

Evidence for covalent attachment of fatty acids to Sindbis virus glycoproteins

(membranes/sodium dodecyl sulfate/polyacrylamide gel/palmitate)

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ABSTRACT Selective binding of lipid to glycoprotein was detected when [³H]palmitate-labeled Sindbis virus particles or viral-infected cells were disrupted by heating with sodium dodecyl sulfate, and glycoproteins were isolated by electrophoresis in sodium dodecyl sulfate/10% polyacrylamide gels. The smaller glycoprotein (E2) retained 2 to 3 times more labeled lipid than did the larger E1 glycoprotein, and the cell-associated glycoprotein precursor (PE2) bound even less lipid. No lipid was associated with the nonglycosylated glycoproteins that accumulated in infected cells treated with tunicamycin. The labeled lipid remained bound to the glycoproteins after exhaustive extraction with chloroform/methanol of virus particles, infected-cell extracts, or isolated glycoproteins, but it could be extracted by chloroform/methanol after treating glycoproteins with mild alkali. Analysis by gas/liquid chromatography showed that 60% of the label was in palmitate and the balance of label was distributed between oleate and stearate. There were approximately 2 mol of fatty acid bound per mol of E1 glycoprotein. Proteolysis of the fatty acid-labeled glycoprotein with pepsin, thermolysin, and Pronase degraded the polypeptide to fragments that retained the fatty acids in an alkali-labile state. These data suggest that a covalent attachment of fatty acid may occur during maturation of the viral glycoproteins.

We have been studying the formation and processing of viral-specific proteins in tissue culture cells infected with Sindbis virus, a small enveloped RNA virus of the family togaviridae. A critical stage in the late events of replication of this virus is a proteolytic cleavage that alters the structure of one of the two viral-specific glycoproteins (1), and this reaction is essential for release of virus particles from the cell (2-5). In the process of studying the glycoprotein cleavage reaction we made the serendipitous observation that glycoproteins isolated by sodium dodecyl sulfate (NaDodSO₄)/10% polyacrylamide gel electrophoresis after disruption of virus by NaDodSO₄ appeared to retain small amounts of lipid (6). We have investigated this glycoprotein-lipid complex in more detail and describe our initial results in this communication.

Two significant observations have emerged from these studies: (i) the lipid remained associated with glycoprotein despite strong denaturing conditions and proteolytic digestion but could be recovered as the methyl ester of fatty acid after mild alkali treatment in methanol, a procedure that also released fatty acid from proteolytic digestion products of the glycoproteins, and (ii) this binding of fatty acid was affected by changes in the glycoprotein structure. These data indicate that fatty acid may be covalently attached to viral glycoprotein and this modification may play an important role in the maturation of viral glycoprotein. Preliminary reports of this work have been presented (7, 8).

MATERIAL AND METHODS

Virus and Cells. The procedures for growing Sindbis virus in cultures of chicken embryo cells have been described (4). Virus was harvested from monolayers 9-16 hr after infection and was purified by isopycnic centrifugation in 30-50% sucrose gradients containing 0.05 M Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 M NaCl, and 0.1% bovine serum albumin. A 3-hr centrifugation in the Spinco SW27 rotor at 25,000 rpm was sufficient to band the virus. The samples from the gradient that contained virus were diluted 1:4 with buffer, and the virus was recovered as a pellet after centrifugation for 2 hr at 120,000 × g. Eagle's minimum essential medium supplemented with 3% fetal calf serum was used for growing the cells. Sindbis virus ts 23 was provided by D. Brown (University of Texas).

Chemicals and Isotopes. L-[³⁵S]Methionine (700-900 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was from Amersham and [9,10-³H (n)]palmitic acid (23.5 Ci/mmol) was from New England Nuclear. Solvents used for lipid analysis were from Mallinckrodt and reference lipids were from Sigma. Reagents for acrylamide gels were from Bio-Rad.

