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The molecular organization of the influenza virus surface. Studies using photoreactive and fluorescent labeled phospholipid probes

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The membrane structures of remantadin-sensitive and remantadin-resistant influenza virus strains were studied using a photoreactive fatty acid as well as analogues of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, carrying a fluorescent or photoreactive reporter group at the end of one of the aliphatic chains. The results obtained demonstrated for the first time that the phospholipids of the viral membrane form lateral domains differing by the fluidity of their hydrocarbon chains and, probably, by the head-group composition of the lipids. The hemagglutinin small subunit (HA_2) was shown to protrude into the apolar region of the phospholipid bilayer, whereas the M_1 protein makes contact only with the inner surface. In the remantadin-sensitive virions the heavy hemagglutinin chain (HA_1) appears not to be in contact with the lipid bilayer, whereas in the remantadin-resistant strain HA_1 has a hydrophobic segment that proved to be inserted into the bilayer.

Introduction

The influenza virus envelope is formed of a lipid bilayer and two glycoproteins, hemagglutinin and neuraminidase, forming spikes protruding into the outer medium. The hemagglutinin monomer consists of two subunits: a small one (HA_2) which is partly inserted into the lipid bilayer, and a large one (HA_1) which carries the main part of the oligosaccharide residues. The inner surface of the viral membrane is in contact with the matrix protein (M_1 protein) which in turn contacts with the nucleocapside (a related M_2 protein has recently been shown to be exposed on the surface of infected cells [1]). Although the influenza virus envelope has been studied using a variety of methods, many details of its structure remain obscure. Particularly, the extent of insertion of the surface glycoproteins and the M_1 protein into the bilayer is unknown and no data regarding the lateral distribution of the phospholipids on the viral surface are available.

In order to elucidate the localization of different lipid classes and proteins in the influenza virus membrane we employed photoreactive and fluorescent probing methods. Fluorescent and photoreactive (photoactivatable) probes are widely used in membrane studies (see, for example, reviews,

Abbreviations: FPV_R, fowl plague virus, remantadin-resistant strain; FPV_S, fowl plague virus, remantadin-sensitive strain; HA, hemagglutinin; HA_1, heavy hemagglutinin chain; HA_2, light hemagglutinin chain; M, matrix protein; NA, neuraminidase; NAP, 2-nitro-4-azidophenyl; NP, nucleocapside; PA, PB, nucleoproteins.

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Refs. 2, 3). The probes (I–V) employed in the present study are photoreactive and fluorescent analogues of the three main viral phospholipid components: phosphatidylcholine, phosphatidylethanolamine and sphingomyelin (Fig. 1). Since these probes closely resemble natural lipids [4] they may be expected to provide information about the behavior of their corresponding native prototypes on the virus surface. Remantadin (1-(1-adamantyl)ethylamine) and the closely related amantadin (1-adamantylamine) are antiviral drugs used extensively in the prophylaxis and therapy of influenza A infections (reviewed in Ref. 5). Both substances are primary symmetrical cycloaliphatic amines with strong hydrophobic properties. Remantadin and amantadin are known to prevent influenza virus replication by blocking virus uncoating which occurs in the endocytic vacuoles [6]. Because these drugs are thought to act on the virus membrane, we have studied in a comparative manner the membrane structure of a remantadin-sensitive and remantadin-resistant strains of the classical fowl plague virus (FPV).

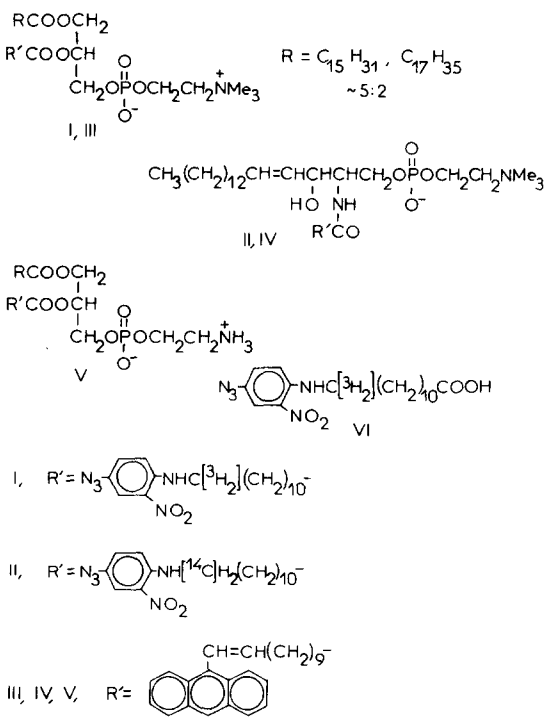

Fig. 1. Structures of photoactivatable and fluorescent lipid probes.

