

Retrograde Transport from the Golgi Region to the Endoplasmic Reticulum Is Sensitive to GTP γ S

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Abstract. The involvement of GTP-binding proteins in the intracellular transport of the secretory glycoprotein α_1 -antitrypsin was investigated in streptolysin O-permeabilized HepG2 cells. This permeabilization procedure allows ready access to the intracellular milieu of the membrane-impermeant, nonhydrolyzable GTP analog GTP γ S. In streptolysin O-permeabilized HepG2 cells, the constitutive secretory pathway remains func-

tional and is sensitive to GTP γ S. Exposure of HepG2 cells to brefeldin A resulted in redistribution of Golgi-resident glycosyltransferases (including both $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ sialyltransferases) to the ER. This redistribution was sensitive to GTP γ S. Our results suggest that GTP-binding proteins are involved in the regulation not only of the anterograde, but also of the retrograde, pathway.

INTRACELLULAR transport of glycoproteins is accomplished by the formation, targeting, and fusion of transport vesicles. Newly synthesized glycoproteins enter the endoplasmic reticulum (ER) and travel in a vectorial fashion through the Golgi cisternae to the *trans*-Golgi network (TGN).¹ They are then routed to the plasma membrane via the constitutive secretory pathway, or to other organelles such as lysosomes (Griffiths and Simons, 1986). Retrieval systems deliver membrane constituents back to the compartments from which they were originally derived, to maintain a balance between the donor and acceptor membranes involved in intracellular transport (Farquhar, 1985). For traffic from the Golgi region to the ER (retrograde transport), the existence of this retrieval pathway is revealed by the action of the antibiotic brefeldin A (BFA). In a cell-free system, BFA prevents the assembly of nonclathrin-coated vesicles from Golgi cisternae (Orci et al., 1991). In murine T cells, BFA blocks the movement of newly synthesized proteins from the ER to the Golgi region, yet leaves the retrograde pathway untouched (Lippincott-Schwartz et al., 1989). The result of BFA treatment is a dramatic redistribution of Golgi constituents to the ER (Lippincott-Schwartz et al., 1989; Lippincott-Schwartz et al., 1990).

In intact cells, the plasma membrane imposes limitations on the analysis of intracellular glycoprotein traffic. A thorough study of vesicular transport requires the use of cell-free or permeabilized cell systems that permit manipulation of the intracellular constituents (Simons and Virta, 1987; Bennett et al., 1989; Beckers et al., 1989). The biochem-

ical reconstitution of different stages of intracellular protein transport has been achieved and has revealed several cellular components that participate in vesicle fusion (Balch et al., 1984; Wattenberg and Rothman, 1986; Melançon et al., 1987; Beckers et al., 1989; Diaz et al., 1989; Clary et al., 1990; Wattenberg et al., 1990; Serafini et al., 1991). The low molecular weight GTP-binding proteins of the ras/rab gene family, of which a great multiplicity have now been identified, have come to the fore as strong candidates for regulators of intracellular traffic in yeast (Salminen and Novick, 1987; Segev et al., 1988) and higher eukaryotes (Zahraoui et al., 1989; Pluttner et al., 1990; Chavrier et al., 1990; Goud et al., 1990; Bailly et al., 1991; Van der Sluijs et al., 1991; Johnston et al., 1991). Their role in intracellular transport is thought to be the regulation of budding and fusion events. Control of vectorial transport is mediated by small GTP-binding proteins that have been proposed to cycle between a membrane-associated and a soluble form, which would require hydrolysis of GTP (Bourne, 1988). Interference in GTP hydrolysis by the presence of nonhydrolyzable GTP analogues, such as GTP γ S, thus is predicted to and indeed inhibits the regulatory action of GTP-binding proteins (Beckers and Balch, 1989; Melançon et al., 1987; Miller and Moore, 1991). Several of the subcellular compartments along the constitutive secretory pathway are endowed with a unique set of enzymes that can modify glycoproteins en route. Specifically, N-linked glycoproteins are translocated across the ER membrane and receive high mannose-type oligosaccharides cotranslationally. Trimming and remodeling of the oligosaccharides to complex-type oligosaccharides start in the ER and are completed in the TGN (Kornfeld and Kornfeld, 1985; Roth, 1987). N-linked glycan modifications can thus report on the compartments through which the glycoproteins have traveled.

1. **Abbreviations used in this paper:** AA, acrylamide; BFA, brefeldin A; Endo H, endoglycosidase H; LDH, lactate dehydrogenase; NANase, neuraminidase; PNGase, glycopeptidase F; SNA, *Sambucus nigra*; Strep O, streptolysin O; TGN, *trans*-Golgi network.

To study the involvement of GTP-binding proteins in intracellular transport of N-linked glycoproteins we used a permeabilized cell system, using the bacterial cytolysin streptolysin O (Strep O), which allows access of the membrane-impermeant agent GTP γ S to the cytosol. We show that in Strep O-permeabilized HepG2 cells, the entire constitutive secretory pathway remains operational and is sensitive to GTP γ S, as monitored by modification of N-linked glycans, and secretion of the reporter glycoprotein α_1 -antitrypsin. We provide evidence that in Strep O-permeabilized cells the retrograde pathway from the Golgi region to the ER is sensitive to GTP γ S, and therefore is likely to be controlled by GTP-binding proteins.

Materials and Methods

Materials

BFA was kindly provided by Sandoz Ltd. (Basel, Switzerland). [³⁵S]Methionine (>1,000 Ci/mmol) and [α -³²P]GTP (3,000 Ci/mmol) were obtained from Amersham International (Buckinghamshire, UK), and [2-³H]mannose (20 Ci/mmol) was from New England Nuclear, Du Pont Co. (Wilmington, DE). Reduced Strep O was obtained from Wellcome Diagnostics, (Beckenham, UK); the rabbit anti-human α_1 -antitrypsin antiserum was from the Central Laboratory of the Blood Bank Transfusion Service (Amsterdam, The Netherlands); and endoglycosidase H (Endo H) and glycopeptidase F (PNGase) were from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). Neuraminidase (NANase) (type VIII) and GTP γ S were from Sigma Chemical Co. (St. Louis, MO).

Cytosol was prepared from rat livers. Rat livers were washed with 20 mM Hepes, pH 7.4, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 10 μ g/ml leupeptin, 1 mM DTT and 0°C and homogenized using a potter (30 strokes with a teflon pestle) in 250 mM sucrose, 10 mM Tris-HCl, pH 7.2, 1 mM PMSF, and 10 μ g/ml leupeptin at 0°C. The homogenate was filtered through a cheese cloth, centrifuged 15 min at 1,000 g at 4°C, followed by 15 min at 3,000 g at 4°C. The supernatant was filtered through a cheese cloth and then centrifuged 2 h at 100,000 g at 4°C. The resulting supernatant was taken as the cytosol preparation (22–26 mg protein/ml), frozen in liquid N₂, and stored at -70°C.

Cells

The human hepatoma cell line HepG2 was cultured in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories) and antibiotics.

Preparation of Permeabilized Cells

Cells were incubated with Strep O in permeabilization buffer (137 mM NaCl, 2.7 mM KCl, 2 mM EGTA, 1 mM CaCl₂, 2 mM MgCl₂, 20 mM Pipes, pH 7.3) at the concentrations indicated for 7 min at 0°C. Unbound Strep O was removed by washing twice with concentrated (1.3 \times) permeabilization buffer at 0°C. Cells were then transferred to (15°C \rightarrow) 37°C to initiate pore formation, under hypotonic conditions in 0.8 \times transport buffer (90 mM KCl, 2 mM MgCl₂, 0.8 mM CaCl₂, 1.6 mM EGTA, 20 mM Hepes, pH 7.3) for 5 min. Concentrated (2.8 \times) transport buffer was added to restore to isotonic conditions. Permeabilization performed under isotonic conditions gave results that were qualitatively and quantitatively similar.

Quantitation of Lactate Dehydrogenase Release

The release of lactate dehydrogenase (LDH) from permeabilized cells was quantitated by adding aliquots of the cell supernatants to be tested to a freshly prepared reaction buffer (50 mM sodium phosphate, pH 7.4, 0.6 mM pyruvate, 0.2 mM NADH) and measuring the decrease in absorption of NADH by conversion of NADH to NAD⁺ spectrophotometrically at 340 nm. The initial slope (d[NADH]/dt) was determined and taken as an arbitrary measure for the amount of LDH present. The initial slope of d[NADH]/dt of control cells, lysed with 0.1% (vol/vol) NP-40, was taken as the 100% value for release of LDH.

