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Fatty Chains of Different Lipid Classes of Semliki Forest Virus and Host Cell Membranes

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Semliki Forest virus was grown in BHK-21 cells. The major classes of phospho- and glycolipids of the virus were analyzed for the compositions of fatty acids, aldehydes, and sphingosine bases, and the major glycerophospholipids were analyzed for the relative proportions of alkenyl-acyl, alkyl-acyl, and diacyl forms. All viral lipid classes proved to be mixtures of several molecular species. Each class contained a characteristic mixture of fatty chains, which was different in all other classes. All viral lipid classes resembled their counterparts of the host plasma membrane and also those of the endoplasmic reticulum. The gangliosides of the virus and the plasma membrane proved to be similar even at the level of individual molecular species. The number of certain lipid molecules in an average virion was less than the number of the protein molecules.

The lipoprotein envelope of group A arboviruses consists of about one-third of lipids and two-thirds of protein (19, 24). The lipids form a bilayer structure (10), which is beneath the hemagglutinating protein layer (6, 18). The hemagglutinin is a glycoprotein with an apparent molecular weight of about 55,000 (4, 12, 28, 29, 30). Recently Schlesinger and co-workers (27) have succeeded in separating the envelope protein in two components by discontinuous disc electrophoresis; both of these polypeptides are glycosylated, and their amount is about equal in the viral envelope.

In spite of its peripheral location, the envelope protein can be regarded as membrane protein, since it binds detergents in the same fashion as delipidated proteins from erythrocyte stroma and delipidated serum lipoproteins (9a). Thus the envelope of arbo A viruses offers a simple model for study of protein-lipid interactions. If these interactions have any structural specificity, the composition of the viral lipids should reveal it.

Our group studied Semliki Forest virus (SFV), a member of the group A arboviruses, which obtains its envelope from the host cell plasma membrane during the budding process (1). We have previously shown that this virus is very similar to its host cell plasma membrane in the lipid class composition (24), although the two membranes have very different proteins (8). The similarity of the polar groups of the lipids does not exclude the possibility of specific lipid compositions at the level of fatty acids or individual molecular species of the major lipid classes. In the present study we have thus analyzed the fatty acid compositions of all major lipid classes of this virus. Analogous data are reported also for the alkenyl groups of the viral plasmalogens and for the sphingosine bases of the viral sphingolipids. The compositions of the viral lipid classes were compared with those of the host cell plasma membrane. We did not detect any significant differences between the virus and the plasma membrane.

MATERIALS AND METHODS

Cells. Two clones of BHK-21 cells, WI-2 and C-13, were studied. The cells were grown to full monolayers in BHK tissue culture medium containing 10% heat-inactivated calf serum and 10% tryptose phosphate broth.

Virus. A prototype strain of SFV was grown in BHK-21 cells, and purified by gradient centrifugation as described by Kääriäinen and co-workers (11).

Membranes. Plasma membranes and endoplasmic reticulum membranes were isolated from BHK-21 cell homogenates by the method of Wallach and Kamat (31) as described by Gahmberg and Simons (9).

Isolation of the major lipid fractions. The phospholipid and the ganglioside fractions studied were those described previously (24, 25).

Separation of glycerophospholipid classes on TLC. The phospholipids were separated into the major classes by two-dimensional thin-layer chromatography (TLC) as described previously (24). The only difference was that the plates were prewashed and re-

activated. The lipids were located on the plates with dichlorofluorescein.

Hydrolysis of phospholipase A_2. The enzymatic hydrolysis with snake venom phospholipase A_2 was carried out, and the liberated fatty acids were isolated as described elsewhere (21).

Alkali-stable phospholipids. These were measured essentially as described elsewhere (22). The method involves mild alkaline methanolysis which cleaves carboxylic ester groups but no other linkages of the phospholipid molecules. The methanolysate is then made slightly acid and partitioned in the biphasic Folch system chloroform-methanol-water (8:4:3). The chloroform-rich layer of this system contains intact sphingomyelins and alkenyl, as well as alkyl-derivatives of glyceryl phosphoryl ethanolamine (GPE), glyceryl phosphoryl choline, (GPC), glyceryl phosphoryl serine (GPS), and glyceryl phosphoryl inositol (GPI). Phosphorus analysis of this stable fraction gives the sum of all these lipids.

