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Site-Specific Mutagenesis Identifies Three Cysteine Residues in the Cytoplasmic Tail as Acylation Sites of Influenza Virus Hemagglutinin

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The hemagglutinin (HA) of influenza virus is a type I transmembrane glycoprotein which is acylated with long-chain fatty acids. In this study we have used oligonucleotide-directed mutagenesis of cloned cDNA and a simian virus 40 expression system to determine the fatty acid binding site in HA and to examine possible functions of covalently linked fatty acids. The results show that the HA is acylated through thioester linkages at three highly conserved cysteine residues located in the cytoplasmic domain and at the carboxy-terminal end of the transmembrane region, whereas a cysteine located in the middle of the membrane-spanning domain is not acylated. Mutants lacking fatty acids at individual or all three attachment sites acquire endoglycosidase H-resistant oligosaccharide side chains, are cleaved into HA_1 and HA_2 subunits, and are transported to the plasma membrane at rates similar to that of wild-type HA. All mutants are membrane bound and not secreted into the medium. These results exclude transport signal and membrane-anchoring functions of covalently linked fatty acids for this integral membrane glycoprotein. Furthermore, lack of acylation has no obvious influence on the biological activities of HA: cells expressing fatty acid-free HA bind to and, after brief exposure to mildly acidic pH, fuse with erythrocytes; the HA-induced polykaryon formation is not impaired, either. Other possible functions of covalently linked fatty acids in integral membrane glycoproteins which cannot be examined in conventional cDNA expression systems are discussed.

The hemagglutinin (HA) of influenza virus is the major viral antigen and has receptor-binding and fusion activities which are necessary for initiating viral infection on the cellular level (for recent reviews, see references 15 and 50). Besides its important role in virus infectivity, HA is among the best-characterized membrane glycoproteins. It consists of a large N-terminal ectodomain that carries the receptor-binding and fusion properties, a stretch of hydrophobic amino acids presumed to traverse the membrane bilayer, and a short cytoplasmic domain. The amino acid sequences of different HA subtypes have been determined (6), the conformation of the protein has been studied by X-ray crystallography (51), and the structures of oligosaccharides attached to individual glycosylation sites have been elucidated for the H7 subtype (14).

HA is synthesized on membrane-bound ribosomes and is translocated into the lumen of the endoplasmic reticulum, where signal peptide cleavage and core glycosylation take place. HA is assembled into trimers soon after synthesis, before the molecules leave the endoplasmic reticulum (3, 7, 40). During its transport through the Golgi complex to the plasma membrane, HA undergoes extensive posttranslational modifications including trimming of carbohydrate side chains, terminal glycosylation, and proteolytic cleavage into the N-terminal HA_1 and the membrane-spanning HA_2 fragments, which are still linked by disulfide bonds. Cleavage is a prerequisite for viral infectivity and pathogenicity, because it activates the fusion ability of HA (16, 21). Although the structural requirements for and biological functions of these protein modifications have been extensively studied recently (15), little is known about another modification of HA, the covalent attachment of fatty acids. Fatty acid acylation has been described for many viral and cellular proteins (for recent reviews, see references 9 and 37). On the basis of chemical stability of the fatty acid linkage and the observation that different fatty acids are used for acylation, two types of this protein modification, myristoylation and palmitoylation, are currently distinguished. Myristoylated proteins contain exclusively the 14-carbon chain in an amide bond, whereas palmitoylated polypeptides are acylated mainly with palmitic acid (36) but also with stearic acid (46) in an ester-type linkage. The fatty acid linkage site in myristoylated proteins has been clearly established as the amino group of an N-terminal glycine residue (for a review, see reference 45), whereas palmitic acid is either linked to serine/threonine residues via oxyester bonds (43) or to cysteine residues through thioester linkage (1, 12, 17, 31, 34).

HA of influenza virus contains only ester-bound fatty acids which are linked to the small subunit HA_2 near its membrane-spanning domain (35). By comparison with model esters, the linkage in HA has been suggested to be of the oxyester rather than the thioester type (38). It has also been proposed that fatty acids contribute to the fusogenic properties of HA, because this activity is drastically impaired after deacylation of the protein with hydroxylamine (20, 38). Finally, it was of interest to find out if acylation serves as a signal for targeting HA to the cell surface.

To prove these concepts, we have analyzed in the present study HA of fowl plague virus [influenza virus A/FPV/Rostock/34 (H7N1)] by employing oligonucleotide-directed mutagenesis of cloned cDNA and a simian virus 40 (SV40) expression system. The results show that HA contains its fatty acids in thioester linkages. The attachment sites are

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cysteine 551 at the carboxy-terminal end of the transmembrane domain and cysteines 559 and 562 in the cytoplasmic tail. Lack of acylation has no influence on intracellular transport and the biological activities of the HA molecule.

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MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis. The construction of a cDNA of the HA gene from FPV (influenza virus A/FPV/Rostock/34, H7 subtype) has been described elsewhere (18). The cDNA was adapted with *Bgl*II linkers and cloned into the replicative-form DNA of M13mp11 by using standard techniques in such a manner that the noncoding strand of the HA cDNA was packaged into progeny M13 phages. Oligonucleotide-directed mutagenesis was done with a commercial kit (Amersham, Braunschweig, Germany) based on the phosphorothioate method (44) using single-strand DNA of M13mp11-HA as template. M13mp11-HA phages with only the desired mutation in the HA gene were selected by sequencing the single-strand DNA by the chain termination method of Sanger et al. (32). The oligonucleotide primers used for mutagenesis and sequencing are available on request. The HA gene was excised from the phage replicative-form DNA with *Bgl*II and ligated into the compatible *Bam*HI site of the SV40 expression vector pa11svl3 (10). The orientation of the HA cDNA appropriate for expression was checked by analytical digestion with *Pvu*II, and the nucleotide sequence of the HA gene was again verified.

