

## The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells

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**Tight junctions in epithelial cells have been postulated to act as barriers inhibiting lateral diffusion of lipids and proteins between the apical and basolateral plasma membrane domains. To study the fence function of the tight junction in more detail, we have fused liposomes containing the fluorescent phospholipid N-Rh-PE into the apical plasma membrane of MDCK cells. Liposome fusion was induced by low pH and mediated by the influenza virus hemagglutinin, which was expressed on the apical cell surface after viral infection. Redistribution of N-Rh-PE to the basolateral surface, monitored at 0°C by fluorescence microscopy, appeared to be dependent on the transbilayer orientation of the fluorescent lipids in the plasma membrane. Asymmetric liposomes containing over 85% of the N-Rh-PE in the external bilayer leaflet, as shown by a phospholipase A<sub>2</sub> assay, were generated by octyl β-D-glucoside dialysis. When these asymmetric liposomes were fused with the apical plasma membrane, fluorescent lipid did not move to the basolateral side. Symmetric liposomes which contained the marker in both leaflets were obtained by freeze-thawing asymmetric liposomes or by reverse-phase evaporation. Upon fusion of these with the apical membrane, redistribution to the basolateral membrane occurred immediately. Redistribution could be observed with asymmetric liposomes only when the tight junctions were opened by incubation in a Ca<sup>2+</sup>-free medium. During the normal experimental manipulations the tight junctions remained intact since a high trans-epithelial electrical resistance was maintained over the cell monolayer. We conclude that the tight junction acts as a diffusion barrier for the fluorescent phospholipid N-Rh-PE in the exoplasmic leaflet of the plasma membrane but not in the cytoplasmic leaflet.**

**Key words:** tight junction/lipid/lateral diffusion/lipid asymmetry/epithelial cell

### 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. Although part of a continuous membrane, the two plasma membrane domains display unique protein and lipid compositions (see Simons and Fuller, 1985). The domains are separated by a specific zone of cell-cell contact which encircles the top of each cell, the tight junction or the zonula occludens; it has been suggested that this junction plays a role in the maintenance of the compositional differences between the two domains by forming a diffusion barrier in the plane of the membrane (see Diamond, 1977).

The tight junction, or zonula occludens, connects the apices of neighbouring cells and seals the spaces between cells. This sealing function has been well characterized. Although the degree of permeability of the tight junctions varies in different epithelia, the tight junctions have been reported to be essentially impermeable to molecules with radii  $\geq 15$  Å (Cereijido *et al.*, 1978, Madara and Dharmasathaphorn, 1985). In MDCK cells they display a characteristic pattern of cation selectivity which makes them behave as pores with hydrated negative sites (Cereijido *et al.*, 1978). The impermeability to anions also suggests that surface molecules with negative charges (phospholipids) cannot pass through.

The molecular arrangement at the membrane contact sites of the tight junction is not understood. No proteins directly associated with the tight junction have yet been identified although the evidence shows that proteins are involved in its function (Griep *et al.*, 1983; Stevenson and Goodenough, 1984; Gumbiner and Simons, 1986). Models for the structure of the tight junction have been devised on the basis of its ultrastructural features (see Hirokawa, 1982; Pinto da Silva and Kachar, 1982). At the site of the tight junctions the intercellular space is occluded and the two apposed plasma membranes seem to have fused or merged (Farquhar and Palade, 1963). In freeze fracture replicas a complementary pattern of anastomosing strands and furrows is observed. Until recently it was assumed that these strands had a proteinaceous nature (e.g. Hirokawa, 1982). However, it has also been argued that the complementarity of strands and furrows can only be explained if each strand were to consist of an intramembranous hexagonal cylinder of lipids (Pinto da Silva and Kachar, 1982; Kachar and Reese, 1982). In this lipid model of the zonula occludens, the cytoplasmic leaflet of the plasma membrane is continuous from the apical to the basolateral plasma membrane, while the exoplasmic leaflets are not; they are interrupted by the hexagonal lipid cylinder. However, the exoplasmic leaflets are postulated to be in continuity with the exoplasmic leaflets of the plasma membrane of the adjacent cells.

The lipid model of the tight junction has interesting predictions for the diffusion of membrane components. Membrane spanning proteins would not be able to pass through from either side. Passage of membrane components which are situated in only one leaflet of the plasma membrane (e.g. lipid molecules) would depend on their orientation in the bilayer. They would diffuse through the continuous cytoplasmic leaflet, but in the exoplasmic bilayer leaflet the tight junction would act as a barrier for movement to the other side of the cell. At the same time lipid molecules in the external leaflet would be able to diffuse through a continuous lipid monolayer from one cell to another.

In this paper we apply fusion of symmetric or asymmetric liposomes to introduce fluorescent lipids into either the exoplasmic or into both leaflets of the apical membrane of Madin-Darby canine kidney (MDCK) cells. Two strains of MDCK cells were grown as monolayers on permeable supports. Strain I and strain

**Table I.** Unique [<sup>32</sup>P]phospholipid composition of MDCK strain I cells and of the viruses isolated from the basolateral (VSV) or apical (FPV) cell surface

	Cells (%) <sup>a</sup>	VSV (%)	FPV (%)
SPH	7.7 ± 0.3	15.6 ± 1.1	20.5 ± 1.2
PC	62.5 ± 1.4	28.9 ± 1.2	9.6 ± 1.9
PI	5.2 ± 0.5	3.2 ± 0.4	2.8 ± 0.6
PS	4.2 ± 0.3	18.9 ± 0.2	22.4 ± 1.8
PE	20.1 ± 0.7	33.5 ± 1.9	44.8 ± 2.1

<sup>a</sup>A monolayer of MDCK strain I cells was grown on nitrocellulose filters with a pore size of 3 μm in mini-Marbrook chambers as described previously (Fuller *et al.*, 1984). After 3 days the medium was replaced by serum-free medium (Taub *et al.*, 1979, but with additional 50 nM Na<sub>2</sub>SeO<sub>3</sub>) containing 0.1 mM phosphate and 1 mCi <sup>32</sup>P/ml. After 36 h the monolayers were infected either with fowl plague virus (FPV) or with vesicular stomatitis virus (VSV). The <sup>32</sup>P medium was added back to the cells for 5 h, after which it was replaced by new serum-free medium. At 11 h after infection the virus was harvested from the apical or basolateral medium respectively, and purified on triplicate potassium tartrate gradients. Phospholipids from the two peak fractions were extracted, analyzed by h.p.t.l.c. and quantitated (van Meer and Simons, 1982). The numbers give the percentage of total lipid <sup>32</sup>P<sub>i</sub> present in each phospholipid class and are followed by the sample standard deviation (*n* = 6).

II MDCK cells (see Hansson *et al.*, 1986) differ in tight junction properties in that they display different trans-epithelial electrical resistances. A confluent monolayer of MDCK strain II cells typically displays a trans-epithelial electrical resistance between 100–300 ohm.cm<sup>2</sup>, while MDCK strain I cells develop resistances of >2000 ohm.cm<sup>2</sup>. Both MDCK strains appear equally polar in their distribution of cell surface proteins (see Simons and Fuller, 1985; S.Fuller and K.Simons, submitted). The results suggest that the tight junction forms a diffusion barrier for lipids exclusively in the exoplasmic bilayer leaflet of the plasma membrane.

