Glycolipid and Glycoprotein Transport through The Golgi Complex Are Similar Biochemically and Kinetically. Reconstitution of Glycolipid Transport in a Cell Free System

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Abstract. Glycolipid transport between compartments of the Golgi apparatus has been reconstituted in a cell free system. Transport of lactosylceramide (galactose β1-4-glucose-ceramide) was followed from a donor to an acceptor Golgi population. The major glycolipid in CHO cells is G_{M3} (sialic acid α2-3 galactose β1-4-glucose-ceramide). Donor membranes were derived from a Chinese hamster ovary (CHO) cell mutant (Lec2) deficient in the Golgi CMP-sialic acid transporter, and therefore contained lactosylceramide as the predominant glycolipid. Acceptor Golgi apparatus was prepared from another mutant, Lec8, which is defective in UDP-Gal transport. Thus, glucosylceramide is the major glycolipid in Lec8 cells. Transport was measured by the incorporation of labeled sialic acid into lactosylceramide (present originally in the donor) by transport to acceptor membranes, forming G_{M3}. This incorporation was dependent on ATP, cytosolic components, intact membranes, and elevated temperature. Donor membranes were prepared from Lec2 cells infected with vesicular stomatitus virus (VSV). These membranes therefore contain the VSV membrane glycoprotein, G protein. Donor membranes derived from VSV-infected cells could then be used to monitor both glycolipid and glycoprotein transport. Transport of these two types of molecules between Golgi compartments was compared biochemically and kinetically. Glycolipid transport required the *N*-ethylmaleimide sensitive factor previously shown to act in glycoprotein transport (Glick, B. S., and J. E. Rothman. 1987. *Nature [Lond.].* 326:309–312; Rothman, J. E. 1987. *J. Biol. Chem.* 262:12502–12510). GTPγS inhibited glycolipid and glycoprotein transport similarly. The kinetics of transport of glycolipid and glycoprotein were also compared. The kinetics of transport to the end of the pathway were similar, as were the kinetics of movement into a defined transport intermediate. It is concluded that glycolipid and glycoprotein transport through the Golgi occur by similar if not identical mechanisms.

THE mechanisms that transport lipids between intracellular organelles are not well understood. This contrasts to the detailed picture that is emerging for protein trafficking. This difference is partly because the distribution of lipids among intracellular membranes does not generally show the absolute specificity that protein distribution does. Secondly, the movement and functioning of lipids is dominated by subtle physical/chemical properties, such as acyl chain mobility, and not by the sequence specificity that can be imparted by an amino acid chain. The distribution of lipids in membrane biogenesis has been covered recently in several excellent reviews (Sleight, 1987; Bishop and Bell, 1988; van Meer, 1989).

Some lipids, in particular phospholipids, may be transported by monomeric diffusion facilitated by exchange proteins. Phosphatidylethanolamine and phosphatidylcholine are in large part transported from their site of synthesis in the ER to the plasma membrane without an appreciable lag time (Sleight and Pagano, 1983; Kaplan and Simoni, 1985*a*) and independent of cellular energy. On the other hand, the characteristics of cholesterol and sphingolipid transport are consistent with a vesicular mechanism. For both, there is a marked lag time after synthesis before they appear at the plasma membrane (Miller-Podraza and Fishman, 1982; DeGrella and Simoni, 1982; Lange and Matthies, 1984; Kaplan and Simoni, 1985*b*). Cholesterol transport is sensitive to inhibitors of metabolic energy production (DeGrella and Simoni, 1982; Kaplan and Simoni, 1985*b*). This energy requirement has not been demonstrated for sphingolipid movement. The involvement of the Golgi apparatus in sphingolipid transport was demonstrated in an elegant series of studies by Pagano and associates using fluorescent analogues of ceramide that are incorporated into sphingomyelin and glucosylceramide (Lipsky and Pagano, 1983; Lipsky and Pagano, 1985).

Sphingolipids are a particularly interesting group of lipid molecules. They exhibit a very tight transmembrane asymmetry; they are mostly if not exclusively, found on the exoplasmic surface (i.e., lumenal in the Golgi) (for review, see Op den Kamp, 1979; van Meer, 1989). This is a consequence

of the lumenal orientation in the Golgi of the enzymes that transfer choline or sugars to ceramide to form sphingomyelin and glycolipids respectively (Lipsky and Pagano, 1985; and references therein; Keenan et al., 1974). There is apparently little or no flip/flop of these molecules between leaflets. The bulk of gangliosides are found on the plasma membrane (Miller-Podraza et al., 1982). Moreover, in polarized epithelia both sphingomyelin and glycosphingolipids are concentrated into the apical membrane domain (van Meer and Simons, 1986; and references therein). The ability to be targeted in this way may be related to the physical properties of these lipids. They can form intermolecular hydrogen bonds (Pascher, 1976), which can result in the formation of sphingolipid aggregates (Rintoul et al., 1979). In addition, sphingolipids exhibit a very high affinity for cholesterol (de Kruyff et al., 1973). Because of these properties, sphingolipids have been implicated in targeting of both proteins (van Meer and Simons, 1986) and cholesterol (Wattenberg and Silbert, 1983) to the cell surface.

The experimental evidence cited above, along with the lumenal orientation of the molecules, strongly suggests that sphingolipids are transported by a vesicular mechanism. The simplest model would be transport via the carriers that bring proteins to the plasma membrane. This is incorporated into the "bulk flow" model of transport (for review, see Pfeffer and Rothman, 1987). In such a system there is continuous vesicular movement through the Golgi and on to the plasma membrane. These vesicles pinch off a nonselective sample of the membrane and transport it to the next organelle in the pathway. Only molecules that are selectively retained in an organelle are excluded from the vesicles.

