On the Structure of the Acyl Linkage and the Function of Fatty Acyl Chains in the Influenza Virus Haemagglutinin and the Glycoproteins of Semliki Forest Virus

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SUMMARY

The acylation of the haemagglutinin (HA) of different influenza viruses and of the envelope glycoproteins of Semliki Forest virus (SFV) were analysed. The fatty acid linkage in these acylproteins was found to be resistant to a variety of organic solvents and combinations of these, even after pretreatment with various detergents. Fatty acids are released from influenza virus HA at a pH value between 11.8 and 12.1 at room temperature. Although this mild alkaline cleavage occurs rapidly, the release of fatty acids by treatment with hydroxylamine is time-, temperature- and concentration-dependent. By comparison with model esters the linkage in HA is suggested to be of the oxygenester type rather than a thioester linkage. To assay for possible functions of protein-bound fatty acids the biological activities of influenza virus (A/FPV/Rostock/34) and its solubilized spike glycoproteins were measured after deacylation. While viral haemagglutination activity was not hampered at all, its ability to haemolyse erythrocytes and infectivity were drastically reduced. Likewise, viral spike glycoproteins solubilized with detergents failed to induce haemolysis at low pH when fatty acids had been cleaved off. These results indicate the possible involvement of protein-bound fatty acids in fusion induction through the acylated fusogenic spike glycoproteins.

INTRODUCTION

The post-translational modification by fatty acid acylation originally described for Sindbis virus (Schmidt et al., 1979) has been reported for certain viral glycoproteins of many enveloped RNA and DNA viruses (for a review, see Schmidt, 1983), a number of transforming proteins (e.g. Seflon et al., 1982; Gallick & Arlinghaus, 1984; Schultz & Oroszlan, 1984) and many cellular membrane-associated polypeptides including the transferrin receptor, histocompatibility antigens, rhodopsin and recently also actin (Schlesinger et al., 1980; Omary & Trowbridge, 1981a; Carr et al., 1982; Aitken et al., 1982; Keenan et al., 1982; Slomiany et al., 1983; Stoffel et al., 1983; O'Brien & Zatz, 1984; Kaufman et al., 1984; Bolanowski et al., 1984; Fergusson & Cross, 1984; Koch & Hämmerling, 1985; Olson et al., 1984; Stadler et al., 1985). Except for a few acylproteins with blocked amino termini (Schultz & Oroszlan, 1984) and lipophillin (Stoffel et al., 1983), no acyl linkage sites could be identified with acylproteins derived from viral envelopes. Likewise, hardly anything is known about the biological functions of protein-bound fatty acids although various hypotheses are being discussed (Schmidt et al., 1979; Schultz et al., 1985; Slomiany et al., 1983; Klockmann & Deppert, 1983; Henning & Lange-Mutschler, 1983; for review, see Schmidt, 1983).

In this communication we compare the stability of fatty acid linkages in the influenza virus haemagglutinin (HA) and Semliki Forest virus (SFV) glycoproteins with that of model esters. The results demonstrate a resemblance between the acyl chains in HA and in oxygenesters, indicating that protein-bound fatty acids are linked to serine or threonine rather than to cysteine residues. In search of functions for acyl chains in viral envelope glycoproteins, we compared the biological activities of acylated and fatty acid-free influenza virus HA and SFV glycoproteins.
Our finding that deacylation of these viral acylproteins leads to a marked reduction of their haemolytic activity indicates a possible involvement of protein-bound fatty acids in the induction of membrane fusion.

METHODS

Cells and viruses. The following viruses were used: influenza viruses A/fowl plague virus/Rostock 34 (H7N1) (FPV) and A/chick/N/Germany/49 (H10N7) (N) and the alphavirus SFV. Myxoviruses were grown in embryonated eggs and SFV in baby hamster kidney (BHK) cells. Virus was purified by differential centrifugation using standard techniques. For labelling experiments virus was usually grown overnight in monolayer chicken embryo cells in Dulbecco’s MEM with 2% foetal calf serum and with 10 to 15 μCi/ml [3H]palmitic acid or with 2 μCi/ml 14C-protein hydrolysate. After removal of cell debris from the culture fluid by low-speed centrifugation the virus was sedimented in a Beckman SW41Ti rotor for 2 h at 36000 r.p.m. and suspended in phosphate-buffered saline (PBS) or PIPES buffer (see below).