Pulse-Chase Experiments

Cells grown to 80–90% confluency were labeled, as indicated, with [³⁵S]methionine at 37°C, treated with Strep O at 0°C as described above, and then chased at 37°C. For reconstitution in the permeabilized cell system, ATP was provided as an ATP-generating system (2.2 mM ATP, 0.44 mM GTP, 22.2 mM creatine phosphate, 31.8 U/ml creatine phosphate kinase; CTP was added instead of GTP when GTP γ S was included, exhibiting equivalent ATP regenerating efficiency), and 3.2 mg/ml cytosol was added. The supernatant containing the secreted proteins was collected and the cells were lysed in NP-40 containing lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5% [vol/vol] NP-40). The glycoprotein α_1 -antitrypsin was recovered from the culture medium and lysates by immunoprecipitation using rabbit anti-human α_1 -antitrypsin antibody and fixed *Staphylococcus aureus*. The immune complexes were washed 4 \times with NET buffer (50 mM Tris-HCl, pH 7.4, 0.5% [vol/vol] NP-40, 5 mM EDTA, 150 mM NaCl), and stored either as dry pellets at -20°C, or analyzed directly.

Biochemical Analysis

The immunoprecipitated α_1 -antitrypsin was analyzed by SDS-PAGE (10% AA) (Laemmli, 1970), one-dimensional IEF gels (Neeffjes et al., 1986), and two-dimensional SDS-PAGE (10% acrylamide) (O'Farrell, 1975). In case of digestions with Endo H, the immunoprecipitate pellets were resuspended in 20 μ l Endo H digestion buffer (50 mM sodium citrate, pH 5.5, containing 0.2% [wt/vol] SDS) and heated for 5 min at 95°C. Digestions were performed with 1.3–2.0 mU of Endo H for 20–24 h at 37°C while shaking. The reaction was terminated with SDS sample buffer. NANase digestions were performed by resuspending the immunoprecipitates in NANase digestion buffer (50 mM NaAc, pH 6.5, 1 mM CaCl₂) containing 5 U/ml NANase. Digestions were carried out for 4–6 h at 37°C while shaking. The reaction was terminated with IEF sample buffer.

Detection of Low Molecular Weight GTP-binding Proteins by Western Blot

The assay for low molecular weight GTP-binding proteins was carried out as described by Lapetina and Reep (1987). Cells were treated with Strep O or 1 \times permeabilization buffer at 0°C for 7 min and then transferred to 37°C for 75 min. The supernatants were collected and the cells were lysed in NP-40 lysis buffer. Proteins from total cell lysates and supernatants were concentrated with 15% TCA and then separated by SDS-PAGE (15% AA) and electrophoretically transferred to nitrocellulose sheets. The nitrocellulose blots were preincubated in binding buffer (50 mM Tris-HCl, pH 7.5, 0.3% [vol/vol] Tween 20, 5 mM MgCl₂, 1 mM EDTA) for 30 min at 25°C, and then incubated with 2 μ Ci/ml [α -³²P]GTP in binding buffer for 60–90 min. To assess the specificity of binding of [α -³²P]GTP, a parallel incubation including 1 μ M of unlabeled GTP was carried out. The blots were rinsed by several changes of binding buffer over 1–2 h and air dried. [α -³²P]GTP-binding polypeptides were visualized by autoradiography.

Lectin Affinity Chromatography of Sialic Acid-containing Oligosaccharides

Cells grown to confluency were labeled with [2-³H]mannose for 30, 120, and 240 min in the absence and presence of BFA. The cells were lysed with NP-40-containing lysis buffer. Glycoproteins were precipitated from total cell lysates with 5% TCA at 0°C and their oligosaccharides were released by PNGase treatment as described by the manufacturer (Boehringer Mannheim Diagnostics, Inc.). The released oligosaccharides were separated by ion-exchange chromatography on a Mono Q column using an HPLC system (Pharmacia Fine Chemicals, Piscataway, NJ) eluted with 10 mM BisTris, pH 6.8, followed by a discontinuous gradient from 0 to 120 mM NaCl. Fractions containing oligosaccharides carrying sialic acids of the BFA-treated cells were pooled, as were the corresponding fractions of control cells, eluting between positions of marker oligosaccharides with 1 and 3 sialic acids (derived from α_1 acid glycoprotein). After dialysis against double-distilled water, they were analyzed by affinity chromatography using an α_2 - \rightarrow 6 sialic acid-specific lectin from *Sambucus nigra* (SNA) (Shibuya et al., 1987) immobilized on tresyl-activated agarose (Pierce Chemical Co., Rockford, IL). The SNA column was equilibrated in PBS, pH 7.4, and eluted stepwise with 5 mM lactose (to release the retarded oligosaccharides), and a discontinuous gradient from 5 to 250 mM lactose (to release the bound oligosaccharides) using an HPLC system.

Analysis of Nucleotides and Nucleotide Sugars

10^7 cells grown to 90% confluency were treated with Strep O (0, 0.3, and 0.5 U/ml) and chased for 75 min at 37°C. Nucleotides and nucleotide sugars were extracted with ice-cold ethanol (75% [vol/vol]) followed by delipidation with chloroform/methanol/water (2:1:0.6, vol/vol/vol) (Pels-Rijcken et al., 1990). Extracts were evaporated to dryness and redissolved in 200 μ l double-distilled water. Half of the volume was analyzed by HPLC on an Ultrasilax column (4.6 \times 25 cm, particle size 10 μ m; Beckman Instruments, Palo Alto, CA) developed with a phosphate buffer concentration gradient at a flow rate of 1.5 ml/min; low-concentration buffer (A) was 5 mM $\text{H}_3\text{PO}_4/\text{KH}_2\text{PO}_4$, pH 4.0, and the high-concentration buffer (B) was 0.5 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 4.5. A three-step linear gradient was used, starting with buffer A and an increase in B at 0.3% per min for 30 min, followed by 2.5% per min for 15 min and 5% per min for 10 min until 99% B was reached, which was maintained till the end of the run (85 min).

Electron Microscopy

Intact and Strep O-permeabilized cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed with OsO_4 (1% [wt/vol]), and embedded according to routine methods. Thin sections, stained with uranyl acetate and lead, were observed in an EM 310 (Philips Electronic Instruments, Inc., Mahwah, NJ).

Results

Permeabilization of HepG2 Cells

HepG2 cells were permeabilized by a two-step procedure using the bacterial toxin Strep O, which binds tightly to the plasma membrane in a temperature-independent way, and produces pores of uniform size in a temperature-dependent fashion by complexation with cholesterol (Buckingham and Duncan, 1983; Duncan and Schlegel, 1975). Pore formation is considered irreversible (Kanbayashi et al., 1972; Duncan and Schlegel, 1975). Binding of Strep O was carried out at 0°C and unbound Strep O was removed by washing. Pore formation was induced in toxin-exposed cells by shifting them to 37°C. Permeabilization can thus be restricted largely, if not exclusively, to the plasma membrane. The pores formed by Strep O permitted the entry of the membrane-impermeant dye trypan blue (data not shown). The cytosolic enzyme LDH (140 kD) was released from the Strep O-permeabilized cells in a Strep O concentration- (Fig. 1 *a*) and temperature-dependent fashion (Fig. 1 *b*). Treatment of HepG2 cells with Strep O resulted also in some loss of other cytosolic components such as low molecular weight GTP-binding proteins (Fig. 1 *c*). For further experiments we chose 0.5 U/ml of Strep O, a concentration at which permeabilization was efficient (>98% permeable) as measured by trypan blue staining, while only a minor fraction of the LDH (Fig. 1 *a*), GTP-binding protein (Fig. 1 *c*), and nucleotide/nucleotide sugar content (Fig. 1 *d*) was released. Based on estimates of the cell volume of HepG2 cells using EM and quantitation of the HPLC profiles (Fig. 1 *d*), the intracellular concentration of GTP in intact HepG2 cells is \sim 0.5 mM. In Strep O-permeabilized cells the reduction in intracellular nucleotide and nucleotide sugars is quite modest, being most pronounced for GTP (estimated twofold reduction, see Fig. 1 *d*). Permeabilization under mildly hypotonic conditions gave results equivalent to those observed under isotonic conditions (data not shown). Cells exposed to Strep O and kept under isotonic conditions largely retained the morphology of untreated cells as analyzed by EM (Fig. 2).

The Entire Secretory Pathway Can Be Reconstituted in Strep O-permeabilized Cells

The constitutive secretory pathway was monitored in pulse-chase experiments by following the biosynthesis, processing, and secretion of the glycoprotein α_1 -antitrypsin. It is a glycoprotein of 51 kD carrying in its mature form three N-linked glycans of the complex type. In all experiments, the ER-resident α_1 -antitrypsin was labeled by a brief pulse with [^{35}S]methionine. Transport from the ER to the TGN results in generation of mature α_1 -antitrypsin as indicated by a shift in molecular weight, due to conversion of high mannose-type to sialic acid-containing complex-type oligosaccharides (Fig. 3 *a*). In Strep O-permeabilized HepG2 cells, the extent of transport to the TGN was greatly diminished. Partial reconstitution could be achieved by addition of ATP, provided as an ATP-generating system, and could be further improved by addition of rat liver cytosol (Fig. 3 *a*).

