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Introduction of Intersubunit Disulfide Bonds in the Membrane-Distal Region of the Influenza Hemagglutinin Abolishes Membrane Fusion Activity

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Summary

Influenza virus hemagglutinin (HA) mediates viral entry into cells by a low pH–induced membrane fusion event in endosomes. A number of structural changes occur throughout the length of HA at the pH of fusion. To probe their significance and their necessity for fusion activity, we have prepared a site-directed mutant HA containing novel intersubunit disulfide bonds designed to cross-link covalently the membrane-distal domains of the trimer. These mutations inhibited the low pH–induced conformational changes and prevented HA-mediated membrane fusion; conditions that reduced the novel disulfide bonds restored membrane fusion activity. We conclude that structural rearrangements in the membrane distal region of the HA are required for membrane fusion activity.

Introduction

Infection of cells by viruses with lipid membranes appears to involve fusion of cellular and viral membranes. For viruses such as influenza, fusion occurs in endosomes formed following virus–receptor interactions at the cell surface. The fusion potential of the infecting virus is activated at endosomal pH (reviewed in White et al., 1983; Wiley and Skehel, 1987), and the influenza virus component involved in both receptor binding and membrane fusion is the hemagglutinin (HA) membrane glycoprotein. A number of studies have addressed the role of this molecule in the fusion process and the mechanism of its activation at low pH (reviewed in Wiley and Skehel, 1987).

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The HA is a trimer of identical subunits each of which contains two polypeptide chains, HA_1 and HA_2, produced by proteolysis of a biosynthetic precursor, HA_0 (Klenk et al., 1975; Lazarowitz and Choppin, 1975; Skehel and Waterfield, 1975). Incubation of viruses or isolated HAs at the pH of fusion results in irreversible changes in the conformation of HA, which expose hydrophobic residues at the amino terminus of HA_2, sometimes called the "fusion peptide" (Wiley and Skehel, 1987). In this conformation the HA becomes extremely susceptible to proteolysis at specific residues, but circular dichroism studies and monoclonal antibody binding analyses indicate that the molecule is not simply denatured and suggest that the low pH–induced changes in structure predominantly involve the movement of molecular domains relative to each other (Skehel et al., 1982; Daniels et al., 1983, 1985; Webster et al., 1983; Yewdell et al., 1983; Doms et al., 1985; Jackson and Nestorowicz, 1985; White and Wilson, 1987). In particular, interactions between the membrane-distal globular regions of the molecule, which contain the receptor binding sites and epitopes for infectivity-neutralizing monoclonal antibodies, are perturbed specifically at the pH of fusion (Wiley and Skehel, 1987). Such observations suggest that structural changes occur throughout the molecule, not simply in the region of the fusion peptide, which is about 100 Å nearer the virus membrane than the receptor binding site. This suggestion is consistent with the locations of amino acid sequence changes in mutant HAs that fuse membranes at higher pH than wild type (Daniels et al., 1985, 1987). These mutants contain amino acid substitutions in the interfaces between subunits of the trimer throughout the length of the molecule, and the results of structural analysis of a mutant HA (Weis et al., 1990) indicate that such substitutions influence the pH of fusion by modifying the structure of the HA locally.

We have attempted here to determine the significance for membrane fusion of structural changes that occur in the membrane-distal globular domain. Specifically, we have determined the fusion activity, as a function of pH, of a mutant HA containing novel cysteine residues at HA_1-212 and HA_1-216, located appropriately to form three disulfide bonds that covalently cross-link the three domains (Figure 1). We have shown using this mutant that restricting the relative mobility of the subunits in this region of the molecule prevents membrane fusion. We discuss the implications of these results for considerations of the changes in HA structure required for membrane fusion activity.

Results

The Design of the Site-Directed Mutant

The mutant HA that we have constructed contains three identical novel intersubunit disulfide bonds between residues HA_1-212 and HA_1-216. The positions where novel disulfide bonds might be created were determined with the

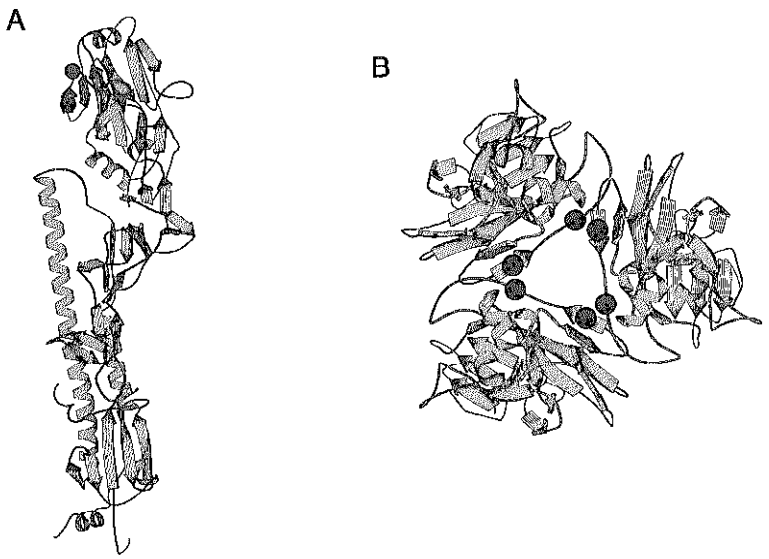

Figure 1. Schematic Diagrams of X-31 HA That Indicate the Locations of the Amino Acid Substitutions Introduced by Site-Specific Mutagenesis

(A) HA monomer. The trimeric interface is to the left, and the viral membrane would be at the base of the figure.

(B) View from the top of the trimer showing the locations of the two amino acid substitutions. Closed circles indicate the locations of T HA_1-212 and N HA_1-216, which are substituted by cysteines in the mutant HA.

protein engineering program Proteus (Pabo and Suchanek, 1986). A series of 73 known disulfide bond conformations from protein structures determined by X-ray crystallography were tested at each position on the HA_1 chain to see whether they could span to any residue on the HA_1 chain of a second subunit. Between HA_1-212 and HA_1-216 on an adjacent subunit, disulfide bond conformations like those of crambin (Cys-3–Cys-40) or phospholipase A2 (Cys-27–Cys-123) could be accommodated. Other possibilities generated included residues Pro-221 or Ala-220, which were not selected for mutagenesis because of the unusual geometry of proline and because both 220 and 221 are conserved residues in all known HA sequences.

The mutations were constructed by oligonucleotide mismatch mutagenesis (Oostra et al., 1983), and the protein was expressed constitutively in $DHFR^-$ Chinese hamster ovary (CHO) cells after amplification by selection with methotrexate (Kaufman and Sharpe, 1982) and in vaccinia recombinant–infected CV1 cells.

