Effects of Exposure to Low pH on the Lateral Mobility of Influenza Hemagglutinin Expressed at the Cell Surface: Correlation between Mobility Inhibition and Inactivation

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ABSTRACT: To investigate the possible role of viral glycoprotein mobility in membrane fusion, fluorescence photobleaching recovery was employed to study the effects of exposure to mildly acidic pH (required to convert many viral fusion proteins to the fusion-active form) on the lateral mobility of influenza hemagglutinin (HA) proteins expressed at the surface of transfected cells. HA proteins from two different strains were compared: X:31 HA, which is activated by a brief exposure to pH 4.9 but is irreversibly inactivated at longer exposure times, and HA from A/Japan/305/57, which is relatively stable to inactivation at this pH [Puri, A., Booy, F. P., Doms, R. W., White, J. M., & Blumenthal, R. (1990) *J. Virol. 64,* 3824–3832]. The HA proteins from both strains, expressed in CV-1 cells using VS-40 vectors, exhibited relatively unrestricted lateral diffusion at the cell surface. The high mobility persisted following a brief exposure (1 min) to pH 4.9 to mediate conversion to the fusogenic state. Longer times (up to 15 min) of preincubation at pH 4.9 inhibited the lateral mobility of X:31 HA (the lateral diffusion rate was markedly reduced, followed by immobilization) but not of A/Japan HA, whose fusion activity is resistant to such treatment. Inhibition of the lateral mobility of X:31 HA due to preincubation at low pH was not specific to the CV-1 cells and was found also in a CHO cell line stably expressing this protein. The results presented demonstrate a close correlation between loss of mobility and inactivation of fusogenic activity, in accord with the notion that lateral motion of the HA proteins is required for fusion. We propose that the inactivation of X:31 HA following prolonged incubation at low pH involves a type of enhanced aggregation that leads to immobilization.

The mechanism of membrane fusion events is still unknown, in spite of their pivotal role in a variety of cellular processes. The best characterized fusion process involving biological membranes is that mediated by viral envelope glycoproteins (Marsh & Helenius, 1989; Stegmann et al., 1989; White, 1990; Wilschut & Hoekstra, 1990). The most extensively characterized viral fusion protein is the hemagglutinin protein (HA)[1] of influenza virus. This homotrimeric viral spike glycoprotein (Wiley & Skehel, 1987) contains three monomers, each consisting of two disulfide-linked polypeptides (HA1 and HA2) derived from the precursor HA0 by proteolytic cleavage (Klenk et al., 1975). As in many other families of enveloped viruses, fusion mediated by influenza HA requires a conformational change induced by a brief exposure to mildly acidic pH (Marsh & Helenius, 1989; White, 1990). This exposes the hydrophobic amino terminus of HA2 (termed the "fusion peptide") (White & Wilson, 1987), which can then interact with the target lipid bilayer (Harter et al., 1988, 1989; Brunner et al., 1991; Stegmann et al., 1991). However, in the absence of a target membrane, exposure of either virions or cells expressing HA to low pH was shown to inactivate the fusion activity of HA of some influenza subtypes (H1 and H3 subtypes, including the X:31 and X:47 strains) but not of others (A/Japan/305/57, H2 subtype) (Sato et al., 1983; Junankar & Cherry, 1986; Stegmann et al., 1987; Puri et al., 1990).

While there is accumulating evidence for the involvement of the "fusion peptide" in the fusion event, there are also indications that additional requirements have to be met to enable fusion. We have demonstrated that lateral motion of the viral envelope proteins in the target cell membrane is obligatory for the induction of cell–cell fusion by Sendai virus (Aroeti & Henis, 1988; Henis et al., 1989). Such information is not yet available for the influenza HA, but numerous independent studies suggest that several HA trimers have to interact at the fusion site (Morris et al., 1989; Ellens et al., 1990; Stegmann et al., 1990), a phenomenon that would require their lateral motion. Furthermore, on the basis of the effect of exposing virions (X:47 strain) to low pH on the rotational motion of the spike glycoproteins, it was proposed that rational mobility of HA in the viral envelope is significant for its functional properties (Junankar & Cherry, 1986).

