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Acylation of Viral Spike Glycoproteins: A Feature of Enveloped RNA Viruses

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The covalent attachment of fatty acids to the glycoproteins of orthomyxo-, paramyxovirus, and coronavirus was studied. All enveloped viruses analyzed afford covalently bound fatty acid in at least one species of their spike glycoproteins. No internal components of the viruses studied including the hydrophobic M proteins of myxoviruses and rhabdoviruses contained fatty acid. Analysis of myxovirus particles devoid of the exposed portions of their spikes revealed that fatty acids are linked to the hydrophobic tail fragment of the glycoprotein which is associated with the viral lipid bilayer. With influenza virus hemagglutinin the fatty acid attachment site could be located at the cyanogen bromide peptide of the small subunit (HAe) which contains the membrane-embedded region of the polypeptide. The binding of fatty acids to viral glycoproteins occurs in a wide range of host cells including mammalian, avian, and insect cells.

INTRODUCTION

Enveloped viruses contain glycoproteins which are associated with the viral lipid bilayer (for references see Rott and Klenk, 1977). These glycoproteins form protrusions called spikes which initiate the interactions between the virion and the host cell membrane leading to infection (Homma and Ohuchi, 1973; Klenk et al., 1975; Huang et al., 1980; Utermann and Simons, 1974). Major progress was made recently in studying the structure and biosynthesis of viral spike glycoproteins, especially of influenza virus hemagglutinin (Wilson et al., 1981; Ward, 1981; Klenk and Rott, 1980). While the amino acid sequence and the composition and attachment sites for oligosaccharides of some envelope glycoproteins of influenza-, alpha-, and a rhabdovirus have been well established (Porter et al., 1979; Verhoeven et al., 1980; Ward, 1981; Rice and Strauss, 1981; Rose et al., 1980; Etchison and Holland, 1974; Garoff et al., 1980; Schwarz et al., 1977; Burge and Strauss, 1970; Pesonen and Renkonen, 1976), a new structural feature of the spikes of vesicular stomatitis and Sindbis virus was only recently detected. With these viruses covalent binding of fatty acids to their envelope glycoproteins G, or E1 and E2 was discovered (Schmidt et al., 1979; Schmidt and Schlesinger, 1980a). Studies on the biosynthesis of fatty acid carrying glycoproteins shed some light on the acylation event itself. It could be shown that fatty acid binding is a late event during the biosynthesis of these glycoproteins (Schmidt and Schlesinger, 1980b).

Little is known, however, about the location of the attachment site for fatty acids in the respective molecules. Furthermore, it was of interest to extend the study on fatty acid binding to glycoproteins of other enveloped viruses. It was thereby expected to gain information on the specificity of the phenomenon with respect to the virus as well as the host cell species utilized to grow the virus. The data presented in this communication reveal that fatty acids are bound to at least one of the spike glycoproteins of a large variety of enveloped viruses grown in different host cells indicating that acylation may be a general feature of enveloped viruses.

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MATERIALS AND METHODS

Viruses and Cells

Stocks of myxoviruses were grown in the allantoic cavity of 11-day-old chick embryos. For infection of chick embryo cells a multiplicity of infection of 50 PFU/cell was employed.

Avian influenza virus. A/FPV/Rostock (H7N1) (FPV), A/FPV/Dutch/27 (H7N7) (Dutch), A/chick/N/Germany/49 (H10N7), (virus N).

Human influenza virus. A/PR/8/34 (H1N1), A/FM/1/47 (H1N1), A/Singapore/11/57 (H2N2), A/Aichi/2/68 (X:31) (H3N2) (Verhoeyen et al., 1980), A/Victoria/3/75 (X:47) (H3N2) (Min-Jou et al., 1980). All human influenza strains were kindly provided by Michaela Orlich.

For labeling experiments human influenza viruses were propagated in chick embryo cells in the presence of trypsin (Klenk et al., 1975).

Paramyxoviruses. Newcastle disease virus (NDV) strains Italia, Ulster, and La Sota (Nagai et al., 1976).