Isolation of subviral components

Spike rosettes. The glycoprotein spikes were prepared according to Sato et al. (1983). Usually, 1 to 2 ml of FPV with an HA titre of 216 in 135 mM-NaCl, 10 mM-PIPES pH 7.4 (PIPES buffer) were mixed with 14C-amino acid-labelled FPV (about 50000 c.p.m.) and treated for 1 h at room temperature with 1% octyl-β-D-glucoside. The nucleocapsid particles were sedimented at 35000 r.p.m. for 90 min in a Beckman SW55Ti rotor with microadapters and the supernatant detergent extracts adjusted to 10% sucrose. The sample was then run on a continuous gradient of 20 to 50% sucrose in PIPES buffer (see below). After monitoring aliquots for HA activity, protein content and radioactivity, peak fractions were pooled and dialysed and pooled for 2 days against 140 mM-NaCl (pH adjusted to 7.5 with dilute NH4OH).

Viral envelope lipids. [3H]Palmitic acid-labelled virus particles were subjected to duplicate extractions with chloroform–methanol (2:1, v/v) according to Folch et al. (1957). Lipid extracts were analysed by thin-layer chromatography (TLC) and scanning as reported previously (Schmidt, 1982b) using Berthold equipment (Wildbad, F.R.G.).

Fatty acid cleavage experiments. Deacylation of viral spike glycoproteins in intact particles and in detergent extracts from viral particles was done by treatment with various concentrations of hydroxylamine, pH 6.6, for the durations and at the temperatures indicated with the experimental data below. To test for biological activities after this deacylation procedure aliquots of the samples were either analysed immediately after extensive dilution or after dialysis overnight against PBS. To cleave acyl chains from the model esters, O-acetylserine, O-palmitoylserine, glycerophospholipids and [14C]palmitoyl-coenzyme A were either treated alone or combined in either aqueous or ethanolic solution and incubated with hydroxylamine or sodium hydroxide as indicated in the legends. Aliquots of the incubation mixtures were spotted on either silica- or cellulose-coated thin layer plates from Merck. The solvent mixtures for TLC are given in the figure legends. Detection of the substances was by iodine or ninhydrin staining or by radiochromatogram scanning with a Linear Analyzer LB 2842 from Berthold.

Haemolysis test. Haemolysis of chicken erythrocytes was determined spectrophotometrically according to Huang et al. (1981). Routinely, samples of 200 μl were adsorbed for 10 min at 4 °C to erythrocytes in 0.9% NaCl in a total volume of 1:2 ml before the pH was adjusted to a value of 5-5 by adding 200 μl of a 1 M-sodium acetate buffer pH 5-5 which results in a sevenfold dilution of the original sample. After incubation at 37 °C for 30 min the samples were centrifuged for 5 min at 30000 r.p.m. and the optical densities of the supernatants recorded at 540 nm.

Haemagglutination inhibition (HAI) test and plaque assay. Standard procedures were used throughout using microtitre plates for the HA and HAI1 tests with a 50 μl sample volume. Monoclonal antibodies directed against different epitopes of HA were prepared by Dr J. J. Skehel, Mill Hill, London, U.K. and provided by Dr C. Scholtissek, Giessen, F.R.G. Plaque assays were performed in chicken embryo fibroblasts for myxoviruses and BHK cells for SFV.

Polyacrylamide gel electrophoresis. Proteins were dissolved in sample buffer to give final concentrations of 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 62.5 mM-Tris–HCl pH 6.8 and 0.002% bromophenol blue tracking dye. Samples were boiled for 5 min and run on 12% polyacrylamide gels containing 6 M-urea according to Laemmli (1970) as described before (Schmidt, 1982c). For the separation of SFV proteins, 2-mercaptoethanol was replaced by water in the sample buffer. Gels were stained with Coomassie Brilliant Blue and/or fluorographed according to Bonner & Laskey (1974) with exposure times of less than 3 weeks.

