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# The influenza hemagglutinin precursor as an acid-sensitive probe of the biosynthetic pathway

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**The hemagglutinin of influenza virus (HA), an acid-activated membrane fusion protein, is synthesized in the endoplasmic reticulum and transported through the Golgi complex to the cell surface of infected cells as an uncleaved, fusion-incompetent precursor, HA0. The mature, proteolytically activated HA is known to undergo a rapid, irreversible, acid-induced conformational change which mediates membrane fusion and virus penetration. On the basis of antigenic modifications and the acquisition of trypsin susceptibility, we demonstrate here that HA0, while unable to cause fusion, is acid sensitive. It undergoes irreversible conformational changes quite similar to those of HA at mildly acidic pH (pH <6.0). The ectodomain of HA0 does not, however, acquire hydrophobic properties and the changes occur in a less concerted manner (the pH dependence is much broader and the rate of conversion slower). These differences are likely to account for the inability of acid-treated HA0 to trigger membrane fusion. It was shown, moreover, that HA0 acquired its acid-sensitive properties immediately following trimerization in the endoplasmic reticulum. Since HA0 did not convert to the acid form at any point during its intracellular transport, we concluded that the *trans*-Golgi compartment, known to be more acidic than the cytosol and involved in constitutive membrane transport, is not likely to have a pH <6.0.**

*Key words:* hemagglutinin/influenza virus/membrane fusion/protein transport/*trans*-Golgi network

## Introduction

Enveloped animal viruses depend on membrane fusion for penetration into their host cells (see White *et al.*, 1983). Fusion is mediated by virally encoded glycoproteins. The target membranes are either the plasma membrane of the host cell or the limiting membranes of endosomes into which viruses are routed after endocytosis. In cases where penetration occurs exclusively from endosomes, the fusion proteins are activated by the acidic pH in these organelles. Many viral fusion factors such as influenza hemagglutinin (HA) are, in fact, inherently acid sensitive. They undergo distinct, irreversible conformational changes in the pH range 5.0–6.5 that are necessary for membrane fusion and penetration (see White *et al.*, 1983; Kielian and Helenius, 1986; Doms *et al.*, 1987).

Influenza HA is synthesized as an inactive precursor which is post-translationally cleaved to the mature form (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975). The 76-kd precursors, called HA0, are assembled in the endoplasmic reticulum (ER) to trimers and transported via the Golgi complex to the plasma membrane (Copeland *et al.*, 1986; Gething *et al.*, 1986b). Activation to the mature HA occurs just prior to, or coincident with, delivery to the plasma membrane (Klenk *et al.*, 1974; Hay, 1974; Matlin and Simons, 1983). As a result, each mature HA molecule consists of two disulfide-linked glycoproteins, HA1 (58 kd) and HA2 (26 kd). The structure of HA's trimeric water-soluble ectodomain, determined to 3 Å resolution by X-ray crystallography, reveals that each HA spike has two structurally distinct parts: a stem containing a triple-stranded coiled coil of three 76 Å long $\alpha$ helices and a head with three globular, highly folded antiparallel $\beta$-sheet structures (Wilson *et al.*, 1981). When exposed to acidic pH the hydrophobic N terminus of HA2, the N terminus generated by the activating cleavage, becomes exposed and apparently helps mediate the interaction that leads to fusion between the two membranes (Skehel *et al.*, 1982; Doms *et al.*, 1985; Gething *et al.*, 1986a; J.White and I.Wilson, unpublished results).

HA0, in contrast to HA, is not fusion active (Huang *et al.*, 1981; White *et al.*, 1982). Whether this is because it cannot undergo an acid-induced conformational change is not clear. Daniels *et al.* (1983) and Ruigrok *et al.* (1984) have reported that HA0 is not modified by acid treatment, while Jackson and Nestorowicz (1985) and Bächi *et al.* (1985) found that acidic pH can lead to irreversible antigenic changes similar to HA. We have examined the effects of low pH on HA0 for two reasons: (i) differences between HA0 and HA are important for understanding the structural requirements for acid activation and the membrane fusion activity of HA; (ii) if HA0 is indeed acid-sensitive, it provides an intrinsic pH probe for the compartments through which it passes during its transport from the ER to the plasma membrane.

Our results show that HA0 is sensitive to low pH. It undergoes an irreversible conformational change similar to HA but does not expose a new hydrophobic moiety. The results provide an explanation for the lack of fusion activity and indicate moreover that the protein spends a short period of time, if any, in compartments with a pH <6.0 during transport to the cell surface.

## Results

### *Evidence for an acid-induced conformational change in HA0*

The irreversible, acid-induced changes in mature HA can be detected in several ways: the protein becomes sensitive to trypsin and other proteases (Skehel *et al.*, 1982; Doms *et al.*, 1985); antigenic sites located in the tip/interface region are lost and new epitopes are exposed (Webster *et al.*, 1983; Yewdell *et al.*, 1983; Daniels *et al.*, 1983; Doms *et al.*, 1985) and the hydrophilic ectodomain acquires hydrophobic properties (Skehel *et al.*, 1982; Doms *et al.*, 1985). We used these approaches to determine whether HA0 undergoes a similar conformational change in mildly acid pH. Two well characterized influenza strains were used; the X:31 strain, which fuses at pH <5.5 and its variant A:31, which has a somewhat higher pH of fusion (Doms *et al.*, 1986).

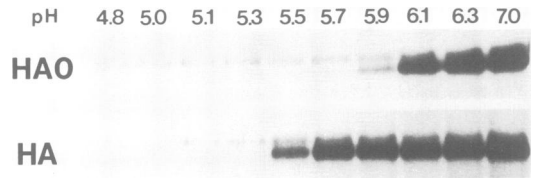


**Fig. 1.** Susceptibility of HA and HA0 to trypsin as a function of pH. Aliquots of [$^{35}$S]methionine-labeled HA0 or HA, isolated in TX100 from CV-1 cells infected with the X:31 wild-type virus, were acidified at various pHs and incubated at 37°C for 30 min. After reneutralization the samples were digested by trypsin in the presence of 0.5% TX100 and precipitated with 10% trichloroacetic acid. The samples were analyzed by SDS–PAGE under non-reducing conditions and radioactive bands were detected by fluorography.

