Temperature- and acceptor-specificity of cell-free vesicular transfer from transitional endoplasmic reticulum to the cis Golgi apparatus

Stephanie DUNKLE,*† Timothy REUST,* D. David NOWACK,† Lisette WAITS,† Mark PAULIK,* Dorothy M. MORRE† and D. James MORRE*†

*Department of Medicinal Chemistry and †Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907, U.S.A.

The temperature dependence and specificity of transfer of membrane constituents from donor transitional endoplasmic reticulum to the cis Golgi apparatus were investigated using a cell-free system from rat liver. The radiolabelled transitional endoplasmic reticulum donors were prepared from slices of rat liver prelabeled with [¹⁴C]leucine. The acceptor Golgi apparatus elements were unlabelled and immobilized on nitrocellulose. When Golgi apparatus stacks were separated by preparative free-flow electrophoresis into subfractions enriched in cisternae derived from the cis, medial and trans portions of the stack respectively, efficient specific transfer was observed only to cis elements. Trans elements were devoid of specific acceptor capacity. Similarly, when transfer was determined as a function of temperature, a transition was observed in transfer activity between 12 °C and 18 °C similar to that seen *in vivo* for formation of the so-called 16 °C cis Golgi-located membrane compartment. Transfer at temperatures below 16 °C and transfer to trans Golgi apparatus compartments at temperatures either above or below 16 °C was similar and unspecific. The unspecific transfer at low temperature was pH independent, whereas specific transfer was greatest at the physiological pH of 7, and was reduced to 10 % and 18 % of that occurring at pH 8 and pH 5.5 respectively. These findings show that the cell-free system derived from rat liver exhibits a high degree of fidelity to transfer *in vivo*, an efficiency approaching that observed *in vivo*, and a nearly absolute acceptor specificity for cis Golgi apparatus. The acceptor-, temperature- and pH-specificity of the cell-free transfer, as well as the saturation kinetics exhibited with respect to acceptor Golgi apparatus, support the concept of transition-vesicle-specific docking sites of finite number associated with cis Golgi apparatus cisternae.

INTRODUCTION

The bulk flow of lipids and proteins between the endoplasmic reticulum and the Golgi apparatus is generally considered to occur via 50–70 nm vesicles [1,2] that bleb from part-rough, part-smooth transitional elements of the endoplasmic reticulum [2] and fuse at the cis face of the Golgi apparatus to form either new Golgi apparatus cisternae or to merge with existing Golgi apparatus membranes [3]. Some of the events in the formation and fusion of these endoplasmic-reticulum-derived transition vesicles can now be reconstituted in cell-free systems employing fractions of varying degrees of purification from rat liver [4–6], cultured cells [7] and yeast [8]. Perforated cells that permit variations in cytosolic conditions and composition as well as the addition of inhibitors to determine the effects on membrane transfer have been employed also [9,10].

The system we describe from liver utilizes transitional endoplasmic reticulum fractions as well as Golgi apparatus purified from rat liver [5]. The use of a completely cell-free system has the advantage of permitting the direct determination of donor and acceptor specificity and the isolation and analysis of the transition-vesicle intermediate [6]. Golgi apparatus, but not endoplasmic reticulum or plasma membrane, function as acceptors [5] and only transitional endoplasmic reticulum (not conventional rough endoplasmic reticulum, for example) functions as an efficient donor [4,5].

In this report we describe experiments to determine the acceptor specificity of different functional domains of the Golgi apparatus. The findings show efficient specific transfer only to cis-derived elements. The specificity of transfer to cis Golgi

elements is shown by a temperature- and pH-dependency that parallels that observed *in vivo*.

MATERIALS AND METHODS

Animals and materials

Rats were 180 g males obtained from Harlan Industries, Inc. (Indianapolis, IN, U.S.A.). Nitrocellulose was from S & S Scientific (Keene, NY, U.S.A.). All chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Isolation of transition elements and preparation of donor endoplasmic reticulum

Radiolabelled transition elements were isolated from 5 g of freshly prepared rat liver slices which were cut by hand with a razor blade and incubated in a final volume of 5 ml of phosphate-buffered saline (PBS), pH 7.2, with 0.5 or 1 mCi of [³H]leucine for 1 h at 37 °C. The slices were collected on a Miracloth (Chicopee Mills, NY, U.S.A.) filter and rinsed to remove unincorporated radioactivity.

As donor, endoplasmic reticulum fractions containing transition elements were isolated as described [4,5]. Livers, or liver slices, were homogenized in 2 vol. of a medium containing 37 mM-Tris/maleate buffer, pH 6.4, containing 0.5 M-sucrose, 5 mM-MgCl₂ and 1 % (w/v) dextran (average molecular mass 225 kDa) for 45 s with a Polytron 20 ST operated at 6000 rev./min. The homogenate was centrifuged for 15 min at 6000 g to remove nuclei, plasma membrane fragments, Golgi apparatus and cell debris, with the supernatant subsequently diluted 1:5 with homogenization medium and centrifuged for

Abbreviations used: PBS, phosphate-buffered saline; Hepes/Mg(OAc₂)/KCl; 30 mM-Hepes, pH 7.0, containing 2.5 mM-magnesium acetate and 30 mM-KCl; BCA, bichinchoninic acid.