Labeling of Cells and Virus. Monolayers of cells in 100-mm dishes were labeled with 4 μCi of [³H]palmitate per ml for 16-24 hr prior to infection. The palmitate was in 80% (vol/vol) ethanol and was added to 5 ml of the Eagle medium so that the final ethanol concentration did not exceed 0.5%. After adsorption of virus at a multiplicity of infection of 100, fresh medium containing labeled palmitate was added.

For labeling of virus-specific proteins in infected cells with [³⁵S]methionine, Eagle's medium lacking methionine was added to cells 4 hr after infection and 25 μCi of [³⁵S]methionine was added 1 hr later for 60 min. For labeling of viral particles, the [³⁵S]methionine was present until the medium was collected 10-15 hr after infection.

Preparation of Viral Components and Cell Samples for Polyacrylamide Gel Electrophoresis. Samples of virus or of isolated glycoproteins were treated with an equal volume of a gel loading buffer consisting of 20% (vol/vol) glycerol, 4% (wt/vol) NaDodSO₄, 10% (vol/vol) mercaptoethanol, 0.125 M Tris-HCl (pH 6.8), and 0.002% bromophenol blue tracking dye. Monolayers of cells were washed three times with cold phosphate-buffered saline and scraped from dishes with 1 ml of cold water into a centrifuge tube. After centrifugation for 10 min at 1000 × g, the cell pellets were suspended in 50 μl of cold water and disrupted by sonication. Debris and unbroken cells were removed by brief centrifugation, and the supernatant fractions were diluted with an equal volume of the gel buffer described above. Prior to loading on gels, samples were boiled for 10 min. Electrophoresis was carried out according to the

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Abbreviation: NaDodSO₄, sodium dodecyl sulfate.

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procedure of Laemmli (9) on slab gels composed of 10% polyacrylamide. Gels were dried and autoradiographed with Kodak XR-5 film. ^3H -labeled samples were analyzed by fluorography according to the procedures of Bonner and Laskey (10).

Three procedures were used for obtaining [^3H]palmitate-labeled glycoproteins free of the viral membranes. (i) Glycoproteins were separated by electrophoresis on preparative polyacrylamide slab gels, and the region of the gel containing ^3H -labeled glycoprotein was cut from the wet gel. After extraction with water at 37°C , the proteins were concentrated and dialyzed by ultrafiltration (Amicon, PM 30 filter) and lyophilized. (ii) Viral particles from cells labeled with [^3H]palmitate were extracted twice at 4°C with 0.05 M potassium phosphate (pH 6.0), containing 0.1% Triton X-100. Only the E1 glycoprotein was released into soluble form (11), and the E2, complexed with the capsid, was recovered in a pellet after centrifugation at 45,000 rpm for 60 min in the Spinco 65 rotor. The isolated E1 was concentrated as described above and lyophilized. Membrane lipid was removed from the protein by 3–5 extractions with 3 ml of chloroform/methanol (2:1) at 23°C for 30 min each time. All ratios of solvents are expressed as vol/vol. Organic extraction was also performed on the E2 glycoprotein in the pellet fraction. (iii) [^3H]Palmitate-labeled virus particles were disrupted with 1% Triton X-100 at 23°C , and the glycoproteins were precipitated with antibodies from rabbits injected with preparations of purified E1 and E2 glycoproteins (unpublished experiments). Precipitates were formed by adding either goat anti-rabbit IgG (Gateway Sera) or preparations of lyophilized *Staphylococcus aureus* (Cowan) (12). These precipitates were thoroughly washed with the Triton buffer and extracted with chloroform/methanol as described above. The radiopurity of glycoprotein–lipid was determined by electrophoretic analysis in NaDodSO₄/polyacrylamide gels.