**Table I.** Response of HA0 and HA to acid

| Probe | Specificity | Epitope | Subunit | HA | | HA0 | |
|---|---|---|---|---|---|---|---|
| | | | | pH | $t_{1/2}$ (min:s) | pH | $t_{1/2}$ (min:s) |
| mAb | | | | | | | |
| N1 | Neutral | Tip | HA1 | 5.5 | 0:11 | 6.1 | 1:30 |
| N2 | Neutral | Tip(B) | HA1 | 5.5 | n.d. | 6.0 | n.d. |
| A1 | Acid | ? | HA2 | 5.5 | 0:10 | 5.4 | 5:00 |
| A2 | Acid | ? | HA1 | 5.5 | n.d. | 5.3 | 5:00 |
| A3 | Acid | 98–106 | HA1 | 5.5 | n.d. | 6.0 | 2:00 |
| Trypsin | Acid | Lys27 | HA1 | 5.5 | 0:10 | 6.1 | 0:30 |
| | | Arg224 | HA1 | | | | |

Methionine-labeled HA0 was isolated in TX100 from CV-1 cells infected with the wild-type X:31. HA was derived from HA0 by mild trypsin digestion. The proteins were incubated with buffers at various pHs for 30 min at 37°C, reneutralized, immunoprecipitated with the indicated monoclonal antibodies or digested with trypsin. The values reflect the pH at which 50% of the molecules were immunoprecipitated. To determine the kinetics of conversion, the proteins were incubated at pH 5.0 for various periods prior to reneutralization and processed as above.

The results with both strains were comparable except that pH-induced effects with A:31 occurred ~0.2 pH units higher. For initial studies, [$^{35}$S]methionine-labeled wild-type HA0 was isolated from infected cells by affinity chromatography and sucrose gradient centrifugation in the presence of Triton X-100 (TX100).

*Trypsin sensitivity and antigenic changes*

The first indication that HA0 underwent an acid-induced change was obtained by monitoring its trypsin sensitivity. In agreement with previous results we found that in its neutral TX100-solubilized form, HA0 was cleaved by trypsin to mature HA but remained resistant to further digestion (Figure 1). In contrast, HA0 that had been acidified (30 min at 37°C) and reneutralized prior to trypsin digestion was found to be fully degraded (Figure 1). Fifty percent was degraded in samples exposed to pH 6.1 while complete digestion to trichloroacetic acid (TCA)-soluble fragments was observed below pH 5.9. When compared with HA, HA0 became irreversibly trypsin sensitive at a pH 0.5 higher (see Figure 1). Results using proteinase K instead of trypsin were identical.

We next took advantage of a panel of conformation-specific monoclonal anti-HA antibodies. Monoclonals N1 and N2 (see Table I) have previously been shown to recognize the tip epitope of neutral, trimeric HA and HA0 (Copeland *et al.*, 1986). Monoclonals A1 and A2, in contrast, bind to acid-treated but

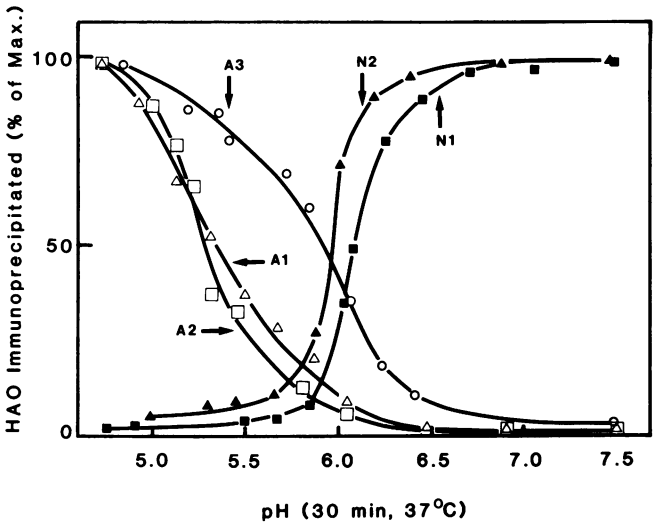


**Fig. 2.** Antigenic changes of TX100-solubilized HA0. Aliquots of [$^{35}$S]-methionine-labeled HA0, purified from CV-1 cells infected with X:31 wild-type virus, were incubated in MNT/TX100 buffer at the indicated pH for 30 min at 37°C. The samples were neutralized and immunoprecipitated as described in Materials and methods. Values are expressed relative to the maximum amount immunoprecipitated at the lowest pH [acid-specific antibodies A1 (△), A2 (□) and A3 (○)] or at the highest pH [neutral-specific antibodies N1 (■) and N2 (▲)].

not neutral HA (Copeland *et al.*, 1986). The binding sites for A1 and A2 have not yet been identified precisely, but they are known to reside in the HA2 and HA1 subunits, respectively. The monoclonal antibody A3, which recognizes a nine amino acid long determinant in HA1 covered in the interface between the globular head domains of the trimer [residues 98–106, referred to as H26D08 by Wilson *et al.* (1984)], has likewise been shown to bind acid-treated HA (J.White and I.Wilson, unpublished results).

We found that monoclonals N1 and N2 were able to precipitate the neutral form of HA0, but not HA0 that had been exposed to pH values <6.0–6.1 (Figure 2). In contrast, antibodies A1, A2 and A3 displayed a strict specificity towards the acid-treated form. With decreasing pH, the determinant recognized by A3 appeared concomitantly with the disappearance of reactivity with antibodies N1 and N2. The change correlated exactly with the acquisition of trypsin sensitivity (cf. Figure 1). In contrast, the determinants recognized by A1 and A2 emerged only after treatment at pH 5.4 and 5.3, respectively. Taken together, these findings indicate that HA0 underwent irreversible conformational changes in two stages; one at a relatively high pH (pH 6.1) and another at a lower pH (pH 5.3–5.5). Only the latter changes in HA0 corresponded in pH dependence to those in HA.

*Temperature dependence and kinetics*

The conformational changes in HA0 were highly temperature dependent. Acid-triggered changes at the tip of the molecule, recorded by disappearance of the N1 epitope, occurred at all temperatures between 0 and 40°C, but the efficiency was reduced at temperatures <35°C (Figure 3). The appearance of the A2 epitope, diagnostic of the late conformational change, was not detected unless acidification was performed at temperatures >30°C. The differences in temperature dependence of the early (pH 6.1) and late (pH 5.4) changes suggest that the structural alterations occurring in the top domain of HA0 required little energy of activation compared to the change leading to exposure

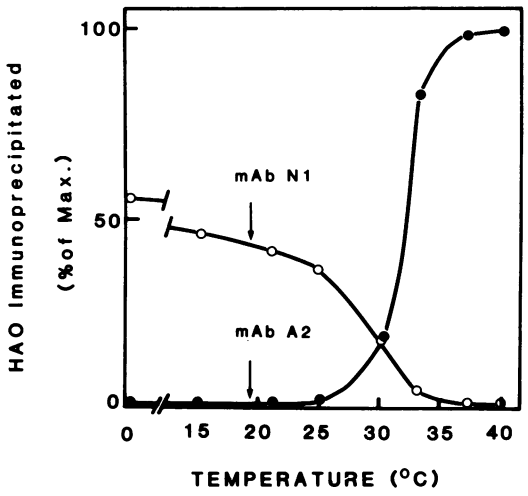


**Fig. 3.** Temperature dependence of HA0's conformational change. Aliquots of [$^{35}$S]methionine-labeled HA0, purified from CV-1 cells infected with X:31 wild-type virus, were incubated with MNT/0.1% TX100 buffer at pH 5.0 for 15 min at the indicated temperature, reneutralized and immunoprecipitated with the acid-specific A2 (●) or neutral-specific N1 (○) monoclonal antibodies. The values are expressed relative to the maximum amount precipitated, i.e. before acidification in the case of N1 or after 15 min at pH 5.0 and 37°C in the case of A2.