Materials. Reagents for PAGE analysis were from Serva, Heidelberg, F.R.G. U-14C-Protein hydrolysate (28 mCi/mmol) and [1-14C]palmitoyl-coenzyme A (55 mCi/mmol) were from Amersham, and [9,10-3H]palmitic acid (23.5 Ci/mmol) was obtained from New England Nuclear. O-Acetylserine and reference substances for TLC were from Sigma, thin layer plates and hydroxylamine were from Merck, Darmstadt, F.R.G. O-Palmitoyl-L-serine was prepared essentially as described by Marinetti & Siani (1984) and octyl-β-D-glucoside was prepared according to Baron & Thompson (1975).
Fatty acyl linkages in viral glycoproteins

Fig. 1. Properties of fatty acids linked to intracellular and viral HA. Cells were infected with influenza virus N and labelled 2 h later with 50 μCi/ml [3H]palmitic acid. Cell lysates were collected at 5 h or labelled virus was harvested at 10 h post-infection. Aliquots were subjected to various treatments as below and the polypeptides subsequently analysed by PAGE and fluorography. Lanes 1, 4 and 11, untreated controls. Lanes 2 and 5, aliquots extracted twice with chloroform–methanol (2:1) and acetone. Lanes 3 and 6, aliquots made 0.2 M in KOH in methanol and neutralized after 10 min at room temperature. Lanes 7 to 10, cell lysates incubated for 60 min at 37 °C after adding water (lanes 7 and 8) or octyl-β-D-glucoside to a 2% concentration (lanes 9 and 10). Aliquots were centrifuged in a Beckman SW55Ti rotor with microadapters for 30 min and supernatants (lanes 8 and 10) and sediments (lanes 7 and 9) were analysed. Lanes 12 to 15, aliquots of [3H]palmitic acid-labelled virus N incubated for 3 min at 37 °C with 0.03, 0.06, 0.5 and 2.0 mg/ml trypsin, respectively.

RESULTS

Characterization of the fatty acid linkage in the influenza virus HA

Two species of avian influenza virus with HA subtypes H7 and H10 were grown in chicken embryo fibroblasts in the presence of [3H]palmitic acid and the infected cells and the purified virus particles treated with organic solvents and/or detergents prior to PAGE analysis and fluorography. As shown for virus N in Fig. 1 (lanes 1 and 4) radioactivity was associated with the viral HA and the envelope lipids. Extractions with organic solvents such as hexane, chloroform, methanol, chloroform–methanol–1 M-HCl (65:25:0.025, by vol.), acetone or mixtures thereof failed to release fatty acids from the protein although the envelope lipids were dissolved completely [shown for chloroform–methanol (2:1, v/v) in lanes 2 and 5]. However, as seen in lanes 3 and 6, treatment with mild alkali (0.2 M-KOH) in methanol cleaved the fatty acids from the HA (lanes 3 and 6). Incubation of [3H]-fatty acid-labelled and virus N-infected cells with detergents did not displace the acyl chains from the intracellular HA polypeptide and accordingly [3H]-labelled HA could be extracted with various detergents. As an example, solubilization with octyl-β-D-glucoside is shown in Fig. 1. Incubation of the cells with water (lanes 7 and 8) or octyl-β-D-glucoside (lanes 9 and 10) and subsequent centrifugation led to a partitioning of fatty acid-labelled HA into the sediment (lane 7) and into the supernatant fraction (lane 10). Very similar results were obtained with FPV-infected cells which contain fatty acids linked to the small fragment (HA₂) of its cleaved HA (see Fig. 3, data not shown).