The studies presented here address whether glycosphingolipids and proteins are transported by the same mechanism. Glycoprotein transport through the Golgi has been extensively studied using a cell free system (for review, see Pfeffer and Rothman, 1987; Goda and Pfeffer, 1989; Wattenberg, 1990). This system uses glycosylation as a marker for transport from one Golgi compartment to the next. Glycoprotein transport requires energy as well as proteins found on the Golgi membranes and in the cytosolic fraction (Balch et al., 1984*a*). One such protein is the *N*-ethylmaleimide sensitive factor (NSF),[1] which acts after the attachment of transport vesicles to the target Golgi (Malhotra et al., 1988). It has been shown to participate in transport from the ER to the Golgi (Beckers et al., 1989) through the medial (Glick and Rothman, 1987) and *trans*-Golgi (Rothman, 1987) compartments and in endocytosis (Diaz et al., 1989). Furthermore, the nonhydrolyzable GTP analogue GTPγs blocks transport by inhibiting the fusion of transport vesicles with the target membrane (Melançon et al., 1987). These diagnostic features provide the means to compare the transport mechanisms of different molecules.

Here I describe the formulation of a cell free assay for the transport of a glycolipid between Golgi compartments. This assay, described in detail in Results, marks transport by using the sequential glycosylation reactions that produce mature glycosphingolipids in the Golgi. Except for the molecules being measured, it is identical to the assay for glycoprotein transport, therefore facilitating a direct comparison between the two. Using these systems, glycolipid and glycoprotein transport were found to be biochemically and kinetically similar.

1. *Abbreviations used in this paper*: G_{M3}, 2,3 sialyl lactosylceramide; LCRI, low cytosol requiring intermediate; NEM, *N*-ethylmaleimide; NSF, NEM sensitive factor; VSV, vesicular stomatitus virus.

Materials and Methods

Materials

[^{3}H]CMP-sialic acid (26 Ci/mMol) was from New England Nuclear (Boston, MA). Triton X-100 was purchased from Sigma Chemical Co. (St. Louis, MO). GTPγS was from Boehringer Mannheim GmbH (Mannheim, FRG). Control IgM, myeloma TEPC 183, was from Bionetics (Charleston, NC). Lipid standards were from Sigma Chemical Co. Ascites fluid containing a monoclonal antibody to vesicular stomatitis virus (VSV) G protein (clone 8G5) (Lefrancois and Lyles, 1982) was a gift from Dr. Leo Lefrancois of this department. Polyclonal rabbit anti-mouse serum was obtained from Cappel Laboratories (Malvern, PA). ENHANCE was purchased from New England Nuclear. X-Omat AR film was from Kodak (Rochester, NY). Thin layer chromatography plates were purchased from E. Merck (Darmstadt, FRG). C-18 Sep Pak cartridges were from Waters Associates (Milford, MA). The purified NSF (Block et al., 1988) and antibody to that protein were generous gifts of P. Weidman and J. Rothman (Department of Molecular Biology, Princeton, NJ). All other materials were purchased from commercial sources and were of reagent grade.

Cells and Virus

Lec2 and Lec8 (American Type Culture Collection, Rockville, MD; Stanley and Siminovitch, 1977) were grown in monolayer culture in a modified MEM containing 10% FCS. Wild-type Chinese hamster ovary (CHO) cells were grown in suspension culture in the same medium. Infections of Lec2 with VSV were performed as previously described (Balch et al., 1984*a*) for clone 15B. Stocks of VSV (Indiana strain) were harvested from infected baby hamster kidney cells as described (Balch et al., 1984*a*).

Membranes and Cytosol

Donor membranes were produced from either uninfected or VSV-infected Lec2 cells essentially as described (Balch and Rothman, 1985) using a stainless steel ball bearing homogenizer to produce homogenates. After enrichment of Golgi membranes by sucrose density centrifugation (Balch and Rothman, 1985), the typical protein concentration was 0.5–1 mg/ml. Acceptor membranes from uninfected Lec8 cells were produced in the same manner.

Cytosol was derived either from wild-type CHO cells or Lec2 cells. Results from the two were indistinguishable. The method of preparation was the same as that reported previously (Balch et al., 1984*a*). Before use, cytosol samples were centrifuged for 30 min at top speed in an Airfuge (Beckman Instruments, Inc., Palo Alto, CA) and the supernatant was taken.

Response to both cytosol and membranes was linear within the range used (data not shown).

Assays for Glycolipid and Glycoprotein Transport

In a standard assay, the indicated amounts of donor and acceptor (usually 10 μl each), cytosol (usually 5 μl), were added to a reaction mixture containing assay buffer (25 mM Hepes/25 mM KCl/2.5 mM Mg-acetate, pH 7.0), an ATP regenerating system (50 μM ATP, 250 μM UTP, 2 mM creatine phosphate, 7.3 IU/ml creatine phosphokinase) and the indicated levels of [^{3}H]CMP-sialic acid, in a total volume of 50 μl. These conditions are similar to those previously reported for glycoprotein transport to the medial Golgi (Balch et al., 1984*a*). Typically, incubations were performed for 2 h at 37°C before terminating the assays for analysis.

