Effects of Altering Palmitylation Sites on Biosynthesis and Function of the Influenza Virus Hemagglutinin

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Mutagenesis studies indicated that the three cytoplasmic cysteines of the influenza virus A/Japan/305/57 hemagglutinin (HA) are all palmitylated, but to an unequal extent. Replacement of all three cysteines abolished palmitylation, but affected neither HA biosynthesis nor function. Palmitate was not required for HA to be incorporated into virions.

The final 12 carboxy-terminal amino acids of most influenza virus hemagglutinin (HA) subtypes contain three cysteines that are among the most highly conserved residues in the sequences of otherwise highly divergent proteins (8, 14). In several subtypes of HA, each of these cysteines is palmitylated (12, 19). There is one report of the A/Japan/305/57 (Japan) HA requiring palmitate for membrane fusion activity (12), although no evidence for impairment of fusion activity has been observed in other HA subtypes when one or more of the cytoplasmic cysteines are changed to another amino acid (15, 17, 19). No other biological consequence of mutation of the cytoplasmic cysteines of HA has been reported. Since in the absence of selective pressure the genes of influenza virus do not maintain the strong sequence conservation observed for the cytoplasmic cysteines of HA (16), the failure to find a significant biological role for palmitylation of these cysteines creates a paradox. Palmitylation of so many residues within a short sequence must affect local polypeptide structure, probably pulling the cytoplasmic sequences back to the lipid bilayer and certainly influencing the accessibility of the cytoplasmic cysteines to the cytosol. If palmitylation itself is not required, how might the covalently modified cysteine side chains participate in other necessary interactions, as suggested by their strong conservation through viral evolution?

We have investigated palmitate incorporation into the wild-type and mutant HAs of the Japan HA in which the cysteines have been mutated to serine, tyrosine, or tryptophan. The cytoplasmic sequences and nomenclature for these mutants are shown in Table 1. The mutant HA cDNAs were prepared by oligonucleotide-directed mutagenesis (6) and were expressed under the control of the late promoter in recombinant simian virus 40 (SV40) vectors (3, 5). CV-1 cells were infected with recombinant SV40 vectors expressing each of these mutated HAs and 30 to 36 h later were labeled with ³⁵S-amino acids or with [³H]palmitate for 4 h. After the labeling period, the proteins were isolated by immunoprecipitation and analyzed by gel electrophoresis and autoradiography. The ratio of palmitate incorporation to ³⁵S incorporation was determined for each protein and compared with that of the wild-type HA (Table 1). Each of the three cysteines of the Japan HA received palmitate; however, in contrast to a previous report (12), levels of incorporation into each of the three sites were not equal. The cysteines at

positions 543 and 546 appeared to incorporate most of the palmitate, with position 546 incorporating at least half of the fatty acid and position 536 incorporating only 10%. In addition, for three mutants (CSC, CWC, and CYC) the ratio of palmitate to amino acid labeling varied according to the amino acid substituted at position 543 (Table 1). These observations suggested that some cytoplasmic cysteines of HA normally lack palmitate and that amino acid changes at one cysteine affect the efficiency of palmitylation at the other sites, presumably by changing the peptide structure in the HA tail. The nonuniform palmitylation of HA could be due to variations in the extent of palmitylation of individual monomers of the HA trimer or to different populations of HA trimers. Since these possibilities would be extremely difficult to distinguish experimentally, they were not investigated further.

To determine whether the extent of palmitylation influences the intracellular transport of the Japan virus HA, the kinetics and extent of trimerization and intracellular transport of HAs lacking one or more palmitylation sites were measured by techniques published previously (1, 9) (Table 2). None of the mutants, including the SSS protein that lacks palmitate, were transported differently from wild-type HA, confirming previous observations on the rate of surface arrival reported by Naeve and Williams (12), who introduced different amino acid changes at the same sites.

The HA protein has a well-characterized membrane fusion activity (21). In four published investigations, only the fusion activity of the Japan H2 HA was found to be affected by loss of a palmitylation site (12), whereas mutation of the analogous site in H3 (15, 17) and H7 (19) HAs had no effect on fusion. Here we have investigated the fusion activity of the Japan HA from the cDNA used by Naeve and Williams expressed by the same SV40 late-replacement vector in the same CV-1 cell type. We observed that CV-1 cells expressing acylation-free mutant HAs (SSS or SYS) induced the formation of syncytia as well as the wild-type Japan HA did (Fig. 1).

