Studies on the Mechanism of Membrane Fusion: Site-specific Mutagenesis of the Hemagglutinin of Influenza Virus

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Abstract. Oligonucleotide-directed mutagenesis of a cDNA encoding the hemagglutinin of influenza virus has been used to introduce single base changes into the sequence that codes for the conserved apolar “fusion peptide” at the amino-terminus of the HA2 subunit. The mutant sequences replaced the wild-type gene in SV40-HA recombinant virus vectors, and the altered HA proteins were expressed in simian cells. Three mutants have been constructed that introduce single, nonconservative amino acid changes in the fusion peptide, and three fusion phenotypes were observed: substitution of glutamic acid for the glycine residue at position 4 in HA2 raised the threshold pH and decreased the efficiency of fusion; and, finally, extension of the hydrophobic stretch by replacement of the glutamic acid at position 11 with glycine yielded a mutant protein that induced fusion of erythrocytes with cells with the same efficiency and pH profile as the wild-type protein. However, the ability of this mutant to induce polykaryon formation was greatly impaired. Nevertheless, all the mutant proteins underwent a pH-dependent conformational change and bound to liposomes. These results are discussed in terms of the mechanism of HA-induced membrane fusion.
HA (6, 39). Such experiments using cells infected with different influenza viruses have shown that HA-induced cell fusion displays a characteristic pH profile, with the threshold pH varying between 5 and 6 depending on the viral origin of the HA molecule (16, 49). To confirm the central role of HA in the fusion mechanism, we have previously used cells that express HA from a cloned copy of the HA gene inserted into recombinant SV40 or BPV vectors to demonstrate that the HA molecule displays fusion activity in the absence of any other influenza virus-encoded components (39, 48). The HA undergoes a conformational change with a pH dependence that parallels that of the fusion activity (5, 44). This conformational change can be monitored by following the acquisition of protease sensitivity by an ectodomain fragment of HA (BHA) released from the membrane by treatment with bromelain (2) and by the ability of this fragment to bind to lipid vesicles or detergents or to aggregate in lipid- or detergent-free solutions (5, 44).

Two genetic approaches are currently being employed to analyze the mechanism of HA-mediated fusion and to identify particular amino acids in HA that are directly involved in the fusion process. The first involves detection or selection of variant viruses that induce fusion with raised pH thresholds. Sequence analysis of the HA from each variant locates altered residues that play a role in the pH dependence of fusion (4, 5a, 38). The second approach, illustrated in this paper, involves the use of site-specific mutagenesis of the cloned HA gene to alter the nucleotide sequence that codes for selected residues of the fusion peptide in the amino-terminus of HA2. Expression of the mutant genes in simian cells has confirmed the central role of the fusion peptide and provided insights into the fusion mechanism.

Materials and Methods

Recombinant DNA Techniques

Oligonucleotides were synthesized using phosphotriester chemistry on a Biosearch Synthesizer (Biosearch, San Rafael, CA). Buffers and reaction conditions for restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were those listed by the commercial source, New England Biolabs (Beverly, MA). Isolation of DNA fragments and preparation of plasmid DNAs were carried out as described by Maniatis et al. (26). Transformation of Escherichia coli strain DH-1 was carried out according to Hanahan (13). Transfer of E. coli strain TG-1 with M13 phage DNA, infection of TG-1 cells with M13 phage virions, and purification of single- and double-stranded DNA molecules. Following either procedure, plaques of phage that contained mutant HA sequences were identified by hybridizing phage DNA adsorbed onto nitrocellulose circles with HP labeled mutagenic oligonucleotide and then washing the filters at increasing temperatures until the oligonucleotide dissociated from wild-type DNA but remained bound to mutant DNA. Phages that showed positive hybridization (e.g., see Fig. 2) were plaque purified, and single-stranded DNA was purified for use as template for sequence analysis by the technique of chain termination (40, 41) using the HA sequence primer shown in Fig. 1. To construct the double mutants, single-stranded DNA from the M11 mutant was used as template for further mutagenesis using the M1 or M4 oligonucleotides. Once the desired base substitution(s) was (were) confirmed, the double-stranded replicative form of each mutant phage DNA was prepared and purified by centrifugation on CsCl gradients.

Reconstruction of SV40-HA Recombinant Genomes Containing Mutant HA Sequences

As shown in Fig. 2, the double-stranded phage DNAs were digested with EcoRI and the 504-bp RI fragments containing mutant HA sequences were purified by gel electrophoresis. EcoRI digestion was also performed on pH5VHEA3, a recombinant SV40-HA genome that contains full-length, wild-type HA sequences and differs only from the vector previously described (7, 9) from the fragment previously described (7, 9) from the unique Bam HI site into plasmid pUC2 (a gift of J. Jenkins and A. Cowie, Imperial Cancer Research Fund, London) that lacks an EcoRI site. Gel electrophoresis was used to purify the large EcoRI fragment that contained the SV40 and plasmid sequences and the HA sequences except for the 504-bp restriction fragment. This fragment was ligated with the various small EcoRI fragments containing the mutant sequences, and the recircularized SV40-HA plasmid was used to transform E. coli DH-1. Bacterial clones harboring plasmids that contained the 504-bp sequence were identified by hybridization (27) to the probe used for hybridization. Finally, plasmids that contained the EcoRI fragment in the correct orientation that maintained the HA reading frame were identified by analysis using agarose gel electrophoresis of restriction fragments generated by digestion with XmaI restriction endonuclease. To confirm that the various SV40-HA recombinant genomes contained the desired mutations, the 504-bp EcoRI fragments were excised and recloned into M13mp11, and sequence analysis was performed by the chain termination procedure (40, 41) using the HA sequence primer.

