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Sphingolipid transport from the trans-Golgi network to the apical surface in permeabilized MDCK cells

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We have measured the transport of de novo synthesized fluorescent analogs of sphingomyelin and glucosylceramide from the trans-Golgi network (TGN) to the apical membrane in basolaterally permeabilized Madin–Darby canine kidney (MDCK) cells. Sphingolipid transport was temperature, ATP and cytosol dependent. Introduction of bovine serum albumin (BSA), which binds fluorescent sphingolipid monomer, into the permeabilized cells, did not affect lipid transport to the apical membrane. Both fluorescent sphingomyelin and glucosylceramide analogs were localized to the lumenal bilayer leaflet of isolated TGN-derived vesicles. These results strongly suggest that both sphingolipids are transported from the TGN to the apical membrane via vesicular traffic.

Sphingolipid transport; Fluorescent lipid analog; Streptolysin O; Trans Golgi network; Permeabilized cell; Apical membrane; MDCK cell

1. INTRODUCTION

The plasma membrane of epithelial cells is differentiated into two domains, the apical membrane lining the epithelial lumen and the basolateral domain which faces the blood supply of the tissue [1,2]. Both the vectorial transport and barrier functions of epithelia are dependent on the polarized distribution of proteins and lipids between the apical and basolateral membrane domains. In Madin–Darby canine kidney (MDCK) cells, newly synthesized apicol and basolateral proteins are sorted from each other in the trans-Golgi network (TGN) [3,4]. The sorting of newly synthesized sphingolipids en route to the epithelical cell surface also takes place intracellularly in the Golgi complex, raising the possibility that protein and lipid sorting are directly connected events [5].

In this paper, we used permeabilized MDCK cells [6] to study the transport of newly synthesized sphingolipids from the TGN to the apical surface. The results strongly suggest that vesicular carriers are responsible for sphingolipid transport as previously shown for proteins [7].

Abbreviations: BSA, Bovine serum albumin; C_6-NBD-Cer, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminocaproyl D-erythro-sphingosine; C_6-NBD-GlcCer, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminocaproyl sphingosine glucoside; C_6-NBD-SM, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminocaproyl sphingosine-1-phosphocholine; HA, Hemagglutinin; LDH, Lactate dehydrogenase; MDCK, Madin Darby canine kidney; SLO, Streptolysin O; TGN, trans-Golgi network.

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2. EXPERIMENTAL

2.1. *Incubation of cells with* N-*(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminocaproyl* D-*erythro-sphingosine (*C_6*-NBD-Cer)/bovine serum albumin (BSA)*

Cells grown on 24-mm diameter, 0.4 μm pore size, premounted Transwell polycarbonate filters (a kind gift from Hank Lane, Costar, Cambridge, MA) were washed twice with water bath medium (Eagle's minimal essential medium with Earle's salts containing 10 mM HEPES, pH 7.3, and 0.35 g/l sodium bicarbonate). The filters were then placed in a six-well culture dish, and 2.6 ml water bath medium was added to the basal side and 1 ml 25 μM C_6-NBD-Cer/BSA complex [8,9] in water bath medium was added to the apical side of the filter. The filters were incubated in a 20°C water bath for 60 min at which time the apical and basal media were replaced with water bath medium containing 1% defatted BSA (1 ml to the apical and 2.6 ml to the basal side). The filters were incubated for 60 min at 20°C. The medium was then replaced with fresh water bath medium containing 1% defatted BSA, and filters were incubated for an additional 60 min at 20°C.

2.2. *Cell-permeabilization procedure*

Filter grown cells were washed twice with ice-cold KOAc buffer (25 mM HEPES, pH 7.4, 115 mM potassium acetate, 2.5 mM $MgCl_2$). Then the basal side of the filter was placed on a 50 μl drop of incubation buffer (25 mM HEPES, pH 7.4, 115 mM potassium acetate, 2.5 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 2.5 mM $CaCO_3$) containing 0.5 μg streptolysin-O (SLO) on parafilm, which was placed on a metal plate on ice. 100 μl ice-cold incubation buffer was added to the apical surface and cells were incubated for 10 min. Cell filters were then washed three times with ice-cold KOAc buffer and transferred to a six-well culture dish. Incubation buffer, 1 ml and 2 ml, was added to the apical and basal side, respectively, and cells were incubated for 40 min in a 20°C water bath.