Analysis of Transport Assays

To measure the amount of [^{3}H]sialic acid incorporated into 2,3 sialyl lactosylceramide (G_{M3})50-μl transport reactions were extracted three times with 0.3 ml of $CHCl_3$:methanol (1:1). Insoluble material was pelleted between extractions with a 3-min centrifugation at 12,000 rpm in a microfuge (Beckman Instruments, Inc.). The extract was dried down under vacuum in a Speed Vac (Savant Instruments, Inc., Hicksville, NY). To remove the

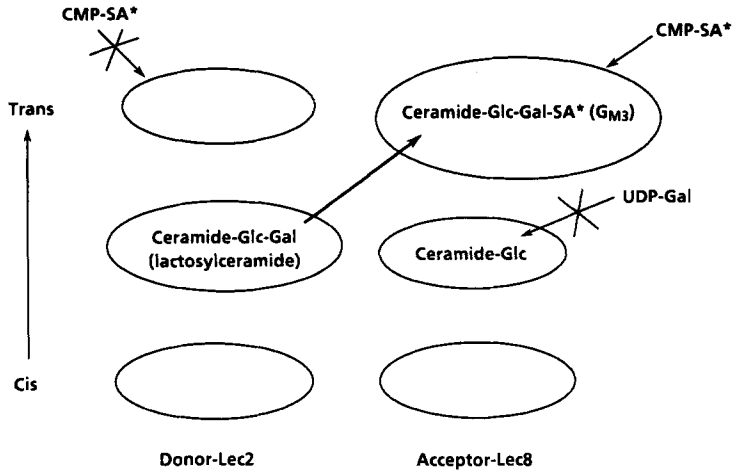

Figure 1. The assay for intercompartmental glycolipid transport. Transport of lactosylceramide is measured between donor and acceptor Golgi populations. Donor is from Lec2, a glycosylation mutant lacking the Golgi CMP-sialic acid transporter and therefore deficient in all sialylated glycoconjugates including the ganglioside G_{M3} (ceramide-Glc-Gal-SA). The predominant glycolipid in these cells is therefore lactosylceramide (ceramide-Glc-Gal). Acceptor is from Lec8, a mutant lacking the UDP-galactose transporter (Deutscher and Hirschberg, 1986). The major glycolipid in these cells is glucosylceramide, which is not a substrate for the sialyltransferase that converts lactosylceramide to G_{M3}. If lactosylceramide is transported from donor to acceptor, it will be sialylated. Transport is quantitated in the assay by the inclusion of [^{3}H]CMP sialic acid in the incubation and measurement of the incorporation of [^{3}H]sialic acid into G_{M3}.

bulk of the unincorporated tritium, as well as to eliminate contaminants interfering with thin layer chromatography, a purification was performed using Sep Pak C-18 cartridges (Waters Associates). Cartridges were prewashed with 5 ml of methanol, and then 5 ml of water. Samples were taken up in 1 ml of water with vigorous vortexing and applied to the cartridges. This was repeated. The cartridges were then washed with 5 ml of water, and the excess water was blown out with air. Gangliosides were eluted with 5 ml of $CHCl_3$:MeOH (1:1). This eluate was dried under nitrogen at 37°C.

