Posttranslational Oligomerization and Cooperative Acid Activation of Mixed Influenza Hemagglutinin Trimers

François Boulay, Robert W. Doms, Robert G. Webster,* and Ari Helenius
Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and * Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101

Abstract. The influenza virus hemagglutinin (HA) is a well-characterized integral membrane glycoprotein composed of three identical subunits. We have analyzed the formation of mixed trimers in cells expressing two different HA gene products. The results show efficient and essentially random assembly of functional hybrid trimers provided that the HAs are from the same HA subtype. Trimerization is thus a posttranslational event, and subunits are recruited randomly from a common pool of monomers in the endoplasmic reticulum. Mixed trimers were not observed between HAs derived from different subtypes, indicating that the trimerization event is sequence specific. Mixed trimers containing mutant subunits were, moreover, used to establish that the acid-induced conformational change involved in the membrane fusion activity of HA is a highly cooperative event.

The majority of plasma membrane proteins in eukaryotic cells are homo- or heterooligomeric. In most instances, oligomeric assembly seems to occur early in the secretory pathway. Oligomerization is, in fact, often a prerequisite for efficient transport of these proteins from the endoplasmic reticulum (ER) (Arce-Gomez et al., 1978; Sege et al., 1981; Germain et al., 1985; Gething et al., 1986b; Copeland et al., 1986; Doms et al., 1988a, Smith et al., 1987). In the case of the influenza hemagglutinin precursor, HA0, trimers are observed 5-10 min after synthesis and a few minutes before trimming of the N-linked oligosaccharides in the cis-Golgi (Copeland et al., 1986; Gething et al., 1986b; Copeland, C. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius, manuscript submitted for publication).

The hemagglutinin is ideally suited for studies on the mechanisms and properties of oligomerization. (a) The crystal structure of the ectodomain is known at 3 Å resolution for the A/Aichi/2/68 HA (Wilson et al., 1981); (b) Numerous human and animal derived virus isolates have been analyzed and classified into well-defined subtypes on the basis of their antigenic properties and amino acid sequence (Air, 1981; Webster et al., 1982). (c) Specific monoclonal antibodies are available to well-defined epitopes (Webster and Laver, 1980; Gerhard et al., 1981; Wiley et al., 1981). (d) The intracellular transport and posttranslational processing of hemagglutinin (HA) have been analyzed in detail (Klenk et al., 1975; Matlin and Simons, 1983; Rodriguez-Boulan et al., 1984). (e) Mutants with a variety of phenotypic changes have been identified and characterized (Daniels et al., 1985; Doms et al., 1986; Nakajima et al., 1986; Schuy et al., 1986). (f) The function of the mature trimeric HA in acid-activated membrane fusion has been extensively studied (Maeda et al., 1981; White et al., 1982; Doms et al., 1985; Gething et al., 1986a; Stegmann et al., 1986; Boulay et al., 1987).

HA is initially synthesized in the endoplasmic reticulum as a 76-kD precursor, HA0, which is transported as a trimer to the plasma membrane via the Golgi complex. Just before or during delivery to the plasma membrane of cells that have the appropriate proteases, HA0 is converted to the mature HA by a proteolytic cleavage generating two disulfide-bonded polypeptides, HA1 and HA2 (Klenk et al., 1974; Hay, 1974; Matlin and Simons, 1983). The activating cleavage is essential for the membrane fusion activity of HA that is expressed at low pH (Huang et al., 1981; White et al., 1982).

The x-ray structure of HAs trimeric ectodomain reveals that each spike has two structurally distinct parts: a fibrous stem containing a triple-stranded coiled coil of three 76-Å-long alpha helices and a head with three globular structures (Wilson et al., 1981). There are numerous noncovalent interactions between the adjoining subunits over the entire length of the spike. Most of the intersubunit salt bridges and hydrophobic interactions are between the HA2 chains which form the stem of the trimer. The importance of these interactions for trimer stability has been confirmed by analyzing membrane fusion variants that display increased sensitivity to acid pH and to heat (Rott et al., 1984; Daniels et al., 1985; Doms et al., 1986; Ruigrok et al., 1986a).

1. Abbreviations used in this paper: ER, endoplasmic reticulum; HA, hemagglutinin; wt, wild type.
While the overall structure of the mature HA is exceptionally well characterized, the events involved in its assembly from monomers in the ER remain unclear. To understand how the assembly process works, several central questions must be considered. Does assembly begin immediately after biosynthesis, does it occur between products of the same polypeptide, and does it parallel the mechanisms by which soluble oligomeric proteins form? How do HA0 subunits recognize each other as appropriate assembly partners? Does assembly proceed through a series of unstable, but defined intermediates (see King et al., 1986)? Are cellular factors involved in folding and assembly? What are the structural requirements for the formation of stable trimers? To address some of these questions, we have introduced a new experimental approach: the analysis of mixed HA trimers. We demonstrate that mixed trimers are formed in cells that express more than one type of hemagglutinin and that they form randomly from a common pool of monomers. Taking advantage of sequence differences in HAS derived from different influenza strains, we have shown that close homology is functional protein that consists of heterologous subunits.

Materials and Methods

Materials

Chemicals and Reagents. The L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (TPCK-trypsin) and soybean trypsin inhibitor (STI) were purchased from Sigma Chemical Co., St. Louis, MO. Fixed S. aureus and FITC-conjugated streptavidin were obtained from Zymed Corp., San Francisco, CA, and affinity-purified rhodamine-conjugated goat F(ab)2 anti-mouse IgM + IgG was from Tago Inc., Burlingame, CA. NHS-LC-Biotin, a long chain analogue of N-hydroxysuccinimide ester of biotin was provided by Enzo Biochem Inc., New York, NY. Reverse transcriptase from avian myeloblastosis virus was obtained from Boehringer-Manheim Biochemicals, Indianapolis, IN. [35S]Methionine was provided by Amersham Corp., Arlington Heights, IL, and had a specific activity of >800 Ci/mmol.

Two mAbs, II/4 and NI, against the A/Aichi/2/68 strain of influenza HA (Webster, R., unpublished; Copeland et al., 1986), a polyclonal antibody against the A/Japan/305/57 strain (Dowsey et al., 1987), and a mAb to the HA of A/Seal/Mass/I/80 (Kida et al., 1982) were principally used in this study.