Mature α_1 -antitrypsin, formed in control cells, is resistant to digestion with Endo H (Fig. 3 *b*). In Strep O-permeabilized cells, α_1 -antitrypsin was resolved in a fully Endo H-sensitive and a partial Endo H-resistant form (Fig. 3 *b*). Inclusion of ATP and cytosol in permeabilized cells resulted in the appearance of additional Endo H-resistant bands, presumably due to an increase in the extent of maturation as a consequence of reconstitution of transport (Fig. 3 *b*). This process could include the addition of both neutral sugars and sialic acid residues. Modification of oligosaccharides in permeabilized cells is clearly less complete than that seen in control cells.

In permeabilized HepG2 cells α_1 -antitrypsin did acquire sialic acids, although considerably less than in control cells, as revealed by IEF (Fig. 3 *c*). The extent of sialylation could be improved (Fig. 3 *c*), in particular for the partially sialylated forms (1–3 sialic acids) and to a lesser extent for the form with 4–8 sialic acids by inclusion of ATP and cytosol (Fig. 3, *c* and *d*).

Secretion was detected by appearance of α_1 -antitrypsin in the medium (supernatant) after 75 min of chase in control cells, but was not detectable in the Strep O-permeabilized cells unless ATP and cytosol were added (Fig. 4 *b*). Reconstituted secretion displayed both time and temperature dependency (Fig. 4 *b*).

Transport of α_1 -Antitrypsin in Permeabilized but Not Intact Cells Is Susceptible to the Inhibitory Action of $\text{GTP}\gamma\text{S}$

In control cells transport from ER to TGN was affected neither by the presence of ATP and cytosol nor by inclusion of the membrane-impermeant agent $\text{GTP}\gamma\text{S}$ (Fig. 4 *a*). Unlike intact cells, Strep O-permeabilized cells were accessible to $\text{GTP}\gamma\text{S}$. $\text{GTP}\gamma\text{S}$ completely suppressed transport of α_1 -antitrypsin to the TGN (Fig. 4 *a*) and secretion (Fig. 4 *b*). In permeabilized cells exposed to $\text{GTP}\gamma\text{S}$, all α_1 -antitrypsin remained in the Endo H-sensitive form (Fig. 3 *b*).

The effects exerted by $\text{GTP}\gamma\text{S}$ were dependent on the concentration and the time of addition during the 75-min chase. To achieve maximal inhibition of transport to TGN a high concentration (1 mM) of $\text{GTP}\gamma\text{S}$ was required (Fig. 4 *c*). Sensitivity of transport to $\text{GTP}\gamma\text{S}$ could not be improved by pretreatment of permeabilized cells with EGTA (data not

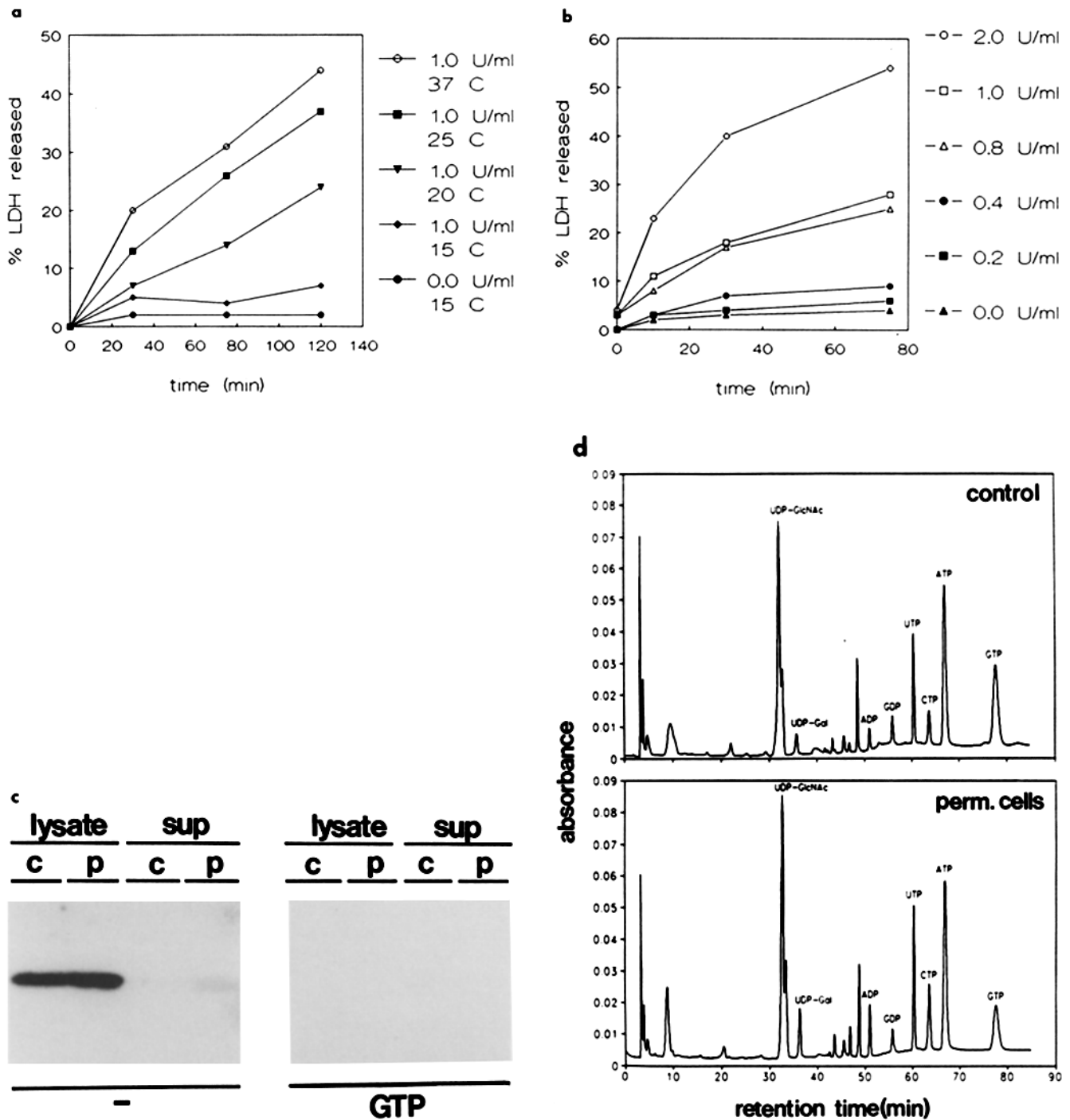


Figure 1. Strep O-permeabilized HepG2 cells release cytosolic components in a Strep O concentration- and temperature-dependent fashion. (a) The percent LDH released in the medium of cells treated with 0–2 U/ml Strep O was measured after 10, 30, and 75 min at 37°C and related to the total amount of LDH present in untreated HepG2 cells. (b) The percent LDH in the medium of untreated and 1.0 U/ml Strep O-treated cells was measured after 30, 75, and 120 min at the temperatures indicated. (c) The presence of GTP-binding proteins in lysates and supernatants (*sup*) of control cells (*c*) and Strep O-permeabilized cells (*p*) was analyzed 75 min after Strep O treatment by a [α - 32 P]GTP binding assay described in Materials and Methods (*left*). Binding of [α - 32 P]GTP could be inhibited by inclusion of 1 μ M unlabeled GTP (*right*). (d) HPLC pattern of nucleotides and nucleotide sugars. Conditions were as described in Materials and Methods. Profiles of UV-absorbing compounds were derived from control cells and cells permeabilized with 0.5 U/ml Strep O (*perm. cells*). (No differences were observed when comparing ethanolic extracts from cells permeabilized with 0.3 or 0.5 U/ml of Strep O.)

shown). Presence of 0.5 and 1.0 mM of GTP γ S at the beginning of the chase (0 min) completely suppressed transport to TGN (Fig. 4 c), whereas addition at later times (15 min) resulted in partial loss of inhibitory effects (Fig. 4 c). The

concentrations of GTP γ S required for inhibition should be seen against the GTP concentration likely to be present locally. The results presented in Fig. 1 d argue that these levels may be high, even in permeabilized cells, but they can not

