody diluted 1:6000 in blocking solution for 1 h at room temperature. Polyclonal antibodies were detected with goat anti-rabbit HRP (Bio-Rad), and monoclonal antibodies were detected with goat anti-mouse horseradish peroxidase (Bio-Rad). The blots were developed with ECL Plus (Amersham Pharmacia Bio- tech) and exposed to X-AR5 film (Eastman Kodak Co.).

Indirect immunofluorescence was performed with NRK cells grown on flame-sterilized 12-mm glass coverslips. The cells were fixed in methanol for 15 min at -20°C, washed four times in TBS, pH 8.0, and treated with 6 M urea in TBS for 5 min at room temperature. After urea treatment, the cells were washed in TBS and blocked in 5% nonfat dry milk at room temperature for 30 min. The cells were incubated in primary antibody in blocking solution overnight at room temperature, and all secondary antibodies were applied in blocking solution for 1 h at room temperature. Mouse monoclonal antibodies were detected with Texas red-conjugated goat anti-mouse antibodies, and rabbit polyclonal antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies. For GTC-90 detection, after overnight incubation with affinity-purified antibody, the coverslips were first washed with blocking solution, incubated with biotinylated goat anti-rabbit antibodies for 1 h at room temperature, washed with blocking solution, and finally incubated with fluorescein isothiocyanate-labeled avidin for 1 h at room temperature. Confocal images were obtained using a Zeiss LSM 510 laser confocal microscope.

For immunoprecipitation of GTC, Madin-Darby bovine kidney (MDBK) cells in 100-mm plates were grown to 80% confluency in 100 ml of Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum, and then incubated for 16 h in 90% Dulbecco’s modified Eagle’s medium without Cys and Met, 10% fetal calf serum, and 50 μCi Tran35S-label (ICN)/ml. The cells were washed once in 1× phosphate-buffered saline and then scraped off the plate in 750 μl of IP buffer (T/D/G, pH 8.0, with 50 mM KCl) containing the protease inhibitor mixture used for preparing BBC (12). The cell suspension was homogenized in a 2-ml Dounce homogenizer with 10 strokes of the B pestle and centrifuged for 20 min at 4°C in a microcentrifuge. The membrane pellet was solubilized in 1.2 ml of IP buffer plus 1% Nonidet P-40 and clarified in a microcentrifuge. The membrane lysate (prepared as above) and the supernatant were collected by side puncture of the tube with a 19-gauge needle attached to a 10-ml syringe. The fractions from the first and second gradients were subjected to SDS-8% polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and immunoblotted with anti-GTC-90, anti-p115, and anti-mannosidase II antibodies.

**RESULTS**

**In Vitro Cis to Medial Intra-Golgi Transport Requires Unidentified Cytosolic Components**—As indicated above, several cytosolic and peripheral membrane proteins involved in vesicular transport have been purified using an in vitro system that reconstitutes protein trafficking from the cis to medial Golgi compartments (12, 22, 44, 45, 58). The assay entails incubating together two types of isolated Golgi membranes, termed donor and acceptor, in the presence of cytosol and ATP. The donor membranes are isolated from VSV-infected Chinese hamster ovary (Lec1) cells, which have an inactivating mutation in the N-acetylglucosaminyltransferase I gene (59) that encodes a medial Golgi glycosyltransferase (60). The acceptor membranes are isolated from uninfected wild-type Chinese hamster ovary cells. Transport is measured by monitoring the addition of [3H]N-acetylglucosamine to the viral protein VSV-G, which can only occur upon fusion of VSV-G-containing membranes from the donor Golgi with the N-acetylglucosaminyltransferase I-containing acceptor Golgi.

