The EMBO Journal Vol.1 No.7 pp.847-852, 1982

Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cells have unique phospholipid compositions

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Communicated by K.Simons
Received on 1 July 1982

Influenza virus and vesicular stomatitis virus (VSV) obtain their lipid envelope by budding through the plasma membrane of infected cells. When monolayers of Madin-Darby canine kidney (MDCK) cells, a polarized epithelial cell line, are infected with fowl plague virus (FPV), an avian influenza virus, or with VSV, new FPV buds through the apical plasma membrane whereas VSV progeny is formed by budding through the basolateral plasma membrane. FPV and VSV were isolated from MDCK host cells prelabeled with [^{32}P]orthophosphate and their phospholipid compositions were compared. Infection was carried out at 31°C to delay cytopathic effects of the virus infection, which lead to depolarization of the cell surface. ^{32}P-labeled FPV was isolated from the culture medium, whereas ^{32}P-labeled VSV was released from below the cell monolayer by scraping the cells from the culture dish 8 h after infection. At this time little VSV was found in the culture medium, indicating that the cells were still polarized. The phospholipid composition of the two viruses was distinctly different. FPV was enriched in phosphatidylethanolamine and phosphatidylserine and VSV in phosphatidylcholine, sphingomyelin, and phosphatidylinositol. When MDCK cells were trypsinized after infection and replated, non-infected control cells attached to reform a confluent monolayer within 4 h, whereas infected cells remained in suspension. FPV and VSV could be isolated from the cells in suspension and under these conditions the phospholipid composition of the two viruses was very similar. We conclude that the two viruses obtain their lipids from the plasma membrane in the same way and that the different phospholipid compositions of the viruses from polarized cells reflect differences in the phospholipid composition of the two plasma membrane domains.

Key words: epithelial cells/lipid composition/plasma membrane/polarized cells/virus

Introduction

Epithelial cells exhibit polarity in structure and function. They form a single layer of cells connected to each other by a junctional complex. The tight junctions of this complex form the boundary between two specialized domains of the plasma membrane of these cells, the apical surface domain, often carrying microvilli at the luminal side, and the basolateral domain facing the internal milieu. The apical plasma membrane domain has a protein composition that is clearly different from that of the basolateral domain (see Rodriguez Boulan, 1982). In intestinal epithelial cells the two surface domains have also been shown to have different lipid compositions (Forstner *et al.*, 1968; Douglas *et al.*, 1972; Kawai *et al.*, 1974; Brasitus and Schachter, 1980). The differences in composition between the apical and the basolateral domains reflect vectorial functions, such as unidirectional transport of ions and fluid across an epithelial cell layer.

The Madin-Darby canine kidney (MDCK) cell line (Madin and Darby, 1958) resembles transporting epithelia in many of its properties (Misfeldt *et al.*, 1976; Cereijido *et al.*, 1978a, 1978b; Louvard, 1980). MDCK cells form tight junctions between adjacent cells in culture, and exhibit vectorial transport of ions and fluid. Also, the polarized distribution of proteins is well established (Richardson and Simmons, 1979; Louvard, 1980). Studies on the distribution of the different lipid molecules in the plasma membrane domains have been hampered by the lack of adequate cell fractionation techniques. To overcome these problems, we have made use of enveloped viruses to study the phospholipid composition of the two surface domains of MDCK cells. It is well known that viruses which obtain their envelopes by budding through the plasma membrane of their host cells have lipid compositions reflecting those of the cell plasma membrane (see Patzer *et al.*, 1979). Moreover, Rodriguez Boulan and Sabatini (1978) have shown that when MDCK cells were infected with influenza virus or with vesicular stomatitis virus (VSV), the two viruses matured by budding from the two opposed cell surface domains; influenza virus budded from the apical membrane whereas VSV was released through the basolateral membrane domain.

Our experimental protocol was to prelabel the MDCK cells with [^{32}P]orthophosphate until the phospholipid classes were uniformly labeled (Quigley *et al.*, 1971, 1972), then to infect the cells with fowl plague virus (FPV), an avian influenza virus, or with VSV, to isolate the virus budding from either domain, and analyze its phospholipid composition. Our results show that their phospholipids are significantly different.