## Results

### *MDCK strain I cells on permeable supports display a polarized distribution of lipids*

MDCK strain II cells grown on solid supports have a polarized phospholipid distribution (van Meer and Simons, 1982). Here, we tested whether the phospholipids of the MDCK strain I cells are polarized when grown on a permeable support. As in the earlier experiments with strain II cells, we made use of enveloped viruses, fowl plague virus (FPV) or vesicular stomatitis virus (VSV), which bud exclusively from the apical and basolateral surface of these cells respectively (Rodriguez Boulan and Sabatini, 1978), and thereby sample the lipid composition of the respective plasma membrane domain.

Monolayers of MDCK strain I cells on nitrocellulose filters with a pore size of 3 μm were infected with FPV or in parallel experiments with VSV. Eleven hours after infection, FPV was harvested from the apical medium and VSV, which is able to pass through the 3 μm pores of the filter, from the basal medium. The electrical resistance remained >1000 ohm.cm<sup>2</sup> throughout the experiment, implying that most of the tight junctions were intact. The phospholipid composition of the viruses and the total cells is compared in Table I. Both viruses had a typical plasma membrane composition in that they possessed far less phosphatidylcholine (PC) and more sphingomyelin (SPH) and phosphatidylserine (PS) than the total cells. Comparing FPV, the apical virus, with VSV, the virus representative of the basolateral membrane, the most striking difference was a 4-fold depletion of PC

**Table II.** Identical [<sup>32</sup>P]phospholipid composition of viruses from baby hamster kidney (BHK) cells which do not have a polarized plasma membrane<sup>a</sup>

	VSV (%) <sup>b</sup>	FPV (%)
SPH	17.6 ± 0.3	18.0 ± 0.5
PC	38.3 ± 0.4	38.5 ± 1.3
PI	10.1 ± 1.4	8.2 ± 0.7
PS	7.0 ± 1.3	6.7 ± 0.3
PE	27.1 ± 0.6	28.6 ± 0.3

<sup>a</sup>Subconfluent BHK cells were grown in 40 mm diameter plastic dishes for 24 h, labeled with 1 mCi <sup>32</sup>P/ml for 24 h and infected with either VSV or FPV as described before for MDCK cells (van Meer and Simons, 1982). After 10 h of infection the virus was harvested and purified from the culture medium.

<sup>b</sup>The [<sup>32</sup>P]phospholipids of the two peak fractions of duplicate gradients were quantitated as described in Table I (*n* = 4).

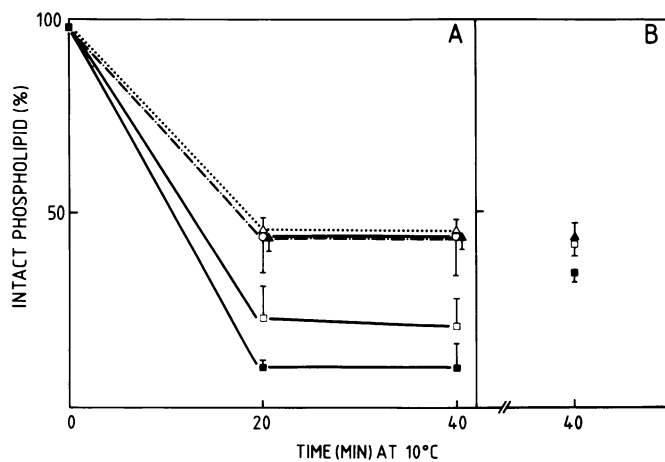
in the apical membrane as compared to phosphatidylethanolamine (PE), a difference also observed in MDCK strain II cells on plastic (van Meer and Simons, 1982). The major differences between the two systems was a reversed polarity of SPH and a higher content of PC in the plasma membrane of MDCK strain I cells on filters.

To exclude the possibility that the differences in phospholipid composition between FPV and VSV reflect a virus-mediated selection of phospholipids during the budding process, the same viruses were allowed to bud from baby hamster kidney (BHK) cells which do not have a polarized plasma membrane. Under these conditions the phospholipid compositions of the viruses (Table II) were very similar, demonstrating that they incorporate the phospholipids present in the plasma membrane of the host cells into the viral envelope essentially without selection (see Klenk and Chopin, 1970a, 1970b).

### *Fusion of liposomes into the apical membrane of filter-grown MDCK cells*

We used an influenza virus hemagglutinin (HA)-mediated fusion protocol to insert water-insoluble fluorescent phospholipids (Struck *et al.*, 1981) directly into both leaflets of the apical plasma membrane of filter-grown cells (van Meer and Simons, 1983; van Meer *et al.*, 1985). This comprises infection of cells with influenza virus, binding of liposomes to the viral HA expressed on the cell surface, and fusion by a 60-s incubation at pH 5.0 and 37°C. The amount of cell-associated liposomes was quantitated by counting a filter with cell monolayer for liposomal radioactivity. To monitor the extent of fusion we followed the hydrolysis of liposomal cholesterol [<sup>14</sup>C]oleate, which is mediated by a cellular enzyme and occurs only when the cholesterol oleate has been incorporated into the cellular plasma membrane (van Meer *et al.*, 1985).

Routinely after the fusion procedure (see Materials and methods; and van Meer *et al.*, 1985), 0.08 nmol liposomal phospholipid was found associated to a monolayer of 2.6 × 10<sup>6</sup> MDCK strain I cells and 0.2 nmol liposomal phospholipid to 3.2 × 10<sup>6</sup> MDCK strain II cells. Of these cell-associated liposomes 40–55% had actually fused with the apical membrane as was determined by the cholesterol oleate hydrolysis. The apical surface area of a monolayer of strain I and strain II cells on a filter measures 5.8 and 9.6 cm<sup>2</sup> respectively (von Bonsdorff *et al.*, 1985). Taking 90 Å<sup>2</sup> for a cholesterol–phospholipid pair, it can be calculated that in this protocol 1.5–2 and 2–3% of additional lipids are fused into the apical cell membrane of strain I and strain II cells respectively. Since the liposomes contained



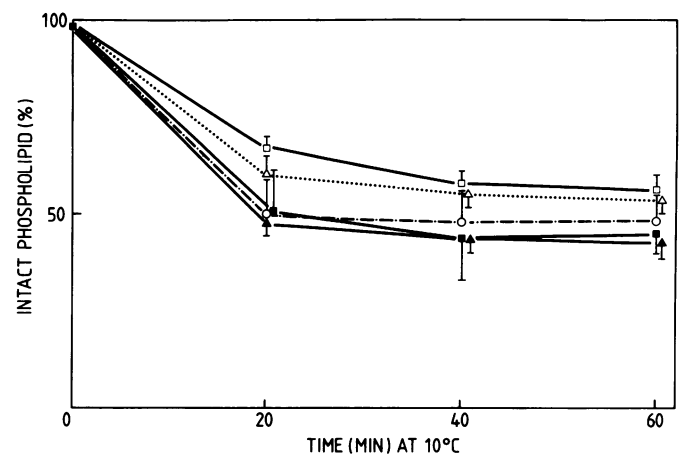
**Fig. 1.** Asymmetric orientation of N-Rh-PE in octylglucoside liposomes as measured by phospholipase  $A_2$  hydrolysis (A) and abolishment of this asymmetry by freeze-thawing (B). For each time-point large unilamellar liposomes (80 nmol total lipid) prepared by octyl  $\beta$ -D-glucoside dialysis, before (panel A) or after freeze-thawing (panel B) as described in the text, were incubated with 8 IU of phospholipase  $A_2$  from *Naja naja* at 10°C. Hydrolysis was stopped by EDTA and the lipids were extracted and analyzed as described in Materials and methods. The curves are the mean of three independent experiments with duplicate samples each. The bar indicates the SD ( $n = 6$ ).  $\Delta$ , egg PE;  $\circ$ , egg PC;  $\blacktriangle$ , [ $^{14}$ C]DOPE;  $\blacksquare$ , N-NBD-PE;  $\square$ , N-Rh-PE.