The uncleaved precursor form (HA0) of A/Japan HA was reported to show high lateral mobility when expressed in the plasma membrane of transfected cells (Ellens et al., 1990; Fire et al., 1991). However, the dynamic properties of the HA proteins in the fusion-relevant state (i.e., after cleavage of HA0 and following the low-pH-mediated conformational change) were not studied. In the present work, we employed fluorescence photobleaching recovery (FPR) to characterize the effects of exposure to low pH on the lateral mobility of HA from two different strains in the plasma membrane of transfected cells: X:31 HA (X:31 is a recombinant strain bearing the HA from the A/Aichi/68 strain; Kilbourne, 1969), which is inactivated following prolonged exposure to mildly acidic pH, and A/Japan/305/57 HA, which is relatively

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[1] Abbreviations: HA, influenza hemagglutinin protein; FPR, fluorescence photobleaching recovery; TMR, tetramethylrhodamine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; D, lateral diffusion coefficient; R_f, mobile fraction.

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insensitive to such exposure. The results of these studies demonstrate that the HA proteins from both strains are free to diffuse in the cellular plasma membrane after the low-pH-mediated conformational change required for their activation and suggest that the inactivation of X:31 HA at longer exposure times is due to enhanced aggregation leading to immobilization. The immobilization is correlated with loss of fusogenic activity, in accord with the notion that lateral motion of the HA proteins is required for fusion.

MATERIALS AND METHODS

Reagents. Tetramethylrhodamine (TMR) 5,6-isothiocyanate was obtained from Molecular Probes (Eugene, OR). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). Trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Polyclonal rabbit antiserum against the HA protein of A/Japan/305/57 was kindly provided by Dr. Michael Roth (University of Texas Southwestern Medical Center, Dallas, TX); antiserum against X:31 HA was raised in rabbits against virions of the X:31 strain. Monovalent Fab′ fragments labeled with TMR isothiocyanate (TMR-Fab′) were prepared from the IgG fractions as described (Henis et al., 1985), following standard labeling procedures (Brandtzaeg, 1973). The Fab′ preparation was free of contamination of $F(ab')_2$ or IgG, as judged by SDS–PAGE under nonreducing conditions. All other reagents were of the highest purity available.

Cells. CV-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and used between passage 3 and 15. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Biological Industries, Beth Haemek, Israel). These cells were used for growing SV-40 recombinant virus stocks and for the experiments employing transient expression of HA.

CHO-K1 cells (American Type Culture Collection) stably expressing X:31 HA were generated in the laboratory of Dr. Judith White (Danieli & White, unpublished work) using the gene amplification vector pEE14 (Celltech Ltd., Berkshire, England) that allows expression of the cDNA under the control of human cytomegalovirus 5′ sequences and contains a glutamine synthetase selectable marker gene (Bebbington et al., 1992). In the presence of methionine sulfoximine, a competitive inhibitor of glutamine synthetase, coamplification of the HA gene is obtained. The cell line generated in this manner, HA300a, was grown in Glasgow minimum essential medium supplemented with the following: nonessential amino acids at 100 μM each (Gibco, Grand Island, NY), glutamate and asparagine (500 μM; Sigma), pyruvate (1 mM; Gibco), nucleosides (30 μM adenosine, guanosine, cytidine, and uridine; 10 μM thymidine), 10% FCS, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), and 300 μM L-methionine sulfoximine (Sigma).

Recombinant Virus Stocks and Infection Procedures. The transient expression studies in CV-1 cells employed the following plasmids, based on SV-40 vectors containing pBR322 derivatives: pKSVEHA, which contains the full-length cDNA encoding the A/Japan/305/57 HA protein (Roth et al., 1986; kindly provided by Dr. Michael Roth); pSVX38, an analogous vector encoding the X:31 HA, generated and generously provided by Dr. M.-J. Gething, University of Texas Southwestern Medical Center at Dallas; and d11055, helper virus DNA providing the late SV-40 genes for the recombinant virus stocks (Pipas et al., 1980).

The plasmid DNAs containing the HA genes were digested with *KpnI* to remove the pBR322 derivative and recircularized under dilute ligation conditions (3 μg/mL). The resulting vectors were transfected into CV-1 cells in subconfluent monolayers, together with an equal amount of d11055 DNA. Transfection and development of high-titer virus stocks were as described (Doyle et al., 1985). For transient expression experiments, slightly subconfluent CV-1 cells were infected with 1:5–1:10 dilutions of second passage virus stocks (Doyle et al., 1985); the lateral mobility measurements were performed 36–40 h later.