† To whom correspondence should be addressed.

10 min at 10000 *g* to remove mitochondria. The supernatant containing the endoplasmic reticulum was layered on to a discontinuous sucrose gradient consisting of 2.0, 1.5 and 1.3 M-sucrose layers. The radiolabelled material from the 1.3 M-sucrose/supernatant interface was collected and pelleted by centrifugation as the starting material for the donor fraction. The yield was approx. 2 mg/10 g of liver.

Isolation of Golgi apparatus and preparation of acceptor membrane

As the acceptor membrane, highly purified Golgi apparatus membranes isolated from the same, but non-radiolabelled, livers as the donor fraction were adsorbed on to nitrocellulose strips. Golgi apparatus elements, purified as described [11], were resuspended at a final concentration of 1–2 mg of protein/ml in 30 mM-Hepes, pH 7.0, containing 2.5 mM-magnesium acetate and 30 mM-KCl [Hepes/Mg(OAc)₂/KCl] and incubated in the presence of 1 cm² nitrocellulose strips (10 strips/ml) at 4 °C for 1 h with continuous shaking. The strips were transferred to 5% (w/v) BSA in Hepes/Mg(OAc)₂/KCl and incubated for 1 h at 4 °C with shaking. The strips, each loaded with 50–100 µg of Golgi apparatus protein, were rinsed through four changes of Hepes/Mg(OAc)₂/KCl and blotted before transfer to the incubation medium.

Cell-free transfer

The reconstituted cell-free system (1 ml volume) contained 250 µl of resuspended radiolabelled transition-element membrane (3 mg/ml), 250 µl of cytosol, 250 µl of an ATP-regenerating system consisting of 30 mM-Hepes/2.5 mM-Mg(OAc)₂/30 mM-KCl/65 µM-ATP/300 µM-UTP/2 mM-creatine phosphate/10 units of creatine phosphokinase (rabbit muscle) per ml (final pH, 7.0) and 250 µl of Hepes/Mg(OAc)₂/KCl, pH 7.0. Each donor preparation contained, in a final volume of 1 ml of the complete incubation medium, between 60000 and 120000 d.p.m. and approx. 750 µg of protein. The cytosolic fraction was the > 10 kDa fraction prepared by filtration of a microsome-free supernatant (90000 *g* for 60 min) from rat liver through a Centricon YM filter (Amicon). Two identical membrane-containing systems were incubated in parallel. One complete mixture was maintained at 4 °C as a control, and the other complete mixture was incubated at 37 °C.

Incubations were in 8 ml glass shell vials, with three strips per vial. The strips were arranged vertically in the vial in the form of a triangle around the circumference. All solutions were maintained at 4 °C until initiation of the reaction by transfer to 37 °C. At the end of the incubation, the strips were rinsed through four changes of Hepes/Mg(OAc)₂/KCl, blotted on Whatman No. 1 filter paper and dried. The dried strips were placed individually in scintillation counter vials and, after addition of 10 ml of aqueous counting scintillant (ACS, Amersham, Bucks., U.K.), radioactivity levels were determined.

All values were corrected for unspecific absorption of radioactivity at *t* = 0 (approx. 0–25 c.p.m./min per strip). These control strips, loaded with acceptor, were handled in exactly the same way as the experimental strips. They were immersed in the donor mixture for several seconds but were not incubated. Specific transfer was calculated as transfer at 37 °C minus transfer at 4 °C (≤ 20% that at 37 °C).

Golgi apparatus subfractionation

The separation of Golgi apparatus membranes into fractions enriched in cis, medial and trans elements was accomplished by preparative free-flow electrophoresis [12]. Liver Golgi apparatus cisternae exhibit a cis to trans gradient of increasing electrophoretic mobility [12] and thus are separated by this technique.

The chamber buffer contained 10 mM-triethanolamine, 10 mM-acetic acid, 5 mM-glucose, 0.25 M-sucrose and 0.5 mM-MgCl₂, pH 6.5, at an osmolality of 270 mosM, and conductivity of $5.9 \times 10^2 \mu\text{mho}$. The electrode buffer contained 100 mM-triethanolamine and 100 mM-acetic acid, pH 6.5. The conditions for electrophoresis were 156–170 mA (constant current), $130 \pm 10\%$ V/cm; buffer flow of 2.75 ml/fraction per h; sample injection rate of 3.5 ml/h; temperature of 6 °C using a VAP-22 continuous free-flow electrophoresis unit (Bender & Hobein, Munich, F.R.G.). The absorbance was determined at 280 nm for all electrophoretic fractions. Fractions were pooled and concentrated by centrifugation at 85 000 *g* for 30 min (Beckman SW-28 rotor) to divide the electrophoretic separations into either three or four regions of differing electrophoretic mobility but of approx. equal protein content as illustrated in Fig. 1. Procedures for estimation of marker enzymes and for cytochemical determinations or origin within the polarity axis of the Golgi apparatus were as described [12–16].