Results

In our initial experiments, we found that when MDCK cells had been infected with VSV there was a significant cytopathic effect and it was not possible to recover progeny virus in amounts large enough for lipid assays, before cells detached from the substrate. Therefore, we prelabeled the phospholipids of the MDCK cells before virus infection with [^{32}P]-orthophosphate and used the distribution of the label over the different phospholipid classes to determine their relative amounts in each virus (Quigley *et al.*, 1971, 1972). The incorporation of [^{32}P]orthophosphate was optimized by lowering the phosphate in the media to 0.3 mM instead of the usual 1 mM. A lower concentration of 0.1 mM could not be used because, at this concentration, the cells started to detach from the dishes 6 h after FPV infection at 37°C. Also, exchange reactions with the unlabeled phospholipids present in serum, mainly phosphatidylcholine (PC), interfere with equilibrium labeling of the plasma membrane especially. A serum-free, hormone-supplemented medium, developed for MDCK cells (Taub *et al.*, 1979) was used during the ^{32}P-labeling and the subsequent viral infections. The cells were prelabeled with ^{32}P for 24 h, after which the cells were infected with the viruses in

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 0261-4189/82/0107-0847$2.00/0.

the subsequent experiments. At this time the individual phospholipid classes in the cells had similar specific activities (Table I). Sphingomyelin (SPH) was the exception, in that its specific activity was still somewhat lower than that of the others. These data are in agreement with previous results of Quigley *et al.* (1972). The PC content of the cells in this study (35%) is considerably lower than those reported in other studies (45–65%, Klenk and Choppin, 1969, 1970a). This is because our cells have been grown for 24 h in a culture medium without serum. Serum lipoproteins contain a very high amount of PC (see Spector *et al.*, 1981), which is readily taken up by the cells and may account for as much as 50% of the total cellular PC (Esko *et al.*, 1982). Growth of the cells in the serum-free medium resulted in a decrease of the percentage of PC from 50 to 35% and an increase in the percentage of the other phospholipids except for SPH, which remained constant.

Our first experiments showed that when cells had been infected with VSV or FPV and then incubated at 37°C for 8 h, new ^{32}P-labeled virus could be isolated (Figure 1B). However, VSV was found predominantly in the culture medium rather than in its expected localization under the monolayer, where it originally buds (Rodriguez Boulan and Sabatini, 1978). Parallel light microscopy showed that, at this time, >50% of the VSV-infected cells had detached from the bottom of the culture dish and most of the other cells were rounding up. In contrast, FPV-infected cells still formed a confluent monolayer at 8 h after infection. Under these experimental conditions the phospholipid compositions of VSV and FPV were similar although some clear differences were found (Table II).

When VSV-infected cells start rounding off and lose their junctions, the polar distribution of the viral spike glycoproteins is gradually lost (Roth and Compans, 1981). In order to obtain VSV from polarized cells, it was necessary to isolate VSV that had been produced before depolarization occurred. This was possible when the cells were grown at 31°C after infection instead of 37°C. This decrease in temperature produced a significant delay in the cytopathic effects of VSV infection. At 37°C the VSV infected cells started to round up 4.5–5 h after infection, whereas at 31°C this effect was not observed until 10.5 h after infection. We were able to show that at 8 h post-infection almost all of the VSV could be isolated from beneath the cell monolayer (Figures 1C, 1D). This is the expected result if most of the tight junctions were