Treatment and Labeling of Cells Expressing HA. For measurements of the lateral mobility of the HA0 forms of X:31 or A/Japan HA, cells were grown on glass cover slips. At 36–40 h postinfection (for CV-1 cells) or 2 days after splitting (for HA300a cells), they were washed twice by Hanks' balanced salt solution (HBSS) devoid of phenol red and bicarbonate (Biological Industries) and supplemented with 20 mM HEPES and 2% BSA (HBSS/HEPES/BSA, pH 7.2–7.3); they were then incubated in the same buffer with the appropriate TMR-Fab′ (100 μg/mL, 45 min, 4 °C). After being rinsed three times, the cells were taken for the lateral mobility measurements in the same buffer equilibrated at the desired temperature.

For measuring the lateral mobility of the HA proteins after the low-pH-mediated conformational change, the cells were treated prior to labeling as described by Puri et al. (1990). Briefly, cleavage of HA0 was achieved by a 10-min incubation at 22 °C with 5 μg/mL trypsin in serum-free DMEM. After being washed twice with the medium containing 10% FCS, cells were taken for TMR-Fab′ labeling as above or incubated for various periods at 37 °C with HBSS containing 10 mM HEPES and 10 mM 4-morpholineethanesulfonic acid (MES), pH 4.9; the acidic buffer solution was replaced with a fresh one after 20 s. The cells were washed twice with the neutral-pH HBSS/HEPES/BSA and subjected to TMR-Fab′ labeling as above. In some cases (see text), the labeling with antibody preceded the trypsin and low-pH treatment.

Fluorescence Photobleaching Recovery. Lateral diffusion coefficients (D) and mobile fractions (R_f) of TMR-Fab′ labeled HA proteins on the cell surface were measured by FPR (Axelrod et al., 1976; Koppel et al., 1976) with the apparatus described earlier (Henis & Gutman, 1983). The bleaching conditions employed in the FPR studies were shown not to alter the lateral mobilities measured (Wolf et al., 1980; Koppel & Sheetz, 1981). After cells expressing HA were labeled with TMR-Fab′ as described in the former section, the cover slip carrying the cells was placed (cells facing downward) over a serological slide with a well filled with HBSS/HEPES/BSA equilibrated at the desired temperature. A thermostated microscope stage maintained the sample temperature constant throughout the FPR experiments, performed within 30 min of the labeling. The endocytosis of HA, which is a protein that is excluded from coated pits, is negligible during this period (Lazarovits & Roth, 1988; Fire et al., 1991). The monitoring laser beam (Coherent Innova 70 argon ion laser; 529.5 nm, 1 μW) was focused through the microscope (Zeiss Universal) to a Gaussian radius of 0.61 ± 0.02 μm. A brief pulse (5 mW, 30 ms) bleached 50–70% of the fluorescence in the illuminated region. The time course of the fluorescence recovery was followed by the attenuated monitoring beam. D and R_f were extracted from these curves by nonlinear regression analysis (Petersen et al., 1986). Incomplete fluorescence recovery was interpreted to represent fluorophores that are

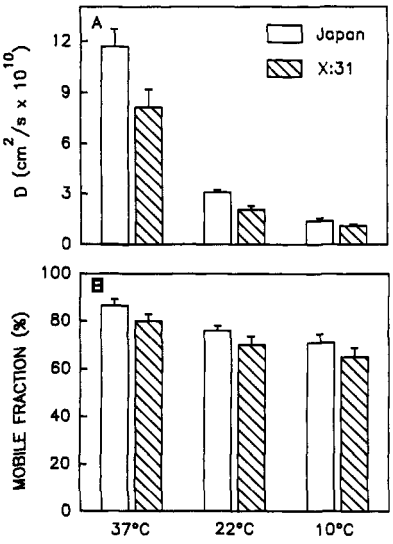

FIGURE 1: Lateral diffusion of HA0 (A/Japan and X:31 strains) on the surface of CV-1 cells. Cells were infected with recombinant SV-40 virions containing the cDNAs encoding HA0 of either A/Japan or X:31, grown on glass cover slips for 36–40 h, and labeled with the appropriate TMR-Fab′ as described under Materials and Methods. The FPR measurements were performed at either 37, 22, or 10 °C (see Materials and Methods). Each bar is the mean ± SE of 33–47 measurements. (A) D values; (B) R_f values.

immobile on the FPR experimental time scale ($D \leq 5 \times 10^{-12}$ cm²/s).

