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NADH-activated cell-free transfer between Golgi apparatus and plasma membranes of rat liver

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This report concerns development of a cell-free system from rat liver to study transport of membrane constituents from the Golgi apparatus to the plasma membrane. Highly purified Golgi apparatus as donor and a mixture of sheets and vesicles as plasma membrane acceptor fractions were combined to analyze requirements for lipid and protein transport. In the reconstituted system, the Golgi apparatus donor was in suspension. To measure transfer, membrane constituents of the donor membranes were radiolabeled with [^{3}H]acetate (lipids) or [^{3}H]leucine (proteins). The plasma membrane vesicles were used as the acceptor and were unlabeled and immobilized on nitrocellulose for ease of recovery and analysis. The reconstituted cell-free transfer was dependent on temperature, but even at 37°C, the amount of transfer did not increase with added ATP, was not specific for any particular membrane fraction or subfraction nor was it facilitated by cytosol. ATP was without effect both in the presence or absence of a cytosolic fraction capable of the support of cell-free transfer in other systems. In contrast to results with ATP, NADH added to the reconstituted system resulted in an increased amount of transfer. A further increase in transfer was obtained with NADH plus a mixture of ascorbate and dehydroascorbate to generate ascorbate free radical. The transfer of labeled membrane constituents from the Golgi apparatus to the plasma membrane supported by NADH plus ascorbate radical was stimulated by a cytosol fraction enriched in < 10 kDa components. This was without effect in the absence of NADH/ascorbate radical or with ATP as the energy source. Specific transfer was inhibited by both N-ethylmaleimide and GTPγS. The findings point to the possibility of redox activities associated with the trans region of the Golgi apparatus as potentially involved in the transport of membrane vesicles from the Golgi apparatus to the cytoplasmic surface of the plasma membrane.

Introduction

In previous work, we have utilized a cell-free system derived from rat liver to study transport of lipids and proteins from radiolabeled transitional endoplasmic reticulum to non-radioactive Golgi apparatus membranes immobilized on nitrocellulose strips as acceptor [1–3]. In the presence of ATP and a cytosolic fraction, transport occurs via 50 to 70 nm transition vesicles [2].

The step between the Golgi apparatus and the plasma membrane in situ also involves transfer of membrane constituents via vesicular intermediates. Both uncoated, product-carrying secretory vesicles and a class of small, clathrin-coated vesicles participate [4].

Despite the success in the reconstitution of the transfer step between the endoplasmic reticulum and the Golgi apparatus in completely cell-free systems [1–3,5] and in the cell-free reconstitution of various endocytic events [6], the step between the Golgi apparatus and the plasma membrane has proven more difficult to reproduce in a cell-free environment. Release of secretory proteins into solution has been demonstrated by Salamero et al. [7] using isolated rat liver Golgi apparatus immobilized on polystyrene microspheres. However, no ATP-dependency or transfer

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Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiophosphate); NEM, *N*-ethylmaleimide; NADH, reduced nicotinamide dinucleotide; A; ascorbate free radical generated from an equimolar mixture of ascorbate and dehydroascorbate in 0.1 M imidazole buffer (pH 7.0).

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of membrane proteins or lipids to the plasma membrane were shown.

In this report, we describe initial efforts to reconstitute cell-free trafficking of membrane constituents between the Golgi apparatus and the plasma membrane. Transfer was dependent but not stimulated by ATP. In contrast to results with ATP, transfer was stimulated by NADH and especially by NADH in combination with ascorbate free radical. The results are consistent with an involvement of electron transfer reactions previously described for trans Golgi apparatus [8] and for coated vesicles [9] in the dynamics of vesicular transport between the Golgi apparatus and the cell surface.

Materials and Methods

Animals, radiolabeling of liver slices and preparation of homogenates. For preparation of fractions, livers from male rats weighing approx. 180 g (Harlan Industries, Inc., Indianapolis, IN) were decapitated and the carcasses drained of blood. Livers were excised and slices were cut by hand with a razor blade as described [10]. The slices were incubated with 1 mCi [^{3}H]acetate or [^{3}H]leucine in 5 ml phosphate-buffered saline (pH 6.2) for 1 h at 37°C, collected on a Miracloth (Chicopee Mills, NY) filter and rinsed to remove unincorporated radioactivity.