2.3. *Incubation conditions*

Following permeabilization, excess moisture was removed from the filter by blotting the basal side of the Transwell filter to a filter paper. Then the basal side of the filter was placed on 100 μl ice-cold incubation buffer containing either an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 50 μg/ml creatine kinase) or an ATP-depleting system (0.5 mg/ml hexokinase, 12.5 mM glucose) and 5–7 mg/ml MDCK cytosol, which was layered on parafilm in a humid chamber. The chamber was incubated on ice for 30 min and then 500 μl prewarmed incubation buffer was added to the apical side and the cells were incubated at 37°C.

2.4. *Measurement of lipid transport*

After incubation, cell filters were incubated for 30 min at 7°C with back-exchange buffer (25 mM HEPES, pH 7.4, 115 mM potassium acetate, 0.9 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM DTT, 1% defatted BSA) which was added to the apical (1 ml) and/or basal (1 ml) side of the filter. The extraction was repeated by adding fresh back-exchange buffer. After back-exchange, the cells were scraped from the filter after the addition of 1 ml back-exchange medium without BSA. In order to examine the effect of BSA on lipid transport, 1 ml incubation buffer containing 1% defatted BSA was added apically during the 37°C incubation. Lipids were extracted from both back-exchange medium and cells. Extracted lipids were separated by TLC and quantified [5,10].

2.5. *Measurement of protein transport*

Cells were infected with influenze virus strain N (A/chick/Germany/49, Hav 2 Ne1), pulse-labeled and incubated as described [7,11]. After basolateral permeabilization and 37°C incubation, the delivery of hemagglutinin (HA) to the apical surface was measured by its accessibility to exogenously added trypsin [11,12]. Following the trypsin treatment, the cells were lysed and the proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The gels were fixed, dried and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). The HA band intensities were calculated and percent of HA transported was calculated as [(HA at 4°C − HA at 37°C)/(HA at 4°C)] × 100.

2.6. *Cell perforation by filter stripping and incubation of released vesicles with BSA*

Cells grown on 100-mm filters were labeled with C_6-NBD-Cer/BSA and perforated [7,13]. After perforation, the filter culture was transferred to a 100 mm tissue culture dish containing 10 ml incubation buffer without $CaCO_3$ containing an ATP-regenerating system. After incubation at 37°C for 1 h, the incubation medium was collected and clarified by centrifugation for 10 min at 1,500 rpm. The supernatant was divided into two parts and 1% defatted BSA (final concentration) was added to one portion. The supernatant was incubated for 1 h at 4°C and the membranes were pelleted by centrifugation for 1 h at 200,000 × g_{av} and 4°C. The lipids were extracted and separated by TLC.

2.7. *Other procedures*

Highly purified SLO [14] was prepared as described previously [14]. MDCK II cells were grown as described previously [7,13]. Protein concentration was determined using micro bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with BSA as a standard. Lactate dehydrogenase (LDH) was measured by the method of Bergmeyer and Bernt [15]. MDCK cytosol was prepared as described [16] using incubation buffer instead of cytosol buffer. Electron microscopy was performed as described [17].

3. RESULTS AND DISCUSSION

SLO is a 69 kDa sulfhydryl-activated cytolysin, which appears to bind specifically to cholesterol and create pores in membranes [18]. At low temperature, the toxin binds to the surface without causing permeabilization. When cells are warmed after washing away excess toxin, pore information is induced [19]. Using this two-step procedure, Gravotta et al. [6] succeeded in permeabilizing the apical or basolateral domain of MDCK cells selectively. We have modified their procedure in order to deplete endogenous cytosol. Thus, the transport of both protein and lipids from the TGN to the plasma membrane required the addition of exogenous cytosol (see below). When SLO was applied to the basal side of filter-grown MDCK cells at 4°C for 10 min, time-dependent release of LDH activity was observed at 20°C (Fig. 1A). LDH was released only to the basal side. The release was dependent on the concentration of SLO and 5 μg/ml SLO was required to release 50% LDH within 40 min (Fig. 1B). Transmission electron micrographs of permeabilized cells indicate intact apical membranes

