JOURNAL OF VIROLOGY, Nov. 1995, p. 6643–6651 Vol. 69, No. 11
0022-538X/95/$04.00+0

Studies of the Membrane Fusion Activities of Fusion Peptide Mutants of Influenza Virus Hemagglutinin

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Received 25 May 1995/Accepted 4 August 1995

Influenza virus hemagglutinin (HA) fuses membranes at endosomal pH by a process which involves extrusion of the NH_2-terminal region of HA_2, the fusion peptide, from its buried location in the native trimer. We have examined the amino acid sequence requirements for a functional fusion peptide by determining the fusion capacities of site-specific mutant HAs expressed by using vaccinia virus recombinants and of synthetic peptide analogs of the mutant fusion peptides. The results indicate that for efficient fusion, alanine can to some extent substitute for the NH_2-terminal glycine of the wild-type fusion peptide but that serine, histidine, leucine, isoleucine, or phenylalanine cannot. In addition, mutants containing shorter fusion peptides as a result of single amino acid deletions are inactive, as is a mutant containing an alanine instead of a glycine at HA_2 residue 8. Substitution of the glycine at HA_2 residue 4 with an alanine increases the pH of fusion, and valine-for-glutamate substitutions at HA_2 residues 11 and 15 are without effect. We confirm previous reports on the need for specific HA_0 cleavage to generate functional HAs, and we show that both inappropriately cleaved HA and mutant HAs, irrespective of their fusion capacities, upon incubation at low pH undergo the structural transition required for fusion.

Influenza virus hemagglutinin (HA) has two functions in the initial stages of virus infection. It is responsible for binding virus to sialylated cell surface receptors, and following endocytosis, it is activated at endosomal pH to mediate the fusion of viral and endosomal membranes, which allows viral nucleocapsids to enter the cell (39). The HA is a trimer of identical subunits, each of which contains two polypeptide chains, HA_1 and HA_2. These are formed by the proteolytic cleavage of a biosynthetic precursor, HA_0, which generates the COOH terminus of HA_1 and the NH_2 terminus of HA_2. In the X-31 (H3 subtype) HA structure (40), the COOH-terminal HA_1 residue, threonine 328 ($T328_1$), is approximately 20 Å (2 nm) from the HA_2 NH_2 terminus, indicating a change in structure upon cleavage of HA_0. As has been shown for a number of viral fusion glycoprotein precursors (14), the cleavage of HA_0 is necessary for the activation of fusion activity and consequently for virus infectivity (11, 12, 15, 17, 20, 38). Cleavage of the HAs of most influenza viruses is mediated extracellularly, possibly by proteases such as those secreted by Clara cells in the lung (13) or by coinfecting bacteria (26, 31), and occurs COOH terminally to an arginine residue (R329 in X-31 HA_0) which is subsequently removed from the COOH terminus of HA_1 (7). In the HA_0s of highly pathogenic viruses, however, the conserved arginine is part of a polybasic sequence, such as KREKR in fowl plague virus HA_0 (2). These HA_0s are cleaved intracellularly in a variety of cell types by subtilisin-like proteases, such as furin or PC6 (10, 30, 35).

With H1, H3, and H10 subtype viruses, in vitro cleavage of HA_0 with enzymes of different specificities yields NH_2-terminal sequences different from the highly conserved NH_2-terminal sequence of HA_2, e.g., thermolysin and chymotrypsin truncate HA_2 by one and three residues, respectively (6). These cleavages fail to activate infectivity and indicate a requirement for specific HA_0 cleavage. Together with observations of the structure and properties of the NH_2 terminus of HA_2 derived from mutant selection (5, 22) and of site-specific mutagenesis (8, 34), protein chemical studies (24), and direct analyses of the membrane fusion properties of analogous synthetic peptides (18, 21, 36), they have contributed to the designation of this region as the fusion peptide. Conserved hydrophobic NH_2-terminal fusion peptides have been identified in the fusogenic glycoproteins of a number of enveloped viruses. However, for none of these, including the influenza virus HA, is the mechanism of fusion understood, and details concerning the role of fusion peptides in the fusion process are not available.

Currently, influenza virus HA provides the best model system for studies of virus membrane fusion, as structural information is available for both the native protein (40) and the conformation of the molecule at the pH of fusion (3). In native HA, the fusion peptide is buried in the center of the trimer, about 30 Å (3 nm) from the virus membrane (40). Activation of HA membrane fusion potential at endosomal pH requires its extrusion from this buried location, whereupon it can interact with the target membrane as a result of extensive molecular rearrangements (3).

The studies reported here were designed to investigate further the dependence of membrane fusion activity on the sequence of the fusion peptide. Sequence data for HAs of numerous strains and mutants of influenza A virus reveal certain common features of the conserved NH_2-terminal domain of HA_2; in vivo proteolytic cleavage of the HA_0 precursor results in a fusion peptide of defined length, charged glutamic acid residues are present within this predominantly hydrophobic domain, and conserved glycine residues are interspersed throughout the region, a common characteristic of most viral glycoprotein fusion peptide sequences. Previous reports (5, 8) have shown that substitutions of particular residues in the HA_2 NH_2-terminal region can lead to changes in membrane fusion properties, suggesting that these residues may be important for

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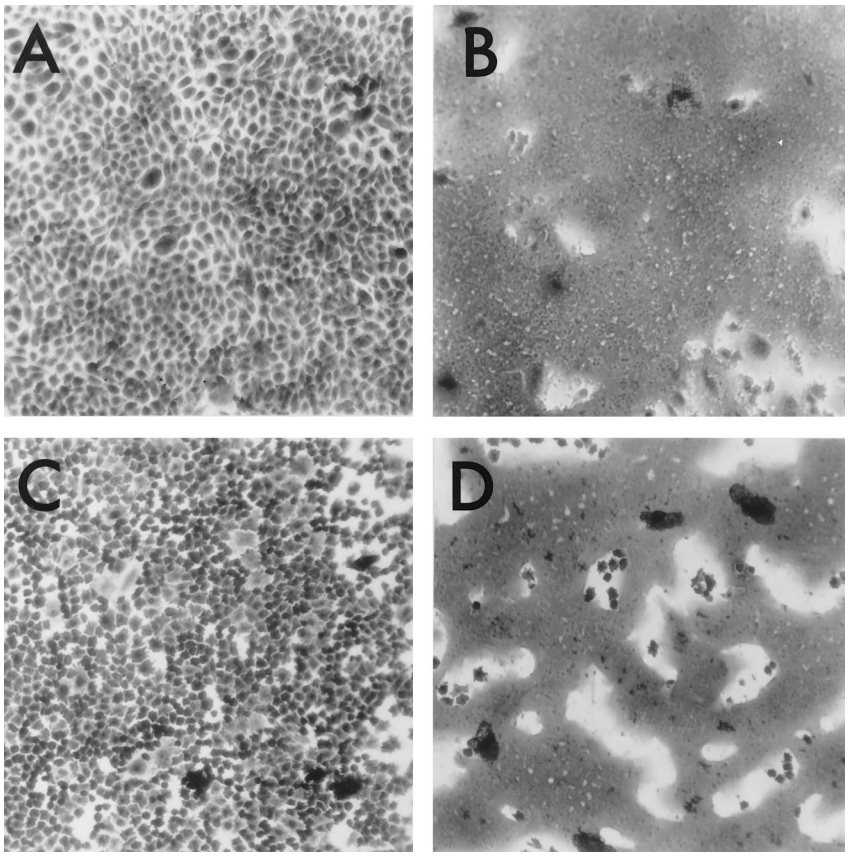