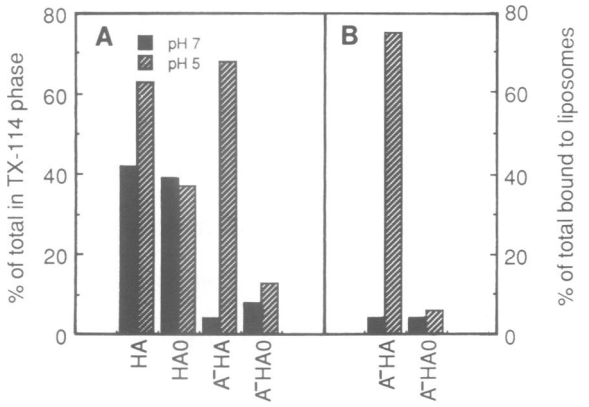


**Fig. 4.** Partitioning in TX114 and binding to liposomes of HA, HA0, A$^-$HA and A$^-$HA0. Aliquots of [$^{35}$S]methionine-labeled proteins purified from CV-1 cells infected with SVEXHA-A$^-$ vector were incubated at pH 7.0 or 5.0 (30 min, 37°C), neutralized and used for detergent partitioning or binding to liposomes. (**A**) Partitioning in TX114 was carried out by the method of Bordier (1981). The amount of radioactivity in each phase was counted. (**B**) The extent of protein bound to liposomes was determined by centrifugation in flotation gradients as previously described by Doms *et al.* (1985).

of the A2 determinant. The temperature dependence of the conformational alterations may explain, at least in part, the previous reports which failed to detect conformational changes in HA0 at acidic pH (Daniels *et al.*, 1983; Ruigrok *et al.*, 1984). The experiments may have been performed at room temperature or below.

To determine the kinetics of the conformational changes at 37°C, HA0 solubilized in TX100 was incubated at pH 5.0 for various times before reneutralization and digestion with trypsin or immunoprecipitation with the conformation-specific monoclonal antibodies. The rate of conversion differed with the assay used, as shown by the half conversion ($t_{1/2}$) values listed in Table I. With a $t_{1/2}$ of 30 s, the acquisition of trypsin susceptibility proved to be the fastest indicator of the conformational change. It was followed by the loss of the N1 epitope in the top domain of the molecule ($t_{1/2}$ = 1.5 min), the unmasking of peptide 98–106 at the interface ($t_{1/2}$ = 2.0 min), and exposure of the antigenic determinants recognized by A1 and A2 ($t_{1/2}$ = 5 min). The irreversible changes in HA0 thus occurred in a sequential fashion starting at the top of the molecule. In contrast to HA0, HA changed its conformation in a faster and more concerted manner. The conversion of HA, monitored by the different assays, was essentially completed within 10–15 s (Table I).

The data thus revealed that TX100-solubilized HA0 passed through at least two structural alterations as the pH was reduced. The first, involving more distal portions of the trimer, occurred at pH 6.1 and displayed fast kinetics. The second took place around pH 5.4, exposing previously hidden epitopes in both HA1 and HA2. The latter changes correlated approximately with the fusion pH of the mature HA. Although mature HA may pass through similar intermediate steps (J.White and I.Wilson, unpublished results), our data indicated that the changes in HA must occur within much narrower pH and kinetic windows.

*Effect of pH on the solubility properties of HA0*

While both HA and HA0 have acid-induced conformational changes, only HA is able to catalyze membrane fusion (see Doms *et al.*, 1987). To define the structural basis for this functional difference, we examined the effects of acid pH on the solubility properties of HA0. The conformational changes in HA are known to result in the exposure of hydrophobic moieties which contain the highly conserved, apolar amino terminus of HA2 (Gething *et al.*, 1986a; J.White and I.Wilson, unpublished results). This peptide moiety is thought to play a critical role in membrane fusion. Since HA0 and HA are hydrophobic membrane proteins, and thus insoluble in water, studies on changes in solubility properties were best performed using water-soluble ectodomain fragments that lack the hydrophobic C-terminal membrane anchors. Such fragments can be generated from HA by bromelain digestion (Brand and Skehel, 1972). The water-soluble ectodomain fragment, termed BHA, comprises >90% of HA's mass and responds to acid pH in a manner nearly identical to HA (Skehel *et al.*, 1982; Doms *et al.*, 1985) although differences in trimer stability have been described (Nestorowicz *et al.*, 1985; Doms and Helenius, 1986; Boulay *et al.*, 1987). Exposure of the amino terminus of HA2 and perhaps other apolar regions imparts hydrophobic character to the acidified molecule as judged by its ability to aggregate as protein–protein rosettes and to bind both lipid vesicles and non-ionic detergents (Skehel *et al.*, 1982; Doms *et al.*, 1985).

A water-soluble form of HA0 cannot be obtained by bromelain treatment because the enzyme, in addition to removing the membrane anchor, converts HA0 to HA. To circumvent this problem, we expressed a cDNA clone of the HA gene which lacked the sequences encoding the transmembrane and cytoplasmic domains. The truncated HA0, called anchor minus HA0 (A$^-$HA0), was expressed in CV-1 cells by use of an SV40 late-replacement vector (Gething and Sambrook, 1982; Doyle *et al.*, 1986). A$^-$HA0 is secreted into the medium mainly as monomers, but small amounts of 9S trimers were also obtained (Copeland *et al.*, 1986). The trimers were isolated and characterized as such by the trypsin-resistance assay.

We found that the trimeric pH 7 forms of A$^-$HA0 and A$^-$HA (like BHA) were resistant to digestion with either trypsin or proteinase K. They separated into the aqueous phase during TX114 partitioning (Figure 4a) and they did not bind to

liposomes (Figure 4b). When incubated at pH 5.0 (37°C, 30 min), both became susceptible to trypsin degradation (not shown). However, only $A^-HA$ acquired hydrophobic properties. $A^-HA0$ was unable to bind to liposomes and did not partition into the detergent phase under any conditions. We also performed TX114 partitioning on intact HA and HA0. At neutral pH, ~40% of both forms were recovered in the detergent fraction, owing to the hydrophobic character of the transmembrane domains. After acid treatment, a greater fraction of HA (62%), but not HA0, was recovered in the detergent phase.

Thus, the ectodomain of HA0 did not acquire hydrophobic properties during acid treatment. This fundamental difference between HA0 and HA indicated that the so called 'fusion peptide' must be present as a free N-terminal peptide in order for the fusion protein to expose a new hydrophobic domain at acid pH.