Methods

Cells and Viruses. BHK-21 cells (a baby hamster kidney cell line) were grown to 90% confluency in Glasgow minimum essential medium (GMEM) supplemented with 5% FCS and 10% tryptic phosphate broth. The virus strains used in this study included the laboratory selected antigenic variants Meg and Flo of A/Memphis/71 (H3N2) (Webster and Laver, 1980), a naturally occurring high pH fusion variant of A/Aichi/2/68 (H3N2) termed A31 (Doms et al., 1986), A/Japan/305/57 (H2N2), and A/Seal/ Mass/I/80 (1977). All viruses were grown in 11- to 12-day embryonated chicken eggs (Stephenson and Dimmock, 1974). Infectious saline fluids were used in most experiments. The Flo antigenic variant was obtained in quantity as previously described (Doms et al., 1985), and further purified on a 30-60% discontinuous sucrose gradient centrifugation in a Beckman SW28 rotor (24,000 rpm for 90 min).

Preparation of Viral RNA and Nucleotide Sequencing. Template RNA from the Flo antigenic variant was isolated by treatment of purified virus with proteinase K and sodium dodecyl sulfate followed by extraction with phenol:chloroform (1:1) as described by Palenc and Schlumberg (1976). A nucleotide primer AGACTGAATCTGG (3') complementary to the nucleotide sequence 525-539, numbered according to the positive strand sequence of the Memphis/1/71 HA gene segment (Newton et al., 1983), was synthesized and used for reverse transcriptase extension reactions. More than 130 nucleotides coding for the antigenic epitope "B" (Wiley et al., 1981) were sequenced using the dideoxy chain termination method of Sanger et al. (1977) as modified by Air (1979). Due to difficulty in reading the third base, it was not clear if threonine 192 (ACC) had been substituted with isoleucine (ATC) or methionine (ATG). However, point mutations occur in the virus population with a frequency of 10-5, and the selection procedure consisted of a single passage in embryonated eggs after neutralization of the parent virus with a mAb (Webster and Laver, 1980). Thus, the change was more likely to be isoleucine because it involves a single base change (C~T), whereas the change to methionine would require two successive mutations (C~T and C~G).

Viral Infection, Labeling, and Chase Conditions. For single or double infection, BHK-21 cells were incubated at 4°C and for 1 h with infectious allantoic fluids diluted in RPMI containing 5 mM Heps, pH 6.8, and 0.2% BSA. Viral infection was initiated by warming the cells at 37°C in a CO2 incubator and at 1 h postinfection complete growth medium was added to the cells. At 5 h postinfection, the cells were washed with PBS and labeled for 30 min with [35S]methionine (800 uCi/ml). To initiate a chase the cells were washed and incubated in fresh growth medium with additional l-methionine (2 mM) for 60 min.

Immunofluorescent Staining. Monolayers of BHK-21 cells growing on glass coverslips and doubly infected with Meg and Flo variants were taken at 6 h postinfection and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. The excess fixative was quenched with 50 mM ammonium chloride and the cells washed extensively with PBS supplemented with 0.2% gelatin. Indirect immunofluorescence staining by use of monoclonal anti-Flo-HA antibody II/4 followed by rhodamine-conjugated goat anti-mouse IgG was performed as described previously (Copeland et al., 1986).

The Journal of Cell Biology, Volume 106, 1988 630

Figure 1. Random trimerization in cells expressing two closely related HAS. In a cell that expresses two distinct HAS (X and Y), four trimer compositions are possible: XXX, XXY, XYX, and YYY. If trimerization is random and if p is the fraction of monomer X, then the fraction of trimers consisting solely of X subunits is given by x(p) = p3 (three solid circles). (1-p) represents then the fraction of monomers Y, therefore the fraction of trimers consisting of 3 Y subunits is y(p) = (1-p)3 (three open circles), whereas the proportion of heterotrimers is expressed by h(p) = 3p(1-p). For any value of P > 0 or <1, there are two heterotrimer subpopulations, XXY and YYX. The frequency with which XXY occurs can be expressed as h(p) = 3p(1-p)2 (two solid circles and one open) while the frequency with which YYX occurs is h(p) = 3p(1-p)2 (two open circles and one solid). Note that the maximum fraction of heterotrimers appears for p = 0.5 (h = 75%), whereas the maximum for the heterotrimer subpopulations XXY and YYX occur for p = 0.66 (h1 = 44.4%) and p = 0.33 (h2 = 44.4%), respectively.
Double staining was achieved with biotinylated monoclonal anti-Meg-HA antibody (Biotin-N1) followed by fluorescein-conjugated streptavidin.

**Hemagglutinin Purification and Immunoprecipitation.** [35S]Methionine-labeled HAO was prepared from singly or doubly infected cells as described above. HA was derived from HAO by digestion of cell lysates with 40 µg TPCK-trypsin/ml (final concentration) for 30 min at 0°C, followed by a fivefold excess of soybean trypsin inhibitor (STI). Both HA and HAO were purified in 0.1% Triton X100 (TX100) by affinity chromatography on ricin-Sepharose as previously described (Doms and Helenius, 1986). HAO from both variants, Meg and Flo, were found to bind to ricin equally well.

Complete activation of HAO, yielding HA1 and HA2, was established under reducing conditions by SDS-PAGE according to Laemmli (1970). Fluorography was performed on gels impregnated with salicylic acid (Chamberlain, 1979) using Kodak XAR-5 film preflashed with orange light.

Immunoprecipitations of HAO and HA from cell lysates or after ricin purification were performed with mono- or polyclonal antibodies as described previously (Copeland et al., 1986), except that the washing of the S. aureus immunocomplex was limited to one wash with PBS containing 0.1% Triton X100. The immunoprecipitated material was solubilized in sample buffer and boiled for 5 min (with or without reducing agents as indicated) before SDS-PAGE or liquid scintillation counting. As seen in Fig. 2 and 5, HAO is not fully denatured even when boiled in SDS (Doms and Helenius, 1986), giving monomer, dimer, and trimer bands. The protein can be completely denatured only following activation to HA and under reducing conditions.

**Results**

**Formation of Mixed Trimers**

We first determined whether HA0 trimers are assembled preferentially from subunits synthesized by the same polypeptide or randomly from a mixed pool of monomers. For this we coexpressed two HA gene products in the same cells and determined to what extent they assembled into mixed trimers. If trimerization is random then 75% of the trimers should be hybrids when two HA0s are expressed at equal levels in a cell. However, if the expression is not equal a lower fraction of hybrid trimers would be expected. The distribution between the four possible trimer combinations (XXX, XXY, YXY, and YYY) can be easily calculated for a variety of expression ratios as shown in Fig. 1.