Transfection of CV-1 cells and generation of a high-titer SV40-HA virus stock. Pa11svl3, containing the HA gene under the control of the late SV40 promoter, and the SV40 helper plasmid *dl* 1055 (4), which has a deletion in the SV40 T antigen, were cut with *Sac*I (pa11svl3) or *Bam*HI (*dl*1055) to remove bacterial parts of the DNA. The SV40-HA recombinant genome and SV40 helper genome were recircularized under dilute ligation conditions (3 μg/ml). A 200-μg portion of each DNA was cotransfected into subconfluent CV-1 monkey cells grown on 6-cm-diameter plates using either DEAE-dextran (500 μg/ml) and chloroquine (100 μM) as described by Doyle et al. (4) or Lipofectin (BRL, Berlin, Germany) as described by the manufacturer. Three to 5 days later, the cell monolayer was dispersed with trypsin and transferred together with an equal amount of untransfected CV-1 cells to a 250-ml cell culture flask. The flasks were incubated for 4 to 7 days until most cells showed cytopathological effects caused by the SV40 infection. The monolayer in medium was subjected to two cycles of freezing and thawing, cell debris was pelleted, and the resulting supernatant was used for expression of the HA gene.

Infection of CV-1 cells with recombinant SV40-HA virus stock and metabolic labeling experiments. A 500-μl portion of the recombinant SV40-HA virus stock was added to CV-1 cells grown to 90% confluency on 2.5-cm-diameter plates. One hour later, 2 ml of Dulbecco's medium with 2% fetal calf serum was added. Labeling experiments were started at approximately 48 h postinfection (p.i.). Labeling with [9,10(*n*)-^{3}H]palmitic acid (Amersham; 54 Ci/mmol, 1 mCi/ml of Dulbecco's medium), D-[6-^{3}H]glucosamine (Amersham; 20 to 40 Ci/mmol, 100 μCi/ml of Dulbecco's medium with 10 mM fructose instead of glucose), and L-[^{35}S]methionine (Amersham; 1,000 Ci/mmol, 10 μCi/ml of Dulbecco's medium without methionine) was done for 4 h. For investigations on the intracellular transport of HA, infected cells were pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 15 min and chased by adding unlabeled methionine to a final concentration of 10 mM. After being labeled, cells were lysed with 500 μl of RIPA buffer (1% Triton X-100, 1% desoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 20 mM Tris, 10 mM EDTA, 10 mM iodoacetamide), and unlysed material was pelleted for 30 min at 100,000 × *g*. Anti-FPV rabbit serum (1 μl), which precipitates all antigenic forms of HA, and 20 μl of a slurry of protein A–Sepharose Cl-4B (Sigma, St. Louis, Mo.) were added to the supernatant and incubated overnight at 4°C. Protein A-Sepharose with bound antigen-antibody complexes was washed four times with RIPA buffer. Samples were boiled for 2 min in electrophoresis sample buffer with or without 5% mercaptoethanol and analyzed by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. Fluorography was done with En^{3}Hance (Amersham) as described by the manufacturer. For endoglycosidase-H (endo-H) and endo-F digestions, immunoprecipitated samples were boiled for 2 min in 50 mM phosphate buffer containing 0.5% mercaptoethanol and 0.1% SDS, divided into three aliquots, and digested with endo-H (1 μl; Boehringer, Mannheim, Germany) or endo-F (1 μl) or mock digested for 16 h at 37°C prior to electrophoresis and fluorography.

Indirect immunofluorescence. At 48 h after infection with recombinant SV40-HA virus, CV-1 cells grown on cover slips were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Unreacted paraformaldehyde was quenched with 0.1 M glycine in PBS for 15 min. Cells were then either made permeable with 0.1% Triton X-100 in PBS for 15 min (intracellular immunofluorescence) or left untreated. The first antibody (anti-FPV rabbit serum) and the second antibody (rhodamine-conjugated swine immunoglobulin to rabbit immunoglobulin) (DAKOPATS, Hamburg, Germany), both at a dilution of 1:400 in PBS containing 2% bovine serum albumin, were adsorbed successively to the cells for 1 h. Unbound antibody was washed off with PBS at every step, and cover slips were mounted in Fluroprep (bioMerieux, Marcy l'Etoile, France). Cells were examined under a microscope (Zeiss Axiophot) with UV optics, and the results were recorded photographically at ×630 magnification.

Hemadsorption assay. At 48 h after infection with recombinant SV40-HA virus, monolayers of CV-1 cells were washed with PBS, and chicken erythrocytes (1% in 0.9 M NaCl) were added. After 30 min at room temperature, unbound erythrocytes were washed off with PBS, and cells with bound erythrocytes were photographed under a microscope at ×630 magnification.

Cell-erythrocyte fusion assay. Hemadsorption was performed as described above, except that guinea pig erythrocytes were used. To convert HA into a fusion-competent conformation, cells with bound erythrocytes were treated with prewarmed pH 5 medium (10 mM morpholineethanesulfonic acid–10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] in PBS) for 3 min (11). Controls were exposed to the same medium at pH 7. Then the media were replaced by Dulbecco's medium, and the cells were returned to the incubator for 20 to 60 min. The progress of fusion was noted every 10 min. Results were recorded photographically under a microscope at ×630 magnification.

Cell-cell fusion assay. At 48 h after infection with recombinant SV40-HA virus, monolayers of CV-1 cells were washed several times with PBS and treated with pH 5 or pH 7 buffer for 3 min at 37°C. The buffer was washed off, and the cells were incubated in Dulbecco's medium for 6 to 8 h.