Materials and Methods

Influenza type A virus (fowl plague virus) was grown in chicken fibroblasts and purified as described in Ref. 7. The purity of the virus preparations was controlled by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the Laemmli system [8] which showed the absence of contaminating proteins. Virus preparations were also examined by electron microscopy which revealed only homogeneous particles 80–100 nm in diameter.

The syntheses of the photoactivatable lipid probes (I), (II) and (VI) have been described [9,10]. The specific radioactivity of these probes was respectively 900, 48 and 900 mCi/mmol. The anthrylvinyl labeled phospholipids (III–V) were synthesized according to Refs. 11–13.

Suspensions of FPV_S and FPV_R were obtained as described in [14]. The protein content of the virus suspensions was determined by the method of Lowry et al. in the presence of sodium deoxycholate [15]. For densitometric determinations, the zones obtained after electrophoresis of the viral proteins in polyacrylamide gel were stained with Coomassie brilliant blue R-250 and the absorbance at 595 nm was measured using a Gilford 2400-2 spectrophotometer. The phospholipids of the viruses were determined after thin-layer chromatography by Bartlett's method [16]; cholesterol was analyzed using gas-liquid chromatography [17].

Vesicles from the virus total lipids were prepared by sonication of lipids obtained by Folch extraction [18] of the virus, in 50 mM Tris-HCl buffer (pH 7.4) (4 × 5 min with external cooling).

For fluorescent labeling, 0.1% ethanolic probe solution was added to an aliquot of virus or vesicle suspension with intense stirring, to a final probe/phospholipid ratio of 1 : 100. The mixture was incubated for 2 h at 36.5°C before measurement; the final ethanol concentration in the sample did not exceed 0.6%.

Fluorescence spectra (uncorrected) were recorded on an Aminco SPF-1000 fluorimeter equipped with a thermostated cell, in quartz

cuvettes 10–10 mm, band-width 5 nm for both excitation and emission. Fluorescence polarization was calculated by the equation:

$$P = \frac{(I_{\parallel} - I_{\parallel}^{0}) - (I_{\perp} - I_{\perp}^{0})}{(I_{\parallel} - I_{\parallel}^{0}) + (I_{\perp} - I_{\perp}^{0})},$$

fluoresence intensities being measured at 370 nm in excitation spectra (emission 440 nm) of samples with the probe (I) and without probe (I^0) with parallel ($_{\parallel}$) and crossed ($_{\perp}$) polarizer and analyzer.

For photoreactive labeling studies, solutions of the photoactivatable probes in dimethylsulfoxide were added under argon to virus suspensions in 50 mM Tris-HCl buffer (pH 7.4) and incubated for 3.5 h at 37°C. The final probe concentration was about 1 mol probe per 100 mol of the corresponding viral phospholipid; the dimethylsulfoxide concentration in the sample did not exceed 1%. Incorporation of the probes was followed by ultracentrifugation at $100\,000 \times g$ for 30 min at 4°C on a MOM 3180 centrifuge with subsequent determination of the supernatant radioactivity.

For photolabeling, the virus suspension was transferred into a Pyrex vessel (diameter 8 mm) non-transmittant at $\lambda < 300$ nm. The sample was irradiated for 35 min with cooling and argon bubbling using a VIO-1 mercury lamp (power 50 W, emission maximum 365 nm) at the distance of 7 cm, and then was twice delipidated by Folch extraction [18]. The aqueous suspension was evaporated in vacuo at 40°C and the residue was solubilized using a buffer containing 2% sodium dodecylsulfate and 5% β-mercaptoethanol. The solubilized proteins were electrophorized in 12.5% polyacrylamide gel in the system of Laemmli [8]. After staining with Coomassie brilliant blue R-250 the gel was cut into equal parts of 2–3 mm width which were solubilized at 37°C using 30% H_2O_2 (for ^{14}C-labeled probe, 1% ammonia was added) and the radioactivity was determined using a Unisolve-1 scintillator (Koch-Light) and a Beckman LS 9800 counter.