The Oligomeric State and Polypeptide Composition of the Expressed Hemagglutinins

Sucrose density gradient centrifugation of the wild-type and mutant HAs extracted from CHO cells by nonionic detergent indicated that both had sedimentation coefficients of 9S, indistinguishable from HA trimers extracted from purified virus particles. Polyacrylamide gel electrophoretic analyses under nonreducing conditions of wild-type HA immunoprecipitated from detergent-lysed cells resolved three polypeptides, the most abundant of molecular weight 75 kd and two minor polypeptides of molecular weights 150 kd and 230 kd (Figure 2). By comparison with previously published results (Doms and Helenius, 1986), these are equivalent to monomers, dimers, and trimers of the HA subunit. Similar analyses of the unreduced mutant HA revealed a major polypeptide of molecular weight 230 kd and a minor polypeptide (about 10%) of molecular weight 75 kd, indicating that the mutant HA was expressed predominantly as a covalently linked trimer. Neither wild-type nor mutant HAs labeled for a shorter period (20 min compared with 18 hr) formed oligomers, and the immunoprecipitated products separated into two components of similar size, 75 kd, on polyacrylamide gels (Figure 2). This was a common observation, under the nonreducing conditions of analysis, which we have not investigated further. Under reducing conditions both wild-type and mutant HAs labeled for 20 min had molecular weights of 73 kd (Figure 2). Longer labeling (18 hr) led to the production of a single wild-type HA polypeptide of molecular weight 75 kd, but mutant HA was found to separate into two polypeptides, one of molecular weight 75 kd, the other 73 kd. These results suggested that the 75 kd polypeptide was produced

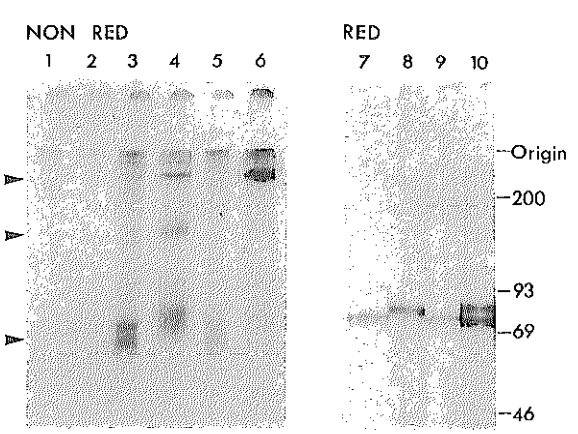

Figure 2. PAGE of the Wild-Type and Mutant HA Molecules Expressed in CHO Cells

Control CHO cells (lanes 1 and 2) or cells expressing wild-type HA (lanes 3, 4, 7, and 8) or mutant HA (lanes 5, 6, 9, and 10) were labeled with [^{35}S]methionine for 20 min (lanes 1, 3, 5, 7, and 9) or 18 hr (lanes 2, 4, 6, 8, and 10). The HAs were immunoprecipitated from cell lysates and were analyzed by 7% SDS–PAGE under either nonreducing (lanes 1–6) or reducing (lanes 7–10) conditions. The arrows to the left of the figure indicate HA monomer, dimer, and trimer. The numbers to the right are molecular weight markers.

as a result of posttranslational modification and that only approximately 50% of the mutant HA polypeptides were completely modified. We therefore examined the differences between the 75 kd and 73 kd polypeptide components of the 18 hr–labeled mutant HA by analyzing their acylation and glycosylation.

Posttranslational Modifications of the Hemagglutinins

Incubation of the ^{35}S-labeled wild-type and mutant HAs with trypsin specifically generated the HA_1 and HA_2 components of the HA subunit, indicating their native conformations (Figure 3A). However, two HA_2 polypeptides that differed in molecular weight by about 2000 were detected in the mutant HA, indicating that the difference in the molecular weights of the 75 kd and 73 kd polypeptides was due to a difference in the size of their HA_2 components. In agreement with previous observations with X-31 HA (Steinhauer et al., 1991), palmitoylation of the HA was restricted to the HA_2 polypeptides. However, the smaller HA_2 polypeptide derived from the mutant HA was found not to be palmitoylated (Figure 3A). Analyses of the composition of the carbohydrate side chains of the HAs were made by labeling with either mannose or fucose (Figure 3B) and by glycopeptidase F digestion. The results of these experiments indicated that 20 min–labeled wild-type and mutant HAs were mannosylated but not fucosylated, in agreement with the conclusion that these short-labeled products were incompletely modified. As expected, the 18 hr–labeled polypeptides were also mannosylated, but unlike the 75 kd polypeptides, the 73 kd polypeptide of mutant HA was not fucosylated. In addition, when 18 hr–labeled wild-type and mutant HAs were deglycosylated using glycopeptidase F, single polypeptides with the same molecular weight of 60 kd were produced, confirming that the size difference between the 75 kd and 73 kd mutant HA components were due to differences in glycosylation. Furthermore, a longer exposure of the autoradiogram (not shown) indicated that the smaller HA_2 component of the mutant HA was neither mannosylated nor fucosylated. However, these differences in glycosylation between wild-type and mutant HAs were not due to abnormalities in carbohydrate processing by the CHO cells expressing the mutant HA, since these cells could be superinfected with A/PR/8/34 influenza virus (H1N1) and shown to produce fully glycosylated viral HA.

We also observed that the 73 kd component was not expressed at the cell surface. When intact cells expressing mutant HA were treated with trypsin (Figure 4), only the 75 kd polypeptide was cleaved to HA_1 and HA_2. However, when cell lysates were treated similarly, both 75 kd and 73 kd HA polypeptides were cleaved. Furthermore, the 73 kd