Our strategy to determine the degree of mixed assembly relied on the efficient coinfection of cells by two viruses with nearly identical HAs and the selective recognition of “antigenic tags” by mAbs. Two antigenic variants of influenza A/Memphis/1/71, called Meg and Flo, were chosen for this experiment because they bear 98% sequence homology with the A/Aichi/2/68 (X31) strain for which the three-dimensional structure of the HA ectodomain is known (Wilson et al., 1981). Like numerous other variants described in the influenza literature, Meg and Flo were originally obtained by growing wild type (wt) virus in the presence of neutralizing mAb (Webster and Laver, 1980). Variants which escape neutralization occur at a frequency of 10⁻⁵ and do not bind to the mAb used for their selection. Such changes in antigenicity are almost invariably related to single amino acid changes in one of four antibody binding sites on the surface of the HA subunit (Webster et al., 1983).

Compared to wt HA, the HA of Meg possesses a single sequence change in the “loop” epitope (site “A”, Wiley et al., 1981); Gly 144 is replaced with an Asp (Webster and Laver, 1980). Flo HA possessed a change in another antibody binding site, the “tip” (also called “B”), judging by its inability to bind several antibodies to this region (Webster and Laver, 1980). The sequencing of the Flo HA gene region coding for the “tip” epitope revealed that a single amino acid substitution at position 192 (Table I) might be involved in the antigenic change. The x-ray structure indicated that the substitutions in Meg and Flo were both well removed from the subunit interfaces of the trimeric HA, and thus unlikely to affect trimer assembly (Table I).

As shown in Fig. 2, the immunoprecipitations from cells infected with only one antigenic variant demonstrated that

---

**Table I. Amino Acid Differences between Fusion Variant A31 (A/Aichi/2/68) and the Flo and Meg Antigenic Variants of A/Memphis/1/71**

| Amino acid position | Subunit | Aichi/2/68 A31 variant | Memphis/1/71 Flo* and Meg variants |
|---------------------|---------|------------------------|----------------------------------|
| 2                   | HA1     | Asp                    | Tyr                              |
| 31                  | HA1     | Asp                    | Asn                              |
| 78                  | HA1     | Val                    | Gly                              |
| 144                 | HA1     | Asp (Gly in wt)        | Asp (in Meg)                     |
| 158                 | HA1     | Gly                    | Gly (in Flo)                     |
| 182                 | HA1     | Ile                    | Val                              |
| 192                 | HA1     | Thr                    | Thr (in Meg)                     |
| 215                 | HA1     | Leu                    | Pro                              |
| 132                 | HA2     | Asn (Asp in wt)        | Asp                              |

Amino acid differences between the fusion variant A31 (A/Aichi/2/68) and A/Memphis/1/71 are shown. The Meg antigenic variant of A/Memphis, like A31, contains Asp at position 144. This change in the loop region (antigenic site A) prevents binding of Mab 11/4 to both Meg and A31. The Flow antigenic variant of A/Memphis contains a substitution at position 192, in the tip (site B) region. Due to difficulty in reading the third base, it was not clear if threonine 192 (ACC) had been substituted with isoleucine (ATC) or methionine (ATG). Finally, the aspartic acid at position 132 of HA2 is replaced with an asparagine in the fusion variant A31, disrupting a salt bridge with arginine 124 of an adjoining subunit. This enables A31 HA to respond to acid at a pH 0.4 higher than the wt A/Aichi.

*Because the Flo HA gene has not been sequenced entirely, we cannot rule out the presence of other point mutations. We consider additional changes to be unlikely, however, since the variant was obtained by single passage of the parent virus through embryonated eggs in the presence of the neutralizing mAb.
antibody 11/4 was specific for Flo HA0 and antibody N1 for Meg HA0. The immunoprecipitated HA0 appeared as three bands (HA0 monomers, dimers, and trimers) reflecting the relative SDS-resistance of HA0 trimers (Doms and Helenius, 1986). Two other antibodies, N2 and a rabbit polyclonal antiserum (Copeland et al., 1986), reacted equally well with both Meg and Flo HA0. All immunoprecipitations were quantitative because a second round of precipitations using each of the monoclonals or the polyclonal brought down >90% of the HA0 produced in doubly infected cells (Copeland et al., 1986).

To determine whether mixed Meg-Flo trimers were present in doubly infected cells, the [35S]methionine-labeled HA0 was purified by ricin affinity chromatography. Only galactosylated HA0 (i.e., HA0 which has passed through the Golgi complex) adsorbs to this lectin. Because only trimeric HA0 is transported through the Golgi complex (Gething et al., 1986b; Copeland et al., 1986), ricin affinity chromatography represented a rapid and quantitative way to separate trimeric from monomeric HA0. Aliquots of the isolated HA0 trimers were next subjected to immunoprecipitation with the various antibodies. Immunoprecipitates were analyzed by SDS-PAGE and quantitated by scintillation counting.

Table II shows the results of two double infection experiments. The HA0 trimers were isolated from cell cultures in which both subunits were produced in nearly equivalent amounts and 95% of all cells were doubly infected. The antibodies to Meg (N1) or Flo (11/4) each precipitated ~80% of the HA0 pulled down by a mixture of N1/4 and N1 or by the polyclonal antibody. From this we can calculate that 62% of the trimers were mixed, i.e., they contained both Meg and Flo subunits. The remaining trimers were distributed between Meg and Flo homotrimers. In five additional experiments the fraction of mixed trimers ranged from 53 to 62%.

To rule out the trivial possibility that hybrid trimers arose through reshuffling of subunits during solubilization and immobilization of the HA0 via ricin-Sepharose affinity chromatography and aliquots were immunoprecipitated with the mAbs 11/4 and N1 (specific for Meg HA) followed by fluorescein-conjugated streptavidin. (Left) Rhodamine-stained cells demonstrating the presence of Flo HA at the plasma membrane. (Right) The corresponding fluorescein staining with the anti-Meg HA antibody. >95% of the cells expressed both HA types. No cross-reactivity was seen with cells infected with only one antigenic variant (see also Fig. 2).

Table II. Formation of Mixed Trimers between Closely Related HAs

| cpms [35S]HA0 immunoprecipitated | % of total trimers |
|----------------------------------|-------------------|
| 11/4 (Flo) N1 (Meg) 11/4+N1 (F+M) | Cells doubly infected |
|                                 | FFM  | FMM  | MMM |
|                                 | %    |      |      |
| 8,700 7,725 10,100 95           | 24   | 62   | 14   |
| (17) (71) (12)                  |      |      |      |
| 16,853 17,695 21,361 95         | 21   | 62   | 17   |
| (14) (72) (14)                  |      |      |      |

HFK-21 cells were simultaneously infected with the A/Memphis antigenic variant Meg and Flo. After 5 h, the cells were pulse labeled with [35S]methionine and chased for 60 min. The HA0 trimers were purified by ricin-affinity chromatography and aliquots were immunoprecipitated with the mAbs 11/4 (specific for Flo HA0), N1 (specific for Meg HA0), or both. The amount precipitated by the combination of 11/4 and N1 was defined as the total (T) amount of HA present, and was in good agreement with the amount precipitated by a rabbit polyclonal antibody. The different populations were quantified as follows: % Flo homotrimer = [T-N1]/T; % Meg homotrimer = [T-11/4]/T; % heterotrimers = [N1 + 11/4 - T]/T; where N1 and 11/4 represent the cpm's of [35S]HA0 immunoprecipitated by each antibody. The values in parentheses are the predicted values if we assume that each cell expresses equal amounts of each subunit and if trimerization is random. The values have been corrected to reflect the fact that 5% of the cells were singly infected with Flo in the first experiment, and approximately 2.5% were singly infected by each of the viruses in the second.