Quantitation of alkenyl-acyl forms of different glycerophospholipid classes. Total phospholipids were subjected to quantitative two-dimensional TLC. Another sample of the same phospholipids was subjected to mild acid methanolysis under conditions described elsewhere (22); this treatment cleaves the alkenyl groups. The methanolysate was then subjected to quantitative two-dimensional TLC as above. The decrease of the spots containing the two-chain derivatives of GPE, GPC, GPS, or GPI indicated the amount of corresponding alkenyl derivatives.

Quantitation of alkyl-acyl GPE and -GPC. Total lipids were subjected to mild acid methanolysis and subsequently to mild alkaline methanolysis as described elsewhere (22); this treatment cleaves the alkenyl and the acyl groups. The methanolysate was then subjected to one-dimensional TLC on silica gel plates with chloroform-methanol-ammonia-water (60:50:2.5:2.5) as solvent. The spots of alkyl GPE and alkyl-GPC were recovered, and their phosphorus content was measured.

Purification of the sphingolipid classes. Sphingomyelin isolated from the two-dimensional TLC plates was contaminated by monoacyl GPE. This contamination was eliminated by mild alkaline methanolysis, after which the sphingomyelins were isolated by preparative TLC. The crude gangliosides were separated from the remaining traces of phospholipids by Florisil chromatography of the acetylated glycolipids (26).

Preparation of GLC samples and analysis of fatty acids and sphingosine bases. The TLC spots containing the major glycerophospholipid classes were scraped from the plates, and their fatty acids were converted to methyl esters by heating with methanol and sulfuric acid. The methyl esters were isolated from the reaction mixture as described previously (21). Methyl esters of the sphingomyelin and ganglioside fatty acids were prepared also by heating the samples with methanol-sulfuric acid (94:6) overnight at 70 C. Gas-liquid chromatography (GLC) of the fatty acid methyl esters was carried out essentially as described elsewhere (21). Dimethyl acetals derived from the alkenyl groups were isolated from mild acid methanolysates and analyzed by GLC as described elsewhere (22). The long-chain bases of sphingomyelins were obtained by converting the phospholipids into ceramides with phospholipase C as described previously (21). The ceramides were hydrolyzed with 1 N KOH in methanol-water as described by Carter and co-workers (5). The long-chain bases of gangliosides were obtained after hydrolysis with barium hydroxide in dioxane-water (17). The liberated long chain bases were analyzed on GLC as *N*-acetyl-*O*-trimethylsilyl ethers as described elsewhere (23).

RESULTS

Fatty acids of glycerophospholipids. The fatty acid composition of the major glycerophospholipids of SFV, of host cells and their plasma membranes and endoplasmic reticulum are shown in Table 1. Oleic acid (18:1) was a major component in all phospholipid classes, but otherwise each class had a specific fatty acid composition which was dissimilar to that of all others. The GPC lipids contained more palmitic than stearic acid; they were also poor in long-chain polyenoic acids. The GPS lipids in turn had a low palmitic, and a high stearic acid content. The GPI lipid, too, had a high stearic acid content, but in addition they were rich in the polyenoic acids. The GPE lipids contained a low amount of both palmitic and stearic acids. Cardiolipin, finally, was the only lipid rich in linoleic acid (18:2).

When the composition of the viral lipids is compared with that of the whole host cells and the isolated plasma membrane and endoplasmic reticulum fractions, a surprising similarity can be seen. There seems to be only one difference: the GPC lipids of the virus and plasma membrane contained more palmitic acid (and probably a little less oleic acid) than their counterparts in the endoplasmic reticulum.

Hydrolysis with phospholipase A_2 liberated only unsaturated fatty acids from the viral GPS and GPE lipids (Table 2). This suggests that the saturated fatty acids were located on C-1 positions of the glycerol moiety in these lipids. On the other hand, the viral GPC lipids released significant amounts of palmitic acid upon the enzymatic hydrolysis; this suggests that they contained palmitic acid both on C-1 and C-2 of the glycerol moiety. Oleic acid, too, seemed to be present in both positions of the GPC-lipids, but stearic acid was present only on C-1. The data of Tables 1 and 2 show that none of the viral glycerophospholipid classes comprises a single molecular species; on the contrary, they are all complex mixtures of several molecular species.