### *HA0 as a biosynthetic pH probe*

The acid sensitivity of HA0 raised a question about the pH in the secretory pathway through which the molecule passes during intracellular transport. Several reports have shown that *trans*-elements of the Golgi complex are acidic. Certain carboxylic ionophores (e.g. monensin) and acidotropic bases (ammonium chloride) disrupt Golgi functions (see Tartakoff, 1983; Matlin, 1986a), a proton pump has been detected in Golgi fractions from liver (Glickman *et al.*, 1983; Zhang and Schneider, 1983), and the *trans*-Golgi accumulates acidotropic amines as shown by electron microscopic immunocytochemistry (Anderson and Pathak, 1985; Schwartz *et al.*, 1985). Since HA0 is intrinsically acid sensitive (especially the A31 variant HA0), how then is it able to pass through an acidic *trans*-Golgi compartment without irreversibly converting to the acid form? There is no doubt that the HA0 traverses the *trans* compartments of the Golgi (Matlin and Simons, 1983; Rodriguez-Boulan *et al.*, 1984), and cell surface HA0 is in the neutral conformation as judged by its trypsin resistance and antigenic structure (Copeland *et al.*, 1986). In addressing this apparent paradox it was first necessary to show that intracellular forms of HA0 were indeed acid sensitive.

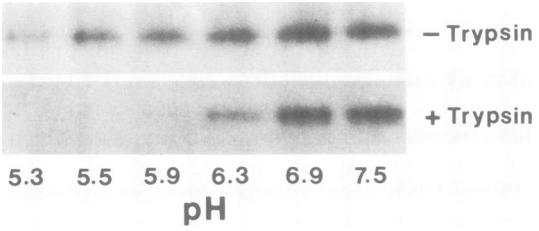


**Fig. 5.** Trypsin sensitivity of intracellular HA0. CV-1 cells grown to confluency in a 35-mm Petri dish were infected with A:31 variant influenza virus for 12 h, pulse-labeled with [$^{35}$S]methionine for 5 min at 37°C, washed with PBS and incubated in medium with unlabeled methionine at 37°C (chased) for 15 min. The cells were solubilized in PBS/0.5% TX100/0.4% BSA and aliquots were acidified at various pHs for 8 min at 37°C. The samples were neutralized and half of each aliquot was treated with TPCK-trypsin (0.1 mg/ml) for 30 min at 37°C. After stopping the reaction with trypsin inhibitor, the non-digested hemagglutinin was immunoprecipitated with a polyclonal antibody and analyzed by gel electrophoresis under non-reducing conditions and autoradiography.

To analyze the acid sensitivity of intracellular HA0, we labeled A:31 variant-infected cells with [$^{35}$S]methionine for 5 min, chased with cold methionine for 15 or 24 min, lysed the cells in TX100 and incubated aliquots at different pH prior to trypsin digestion (Figure 5). Not only did the HA0 in both samples become trypsin sensitive after acidification, but the pH dependence of conversion to the trypsin-sensitive form was similar to that of cell surface-derived, solubilized HA0 (Figure 1). The sample chased for 15 min was particularly important because the majority of HA0 was still in the endoglycosidase H-sensitive form (i.e. had trimerized and left the ER but had not yet passed into the medial Golgi). These results indicated that intracellular HA0, at least in detergent-solubilized form, responded to acid with similar pH dependence as the cell surface form.

### *Acid sensitivity of membrane-anchored HA0*

We next tested whether the conformational changes observed with TX100-solubilized HA0 were similar to the changes observed when the protein was anchored to a biological membrane. A:31 variant HA0 expressed on the surface of CV-1 cells was examined by indirect immunofluorescence before and after acid treatment. When cells expressing HA0 were acidified (37°C, 10 min at pH 5.5) prior to fixation at pH 5.5 and immunofluorescence, strong staining with the anti-peptide monoclonal A3 (see Table I) could be observed (Figure 6a). The expression of the epitope was neither reversible nor dependent on fixation since

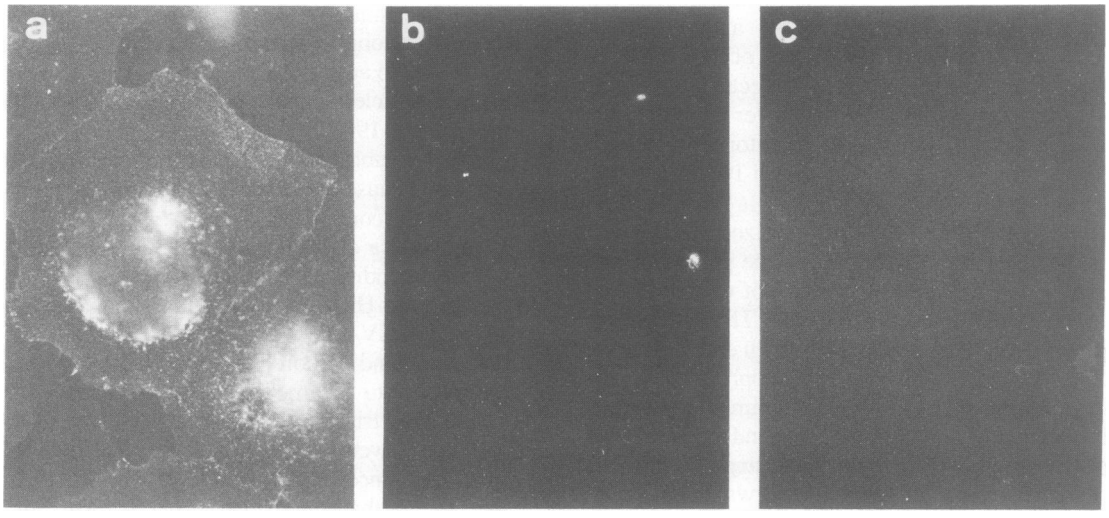


**Fig. 6.** Immunofluorescent staining of cell surface HA0 with the acid-specific monoclonal antibody A3. CV-1 cells expressing HA0 from the A:31 variant influenza virus at the plasma membrane were kept at neutral pH (**c**) or acidified to pH 5.5 for 10 min at 37°C (**a**) or 0°C (**b**) and then fixed in 3% paraformaldehyde for 15 min under the same conditions. The fixed cells were processed for immunofluorescence using the acid-specific monoclonal antibody A3.