Determination of protein content

Proteins were determined by the BCA procedure [17]. Nitrocellulose strips, before blocking by transfer to 5% (w/v) BSA, were added directly to the BCA reagent for staining of the membrane proteins absorbed to the strips. A blank strip served as a control.

Electron microscopy and immunocytochemistry

Material was prepared for electron microscopy by fixation in 2.5% (w/v) glutaraldehyde in 0.1 M-sodium phosphate buffer, pH 7.2, followed by exposure to osmium tetroxide in the same buffer. Dehydration was through an acetone series followed by embedding in Epon. Thin sections were observed and photographed using a Philips EM 200 electron microscope.

Antisera to transition vesicles were as described [6]. The processing of electron microscope preparations for immunocytochemistry was according to Brown *et al.* [18] and Ohtsuki *et al.* [19] but using, instead of peroxidase-linked antibody, antibody complexed with colloidal gold according to Slot & Geuze [20]. Acceptor strips to which guinea-pig liver Golgi apparatus had been bound were incubated for 15 min at 37 °C in the presence of a transition-vesicle-enriched fraction from rat. The strips were fixed immediately after incubation with donor fraction in 2% (w/v) paraformaldehyde with 0.1% glutaraldehyde in PBS for 30 min at room temperature, rinsed several times with PBS, and treated with 0.1 M-NH₄Cl in PBS for 45 min to block free aldehyde groups [21]. After two additional PBS rinses, the strips were transferred to antibody diluted 1:10 with Hepes buffer containing 0.1% BSA (Hepes/BSA) at 4 °C for 1 h. The strips were then washed five times for 5 min each with Hepes/BSA and transferred to a 1:15 dilution of 6 nm colloidal gold particles complexed to goat anti-(rabbit IgG) antibody [20] at 4 °C for 1 h, followed again by washing five times for 5 min each. Lastly, the strips were fixed in 2% (w/v) glutaraldehyde containing 5% (w/v) sucrose and 0.1 M-sodium phosphate, pH 7.2, for 1 h followed by washing, reaction with osmium tetroxide, and Epon embedding. Controls were without transition vesicles or without primary antibody.

RESULTS

In the system described with radiolabelled transitional endoplasmic reticulum donor, transfer to unlabelled Golgi apparatus acceptor immobilized on nitrocellulose was ATP- and cytosol-dependent and proportional to time for up to 60 min of transfer. The Golgi apparatus acceptor was characterized by analysis of enzyme markers and by morphometry and was less

Table 1. Specific activities of marker enzymes for Golgi apparatus of rat liver and estimates of fraction purity

Units of specific activity are $\mu\text{moles/h}$ per mg of protein for succinic dehydrogenase measured as succinate-INT-reductase, μmoles of inorganic phosphorus released/h per mg of protein from 5'-AMP or glucose-6-phosphate for 5'-nucleotidase and glucose-6-phosphatase respectively, and nmoles of galactose transferred to ovomucoid/h per mg of protein for galactosyltransferase. Results are means $\pm$ s.d. from 3–5 different membrane preparations. Present in only trace amounts were latent β -glycerolphosphate phosphatase (lysosomes), uricase acid (peroxisomes) and DNA (nuclei). The numbers in parentheses are the percentage values of contaminating components based on specific activities of the marker enzymes in the fractions analysed.

Isolated cell fraction	Endoplasmic reticulum (glucose-6-phosphatase)	Golgi apparatus (galactosyl transferase)	Plasma membrane (5'-nucleotidase)	Mitochondria (succinate-INT reductase)	Fraction purity (%)	
					Enzymic markers	Morphometry
Total homogenate	3.0 ± 1.0	2 ± 1	3.0 ± 0.5	2.0 ± 0.6	—	—
Golgi apparatus	1.5 ± 0.2 (8)	283 ± 65	2.5 ± 0.6 (6)	0.2 ± 0.1 (1)	85	86*

* Values are from 15 electron micrographs photographed at random and averaged from five different membrane preparations.