Generation of SV40-HA Virus Stocks and Infection of Simian CV-1 Cells

50 µg plasmid DNA was digested with BamHI and the larger fragment, corresponding to the SV40-mutant HA recombinant genome, was isolated by agarose gel electrophoresis, recircularized under dilute ligation conditions (3 µg/ml), and transfected into subconfluent monolayers of CV-1 cells together with an equal amount of DNA from the helper virus d1055 (32). The procedures used for the transfection and development of high titer virus stocks were exactly as previously described (7). CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 250 µU/ml penicillin, and 100 µg/ml streptomycin sulfate. For assay, slightly subconfluent monolayers of CV-1 cells were infected with the SV40-HA recombinant viruses containing either wild-type or mutant HA genes. Second passage virus stocks were used at a 1/10 dilution in DMEM (0.3 µl per 5-cm dish of cells or 0.1 ml per well in 12-well Costar cluster plates; Costar, Cambridge, MA). At 1.5 h at 37°C, DMEM plus 10% fetal calf serum was added and the cells were incubated for 36-60 hr before assays were performed.
Characterization of Wild-type and Mutant HAs Expressed in CV-1 Cells

To quantitate the amount of HA protein produced from the recombinant genomes, cell extracts were prepared 48 and 60 h after infection, and radioimmunoassays were performed as described previously (9) using an IgG fraction purified from a high-titer rabbit anti-HA serum. Standard curves were generated using HA that had been purified to homogeneity from A/Japan/305/57 virions and whose concentration had been determined by amino acid analysis (Cope- land, C., unpublished results). To compare the size of the wild-type and mutant HAs, infected cell monolayers (36 h after infection) were labeled with [35S]methionine in the presence of tunicamycin. Aliquots of the cell extracts produced in this manner were trimeric as judged by their position on sucrose gradients as 9S peaks (2); all preparations were >90% pure by examination on SDS PAGE. A linear diagram of the HA molecule shows the three hydrophobic domains of the protein as cross-hatched regions. The amino acid sequence and the encoding nucleic acid sequence are shown for the region of HA that includes the fusion peptide. The mismatched oligonucleotides that were chosen to introduce specific amino acid changes in this region are shown, together with the matched oligonucleotide that was used as primer for DNA sequence analysis (see Materials and Methods).

Figure 1. Oligonucleotide-directed mutagenesis of the fusion peptide of HA. A linear diagram of the HA molecule shows the three hydrophobic domains of the protein as cross-hatched regions. The amino acid sequence and the encoding nucleic acid sequence are shown for the region of HA that includes the fusion peptide. The mismatched oligonucleotides that were chosen to introduce specific amino acid changes in this region are shown, together with the matched oligonucleotide that was used as primer for DNA sequence analysis (see Materials and Methods).

HA Assays

Infected cell monolayers were incubated with guinea pig or human RBCs (a 1% solution in phosphate-buffered saline [PBS]) for 15 min at room temperature. The monolayers were then washed with PBS until all the loose erythrocytes were removed and examined by light microscopy.

Cell-Cell Fusion Assays

The capacity of CV-1 cells infected with the various SV40-HA viruses to fuse together to form polykaryons was assayed as previously described (48). 54-60 h after infection (when parallel cultures showed >90% RBC binding), the cells were treated with 5 μg/ml trypsin to cleave HA0 into HA1 and HA2. The monolayers were then treated for 60 s at 37°C with fusion medium (PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM Hepes) and, C., unpublished results). To compare the size of the wild-type and mutant HAs, infected cell monolayers (36 h after infection) were labeled with [35S]methionine in the presence of tunicamycin (4 μg/ml; Calbiochem-Behring Corp., San Diego, CA) for 2 h before labeling for 15 min with [35S]methionine in the presence of tunicamycin. Aliquots of the cell extracts were immunoprecipitated using anti-HA serum, and the products were separated by SDS PAGE as described previously (9).

RBC-Cell Fusion Assays

The ability of HA-expressing cells to fuse with RBCs was assayed as previously described (6). In brief, 48-54 h after infection the cells were trypsinized as above to cleave HA0 to the fusion-active form. The cells were then washed twice in PBS and allowed to bind for 15 min at room temperature to human RBCs previously loaded with horseradish peroxidase (HRP). Excess RBCs were aspirated, and 2 ml of fusion medium of the indicated pH (prewarmed to 37°C) was added to each well. After the cells had been treated for 60 s at 37°C the fusion solution was aspirated and the cells were incubated in culture medium for 45 min in a CO2 incubator. Unless otherwise stated the cells were then fixed in 2.5% glutaraldehyde and stained with diaminobenzidine (DAB) as previously described (6). The coverslips were mounted and viewed by light microscopy. The percentage of DAB-positive cells was then determined by scoring at least four fields per coverslip; each field contained ~100 cells.

To quantitate the fusion of CV-1 cells with loaded erythrocytes more rigorously, unfixed cells were treated for 30 min at 37°C with 15 mg/ml neuraminidase to remove RBCs that were bound but not fused to the CV-1 cells. Cell extracts were then prepared with Nonidet P-40 and assayed for HRP activity by the method of Steinman and Cohn (45).