1 mol % of *N*-(lissamine rhodamine-B-sulfonyl)dioleoyl phosphatidylethanolamine, N-Rh-PE, calculation shows that on average  $3.7 \times 10^5$  and  $7.5 \times 10^5$  N-Rh-PE molecules became cell-associated and  $1.5 - 2 \times 10^5$  or  $3 - 4 \times 10^5$  N-Rh-PE molecules became fused per cell for strain I and strain II respectively.

#### Transbilayer distribution of the fluorescent phospholipids *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoyl phosphatidylethanolamine (N-NBD-PE) and N-Rh-PE in large unilamellar liposomes

To probe whether the tight junctions inhibit lipid diffusion from the apical to the basolateral surface, we used the fluorescent phospholipid N-Rh-PE which was incorporated into the liposomes. During fusion of liposomes with the plasma membrane the external leaflet of the liposome should become continuous with the exoplasmic leaflet of the plasma membrane, whereas the internal leaflet of the liposome should merge with the cytoplasmic leaflet. Since the fluorescent phospholipid molecules might display a different behavior depending on whether they were inserted into the exoplasmic or the cytoplasmic leaflet of the plasma membrane, we used phospholipase  $A_2$  to assay the transbilayer orientation of N-Rh-PE in the liposomes (Sundler *et al.*, 1978; Wilschut *et al.*, 1979; Kumar and Gupta, 1985). It should be noted that N-Rh-PE constituted only 1 mol % of the total liposomal lipids.

We first tested large unilamellar liposomes prepared by octylglucoside dialysis, which have been used in previous studies (van Meer *et al.*, 1985). At 10°C phospholipase  $A_2$  rapidly hydrolyzed the matrix PC and PE and the [ $^{14}$ C]dioleoyl phosphatidylethanolamine (DOPE) (Figure 1A), reaching a plateau of 55% hydrolysis after 20 min at 10°C. The addition of fresh phospholipase  $A_2$  and  $Ca^{2+}$  did not result in higher levels of hydrolysis. We conclude that PC and PE are about equally distributed over the two leaflets of the bilayer. In striking contrast, the hydrolysis of the fluorescent phospholipids N-NBD-PE and N-Rh-PE did not level off at 50% but reached levels of 95 and 85% respectively. N-NBD-PE and N-Rh-PE are thus highly enriched in the external leaflet of octylglucoside dialysis liposomes.



**Fig. 2.** N-NBD-PE and N-Rh-PE are symmetrically localized in REVs. For each time-point large unilamellar liposomes (80 nmol total lipid), prepared by reverse-phase evaporation and sized through a 0.2- $\mu$ m polycarbonate filter, were incubated with 8 IU of phospholipase  $A_2$ . For treatment and meaning of the symbols see Figure 1.

To exclude the possibility that any residual octylglucoside caused transbilayer movement selectively of N-NBD-PE and N-Rh-PE, liposomes were redialyzed three times for 4 h, or deoxycholate was used instead of octylglucoside. The hydrolysis pattern did not change. Size heterogeneity of the liposomes did not play a role since after a 60-min spin at 100 000  $g$  max pellet and supernatant displayed identical properties. The arrangement of most of the N-NBD-PE and N-Rh-PE in the external leaflet is most likely due to an insertion of the fluorescent lipids after closed bilayer structures had formed during detergent dialysis (Helenius *et al.*, 1981).

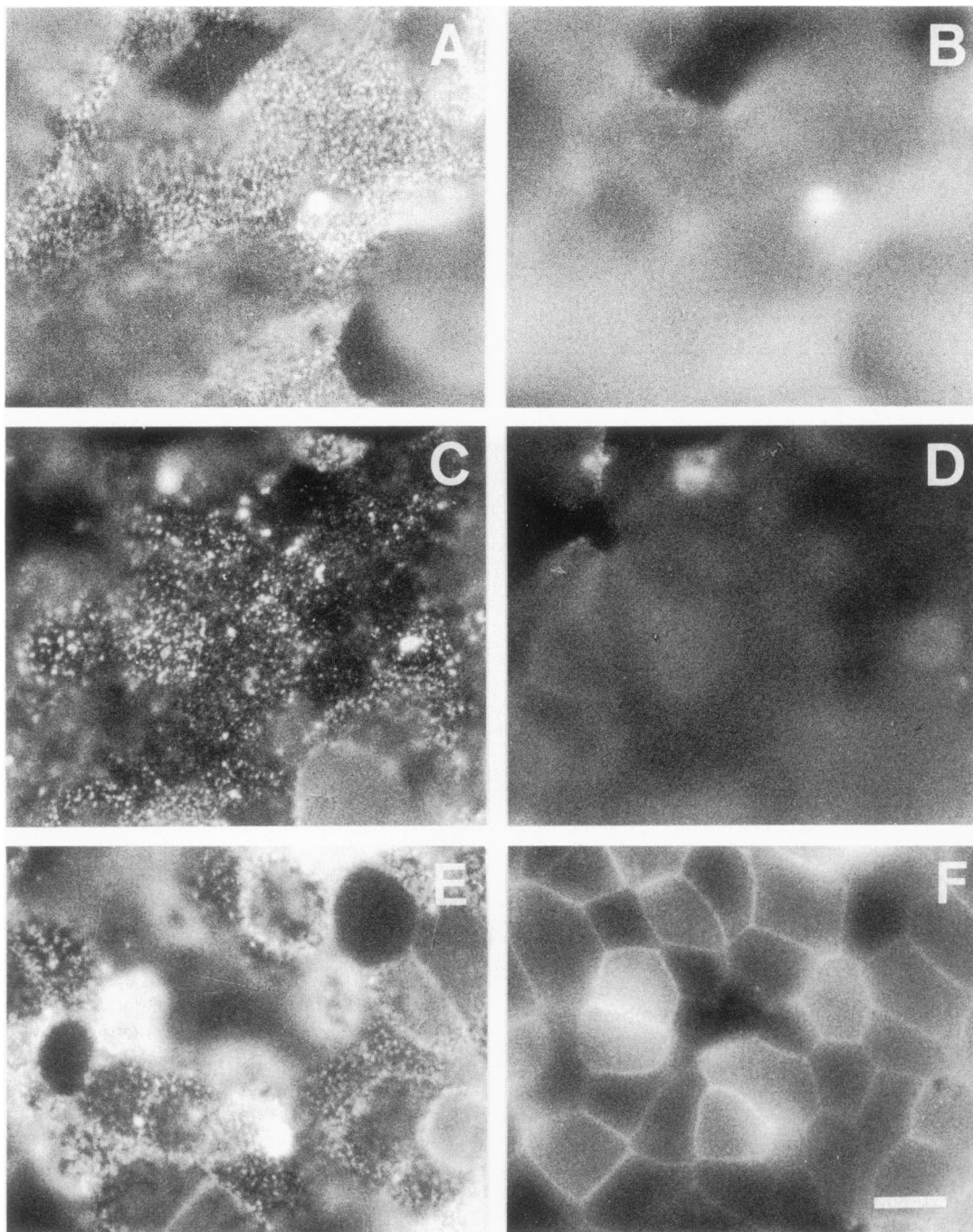
The highly asymmetrical distribution of the fluorescent phospholipids could be largely abolished by freeze-thawing. Ten cycles of freezing in a  $-70^\circ\text{C}$  freezer and thawing on the bench fused the liposomes into larger structures, observed as a change in optical diffraction. After sizing these by extrusion through a polycarbonate filter with a pore diameter of 0.2  $\mu\text{m}$ , the resulting liposomes were found to be symmetric: the hydrolysis of both N-NBD-PE and N-Rh-PE closely followed that of the [ $^{14}$ C]DOPE (Figure 1B).