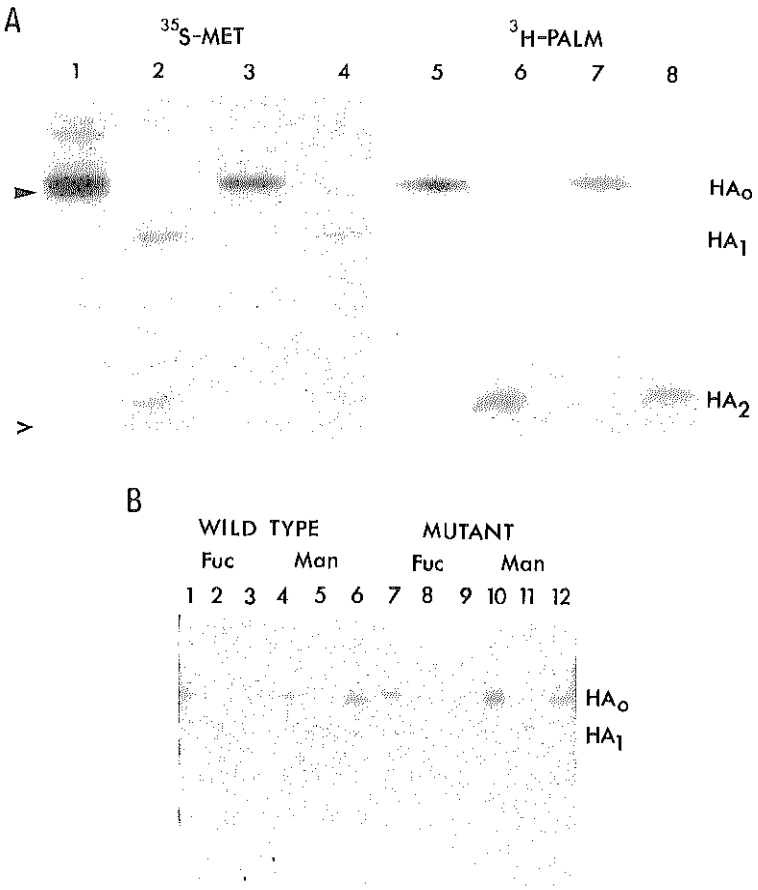

Figure 3. Posttranslational Modifications of HA

(A) (Lanes 1–4) Demonstration of the cleavage of wild-type and mutant HA_0 into HA_1 and HA_2 by trypsin. CHO cells expressing HA were labeled for 18 hr with [^{35}S]methionine, and cell lysates were incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of trypsin (2.5 μg/ml). Trypsin inhibitor (2.5 μg/ml), was added, and the HA was immunoprecipitated and analyzed on a 12% polyacrylamide gel under reducing conditions. Mutant HA, lanes 1 and 2; wild-type HA, lanes 3 and 4. A solid arrowhead denotes the 73 kd mutant HA_0 and an open arrowhead the smaller HA_2 component. (Lanes 5–8) Palmitoylation of wild-type and mutant HAs. CHO cells expressing HAs were labeled with ^{3}H-palmitate for 18 hr, and the HAs were immunoprecipitated from cell lysates that had been incubated in the presence (lanes 6 and 8) or absence (lanes 5 and 7) of trypsin and trypsin inhibitor. Mutant HA, lanes 5 and 6; wild-type HA, lanes 7 and 8. Note the absence of palmitoylated 73 kd mutant HA_0 and of the smaller HA_2 component.

(B) Glycosylation of wild-type and mutant HAs. CHO cells expressing wild-type (lanes 1–6) or mutant HA (lanes 7–12) were labeled for 18 hr or 20 min with ^{3}H-fucose (lanes 1–3 and 7–9) or ^{3}H-mannose (lanes 4–6 and 10–12). HA was immunoprecipitated from cell lysates and analyzed by PAGE on a 12% polyacrylamide gel under reducing conditions. Lanes 1, 4, 7, and 10 contain 18 hr–labeled products; lanes 2, 5, 8, and 11 contain lysates that were treated with trypsin (2.5 μg/ml, 10 min at 37°C) and trypsin inhibitor (2.5 μg/ml); and lanes 3, 6, 9, and 12 contain 20 min–labeled products.

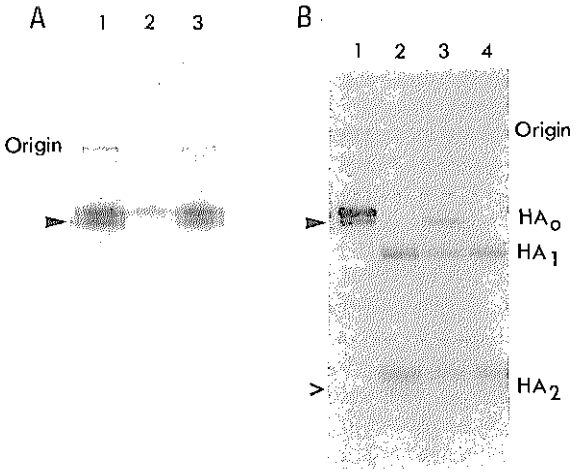

Figure 4. Demonstration That the 73 kd Mutant HA Is Incorrectly Folded and Is Not Expressed at the Cell Surface

(A) Immunoprecipitation of mutant HA with a conformation-specific monoclonal antibody. CHO cells expressing mutant HA were labeled with [^{35}S]methionine for 18 hr. HA was immunoprecipitated from cell lysates using polyclonal anti-BHA rabbit antiserum (lane 1) or conformation-specific monoclonal antibody HC67 (lane 2) or HC3 (lane 3) and analyzed under reducing conditions on 10% polyacrylamide gels. The arrow denotes the 73 kd HA.

(B) Trypsin treatment of cells expressing mutant HA shows that the 73 kd species is not expressed on the cell surface. CHO cells expressing mutant HA were labeled as described in (A). The HA was immunoprecipitated from the cell lysate using anti-BHA rabbit antiserum (lane 1). Lane 2 contains HA immunoprecipitated from cell lysate, which had been incubated with trypsin (2.5 μg/ml, 5 min, 37°C) and then with trypsin inhibitor (2.5 μg/ml). Lane 3 contains HA immunoprecipitated from cell lysates prepared from cells that had been incubated with trypsin and then trypsin inhibitor before cell lysis. Lane 4 contains immunoprecipitated HA prepared in the same way as the lane 3 sample, but additionally digested with trypsin following cell lysis. A solid arrowhead denotes the 73 kd HA_0, and an open arrowhead denotes the smaller HA_2 component.

polypeptide was found not to be immunoprecipitated by the conformation-specific monoclonal antibody HC67 (Figure 4).

We therefore conclude that the 73 kd polypeptide produced in mutant HA–expressing cells represents incompletely modified HA, incorrectly folded and not expressed at the cell surface.

Finally, the relative proportions of the 75 and 73 kd glycopolypeptides produced in mutant HA–expressing cells could be altered by varying the incubation temperature. At 37°C equal amounts of both glycopolypeptides were produced, at 32°C the products were almost exclusively the larger glycopolypeptides, and at 39°C predominantly the 73 kd glycopolypeptide was produced. These results are consistent with the smaller glycopolypeptide being incorrectly folded, albeit trimeric: the proportion of this species increasing with increasing incubation temperatures. Pulse–chase experiments indicated that the half-life of the smaller component was about one-third that of the 75 kd species (8 hr vs. 24 hr).

In considering the results that follow it should be noted that since the aberrant 73 kd HA was not expressed on the cell surface, its presence did not affect the results of the antibody-binding or cell fusion experiments.

The Antigenic Properties of the Surface-Expressed HAs

Immunoprecipitation and ELISA experiments showed that both mutant and wild-type HA molecules expressed on CHO cells bind to conformation-specific anti-HA monoclonal antibodies. The ELISA results are shown in Table 1. Only one of the antibodies (HC31(198)), which bound to the wild-type HA, did not appear to bind to the mutant HA. The region recognized by HC31 contains residue 198, which is about 15 Å from the site of the novel disulfide bond (Wilson et al., 1981), and the lack of binding suggests a localized difference in the structure of the mutant HA in this region. Overall, therefore, these data indicate that the mutant HA molecules resemble the wild-type HA in binding to most antibodies, but that the HA with disulfide bonds between residues 212 and 216 can be distinguished from wild type by a number of antibodies that recognize a region of the HA_1–HA_1 interface near the location of the introduced substitutions.

Analyses of the pH-Dependent Conformational Changes in the Expressed Hemagglutinins

Antibody-Binding Studies

The low pH–induced changes in HA structure were monitored by comparing the binding of monoclonal antibodies that recognize both neutral and low pH HA, e.g., HC3(144), to that of antibodies that only bind to the neutral form, e.g., HC31(198) or HC67(156, 193).