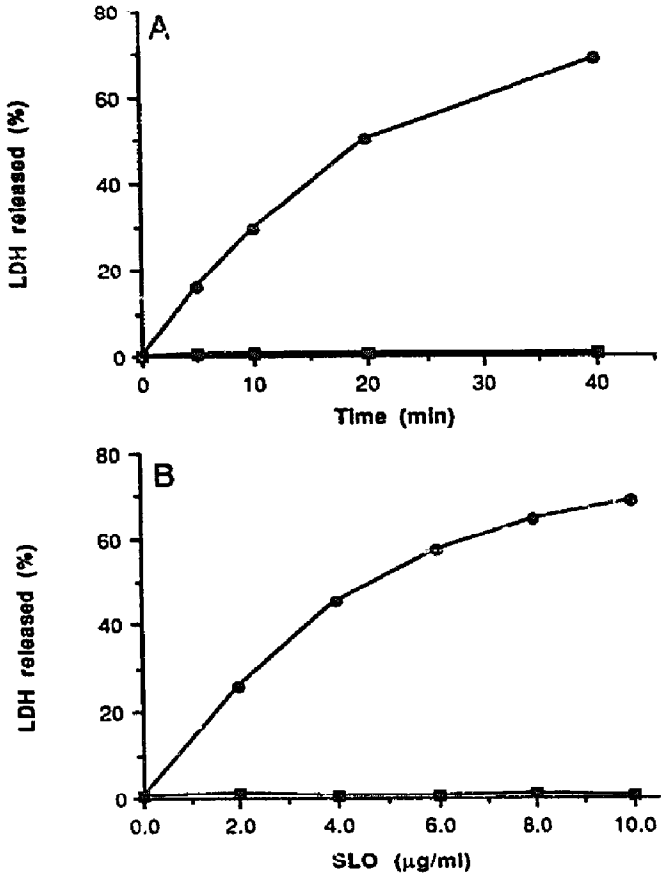

Fig. 1. LDH is released only into the basal medium in basolaterally permeabilized MDCK cells. (A) MDCK cells were basolaterally permeabilized with 10 μg/ml SLO as described in Section 2. At appropriate intervals during 20°C incubation, LDH activity of apical (■) and basal (●) medium and of the cells on the filter were measured. Cell filters were incubated in 0.1% Triton X-100 in KOAc buffer for 30 min at 4°C before measuring enzyme activity. (B) Various concentrations of SLO were added to the basal side of filter grown cells at 4°C for 10 min. Cells were then washed and incubated for 40 min at 20°C. After incubation, LDH activity of apical (■), basal (●) medium and filter-grown cells was measured.

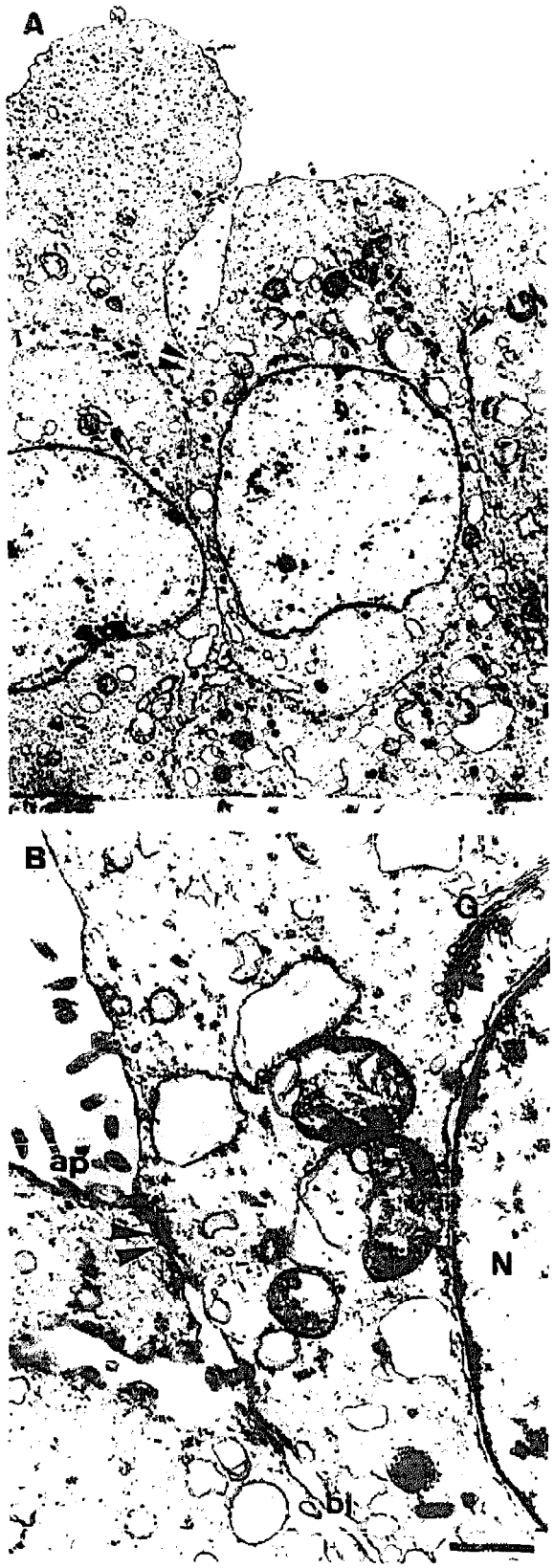

Fig. 2. Electron microscopy of basolaterally permeabilized cells. Cells were permeabilized using SLO as described in Section 2. The arrowheads in (A) indicate sites of tight junctions. The junction indicated by double arrowheads in (A) is shown at higher magnification in (B). Note that the Golgi cisternae (G) are well preserved. N, nucleus; ap, apical plasma membrane; bl, basolateral plasma membrane. Bars = 1 μm.