Symmetrical liposomes of the same lipid composition and size could also be prepared by reverse-phase evaporation. When the reverse-phase evaporation vesicles (REVs) were subjected to phospholipase  $A_2$ , the hydrolysis of the matrix PC, the matrix PE and [ $^{14}$ C]DOPE reached an equilibrium at 50% and now also the fluorescent phospholipids N-NBD-PE and N-Rh-PE were essentially localized symmetrically (Figure 2).

The different lipid asymmetries in the various types of liposomes made it possible to fuse the fluorescent phospholipids either mainly into the exoplasmic leaflet or into both leaflets. With the octylglucoside liposomes one should insert 85–95% of the fluorescent phospholipids into the exoplasmic leaflet of the apical plasma membrane as compared to 5–15% into the cytoplasmic leaflet, whereas symmetrical insertion into both leaflets should result from fusing freeze-thawed liposomes or REVs.

#### Redistribution of N-Rh-PE from the apical to the basolateral surface

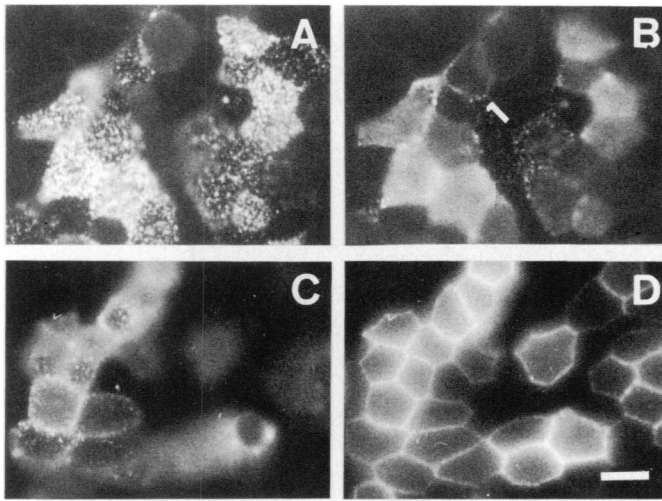
**Octylglucoside liposomes.** When asymmetric octylglucoside liposomes containing N-Rh-PE were allowed to bind to a monolayer of filter-grown MDCK strain II cells, a characteristic dotted pattern was observed under the fluorescence microscope (Figure



**Fig. 3.** N-Rh-PE does not diffuse to the basolateral surface when fused into the exoplasmic leaflet of the apical plasma membrane of MDCK strain II cells. Octylglucoside liposomes containing N-Rh-PE predominantly in the external bilayer leaflet (Figure 1) were bound at 0°C to the influenza virus HA expressed on the apical surface of MDCK strain II cells after viral infection (panels A and B). Liposome-plasma membrane fusion was induced by incubation for 60 s at pH 5.0 and 37°C (panels C and D). No redistribution to the basolateral surface is observed, although 40% of the cell-associated liposomes had fused, as assayed by cholesterol oleate hydrolysis (van Meer *et al.*, 1985). N-Rh-PE only diffuses to the basolateral surface after opening of the tight junctions with Ca<sup>2+</sup>-free medium for 60 s at 37°C (panels E and F). The microscope was focused at the apical cell surface (panels A, C and E) or halfway down the lateral surface (panels B, D and F). Bar = 10 μm.

3A and B). Combining the amount of binding with the liposome size shows that an average 260 liposomes were bound per cell. Thus the dots cannot represent individual liposomes but probably reflect binding to microvilli on the cell surface (Rodriguez Boulan, 1983). Upon fusion the pattern of fluorescence did not change (Figure 3C and D), but part of the cell-associated liposomes were lost during the fusion step (van Meer and Simons, 1983). All fluorescence was found on the apical surface and remained there

for hours at 0°C. No sharp pattern of fluorescence was observed when the focal plane was lowered to the lateral side of the cells. A strikingly different pattern was observed immediately after the tight junctions were opened by a 60-s incubation at 37°C in complete medium without Ca<sup>2+</sup> (Figure 3E and F). Now when the microscope was focused below the apical plane a honeycomb pattern of sharp fluorescent lines appeared, typical of lateral membrane staining (Fuller *et al.*, 1984), which implies



**Fig. 4.** R18 in octylglucoside liposomes is properly fused into the apical plasma membrane of MDCK strain II cells and diffuses to the basolateral surface. Octylglucoside liposomes containing R18 were bound (panels A and B) or fused (panels C and D) with MDCK cells as described in the legend to Figure 3. Fusion was 50% of the cell-associated liposomes. Note that not all cells are infected. Binding and fusion are restricted to those cells that are. The monolayers in all the experiments displayed (Figures 3–6) are completely confluent. Bar = 10  $\mu\text{m}$ .

that the fluorescent lipids under these conditions had diffused to the basolateral surface. The incubation in  $\text{Ca}^{2+}$ -free medium did not increase the level of fusion. Incubation at  $37^\circ\text{C}$  in a  $\text{Ca}^{2+}$ -containing medium did not result in any redistribution to the lateral surface. We conclude that the tight junction acts as a diffusion barrier for N-Rh-PE when this lipid is inserted predominantly into the exoplasmic leaflet of the apical plasma membrane by fusion of octylglucoside liposomes.

The lack of redistribution to the lateral surface was dependent on the properties of the fluorescent lipid probe. When N-Rh-PE in the liposomes was replaced by a more artificial probe, octadecylrhodamine B chloride (R18), this fluorescent lipid, which does not spontaneously exchange through the aqueous phase (Hoekstra *et al.*, 1984), remained localized on the apical cell surface after liposome binding. However, after fusion at pH 5, R18 redistributed to the basolateral membrane (Figure 4), implying a correct insertion into the plasma membrane during the fusion process. The transbilayer distribution of R18 in the liposomes could not be assessed, but with both N-Rh-PE- and R18-containing liposomes 40–50% of the cell-associated liposomes had been fused, as shown by the cholesterol oleate hydrolysis assay.

*Freeze-thawed octylglucoside liposomes and reverse-phase evaporation vesicles (REV)s.* To test whether the tight junction acts as a barrier to lipid diffusion in both leaflets of the plasma membrane or in the exoplasmic leaflet only, we subsequently fused freeze-thawed octylglucoside liposomes or REV)s into the plasma membrane of the MDCK strain II cells. Since in these liposomes N-Rh-PE is symmetrically distributed across the bilayer, the lipid probe should be present in both leaflets of the plasma membrane after fusion.

When freeze-thawed octylglucoside liposomes were bound to a monolayer of MDCK strain II cells a dotted pattern was observed (Figure 5A and B), which was very similar to that of bound octylglucoside liposomes (Figure 3A and B). The fusion assay showed that no fusion had occurred. Upon subsequent fusion of the freeze-thawed liposomes or of REV)s to the apical plasma membrane (Figure 5C–F), however, the resulting fluorescence

pattern was very different from that after octylglucoside liposome fusion (Figure 3C and D). In contrast to the latter situation, a pattern of fluorescent lines was observed at the level of the lateral membrane, suggesting that part of the N-Rh-PE had diffused through the tight junction at  $0^\circ\text{C}$ . We interpret this immediate redistribution of N-Rh-PE to the lateral surface after fusion of symmetric liposomes as evidence for the absence of a lipid diffusion barrier in the cytoplasmic leaflet of the plasma membrane. Opening of the tight junctions resulted in an increase in the amount of fluorescent label seen on the basolateral side, as if in addition to the N-Rh-PE in the cytoplasmic leaflet of the apical membrane, also the N-Rh-PE in the exoplasmic leaflet diffused to the lateral membrane (not shown).