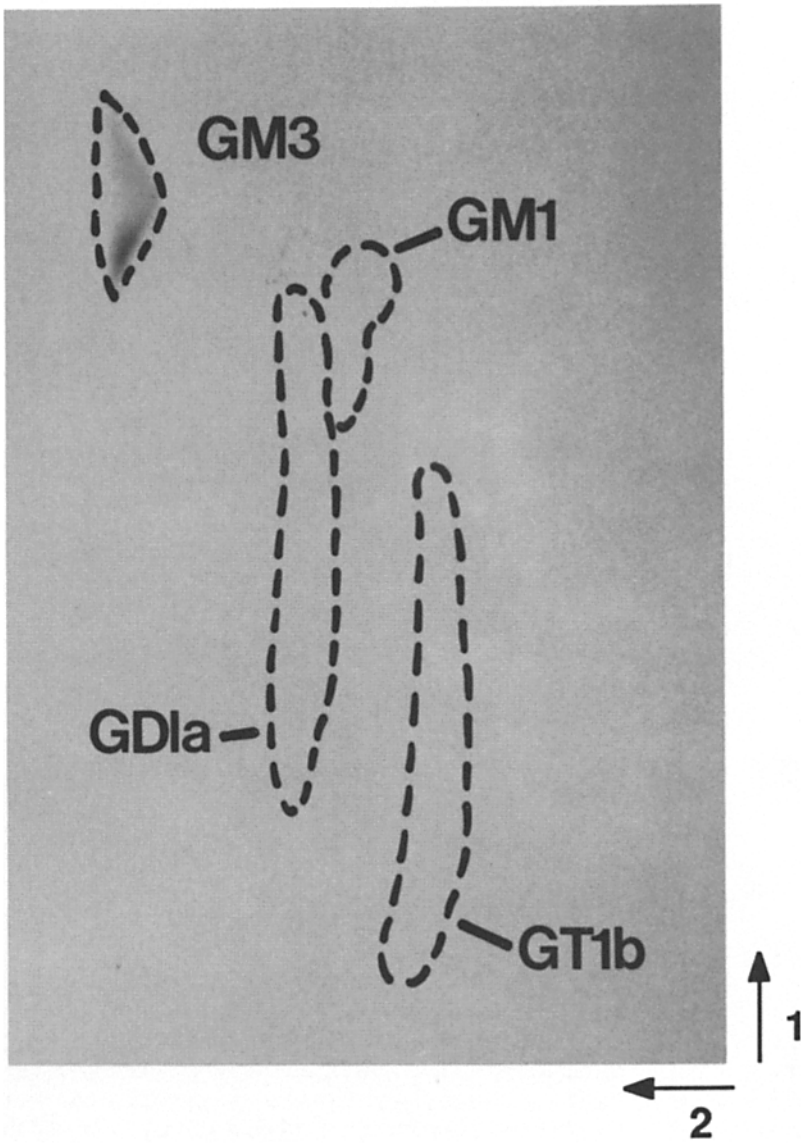

Figure 2. Under assay conditions, radioactivity is incorporated into chromatographically authentic G_{M3}. 20 μl each of donor and acceptor (~20 μg membrane protein), 10 μl of CHO cell cytosol (~50 μg protein), using the buffer and ATP regenerating system described in Materials and Methods, with 4 μCi [^{3}H]CMP sialic acid, in a total volume of 200 μl was incubated for 2 h at 37°C. The incubation mixture was extracted as described in Materials and Methods and analyzed by two-dimensional thin layer chromatography essentially as described by Tanno et al. (1988), except that the thin layer plates used were silica gel 60. The first dimension was developed twice in $CHCl_3$/MeOH/0.2% $CaCl_2$ (65:35:8) and the second dimension in *N*-propanol/NH_3OH (28%)/H_2O (75:5:25). As internal standards 20 μg each of G_{M3}, G_{M1}, G_{Dla}, and G_{T1b} were also included in the radioactive sample spotted. After developing the plate, it was sprayed with scintillant as described and autoradiographed for 7 d. The plate was then sprayed with 50% H_2SO_4 and heated at 110°C for 1 h for visualization of standards. The identity of the standards (*dotted lines*) was established by comparison to each standard run separately under identical conditions. Labeled arrows indicate the first and second dimensions of development.

Samples were taken up in 100 μl of $CHCl_3$:MeOH (1:1) of which 80 μl was spotted onto thin layer plates (Silica gel 60, 20 cm) in the preconcentration zone. Plates were developed in $CHCl_3$:MeOH:0.5% $CaCl_2$ (55:45:10). After development, plates were thoroughly dried and saturated by spraying with ENHANCE (New England Nuclear). These were then dried and visualized by autoradiography. A lane containing glycolipid standards was routinely included. This lane was cut off and visualized by spraying with 50% sulfuric acid and heating at 110°C. Autoradiograms were used as templates to localize the labeled G_{M3} and the appropriate areas were scraped into scintillation vials and counted in 10 ml of Ready Safe (Beckman Instruments, Inc.). The labeled spots always co-migrated with the G_{M3} standard at an Rf of ~0.47.

Incorporation of [^{3}H]sialic acid into VSV G protein was determined by immunoprecipitation onto filters by a modification of the method previously described (Balch et al., 1984*a*). 50 μl of stop buffer (50 mM Tris/0.2 M NaCl/5 mM EDTA/1% Triton X-100/1% NaCholate, pH 7.4) was added to a 50 μl assay. To this was added 0.6 μl of anti-G protein monoclonal 8G5 (as ascites fluid) and 7 μl of rabbit anti-mouse antiserum. This was incubated for 15 min at 37°C before filtration as described (Balch et al., 1984*a*).

Results

The Assay for Glycolipid Transport

The assay for transport of glycolipid between donor and acceptor Golgi populations uses glycosylation as a marker for transit (Fig. 1). The logic of the assay is borrowed from well developed cell free systems measuring glycoprotein transport (Balch and Rothman, 1984; Rothman, 1987). Donor membranes were prepared from Lec2, a CHO cell mutant deficient in the Golgi transporter for CMP-sialic acid (Deutscher et al., 1984). Therefore, all glycoconjugates in these cells are lacking sialic acid. The major glycolipid in Lec2 cells is lactosylceramide (Stanley, 1980) instead of the ganglioside G_{M3} (sialic acid 2,3-Gal-Glc-ceramide) that is produced in wild-type cells. Acceptor Golgi was prepared from Lec8, which lacks an active UDP-galactose transporter (Deutscher and Hirschberg, 1986). The major glycolipid in these cells is glucosylceramide (Stanley, 1980). Thus, acceptor has the machinery to sialylate lactosylceramide to G_{M3}, but produces no G_{M3} because it lacks the proximal substrate for the sialyltransferase. If lactosylceramide is transported from donor to acceptor, it will be sialylated. This was measured by including [^{3}H]CMP-sialic acid in the assay, and then measuring the incorporation of radioactivity into G_{M3}. In a standard assay, donor and acceptor membranes are mixed with CHO cell cytosol in a buffer containing CHO cell cytosol, an ATP regenerating system, and [^{3}H]CMP-sialic acid. After incubation (generally for 2 h) the mixture is extracted and the ganglioside G_{M3} is isolated by thin layer chromatography.

Although Fig. 1 depicts lactosylceramide traveling from a medial compartment in the donor Golgi to a *trans* segment in acceptor, neither the compartment of origin of the lactosylceramide nor the location of the sialyltransferase have been determined. Currently, then, it can only be stated with certainty that transport is between Golgi populations; the precise locale of delivery awaits localization of the lactosylceramide sialyltransferase.

Routinely, analysis of the glycolipid transport assays was performed using one dimensional thin layer chromatography. However, it was important to ensure that the spot that co-migrated with G_{M3} in one dimension was truly G_{M3}. A transport reaction was carried out, extracted, and analyzed by two-dimensional thin layer chromatography. The autoradiogram shown in Fig. 2 illustrates that the labeled product of the reaction co-migrates exactly with G_{M3}, and not with any other ganglioside.