Preparation of BHA Fragment from CV-1 Cells Infected with SV40-HA Vectors

48 h after infection with SV40-HA vectors containing wild-type or mutant HA genes, 10-cm dishes of CV-1 cells were washed with PBS and placed into methionine-free medium supplemented with 0.5 mM [35S]methionine. After they were labeled overnight, the cells were washed twice with PBS and lysed with 1 ml of 1% Triton X-100 in 0.1 M Tris-HCl, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride and 1 mM aprotinin (both from Sigma Chemical Co., St. Louis, MO). The lysate was digested with 0.1 mg/ml bromelain for 16 h at room temperature in the presence of 20 mM 2-mercaptoethanol. The digestion was stopped by the addition of 1 mM N-ethylmaleimide, and the lysate was passed over a rincere-Sepharose affinity column as previously described (5). The bound material, most of which was BHA, was eluted with 0.2 M galactose, concentrated in an Amicon ultrafiltration cell (XM50 filter, Amicon Inc., Birmingham, AL) in a 1:1:1:5:0.3 molar ratio. Trace amounts of [35S]-BHA (~15,000 cpm) in 10 μl were added to 90 μl of liposomes (4.5 mM in phospholipid). The solution was incubated at 37°C, and the pH was adjusted...
Figure 2. Construction of SV40-HA vectors containing mutant HA genes. The EcoRI restriction fragment of the Japan HA gene (10) was cloned into M13mp11 phage DNA, and the single stranded virion RNA was used as template for mutagenesis by the double primer method (55) using each of the mismatched oligonucleotides shown in Fig. 1. Phage plaques containing mutant HA sequences were identified by hybridization with 32p-labeled oligonucleotide. The EcoRI restriction fragments containing the mutant sequences were excised from the double-stranded replicative form of the phage DNAs and used to replace the equivalent wild-type sequences in the SVEHA3 vector. Details are given in Materials and Methods.

by the addition of pretitrated volumes of 0.5 M acetic acid. After 30 min at 37°C, the solution was neutralized with NaOH and 100 µl of 67% sucrose was added to bring the final sucrose concentration to 33%. This solution was overlaid with 300 µl of 25% sucrose and 200 µl of 10% sucrose in a 700-µl clear ultracentrifuge tube (Beckman Instruments Inc.). All sucrose solutions were wt/vol in 20 mM 2-(N-morpholino)ethane sulfonic acid, 130 mM NaCl, pH 7.0 unless otherwise stated. After centrifugation at 200,000 × g for 3 h, 7 × 100 µl fractions were collected and counted for 125I. Counts in the top half of
the plasma membrane. Intact monolayers of labeled, infected cells were labeled with \[^{35}S\]methionine for 15 min and then incubated for 2 h more to allow expression of the terminally glycosylated proteins on the cell surface. Since the species of HA produced in CV-1 cells is the uncleaved HA0 (9, 48) it was possible to estimate the amount of HA that had reached the plasma membrane. Intact monolayers of labeled, infected cells were treated with trypsin to cleave HA0 into HA1 and HA2 subunits before cell extracts were prepared, immunoprecipitated, and analyzed by electrophoresis through SDS polyacrylamide gels. The results shown in Fig. 3 confirm that the wild-type and mutant HAs were expressed at very similar levels and indicate that the mutant proteins were transported to the cell surface with the same efficiency as the wild-type.

Proteinase K Digestions

BHA was incubated with liposomes as described above. After neutralization, an equal volume of 0.2 mg/ml proteinase K (Worthington Biochemical Corp., Freehold, NJ) in PBS containing 0.5 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\) was added. The digestion was allowed to proceed for 30 min at 37°C, pH 7.4, and was then terminated by the addition of 200 \(\mu\)l of ice-cold 20% trichloroacetic acid (TCA). The TCA-precipitable material was washed with cold acetone to remove lipid and was prepared for SDS PAGE by solubilization in 50 \(\mu\)l Laemmli sample buffer. Aliquots were counted for the content of \[^{35}S\]labeled protein.

Results

Construction of SV40-HA Recombinant Virus Vectors That Express Mutant HA Proteins

The hydrophobic “fusion peptide” at the amino-terminus of the HA subunit is the most highly conserved region of the HA molecule, suggesting that it plays a critical role in the infectious cycle of influenza. To confirm a direct role for this sequence in the fusion activity and to probe the mechanism of HA-mediated membrane fusion, we have used oligonucleotide-directed mutagenesis to engineer mutant HA proteins with specific nonconservative amino acid changes in the fusion peptide. These amino acid alterations were achieved by introducing single nucleotide alterations into the DNA sequence that encodes the fusion peptide (Fig. 1). Three oligonucleotides were designed, synthesized using phosphate triester chemistry, and used to introduce single mutations in HA sequences that had been cloned into an M13 vector (see Materials and Methods for a description of the mutagenesis procedures). To construct the double mutations the M1 and M4 alterations were introduced on the background of the M11 mutant gene. Once the presence of the desired mutations had been confirmed by DNA sequence analysis, restriction fragments containing each altered sequence were used to replace wild-type sequences in an SV40-HA recombinant virus vector. High-titer virus stocks containing the recombinant genomes were developed and used to infect simian CV-1 cells for expression of the wild-type and mutant HA proteins (see references 7 and 9 and Materials and Methods).

Synthesis and Cell Surface Expression of Wild-type and Mutant HAs

Before analyzing the fusion phenotype of the mutant HA proteins, we performed experiments to characterize the HA molecules and to ensure that the mutants were not grossly altered in their structure or level of expression. The amounts of HA antigen present in CV-1 cells at late times after infection with the mutant and wild-type SV40-HA recombinant viruses were quantitated by solid-phase radioimmunoassay. All mutants produced amounts of HA comparable to that expressed from the original SVEHA3 (wild-type) recombinant vector, i.e., \(\sim10^8\) molecules per infected cell at 60 h after infection (9). To characterize further the mutant HAs, infected cells were labeled with \[^{35}S\]methionine for 15 min and then incubated for 2 h more to allow expression of the terminally glycosylated proteins on the cell surface. Since the species of HA produced in CV-1 cells is the uncleaved HA0 (9, 48) it was possible to estimate the amount of HA that had reached the plasma membrane. Intact monolayers of labeled, infected cells provided further evidence of efficient cell surface expression. Approximately 48 h after infection with wild-type or mutant viruses, \(>95\%\) of the CV-1 cells displayed binding of erythrocytes (see Fig. 5).