Relative amounts of alkenyl-acyl, alkyl-acyl, and diacyl forms within the glycerophospholipid classes. Mild alkaline methanolysis revealed 40% alkali-stable components in the viral phospholipids. This fraction contains the sphingomyelins and the ether phospholipids of alkenyl-acyl and

TABLE 1. *Fatty acid composition of major glycerophospholipid classes of Semliki Forest virus and of host cells and their membrane fractions*[a]

Glycerophospholipids	16:0 (%)	16:1 (%)	18:0 (%)	18:1 (%)	18:2 (%)	Polyenes (%)
GPC lipids						
SFV[b]	38	6.3	8.7	38	5.7	1.2
PM[c]	31 ± 4	6.4 ± 1	7.9 ± 1	45 ± 3	5.6 ± 1	1.8 ± 1
ER[c]	22 ± 3	7.1 ± 1	6.4 ± 1	51 ± 5	8.2 ± 2	2.1 ± 1
Cells[d]	22	9.0	6.8	46	12	0.9
GPS lipids						
SFV[b]	5.3	4.1	34	46	7.0	2.5
PM[c]	4.4 ± 2	2.8 ± 1	29 ± 2	53 ± 3	5.5 ± 2	3.6 ± 4
ER[c]	4.8 ± 3	1.3 ± 1	28 ± 3	50 ± 5	4.5 ± 1	8.7 ± 6
Cells[d]	5.6	2.4	31	44	10	4.9
GPI lipids						
SFV[e,f]	7.9	3.0	42	16	4.2	27
PM[c,e]	6.7 ± 1	2.3 ± 1	38 ± 2	30 ± 4	2.9 ± 1	18 ± 5
ER[c]	3.3 ± 1	1.4 ± 1	36 ± 3	27 ± 3	3.3 ± 1	28
Cells[d]	8.7	2.8	37	20	3.7	25
GPE lipids						
SFV[b]	4.3	1.6	5.2	43	10	23
PM[c]	5.1 ± 1	2.4 ± 1	8.3 ± 5	50 ± 4	8.3 ± 2	11 ± 6
ER[c]	5.2 ± 1	3.4 ± 1	12 ± 5	53 ± 10	8.8 ± 1	6.9 ± 3
Cells[d]	4.6	3.0	15	41	12	14
Cardiolipin cells[d]	2.3	7.4	2.1	40	42	3.2

[a] Abbreviations: GPC, glyceryl phosphoryl choline; GPS, glyceryl phosphoryl serine; GPI, glyceryl phosphoryl inositol; GPE, glyceryl phosphoryl ethanolamine; SFV, Semliki Forest virus; PM, plasma membranes; ER, endoplasmic reticulum; cells, BHK-21 cells. 16:0 palmitic acid; 16:1 hexadecenoic acid; 18:0 stearic acid; 18:1 octadecenoic acids; 18:2 octadecadienoic acids; polyenes, polyenoic acids.

[b] Mean values of SFV grown in Wi-2 and C-13 clones of BHK-21 cells. The two samples agreed within 15% of the means of all major components except in the dienoic and polyenoic fatty acids of the GPE-lipids.

[c] Mean values (± standard deviation) of membranes isolated from Wi-2 and C-13 clones of BHK-21 cells. Four samples of Wi-2 cells and one sample of C-13 cells were analyzed. The two clones of cells gave similar results.

[d] Mean values of Wi-2 and C-13 clones of BHK-21 cells. The two samples agreed within 15% of the means of all major components except in the polyenoic acids of the GPI and GPE lipids.

[e] SFV grown in BHK-21-Wi-2 cells (one sample).

[f] These values are more uncertain than the others because of the very small amount of GPI lipids in the virus and plasma membrane. Therefore, much attention should not be paid to the apparent differences between the virus and the plasma membrane.

alkyl-acyl type. The viral phospholipids contain 21% of sphingomyelins (24). Thus by difference they contained 19% of the ether phospholipids. Therefore, the viral glycerophospholipids comprised 24% ether lipids and 76% diacyl lipids (Table 3). Similar analysis of the host cells and the isolated membrane fractions showed that the plasma membrane resembled the virus, whereas the endoplasmic reticulum and the whole cells contained slightly less of the ether lipids.