RESULTS

HA0 from both X:31 and A/Japan Strains Show High Lateral Mobility on CV-1 Cells. The first step in characterizing the differential effects of exposure to mildly acidic pH on X:31 and A/Japan HA proteins was to determine the "baseline" level of their lateral mobility in the cell membrane—namely, the lateral diffusion of the uncleaved precursor forms (HA0). To enable a direct comparison under identical conditions, CV-1 cells expressing these proteins were labeled in the cold by the appropriate TMR-Fab′ fragments; the use of monovalent fragments is required to avoid any possible cross-linking that may otherwise affect the aggregation state and the lateral diffusion of the protein antigen. The lateral diffusion of the labeled proteins was measured by FPR at several temperatures (37, 22, and 10 °C) (Figure 1). At all temperatures, X:31 HA0 exhibited relatively high D and R_f values, which were only slightly lower than those of A/Japan HA0. These values are in the same range measured on NIH3T3 cells stably expressing A/Japan HA0 (Ellens et al., 1990). Thus, the lateral diffusion of the uncleaved HA0 from both virus strains is relatively unrestricted and is in the higher range observed for membrane proteins at the surface of mammalian cells (Edidin, 1987; Jacobson et al., 1987).

Exposure to Low pH Affects Differently the Mobility of HA from the Two Influenza Strains. The effects of exposure to a mildly acidic pH (4.9) on the lateral mobility of the HA proteins is of special interest in view of the fact that the fusogenic form of HA is obtained by a specific proteolytic cleavage followed by a conformational change mediated by the low pH [reviewed in Marsh and Helenius (1989) and White (1990)]. Furthermore, prolonged exposure to this pH in the absence of a target membrane inactivates the fusogenic activity of, e.g., X:31 HA but not of the A/Japan HA (Sato et al., 1983; Junankar & Cherry, 1986; Stegmann et al., 1987; Puri et al., 1990). We have therefore examined the effects of incubating CV-1 cells expressing either X:31 HA or

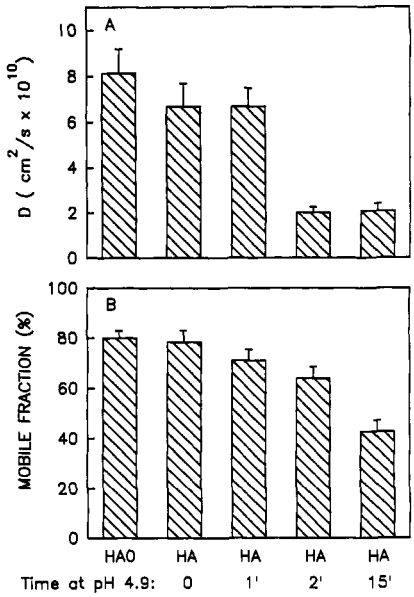

FIGURE 2: Effect of incubation at pH 4.9 on the lateral mobility of X:31 HA in the plasma membrane of CV-1 cells. The experiments were conducted at 37 °C on CV-1 cells expressing X:31 HA, as described in Figure 1. Except for the experiment shown in the first bar, HA0 on the cell surface was cleaved by trypsin (HA designates the cleaved protein), and the cells were exposed for the indicated times (minutes) to pH 4.9 at 37 °C as described under Materials and Methods. Incubation at pH 4.9 without prior cleavage to HA had no detectable effect on the lateral diffusion of HA0. After return to the neutral pH buffer, the cells were labeled with TMR-Fab′ and taken for the FPR measurements. Each bar is the mean ± SE of 34–47 measurements. (A) D values; (B) R_f values.

A/Japan HA (precleaved by trypsin as described under Materials and Methods) at pH 4.9 on the lateral mobility of the HA proteins. In the case of X:31 HA (Figure 2), trypsin cleavage alone (to the HA1–HA2 disulfide-linked form) did not alter significantly the lateral diffusion of the protein, and further incubation for 1 min at pH 4.9 resulted only in a very slight reduction in the mobile fraction. However, 2 min at this pH already led to a marked drop (3–4-fold) in the D value, concurrent with a small but significant drop in R_f (from 78–80% for HA0 or for trypsin-cleaved HA to 64%). Longer incubation at pH 4.9 (15 min) did not affect further the D value; however, the mobile fraction continued to drop to about half of the original value (Figure 2). This time scale is similar to that observed for the effect of low-pH preincubation of CV-1 cells expressing X:31 HA on their ability to fuse with human erythrocytes: a 2-min preincubation (pH 4.9, 37 °C) had a mild effect on the fusion process (the half-time increased by a factor of 2.5, accompanied by a 20% reduction in the maximal fusion level), while preincubation for 15 min completely abolished the fusogenic activity (Puri et al., 1990).