Structural Comparison of Wild-type and Mutant HA Proteins

Further evidence that the site-specific mutagenesis of HA had caused no gross alterations in the protein structure was provided by the fact that all the mutant proteins were recognized by a panel of monoclonal antibodies directed against the A/Japan/305/57 HA and a closely related HA from the H2 virus subtype A/Guigyang/1/57 (32). In particular, all of the mutant HAs were precipitated by two of the monoclonal antibodies that recognize native HA but do not recognize the denatured molecule (results not shown). Furthermore, soluble BHA fragments prepared from cells expressing the mutant proteins migrated on sucrose gradients with the mobility (9S) characteristic of the native trimeric ectodomain (data not shown). Finally, the mutant BHAs all displayed similar resistance to proteolytic degradation at neutral pH, a criterion for the intact structure (see Fig. 7).

Although the HA0 and HA1 bands of all the proteins had very similar or identical mobilities, minor differences were detected in the mobilities of the HA2 bands. The magnitude of these differences varied from experiment to experiment, with the maximum deviation from the mobility of the wild-type HA2 corresponding to a molecular weight difference \(\sim1,000-2,000\). Similar alterations in mobility have been observed when single amino acid changes have been introduced into small proteins such as p21 ras (30, 42) or subtilisin (Wells, J., personal communication). Alternatively, it is possible that these mobility differences might reflect minor alterations in the addition of terminal sugars to the single oligosaccharide side chain on HA2. To investigate this possibility, wild-type and mutant HAs were isolated from vector infected cells labeled for 10 min so as to permit core glycosylation but not terminal glycosylation, or from cells labeled in the presence of tunicamycin (46) to prevent any core glycosylation of HA. When the \[^{35}S\]methionine-labeled HAs were treated with trypsin before SDS PAGE, the HA2 bands had very similar mobilities (results not shown). This result indicated that the mobility differences were manifested only after terminal glycosylation occurred. It is noteworthy that in the threedimensional structure of the HA molecule deduced by Wilson et al. (51), the single oligosaccharide chain attached to asparagine residue 154 in HA2 is situated adjacent to the fusion peptide.

Fusion Activities of Mutant HAs

We have previously shown that CV-1 cells that express HA on their plasma membranes can be induced to fuse with each other (48) or with RBCs (6) after a brief exposure to low pH;
polykaryon formation appears to require more HA molecules per cell than does RBC–cell fusion (White, J., unpublished observations). We have used both types of fusion assays to monitor the effects of substitutions of amino acids in the fusion peptide.

To analyze the ability of the mutant HAs to elicit RBC-cell fusion, CV-1 cells infected with the various recombinant viruses were treated with trypsin, and human erythrocytes loaded with HRP were allowed to bind to the cells. The pH was then transiently decreased to values between 4.8 and 5.6. Fusion-mediated delivery of the RBC contents into CV-1 cells was monitored by staining fixed cells with DAB, or by measuring peroxidase activity in cell extracts. The results of these experiments are illustrated as light micrographs of fixed and stained cells in Fig. 4, and shown graphically in Fig. 5. HRP delivery into cells expressing the wild-type HA followed a pH dependence identical to that reported previously for fusion of baby hamster kidney cells by the Japan strain of influenza virus (49, 50). The threshold pH for fusion was ~5.3, and delivery of HRP into 50% of cells occurred at pH 5.1. At pH 4.8, 80–90% of the cells had received HRP. Cells that expressed the M11 mutant HA showed an almost identical profile, which indicated that replacing the glutamic acid at position 11 with glycine had no effect on the pH-dependence or efficiency of RBC-cell fusion. By contrast, both the pH profile and efficiency of RBC-cell fusion were altered for cells expressing the M4 HA (glycine to glutamic acid). The threshold pH for RBC-cell fusion was elevated to approximately pH 5.7. Maximum fusion was obtained at pH 5.3, with ~45% of the cells showing cytoplasmic staining with DAB. The M1 mutant protein (glycine to glutamic acid) had the striking effect of abolishing the fusion activity of HA; cells expressing the M1 protein showed little or no DAB staining at any pH, even though they bound RBCs as efficiently as the cells that expressed wild-type HA. Finally, the RBC-cell fusion characteristics of the M1/11 and M4/11 double mutants resembled those of the M1 and M4 parents. Thus the secondary M11 mutation apparently contributed little to the RBC-cell fusion phenotype.

To quantitate directly the amount of HRP enzyme that had been delivered at pH 5.0 into cells expressing the mutant and wild-type HAs, it was necessary to remove the HRP-loaded erythrocytes that were bound to the infected CV-1 cells but had not undergone the fusion reaction. This was achieved using neuraminidase, and then cell extracts were prepared and cell-associated HRP activity was measured using a spectrophotometric assay. The results summarized in Table I resemble those obtained by counting stained cells (Fig. 5). Mutants M1 and M1/11 lacked any fusion capability; the fusion activities of M4 and M4/11 were ~60% of the wild-type level and that of the M11 mutant was ~90% of the wild-type activity.

In a parallel study using polykaryon formation as an assay for the HA-mediated membrane fusion, cells expressing the
Figure 4. Low pH-induced fusion of RBCs with CV-1 cells expressing wild-type or mutant HA glycoproteins. CV-1 cells that had been infected for 54 h with SV40-HA recombinant vectors containing wild-type or mutant HA genes were treated with trypsin to cleave cell-surface HA into HA1 and HA2 subunits. Human erythrocytes loaded with HRP were bound to the cells and then the pH of the medium was transiently decreased to 5.6, 5.3, or 5.0. After incubation in culture medium for 45 min the cells were fixed with glutaraldehyde and stained with DAB to identify CV-1 cells that had fused with HRP-loaded erythrocytes. Bar, 100 μm.