FIG. 1. Heterokaryon formation by cells expressing HA cleaved by trypsin and/or thermolysin. CHO cells constitutively expressing HA_0 were treated with (A) no proteases, (B) 2.5 μg of trypsin per ml for 5 min, (C) 100 μg of thermolysin per ml for 15 min, and (D) 2.5 μg of trypsin per ml for 5 min and then with 100 μg of thermolysin per ml for 15 min. Cells were then incubated at pH 5.1 and 37°C for 1 min and then for 1 h in complete minimal essential medium prior to fixing and staining.

either the stability of the native structure or a direct role of the NH_2-terminal domain in fusion activity.

Here, we address in more detail the sequence requirements of the HA fusion peptide for stability of the native molecule, for lipid association, and for membrane fusion capability. Specifically, we address the requirements for charge and length of the fusion peptide as well as the significance of interspersed glycine residues, with particular attention to the HA_2 NH_2-terminal glycine. We confirm the observations of Garten et al. (6) that the HA_2 polypeptide formed by thermolytic digestion of HA_0 lacks the NH_2-terminal glycine, and we extend them to show that even though the HA has no fusion activity, its structure changes characteristically at low pH. We compare the pH of extrusion of the modified fusion peptide and the pH at which it associates with liposomes with the properties of trypsin-cleaved HA; we determine the importance of the sequence in this region of HA_0 for cell surface expression, HA_0 cleavability, the pH of fusion peptide extrusion, and the pH of fusion by using a series of site-specific mutant HAs; and we compare the fusion activities of synthetic peptide analogs of the mutant fusion peptides with the fusion activities of the mutant HAs.

MATERIALS AND METHODS

Mutagenesis and expression of HAs. Site-specific mutagenesis was carried out by the method of Kunkel et al. (16). The genes for wild-type (WT) and mutant HAs were cloned into vaccinia virus expression vectors that direct transcription from the 7.5K early-late promoter (32) or the cowpox virus p160 late promoter (23), and recombinants were generated with the Copenhagen strain of vaccinia virus as described previously (19). HA-expressing recombinant viruses were plaque purified twice before use in experiments. CHO cells constitutively expressing WT HA are described elsewhere (9).

Conformational change and membrane fusion assays. Conformational change assays by enzyme-linked immunosorbent assays (ELISA) were done with recombinant vaccinia virus-infected HeLa cells at 8 h postinfection as described previously (29). Heterokaryon formation assays were done by using recombinant vaccinia virus-infected BHK cells as described previously (28). Heterokaryon formation assays using the WT HA-expressing CHO cells were done as described elsewhere (9), except that low pH treatment was for 1 min. To estimate the trypsin cleavability of HA_0, CV1 cell monolayers were infected with recombinant vaccinia viruses, and at 12 h postinfection, the cells were washed with phosphate-buffered saline (PBS) and treated with trypsin at 5 μg/ml for 10 min at 37°C, trypsin inhibitor was added (final concentration, 5 μg/ml), the cells were washed

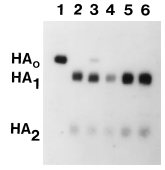

FIG. 2. Cleavage of detergent-extracted rosettes of HA_0 by trypsin and/or thermolysin. The treatments were as follows: lane 1, control rosettes with no proteases; lane 2, 5 μg of trypsin per ml for 10 min; lane 3, 5 μg of thermolysin per ml for 30 min; lane 4, 25 μg of thermolysin per ml for 30 min; lane 5, 5 μg of trypsin per ml for 10 min and then 5 μg of thermolysin per ml for 30 min; and lane 6, 5 μg of trypsin per ml for 10 min and then 25 μg of thermolysin per ml for 30 min. Electrophoresis was done under reducing conditions and was followed by immunoblotting.

TABLE 1. Hemolysis mediated by detergent-extracted rosettes of HA cleaved with trypsin and/or thermolysin[a]

Treatment	% Hemolysis
No treatment	1
Trypsin (5 μg/ml for 10 min)	78
Thermolysin (5 μg/ml for 30 min)	1
Thermolysin (25 μg/ml for 30 min)	1
Trypsin (5 μg/ml for 10 min), then thermolysin (5 μg/ml for 30 min)	81
Trypsin (5 μg/ml for 10 min), then thermolysin (25 μg/ml for 30 min)	84

[a] Detergent-extracted rosettes of HA (25 μg/ml) in 50 μl of PBS were treated as described above at 37°C. Tryptic digestion was stopped by adding an equimolar amount of trypsin inhibitor, and thermolytic digestion was stopped by adding 1 mM *o*-phenanthroline. The rosettes were added to 0.5 ml of 2% (vol/vol) human blood in PBS, and the pH was adjusted to 5.1 with 0.15 M citrate buffer (pH 3.5). The OD_{520} of a 1,000 × *g* supernatant was measured after a 30-min incubation at 37°C. A 100% hemolysis was defined as the OD_{520} of erythrocytes incubated with 0.5% (wt/vol) Brij 36T at pH 5.1. Background hemolysis obtained with 50 μl of PBS was less than 5% and has been subtracted from the values given.

again, and lysates were prepared. Lysates were separated by electrophoresis on 12% polyacrylamide gels under reducing conditions, and immunoblotting was performed with anti-HA rabbit polyclonal antibody and ^{125}I-labelled donkey anti-rabbit second antibody. HA rosettes were prepared and hemolysis assays were done essentially as described previously (9). Equal quantities (25 μg) of rosettes (untreated or trypsin treated) were incubated with erythrocytes for 60 min at pH 5.2 or 7.2, and the optical density at 520 nm (OD_{520}) of the 1,000 × *g* supernatants was determined.