Two thirds of the viral GPE lipids proved to be of the alkenyl-acyl type, but the other classes contained only a few percent of these molecules (Table 3). The host plasma membrane was similar to the virus. The endoplasmic reticulum and the whole cells contained slightly less of alkenyl-acyl GPE lipids, but otherwise resembled the virus. These data show that about 90% of the alkenyl groups of the virus and of the host were present in the GPE lipids. The large amount of alkenyl chains in the GPE lipids explains the characteristically low amounts of palmitic and stearic acids in these lipids; the saturated acids are simply substituted by the alkenyl groups.

The alkyl-acyl lipids of the virus and the host were concentrated into the GPE and GPC lipids.

TABLE 2. *Composition of fatty acid mixtures liberated from Semliki Forest virus phospholipids with phospholipase A_2*[a]

Lipids	16:0 (%)	16:1 (%)	18:0 (%)	18:1 (%)	18:2 (%)	Polyenes
GPC	49	6.6	1.3	39	3.6	Trace
GPS	0	4.9	0	78	10	7.0
GPE	0	2.5	0	76	15	7.4

[a] Abbreviations as in Table 1. Results of one analysis (Wi-2 cells).

TABLE 3. *Distribution of subclasses in different glycerophospholipids of Semliki Forest virus and of host cells and their membrane fractions*[a]

Glycerophospholipids	Alkenyl-acyl form	Alkyl-acyl form	Diacyl form
All glycerophospholipids			
SFV[b]	24[c]		76
PM[b]	27[c]		73
ER[b]	20[c]		80
Cells[d]	19[c]		81
GPC lipids			
SFV[d]	2	10[b]	88
PM[b]	1		99[c]
ER[b]	0		100[c]
Cells[e]	4 ± 3	13[b]	83
GPS lipids			
SFV[d]	2		98[c]
PM[b]	0		100[c]
ER[b]	0		100[c]
Cells[e]	1 ± 2		99[c]
GPI lipids			
SFV[d]	0		100[c]
Cells[e]	2 ± 4		98[c]
GPE lipids			
SFV[d]	63	7[b]	30
PM[b]	59		41[c]
ER[b]	40		60[c]
Cells[e]	48 ± 5	9[b]	42

[a] Abbreviations as in Table 1.
[b] Result of one sample (Wi-2 cells).
[c] Includes also the alkyl-acyl form.
[d] Mean of two samples (Wi-2 cells).
[e] Mean ± standard deviation of four samples (Wi-2 cells).

Quantitative analysis showed that the viral GPE lipids contained 7% and the GPC lipids 10% of alkyl-acyl lipids (Table 3). The host cell lipids were similar.

GLC analysis of the alkenyl chains of the viral phospholipids showed that most of the alkenyl chains were saturated and contained 16 to 18 carbon atoms (Table 4). The alkenyl groups of the host cell phospholipids resembled those of the virus.

Fatty chains of sphingolipids. The sphingolipids of SFV and the host cell membranes had a rather different set of component fatty acids than the glycerophospholipids. They were characterized by a high content of saturated and monoenoic acids, by a high content of C_{20}-C_{24} acids, and by absence of polyenoic acids (Table 5). These features are quite common in animal sphingolipids. The gangliosides seemed to contain less palmitic and more oleic and stearic acids than the sphingomyelins.

The viral gangliosides had a fatty acid composition similar to their counterparts of host plasma membrane and endoplasmic reticulum (Table 5). Even the spingomyelins of SFV resembled those of plasma membrane and endoplasmic reticulum. The long-chain base fraction of the viral gangliosides contained only one major component which was identified as erythro-Δ4-trans-sphingenine. Also host cell gangliosides and sphingomyelins contained this molecule as their only long-chain base. As all sphingolipid molecules contained the same long-chain base, the data in Table 5 suggest that the viral gangliosides and sphingomyelins were

TABLE 4. *Composition of dimethyl acetals derived from the alkenyl groups of phospholipids of Semliki Forest virus and host cells*[a]

Determination	16:0 (%)	17:0 (%)	18:0 (%)	18:1 (%)
SFV	33	6	22	38
Cells	31	6	24	39

[a] Abbreviations are as in Table 1, except that 16:0 refers to palmitaldehyde dimethyl acetal etc. Results of one analysis.