Wild-type or mutant HAs were treated with trypsin to activate HA, and then the pH was transiently decreased to 5.6, 5.3, or 5.0 (see Materials and Methods). The cells were then returned to normal medium and incubated for several hours to facilitate visualization of polykaryon formation. Representative light micrographs of cells from such an experiment are shown in Fig. 6. The results obtained with cells expressing the M1 and M4 mutant HAs paralleled those seen in RBC-cell fusion assays. The M1 HA induced no polykaryon formation at any pH (data not shown), a result consistent with the inability of M1 to mediate HRP delivery. Cells expressing the M4 mutant fused with a higher pH threshold (pH 5.6). Approximately 50% fusion was achieved at pH 5.3, but the level of fusion was not further increased at lower pH values. The most surprising result was obtained with cells that expressed the M11 mutant. M11 HA could not induce polykaryon formation at or above pH 5.0, despite being able to mediate RBC-cell fusion with the same pH profile and efficiency as wild-type HA. Further experiments at even lower pH (data not shown) revealed that very limited polykaryon formation (~10%) could be induced by M11 HA at pH 4.6 or 4.4 (the lowest pH at which the assay could be performed). The fact that the M4/11 double mutant HA also lacked cell-cell fusion activity at pH 5.0 (data not shown) indicated that the amino acid alteration in M11 was dominant in preventing polykaryon formation. As expected, the combination of the M1 and M11 mutations, which individually abolished cell-cell fusion activity at pH 5.0, resulted in a double mutant that induced no polykaryon formation.

Effect of the Mutations on the Conformational Change in HA

The HA trimer has been shown to undergo a conformational change under the conditions that trigger membrane fusion (5, 44). The change, which is irreversible, exposes a hydrophobic domain in HA2 which is probably the highly conserved amino-terminal peptide of HA2 (3). A water-soluble fragment of HA, termed BHA, undergoes a conformational change at pH 5.0, resulted in a double mutant that induced no polykaryon formation.
then fixed with glutaraldehyde, stained with DAB, and scored for the extent of fusion as described in Materials and Methods.

For their ability to fuse with HRP-loaded erythrocytes. The cells were containing the wild-type or mutant HA genes. 54 h after infection, Figure 5. Conversion of the wild-type BHA to the protease-sensitive form was first detected after treatment at pH 5.8 and was essentially complete at pH 4.8.50% conversion of the BHA was obtained after incubation at pH 5.2, which is 0.1 pH unit higher than the midpoint of HA-induced fusion (Fig. 5). Analysis of the BHA by SDS PAGE demonstrated that both HA1 and HA2 were digested and that after protease treatment following incubation for 30 min at pH 4.8, >80% of the [35S]methionine in BHA became TCA soluble. All of the mutant proteins were resistant to protease at neutral pH and could undergo the conformational change, with maximal protease sensitivity achieved after incubation at pH 4.8. The protease sensitivity of the M11 and M11/11 BHAs followed the same pH profiles as those of the wild-type protein. The conformational change in the M4 and M4/11 BHAs occurred more readily than that in the wild-type protein. The pH profiles of protease sensitivity were shifted 0.3 U in the alkaline direction; 50% of the mutant BHA molecules converted to the sensitive form after treatment at pH 5.5. Finally, the conformational change in M11 BHA occurred at lower pH than in the wild-type, since the protease sensitivity profile was shifted 0.2 pH U in the acid direction.

Lipid Binding Properties of Wild-type and Mutant BHAs

A second consequence of the low pH-induced conformational change in BHA is that the molecule acquires amphiphilic properties (5, 44). As a result the fragment will bind to other amphiphiles, such as nonionic detergents and artificial lipid bilayers. Having determined that both the wild-type and mutant BHAs could undergo a low pH-induced conformational change, we assessed the capacities of the proteins to bind to liposomes during incubation in solutions of various pH. After neutralization, the extent of binding was determined using flotation gradients. The results are shown in Fig. 7B. The extent of binding of the various BHAs to liposomes varied with pH in a manner identical to that observed for the protease sensitivity of each molecule.

We have previously demonstrated that the association of BHA (from the X31 strain of influenza) with liposomes is hydrophobic in nature (5). Once bound, the protein cannot be removed by high or low salt, chaotropic agents, or urea. It can, however, be eluted by elevated pH (>10.5). The wild-type and mutant BHAs originating from the Japan strain of virus (Table II) showed identical characteristics in their interaction with lipid bilayers. Once bound to liposomes, these BHA molecules could be removed only by treatment at high pH. All the molecules could rebind to lipid when returned to neutral pH. Thus we could not detect any differences in the nature of the interaction with liposomes of the wild-type or the mutant proteins.

Kinetics of the Conformational Change in BHA

To investigate the kinetics of development of protease sensitivity and lipid binding capacity, trace quantities of [35S]methionine-labeled BHAs were incubated with liposomes at pH 4.8 for various times at 37°C. Aliquots were removed and tested for protease sensitivity or lipid binding as described above. In Fig. 8 we display the results for the wild-type and the M1 and M4 mutant BHAs. For the wild-type, M4 and M11 molecules, the kinetics of conversion to protease sensitivity closely parallels those of acquisition of amphiphilic character. M11 BHA showed the same kinetics as the wild-type, with 50% of the maximal values reached 2 min after acidification. M4 BHA converted to the low pH form more rapidly. M1 BHA was the only protein that displayed different kinetics for protease sensitivity and lipid binding. Compared with the wild-type BHA, M1 displayed a slower rate of conversion to protease sensitivity, and an even slower rate of interaction with lipid, suggesting that its mode of interaction with the target bilayer may be altered from that of the wild-type HA or the other mutant proteins.

