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Reconstitution of Golgi vesicle CMP-sialic acid and adenosine 3′-phosphate 5′-phosphosulfate transport into proteoliposomes

(membrane topography/glycoproteins/glycolipids/glycosaminoglycans/rat liver)

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ABSTRACT **We have previously shown that Golgi apparatus vesicles transport nucleotide sugars and nucleotide sulfate into their lumen. These transport activities are organelle and substrate specific and are characterized by apparent K_m for nucleotide derivatives in the low micromolar range. As part of our goal of purifying and characterizing the above transport proteins, we have reconstituted a protein extract from rat liver Golgi membranes into phosphatidylcholine liposomes. The resulting proteoliposomes transport CMP-*N*-acetylneuraminic acid (CMP-AcNeu) and adenosine 3′-phosphate 5′-phosphosulfate with very similar affinity and inhibition characteristics as intact Golgi vesicles. Sialic acid and sodium sulfate, which are transported only very slowly into the lumen of Golgi vesicles, are transported at low rates by the reconstituted proteoliposomes. Neither rough endoplasmic reticulum-derived vesicles nor proteoliposomes made from proteins of the rough endoplasmic reticulum transport CMP-AcNeu. The above results demonstrate that this reconstituted system can be used for further purification and characterization of nucleotide sugar and nucleotide sulfate translocator proteins. This approach should also be useful to study membrane transport proteins of lysosomes and endosomes.**

Previous studies *in vivo* and *in vitro* using purified Golgi apparatus vesicles have provided evidence for the existence of nucleotide sugar and nucleotide sulfate transporter proteins in the Golgi membrane (1). The role of these transporters is to translocate the above nucleotide derivatives from their sites of synthesis in the cytosol [except for CMP-*N*-acetylneuraminic acid (CMP-AcNeu), which is synthesized in the nucleus] (2) into the lumen of the Golgi apparatus, where glycosylation and sulfation of proteins and lipids are known to occur (3–5). The principal characteristics of the transport *in vitro* of nucleotide sugars and nucleotide sulfate into Golgi vesicles are organelle specificity, solute specificity, solute affinity for and saturability of the transport process (apparent K_m), and inhibition by solute analogs and other compounds (1).

The mechanism of translocation appears to be via an antiporter: a coupled, equimolar exchange occurring with the corresponding luminal nucleoside monophosphate (6). The physiologic relevance of these transport reactions was demonstrated by the characterization of mutant Chinese hamster ovary cells specifically defective in Golgi transport of CMP-AcNeu and UDP-Gal and that showed a corresponding defect in glycosylation of proteins and lipids *in vivo* (7, 8).

Purification and characterization of the above transport proteins is necessary to (*i*) determine their localization within the Golgi apparatus itself and their relationship with the corresponding glycosyltransferases, (*ii*) understand the mechanism of transport, and (*iii*) determine whether or not the transporters are subject to regulation and thereby affect the extent of glycosylation and sulfation of lipids and proteins.

One would expect the above described transport characteristics to prevail in a successful reconstitution of Golgi membrane proteins into liposomes. Our strategy for this goal was to solubilize the bulk of Golgi membrane proteins with detergent and to incorporate them into phosphatidylcholine liposomes. The resulting proteoliposomes were able to transport CMP-AcNeu and adenosine 3′-phosphate 5′-phosphosulfate (PAPS) with affinity and inhibition characteristics very similar to those of intact Golgi vesicles, demonstrating that this system can be used for further purification and characterization of the above nucleotide sugar and nucleotide sulfate translocator proteins.

MATERIALS AND METHODS

Radioactive Materials. The following radioactive materials were purchased from DuPont, New England Nuclear: CMP-[9-^{3}H]AcNeu, 13.6 Ci/mmol (1 Ci = 37 GBq); [α-^{32}P]ATP, 3000 Ci/mmol; GDP-[3,4-^{3}H]Man, 29.1 Ci/mmol; UDP-[6-^{3}H]GlcNAc, 13.9 Ci/mmol; L-α-1-palmitoyl-2-oleoyl-[*oleoyl*-1-^{14}C]phosphatidylcholine, 52.6 mCi/mmol; [*fructose*-1-^{3}H-(N)]sucrose, 13.6 Ci/mmol; [^{35}S]PAPS, 2.5 Ci/mmol; $Na_2{}^{35}SO_4$, 805 Ci/mmol. [9-^{3}H]AcNeu, 0.5 Ci/mmol, was prepared as described (9). [5′-^{32}P]PAPS, 1.4 Ci/mmol, was prepared as described using [α-^{32}P]ATP as precursor (10).