We found that assays performed in the presence of functionally saturating amounts of NSF, SNAP-25, and p115 still required the addition of cytosol for optimal activity, suggesting that at least one other factor was required, as had been previously suggested (12). Specifically, bovine brain cytosol stimulated the assay about 6-fold over background (Fig. 1A). To determine whether multiple activities might be present in cytosol, it was chromatographed on a Mono-Q anion exchange column, and the fractions were tested for activity in the transport assay. We found that at least two activities were present in cytosol, one that flowed through the column and one that bound and could be eluted with a KCl gradient (Fig. 1B). The simultaneous addition of both the flow-through and bound activities to the assay resulted in a signal that was approximately equal to the sum of the signals obtained with the individual fractions (Fig. 1B). Subsequent size exclusion chromatography of the flow-through fractions revealed that it predominantly contained low molecular weight factors possessing transport activity, one of which was phosphatidylinositol transfer protein (PITPα) (58) and another that may be the recently described protein p16 (22). In contrast, size exclusion chromatography of the bound activity revealed that it was composed of high molecular weight factors (data not shown), indicating that the flow-through and bound activities are distinct. This report describes the purification and characterization of one of the high molecular weight activities that bound to Mono-Q.

The response of the assay to either of the two activities suggests that some of the other component(s) are present in the system, perhaps associated with the Golgi membranes, which were not treated with salt to strip peripheral membrane proteins as had been done in previous assay systems (12, 44). This
activity sedimented at about 13 S and coincided with a hetero-
the last step of the purification, velocity sedimentation, the
Stokes radius of the activity of about 120–130 Å (Fig. 2). At
before the largest molecular weight standard. Extrapolation
eluted from the Superose 6 size exclusion column significantly
During the course of the purification, we found that the activity
followed by velocity sedimentation through a glycerol gradient.
flow-through was then fractionated by DEAE-Sepharose anion
exchange, Cibacron Blue-3GA dye affinity, butyl-Sepharose hy-
drophobic interaction, ceramic hydroxylapatite, Superose 6 size
exchange, Cibacron Blue-3GA dye affinity, butyl-Sepharose hy-
Sulfate precipitation, SP-Sepharose cation exchange, DEAE-Sepharose
Steps (DEAE-Sepharose, Superose 6, and the glycerol gradient)
steps (DEAE-Sepharose, Superose 6, and the glycerol gradient)
noteworthy point is that the Superose 6 size exclusion step near
the end of the purification separates the activity from the bulk
of the protein, resulting in a precipitous drop in protein con-
centration. This necessitated the addition of an exogenous pro-
tein to reduce nonspecific losses and perhaps to stabilize the
activity. For this purpose, we employed highly purified BSA,
which has no affect on transport activity (data not shown) and
can be efficiently removed at the last step of the purification
(Figs. 3 and 4). Fig. 4A shows a silver-stained gel of fractions of each step of
the purification. Quantitation of activity and protein yields is
shown in Table 1. It should be noted that the activity measure-
ments early in the purification are not indicative of the specific
activity of GTC alone, because several other activities that also
affect the assay were present, as described previously. For this
reason, the “specific” activity does not significantly increase
until the last few steps of the purification when GTC has been
separated from other components that affect the in vitro sys-
tem. Thus, the -fold purification of GTC is greatly underesti-
 gated. Another issue that affected the determination of GTC-
specific activity was the addition of BSA at the later stages of
the purification, which precluded an accurate determination
of GTC protein concentration and thus specific activity. Exami-
nation of the protein yield and protein profiles of fractions
through the purification indicated that only a few purification
steps (DEAE-Sepharose, Superose 6, and the glycerol gradient)
seem to be highly effective (Fig. 4A). Nevertheless, attempts to
observation also indicates that the assay conditions are such
that there is not one true “rate-limiting step.” Rather, the
biochemical flux through the pathway, i.e. the movement of
VSV-G protein through all of the biochemical intermediates
that are required for its ultimate glycosylation by GlcNAc
transferase, can be affected by the concentration of more than
one enzyme, as is often the case in a series of biochemical
reactions (61).