Discussion

In past studies, enveloped viruses have been useful probes for many dynamic aspects of biological membranes such as receptor-mediated endocytosis (reviewed in reference 14) and

Table 1. Relative Fusion Efficiencies at pH 5.0 of Wild-type and Mutant HAs

| HA protein expressed by CV-1 cells | Cell-associated HRP activity after RBC-cell fusion at pH 5.0* | No. of determinations |
|-----------------------------------|-------------------------------------------------------------|-----------------------|
| Wild-type HA                      | 1.00                                                        | 4                     |
| M1 HA                             | 0.04 ± 0.02                                                 | 2                     |
| M4 HA                             | 0.64 ± 0.09                                                 | 3                     |
| M11 HA                            | 0.91 ± 0.11                                                 | 4                     |
| M1/11 HA                          | 0.07 ± 0.04                                                 | 3                     |
| M4/11 HA                          | 0.58 ± 0.16                                                 | 4                     |

* CV-1 cells were infected for 48 h with SV40-HA vectors expressing wild-type or mutant HA genes, treated with trypsin, and allowed to bind RBCs that had been preloaded with HRP. The cells were then fixed with glutaraldehyde, stained with DAB, and scored for the extent of fusion as described in Materials and Methods.

Figure 5. pH profiles of RBC-cell fusion induced by wild-type and mutant HA proteins. CV-1 cells were infected with SV40-HA vectors containing the wild-type or mutant HA genes. 54 h after infection, the cells were treated with trypsin and tested at different pH values for their ability to fuse with HRP-loaded erythrocytes. The cells were then fixed with glutaraldehyde, stained with DAB, and scored for the extent of fusion as described in Materials and Methods.
the biosynthesis, assembly, and intracellular transport of integral membrane proteins (see for example references 18, 24, 36, and 43). Similarly, in recent years, the fusion reactions that mediate infections by such viruses have increasingly been used as models of cellular membrane fusion events (reviewed in reference 49).

Although fusion between lipid bilayers is undoubtedly an extremely complex event in molecular terms, every fusion reaction can be considered in three simple stages: close approach of the interacting membranes, lipid layer destabilization, and reformation of one united bilayer. Since two membranes experience an exponentially increasing repulsive force as they approach closer than ~20 Å (33), the first phase of any membrane fusion event is energetically highly unfavorable. Mechanisms must therefore exist to overcome this formidable obstacle before two bilayers can fuse. Our previous work has suggested how the HA glycoprotein of influenza virus may facilitate the first phase of fusion by binding firmly and simultaneously to both of the fusing membranes (48). The interaction with the virus or host cell membrane involves the hydrophobic transmembrane “anchor” near the carboxyterminus of the HA polypeptide; the interaction with the target membrane involves the highly conserved apolar peptide at the amino-terminus of the HA2 subunit, which becomes exposed only at low pH (3, 5, 44).

Recent studies of variant influenza viruses that mediate fusion at elevated pH values have identified amino acid residues in the HA molecule that stabilize the structure of HA at neutral pH (4, 5a, 38). Substitution of these residues appears to lower the energy barrier necessary for the low pH–induced conformational transition to the fusion-active state. Some of these residues were located along the interface between the individual subunits of the HA trimer (4, 5a), whereas others, including three amino acids located within the fusion peptide, stabilized the unexposed location of the amino-terminus of HA2 (4, 38). It was notable that unlike most of the mutated residues analyzed in the variant HAs, the three amino acids located in the fusion peptide had undergone conservative substitutions; none had been altered to charged amino acids (4). This may have been a consequence of the selection procedures used; the population of variant viruses would not by definition include mutant viruses that could not carry out the fusion reaction at any pH. Thus, a different strategy was necessary to produce and characterize mutant HAs that might be inactive or disabled for fusion instead of or as well as altered in their pH dependence. In an attempt to find such mutants and to probe the consequences of altering the hydrophobicity and length of the fusion peptide, we have constructed and characterized five genetically engineered HA molecules that contain specific, nonconservative amino acid changes in the fusion peptide.

Oligonucleotide-directed mutagenesis was used to introduce single nucleotide changes into a cloned copy of the gene encoding the HA from the A/Japan/305/57 strain of influenza virus. The amino acid alterations that were introduced are shown in Fig. 1, and the rationale for our choices was as...
determined. One of which was assayed immediately for liposome binding as described in Materials and Methods. For determination of the pH dependence of conversion to sensitivity to digestion with protease, the labeled BHAs were incubated at the indicated pH for 30 min at 37°C, neutralized, and digested with 0.1 mg/ml proteinase K before analysis of the quantity of TCA-precipitable radioactivity. For determination of the pH dependence of acquisition of liposome binding capacity, the BHAs were incubated in the presence of liposomes at the indicated pH for 30 min at 37°C, after which the solution was neutralized and centrifuged on a flotation gradient to determine the amount of lipid-bound protein.

