JOURNAL OF VIROLOGY, Feb. 1993, p. 933–942
0022-538X/93/020933-10$02.00/0

Vol. 67, No. 2

Mutations in or near the Fusion Peptide of the Influenza Virus Hemagglutinin Affect an Antigenic Site in the Globular Region

JONATHAN W. YEWDELL,[1]* ALEX TAYLOR,[2] AMY YELLEN,[2] ANDREW CATON,[2] WALTER GERHARD,[2] AND THOMAS BÄCHI[3]

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[1]; Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104[2]; and Laboratory of Electron Microscopy, University of Zurich, Zurich, Switzerland CH 8028[3]

Received 20 August 1992/Accepted 2 November 1992

We previously described a monoclonal antibody (Y8-10C2) that binds influenza virus hemagglutinin (HA) monomers but not native trimers. In this study, we demonstrated that Y8-10C2 binds to the globular domain of HA and found evidence that its epitope is located at the interface of adjacent subunits. We further showed that at elevated temperatures, the Y8-10C2 epitope is transiently exposed in trimers for antibody binding. Introduction of intrasubunit chemical cross-links into HA reversibly inhibited both Y8-10C2 binding to trimers at elevated temperatures and viral fusion activity, indicating that exposure of the epitope requires the normal conformational flexibility of the molecule. Prolonged incubation of Y8-10C2 with virus at an elevated temperature resulted in neutralization of viral infectivity, allowing selection of neutralization-resistant virus mutants. Mutants were divided into two classes based on a radioimmunoassay in which the virus is attached to polyvinyl: those with reduced affinity for Y8-10C2 or other monoclonal antibodies specific for the globular domain and those with no alteration in their interaction with Y8-10C2 or other antibodies. DNA sequencing of HA genes revealed that the first type of mutants possessed single amino acid substitutions in the Y8-10C2 epitope itself, while remarkably, the second type of mutants possessed single amino acid substitutions in or near the fusion peptide of the HA, which is located in the stem of the HA at a considerable distance from the Y8-10C2 epitope. These findings indicate that the conformational flexibility of the HA affects its antigenicity and that single amino acid substitutions in or near the fusion peptide influence the flexibility of the globular domains.

The influenza virus hemagglutinin (HA) is a type I anchored membrane glycoprotein whose three-dimensional structure, function, antigenicity, and biosynthesis have been extensively characterized (17, 22, 25, 27). By virtue of its affinity for sialic acid residues, HA attaches influenza virus to the cell surface. Following internalization of the virus into cellular acidic endosomes, HA mediates fusion of viral and cellular membranes (14, 15).

HA monomers derived from A/Puerto Rico/8/34 (H1N1) (PR8) assemble into trimers shortly after their export from the endoplasmic reticulum, probably in a pre-Golgi complex intermediate compartment (33). When analyzed under nonreducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), HA trimers exist as a mixture of monomers, dimers, and trimers (20). The stability of dimers and trimers under these conditions is due to either interchain disulfide bonds between cysteine residues present in the carboxy terminus of the HA or strong interactions in this region that are disrupted by detergent binding only after disulfide bonds are broken. During the late stages of its transport to the cell surface, HA is proteolytically cleaved in certain cell types into disulfide-linked subunits. The carboxy-terminal subunit, termed HA2, contains the cytosolic and transmembrane domains, an extended chain that constitutes the bulk of the HA stem, and at its amino terminus, the sequence of 10 amino acids that mediates the fusion of viral and cellular membranes. The amino-terminal HA1 subunit contains an extended portion that contributes to the fibrous stem of the HA and a globular domain resting atop the stem containing the sialic acid-binding sites and the surrounding four antigenic sites recognized by antibodies able to neutralize viral infectivity. Two of these sites, termed Sa and Sb, are located at the tip of the globular domain surrounding the sialic acid-binding site, while two others, termed Ca and Cb, are located more towards the stem (1).

Exposure of the HA to the mildly acid conditions of the endosome (between pH 5 and pH 6) triggers irreversible conformational alterations in the HA that coincide with activation of viral fusion activity (21). Largely on the basis of studies with monoclonal antibodies (MAbs), it is believed that acid-induced conformational alterations in the HA result in a decrease in the interactions between adjacent monomers without greatly altering the structure of the monomers themselves (3, 8, 16, 23, 26, 31). It appears that the separation of monomers is essential for triggering of viral fusion activity (7, 10). We previously described an HA-specific MAb, termed Y8-10C2, that binds to virions only after they have been exposed to the same acid conditions that trigger viral fusion activity (31). Subsequently, we found that the ability of Y8-10C2 to bind to native HA is lost when the HA trimerizes during its biosynthesis (33). Thus, binding of Y8-10C2 to native HA is a sensitive probe of the proximity of adjacent globular domains within a trimer.

Here we show that Y8-10C2 binds trimeric HA at elevated temperatures and that this binding depends on the conformational flexibility of the HA, inasmuch as it is reversibly inhibited by introduction of chemical cross-links into the HA. We further show that single amino acid substitutions in

* Corresponding author.

and around the fusion peptide alter the conformational flexibility of the HA, as detected by the resistance of mutant HAs to Y8-10C2 binding at elevated temperatures.

MATERIALS AND METHODS

Chemical cross-linking of viruses. Dimethyl adipimate, dimethyl suberimidate, dimethyl-3,3′-dithio*bis*propionimidate (DTBP), and ethylene glycol*bis*(succinimidyl succinate) were purchased from Pierce Chemical. Dimethyl adipimate, dimethyl suberimidate, and DTBP were dissolved in 0.2 M triethanolamine HCl (pH 8.5) and incubated with purified PR8 at 100,000 hemagglutinating units/ml at room temperature. As a control, virus was routinely incubated in triethanolamine in the absence of chemical cross-linkers. Cross-linking was stopped by addition of excess glycine in phosphate-buffered saline (PBS). Cross-links in DTBP-treated virus were reversed by incubating virus with dithioerythritol for 1 h at 37°C. Iodoacetamide at a 50% excess over dithioerythritol was added to alkylate free sulfhydryl groups, and bovine serum albumin was then added to quench unreacted iodoacetamide. Ethylene glycol*bis*(succinimidyl succinate) was dissolved in sodium phosphate buffer and incubated with purified PR8 as described above, and the reaction was stopped by addition of excess glycine. Ethylene glycol*bis*(succinimidyl succinate) cross-links were reversed by adjusting the virus to 1 M hydroxylamine HCl (pH 8.5) and incubating it overnight at room temperature. Excess bovine serum albumin was added to minimize the residual activity of reagents. Prior to assay of samples for biological activity, the volumes of all samples were adjusted to contain equal concentrations of viral hemagglutinating units.

Hemagglutination titrations. HA, hemagglutination inhibition (HI), and hemolysis activities were measured by using human erythrocytes as previously described (31). Acid treatment of virus was performed as previously described (31).