#### *Redistribution of N-Rh-PE occurs in the presence of intact tight junctions*

We also performed a series of experiments with filter-grown strain I MDCK cells using conditions which were as mild as possible to avoid perturbing the functions of the tight junctions. The integrity of the tight junctions is evaluated more easily with these cells since the trans-epithelial resistance is >10-fold higher than with MDCK strain II cells. Furthermore, trypsin treatment can be omitted from the fusion protocol with strain I MDCK cells, for reasons described under Materials and methods. Monolayers having a trans-epithelial resistance of  $4500 \pm 1500 \text{ ohm.cm}^2$  (mean  $\pm$  SD,  $n = 6$ ) were infected with 5 p.f.u./cell of influenza virus instead of the usual 20 p.f.u. This resulted in a mosaic of infected and non-infected cells. At 4 instead of 5 h after infection, liposomes (octylglucoside liposomes or REV)s were allowed to bind to the cells on ice. After 30 min of binding, fusion of the liposomes was induced by an incubation for 60 sec at pH 5.0 and  $37^\circ\text{C}$ , after which the monolayer was washed in pH 7.4 medium. The electrical resistance of the cell monolayers after all these manipulations was  $1190 \pm 180 \text{ ohm.cm}^2$  ( $n = 10$ ), which implies that most of the tight junctions were still intact (see Fuller and Simons, submitted). After the measurement the cells were immediately studied by fluorescence microscopy and the morphological results are illustrated in Figure 6. The experiment confirmed the previous experiments on strain II cells. After fusion of octylglucoside liposomes, redistribution of fluorescence to the basolateral surface did not occur (figure 6A and B) unless the tight junctions were opened (Figure 6C and D). When REV)s were fused to the apical surface, redistribution occurred immediately (Figure 6E and F).

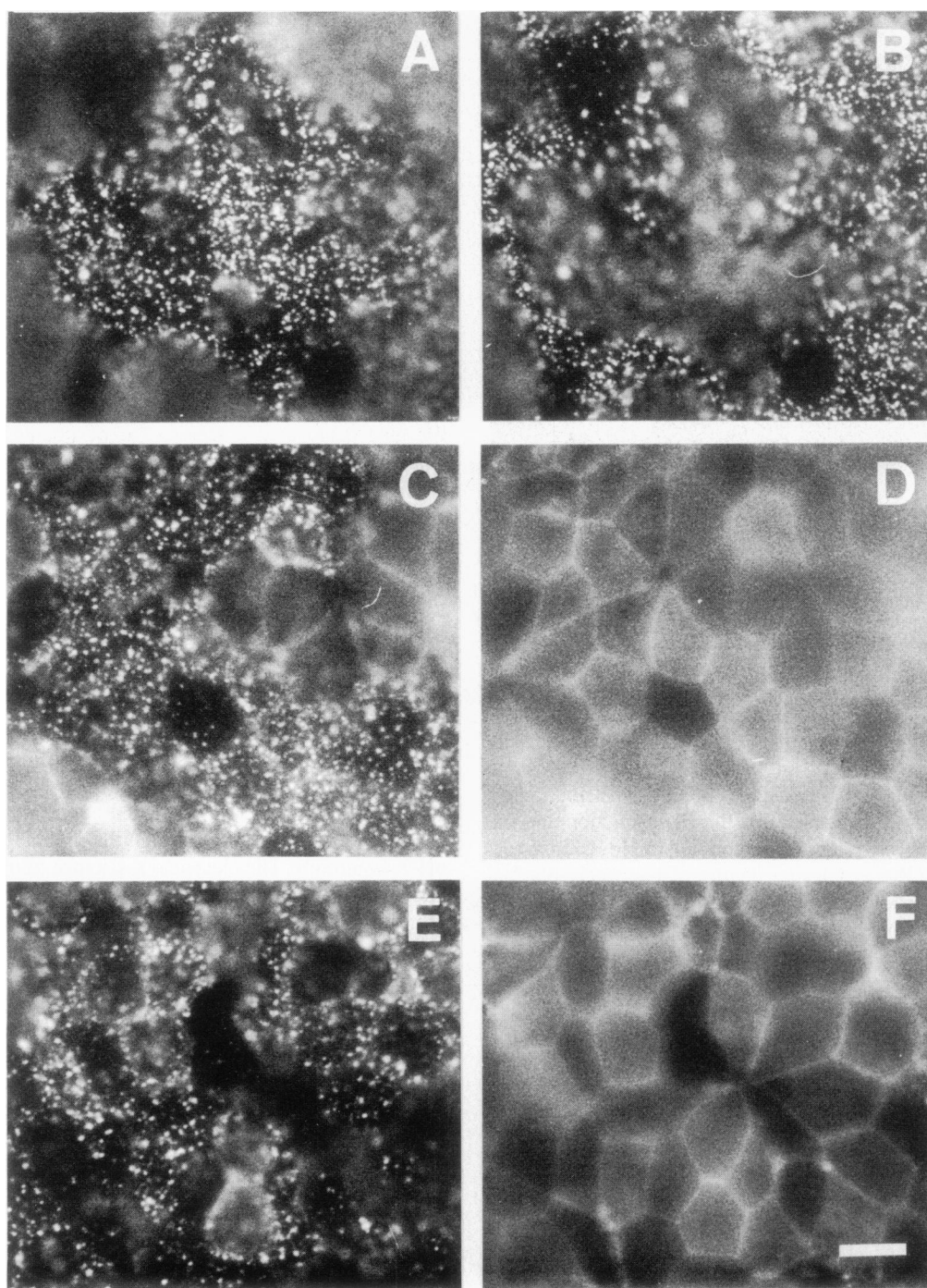
## Discussion

How are domains formed and maintained within continuous membranes? Epithelial cells provide intriguing examples of such a problem of membrane differentiation. In these studies we asked whether or not both of the two leaflets of the apical and basolateral plasma membrane lipid bilayers are interrupted by a diffusion barrier in the plane of the membrane. Our results suggest that only the exoplasmic leaflets are separated from each other, whereas the cytoplasmic leaflets communicate freely.