TABLE 5. *Fatty acid composition of major sphingolipid classes of Semliki Forest virus and of the isolated plasma membrane and endoplasmic reticulum fractions of the host cells*[a]

Determination	16:0 (%)	16:1 (%)	18:0 (%)	18:1 (%)	22:0 (%)	23:0 (%)	24:0 (%)	24:1 (%)
Gangliosides								
SFV[b]	46	6.4	6.4	8.3	5.4	2.4	6.6	13
PM[c]	41	3.6	6.2	5.9	5.0	3.3	8.2	16
ER[c]	46	5.8	8.5	5.2	6.8	3.8	6.8	10
Sphingomyelins								
SFV[b]	74	1.6	0.7	TR	3.7	TR	5.5	14
PM[c]	69	TR	TR	TR	9.7	TR	TR	22
ER[c]	78	1.4	TR	TR	6.6	TR	5.9	7.3

[a] Abbreviations as in Table 1; TR = trace.
[b] Mean values of two samples (Wi-2 cells).
[c] Result on one sample (Wi-2 cells).

similar to their plasma membrane counterparts at the level of individual molecular species.

DISCUSSION

Several groups have studied the lipid class composition of the envelopes of ribonucleic acid (RNA) viruses known to bud through the host cell plasma membrane. Blough and Lawson (2), Blough (3), David (7), and McSharry and Wagner (16) have suggested that the viral lipid composition reflects the lipid affinities of the viral envelope proteins. On the other hand, Klenk and Choppin (13–15) found that parainfluenza virus SV 5 grown in four different hosts had widely different lipid compositions; thus the lipids of this virus cannot be determined primarily by the affinities of the viral peptides. Quigley and co-workers (20) found that six different RNA viruses grown in the same host had very similar lipid class profiles. This finding, too, speaks against specific lipid affinities in the viral polypeptides.

The lipid class compositions of these viruses resemble those of the host cell plasma membranes (7, 13–16, 19, 20, 24). So far no direct comparisons have been reported on the fatty acid compositions of different lipid classes in these viruses and the host cell plasma membranes. Our present data show that the fatty acids of the different lipid classes are quite similar in the two membranes. This similarity prevails also in the alkenyl groups of the plasmalogens. The gangliosides are similar in the two membranes even at the level of individual molecular species. Consequently the viral envelope proteins and the plasma membrane proteins resemble each other in their affinities for the different lipid structures—the different lipid classes of influenza virus, too, resemble those of SFV in several of the major features of fatty acid composition (3).

Our data show further that SFV contains some lipids in so small quantities that there are fewer than one molecule of them for every envelope protein molecule. This finding, too, implies certain nonspecificity in the lipid affinities of the viral envelope proteins. Our present estimate is that there are about 25 polar lipid molecules for one envelope protein molecule in the virus (O. Renkonen, Proc. Golden Jubilee Symp. Dept. Biochem., Bangalore, *in press*). Therefore, all the minor lipids not exceeding a 4% level are present in less than equimolar amounts when compared to the envelope proteins. Examples of such lipids are all GPI lipids, alkenyl-acyl GPC lipids, alkyl-acyl GPE lipids, stearoyl-ganglioside and lignoceryl-sphingomyelin. These and other small components together form a sizeable fraction of the viral lipids.

An additional example of nonspecificity in the lipid affinities of the envelope polypeptides was observed recently by Gahmberg et al. (C. G. Galmberg et al., Virology, *in press*), who studied the exposed amino groups, and found that diacyl GPE lipids and alkenyl-acyl GPE lipids may be randomly distributed in SFV envelope.

The fatty chain compositions in the individual lipid classes in the virus, in the plasma membrane, in the endoplasmic reticulum, and in the whole cells were, for the most part, so similar that the present data are of little help in tracing the origin of the viral lipids.

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