Release and Analysis of Lipid Bound to Glycoprotein. Preparations of glycoproteins freed of membrane lipids were incubated with 1 ml of 0.1 M KOH in methanol for 20 min at 23°C . The mixture was acidified with 1 M HCl, and chloroform and water were added to yield a ratio of CHCl₃/CH₃OH/H₂O of 8:4:3. After vigorous mixing and subsequent centrifugation, the upper phase was removed and the lower phase was washed three times with 1 ml of CHCl₃/CH₃OH/H₂O at 1:10:10 (13). Radioactivity was recovered in the lower phase and analyzed by gas/liquid chromatography (see below) or thin-layer chromatography on precoated plates (Sil-GHL, Analtech, Newark, NJ) with the following solvents: chloroform/methanol/water (65:25:4) or hexane/diethyl ether/acetic acid (70:30:10). The thin-layer plates were scraped and counted in a xylene-based scintillant (Aquasol, Amersham). Reference lipids were detected by staining with iodine or with UV light after spraying with 0.2% 2', 7'-dichlorofluorescein in ethanol. In some cases, samples were treated with methanolic 1 M HCl (10 M H₂O) at 80°C for 16 hr prior to extraction as described above (14).

For analysis by gas/liquid chromatography, the samples were methylated to completion with diazomethane in diethylether. The dried samples were dissolved in hexane, and aliquots were taken for determination of ^3H . More than 80% of the input label was recovered in the hexane. Methylated samples, mixed with reference methylated fatty acids, were analyzed in a Hewlett Packard 5711A chromatograph equipped with a gas effluent splitter. Analyses were performed at 170°C with an ethylene glycol succinate column at a flow rate of 40 ml/min. Fractions for radioactivity determination were collected at 1-min intervals.

Proteolytic Digestions. Approximately 3 mg each of E1 and E2 containing [^3H]palmitate were exhaustively extracted with chloroform/methanol (2:1), and the residue was digested with 50 μg of pepsin (Worthington) in 1 ml of 5% formic acid. After

6 hr at 23°C , an additional 50 μg of the protease was added and the reaction was continued for another 12 hr. The mixtures were neutralized to pH 7.0 by adding reagent NH₄OH, and 50 μg of thermolysin (Calbiochem) was added. After 6 hr at 37°C , another 50 μg of thermolysin was added and incubation was continued for 12 hr at 37°C . Analysis by NaDodSO₄/10% polyacrylamide gel electrophoresis indicated that the proteins had been degraded to small fragments. The digested material was lyophilized and extracted with chloroform/methanol (2:1). After several extractions, the residues were treated with 0.1% phosphoric acid in 0.1% amyl alcohol. The insoluble core was further digested with Pronase (Sigma) in the presence of toluene for 100 hr at 37°C (15). These samples were lyophilized and extracted with chloroform/methanol.

The various extracts of protease-digested samples were analyzed by thin-layer chromatography with chloroform/methanol/H₂O (65:25:4) as solvent. The chromatograms were sprayed with ninhydrin (Sigma) and analyzed for radioactivity by scraping bands into a xylene-based scintillant (Aquasol, Amersham).

RESULTS

Detection of Lipid Bound to Glycoprotein. Our first indication that a stable glycoprotein–lipid complex exists in Sindbis virus particles came from an examination of purified Sindbis virus particles that had been grown in chicken embryo fibroblasts labeled with [^3H]palmitate prior to and during infection. When a sample of this labeled virus was denatured with NaDodSO₄ and mercaptoethanol and electrophoresed in a NaDodSO₄/10% polyacrylamide slab gel, about 5% of the label was detected in two bands that comigrated with viral glycoproteins E1 and E2 (Fig. 1, gel A). The balance of the label migrated with the tracking dye marker to the front of the gel. There was no ^3H label in the region of the gel corresponding to the viral capsid protein. Thus, we considered it unlikely that the [^3H]palmitate had been degraded in the cell and reutilized as amino acids or as carbohydrate. Furthermore, the label was unequally distributed between the glycoproteins: E2 contained about twice as much label as E1. These glycoproteins are present in equivalent amounts in the virions and contain almost equal amounts of carbohydrate (16). More direct evidence that the ^3H label is in lipid is presented below.