Table 2 shows results obtained for the binding of the conformation-specific antibodies to cells expressing wild-type or mutant HA molecules. The cells were treated with trypsin to cleave HA_0 to HA_1 and HA_2 and were then incubated at either pH 7 or pH 5. The binding to wild-type HA–expressing cells of antibodies that only recognize native HA was decreased following incubation at pH 5. However, no decrease in binding was seen with the mutant HA–expressing cells, indicating that the structure of this HA had not changed at the lower pH. In addition, the binding

Table 1. Binding of Various Monoclonal Antibodies to HA-Expressing Cells

Antibody	Wild-Type HA	Mutant HA
HC3 (144)	1.00	1.00
HC100 (63)	0.82	0.78
HC67 (156,193)	0.69	0.74
HC263 (158)	0.90	0.58
HC2 (188)	0.39	0.20
HC68 (193)	0.72	0.80
HC31 (198)	0.44	0.02
HC221 (199)	0.42	0.33

All ELISA assays were done on cells expressing HA that had been cleaved into HA_1 and HA_2 by incubation with 2.5 μg/ml trypsin for 10 min at 37° C. Results are expressed as ratios compared to binding to HC3. The residues on HA_1 recognized by each antibody are shown in parentheses (Daniels et al., 1983).

Table 2. Low pH–Induced Conformational Changes in HA As Detected by Conformation-Specific Monoclonal Antibodies

		+Trypsin		−Trypsin	
HA	Antibody	pH 7	pH 5	pH 7	pH 5
Wild Type	HC67 (156,193)	0.65	0.11	0.57	0.73
	HC68 (193)	0.64	0.08	0.52	0.66
	HC31 (198)	0.51	0.15	0.39	0.46
	HC221 (199)	0.42	0.15	0.38	0.49
	pH12 (HA2)[a]	0.26	0.77	0.29	0.31
Mutant	HC67 (156,193)	0.73	0.69	0.74	0.72
	HC68 (193)	0.80	0.79	0.77	0.82
	HC31 (198)	0.02	0.04	0.04	0.04
	HC221 (199)	0.33	0.32	0.31	0.38
	pH12 (HA2)	0.10	0.10	0.10	0.90

All ELISA results were corrected for the amount of HA expressed on the cell surface by dividing the OD obtained by that obtained with HC3 (144) (an antibody that recognizes native and low pH HA equally well). Trypsin cleavage of the HA_0 to HA_1 and HA_2 was done with 2.5 μg/ml TLCK-treated trypsin for 10 min at 37° C.

[a] A monoclonal antibody that recognizes low pH–treated HA but not native HA. The antibody recognizes HA_2 (although not the 38 amino-terminal residues), as determined by immunoblotting.

of an antibody, pH12, that only recognizes HA in the low pH conformation was increased for pH 5–treated wild-type HA but not for the mutant HA. Without trypsin treatment, no changes in the binding of any of these antibodies were seen to any of the HA-expressing cells, consistent with the observations that unlike cleaved HA, HA_0 does not mediate membrane fusion and does not undergo a pH-dependent conformational change (Daniels et al., 1983).

Proteolysis Experiments

The structural changes in HA at the pH of fusion have been observed to result in changes in the susceptibility of the molecule to proteolytic digestion. The low pH–induced

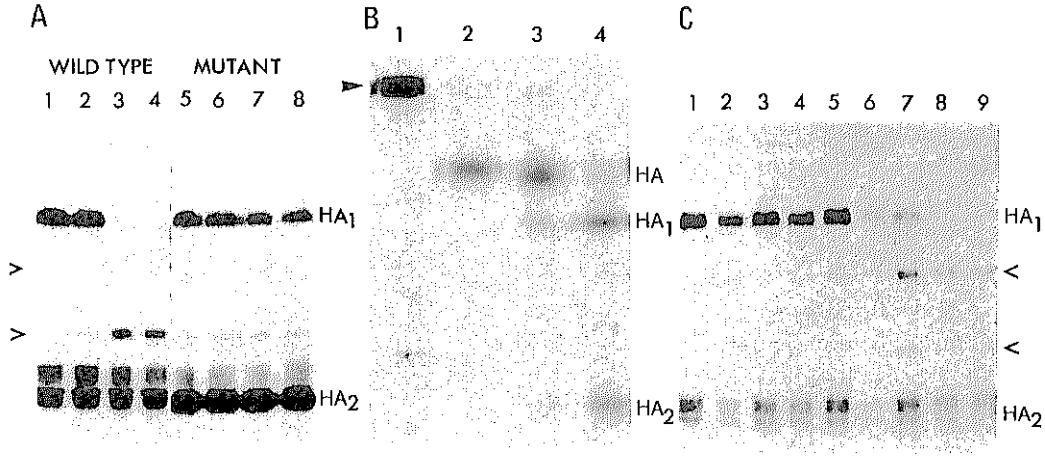

Figure 6. Comparison of the pH-Dependent Conformational Changes in Wild-Type and Mutant HA As Determined by Susceptibility of the HAs to Proteolysis by Trypsin

(A) Absence of pH-induced conformational changes in the nonreduced mutant HA. CHO cells expressing wild-type or HA were labeled for 4 hr with [^{35}S]methionine and then incubated for 1 hr in methionine-supplemented medium. Lysates of these cells were incubated with trypsin to cleave HA_0 into HA_1 and HA_2 and then incubated at different pH for 2 min at 37°C. After neutralization, they were incubated with trypsin (5 μg/ml) for 10 min at 23°C, the digestion was stopped by adding trypsin inhibitor, and the products were immunoprecipitated and analyzed by PAGE (10% gels) under reducing conditions. The gel lanes contain: wild-type HA incubated at pH 7.7 (lane 1); pH 5.5 (lane 2); pH 4.9 (lane 3); pH 4.8 (lane 4); mutant HA incubated at pH 7.7 (lane 5); pH 5.5 (lane 6); pH 4.9 (lane 7); pH 4.8 (lane 8). The products of digestion that are formed at the pH of fusion are indicated by the arrows.

(B) Conditions for reduction and alkylation of mutant HA. CHO cells expressing mutant HA were labeled with [^{35}S]methionine for 18 hr. Cell lysates were incubated at 37°C for 30 min, either without (lane 1) or with DTT: lane 2, 25 mM DTT; lane 3, 50 mM DTT. They were then alkylated using iodoacetamide (125 mM) at 20°C for 10 min and incubated with trypsin (2.5 μg/ml) to cleave HA_0 into HA_1 and HA_2. Lysate incubated with trypsin before reduction by DTT (25 mM) and alkylation is shown in lane 4. HA was immunoprecipitated from all four lysates and analyzed by PAGE using 7% gels under nonreducing conditions. The unreduced trimer in lane 1 is indicated by the arrow.

(C) Demonstration that reduction and alkylation of mutant HA restores its ability to undergo a low pH–induced conformational change. [^{35}S]Methionine-labeled CHO cell lysates were prepared as described in (A). They were reduced with DTT (25 mM), alkylated with iodoacetamide (125 mM), and treated with trypsin. Aliquots were then incubated at different pH, neutralized, and incubated with trypsin and then trypsin inhibitor as in (A). They were then immunoprecipitated and analyzed as in (A). Lane 1, pH 6.2; lane 2, pH 6.0; lane 3, pH 5.5; lane 4, pH 5.4; lane 5, pH 5.3; lane 6, pH 5.2; lane 7, pH 5.1; lane 8, pH 5.0; lane 9, pH 4.9. The products of digestion are shown by arrows.

conformational changes in the HAs were therefore also measured by monitoring the susceptibility of their HA_1 glycopolypeptides to trypsin (Skehel et al., 1982). The results in Figure 5A show that the HA_1 of wild-type HA expressed in CHO cells became susceptible to proteolysis after incubation below pH 5.5. By contrast, the mutant HA was not susceptible to digestion even at pH 4.8, indicating, in agreement with the antibody-binding experiments, that the conformation of the mutant HA does not change at low pH.