Fig. 1. Free-flow electrophoretic separation of unstacked Golgi apparatus as described [12]

The figure shows the average absorbance profile at 280 nm of the electrophoretic separations analysed and the manner in which the electrophoretic separations were pooled to obtain the different acceptor fractions. Cisternae from the cis-Golgi-apparatus face were recovered in fractions 46–57 nearest the cathode. Fractions enriched in trans cisternae were from the most electrophoretic fractions 35–41 nearest the anode. Cisternae of intermediate electrophoretic mobility (42–45) were collected as representing medial cisternae. Cytochemical and biochemical criteria for identification and assignment of the different Golgi-apparatus fractions are given in detail in the references [12,14–16].

than 15% contaminated by endoplasmic reticulum (8%), plasma membranes (6%), mitochondria (1%), lysosomes (trace), peroxisomes (trace) or nuclei (not detected) (Table 1). Unspecific absorption determined using incubated nitrocellulose strips loaded only with albumin was small (0.3% transfer per 1 cm^2 of strip) compared with transfer with acceptor present.

Golgi apparatus subfractionation and determination of acceptor specificity

Preparative free-flow electrophoresis was used to subfractionate Golgi apparatus into fractions enriched in cis, trans, or medial cisternae (Fig. 1). The fractions designated trans were enriched in the Golgi apparatus markers thiamine pyrophosphatase [22], sialyltransferase [23], galactosyltransferase [23] and a H^+ -ATPase [22]. NADPase, a marker located cytochemically in the mid region of Golgi apparatus, co-migrated

with the centre of the separation marking these cisternae as medial [15]. The fractions nearest the cathode reduced osmium tetroxide according to the classic method of Friend & Murray [13], a characteristic restricted to cis Golgi apparatus membranes [12,14].

Based on analyses of trans-face marker enzymes galactosyl- and sialyltransferases, thiamine pyrophosphatase and dipeptidyl-peptidase IV, the cis Golgi apparatus fractions were less than 25% contaminated by trans elements. Similarly, measurements of NADH-cytochrome *c* reductase and nucleoside diphosphatase activities suggested the trans Golgi apparatus fractions to be less than 25% contaminated by cis Golgi apparatus elements. The starting Golgi apparatus, based on morphometry and analyses of marker enzymes, had a combined contamination of endoplasmic reticulum (glucose-6-phosphatase), mitochondria (succinate-INT-reductase), plasma membrane (5'-nucleotidase), lysosomes (latent acid β -glycerolphosphate phosphatase), peroxisomes (uricase) and nuclei (DNA) of no more than 15% (Table 1). Electron microscopy of the individual electrophoretic fractions, as well as analyses of marker enzymes, verified that neither the cis- nor the trans-enriched fractions were preferentially contaminated by non-Golgi apparatus membranes such as lysosomes, plasma membranes or endoplasmic reticulum fragments.

The yield of each of the differential cisternal fractions from 10 mg of total Golgi apparatus protein were, on average, cis 2.5 mg, medial 4.0 mg and trans 1.5 mg. The recovery was about 85%.

The donor fraction was characterized both from morphology and marker enzymes and consisted of 85% part-rough/part-smooth endoplasmic reticulum membranes. Possible contaminants included 4% Golgi apparatus (galactosyl transferase), 3% mitochondria (succinate-INT-reductase) and 8% plasma membrane (5'-nucleotidase).

Acceptor specificity

When assayed for transfer activity at 37 °C under conditions where transfer was saturable and proportional to acceptor protein, efficient specific transfer occurred only with the cis-derived fraction as acceptor and to a lesser extent with adjacent medial cisternal fractions, which may contain cis-derived membranes (Figs. 2 and 3). When measured at 4 °C (Fig. 3b), transfer was essentially equal for all Golgi apparatus subfractions and approx. equivalent to that observed with the trans fractions at 37 °C. Values of about 3500 d.p.m./mg of acceptor protein were reached for specific transfer to cis Golgi apparatus acceptor.

Fig. 2. Acceptor specificity of cell-free transfer comparing different Golgi apparatus fractions prepared by free-flow electrophoresis as described [12]

Transitional endoplasmic reticulum was isolated from liver slices prelabeled with 0.5 mCi of [³H]leucine and incubated for the times indicated with different Golgi apparatus subfractions as acceptors. The latter were immobilized on nitrocellulose strips at equal protein amounts of $70 \pm 10 \mu\text{g}$ per strip. Each determination consisted of the average of three different acceptor strips. The experiment was repeated three times to obtain a mean $\pm$ S.D. for the three experiments. Transfer was greatest in the electrophoretic fractions enriched in cis Golgi apparatus and least in electrophoretic fractions enriched in trans Golgi apparatus.

These levels of specific transfer represented between 0.25 and 0.5% of the total donor radioactivity per strip. In these experiments, trans-derived Golgi fractions used as acceptor exhibited essentially no specific transfer (transfer at 37 °C minus transfer at 4 °C).