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and apparently normal intercellular junctions after SLO treatment (Fig. 2).

Using basolaterally permeabilized cells, we have measured the transport of both protein and lipids from the TGN to the plasma membrane. For protein transport, we have measured the delivery of influenza virus HA to the apical membrane. Filter-grown MDCK cells were infected with influenza N virus, pulse-labeled and incubated at 19.5°C for 90 min. During the 19.5°C incubation, HA is accumulated in the TGN [11]. During the 37°C incubation after basolateral permeabilization, HA was transported to the apical membrane. HA transport was dependent on temperature, ATP, and cytosol (Fig. 3).

For lipid transport, the cells were labeled with C_6-NBD-Cer and incubated for 2 h at 19.5°C before being permeabilized basolaterally. During this incubation, C_6-NBD-Cer is metabolized to *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminocaproyl sphingosine-1-phosphocholine (C_6-NBD-SM) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminocaproyl sphingosine glucoside (C_6-NBD-GlcCer), which accumulate in the TGN [5]. Transport of fluorescent sphingolipids to the apical membrane was assayed by extracting the plasma membrane lipids into BSA. First we asked whether lipid transport to the apical and basolateral membranes could be measured separately in permeabilized cells. To test this, BSA was selectively added to either the apical or the basolateral surface after transport (Table I). The

Table I

C_6-NBD-GlcCer and C_6-NBD-SM on one surface are not depleted by BSA present in the medium on the opposite side in permeabilized cells

Medium	C_6-NBD-SM		C_6-NBD-GlcCer	
	% Transported			
	Incubation 1	Incubation 2	Incubation 1	Incubation 2
I				
Apical	8.72 ± 0.32	1.50 ± 0.05	21.07 ± 0.81	5.35 ± 0.15
Basolateral	5.48 ± 0.69	2.60 ± 0.21	14.95 ± 1.20	12.42 ± 0.23
II				
Apical	9.41 ± 0.85	1.58 ± 0.05	22.20 ± 1.48	5.93 ± 0.80
Basolateral	0.62 ± 0.27	4.21 ± 0.61	2.55 ± 0.09	14.28 ± 0.34
III				
Apical	2.88 ± 0.50	6.00 ± 0.24	4.98 ± 1.51	17.91 ± 1.26
Basolateral	5.42 ± 0.61	2.83 ± 0.36	12.71 ± 3.46	11.36 ± 1.45

Cells were incubated with C_6-NBD-Cer and permeabilized (see Section 2). After transport, the cells were incubated (incubation 1) selectively at 7°C in the presence of BSA on the apical (II) or the basal side (III). After 0.5 h, BSA was added to both sides for 0.5 h at 7°C (incubation 2). Control filters were incubated twice with BSA on both sides for 0.5 + 0.5 h. Values are the mean ± S.D. of three independent experiments.

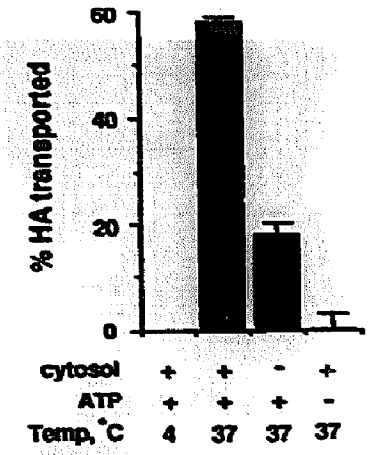

Fig. 3. HA transport in permeabilized MDCK cells. Cells were infected, pulse labeled, permeabilized and preincubated at 4°C as described in section 2. The permeabilized cells were then incubated for 45 min at 4°C or 37°C under various conditions. After transport, the apical surface was treated with trypsin and the amount of HA transported to the cell surface was calculated as described in Section 2. The bars indicate the variation of duplicate experiments.