Figure 2. Specificity of anti-hemagglutinin antibodies. [35S]Methionine-labeled hemagglutinin (HA0) from the A/Memphis/1/71 antigenic variants Meg and Flo were isolated from TX100 lysates of infected BHK-21 cells by ricin-Sepharose affinity chromatography. Aliquots were immunoprecipitated by monoclonal antibodies N1 and 11/4 or by a polyclonal antibody (P) which recognizes both Meg and Flo. The precipitates were analyzed by SDS-PAGE and fluorography.

Figure 3. Immunofluorescent staining of doubly infected BHK-21 cells. The cells were treated with antibody 11/4 (specific for Flo HA) followed by a rhodamine-conjugated goat anti-mouse antibody. After washing, the preparation was incubated with biotinylated antibody N1 (specific for Meg HA) followed by fluorescein-conjugated streptavidin. (Left) Rhodamine-stained cells demonstrating the presence of Flo HA at the plasma membrane. (Right) The corresponding fluorescein staining with the anti-Meg HA antibody. >95% of the cells expressed both HA types. No cross-reactivity was seen with cells infected with only one antigenic variant (see also Fig. 2).
munoprecipitation, we mixed equal amounts of Flo and Meg homotrimers obtained from lysates of singly infected cells. The variant specific antibodies N1 and M1/4 each precipitated 50% of the HA0, indicating that subunits were not exchanged between HA0 trimers after solubilization.

The results indicated that the fraction of hybrid trimers in doubly infected cells approached the value expected from a random trimerization process (Fig. 1). The small apparent deviation from randomness most likely reflected heterogeneity in expression of the two variant HA0s in individual cells. Double immunofluorescence allowed us to correct the values for the small number of cells (usually ≤5%) that were only infected with a single virus strain and therefore only produced homotrimers. This correction is included in the percent values shown in brackets in Table II. It was not possible, however, to correct for uneven expression of Meg and Flo in doubly infected cells. That Meg and Flo were not equally expressed in all cells was shown by double immunofluorescence (Fig. 3 and unpublished observations). As shown in Fig. 1, deviation from equal expression results in a lower heterotrimer value.

We concluded from these results that most of the trimers must have formed posttranslationally from subunits recruited from a mixed HA0 pool and that preferential association between HA0 subunits synthesized by the same polysome was unlikely.

**Hemagglutinins of Different Subtypes Fail to Form Mixed Trimers**

The structural requirements for correct trimerization were next investigated by coexpressing different influenza A HA subtypes. There are 13 known HA0 subtypes, many of which contain numerous known strains (Air, 1981; Webster et al., 1982). The subtypes, initially defined by serological means, have now been extensively compared in terms of nucleotide sequence. While considerable sequence differences occur (42–49% homology), highly conserved features such as all 12 cysteine residues in the ectodomain, the amino terminal fusion sequence, residues in the sialic acid binding site, and other amino acids involved in intersubunit contacts suggest that the different subtypes share a common overall molecular structure (Air and Laver, 1986; Wiley and Skehel, 1987). Strains within subtypes show greater homology, with differences largely confined to the antigenic regions of HA1 (Webster et al., 1983).

We coninfected cells with A/Japan/305/57 (H2 subtype) and A/Memphis/1/71 (H3 subtype) or with A/Japan and A/Seal/Mass/1/80 (H7 subtype). After pulse labeling and a 60-min chase, the HA0s were immunoprecipitated with mono- or polyclonal antibodies specific for a given subtype. (Lanes 1 and 3) Polyclonal antisera against A/Japan. (Lanes 2 and 4) Monoclonal antibodies against A/Memphis and A/Seal, respectively. The immunoprecipitated material was analyzed by SDS-PAGE and fluorography. Although the cells were doubly infected as judged by double immunofluorescent staining, no co-precipitation of one subtype by an antibody to the other was observed.

**Mixed Trimers between wt and Fusion Variant HA**

Influenza HA undergoes an irreversible conformational change at acid pH, and the acid form mediates fusion between the viral and target membranes (see Doms et al., 1988b; Wiley and Skehel, 1987). This change involves partial dissociation of the HA ectodomain and exposure of the hydrophobic fusion sequence (Doms et al., 1988b; Wiley and Skehel, 1987). The threshold pH at which HAs from different influenza virus subtypes and strains undergo the change and mediate membrane fusion varies between pH 5.0 and 6.0 (Beyer et al., 1986). Variants that catalyze fusion at a pH higher than wt have been identified within the H3 subtype (Rott et al., 1984; Daniels et al., 1985; Doms et al., 1986). The differential responses are often the result of single amino acid substitutions which disrupt subunit interactions or the structure around the highly conserved NH2-terminal fusion peptide of HA2.

To determine how a mixed HA trimer behaves at acid pH, we analyzed the acid-induced conformational changes and fusion activity of trimers that contained HA0 subunits from viruses with different pH sensitivity. Two antigenically distinct HAs within the H3 subtype were used: Flo (introduced above) and A31, a variant which catalyzes fusion at a pH 0.4 units higher than Flo (Doms et al., 1986). A31 has a substitution at position 132 in HA2 (Asn for Asp), which eliminates a highly conserved salt bridge with Arg 124 of the adjoining HA2 subunit. This change is responsible for A31's increased sensitivity to acid (Doms et al., 1986). Flo, like wt HA, has Asp at position 132. As shown in Table I, there are eight other differences between Flo and A31 all within the HA1 polypeptide, most of them well removed from the subunit interface. The additional differences are unlikely to be crucial for oligomerization or pH sensitivity.