As specific transfers of between 0.25 and 0.5% were obtained per 1 cm² of nitrocellulose strip, we utilized three such 1 cm² nitrocellulose strips per time point which was sufficient to generate statistically reliable measurements of transfer. However, up to 10 strips per reaction could be accommodated without loss of transfer efficiency per strip. With 10 strips per reaction, total specific transfer values of between 2.5 and 5% per h for the isolated transfer system were obtained. These percentages were comparable with those observed *in vivo* [24] or with liver slices [25] for endoplasmic reticulum to Golgi apparatus transfer, both in terms of overall efficiency and absolute amounts of radioactivity transferred (see the Discussion section).

A preference for transition vesicles fusing with cis Golgi apparatus elements received confirmation from electron microscopic immunocytochemistry using a heterologous transfer system (Fig. 4). Transition vesicles generated from rat liver were combined with Golgi apparatus elements from another species, guinea-pig, which were not cross-reactive with the liver antisera [6]. In the heterologous system, transfer of vesicles was observed as small patches or tesserae of immunoreactive membrane incorporated into the unreactive guinea-pig Golgi apparatus cisternae. These were much more prevalent with cis-derived fractions (Fig. 4a) than with mature secretory vesicles or with the trans Golgi apparatus network. (Fig. 4b).

In these experiments all the gold particles, both free and membrane-associated, were counted on 10 thin sections of

Fig. 3. Cell-free transfer of radioactivity from radiolabelled transitional endoplasmic reticulum to acceptor-Golgi-apparatus subfractions (50 μg /nitrocellulose strip) comparing transfer at 37 °C (a) and transfer at 4 °C (b)

Results are from duplicate determinations $\pm$ mean deviations. (a) Transfer at 37 °C was greatest with the electrophoretic fractions enriched in trans Golgi apparatus. The inset shows the absorbance profile of 280 nm of the electrophoretic separation and the fractions pooled for analysis. (b) Transfer at 4 °C was approx. equal for all subfractions and equivalent to that with the trans-derived fraction at 37 °C.

nitrocellulose strips for preparations with and without added transition vesicles and with and without primary antibody. The number of gold particles associated with the vesicles or membrane tesserae attached to Golgi stacks in the absence of added transition vesicles or without primary antibody was only about a fiftieth of that to which both transition vesicles and primary antibody were added. Cisternal elements and secretory vesicles, having morphological characteristics consistent with a trans derivation, were normally devoid of attached transition vesicles and marked only by occasional gold particles with a frequency below that of the control preparations.

Temperature dependency of transfer

With saturating amounts of total Golgi apparatus as acceptor, transfers on average were linear for up to 60 min (Fig. 5) before Golgi-apparatus-acceptor sites became rate limiting. Best-fit curves were obtained under such conditions for a range of

Fig. 4. Immunocytochemical verification of the specificity for transition-vesicle-transfer Golgi apparatus elements immobilized on nitrocellulose strips comparing *cis*- (a) and *trans*-derived elements (b)

The donor fractions (transition vesicles) were from rat liver and acceptor Golgi apparatus elements were from guinea-pig. Except for normal osmium tetroxide fixation, membranes were not contrasted. Gold-labelling was carried out on the strips before fixation. Antigenic sites (to rat transition vesicles) marked by 6 nm gold (arrows) and indicative of fusion of immunoreactive rat-derived membranes with the normally unreactive Golgi apparatus cisternae from guinea-pig were most evident with *cis*-derived fractions (a). Some gold particles were associated with attached but incompletely fused vesicles as well. With preparations of *trans* Golgi apparatus elements, either mature secretory vesicles (sv) with small lipoprotein particles or the *trans* Golgi apparatus network (TGN), there was little or no evidence of vesicle fusion. In control preparations, where all reactants were present but from which transition vesicles were omitted (not illustrated), essentially no gold particles were associated with Golgi apparatus membranes or with small vesicles. As shown by measurements with radio-iodinated membranes [6], transfer in the heterologous system was nearly as efficient as with the homologous system. Scale bar = 0.1 μ m.

temperatures between 4 °C and 37 °C. Transfer increased as a function of temperature (Fig. 6), but was characterized by a sharp and highly significant increase in transfer between 12 °C and 18 °C. The results, which are expressed as percent transfer per nitrocellulose strip to correct for small differences in the amount of radioactive donor (Fig. 6, inset), also showed the transition. Transfer at temperatures of 4 °C, 8 °C and 12 °C averaged 0.13 % and were not significantly different from each other. Transfers at temperatures of 18 °C, 20 °C, 25 °C and 37 °C averaged 0.32 % and were not different from each other, although the mean for each temperature did increase numerically from 0.28 % at 18 °C to 0.36 % at 37 °C. A temperature of 16 °C appeared to mark the mid-point of the rather sharp temperature transition of vesicle transfer.

pH

As a further indication of the specificity of transfer measured

at 37 °C compared with that at 4 °C, transfer was measured as a function of pH. At 37 °C transfer showed an optimum at around pH 7.0 (Table 2). At 4 °C, transfer was only 23 % of that at 37 °C and pH 7.0, and was independent of pH. In contrast, specific transfer (transfer at 37 °C minus transfer at 4 °C) declined to a value 19 % of that at pH 7.0 when incubated at pH 5.5, and to 6 % of that at pH 7.0 when incubated at pH 8.0. Specific transfer at pH 6.0 was about half that observed at pH 7.0.