Purification of a High Molecular Weight Complex That Stimulates in Vitro Intra-Golgi Transport—In the fist step of the purification of the high molecular weight Mono-Q bound activity, bovine brain cytosol (BBC-50), B, at least two distinct activities are separable by anion exchange chromatography. BBC-50 was dialyzed into 25 mM Tris-Cl, pH 8.0, 50 mM KCl, 1 mM DTT, 10% glycerol (w/v), diluted to 5.0 mg/ml, and chromatographed on a Mono-Q anion exchange column in a flow-through (fractions 4–18) and bound fractions (fractions 28–45). Fractions (10 µl) were assayed for transport-stimulating activity.

Buffer, the assay background; Load, the activity of 10 µl of the column load. Subsequent size exclusion chromatography of the flow-through and bound activities indicated that they chromatographed at vastly different molecular sizes and were thus distinct (data not shown).

FIG. 1. Transport from the cis to medial Golgi requires at least two cytosolic factors in addition to NSF, SNAPs, and p115. A, in vitro transport assays (see “Materials and Methods”) were performed in the presence of functionally saturating amounts of NSF (25 ng), α-SNAP (200 ng), and p115 (125 ng) with either transport assay buffer or a serial dilution of a 50% ammonium sulfate precipitate of bovine brain cytosol (BBC-50). B, at least two distinct activities are separable by anion exchange chromatography. BBC-50 was dialyzed into 25 mM Tris-Cl, pH 8.0, 50 mM KCl, 1 mM DTT, 10% glycerol (w/v), diluted to 5.0 mg/ml, and chromatographed on a Mono-Q anion exchange column into flow-through (fractions 4–18) and bound fractions (fractions 28–45). Fractions (10 µl) were assayed for transport-stimulating activity. Buffer, the assay background; Load, the activity of 10 µl of the column load. Subsequent size exclusion chromatography of the flow-through and bound activities indicated that they chromatographed at vastly different molecular sizes and were thus distinct (data not shown).

Fig. 2. Superose 6 size exclusion chromatography of GTC. Partially purified material that had been subjected to 30% ammonium sulfate precipitation, SP-Sepharose cation exchange, DEAE-Sepharose anion exchange, Cibacron Blue-3GA dye affinity, butyl-Sepharose hydrophobic interaction, and ceramic hydroxylapatite chromatographic steps was concentrated and sieved through a 24-ml Superose 6 column. Fractions (10 µl), as well as buffer and the column load (3 µl), were assayed for activity. Molecular size standards (bovine thymus thyroglobulin (669 kDa, 85 Å), horse spleen ferritin (440 kDa, 61 Å), and bovine liver catalase (232 kDa, 52.2 Å)) were chromatographed subsequently under the same conditions and used to estimate the Stokes radius.

At the end of the purification, the complex is purified to apparent homogeneity (Fig. 3A), although there is frequently some contamination from other proteins (Fig. 3B). Nevertheless, transport activity invariably fractionated only with the complex and not with any of the minor contaminants. Another noteworthy point is that the Superose 6 size exclusion step near the end of the purification separates the activity from the bulk of the protein, resulting in a precipitous drop in protein concentration. This necessitated the addition of an exogenous protein to reduce nonspecific losses and perhaps to stabilize the activity. For this purpose, we employed highly purified BSA, which has no affect on transport activity (data not shown) and can be efficiently removed at the last step of the purification (Figs. 3 and 4).

Fig. 4A shows a silver-stained gel of fractions of each step of the purification. Quantitation of activity and protein yields is shown in Table 1. It should be noted that the activity measurements early in the purification are not indicative of the specific activity of GTC alone, because several other activities that also affect the assay were present, as described previously. For this reason, the "specific" activity does not significantly increase until the last few steps of the purification when GTC has been separated from other components that affect the in vitro system. Thus, the -fold purification of GTC is greatly underestimated. Another issue that affected the determination of GTC-specific activity was the addition of BSA at the later stages of the purification, which precluded an accurate determination of GTC protein concentration and thus specific activity. Examination of the protein yield and protein profiles of fractions through the purification indicated that only a few purification steps (DEAE-Sepharose, Superose 6, and the glycerol gradient) seem to be highly effective (Fig. 4A). Nevertheless, attempts to
FIG. 3. Velocity sedimentation of GTC. As a final step in the purification, the active fractions were sedimented through 10–25% glycerol gradients (see "Materials and Methods"). After centrifugation, the gradients were fractionated, and 2.5% of the load and fractions were analyzed by SDS-10% polyacrylamide gel electrophoresis and silver staining. S value standards (indicated across the top) from a parallel gradient were visualized by Coomassie staining. Undialyzed fractions results in an increase in the glycerol concentration in the assay by 3.0% to 7.5%. Assay background (bg., the signal obtained with buffer rather than a gradient fraction) was 200 cpm and has been subtracted. Two independent purifications are shown. The purity of the complex obtained was variable, often being homogeneous (Fig. 3A) but sometimes showing minor contamination (Fig. 3B).