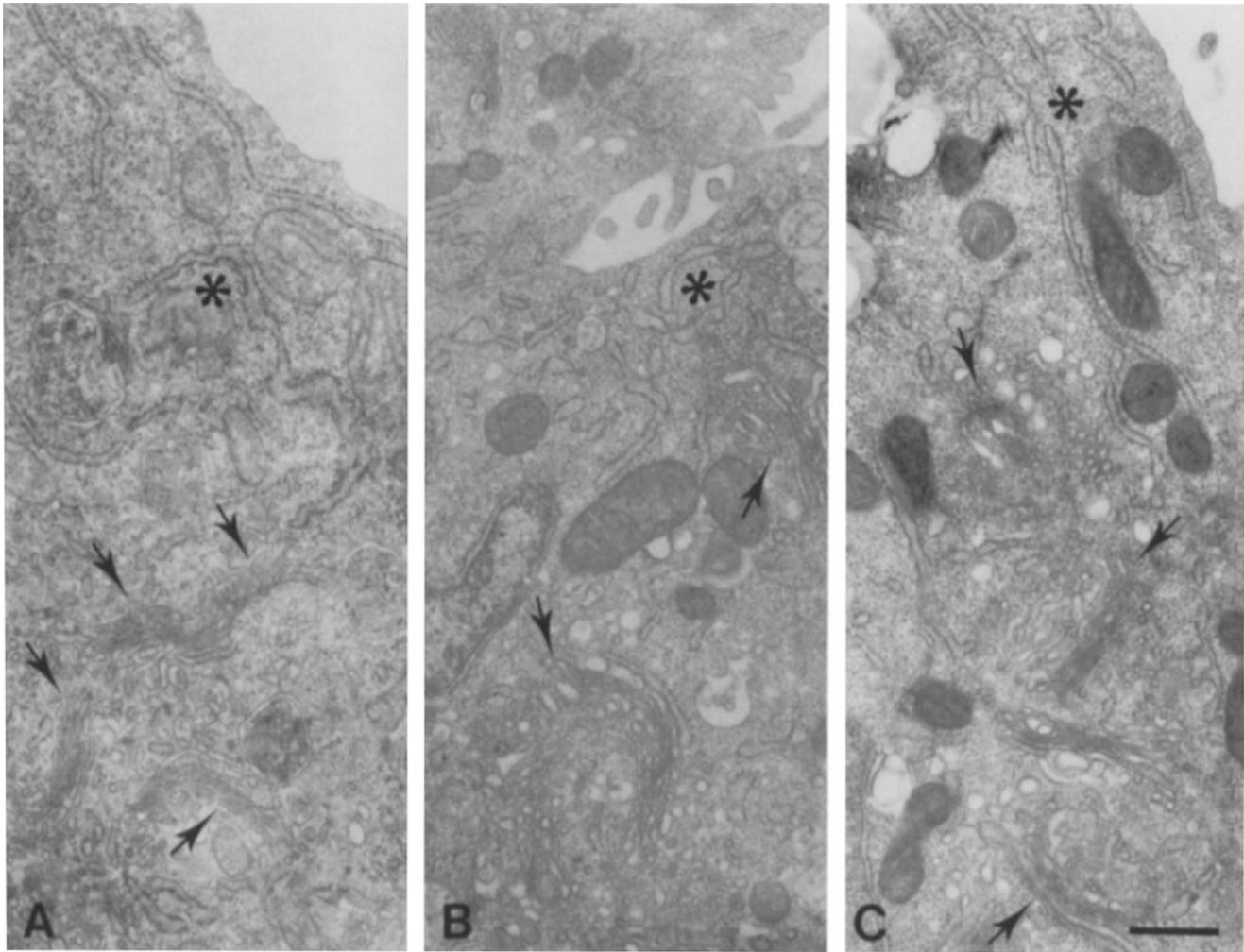


Figure 2. Morphology of intact and Strep O-permeabilized HepG2 cells analyzed by EM. The Golgi (arrows) and ER (asterisk) regions from untreated (A) and Strep O-permeabilized cells (using 0.4 U/ml Strep O), 1 h (B) and 2 h (C) after pore formation at 37°C, are indicated. Cells were kept under isotonic conditions until fixation.

be measured accurately in the proximity of the subcellular compartments concerned. When transport was partially blocked (as seen particularly clearly by addition of 0.1 mM GTP γ S at 0 min, and of 0.5 mM GTP γ S at 15 min), an α_1 -antitrypsin of slightly higher molecular weight was generated (Fig. 4 c). Inhibition of intracellular transport by GTP γ S could lead to a prolonged exposure to glycosyltransferases in compartments where α_1 -antitrypsin was trapped, and might explain this result.

BFA Induces Redistribution of Compartment(s) Containing $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ Sialyltransferases

Having shown that the anterograde pathway in Strep O-permeabilized cells is sensitive to GTP γ S, the preservation of subcellular organization as seen by EM (Fig. 2) justified investigating the sensitivity to GTP γ S of the retrograde pathway from the Golgi region to the ER. To study the retrograde pathway, the fungal antibiotic BFA was used. BFA blocks exit of proteins out of the ER while the transport from the Golgi region to the ER remains intact (Lippincott-Schwartz et al., 1990). When BFA is applied to intact HepG2 cells at the be-

ginning of a pulse with [³⁵S]methionine, an increase in molecular weight is observed for α_1 -antitrypsin at increasing times of exposure to BFA, concomitant with the acquisition of Endo H resistance (Fig. 5 a). Thus, in the presence of BFA, α_1 -antitrypsin is exposed in the ER to the action of otherwise Golgi-resident glycosyltransferases. BFA is not metabolized (see Fujiwara et al., 1988) under our experimental conditions. When the supernatant of a 4-h incubation of HepG2 cells with 10 μ g/ml of BFA was transferred to a fresh dish of pulse-labeled cells, the effects of this supernatant on N-linked glycan conversions were indistinguishable from freshly added BFA, and complete acquisition of Endo H resistance was observed (data not shown).

In control cells, full maturation of α_1 -antitrypsin results in a change in isoelectric point due to the acquisition of sialic acids, as verified by digestions with NANase (Fig. 5 b). In BFA-treated intact cells, α_1 -antitrypsin also acquires sialic acids (Fig. 5 b and Table I). In the presence of BFA, 51% of all α -antitrypsin acquired sialic acids after 4 h of treatment with BFA (Fig. 5 b and Table I). In the presence of 0.2 mM cycloheximide and BFA, comparable amounts of α_1 -antitrypsin received sialic acids (Fig. 5 b and Table I), ruling

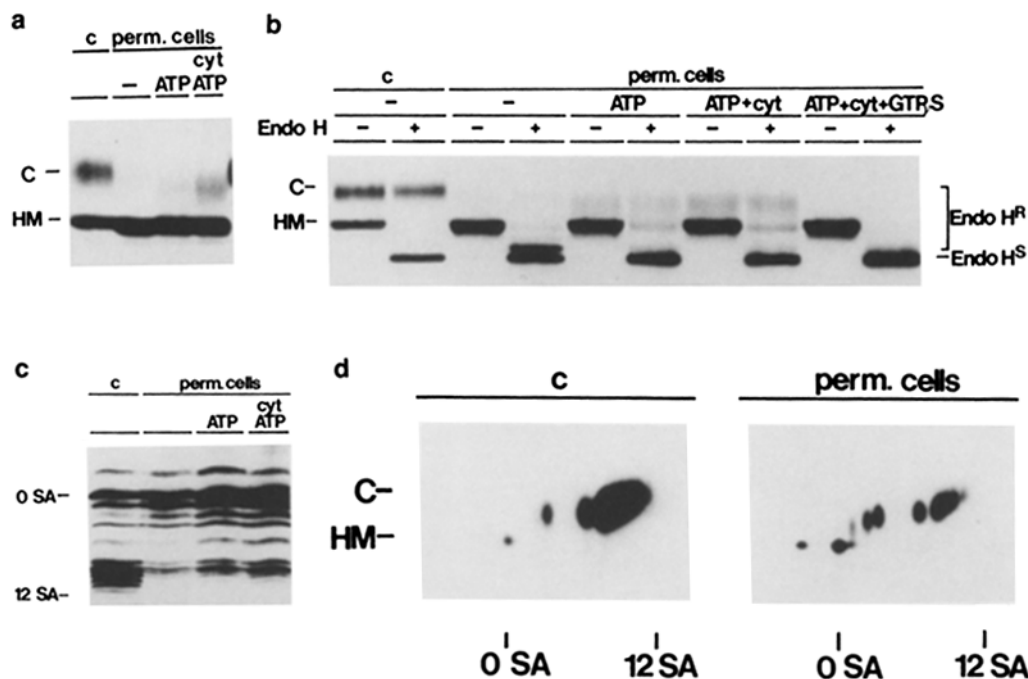


Figure 3. Reconstitution of intracellular transport of α_1 -antitrypsin in Strep O-permeabilized HepG2 cells. Positions of migration of α_1 -antitrypsin carrying high mannose-type (HM) and complex-type (C) oligosaccharides are indicated. (a) Cells were pulse-labeled with [35 S]methionine for 7 min at 37°C, treated with Strep O for 7 min at 0°C, and chased for 75 min at 37°C. ATP provided as an ATP-generating system and cytosol were added where indicated, at the beginning of the chase. α_1 -antitrypsin isolated by immunoprecipitation from the cell lysates was analyzed by SDS-PAGE (10% AA). (b) α_1 -Antitrypsin recovered from a pulse-chase experiment as in a was digested with Endo H where indicated. 1.0 mM GTP γ S was added at the beginning of the chase

where indicated. (c) α_1 -Anti-trypsin isolated from a pulse-chase experiment as in a was analyzed by one-dimensional IEF. (d) α_1 -Antitrypsin recovered as in a from intact and permeabilized cells, supplemented with ATP and cytosol, were analyzed by two-dimensional SDS-PAGE (10% AA).