The lipid that remained bound to glycoprotein despite denaturation with NaDodSO₄ could not be extracted from viral

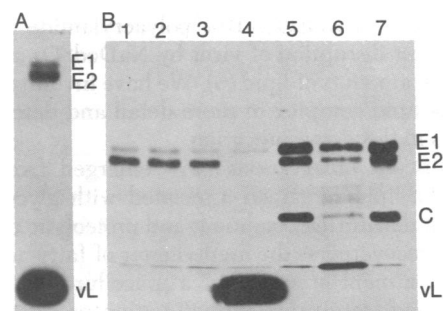


FIG. 1. Fluorogram of NaDodSO₄/polyacrylamide slab gels containing samples of Sindbis virus particles labeled with [^3H]palmitate (gel A and gel B, lanes 1–4) or with [^3S]methionine (gel B, lanes 5–7). Prior to their electrophoretic analysis, the samples were treated as follows. Lanes: 1 (control), 30 min at 37°C in water and extracted with chloroform/methanol; 2, 30 min at 37°C in 8 M urea and extracted with chloroform/methanol; 3, 10 min at 100°C in 10% NaDodSO₄ and extracted with chloroform/methanol; 4, 60 min at 37°C in 5% sodium deoxycholate; 5, 60 min at 37°C in water; 6, 60 min at 37°C in 5% sodium deoxycholate; 7, nontreated. E1 and E2 are the viral envelope proteins and C is the viral capsid protein. The viral membrane lipid (vL) migrates with the dye front in these gels.

particles by chloroform/methanol even if these particles were pretreated with NaDodSO₄ or 8 M urea (Fig 1 gel B, lanes 1-3). Incubation of the lipid-labeled viral particles with deoxycholate appeared to release lipid, especially from the E2 glycoprotein (lane 4). This observation suggested to us initially that the lipid was noncovalently linked to the protein; however we subsequently discovered that the deoxycholate treatment leads to a degradation of the E2 glycoprotein (lane 6). Thus, the loss of lipid from the E2 protein was not due to an exchange of labeled lipid for deoxycholate, but was the result of an apparent proteolytic activity in the particle that was activated by the detergent treatment.

Sindbis virus glycoproteins can be separated from each other by treating particles with Triton X-100 in various buffers (11). At low ionic strength at pH 6.0, the E1 glycoprotein was released from the particle and the E2 protein was recovered in a pellet fraction. The glycoproteins could also be isolated and freed from the membranes by precipitating Triton-disrupted particles with antibodies directed against the glycoproteins. Attempts to extract the labeled lipid from these different preparations of glycoproteins with chloroform/methanol were unsuccessful.

Identity of the Glycoprotein-Bound Lipid. The only reproducible method that freed the lipid into an organic solvent-extractable form was hydrolysis of the complex with mild alkali in methanol at room temperature or with HCl in methanol (1 M for 16 hr at 80°C or 0.1 M for 10 min at 100°C). The latter treatment, however, degraded the protein completely. The lipid released under these conditions was analyzed by radio gas/liquid chromatography after the samples were methylated with diazomethane. The major fraction of lipid that was released by acid and alkali from different preparations of glycoproteins had the same retention time as a methylated palmitic acid reference substance. Smaller amounts of radioactivity were detected as stearic acid and oleic acid (Fig. 2 A and B).

From the distribution of radioactivity between viral glycoprotein and membrane (Fig. 1, gel A) we estimated that the E1 protein contained 1-2 mol of lipid per mol of protein. To obtain additional data on the stoichiometry of lipid bound to glycoprotein, a sample of E1 from an unlabeled virus preparation was isolated and freed of nonbound lipid as described above. After the protein was treated with methanolic KOH, the fatty acid was extracted into organic solvent and analyzed by gas/liquid chromatography (Fig. 2C). A known amount of methyl palmitate was included in one of the analyses to confirm the position and quantitation of the fatty acid derived from the protein (Fig. 2D). The aqueous layer together with washes of the organic layer was dialyzed against water and lyophilized, and the protein was dissolved in 1% NaDodSO₄. Protein was determined by the Lowry method (17), with 1 mg of bovine serum albumin in 1 ml of 1% NaDodSO₄ as a standard. The E1 sample yielded 1.6 mg of protein (32 nmol) in the aqueous layer and 59 ± 10 nmol of methyl palmitate in the organic phase. Thus, there was 2 mol of fatty acid per mol of polypeptide in this sample of nonradioactive virus.