Metabolic labelling and immunoprecipitation. MDCK or L929 cells were infected with PR8 wild-type or variant viruses for 5 h, labeled with [^{35}S]methionine, incubated for 0 to 60 min at 37°C in medium containing excess unlabeled methionine, and extracted with Triton X-100. All procedures were performed as previously described (33). Detergent extracts were immunoprecipitated as previously described (33) and were analyzed on polyacrylamide gels under nonreducing or reducing conditions by using the buffer system of Laemmli (12). After fixation, gels were soaked in Amplify (Amersham), dried, and fluorographed at −70°C, using preflashed film. Densitometry was performed by using a Bio-Rad 620 video densitomer. For cross-linking, extracts were derived from cells labeled for 30 min with [^{35}S]methionine and chased for 1 h at 37°C. Extracts were adjusted to the indicated concentration of DTBP by using a stock solution of DTBP at 5 mg/ml in 0.2 M triethanolamine HCl (pH 8.5). After 3 h at room temperature, the cross-linker was quenched by addition of excess glycine, and free sulfhydryls were alkylated by addition of *N*-ethylmaleimide. In the absence of *N*-ethylmaleimide, disulfides in DTBP-treated HA were reduced simply by boiling in SDS-PAGE sample buffer in the absence of reducing reagents. Cross-linked extracts were incubated with MAbs for 2 h at 45°C, and immune complexes collected with protein A-Sepharose were analyzed by SDS-PAGE as described above. For trypsin treatment, extracts derived from virus-infected cells labeled with [^{35}S]methionine for 10 min were incubated with trypsin at 10 μg/ml for 30 min at room temperature. Trypsin was inactivated by adding phenylmethylsulfonyl fluoride to a concentration of 1 mM and egg white trypsin inhibitor to a concentration of 10 μg/ml. Extracts were then immunoprecipitated by addition of protein A-Sepharose that had been preloaded with MAbs and analyzed by SDS-PAGE performed under reducing conditions by using a separating gel containing 12% acrylamide.

Nucleotide sequence analysis of virion RNA. The coding regions of HA genes of variant viruses and the wild-type stock they were selected from were completely sequenced by using the dideoxy method as previously described (1). The amino acid sequence of the HA1 region of the wild-type virus was identical to that reported previously (1). The amino acid sequence of the HA2 region of the wild type and the YV1 and YV10 variants all differed from the published sequence for PR8 at a single residue, position 54, in which T was substituted for S.

RIAs. Antigenic analysis of variant viruses was performed as previously described (1, 32). Briefly, viruses were adsorbed to 96-well polyvinyl plates by drying overnight and tested in a two-step radioimmunoassay (RIA) for the capacity to bind a standard dilution of hybridoma culture fluids containing approximately 1 ng of antibody per ml. Binding was related to a standard curve generated in the same experiment by testing the binding of twofold dilutions of the standard antibody dilution with the wild-type virus.

Variant selection with Y8-10C2. PR8 virus grown in the allantoic cavities of embryonated chicken eggs was incubated for 18 h at 37°C with Y8-10C2 or a control MAb specific for Sendai virus. Dilutions of the mixture were added to allantois on shell fragments (32) and incubated for 1 h at 37°C. The inoculum was then removed, and the fragments were washed twice and then cultured at 37°C for 4 days. Cultures were assessed for virus growth by hemagglutination titration. Cultures exhibiting virus growth were grown in the allantoic cavities of eggs and analyzed for reactivity with Y8-10C2 by RIA and HI assay. Viruses with altered phenotypes were cloned by limiting dilution in AOS cultures, grown in large quantities in eggs, and purified by velocity centrifugation in sucrose gradients.

Western blotting. Purified virus was separated by SDS-PAGE performed under reducing conditions with a 9% separating gel. Proteins were transferred to 0.22-μm-pore-size nitrocellulose by overnight blotting using a current of 150 mA. Filters were blocked by incubation with PBS–0.05% Tween 20 and cut into strips. Lanes were located by inclusion in each sample of molecular weight markers that had been danyslated. Strips were incubated for at least 16 h with MAb-containing culture fluids diluted 1/10 in PBS–0.05% Tween 20 and washed for several h with PBS–0.05% Tween 20. Strips were then incubated with a radioiodinated rat MAb specific for mouse κ chains, washed with PBS–0.05% Tween 20, and fluorographed, using Kodak XAR film and intensifying screens.

RESULTS

Definition of the Y8-10C2 epitope. The HA1 and HA2 chains of HA derived from egg-grown virus can be separated by SDS-PAGE under reducing conditions since eggs possess a protease that cleaves the HA during biosynthesis. Western blotting (immunoblotting) of egg-grown virus demonstrated that Y8-10C2 binds the HA1 chain and also some uncleaved HA (Fig. 1A, lane 3). The specificity of this reaction was demonstrated by the very low signal obtained with a control MAb (lane 2) and the strong reaction of an HA2-specific antibody with HA2 (lane 1) (the higher-molecular-weight

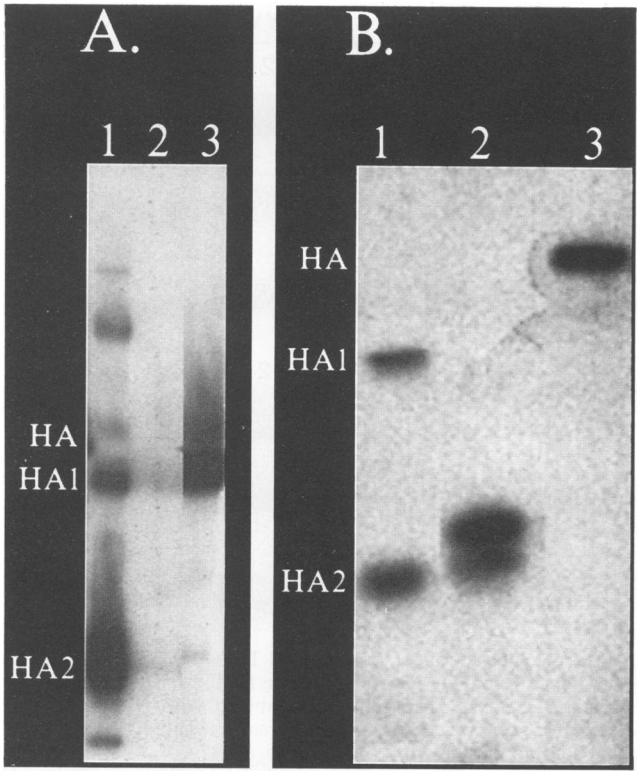

FIG. 1. Specificity of Y8-10C2 for the globular domain of HA1. (A) Viral proteins separated by SDS-PAGE under reducing conditions were detected in Western blotting by HA2-specific MAb H18-L10 (lane 1), a control antibody specific for Sendai virus (lane 2), or Y8-10C2 (lane 3). (B) A detergent extract from PR8-infected L929 cells labeled for 10 min with [^{35}S]methionine was left untreated (lane 3) or was treated with trypsin (lanes 1 and 2) and then immunoprecipitated with trimer-specific MAb H17-L2 (lanes 1 and 3) or Y8-10C2 (lane 2).