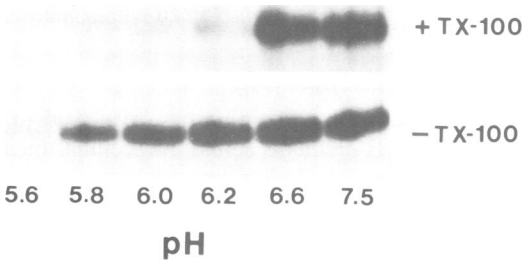


**Fig. 7.** Susceptibility of HA0 virus to trypsin after acidification to various pHs in the presence or absence of TX100. Aliquots of [$^{35}$S]-methionine-labeled HA0 A:31 variant influenza virus were acidified at various pHs in the presence or absence of 0.5% TX100 for 10 min at 37°C, reneutralized and digested by trypsin as described in Materials and methods. The samples were then precipitated with 10% trichloroacetic acid and processed for gel electrophoresis under non-reducing conditions. The trypsin-resistant HA was detected by fluorography and the percentage of HA0 digested was calculated after scanning of the film. The amount digested in the absence of TX100 at pH 5.8 was 32%, while 23% was digested afer pH 6.0 incubation.

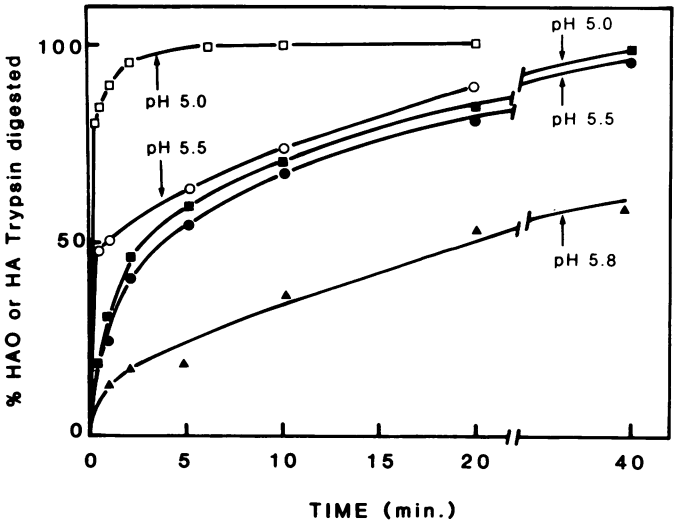


**Fig. 8.** Kinetics of conformational change of membrane-anchored HA0 assayed by trypsin susceptibility. Aliquots of [$^{35}$S]methionine-labeled A:31 HA virus (□,○) or A:31 HA0 virus (■,● and ▲) were incubated at 37°C for various periods of time at the indicated pH. At the indicated time point, aliquots were withdrawn, neutralized and submitted to trypsin digestion in the presence of 0.5% TX100. Samples were then precipitated with 10% trichloroacetic acid and processed for gel electrophoresis under non-reducing conditions. The percentage of protein digested was calculated after excision and counting of the polyacrylamide band according to Walter *et al.* (1979).

fluorescence was observed if antibody binding was performed on reneutralized cells or at pH 5.5 before or after fixation. However, fluorescence was not observed without prior acidification (Figure 6c) or when acidification was performed at 4°C (Figure 6b). This confirmed that acid treatment of membrane-bound HA0 converts the protein into an acid conformation and that the conversion is temperature dependent.

Surprisingly, immunofluorescence with the neutral specific monoclonals N1 and N2 was not fully abolished by a similar acid treatment, nor was reactivity with the acid-specific antibodies A1 and A2 readily detected (not shown). These findings contrasted with the detergent-solubilized HA0 and mature cell surface HA, both of which lost the N1 and N2 epitopes and gained the A1 and A2 determinants after similar treatment (Figure 2 and Copeland *et al.*, 1986). The immunofluorescence observations thus suggested that although membrane-anchored HA0 underwent an acid-induced conformational change, the change was different from that in detergent solution.

This prompted us to characterize the changes in membrane-bound HA0 more quantitatively. We used intact, [$^{35}$S]-methionine-labeled A:31 variant virus in which the HA0 had not been proteolytically activated. The virus was first exposed to different pH buffers with or without prior addition of TX100. It was then neutralized, digested with trypsin and analyzed by SDS−PAGE. As shown in Figure 7, the HA0 in the viral membrane became sensitive to trypsin at a lower pH than TX100-solublized HA0. Whereas the detergent form was already fully sensitive at pH 6.2, the membrane form had to be acidified to ≤pH 5.8 for comparable effect. Analysis of conversion kinetics (Figure 8) indicated, moreover, that membrane-associated HA0 converted to the trypsin-sensitive form more slowly than detergent-solubilized HA0 and displayed biphasic kinetics (Figure 8). The rate of conversion was affected by the pH used; at pH 5.5, 30% of the membrane-bound HA0 was converted within 1.5 min; the same extent of conversion at pH 5.8 took 8 min. We observed that the nine amino acid peptide (residues 96−106 at the interface of the globular domain) recognized by the A3 antibody, was exposed much more slowly (with a $t_{1/2}$ = 20 min) at pH 5.5. Similar results were observed for the disappearance of N1 and N2 epitopes (not shown). The antibody binding results thus suggested that the irreversible dissociation of the top domains was relatively slow in membrane-bound HA0.

Taken together the results showed that membrane-bound HA0 converts irreversibly to a new conformation when exposed to pH values <pH 6.0. It is apparently more resistant to the effects of acid than its TX100-solubilized counterpart; it converts more slowly and the dissociation of the ectodomain is not as extensive. Viewed in the context of Golgi acidity, the irreversible changes in HA0 below pH 6.0 suggested that the *trans*-Golgi compartments are not very acidic and/or that the HA0 traverses these compartments very rapidly (see Discussion).

## Discussion

### *Precursors for viral fusion factors*

Viral fusion proteins are, as a rule, synthesized as precursors that are cleaved to the mature protein late in the secretory pathway (see Dubois-Dalcq *et al.*, 1984). For influenza HA, the paramyxovirus F protein and the E2 glycoprotein of Corona virus there is good evidence that the uncleaved precursors are not fusion active (White *et al.*, 1983; Holmes *et al.*, 1984). There are several possible reasons why a 'proform' of these fusion factors may be needed. (i) A hydrophobic fusion sequence may only be translocated correctly to the lumen of the ER if it is part of a continuing polypeptide chain. A recent study on the paramyxovirus F protein (Paterson and Lamb, 1987) suggests that the activating cleavage increases the hydrophobicity of the fusion peptide enabling it to interact stably with a membrane. (ii) Uncleaved polypeptide chains may be required for correct folding and assembly of the proteins in the ER. (iii) Cleavage may be needed to generate a metastable conformation necessary for the cooperative changes required for fusion. (iv) An inactive precursor may be needed to avoid premature fusion. Fusion during exocytic transport could, for instance, lead to loss of the fusion factor and damage to cellular organelles.

These explanations are not mutually exclusive; the rationale for the existence of precursors may, in fact, be quite complex. It is of interest that all viral fusion proteins do not need proteolytic

activation; the vesicular stomatitis virus (VSV) G-protein is an example of an acid-activated fusion factor which is not cleaved. Unlike other viral fusion factors the change undergone by VSV-G may, however, be reversible (Crimmins *et al.*, 1983; R.W.Doms, D.Keller, A.Helenius and W. Balch, submitted).