Isolation of Golgi Vesicles and Preparation of a Golgi Protein Extract. Golgi vesicles were isolated from ≈100 g (wet weight) of livers from Sprague–Dawley rats (11). Golgi protein (60–70 mg) was obtained and was enriched 60-fold, over homogenate, in sialyltransferase specific activity (25% yield). The protein was suspended at 2 mg of protein per ml in buffer A [10 mM Tris·HCl, pH 7.0/0.3 M sucrose/1 mM $MgCl_2$/1 mM dithiothreitol/20% (vol/vol) glycerol]. Triton X-100 was added (from a 10% stock solution in buffer A) to a final concentration of 0.5%. The mixture was incubated at 4°C for 2 min, with occasional mild stirring followed by centrifugation at 50,000 rpm for 1 hr in a Beckman 50 Ti rotor. The supernate, which contained 80% of the Golgi vesicles' proteins and 83% of the Golgi vesicles' sialyltransferase activity, was saved. The Triton X-100 insoluble pellet contained 20% of the Golgi vesicles' protein and 17% of the Golgi vesicles' sialyltransferase activity. Detergent was removed from the supernate by two extractions at 4°C with Bio-Beads SM-2 (0.3 g/ml; Bio-Rad). Each extraction was for 2 hr. The detergent-depleted suspension was concentrated to 10 mg of protein per ml by ultrafiltration through a Centripep 30 filter (Amicon). This concentrate was stored in aliquots at −70°C.

Preparation of Liposomes. Egg yolk phosphatidylcholine (30 mg) (Sigma) in hexane was dried on the walls of a 10-ml glass ampule with nitrogen gas. The sample was then ly-

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Abbreviations: AcNeu, *N*-acetylneuraminic acid; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate.

ophilized overnight. The dried lipid was resuspended in 1 ml of buffer B (10 mM Tris·HCl, pH 7.0/0.3 M sucrose/1 mM $MgCl_2$/1 mM dithiothreitol/1% glycerol). The resulting milky emulsion was sonicated (Heat Systems Ultrasonics model W-225 with cuphorn device, 30–35% of maximum output) until a clear suspension was obtained. Electron microscopic and particle sizing analyses showed that all phosphatidylcholine was in unilamellar liposomes.

Reconstitution of Golgi Protein into Phosphatidylcholine Liposomes. The freeze–thawing procedure described by Kasahara and Hinkle was used (12). One milliliter of liposomes (30 mg of lipid in buffer B) was mixed by gentle stirring with 1 mg (0.1 ml) of the Golgi protein extract in buffer A. The mixture was then quickly frozen in a dry ice/acetone bath and allowed to thaw at room temperature (15 min). This cycle was repeated five times. After the fifth thawing, the mixture was sonicated for 10 sec and applied to a Sephadex G-50 column (20 × 1 cm; Sigma, fine) and eluted with buffer B. Fractions of 1.5 ml were collected. Proteoliposomes eluted in the void volume and were used for transport assays without further dilution.

Transport Assay. A typical incubation mixture contained 0.4 ml of Sephadex-filtered proteoliposomes (70 μg of protein) and 0.1 ml of buffer B with the radiolabeled solute whose transport was being assayed (last addition). The reaction time was 5 min. After incubation, the reaction mixture was applied to a Sephadex G-50 column (20 × 1 cm) and eluted with buffer B. Fractions of 1.5 ml were collected and their radioactivity was determined by liquid scintillation spectrometry. Solutes within proteoliposomes eluted in the void volume while free solutes eluted later.