Table II. Effect of High Salt and High pH on the Binding of BHAs to Liposomes

|                | % BHA bound to lipid after 30 min, pH 4.8 | % BHA eluted by 1 M KCl | % BHA eluted at pH 11 | % BHA re-bound after shift from pH 11 to pH 7 |
|----------------|----------------------------------------|------------------------|----------------------|---------------------------------------------|
| Wild-type BHA  | 78                                     | 7                      | 58                   | 100                                         |
| M1 BHA         | 57                                     | 14                     | 65                   | 108                                         |
| M4 BHA         | 79                                     | 8                      | 63                   | 97                                          |
| M11 BHA        | 79                                     | 10                     | 58                   | 104                                         |
| M1/11 BHA      | 70                                     | 12                     | 62                   | 88                                          |
| M4/11 BHA      | 87                                     | 10                     | ND                   | ND                                          |

Wild-type BHAs were incubated with liposomes at pH 4.8 for 30 min at 37°C. The solutions were then neutralized and divided into four aliquots, one of which was assayed immediately for liposome binding as described in Fig. 7. The remaining aliquots were treated with either salt or base as follows before the amount of lipid-associated protein was determined. 4 M KCl was added to one aliquot to bring the final salt concentration to 1 M. This solution was incubated for 15 min at 37°C before centrifugation on a flotation gradient that also contained 1 M KCl. After the remaining two aliquots were incubated at pH 11 for 15 min, one was assayed for lipid-bound protein in a gradient maintained at pH 11. The final aliquot was returned to pH 7 for 15 min before being assayed for lipid binding in a flotation gradient at pH 7. ND, not determined.

In the neutral conformation of the HA molecule, the fusion peptide is tucked into the interface between the subunits of the trimer. The amino nitrogen of residue 1 (Gly) and the amide nitrogens of residues 4, 5, and 6 (Gly-Ala-Ile) of HA2 form hydrogen bonds to oxygen atoms of residue 112 (Asp) in the long helix of HA2 (4, 51). Substitution of glutamic acid for glycine residues at positions 1 and 4 (mutants M1 and M4) introduces negative charges into the fusion peptide which might disrupt the normal hydrogen bonding interactions. In the second instance, the M4 substitution would significantly shorten the stretch of apolar amino acids. In the Japan HA2 subunit, the stretch of hydrophobic residues is terminated by a glutamic acid residue at position 11. Substitution of this amino acid by a glycine (mutant M11) extends the apolar sequence to 18 residues until the aspartic acid at position 19, permitting additional testing of the role played by the length of the hydrophobic sequence. The role of the positioning of the hydrophobic sequence was tested by constructing the double mutants (Glu4/Gly11 (M1/11) and Glu4/Gly11 (M4/11)) which contain internal apolar stretches of 17 or 14 residues, respectively.

The wild-type and mutant genes were expressed in simian cells using SV40-based vectors, and the HA molecules were assayed for fusion activity and for the ability to undergo the characteristic low pH-induced conformational change. The results of these experiments are summarized in Table III. The wild-type HA protein undergoes a rapid conversion at low pH into a conformation that can interact with lipid and that is sensitive to degradation with protease. Concomitant with this conformational change, which occurs at a characteristic pH threshold, wild-type HA expressed on the surface of a CV-1 cell can mediate fusion either between the CV-1 cell and RBCs, or between adjacent CV-1 cells to form polykaryons. This fusion occurs with high efficiency; at pH 5.0, >90% of the CV-1 cells expressing the wild-type protein fuse with target lipid bilayers. Examination of the results obtained with the mutant HA proteins reveals three different fusion phenotypes and provides insights into the roles of HA in the fusion reaction.

**M1 HA**

The substitution of a glutamic acid for the glycine residue at the amino-terminus of the fusion peptide abolished all of the fusion activity of the HA protein. However, this protein could still undergo a conformational change at low pH that resulted in protease sensitivity and lipid binding capability. The lower pH at which this conformational change occurred, and the greater time taken at pH 4.8 for the conversion to be manifested, suggested that the mutation had in some way stabilized a neutral conformation of the protein. However, once the mutant protein became bound to liposomes, its interaction with lipid had the same characteristics as that of the wild-type protein. This result makes it unlikely that the mutant protein is interacting in a nonspecific fashion with the lipid bilayer and suggests that the binding of HA to both membranes is necessary but not sufficient for fusion to occur. Thus it appears that HA may play an additional role in destabilization of the lipid bilayer during fusion.
M4 HA

The substitution of a glutamic acid for the glycine residue at position 4 of the fusion peptide (which decreases the length of the apolar stretch to six amino acids) accelerated the rate and raised the threshold pH at which the low pH-induced conformational change occurred in the mutant protein. Thus, the mutation had destabilized the neutral conformation of HA in a similar fashion to the amino acid alterations identified in the variant viruses selected in previous studies (4, 5a, 38). The raised pH threshold for the conformational change was reflected in a raised pH threshold for RBC-cell or cell-cell fusion. However, the fusion mediated by this mutant HA never became as efficient as that caused by the wild-type protein; even at pH <5.0 only 50% fusion was observed. This result suggests that the amino acid at position 4 in HA2 may play two roles—a structural role in maintaining the fusion peptide in its neutral conformation (4) and another role in the lipid destabilization phase of fusion. Whether the decreased efficiency of fusion is a direct result of the specific substitution at position 4 in the fusion peptide or whether it is a consequence of interruption of the stretch of apolar residues by a charged amino acid remains to be tested. No HA molecules isolated from fusion-active influenza viruses (either naturally occurring “wild-type” influenza A viruses (21) or fusion variants [4]) contain polar residues within the fusion peptide, perhaps because variants such as M4 would be at a competitive disadvantage during infection as a result of their reduced fusion activity. Unfortunately, we are at present unable to convert the mutated HA cDNAs into viral RNA genes and thus cannot assay the ability of influenza virions containing M4 HA to enter the cell and mediate infection.