Chromatographic analyses were also carried out on samples that had been hydrolyzed with methanolic KOH and not further methylated. The labeled fatty acids were recovered as the methylated esters (Fig. 3). This direct methanolysis showed that protein-bound palmitate was indeed linked in an ester bond.

To determine if the released fatty acids were esterified to phospholipids, a preparation of ³²P-labeled Sindbis virions (≈10⁶ cpm/mg of virus, kindly provided by S. Monroe) was analyzed by NaDodSO₄/10% polyacrylamide gel electrophoresis. Virtually no radioactivity was found in the E1 or E2 protein bands but viral phospholipid was heavily labeled (data not shown). Thus, it is unlikely that the fatty acids were bound to glycoproteins in a phospholipid.

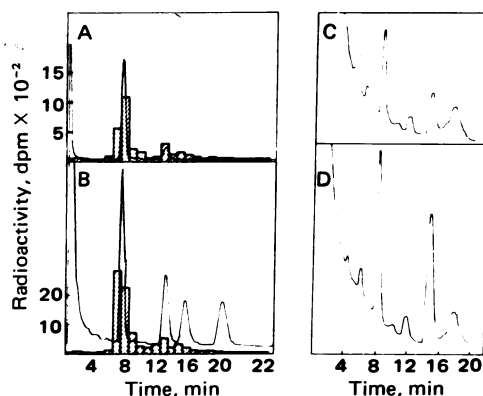


FIG. 2. Analysis of [³H]palmitate-labeled lipid by gas/liquid chromatography after release from purified viral glycoprotein by HCl or KOH in methanol. The samples were methylated with diazomethane and injected into a Hewlett-Packard gas/liquid chromatograph with a splitter device. About 80% of the injected material was split off and fractionated in 1-min intervals. The radioactivity in the condensates was analyzed in a scintillation spectrophotometer. The peaks indicate the standard methylated fatty acids that were injected together with the radioactive samples; their retention times, beginning at 5 min, increased in the order C-16, C-18, C-18:1, C-18:2, and C-18:3. (A) HCl released fatty acids from [³H]palmitate-labeled immunoprecipitates of E2. (B) KOH released fatty acids from [³H]palmitate-labeled E1 and E2 extracted from a polyacrylamide slab gel electropherogram immediately after electrophoresis without drying the gel. (C) KOH released fatty acids from unlabeled E1 (see text for details). (D) The same as C but 0.8 μg of standard methyl palmitate and 0.66 μg of standard methyl stearate were included in the sample. Hatched areas indicate radioactivity.

Evidence for a Covalent Linkage of Fatty Acid to Glycoprotein. The inability to release the lipid component from protein that had been subjected to strong denaturants and the ease of dissociating lipid by mild methanolic alkali suggest that the fatty acids recovered by the latter treatment might be acylated to the protein, possibly directly to an amino acid hydroxyl group. There was, however, the possibility that the labeled fatty acid was in lipid noncovalently bound to a cryptic region of the glycoprotein and thus inaccessible to organic solvent, despite various procedures that denature and unfold the protein. To determine the type of glycoprotein-fatty acid linkage, we extracted preparations of glycoproteins exhaustively with chloroform/methanol and then digested them with pepsin, thermolysin, and Pronase. After proteolysis, 69% of the fatty