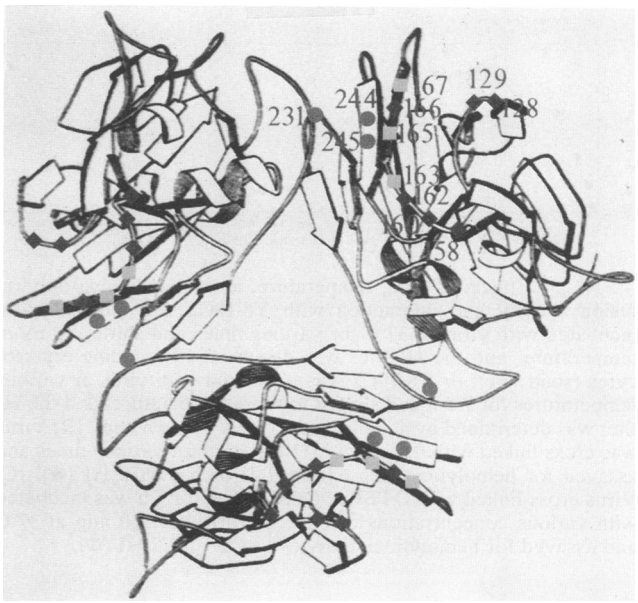

FIG. 2. Localization of the Y8-10C2 epitope. Superimposed on the three-dimensional structure of the HA are the residues identified by analysis of MAb-selected antigenic variants that influence the binding of Sa site-specific MAbs. Diamonds represent residues whose substitutions reduce the affinity of binding of other Sa site-specific MAbs without affecting Y8-10C2 binding to the HA. Squares represent residues whose substitutions reduce the affinity of Y8-10C2 and other Sa site-specific MAbs for HA binding. Circles represent residues whose substitutions reduce the affinity of Y8-10C2 without affecting the binding of other Sa site-specific antibodies able to interact with trimers.

forms detected by this antibody probably represent HA2 homomultimers as well as uncleaved HA; the species comigrating with HA1 probably represents HA2 dimers).

The localization of the Y8-10C2 epitope in the HA1 chain was further defined by immunoprecipitation of trypsin-treated detergent extracts derived from [^{35}S]methionine-labeled PR8-infected cells. Under these conditions, Y8-10C2 precipitated tryptic fragments with molecular masses of 26 and 30 kDa (Fig. 1B, lane 2). This is consistent with prior findings that HA monomers are susceptible to protease digestion (2, 6). This fragment must be derived from HA1, since it migrates more slowly than the intact HA2 chain (22 kDa, lane 1). Aside from the initiating Met, which is cleaved during or shortly after HA translocation into the endoplasmic reticulum, the only Met residues in HA1 are at positions 230, 274, and 316. Given that the fragments consist of approximately 180 residues and HA1 extends to position 329, this would limit the start of the fragment to residues 50 and 150 and the end to residues 230 to 329.

The localization of the Y8-10C2 epitope to this fragment agrees with our previous analysis of the binding of Y8-10C2 to a series of viruses with defined point mutations in the globular domain of the HA. These variants were selected by virtue of their resistance to neutralization mediated by HA-specific MAbs. Amino acid alterations at residues 163, 165, and 167 reduce Y8-10C2 binding to nondetectable levels in indirect radioimmunoassays (1), while amino acid alterations at residues 207 (1), 244 (30), and 245 (see below) have a partial effect on antibody binding. On the basis of the following findings, we believe that the residues identified by this analysis are in direct contact with the Y8-10C2 antigen-binding site and do not influence antibody binding by conformational effects. (i) As seen in Fig. 2, these residues are all present on the surface of the HA, forming a contiguous area of a size easily accommodated by an antibody-binding site (13). (ii) Mutations in these residues affect binding of only a subset of antibodies that map to the Sa site and do not affect the binding of numerous other antibodies specific for the other antigenic sites in the globular domain (1). (iii) The same amino acid alterations also reduce the binding of Y8-10C2 to the denatured HA (29a) (although the epitope recognized by Y8-10C2 is destroyed by denaturation, it is able to reform slowly in the presence of Y8-10C2, which probably accounts for the binding of Y8-10C2 to HA1 chains in Western blots). If the amino acid alterations did alter the Y8-10C2 epitope at a distance, it seems highly unlikely that they would cause similar alterations in both native HA and partially refolded HA.

If this analysis is correct, this means that some of the amino acids contacted by Y8-10C2 also serve as contact residues for MAbs that bind to native trimers. The likely answer to this paradox lies in the orientation of the antibodies in binding to these residues. Figure 2 locates the residues that alter the binding of "Sa" site-specific antibodies in the three-dimensional structure of a related HA molecule (28,

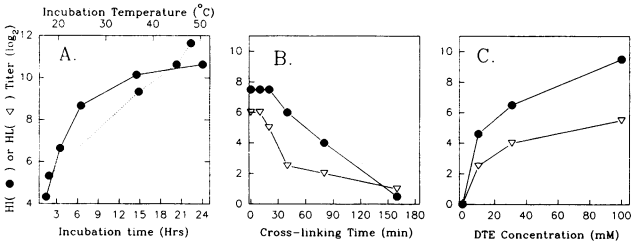

FIG. 3. Effects of time, temperature, and cross-linking on viral fusion activity and interaction with Y8-10C2. (A) Y8-10C2 was incubated with virus at 37°C for various times and shifted to room temperature, and the HI titer was determined by adding erythrocytes (solid line), or Y8-10C2 was incubated with virus at various temperatures for 7.5 h and shifted to room temperature, and the HI titer was determined by adding erythrocytes (broken line). (B) Virus was cross-linked with 0.5 mg of DTBP per ml for various times and assayed for hemolytic (HL) activity (▽) or Y8-10C2 HI (●). (C) Virus cross-linked with DTBP at 0.625 mg/ml for 3 h was incubated with various concentrations of dithioerythritol for 30 min at 37°C and assayed for hemolytic activity (▽) or Y8-10C2 HI (●).

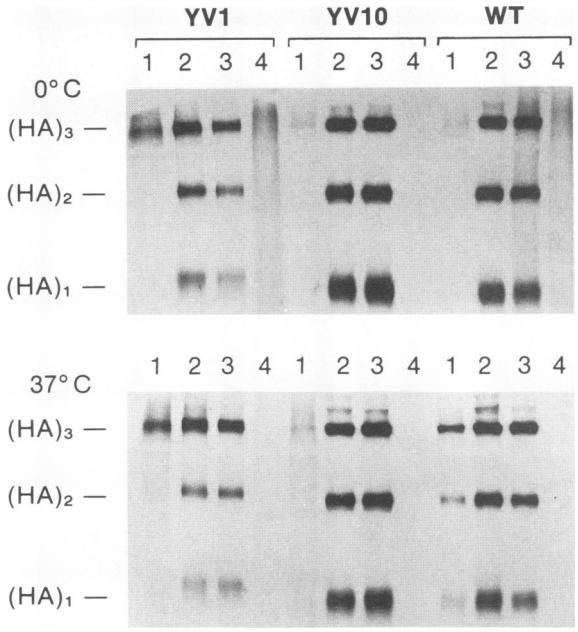

FIG. 4. Interaction of radiolabeled HA with Y8-10C2. MDCK cells infected for five h with wild-type (WT) or Y8-10C2 selected-variant PR8 virus were pulse-radiolabeled with [^{35}S]methionine, chased for 60 min at 37°C, and detergent extracted. Extracts were incubated for 18 h with MAbs at 0 or 37°C, and the immune complexes were collected on protein A-Sepharose and analyzed by SDS-PAGE. HA migrated as a mixture of monomers, dimers, and trimers since it was electrophoresed under nonreducing conditions. Lanes: 1, Y8-10C2; 2, H2-4C2; 3, H17-L2; 4, control MAb.