remove other steps from the purification resulted in a significant loss of purity of the final material.

GTC purified from cytosol appears to be composed of seven polypeptide subunits with molecular masses of approximately 110, 109, 90, 86, 82, 76, and 71 kDa (Fig. 4B, Standard Purification). Although the staining intensities of the individual components differ with respect to each other, the profile is highly reproducible using several staining techniques (silver, Coomassie, and copper) as well as in multiple purifications. It is possible, however, that some of the subunits could be proteolytic fragments that remain associated with the complex and resist further degradation as has been observed with other multisubunit complexes such as coatomer (63, 64) and the "exo" complex in the yeast Saccharomyces cerevisiae (65).

The 90-kDa Subunit (GTC-90) Is Encoded by an Alternatively Spliced mRNA—The amino acid sequences of three tryptic peptides from the 90-kDa subunit (GTC-90) were determined by either Edman degradation or mass spectroscopy. None of the sequences showed significant homology to known proteins; however, they were homologous to a set of overlapping human cDNA clones (52) in the EST data base (dBEST), which together contained all three peptides and had identical 3'-ends (Fig. 5A). Sequencing of the ESTs revealed that the largest cDNA (EST 626427, derived from a HeLa cell library) encodes approximately 71% of the predicted open reading frame (see below), whereas a second cDNA (EST 53376, derived from a human infant brain library) encodes approximately 43% of the predicted open reading frame. The overlapping sequence of the two ESTs are identical except for one region (encoding amino acids 557–593), which is absent in EST 626427, suggesting that GTC-90 may be alternatively spliced. Further searches in the dBEST database revealed several ESTs from various tissues that encode human and mouse homologs of GTC-90 and have different splicing patterns over this putative alternatively spliced region (Fig. 5C).

Based on the sequence of the human brain EST through the alternatively spliced region and the fact that GTC-90 from purified bovine brain GTC contains a peptide encoded by exon 16, the most likely brain cDNA was constructed (see "Materials and Methods"). The putative cDNA contains exons 1–14, exon 16, and exons 19–24. Analysis of the first ATG in the open reading frame indicates that it is in the appropriate context for translation initiation (66). The cDNA encodes a protein of 839 amino acids (Fig. 6), which encompasses the three tryptic peptides obtained from purified bovine brain GTC-90 and has a predicted molecular mass of 92,700 Da and a pI of 6.57. The predicted molecular mass is in close agreement with the apparent molecular mass of GTC-90 on SDS-PAGE.

Data base searches indicate that GTC-90 is a novel protein that has yet to be described. There are putative homologs in Drosophila melanogaster, Caenorhabditis elegans, and Arabidopsis thaliana EST data bases. However, we were surprised to find no convincing homolog in the S. cerevisiae genome, which has been completely sequenced, since most vesicular transport factors have functional homologs in yeast that are recognizable at the primary sequence level.

GTC-90 Is Enriched during the Purification and Exists Exclusively in a Complex—While the enrichment of the complex through the purification was difficult to assess based on activity (see above and Table I), immunoblot analysis of the fractions over the course of the purification with an affinity-purified anti-GTC-90 antibody showed that GTC-90 was enriched as the purification proceeded (Fig. 7A). The antibody also recognized a second protein in cytosol (Fig. 7A, lane 3–BBC), but, only one of these cross-reactive proteins is present in GTC, since the lower molecular weight protein is removed early in the purification.