Liver slices were homogenized in 37.5 mM Tris-maleate (pH 6.4) containing 0.5 M sucrose, 1% dextran (average mass 225 kDa, Sigma Chemical Company), and 5 mM $MgCl_2$ for 45 s with a Polytron 20 ST operated at 6000 rpm. The top 1/3 to 1/2 of the resultant pellet was resuspended in supernatant and layered over 1.2 M sucrose followed by centrifugation for 30 min at $100\,000 \times g$ as described [11]. The Golgi apparatus were collected from the 1.2 M sucrose interface, diluted with homogenization medium, collected by centrifugation (20 min, $6000 \times g$) and resuspended at a final concentration of 1–2 mg protein/ml in 33 mM Hepes (pH 7.0) containing 2.5 mM magnesium acetate and 33 mM KCl (Hepes/$Mg(OAc)_2$/KCl).

Plasma membrane acceptor. For the acceptor fraction, the bottom two-thirds of the $6000 \times g$ pellet was resuspended in 5 ml of 1 mM $NaHCO_3$ and gently homogenized with a conical glass/glass Dounce homogenizer to resuspend the membranes and break unbroken cells. An additional 5 ml of 1 mM $NaHCO_3$ was added and the homogenate was centrifuged at $4500 \times g$ for 10 min. The top half of the resulting pellet was used for isolation of plasma membrane by aqueous two-phase partition as described [12].

The two-phase system contained 6.6% (w/w) Dextran T-500, 6.6% (w/w) poly(ethylene glycol) 3350, 0.25 M sucrose, 5 mM phosphate buffer (pH 7.2) and resuspended membranes. The two-phase system was then mixed thoroughly by inversions of the tubes in the cold (4°C). The phases were separated by centrifugation for 5 min at $1000 \times g$, diluted with 5 volumes of 1 mM $NaHCO_3$ and pelleted by centrifugation for 30 min at $16\,000 \times g$.

To immobilize the acceptor membranes on nitrocellulose (Nytran, S&S Scientific, Keene, NY), the purified plasma membranes were resuspended at a final concentration of 1–2 mg of protein/ml in Hepes/$Mg(OAc)_2$/KCl. 1 cm^2 strips were incubated in scintillation vials, 25 strips/ml, for 30 min at 4°C with continuous shaking. The plasma membrane solution was removed and 2.5 ml of 5% BSA in Hepes/$Mg(OAc)_2$/KCl were added to the strips followed by a further incubation at 4°C for 30 min to block binding sites not occupied by plasma membrane. The strips, each loaded with approximately 30 μg of plasma membrane proteins, were rinsed through three changes of Hepes/$Mg(OAc)_2$/KCl and added to the reconstituted transfer system.

Reconstituted transfer system. The incubation medium (1 ml volume) contained 250 μl of resuspended radiolabeled Golgi apparatus membranes (1–2 mg/ml), 250 μl of cytosol (0.5 mg of protein), with or without 250 μl of an ATP-regenerating system (30 mM Hepes, 2.5 mM $Mg(OAc)_2$, 30 mM KCl, 80 μM ATP, 300 μM UTP, 2 mM creatine phosphate, 10 units of creatine phosphokinase (rabbit muscle) per ml (final pH 7.0) where indicated) with or without 250 μl of 0.4 mM of an equal mixture of ascorbate and dehydroascorbate freshly prepared in 0.1 M imidazole buffer (pH 7.0), with or without NADH or other additions. With no other additions, 250 μl of Hepes/$Mg(OAc)_2$/KCl (pH 7.0) were added. The cytosol fraction was the > 10 kDa molecular mass fraction prepared by filtration of a microsome-free supernatant ($90\,000 \times g$ for 60 min) of rat liver through a Centricon YM-10 filter (Amicon). Two membrane-containing systems were incubated in parallel. A complete mixture was maintained at 37°C to measure transfer. A mixture with no added ATP or cytosol was incubated at 37°C as a control. Incubations were in 8 ml glass shell vials, four to six strips/vial. Determinations were in duplicate and all experiments were repeated two or three times with consistent results. The incubation mixtures were maintained at 4°C until initiation of the reaction by transfer to 37 or 4°C. At the end of the incubation, the strips were rinsed through three changes of cold Hepes/$Mg(OAc)_2$/KCl and placed individually in scintillation vials. After addition of 5 ml of aqueous scintillant (ACS, Amersham), radioactivity was determined.