#### *The tight junction as a diffusion barrier for lipids*

Spiegel *et al.* (1985) recently reported that the ganglioside  $\text{G}_{\text{M1}}$  present on the apical surface of MDCK cells does not equilibrate with the basolateral surface. Previously, Dragsten *et al.* (1981) observed that certain fluorescent amphiphiles, added in ethanolic solution to the apical surface of an epithelial monolayer grown on a solid support, remained on the apical surface while other amphiphiles distributed to the lateral surface. A problem in the

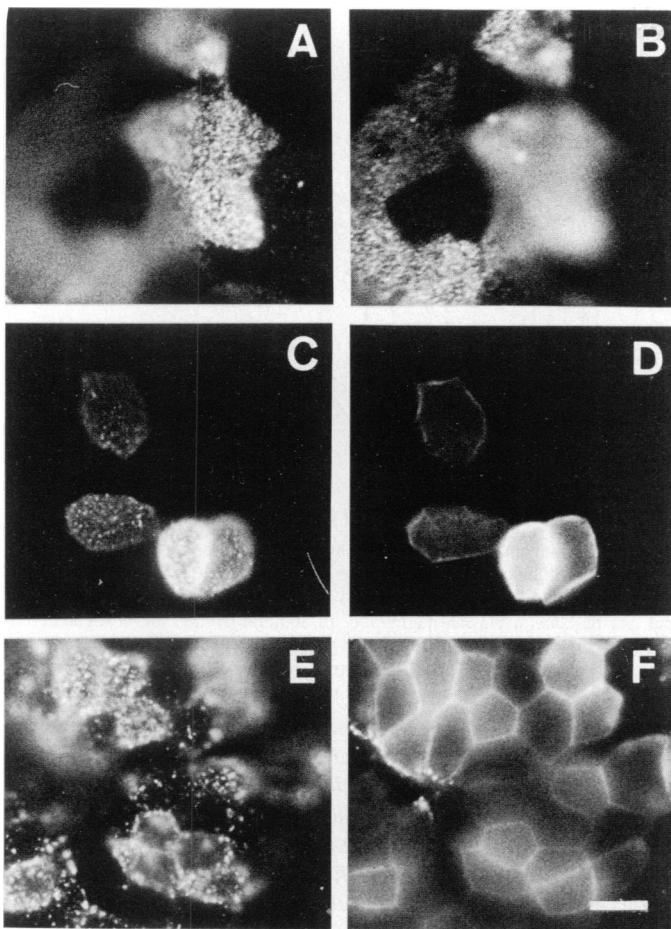




**Fig. 5.** N-Rh-PE fused into both leaflets of the apical plasma membrane domain of MDCK strain II cells immediately diffuses to the basolateral domain. Freeze-thawed octylglucoside liposomes (A–D) or reverse-phase evaporation vesicles (REVs) (E and F) in which N-Rh-PE is equally distributed over both leaflets (Figures 1 and 2) were bound to (panels A and B) or fused with (panels C–F) the apical membrane of MDCK cells as described in the legend to Figure 3. Photograph B has been taken at a level just below photograph A, which is evident from the fact that the apical surface of neighboring cells out of focus in A is focused in B. No sharp staining pattern is observed at the lateral focal plane before the fusion (A and B). However, immediately after the fusion of freeze-thawed octylglucoside liposomes (C and D; 45% fusion) or REVs (E and F; 50% fusion) a lateral staining pattern is easily observed (D and F). Bar = 10  $\mu\text{m}$ .

interpretation of the latter experiments is that they were performed at 20°C. At this temperature endocytosis and transcytosis are not inhibited, and these processes may cause redistribution to the lateral surface independent of lateral diffusion (Pesonen *et al.*, 1984). Moreover, since the lipid probes used were water-soluble, it was not possible to exclude the possibility that equilibration between the plasma membrane domains occurred by monomer exchange through the aqueous cytoplasm.

We have tried to overcome these problems by a more direct experimental approach to the question of tight junction permeability. First of all, we chose as an experimental system two strains of MDCK cells grown on permeable supports, which have been carefully studied for basal and apical protein polarity (Simons and Fuller, 1985), and here we show that the phospholipids also have a polarized distribution. By growing MDCK cells on filters, conditions are achieved which closely



**Fig. 6.** Localization of N-Rh-PE fused into the apical plasma membrane of MDCK strain I cells which maintained a high transepithelial electrical resistance ( $>1000 \text{ ohm.cm}^2$ ) throughout the experiment. N-Rh-PE inserted mainly into the exoplasmic bilayer leaflet by means of octylglucoside liposome fusion does not diffuse to the basolateral plasma membrane (panels A and B) unless the tight junctions are opened (panels C and D). When N-Rh-PE was inserted into both bilayer leaflets by REV fusion, immediate redistribution to the basolateral plasma membrane occurred (panels E and F). For details see the legend to Figure 3. Bar =  $10 \mu\text{m}$ .

mimic those prevailing *in vivo*. On filters the cells feed from the basal side and form a monolayer of cuboidal cells, which is more stable to experimental manipulations. Second, we have developed methods to fuse labeled lipids into the apical plasma membrane domain (van Meer and Simons, 1983; van Meer *et al.*, 1985). Because of this we were able to use a fluorescent phospholipid that in its water insolubility is more natural than the analogs used before. The fusion reaction also made it possible to insert the fluorescent lipid into both leaflets of the plasma membrane bilayer by the use of symmetric liposomes or primarily into the exoplasmic leaflet by fusing liposomes where the fluorescent lipid was enriched in the external leaflet. Third, in the present system the behavior of the fluorescent lipid could be studied at  $0^\circ\text{C}$ , at which temperature diffusion should occur but vesicular transport is completely inhibited. The fusion takes place within 1 min at  $37^\circ\text{C}$ , which is too short for transcytosis to play a role in the transport to the basolateral surface (Pesonen *et al.*, 1984; von Bonsdorff *et al.*, 1985). Finally, we were able to assess the intactness of the tight junction by two criteria. Not only did the cell monolayers maintain a high trans-epithelial electrical

resistance (Cereijido *et al.*, 1978; Gumbiner and Simons, 1986) during the manipulations, but in addition lipid polarity was preserved as judged by the lipid composition of viruses produced from the infected MDCK cells (Table I). A disadvantage which the present approach shares with previous studies is that it represents a qualitative study. Nevertheless, we were able to observe clear differences in the behavior of implanted N-Rh-PE in MDCK cells. When fused into the exoplasmic leaflet of the apical plasma membrane N-Rh-PE did not distribute to the lateral plasma membrane unless the junctions were opened. However, when N-Rh-PE was fused into both exoplasmic and cytoplasmic leaflets of the bilayer, a fraction of the fluorescent phospholipid immediately passed to the basolateral plasma membrane domain. N-NBD-PE, a fluorescent phospholipid with an identical PE backbone to the N-Rh-PE but carrying a different fluorescent group displayed a behavior identical to that of N-Rh-PE. This was more difficult to document since N-NBD-PE easily bleaches under the microscope.

#### *Lipid organization in the epithelial plasma membrane*

What does this imply for the organization of the lipid bilayers in the apical and basolateral plasma membrane domains? The most careful studies concerning the lipid composition of apical and basolateral domains have been performed on intestinal cells. In these cells the major differences in lipid composition between the plasma membrane domains was a 2- to 4-fold enrichment of glycosphingolipids in the apical membrane and a 2- to 4-fold depletion of PC (Forstner and Wherrett, 1973; Kawai *et al.*, 1974; Brasitus and Schachter, 1980). The amount of the second major phospholipid, PE, as a percentage of total lipid was equal in the two domains. The difference in phospholipid composition between the two domains in both strains of MDCK cells are very similar to those observed in the intestinal cells (Kawai *et al.*, 1974). In addition, recent studies (Nichols *et al.*, 1985; Hansson *et al.*, 1986) show that MDCK cells have a very high content of glycosphingolipids which, like in intestinal cells, have been localized to the apical but not basolateral domain of MDCK cells (Spiegel *et al.*, 1985; Hansson *et al.*, 1986) and other kidney epithelia (Zalc *et al.*, 1978; Turner *et al.*, 1985). Glycosphingolipids have always been localized in the exoplasmic leaflet (Op den Kamp, 1979). Whether the exoplasmic surface of the apical plasma membrane domain in MDCK cells, as in intestinal cells (Forstner and Wherrett, 1973; Kawai *et al.*, 1974; Brasitus and Schachter, 1980) is almost exclusively covered by glycosphingolipids is the subject of our present research.