The ectodomain of HA solubilized by bromelain (BHA) was prepared from the membranes of recombinant vaccinia virus-infected cells, and aggregation experiments were done as described previously (9). Lipid association experiments were done by incubating BHA with small unilamellar liposomes at pH 7.0 or 5.0. The mixture was made to 30% (wt/vol) sucrose and was layered under a 10% sucrose solution in PBS, PBS was layered on top, and the liposomes and any associated protein were floated by centrifugation at 100,000 × *g* for 18 h at 4°C.

Peptide synthesis and hemolysis assays. Peptides were made by the F-moc procedure (1) on an Applied Biosystems 430A peptide synthesizer. High-performance liquid chromatography showed them to be >95% pure. Hemolysis was done with stock solutions of peptides in dimethyl sulfoxide as described elsewhere (36).

RESULTS

Properties of thermolysin-cleaved HA. We have shown previously (9) that CHO cells transfected with cDNA for HA constitutively express the precursor HA_0 at the cell surface and that incubation with trypsin leads to the formation of HA_1 and

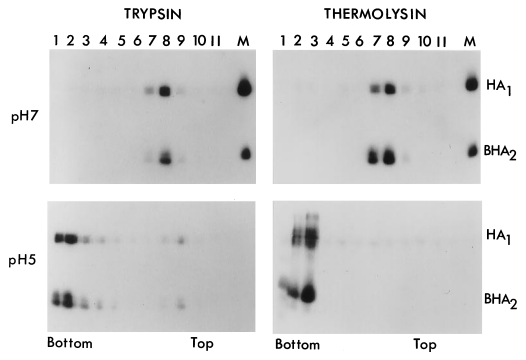

FIG. 3. Aggregation of trypsin-cleaved and thermolysin-cleaved BHA at low pH. Trypsin- and thermolysin-cleaved BHAs were incubated at pH 7 or 5 for 10 min at 37°C, neutralized, and then subjected to sucrose density gradient centrifugation. Lane 1 is the bottom of the gradient and lane 11 is the top. Lane M contains viral BHA as a marker. Electrophoresis was done under reducing conditions.

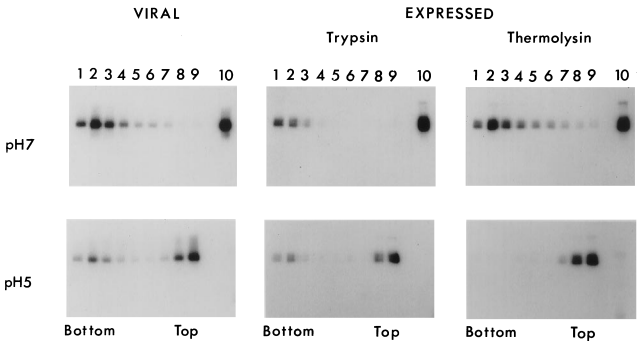

FIG. 4. Association of viral BHA, trypsin-cleaved expressed BHA, and thermolysin-cleaved expressed BHA with liposomes at low pH. BHA preparations were incubated with liposomes at pH 7 and 5 for 10 min at 37°C, neutralized, and subjected to sucrose density gradient centrifugation. The bottom of the gradient contains BHA not associated with liposomes, and the top contains liposomes and liposome-associated BHA. Lane 10 contains marker protein. The gel was run under nonreducing conditions; therefore, the BHA migrates as a single band.

HA_2, which is required for the activation of HA-mediated membrane fusion at acid pH. We have used these cells to show that the inability of thermolysin to activate virus infectivity as demonstrated by Garten et al. (6) is due to the absence of fusion activity associated with HAs cleaved by this protease. One assay for fusion activity is by observing the formation of heterokaryons with decreasing pH. Extensive heterokaryon formation was observed when trypsin-treated cells were incubated at 37°C, pH 5.1, for 1 min (Fig. 1). HA_0 cleaved with thermolysin rather than trypsin had no activity under these conditions. Heterokaryons were seen with cells first treated with trypsin and then with thermolysin, showing that thermolysin treatment did not adversely affect cell integrity or nonspecifically inhibit fusion activity. By ELISA, we also monitored the changes in HA structure required for fusion by using monoclonal antibodies that bind to native HA rather than HA in the fusion pH conformation; antibody HC67 recognizes residues at the membrane distal trimeric interface and only

TABLE 2. Amino acid sequences of HA fusion peptide mutants

HA[a]	Amino acid at position:																
	329		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
WT	R		G	L	F	G	A	I	A	G	F	I	E	N	G	W	E	G
$E15_2V$	-[b]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-
$E11_2V$ and $E15_2V$	-		-	-	-	-	-	-	-	-	-	-	V	-	-	-	V	-
$G8_2A$	-		-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
$G4_2A$	-		-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-
$G1_2A$	-		A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G1_2F$	-		F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G1_2H$	-		H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G1_2I$	-		I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G1_2L$	-		L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G1_2S$	-		S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$A1_2$ insertion	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$A2_2$ insertion	-	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$\Delta G1_2$	-		Δ[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$\Delta L2_2$	-		-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] HA nomenclature indicates amino acid substitutions. As an example, $E15_2V$ refers to a glutamate-to-valine substitution at position 15 for the HA_2 subunit.
[b] -, no substitution.
[c] Δ, deletion.