29). Residues that affect the binding of Y8-10C2 and other MAbs are squares, residues that affect only the binding of Y8-10C2 are circles, and those that affect only the binding of trimer reactive antibodies are diamonds. It is evident that trimer reactive antibodies recognize the external side of the Sa site, while Y8-10C2 reacts with the internal side, i.e., the side that faces the adjacent monomer. It is not difficult to imagine that access of Y8-10C2 to its epitope in trimers requires separation of adjacent monomers, which can be achieved by exposure of HA to pH 5 or, as discussed below, the partial denaturation of HA that occurs when the virus is dried onto polyvinyl for RIA.

Effect of temperature on Y8-10C2 binding. Y8-10C2 does not bind native trimeric HA present on virions, as detected by either HI assays or direct electron microscopic examination of negatively stained virus preparations (31). Y8-10C2 also does not bind metabolically radiolabeled HA trimers present in detergent lysates (33). All of these assays entail short (1 to 2 h) incubations at or below room temperature. By incubating Y8-10C2 with virus for extended periods at elevated temperatures, however, binding to HA can be detected by a number of methods.

As seen in Fig. 3A, by increasing the time or temperature of incubation of Y8-10C2 with the virus, viral hemagglutination was inhibited. At 37°C, HI titers reached plateau values only after incubation for 24 h prior to addition of erythrocytes (Fig. 3A, solid line). With incubation for 7.5 h, HI titers increased linearly with temperature (Fig. 3A, dashed line). Although Y8-10C2 specifically mediated HI under these conditions, it is important to note that the efficiency of this process on a molar basis was approximately 100-fold less than that of other antibodies specific for the globular domain or, for that matter, its own interaction with acid-treated virus (31).

Temperature-dependent binding of Y8-10C2 to HA was confirmed by a biochemical technique. Detergent extracts from [^{35}S]methionine-labeled PR8-infected MDCK cells were incubated with Y8-10C2 for 16 h at 37 or 4°C, and the amount of bound HA was determined by SDS-PAGE following addition of protein A-Sepharose to collect immune complexes. To minimize the amount of monomers present in the extracts, extracts were derived from cells incubated for 60 min at 37°C after pulse-radiolabeling. To guarantee further that immunoprecipitated material represented trimeric HA, SDS-PAGE was performed under nonreducing conditions, since PR8 trimers migrate as a mixture of monomers, dimers, and trimers in the absence of a reducing agent. As observed previously (33), Y8-10C2 incubated for 16 h at 0°C precipitated minimal amounts of HA (Fig. 4, top, WT lane 1). Under the same conditions, other HA-specific MAbs immunoprecipitated large amounts of HA (WT lanes 2 and 3). When Y8-10C2 was incubated with extracts for 16 h at 37°C, the amount of HA bound increased approximately 12-fold (as estimated by densitometry) (bottom, WT, lane 1). By contrast, elevated temperature did not influence the amount of HA precipitated by the other MAbs, except perhaps to decrease it slightly. The specificity of the immunoprecipitations is indicated by the absence of HA when a MAb specific for Sendai virus was used (lane 4).

The temperature-dependent binding of Y8-10C2 in these assays was due to conformational alterations in the antigen rather than in the antibody, since prior findings demonstrated that Y8-10C2 binding to HA monomers or acid-treated HA occurs in a temperature-independent manner (31). Furthermore, these conformational alterations are transient in nature, since preincubation of the virus or detergent-solubilized HA trimers at 37°C did not increase Y8-10C2 binding once the temperature was decreased (data not shown).

Chemical cross-linking of virus reversibly inhibits Y8-10C2 binding. The temperature dependence of Y8-10C2 binding to native HA, taken together with the location of the Y8-10C2 epitope, suggested to us that exposure of the Y8-10C2

epitope for antibody binding is related to the mobility of the globular heads relative to each other. To test this, we examined the effect of chemical-cross linkers on virus binding to Y8-10C2.

By using the reversible homobifunctional cross-linker DTBP, Y8-10C2-mediated HI was reduced in a manner dependent on the time of incubation and the DTBP concentration. With DTBP at 0.5 mg/ml, Y8-10C2-mediated HI was reduced more than 100-fold following cross-linking for 160 min (Fig. 3B). Importantly, DTBP treatment did not reduce either the hemagglutination activity of the virus or the HI titers of 10 other MAbs tested (including 7 that recognize the external ridge of the Sa site [data not shown]). It did, however, reduce viral hemolytic activity in parallel with its effect on Y8-10C2-mediated HI (Fig. 3). Similar results were obtained by using another reversible cross-linker, ethylene glycol*bis*(succinimydyl succinate), and two irreversible chemical cross-linkers, dimethyl superimidate and dimethyl adipimidate (data not shown).

The effect of DTBP on Y8-10C2 binding and viral fusion activity was reversed completely by reduction of the disulfide bridge connecting the DTBP active groups (Fig. 3C). This demonstrates that the effects of DTBP treatment are due to introduction of cross-links into the HA and not chemical modification of residues in the Y8-10C2 epitope.

The effect of DTBP on Y8-10C2 binding to trimers was confirmed by biochemical methods. Detergent extracts from [^{35}S]methionine-labeled PR8-infected cells were cross-linked for 3 h with DTBP at 0.05, 0.25, and 0.5 mg/ml and then incubated for 2 h at 45°C with Y8-10C2 or an Sa site-specific control MAb that binds trimers in a temperature-independent manner. Immune complexes collected by protein A-Sepharose were then analyzed by SDS-PAGE performed under nonreducing conditions (Fig. 5), and the amount of HA species precipitated was estimated by densitometry (Fig. 6). With increasing cross-linker concentrations, the amount of HA reactive with the control trimer-reactive MAb remained relatively constant and the proportion of HA migrating as trimers increased from 35 to 68%. By contrast, Y8-10C2 binding to trimers was inhibited in a cross-linker concentration-dependent manner to a maximum of 70% at the highest cross-linker concentration used. At the same time, the proportion migrating as trimers increased from 52 of the total to 82%. This provides direct evidence that introduction of cross-links into the HA reduces the capacity of HA trimers to interact with Y8-10C2 at elevated temperatures.

Selection of mutant viruses resistant to Y8-10C2-mediated neutralization. Having found the temperature dependence of Y8-10C2-mediated HI, we thought it important to re-examine the ability of Y8-10C2 to neutralize viral infectivity. Coincubation of Y8-10C2 with virus for 18 h at 20°C prior to addition of the virus-antibody mixture to allantois on chicken eggshell fragment cultures resulted in a slight reduction of viral infectivity versus incubation with a control antibody (from $10^{7.5}$ to $10^{6.8}$ infectious doses per ml). By contrast, preincubation of Y8-10C2 with virus at 37°C decreased infectivity at least 3,000-fold versus the control ascites. The term "at least" is used advisedly, since incubation of influenza virus with an overneutralizing concentration of MAb results in growth of neutralization-resistant variants at high virus inputs (5), masking the true antibody-neutralizing potency. In previous studies, without exception, these variants displayed reduced binding to the selecting antibody when tested in solid-phase RIAs; this is attributable to amino acid alterations within the HA1 chain (1).