**Formation of Mixed wt and Fusion Variant Trimers.** Because the A31 HA contains the same sequence change as Meg in position 144 of HA1, we were able to employ the mAbs M1/4 (anti Flo) and M1 (anti A31) to discriminate be-
between A31 and Flo (Fig. 6 a). Furthermore, the HA1 polypeptide of Flo migrated somewhat faster in SDS-PAGE than HA1 from A31. This provided an additional method for distinguishing between the two HA gene products (Fig. 5, lanes 3 and 4). To take advantage of this, we cleaved the HA0 to HA and separated HA1 and HA2 on gels after reduction. The difference in apparent molecular weight was not seen under nonreducing conditions (Fig. 5, lanes 1 and 2).

When affinity-purified HA from cells singly infected with Flo or A31 were mixed together before immunoprecipitation with II/4 or N1, no co-precipitation of A31 HA by II/4 (and conversely of Flo HA by N1) was observed (Fig. 6 a, lane 3). This demonstrated that the antibodies were specific and that there were no interactions or exchanges between detergent solubilized Flo and A31 trimers. Formation of mixed trimers was, however, easily detected in cells simultaneously infected with both viruses. Each monoclonal precipitated a mixture of Flo and A31 HA as seen by the two HA1 bands in Fig. 6 b (lanes 1 and 3). Moreover, we found that after a first precipitation with II/4, a second immunoprecipitation with N1 pulled down a single band representing the A31 homotrimer population (lane 2). Conversely, II/4 precipitated only Flo HA1 after prior immunoprecipitation with N1. By quantifying the immunoprecipitated materials we found that the HA contained 48% Flo:A31 heterotrimers, 31% A31 homotrimers, and 21% Flo homotrimers. The agreement between the theoretical (Fig. 1) and experimental values was quite good.

The large fraction of mixed trimers indicated that the nine amino acid sequence differences (see Table I) were not sufficient to prevent stable association between A31 and Flo HA. It suggested, moreover, that whereas oligomerization was unlikely between HAs of different subtypes, it could occur between hemagglutinins within a subtype.

**Response of Mixed Flo:A31 Trimers to Acid pH.** Having shown that hybrid trimers comprised a large fraction in cells infected with Flo and A31, we were able to examine the response of the mixed trimers to acid pH. This analysis was made somewhat complicated by the fact that cells which express nearly equal amounts of the two subunits contain four populations of trimers (XXX, XXY, XYY, and YYY; Fig. 1). We therefore altered the infection conditions to dramatically reduce the level of two of the four combinations. Fig. 1 shows that if one HA type (X) is in threefold excess over the other (Y), then the resulting trimers should contain 42% XXX, 42% XXY, 14% XYY, and 2% YYY. To obtain this simplified distribution, we infected cells with a 3:1 excess of A31 over Flo. Quantitative immunoprecipitations on affinity-purified HA and SDS-PAGE showed that the resulting trimers consisted of 54% mixed trimers, 44% A31 homotrimers, and 2% Flo homotrimers (Table III). Of the mixed trimers ~75% were expected to contain two A31 subunits (Fig. 1).

To determine the response of the mixed trimers to acid pH, aliquots of purified [35S]methionine-labeled HA from the doubly infected cells were acidified to various degrees, reneutralized, and digested with trypsin. Previous studies
Table III. Relationship between Infection Conditions and Trimer Distribution in Doubly Infected Cells

| Monomers | Ratio | % of total trimers |
|----------|-------|--------------------|
| X        | Y     | Flo:A31            | XXX | XXY+ | XYY | YYY |
| Flo      | A31   | 1:3                | 2   | 54   | 44  |
| Flo      | A31   | 1:2                | 8   | 55   | 37  |
| Flo      | A31   | 1:2                | 11  | 51   | 38  |
| Flo      | A31   | 1:1.5              | 21  | 48   | 31  |
| Flo      | A31   | 1:1                | 25  | 52   | 23  |
| Flo      | A31   | 1:0.2              | 80  | 20   | <1  |

To determine if the proportions of homo- and heterotrimers could be varied by altering the amount and ratios of the input viruses, BHK-21 cells were first infected with the A/Memphis antigenic variant FIo so that >95% of the cells were infected. After 1 h, variable amounts of the A/Aichi fusion variant A31 (from 0.2 to 3 the amount of Flo) were added. After an additional 4 h, the cells were labeled and [35S]methionine-labeled HA0 trimers were purified on ricin-Sepharose as described in Materials and Methods. Aliquots were immunoprecipitated with the Flo specific monoclonal antibody 11/4, the A31 specific antibody N1, or both. The trimer distributions were determined from the equations defined in Table II.

have shown that only the acid conformation of HA is trypsin-sensitive (Skehel et al., 1982; Boulay et al., 1987). Fig. 7 (inset) shows that Flo homotrimers obtained from singly infected cells converted to the trypsin-sensitive conformation at pH values below 5.5, whereas A31 homotrimers converted at a pH of 5.8 or below. The HA from the doubly infected cells showed, in contrast, a mixed phenotype. About 40% of the trimers (representing A31 homotrimers) converted at 5.8 and the remaining HA trimers (representing hybrid molecules) at a pH below 5.7. The results indicated that the mixed trimers displayed a pH sensitivity intermediate between Flo and A31 homotrimers. Given the excessive expression of A31 under the infection conditions used above, the majority of the hybrid trimers were expected to contain two variant (A31) subunits.

To enrich for trimers with two wild type (Flo) subunits, cells were infected with a 5:1 excess of Flo over A31. In one such experiment, we found that the HA from doubly infected cells consisted of 20% mixed trimers, <1% A31 homotrimers, and 80% Flo homotrimers (Table III). 90% of the hybrid trimers could be expected to contain two Flo subunits (Fig. 1). In view of the detection problem caused by the large population of trypsin-resistant Flo homotrimers at pH>5.4, an alternative assay was used to analyze the response of these mixed trimers to acid pH. We used the A31 specific antibody N1 to specifically monitor the acid-induced conformational change of the mixed trimers to acid pH. This antibody was particularly well suited because it does not react with Flo and it only reacts with the neutral conformation of A31 HA (Copeland et al., 1986; Boulay et al., 1987). Thus, when applied to the acidified and reneutralized HA, it precipitated mixed trimers and A31 homotrimers still in the neutral conformation. Because A31 homotrimers were virtually absent in the mixture, this antibody was in essence a reagent specific for heterotrimers in their neutral conformation. The loss of the N1 epitope at acid pH has been shown to correlate exactly with the acquisition of trypsin sensitivity (Boulay et al., 1987). It could therefore be compared directly with the trypsin assay used above.