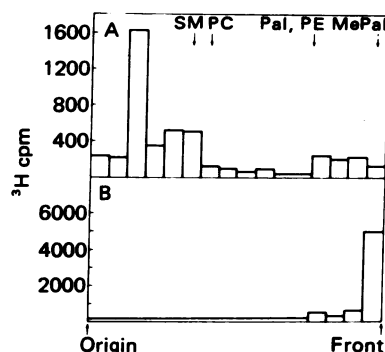


FIG. 3. Thin-layer chromatography of proteolytic digestion products from E1 that were soluble in chloroform/methanol (2:1). (B) Prior to analysis, the sample was treated with mild alkali. (A) The sample was carried through a similar extraction but not subjected to hydrolysis treatment. The chromatography solvent was chloroform/methanol/water (65:25:4). Reference lipids are sphingomyelin (SM), phosphatidylcholine (PC), palmitic acid (Pal), phosphatidylethanolamine (PE), and methylpalmitate (MePal).

acid bound to E1 became extractable by organic solvent and 14% was soluble in phosphoric acid. Only 30% of the E2-bound fatty acid was extractable by organic solvent and 14% was soluble in H₂O. The organic solvent-extracted digestion products were separated by thin-layer chromatography, and the major fraction of label from E1 fragments was present in a single band (Fig. 3). The labeled fatty acid in this fragment was hydrolyzed to methylated fatty acids by alkali. The mobility of the labeled lipid in the chloroform/methanol/water solvent did not correspond to any of the major cellular phospholipids or sphingomyelin.

Formation of Lipid-Protein Complex in Viral-Infected Cells. During the replication of Sindbis virus, the E2 glycoprotein initially appears as a larger precursor, PE2 (1). To determine the amount of lipid associated with PE2, we prelabeled cells with [³H]palmitate, infected the monolayers with virus, and harvested cells 5 hr after infection. Extracts were prepared by sonication and samples were boiled in NaDodSO₄/mercaptoethanol prior to NaDodSO₄ gel analysis. All three glycoproteins contained ³H, but PE2 had much lower amounts of lipid bound than did E1 or E2 (Fig. 4 *lower left*). At the level of detection analyzed in these gels, no discrete bands of ³H were found in the mock-infected cells or infected cells at early times after infection (data not shown). The pattern of lipid binding observed in the extracts of infected cells could reflect relative amounts of PE2 and E2 at steady state levels during viral replication. This is not the case, however, because the distribution of [³⁵S]methionine among the three glycoproteins showed that there were roughly equivalent amounts of PE2 and E2 at steady state (Fig. 4 *upper left*). Thus, conversion of PE2 to E2 might be required in order for lipid to bind to this glycoprotein. Other data have shown, however, that under some conditions PE2 was capable of binding almost as much lipid as E2. For example, in cells infected with the temperature-sensitive Sindbis mutant ts20 at the nonpermissive temperature, PE2 was not converted to E2 (2, 4), but this PE2 bound almost as much lipid as did the

E2 of cells grown at the permissive temperature (Fig. 4 *center*). It is noteworthy that in cells replicating the ts10 mutant, a member of a different complementation group of ts mutants (18), PE2 conversion was also blocked, but the binding of lipid to PE2 was more similar to that of the wild-type virus (Fig. 4 *right*). Similar data (not shown) were obtained with the ts23 mutant, which belongs to the same complementation group as ts10 (18).

Formation of the lipid-protein complex was also inhibited when the maturation of glycoproteins was blocked by tunicamycin, an antibiotic that inhibits glycosylation of viral membrane proteins (19–21). In the presence of tunicamycin, the two Sindbis glycoproteins appeared as a doublet with mobilities between the mature E1 and E2 glycoproteins, and no ³H-labeled lipid was detected in these bands (data not shown). In the absence of glycosylation, the Sindbis glycoproteins migrate faster in NaDodSO₄ gels (22, 23).