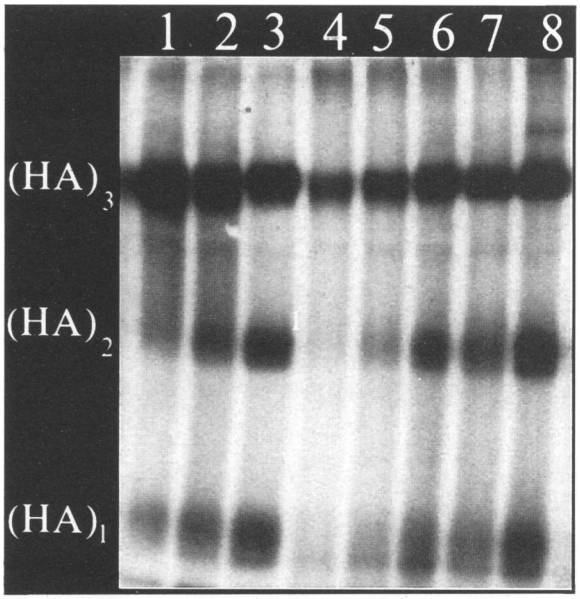

FIG. 5. Binding of Y8-10C2 to cross-linked HA in detergent extracts. A detergent extract from PR8-infected MDCK cells was left untreated (lanes 7 and 8) or was treated with DTBP at 0.05 (lanes 3 and 6), 0.25 (lanes 2 and 5), or 0.5 (lanes 1 and 4) mg/ml. Lysates were incubated for 2 h at 45°C with Y8-10C2 (lanes 4 to 7) or trimer-specific MAb H2-4B3 (lanes 1 to 3 and 8), and immune complexes were analyzed by SDS-PAGE performed under nonreducing conditions. The three species correspond to HA trimers $(HA)_3$, dimers $(HA)_2$, and monomers $(HA)_1$.

Six virus isolates from wells demonstrating virus growth in the presence of Y8-10C2 following 18 h of preincubation at 37°C were cloned by limiting dilution, grown in embryonated eggs, and purified for further analysis. Each of the isolates demonstrated twofold or greater resistance, relative to the wild-type virus, to Y8-10C2-mediated HI following virus-antibody incubation for 24 h at 37°C (Table 1). Antigenic analysis was performed by testing the binding of Y8-10C2 to virus adsorbed to polyvinyl in an indirect RIA (Fig. 6). As we and many others have noted in previous studies, binding of antigen to polyvinyl results in partial denaturation of the antigen; thus, adsorption of the virus to polyvinyl exposes the Y8-10C2 epitope on at least a subpopulation of HA. Two of the variants (YV3 and YV9) demonstrated a large decrease in avidity for the selecting antibody (Y8-10C2), which is typical for antibody escape mutants. Two other variants (YV2 and YV4) demonstrated a major decrease in avidity with one antibody (Sa9) and moderate decreases with several other antibodies. Of the two remaining variants, one (YV10) demonstrated a moderate decrease in affinity with a single antibody (Sa14), while the other (YV1) demonstrated no significant decrease with any of the 53 MAbs tested that are specific for the globular domain.

The mutations responsible for the altered interaction of four of the variants with Y8-10C2 binding were located by sequencing the entire coding region of HA genes derived from YV1, YV4, YV9, YV10, and the wild-type virus used for variant selection. Each variant possesses a point mutation resulting in a single amino acid substitution (Fig. 6). Variants with antigenic alterations detected by multiple

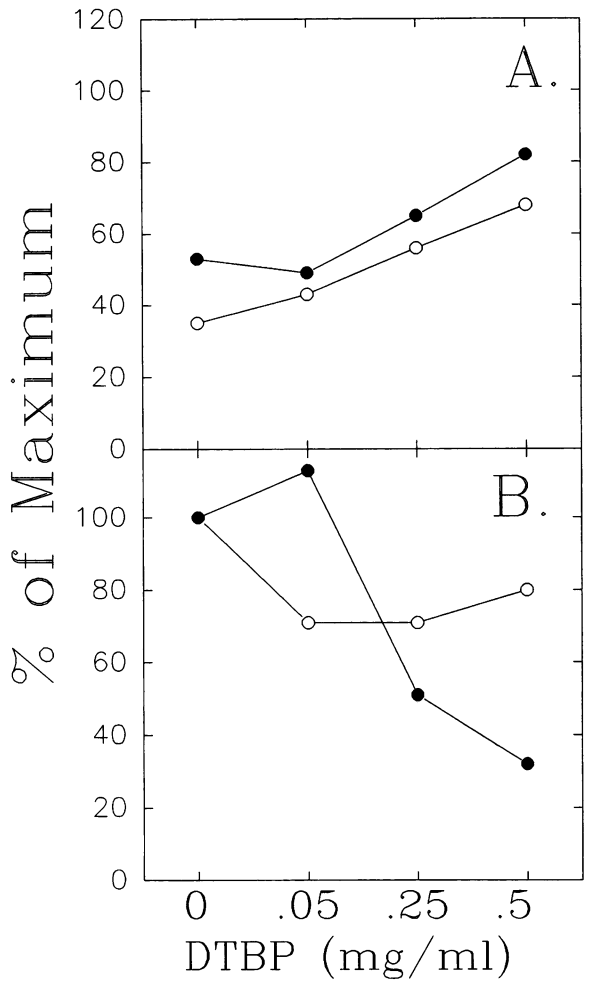

FIG. 6. Quantitation of binding of Y8-10C2 to cross-linked HA in detergent extracts. Densitometric estimations of the amounts of various HA species present in the fluorograph shown in Fig. 5 are plotted versus the DTBP concentration used to cross-link detergent-solubilized radiolabeled HA. (A) Percentage of H2-4B3-reactive (○) or Y8-10C2-reactive (●) HA migrating as trimers. (B) Percentage of H2-4B3-reactive (○) or Y8-10C2-reactive (●) HA precipitated from cross-linked versus untreated virus.

antibodies specific for Sa and Ca sites possess an amino acid substitution at position 245. Not surprisingly, the conservative replacement of Ile with Met (YV4) has less of an effect on HA antigenicity than its replacement with Lys, which introduces a positive charge at this position (YV9). As discussed above, these mutations are consistent with the localization of the Y8-10C2 epitope. By contrast, the locations of the mutations in YV1 and YV10 are quite surprising, as they are either in the fusion peptide itself or in a nearby region. YV10 has a Phe-to-Ile substitution at position 3 in the HA2 chain ($HA2_3$). YV1 has a Tyr-to-Cys substitution at position 17 in the HA1 chain ($HA1_{17}$).

Further characterization of YV1 and YV10 mutants. The phenotype of YV1 and YV10 antigenic variants was completely unexpected, as all previous escape variants demonstrated reduced affinity for the selecting antibody in the RIA and possessed amino acid substitutions in the epitope defined by antigenic analysis. Although the antigenicity of the

TABLE 1. HI titers of variants selected by Y8-10C2[a]

Virus	Relative HI titer (%)
Wild type	100
YV1	<6
YV2	25
YV3	<6
Y4	50
YV9	<6
YV10	<6

[a] Purified variants were incubated with twofold dilutions of Y8-10C2 containing ascites fluid for 24 h at 37°C prior to addition of chicken erythrocytes. HI titers are expressed as percentages of Y8-10C2 (= 100%) inhibition of agglutination of wild-type virus.

globular domains of YV1 and YV10 was altered to a very minor extent as detected by RIA, these viruses demonstrated a greater than 16-fold titer decrease in the HI assay (Table 1). To confirm that these isolates interact with Y8-10C2 in an altered manner, $\log_{10}$ dilutions of virus were incubated with Y8-10C2 at 37°C for 16 h and then tested for the ability to infect MDCK cells. While Y8-10C2 reduced the infectivity of the wild-type virus by 30-fold, it reduced the titer of YV1 by only 10-fold and failed to reduce the infectivity of YV10.