Characterization of Proteoliposomes. We determined whether or not the transport activity of proteoliposomes was in a vesicle population generated after freeze–thawing of phosphatidylcholine liposomes and Golgi proteins. Reconstituted and not reconstituted mixtures of these components were fractionated according to size on a Sepharose CL-4B column (100 × 1 cm; Pharmacia). The column was eluted with buffer B. Fractions of 3 ml were collected. The lipid content of the fractions was determined by adding [^{14}C]phosphatidylcholine to the dry lipid before swelling in buffer B. The protein content was followed by adding radiolabeled Golgi protein. This was obtained by incubation of Golgi vesicles with UDP-[^{3}H]GlcNAc or CMP-[^{3}H]AcNeu prior to solubilization of the protein with Triton X-100. Proteoliposomes eluted in the void volume and liposomes were included. Both fractions were pooled and analyzed for their size. This was done by electron microscopy of samples fixed with glutaraldehyde and stained with uranyl acetate and also by using a Coulter-4-submicron particle counter. Both pools were also assayed for their CMP-AcNeu transport activity.

To determine the internal volume of proteoliposomes, liposomes were prepared with a known amount of [^{3}H]-sucrose in buffer B prior to reconstitution with a protein extract. After reconstitution and fractionation of proteoliposomes on a Sephadex G-50 column, [^{3}H]sucrose eluting with proteoliposomes was determined. This value divided by the total radioactivity of the preparation is the fraction of the total volume that is inside proteoliposomes.

Other Analytical Methods. Transport of CMP-AcNeu and PAPS into Golgi vesicles was done as described (13). Protein was assayed by the method of Schaffner and Weissman (14). Sialyltransferase was assayed as reported (5).

RESULTS

Golgi Proteoliposomes are Selective in Solute Transport. Proteoliposomes were prepared by using Triton X-100 solubilized bulk Golgi membrane proteins and phosphatidylcholine liposomes. These proteoliposomes were able to transport CMP-AcNeu (Table 1). Transport was temperature dependent and highly selective (Table 1): AcNeu and GDP-Man, which are transported very slowly, if at all, across Golgi vesicle membranes (9, 15), showed a similar behavior with Golgi proteoliposomes. Proteoliposomes prepared with membrane proteins from the rough endoplasmic reticulum were inactive toward transport of CMP-AcNeu, similar to rough endoplasmic reticulum vesicles (16). Freeze–thawed liposomes alone or solubilized Golgi membrane proteins, from which Triton X-100 had been removed, were inactive in the transport of CMP-AcNeu. Together, these experiments demonstrate that Golgi proteoliposomes can translocate CMP-AcNeu into their lumen.

Table 1. Proteoliposomes are selective in solute transport

Solute	t, °C	Conditions	Transport, pmol per mg of protein
CMP-[^{3}H]AcNeu (2 μM)	30	Golgi proteolip.	68.0
	0	Golgi proteolip.	14.3
	30	Golgi protein alone	2.4
	30	Lipids alone	1.0
[^{3}H]AcNeu (2 μM)	30	Golgi proteolip.	4.3
GDP-[^{3}H]Man (2 μM)	30	Golgi proteolip.	5.3
CMP[^{3}H]AcNeu (2 μM)	30	RER proteolip.	1.0
[5′-^{32}P]PAPS (1 μM)	30	Golgi proteolip.	67.5
	0	Golgi proteolip.	8.8
$Na_2{}^{35}SO_4$ (1 μM)	30	Golgi proteolip.	0.7

Proteoliposomes were incubated with different solutes. In the first seven experiments, proteoliposomes were preloaded with 100 μM 5′-CMP; in the last three experiments, they were not. Results are average of two independent determinations. RER, rough endoplasmic reticulum.