TABLE 3. Analysis by ELISA of HA conformational change[a]

HA	Ratio of HC67 to HC3 reactivity at pH:									
	7.4	6.4	6.2	6.0	5.8	5.6	5.4	5.2	5.0	4.8
WT	0.65	0.71	0.69	0.71	0.70	0.59	0.27	0.16	0.21	0.25
WT	0.91	0.86	0.85	0.93	0.92	0.87	0.25	0.25	0.30	0.39
WT	0.86	0.91	0.91	0.86	0.98	0.75	0.24	0.27	0.23	0.26
WT	0.78	0.77	0.78	0.89	1.07	0.97	0.39	0.34	0.37	0.36
$E15_2V$	0.78	0.80	0.81	0.75	0.77	0.72	0.36	0.22	0.23	0.31
$E11_2V$ and $E15_2V$	0.87	0.88	0.80	0.82	0.79	0.79	0.36	0.23	0.24	0.26
$G8_2A$	0.69	0.73	0.70	0.39	0.18	0.12	0.10	0.11	0.11	0.10
$G4_2A$	0.83	0.82	0.80	0.58	0.09	0.11	0.13	0.12	0.15	0.20
$G1_2A$	0.56	0.64	0.61	0.63	0.58	0.22	0.11	0.06	0.05	0.07
$G1_2F$	0.84	0.79	0.80	0.81	0.47	0.12	0.09	0.11	0.11	0.14
$G1_2H$	0.43	0.51	0.51	0.52	0.26	0.10	0.06	0.06	0.06	0.10
$G1_2I$	0.61	0.61	0.58	0.60	0.24	0.09	0.07	0.05	0.14	0.06
$G1_2L$	0.93	0.88	0.88	0.86	0.56	0.25	0.15	0.09	0.12	0.14
$G1_2S$	0.43	0.43	0.43	0.41	0.46	0.13	0.04	0.03	0.04	0.11
$\Delta L2_2$	0.89	0.78	0.87	0.84	0.22	0.10	0.09	0.07	0.07	0.07
$\Delta G1_2$	0.50	0.49	0.57	0.54	0.15	0.05	0.03	0.04	0.05	0.06
$A1_2$ insertion	0.74	0.81	0.79	0.80	0.75	0.81	0.84	0.83	0.75	0.79
$A1_2$ insertion	0.81	0.69	0.75	0.70	0.68	0.76	0.64	0.75	0.75	0.68
$A2_2$ insertion	0.85	0.78	0.80	0.83	0.75	0.61	0.60	0.60	0.57	0.57
$A2_2$ insertion	0.62	0.67	0.74	0.63	0.44	0.41	0.34	0.31	0.22	0.26

[a] HC67 reacts only to the native HA, whereas HC3 reacts to both the native and the low pH conformation. Surface expression levels relative to that of the WT, as judged by HC3 reactivity at neutral pH, were determined to be as follows: $E15_2V$, 84%; $E11_2V$ and $E15_2V$, 121%; $G8_2A$, 101%; $G4_2A$, 92%; $G1_2A$, 91%; $G1_2F$, 96%; $G1_2H$, 112%; $G1_2I$, 118%; $G1_2L$, 104%; $G1_2S$, 87%; $\Delta L2_2$, 90%; $\Delta G1_2$, 120%; $A1_2$ insertion, 128%; $A2_2$ insertion, 105%.

binds to native HA, while antibody HC3 binds to HA in both the native and the low pH conformation (4). ELISA analyses showed that the changes in HA structure required for fusion occurred at pH 5.3 at 37°C following trypsin cleavage; changes in thermolysin-cleaved HA were detected at pH 5.65.

We used CV1 cells infected with a vaccinia virus recombinant containing the cDNA for HA to prepare larger quantities of HA_0 for more detailed analyses of the properties of thermolysin-cleaved HA. HA_0 rosettes were prepared by dialysis of purified HA following the detergent extraction of infected cell membranes. As can be seen from Fig. 2, treatment with trypsin at 5 μg/ml for 10 min efficiently cleaved HA_0 into HA_1 and HA_2; 90% cleavage of HA_0 was seen after 30 min of incubation with thermolysin (5 μg/ml), and cleavage was complete with thermolysin at 25 μg/ml. The ability of HA rosettes to lyse erythrocytes at low pH is another assay that correlates with membrane fusion activity (25). The results in Table 1 show that unlike trypsin-cleaved HA, thermolysin-cleaved HA did not mediate hemolysis at low pH, confirming the results of the heterokaryon formation experiments described above. Rosettes of HA cleaved with trypsin before incubation with thermolysin retained their hemolytic activity.

To determine the sites of tryptic and thermolytic cleavage, the NH_2-terminal sequences of the HA_2 polypeptides were determined. Infected cell membranes containing HA_0 were incubated at 37°C without enzymes, with trypsin, or with

TABLE 4. Analysis by ELISA of HA conformational change[a]

HA	Ratio of HC67 to HC3 reactivity at pH:									
	6.2	6.1	6.0	5.9	5.8	5.7	5.6	5.5	5.4	5.3
WT	0.79	0.90	0.85	0.90	0.84	0.87	0.88	0.76	0.32	0.21
WT	0.72	0.77	0.81	0.76	0.75	0.77	0.80	0.64	0.39	0.22
WT		0.82	0.85	0.91	0.82	0.86	0.86	0.79	0.35	0.27
$E15_2V$	0.87	1.00	0.95	0.98	1.00	0.83	0.87	0.74	0.56	0.26
$E11_2V$ and $E15_2V$	0.74	0.73	0.75	0.72	0.75	0.71	0.71	0.69	0.50	0.21
$G8_2A$	0.85	0.82	0.79	0.56	0.29	0.23	0.22	0.18	0.14	0.16
$G8_2A$	1.00	0.81	0.57	0.44	0.20	0.23	0.17	0.20	0.14	0.17
$G4_2A$	0.73	0.81	0.82	0.63	0.34	0.13	0.11	0.12	0.09	0.13
$G4_2A$	0.62	0.61	0.59	0.41	0.25	0.16	0.18	0.16	0.13	0.16
$G1_2A$	1.05	1.00	0.89	0.88	0.87	0.90	0.71	0.19	0.13	0.12
$G1_2A$	0.85	0.76	0.71	0.72	0.78	0.70	0.55	0.30	0.23	0.10
$G1_2F$	0.94	0.96	0.91	0.90	0.87	0.49	0.11	0.09	0.07	0.07
$G1_2H$	0.77	0.78	0.79	0.78	0.79	0.48	0.14	0.10	0.08	0.06
$G1_2I$	0.79	0.81	0.84	0.83	0.80	0.41	0.18	0.13	0.10	0.08
$G1_2L$	0.62	0.68	0.76	0.71	0.72	0.40	0.16	0.12	0.07	0.08
$G1_2S$	0.77	0.80	0.79	0.80	0.87	0.81	0.71	0.29	0.15	0.14
$\Delta G1_2$	0.77	0.77	0.82	0.83	0.77	0.45	0.10	0.08	0.07	0.07
$\Delta L2_2$	0.66	0.67	0.77	0.70	0.58	0.13	0.06	0.03	0.04	0.09
$A2_2$ insertion		0.74	0.75	0.71	0.58	0.54	0.45	0.46	0.48	0.48

[a] See footnote *a* to Table 3.