As shown in Fig. 7, when 90% of the mixed trimers con-

Figure 7. Response of mixed trimers to acid pH. HA trimers were obtained from singly or doubly infected cells. The double infections were performed with an excess of one virus type over the other (wt Flo or fusion variant A31) to maximize one of the two heterotrimeric subpopulations (XXY or XYY) at the expense of the other (see Fig. 1). The acid sensitivity of the HA was monitored by the loss of the epitope recognized by the mAb N1 or by the acquisition of trypsin sensitivity (inset). In each assay, equal aliquots of purified [35S]methionine labeled HA were acidified at the indicated pH for 30 min at 37°C and then reneutralized before immunoprecipitation with N1 or digestion with trypsin. The amount of HA precipitated was expressed relative to the total precipitated at neutral pH. The graph shows the loss of the N1 epitope by mixed trimers with one A31 subunit (●) when Flo virus was in excess, by a mixture of A31 homotrimers (44%) and hybrids (54%) mostly with two A31 subunits (●) when A31 virus was in excess, and by A31 homotrimers from singly infected cells (○). (Inset) The trypsin assay after acidification and reneutralization. Trypsinization was performed with 100 μg/ml of TPCK-trypsin (30 min, 37°C) after which the resistant HA was precipitated by 10% (wt/vol) TCA and analyzed by SDS-PAGE and fluorography under nonreducing conditions. In the double infection experiment (A31 + Flo), the resulting HA consisted of ~44% A31 homotrimers and 54% mixed trimers, most with two A31 subunits. The Flo homotrimers represented <2% of the total trimer population and so are not seen on the gel.
tained only one A31 subunit, i.e., when cells were doubly infected with an excess of Flo, the pH midpoint was 5.6. This was slightly lower than the pH at which hybrid trimers containing two A31 subunits became trypsin-sensitive (Fig. 7, inset). To be sure that the differential response to acid pH was not due to differences between the trypsin and N1 precipitation assays, we performed immunoprecipitations with N1 on the trimer population obtained with an excess of A31 (54% mixed trimers, 44% A31 homotrimers, and 2% Flo homotrimers; Fig. 7, inset). We found that ~40% of the HA lost the N1 epitope at high pH (pH 5.9) due to the presence of A31 homotrimers, whereas the rest of the population, composed mostly of mixed trimers with two A31 subunits, converted with a pH midpoint of 5.7. These results were identical with the trypsin assay (Fig. 7, inset). Finally, A31 homotrimers from singly infected cells lost the N1 epitope half maximally at pH 5.9. Taken together these experiments confirmed our observation that A31 homotrimers show a midpoint of conversion at pH 5.9 and Flo at pH 5.5 (Doms et al., 1986). By contrast, the mixed trimers expressed pH sensitivities intermediate between the A31 and Flo phenotypes: A31:A31:Flo hybrids converted at pH 5.7 and the A31:Flo: Flo hybrids at pH 5.6.

**Fusion Activity of Mixed Trimers.** We have previously established that the conformational change in HA correlates with its fusion activity (Doms et al., 1985). The midpoint of fusion activity coincides, as a rule, with 80% conversion of HA to the acid form. To determine the pH at which the mixed trimers caused cell-cell fusion, we infected cells with Flo and A31 either alone or together. In doubly infected cells, 3:1 excess of Flo was used to suppress the production of A31 homotrimers which, due to their activity at high pH, would otherwise mask the fusion activity of the hybrid trimers. Monolayers were immersed in buffers of different pH, and cell-cell fusion observed by light microscopy as previously described (White et al., 1981).

The results, documented in Fig. 8, show that maximal fusion between cells infected with A31 alone occurred at pH 5.7, whereas cells infected with Flo alone fused with a half-maximum at pH 5.4 (Fig. 8) and complete fusion at pH 5.3 (not shown). Cells doubly infected with Flo and A31 (so that predominantly A31:Flo:Flo heterotrimers and Flo homotrimers were obtained) showed a half-maximal fusion pH of 5.6 (Fig. 8) and complete fusion at pH 5.5 (not shown). Because the Flo homotrimers were activated only at lower pH values, the result showed that mixed trimers displayed fusion activity at an intermediate pH, coinciding with the conformational change described above.

**Figure 8.** Mixed trimers induce cell-cell fusion at an intermediate pH. BHK-21 cells were singly infected with A31 (left column) or Flo (right column) or doubly infected with Flo and A31 (center column) so that only Flo homotrimers, with a low-fusion pH, and hybrid trimers were produced. Cell-cell fusion was initiated by gentle trypsinization of the cell surface to activate the HA to fusion competent HA. Cells were then treated at various pHs for 5 min at 37°C followed by reneutralization and incubation in complete growth medium. After 90 min, the cells were fixed in methanol, stained with Giemsa stain, and polykaryon formation monitored by light microscopy.
**Discussion**

Of the various co- and posttranslational modifications that proteins undergo, oligomerization is one of the least understood. This is particularly true for membrane proteins. Yet, most mature membrane proteins occur as complexes of more than one polypeptide chain, and their functions are intimately dependent on their quaternary structure (Carlin and Merlie, 1986). The interactions between subunits vary in strength from multiple covalent disulfide bonds to weak, noncovalent interactions that do not withstand detergent solubilization and isolation procedures. In the case of plasma membrane glycoproteins, assembly is observed quite early in the secretary pathway. The site of assembly is usually thought to be the endoplasmic reticulum, from which many proteins cannot exit without having assembled correctly. The rates by which oligomers are formed vary from protein to protein (Carlin and Merlie, 1986), and this may in part explain why they are transported to the surface at widely variable rates.

Among the best studied oligomeric membrane proteins are the vesicular stomatitis virus G protein and the influenza HA. Both are homotrimeric molecules which acquire their final, stable quaternary structure within 5-10 min of synthesis (Gething et al., 1986b; Copeland et al., 1986; Kreis and Lodish, 1986; Doms et al., 1987). In both instances, trimerization has been shown to occur in the endoplasmic reticulum and is one of the prerequisites for transport to the Golgi (Doms et al., 1987b; Copeland, C. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius, manuscript submitted for publication). Mutant forms of G and HA which fail to trimerize, or which trimerize but do not fold correctly, are not transported (Gething et al., 1986b; Doms et al., 1988a).

The use of hybrids containing variant subunits allows the characterization of the functional and structural roles played by individual subunits in complex multimeric proteins. Mixed oligomers, obtained after reconstitution of purified individual subunits, have been used with great success to analyze the catalytic sites in multimeric enzymes such as aspartate transcarbamoylase (Wente and Schachman, 1987). Such a reconstitution approach is not easily applicable to most membrane proteins. Isolation of intact subunits is often difficult and the reconstitution has to be performed in a membrane environment. To obtain hybrid oligomers of HA0, we have here taken advantage of the assembly process in the living cell.