out the possibility that newly synthesized glycosyltransferases were responsible for the carbohydrate modifications observed. The α_1 -antitrypsin produced after 4 h of exposure to BFA was fully Endo H resistant (Fig. 5 a), yet clearly distinct from mature α_1 -antitrypsin seen in control cells (Fig. 5, a and b). Thus conversion to complex-type oligosaccharides does occur but is incomplete. In contrast to a published report where CHO cells were used (Chege and Pfeffer, 1990), we did find sialylation, expressed as the percentage of total α_1 -antitrypsin carrying sialic acids, to be a rather prominent process in BFA-treated HepG2 cells (Table I and Fig. 5 b). The limited extent of sialic acid addition to glycoproteins in BFA-treated CHO cells has been interpreted as evidence that the action of sialyltransferases, considered a *trans*-Golgi/TGN marker, is not affected by BFA treatment. The situation may well differ for other cell types, such as HepG2.

In HepG2 cells both $\alpha_2 \rightarrow 3$ and $\alpha_2 \rightarrow 6$ sialyltransferases are responsible for the linkage of sialic acids to external galactose residues (Campion et al., 1989). In BFA-treated cells, α_1 -antitrypsin receives sialic acids (Fig. 5 b and Table I), but sialylation is incomplete, which might be the result of sequestration of sialyltransferases to compartments with differential sensitivity to BFA-induced redistribution. PNGase-released, sialic acid-containing oligosaccharides from total cell lysates of control and BFA-treated HepG2 cells were analyzed for the type ($\alpha_2 \rightarrow 3$ or $\alpha_2 \rightarrow 6$) of linkage of sialic acids. After 4 h of treatment with BFA, sialic acid-containing oligosaccharides were detectable by ion-exchange chromatography on a Mono Q column (equivalent to 27% of corresponding material from control cells; data not shown), consistent with the appearance of sialylated α_1 -antitrypsin

upon BFA treatment as analyzed by IEF (data not shown). Affinity chromatography using the $\alpha_2 \rightarrow 6$ sialic acid-specific lectin from SNA revealed that $\sim 73\%$ of the sialic acid-containing oligosaccharides from BFA-treated cells does not bind to the column (peak I; Fig. 6 and Table II), suggesting the presence of $\alpha_2 \rightarrow 3$ -linked sialic acids. The bound material (27%) eluted in two peaks corresponding to oligosaccharides containing 1 and ≥ 2 $\alpha_2 \rightarrow 6$ -linked sialic acids (peaks II and III, respectively; Fig. 6 and Table II). Corresponding oligosaccharides derived from control cells show comparable lectin-binding characteristics (Fig. 6). Thus, BFA induces the redistribution of compartment(s) containing both $\alpha_2 \rightarrow 3$ and $\alpha_2 \rightarrow 6$ sialyltransferase activity.

Retrograde Transport from Golgi to ER Is Sensitive to GTP γ S

To examine the effects of GTP γ S on BFA-induced redistribution to ER, GTP γ S and BFA were added simultaneously at the beginning of the chase. The fraction of labeled proteins resident in the ER was maximized by using a very short (1-min) pulse. In Strep O-permeabilized cells, BFA treatment also resulted in the generation of Endo H-resistant α_1 -antitrypsin (Fig. 5 c). Thus, in permeabilized cells Golgi-resident glycosyltransferases also redistributed to the ER, indicating that the retrograde pathway remained functional. Retrograde transport did not show a strict requirement for exogenously added ATP under our experimental conditions (but see Donaldson et al., 1991; Orci et al., 1991). After a 4-h exposure of permeabilized cells to BFA, only 8% of α_1 -antitrypsin remained Endo H sensitive as determined by densitometry of suitably exposed autoradiograms from the experiment shown in Fig. 5 c. Inclusion of GTP γ S inhibited

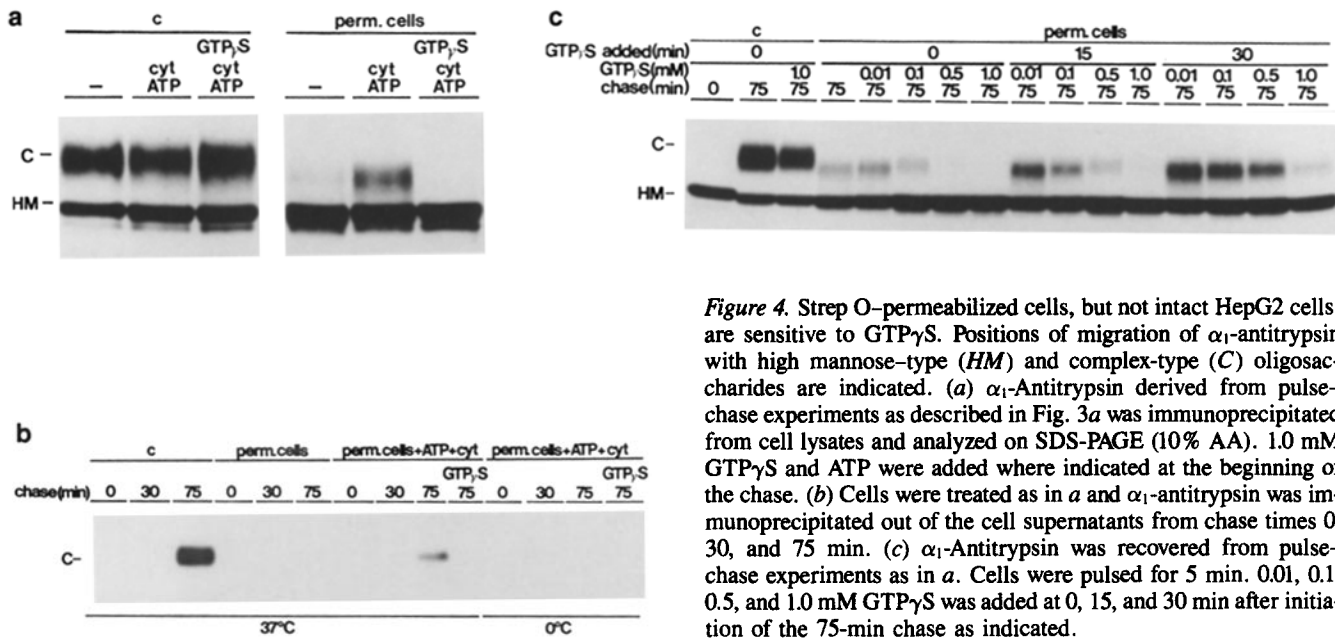


Figure 4. Strep O-permeabilized cells, but not intact HepG2 cells, are sensitive to GTP γ S. Positions of migration of α_1 -antitrypsin with high mannose-type (HM) and complex-type (C) oligosaccharides are indicated. (a) α_1 -Antitrypsin derived from pulse-chase experiments as described in Fig. 3a was immunoprecipitated from cell lysates and analyzed on SDS-PAGE (10% AA). 1.0 mM GTP γ S and ATP were added where indicated at the beginning of the chase. (b) Cells were treated as in a and α_1 -antitrypsin was immunoprecipitated out of the cell supernatants from chase times 0, 30, and 75 min. (c) α_1 -Antitrypsin was recovered from pulse-chase experiments as in a. Cells were pulsed for 5 min. 0.01, 0.1, 0.5, and 1.0 mM GTP γ S was added at 0, 15, and 30 min after initiation of the 75-min chase as indicated.

this acquisition of Endo H resistance: 73% of α_1 -antitrypsin remained Endo H sensitive. GTP γ S had no effect on the action of BFA in intact cells. We conclude that GTP γ S interferes with the redistribution of Golgi-resident glycosyltransferases to the ER in permeabilized cells.