Unlike the situation with X:31 HA, incubation of CV-1 cells expressing A/Japan HA (pH 4.9, 37 °C) for as long as 15 min had no measurable effect on the D value of the viral glycoprotein and only marginally reduced the R_f value (Figure 3). Again, these results are in correlation with the effect of a similar preincubation on the ability of CV-1 cells expressing A/Japan HA to fuse with human erythrocytes: the fusion rate was not affected, and the maximal fusion level attained was only slightly reduced (Puri et al., 1990).

Effect of Exposure to pH 4.9 on the Lateral Mobility of X:31 HA in Stably Expressing CHO Cells. In order to demonstrate that the effects of exposure to low pH on the mobility of X:31 HA is not specific to CV-1 cells, we determined the lateral mobility of the viral glycoprotein in

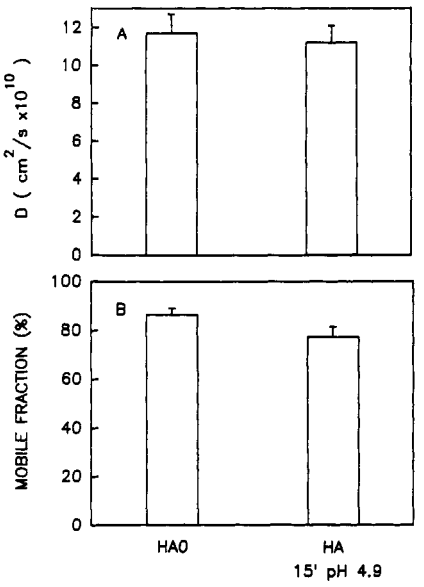

FIGURE 3: Effect of exposure to pH 4.9 on the lateral mobility of A/Japan HA on the surface of CV-1 cells. The experiments were carried out as in Figure 2, except that CV-1 cells expressing A/Japan HA were employed. Each bar is the mean ± SE of 30–40 measurements. (A) D values; (B) R_f values.

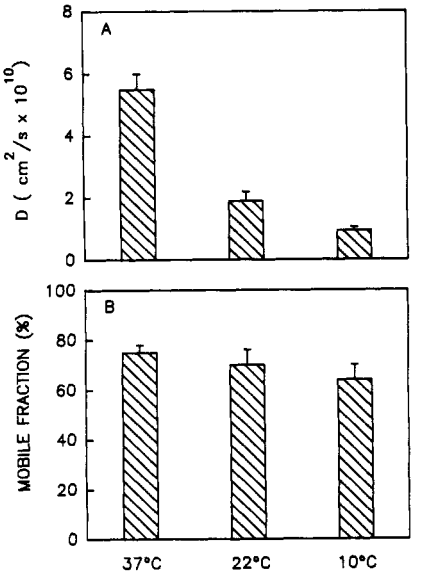

FIGURE 4: Lateral diffusion of HA0 (X:31 strain) on the surface of HA300a cells. HA300a cells, which permamently express X:31 HA0, were labeled with TMR-Fab′ and taken for FPR measurements performed at 37, 22, or 10 °C, as described under Materials and Methods. Each bar is the mean ± SE of 30–43 measurements. (A) D values; (B) R_f values.

the plasma membrane of a stably transfected CHO cell line (HA300a). The lateral diffusion parameters of X:31 HA0 at 37, 22, and 10 °C (Figure 4) showed the same temperature dependence observed in the transiently expressing CV-1 cells and at all temperatures were only somewhat lower than the equivalent values found on CV-1 cells (Figure 1).