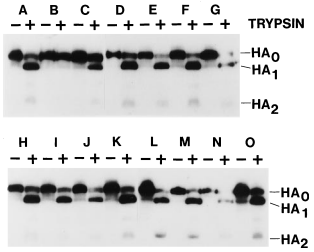

FIG. 5. Western blot (immunoblot) analysis with a reducing 12% polyacrylamide gel of lysates from monolayers of HA-expressing cells following treatment with (+) or without (−) trypsin. (A) WT, (B) $A1_2$ insertion, (C) $A2_2$ insertion, (D) $G1_2A$, (E) $G4_2A$, (F) $G8_2A$, (G) $G1_2F$, (H) $G1_2H$, (I) $G1_2I$, (J) $G1_2L$, (K) $G1_2S$, (L) $E15_2V$, (M) $E11_2V$ and $E15_2V$, (N) $\Delta G1_2$, and (O) $\Delta L2_2$.

thermolysin, and then the soluble ectodomain, BHA, was released from the membranes by bromelain digestion. The HA_2 polypeptides were isolated for analysis by polyacrylamide gel electrophoresis. HA_2 from the trypsin-treated sample had the same sequence, NH_2-GLFGA, as the HA_2 components of infectious virus, whereas the HA_2 of thermolysin-cleaved HA lacked the NH_2-terminal glycine, as did the HA_2 of HA cleaved by bromelain alone.

We also used the soluble BHA preparations to show that the extrusion of the HA_2 NH_2-terminal regions of both thermolysin- and trypsin-cleaved HA occurred at low pH by detecting the formation of aggregates of BHA (Fig. 3). In addition, we showed that both thermolysin- and trypsin-cleaved BHA associated with liposomes at low pH (Fig. 4). The HA aggregation results were confirmed by electron microscopy (data not shown). Together, these experiments show that the inability of thermolysin-cleaved HA to fuse membranes does not appear to result from incorrect folding of the HA on cleavage, from a block in the extrusion of the HA_2 NH_2-terminal region at low pH, or from an inability of the region to associate with lipid bilayers upon extrusion. They suggest that a specific interaction between a functional fusion peptide and the target membrane is required for fusion activity.

Expression and conformational change of mutant HAs. In order to analyze the fusion peptide sequence requirements in more detail, we constructed a series of site-specific mutant HAs listed in Table 2 and expressed them using recombinant vaccinia viruses. All of the mutant HAs were expressed at the surface of infected cells, as evidenced by their reactivity with monoclonal antibodies by ELISA. Analyses of HC3 binding to recombinant-infected cells at neutral pH indicated that the level of cell surface expression of all mutant HAs was similar to that of WT HA, ranging from 84 to 128% of the WT level. $E15_2V$ was expressed at the lowest level, and $A1_2$ insertion was expressed at the highest (Table 3). All except one of the mutant HAs were also cleaved at the surface of infected cells upon incubation with trypsin (Fig. 5); the exception was the mutant with an alanine insertion between HA_0 R329 and G330 (designated as the $A1_2$ insertion in Table 2). ELISA experiments using the conformation-specific antibodies HC67 and HC3 were used to show that all cleaved HAs had the ability to change their structures at low pH (Tables 3 and 4). For WT HA and for all mutants except the uncleavable alanine insertion mutant ($A1_2$ insertion), a clear structural transition pH was defined by the decrease in the ratio of HC67 to HC3 bound (Tables 3 and 4). The structure of the mutant HAs containing valine rather than glutamate at $HA_2$15, or at both $HA_2$11 and $HA_2$15, changed at the same pH as that of WT HA; all other mutations, to different degrees, increased the pH of the structural transition. These results are summarized in Table 5. The results obtained with the $\Delta G1_2$ mutant are in accordance with those obtained with thermolysin-cleaved WT HA which has the identical fusion peptide sequence. In addition, for several mutants ($G1_2A$, $G4_2A$, $G8_2A$, and $\Delta G1_2$), we confirmed the pH of conformational change by using a different assay in which the characteristic susceptibility of HA to tryptic digestion at the pH of fusion (27) was determined (data not shown).

Fusion activity of mutant HAs. The capacity of WT and mutant HAs to mediate membrane fusion was assayed by observing the formation of heterokaryons by HA-expressing cells and by determining the hemolytic activity of HAs isolated by detergent extraction of infected cell membranes. BHK cells infected with the recombinant vaccinia viruses were treated with trypsin at 15 h postinfection to cleave HA_0, washed, incubated at pH 5.0 for 1 min, and then neutralized. Following incubation in complete minimal essential medium for 1 h at 37°C, the cells were fixed and stained with toluidine blue (1%). The results in Fig. 6 show that substitution of valine for glutamic acid at $HA_2$15 or at both $HA_2$11 and $HA_2$15 had no effect on the capacity to form heterokaryons. Mutants with substitutions of alanine for glycine at $HA_2$1 or $HA_2$4 caused heterokaryon formation, but consistently less extensively than that by WT HA. Under these conditions, very little, if any, heterokaryon formation was detected by any other mutant HA or by WT HA which was not treated with trypsin to cleave HA_0. However, increasing the time of incubation at pH 5.0 and the subsequent time of incubation at 37°C showed that mutants $G1_2F$, G1I, $G1_2L$, $G1_2H$, and $G1_2S$ could cause heterokaryon formation at a low but clearly detectable level compared with that observed for the other mutant infected cells, for trypsin-treated vaccinia virus-infected control cells, or for non-trypsin-treated, WT HA-expressing cells. An example of cells expressing the mutant $G1_2F$ is shown in Fig. 7. For all mutants capable of heterokaryon formation, the pH at which this occurred was determined (data not shown). In each case, the pH at which heterokaryon formation was observed mirrored the pH of conformational change as determined by ELISA (Tables 3 to 5).