Discussion

Intracellular protein traffic has been examined not only in intact cells, but also in vitro using cell-free systems (Balch et al., 1984) and "semi-intact" cells produced by the nitrocellulose filter stripping technique (Simons and Virta, 1987; deCurtis and Simons, 1988; Bennett et al., 1989; Podbilewicz and Mellman, 1990). Other methods for permeabilization include the swelling technique (Beckers et al., 1987) and the use of Strep O (Gravotta et al., 1990; Miller and Moore, 1991) as also used in the present study for HepG2 cells. Analysis of glycoprotein traffic is facilitated by the abundant levels of the well-characterized secretory glycoprotein α_1 -antitrypsin produced by HepG2 cells. To min-

imize possible damage to intracellular organelles, low concentrations of Strep O were used. The toxin was allowed to bind to the plasma membrane at 0°C, followed by removal of unbound Strep O before initiation of pore formation at 37°C. Since binding of Strep O to cholesterol is irreversible (Duncan and Schlegel, 1975), pore formation is thus largely restricted to the plasma membrane. Analysis of Strep O-treated cells using EM demonstrated that in permeable cells the intracellular organelles retained the gross morphology as seen in intact cells (Fig. 2). Minor differences in morphology concern the extent of vacuolization, which appears slightly more prominent in the Golgi region of Strep O-permeabilized cells. Such differences could be responsible, at least in part, for our inability to reconstitute N-linked glycan maturation to the level seen in control cells. Membrane-impermeant molecules such as GTP γ S can enter the permeabilized cells, and only a limited release of cytosolic proteins such as lactate dehydrogenase (Fig. 1, a and b) and soluble low molecular weight GTP-binding proteins (Fig. 1 c) is evident, while >98% were permeable as measured by trypan blue staining (data not shown). The levels of nucleotide and nucleotide sugars recovered in an ethanolic extract of control and permeabilized cells revealed minor differences. Only a twofold reduction in the intracellular level of GTP was observed. Permeabilization with Strep O may therefore be considered a relatively mild permeabilization procedure.

Permeabilization of HepG2 cells reduced intracellular transport of the reporter glycoprotein α_1 -antitrypsin, presumably because of loss of essential cytosolic factors, the identity of which remains to be established, but the loss of transport could be partially overcome by the addition of an ATP-generating system and cytosol (Fig. 3 a). The requirement for cytosolic factors was found to be variable. It is noteworthy that there are no strict requirements for ATP and cytosol in intracellular transport in nitrocellulose perforated MDCK and BHK cells (Bennett et al., 1989), which is in

Table I. BFA Induces Acquisition of Sialic Acids

Sample	Chase	Unsialylated	Sialylated
	min	%	%
- BFA	0	100	0
- BFA	120	51	49
- BFA + NANA	120	87	13
+ BFA	240	49	51
+ BFA + NANA	240	76	24
+ BFA + cycloheximide	240	46	54
+ BFA + cycloheximide + NANA	240	78	22

The percent of unsialylated and sialylated α_1 -antitrypsin isolated from cell lysates of control and BFA-treated cells was determined with gel scanning densitometry from Fig. 5 b. The α_1 -antitrypsin produced at 0 min of chase was taken as the fully unsialylated form.

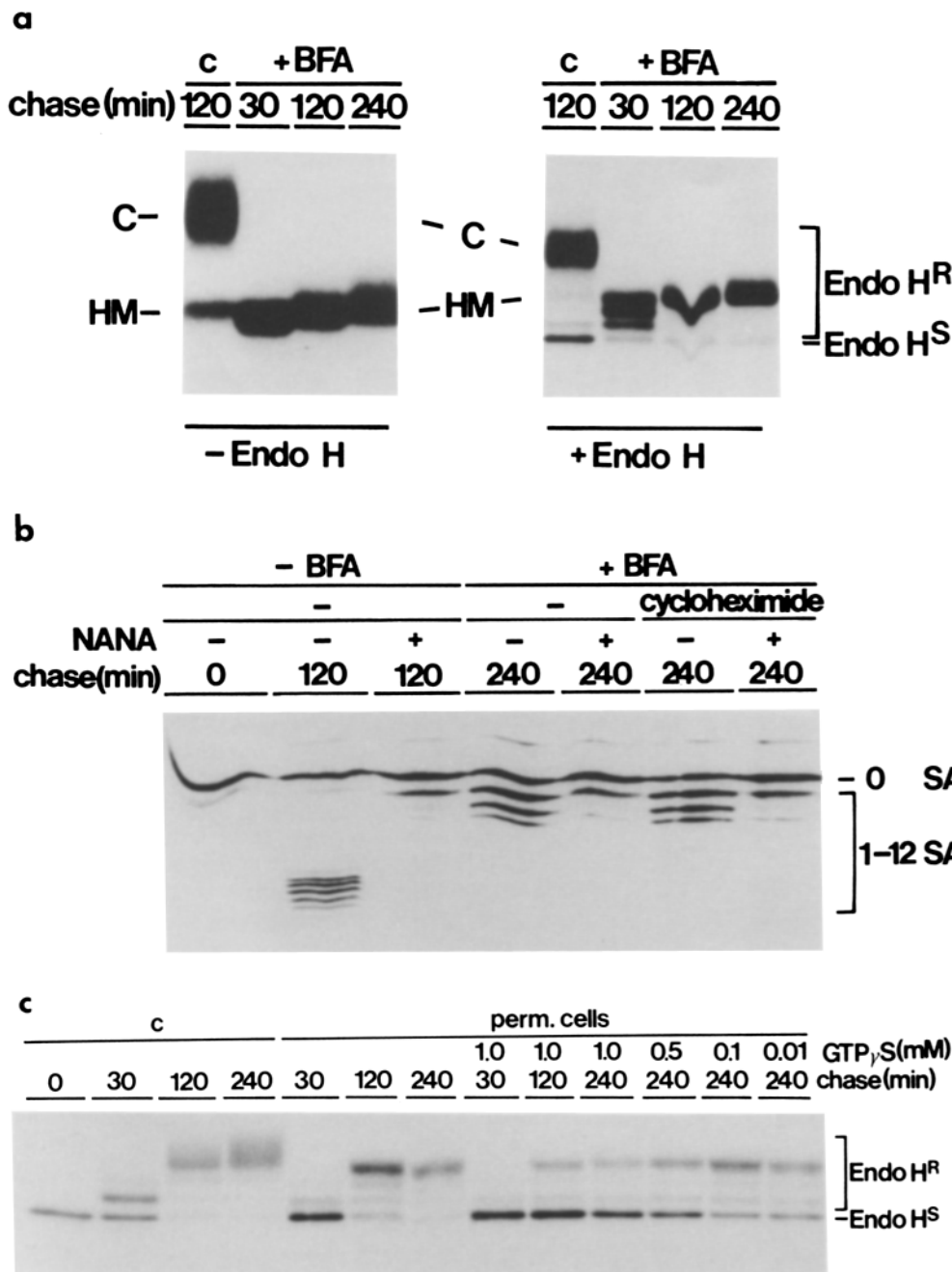


Figure 5. Redistribution of Golgi-resident glycosyltransferases occurs in Strep O-permeabilized HepG2 cells and is sensitive to GTP γ S. (a) Intact cells were pulsed for 10 min with [³⁵S]methionine and chased for 30, 120, and 240 min. 10 μ g/ml BFA was added at the beginning of the pulse and present during the entire pulse-chase experiment. α_1 -Antitrypsin was isolated by immunoprecipitations from the cell lysates and analyzed on SDS-PAGE (10% AA). Digestions were performed with Endo H where indicated. (b) Intact cells were pulsed with [³⁵S]methionine for 2 min and 45 s, followed by a chase of 0, 120, or 240 min. 5 μ g/ml BFA and 0.2 mM cycloheximide were included at the beginning of the chase where indicated. α_1 -Antitrypsin was isolated as in a and digested with NANase before analysis by IEF. (c) Cells were pulsed with [³⁵S]methionine for 1 min. Intact cells (c) and cells treated with Strep O (*perm. cells*) were chased for 0, 30, 120, or 240 min as indicated. 1.0, 0.5, 0.1, and 0.01 mM GTP γ S where indicated and 10 μ g/ml BFA were included at the beginning of the chase. α_1 -Antitrypsin was immunoprecipitated from the cell lysates. All samples were digested with Endo H before analysis on SDS-PAGE (10% AA).

contrast to requirements for transport processes in cell-free systems (Balch et al., 1984). Apparently, such perforated cells retain sufficient levels of cytosolic proteins and perhaps an adequate source of energy essential for transport, unless cytosol is removed by washing before incubation (Bennett et al., 1989).