When virus N which contains uncleaved HA (when grown in chicken embryo cells) was subjected to limited digestion with trypsin, PAGE analysis of the digests revealed that as with all other subtypes analysed previously (Schmidt, 1982b) the H10 subtype fatty acids were restricted to the small fragment of the HA (HA₂) while the large fragment (HA₁) and the second spike glycoprotein, the neuraminidase (NA), were fatty acid-free (Fig. 1, lanes 11 to 15).

Stability of the fatty acid linkage in the influenza virus HA

To gain more information on the chemical nature of the linkage between fatty acids and the HA polypeptide and at the same time to establish a mild procedure for deacylating this protein, the requirements for fatty acid cleavage were studied in more detail. Fig. 2 shows the results of an experiment in which octyl-β-D-glucoside-solubilized [3H]-fatty acid-labelled FPV and
Fig. 2. Fatty acid release from influenza virus HA and glycerophospholipids. [3H]Palmitic acid-labelled FPV was extracted with 2% octyl-β-D-glucoside for 10 min at room temperature and subsequently centrifuged at 16000 r.p.m. for 1 h in a Sorvall SS34 rotor. Aliquots of the supernatant extract (containing the 3H-fatty acid-labelled lipids and glycoproteins as shown in Fig. 1 and 6) were kept at different pH values for 10 min at room temperature and neutralized with HCl before the protein was precipitated with chloroform-methanol (1:1). Proteins were separated by PAGE and radioactivity in the HA2 bands (△) was measured by liquid scintillation counting. Radioactivity in glycerophospholipids (●) was determined after TLC in chloroform/methanol/water (65:35:8) and radiochromatogram scanning. Values for cleaved fatty acids are given as percent of total fatty acid bound in untreated controls, 100% being equivalent to 2500 c.p.m. for HA2 and 15800 c.p.m. for glycerophospholipids.

Reference substances were incubated for 10 min at room temperature at different pH values. The release of 3H-fatty acid from solubilized HA2 was compared to the cleavage of acyl chains from endogenous glycerophospholipids which contained [3H]palmitic acid in oxygenester linkage. As is apparent from the plot in Fig. 2, both substrates were cleaved at similar pH values and at pH 12.5 fatty acid release was almost complete after 2 min (not shown). Incubations at a pH value just below the cleavage point (tested for pH 11.5) did not liberate any fatty acid from the HA2 polypeptide even during prolonged incubation (data not shown). To prove its ester character the protein-bound fatty acids in the viral polypeptides of intact virus particles, in octyl-β-D-glucoside extracts and present in denatured form in SDS-polyacrylamide gels were cleaved by treatment with hydroxylamine (Omary & Trowbridge, 1981b; Magee et al., 1984). It was revealed that in all cases this treatment led to the liberation of fatty acids in a concentration, time- and temperature-dependent manner. As shown in Fig. 3 the fatty acid cleavage began at a 0.32 M concentration if octyl-β-D-glucoside-solubilized HA and SFV E proteins were treated with the reagent (Fig. 3a). When the polypeptide was present in fully denatured form, cleavage with 1 M-NH2OH was complete after 1 h at room temperature (c), but total release of fatty acids took somewhat longer if the HA was still a structural part of intact virus particles (b).

While the above results were sufficient proof for the ester character of the fatty acid linkage in the SFV and influenza virus acylproteins they did not allow discrimination between oxygen- and
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Fig. 3. Cleavage of fatty acids from viral acylproteins with hydroxylamine. (a) [3H]Palmitic acid-labelled SFV (left panel) and FPV (right panel) reacted with 2% octyl-β-D-glucoside and aliquots of the samples subjected to a 1 h treatment with various concentrations of hydroxylamine at 37 °C (molar concentrations indicated above the lanes). The proteins were precipitated with methanol and analysed on 12% SDS-polyacrylamide gels. A fluorogram of a 3 week exposure is shown, with the positions of viral proteins indicated according to a Coomassie Brilliant Blue stain of the same gel. (b) Labelled intact FPV particles treated with 1 M-NH₂OH (pH 6-6) at room temperature for the time periods (min) indicated above the lanes. (c) Identical samples of [3H]palmitic acid-labelled FPV first separated on a polyacrylamide gel and then excised gel strips stained and fluorographed either directly or after a 60 min incubation in 1 M-NH₂OH (pH 6-6). Fluorograms (F) and Coomassie Brilliant Blue staining patterns (C) of the same gels are depicted.