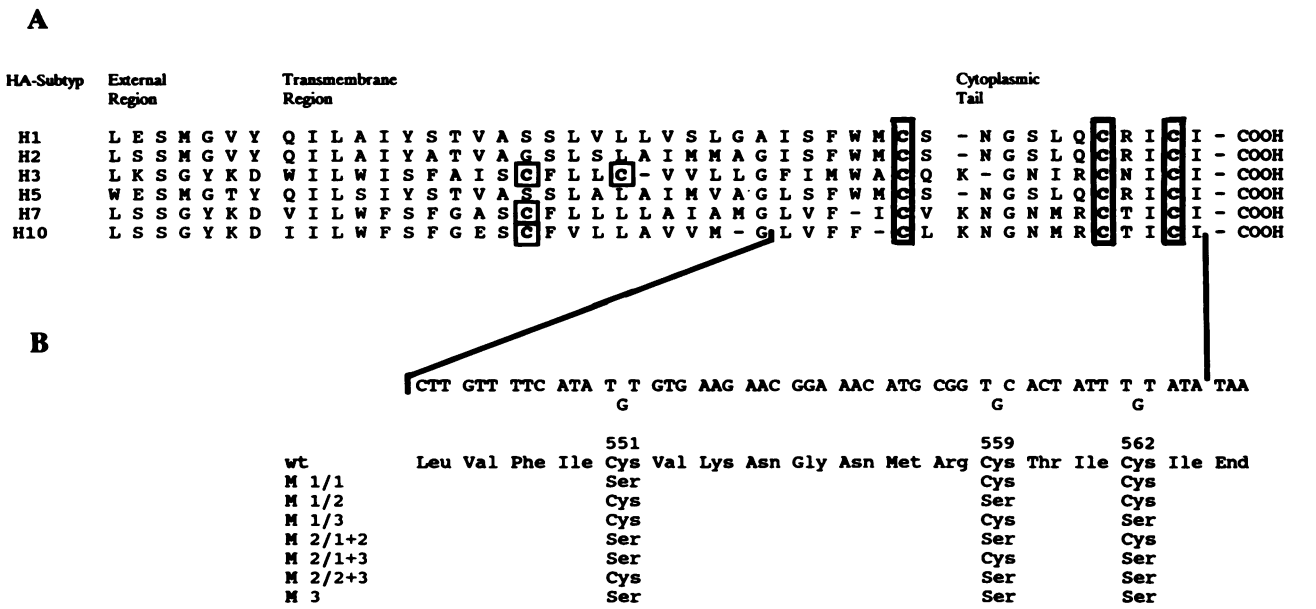

FIG. 1. Cysteine residues at the carboxy terminus of the HA. (A) Amino acid sequences of the transmembrane region and the cytoplasmic domain of different HA subtypes. The three cysteine residues which are conserved through all HA subtypes and cysteine residues in the middle of the transmembrane region, which are present only in the H3, H7, and H10 subtypes, are boxed. The one-letter amino acid code is used. The sequences are aligned according to Feldmann et al. (6). (B) Replacement of cysteine residues by serine using oligonucleotide-directed mutagenesis. The figure shows the coding cDNA and the corresponding amino acid sequence in the C-terminal part of the HA gene of FPV. The triplets encoding cysteine residues were changed stepwise to triplets encoding serine by using site-directed mutagenesis to replace G by C in the cDNA sequence. The abbreviations of the resulting HA mutants and their amino acids at positions 551, 559, and 562 are shown below. Numbering of amino acids is done by the method of Porter et al. (29).

After fixation with methanol at −20°C for 10 min, cells were stained with Giemsa (1:20 in PBS), and polykaryons were photographed under a microscope at ×200 magnification.

RESULTS

Determination of palmitoylation sites of HA. It has been reported that fatty acids which are linked to cysteine residues via thioester bonds are released by treatment with reducing agents (24, 34). The fatty acid linkage in HA of influenza virus is also sensitive to treatment with mercaptoethanol in a concentration-, time-, and temperature-dependent manner (46), which argues for a thioester-type bond to a cysteine residue. Because all cysteine residues in the extracytoplasmic part of HA_2 are involved in inter- and intramolecular disulfide bonds (48), only cysteines in the membrane region or the cytoplasmic domain are possible fatty acid linkage sites. Inspection of published amino acid sequences reveals that three cysteine residues in this region are conserved through all HA subtypes. Two of them are located in the cytoplasmic tail, 7 and 10 amino acids away from the membrane (Cys-559 and Cys-562), and one is placed in the membrane-spanning region adjacent to the cytoplasm (Cys-551). HAs of H3, H7, and H10 subtypes contain one or two additional residues in the middle of their membrane-spanning regions (Fig. 1A).

To determine whether one or more of the conserved cysteine residues are indeed the fatty acid binding site(s), we used oligonucleotide-directed mutagenesis of the cloned cDNA of FPV HA to change the triplets encoding cysteines to triplets encoding serine residues. The cysteine in the middle of the membrane region (Cys-535) was retained in all mutants (Fig. 1B).

Wild-type and mutant HAs were expressed in CV-1 cells with an SV40 expression system (see Materials and Methods) and were labeled with [^{3}H]palmitic acid. Labeling with [^{3}H]glucosamine or [^{35}S]methionine was done in parallel to ensure that wild-type and mutant HAs were synthesized in equal amounts (Fig. 2). The results shown in Fig. 2a and c reveal that FPV HA is cleaved in CV-1 cells into HA_1 and HA_2 subunits in the absence of other viral gene products, as has been observed before when this HA was expressed in insect cells with a baculovirus vector (18). The recombinant exhibits a molecular weight slightly higher than that of HA synthesized in FPV-infected cells. This is probably due to terminal sialylation of the carbohydrate side chains, which can take place in the recombinant system because the FPV neuraminidase is absent.

Labeling with [^{3}H]palmitic acid reveals that wild-type HA is strongly labeled in its HA_2 subunit and in the uncleaved precursor HA, as has been observed for HA synthesized during influenza virus infection (35) (Fig. 2a). In contrast, mutant M3, in which all three conserved cysteines have been replaced by serine residues, is not labeled with [^{3}H]palmitic acid (Fig. 2a and c). To rule out the possibility that M3 contains fatty acids at the residual cysteine in a particularly mercaptoethanol-sensitive linkage, we separated the immunoprecipitates by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. As a control, a truncated HA molecule that lacked the entire cytoplasmic and membrane-spanning regions including nine amino acids of the luminal domain was also analyzed (17a). Figure 2b demonstrates that even under nonreducing conditions, neither mutant M3 nor the anchor-minus mutant (A^-) was labeled with [^{3}H]palmitic acid. These results indicate that fatty acylation of HA is confined to cysteines 551, 559, and 562, whereas the cysteine