Results

Lipid and protein composition of the viruses

The remantadin-sensitive and remantadin-resistant strains of FPV differed strongly in their phospholipid content (Table I). In both strains the three major phospholipids are phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. However, the remantadin-sensitive strain contained 3-times more sphingomyelin than phosphatidylcholine, whereas in the remantadin-resistant virions the two phospholipids were present in about equal amounts. The phosphatidylethanolamine content was also different (6 and 17% in the remantadin-resistant and remantadin-sensitive samples, respectively). The two strains differed also considerably in their cholesterol level (Table I): the remantadin-resistant strain contained 2-times less cholesterol than the remantadin-sensitive virus.

TABLE I

LIPID COMPOSITION OF THE INFLUENZA VIRUS, μg/mg PROTEIN

Lipid	Remantadin-sensitive strain FPV_S	Remantadin-resistant strain FPV_R
Phosphatidylcholine	32 ±2	90±5
Sphingomyelin	115 ±7.5	85±1
Phosphatidyl-ethanolamine	9.4±0.5	35±2.5
Cholesterol	138 ±1.5	57±1

On the other hand, the protein profiles of the two strains were virtually identical (Table II). In comparison with the remantadin-sensitive samples the remantadin-resistant viruses showed a slightly higher content of M_1 protein, and a somewhat lower content of nucleocapside protein and neuraminidase.

TABLE II

PROTEIN CONTENT OF INFLUENZA VIRUS: PERCENT OF THE TOTAL VIRUS PROTEIN

Protein	FPV_S strain	FPV_R strain
PA, PB [a]	1.1	1
HA	2.6	3.6
NP, NA [a]	36	30.6
HA_1	21.3	21.4
HA_2	12	12
M	27	32.2

[a] Proteins are not resolved, their sum is given.

Photolabeling studies

After incubation of the two FPV strains with the photoactivatable lipid probes (I), (II) and (VI) and subsequent ultracentrifugation, 97–99% of the initial radioactivity was found in the sediment. Since incorporation of the fluorescent-labeled lipids (III–V) into the viral membrane is complete after 1.5 h incubation (see below), the same is probably true for photoreactive lipids.

The virus suspensions containing the photoactivatable lipid probes were photolyzed and the unbound lipids were removed by Folch extraction. The residues were solubilized and electrophoresed and the radioactivity of the different zones was measured. The electrophoretic behavior of the influenza virus proteins is well known (see, for example, Ref. 19). Fig. 2 shows a typical electrophoretogram and radioactivity profile. Since any nitrenes in the aqueous phase or at the membrane surface would be trapped by glutathione [20], the photolysis experiment was repeated in the presence of glutathione. This did not alter the total radioactivity of the sample and the label distribution between the protein fractions (data not shown).

The results of photoreactive labeling of the two FPV strains are presented in Table III. These data show that in the case of the remantadin-sensitive virus the light hemagglutinin subunit (HA_2) was modified most extensively, the viral neuraminidase (NA) was labeled to a considerably smaller extent, whereas only insignificant amounts of radioactivity were present in the heavy HA subunit (HA_1) zone.

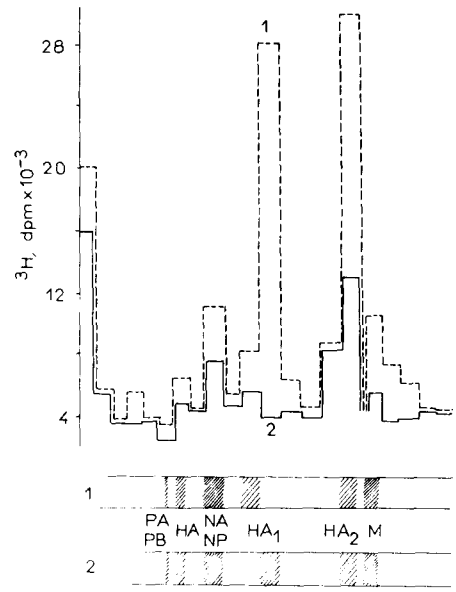

Fig. 2. Electrophoresis (below) and radioactivity profiles (above) of the proteins of FPV_S (1) and FPV_R (2) virions after photolabeling with the phosphatidylcholine probe (I).

The M_1 protein was labeled by the fatty acid probe (VI) to a much higher extent than by the photoreactive phospholipids (I) and (II): after photolysis the amount of radioactivity in the M_1 protein zone was 20% of the total activity in the case of probe (VI) and only 10–13% with probes (I) and (II).