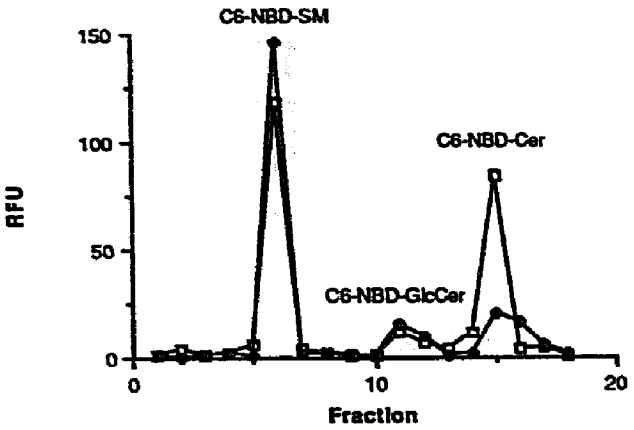

Fig. 5. Distribution of fluorescent sphingolipids in TGN-derived vesicles. Vesicles were isolated from MDCK cells and incubated with BSA as described in Section 2. Lipids were extracted and separated by TLC. □, absence of BSA; ●, presence of BSA.

transport of both C_6-NBD-SM and C_6-NBD-GlcCer to the apical membrane was observed at 37°C in permeabilized cells. The efficiency of transport was 60–70% of that of intact cells after 45 min. Basolaterally applied BSA extracted only a small amount of apically transported fluorescent sphingolipids and vice versa.

We then measured several parameters of lipid transport from the TGN to the apical membrane. Low temperature, ATP depletion and cytosol depletion inhibited both fluorescent sphingomyelin and glucosylceramide transport (Table II). Since NBD-sphingolipids are trapped by BSA, BSA added during transport should inhibit the transport of fluorescent lipid if lipid monomers were transported from the TGN across the cytosol to the plasma membrane. Addition of BSA to the basolateral medium did not significantly affect lipid transport to the apical membrane (Fig. 4). This result makes it unlikely that sphingolipids are transported to the plasma membrane via monomer diffusion.

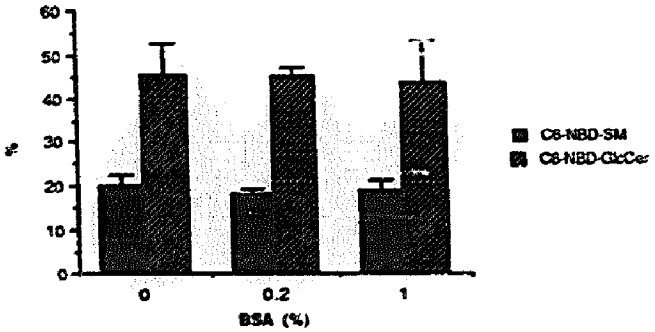

Fig. 4. BSA added to permeabilized basolateral membrane did not inhibit lipid transport to the apical membrane. MDCK cells were incubated with C_6-NBD-Cer and permeabilized. Cytosol and ATP-regenerating system containing various concentrations of BSA was added to the basal side. After 30 min at 4°C, 1 ml ATP-regenerating system containing 1% BSA was added to the apical side and cells were incubated at 37°C for 45 min. After incubation, fluorescent sphingolipids in the medium and cell filters were measured. The bars indicate the S.D. from the mean ($n = 3$).

These results speak in favor of vesicular traffic of fluorescent sphingolipids from the TGN to the apical plasma membrane. Further support for this mechanism was obtained by measuring the topology of fluorescent sphingolipids in the transport vesicles derived from the TGN. A vesicle-enriched fraction obtained from filter-permeabilized cells [7] was incubated with or without BSA. The vesicles were then pelleted and lipid extracted. Neither C_6-NBD-SM nor C_6-NBD-GlcCer were depleted by BSA treatment, whereas the content of C_6-NBD-Cer decreased significantly (Fig. 5). This result indicates that both C_6-NBD-SM and C_6-NBD-GlcCer are oriented towards the luminal leaflet of these vesicles. These data support the involvement of vesicular carriers in the transport of sphingolipid from the TGN to the apical surface. Since sphingomyelin is synthesized on the lumenal leaflet of the Golgi apparatus [20,21] and glucosylceramide is synthesized at the cytoplasmic surface [22–24], our results suggest that transbilayer movement of glucosylceramide from the cytoplasmic surface

Table II

ATP and cytosol dependence of lipid transport to the plasma membrane

Treatment	C_6-NBD-SM	C_6-NBD-GlcCer
Complete	100	100
ATP-depletion	21.1 ± 5.4	19.9 ± 3.8
− Cytosol	34.1 ± 11.9	36.3 ± 12.9

Difference between transport at 37°C and at 4°C under complete condition was taken as 100. Values are the mean ± S.D. of three independent experiments.

to the lumenal leaflet occurs before the vesicles bud from the TGN.

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