Definition of Conditions for Selectively Reducing the Novel Disulfide Bonds in the Mutant HA

Following the above observations, conditions were sought under which the novel disulfide bonds in the mutant HA could be selectively reduced and the free cysteines alkylated. Lysates of mutant HA–expressing cells were incubated with dithiothreitol (DTT) and alkylated with iodoacetamide. Polyacrylamide gel electrophoresis (PAGE) analysis under reducing conditions (Figure 5B) showed that the mutant HA no longer migrated as a covalent trimer, indicating cleavage by DTT of the intersubunit disulfide bonds. When the reduced and alkylated HA was subsequently treated with trypsin in order to cleave it into HA_1 and HA_2, its apparent molecular weight under nonreducing conditions remained 75 kd, showing that reduction and alkylation had not cleaved the natural intrasubunit disulfide bond between HA_1 residue 14 and HA_2 residue 137. Interestingly, if the mutant HA_0 was first treated with trypsin to produce HA_1 and HA_2 before reduction and alkylation, this natural HA_1–HA_2 disulfide bond was broken (Figure 5B). In all other experiments described, therefore, reduction and alkylation always preceded trypsin treatment. Additional experiments were done to define conditions for reduction and alkylation of the intersubunit disulfide bonds of the mutant HA expressed at the cell surface. However, even in the presence of DTT (50 mM) that was extremely toxic to the cells, only about 30% of the bonds were reduced as judged by PAGE analysis.

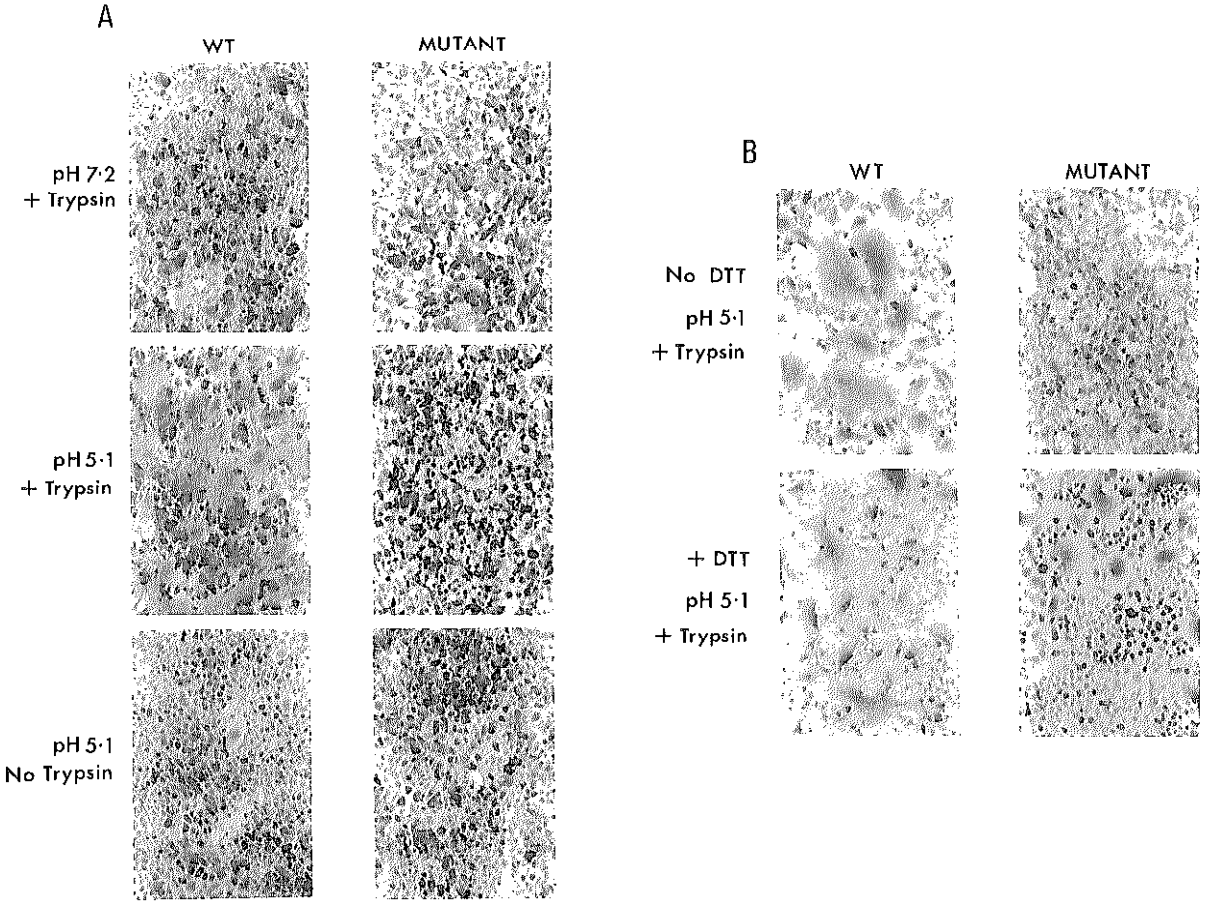

Figure 6. Demonstration by Heterokaryon Formation of Low pH–Induced Cell–Cell Fusion by Cells Expressing Wild-Type and Mutant HAs

(A) The lack of cell–cell fusion activity of cells expressing mutant HA. CHO cells expressing wild-type or mutant HAs were grown to confluence in 24-well tissue culture plates. Where appropriate they were treated with trypsin (2.5 μg/ml), to cleave HA_0 into HA_1 and HA_2, and then with trypsin inhibitor (2.5 μg/ml). Cells were incubated at different pH for 10 min at 37°C and overlaid with Alpha MEM containing fetal calf serum for 2 hr at 37°C. They were then fixed with 2.5% glutaraldehyde for 15 min at 20°C and stained with 1% toluidine blue (w/v).

(B) The acquisition of cell–cell fusion activity by mutant HA-expressing cells following reduction and alkylation. Experimental details as for (A). Cells were incubated with DTT (25 mM) or without DTT for 30 min at 37°C. They were then washed twice in 1 mM iodoacetamide to alkylate free sulfydryl groups and treated with trypsin, then trypsin inhibitor, and incubated at pH 5.1.

When assays of proteolytic susceptibility as a function of pH were done on the fully reduced and alkylated mutant HA, HA_1 became susceptible after incubation at pH 5.2 (Figure 5C). It was therefore concluded that the mutant molecule was able to undergo the conformational change in a manner similar to and at the same pH as wild-type HA once the novel intersubunit disulfide bonds were broken. However, it was not possible to confirm these results using the differential antibody-binding ELISA protocol, since efficient reduction and alkylation was only achieved in the presence of nonionic detergent, which caused cells to detach from the monolayer.