Golgi apparatus subfractionation by preparative free-flow electrophoresis. Preparation of Golgi subfractions was as described [13,14]. The material from the 1.2 M sucrose/homogenate interface was not pelleted but was removed in a final volume of about 5 ml. To the resuspended Golgi apparatus, 3 mg each of crude α-amylase type X-A from *Aspergillus oryzae* (Sigma,

132 units/mg protein) and α-amylase type VIII-A from barley malt (Sigma, 1.7 units α-amylase/mg solid and 2.7 units β-amylase/mg solid) were added. This mixture was incubated for 45 min at 4°C. Unstacking was completed at the end of the incubation by drawing the suspension approx. 40 times in and out of a Pasteur pipette with an inside diameter of about 1 mm at the tip. The resulting mixture was used for the electrophoretic separations as described [14].

Heterologous transfer system and separation of right side-out and inside-out plasma membrane vesicles.

Seeds of soybean (*Glycine max* [L.] Merr. var. Williams) were soaked in tap water 4 to 6 h, planted in moist vermiculite, and grown 4–5 d in darkness as described [15]. 2-cm long segments, cut 5 mm below the cotyledons, were harvested under normal laboratory conditions and used for isolation of membranes.

Hypocotyl segments (25 g) were homogenized in 100 ml of a medium containing 25 mM Tris-Mes (pH 7.5), 300 mM sucrose, 10 mM KCl, and 1 mM $MgCl_2$ by smashing with a mortar and pestle. After filtration through one layer of Miracloth (Chicopee Mills, NY), the filtrate was centrifuged for 10 min at $6000 \times g$ (Sorvall, HB 4 rotor), and the pellet was discarded. The resulting supernatant was centrifuged for 30 min at $40000 \times g$ (Beckman, SW-28 rotor), and the supernatant was discarded. The $40000 \times g$ pellets were resuspended in electrophoresis chamber buffer (see below) for free-flow electrophoresis and centrifuged for 30 min at $40000 \times g$. The final pellets again were resuspended in electrophoresis chamber buffer using about 1 ml per 10 g starting fresh weight of hypocotyl segments.

Right side-out and inside-out vesicles (cytoplasmic side-out) of plasma membranes were separated by free-flow electrophoresis [15]. The electrophoretic chamber buffer contained 10 mM triethanolamine, 10 mM acetic acid, 0.25 M sucrose, 2 mM KCl and 10 μM $CaCl_2$ (pH 7.5). The electrode buffer contained 100 mM triethanolamine and 100 mM acetic acid (pH 7.5). The conditions for electrophoresis were 240 mA (constant current), 110 ± 10 V/cm, buffer flow of 3 ml/fraction per h, sample injection rate of 1 ml/h and temperature of 6°C using a VAP-22 continuous free-flow electrophoresis unit (Bender and Hobein, Munich, Germany). The absorbance was determined at 280 nm for all electrophoretic fractions. Appropriate fractions containing vesicles of the different orientations were pooled and concentrated by centrifugation at $85000 \times g$ for 30 min. The pelleted plasma membranes were resuspended in Hepes/$Mg(OAc)_2$/KCl (pH 7.0) and loaded onto acceptor strips as described above for rat liver plasma membranes.