The effect of incubation at pH 4.9 on the lateral mobility of trypsin-cleaved X:31 HA on the surface of HA300a cells is depicted in Figure 5. In these experiments, the cells were plated on cover slips precoated with fibronectin (30-min incubation with 100 μg/mL fibronectin); in the absence of fibronectin, the CHO cells tended to round up upon exposure to the low pH buffer, resulting in cell motion that interfered with the FPR experiments. The precoating with fibronectin

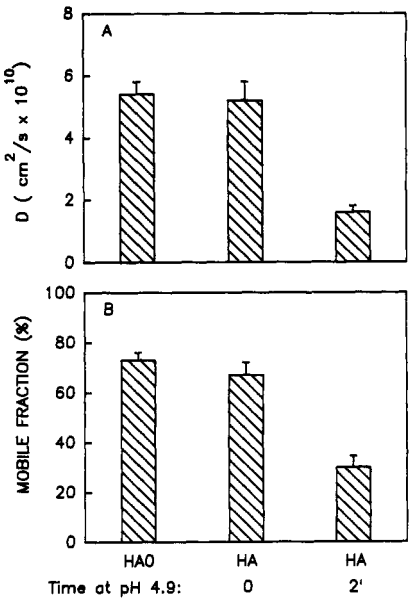

FIGURE 5: Effect of exposure to pH 4.9 on the lateral mobility of X:31 HA on the surface of HA300a cells. The experiments were performed as in Figure 2, except that the labeling with TMR-Fab′ was performed prior to trypsin cleavage and exposure to low pH (where applicable). The indicated times (minutes) represent the time of exposure to pH 4.9 after trypsin cleavage to HA. Incubation of HA0 (2 min) at pH 4.9 without trypsin cleavage did not affect its lateral mobility. Each bar is the mean ± SE of 30–43 measurements. (A) D values; (B) R_f values.

had no effect on the lateral mobility of the viral glycoprotein (compare the first bars in Figure 4 and 5). In order to make the time between the exposure to pH 4.9 and the FPR measurements as short as possible (so that the cells would still be stably attached), the labeling with TMR-Fab′ was performed prior to the trypsin cleavage of HA0. This change in labeling order had no effect on the lateral mobility parameters of uncleaved X:31 HA0 in HA300a cells (compare Figures 4 and 5); furthermore, control experiments employing CV-1 cells transiently expressing X:31 HA (which are stably attached at pH 4.9 also in the absence of fibronectin coating) gave similar results after a 2-min exposure to pH 4.9 whether the labeling was performed before trypsin cleavage or after the exposure to pH 4.9 (not shown). As can be seen in Figure 5, the effects of exposure to low pH on the lateral mobility of X:31 HA on HA300a cells are basically similar to those measured on CV-1 cells: cleavage of HA0 to HA had no significant effect on its lateral mobility, while a 2-min further incubation at pH 4.9 resulted in marked reduction of both D (over 3-fold) and R_f (from 73% to 30%).

DISCUSSION

Lateral motion of fusion-promoting viral envelope proteins was shown to be required for the induction of cell–cell fusion by Sendai virus and was proposed to be important for virally mediated membrane fusion in general (Aroeti & Henis, 1988; 1991; Henis et al., 1989). In the case of influenza virus, this suggestion is in accord with studies indicating that a concerted action of several HA trimers (which have to encounter and interact in the membrane) is involved in the fusogenic activity (Morris et al., 1989; Ellens et al., 1990; Stegmann et al., 1990, 1991), but it was not directly investigated. In the current study, we explored this question by studying the lateral mobility of HA after conversion to the fusogenic state by incubation in a mildly acidic pH, comparing HA proteins that differ in

their sensitivity to inactivation by prolonged exposure to this pH.

The results depicted in Figures 1–3 clearly demonstrate that HA at the fusion-active state (after trypsin cleavage of HA0 and briefly after the low-pH-mediated conformational change) shows high lateral mobility at the surface of CV-1 cells, as required if the lateral motion of the fusion-promoting proteins indeed plays a role in the fusion mechanism. This is evident in the case of A/Japan HA, which continues to show high D and R_f values even after long exposure (15 min) to pH 4.9 (Figure 3). However, it holds also for X:31 HA, since D and R_f of this protein expressed in CV-1 cells did not drop significantly after exposure for 1 min (at 37 °C) to this pH (Figure 2); by this time, the fusion reaction of CV-1 cells expressing X:31 HA with human erythrocytes is over 80% complete (Puri et al., 1990). Thus, even in the case of X:31 HA, the lateral diffusion is inhibited only *after* the fusion reaction has occurred (after exposure of 2 min or more to pH 4.9) (Figure 2).