When analysing the results of the photolabeling experiments one must take into account that nitrenes not only bind to protein molecules but may also initiate radical chain reactions (see, for

TABLE III

PHOTOINITIATED CROSSLINKING OF THE PROBES TO PROTEINS OF REMANTADIN-SENSITIVE AND REMANTADIN-RESISTANT STRAINS OF FPV

Percent of the total probe covalently linked to protein; means of three experiments ± S.D.

Protein	Probe (I)		Probe (II)		Probe (VI)
	FPV_S	PFV_R	FPV_S	FPV_R	FPV_S
PA, PB, HA [a]	8±4	4±1	8±3.5	12±1	21±2
NP, NA [a]	17±2	9±3.5	8±2	6±0.3	6±2
HA_1	9±2	36±3	10±1	24±2	11±1
HA_2	55±5	40±2	60±2	46±3	42±1.5
M	11±1	11±0.5	13±0.5	12±1	20±0.5

[a] total values for the proteins are given.

example, Ref. 21) that will result in the formation of oligomers. Apparently, part of the radioactivity found in the zones corresponding to nucleocapside proteins or uncleaved hemagglutinin may be due to such oligomerization of the lighter proteins and/or lipid molecules.

The binding efficiency of the phosphatidylcholine probe (I) was considerably higher than that of the sphingomyelin probe (II) (binding efficiency is the ratio of total radioactivity of protein zones to the total radioactivity of lipid probe incorporated in the viral membrane).

Upon incubation of the photoactivatable probes with viruses of the FPV_R strain, the probe concentration in the virus membrane was about the same as in the case of the remantadin-sensitive strain, i.e., the concentration of each probe comprised approx. 1% of the amount of the corresponding viral lipid species. However, significant differences were detected after photolysis. With FPV_R viruses the binding efficiency of both probe (I) and probe (II) was higher than with FPV_S virions. Moreover, with FPV_R we observed significant binding of these probes to the heavy hemagglutinin chain HA_1. Such binding was almost absent in the case of FPV_S (see Table III).

Fluorescent studies

After addition of the fluorescent lipid probe (III), (IV) or (V) to the virus suspensions, the initial fluorescence intensity was very low due to self-quenching of the probes in aqueous medium. As the probe was incorporated into the virus membrane, the fluorescence intensity steadily increased and reached a maximal value after 2 h. Evidently this time corresponds to maximal incorporation of the probes into the membranes of the virions.

The fluorescence polarization (P) values of probes (III)–(V) incorporated into the virus membrane differed considerably from those observed when the probes were included into liposomes prepared from the viral total lipids (Fig. 3). In the virus membranes, the P values of probes (III) and (IV) were much higher than in the liposomes; with the phosphatidylethanolamine probe (V) the picture was reversed: in the liposomes this probe showed a higher P value than when inserted into the virus membrane.

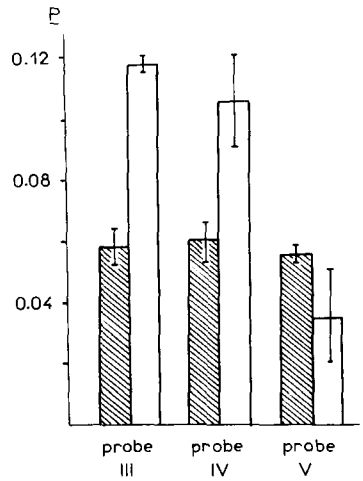

Fig. 3. Steady-state fluorescence polarization of the fluorescent lipid probes (III)–(V) in lipid vesicles from FPV_S total lipids (hatched slabs) and in the viral membrane (empty slabs) at 36.5°C. Data are means of three experiments.

Measurement of the P values of probes (III) and (IV) revealed important differences between the remantadin-sensitive and remantadin-resistant strains. In the latter case the P value of the sphingomyelin probe (IV) was considerably lower than in the former, whereas the phosphatidylcholine probe (III) showed approximately the same P values in both strains.