Studies of the Membrane Fusion Activity of the Expressed Hemagglutinins

Cell–Cell Fusion

Heterokaryon and syncytia formation were initially used to assay the fusion activity of cells expressing wild-type or mutant HA molecules. Confluent monolayers were treated with trypsin to cleave HA_0 into HA_1 and HA_2. The cells were then incubated at different pH, washed with Alpha modified Eagle's medium (MEM), and incubated for an additional 2 hr at 37°C. They were then fixed and stained with 1% toluidine blue. Cells not expressing HA and cells expressing HA but not treated with trypsin were used as controls. The results shown in Figure 6 indicate that cells expressing wild-type HA formed extensive syncytia after trypsin cleavage of HA_0 to HA_1 and HA_2 and incubation at low pH. By contrast, cells expressing the mutant HA did not form significant numbers of heterokaryons. In the absence of trypsin cleavage of HA_0 to HA_1 and HA_2, no heterokaryons were formed at any pH by any of the HA-expressing cells.

To investigate whether the mutant HA became fusogenic after reduction and alkylation, cells were treated with DTT (50 mM) and washed twice with iodoacetamide (1 mM) to alkylate free cysteine residues. (Higher concentrations of iodoacetamide proved too toxic.) The cells were then treated with trypsin and incubated at pH 5.1. The results obtained (Figure 6) indicate that under these conditions, fusion by reduced and alkylated mutant HA was clearly detectable, even though the reduction and alkylation procedures decreased the extent of fusion by wild-type HA. However, estimates of the percentage of nuclei present in heterokaryons indicated that the extent of syncytia formation by the mutant HA–expressing cells was only about 50% of that observed using wild-type HA–expressing cells, reflecting the incomplete reduction of cell surface mutant HA. No fusion was seen with either wild-type or mutant HA–expressing cells in which HA_0 had not been cleaved to HA_1 and HA_2 with trypsin.

Fusion Activity of Detergent-Extracted Hemagglutinin

Rosettes of HA formed by dialysis of detergent-extracted virus are capable of fusing liposomes (Wharton et al., 1986) and causing hemolysis (Sato et al., 1983). To confirm the cell–cell fusion results obtained using mutant HA–expressing cells, we therefore attempted to establish that the mutant HA was capable of mediating fusion efficiently following reduction and alkylation in the presence of detergent, by using a hemolysis assay, which is an accurate quantitative measure of fusion activity (Wharton et al., 1986). To obtain sufficient quantities of HA for such assays, a vaccinia expression system was used as described in the Experimental Procedures. The results obtained (Table 3) indicate that the reduced and alkylated mutant HA caused hemolysis at levels comparable to those obtained with wild-type HA. Unreduced mutant HA caused a small amount of hemolysis (about 10% of the reduced and alkylated mutant HA), probably reflecting the small amount of noncovalently cross-linked mutant HA produced by the cells (Figure 2), which was shown by iodination experiments to be expressed at the cell surface. Treatment with trypsin greatly increased the extent of hemolysis by wild-type and reduced and alkylated mutant HA, but since a small proportion of the HA was cleaved during preparation using this expression system, a low level of hemolysis was observed following incubation of untrypsinized wild-type HA at low pH.

Observations of the Aggregation of Soluble, Bromelain-Released HA at the pH of Fusion

The conformational changes in soluble, bromelain-released HA (BHA) at the pH of fusion result in the aggregation of BHA as a consequence of the exposure of the hydrophobic amino terminus of HA_2 (reviewed in Wiley and Skehel, 1987). To compare the structural changes in the HA_2 amino-terminal region of the mutant HA at low pH with those that occur in wild-type HA, we determined the ability of mutant BHA and wild-type BHA isolated from membranes of vaccinia recombinant–infected cells to aggregate at low pH. The results obtained (Figure 7) indicated that unlike wild-type BHA, which aggregated at pH 5, mutant BHA did not aggregate and sedimented with a sedimentation coefficient of 9S to the same position as BHA incubated at pH 7. Our conclusion from these results is that the fusion peptide of the mutant BHA is not exposed sufficiently to cause aggregation following incubation at pH 5.

Table 3. Hemolysis Caused by Detergent-extracted Rosettes of HA Expressed in Vaccinia Recombinant-Infected CV1 Cells

		+ Trypsin		– Trypsin	
HA	Treatment	pH 7.2	pH 5.2	pH 7.2	pH 5.2
Wild Type	– DTT	0	82	0	21
	+ DTT	0	45	4	3
Mutant	– DTT	0	8	0	1
	+ DTT	0	69	ND	ND
Viral HA	– DTT	2	86	–[a]	–[a]

10 μg HA was incubated with 0.75 ml of 1% human erythrocytes for 6 hr at 37° C. The ODs of the 1000 g supernatants were determined at 520 nm, and results were expressed as percentages compared with the total hemolysis of detergent-lysed erythrocytes. Values were corrected for background hemolysis by incubations lacking HA. This hemolysis was never greater than 5%. Trypsin treatment was for 10 min with 5 μg/ml trypsin at 37° C, and reduction and alkylation of HA using DTT was done as described in the Experimental Procedures section. ND, not determined.

[a] Since viral HA is already cleaved to HA_1 and HA_2, this experiment could not be done.

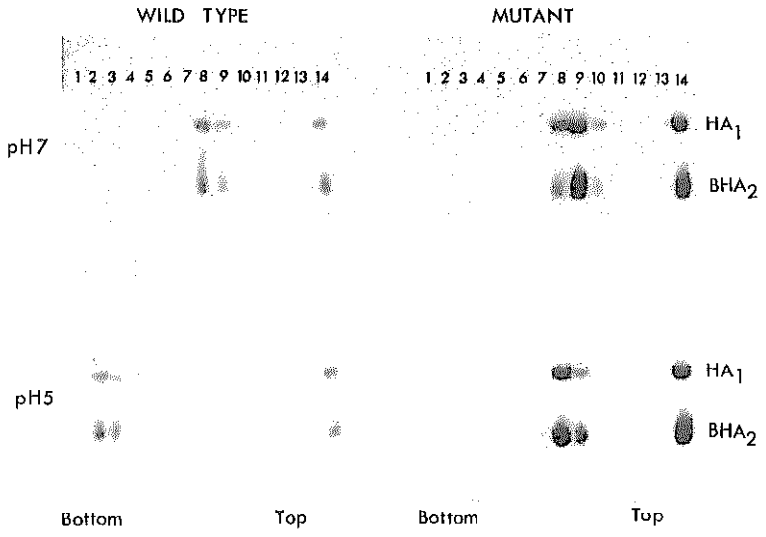

Figure 7. Effects of Incubation at Low pH on the Aggregation State of BHA Isolated from Cells Expressing Wild-Type or Mutant HA As Measured by Sucrose Density Gradient Centrifugation.