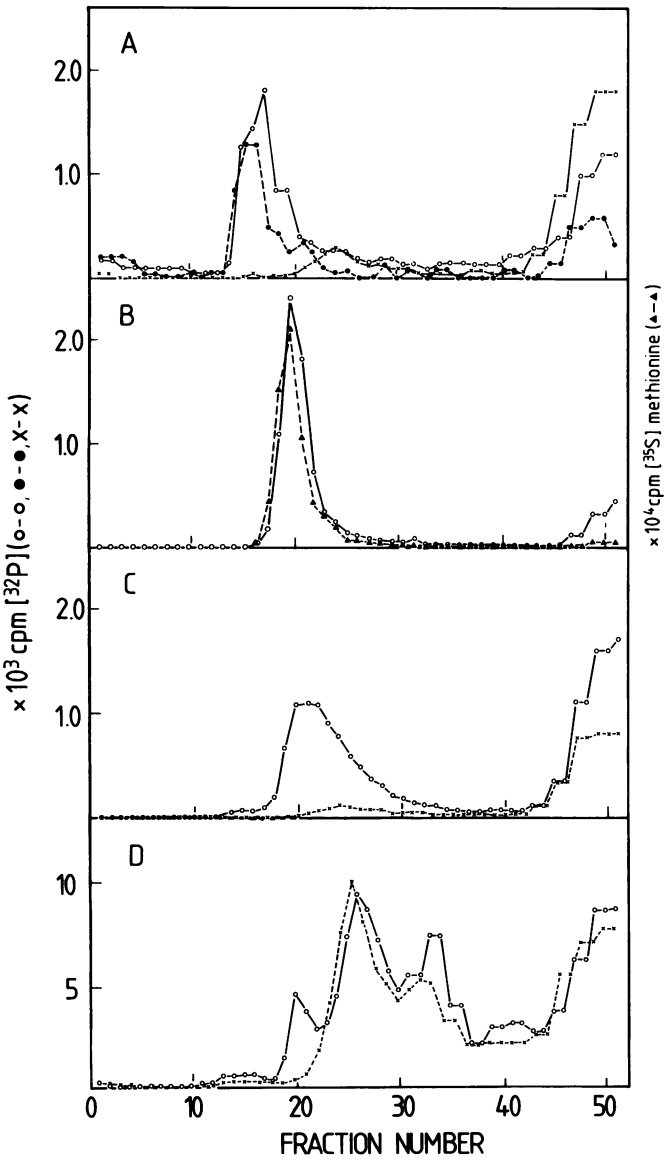

Fig. 1. Isolation of FPV and VSV from MDCK cell monolayers by tartrate gradient centrifugation. The 111 x 10^3 *g* virus pellet was resuspended in 500 μl TN and layered onto a 12 ml 5–40% (w/v) tartrate gradient in TN. The gradient was centrifuged at 4°C at 284 x 10^3 *g* for 2.5 h. Fractions were collected from the bottom and 15 μl aliquots assayed for radioactivity. **A)** FPV was harvested 8 h after infection from the culture medium of a monolayer of MDCK cells incubated at 31°C (○—○). New pre-warmed hormone supplemented 0.3P_i MEM was added to the monolayer and a second batch of FPV was harvested 12.5 h after infection (●—●). The culture medium of a monolayer of non-infected MDCK cells was treated exactly as the FPV containing medium (x—x). Each monolayer had been prelabeled with 1 mCi [^{32}P]orthophosphate. **B)** VSV was harvested 8 h after infection from the culture medium of a monolayer of MDCK cells incubated at 37°C (○—○). The cells had been prelabeled with 1 mCi [^{32}P]orthophosphate. In order to localize the VSV, [^{35}S]VSV was added to the [^{32}P]VSV before it was layered on the gradient (▲—▲). **C)** VSV was harvested 8 h after infection from the culture medium of a monolayer of MDCK cells incubated at 31°C (○—○). The culture medium of a non-infected monolayer was treated exactly as the VSV-containing medium (x—x). Each monolayer had been prelabeled with 3 mCi [^{32}P]orthophosphate. **D)** VSV was harvested 8 h after infection from the MDCK cell monolayer described under **C** after scraping the cells (○—○). Scraping the cells resulted in a considerable ^{32}P background on the gradient but from the radioactivity profile of a monolayer of non-infected cells, treated exactly as the VSV-infected cells (x—x), it is evident that VSV could be harvested without being contaminated by cell debris.