Although analysis of the purification fractions indicated that GTC-90 was enriched as the purification proceeded, we investigated whether GTC-90 is entirely associated with GTC, or if a noncomplexed pool is also present. Superose 6 size exclusion chromatography followed by immunoblot analysis for GTC-90 was utilized for this purpose. GTC-90 exists exclusively in a complex that behaves identically to GTC (Fig. 2 and Fig. 7B). The cross-reactive protein in cytosol also appears to exist in a complex, since it has a Stokes radius of approximately 60 Å (Fig. 7B). The nature of this complex and its potential relationship to GTC remain to be explored.

In Vitro Transport Is Inhibited in the Presence of Anti-GTC-90 Antibodies—To confirm that GTC was the factor responsible for the activity in our purified fractions, we tested...
whether affinity-purified antibodies raised against GTC-90 would inhibit the transport assay. Antibody was added to the assay in the presence of varying amounts of either the starting material of the purification (cytosol) or partially purified GTC (hydroxylapatite pool, CIIT2-1). Inhibition of the assay was evident when 300 ng of anti-GTC-90 was added to an assay driven by cytosol; the signal was reduced approximately 40% (at 100 µg of BBC-30) (Fig. 8A). The antibody was also an effective inhibitor of the assay driven by partially purified GTC, reducing the signal by about 40% (at 6 µg of hydroxylapatite pool) (Fig. 8B). The addition of more than 300 ng of antibody to the assay did not result in further inhibition of transport (data not shown). In general, the signal could be reduced 40–60% upon the addition of antibody to the assay. Comparable levels of preimmune IgG added to the assay failed to inhibit either the cytosol- or hydroxylapatite pool-driven assays, indicating that the inhibition is specific to the anti-GTC-90 antibody (Fig. 8A). Because the antibody recognizes a single protein in the hydroxylapatite pool by immunoblot (Fig. 7A) and all of the detectable GTC-90 is in a complex (Fig. 7B), these results are consistent with GTC being responsible for part of the transport activity present in cytosol. We also attempted to inhibit the transport assay using monovalent Fab fragments generated from the affinity-purified antibodies, but the Fab fragments were not inhibitory (data not shown). Since the Fab fragments could still recognize GTC-90 on immunoblots (data not shown), the lack of inhibition suggests that inhibition of GTC activity by the anti-GTC-90 antibodies requires more than simply interaction of the antigen with the antigen-binding site of the antibody. For example, the bivalent nature of the antibody may be critical to effect GTC-90 cross-linking, or the larger size of the whole antibody relative to the Fab fragment may cause inhibition for steric reasons.

**GTC Affinity-purified from Solubilized 35S-Labeled Membranes Has a Subunit Composition Similar to the Chromatographically Purified Complex**—The composition of GTC was examined by immunoprecipitation in order to determine if the subunits we observe in the purified material co-fractionate using a different method. Because most of the GTC-90 is membrane-associated (data not shown), we affinity-purified GTC from a [35S]cysteine and methionine metabolically labeled MDBK cell membrane fraction solubilized in nonionic detergent. The affinity-purified material contained eight subunits (180, 110, 99, 92, 87, 82, and 71 kDa), while the chromatographically purified material contained seven subunits (Fig. 4B), as mentioned above. Five of these subunits (110, 109, 90, and 82 kDa) appear to be present in both.

The two additional subunits that are present in the chromatographically purified GTC (86 and 76 kDa) and absent in the affinity-purified complex may represent 1) polypeptides that dissociated from the immunopurified complex due to the presence of detergent or antibody, 2) partially proteolyzed subunits that remain associated during the standard purification, or 3) proteins that happen to co-fractionate with GTC. The three additional polypeptides that are present in the affinity-purified GTC (180, 92, and 87 kDa) may be a result of 1) the complex being isolated from a different source (MDBK cells rather than bovine brain), 2) a GTC membrane receptor co-purifying with the complex, since a different subcellular fraction (membranes rather than cytosol) was used for the affinity-purification, or 3) components of GTC that are proteolyzed during the chromatographic purification. The 92- and 87-kDa polypeptides could also be alternatively spliced isoforms of GTC-90 that are not expressed in brain.