For some experiments, it was desirable to compare precisely glycolipid and glycoprotein transport. To accomplish this, donor membranes were prepared from cells infected with VSV. These membranes therefore contained the VSV glycoprotein, G protein, lacking sialic acid. Transport of G protein from donor to acceptor could then be marked by the incorporation of [^{3}H]sialic acid into G protein. The transport of G protein to the *trans*-Golgi apparatus in a cell free system has been previously described and characterized (Rothman, 1987). Glycolipid and protein transport could therefore be measured in identical incubations: only the post-assay analysis differed. Control experiments indicated that glycolipid transport was similar when measured in membranes from infected or uninfected cells (data not shown).

Production of G_{M3} in Transport Assays Requires Donor, Acceptor, ATP, Cytosol, and Intact Membranes

The experiments depicted in Fig. 3 illustrate that the labeling of G_{M3} occurs only under conditions that are expected to support transport. No labeling occurs if either donor or acceptor is omitted from the assay mix. ATP dependence is a hallmark of transport, whereas the simple enzymatic conversion of lactosylceramide to G_{M3} is not ATP dependent. If the ATP regenerating system is not present, labeled G_{M3} is not detected. G_{M3} labeling also requires the presence of a high molecular weight cytosolic fraction. Protein transport through the Golgi requires several cytosolic proteins (Balch et al., 1984*a*; Wattenberg and Rothman, 1986). The cytosol requirement for G_{M3} labeling in vitro is therefore indicative that this is a transport coupled labeling. The addition of Triton X-100 to disrupt the Golgi membranes completely eliminates G_{M3} labeling. In disrupted membranes, both lactosylceramide and sialyltransferase would be exposed to the extralumenal milieu. Under these conditions no labeling is found, presumably because both enzyme and substrate are dilute. Thus, it is unlikely that labeling in the absence of detergent is similarly due to solubilized sialyltransferase acting on exposed lactosylceramide. Finally, no labeling was observed when assays were incubated on ice instead of at 37°C. This strict temperature dependence is also characteristic of vesicular transport. Together, this group of experiments indicate that the labeling of G_{M3} in this assay is an indicator of lactosylceramide transport from donor to acceptor.

Glycolipid Transport Requires N-Ethylmaleimide Sensitive Factor (NSF) for Glycoprotein Transport

NSF was isolated on the basis of its requirement in glycoprotein transport through the Golgi (Glick and Rothman, 1987; Block et al., 1988). A neutralizing monoclonal antibody to NSF was added to reactions measuring either glycolipid or glycoprotein transport (Fig. 4). Addition of the anti-NSF antibody at 6 mg/ml inhibited both glycolipid and glycoprotein transport by ~50%. There seems to be a slight difference in the dose response of glycolipid and glycoprotein transport to the antibody. However, these differences may lie within the

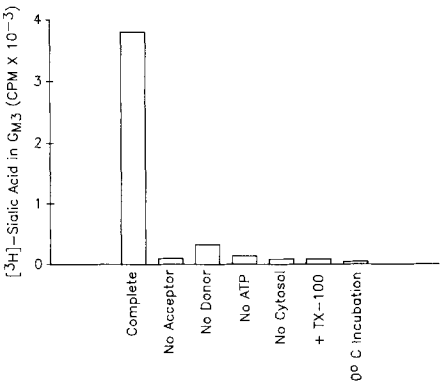

Figure 3. Incorporation of [^{3}H]sialic acid into G_{M3} requires both donor and acceptor, cytosol, ATP, intact membranes, and incubation at elevated temperature. Complete incubations contained 10 μl each of donor and acceptor, 5 μl of CHO cell cytosol, 1 μCi of [^{3}H]CMP sialic acid and buffer, and ATP regenerating system as described in Materials and Methods in a total volume of 50 μl. Incubations were for 2 h at 37°C before extraction and analysis by one-dimensional thin layer chromatography and liquid scintillation counting as described in Materials and Methods. Where indicated, the following modifications were made: acceptor or donor were replaced with equivalent volumes of 35% (wt/vol) sucrose in 10 mM Tris (pH 7.4); the ATP regenerating system (ATP, UTP, creatine phosphokinase, creatine phosphate) was omitted; cytosol was replaced with 25 mM Tris/50 mM KCl; Triton X-100 was added to a final concentration of 0.1%; the incubation was for 2 h on ice instead of at 37°C.

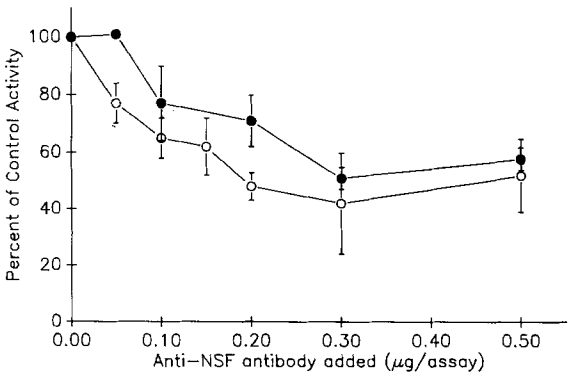

Figure 4. Glycolipid and glycoprotein transport are similarly inhibited by antibody to NSF. Both glycoprotein (*open circles*) and glycolipid (*closed circles*) transport incubations contained 10 μl each of acceptor and VSV-infected donor, 5 μl of CHO cell cytosol, the ATP regenerating system, and a buffer consisting of 25 mM Hepes/2.5 mM MgAc/25 mM KCl/40 mM NaCl in 50 μl. Glycoprotein transport assays (*open circles*) contained 0.5 μCi [^{3}H]CMP sialic acid, whereas glycolipid assays (*closed circles*) contained twice as much radioactivity. Assays were incubated with the indicated amount of antibody against NSF (6.7 mg/ml in 20 mM Tris/150 mM NaCl/1 mM EDTA) for 15 min on ice before initiating the assays for 2 h at 37°C. The volumes of added antibody were compensated by the addition of buffer of the same composition. Values are computed as the percentage of counts in incubations without antibody. Shown are the average of four experiments (±SD). Addition of control antibody (myeloma TEPC183) at similar concentrations had only marginal effect (data not shown).

inherent error of the measurements. Control antibodies had no effect (data not shown). Inhibition of transport was not complete with increased levels of antibody. It has been suggested that some NSF on the membranes is not accessible to the antibodies (P. Weidman, personal communication).