Hemolysis experiments were done with HA rosettes prepared by detergent extraction of membranes from cells infected with recombinant vaccinia viruses expressing WT HA and the mutants $\Delta G1_2$ and $G1_2F$. In separate experiments with

TABLE 5. pH of conformational change

HA	pH of change[a]	ΔpH[b]
WT	5.48	
$E15_2V$	5.45	0.0
$E11_2V$ and $E15_2V$	5.45	0.0
$G8_2A$	5.97	+0.5
$G4_2A$	5.92	+0.4
$G1_2A$	5.63	+0.2
$G1_2F$	5.76	+0.3
$G1_2H$	5.76	+0.3
$G1_2I$	5.79	+0.3
$G1_2L$	5.74	+0.3
$G1_2S$	5.62	+0.1
$\Delta G1_2$	5.79	+0.3
$\Delta L2_2$	5.83	+0.4
$A2_2$ insertion	5.78	+0.3

[a] The pH of conformational change was determined graphically by plotting the data in Tables 3 and 4 against pH. The midpoint of the slope was designated as the pH of conformational change.

[b] Differences in the pH of conformational change relative to that of the WT are given to the nearest 0.1 pH.

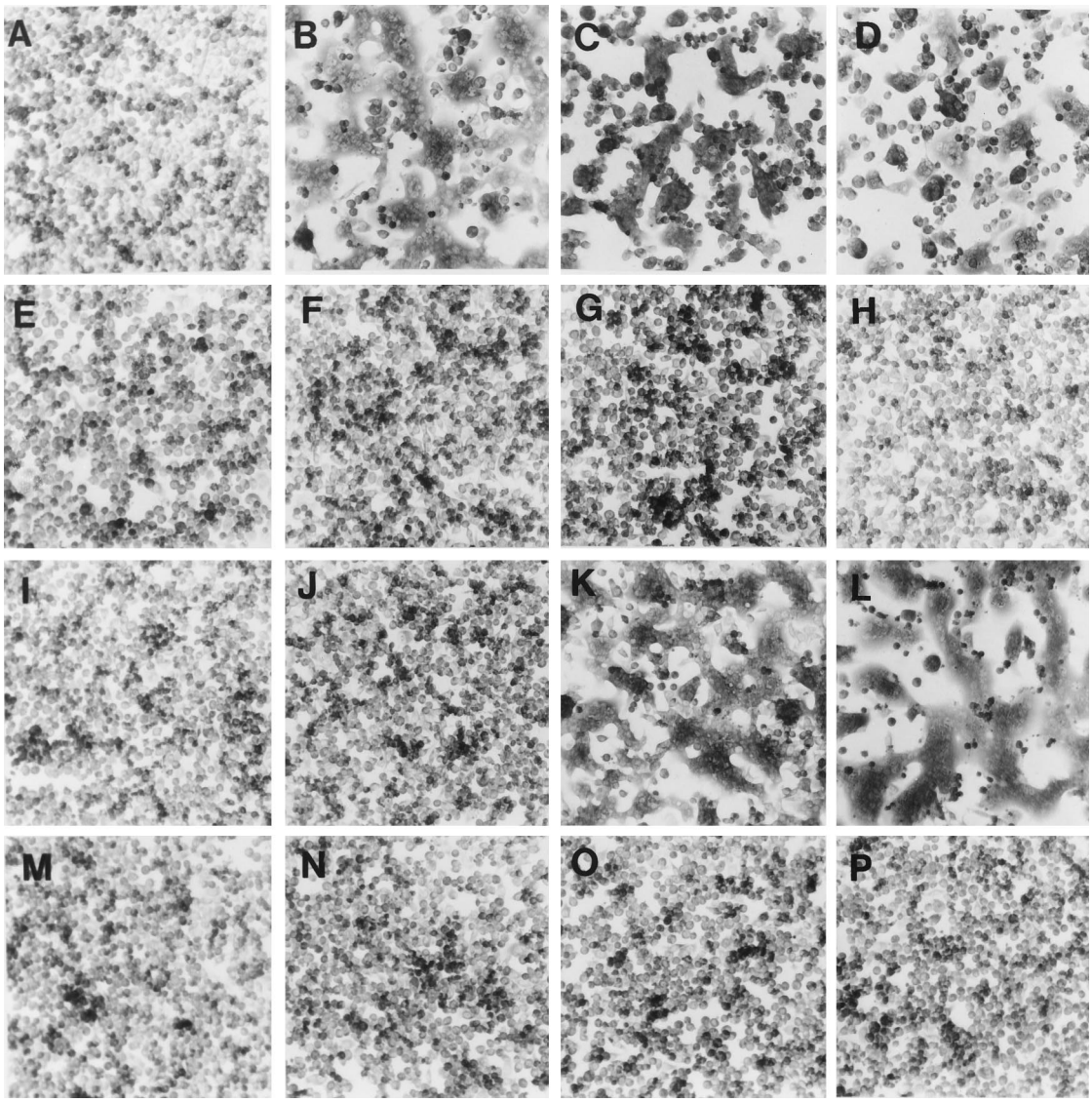

FIG. 6. Heterokaryon formation by HA-expressing BHK cells following incubation at pH 5.0 with (A) WT (no trypsin), (B) WT, (C) $G1_2A$, (D) $G4_2A$, (E) $G8_2A$, (F) $G1_2F$, (G) $G1_2H$, (H) $G1_2I$, (I) $G1_2L$, (J) $G1_2S$, (K) $E15_2V$, (L) $E11_2V$ and $E15_2V$, (M) $\Delta G1_2$, (N) $\Delta L2_2$, (O) $A1_2$ insertion, and (P) $A2_2$ insertion. Cells shown in panels B to P were all trypsin treated prior to pH adjustment.

rosettes formed following the dialysis of sucrose density gradient-purified HAs, hemolysis by the mutant HAs was greatly reduced in comparison with that by the WT HA (Table 6), consistent with the results of heterokaryon formation and the results obtained with thermolysin-treated WT HA rosettes.

Hemolysis mediated by synthetic peptides. We have shown before (36) that the membrane fusion activities of synthetic analogs of mutant fusion peptides correlate with those of corresponding mutant HAs. The results presented in Table 7 indicate that these correlations were extended with synthetic peptides analogous to several of the fusion peptides of the mutant HAs described here. In addition, the peptide analog of the fusion peptide of the uncleaved insertion mutant, i.e., a peptide with an additional alanine residue at the NH_2 terminus of the WT fusion peptide, was found to have hemolytic activity. As was reported previously (36) for such fusion peptide analogs, when any of the peptides studied here were incubated with liposomes or with brominated Brij 96, their fluorescence spectra due to tryptophan showed that they were capable of interacting with membranes and detergents.