Given the availability of virus strains and HA mutants with different, well-characterized HA genes, double infection proved a very convenient way to obtain the coexpression of different subunit types. A high proportion of hybrid trimers were formed in cells which expressed nearly equivalent amounts of two closely related but antigenically distinct HA0's. The efficiency of hybrid trimer formation (62%) approached the theoretically expected value for random subunit association (69-72% hybrids depending on the efficiency of double infection). The apparent deviation from the theoretical value reflected most likely uneven expression of the two HA0 forms in some of the doubly infected cells. As shown in Fig. 1, even slight deviation from equal expression would result in fewer mixed trimers and more homo-trimmers. We concluded that HA0 subunits are recruited from a mixed pool of monomers—a reaction that must therefore take place posttranslationally. The mixed trimers were not compatible with any assembly mechanism that would involve preferential co- or posttranslational association between subunits from the same polysome.

One of the consequences of the posttranslational nature of trimer formation is that individual HA0 subunits must recognize each other among thousands of other ER proteins and selectively associate to form fixed complexes. The fidelity of this reaction is rather impressive given their low concentration in the ER. In BHK-21 cells infected with Semliki Forest virus it has been estimated that the viral spike glycoprotein constitutes <0.3% of the endogenous ER proteins (Quinn et al., 1984). This corresponds to a density of 90 molecules/μm² of ER membrane. The density of HA0 monomers in the ER membrane is most likely in the same range. In light of the relatively low concentration and intermixing of monomers from different polysomes, newly synthesized HA0 must be able to diffuse in the ER membrane. To what extent the association of the subunits with a membrane modulates recognition and assembly is unclear.

To determine the degree of sequence specificity involved in subunit interactions, we tested whether HA0 molecules from different influenza subtypes could form oligomers. Although the HAs from different subtypes are similar in overall structure and function (Air and Laver, 1986), our experiments suggested that hybrid HA0 trimers formed between strains within a subtype but not between different subtypes. While a larger number of strains must be tested to determine the generality of this rule, the results already indicate that trimerization of HA0 is very sequence dependent. It is not enough that the overall structural features retained between subtypes (location of disulfide bonds, N-linked carbohydrates, polar and hydrophobic stretches of sequence, the sialic acid binding site, and the fusion peptide) are conserved. Our results suggest that stable HA trimer formation might require a close steric compatibility at the level of single residues in the interface, particularly for the numerous amino acids involved in stabilizing noncovalent interactions.

This is consistent with x-ray crystallographic data on hemoglobin or antibody–antigen complexes (Amit et al., 1986) as well as with the observed instability of mutant HA0 trimers with point mutations in the subunit interface (Rott et al., 1984; Daniels et al., 1985; Doms et al., 1986; Ruigrok et al., 1986a). These mutations have established that the affinity between subunits can be dramatically affected by relatively small sequence differences. A more detailed analysis of the rules that govern trimerization at the molecular level is in progress using a larger panel of HA isolates and site-specific mutants.

Membrane fusion is a critical step in numerous cellular functions, including vesicle-mediated transport, synaptic transmission, fertilization, and cell division. The only well characterized biological membrane fusion proteins identified thus far are enveloped virus glycoproteins and of these, the influenza HA is the best characterized (White et al., 1983). A variety of studies have revealed that when exposed to acid pH, noncovalent interactions between the HA subunits are disrupted (for reviews see Doms et al., 1988; Wiley and Skehel, 1987). Ectodomain dissociation is associated with the concomitant exposure of previously buried hydrophobic moieties, including the NH₂-terminal peptide of HA2. This highly conserved, hydrophobic “fusion peptide” is thought to bring about the attachment of the two
membranes as well as the subsequent fusion reaction (Doms et al., 1985; Gething et al., 1986a; Doms and Helenius, 1988; Wiley and Skehel, 1987; Doms et al., 1988).

The fusion reaction is cooperative, requiring a critical number of acid activated HA trimers to bring about membrane fusion (Doms et al., 1985). However, it is not known if one, two, or all three subunits in an individual trimer must be in the acid, fusion competent conformation or if the subunits undergo the conformational change in concert. The question of subunit cooperativity is basic to understanding the acid triggered membrane fusion activity. Trimers composed of both fusion competent and incompetent (Gething et al., 1986a) subunits should allow us to determine if one, two, or three fusion active subunits are required to bring about viral and target membrane fusion.

Ongoing studies are aimed at further defining the structural requirements for HA0 trimerization in the ER and for the acid triggered membrane fusion activity. Trimers composed of both fusion competent and incompetent (Gething et al., 1986a) subunits should allow us to determine if one, two, or three fusion active subunits are required to bring about viral and target membrane fusion.

We wish to thank Drs. Ira Mellman, Sandy Schmid, Teresa Burgess, and Connie Copeland for critically reading the manuscript.

This work was supported by National Institutes of Health Grant AI-18599 to A. Helenius and by United States Public Health Research Grant AI-08831 to R. Webster from the National Institute of Allergy and Infectious Disease. Dr. Boulay received a post-doctoral salary from "Le Centre National de la Recherche Scientifique, FRANCE" and a N.A.T.O. fellowship. R. W. Doms was supported by the Medical Scientist Training Program.

Received for publication 10 September 1987 and in revised form 9 November 1987.

References

Air, G. M. 1979. Nucleotide sequence coding for the "signal peptide" and N terminus of the hemagglutinin from an Asian (H2N2) strain of Influenza virus. Virology. 97:468-472.

Air, G. M. 1981. Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. Proc. Natl. Acad. Sci. USA. 78:7639-7643.

Air, G. M., and W. G. Laver. 1986. The molecular basis of antigenic variation in influenza virus. Adv. Virus Res. 31:53-102.

Amit, A. G., R. A. Maruzzu, S. E. V. Phillips, and R. J. Poljak. 1986. Three dimensional structure of an antigen-antibody complex at 2.8 Å resolution. Science (Wash., D.C.). 233:747-753.

Arce-Gomez, B., E. A. Jones, C. J. Barnstable, E. Solomon, and W. F. Bowmer. 1978. The genetic control of HA-A and B antigens in somatic cell hybrids: requirement for beta-2-microglobulin. Tissue Antigens. 11:96-112.

Beyer, F., W. E. P., R. W. H. Ruigrok, H. van Driel, and H. Masurel. 1986. Influenza virus strains with a fusion threshold of pH 5.5 or lower are inhibited by amantadine. Arch. Virol. 90:173-181.

Boulay, F., R. W. Doms, I. Wilson, and A. Helenius. 1987. The influenza hemagglutinin precursor as an acid sensitive probe of the biosynthetic pathway. EMBO J. 6:2643-2650.