To test more directly for the dependence of transport on NSF, membrane bound NSF was inactivated by a mild treatment with NEM (Fig. 5). NEM treatment strongly inhibited activity. When purified NSF is added back to NEM-treated membranes, both glycolipid and glycoprotein transport activity are partially restored. Activity is restored by 26% (relative to the untreated control) over the activity remaining in the NEM-treated membranes when measuring protein transport, and 35% when measuring glycolipid transport. It is not clear whether this difference is significant. The lack of complete recovery by NSF had been noted earlier (Glick and Rothman, 1987). This indicates that there are other membrane components that are partially inactivated by NEM treatment.

Together, these two experiments establish that NSF is required for glycolipid transport as it is for glycoprotein transport.

GTPγS Inhibits Glycolipid and Glycoprotein Transport Identically

GTPγS is known to inhibit protein transport reactions at a step after the attachment of transport vesicles to the acceptor membrane surface, but before the uncoating of the vesicles (Melançon et al., 1987). The experiment shown in Fig. 6 demonstrates that glycolipid transport is also inhibited by GTPγS. Furthermore, the degree of inhibition is identical to that seen with glycoprotein transport using the same membranes. The inhibition of transport by GTPγS was previously shown to depend on a high cytosol to membrane ratio in the assays (Melançon et al., 1987). Because in the assays presented here this ratio could not be optimized for GTPγS inhibition while maintaining a reasonable transport signal, the inhibition of transport by the nucleotide was not complete.

Glycolipid and Glycoprotein Transport Follow Similar Kinetics

If glycolipids and glycoproteins are being co-transported, they should follow the same kinetics of movement. To explore this, transport reactions were begun and aliquots were removed at various times and analyzed for both glycolipid and glycoprotein transport (Fig. 7). This measured the rate of movement from the beginning to the end of the pathway. The time course for glycolipid and glycoprotein transport were similar. Both exhibited a lag time of ~25 min before a linear rate of transport was achieved. This is considerably longer than the 7–10 min lag time for glycoprotein transport to the medial Golgi complex (Balch et al., 1984*a*; Balch et al., 1984*b*). It is also slightly longer than the lag time previously reported for G protein delivery to the *trans* Golgi apparatus (Rothman, 1987). Transport is complete by ~120 min; the half-time of transport was 50–70 min. There were slight differences in the curves during the linear phase of transport.

The lag time measured in the transport experiment described above is due to the time required to fill all of the ki-

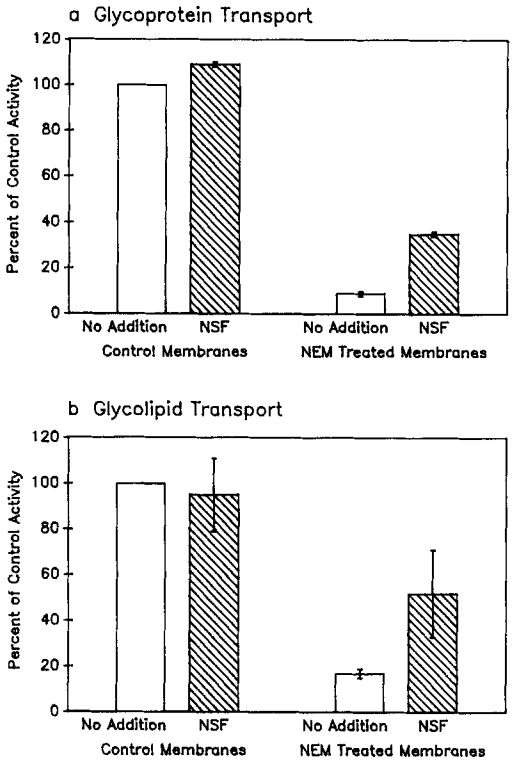

Figure 5. NSF is required for both glycolipid and glycoprotein transport. Analysis of assays for glycolipid and glycoprotein transport were performed on identical incubations containing 10 μl each of acceptor and VSV-infected donor, 5 μl of CHO cell cytosol, 1 μCi of CMP-sialic acid and buffer, and the ATP regenerating system. Assays were incubated for 2 h at 37°C. For NEM treatment of membranes, donor and acceptor were incubated with 0.1 mM NEM for 15 min on ice and then 0.4 mM DTT was added. Control membranes were exposed to DTT before NEM addition. Where indicated, 14 ng of purified NSF was added to the incubations. Values are reported as percentage of counts incorporated into incubations with untreated membranes. Error bars indicate the range of duplicates in a single experiment (protein transport) or standard deviation of four determinations in two experiments (glycolipid transport).

netic intermediates in the process (Balch et al., 1984*b*; Wattenberg et al., 1986). The similar lag time for glycolipid and glycoprotein transport indicates that the kinetics of populating transport intermediates is the same for these two molecules. This was tested more precisely by measuring entry into a specific transport intermediate. Such an intermediate has been characterized in protein transport to the medial Golgi, and is termed the low cytosol requiring intermediate (LCRI) (Wattenberg et al., 1986; Malhotra et al., 1988). This intermediate is formed after transport vesicles have attached to the target membranes, but before fusion. The diagnostic feature of this intermediate is that its formation requires relatively high levels of cytosol, whereas the processing of the LCRI leading to fusion proceeds at fivefold lower levels of cytosol. This is because cytosolic factors required for formation of the LCRI are different from those needed after its formation (Wattenberg and Rothman, 1986; Wattenberg et al., 1990).