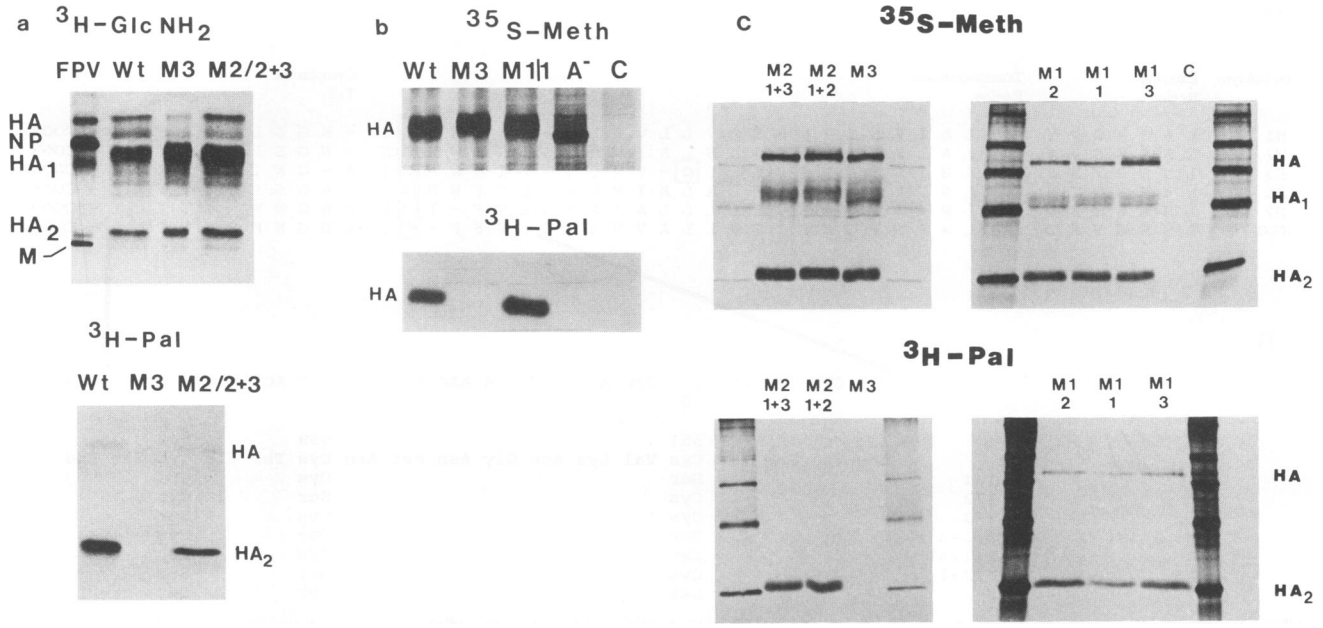

FIG. 2. [^{35}S]methionine, [^{3}H]glucosamine, and [^{3}H]palmitic acid-labeling of wild-type and mutant HAs synthesized in CV-1 cells by using an SV40 expression system. CV-1 cells infected with recombinant SV40-HA were labeled at 48 h p.i. for 4 h with [^{35}S]methionine (^{35}S-Meth), [^{3}H]glucosamine (^{3}H-GlcNH$_2$), and [^{3}H]palmitic acid (^{3}H-Pal). Proteins were immunoprecipitated from cell extracts with anti-FPV rabbit serum, electrophoresed in a 12% polyacrylamide gel using reducing (a and c) or nonreducing (b) conditions, and subjected to fluorography. Abbreviations: FPV, cells infected with FPV and labeled at 16 h p.i. with [^{35}S]methionine; Wt, wild-type HA; A$^-$, anchor-minus mutant; C, cells infected with nonrecombinant SV40. For abbreviations of HA mutants, see Fig. 1B.

in the middle of the membrane-spanning region is not involved.

To decide which of the three carboxy-terminal cysteines are acylated, we also expressed and labeled mutants M1/1, M1/2, M1/3, M2/1+2, M2/1+3 (Fig. 2c), and M2/2+3 (Fig. 2a). The results reveal that replacing one or two cysteines in different combinations is not sufficient to abolish labeling with [^{3}H]palmitic acid. Densitometer tracing of the HA bands and calculating the average ratio of [^{3}H]palmitic acid to [^{35}S]methionine show that [^{3}H]palmitic acid incorporation is reduced by 25 to 35% in the M1 mutants and by 65 to 75% in the M2 mutants relative to incorporation in the wild type. Thus, all three carboxy-terminal cysteines are acylated in wild-type HA at about equimolar rates.

Palmitoylation has no influence on intracellular transport and membrane anchoring of HA. It has been speculated that acylation may be necessary for membrane insertion and targeting of glycoproteins (52). Therefore we investigated intracellular transport of the HA mutants by using either intracellular or cell surface immunofluorescence. The results are shown in Fig. 3 for wild-type and fatty acid-free HA (M3), as well as for the mutants with the cysteine in the membrane-spanning region adjacent to the cytoplasm replaced (M1/1) or with both cytoplasmic cysteines replaced (M2/2+3). Intracellular immunofluorescence revealed bright staining of the perinuclear Golgi region both for wild-type and mutant HAs. This is a typical staining pattern for glycoproteins in transit to the plasma membrane. Cell surface immunofluorescence showed that wild-type as well as mutant HAs are transported to and anchored in the plasma membrane. Furthermore, immunoprecipitation of the supernatant of cell cultures obtained for cells infected with recombinant SV40-HA virus revealed that fatty acid-free HA was not shed into the medium (not shown), indicating that anchoring of HA in membranes is not dependent on covalently linked fatty acids.