Translocation of CMP-AcNeu into Golgi Proteoliposomes Is Saturable. We next determined whether the transport of the CMP-AcNeu into Golgi proteoliposomes was saturable and, if so, whether the affinity of this process was similar to that of Golgi vesicles. Transport of CMP-AcNeu into proteoliposomes was linear with time (up to 6 min) and with protein within a range of 0.05 and 1.2 mg. As shown in Fig. 1, transport of CMP-AcNeu was saturable with an apparent K_m of 1.5 μM (Hofstee plot). This was very similar to the value

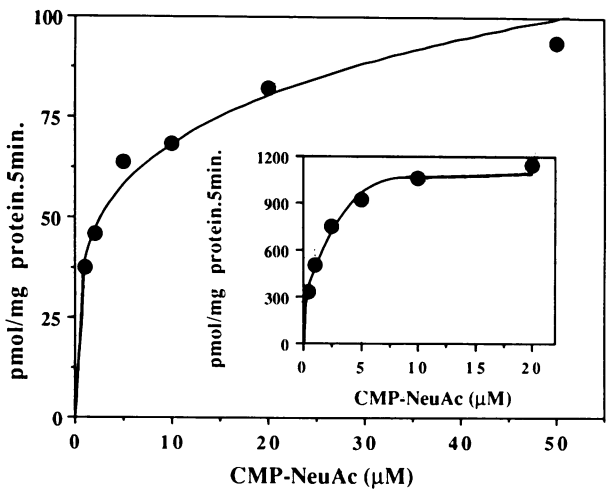


FIG. 1. Rate of CMP-AcNeu transport into proteoliposomes versus concentration. The incubation medium contained CMP-[^{3}H]AcNeu at constant specific activity (500 cpm/pmol). The curve has been corrected for passive diffusion by subtracting the amount of radiolabel associated with proteoliposomes in the presence of 100 μM DIDS in the incubation medium. At 2 μM CMP-AcNeu there were 49.1 pmol per mg of protein without DIDS and 3.4 pmol per mg of protein with DIDS associated with proteoliposomes. (*Inset*) Saturation curve for total transport (solutes and transfer) into Golgi vesicles measured as described (13).

Table 2. Effect of inhibitors on transport of CMP-AcNeu and PAPS into Proteoliposomes and Golgi vesicles

Solute	Inhibitor	Transport activity, % remaining	
		Proteoliposomes	Golgi vesicles
CMP-AcNeu	None	100	100
	10 μM CMP	59	63
	10 μM dCMP	55	46
	10 μM Br-CMP	59	68
	10 μM Br-dCMP	48	32
	20 μM DIDS	10	50
PAPS	None	100	100
	10 μM 3'-AMP	85	95
	10 μM 5'-AMP	24	40
	10 μM 3',5'-ADP	9	5
	20 μM DIDS	54	50

Proteoliposomes (preloaded with 100 μM CMP) were incubated with 2 μM CMP-AcNeu; proteoliposomes without preloading were incubated with 1 μM PAPS. DIDS was preincubated with proteoliposomes and Golgi vesicles for 3 min at 30°C prior to solute addition. Transport of CMP-AcNeu and PAPS without inhibitors was 68 pmol per mg of protein. Results are average of two independent determinations.

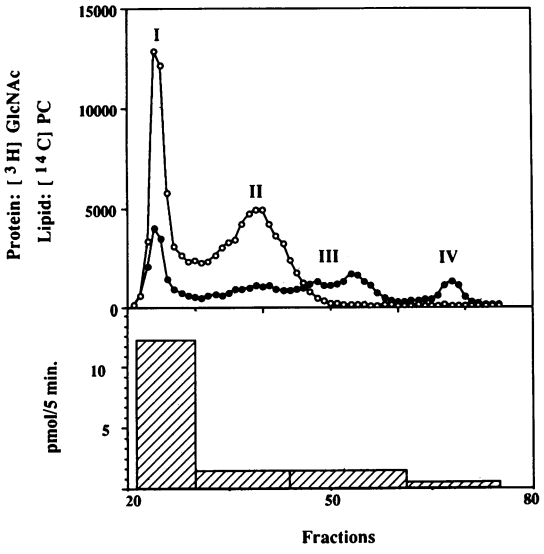
obtained with intact Golgi vesicles (1.4 μM; *Inset*). The V_{max} for solute entry was ≈10 times lower than that for Golgi vesicles (*Inset*). In Golgi vesicles, transfer of sialic acid to endogenous glycoprotein acceptors occurs after transport of CMP-AcNeu into the lumen (16). After CMP-AcNeu transport into proteoliposomes, there was virtually no transfer of sialic acid into endogenous macromolecular acceptors (2 pmol per mg of protein versus 70 pmol per mg of protein observed in Golgi vesicles permeabilized with 0.05% Triton X-100 and incubated with 2 μM CMP-AcNeu without addition of asialofetuin). Sialyltransferases, however, remained active and were distributed equally on both sides of the liposomal bilayer. This was determined by measuring sialyltransferase activity, with asialofetuin as exogenous acceptor in the presence or absence of 0.2% Triton X-100 (data not shown).