Table I. Phospholipid composition and ^{32}P label distribution in the lipids of MDCK cells at the time of infection with virus[a]

	Phosphate distribution (%)	^{32}P label distribution (%)
SPH	12.6 ± 2.0	8.2 ± 2.9
PC	34.8 ± 0.3	35.3 ± 5.2
PI	9.0 ± 1.4	10.9 ± 1.3
PS	7.2 ± 0.1	6.0 ± 0.7
PE	36.4 ± 0.2	39.8 ± 5.9

[a]In four experiments MDCK cells were labeled with [^{32}P]orthophosphate for 24 h and the phospholipids extracted and analyzed as described in Materials and methods. The numbers represent the percentage of the total phospholipid present in each phospholipid class and are followed by the s.d.
After the labeling procedure a monolayer of MDCK cells in a 93 mm diameter dish contained 1.5 x 10^7 cells, 30 nmol phospholipid/10^6 cells and 2.5–3.0 x 10^8 c.p.m. ^{32}P when labeled with 3 mCi [^{32}P]orthophosphate.

intact at the time of virus harvest and provides evidence that VSV had been produced when the cells were still polarized. Analysis of the phospholipid composition of the VSV produced from the basolateral side of the monolayer and parallel data of the phospholipid composition of FPV produced from cells also grown at 31°C and harvested 8 h after infection from the medium in contact with the apical surface (Figure 1A) is shown in Table III. Clear differences between the viruses were observed. VSV contained a significantly higher amount of the choline phospholipids, 1.5-fold more SPH and 4.5-fold more PC, and 3.5-fold more phosphatidylinositol (PI), while FPV possessed relatively more phospholipids containing the amino group, 1.5-fold more phosphatidylethanolamine (PE) and slightly more phosphatidylserine (PS).

It was important to test, in an independent experiment, whether the phospholipid composition of the viruses accurately reflects that of the membrane from which they bud. Therefore, we infected a monolayer of MDCK cells with either VSV or FPV and before virus budding started, at 3 h after infection, the junctions between the cells were broken with EDTA and the cells released from the support by trypsinization as described in the legend to Figure 2. After being replated at 37°C, non-infected control cells formed a confluent monolayer within 4 h, but the infected cells remained in suspension. At 5 h after replating, VSV and FPV could be isolated from the suspensions of freely floating cells that had been infected with the proper virus prior to trypsinization (Figure 2). In contrast to the previous experiments in which VSV and FPV had quite different phospholipid compositions

Table II. Phospholipid composition of VSV and FPV isolated from the culture medium of MDCK cells at 37°C[a]

	VSV (%) Figure 1B	FPV (%)
SPH	19.9 ± 0.7	21.3 ± 0.5
PC	9.2 ± 0.8	2.4 ± 0.1
PI	3.2 ± 0.7	1.3 ± 0.1
PS	16.3 ± 0.7	16.8 ± 0.1
PE	51.4 ± 0.5	58.4 ± 0.5

[a]The cells were grown and the virus harvested and analyzed as described in the legend to Figure 1B and in Materials and methods. The numbers represent the average of two determinations and are followed by the difference of the individual numbers from the mean ($n=2$).

Table III. Phospholipid composition of VSV and FPV harvested from polarized MDCK cells at 31°C[a]

	VSV (%)[b] Figure 1D (○—○)	FPV (%)[c] Figure 1A (○—○)
SPH	26.4 ± 2.4	19.0 ± 1.3
PC	11.3 ± 0.6	2.4 ± 0.0
PI	6.9 ± 1.0	2.1 ± 0.1
PS	17.2 ± 0.9	20.8 ± 0.8
PE	38.4 ± 3.3	55.9 ± 2.2

[a]The growth conditions for the virus preparations are described in the legends to Figures 1A, C, and D. The peak fractions from the gradient were collected and analyzed as in Materials and methods. The numbers represent the percentage of the total phospholipid present in each phospholipid class. They are the mean of four determinations and are followed by the s.d.
[b]VSV was harvested from beneath the monolayer, 8 h after infection.
[c]FPV was harvested from the culture medium, 8 h after infection.