Several mutants of HA that have alterations in the cytoplasmic domain and are expressed at the cell surface but delayed at different stages of their intracellular transport have been described (4). Palmitoylation of HA is a posttranslational event occuring after trimerization but before Golgi-located trimming of carbohydrate side chains and HA cleavage (39, 45a). Therefore, a delay in the transport of fatty acid-free HA through the Golgi complex should result in delayed occurence of endo-H-resistant carbohydrate side chains and of HA cleavage products. To investigate the contribution of covalently linked fatty acids to the rate of intracellular transport of HA, we carried out pulse-chase experiments and digested the immunoprecipitated HA with endo-H to remove high-mannose carbohydrates and with endo-F to remove all N-linked carbohydrates (Fig. 4). The first cleavage products of wild-type HA are seen after 20 min of chase, and cleavage is almost complete after 1 h of chase. The occurrence of endo-H-resistant HA is nearly in phase with cleavage; i.e., the cleavage products HA$_1$ and HA$_2$ are always endo-H resistant, whereas uncleaved HA is predominantly endo-H sensitive. Comparison of the effects of endo-F and endo-H digestion on the HA cleavage products indicates that HA$_1$ and HA$_2$ each contain one high-mannose carbohydrate side chain in the fully processed glycoprotein (14). Essentially the same results were obtained for the HA mutant lacking all acylation sites (M3). Thus, there is no evidence that palmitoylation influences carbohydrate processing or intracellular transport of HA through the Golgi complex. Furthermore, cell surface immunoprecipitation revealed no temporal difference in the appearance of fatty acid-free HA and wild-type HA at the plasma membrane (not shown), which excludes an influence of palmitoylation on

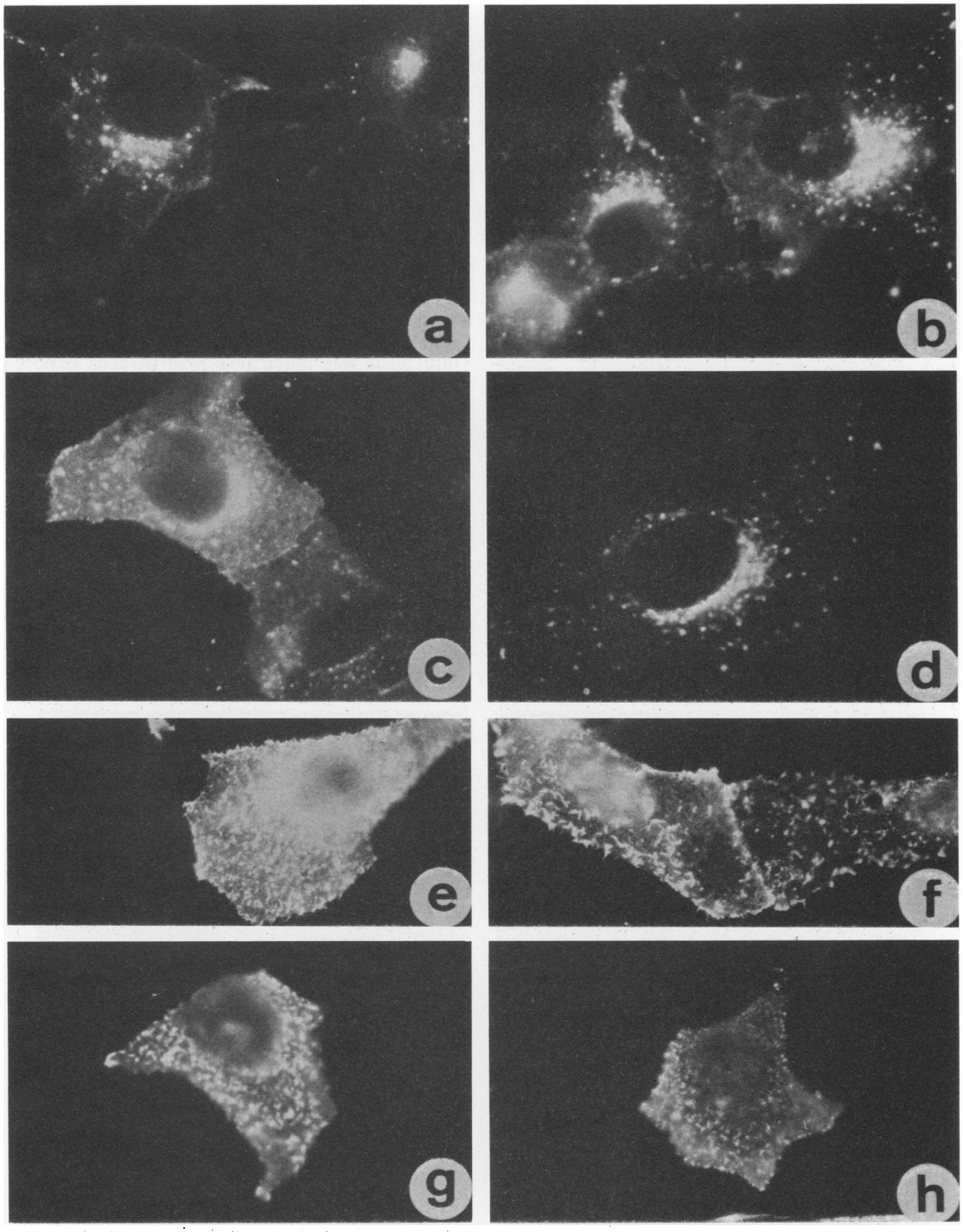

FIG. 3. Indirect immunofluorescence of wild-type and mutant HA. (a to d) Staining of intracellular HA with anti-FPV rabbit serum and rhodamine-conjugated swine immunoglobulins to rabbit immunoglobulins after permeabilization of recombinant SV40-HA-infected cells. (e to h) Staining of cell surface HA without prior permeabilization. a and e, Wild-type HA; b and f, M3; c and g, M2/2+3; d and h, M1/1.

transport from the Golgi complex to the plasma membrane. We have also analyzed trimerization by cross-linking of pulse-chase labeled HA with dithiobissuccinimidyl propionate followed by immunoprecipitation and polyacrylamide gel electrophoresis. Fatty acid-free HA trimerized, and the trimers were stable during 1 h of chase, as was also observed with acylated HA (not shown).