Inhibition of CMP-AcNeu Translocation into Golgi Proteoliposomes and Golgi Vesicles Is Similar. An important control for a reconstituted transport system is the ability of transport inhibitors that are effective with vesicles to remain active with proteoliposomes. The effect of competitive inhibitors as well as noncompetitive inhibitors, such as 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), on CMP-AcNeu transport was examined with Golgi vesicles and proteoliposomes. Table 2 shows that these inhibitors were active, to a similar extent, with proteoliposomes and Golgi vesicles.

Physical Characterization of Proteoliposomes. We next determined that the transport of CMP-AcNeu into proteoliposomes was into a population of vesicles that arose after freeze–thawing of the mixture of liposomes and Golgi proteins, and not into liposomes alone or mixtures of liposomes and Golgi proteins that had not been freeze–thawed. As shown in Fig. 2 (*Left*), the elution profile on a Sephadex CL-4B column of radiolabeled phosphatidylcholine, following proteoliposome reconstitution, showed two peaks. One peak eluted in the void volume (peak I), while the other one was included in the column (peak II). When the Golgi protein and phosphatidylcholine liposomes were not subjected to freeze–thawing cycles but immediately applied to the column, all the radiolabeled phosphatidylcholine was included in the column, as expected for a population of small unilamellar vesicles (data not shown). Together, these results show that the phosphatidylcholine-containing vesicles, eluting in the void volume, are proteoliposomes formed during freeze–thawing.

Fractions containing peaks I (fractions 21–31) and II (fractions 32–34) as well as those containing mainly proteins [peak III (fractions 45–60) and IV (fractions 61–70)] were pooled and assayed separately for CMP-AcNeu transport. As shown in Fig. 2 (*Left*), only the pooled fractions from peak I representing proteoliposomes had significant transport activity. These proteoliposomes appeared by electron microscopy, with negative staining, as a homogenous population of unilamellar vesicles with a mean diameter of 150–200 nm (Fig. 2 *Right*).

CMP-AcNeu Transport into Proteoliposomes Is Stimulated by Luminal CMP. CMP-AcNeu translocation into the lumen of Golgi vesicles has been shown to occur via a coupled exchange with luminal CMP (6). To obtain evidence for such reaction in proteoliposomes, liposomes were prepared in the presence or absence of 5'-CMP in buffer B before swelling. The rate of CMP-AcNeu transport into proteoliposomes containing 100 μM luminal CMP was 70 pmol per mg of