For metabolic labeling NDV was grown in primary chick embryo or in cultured cells of the chorioallantoic membrane (CAM) (Cursiefen and Becht, 1975).

Alphavirus. Semliki Forest virus (SFV or SF in Fig. 9), strain Osterrieth, was grown in BHK, primary chick embryo, human lymphoma (U698 and Ramos), mouse L, and in insect cells derived from larvae of Aedes albopictus. Sindbis virus (HR strain), SFV, and insect cells were kindly provided by Gisela Wengler.

Coronavirus. Mouse hepatitis virus A 59 was grown in the 17 clone 1 line of spontaneously transformed Balb C 3T3 cells according to Sturman et al. (1980). Bovine enteropathogenic corona virus, L9, was grown in bovine fetal thyroid cells according to Storz et al. (1981a).

To prepare fatty acid-labeled virus particles the virus was grown in monolayers or suspensions (human lymphoma) of the above specified cell types usually using Dulbecco’s MEM with 2% fetal calf serum and with 10–50 μCi/ml of [3H]palmitic acid. For labeling with [3H]glucosamine (50 μCi/ml), 14C-protein hydrolysate (2 μCi/ml), or with [3H]proline (2 μCi/ml), Earle’s medium with 10 mM fructose or 10 mM glucose, respectively, was used. If not otherwise stated the virus was propagated overnight at 37°C except for coronaviruses and paramyxoviruses which were grown for 2–3 days. After clarifying the culture fluid from cell debris by low-speed centrifugation, the virus was pelleted by centrifugation usually in a Beckman SW 41 Ti rotor for 2 hr at 36,000 rpm. The pellets were suspended in PBS and prepared for SDS-polyacrylamide gel electrophoresis as described below. Where indicated an additional purification step by centrifugation on sucrose gradients was added.

Isolation of Spike-Free Particles

Purified preparations of FPV and virus N labeled with [3H]palmitic acid were incubated with 0.2 μg of protease in a total volume of 100 μl for 3 min at 37°C. Digestion with TPCK-trypsin (Serva, Heidelberg, West Germany) and other proteases was performed in PBS. Thermolysin (Boehringer, Mannheim, West Germany) was used in the presence of 5 mM Ca2+ and bromelain (Boehringer) was applied in the presence of 5 mM dithiothreitol. The digestion was terminated by chilling the samples, diluting them fivefold and spinning them immediately for 30 min at 45,000 rpm in the Beckman SW 50 rotor using microadapters. After centrifugation supernatants and pellets were prepared for analysis on 15% SDS-polyacrylamide gels. In contrast to the bromelain treatment of the Hong Kong strain of influenza virus which yields intact hemagglutinin (HA) lacking the membrane-bound fragment (Brand and Skehel, 1972; Skehel and Waterfield, 1975), the conditions applied here on the avian influenza viruses lead to the degradation of exposed portions of the hemagglutinin into peptides of heterogeneous size.

Fragmentation with Cyanogen Bromide

Cyanogen bromide (CNBr) cleavage was performed in 70% formic acid using 100
acylation of viral spike glycoproteins

Fig. 1. The polypeptides of fatty acid-labeled avian influenza viruses. Chick embryo cells were infected with FPV (FLU), the Dutch strain of fowl plague virus (D), and with virus N (N) and labeled with [3H]palmitic acid for 1 to 14 hr p.i. Released virus particles were prepared from the culture fluid and run on a 12% acrylamide gel in the presence of 6 M urea. The left panel shows the viral polypeptide pattern after Coomassie staining. The right panel depicts a fluorogram of the same gel which has a smaller size due to shrinkage during treatment for fluorography. Lineup of labeled and stained protein bands was done with the fluorogram and the fluorographed gel which still showed the Coomassie staining pattern. The position of neuraminidase is indicated by arrowheads (▼).

mg CNBr for 2–10 mg of protein. Treatment was for 20 hr at room temperature after which the reaction mixture was lyophilized three times before it was taken up in sample buffer for polyacrylamide gel analysis.