In Strep O-permeabilized cells the entire constitutive secretory pathway from ER to the actual steps of secretion could be reconstituted (Fig. 3 a and Fig. 4 b). In permeabilized HepG2 cells, glycosyltransferases remained active and could still induce acquisition of Endo H resistance of α_1 -antitrypsin (Fig. 3 b). Addition of sialic acids likewise occurred, but the extent of glycan modifications was less than that seen in control cells (Fig. 3 c), as also revealed by analysis of

α_1 -antitrypsin on two-dimensional SDS-PAGE (Fig. 3 d). Release of membrane vesicles through Strep O-induced pores we consider unlikely, as these pores have been reported to be too small (20–30 nm in diameter [Bhakdi et al., 1985]) to allow passage of exocytic vesicles (80–300 nm in diameter [de Curtis and Simons, 1988]). Furthermore, α_1 -antitrypsin secreted by Strep O-permeabilized cells was not sedimentable by centrifugation at 100,000 g and was accessible to antibodies in the absence of detergent (data not shown). It was therefore released in a soluble form. Had the α_1 -antitrypsin in the supernatant from Strep O-permeabilized HepG2 cells arisen from breakage of cells, or indiscriminate release of vesicular material, representation of both Endo H-sensitive and -resistant α_1 -antitrypsin would

Table II. SNA Lectin Affinity Chromatography of Sialic Acid-containing Oligosaccharides Isolated from Total Cell Lysates of Control and BFA-treated Cells

Peak	Control cells		BFA-treated cells	
		%		%
I Unbound no $\alpha 2 \rightarrow 6$ Si		79		73
II Retarded 1 $\alpha 2 \rightarrow 6$ Si		13		15
III Bound ≥ 2 $\alpha 2 \rightarrow 6$ Si		8		12

The percent of unbound, retarded, and bound radioactive sialic acid-containing oligosaccharides was determined from the experiment shown in Fig. 6. The recovered radioactivity is expressed as percent of the total radioactivity.

have been the expected result. That fraction of intracellular transport and glycan modifications supported by the Strep O-permeabilized cell preparation was completely sensitive to GTP γ S (Fig. 4 a, see below). Thus, the contribution to transport and glycan modification of α_1 -antitrypsin by a minor population of intact cells that could have escaped the permeabilization procedure is effectively excluded.

In cell-free systems, the inhibitory action of GTP γ S has been established for vesicular transport from the ER to Golgi region (Beckers and Balch, 1989) and between Golgi cisternae (Melançon et al., 1987). Recently, in Strep O-permeabilized cells, transport of a viral glycoprotein from the TGN to the plasma membrane was reported to be sensitive to GTP γ S (Gravotta et al., 1990; Miller and Moore, 1991). We found that the membrane-impermeant agent GTP γ S inhibited the secretory pathway (Fig. 4, a and b), in accord with current evidence on the involvement of small GTP-binding proteins of the rab gene family in the secretory pathway (Chavrier et al., 1990; Goud et al., 1990; Pluttner et al., 1990). The sensitivity to GTP γ S of N-linked glycan modifications that accompany transport through the TGN was also dependent on the time of addition, indicating that GTP γ S must be present at an early stage of transport for full inhibition (Fig. 4 c). To achieve complete inhibition of transport to TGN, high concentrations of GTP γ S were required (Fig. 4 c). We consider this likely to be a consequence of the use of mildly permeabilized cells. The rate at which these cells lose GTP through diffusion is low (Fig. 1 d), and probably distinct from that observed in more harshly permeabilized cells or cell-free systems. For inhibition of transport of a viral glycoprotein from the TGN to the cell surface in Strep O-permeabilized cells, 100 μ M of GTP γ S was used to achieve maximal inhibition (Gravotta et al., 1990). Penetration of GTP γ S to compartments located at a greater distance from the plasma membrane would be expected to require a steeper gradient of GTP γ S to achieve the requisite local inhibitory concentration.

Not only the anterograde pathway, but also the retrograde pathway from the Golgi region to the ER could be examined using Strep O-permeabilized HepG2 cells. The anterograde and retrograde pathways are responsible for membrane recycling between ER and Golgi cisternae (Farquhar, 1985). The redistribution of Golgi-resident glycosyltransferases to ER in intact HepG2 cells was assessed by acquisition of

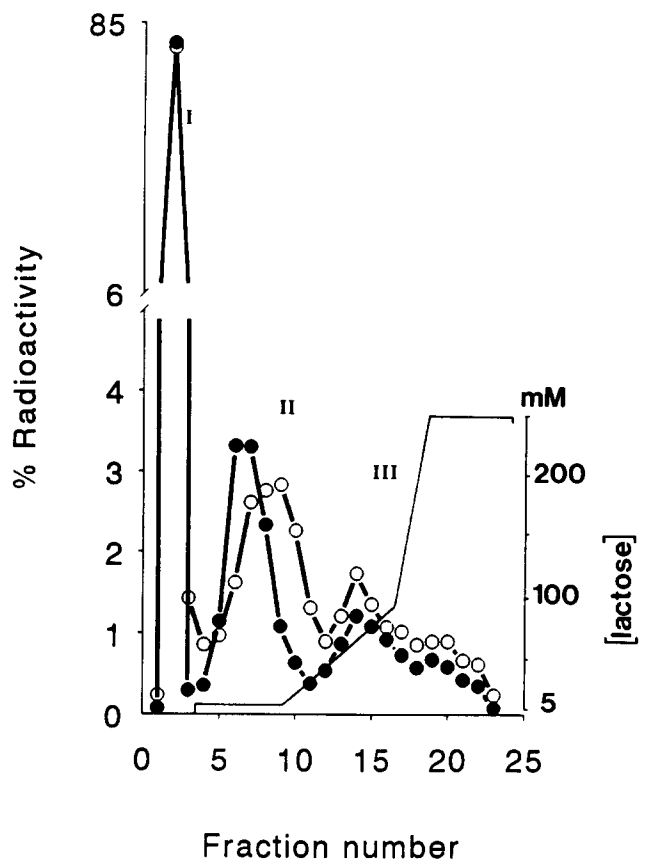


Figure 6. SNA affinity chromatography of sialic acid-containing oligosaccharides isolated from total cell lysates of control and BFA-treated HepG2 cells. Intact HepG2 cells were labeled with [3 H]-mannose for 4 h in absence and presence of BFA. Oligosaccharides were released from the glycoproteins present in total cell lysates by digestion with PNGase and separated by Mono Q exchange chromatography. The sialic acid-containing oligosaccharide fractions from BFA-treated cells (○) and the corresponding control (●) oligosaccharide fractions were analyzed on an $\alpha 2 \rightarrow 6$ -specific SNA lectin column as described in Materials and Methods. The SNA column was developed with 5 mM lactose, followed by a broken gradient from 5 to 250 mM lactose as indicated. Peaks I, II, and III correspond with the unbound, retarded, and bound sialic acid-containing oligosaccharides.

Endo H resistance (Fig. 5 a) and by addition of sialic acids of α_1 -antitrypsin in the ER (Fig. 5 b). In contrast to reports suggesting that *trans*- and TGN-resident glycosyltransferases do not relocate to the ER in BFA-treated cells (largely based on the lack of sialic acid addition) (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Chen et al., 1991), our results suggest that a sialyltransferase-containing compartment does redistribute rather efficiently to ER in the presence of BFA (Fig. 5 b and Fig. 6). Also in BFA-treated MEL cells, sialyltransferases redistribute to the ER (Ulmer and Palade, 1989). Thus, the effects of BFA on redistribution of sialyltransferases can differ between cell types. Nuclear magnetic resonance studies have demonstrated that transferrin, isolated from HepG2 cells, contains N-linked glycans with both $\alpha 2 \rightarrow 3$ - and $\alpha 2 \rightarrow 6$ -linked sialic acids (Campion et al., 1989), and therefore both enzymatic activities must be present. Affinity chromatography of sialic acid-containing oligosac-

charides isolated from total HepG2 cell lysates on the SNA $\alpha 2 \rightarrow 6$ sialic acid-specific lectin revealed that glycoproteins synthesized in BFA-treated HepG2 cells can acquire both $\alpha 2 \rightarrow 3$ - and $\alpha 2 \rightarrow 6$ -linked sialic acids (Fig. 6 and Table II). Both $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ sialyltransferases relocate at least partially to the ER as a consequence of BFA treatment. However, the extent of sialylation of α_1 -antitrypsin in BFA-treated cells is significantly lower than that of the normal mature glycoprotein found in control cells (Fig. 5 b). Sialyltransferases involved in ganglioside biosynthesis in primary cultures of murine cerebellar cells (sialyltransferases type I and II) relocate to ER after treatment with BFA, whereas type IV and V do not (van Echten et al., 1990). Our results show that BFA apparently does not distinguish between $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ sialyltransferase-containing compartments.

In Strep O-permeabilized cells, the retrograde pathway remained operative in the presence of BFA (Fig. 5 c). Addition of GTP γ S to BFA-treated Strep O-permeabilized cells strongly inhibited acquisition of Endo H resistance of α_1 -antitrypsin during chase in a concentration-dependent manner (Fig. 5 c). Therefore, not only the anterograde pathway, but also the retrograde pathway, is sensitive to GTP γ S. While delivery of α_1 -antitrypsin to the TGN in the anterograde pathway could be blocked completely by the early inclusion of GTP γ S, its inhibitory effects on retrograde transport were $\sim 73\%$. The lack of complete inhibition may result from differences in accessibility of the GTP-binding proteins involved, or could reflect more profound differences between the anterograde and retrograde pathways. The latter is known to depend on the integrity of the microtubule system, whereas the former is not (Lippincott-Schwartz et al., 1990). The BFA-induced formation of tubular networks from Golgi cisternae in a cell-free system, considered to be the in vitro equivalents of the membrane tubules mediating retrograde transport in vivo, is not sensitive to GTP γ S (Orci et al., 1991). This lack of inhibition might explain the partial sensitivity of the retrograde pathway to GTP γ S observed in Strep O-permeabilized HepG2 cells. Alternatively, an essential step other than the formation of such tubules might be sensitive to GTP γ S. Our data are in accord with recent findings suggesting the interference of GTP γ S in the BFA-induced dissociation of the 110-kD protein from Golgi membranes, and redistribution of the *cis*/medial Golgi-resident mannosidase II to the ER as monitored by immunofluorescence (Donaldson et al., 1991). Thus, it is likely that GTP-binding proteins are involved in both the anterograde and retrograde pathways of intracellular membrane traffic.