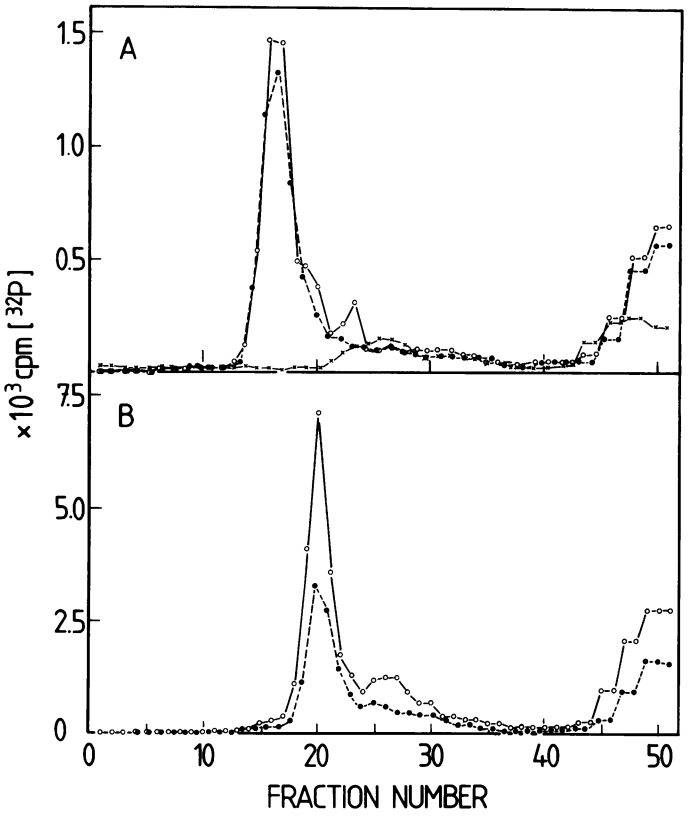

Fig. 2. Isolation of FPV and VSV from MDCK cells trypsinized 3 h after virus infection. Two MDCK cell monolayers, prelabeled with 1.6 mCi [^{32}P]orthophosphate, were infected with either FPV **A)** or VSV **B)**, all as described in Materials and methods. The plates were incubated at 37°C and 3 h after infection the culture medium was replaced by 5 ml trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA/l in Puck's saline). After 5 min (VSV-infected cells) or 10 min (FPV-infected cells) at 20°C, 4 ml trypsin-EDTA solution was discarded and the plates transferred to 37°C until the cells were floating, which was after 20 min for VSV and 40 min for FPV. The cells were subsequently pelleted at 200 *g* for 5 min, carefully resuspended into 5 ml pre-warmed, hormone-supplemented, 0.3 P_i MEM, containing 0.5 mg/ml soybean trypsin inhibitor, and reincubated at 37°C. At 8 h after infection the viruses were harvested from the culture medium as described in Materials and methods and purified on a tartrate gradient as described in the legend to Figure 1. **A)** FPV (○—○, ●—●) and a non-infected cell monolayer (x—x) treated exactly as the FPV-infected monolayer. **B)** VSV (○—○, ●—●). The open and closed circles represent two independent virus preparations.

when budding from polarized cells (Table III), now, under conditions where the cells were rounded up and the plasma membrane most probably not organized in two separate domains, the phospholipid composition of the viruses was very similar (Table IV). These data provide strong evidence in support of the hypothesis that VSV and FPV sample the phospholipids of the host cell plasma membrane and that the differences found between VSV and FPV in Table III actually reflect differences between the basolateral and apical plasma membrane domain, respectively.

Discussion

These experiments show clearly that two viruses budding from the same cell under identical conditions have significantly different phospholipid compositions. The most likely explanation for the differences in Table III is that FPV reflects in its phospholipid composition the apical, and VSV the basolateral, plasma membrane domain and that these two do-

mains of the MDCK cell plasma membrane have a different phospholipid composition. This interpretation is supported by our finding that if infected MDCK cells were depolarized by EDTA and trypsin treatment and replated, VSV and FPV that budded subsequently had similar phospholipid compositions, implying that each virus indeed reflects, in its phospholipid composition, the membrane from which it buds. The small differences (2-fold for PC) still observed between VSV and FPV when budding from depolarized cells (Table IV) are because VSV and FPV have a different effect on the phospholipid metabolism of the cell during the infection or because some cells may still retain a residual polarity after trypsin treatment. A difference in the morphology of the viruses may also play a role in the sense that VSV particles are bullet-shaped and, because of the high curvature of their membrane, they contain more outer monolayer than inner monolayer lipids. That preferential interactions of the viral spike proteins with specific lipids occur is unlikely in view of what is known of viral phospholipid compositions from previous work (see Patzer *et al.*, 1979). The phospholipid composition of enveloped viruses closely reflects that of the plasma membrane of the host cell (see, however, Pessin and Glaser, 1980).