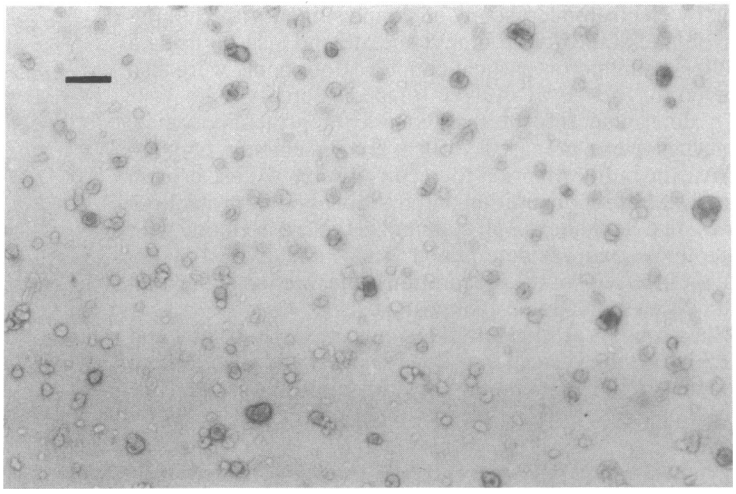


FIG. 2. Size and transport activity of reconstituted proteoliposomes. (*Left*) (*Upper*) Profile (dpm) of [^{14}C]phosphatidylcholine (PC) (○) and [^{3}H]GlcNAc-labeled glycoproteins (●) after reconstitution into proteoliposomes and elution from a Sepharose CL-4B column. Roman numerals indicate the pools taken for the measurements of transport activity. (*Lower*) Transport activity of CMP-[^{3}H]AcNeu of the different pools eluted from the above column. (*Right*) Electron micrograph of pool I. Sample was fixed with glutaraldehyde and negatively stained with uranyl acetate. (×10,000; bar = 1 μm.)

protein while that into proteoliposomes prepared in the absence of CMP was 20 pmol per mg of protein. From the internal volume of these proteoliposomes (11.6 μl per mg of protein) it was calculated that the concentration of CMP-AcNeu in the lumen, following a 5-min incubation at 30°C, was 6 μM, 3 times larger than that of the incubation medium. Thus, proteoliposomes are able to concentrate CMP-AcNeu against a concentration gradient similarly to (although less efficiently than) Golgi vesicles and consistent with an antiport mechanism.

Golgi Proteoliposomes Transport PAPS. To determine whether the above described proteoliposomes were also active in transport of other nucleotide derivatives, we measured their ability to transport PAPS. This is the substrate for protein and proteoglycan sulfotransferases (17) and has been shown to be transported across Golgi vesicle membranes (10).

In preliminary experiments, we found that incubations of Golgi proteoliposomes with [^{35}S]PAPS resulted in 100-fold higher amounts of ^{35}S-labeled solutes associated with proteoliposomes than those incubations with $Na_2{}^{35}SO_4$. In these incubations, the majority (75%) of the radiolabeled sulfur became covalently linked to macromolecules. Because we were unable to quantify what proportion of this transfer occurred on the outside of the proteoliposome membranes, [5′-^{32}P]PAPS was synthesized and used for subsequent transport studies. This substrate measures only transport and will not yield radiolabeled macromolecules upon transfer of the sulfate to endogenous macromolecular acceptors of the proteoliposomes. As shown in Table 1, proteoliposomes were able to transport [5′-^{32}P]PAPS in a temperature-dependent manner. Transport of PAPS into Golgi vesicles and proteoliposomes was inhibited by substrate analogs such as 5′-ADP and 3′,5′-ADP but not 3′-AMP; transport was inhibited by DIDS to a very similar extent (Table 2). Transport of PAPS into proteoliposomes was saturable with an apparent K_m of 2.3 μM (Hofstee plot), a value very similar to that of Golgi vesicles (1.3 μM) (Fig. 3). Over 90% of the radiolabeled solutes within proteoliposomes were [5′-^{32}P]-PAPS as determined by thin-layer chromatography (data not shown). Based on the internal volume of proteoliposomes, it was calculated that PAPS was concentrated 6-fold in their lumen over the concentration in the incubation medium (1 μM).

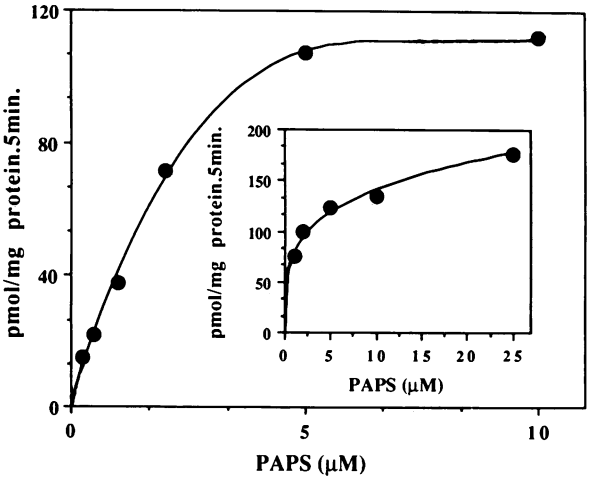


FIG. 3. Rate of PAPS transport into proteoliposomes versus concentration. The incubuation medium for proteoliposomes contained [5′-^{32}P]PAPS at constant specific activity (1000 cpm/pmol). Correction for passive diffusion was done as described in the legend of Fig. 1. At 2 μM PAPS, there were 126.5 pmol per mg of protein associated with proteoliposomes without DIDS and 55 pmol per mg of protein with DIDS. (*Inset*) Saturation curve for total transport into Golgi vesicles measured as described (13).