The inability of Y8-10C2 to block hemagglutination or infection by YV10 or YV1 could reflect a qualitative or quantitative alteration in the interaction of the mutant HAs with Y8-10C2. To distinguish between these possibilities, we examined the interaction of Y8-10C2 with radiolabeled HA present in detergent lysates of MDCK cells infected with the variants (Fig. 4). Similar quantities of HA were recovered from cells infected with either wild-type virus, YV1, or YV10 by using antibodies that recognize typical antigenic sites on the globular domain. When incubated at 0°C, Y8-10C2 immunoprecipitated only small amounts of YV10 HA and slightly greater amounts of YV1 HA. Note that in contrast to HA precipitated by other MAbs, all of the Y8-10C2-reactive YV10 HA migrated as trimers under the nonreducing conditions utilized and is therefore likely to represent an aberrant, possibly misfolded form of HA. Most importantly, while the amount of wild-type HA bound to Y8-10C2 increased 12-fold at 37°C (as estimated by densitometry), the amount of variant HAs precipitated increased only 2-fold.

Taken together, these findings demonstrate that mutations in or near the fusion peptide alter the interaction of HA trimers with Y8-10C2 without altering the interaction of HA monomers or unfolded trimers.

YV1 and YV10 HAs are more easily triggered by acid pH. It has previously been reported that mutations in $HA2_3$ or $HA1_{17}$ in HAs of different subtypes decrease the H^+ concentration needed to trigger HA membrane fusion activity (4, 18). Consistent with these findings, YV1 and YV10 mediated hemolysis at approximately 0.3 pH units higher than the wild-type virus (Fig. 8). Acid triggering of conformational changes leading to inactivation of viral fusion activity of wild-type HA is highly dependent on temperature (9). To determine the temperature dependence of acid triggering of conformational changes, viruses were incubated at pH 5 for 1 h at 0, 22, 32, and 37°C, returned to pH 7, and tested for hemolytic activity (Fig. 8). Conformational alterations in variant HAs were fully triggered at 0°C, while conformational alterations in wild-type HA were triggered

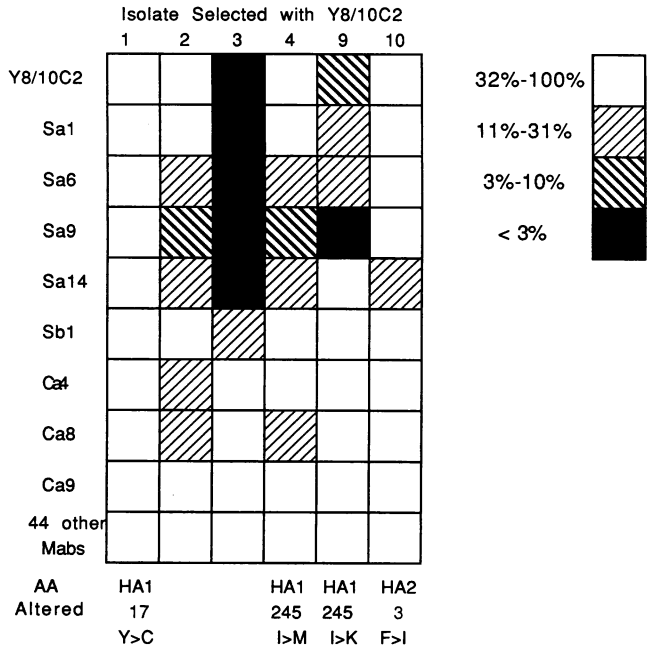

FIG. 7. Antigenic analysis of variant viruses selected by Y8-10C2. The binding of MAbs specific for the globular domain of PR8 to variant viruses dried onto polyvinyl was determined by an indirect RIA using a radioiodinated MAb specific for mouse κ chains. Each antibody was used at a concentration that is subsaturating for its binding to wild-type virus. Antibody binding to variants was related to a standard curve generated in the same assay by the binding of twofold dilutions of the antibody to wild-type virus. Binding is expressed as the dilution required to give the same number of counts with wild-type virus. The entire coding region of the HA genes of four of the variants included in this analysis was determined by dideoxy sequencing. Each variant has a single amino acid (AA) substitution; these are indicated at the bottom. Symbols: □, 32 to 100%; ▨, 11 to 31%; ▧, 3 to 10%; ■, <3%.

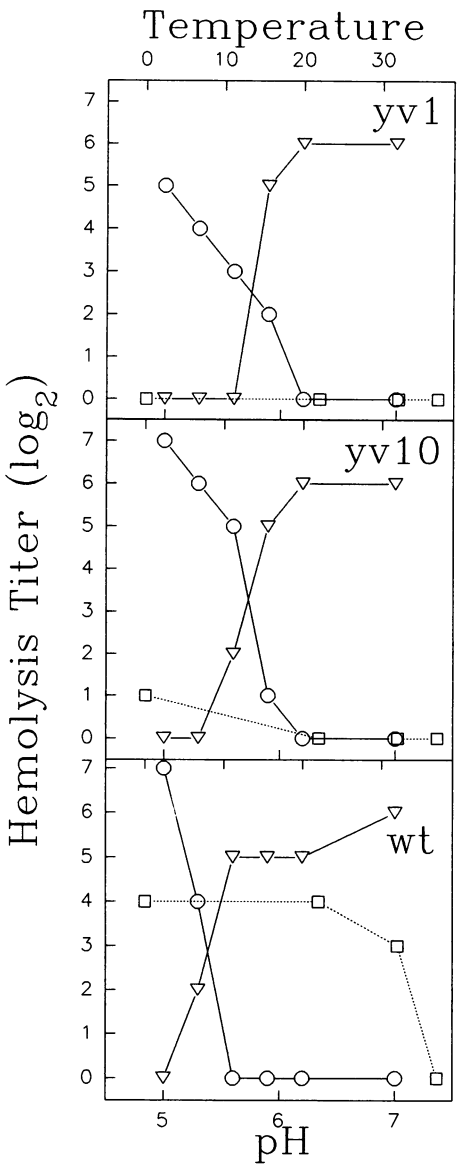

FIG. 8. pH and temperature dependence of triggering of fusion activity and conformational alterations in wild type (wt) and variant viruses. Hemolysis titers were determined for the viruses at various pHs (○). Viruses were treated for 2 h at room temperature at various pHs and neutralized, and their hemolysis activities were determined at pH 5 (▽). Viruses were treated for 1 h at pH 5 and various temperatures and neutralized, and their hemolysis activities were determined at pH 5 (□).

only above 23°C. These findings indicate that each mutation makes the HA unstable at acid pH.