DISCUSSION

A system of specific signals and signal receptors has been proposed to explain vectorial trafficking of membrane constituents [26]. Such a mechanism would be anticipated to operate between the endoplasmic reticulum and the Golgi apparatus. Signal receptors concentrated at entry ports would help to explain, for example, delivery of new membrane materials

Fig. 5. Transfer kinetics as a function of temperature

Transfer of radioactivity was from transitional endoplasmic reticulum prepared from liver slices pre-incubated for 1 h with 0.5 mCi of [^3H]leucine. Unlabelled Golgi apparatus elements were immobilized to nitrocellulose (100 μg /strip). Means are for duplicate determinations from three different experiments (total of six acceptor strips) comparing 8 °C, 18 °C, 25 °C and 37 °C.

specifically to the cis face. That the recognition of the acceptor compartment by donor transition vesicles (docking) is specific for cis Golgi apparatus membranes was demonstrated here for the first time in a completely cell-free system. Since transfer in the cell-free system is saturable and proportional to the amount of cis-derived Golgi apparatus acceptor present at saturation, a finite number of docking sites at the cis Golgi apparatus is indicated as well.

We have determined previously that the donor transition elements from rat liver form transition vesicles when incubated at 37 °C with nucleoside triphosphate, an ATP-regenerating system and cytosol. These vesicles can subsequently be separated from the transitional endoplasmic reticulum by preparative free-flow electrophoresis [6]. Neither the incubated transitional endoplasmic reticulum nor the purified transition vesicles were found to transfer radioactivity to either endoplasmic reticulum or plasma membranes immobilized on nitrocellulose as acceptor [5]. Only with Golgi apparatus [5,6], and specifically with cis Golgi apparatus (Figs. 2 and 3), was efficient specific transfer achieved (see also [5,6] for other membranes). Use of the cis Golgi apparatus as the acceptor significantly increased the specific signal generated by the system. Transfers of several hundred d.p.m. per 1 cm^2 strip were readily obtained. These corresponded to between 0.3% and 0.5% of the total radioactivity of the transitional endoplasmic reticulum fraction. With the addition of multiple strips, transfer values of up to 4% or 5% of the starting radioactivity were achieved. Transfer was ATP-dependent and inhibited by *N*-ethylmaleimide (results not shown). Transfer to strips lacking acceptor (albumin only) was negligible (specific transfer of 0.03% after 120 min), uninfluenced by ATP, was pH independent and exhibited a Q_{10} of 1.5 compared with specific transfer which was pH- and ATP-dependent and exhibited a Q_{10} of > 2 . Transfer at 37 °C was between 4- and 5-fold that at 4 °C.

Specific cell-free transfer is proportional to the number of

Fig. 6. Transfer rate as a function of temperature of the incubation medium determined from time courses as illustrated in Fig. 5

Rates were determined over 60 min from the best-fit slopes of the linear portion (0–60 min) of the time course of transfer for each individual determination. Each determination compared four temperatures, one of which was 37 °C. Results are means of three independent determinations at all eight temperatures $\pm$ S.D. among the three determinations. An abrupt transition was seen between 12 °C and 20 °C. The inset, which presents transfer calculated as a percentage of the amount of radioactivity in the acceptor fraction per strip over 60 min $\pm$ S.D., also shows the transition between 12 °C and 20 °C.

Table 2. Transfer as a function of pH comparing 4 °C and 37 °C

At 37 °C, transfer was optimal at the physiological pH of 7.0 and reduced to 10 and 18% of that of pH 7.0 at pH 8.0 and 5.5 respectively. At 4 °C, transfer was unspecific and little influenced by pH.

Temperature	pH	10 ⁻² x Total transfer (c.p.m./min per mg of protein)
37°	5.5	10.6
	6.0	20.6
	7.0	35.0
	8.0	9.0
4°	5.5	5.2
	6.0	6.1
	7.0	7.2
	8.0	7.2

transition vesicles generated [6,27]. With transition-vesicle-enriched fractions, nearly 50% of the total radioactivity was transferred to cis Golgi apparatus. This corresponded to the proportion of vesicles present based on morphology [6]. However, with transitional endoplasmic reticulum no more than 0.5–1% of the total membrane of the transitional endoplasmic reticulum was represented by blebbing profiles representing transition vesicles after 15 min of incubation with ATP plus cytosol. Thus,

specific transfers of 2.5–5 % of the starting radioactivity represent a practical upper limit of transfer without first artificially concentrating the transition vesicles.