If one assumes that the free diffusion of lipid molecules in the cytoplasmic leaflet of the plasma membrane leads to an identical lipid composition of the cytoplasmic leaflets of the apical and basolateral plasma membrane domains, interesting consequences arise for the transbilayer distribution of the individual lipid classes in the apical and basolateral membranes. If the exoplasmic leaflet of the apical domain was predominantly occupied by glycosphingolipids, the phospholipids of the apical domain would be mainly situated in its cytoplasmic leaflet. The phospholipid composition of the cytoplasmic leaflet of the basolateral membrane would be identical to this, and the distribution of the individual phospholipid classes across the basolateral membrane bilayer could be predicted from the total phospholipid composition of the basolateral membrane. For the two major phospholipid classes this leads to the following results: 65–90% of the PE and only 10–25% of the PC would be localized in the cytoplasmic leaflet (Kawai *et al.*, 1974; van Meer and Simons, 1982; this study). This agrees with the situation reported for the

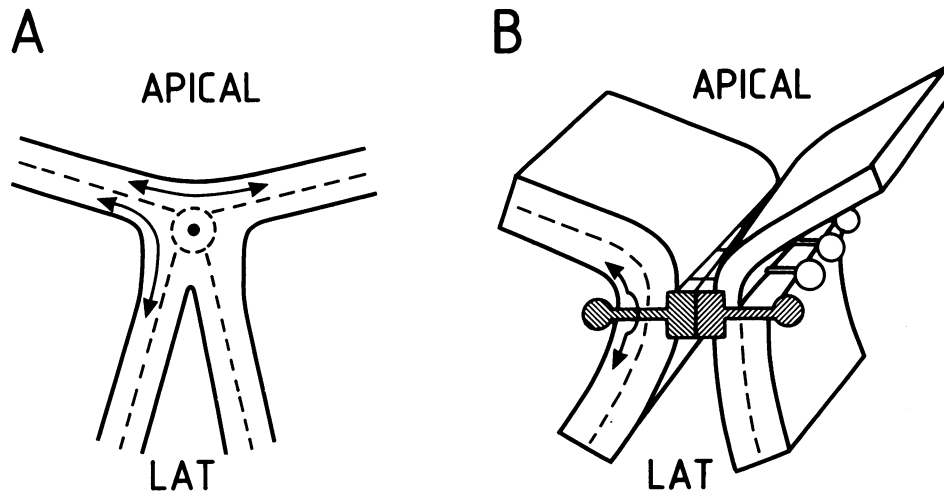


Fig. 7. Two models for tight junction structure with different predictions for lipid diffusion. **Model A** is the hexagonal lipid model proposed by Pinto da Silva and Kachar (1982). **Model B** assumes that the tight junction is made up of proteins spanning the bilayers and interacting with each other to restrict diffusion between the cells and in the exoplasmic leaflet. Both **model A** and **model B** are in agreement with the findings reported in the present paper that free lipid diffusion occurs between the cytoplasmic but not the exoplasmic leaflets of the apical and basolateral plasma membrane. In addition to this, the hexagonal lipid model (**A**) predicts that lipids are free to diffuse between the exoplasmic domains of neighbouring cells. Our findings, reported in a separate paper (van Meer *et al.*, 1986), that neither endogenous nor exogenous lipids were able to diffuse from one epithelial cell to the next, favor model **B**.

plasma membrane of erythrocytes and blood platelets (Bretscher, 1972; Op den Kamp, 1979) and may reflect general principles underlying the phospholipid organization in mammalian plasma membranes.

#### *Glycosphingolipids, sphingomyelin and intracellular lipid sorting*

Apart from an apical enrichment of glycosphingolipids, sphingomyelin (SPH) has been found to be enriched at the cost of PC in the apical membrane of epithelial cells of rat kidney (Hise *et al.*, 1984; Molitoris and Simon, 1985), dog kidney (Carmel *et al.*, 1985), rat intestinal mucosa (Chapelle and Gilles-Baillien, 1983) and rat liver (Kremmer *et al.*, 1976; Meier *et al.*, 1984). This finding is intriguing since SPH is generally found in the exoplasmic leaflet of plasma membranes (Op den Kamp, 1979) and has, in contrast to the other phospholipids, the same lipophilic backbone as the glycosphingolipids.

This has implications for the sorting process which is responsible for the generation of the unique lipid compositions in each plasma membrane domain and which most likely takes place in the trans-Golgi network (Griffiths and Simons, 1986). The sorting process necessarily involves the lateral segregation of unique sets of lipids to the areas of the membrane where the budding of vesicles destined for either plasma membrane domain occurs. A segregation of glycosphingolipids and/or SPH in the non-cytoplasmic leaflet of the membrane of the sorting compartment could be the main factor in generating the observed differences in lipid composition between the two plasma membrane domains. Self-aggregation of glycosphingolipids, which has been reported to occur in glycosphingolipid/phospholipid mixtures *in vitro* (Thompson and Tillack, 1985), may well play a role in such segregation processes.

#### *Models for the structure of the tight junction*

Two strains of MDCK cells were used in this study which have very different properties (Hansson *et al.*, 1986). However, in both strains the junctions behaved as a diffusion barrier in the exoplasmic bilayer leaflet and both strains display a very similar phospholipid polarity which is nearly identical to that in intestinal cells *in vivo*. The properties of the tight junction may therefore be general in epithelia and intimately linked to the nature of their

building blocks. The results presented here are in agreement with the predictions of the hexagonal lipid model for the tight junction regarding the cytoplasmic leaflet of the plasma membrane (Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982). However, more recently we have obtained evidence (van Meer, *et al.*, 1986) that endogenous and exogenous lipids in the external leaflet of the apical plasma membrane domain are unable to diffuse from one epithelial cell to the next under conditions where the tight junctions were shown to be intact. These findings do not agree with the hexagonal lipid model (Figure 7A). Perhaps the most reasonable model is a protein bridge between two cells which, apart from blocking the passage of anions, also blocks the diffusion of proteins and lipids in the exoplasmic leaflet from one plasma membrane domain to the other (Figure 7B). The lipid molecules in the cytoplasmic leaflet would freely diffuse between the protein subunits of the tight junction on the cytoplasmic surface. In the present study we have not addressed the mobility of the fluorescent lipid in the apical membrane. We are presently extending our observations by photobleaching studies and by the use of radiolabeled lipids to quantitate the redistribution observed here.

## Materials and methods

### *Liposomes*

Large unilamellar liposomes (200 nm diameter) were prepared either by octyl- $\beta$ -D-glucoside dialysis ['octylglucoside liposomes'; van Meer *et al.* (1985)] or by reverse-phase evaporation [REVs; Szoka and Papahadjopoulos (1978)] from the following lipid mixture: egg phosphatidylcholine/egg phosphatidylethanolamine/cholesterol/ $\text{Iv}^3$  NeuAc II<sup>3</sup> NeuAc GgOse<sub>4</sub> Cer (G<sub>D1a</sub>) 25:25:50:5, (mol/mol), 1 mol% ( $5 \times 10^5$  c.p.m./ml) cholesterol [ $1\text{-}^{14}\text{C}$ ]oleate and 1 mol% of one of the following fluorescent lipids: N-NBD-PE, N-Rh-PE or R18. For phospholipase experiments the cholesterol [ $1\text{-}^{14}\text{C}$ ]oleate was replaced by  $2.5 \times 10^5$  c.p.m. of [ $^{14}\text{C}$ ]DOPE. The REVs were sized through polycarbonate filters with a pore size of 0.2  $\mu\text{m}$ . The lipid concentration in the final preparations was 1  $\mu\text{mol/ml}$ .