**GTC Co-localizes with Golgi Markers by Subcellular Fractionation**—The intracellular localization of GTC was examined by subcellular fractionation of bovine brain postnuclear supernatant on equilibrium density sucrose gradients (Fig. 9A). Fractions were analyzed by immunoblotting with affinity-purified anti-GTC-90 and with antibodies that recognize either p115, a marker of the cis Golgi apparatus (12, 67), or the medial Golgi marker protein mannosidase II (Fig. 9B). In a first gradient, membranes that collected at the 0.86/1.25 M interface were harvested, adjusted to 1.35 M sucrose, and loaded onto the bottom of a second gradient (Fig. 9A). Immunoblot analysis of the second gradient showed that GTC-90, p115, and mannosidase II floated with the membranes to the 1.0/1.25 M interface (Fig. 9B). This verifies that GTC-90, and therefore GTC, like the peripheral membrane protein p115 (12), has both a cytosolic and a membrane-bound pool. In addition, the results suggest that GTC may be associated with the Golgi. Another
The observation of interest is that the affinity-purified antibody recognizes an additional protein of approximately 70 kDa. This cross-reactive protein, which is different from the one observed in cytosol (compare Fig. 7B to Fig. 9B), is membrane-associated and also fractionates with the Golgi markers. This protein could be a unique species, a degradation product of GTC-90, or the 68-kDa alternatively spliced form of GTC-90.

### TABLE I

Quantitation of the GTC purification

| Protein                  | Volume | Total activity | Specific activity | Purification | Activity yield |
|--------------------------|--------|----------------|-------------------|--------------|----------------|
|                          | mg     | ml             | cpm              | cpm/µg       | fold           | %              |
| Cytosol                  | 31,000 | 3000           | 8.62 × 10^6      | 27.8         | 1              | 100            |
| 30% ammonium sulfate     | 7100   | 228            | 2.46 × 10^6      | 34.6         | 1.2            | 28.5           |
| S-Sepharose pool         | 4900   | 1200           | 2.05 × 10^6      | 41.8         | 1.5            | 28.8           |
| DEAE pool                | 319    | 133            | 1.17 × 10^7      | 36.8         | 1.3            | 28.8           |
| Blue pool                | 103    | 43             | 4.93 × 10^7      | 47.9         | 1.7            | 28.8           |
| Butyl pool               | 39     | 9.0            | 2.45 × 10^8      | 62.2         | 2.2            | 28.8           |
| Hydroxylapatite pool     | 2.9    | 3.0            | 3.71 × 10^6      | 128          | 4.6            | 28.8           |
| Superose 6 pool          | 2.5    | 1.05 × 10^6    | 2.56 × 10^6      | 62.2         | 2.2            | 28.8           |
| Mono-Q pool              | 0.7    | 8.40 × 10^6    | 330              | 1000         | 100            | 28.8           |
| Glycerol gradient pool   | <0.05  | 1.0            | 3.30 × 10^6      | >600         | >24            | 28.8           |

*Protein concentrations were not determined beginning at the Superose 6 step because of the addition of BSA as a stabilizing agent.

**GTC-90 Localizes to the Golgi Apparatus in NRK Cells**

To examine further whether GTC-90 (and GTC) is localized to the Golgi, NRK-52E cells were examined by double label indirect immunofluorescence.