Polyacrylamide Gel Electrophoresis

Samples were dissolved in loading buffer to give final concentrations of 10% glycerol, 2% sodium dodecyl sulfate, 5% mercaptoethanol, 62.5 mM Tris–HCl (pH 6.8), and 0.002% bromophenol blue tracking dye. When nonreducing conditions were required, 2-mercaptoethanol was omitted in the loading buffer. Prior to loading onto gels the samples were usually boiled for 5 min. Electrophoresis was carried out according to Laemmli (1970). Usually 10, 12, and 15% polyacrylamide was used for the separation gels. When influenza polypeptides were separated 6 M urea was present in the separation gel. The gels were dried and autoradiographed with Kodak XR-5 film. Tritium-labeled samples were analyzed by fluorography according to the procedure described by Bonner and Laskey (1974). The protein content in the samples was measured according to Lowry et al. (1951).

Materials

Reagents for polyacrylamide gel electrophoresis were products of Serva, Heidelberg, West Germany. U-14C-labeled protein hydrolysate (285 mCi/mmol),
RESULTS

Fatty Acid Binding to Influenza Virus Hemagglutinin

Avian strains of influenza virus from chick embryo cells grown in the presence of [3H]palmitic acid were isolated from the growth medium and analyzed by SDS-gel analysis. The fluorograms of the labeled polypeptides revealed that only the small subunit of the hemagglutinin, designated HA2, contained labeled fatty acid (Fig. 1). The neuraminidase, the large subunit of the hemagglutinin, designated HA1, and the hydrophobic M protein did not contain labeled palmitate at the level of detection by fluorography. Since influenza particles contain roughly 5–10 times more hemagglutinin than neuraminidase (Schulze, 1972) fatty acid binding to the latter glycoprotein may have escaped detection during gel analysis. Therefore neuraminidase from [3H]palmitic acid labeled FPV was prepared by the method described by Huang et al. (1979). However, this preparation also failed to afford significant radioactivity (not shown). It was therefore concluded that neuraminidase of FPV is free of fatty acids. While analogous results were received with FPV strain Dutch, in the case of virus N and human influenza viruses grown in chick cells fatty acids were found to be attached to the non-cleaved hemagglutinin (HA) (Fig. 1, right panel, lane N). This indicates that cleavage of HA is not required for the biosynthetic acylation. In order to determine to which portions of HA fatty acids were attached, these viruses were labeled in the presence of trypsin in the medium which leads to the specific cleavage of the hemagglutinin to yield HA1 and HA2 (Klenk et al., 1975; Lazarowitz and Choppin, 1975). SDS-gel analysis of particles grown under such conditions revealed that, as with FPV...
and the Dutch strain of fowl plague virus, fatty acid label was bound exclusively to HA₂. This is shown in Fig. 2 for the hemagglutinin subtypes H0, H12, and H13. The same result was obtained also for the HA of H1 subtype in the human influenza strain FM1 (not shown).

**Release of Fatty Acids from the Glycoproteins**

Like all other viral glycoproteins analyzed so far (Schmidt et al., 1979; Schmidt and Schlesinger, 1980a) both the viral and the intracellular form of influenza hemagglutinin contain fatty acid in chloroform–methanol resistant form. In contrast all the lipids of the viral and the cellular membranes are readily extracted with this organic solvent (see lanes 2 and 5 in Fig. 3). Only treatment with 0.2 N potassium hydroxide in methanol at room temperature leads to a release of fatty acids from the hemagglutinin (lanes 3 and 6 in Fig. 3).

For comparison, fatty acid-labeled virus N was treated with aqueous or methanolic potassium hydroxide for various periods of time and subsequently analyzed on acrylamide gels. It was found that the methanolic environment facilitates the release of fatty acid from the hemagglutinin spike glycoprotein. As shown in Fig. 4 a 2-min treatment with methanolic KOH is sufficient to liberate all [³H]palmitic acid radioactivity from the hemagglutinin whereas in the aqueous medium 15–30 min are required to initiate the cleavage of fatty acids from the glycoprotein. This is taken as an indication that the fatty acid attachment site may be closely associated with the viral lipid bilayer. Methanol, in contrast to water, possibly perturbs the viral envelope and thereby renders fatty acid binding portions of the protein more easily accessible to potassium hydroxide.