### *Phospholipase A<sub>2</sub> assay of phospholipid localization*

Liposomes were diluted with three volumes of PBS containing 33 IU/ml of phospholipase A<sub>2</sub> from *Naja naja* (EC 3.1.1.4) and incubated at 10°C. At varying time intervals 320- $\mu\text{l}$  samples (80 nmol total liposomal lipid) were added to 300  $\mu\text{l}$  of an ice-cold 10 mM solution of EDTA in water, pH 5. A control experiment showed that this inhibited hydrolysis by the phospholipase completely.



Lipids were extracted according to Bligh and Dyer (1959), separated by one- or two-dimensional (D) alkaline thin-layer chromatography (t.l.c.) (Renkonen *et al.*, 1972) on 10 × 10 cm<sup>2</sup> high-performance (h.p.) t.l.c. plates (Merck, Darmstadt, FRG) and the individual lipid classes were analyzed as follows.

Firstly, the fluorescent spots representing N-Rh-PE plus N-NBD-PE and lyso N-Rh-PE plus lyso N-NBD-PE were scraped from the plate. The fluorescent lipids were extracted from the silicagel in a mixture of 1 ml water, 2.2 ml methanol and 1 ml chloroform for 30 min at 20°C. The silicagel was subsequently pelleted in a Christ centrifuge at 1500 g max for 10 min and the fluorescence in the supernatant was determined in a MPF-44A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The excitation wavelength was set at 470 nm for NBD and 550 nm for Rh and the emission was measured at 535 and 595 nm respectively. Hydrolysis was calculated from the relative fluorescence intensity of the individual spots.

The t.l.c. plates were subsequently exposed to a film overnight in order to localize the radioactive lipids and their hydrolysis products. The radioactivity in the individual spots was quantitated after scraping as described by van Meer *et al.* (1985). The hydrolysis of dioleoyl [2-<sup>14</sup>C]PE could be calculated from the relative radioactivity in the PE and lyso PE spots, since the label is present in the polar head group.

In parallel samples the lipid spots on the thin-layer plates were visualized by iodine staining and the amounts of unlabeled lipid quantitated by a phosphate determination according to Rouser *et al.* (1970). Hydrolysis of PC and PE was calculated from the relative phosphate content of the lyso PC and lyso PE spots as compared to the PC and PE spots respectively.

N-NBD-PE and N-Rh-PE in detergent solution (10 μM fluorescent lipid and 30 mM octyl β-D-glucoside in PBS) act as substrates for phospholipase A<sub>2</sub> (5 IU/ml). After 15 min at 37°C, they had been completely hydrolyzed as judged by 2-D h.p. t.l.c. The fluorescent hydrolysis product of N-NBD-PE, lyso N-NBD-PE, displayed R<sub>f</sub> values in the alkaline and acidic direction of 0.6 and 0.5 respectively, as compared to 0.8 and 0.9 for the intact N-NBD-PE. For lyso N-Rh-PE these R<sub>f</sub> values were 0.6 and 0.5 respectively, as compared to 0.8 and 0.8 for the parental compound N-Rh-PE.

#### Cells

MDCK strain I and strain II cells were grown on nitrocellulose filters with a pore size of 0.22 μm as described before (Hansson *et al.*, 1986). Experiments were performed on strain I cells on day 4 and on strain II cells on day 3 after seeding.

#### Virus infection

For the production of FPV and VSV, monolayers of the strain I cells on nitrocellulose filters were infected as described before (van Meer and Simons, 1982) except that per filter 4 × 10<sup>7</sup> p.f.u. were added in a volume of 50 μl.

Monolayers of MDCK cells were infected with influenza N virus as described before (van Meer *et al.*, 1985). Cells were used for further experimentation at 4 or 5 h after infection (see text).

#### Fusion of liposomes with the apical membrane of MDCK cells

Monolayers of MDCK strain II cells expressing the viral HA on the cell surface were first treated with trypsin to bring the protein into its fusogenic form (van Meer *et al.*, 1985). For MDCK strain I cells this treatment is not necessary. Pulse-chase experiments in the presence and absence of conditioned medium demonstrated that MDCK strain I cells secrete a protease which cleaves the influenza N virus HA into its fusogenic HA<sub>1</sub> and HA<sub>2</sub> subunits (G. van Meer, unpublished results). As a consequence, a plaque titration of the virus on MDCK strain I cells does not require trypsin. When the surface of MDCK strain I cells was treated with trypsin, this enhanced the amount of liposomes that would subsequently bind to and fuse with the cells 2-fold (not shown). The percentage of the cell-associated liposomes that fused with the cells was, however, not influenced by the trypsin treatment.

Liposomes (10 nmol phospholipid/3 × 10<sup>6</sup> cells) were allowed to bind to the cells at 0°C and liposome-cell fusion was induced by incubation at pH 5 and 37°C for 60 s, after which the filter with the monolayer of cells was immediately removed from the filter holder and cooled to 0°C in a Petri dish containing 2 ml of complete medium (Eagle's minimal essential medium with Earle's salts, without bicarbonate, and supplemented with 10 mM Hepes and 1% fetal calf serum) on an ice-cooled metal plate.

#### Opening of the tight junctions

After the liposome-cell fusion, part of the filters were washed twice in a 'Ca<sup>2+</sup>-free' medium consisting of Eagle's minimal essential medium with Earle's salts, but without Ca<sup>2+</sup> and bicarbonate, supplemented with 10 mM Hepes, 10 mM glutamine and 1% fetal calf serum which had been dialyzed against a 40-fold volume of 150 mM NaCl (12 h), 150 mM NaCl plus 1 mM ethyleneglycol-bis (β-amino-ethylether N,N-tetraacetic acid) (12 h) and twice for 12 h against 150 mM NaCl. They were then incubated for 60 s at 37°C in 100 ml of prewarmed Ca<sup>2+</sup>-free medium, after which they were washed twice

in the same medium containing the normal concentration of Ca<sup>2+</sup> and left on ice. When cells have been infected the junctions open more quickly (cf. Gumbiner and Simons, 1986).

#### Fluorescence microscopy

A Petri dish containing a nitrocellulose filter with a monolayer of cells immersed in medium was transferred from an ice-cooled metal plate onto the stage of a Zeiss photomicroscope III (Oberkochen, FRG) and viewed immediately through a ×40 water immersion objective by epifluorescence. The (living) cells underwent no changes when left on ice and all situations illustrated in the present paper were stable on ice for hours.

#### Sources of reagents

N-NBD-PE and N-Rh-PE were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and R18 was from Molecular Probes, Inc. (Junction City, OR). Egg PC, egg PE, cholesterol, buffer salts and phospholipase A<sub>2</sub> from *Naja naja* were obtained from Sigma Chemical Co. (St Louis, MO). G<sub>D11</sub> was from Supelco, Inc. (Bellefonte, PA). Cholesterol [1-<sup>14</sup>C]oleate (50.8 Ci/mol) and dioleoylphosphatidyl [2-<sup>14</sup>C]ethanolamine (49 Ci/mol) were purchased from Amersham International (Amersham, UK). Octyl β-D-glucoside was from Calbiochem-Behring Corp. (La Jolla, CA). The bath sonicator used for the preparation of REVs was from Laboratory Supplies Co., Inc. (Hicksville, NY) and the polycarbonate filters were obtained from Nuclepore Corp. (Pleasanton, CA). Fetal calf serum was from KC Biologicals (Lenexa, KS). Other chemicals and solvents were of analytical grade and obtained from Merck (Darmstadt, FRG).

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