These percentages of transfer may be comparable with those estimated *in vivo* [24] or with liver slices [25]. *In vivo*, endoplasmic reticulum membranes of rat liver reached specific radioactivity of about 70000 d.p.m./mg of protein over 30 min [25]. A 10 g liver contained 380 mg of endoplasmic reticulum protein [28] giving a total specific radioactivity of 26.6×10^6 d.p.m. in endoplasmic reticulum. A 10 g liver contains about 15 mg of Golgi apparatus [28]. At 30 min, the specific radioactivity had reached about 190000 d.p.m./mg of protein [25], equivalent to 2.85×10^6 d.p.m. per 10 g of liver. By 30 min very little membrane label had left the Golgi apparatus for transit to the plasma membrane so the ratio of radiolabel in the Golgi apparatus to that in the endoplasmic reticulum provides an estimate of about 10 % being transferred over a period of 30 min. Using liver slices, values of 2500 d.p.m. per mg of protein were obtained for endoplasmic reticulum [25] equivalent to 0.95×10^6 d.p.m. per 10 g of liver, and 3500 d.p.m. per mg of Golgi apparatus protein [25] which is equivalent to 0.05×10^6 d.p.m. per 10 g of liver. This gives an estimate of 5.3 transfer over 10–20 min of radiolabel from the endoplasmic reticulum to the Golgi apparatus to the endoplasm.

The targeting of transition vesicles to cis Golgi apparatus furthermore emerges as a property inherent in the interacting membranes and not a characteristic that resides exclusively in the organization of the cytoplasm. Cytoplasmic constituents of the endoplasmic reticulum–Golgi apparatus interface or systems of cytoskeletal guide elements would be expected to be disrupted when reconstituting the cell-free system and would appear to be non-essential for recognition.

An important characteristic pointing to the specificity of the cell-free transfer of membrane from transitional endoplasmic reticulum to the cis Golgi apparatus is the strict temperature dependency of the transfer process. A 16 °C compartment between the endoplasmic reticulum and the Golgi apparatus has been reported for cultured cells infected with temperature-sensitive virus strains [29–31]. Reduced temperature has been shown to affect post-translational processing and secretion in a number of other systems as well [32–35].

This low-temperature block is seen with the cell-free transfer system. Vesicle formation, as determined by morphometry, shows the expected temperature dependency predicted from the Arrhenius equation ($Q_{10} \approx 2$) but not the sharp transition between 12 °C and 20 °C shown by the complete transfer system. Thus, from these results, we are able to conclude that vesicle transfer *in vitro* between endoplasmic reticulum and the cis Golgi apparatus at temperatures of 20 °C and above was physiological and similar to that observed *in situ*, whereas labelling of trans Golgi apparatus was much less affected by temperature and largely unspecific.

The use of the cell-free system largely rules out a temperature effect on cytoskeletal elements. While other factors may contribute as well, preliminary results suggest that transition vesicles formed at low temperatures may be fusion incompetent due to a reduced content of phosphatidylserine and phosphatidylethanolamine (P. Moreau, H. Juguelin, C. Cassagne & D. J. Morré, unpublished work).

As with temperature, the pH dependency of transition-vesicle formation and transfer *in vivo* also provided evidence for its physiological relevance. Optimum formation and transfer were observed at or near the physiological pH of the cytoplasm. In this regard as well, unspecific cell-free transfer at 4 °C was unaffected by pH.

Comparing the three different Golgi apparatus domains, cis, medial and trans, and from the temperature dependency of

transfer and the response to pH, the cell-free transfer *in vitro* of radiolabelled membrane materials from endoplasmic reticulum to Golgi apparatus exhibits strict specificity for cis Golgi apparatus elements. This is suggestive of the operation of a system of interacting signals and signal receptors. Furthermore, the targeting of transition vesicles to cis Golgi apparatus emerges as a property inherent in the interacting membranes and not a characteristic that resides exclusively in the organization of the cytoplasm with the endoplasmic reticulum–Golgi apparatus interface or of systems of cytoskeletal guide elements expected to be disrupted in reconstituting the cell-free system. A strict temperature dependency was exhibited both *in vivo* and *in vitro* where transition-vesicle fusion with the Golgi apparatus was blocked at temperatures below 18 °C. That specific transfer *in vitro* was only to cis-located Golgi apparatus elements and that this transfer showed the same characteristics of temperature dependency as seen *in vivo*, as well as optimum transfer at physiological pH, provides important evidence for the fidelity of the cell-free system as a model suitable for more detailed molecular study.

We thank Keri Safranski for preparation of membranes and Golgi apparatus subfractions and Dorothy Werderitsh for assistance with the electron microscope. Electron microscope and darkroom facilities were provided courtesy of Professor Charles Bracker. Work was supported in part by a grant from the National Institutes of Health GM 44675 (D.J.M., D.M.M.), Phi Beta Psi Sorority (D.M.M.) and Purdue Research Foundation (D.M.M.), 8546-561264 Paper No 13478 of the Purdue University Agricultural Experiment Station, West Lafayette, IN 47907, U.S.A.