Protein determinations. Proteins were determined by the BCA procedure [16]. Nitrocellulose strips before blocking by transfer to 5% bovine serum albumin were added directly to the bicinchoninic acid (BCA) reagent for determination of absorbed proteins. A blank strip served as a control.

Results

Using [^{3}H]acetate labeled Golgi apparatus as donor and unlabeled plasma membrane vesicles as acceptor, both from liver, temperature-dependent transfer was observed (Fig. 1). At 37°C, transfer was about 5-times that of 4°C. However, at either 37°C or at 4°C, neither ATP nor cytosol alone promoted transfer nor did ATP plus cytosol in combination (Fig. 1). The bulk of the radioactivity (> 99%) remained associated with membranes during the 1-h incubation of the reconstituted cell-free system.

The purity of the Golgi apparatus donor was verified from electron microscopy and assays of marker enzyme to be > 85%. Contaminants based on marker enzyme analysis were endoplasmic reticulum (7%), mitochondria (2%) and plasma membranes (2%). The donor concentration was varied over the range 1 to 2 mg/ml (average 1.6 mg/ml). The acceptor plasma membrane also was > 85% pure. By marker enzyme analysis, contaminants were principally mitochondria (8%) and endoplasmic reticulum (3%). Approx. 30 μg plasma membrane protein was bound per 1 cm^2 nitrocellulose strip. Binding sites on the nitrocellulose, not occupied by plasma membranes, were blocked by incubating the loaded strips with 5% albumin. Donor lipids, labeled and transferred, included the phospholipids

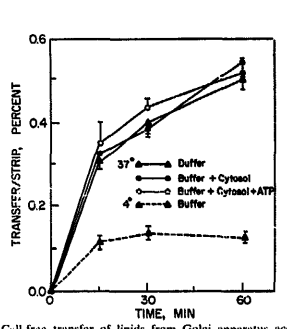

Fig. 1. Cell-free transfer of lipids from Golgi apparatus acceptor radiolabeled with [^{3}H]acetate to unlabeled plasma membrane immobilized on nitrocellulose, comparing 4°C and 37°C, with or without cytosol and with or without ATP. Cell-free transfer was not influenced by ATP (ATP plus ATP regenerating system) or by a > 10 kDa molecular mass supernatant fraction from rat liver (cytosol) prepared by filtration of a microsome-free supernatant of rat liver through a Centricon YM-10 filter.

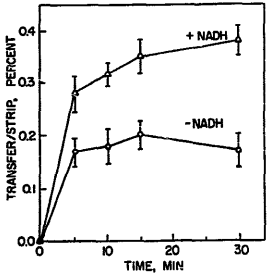

Fig. 2. Cell-free transfer of lipids from Golgi apparatus acceptor radiolabeled with [^{3}H]acetate to unlabeled plasma membrane immobilized on nitrocellulose stimulated by 0.1 mM NADH.

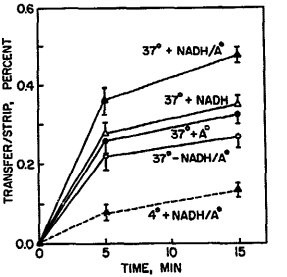

Fig. 3. Cell-free transfer of lipids from [^{3}H]acetate-labeled Golgi apparatus donor to unlabeled plasma membrane acceptor was stimulated by both 0.1 mM NADH and by ascorbate-free radical ($A^{\cdot}$) generated from an equal mixture of 1 mM ascorbate plus 1 mM dehydroascorbate in 0.1 M imidazole buffer (pH 7.0).

phosphatidylcholine (49%), phosphatidylethanolamine (27%), phosphatidylinositol (16%), phosphatidylserine (3%) and sphingomyelin (5%), and sterols. Leucine was incorporated into a representative spectrum of membrane proteins as determined by SDS-PAGE.

Liver plasma membranes were isolated largely as sheets stabilized by junctional complexes bound to the nitrocellulose as thin layers of continuous membranes on the nitrocellulose surface. Measurement of ATPase latency and concanavalin A binding demonstrated that the liver preparations were not dominated by sealed right side-out vesicles. Because of the numerous junctional complexes, many of the fragments were with the cytoplasmic surface accessible to ATP and presumably available for vesicle fusion. The plasma membrane preparations were relatively homogenous when analyzed by preparative free-flow electrophoresis and could not be clearly resolved into right side-out and inside-out vesicle populations.