DISCUSSION

We have shown that Triton X-100 solubilized rat liver Golgi membrane translocator proteins for CMP-AcNeu and PAPS can be incorporated in a functional manner into liposomes. This reconstitution approach should be applicable to the study of other translocator proteins of the Golgi membrane as well as those of lysosomes and endosomes (18).

Important features of the above described reconstitution are the similarity of proteoliposomes compared to Golgi membranes in their solute selectivity, affinity, and specificity toward CMP-AcNeu and PAPS transport inhibitors. These nucleotide derivatives also maintained their transport selectivity across membranes such as those from the endoplasmic reticulum because they were not translocated into proteoliposomes made with proteins from this organelle.

Although the V_{max} for the transport of CMP-AcNeu into proteoliposomes appeared to be 10 times less than that for Golgi vesicles, this value is an underestimate. (*i*) In Golgi vesicles, one-half to two-thirds of the transport of CMP-AcNeu into the lumen is followed by transfer of AcNeu to endogenous macromolecular acceptors; in proteoliposomes, no transfer of AcNeu to endogenous acceptors was seen. This could be the result of disruption by the detergent of the CMP-AcNeu transporter and sialyltransferase complexes (or their acceptors) because we know that sialyltransferases remain active when monitored with exogenous acceptors. (*ii*) Only one-half of the available CMP-AcNeu transporters are oriented in the proteoliposomes with their correct topography. This is inferred from the observation that only half of sialyltransferases in proteoliposomes are luminal (while the remainder face the outside). (*iii*) Approximately 10% of the membrane proteins are actually incorporated into the lipid bilayer of the liposomes. (*iv*) A significant amount of CMP-AcNeu is degraded during incubations with proteoliposomes (40% after 5 min at 30°C), while it remains essentially intact in incubations with Golgi vesicles; therefore, less CMP-AcNeu is available for translocation into proteoliposomes compared to Golgi vesicles.

The mechanism of transport of CMP-AcNeu into the lumen of Golgi vesicles has been described as being an equimolar exchange with luminal CMP. Although proteoliposomes containing luminal CMP showed a 3-fold higher rate of transport per mg of protein than those without CMP, consistent with this antiport mechanism, a number of questions remain unanswered. These include why proteoliposomes without exogenous CMP transport CMP-AcNeu at all. One possibility is that sufficient CMP remains bound to membrane proteins to drive the transport reaction of CMP-AcNeu. Another is that the translocator is a "leaky antiporter" and can complete the transport cycle without counter transport. The same phenomenon appears to occur with the PAPS transport system. Until the transport proteins are further purified, the answers to this question will not be known.

The reconstituted PAPS transport system showed differences with that of CMP-AcNeu: with PAPS, in addition to transport, there was transfer of sulfate to endogenous macromolecular acceptors. This is similar to the behavior with Golgi vesicles, although in proteoliposomes transfer to macromolecular acceptors occurred on both sides of the membrane. Because of this added difficulty, transport of PAPS was routinely measured by using a radioactive label in an atom that was not covalently transferred to endogenous macromolecules (phosphorus). Another difference with the reconstituted CMP-AcNeu transport was the similarity of the V_{max} for PAPS transport into proteoliposomes compared to Golgi vesicles. This is because there is much less breakdown

of PAPS in incubations with proteoliposomes compared to Golgi vesicles. We found, by thin-layer chromatography of the incubation medium, that after 5 min of incubation of Golgi vesicles at 30°C, <5% of the radiolabel was intact PAPS, compared to 30% with proteoliposomes.

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