It is clear, from this and a previous study, that VSV infection leads to depolarization of MDCK cells. Roth and Compans (1981) reported that there was only a short interval at 37°C between the production of the first progeny VSV and opening of the tight junctions and loss of the polarized distribution of the viral spike proteins. We have observed that budding of the first new VSV coincided with rounding up of the cells and opening of their tight junctions. FPV had similar effects but only at later stages of the infection. If the infection and subsequent incubations were performed at 31°C, a considerable slowing down of these cytopathic effects was observed, while virus production was nearly unaffected (Figure 1). Indirect immunofluorescence studies with specific antibodies against VSV and FPV spike glycoproteins (K.Simons, unpublished observations) confirmed that the spike glycoproteins remained polarized longer at 31°C than at 37°C. In addition to the fact that at the time of harvest most VSV was still present below the cell monolayer (see Results), these observations support our conclusion that the VSV and FPV isolated under these conditions (Figure 1) had budded from polarized cells.

VSV harvested at a late stage of depolarization (Figure 1B, Table II) had a phospholipid composition in between those of the polarized VSV and FPV in Table III. Furthermore, an intermediate composition was found for the VSV isolated in the latter experiment from the culture medium (Figure 1C, Table V). Because this VSV was present in the apical culture medium, it had probably budded from regions of the monolayer where the junctions had opened. FPV harvested at 12.5 h after infection (Figure 1A, Table V), at the time when FPV-infected cells showed the first signs of rounding up, also had such an intermediate composition. The cytopathic effects of viral infections therefore have the same randomizing effect on the phospholipid composition of the viruses as a trypsin-EDTA treatment (Table IV). Because the most characteristic consequence of either event is the opening of the tight junctions, the loss of these tight junctions may be the primary cause of the randomization of the phospholipid compositions.

Previous studies on the phospholipid composition of the plasma membrane domains of mammalian epithelial cells yield a general picture that is in good agreement with the results in Table III. Kawai *et al.* (1974) compared the phospholipids in apical and basolateral plasma membrane fractions of mouse intestinal epithelium and found 2-fold more PC and 2-fold less PE in the basolateral domain. Also based on a membrane fractionation, Brasitus and Schachter (1980) reported the presence of 1.75-fold more PC in the basolateral plasma membrane fraction than in brush-border membranes of rat intestinal epithelium.

Klenk and Choppin (1970a, 1970b) have determined the lipid composition of simian virus 5 (SV5) grown on Madin-Darby bovine kidney (MDBK) cells. The virus contained 1.9-fold less PC and 1.5-fold more PE than a total plasma membrane preparation, which can now be understood from the observation that SV5 buds from the apical plasma membrane of the polarized MDBK cells (Rodriguez Boulan and Sabatini, 1978). The observed differences corroborate those from the present study.

The apical plasma membrane of intestinal cells contains, apart from a different phospholipid composition, considerably more cholesterol and even 2- to 10-fold more glycolipids than the basolateral domain (Forstner *et al.*, 1968; Douglas *et al.*, 1972; Kawai *et al.*, 1974; Brasitus and Schachter, 1980). This distribution over the two domains may have interesting consequences for the phospholipid distribution over the two bilayer leaflets in each plasma membrane

Table IV. Phospholipid composition of VSV and FPV harvested from MDCK cells depolarized by trypsinization at 37°C

	VSV (%)[a]	FPV (%)	VSV (%)[b]	FPV (%)
SPH	22.0 ± 1.0	24.1 ± 0.3	27.2 ± 2.9	27.2 ± 0.4
PC	7.7 ± 0.5	3.4 ± 0.3	8.1 ± 0.8	5.2 ± 1.2
PI	2.3 ± 0.2	1.7 ± 0.5	3.2 ± 0.8	3.4 ± 0.2
PS	15.2 ± 0.2	15.4 ± 0.6	15.2 ± 1.6	15.3 ± 0.6
PE	52.9 ± 0.7	55.6 ± 0.6	46.5 ± 2.8	49.1 ± 0.1