In an effort to increase the efficiency of the cell-free transfer, conditions known to influence vesicle acidification in isolated Golgi apparatus from liver were examined. One of these, NADH [17], promoted transfer in the cell-free system using [^{3}H]acetate-labeled Golgi apparatus donor and unlabeled and immobilized plasma membrane as acceptor in the presence of cytosol (Fig. 2). Over 30 min at 37°C, transfer was approximately doubled compared to buffer alone. The optimum concentration of NADH for promotion of transfer was about 0.1 mM. Equivalent transfer was obtained at 0.2 mM NADH and 0.5 mM NADH was inhibitory.

On the assumption that an acceptor limited transfer of electrons from NADH might influence transfer, the cell-free system was further supplemented with ascorbate radical (Fig. 3). Inclusion of the ascorbate radical in the incubation medium stimulated transfer over that of NADH alone. These transfers were all in the presence of a cytosol fraction. As illustrated in Fig. 4, cytosol was largely without effect on transfer with buffer alone or with buffer plus ATP but did augment the stimulation by NADH and ascorbate radical.

With [^{3}H]leucine, to measure transfer primarily of proteins, transfer again was temperature-dependent, nonlinear and not promoted by ATP (Fig. 5). The stimulation of transfer by NADH plus ascorbate radical was seen as well with membranes radiolabeled with [^{3}H]leucine (Table I). As with lipid transfer, cytosol

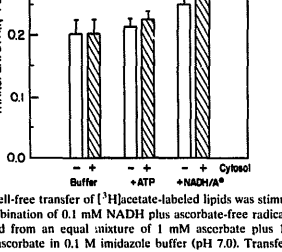

Fig. 4. Cell-free transfer of [^{3}H]acetate-labeled lipids was stimulated by a combination of 0.1 mM NADH plus ascorbate-free radical ($A^{\cdot}$) generated from an equal mixture of 1 mM ascorbate plus 1 mM dehydroascorbate in 0.1 M imidazole buffer (pH 7.0). Transfer was facilitated by the presence of a cytosol fraction (> 10 kDa molecular mass fraction as described in Fig. 1).

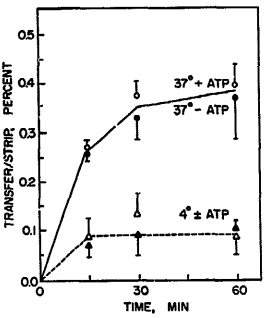

Fig. 5. Cell-free transfer of proteins from Golgi apparatus acceptor radiolabeled with [^{3}H]leucine to unlabeled plasma membrane immobilized on nitrocellulose comparing 4°C and 37°C in the presence of cytosol (see Fig. 1) with and without ATP. As with [^{3}H]acetate-labeled Golgi apparatus, cell-free transfer to plasma membrane was not influenced by ATP.

was beneficial and ATP was without effect even in the presence of cytosol. The radiolabeled proteins transferred specifically in response to ascorbate plus NADH have not been analyzed. They would appear to be some subset of the total radiolabeled proteins of the donor fraction and not exclusively a single secretory protein such as albumin.

To test for a requirement for plasma membrane donor with the cytoplasmic surface exposed, a heterologous transfer system was employed. Homogenates of plant stems, for example, yield populations of sealed plasma membrane vesicles which can be broken by dilution to give both right side-out and inside-out vesicles. The two populations are readily resolved by preparative free-flow electrophoresis. When tested in the cell-free transfer system with Golgi apparatus donor from rat, efficient transfer was obtained only with the preparations of cytoplasmic side-out vesicles (Table II). When Golgi apparatus were subfractionated by preparative free-flow electrophoresis into cis, medial and trans fractions, transfer to immobilized plasma membrane was unspecific and non-linear with time for cis-derived fractions but both specific and linear with time for subfractions enriched in trans elements (Table III). Transfer was stimulated by NADH plus ascorbate radical for trans- and, to a lesser extent, medial-derived elements but not for fractions enriched in cis-derived Golgi apparatus membranes.