*Why does HA0 fail to induce membrane fusion?*

Our results indicated that HA0 trimers, though fusion incompetent, are acid sensitive. The acid responses of HA and HA0 were however different in several respects, and these differences are likely to explain why HA0 is unable to induce fusion.

First, the irreversible conformational changes in HA0 occurred over a wider pH range (pH 6.3−5.3 for HA0 versus pH 5.6−5.3 for HA). HA0 in TX100 solution underwent at least two separate, irreversible changes. The initial change occurred at pH values well above the threshold pH for HA conversion and membrane fusion, the second change coincided approximately with the change in HA. The initial change involved more distal portions of the molecule judging by the exposure of the A3 epitope and the loss of the N1 and N2 determinants; the second change resulted in alterations both in HA1 and HA2, perhaps lower down in the spike. When membrane-bound HA0 was analyzed, the wider pH dependence was also observed, although not as dramatically as in detergent solution.

Second, HA0 responded to acid with markedly slower and less concerted kinetics than HA. At pH 5.0 the changes detectable with our assays ranged in $t_{1/2}$ from 30 s to 5 min with the changes which occurred at higher pH preceding those which required lower pH. In HA the corresponding changes are rapid and remarkably cooperative with a $t_{1/2}$ of ~10 s (see Table I).

Third, the ectodomain of HA0 did not expose a hydrophobic moiety upon acid treatment. This was demonstrated by the inability of $A^{-}HA0$ (Figure 4) to bind non-ionic detergent (TX114) and liposomes. The N terminus of HA2 generated by the proteolytic activation most likely constitutes a part of the hydrophobic moiety which is exposed in HA after acid treatment and plays a crucial role in fusion (Doms *et al.*, 1985; Gething *et al.*, 1986a; J.White and I.Wilson, unpublished results). The lack of a hydrophobic moiety in acid-treated HA0 supports the notion that the N-terminal sequence of HA2 is involved in fusion, and that it has to be present as a free terminal peptide in order to be functional.

*The effect of detergent*

One of our findings was that TX100-solubilized HA0 exhibited more extensive, irreversible conformational alterations at intermediate pH than its membrane-bound form. This observation is not surprising in view of an extensive literature on subtle effects of so called 'non-denaturing' detergents on membrane-bound enzymes and conformation of proteins (Helenius *et al.*, 1979). It is possible that replacing the lipids around the anchor moiety of HA0 by detergent affects the stability of trimeric interactions. Our recent studies have indicated that the anchor portion of the molecule is important for the quaternary structure of HA (Doms and Helenius, 1986).

As our assays record changes in conformation, it is equally possible that the detergent modifies the dynamic properties of HA0. The presence of detergent could, for instance, render a change which is reversible in the absence of detergent, irreversible by stabilizing the altered conformation. As we can only follow irreversible changes, such an effect would be manifested as an increase in pH sensitivity. Whatever the underlying mechanism of detergent action, the results emphasize the need for caution in interpreting results obtained with solubilized membrane proteins.

*The pH of the secretory pathway*

In recent years increasing attention has been paid to the role and generation of pH gradients across intracellular membranes. Acidic organelles include endosomes, phagosomes, lysosomes and secretory vacuoles (see Mellman *et al.*, 1986). There is considerable evidence that *trans*-elements of the Golgi complex are acidic (Anderson and Pathak, 1985; Schwartz *et al.*, 1985). It has been suggested, on the basis of inhibitor studies, that the low pH has a role in regulating membrane transport and molecular sorting in this compartment (see Kelly, 1985; Matlin, 1986a; Griffiths and Simons, 1986). The actual pH in the *trans*-Golgi network is not known, nor is the mode of acidification well understood.

Being acid sensitive, the HA0 serves as an intrinsic pH probe for the secretory pathway. Using pulse−chase experiments in CV-1 cells infected with an SV40 vector and trypsinization of the cell surface (Copeland *et al.*, 1986), we have shown that HA0 transport to the plasma membrane is at least 80% efficient, i.e. ~80% of HA0 molecules synthesized during a 5-min pulse can be detected on the cell surface as intact HA0 trimers, yielding HA1 and HA2 polypeptides but resistant to further trypsin digestion. We have also shown that of the HA0 (20%) that does not reach the surface, half is not transported out of the ER because it is incorrectly folded or trimerized (Copeland *et al.*, 1986). This leaves only 10% for additional losses in the pathway. As the value is relatively low, one can conclude that the majority of HA0 molecules do not spend considerable time in compartments with a pH $<6.0$.

Conversion of this general conclusion to a specific minimum pH value for the most acidic organelle in the transport pathway is, unfortunately, complicated by the relatively slow conversion of membrane-bound HA0 to the acidic form. If the HA0 passes through an acidic compartment very rapidly it may not have time to convert to the acid conformation. In the transfected CV-1 cells it takes, on average, 15 min for HA0 to travel from the medial Golgi compartments where the N-linked carbohydrate chains become resistant to endoglycosidase H to the cell surface (Copeland *et al.*, 1986). A similar time interval has been found in MDCK cells for transit of HA0 accumulated at 20°C in a *trans*-Golgi compartment to the plasma membrane (Matlin and Simons, 1984; Matlin, 1986b). Studies on the synchronized intracellular transport of a temperature-sensitive VSV-G protein in CHO cells have suggested that the time required for transport between the Golgi region and the plasma membrane is 11−15 min (Bergman and Singer, 1983). In BHK-21 cells, Saraste and Kuismanen (1984) have determined by radioimmunoassay that it takes $\leq 10$ min for the first molecules of Semliki Forest virus membrane proteins to travel from a *trans*-Golgi compartment (defined by the 20°C block) to the plasma membrane. Thus, while a precise time spent by these viral membrane glycoproteins in the *trans*-Golgi network is not known, it is probably of the order of several minutes. If only 2 min is spent in an acidic compartment, we can conclude that the pH cannot be much lower than 6.0. The kinetics of conversion in Figure 8 show that if the HA0 would stay in a compartment of pH 5.5 for 2 min no less than 50% of the HA0 could convert to the acid form. At pH 5.8 ~15% could have converted. As our assays could readily detect changes in 10% of the HA0 population, we suggest that the *trans*-

Golgi or other compartments involved in the constitutive transport of HA0 are not very acidic.

Our pH estimates assume that there are no factors present in the Golgi that modulate the acid sensitivity of HA0. Since HA0 is the only influenza gene product in the transfected cells, we can eliminate the possibility of virus-coded modulating factors, but we cannot exclude that the Golgi complex contains cellular factors that suppress the low pH-induced conformational change. It is interesting to mention in this context that several influenza virus ts mutants have been described in which the HA0 exits the ER normally but is not transported beyond the Golgi complex (Naruse *et al.*, 1986). It will be interesting to determine whether the block is related to acid-induced conformational changes. Moreover, mutant tissue culture cell lines with abnormalities in the transport of the Sindbis virus spike glycoproteins have been described as defective in their late Golgi functions (Robbins *et al.*, 1984). The late Golgi defects might be related to an alteration in acidification. Virus mutants and mutant cell lines may provide valuable tools to analyze the role of acidity in the secretory pathway and its effects on acid-sensitive proteins.