M11 HA

Substitution of a glycine residue for the glutamic acid residue at position 11 of HA2 (which increases the length of the hydrophobic sequence to 18 amino acids) had no effect on the rate or pH dependence of the conformational change in the mutant protein. This observation is consistent with the location of this residue on the external surface of the molecule, distant from the trimer interface or the “hinge region” (51). Neither did the mutation have any positive or negative effect on the initial binding of the protein to the target lipid bilayer, suggesting that the length of the apolar sequence is not critical for this interaction. Furthermore, the mutant protein induced RBC-cell fusion with the same pH dependence and efficiency as wild-type HA. However, the M11 HA was greatly impaired in mediating cell-cell fusion. This phenotype provides the first instance of a distinction between RBC-cell fusion and cell-cell fusion. The M11 HA is competent to mediate fusion of the CV-1 and erythrocyte cell membranes over the small areas necessary for injection of HRP into the CV-1 cytoplasm, but except at very low pH it appears to be unable to cause lipid bilayer destabilization over areas sufficient to allow polykaryon formation. It should be noted that the localized fusion that occurs between erythrocytes and cells (6) may be a better model for the infection of cells by fusion of the influenza virus envelope and the endosome membrane (28, 49) than is the extensive membrane coalescence that is assayed by polykaryon formation.

M1/11 and M4/11 HAs

Analysis of the phenotypes of the proteins that contain double mutations confirmed our conclusions that the conformational change in HA and the subsequent interaction of the protein with the target bilayer are separate events from the bilayer destabilization that results in membrane fusion. When two amino acid substitutions are present in the HA molecule, the conformational change and lipid-binding phase of the fusion reaction is dominated by the mutation that destabilizes the native protein structure, causing the threshold pH for the conversion to be raised. By contrast, the mutation that dominates the membrane fusion phase of the reaction is that which shows the most deleterious effects in the single mutants. Thus, in the M1/11 double mutant, the M11 alteration influences the pH at which the conformational change occurs, whereas the M1 substitution dominates in abolishing fusion activity. On the other hand, in the M4/11 double mutant the M4 substitution influences the conformational change and the pH threshold for RBC-cell fusion whereas the M11 alteration dominates in preventing cell-cell fusion. The double mutants also provide evidence that the length and positioning of the apolar stretch of amino acids at the amino terminus of HA2 are not critical for fusion.

Summary

In summary, the mutations that we have introduced into the fusion peptide of HA have allowed us to delineate several stages of the mechanism of HA-mediated membrane fusion. These stages include the low pH-induced conformational change in HA that exposes the fusion peptide; the interaction of the fusion peptide and/or other regions of the HA molecule with the target lipid bilayer; destabilization of the lipid bilayer and membrane coalescence over small areas, and membrane.

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Table III. Summary of the Fusion Phenotypes of the Wild-type and Mutant HA Proteins

|                | Threshold pH of RBC-cell fusion | Threshold pH of cell-cell fusion | Efficiency at pH 5 of RBC-cell fusion | Efficiency at pH 5 of cell-cell fusion | pH at which 50% of BHA is converted to protease sensitivity and lipid binding | Time at pH 4.8 for 50% conversion |
|----------------|-------------------------------|---------------------------------|------------------------------------|------------------------------------|---------------------------------------------------------------------|----------------------------------|
|                | %                             | %                              | %                                 | %                                 |                                                                                     | min                              |
| **WT**        | 5.3                           | 5.3                            | 90                                 | 90                                 | 5.3                                                                                     | 3                                |
| **M1**        | —                             | —                              | 0                                  | 0                                  | 5.0                                                                                     | 6                                |
| **M4**        | 5.7                           | 5.6                            | 50                                 | 50                                 | 5.5                                                                                     | 1                                |
| **M11**       | 5.3                           | 4.6                            | 0                                  | 0                                  | 5.3                                                                                     | ND                               |
| **M1/11**     | —                             | —                              | 0                                  | 0                                  | 5.3                                                                                     | ND                               |
| **M4/11**     | 5.7                           | 4.6                            | 50                                 | 0                                  | 5.5                                                                                     | ND                               |

These values are summarized from the data shown in Figs. 5-8. The experimental details are given in the figure legends and in Materials and Methods. —,—, no fusion detected at any pH tested. ND, not determined.
coalescence and separation over large areas, resulting in polykaryon formation. The M1 mutant provides the first opportunity to separate temporally the conformational change from lipid binding; in all previous studies these events have appeared to occur simultaneously (5, 44). The M1 mutant also allows separation of the lipid bindings and bilayer fusion stages of the process, which indicates that HA does more than simply bring the two membranes close together. It is possible that the fusion peptide must assume a precise structure in the target membrane in order to cause sufficient destabilization to promote fusion. Finally, the M11 mutant provides evidence that HA may also play a role in propagation of fusion over large areas of membrane—a role that is manifested in the in vitro assay systems but not during infection in vivo.

Although this study has provided insights into the sequence of events that lead to fusion, the molecular details of HA’s role in the fusion mechanism remain to be elucidated. Whereas it is possible to use the known three-dimensional structure of the neutral form of HA (51) to interpret the role of individual amino acid substitutions that affect the conversion of the protein into the low pH conformation, it is difficult to interpret the effect of substitutions that affect bilayer destabilization because we as yet have no detailed knowledge of the structure of the low pH form of HA before or after it interacts with the target membrane. Furthermore, we currently have no evidence as to whether this interaction involves solely the fusion peptide, or whether other portions of the HA molecule (such as the amphipathic region at the top of the long alpha-helix of HA2) may also bind to the lipid bilayer. Further mutants with site-directed alterations in this region could be constructed to test this hypothesis. We hope that future experiments that combine the power of recombinant DNA techniques with the elegance of the model system provided by influenza HA will reveal further details of the molecular mechanism of HA-induced fusion. The availability of cloned copies of other viral fusion proteins (1, 20, 31, 34, 37) should now permit similar analyses in other virus systems. Such studies should reveal both the common and the unique features of the mechanisms by which these proteins mediate membrane fusion.

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