HA has recently been shown to be highly mobile in the plasma membrane (4) but, for as-yet-unexplained reasons, is excluded from coated pits (13). Other proteins that resemble HA in this behavior are glycolipid-linked proteins such as Thy-1 that lack cytoplasmic tails (10) and receptors from which the cytoplasmic sequences have been deleted (2, 11). To investigate whether palmitylation might be responsible for the ability of HA to escape the endocytic pathway and the acid bath that would result from internalization, we

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TABLE 1. Incorporation of palmitate into cytoplasmic sequences of mutant HAs

Protein ^a	Amino acid at position ^b :												Palmitate incorporation ^c		
	536	537	538	539	540	541	542	543	544	545	546	547	Expt 1	Expt 2	Expt 3
CCC	C	S	N	G	S	L	Q	C	R	I	C	I	1.0	1.0	1.0
SCC	S	—	—	—	—	—	—	C	—	—	C	—	0.9	0.8	0.8
CSC	C	—	—	—	—	—	—	S	—	—	C	—	0.3	0.2	0.2
CWC	C	—	—	—	—	—	—	W	—	—	C	—	0.5	0.6	
CYC	C	—	—	—	—	—	—	Y	—	—	C	—	0.4	0.5	
CCY	C	—	—	—	—	—	—	C	—	—	Y	—	0.3	0.5	0.4
CY*	C	—	—	—	—	—	—	Y	—	—	*	—	0.1	0.1	0.1
SYS	S	—	—	—	—	—	—	Y	—	—	S	—	0.0	0.0	0.0
SSS	S	—	—	—	—	—	—	S	—	—	S	—		0.0	

^a Proteins are designated by the amino acid found at positions 536, 543, and 546.

^b The sequences shown are predicted to be cytoplasmic by a hydropathy algorithm (7). The wild-type HA (CCC) cytoplasmic amino acid sequence is given in the single-letter code. —, wild-type residue other than cysteine at that position; *, stop codon. The identities of residues changed from the wild type are given.

^c Values are normalized to the ratio of palmitate to ³⁵S-amino acid incorporation for wild-type HA. Values for experiment 1 are the averages of duplicate measurements, and those for experiments 2 and 3 are the averages of triplicate measurements.

compared the internalization rates of wild-type and mutant HAs by published techniques (5, 8). The SSS mutant lacking palmitate was internalized at 0.1%/min, a rate identical to that of HA. Palmitate is not responsible for keeping HA out of coated pits.

Using recombinant vaccinia virus vectors expressing the A/Udorn/72 (Udorn) H3 HA, Simpson and Lamb (15) have reported that deletion of the carboxy-terminal 10 amino acids, including two palmitylation sites, results in a 100-fold decrease in infectivity of pseudotyped A/WSN/33 (WSN) ts61S virus. Mutation of the final two cysteines alone of Udorn HA has a modest effect, if any. However, the first of the three palmitylation sites was not changed in those studies, nor was the extent of incorporation of palmitate measured for the different Udorn HA mutants. To determine whether Japan HA with altered cysteine residues could complement the ts61S virus, which does not transport HA to the plasma membrane at 39.5°C (14, 18), CV-1 cells were first infected with SV40 vectors to express either wild-type HA (CCC) or mutants that lack all the potential palmitylation sites (SSS or SYS). At 30 to 32 h postinfection, cells were superinfected with ts61S virus at 32.5°C at a multiplicity of infection of 10. Cells were incubated at 39.5°C for 8 to 10 h, and either the virus was purified from culture medium on 20 to 60% sucrose gradients or virus titers were determined by plaque assay on MDCK cells at 32.5°C. Cultures infected with ts61S virus alone at 39.5 or 32.5°C served as controls. Figure 2 shows that only at the permissive temperature (32.5°C) could ts61S HA be recovered from sucrose gradi-

TABLE 2. Folding and intracellular transport of HA mutants

Protein	Half-life of sensitivity to trypsin (min) ^a	Extent of folding at 20 min ^b	Half-life of arrival at cell surface (min) ^c	% of proteins at surface at 120 min
CCC	11.5, 14.4	0.77	26	93
CYC	12.1, 13.2	0.81	27	93
CYS	11.7, 14.0	0.78	27	94
SYS	12.6, 12.8	0.79	28	97
SSS	12.4, 13.2	0.80	30	98
SCC	12.5, 15.2	0.77	30	98

^a Values are minutes after a 2-min pulse of radioactive amino acids.

^b Values are the averages of two experiments.

^c Values are minutes after a 5-min pulse with radioactive amino acids.

FIG. 1. Cell-cell fusion mediated by HA mutants SYS (A) and SSS (B) lacking fatty acids. Mutant HAs on the surfaces of CV-1 cells infected with recombinant SV40 vectors were activated for fusion by brief treatment with trypsin and exposure to pH 5.0. Eight hours later, cell monolayers were fixed with methanol, stained with Giemsa, and photographed at a magnification of $\times 200$. The cell monolayers contained many aggregates of nuclei sitting in a common cytoplasm, indicating that syncytia had formed.