To begin to distinguish between aggregation and bona fide membrane fusions, the responses of specific transfer (transfer at 37°C minus transfer at 4°C) to the inhibitors GTPγS and *N*-ethylmaleimide (NEM) were determined (Table IV). With both inhibitors, specific

TABLE I

Cell-free transfer over 15 min of proteins from Golgi apparatus donor radiolabeled with [^{3}H]leucine to unlabeled plasma membrane acceptor immobilized on nitrocellulose

Transfer was temperature-dependent and at 37°C was stimulated by NADH (0.1 mM) and ascorbate-free radical (A·).

Addition	Temperature (°C)	Transfer per cm^2 nitrocellulose strip (%) [a]
None	4	0.07 ± 0.02 [c]
	37	0.34 ± 0.03 [d]
+ ATP	37	0.36 ± 0.02 [d]
+ NADH	37	0.48 ± 0.02 [c]
+ NADH + A· [b]	37	0.65 ± 0.05 [f]

[a] Numbers not followed by the same letters were significantly different ($P < 0.05$).
[b] Generated from an equal mixture of 1 mM ascorbate plus 1 mM dehydroascorbate in 0.1 M imidazole buffer (pH 7.0).

TABLE II

Transfer of [^{3}H]acetate-labeled lipids from Golgi apparatus donor to right side-out and inside-out plasma membrane acceptor vesicles prepared from dark-grown soybean seedlings and immobilized on nitrocellulose

Plasma membrane acceptor vesicles	Concentration		Transfer per cm^2 strip (%) [a]
	NADH	ascorbate radical [b]	
Cytoplasmic side-in	none	none	0.08 [c]
	0.1 mM	1 mM	0.05 [d]
Cytoplasmic side-out	none	none	0.12 [c]
	0.1 mM	1 mM	0.25 [e]

[a] Percent transfer was over 15 min (20 min–5 min). Numbers not followed by the same letter were statistically significant ($P < 0.01$).
[b] Generated from an equal mixture of 1 mM ascorbate plus 1 mM dehydroascorbate in 0.1 M imidazole buffer, pH 7.0.

TABLE III

Comparison of cis, medial, and trans Golgi apparatus elements as donor for the NADH (0.1 mM) + ascorbate-free radical (A·)-mediated transfer of [^{3}H]acetate-labeled lipids to plasma membrane acceptor immobilized to nitrocellulose

Values are percent transfer per cm^2 strip after 30 min of incubation.

Additions	Donor			
	Golgi apparatus			plasma membrane
	cis	medial	trans	
None	0.44 ± 0.06	0.30 ± 0.10	0.58 ± 0.06	0.42
Cytosol	0.45 ± 0.06	0.52 ± 0.08	0.52 ± 0.05	0.34
ATP + cytosol	0.45 ± 0.05	0.56 ± 0.07	0.53 ± 0.08	0.38
NADH + A· [a] + cytosol	0.45 ± 0.04	0.80 ± 0.04	1.3 ± 0.13	0.32

[a] Generated from an equal mixture of 1 mM ascorbate plus 1 mM dehydroascorbate in 0.1 M imidazole buffer (pH 7.0).

TABLE IV

Specific cell-free transfer (37°C–4°C) over 15 min of constituents from Golgi apparatus donor radiolabeled with [^{3}H]acetate to unlabeled plasma membrane acceptor immobilized on nitrocellulose

Transfer was in the presence of a complete system containing 0.1 mM NADH and ascorbate free radical generated from an equal mixture of 1 mM ascorbate plus 1 mM dehydroascorbate in 0.1 M imidazole buffer (pH 7.0) plus the standard ATP + ATP regenerating system. Results are from three independent determinations ± S.D.