**FIG. 5.** GTC-90 is alternatively spliced. A, one of the GTC-90 ESTs (number 626427) does not contain a region (the area enclosed by the dashed lines) that encodes one of the GTC-90 tryptic peptides (Pep. 1), which is present in another GTC-90 EST (number 53576). B, a schematic representation of the intron/exon structure of a region of human chromosome seven (7q31.1) encompassing the alternatively spliced region, exons 14–19 (contained in bacterial artificial chromosome RG363E19). C, BLAST searches of the dBEST data base revealed four human ESTs and one mouse EST whose nucleotide sequences differ in the GTC-90 alternatively spliced region. For one EST, clone 727321, the 5’-end of the cDNA is within the alternatively spliced region. The clones were from the following cDNA libraries: G3204, human fetal heart, GenBank™ accession number R58366; 53576, human infant brain, GenBank™ no. R5430 (I.M.A.G.E. Consortium); 626427, HeLa cell, GenBank™ no. AA188905 (Stratagene, La Jolla CA); 727321, human testis, GenBank™ no. AA292919 (I.M.A.G.E. Consortium); 1247363, mouse mammary gland, GenBank™ no. AA839785 (I.M.A.G.E. Consortium).

**FIG. 6.** Predicted amino acid sequence of GTC-90. Predicted amino acid sequence of the putative GTC-90 full-length brain cDNA. The thick underlines indicate bovine GTC-90 tryptic peptide sequences that are identical to the predicted human protein sequence. The thin underline indicates a bovine GTC-90 tryptic peptide sequence (VLQTQPSYVR) that was 81% identical and 100% similar to the predicted human protein sequence. The two mismatches are with amino acid residues that could not be determined with a high degree of confidence during peptide sequencing, although the difference could be accounted for by conservative substitutions between human and bovine proteins.
GTC-90 or enhance accessibility of the antibody to GTC-90 (68). Fig. 10A shows that GTC-90 is localized in the perinuclear region in a pattern very similar to that of p115. To confirm that the urea treatment did not have an adverse affect on immunofluorescent analysis of the Golgi, we also examined the colocalization of p115 with the medial Golgi marker mannosidase II. As expected from previous reports, p115 was localized adjacent to, but not precisely colocalized with, mannosidase II (67), indicating that the urea treatment does not interfere with the analysis.

Close examination of the GTC-90/p115 double labeling indicated that although the two proteins are both present in the perinuclear region, they do not precisely co-localize (Fig. 10B). Whereas a reticular pattern was obtained for p115, the stain-
**FIG. 10.** GTC-90 localizes to the Golgi by indirect immunofluorescence microscopy. 

**A**, low magnification confocal image of NRK cells incubated with anti-p115 monoclonal antibody (3A10, 1:1000) and either anti-mannosidase II polyclonal antibody (1:5000) or affinity-purified anti-GTC-90 polyclonal antibody (1:2).

**B**, higher magnification confocal images using the same conditions as **A**.

*Fig. 10. GTC-90 localizes to the Golgi by indirect immunofluorescence microscopy.* A, low magnification confocal image of NRK cells incubated with anti-p115 monoclonal antibody (3A10, 1:1000) and either anti-mannosidase II polyclonal antibody (1:5000) or affinity-purified anti-GTC-90 polyclonal antibody (1:2). B, higher magnification confocal images using the same conditions as A.
ing pattern of GTC-90 appears to be more punctate in nature, raising the possibility that p115 and GTC-90 reside within closely apposed but distinct regions of the Golgi stack. The absence of a clearly evident cytoplasmic pool of GTC-90 by immunofluorescence may be due to either a higher concentration of GTC-90 on the Golgi relative to cytosol, as is found with other Golgi peripheral membrane proteins, or depletion of cytosolic GTC-90 by the somewhat stringent washing conditions required to obtain the immunofluorescent signal.

Since the anti-GTC-90 recognizes more than one membrane-associated protein by immunoblot (Fig. 9B), immunofluorescence was also performed using a second anti-GTC-90 antibody that does not recognize this additional 70-kDa band (data not shown). The localization of GTC-90 using the second antibody was indistinguishable from the first (data not shown). These results, taken together with the size exclusion and fractionation results, indicate that that GTC exists in both cytosolic and Golgi-associated pools.