FIG. 2. Fluorogram of immunoprecipitates of gradient fractions containing virus. Mutant Japan virus HAs are incorporated into *ts61S* virions at 39.5°C. Virus-infected CV-1 cells were labeled with ³⁵S-amino acids, and viruses were isolated from culture supernatants by centrifugation on sucrose gradients. Lanes marked *ts61S* were infected with the temperature-sensitive A/WSN/33 virus alone at the temperature indicated. Lanes marked *ts61S* mut were infected first with recombinant SV40 vectors expressing the CCC (lane 3), SYS (lane 4), or SSS (lane 5) Japan virus proteins, and then were superinfected 36 h later with *ts61S* virus at a multiplicity of infection of 10. The uncleaved hemagglutinin (HAo), the nucleoprotein (NP), and the matrix protein (M) are indicated. Proteins in lanes 1 and 2 were precipitated with anti A/WSN/33 rabbit serum prepared against whole virus. Proteins in lanes 3 through 5 were precipitated with an anti-Japan virus rabbit serum that cross-reacts with the A/WSN/33 NP but does not recognize the A/WSN/33 HA.

ents (lanes 1 and 2). At the nonpermissive temperature, HA CCC, HA SYS, and HA SSS (Fig. 2, lanes 3, 4, and 5, respectively) were incorporated into *ts61S* virus. A band that migrated faster than the WSN nucleoprotein that cross-reacted with antisera to either WSN or Japan virus, and that appeared in the absence of SV40 infections, is probably a truncated form of nucleoprotein that has been observed by others (15).

Both wild-type and mutant Japan virus HAs produced by SV40 vectors were incorporated into pseudotype *ts61S* viruses that were capable of infecting cells at 39.5°C but could only be propagated into virus plaques at 32.5°C (Table 3). No significant difference in infectivity was observed for any of the mutant Japan HAs, indicating that modification with palmitate is not required for incorporation of HA into infectious virions. Similar findings were reported recently for the G glycoprotein of vesicular stomatitis virus (20). At 39.5°C, *ts61S* virus produced a low background titer that was probably caused by experimental manipulations, since the infecting virus stock showed a 6-log-unit decrease in titer when virus grown at 32.5°C was compared with virus cultured at 39.5°C. It is important to note that although the titer of *ts61S* virus grown at the permissive temperature was only 4×10^4 PFU/ml, this represents a harvest only of virions produced during the first 4 to 6 h of influenza virus production. WSN virus grown at 37°C for the same period in CV-1 cells in the absence of SV40 produced a similar low titer of virus (Table 3), indicating that complementation of *ts61S* virus by SV40-vector-produced HAs was as efficient as incorporation of WSN HA produced by influenza virus under the same conditions. We did not attempt to prolong the time of infection because cytopathic effects occur much earlier in CV-1 cells doubly infected with SV40 vectors and

TABLE 3. Complementation of *ts61S* virus with wild-type or mutant Japan HA

Protein ^a	Surface expression ^b	Titer ^c at temp of:	
		32.5°C (permissive)	39.5°C (nonpermissive)
CCC	1.0	$(9.5 \pm 1.2) \times 10^3$	0
SCC	1.2	$(8.0 \pm 1.0) \times 10^3$	0
SSS	1.3	$(10.0 \pm 1.0) \times 10^3$	0
CYC	1.2	$(8.2 \pm 1.2) \times 10^3$	0
CYS	1.2	$(9.0 \pm 1.1) \times 10^3$	0
SYS	1.3	$(8.0 \pm 1.0) \times 10^3$	0
<i>ts61S</i> at 32.5°C	ND	$(5 \pm 4) \times 10^4$	$(3.0 \pm 2) \times 10^1$
<i>ts61S</i> at 32.5°C	ND	$(5 \pm 4) \times 10^4$	$(3.0 \pm 2) \times 10^1$
<i>ts61S</i> at 39.5°C	ND	$(6.9 \pm 2.0) \times 10^1$	0
WSN at 37°C	ND	$(5 \pm 0.5) \times 10^4$	

^a Japan virus HAs were produced by recombinant SV40 vectors in CV-1 cells. *ts61S* HA was produced by infection of CV-1 cells with the temperature-sensitive influenza virus at the temperatures shown.

^b HA protein expression for mutant HAs is normalized to that of Japan virus. ND, not determined.

^c Expressed as PFU per milliliter of culture supernatants. Values are the averages of three experiments with standard deviations shown.

influenza viruses, complicating comparisons of infectivity of viruses produced during longer periods of infection.

Our results extend and confirm previous observations that palmitoylation of the highly conserved cytoplasmic cysteines of influenza virus HA is not required for biological function, at least in the laboratory. Furthermore, our finding that palmitoylation of the HA cysteines is incomplete resolves the paradox of how palmitate addition can be simultaneously unnecessary but not inhibitory. A simple explanation is that the HA cytoplasmic cysteines are conserved for an as-yet-unknown reason and that most HA trimers contain an unmodified cysteine (or cysteines) capable of participating in that function.

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