REFERENCES

1. Zeigel, R. F. & Dalton, A. J. (1962) *J. Cell Biol.* **15**, 45–54
2. Morré, D. J., Mollenhauer, H. H. & Bracker, C. E. (1971) in *Results and Problems in Cell Differentiation: Origin and Continuity of Cell Organelles* (Reinert, J. & Ursprung, H., eds.), vol. 2, pp. 82–126, Springer Verlag, Berlin, New York, Heidelberg
3. Morré, D. J., Kartenbeck, J. & Franke, W. W. (1979) *Biochim. Biophys. Acta* **559**, 71–152
4. Morré, D. J., Paulik, M. & Nowack, D. (1986) *Protoplasma* **132**, 110–113
5. Nowack, D. D., Morré, D. M., Paulik, M., Keenan, T. W. & Morré, D. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6098–6102
6. Paulik, M., Nowack, D. D. & Morré, D. J. (1988) *J. Biol. Chem.* **263**, 17738–17748
7. Beckers, C. J., Keller, D. S. & Balch, W. E. (1987) *Cell* (Cambridge, Mass.) **50**, 523–534
8. Haselbeck, A. & Shekman, R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2017–2021
9. Balch, W. E., Elliot, M. M. & Keller, D. S. (1986) *J. Biol. Chem.* **31**, 14861–14869
10. Simons, K. & Virta, H. (1987) *EMBO J.* **6**, 2241–2247
11. Morré, D. J., Cheetham, R. D., Nyquist, S. E. & Ovtracht, L. (1972) *Prep. Biochem.* **2**, 61–69
12. Morré, D. J., Morré, D. M. & Heidrich, H. G. (1983) *Eur. J. Cell Biol.* **31**, 263–273
13. Friend, D. & Murray, M. J. (1965) *Am. J. Anat.* **117**, 135–149
14. Morré, D. J., Creek, K. E., Matyas, G. R., Minnifield, N., Sun, I., Baudoin, P., Morré, D. M. & Crane, F. L. (1984) *BioTechniques* **2**, 224–233
15. Navas, P., Minnifield, N., Sun, I. & Morré, D. J. (1986) *Biochim. Biophys. Acta* **881**, 1–9
16. Matyas, G. R. & Morré, D. J. (1983) *J. Histochem. Cytochem.* **31**, 1175–1182
17. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, E. K., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **100**, 76–85
18. Brown, W. J., Constaninescu, E. & Farquhar, M. G. (1984) *J. Cell Biol.* **99**, 320–326
19. Ohtsuki, I. R., Manzi, R. M., Palade, G. E. & Jamieson, J. D. (1978) *Biol. Cell* **31**, 119–126
20. Slot, J. W. & Geuze, H. J. (1985) *Eur. J. Cell Biol.* **38**, 87–93

21. Roth, J. & Berger, E. G. (1982) *J. Cell Biol.* **93**, 223–229
22. Brightman, A. O., Navas, P., Minnifield, N. M. & Morré, D. J. (1992) *Biochim. Biophys. Acta* **1104**, 188–194
23. Hartel-Schenk, S., Minnifield, N., Reutter, W., Hanski, C., Bauer, C. & Morré, D. J. (1991) *Biochim. Biophys. Acta* **115**, 108–122
24. Morré, D. M., Morré, D. J., Bowen, S., Reutter, W. & Windel, K. (1988) *Eur. J. Cell Biol.* **46**, 307–315
25. Morré, D. J., Morré, D. M., Mollenhauer, H. H. & Reutter, W. (1987) *Eur. J. Cell Biol.* **43**, 235–242
26. Palade, G. E. (1983) *Methods Enzymol.* **69**, 24–55
27. Nowack, D. D., Paulik, M., Morré, D. J. & Morré, D. M. (1990) *Biochim. Biophys. Acta* **1051**, 250–258
28. Jelsema, C. J. & Morré, D. J. (1978) *J. Biol. Chem.* **253**, 7960–7971
29. Saraste, J. & Kuismanen, E. (1984) *Cell (Cambridge, Mass)* **38**, 535–549
30. Holmes, K. V., Collier, E. W. & Sturman, L. S. (1981) *Virology* **115**, 334–344
31. Tooze, S., Tooze, J. & Warren, G. (1988) *J. Cell Biol.* **33**, 291–293
32. Tartakoff, A. M. (1986) *EMBO J.* **5**, 1477–1482
33. Fries, E. & Lindstrom, E. (1986) *Biochem. J.* **237**, 33–39
34. Saraste, J., Palade, G. E. & Farquhar, M. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6425–6429
35. Morré, D. J., Minnifield, N. & Paulik, M. (1989) *Biol. Cell. (Paris)* **67**, 51–60

Received 1 May 1992/23 June 1992; accepted 13 July 1992