Palmitoylation has no influence on biological activities of HA. HA is responsible for virus attachment to sialic acid-containing receptors on the host cell and for fusion of the

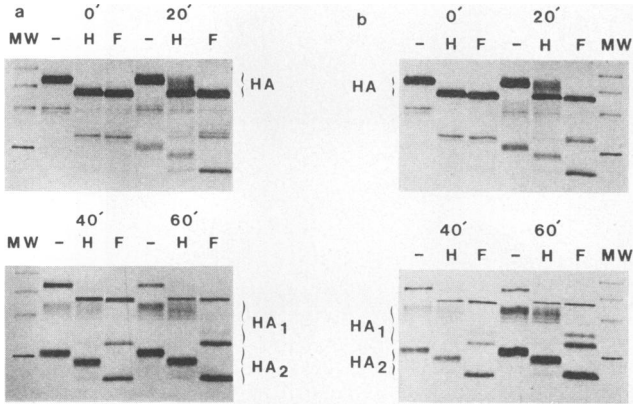

FIG. 4. Rate of cleavage and acquisition of complex carbohydrate side chains by wild-type HA (a) and fatty acid-free HA (b). At 48 h after infection with recombinant SV40-HA virus, CV-1 cells were pulse-labeled for 15 min with [^{35}S]methionine and chased for 0, 20, 40, or 60 min in the presence of 10 mM unlabeled methionine. Immunoprecipitated HA was digested with endo-H (H) to cleave high-mannose oligosaccharide side chains or with endo-F (F) to remove all carbohydrate side chains or was mock digested (−) prior to electrophoresis under reducing conditions and fluorography. Lane MW, ^{14}C-labeled molecular mass markers: phosphorylase, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; and carbonic anhydrase, 30 kDa.

viral envelope with cellular membranes. To throw light on a possible contribution of covalently linked fatty acids to these activities, we have measured the ability of HA to bind to erythrocytes (hemadsorption) and to cause hemolysis and cell fusion at low pH (11, 49).

Figure 5 shows that CV-1 cells expressing wild-type HA as well as those expressing mutant HAs are all able to bind erythrocytes. This reveals that lack of acylation has no influence on the receptor-binding properties of HA. To investigate the contribution of fatty acids to the fusion properties of HA, CV-1 cells infected with the SV40 mutant M3 were briefly exposed either to pH 5 or to pH 7 medium after hemadsorption. After withdrawal of pH 5 medium and incubation in growth medium, the amount of bound erythrocytes per recombinant SV40-HA virus-infected cell culture plate was much less than in cells exposed to pH 7 medium. Erythrocytes still bound to HA-expressing cells were enlarged and had lost their discoidal appearance (Fig. 6, left panels). These results indicate that hemolysis occurs after fusion of erythrocytes to cells expressing nonacylated HA.

Furthermore, 6 to 8 h after pH 5 treatment, cells expressing wild-type HA as well as those expressing fatty acid-free HA revealed strong polykaryon formation (Fig. 6, middle and right panels). Calculating the average number of polykaryons per microscopic field revealed no difference in the level of fusion activity between wild-type and fatty acid-free

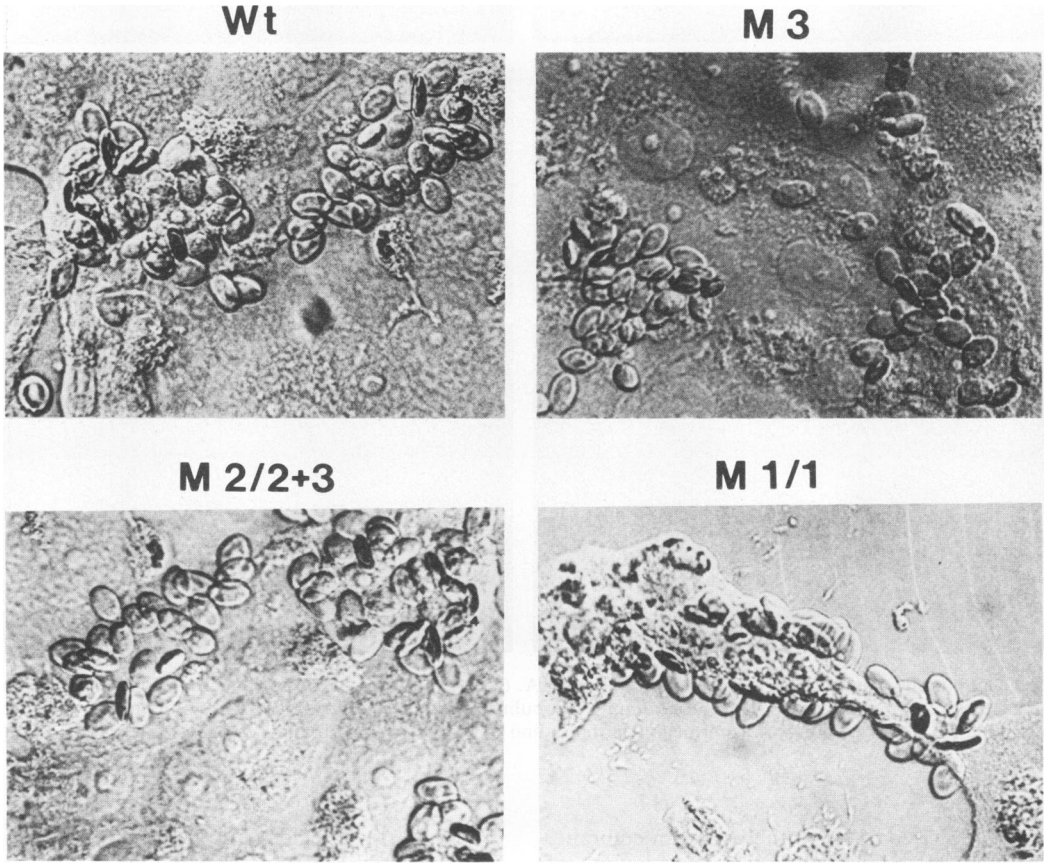

FIG. 5. Hemadsorption of chicken erythrocytes to CV-1 cells expressing wild-type and mutant HAs. At 48 h after infection with recombinant SV40-HA virus, CV-1 cells were overlaid with a 1% suspension of chicken erythrocytes. After 30 min, unbound erythrocytes were removed by being washed with PBS. Wt, Wild-type HA. For abbreviations of the HA mutants, see Fig. 1B.