## Materials and methods

*Reagents*

The L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (TPCK-trypsin), soybean trypsin inhibitor (STI) and neuramindase from *Clostridium perfringens* were purchased from Sigma Chemical Co (St Louis, MO). Fixed *Staphylococcus aureus* was obtained from Zymed Corp. (South San Francisco, CA). Affinity-purified FITC-conjugated goat F(ab')2 anti-mouse IgM + IgG and goat anti-mouse IgG were obtained from Tago Inc. (Burlingame, CA). [$^{35}$S]-Methionine was provided by Amersham Corp. (Arlington Heights, IL) and had a specific activity of >800 Ci/mmol.

*Cells and viruses*

CV-1 cells (a monkey kidney cell line) were cultured as described previously (Doxsey *et al.*, 1985). Primary chicken embryo fibroblasts (CEF), obtained from 8- to 10-day-old chick embryos, were grown to 80% confluency in Glasgow minimum essential medium (G-MEM) supplemented with 7.5% fetal calf serum, 1.5% chicken serum and 10% tryptose phosphate broth. Both wild-type X:31 (H3N2) influenza virus and the fusion variant A:31 were propagated from the plaque-purified inoculum by two passages in 11-day-old embryonated chicken eggs (Doms *et al.*, 1985).

*Growth and purification of radioactive virus*

For production of the fusion variant A:31, a monolayer of CEF in an 85-mm dish was infected at 37°C with 500 μl of allantoic fluid from infected eggs diluted in 2.5 ml of Dulbecco's modified Eagle medium (DMEM) buffered with 10 mM Hepes. After 1 h, cells were supplemented with 7.5 ml of DMEM containing 10% fetal calf serum plus 10 mM Hepes and then, at 5 h post-infection, washed twice with 10 ml phosphate-buffered saline (PBS) and incubated for 18 h at 37°C with 1 mCi of [$^{35}$S]methionine in 5 ml of methionine-free medium supplemented with 10 mM Hepes and 125 μl of G-MEM. After collecting the medium, the cell monolayer was treated with 4 mg of neuraminidase in 2 ml DMEM, 10 mM Hepes for 30 min at 37°C with gentle shaking after which the pre- and post-neuraminidase supernatants were centrifuged for 5 min at 1000 *g* to remove cell debris, layered on a 30–60% sucrose step gradient in MNT buffer (NaCl 100 mM, Tris 50 mM, MES 20 mM pH 7.5) and centrifuged for 90 min at 4°C in an SW28 rotor at 24 000 r.p.m. The [$^{35}$S]methionine-labeled virus, collected at the interface of the two sucrose layers, contained exclusively uncleaved hemagglutinin (HA0) as judged by the absence of HA1 and HA2 polypeptides after SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Virus containing mature HA was obtained by incorporating 0.5 μg TPCK-trypsin/ml of medium during the overnight radiolabeling. Under these conditions the hemagglutinin was exclusively recovered in its cleaved form as evidenced by the presence of radioactive HA1 and HA2 polypeptides after SDS–PAGE under reducing conditions.

*Preparation of radioactively labelled HA0 and HA*

[$^{35}$S]Methionine-labeled HA0 was prepared by infecting confluent CV-1 cells in an 85-mm dish with 500 μl of allantoic fluid in 2.5 ml of DMEM and purified in 0.1% TX100 by affinity chromatography on a ricin column followed by a 5–25% (w/v) sucrose gradient as previously described (Doms and Helenius, 1986). HA was prepared from purified HA0 by gentle digestion with 10 μg TPCK-trypsin/ml (final concentraiton) for 10 min at 37°C followed by a 5-fold weight excess of STI. Complete activation, yielding HA1 and HA2, was established by SDS–PAGE under reducing conditions.

*Preparation of anchor minus HA and HA0*

CV-1 cells were infected with an SV40 late replacement vector containing a truncated HA gene of X:31 (SVEXHA-A$^-$) lacking the sequences encoding the transmembrane and cytoplasmic domains of HA (Doyle *et al.*, 1986). After 48 h the cells were labeled overnight with [$^{35}$S]methionine. Anchor minus HA was obtained by trypsin digestion of the anchor minus HA0 precursor purified by ricin affinity chromatography and sucrose density centrifugation as described above.

*Immunoprecipitation*

Monoclonal antibodies specific to the neutral (N1,N2) or acid (A1,A2) conformations of HA have been previously described (Copeland *et al.*, 1986). A monoclonal antibody raised against a synthetic peptide corresponding to residues 75–110 in the HA1 subunit of HA and which recognizes a determinant in residues 98–106 has also been described (Wilson *et al.*, 1984). This monoclonal, H26D08, will be referred to in this paper as A3. Immunoprecipitations of purified $^{35}$S-labelled HA0, HA0 virus, HA, or HA virus were carried out essentially as described by Copeland *et al.* (1986). SDS–PAGE was after Laemmli (1970) and fluorography performed on gels impregnated with salicylic acid (Chamberlain, 1979) using Kodak XAR-5 film pre-flashed with orange light. Quantification of the radioactive bands located by fluorography was determined after excision and solubilization according to Walter *et al.* (1979).

*Immunofluorescence*

CV-1 cells on coverslips were infected with influenza virus as described above. After 14 h of infection the cells were incubated for 10 min at the indicated pH and temperature (Figure 6) in PBS with 10 mM MES (all PBS solutions were supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Fixation in 3% paraformaldehyde and processing for immunofluorescence was performed essentially as described previously (Copeland *et al.*, 1986).

*Detergent partitioning and liposome binding*

Phase separation in TX114 was performed according to the method of Bordier (1981). Liposomes consisting of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, cholesterol and phosphatidic acid in ratios of 1:1:1:1.5:0.2 were prepared according to White and Helenius (1980). Anchor minus HA or HA0 was incubated with liposomes at pH 5.0 for 15 min at 37°C. Liposome-bound protein was separated from unbound material by centrifugation (Doms *et al.*, 1985).