One way to approach this problem is to compare the stability of the fatty acid linkages in viral acylproteins with those in O-palmitoylserine, O-acetylserine (both oxygenesters) and fatty acyl-coenzyme A (a thioester). As demonstrated in Fig. 4 the conditions for cleavage of the fatty acid linkages in these model esters differed with the oxygenester being less
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Fig. 4. Comparative cleavage of oxygenester and thioester linkages in model substances. A mixture of 50 μg O-palmitoylserine (PalSer) or O-acetylserine and [1-14C]palmitoyl-coenzyme A (PalCoA) (total of 10000 c.p.m.) in aqueous solution was subjected to the various molar concentrations of hydroxylamine as indicated. After a 60 min incubation at 37 °C, aliquots were spotted on cellulose-coated TLC plates and run for 4 h in butanol/acetic acid/water (50:20:30) from S to F. Oleic acid, serine, PalSer, O-acetylserine and [1-14C]palmitoyl-coenzyme A were run as authentic references. Staining was with iodine for the fatty acid and with ninhydrin for all others. All radiolabelled substances were detected by radiochromatogram scanning. A stained TLC plate is depicted on the right; the scans of the respective lanes and the positions of PalSer, PalCoA, palmitic acid (Pal) and its hydroxamate ester (PalHM) are shown in the left panel.

Table 1. Hydroxylamine inhibition of haemolysis by FPV*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control</th>
<th>NH$_2$OH</th>
<th>pH</th>
<th>Control</th>
<th>NH$_2$OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV</td>
<td>0.413</td>
<td>0.060</td>
<td>5.5</td>
<td>2$^a$</td>
<td>2$^a$</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>0.006</td>
<td>6.8</td>
<td>2$^a$</td>
<td>2$^a$</td>
</tr>
</tbody>
</table>

* NH$_2$OH treatment was at 1 M concentration for 30 min at 37 °C. Samples were diluted 1:10 before aliquots were measured.

Influence of fatty acid binding on biological activities on influenza virus and SFV glycoproteins

While it is clear that not all species of viral envelope glycoproteins contain fatty acid, it has been shown that acylated glycoproteins are generally fusogenic (see Schmidt, 1983). One approach to test for a possible involvement of fatty acids in fusion induction is to study the influence of deacylation on the haemolytic activity of virus particles or their envelope glycoproteins. As seen in Table 1, after a 30 min treatment with hydroxylamine at 37 °C pH-dependent haemolysis of FPV was drastically inhibited, whereas HA activity was not affected at all. The incubation of influenza virus as well as SFV with hydroxylamine also led to a drastic reduction of their infectivity (not shown), a property of this drug reported before for various enveloped viruses in different contexts (Schäfer & Rott, 1962; Scholtissek & Rott, 1963; Newlin & Bussell, 1975). With both viruses this inhibition of biological activity correlated with a release
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Fig. 5. Correlation of inhibition of haemolysis with fatty acid release from viral acylated spike glycoproteins. [3H]Palmitic acid-labelled preparations of SFV or FPV were incubated for 1 h at 37 °C with 1 M-hydroxylamine (+) or 1 M-Tris–HCl buffer (−), both pH 6.6. The samples were diluted 1:10 with PBS and aliquots were taken for PAGE and haemolysis. The values given above the lanes represent the OD₅₄₆ in haemolysis tests. After PAGE the gel was first stained with Coomassie Brilliant Blue and then fluorographed. The fluorogram is shown overlapping the staining pattern to facilitate identification of the fatty acid-labelled protein species.

Likewise, no alterations of viral morphology could be detected by electron microscopy of negatively stained hydroxylamine-treated virus samples, nor during centrifugations on sucrose density gradients (results not shown). Thus it was concluded that disintegration of the viral lipid envelope during treatment with NH₂OH was most unlikely.