BHA was prepared from wild-type and mutant HA–expressing CV1 cells as described in Experimental Procedures. Aliquots (1 ml) of BHA (approximately 20 μg protein) in PBS were incubated at either pH 7 or pH 5 for 10 min at 37°C. The samples were neutralized and centrifuged at 35K for 16 hr (Beckman SW41 rotor) in a continuous 10%–30% sucrose gradient with a 1 ml 60% sucrose cushion. Gradient fractions (1–13) were analyzed by PAGE using 12% gels under reducing conditions, and the BHA was detected by immunoblotting using anti-BHA rabbit antiserum and ^{125}I-labeled goat anti-rabbit antiserum (Amersham). Fraction 1 contained pelleted material, fraction 2 contained protein in the 60% sucrose cushion, and fraction 13 was from the top of the gradient. Lane 14 contains an aliquot of the glycoprotein that was loaded on the gradient.

Discussion

The fusion of influenza virus and cellular membranes required for transfer of the virion genome–transcriptase complex into the cell occurs in endosomes and is activated at the low pH of these cellular compartments (for review see White et al., 1983). The virus membrane component that mediates fusion is the HA, and a number of studies have indicated that at the pH of fusion the structure of this molecule is irreversibly modified (for review see Wiley and Skehel, 1987). Investigations of the mechanism of membrane fusion have, therefore, included analyses of these changes in structure in attempts to understand their significance for the process of fusion.

The Conformational Change

Initial experiments with X-31 HA indicated that at the pH of fusion, membrane-anchorless, soluble HA, BHA, aggregated, or in the presence of lipids or detergents acquired the ability to bind to them (Skehel et al., 1982). These hydrophobic reactions were subsequently shown to involve the conserved hydrophobic amino-terminal region of HA_2 referred to as the fusion peptide (Ruigrok et al., 1988; Brunner, 1989), and it was concluded that at the pH of fusion this region of the molecule was extruded from its buried location in the native trimer. In addition, specifically at the pH of fusion, HA was observed to become extremely susceptible to proteolysis, and analyses of tryptic digestion products by chemical cross-linking and sedimentation showed that, although HA_2 remained trimeric, the membrane-distal HA_1 domain, released by proteolysis, had dissociated into monomers. Possibly as a consequence of this dissociation at the HA_1–HA_1 interfaces, differences in reactivity with HA_1–specific monoclonal and anti-peptide antibodies were also observed (Daniels et al., 1983; Webster et al., 1983; Yewdell et al., 1983; Jackson and Nestorowicz, 1985; White and Wilson, 1987). These results and the mutations discovered in the interface between the membrane-distal domains of HA_1 that affected identically both the pH of membrane fusion and the conformational changes required to expose the fusion peptide (Daniels et al., 1985, 1987) led to the conclusion that at the pH of fusion, structural changes also occurred in the membrane-distal regions of the molecule and were therefore not restricted to the region containing the amino terminus of HA_2.

More recently, however, experiments by Puri et al. (1990) suggested that the structural changes induced in the HAs of different influenza viruses at the pH of fusion may differ in detail, since the results of electron microscopic observations in particular indicated less extensive changes in the appearance of HAs on A/Japan/305/57 (H2N2) virus particles incubated at low pH than those observed for HA on X-31 virus (Ruigrok et al., 1986). Furthermore, the results of a recent study of X-31-mediated fusion at 0°C (Stegmann et al., 1990) suggested that, at least as judged by antigenic analyses and electron microscopy, the structural changes in the membrane-distal domain observed at 20°C and 37°C did not occur at low temperature.

A general conclusion drawn from all these studies, then, is that extrusion of the amino terminus of HA_2 occurs at the pH of fusion, and it also seems to be agreed, but not proven, that as the "fusion peptide" this region of HA is involved in fusion activity. However, whether any or all of the additional changes in structure that have been observed are required for this extrusion or for the process of fusion itself or to what extent they are secondary to fusion activation are currently the objects of discussion.

Consequences for Membrane Fusion

In the study reported here we attempt to obtain information relevant to this discussion by experiments involving site-directed mutagenesis of HA in which we address specifically the structural changes in the membrane-distal globular domain of HA required for fusion activation. We report results obtained with a mutant HA in which substitution

of two residues by cysteines allow the formation of three disulfide bonds, which cross-link the domain (Figure 1). Both amino acid substitutions are accommodated in the native structure: they do not prevent trimerization, as shown by sedimentation analyses; they have no effect on receptor binding, as judged by hemadsorption assays (Pfeifer et al., unpublished data), they allow only the specific cleavage by trypsin of the precursor HA_0 into HA_1 and HA_2; and they are without effect on the binding of the majority of conformation-specific monoclonal antibodies.

Covalent cross-linking of the molecule by disulfide bonds prevents the changes in structure that are shown to occur in wild-type HA at the pH of fusion, prevents extrusion of the amino terminus of HA_2, and prevents membrane fusion. Reduction of the disulfide bonds allows activation of fusion, which occurs at the same pH as for wild-type HA. Together these results clearly indicate that when structural rearrangements in the membrane-distal region of a fusion-competent HA are prevented, HA-mediated membrane fusion is blocked. They therefore support the conclusion that such rearrangements are required for activation of membrane fusion and not merely for a secondary "built-in deactivation process" as recently suggested (Stegmann et al., 1990). How do they relate to observations that other HAs, or X-31 HA under conditions of fusion at low temperature, appear not to change in structure as extensively as X-31 HA under physiological conditions? The extent of the conformational changes required for fusion cannot be directly addressed, because the experimental methods used—antibody binding, electron microscopy, and sensitivity of HA to proteolysis—have not yet defined the extent of changes found in the fusion-active conformation, and high resolution structural data are only available for HA in the native, pH 7 conformation. Although structural data are only available for the X-31 HA, it should also now be possible to construct disulfide bonds in the HAs of other subtypes to assess the changes required in their conformations for membrane fusion. It appears most likely, however, from our results, that molecular movements in the membrane-distal region will be required generally for fusion and should be considered in proposals for the mechanism of HA-mediated membrane fusion.

Consequences for Inhibition of Infectivity

Based on the observations that cross-linking of the membrane-distal domain prevents membrane fusion, it may be possible to select reagents that, by stabilizing the interface in this region, would also inhibit fusion and as a consequence have antiviral activity. An assay to screen for such compounds could, for example, be constructed using monoclonal antibodies that recognize specifically either the low pH or the neutral pH forms of the HA. A formally similar strategy led to the development of compounds designed to stabilize the structure of oxyhemoglobin in preference to the deoxy molecule for the treatment of sickle cell anemia (Beddell et al., 1984).

Experimental Procedures

Construction and Expression of Mutant HAs

The cDNA for the HA of the X-31 strain of Influenza virus (Verhoeyen et al., 1980) was mutated using synthetic oligonucleotide primers according to the procedure of Oostra et al. (1983). The mutations were confirmed by in situ colony hybridization and nucleotide sequencing (Chen and Seeberg, 1985). CHO cells lacking the dihydrofolate reductase gene (kindly provided by A. Dorner, Genetics Institute) were cotransfected with plasmids containing the HA gene, pMT2-HA (Kaufman et al., 1989), and the gene for neomycin resistance, pSV2-Neo, using calcium phosphate precipitation. Cells were selected with G418 (Geneticin, Gibco) and nucleoside-free medium Alpha MEM (Gibco). HA expression was monitored by fluorescence-activated cell sorting analyses using rabbit anti-X-31 serum and goat anti-rabbit immunoglobulin G fluorescein isothiocyanate. Methotrexate ((+)-amethopterin, Sigma) was used to select for amplification (Kaufman and Sharpe, 1982). Cells were routinely cultured in nucleoside-free Alpha MEM supplemented with 0.5 mg/ml Geneticin, penicillin, streptomycin, and 10% (v/v) heat-inactivated dialyzed fetal calf serum (Gibco).