Reconstruction Experiment. It is possible that the apparent covalent binding of fatty acid to glycoprotein was the result of our experimental procedure and that the fatty acid binding occurred during extraction of the infected cell or in dissociating virions. To test for this kind of artifact we prepared samples in which [³H]palmitate-labeled *mock-infected* cells were mixed with *nonlabeled infected cells* prior to sonication and also afterwards but prior to the NaDodSO₄ treatment. No ³H label was found with the virus glycoproteins by NaDodSO₄/10% polyacrylamide gel electrophoresis. In another test, 50 μg of unlabeled viral particles was incubated with 5 μCi of [³H]palmitate at 23°C for 12 hr followed by 2 hr at 37°C, and samples were analyzed by NaDodSO₄/10% polyacrylamide gel electrophoresis after boiling in loading buffer for 10 min. Less than 0.2% of the radioactivity was recovered in the region of the gel containing the glycoproteins. These results rule out an artifactual binding of fatty acid to glycoprotein.

DISCUSSION

The results presented in this paper provide evidence for a covalent attachment of fatty acids to eukaryotic cell glycoproteins. Two previous reports have described small lipophilic proteins (called proteolipids) that contain esterified fatty acids. One of these is a subunit of myelin (24) and the other is a subunit of the Ca²⁺, Mg²⁺ ATPase from sarcoplasm reticulum (25). Both proteins are extractable into chloroform/methanol, and the fatty acid is released from the protein by mild alkaline hydrolysis. One to two moles of fatty acid were present in a mole of the sarcoplasm reticulum proteolipid, and we found similar values for the Sindbis virus glycoprotein E1. The linkage between fatty acid and protein in the proteolipids is presumed to be covalent, but the component to which fatty acid is bound has not been determined for either of these proteins. Covalent attachment of lipid to protein has been established for a prokaryotic membrane protein in which a diglyceride moiety is linked through a sulfhydryl group to the amino-terminal residue of the protein (26). Membrane-associated penicillinase in *Bacillus licheniformis* contains a phosphatidic acid covalently linked to the amino-terminal serine residue of the polypeptide chain (27). It is unlikely that these types of lipid-protein bonds are present in the viral glycoproteins because the amino-terminal residues have been identified as tyrosine for E1 and serine for E2 (28), and less than 0.1 mol of phosphate per mol of protein has been detected in these proteins (29). We also found insignificant amounts of phosphate in the glycoproteins. Our data are consistent, however, with a model in which the fatty acids are covalently linked via an ester bond to the protein, possibly through the hydroxyl group of an amino acid such as serine, threonine, or tyrosine. It is unlikely that the fatty acids are bound to the oligosaccharides of the glycoprotein because we