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References

- Bailey, E., M. McCaffrey, N. Touchot, A. Zahraoui, B. Goud, and M. Bornens. 1991. Phosphorylation of two small GTP-binding proteins of the Rab family by p34^{cdc2}. *Nature (Lond.)*. 350:715-718.
- Balch, W. E., B. S. Glick, and J. E. Rothman. 1984. Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell*. 39:525-536.
- Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245-1256.
- Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell*. 50:523-534.
- Beckers, C. J. M., M. R. Block, B. S. Glick, J. E. Rothman, and W. E. Balch. 1989. Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature (Lond.)*. 339:397-398.
- Bennett, M. F., A. Wandinger-Ness, I. deCurtis, C. Anthony, and K. Simons. 1989. Perforated cells for studying intracellular membrane transport. *Methods Cell Biol.* 31:103-126.
- Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit. 1985. Mechanisms of membrane damage by streptolysin O. *Infect. Immun.* 47:52-60.
- Bourne, H. R. 1988. Do GTPase direct membrane traffic in secretion? *Cell*. 53:669-671.
- Buckingham, L., and J. L. Duncan. 1983. Approximate dimensions of membrane lesions produced by streptolysin S and streptolysin O. *Biochim. Biophys. Acta*. 729:115-122.
- Campion, B., D. Legler, J.-M. Wieruszkeski, J. Montreuil, and G. Spik. 1989. Presence of fucosylated triantennary, tetraantennary and pentaantennary glycans in transferrin synthesized by the human hepatoma cell line HepG2. *Eur. J. Biochem.* 184:405-413.
- Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to the exocytic and endocytic compartments. *Cell*. 62:317-329.
- Chege, N. W., and S. R. Pfeffer. 1990. Compartmentation of the Golgi complex: brefeldin A distinguishes *trans*-Golgi cisternae from the *trans*-Golgi network. *J. Cell Biol.* 111:893-899.
- Chen, S.-Y., Y. Matsuoka, and R. W. Compans. 1991. Assembly and polarized release of punta toro virus and effects of Brefeldin A. *J. Virol.* 65:1427-1439.
- Clary, D. O., J. C. Griff, and J. E. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell*. 61:709-721.
- deCurtis, I., and K. Simons. 1988. Dissection of Semliki forest virus glycoprotein delivery from the *trans*-Golgi network to the cell surface in permeabilized BHK cells. *Proc. Natl. Acad. Sci. USA*. 85:8052-8056.
- Diaz, R., L. S. Mayorga, P. J. Weidman, J. E. Rothman, and P. D. Stahl. 1989. Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature (Lond.)*. 339:398-400.
- Doms, R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* 109:61-72.
- Donaldson, J. G., J. Lippincott-Schwartz, and R. D. Klausner. 1991. Guanine nucleotides modulate the effects of brefeldin A in semi-permeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* 112:579-588.
- Duncan, J. L., and R. Schlegel. 1975. Effect of streptolysin O on erythrocyte membranes, liposomes, and lipid dispersions. *J. Cell Biol.* 67:160-173.
- Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447-488.
- Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. 1989. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* 263:18545-18552.
- Goud, B., A. Zahraoui, A. Tavitian, and J. Saraste. 1990. Small GTP-binding proteins associated with Golgi cisternae. *Nature (Lond.)*. 345:553-556.
- Gravotta, D., M. Adesnik, and D. D. Sabatini. 1990. Transport of influenza HA from the *trans*-Golgi network to the apical surface of MDCK cells permeabilized in their basolateral plasma membranes: energy dependence and involvement of GTP-binding proteins. *J. Cell Biol.* 111:2893-2908.
- Griffiths, G., and K. Simons. 1986. The *trans* Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC)*. 234:438-443.
- Johnston, P. A., B. T. Archer III, K. Robinson, G. A. Mignery, R. Jahn, and T. C. Südhof. 1991. Rab3A attachment to the synaptic vesicle membrane mediated by a conserved poly-isoprenylated carboxy-terminal sequence. *Neuron*. 7:101-109.
- Kanbayashi, Y., M. Hotta, and J. Koyama. 1972. Kinetic study on streptolysin O. *J. Biochem.* 71:227-237.
- Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lapetina, E. G., and B. R. Reep. 1987. Specific binding of [α -³²P]GTP to cytosolic and membrane-bound proteins of human platelets correlates with the activation of phospholipase C. *Proc. Natl. Acad. Sci. USA*. 84:2261-2265.
- Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacio, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell*. 56:801-813.
- Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retro-

- grade transport of proteins into the ER in the presence of brefeldin A suggest an ER recycling pathway. *Cell*. 60:821-836.
- Melançon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Seratini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell*. 51:1053-1062.
- Miller, S. G., and H. P. H. Moore. 1991. Reconstitution of constitutive secretion using semi-intact cells: regulation by GTP but not calcium. *J. Cell Biol.* 112:39-54.
- Neeffes, J. J., B. S. Breur-Vriesendorp, G. A. van Seventer, P. Iványi, and H. L. Ploegh. 1986. An improved biochemical method for the analysis of HLA class I antigens. Definition of new HLA class I subtypes. *Hum. Immunol.* 16:169-181.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell*. 64:1183-1195.
- Pels-Rijcken, W. R., G. J. M. Hooghwinkel, and W. Ferwerda. 1990. Pyrimidine metabolism and sugar nucleotide synthesis in rat liver. *Biochem. J.* 266:777-783.
- Pluttner, H., R. Schwaninger, S. Pind, and W. E. Balch. 1990. Synthetic peptides of the *rab* effector domain inhibit vesicular transport through the secretory pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2375-2383.
- Podbilewicz, B., and I. Mellman. 1990. ATP and cytosol requirements for transferrin recycling in intact and disrupted MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3477-3487.
- Roth, J. 1987. Subcellular organization of glycosylation in mammalian cells. *Biochim. Biophys. Acta.* 906:405-436.
- Salminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell*. 49:527-538.
- Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretory machinery. *Cell*. 52:915-924.
- Serafini, T., G. Stenbeck, A. Brecht, F. Lottspeich, L. Orci, J. E. Rothman, and F. T. Wieland. 1991. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein β -adaptin. *Nature (Lond.)*. 349:215-220.
- Shibuya, N., I. J. Goldstein, W. F. Broekaert, M. Nsimba-Lubaki, B. Peeters, and W. J. Peumans. 1987. The Elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α 2-6)Gal/GalNAc sequence. *J. Biol. Chem.* 262:1596-1601.
- Simons, K., and H. Virta. 1987. Perforated MDCK cells support intracellular transport. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2241-2247.
- Ulmer, J. B., and G. E. Palade. 1989. Targeting and processing of glycoporphins in murine erythroleukemia cells: use of brefeldin A as a perturbant of intracellular traffic. *Proc. Natl. Acad. Sci. USA.* 86:6992-6996.
- Van der Sluijs, P., M. Hull, A. Zahraoui, A. Tavitian, B. Goud, and I. Mellman. 1991. The small GTP-binding protein rab4 is associated with early endosomes. *Proc. Natl. Acad. Sci. USA.* 88:6313-6317.
- van Echten, G., H. Iber, H. Stotz, A. Takatsuki, and K. Sandhoff. 1990. Uncoupling of ganglioside biosynthesis by brefeldin A. *Eur. J. Cell Biol.* 51:135-139.
- Wattenberg, B. W., and J. E. Rothman. 1986. Multiple cytosolic components promote intra-Golgi protein transport. Resolution of a protein acting at a late stage, prior to membrane fusion. *J. Biol. Chem.* 261:2208-2213.
- Wattenberg, B. W., R. R. Hirsch, L. W. LeCreux, and M. P. White. 1990. Identification of a 25 kD protein from yeast cytosol that operates in a prefusion step of vesicular transport between compartments of the Golgi. *J. Cell Biol.* 110:947-954.
- Zahraoui, A., N. Touchot, P. Chardin, and A. Tavitian. 1989. The human *rab* genes encode a family of GTP-binding proteins related to yeast YPT1 and Sec4 products involved in secretion. *J. Biol. Chem.* 264:12394-12401.