Deacylation of isolated glycoproteins

To prove that NH₂OH exerts one of its inhibitory effects on the virus by acting on the acylated viral spike glycoproteins, the envelope proteins of FPV were isolated according to Sato et al. (1983) and their biological activities determined after deacylation with hydroxylamine. Fig. 6(a) depicts the distribution of ¹⁴C-amino acid-labelled proteins in a sucrose gradient after centrifugation of an octyl-β-D-glucoside extract from ¹⁴C-labelled FPV. Fig. 6(b) gives a comparison of FPV proteins in the extract and the gradient fraction containing the spike of ³H-fatty acids from the acylated viral spike glycoproteins. Analysis of [³H]palmitic acid-labelled SFV and FPV before and after hydroxylamine treatment for 60 min at 37 °C on SDS–polyacrylamide showed that most ³H-fatty acid had been lost from the respective viral glycoproteins (E and HA₂) and that accordingly haemolysis titres of the same samples were decreased (Fig. 5). However, as judged from the Coomassie Brilliant Blue staining pattern of the same gels, migration of NH₂OH-treated proteins was not different from that of their untreated controls (Fig. 5) and no changes in the lipid composition of the NH₂OH-treated viruses could be detected during the analysis of [³H]palmitic acid-labelled lipids extracted from the viral envelopes (not shown).

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Fig. 6. Preparation of biologically active influenza glycoprotein spikes. FPV labelled with ¹⁴C-amino acids was mixed with egg-grown virus and extracted with octyl-β-D-glucoside (OG). The OG extracts were run on gradients of 20 to 50% sucrose in PIPES buffer pH 7.4, in a Beckman SW41 Ti rotor at 30000 r.p.m. for 18 h. (a) Distribution of radioactivity in the gradient fractions. Fractions 8 and 9 containing spike rosettes were combined, dialysed extensively and aliquots analysed by PAGE (b) against samples from the original OG extract and reference virus. A Coomassie Brilliant Blue-stained pattern of this analysis is shown on the right. OGP, Pellet after extraction with OG; OGS, supernatant after extraction with OG; ROS, spike rosettes.

Table 2. Dose-dependent inhibition of FPV and influenza virus spike rosettes-induced haemolysis by hydroxylamine*

<table>
<thead>
<tr>
<th>Sample</th>
<th>NH₂OH (M)</th>
<th>Haemolysis (OD₅₄₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV (2¹⁲ HAU)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0</td>
<td>0.598</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.268</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.090</td>
</tr>
<tr>
<td>Spike rosettes (2¹¹ HAU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.413</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.240</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.004</td>
</tr>
<tr>
<td>Spike rosettes trypsin-treated†</td>
<td>0</td>
<td>0.061</td>
</tr>
</tbody>
</table>

* FPV and the solubilized glycoproteins (spike rosettes) were subjected to a 30 min treatment at 37 °C with various concentrations of NH₂OH, pH 6.5.
† Sixty min at 37 °C.

Table 3. Time dependence of inhibition by hydroxylamine of haemolysis by influenza spikes

<table>
<thead>
<tr>
<th>Duration of treatment with NH₂OH (min)*</th>
<th>Haemolysis (OD₅₄₀)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.729</td>
</tr>
<tr>
<td>10</td>
<td>0.434</td>
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<tr>
<td>30</td>
<td>0.343</td>
</tr>
<tr>
<td>150</td>
<td>0.124</td>
</tr>
<tr>
<td>360</td>
<td>0.130</td>
</tr>
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</table>

* Treatment of 2¹⁵ HAU of spike rosettes was at 37 °C with 1 M-NH₂OH, pH 6.5. The control (0 min) was diluted immediately after addition of hydroxylamine.
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Table 4. Reactivity of antibodies with hydroxylamine-treated FPV as tested in the HA inhibition assay*