Immunoprecipitation of [^{35}S]Methionine-Labeled HA

Cells expressing HA were labeled with [^{35}S]methionine in methionine-free Alpha MEM supplemented with dialyzed fetal calf serum for the times stated in the figure legends. After the appropriate chase period (done in medium supplemented with 5 mM methionine), the cells were washed twice in phosphate-buffered saline (PBS) and the membranes solubilized with 1% (v/v) Triton X-100, 0.15 M NaCl, 50 mM Tris (pH 7.4), and 1 mM EDTA. The HA was immunoprecipitated as described by Belsham et al. (1986) using Pansorbin (Calbiochem) and rabbit anti-BHA serum. SDS-PAGE was done according to Laemmli (1970). In the reducing conditions referred to in the figure legends and the text, samples were boiled for 5 min in sample buffer containing 150 mM β-mercaptoethanol. Following electrophoresis, gels were treated with Amplify (Amersham International), dried, and exposed to X-ray film.

Analyses of Posttranslational Modifications of Cell-Expressed HAs

For palmitoylation experiments cells were labeled for 18 hr with [9,10(n)^{3}H]-palmitic acid (Amersham) (200 μCi/ml) in Alpha MEM containing dialyzed fetal calf serum and 5 mM sodium pyruvate. For glycosylation experiments cells were labeled for 30 min or 18 hr with either L-[6-^{3}H] fucose (20 μCi/ml) or D-[2-^{3}H] mannose (20 μCi/ml) in Alpha MEM containing 10% dialyzed fetal calf serum and one-tenth of the normal glucose concentration. Cell lysates were prepared and analyzed as described in the previous section. Digestions with glycopeptidase F involved incubating lysates with enzyme (0.4 U) (Boehringer) for 18 hr at 37°C in buffer containing 100 mM glycine, 20 mM HEPES, 5 mM EDTA, 1% β-mercaptoethanol, 0.5% Brij 36T (pH 7.8).

Conformational Change Assays

Susceptibility to Proteolysis

[^{35}S]Methionine-labeled cell lysates were incubated with 2.5 μg/ml TPCK-treated trypsin (Sigma) for 10 min at 37°C to cleave precursor HA_0 to HA_1 and HA_2. The reaction was stopped with an equal amount of soybean trypsin inhibitor. Susceptibility of low pH–treated HA to trypsin was determined as described previously (Skehel et al., 1982). Aliquots of labeled cell lysates were incubated at the appropriate pH for 2 min at 37°C, neutralized, and incubated with 5 μg/ml trypsin for 10 min at 23°C. Reactions were stopped by adding an equal amount of trypsin inhibitor, and the digests were immunoprecipitated and analyzed by SDS–PAGE. The mutant HA was reduced by the addition of 25 mM DTT to the cell lysate. After incubation for 30 min at 37°C, the reduced cysteines were alkylated by incubation with 125 mM iodoacetamide for 15 min at 23°C while maintaining the pH at 8 by addition of Tris buffer.

ELISA Experiments on HA-Expressing Cells

HA-expressing cells were grown in 96-well microtiter tissue culture plates (Falcon). HA_0 was cleaved by incubation of cell monolayers with 2.5 μg/ml trypsin for 10 min at 37°C, and low pH treatment was for 10 min at 37°C. The cells were fixed at 4°C for 18 hr with 0.05% (v/v) glutaraldehyde in PBS, and ELISA determinations were done on surface-expressed HA as described by Belshe et al. (1988) using protein A–peroxidase conjugate and 3,3′,5,5′-tetramethylbenzine as substrate. By comparison of the binding of monoclonal antibodies that recognize both native and low pH–treated HA with those that recognize

only native HA, a determination of the extent of low pH–induced conformational change in HA was made.

Vaccinia Virus Expression of HA

Mutant HA and wild-type sequences were cloned into the vaccinia virus expression vector p2100 (generously provided by D. J. Pickup, Dept. of Microbiology, Duke University Medical Center). This vector is a derivative of p1200, which directs transcription from the cis-acting element (CAE1) of the gene coding for the 160 kd major late gene product of cowpox virus (Patel et al., 1988). The Copenhagen strain of vaccinia was used to generate HA-expressing recombinant viruses as described previously (Mackett et al., 1984). CV1 cells were used for virus propagation and expression of HA. Cells were infected at an MOI of 3 and harvested 36 hr postinfection.

Preparation of HA Rosettes and Hemolysis Assays

Membranes of the vaccinia-infected cells were prepared by flotation through sucrose gradients as described below. They were reduced and alkylated as described above, and solubilized in 1% (w/v) Brij 36T. The HA was purified and exchanged into 1% (w/v) octyl-β-D-glucopyranoside on a 5%–25% sucrose gradient as described previously (Wharton et al., 1986). HA-containing fractions were identified by immunoblotting, subjected to another round of reduction and alkylation, and HA rosettes were made by exhaustive dialysis against PBS. After concentration to 20 μg/ml, 0.5 ml of rosettes was incubated with 0.25 ml of human erythrocytes (final hematocrit 1%) for 6 hr at 37°C, and the percentage hemolysis was determined as described previously (Daniels et al., 1985; Wharton et al., 1986).

Preparation of BHA from HA-Expressing Cells

HA-expressing cells (1–2 g wet weight) were homogenized using a Dounce homogenizer in 10 mM Tris (pH 7.2), 2 mM $MgCl_2$ with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μM E-64 [L-trans-epoxysuccinyl leucylamido (4-guanido)-butane], 1 μM pepstatin, and 1 μM bestatin). Sucrose was added to a final concentration of 70% (w/v), and membranes were prepared by flotation in a discontinuous 0%, 35%, 55%, 70% sucrose gradient in 10 mM Tris (pH 7.2), 1 mM EDTA, and protease inhibitors. After centrifugation at 20,000 rpm for 2 hr (Beckman SW28 rotor), the plasma membrane fraction (at the 0%–35% interface) and the smooth membrane fraction (at the 35%–55% interface) were pooled and washed repeatedly in 10 mM Tris buffer (pH 7.2), 1 mM EDTA. The membranes were resuspended in 2 ml of PBS, and the HA_0 was cleaved to HA_1 and HA_2 by treatment with 5 μg/ml trypsin for 10 min at 37°C. The reaction was stopped by the addition of trypsin inhibitor. The membranes were then pelleted, resuspended in 1 ml of 0.15 M NaCl, 50 mM Tris (pH 8), and 50 mM β–mercaptoethanol, and then digested with 100 μg/ml bromelain (Sigma cat. no. B2252) for 18 hr at 37°C. The digestion was stopped by incubation with 100 mM iodoacetamide, and the BHA was purified on 5%–25% (w/v) sucrose gradients (Brand and Skehel, 1972). BHA-containing fractions (as detected by immunoblotting) were pooled and concentrated to approximately 20 μg/ml.

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