![Image of gel showing release of fatty acids from glycoproteins](image-url)

**Fig. 3.** Properties of protein-bound fatty acid. Virus N or lysates of virus N-infected chick embryo cells (4 hr p.i.) labeled with [³H]palmitic acid were left untreated (1, 4), extracted with chloroform/methanol (2, 5), or treated with 0.2 N KOH in methanol for 15 min at room temperature (3, 6). After this treatment polypeptide analysis on an SDS-acrylamide gel was performed.
It should be noted that alkali treatment has no effect on the polypeptide backbone of the glycoprotein itself (see Fig. 4, right panel).

**Location of Fatty Acid Attachment Sites**

To test the hypothesis that fatty acids are linked to a region of the polypeptide which is associated with the viral membrane $[^{3}H]$palmitic acid-labeled particles of FPV and virus N were treated with protease to digest the spike glycoproteins. Since membrane-associated portions of the hemagglutinin are protected from proteolytic degradation (Brand and Skehel, 1972; Schulze, 1972; Klenk et al., 1972) fatty acid-containing peptides should be present in preparations of spikeless particles if the above hypothesis is correct. Figure 5 shows that this is indeed the case for FPV. After treatment with bromelain $[^{3}H]$palmitic acid-labeled peptides were only detected in “naked” particles and no $^{3}H$-labeled fragments of the hemagglutinin could be detected in the supernatant after centrifugation. Under the same conditions trypsin and thermolysin failed to lead to complete digestion of the exposed hemagglutinin regions thus yielding virus particles with shortened but still fatty acid-labeled HA spikes embedded in the viral lipid bilayer (Fig. 5, lanes “Try” and “Th”).

Further support for the location of fatty acids at the carboxyterminal region of viral glycoproteins comes from an analysis of cyanogen bromide fragments generated from $[^{3}H]$palmitic acid- or $[^{14}C]$proline-labeled HA$_{2}$ of FPV. The only proline residue in HA$_{2}$ is located at position 175, close to the membrane-embedded portion of the polypeptide suggested to extend from position 185 to 208 (Porter et al., 1979).
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a more direct approach to determine the fatty acid linkage region in the primary sequence, it was tested whether proline and palmitic acid are located on the same cyanogen bromide fragment of the total of six peptides to be expected. As shown in Fig. 6 this is indeed the case. Separation of the labeled fragments on polyacrylamide gradient gels revealed that [3H]-palmitic acid- and [14C]proline-labeled CNBr peptides of HA2 both migrated to a position corresponding to a molecular weight of about 6000 daltons. Although sequence data on this peptide are not yet available this result gives strong support to the hypothesis that the fatty acid attachment sites of the influenza hemagglutinin are located close or even inside the lipid bilayer.

Fatty Acid Binding to Spike Glycoproteins of Corona-, Alpha-, and Paramyxovirus

It was of interest to investigate the distribution of fatty acid binding with other enveloped viruses. Therefore in addition to orthomyxoviruses described above, also coronaviruses, an alphavirus, and paramyxoviruses were studied utilizing a variety of different host cells of mammalian, avian, or arthropode origin. Figure 7 shows the glycoprotein pattern in a 10% polyacrylamide gel of three different strains of Newcastle disease virus (NDV) which were grown in cultured cells of the chorioallantoic membrane (CAM) of chicken (Cursiefen and Becht, 1975; Nagai et al., 1976). As apparent from the right half of the figure, fatty acids are predominantly bound to the fusion protein (F) in all three strains analyzed. The nonpathogenic NDV-Ulster which contains both some cleaved (F₁) and mainly the noncleaved form of the F protein (F₀) shows fatty acid binding to both forms of this spike glycoprotein. Only faint labeling of the other protein component of the envelope of NDV, the HN glycoprotein, was detected which may be due to metabolic conversion of some palmitic acid to amino acids or sugars since these low levels of radioactivity in contrast to the bulk radioactivity in the F protein are resistant to treatment with mild alkali in methanol.