**DISCUSSION**

We have used an in vitro system that reconstitutes transport between the cis and medial Golgi cisternae to identify and purify a novel protein complex that stimulates this protein trafficking event. The complex, which we have termed GTC, appears to be composed of at least five subunits ranging from 71 to 110 kDa in molecular mass. Peptide sequence data from the 90-kDa subunit (GTC-90) allowed identification of a number of putative cDNAs as well as the human genomic sequence that encodes the protein, which, in turn indicated that GTC-90 can be alternatively spliced. GTC-90 is found exclusively in the GTC complex, is present in both membrane and cytoplasmic pools, and localizes to the Golgi by immunofluorescence in NRK-52E cells. The variety of cDNA isoforms that were observed might reflect tissue-specific variants of the protein or provide a means by which a putative heterogeneous population of GTC-related complexes act at different locations in the secretory pathway within one cell.

The precise localization of GTC within the Golgi region remains to be determined. It is noteworthy, however, that confocal microscopy indicates that it is closely apposed to, but not completely coincident with, p115, which is thought to reside predominately on the cis side of the Golgi (67). It will be interesting to examine whether the alternatively spliced GTC-90 isoforms that are evident upon cDNA analysis are also present on the Golgi and, if so, whether their location within the stack is isoform-specific. In this regard, it is noteworthy that our affinity-purified anti-GTC-90 antiserum detects an approximately 70-kDa protein that cofractionates with the Golgi complex on sucrose gradients. A GTC-90-related protein of approximately this size has been predicted based on analysis of one of the alternatively spliced cDNAs, which bears an exon with a stop codon, resulting in a smaller protein.

The subunit composition of GTC is reminiscent of that of another multiprotein complex involved in secretion, termed the Exocyst. This protein complex has seven subunits (Sec6p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p) and is involved in the docking and/or fusion of post-Golgi secretory vesicles to the plasma membrane in the yeast S. cerevisiae (65, 69). The mammalian homolog of the exocyst has also been characterized and found to be localized to the plasma membrane (70). The molecular masses of the mammalian exocyst subunits are similar to those of GTC (rsec8, 110 kDa; rsec6, 106 kDa; rsec5, 102 kDa; rsec15, 96 kDa; rsec6, 86 kDa; exo84, 84 kDa; rexo70, 79 kDa; rsec10, 71 kDa). Given that the subunit composition of GTC is similar to the mammalian exocyst, we examined whether the two complexes were related by immuno blotting fractions through the GTC purification (see Figs. 4A and 7A) with antibodies raised against the 110-kDa rsec8 protein. The results showed that rsec8 separates from GTC during the course of purification (data not shown), and thus the two complexes are distinct. Perhaps more telling, the primary sequence of GTC-90 does not correspond to any of the mammalian exocyst subunits, most of which have been recently described (71). Moreover, GTC localizes to the Golgi, whereas the exocyst localizes to the plasma membrane. It is still possible, however, that in the Golgi, GTC might be an exocyst-like complex that functions in the early secretory pathway.

It is noteworthy that the sequence of GTC-90 is not significantly homologous to any yeast open reading frame. This is unusual, because most transport factors are highly evolutionarily conserved (72). It is possible that GTC is absent in yeast or that there is a functional homolog to GTC-90 in yeast whose sequence homology is not significant enough to be clearly evident. Peptide sequencing and cloning of the remaining GTC subunits may help address this question; while GTC-90 may not have structural homologs in yeast, the same may not be true for other subunits.

This report has described the purification and initial characterization of a novel complex of proteins, termed GTC, that stimulates in vitro intra-Golgi transport. Although GTC was purified based on its activity in the in vitro transport assay, its precise role in facilitating protein traffic remains to be determined. Since the system appears to be the most sensitive to the late steps of transport, i.e., docking and fusion of VSV-G bearing vesicles and the subsequent glycosylation of the transferred VSV-G protein, it is likely that GTC affects one of these biochemical events. Further characterization of GTC-90 and its alternatively spliced isoforms as well as characterization of the other GTC subunits will help to define GTC’s precise role in intracellular protein traffic.

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