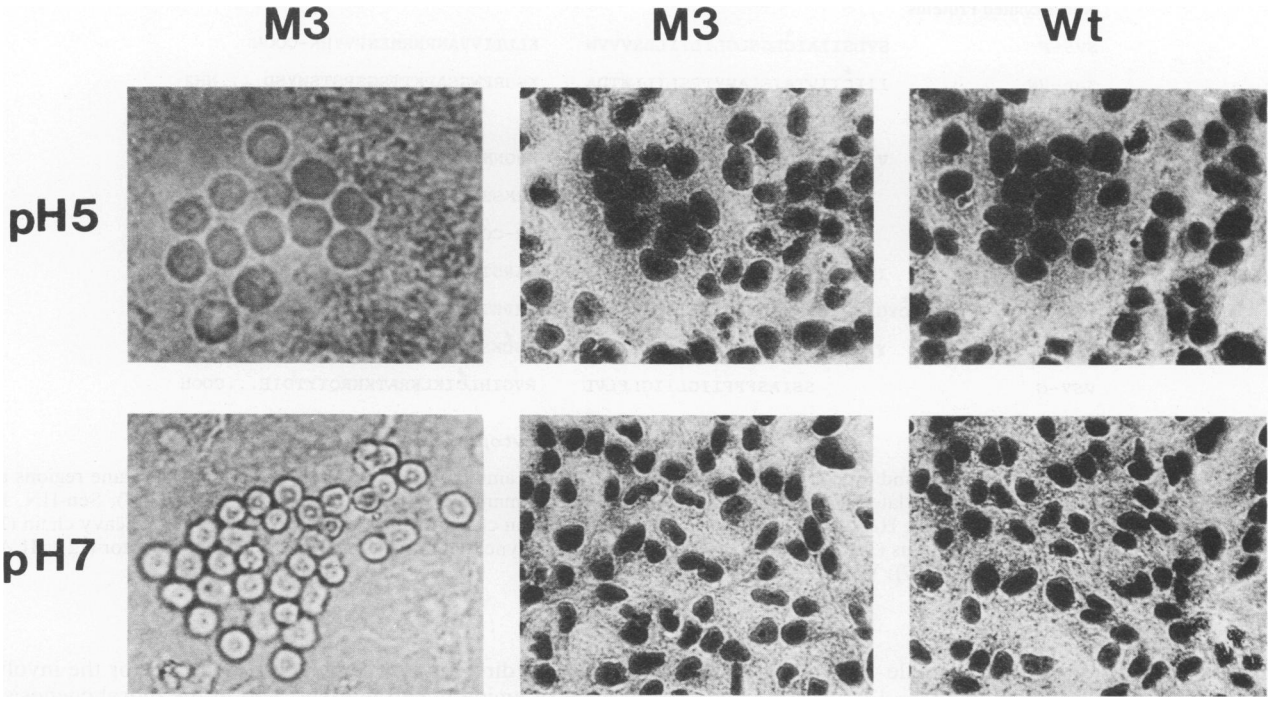

FIG. 6. Fusion activity of fatty acid-free HA expressed on the surface of CV-1 cells. Left, Fusion with erythrocytes. After hemadsorption of guinea pig erythrocytes to CV-1 cells infected with SV40-HA virus, monolayers with bound erythrocytes were briefly treated with pH 7 or pH 5 medium and then incubated in Dulbecco's medium for 60 min. Middle and right, Polykaryon formation. CV-1 cells infected with recombinant SV40-HA were briefly treated with pH 5 or pH 7 medium at 48 h p.i. and then incubated in Dulbecco's medium for 6 h. Monolayers were fixed with methanol and stained with Giemsa. M3, Fatty acid-free HA; Wt, wild-type HA.

HAs. Thus the fusion capacity of FPV HA appears not to depend on acylation.

DISCUSSION

Using oligonucleotide-directed mutagenesis of cloned cDNA and an SV40 expression system, it was established that the HA of influenza virus is acylated exclusively at cysteine residues and that O-ester linkages to serine and threonine do not occur. Three conserved cysteines could be determined as fatty acid attachment sites in the cytoplasmic tail and in the membrane-spanning region adjacent to the cytoplasmic domain.

Comparison of the palmitoylated cysteines of HA with those reported for other viral and cellular membrane glycoproteins of both type I and type II reveals that whenever such proteins contain acylation sites, the sites are clustered around the borderline between transmembrane domain and cytoplasmic tail (Fig. 7). This topography indicates that the enzyme responsible for acylation, which has not been purified yet but is known to be membrane bound and of cellular origin (2), must be oriented with its active center towards the cytoplasm. Assuming that positively charged amino acids mark the membrane boundary of the cytoplasmic tail, it appears that cysteine residues up to 10 amino acids away from the membrane (Cys-562 of HA) can still serve as acylation sites, whereas this is no longer possible if the distance becomes too large, as is the case with human class I histocompatibility antigen B7. Likewise, cystein residues of the transmembrane domain are still acylated, if they are only one (human class I histocompatibility antigen B7, respiratory syncytial virus F protein, and transferrin receptor), two (Cys-551 of HA), four (Semliki Forest virus E1 protein), or six (transferrin receptor) amino acids away from the cytoplasmic tail. However, cysteines located in the middle (Cys-545 of HA and SV5 F protein) or at the opposite end (transferrin receptor or Sendai virus HN protein) of the transmembrane region are not acylated, indicating that a potential acylation site is not accessible to the acyltransferase if it is buried deep enough in the lipid bilayer.

Inspection of the amino acids in the vicinity of the acylated cysteine residues of these glycoproteins reveals no obvious consensus signal for acylation such as has been established, for instance, in the case of N-linked glycosylation. Thus it appears that a transmembrane protein is acylated whenever cysteine residues are present in the region just described, where they are accessible to the acyltransferase.