The time course of entry of glycolipids and glycoproteins into the LCRI was measured in a dilution assay (Fig. 7). Transport reactions were incubated under standard conditions for the time t indicated on the abscissa. At that time, an aliquot was removed and diluted fivefold with a reaction mix complete except for cytosol. This diluted aliquot was then incubated for 180-t minutes, to allow the molecules that had reached the LCRI to progress to the end of the pathway. Again, the kinetics of transport of glycolipids and glycoproteins were very similar with only slight differences in the linear phase. Entry into this intermediate proceeds without a lag time. This indicates that the lag time observed in the standard assay is due primarily to steps that come after the formation of the LCRI. Entry into the LCRI is complete after 90 min; three-quarters of the time required for all glycolipid or glycoprotein to reach the very end of the pathway. This contrasts to measurements of the same kinetic step in transport to the medial Golgi. In the latter case, entry into the LCRI is complete after only 30% of the time required for glycoprotein to completely transit the pathway (Wattenberg et al., 1986).

Discussion

A pivotal technique in attaining a detailed understanding of transport through the secretory pathway is the reconstitution of transport in cell free systems. Described here is the reconstitution of glycolipid transport. This complements the already well-characterized glycoprotein transport assay. Glycolipid transport through the Golgi is measured by a transport coupled glycosylation reaction. This is similar to the way in which glycoprotein transport is measured in a previously established cell free system (Balch et al., 1984*a*). The ATP and cytosol dependence of the assay, and the detergent and NEM sensitivity of the membranes used, ensure that the assay measures vesicular transport and not simply enzymatic glycosylation of lactosylceramide in leaky membranes. Under similar conditions, measuring protein transport, it has been

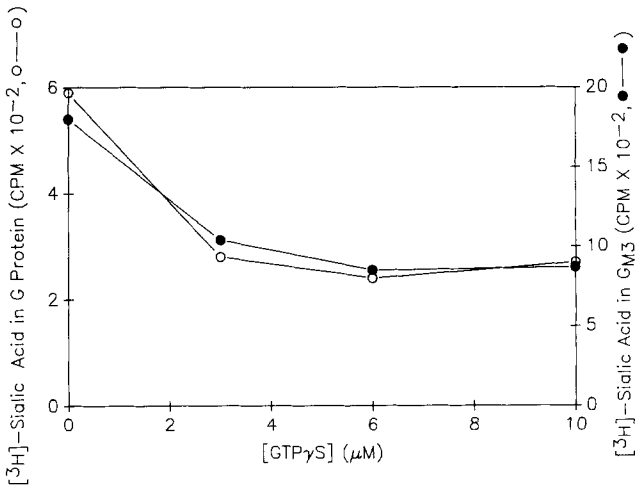

Figure 6. GTPγS is similarly inhibitory for glycolipid and glycoprotein transport. Incubations for analysis of glycolipid (*closed circles, right ordinate*) and glycoprotein (*open circles, left ordinate*) transport were performed identically with 5 μl each of acceptor and VSV-infected donor, 5 μl of CHO cell cytosol, 1 μCi of [^{3}H]CMP sialic acid, buffer, and the ATP regeneration system. GTPγS was added to the indicated final concentrations. Assays were incubated for 2 h at 37°C. Shown are the mean of triplicate samples.

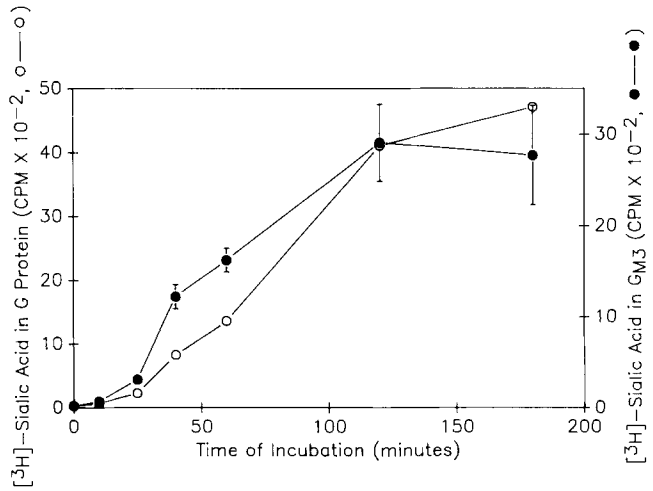

Figure 7. The time course of glycolipid and glycoprotein transport to the end of the transport pathway are similar. Incubations were initiated at $t = 0$, and 50-μl aliquots were removed at each indicated time point and placed on dry ice. At the conclusion of the time course, all samples were thawed and analyzed for transport of either glycolipid (*right ordinate, solid circles*) or VSV G protein (*left ordinate, open circles*). Incubations contained, for each time point, 10 μl of acceptor and 10 μl of VSV-infected donor, 5 μl of CHO cell cytosol, buffer and the ATP regenerating system, and either 0.5 (glycoprotein transport) or 1.0 (glycolipid transport) μCi [^{3}H]CMP sialic acid in 50 μl. Shown are the mean of duplicates (±range).