During the course of hemolysis and hemagglutination assays, we noticed that the HA titer of acid-treated YV1 appeared to drop when assays were performed at 37°C. By simply performing hemagglutination assays at various temperatures, we confirmed that hemagglutination mediated by the YV1 HA in its acid conformation was temperature sensitive (Table 2), demonstrating 25 and 4%, HA activities at 20 and 37°C that were respectively, of its activity at 0°C. This loss of HA activity was almost completely reversible, as demonstrated by the greater 10-fold increase in HA activity achieved simply by resuspending erythrocytes incubated with YV1 at 37°C and allowing YV1 to interact at 0°C (Table 2). Thus, in addition to its effects on native HA, the Tyr-to-Cys substitution at position 17 causes reversible conformational alterations in acid-treated HA that greatly decrease HA receptor-binding activity.

DISCUSSION

In the present study, we found that Y8-10C2 binding to native trimers was directly dependent on temperature. The slow kinetics of the interaction and the high antibody concentrations required indicate that exposure of the epitope in a manner sufficient for interaction with antibody is a relatively infrequent event, even at temperatures as high as 48°C. We physically located the Y8-10C2 epitope in the globular portion of the HA1 chain by Western blotting and immunoprecipitation of tryptic fragments. Moreover, on the basis of the effect of amino acid substitutions in the globular domain on Y8-10C2 binding, we further localized the Y8-10C2 epitope to the trimer interface of monomers near the tip of the globular domain. On the basis of this information and previous findings that Y8-10C2 binds monomers and acid-treated trimers, we conclude that the temperature-dependent conformational alteration detected by Y8-10C2 is separation

TABLE 2. Hemagglutination by the YV10 HA in its acid conformation is temperature sensitive[a]

pH 5-treated virus	Relative HA activity (%) at a temp(s) (°C) of:			
	0	20	37	37 then 0
Wild type	100	100	100	71
YV10	100	71	50	71
YV1	100	25	4	50

[a] Viruses were incubated at pH 5 for 60 min at room temperature and returned to neutral pH. They were then tested for the ability to agglutinate human erythrocytes at various temperatures. HA titers were determined after 1 h of incubation. At this time, virus erythrocyte mixtures from the 0 and 37°C incubations were resuspended and allowed to resettle for another hour at 37 or 0°C, respectively.

of the globular domains to allow access to the Y8-10C2 epitope. These findings are consistent with a prior report that at elevated temperatures (50°C and higher, depending on the HA examined), HA undergoes conformational changes similar to those induced by acid and is even capable of mediating membrane fusion (19).

The validity of this interpretation depends, of course, on the accuracy of our localization of the Y8-10C2 epitope to the trimer interface by analysis of mutant HAs with defined amino acid substitutions. This method is probably the most widely used approach for locating discontinuous antigenic determinants in proteins. Implicit in this approach is the assumption that the altered amino acids act by directly decreasing the free energy of interaction with the antibody by either (i) introducing improper electrostatic charges or bulk into a critical region of interaction, (ii) altering the local solvent structure, or (iii) inducing very local conformational alterations. The utility of this approach is supported by two types of direct crystallographic evidence. (i) Solution of the three-dimensional structures of closely related antigens, including the HA (11), generally reveals that single residue replacements do not greatly alter structure. (ii) Most directly, the structures of the five antibody-antigen complexes solved to date consistently demonstrate that residues identified by variant analysis are, in fact, contacted by antibody (reviewed in reference 13).

While antigenic variant analysis is clearly a powerful and useful technique for physical mapping of antigenic determinants, one of the major points of this report is that mutations in residues located outside of the determinant can alter the interaction of antibody with the antigenic site without altering the interaction of other conformation-specific antibodies that bind to overlapping antigenic sites. In the present study, we show that such "extra-determinant" mutations in an oligomeric protein can alter the interaction of subunits such that a cryptic antigenic site is not accessible to antibody binding. Obviously, this is only one of many plausible mechanisms by which amino acid substitutions could alter epitope structure at a distance without causing major structural alterations in the protein. The important practical point is that there may not always be a perfect correlation between those amino acid residues that can alter antibody binding and residues that actually make up the antigenic site. Therefore, it is essential that the mapping of epitopes by variant analysis be confirmed by an additional approach. In the case of Y8-10C2, variant analysis performed with native HA could be correlated with variant analysis performed with alternative forms of the protein, including denatured protein (29a), which differs so extensively from native HA in structure that it is highly unlikely that amino acid substitutions could act in a parallel manner to induce long-range conformational alterations in the epitope that decrease antibody binding.

Our conclusion that Y8-10C2 binding to native trimers reflects thermal gyrations of the globular domains that temporarily expose residues near the trimer interface is supported by the ability of chemical cross-linkers to abrogate Y8-10C2 binding. On the basis of the behavior of cross-linked detergent-extracted HA (Fig. 5), it is clear that Y8-10C2 binding can be blocked by cross-linking residues within individual trimers. The parallel behavior of Y8-10C2 binding and viral fusion activity observed upon viral cross-linking and reversal with DTBP are consistent with recent reports that separation of the globular domains is required for HA-mediated fusion activity (7, 10).

By using Y8-10C2 to neutralize virus at 37°C, we were able to select two classes of antigenic variants. The first class of variants are typical of antibody escape mutants in demonstrating antigenic alterations detected by a number of antibodies when virus is attached to polyvinyl for indirect RIA. Two of the members of this set (YV2 and YV4) are somewhat unusual in demonstrating little change in binding to the selecting antibody (Y8-10C2) in the RIA. This finding correlates with the minor reduction in Y8-10C2-mediated HI observed with these variants (four- and twofold, respectively). These variants thus appear to have subtle alterations in the Sa epitope that are nonetheless sufficient to allow the variants to escape the relatively inefficient neutralization mediated by Y8-10C2 at 37°C. The conservative nature of the mutation in YV4 (Ile to Met) is consistent with this conclusion, and it is worth noting that this precise substitution has not been found in any of the other PR8 variants we have sequenced with single point mutations (1). Another factor contributing to the relatively minor effect that the Ile-to-Met substitution has on Y8-10C2 binding could be that position 245 is at the edge of the Y8-10C2 epitope, since even a nonconservative substitution at this position (Ile to Lys in YV9) results in only a partial decrease in Y8-10C2 binding in the RIA.

The second class of antigenic variants selected by Y8-10C2 are novel in possessing single residue substitutions located a considerable distance from the antibody-binding site. Y8-10C2 maintains its ability to bind these variants once its epitope is exposed by attachment to polyvinyl and, indeed, binds to [^{35}S]methionine-labeled monomers in detergent extracts from cells infected with the variants (29b). Thus, the amino acid substitutions do not alter the structure of the Y8-10C2 epitope but, rather, appear to limit the access of Y8-10C2 to its epitope by limiting the range of motion of the globular domains relative to each other at 37°C. To understand how the mutations affect HA structure, it is necessary to have detailed information regarding the location and interactions of the residues within the HA. Although the three-dimensional structure of the PR8 HA has not been determined, it is likely that the basic elements of the structure are similar to those of the H3 HA structure, which has been determined at high resolution by X-ray crystallography. $HA1_{17}$ of the H3 HA is located in sufficient proximity to the fusion peptide to interact with several of its residues and, indeed, participates in hydrogen bonds through water molecules with $HA2_6$ and $HA2_{10}$. Thus, it appears that both mutations that decrease the mobility of the globular domains act by altering the interactions of the fusion peptide. This suggests that this region of the HA plays a critical role in governing the overall conformational flexibility of the molecule. The substitutions need not cause large conformational alterations in the region of the fusion peptide

to alter the function of HA. This conclusion is based on the three-dimensional structure of a mutant HA with an Asp-to-Gly substitution in $HA2_{112}$ (a residue that interacts with multiple residues in the fusion peptide) that results in its being triggered 0.4 pH unit higher than the wild-type virus (24). The structure surrounding this residue was unchanged, and it appeared that the increased pH sensitivity was simply due to loss of the four hydrogen bonds that normally help to stabilize the fusion peptide. It is also worth noting that the effects of mutations at positions $HA1_{17}$ and $HA2_3$ on the exposure of the Y8-10C2 epitope occur independently of cleavage of HA into HA1 and HA2 subunits, since they affect Y8-10C2 binding to egg-grown virus (in which all of the HA is cleaved) and to HA in infected-MDCK lysates (in which none of the HA is cleaved).