Little is known about the biological significance of ester-linked fatty acids in integral membrane glycoproteins. Here we have shown that palmitoylation has no influence on the intracellular transport of the HA. HA without any covalently linked fatty acids trimerizes, acquires endo-H-resistant carbohydrate chains, is cleaved into HA_1 and HA_2, and is transported to the plasma membrane at the same rate as authentic HA. This confirms and extends former studies using nonacylated recombinant cDNA mutants of the G protein from vesicular stomatitis virus (VSV) (31) and of the human transferrin receptor (12). Furthermore, fatty acid-free HA is still embedded in the membrane and is not secreted into the medium. Therefore, the stretch of hydrophobic amino acids at the C-terminal part of HA is sufficient to anchor the molecule in the membrane, whereas peripheral

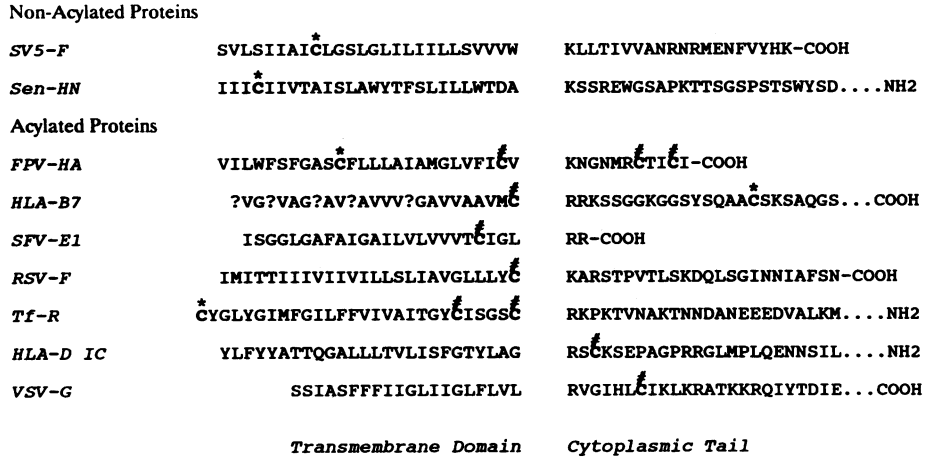

FIG. 7. Palmitoylation sites of type I and type II transmembrane proteins. The amino acid sequences of the transmembrane regions and the cytoplasmic tails are shown, with acylated (#) and nonacylated (*) cysteines marked. SV5-F, F protein of SV5 (25, 47); Sen-HN, HN protein of Sendai virus (41, 47); FPV-HA, HA of FPV (this work); HLA-B7, human class I histocompatibility antigen B7, heavy chain (13); SFV-E1, E1 protein of Semliki Forest virus (34); RSV-F, F protein of respiratory syncytial virus (1); Tf-R, transferrin receptor (12); HLA-D IC, HLA-D-associated invariant chain (17); VSV-G, G protein VSV (31).

membrane proteins often require amide- or ester-linked fatty acids for their membrane binding (26, 42).

We were also unable to establish that lack of acylation had an influence on the role of HA as an initiator of infection. Fatty acid-free HA has receptor-binding and fusion activities. The concept that covalently linked fatty acids are not a general requirement for viral glycoproteins to express their fusion activity is further supported by the observation that the fusion protein of Sendai virus is regularly not acylated (47). The impairment of fusion activity that has been observed after cleavage of fatty acids from HA with hydroxylamine (20) may be due to alterations in the conformation of HA by this rather harsh treatment.

Thus, the function of covalently linked fatty acids in HA remains elusive. However, the fact that the three acylated cysteine residues are conserved through all HA subtypes and through different isolates of the same subtype argues in favor of a crucial function of fatty acylation, and the precise localization of the attachment sites now allows some reasonable speculations in this respect. For instance, it is reasonable to assume that the hydrocarbon chains of the fatty acids penetrate the membrane bilayer as previously suggested for the acyl chain of the VSV G protein (27). In this regard it is noteworthy that Cys-562 in HA is surrounded by two hydrophobic amino acids (isoleucine), which are also conserved through all HA subtypes (cf. Fig. 1A). This portion of the cytoplasmic tail may loop back to the membrane, as has been proposed for the fatty acids linked to the cytoplasmic tail of bovine rhodopsin (24). Alternatively, the carbon chain of fatty acids linked to different cysteine residues of the same molecule or to different molecules of the trimeric HA glycoprotein may interact intimately with each other. In both cases, fatty acids should have an important influence on the conformation of the cytoplasmic tail of acylated glycoproteins. This special conformation may be necessary to bring about protein-protein interactions between the cytoplasmic domains of HA and other virus-encoded membrane proteins which are required to facilitate the packing of HA molecules into new virus particles. An influenza virus protein that has been suggested to be involved in packaging is M2 (19), and it is interesting to note that M2 contains also fatty acids (45a). Whereas direct evidence has been obtained for the involvement of amide-linked myristic acid in the morphogenesis of new virus particles (8, 22, 30), experimental data pointing to a similar function of ester-linked palmitic acid are rather indirect. Using cerulenin, a putative inhibitor of the acyltransferase, Schlesinger and Malfer (33) have shown that fatty acid-free VSV G protein is transported to the plasma membrane but is only poorly incorporated into new virus particles. Regarding the fact that viruses rely largely on cellular mechanisms for propagation, a recent report of Pfanner and co-workers (28) is noteworthy. These authors have shown that fatty acyl coenzyme A is necessary to promote budding of intracellular transport vesicles, a process which morphologically resembles budding of virus particles. They suggest that acylation of an unknown cellular protein is a presupposition for this process. O'Dowd et al. (23) reported that deletion of a fatty acid linkage site in the human β2 adrenergic receptor leads to the uncoupling of signal transduction between receptor and adenyl cyclase via G proteins, which supports the assumption that fatty acids are indeed involved in protein-protein interactions. Because techniques to introduce site-specific mutations into the genome of influenza virus are now available (5), studying the contribution of HA-linked fatty acids to the morphogenesis of new virus particles is now amenable to experimental approaches.

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ADDENDUM

After submission of this manuscript, a similar study was published by Naeve and Williams (22a). By subjecting H2 HA to site-specific mutagenesis, those authors also observed that each of the cysteines at positions 551, 559, and 562 could serve as an acylation site. In contrast to the results reported

here, however, H2 HA lacking fatty acids was found to have lost its fusing activity. Except for the possibility that there are strain-dependent differences in fatty acid requirement, we have no explanation for these conflicting observations.

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