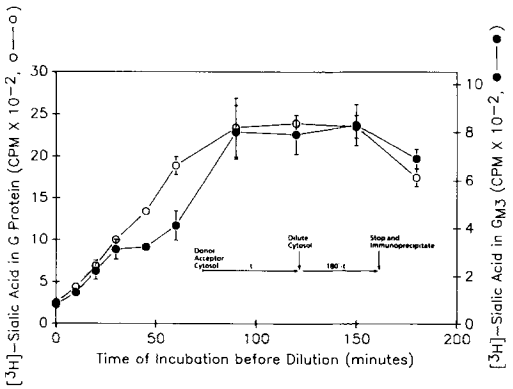

Figure 8. The kinetics of transport into the LCRI is similar for glycoprotein and glycolipid. Measurement of entry into the LCRI is accomplished in a two-step assay. In the first incubation, for each time point, a mixture was made of 5 μl each of acceptor and VSV-infected donor, buffer, and the ATP regeneration system, 1.25 μl CHO cell cytosol, and 0.5 μCi CMP-sialic acid in a volume of 25 μl. After incubation for the indicated times, 25 μl aliquots were removed and diluted into 100 μl of incubation buffer with the ATP regenerating system, 0.5 μCi/25 μl of CMP-sialic acid, and 10 μl/25 μl of 35% sucrose to balance the sucrose contained in the membrane fractions. The diluted samples were then further incubated for 180-t minutes. Samples were then analyzed for glycolipid transport (*right ordinate, solid circles*) or VSV G protein transport (*left ordinate, open circles*). Shown are the mean of duplicates (±range).

shown that glycosylation does not arise from the wholesale fusion of membranes, or from transfer of the glycosyltransferase from acceptor to donor (Braell et al., 1984). The sialyltransferase adding sialic acid to glycoproteins has been localized to the *trans*-Golgi complex of several (but not all) cell types (for review, see Paulson and Colley, 1989). It is reasonably certain that the destination of the measured protein transport is therefore the *trans*-Golgi complex. The localization of the glycolipid sialyltransferase, however, is unknown. By analogy, it seems reasonable that the glycolipid sialyltransferase is similarly located in a *trans* compartment, but at present it can only be said that the observed transport is between Golgi compartments.

A simple calculation illustrates that this assay measures the bulk of glycolipid transport, and not a minor side-pathway. The concentration of ganglioside sialic acid in rat liver Golgi complex is 0.25 nmol/mg of protein (Matyas and Morré, 1987). The concentration of viral spike glycoprotein (determined for the Semliki Forest virus spike glycoprotein in baby hamster kidney cells) in the Golgi apparatus is ~1% (Griffiths et al., 1983); for VSV G protein (molecular mass, 65,000 D) this translates to 0.15 nmol/mg. Therefore, nearly equivalent amounts of [^{3}H]sialic acid should be incorporated into G_{M3} and G protein. This was what was observed (Fig. 6–8). There was some variability in the relative incorporation of label into the two molecules, possibly due to differences in VSV infection; however, incorporation of sialic acid into G_{M3} was generally within twofold of the incorporation into G protein. Considering that more than one sialic acid can be incorporated into the G protein oligosaccharide, this comparison can only underestimate the relative ratio of G_{M3} to G protein transport.

Having established a cell free assay for glycolipid transport, glycolipid, and glycoprotein transport could be directly compared biochemically and kinetically. An advantage of this system is that the same membranes can be used for both assays. Two biochemical parameters were measured, the dependence on NSF and inhibition by GTPγS. The time course of movement to the end of the pathway, as well as transport into a defined transport intermediate (LCRI), was also determined. In each of these tests, glycolipid and glycoprotein transport were very similar. The slight differences that were observed could well fall within experimental variability. These data strongly argue against any nonvesicular route for glycolipid transport, such as diffusion through the aqueous space, or lateral diffusion in the membrane through bridges between Golgi compartments.

In a comparative study of this kind, it is impossible to establish with certainty that glycolipids and glycoproteins use exactly the same mechanism. This knowledge will await the isolation of transport vesicles that can be shown to carry both types of cargo. The similarity of biochemistry and kinetics reported here, however, makes such a shared machinery seem likely. This fits well with the bulk flow model of transport. That two molecules, differing vastly in size and other physical properties, have similar requirements and kinetics of transport suggests that nonspecific samples of membrane are being transported through the pathway.

The indication that glycolipid and glycoprotein are co-transported through the Golgi apparatus has interesting implications for lipid and protein sorting. It has been suggested that the self-aggregating properties of sphingolipids may be involved in the protein sorting that occurs upon exit from the Golgi apparatus in polarized epithelial (van Meer and Si-

mons, 1986). The co-transport of glycolipid and glycoprotein supports this possibility. The high affinity of cholesterol for sphingolipids suggests that cholesterol might be transported by the same vesicular mechanism. Movement of cholesterol to the cell surface follows kinetics that are similar to that of a bulk membrane marker and indicative of vesicular transport (DeGrella and Simoni, 1982; Kaplan and Simoni, 1985*b*). However, Urbani and Simoni (1990) recently reported that cholesterol movement is not affected by Brefeldin A, an antibiotic known to disrupt Golgi function and interfere with protein egress from the ER. It remains possible that cholesterol enters the vesicular pathway late in the Golgi complex, possibly at the site of sphingolipid synthesis. The ability to measure glycolipid transport in a cell free system should make it possible to test some of these concepts.

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