<table>
<thead>
<tr>
<th>Type of antibody</th>
<th>Control (PBS)</th>
<th>NH₂OH (1 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-FPV</td>
<td>4096</td>
<td>8192</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>1024</td>
<td>2048</td>
</tr>
<tr>
<td>HC 1</td>
<td>3200/3200</td>
<td>3200/1600</td>
</tr>
<tr>
<td>HC 2</td>
<td>3200/3200</td>
<td>3200/3200</td>
</tr>
<tr>
<td>HC 10</td>
<td>100/100</td>
<td>50/100</td>
</tr>
<tr>
<td>HC 61</td>
<td>3200/1600</td>
<td>800/800</td>
</tr>
</tbody>
</table>

* FPV with an HA titre of 2¹⁶ was incubated for 16 h with 1 M-NH₂OH pH 6·5 or PBS at room temperature. After dilution 50 µl of the virus (8 HAU) were reacted with the different antibody preparations and the inhibition titres scored in the standard HA test. Polyclonal antisera directed against FPV and purified HA were kind gifts from M. Orlich. Monoclonal antibodies (HC series) directed against different epitopes of HA were prepared by Dr J. J. Skehel.

† Duplicate titration results are shown for monoclonal antibodies.

Table 5. Effect of hydroxylamine on neuraminidase activity of FPV*

<table>
<thead>
<tr>
<th>FPV (HAU)</th>
<th>Control (PBS)</th>
<th>NH₂OH-treated (1 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>128</td>
<td>0·407/0·317</td>
<td>0·465/0·334</td>
</tr>
<tr>
<td>256</td>
<td>0·666/0·537</td>
<td>0·780/0·518</td>
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</tbody>
</table>

* FPV prepared as for the experiment shown in Table 4 was utilized for the neuraminidase test according to the standard procedure described by Seto & Rott (1966). The test was run in duplicate.

trypsin prior to the haemolysis test. As can be seen in the table, this treatment abolished haemolytic activity, suggesting that intact proteins are required. Also, the glycoprotein preparation failed to express this fusion activity when incubated with chicken erythrocytes at neutral pH (see Table 1). In addition, Table 3 shows that as with the time dependence of fatty acid release from the HA (see Fig. 3), inhibition also occurred as a function of incubation time.

Although the lack of any effect of hydroxylamine on the HA activity of virus or preparations of spike glycoproteins argued against drastic conformational changes, more subtle tests were applied to exclude this possibility. As demonstrated in Table 4, the potential of a series of HA-directed monoclonal antibodies to inhibit HA activity of FPV was not greatly changed after cleavage of HA-bound fatty acids with NH₂OH.

It was also of interest to assay for neuraminidase activity of hydroxylamine-treated influenza virus. In addition to serving the above purpose this test helps to investigate the possibility that the reduction in haemolytic activity is caused by an impairment of neuraminidase which may be required for membrane fusion (Huang et al., 1980). The results shown in Table 5 demonstrate that NH₂OH had no influence on the neuraminidase activity of FPV, which emphasized that both a general effect of our deacylation procedure on the externally exposed glycoprotein structure and an inhibition of haemolysis via neuraminidase are most unlikely.

DISCUSSION

Although acylation of viral proteins with long chain fatty acids has been known for some years, we are still in search of both the precise linkage site of the acyl chains and the functional relevance of protein-bound fatty acids. This communication presents results which contribute to both aspects of this phenomenon, mainly for the acyl proteins of an influenza virus but also of SFV.
Fig. 7. Potential acylation sites in the membrane-spanning regions of viral membrane glycoproteins. The amino acid sequences of the HA, of influenza virus subtypes H1, H2 and H3 were deduced from nucleotide sequences published by Hitte et al. (1981), Gething et al. (1980), and Sleigh et al. (1980), respectively, and those of subtypes H5 and H7 published by Porter et al. (1979). The sequences shown for SFV E1 and E2 are from Garoff et al. (1980), and for the fusion protein of simian virus 5 (SV5) from Paterson et al. (1984). Extending from the membrane (indicated by vertical lines), 10 amino acids are shown on the amino terminal side of the polypeptides and the full sequence of the internal amino acids towards the carboxy terminus are given. Serine and threonine residues are marked by high and low marks respectively and cysteine residues are indicated by hatch marks (II).