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## References

Anderson,R.G.W. and Pathak,R.K. (1985) *Cell*, **40**, 635–643.
Bächi,T., Gerhard,W. and Yewdell,J.W. (1985) *J. Virol.*, **55**, 307–313.
Bergmann,J.E. and Singer,S.J. (1983) *J. Cell Biol.*, **97**, 1777–1787.
Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.
Boulay,F., Doms,R.W. and Helenius,A. (1987) In Brinton,M.A. and Ruickert, R.R. (eds), *Positive Strand RNA Viruses*. Alan R.Liss, 41 East 11th Street, New York, Vol. 54, pp. 103–112.
Brand,C.M. and Skehel,J.J. (1972) *Nature, New Biol.*, **238**, 145–147.
Chamberlain,J. (1979) *Anal. Biochem.*, **98**, 132–135.
Copeland,C.S., Doms,R.W., Bolzau,E.M., Webster,R.G. and Helenius,A. (1986) *J. Cell Biol.*, **103**, 1179–1191.
Crimmins,D.L., Mehard,W.B. and Schlessinger,S. (1983) *Biochemistry*, **22**, 5790–5796.
Daniels,R.S., Douglas,A.R., Skehel,J.J. and Wiley,D.C. (1983) *J. Gen. Virol.*, **64**, 1657–1662.
Doms,R.W. and Helenius,A. (1986) *J. Virol.*, **60**, 833–839.
Doms,R.W., Helenius,A. and White,J. (1985) *J. Biol. Chem.*, **260**, 2973–2981.
Doms,R.W., Gething,M.-J., Henneberry,J., White,J. and Helenius,A. (1986) *J. Virol.*, **57**, 603–613.
Doms,R.W., White,J., Boulay,F. and Helenius,A. (1987) In Wilschut,J. and Hoekstra,D. (eds), *Cellular Membrane Fusion*. Marcel Dekker, Amsterdam, in press.
Doxsey,S.J., Sambrook,J., Helenius,A. and White,J. (1985) *J. Cell Biol.*, **101**, 19–27.

Doyle,C.J., Sambrook,J. and Gething,M.J. (1986) *J. Cell Biol.*, **103**, 1193–1204.
Dubois-Dalcq,M., Holmes,K.V. and Rentier,B. (1984) *Assembly of Enveloped RNA Viruses*. Springer-Verlag, Vienna.
Gething,M.-J. and Sambrook,J. (1982) *Nature*, **300**, 598–603.
Gething,M.-J., Doms,R.W., York,D. and White,J. (1986a) *J. Cell Biol.*, **102**, 11–23.
Gething,M.-J., McCammon,K. and Sambrook,J. (1986b) *Cell*, **46**, 939–950.
Glickman,J., Croen,K., Kelly,S. and Al-Awqati,Q. (1983) *J. Cell. Biol.*, **97**, 1303–1308.
Griffiths,G. and Simons,K. (1986) *Science*, **234**, 438–443.
Hay,A.J. (1974) *Virology*, **60**, 398–418.
Helenius,A., McCaslin,D.R., Fries,E. and Tanford,C. (1979) *Methods Enzymol.*, **56**, 734–749.
Holmes,K.V., Frana,M.F., Robbins,S.G. and Sturman,L.S. (1984) In Rottier,P.J.M,. van der Zeijst,B.A.M. and Horzinek,M. (eds), *Coronaviruses: Molecular Biology and Pathogenesis*. Plenum Press, New York, pp. 37–52.
Huang,R.T.C., Rott,R. and Klenk,H.D. (1981) *Virology*, **110**, 243–247.
Jackson,D.C. and Nestorowicz,A. (1985) *Virology*, **145**, 72–83.
Kelly,R.B. (1985) *Science*, **230**, 25–32.
Kielian,M. and Helenius,A. (1986) In Schlesinger,S. and Schlesinger,M.J. (eds), *The Togaviridae and Flaviviridae*. Plenum Publishing Corporation.
Klenk,H.D,. Wöllert,W., Rott,R. and Scholtissek,C. (1974) *Virology*, **57**, 28–41.
Klenk,H.D., Rott,R., Orlich,M. and Blodorn,J. (1975) *Virology*, **68**, 426–439.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lazarowitz,S. and Choppin,P. (1975) *Virology*, **68**, 440–454.
Matlin,K.S. (1986a) *J. Cell. Biol.*, **103**, 2565–2568.
Matlin,K.S. (1986b) *J. Biol. Chem.*, **261**, 15172–15178.
Matlin,K.S. and Simons,K. (1983) *Cell*, **34**, 233–243.
Matlin,K.S. and Simons,K. (1984) *J. Cell Biol.*, **99**, 2131–2139.
Mellman,I., Fuchs,R. and Helenius,A. (1986) *Annu. Rev. Biochem.*, **55**, 663–700.
Naruse,H., Scholtissek,C. and Klenk,H.D. (1986) *Virus Res.*, **5**, 293–305.
Nestorowicz,A., Laver,G. and Jackson,D.C. (1985) *J. Gen. Virol.*, **66**, 1687–1695.
Paterson,R. and Lamb,R.A. (1987) *Cell*, **48**, 441–452.
Robbins,A.R., Oliver,C., Bateman,J.L., Krag,S.S., Galloway,C.J. and Mellman,I. (1984) *J. Cell Biol.*, **99**, 1296–1308.
Rodriguez-Boulan,E., Paskiet,K.T., Salas,P.J.I. and Bard,E. (1984) *J. Cell Biol.*, **98**, 308–319.
Ruigrok,R.W.H., Cremers,F.M., Beyer,W.E.P. and de Ronde Verloop,F.M. (1984) *Arch. Virol.*, **82**, 181–194.
Ruigrok,R.W.H., Wrigley,N.G., Calder,L.J., Cusak,S., Wharton,S.A., Brown,E.B. and Skehel,J.J. (1986) *EMBO J.*, **5**, 41–49.
Saraste,J. and Kuismanen,E. (1984) *Cell*, **38**, 535–549.
Schwartz,A.L., Strous,G.J.A.M., Slot,J.W. and Geuze,H.J. (1985) *EMBO J.*, **4**, 899–904.
Skehel,J.J., Bayley,P.M., Brown,E.B., Martin,S.R., Waterfield,M.D., White, J.M., Wilson,I.A. and Wiley,D.C. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 968–972.
Tartakoff,A.M. (1983) *Cell*, **32**, 1026–1028.
Watler,P., Jackson,R.C., Marcus,M.M., Lingappa,V.R. and Blobel,G. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1795–1799.
Webster,R.G., Brown,L.E. and Jackson,D.C. (1983) *Virology*, **126**, 587–599.
White,J. and Helenius,A. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3273–3277.
White,J., Helenius,A. and Gething,M.J. (1982) *Nature*, **300**, 658–659.
White,J., Kielian,M. and Helenius,A. (1983) *Q. Rev. Biophys.*, **16**, 151–195.
Wilson,I.A., Skehel,J.J. and Wiley,D.C. (1981) *Nature*, **292**, 366–373.
Wilson,I.A., Niman,H.L., Houghten,R.A., Cherenson,A.R., Connolly,M.L. and Lerner,R.A. (1984) *Cell*, **37**, 767–778.
Yewdell,J.W., Gerhard,W. and Bächi,T. (1983) *J. Virol.*, **48**, 239–248.
Zhang,F. and Schneider,D.L. (1983) *Biochem. Biophys. Res. Commun.*, **114**, 620–625.

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