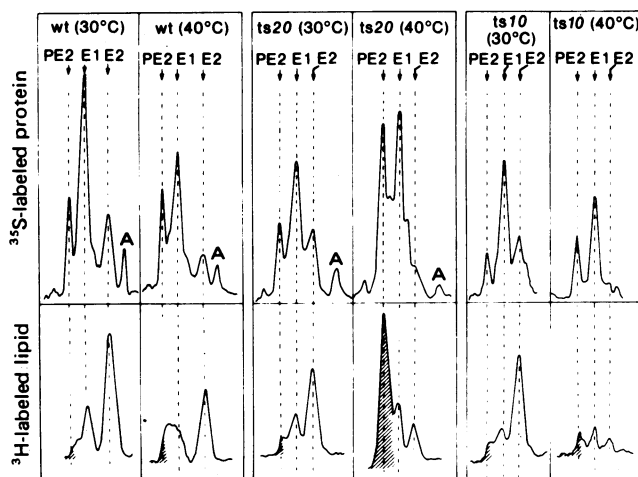


FIG. 4. Densitometric traces of fluorograms from a NaDodSO₄/polyacrylamide slab gel containing extracts of cells infected with wild-type (wt) and temperature-sensitive (ts) Sindbis virus. (*Upper*) Traces from slab gel lanes that contained infected cell extracts labeled with [³⁵S]methionine. (*Lower*) Traces from lanes from the same slab gel that contained cell extracts labeled with [³H]palmitate. Only the region of the fluorograms containing the viral envelope glycoproteins (PE2, E1, and E2) are shown; A refers to the actin band. Cells were grown in 60-mm dishes and half were labeled at 37°C with 20 μCi of [³H]palmitate for 12 hr prior to infection. Infected cells (multiplicity of infection, 100) were harvested 5 hr after infection at 40°C or after 7.5 hr at 30°C. [³H]palmitate (10 μCi) was present during infection and [³⁵S]methionine (5 μCi per dish) was added in medium minus methionine 3 hr after infection at 40°C or 5 hr after infection at 30°C. Hatched areas indicate lipid label in the PE2 glycoprotein.

have detected the same type of bound fatty acid in a nonglycosylated form of the vesicular stomatitis virus (unpublished results).

Because covalent peptide-lipid bonds have been detected in only a few cases, we were reluctant to initially consider a covalent linkage between the glycoprotein and lipid. Furthermore, tightly bound, noncovalent lipid-protein complexes have been observed in various proteins and enzymes that function in cell membranes (reviewed in ref. 30), and some of these require strong denaturing conditions in order to extract lipid. In addition, two observations from our initial studies support a noncovalent type of bond. One of these is the result with deoxycholate treatment of lipid-labeled particles in which labeled lipid from the E2 glycoprotein band was lost (see Fig. 1 gel B, lane 4). However, that result is shown here to be due to an apparent proteolytic degradation of the protein itself. In another series of experiments, we purified the glycoprotein by eluting the lipid-labeled proteins from NaDodSO₄/10% polyacrylamide slab gels that had been dried with heat under reduced pressure. Almost all the label in these preparations was extracted by organic solvent directly from the lyophilized extracts and it was identified as ceramide by its migration in several solvent systems (7). Since all other purification methods have failed to yield organic-extractable lipid and the label can be recovered as the methyl ester of fatty acid, we attribute these early results to artifact generated by the isolation procedure. We should note also that the fatty acid in ceramide is not released by mild alkaline hydrolysis.

The attachment of fatty acid to viral glycoproteins appears to be a "late" stage in the maturation of the viral glycoproteins, possibly at the time these proteins migrate to the plasma membrane. The nonglycosylated proteins that accumulate in tunicamycin-treated cells do not reach the cells' surface (23) and these proteins do not bind the fatty acid. The PE2 precursor protein in cells replicating wild-type virus also binds very little fatty acid compared to the E2 product, and PE2 does not appear to be on the surface of the infected cell (31). However, PE2 is capable of binding fatty acid in cells replicating the ts20 virus that is temperature sensitive in the PE2 to E2 conversion. Cells infected with this mutant at nonpermissive temperatures exhibit viral nucleocapsids aligned at the plasma membrane, but budding does not occur (32). In contrast, cells infected with mutants ts23 and ts10, which are in a different complementation group (18), show nucleocapsids scattered throughout the cytoplasm (32). Perhaps the attachment of fatty acid to the PE2 precursor provides a "receptor" or signal for the interaction of the nucleocapsid with the plasma membrane at sites containing viral glycoproteins. Another possible function of the fatty acid residue might be to provide a type of anchor that could bind these glycoproteins more strongly to the membrane. Both glycoproteins appear to have portions of their polypeptides embedded in the membrane (33).

Sindbis viral glycoproteins are not unique with respect to the binding of fatty acid. We have detected fatty acid bound to the glycoprotein of vesicular stomatitis virus, the hemagglutinin of influenza virus, and in proteins from uninfected cells (unpublished data). The biochemical mechanism for attachment of the fatty acid is unknown. A coenzyme A-linked fatty acid might be directly coupled to protein via an acyl transferase of the type recently described in rat microsomes (34), or the fatty acid could be transferred from membrane lipid. The role that these fatty acids may play in glycoprotein structure and function is clearly a most intriguing one, and this additional posttranslational modification of glycoprotein could be crucial not only for the final stages of viral assembly but for many cellular functions as well.

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