Discussion

Mixoviruses acquire their lipids from the host cell [22]. Nonetheless, the influenza virus membrane was shown to differ in its lipid composition from the plasma membrane of the host mainly by a higher content of sphingomyelin and cholesterol and a lower content of phosphatidylcholine [23,24]. Analogous trends were found in the present investigation. Attention should be drawn to the fact that the remantadin-sensitive FPV_S virus differs from the remantadin-resistant FPV_R strain by a much higher cholesterol content. Such high cholesterol/phospholipid ratios are quite rare in cell membranes [25]. The relatively low P values of the probes in this case may indicate that the probes are partly excluded from cholesterol-rich domains. Since both strains were grown under completely identical conditions, the difference in cholesterol contents indicates that some protein

component of the two strains must differ in its affinity for cholesterol.

The transmembrane asymmetry of the influenza virus phospholipids has been investigated [26]; however, data regarding their lateral distribution in the plane of the membrane are lacking. In the present study such information was obtained using photoactivatable or fluorescent-labeled phospholipids incorporated into the intact virus membrane. Because lipid probes differ from natural lipids only in the structure of one of the apolar chains they may be expected to mimic the behavior of their natural counterparts in the influenza virus membrane [4]. Since in the latter transmembrane migration of the phospholipids ('flip-flop') occurs only very slowly with half-times ranging from 10 to 30 days [26], the probes will report predominantly on the lipids of the outer shell of the viral membrane.

Although the fluorescent and photoactivatable lipids of the present study closely resemble natural phospholipids, they still will to some degree disturb the molecular organization of the viral membrane as in the case with any extrinsic membrane probe. In order to minimize the uncertainties arising from such perturbations we have adopted an experimental strategy, the so called 'two probes technique' [4]. According to this approach, two or more lipid probes with the same reporter group and different headgroups are independently introduced into the membrane, and their behavior is compared. Since the perturbations induced by such different lipid probes and other distorting factors (e.g., systematic errors in measurements of P values) are practically the same, any differences in their behavior must be due to differences in their polar headgroups. This allows to reveal peculiarities of the domain organization and lipid–protein interactions depending on the lipid headgroup structure.

As can be seen from Fig. 3, after incorporation of the three fluorescent lipids into the virus they show different fluorescence polarization (P) values: those of the phosphatidylcholine (III) and sphingomyelin (IV) probes considerably exceed the P value of the phosphatidylethanolamine probe (V).

As is well known, the fluorescence polarization of a probe reflects the fluidity state of environment of its chromophore. Therefore, the fact that the cholinephosphatide and phosphatidylethanolamine probes showed similar P values in lipid vesicles but different ones when inserted into the viral membrane indicates that in the latter case they reside in different environments, i.e., in different domains of the membrane. This suggests that the viral lipids distribute non-randomly in the plane of the membrane. Judging from the P values, the rigidity of the domains sampling the phosphatidylcholine (III) and sphingomyelin (IV) probes is comparable with the rigidity of lipid chains in the gel state; the domains sampling the phosphatidylethanolamine (V) probe must be much more fluid. Since in vesicles prepared from the viral total lipids the three probes show much closer P values than in the viral membrane (Fig. 3) the differences of P magnitudes observed when the probes are incorporated into the virus must be due to their interaction with viral proteins. Apparently, phosphatidylethanolamine is excluded from boundary lipids surrounding viral proteins. Taking into account the structural similarity between the probes (III)–(V) and natural phospholipids, we suppose that the different behavior of the phospholipid probes reflects differences in the interaction of their native prototypes with viral proteins that are in contact with the lipid bilayer, i.e. with the M protein, hemagglutinin and neuraminidase.

Further information on the interaction of these proteins with different phospholipid classes of the viral membrane was obtained using the photoactivatable lipid probes (I) and (II). The use in a comparative manner of two different lipid analogues with the same photoreactive group permits minimization of the influence of perturbing factors (cross-linking between lipid molecules, radical side reactions, etc.), because these factors are the same, or very similar, for both probes.

As can be seen from the data of Table III, upon photolysis of FPV_S viruses containing photoactivatable phospholipids the latter crosslink preferentially to the light hemagglutinin subunit (HA_2) and only to a much smaller extent to HA_1, neuraminidase and M_1 protein. As was pointed out above, the phospholipids (I) and (II) may be expected to reside predominantly in the outer shell of the viral lipid bilayer. Their photoreactive NAP

groups are most probably located near the centre of the bilayer, as has been demonstrated for these probes introduced into dimyristoylphosphatidylcholine vesicles [9]. Thus, our photolabeling results are in accord with earlier data indicating that the hydrophobic part of the HA_2 chain is deeply inserted into the lipid bilayer of the virus membrane [27].