Addition	Concentration	Specific transfer (37°C–4°C) per cm^2 nitrocellulose strip (%) [a]
None	–	0.21 ± 0.02 [c]
N-Ethyl-maleimide [b]	1 mM	0.06 ± 0.05 [d]
GTPγS	20 μM	0.02 ± 0.01 [d]

[a] Numbers not followed by the same letters were significantly different ($P < 0.01$).

[b] The donor membranes were preincubated 15 min with the *N*-ethylmaleimide prior to addition to the complete reaction mixture.

transfer was substantially reduced (70% by NEM and 90% by GTPγS).

Discussion

Cell-free systems provide opportunities for exploration of the roles of specific cytosolic factors and membrane constituents in vesicle formation, transfer and docking and in the overall control of eukaryotic membrane trafficking not offered by whole cells and tissues. Success with completely cell-free systems has been considerable with inter-Golgi apparatus trafficking [18–20] and endocytic events [6] and between the endoplasmic reticulum and the Golgi apparatus [1–3,5]. Additionally, permeabilized cells have been used to advantage to investigate the steps and the order of events involved in the processing of vesicular stomatitis virus G protein [21]. However, there has been little or no work with the step between the Golgi apparatus and the plasma membrane.

Most current cell-free assays rely almost entirely on measurement of the completion of specific processing steps as evidence for transfer. This has the advantage in many instances of simultaneously demonstrating attachment and fusion since mixing of constituents at the luminal aspects of the two interacting compartments is required to generate a signal. However, this type of assay has been difficult to devise for mixtures of plasma membrane sheets and vesicles.

The transfer assay developed in our laboratory uses a different principle. The acceptor membranes are unlabeled and immobilized on nitrocellulose [1]. Using a radioactive donor, transfer of radioactivity dependent on time, temperature, cytosolic factors and an energy source (e.g., ATP) can be readily measured [1]. It is also possible to combine the assay with other types of analyses to demonstrate fusion of membranes and/or specific processing events [2]. The assay is completely general and can be utilized in heterologous transfer systems, for example, between acceptor and donor compartments of different species [22].

When applied to transfers between the Golgi apparatus and the plasma membrane, especially when transfer of radiolabeled lipids were followed, results were encouraging in that transfer was time- and temperature-dependent but appeared to be unspecific in that neither ATP nor cytosolic factors, alone or in combination, were required for transfer. Similar results were obtained for plants [23]. Both these studies and the experiments reported here were with a cytosolic fraction and ATP concentration plus an ATP-regenerating system verified to be optimal for cell-free transfer between the endoplasmic reticulum and the Golgi apparatus [1] and for inter-Golgi apparatus transfers [18–20]. Specifically a > 10 kDa cytosolic fraction prepared by YM-10 (Centricon) filtration of a microsome-free rat liver supernatant was used.

In contrast to the results with ATP, the stimulations by NADH and especially those by NADH plus ascorbate radical were specific and facilitated by addition of a cytosol fraction prepared from liver homogenates by high speed centrifugation to remove membranes and particles and concentration by ultrafiltration to provide an enrichment of > 10 kDa constituents as utilized previously for endoplasmic reticulum to Golgi apparatus transfers [1–3]. Barr et al. [17] had previously shown that NADH in parallel with ATP was able to support vesicle acidification using isolated rat liver Golgi apparatus.

Golgi apparatus cisternae contain ascorbate within the cisternal lumens even after isolation and generate ascorbate free radical when incubated with NADH [8]. The cisternal membranes also are enriched in the enzyme NADH–ascorbate free radical oxidoreductase and this activity increases cis to trans and is more active in trans elements [8]. The activity is found as well in coated vesicles from liver when the activity is diminished by extraction of clathrin and enhanced by addition of clathrin [9].