Semliki Forest virus grown in chick embryo cells in the presence of tritiated fatty acid had a polypeptide pattern very similar to that of Sindbis virus with which fatty acid binding to glycoproteins was discovered (Schmidt et al., 1979). Figure 8 shows that both E2 and E1 contain labeled fatty acid while the capsid protein
is free of palmitic acid. Utilizing nonreducing conditions for the separation of E1 and E2 (see Coomassie blue stained gel, left half of figure) (Kalkkinen et al., 1980) it was possible to determine the relative distribution of labeled fatty acid in the two SFV glycoproteins E1 and E2. The glycoproteins were eluted from the gel and the protein content and radioactivity measured in the eluates. Values of $1.9 \times 10^6$ cpm/mg E2 and $3.4 \times 10^4$ cpm/mg of E1 were obtained. The E2 glycoprotein thus contains five times more fatty acid than the E1 glycoprotein. The glycoproteins of Semliki Forest virus bind fatty acid not only in chick cells but also in hamster (BHK-21), human (lymphoma lines U698 and Ramos), and insect cells (Aedes albopictus) (Fig. 9). These results indicate a wide distribution of the enzymes responsible for the acylation of viral glycoprotein.

Coronaviruses also show fatty acid binding to envelope protein. As in the case of ortho- and parainfluenza viruses only one of the two glycoproteins of coronavirus is acylated, namely the E2 which is believed to be responsible for the fusion activity of the virus (Sturman et al., 1980; K. Holmes, personal communication). The results are illustrated by the fluorogram of bovine coronavirus polypeptides after labeling with $^3$H-palmitic acid and with $^{14}$C-labeled protein hydrolysate (Fig. 10). While the aggregates of E2 (E2 ag) are heavily labeled with fatty acid only trace amounts of radioactivity are detectable in the E1 glycoprotein. Since the nucleoprotein of coronavirus (designated N) also contains some radioactivity it is reasonable to assume that some of the $^3$H label may have been converted to metabolites other than fatty acids which are incorporated into N and E1. While bovine co-
ronavirus L9 was grown in bovine fetal thyroid cells (Storz et al., 1981a; Storz et al., 1981b), the A59 strain of murine coronavirus was grown in a line of spontaneously transformed Balb/c 3T3 cells. Nevertheless, the same results were obtained for both coronaviruses. Only E2 (and E3) were labeled with fatty acids but not the other envelope glycoprotein E1 and the nucleoprotein N (Niemann and Klenk, 1981).

**DISCUSSION**

The data presented in this communication show that fatty acid binding to viral glycoproteins is a widespread phenomenon which occurs in all major groups of enveloped RNA viruses including retroviruses (Schmidt, Schneider, and Hunsmann, preliminary results). Fatty acids become attached to the glycoproteins of a given enveloped virus in a variety of different host cells including mammalian, avian, and insect cells. The wide distribution of this modification of glycoproteins among envelope viruses and their host cells indicates that protein acylation must be of important biological significance.

What may be the functions of fatty acid chains linked to the polypeptide backbone of viral glycoproteins? The first clue comes from studies on the topography of the acylation sites in the influenza hemagglutinin, the VSV G protein (Schmidt and Schlesinger, 1980a; Petri and Wagner, 1981) and Sindbis virus E1 and E2 (Rice and Strauss, 1981, E. G. Strauss, personal communication; Schmidt and Schlesinger, unpublished). With these glycoproteins it could be shown by controlled proteolysis and in the case of FPV hemagglutinin by cyanogen bromide fragmentation that fatty acids are linked to regions of the polypeptide which are located in immediate vicinity or even inside the viral lipid

**FIG. 7.** Fatty acid binding to Newcastle disease virus polypeptides. The strains Italian, La Sota, and Ulster of NDV were labeled in cells of the chorioallantoic membrane (CAM) with [3H]palmitic acid (right panel) and [3H]glucosamine (left panel). The viral polypeptides were separated on a 10% SDS-acrylamide gel which was subsequently subjected to fluorography.