DISCUSSION

In these studies of the structural properties of the HA fusion peptide, we have confirmed and extended a number of conclusions made previously from experiments with mutant HAs (8, 34). We have shown that cosubstitution of the glutamates at $HA_2$11 and $HA_2$15 with valines affected neither the capacity for nor the pH of heterokaryon formation, and thus there is no requirement for an acidic residue within the first 15 residues of the HA fusion peptide. It has been reported before (8) that a glutamate-to-glycine substitution at residue 11 of A/Japan/305/57 (H2 subtype) HA similarly had no effect on the pH of conformational change and that erythrocyte fusion was indistinguishable from that with WT HA. However, these studies suggested that glycine at this position was detrimental for

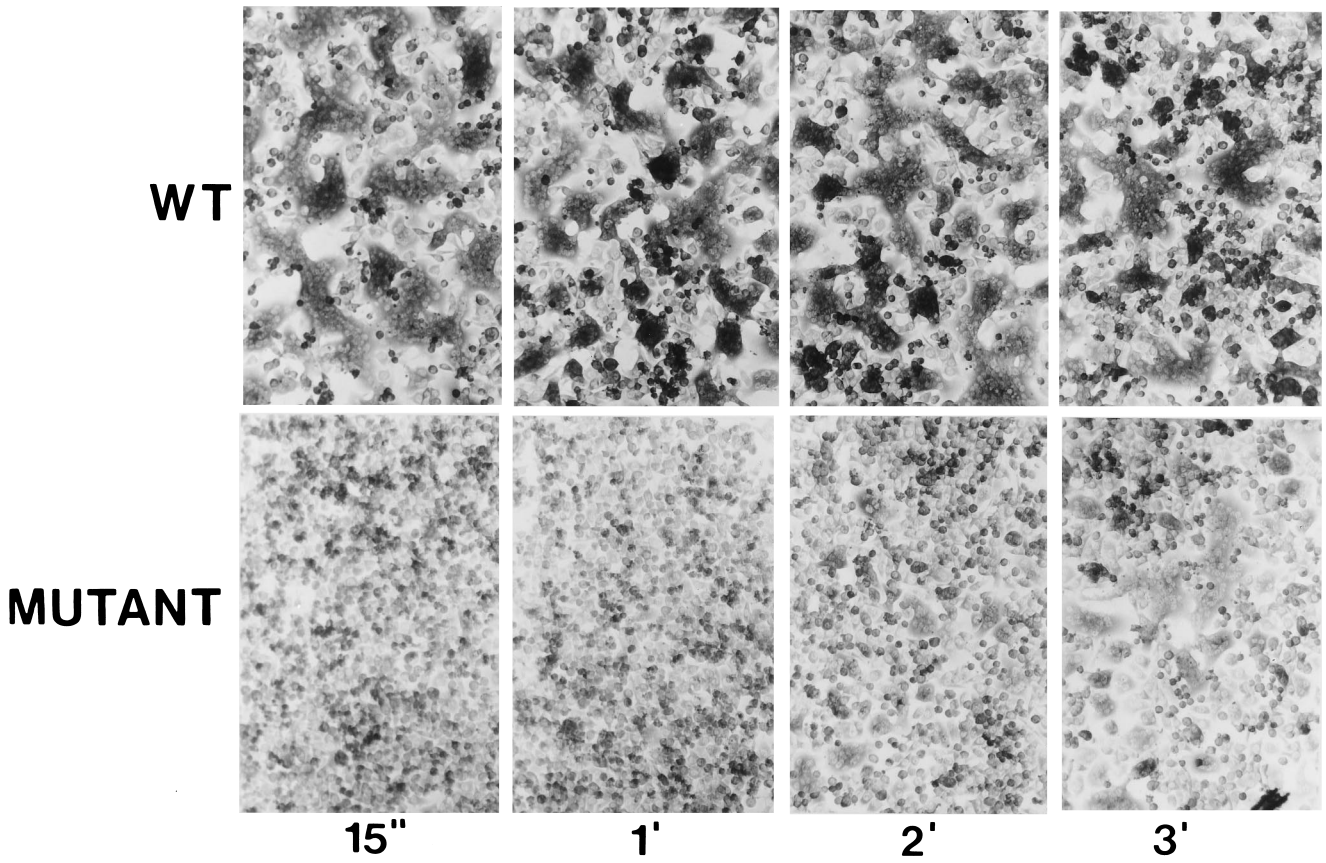

FIG. 7. Heterokaryon formation by BHK cells expressing WT HA or mutant $G1_2F$ HA following incubation at pH 5.0 for the indicated times.

cell-cell fusion activity. It was also reported (8) that glycine-to-glutamate substitutions at $HA_2$1 and $HA_2$4 each affected membrane fusion properties, and we have confirmed the importance of glycine residues at these positions for HA stability and fusion activity by substituting them individually with alanine. Fusion was allowed in both cases, but the stability of the HAs decreased, as judged by their elevated pH of fusion, indicating the importance of the glycine residues for the structure of the native WT HA. Our observations from heterokaryon formation and hemolysis assays using synthetic peptides that the $G1_2A$ mutation causes a decrease in fusion activity are in agreement with the results of Walker and Kawaoka (34), who reported that this substitution in an HA of the H5 subtype reduced fusion activity to 40% of that of the WT. We have also shown here that a glycine-to-alanine substitution at $HA_2$8 not only decreases the stability of the native HA but also inhibits the formation of heterokaryons. Glycines are found at this approximate 4-residue spacing in the fusion peptides of other virus fusion proteins, suggesting some common feature of their native structures or perhaps an additional importance for these residues in the fusion-active structures.

We have also extended our previous correlations between the fusion capacities of synthetic peptide analogs of mutant fusion peptides and corresponding HA mutants, not only for

TABLE 6. Hemolysis caused by detergent-extracted rosettes of HA

HA	% Hemolysis[a]			
	With trypsin		Without trypsin	
	pH 7.2	pH 5.2	pH 7.2	pH 5.2
WT	2	74	1	12
$\Delta G1_2$	2	16	1	8
WT	2	75	2	3
$G1_2F$	2	5	2	4

[a] Results of two experiments are expressed as percentages of the total hemolysis caused by incubation of an equivalent aliquot of erythrocytes in 1% Brij 36T.