The stimulation of transfer in the cell-free system afforded by NADH plus ascorbate free radical would imply a role for electron transport as a means to energize the transfer of membranes between the Golgi apparatus and the plasma membrane. ATP added to the mixture of NADH plus ascorbate radical was without effect. A contribution of small amounts of endoplasmic reticulum which may be present in the fraction is unlikely since endoplasmic reticulum contaminants would concentrate with the cis- rather than trans-derived membranes upon free-flow electrophoresis. Transfer activity of the cis Golgi apparatus fractions

which would contain any contaminating endoplasmic reticulum was unresponsive to NADH plus ascorbate (Table IV).

In the cell, vesicles or other forms of carriers of membrane constituents coming from the Golgi apparatus would be directed only to the cytoplasmic aspect of the plasma membrane. With the liver membranes used in this study both latency and reactivity of concanavalin A-peroxidase demonstrated that many cytoplasmic membrane surfaces were exposed. To obtain defined subpopulations enriched in either right side-out or inside-out vesicles, a heterologous transfer system was employed. Inside-out (cytoplasmic side-out) vesicles from stems of dark-grown soybean seedlings [15] were more active as acceptor membranes for transfer from Golgi apparatus of rat liver than were the corresponding cytoplasmic side-in (right side-out) vesicle preparations.

The NADH–ascorbate free radical reductase of the liver Golgi apparatus appears to be located dominantly at the trans face [8]. Similarly when Golgi apparatus subfractions enriched in cis, medial or trans elements [13,14] were compared, transfer was greatest with medial and trans fractions whereas the response to NADH plus ascorbate radical was greatest with the fractions enriched in trans elements. In these studies, trans elements were defined as those Golgi apparatus subfractions recovered from free-flow electrophoretic separations (25–35% of the total Golgi apparatus membranes) having the greatest electrophoretic mobility and enriched in galactosyltransferase, sialyltransferase, thiamine pyrophophatase and other markers found predominantly or exclusively at the trans or exit face of the Golgi apparatus [13,14]. This is the Golgi apparatus region from which membrane materials transported to the plasma membrane would normally be expected to be derived [4]. Thus, the transfer observed between Golgi apparatus and plasma membrane stimulated by NADH plus ascorbate represents a very distinct system from that between the transitional endoplasmic reticulum and the cis Golgi apparatus. The latter is energized by ATP and trans Golgi apparatus membranes are ineffective as the acceptor [24].

An energy requirement for late stages of post translational processing, presumably at or near the trans Golgi, has been deduced from studies using the protonophone, carbonyi cyanide *m*-chlorophenylhydrazone (CCCP) [25,26]. An ATP-dependence of transport from the trans Golgi apparatus and the plasma membrane has been demonstrated as well with semiintact (permeabilized) cells (Refs. 27, 28 and references cited). Using a cell-free in vitro budding assay, Tooze and Huttner [29] found the formation of post-Golgi apparatus vesicles in PC 12 cells to be dependent upon ATP and that the formation of both regulated and constitutive Golgi apparatus vesicles was inhibited by about 60% by GTPγS [30]. However, in none of the studies has cell-free transport to the plasma membrane been successfully reconstituted. While it is clear that transport from the Golgi apparatus to the cell surface in situ exhibits an essential requirement for ATP at one or more steps, those steps which are rate limiting in the liver transfer assay described here are unknown but are clearly different from the ATP + cytosol-requirement for endoplasmic reticulum to Golgi apparatus, for example [21]. Whatever is limiting cell-free Golgi apparatus to plasma membrane transfer using isolated cell components, as described in this report, it is not ATP but can apparently be partly overcome by the NADH plus ascorbate radical addition.

The transfers stimulated by NADH plus ascorbate radical are apparently not simply a result of unspecific aggregation since specific transfer is inhibited both by *N*-ethylmaleimide and GTPγS. These two inhibitors have been used previously to block cell-free transfer among successive Golgi apparatus cisternae [31–33] and endoplasmic reticulum to Golgi apparatus transport in perforated cell systems [34]. Generally, *N*-ethylmaleimide blocks both transfer and processing (vesicle formation and fusion) [31,32] whereas GTPγS may inhibit processing (fusion) preferentially. The latter represents a step where a requirement for GTP hydrolysis has been implicated [33,34].

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