**FIG. 8.** Fatty acid binding to Semliki Forest virus polypeptides. The Osterrieth strain of SFV was grown in chick embryo cells in the presence of [3H]palmitic acid. The virus particles isolated from the culture fluid were treated for SDS-gel electrophoresis under reducing (R) or nonreducing (NR) conditions. The right panel depicts a fluorogram which has a smaller size due to shrinkage of the gel during treatment for fluorography. Lineup of labeled and stained protein bands was done with the fluorogram and the fluorographed gel which still showed the Coomassie staining pattern.
FIG. 9. Polypeptides of togaviruses grown in insect cells. Sindbis virus (SIN) or Semliki Forest virus (SF) was grown in cultured cells of *Aedes albopictus* labeled with protein hydrolysate ($^{14}$C-AA) or palmitic acid ($^{3}$H-PAL) in Dulbecco's medium containing 1% serum and 0.2 μg/ml actinomycin. At 24 hr p.i. virus was harvested from the culture medium by sedimentation at 40,000 rpm for 1 hr in a Beckman SW55 Ti rotor. The pellets were dissolved in loading buffer and run on a 10% SDS–acrylamide gel. The positions of viral capsid protein (C), envelope lipid (L), and the glycoproteins (E1 + E2) are indicated in the depicted fluorogram.

bilayer. This suggests a possible anchoring function for the protein-bound acyl chains. In addition, acylation could be used as a biochemical regulator to modulate membrane affinity of glycoproteins during their synthesis. Fatty acid attachment could supplement the hydrophobicity of membrane-bound protein segments. This would occur not only by the addition of hydrophobic acyl chains but also by “masking” hydrophilic hydroxyl groups of serine residues, shown to be the most probable binding sites for acyl chains (Schmidt and Schlesinger, 1980a; Schmidt, unpublished). This hypothesis is supported by experiments of Huang et al. (1979), who found that neuraminidase of influenza virus and neuraminidase-hemagglutinin (HN) of NDV, both free of fatty acid, incorporated very poorly into liposomes when compared with the acylated FPV hemagglutinin or with the F protein of NDV. More direct evidence that fatty acid linkage to proteins increases its membrane affinity stems from a report by Huang et al. (1981), who modified monoclonal antibodies by chemical acylation. While nontreated IgG could not be incorporated into liposomes, the acylated form was readily incorporated into artificial membranes. To test this hypothesis, experiments are in progress in which viral glycoproteins are deacylated enzymatically in order to com-

FIG. 10. Fatty acid binding to polypeptides of a bovine coronavirus. Coronavirus L9 was labeled with $^{3}$H-palmitic acid and $^{14}$C-protein hydrolysate in bovine fetal thyroid cells. Labeled particles were analyzed for radiolabeled polypeptides in a 10% SDS–acrylamide gel.
pare their functional properties with those of the acylated species.

Another possible function of acylation could be a biosynthetic one. Recently Zilberstein et al. (1980) described a VSV mutant which produced almost fully glycosylated G protein at nonpermissive temperature indicating that its transport from the rough endoplasmic reticulum to the Golgi apparatus functions normally. This G protein failed to reach the plasma membrane and was also not acylated. These results were interpreted to indicate that acylation may be essential for intracellular transport of certain glycoproteins from the Golgi apparatus to the cell surface. While similar results were also obtained with influenza ts mutants (Schmidt and Klenk, unpublished), it is still unclear whether in these mutant glycoproteins the lack of acylation alone or additional defects are the reason for the block of intracellular transport.

Recently acylation was also detected in membrane proteins of noninfected cells (Schlesinger et al., 1980). This finding led to the presumption that post-translational modification of polypeptides by covalent linkage of fatty acids may be a general feature of membrane proteins. Current research on the structure and function of acylated viral glycoproteins, which represent excellent model systems, will help to understand the properties of membrane proteins and their interactions with the lipid bilayer.

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