[a]The growth conditions for the different virus preparations are described in the legend to Figure 2. The phospholipids of the peak fractions were extracted and analyzed as described in Materials and methods. The numbers represent the percentage of the total phospholipid present in each phospholipid class. Each column represents the mean of the phospholipid composition of the two preparations of each virus (Figure 2), determined in duplicate. The numbers are followed by the standard deviation ($n=4$).
[b]Phospholipid compositions of viruses grown in an experiment identical to that described in the legend to Figure 2 with the only difference that in this experiment each plate of cells had been prelabeled with 0.5 mCi [^{32}P]orthophosphate in medium containing 0.6 mM phosphate and that the viruses were harvested 10 h after infection. The numbers are the average of a determination in duplicate and are followed by the difference of the individual numbers from the mean ($n=2$).

Table V. Phospholipid composition of VSV and FPV harvested at the onset of cell depolarization at 31°C[a]

	VSV[b] Figure 1C (○—○)	FPV[c] Figure 1A (●—●)
SPH	25.7 ± 3.0	25.4 ± 1.5
PC	7.8 ± 0.6	3.7 ± 0.7
PI	5.4 ± 0.4	3.2 ± 0.3
PS	17.8 ± 0.4	21.0 ± 1.2
PE	43.4 ± 1.4	46.9 ± 2.3

[a]See footnotes to Table III.
[b]VSV was collected from the culture medium, 8 h after infection.
[c]FPV was collected from the culture medium, 12.5 h after infection.

domain. Lipid asymmetry has been found to be an intrinsic property of most biological membranes studied (see Op den Kamp, 1979) and glycolipids are located, in general, in the non-cytoplasmic monolayer of the lipid bilayer (see Gahmberg, 1981). Viruses budding from either the apical or the basolateral domain of MDCK cells would be well suited as probes for studies of phospholipid asymmetry.

Little is known of how the differences in lipid composition are generated between the apical and basolateral surface domains and how they are maintained. It is likely that the tight junction not only acts as barrier to prevent lateral diffusion of proteins (Pisam and Ripoche, 1976; Hoi Sang *et al.*, 1979; Ziomek *et al.*, 1980) but also of lipid molecules from one membrane domain to the other. Two models (Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982) based on the morphology of tight junctions (for a review see the latter papers) and model membranes (for a review see Verkley, 1980) predict free diffusion in a continuous cytoplasmic lipid monolayer and a diffusion barrier in a discontinuous outer lipid monolayer. Our present results show that the apical and basolateral plasma membrane domains of MDCK cells have different phospholipid compositions and we are now testing in MDCK cells the postulated models for tight junction structure and their functional consequences.

Materials and methods

Cells

MDCK cells were grown and passaged as described by Matlin *et al.* (1981). To obtain ^{32}P-labeled viruses, trypsin-EDTA released cells were plated at a density of 5 x 10^4 cells/cm^2 in 93 mm diameter plastic dishes (Nunc, Algade, Denmark) and grown in Eagle's minimal essential medium (Eagle's MEM) supplemented with 10 mM HEPES, pH 7.3, 5% (v/v) fetal calf serum, penicillin (110 U/ml), streptomycin (100 μg/ml), and fungizone (0.025 μg/ml). After 48 h, when the cells were 90–95% confluent, routinely during 24 h, 3 mCi carrier-free [^{32}P]orthophosphate (Radiochemical Centre, Amersham, UK) was added per plate in a serum-free growth medium consisting of Eagle's MEM containing 0.3 mM instead of the usual 1 mM phosphate and supplemented with HEPES and antibiotics as above and additionally with hormones as described by Taub *et al.* (1979).

Viruses

Unlabeled FPV and VSV were used for the infections. The necessary stock viruses and the ^{35}S-labeled virus used in the experiments were all obtained as described previously (Matlin *et al.*, 1981, 1982).