Measurements of the crosslinking of the photoreactive lipids to neuraminidase (NA) was complicated by the fact that NA could not be separated from the nucleocapside (NP) protein by electrophoresis (Fig. 2a). However, since in intact viruses the nucleocapside does not contact with the lipids, being separated from the bilayer by the M_1 protein, one may safely assume that the radioactivity of the NP-NA zone is due to binding of the photoactivatable lipids to neuraminidase. This glycoprotein comprises only 3–7% of the viral total protein [28], and Table III demonstrates that the relative level of photoreactive labeling of neuraminidase is considerably higher than that of HA_1 (in FPV_S strain) and M_1 protein. Evidently neuraminidase protrudes into the apolar region of the bilayer, a conclusion that is supported by data showing that the N-terminal of the neuraminidase chain is attached to a hydrophobic fragment of 30 non-charged amino-acid residues [29].

The data of Table III show also that for the FPV_S strain the crosslinking efficiency of the phosphatidylcholine probe (I) to neuraminidase is much higher than that of the sphingomyelin probe (II) although both probes crosslink with approximately the same efficiency to HA_2. This lends further support to our conclusion that phosphatidylcholine and sphingomyelin distribute non-randomly in the plane of the viral membrane.

The M_1 protein is known to interact both with the nucleocapside and the lipids of the viral membrane [30–32]. The results of the present study suggest that in the intact virions the M_1 protein probably does not protrude into the central region of the bilayer. This supposition was supported by photolabeling data obtained with the photoactivatable fatty acid (VI). Fatty acids are known to undergo rapid flip-flop when incorporated into phospholipid bilayer [33]. One can therefore expect that, in contrast to the phospholipid probes (I) and (II), the photoactivatable acid (VI) will rapidly distribute between the outer and inner shells of the viral bilayer membrane. Indeed, the acid (VI) proved to bind to the M_1 protein with considerably higher efficiency than the photoreactive phospholipids. This fact suggests that the M_1 protein contacts only with the inner lipid shell and does not reach the centre of the bilayer. Earlier an analogous conclusion was drawn with respect to the M protein of vesicular stomatitis virus [34].

With the remantadin-resistant FPV_R strain the crosslinking efficiency of all three photoactivatable probes was significantly higher than in the case of the remantadin-sensitive FPV_S strain (see Table III). At present we have no convincing explanation for this difference. The following causes may be considered: (a) the lipid bilayer of the FPV_s viruses is more permeable to water, which quenches the nitrenes; (b) in the membrane of the FPV_s viruses the probes partly form separate phases, giving rise to higher lipid-lipid and concomitantly lower lipid-protein crosslinking. Taking into account the lipid composition of the two strains, the first explanation appears unlikely because the high cholesterol and sphingomyelin content of the FPV_R viruses could be expected to lower the water permeability of the viral membrane.

An other important difference of the two influenza virus strains is that in the case of FPV_R the photoactivatable phospholipids (I) and (II) bind not only to the HA_2 chain but also to the HA_1 subunit of hemagglutinin. This is a surprising fact, because the HA_1 subunit consists mainly of polar amino acids and contains the major part of the hemagglutinin sugar chains [35] and thus must be highly hydrophilic. However, the N-terminal of the hemagglutinin precursor is known to be attached to a leader, a short polypeptide of 18 hydrophobic amino acids [35]. As in the case with other secretable proteins, the leader is split off during the assembly of virus. Probably, in the FPV_R virions the hydrophobic HA leader is retained and as a result the *N*-terminal of HA_1 chain becomes inserted into lipid bilayer. Such assumption seems to be inconsistent with the data of Fig. 2 which shows that the HA_1 unit of FPV_S has a smaller electrophoretic mobility than that of FPV_R. Such a difference might be due to different degrees of glycosylation. This suggestion is sup-

ported by the fact that the hemagglutination titer of the FPV_R strain is 2–3 times less than that of the FPV_S virus (data not shown).

Other causes of the higher hydrophobicity of the HA_1 subunit of the FPV_R strain cannot be excluded at the moment. One possibility is that the HA_1 subunit of the FPV_R strain carries one or several fatty acid residues. However, for the FPV_S strain this was shown not to be the case [36]. Still another possibility is that the HA_1 unit of the FPV_R strain contains more nonpolar amino-acid residues than that of the FPV_S virions. To select between these possibilities, further studies involving experiments with individual viral proteins inserted in the lipid bilayer are in progress.

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