In contrast to many residues in the HA, positions $HA1_{17}$ and $HA2_3$ are highly conserved among natural influenza virus isolates. $HA1_{17}$ is conserved between all H1 and H2 HAs sequenced, while $HA2_3$ is conserved among all of the natural isolates sequenced. As both YV1 and YV10 grow to high titers in eggs, it is clear that substitutions in these residues do not grossly alter the ability of the virus to replicate in ovo. Thus, other factors must account for the high conservation of these residues in vivo. In the course of infection and attendant inflammation, influenza virus virions might be exposed to pHs between 5 and 6 in extracellular fluids that would inactivate virions with mutations that decrease the H^+ concentration needed to trigger conformational alterations. Alternatively, alterations in position 17 have been found to alter the ability of cellular proteases to cleave the HA into HA1 and HA2 subunits (18). As this is essential for fusion activity and, consequently, viral infectivity, it is possible that mutations in the fusion peptide and nearby residues prevent cleavage of the HA by proteases present in cells that produce virus in infected individuals.

REFERENCES

1. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31**:417–427.
2. **Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius.** 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J. Cell Biol. **103**:1179–1191.
3. **Daniels, R. S., A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1983. Analyses of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion. J. Gen. Virol. **64**:1657–1662.
4. **Daniels, R. S., J. C. Downie, A. J. Hay, M. Knossow, J. J. Skehel, M. L. Wang, and D. C. Wiley.** 1985. Fusion mutants of the influenza virus hemagglutinin glycoprotein. Cell **40**:431–439.
5. **Gerhard, W., and R. G. Webster.** 1978. Antigenic drift in influenza A viruses. I. Selection and characterization of antigenic variants of A/PR/8/34 (HON1) influenza virus with monoclonal antibodies. J. Exp. Med. **148**:383–392.
6. **Gething, M.-J., K. McCammon, and G. Sambrook.** 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46**:939–950.
7. **Godley, L., J. Pfeifer, D. Steinhauer, B. Ely, G. Shaw, R. Kaufmann, E. Suchanek, C. Pabo, J. J. Skehel, D. C. Wiley, and S. Wharton.** 1992. Introduction of intersubunit disulfide bonds in the membrane-distal region of the influenza hemagglutinin abolishes membrane fusion activity. Cell **68**:635–645.
8. **Jackson, D. C., and A. Nestorowicz.** 1985. Antigenic determinants of influenza virus hemagglutinin. XI. Conformational changes detected by monoclonal antibodies. Virology **145**:72–83.
9. **Junakar, P. R., and R. J. Cherry.** 1986. Temperature and pH dependence of the haemolytic activity of influenza virus and of the rotational mobility of the spike glycoprotein. Biochim. Biophys. Acta **854**:198–206.
10. **Kemble, G. W., D. L. Bodian, J. Rose, I. A. Wilson, and J. M. White.** 1992. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. J. Virol. **66**:4940–4950.
11. **Knossow, M., R. S. Daniels, A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1984. Three-dimensional structure of an antigenic mutant of the influenza virus haemagglutinin. Nature (London) **311**:678–680.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Laver, W. G., G. M. Air, R. G. Webster, and S. J. Smith-Gill.** 1990. Epitopes on protein antigens: misconceptions and realities. Cell **61**:553–556.
14. **Maeda, T., and S. Ohnishi.** 1980. Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett. **122**:283–287.
15. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. **91**:601–613.
16. **Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature (London) **304**:76–78.
17. **Roth, M. G., M.-J. Gething, and J. Sambrook.** 1989. Membrane insertion and intracellular transport of influenza virus glycoproteins, p. 219–268. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
18. **Rott, R., M. Orlich, H.-D. Klenk, M. L. Wang, J. J. Skehel, and D. C. Wiley.** 1984. Studies on the adaptation of influenza viruses to MDCK cells. EMBO J. **3**:3329–3332.
19. **Ruigrok, R. W., S. R. Martin, S. A. Wharton, J. J. Skehel, P. M. Bayley, and D. C. Wiley.** 1986. Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. Virology **155**:484–497.
20. **Selimova, L. M., G. G. Mukazhanova, V. M. Zaides, and V. M. Zhdanov.** 1986. Intermolecular covalent (disulphide) bonds in peplomers of influenza virus hemagglutinin. Vopr. Virusol. **2**:163–169.
21. **Skehel, J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA **79**:968–972.
22. **Stegmann, T., R. W. Doms, and A. Helenius.** 1989. Protein-mediated membrane fusion. Annu. Rev. Biophys. Biophys. Chem. **18**:187–211.
23. **Webster, R. G., L. E. Brown, and D. C. Jackson.** 1983. Changes in the antigenicity of the hemagglutinin molecule of H3 influenza virus at acidic pH. Virology **126**:587–599.
24. **Weis, W. I., S. C. Cusack, J. H. Brown, R. S. Daniels, J. J. Skehel, and D. C. Wiley.** 1990. The structure of a membrane fusion mutant of the influenza virus haemagglutinin. EMBO J. **9**:17–24.
25. **Wharton, S. A., W. Weis, J. J. Skehel, and D. C. Wiley.** 1989. Stucture, function, and antigenicity of the hemagglutinin of influenza virus, p. 153–218. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
26. **White, J. M., and I. A. Wilson.** 1987. Anti-peptide antibodies detect steps in a protein conformational change: low-pH activation of the influenza virus hemagglutinin. J. Cell Biol. **105**:2887–2896.
27. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56**:365–394.
28. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289**:373–378.
29. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of

the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature (London) **289**:366–373.

29a. **Yewdell, J. W., et al.** Submitted for publication.

29b. **Yewdell, J. W.** Unpublished observations.

30. **Yewdell, J. W., A. J. Caton, and W. Gerhard.** 1986. Selection of influenza virus adsorptive mutants by growth in the presence of a mixture of monoclonal antihemagglutinin antibodies. J. Virol. **57**:623–628.

31. **Yewdell, J. W., W. Gerhard, and T. Bächi.** 1983. Monoclonal anti-hemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/8/34-mediated hemolysis. J. Virol. **48**:239–248.

32. **Yewdell, J. W., R. G. Webster, and W. U. Gerhard.** 1979. Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. Nature (London) **279**:246–248.

33. **Yewdell, J. W., A. Yellen, and T. Bächi.** 1988. Monoclonal antibodies localize events in the folding, assembly, and intracellular transport of the influenza virus hemagglutinin glycoprotein. Cell **52**:843–852.