Infection and purification of the newly produced virus

Confluent monolayers of ^{32}P-prelabeled MDCK cells in 93 mm diameter dishes were infected with 20 plaque-forming units (p.f.u.) of FPV or VSV per cell suspended in 1.3 ml unlabeled serum-free medium. The viruses were allowed to adsorb for 1 h at 37°C after which the inoculum was replaced by the previous ^{32}P-containing serum-free medium, which had been stored at 37°C under 5% CO_2 during the infection. Subsequent incubation took place at either 37°C or 31°C depending on the experimental protocol. At 3 h post-infection the medium was aspirated and fresh unlabeled serum-free medium was added. After different time intervals the medium was taken from a plate of cells and the cell monolayer was released from the plate into an equal volume of medium with a disposable scraper at 0°C. Cells were pelleted from both the medium and the scraped cell suspension at 200 *g* for 5 min. An additional spin of 7000 *g* for 15 min at 4°C was used to pellet remaining cell debris. Subsequently the viruses were pelleted through 2 ml of 50% (w/v) glycerol in a buffer containing 0.1 M NaCl and 0.05 M Tris (hydroxymethyl) aminomethane, pH 7.4 with HCl (TN) at 111 x 10^3 *g* for 90 min at 4°C and the pellet was stored overnight in 250 μl, put onto a 12 ml continuous 5–40% (w/v) potassium tartrate gradient in TN and spun for 150 min at 284 x 10^3 *g* in an SW40 Beckman rotor (Beckman Instruments, Munich, FRG). The gradient was then fractionated into 250 μl aliquots using a peristaltic pump (Ismatec, SA, Zürich, Switzerland) and the protein content of the fractions was monitored by a Uvicord spectrophotometer equipped with a flow cell (LKB, Bromma, Sweden) at 280 nm. The radioactive viruses were localized by assaying radioactivity of a 15 μl sample of each fraction in a Mark III liquid scintillation spectrometer (Searle Analytic, Des Plaines, IL) in the double label mode.

When the cells had been incubated at 37°C, 8 h after infection, both FPV and VSV could be isolated from the culture medium and gave a single radioactivity peak on the gradient. To identify this peak, 100 μg of unlabeled FPV and a batch of ^{35}S-labeled VSV (prepared as described in Matlin *et al.*, 1982) were added to the FPV and VSV gradients, respectively. The protein pattern of the unlabeled FPV coincided with the radioactivity distribution over the fractions, identifying this as FPV (not shown) and in the VSV gradient the [^{35}S]VSV banded at the same position as the ^{32}P radioactivity (Figure 2b) indicating that this ^{32}P peak represents [^{32}P]VSV.

Phospholipid analyses

Cell pellets and virus containing fractions from the gradients were extracted using the procedure of Bligh and Dyer (1959). Carrier lipid (200 nmol phospholipid phosphorus from human erythrocytes supplemented with additional PI) was added to radioactive samples. When cells had to be extracted, the Bligh and Dyer one-phase system was sonicated for 2 min at 4°C under nitrogen with a Branson sonifier (output 65 W). The lipid extract was subsequently spotted onto precoated HP t.l.c. plates (Merck, Darmstadt, FRG) and the plates were developed using a two-dimensional solvent system, essentially according to Renkonen *et al.* (1972). The first solvent was composed of chloroform-methanol-14 M ammonia (65:25:5 v/v) and the second of chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5 v/v). The phospholipid spots were visualized by staining with iodine, scraped off, and counted. Only the five major phospholipid classes that accounted for ~97% of the total ^{32}P counts on the thin layer plates were analyzed. Phosphate in unlabeled phospholipids was determined according to Rouser *et al.* (1970).

Sources of reagents

Cell culture media and reagents were purchased from Gibco Biocult (Glasgow, UK). Fetal calf serum was obtained from Boehringer Mannheim (Mannheim, FRG) and the hormones from Sigma (St Louis, MO). Chemicals and solvents were of *pro analysi* quality and obtained from Merck (Darmstadt, FRG).

Acknowledgements

The authors are grateful to Karl Matlin, Kathryn Howell, and Daniel Louvard for helpful discussions and for critical reading of the manuscript, to Hilkka Virta who provided excellent technical assistance, and to Marianne Remy for typing the manuscript.

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