Our comparison of the properties of fatty acid linkages in viral acylproteins and model esters proves the ester character of the acyl linkage in these viral proteins. Furthermore, they reveal a clear distinction between the two possible binding types, oxygenester and thioester linkage. While thioesters, as exemplified by palmitoyl-coenzyme A, are cleaved after a 1 h treatment at 37 °C with 0.075 M-hydroxylamine, the oxygenesters O-acetylserine and O-palmitoylserine are not degraded under the same conditions (Fig. 4). Like these oxygenesters the viral glycoproteins also resist a comparable treatment and begin to release their covalently bound fatty acids only after much longer incubation times or at higher concentrations of hydroxylamine (Fig. 3). Thus, although some evidence with acylproteins of Sindbis and vesicular stomatitis viruses may point to cysteine residues as acylation sites (Magee et al., 1984; Rose et al., 1984), for the acylproteins studied here we consider serine or threonine residues in the polypeptide chain as the most likely binding partners for fatty acid. It has been determined for a number of different viral acylproteins that fatty acids are topologically located inside or close to the membrane-spanning region of the respective spike glycoprotein (Schlesinger et al., 1981; Petri & Wagner, 1980; Rice et al., 1981; Schmidt, 1982a, b). Inspection of the published sequences of these polypeptide regions indicates that the proportion of hydroxyamino acids in the membrane-spanning polypeptide segment is quite high towards the outward pointing leaflet of the viral lipid layer or just outside the membrane. In contrast, cysteine residues show the opposite distribution (Fig. 7). Thus, long chain fatty acids could potentially reach out of the viral lipid bilayer and interact with heterologous membranes as for instance during infection of a host cell, a process which requires membrane fusion to occur at some stage, only if bound to serine or threonine.

To investigate a possible involvement of protein-bound acyl chains in membrane fusion, the haemolytic activity of acylated and fatty acid-free spike glycoproteins were compared. The results given in Tables 1 to 3 and in Fig. 5 clearly indicate that cleavage of fatty acids from the spike glycoprotein with hydroxylamine leads to a severe reduction of haemolytic activity. Like the fatty acid cleavage itself, the inhibition of this activity by NH$_2$OH is time-, concentration- and temperature-dependent and works with both intact virus particles and the isolated viral acylproteins. Taken together, these latter results, the above considerations on the fatty acid
linkage site and the fact that nearly all acylproteins of enveloped viruses known so far are fusogenic (Schmidt, 1982a, 1983) indeed point to a critical participation of protein-bound acyl chains in fusion induction, although other mechanisms are also possible. It is known that hydroxylamine has other effects on the virus, involving its nucleic acid (Franklin & Wecker, 1959; Freese et al., 1961; Schäfer & Rott, 1962; Scholtissek & Rott, 1963). It may also be possible that this reagent affects the solubilized glycoproteins (Deselnicu et al., 1973) in an as yet unidentified manner not detectable by our control experiments. However, it is important to know that neither the primary structure nor the general conformation at least of the exposed portions of the spike glycoproteins are drastically altered (see Tables 4 and 5) by the NH₂OH treatment applied in this study. In this respect it is also noteworthy that SFV E1 after cleavage of unidentified manner not detectable by our control experiments. However, it is important to

Nevertheless, more data will be necessary to prove a direct or indirect participation of polypeptide-bound fatty acids in the triggering of fusion. Although haemolysis is frequently used as a parameter for membrane fusion (Huang et al., 1981; Sato et al., 1983) one has to bear in mind that the mechanism of haemolysis requires more than just the lipid bilayers to merge. To account for these possibilities we are presently testing alternative deacylation procedures and are establishing an experimental system in which the acylprotein-mediated fusion process itself can be quantified under various closely controlled conditions.

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REFERENCES


Fatty acyl linkages in viral glycoproteins


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