Carlton, B. E., and J. P. Merlie. 1986. Assembly of multisubunit membrane proteins. In Protein Compartmentalization. A. W. Strauss, I. Boime, and G. Kreil, editors. Springer-Verlag. New York. 71-86.
Chamberlain, J. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor sodium salicylate. Anal. Biochem. 98:132-135.

Caspersen, 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.

Daniels, R. S., J. C. Downie, A. J. Hay, M. Knossow, J. J. Skehel, M. L. Wang, and J. C. Virus. 1985. Fusion mutants of the influenza virus hemagglutinin glycoprotein. Cell. 40:431-439.

Doms, R. W., and A. Helenius. 1986. The quaternary structure of the influenza virus hemagglutinin after acid treatment. J. Virol. 60:833-839.

Doms, R. W., and A. Helenius. 1988. Properties of a viral fusion protein. In Molecular Mechanism of Membrane Fusion. S. Okbi, editor. Plenum Press, New York. In press.

Doms, R. W., J.-J. Gething, J. Hennebery, J. White, and A. Helenius. 1986. Variant influenza virus hemagglutinin that induces fusion at elevated pH. J. Virol. 57:603-613.

Doms, R. W., A. Helenius, and J. White. 1985. Membrane fusion activity of the influenza virus hemagglutinin. J. Biol. Chem. 260:2973-2981.

Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch. 1987. Role for ATP in regulating the assembly and transport of Vescicular Stomatitis Virus G protein trimers. J. Cell Biol. In press.

Doms, R. W., J. White, F. Boulay, and A. Helenius. 1988. Cellular Membrane Fusion. J. Wilschut and D. Hockstra, editors. Marcel Dekker, Amsterdam. 105:1957-1969.

Doxsey, S. J., F. M. Brodsky, S. Blank, and A. Helenius. 1987. Inhibition of endocytosis by anti-clathrin antibodies. Cell. 50:453-463.

Gerhard, W., J. Yewdell, M. E. Frankel, and R. Webster. 1981. Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. Nature (Lond.). 290:713-716.

Germain, R. N., R. W. Bentley, and H. Quill. 1985. Influence of allelic polymorphism on the assembly and surface expression of Class II MHC (Ia) molecules. Cell. 40:433-439.

Gething, M.-J., K. McCammon, and J. Sambrook. 1986b. Expression of wild type influenza virus proteins in intracellular membranes II. Biochemical studies. J. Cell Biol. 98:2142-2147.

Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis virus glycoprotein to the cell surface. Cell. 46:929-937.

Nakajima, S., D. J. Brown, M. Ueda, K. Nakajima, A. Sugiura, A. K. Pattnaik, and D. P. Nayak. 1986. Identification of the defects in the hemagglutinin gene of two temperature-sensitive mutants of A/WSN/33 influenza virus. Virology. 154:279-285.

Newton, S. E., G. M. Air, R. G. Webster, and W. G. Laver. 1983. Sequence of the hemagglutinin gene of influenza virus A/Memphis/1/71 and previously uncharacterized monoclonal antibody-derived variants. Virology. 128:495-501.

Palese, P., and J. L. Schumman. 1976. Differences in RNA patterns of influenza A viruses. J. Virol. 17:876-884.

Quinn, P., G. Griffiths, and G. Warren. 1984. Density of newly synthesized plasma membrane proteins in intracellular membranes II. Biochemical studies. J. Cell Biol. 98:2142-2147.

Roth, 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 (Eur. Mol. Biol. Organ.) J. 3:329-3332.

Rugier, R. W. H., S. A. Martin, S. A. Wharton, J. J. Skehel, P. M. Bayley, and D. C. Wiley. 1986a. Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. Virology. 155:484-504.

Rugier, R. W. H., N. G. Wrigglesworth, J. L. Calder, S. Cussack, S. A. Wharton, E. B. Brown, and J. J. Skehel. 1986b. Electron microscopy of the low pH structure of influenza virus hemagglutinin. EMBO (Eur. Mol. Biol. Organ.) J. 5:41-49.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463-5467.

Schuy, C. W., W. K. Kuroda, C. Scholtissek, W. Garten, and H.-D. Klenk. 1986. Mutations blocking the transport of the influenza virus hemagglutinin between the rough endoplasmic reticulum and the golgi apparatus. EMBO (Eur. Mol. Biol. Organ.) J. 5:2831-2836.

Sege, K., L. Rask, and P. A. Peterson. 1981. Role of β2-microglobulin in the intracellular processing of HLA antigens. Biochemistry. 20:4523-4530.

Skehel, J., P. Bayley, E. Brown, S. Martin, M. Waterfield, J. White, J. 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.

Smith, M. M., J. Linström, and J. P. Merrie. 1987. Formation of the alphab-toxin binding site and assembly of the nicotinic acetylcholine receptor subunits occur in the endoplasmic reticulum. J. Cell Biol. 262:4367-4376.

Stepmann, T., D. Hockstra, G. Scherphof, and J. Wilschut. 1986. Fusion activity of influenza virus: a comparison between biological and artificial target membrane vesicles. J. Biol. Chem. 261:10966-10969.

Stephenson, J. R., and N. J. Dimmock. 1974. Inhibition of the processing of ribosomal RNA in avian cells infected with an influenza virus. Biochim. Biophys. Acta. 361:198-208.

Webster, R. G., and W. G. Laver. 1980. Determination of the number of non-overlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology. 104:139-148.

Webster, R. G., W. G. Laver, and G. M. Air. 1983. Antigenic variation among type A influenza viruses. In Genetics of the Influenza Viruses. P. Palese and D. W. Kingsbury, editors. Springer-Verlag, Berlin. 127-168.

Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild. 1982. Molecular mechanisms of variation of influenza viruses. Nature (Lond.). 296:115-121.

Wente, S. R., and H. K. Schachman. 1987. Shared active sites in oligomeric enzymes: model studies with defective mutants of aspartate transcarbamoylase produced by site-directed mutagens. Proc. Natl. Acad. Sci. USA. 84:31-35.

White, J., A. Helenius, and M.-J. Gething. 1982. Haemagglutinin of influenza virus expressed from a cloned gene product promotes membrane fusion. Nature (Lond.). 300:658-659.

White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. Q. Rev. Biophys. 16:151-195.

White, J. K. McFall, and A. Helenius. 1981. Cell fusion by Semliki Forest, Influenza, and Vescicular Stomatitis viruses. J. Cell Biol. 89:674-679.

Wiely, 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.

Wiely, 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 (Lond.). 289:373-378.

Wilson, I., J. Skehel, and D. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature (Lond.). 292:366-373.