TABLE 7. Hemolytic activity at pH 5.1 of synthetic peptides with sequences corresponding to those of the fusion peptides of mutant HAs[a]

Synthetic peptide	% Hemolysis
WT	91
$\Delta G1_2$	1
$A2_2$ insertion	6
$A1_2$ insertion	78
$G1_2I$	1
$G1_2F$	1
$G1_2A$	49
$\Delta L2_2$	8

[a] Two microliters of a stock solution of peptide (5 mg/ml in dimethyl sulfoxide) was added to 0.5 ml of 2% human blood in PBS. The pH was adjusted to 5.1 with 0.15 M citrate buffer (pH 3.5), and the OD_{520} of a 1,000 × *g* supernatant was measured after a 20-min incubation at 37°C. A 100% hemolysis was defined as the OD_{520} of erythrocytes incubated with 0.5% Brij 36T at pH 5.1. Background values (2 μl of dimethyl sulfoxide) are subtracted from the values shown. Background values were less than 5%.

TABLE 8. Amino acid sequences of HA with fusion activity

HA_2 residue											Reference
1	2	3	4	5	6	7	8	9	10	11	
G	L	F	G	A	I	A	G	F	I	E	33[b]
A	-[a]	-	-	-	-	-	-	-	-	-	34, this paper
L	F	L	-	-	-	-	-	-	-	-	22
L	L	L	-	-	-	-	-	-	-	-	22
L	I	L	-	-	-	-	-	-	-	-	22
-	I	-	-	-	-	-	-	-	-	-	33
-	-	L	-	-	-	-	-	-	-	-	5
-	-	-	A	-	-	-	-	-	-	-	This paper
-	-	-	E	-	-	-	-	-	-	-	8
-	-	-	-	-	M	-	-	-	-	-	5
-	-	-	-	-	-	-	-	L	-	-	5
-	-	-	-	-	-	-	-	-	-	V	This paper

[a] -, no substitution.
[b] WT HA.

amino acid substitutions but also for fusion peptide length. Our results with both synthetic peptides and mutant HAs suggest that only alanine may be an acceptable substituent for the NH_2-terminal glycine. All other substitutions analyzed greatly decreased fusion activity, which is consistent with the previous reports concerning a glutamate substitution at this position (8) and removes the possibility that a negatively charged residue was exclusively responsible for this fusion-negative phenotype. The length of the fusion peptide seems also to be an important factor, since insertion of alanine at $HA_2$2 and deletion of the NH_2-terminal glycine or the leucine at $HA_2$2 also prevent fusion. By contrast, the synthetic peptide with an additional alanine at the NH_2 terminus, NH_2-AGLF, was fusion active. However, we could not compare this with the equivalent insertion mutant, since when it is expressed, this mutant HA was not cleaved by trypsin into HA_1 and HA_2 and thus was not fusogenic. It remains a possibility, therefore, that HAs with longer fusion peptides such as this would be capable of fusion if properly cleaved.

For thermolysin-digested BHA, which lacks the N-terminal glycine residue, we showed that the lack of membrane fusion activity was not due to an inability to associate with lipid, a finding similar to results obtained previously for the glycine-to-glutamic acid mutant at the NH_2 terminus of HA_2 (8). Likewise, all synthetic peptides analyzed, including nonfusogenic ones, were capable of interacting with detergent and membranes. These results are similar to those reported for other peptide analogs (36).

The HA_2 NH_2-terminal fusion peptide domain is the most highly conserved region in the HA. However, it is clear, particularly from site-specific mutagenesis experiments (8, 34) and mutant selection studies (5, 22), that a number of residues in this region can be substituted without a loss of fusion activity (Table 8). There appear to be two molecular requirements for the biological activity of fusion peptides. First, following cleavage, HAs must fold to form a stable structure in which the fusion peptide is buried in the trimer at neutral pH and can be extruded at low pH to participate in membrane fusion. Second, on extrusion, the fusion peptide must assume a fusion-active conformation, with the ability to interact with lipid membranes in such a way as to cause fusion. Our studies demonstrate that neither a particular NH_2-terminal residue nor a particular fusion peptide length seems to be necessary for the folding of the HA, as judged by conformation-specific monoclonal antibody binding to mutant HAs. They also appear to be unnecessary for the changes in HA structure required for fusion, as judged by changes in monoclonal antibody binding and by proteolytic digestion of mutant HAs in the low pH conformation, or for mutant fusion peptide extrusion, as judged by low pH-dependent aggregation and lipid association of BHA prepared from trypsin- or thermolysin-cleaved HA_0. Both a defined length and a specific NH_2-terminal residue may be necessary for a functional association of the fusion peptide with lipid, which has not as yet been defined and which may influence the efficiency of the fusion process. Clearly, there are differences in the efficiencies of fusion displayed by the fusion proteins of different viruses which contain fusion peptides with amino acid sequences similar to but different from that of the influenza virus HA. For example, Sendai virus is much less efficient as a fusogen than influenza virus (37), and these differences may be related to differences in the structures that their fusion peptides assume or the precise way in which they interact with membranes. It is also possible that differences in fusion rate or efficiency may account for the observation that HA mutants dependent on cleavage by thermolysin are infectious (22). The recent studies of Orlich and Rott (22) clearly show that HA mutants of A/Seal/Mass/80 virus (H7 subtype) with fusion peptide NH^2-terminal sequences LFLG, LILG, and LLLG and in which the length of the fusion peptides has been maintained by leucine insertion are viable when cleaved by thermolysin. These observations are consistent with the conclusion that the length of the fusion peptide may be important for fusion. However, the findings that the NH_2-terminal residue of the fusion peptide of an infectious virus can be leucine appear to differ from our observation that efficient membrane fusion requires either a glycine or an alanine residue at this position, although none of the thermolysin-dependent mutants had the exact amino acid sequence as the mutant in our study, with leucine at the NH_2 terminus. Examination of these mutants is clearly necessary to understand the differences between in vivo and in vitro assays of fusion, which may explain the apparent contradiction, since the latter are essential for studies of the mechanism of HA-mediated membrane fusion. It may be that specific sequence changes affect the secondary structure of the fusion peptide or the manner of membrane interaction. The elucidation provided by these and other studies of the sequence requirements for fusion activity, including the knowledge that synthetic peptides can mimic the fusion properties of intact HAs, should provide a framework for the design of experiments on the mechanism of membrane fusion.

ACKNOWLEDGMENTS

We thank Carol Newman, David Stevens, Rose Gonsalves, Lesley Calder, and Alan Douglas for excellent technical assistance; Alan Harris for amino acid sequencing; and Peter Fletcher for peptide synthesis.

This work was supported by the MRC and NIH (A1-13654 to D.C.W.). D.C.W. is an investigator of the Howard Hughes Medical Institute.

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