The notion that the lateral motion of HA plays an essential role in the fusion process is strongly supported by the marked correlation between the inhibition of HA lateral mobility and the inactivation of its fusogenic activity. The fusion-promoting activity of A/Japan HA expressed in CV-1 cells was only slightly affected by a 15-min exposure to pH 4.9 (Puri et al., 1990; Ellens et al., 1990), in accord with the marginal effect of such treatment on its lateral mobility (Figure 3). On the other hand, incubation of cells (CV-1 or CHO) expressing X:31 HA at pH 4.9 for periods of 2 min or longer markedly reduced the lateral mobility parameters of the protein (Figure 2 and 5), in correlation with the inhibition of fusion of cells expressing this protein with human erythrocytes under similar conditions (Puri et al., 1990). Interestingly, the reduction in D of X:31 HA at the surface of CV-1 cells after 2 min at pH 4.9 correlates with a slower rate of fusion with human red blood cells, while the reduction in R_f that takes place after a longer preincubation occurs in parallel with abolishment of the fusogenic activity altogether (Figure 2; Puri et al., 1990). This pattern is expected if lateral diffusion of HA proteins is the rate-limiting step in the fusion process measured.

The need for lateral mobility of fusion proteins (in this case, HA) may reflect the participation of several HA trimers in the active fusion complex, thereby requiring their relative motion to enable formation of the final fusion complex. This notion is in accord with studies that demonstrated tight correlations between the lateral and rotational mobilities of Sendai virus glycoproteins and their fusogenic activity (Aroeti & Henis, 1988; Henis et al., 1989; Aroeti et al., 1990). It is also in agreement with kinetic studies which suggested that more than one HA trimer take a part in the fusion reaction (Morris et al., 1989; Ellens et al., 1990) and that the lag phase preceding fusion may represent the time needed for bound trimers to assemble into a functional fusion complex (Stegmann et al., 1990, 1991; Spruce et al., 1989). Concerted action of several HA trimers was also implied by patch-clamp studies, indicating pore formation (which requires the assembly of several membrane-spanning proteins) between the fusing membranes at the early stages of the fusion process (Spruce et al., 1989). An alternative possibility is that the fusogenic proteins have to reach a threshold of high local density at the region of contact with the target membrane, for which their lateral mobility would be needed; indeed, accumulation of viral envelope proteins at contact regions between fusing cells was detected during the fusion of human erythrocytes by Sendai virions (Aroeti & Henis, 1991). It should be noted that these two alternatives may be related, since an elevated local density could enhance the tendency of the fusion proteins to form higher complexes.

How might prolonged incubation at low pH induce inactivation of the fusion activity of X:31 HA? The sequence of the effects on X:31 HA mobility (Figure 2) provides a clue for the possible mechanism. After trypsin cleavage, incubation at low pH was proposed to induce at the first stage exposure of the fusion peptides and formation of a fusion-active conformation (White & Wilson, 1987; Puri et al., 1990; Kemble et al., 1992). The finding that after 1 min at pH 4.9 (sufficient for 80% completion of CV1-erythrocyte fusion and thus for formation of the fusion-active state) D and R_f of X:31 HA are not altered significantly (Figure 2) suggests that the reduction in HA mobility (and the concomitant loss of fusion activity) is due to another process that occurs after longer incubation times at low pH. This process, which may require the second stage of the conformational change during which the heads of the trimer were proposed to dissociate substantially (White & Wilson, 1987; Stegmann et al., 1989; Puri et al., 1990; Kemble et al., 1992), most likely involves the formation of large aggregates containing HA (larger and distinct from the fusion complexes mentioned above). This suggestion is based on the first effect detected being a reduction in D, which is followed only later by a drop in the mobile fraction. Midsize aggregates are still expected to diffuse, in view of the weak dependence (logarithmic) of the lateral diffusion of transmembrane proteins on molecular size (Saffman & Delbruck, 1975). However, increased size may result in enhanced steric hindrance due to interactions with other membrane proteins or membrane-associated cellular structures, leading to slower diffusion rates. As the aggregates grow larger, they will finally become essentially immobile, as was observed following antibody-mediated cross-linking and patching of specific membrane receptors (Henis et al., 1990). The notion of irreversible aggregation upon prolonged exposure of X:31 HA to low pH is supported by the disordered HA spike morphology induced following incubation at low pH in X:31 virions but not in the A/Japan strain (Doms et al., 1985; Puri et al., 1990); negative-stain electron microscopy indicates that HA molecules in the viral particles do not dissociate upon acidification and may form larger aggregates in the plane of the viral membrane (Doms & Helenius, 1986). It is also in accord with rotational mobility studies on the spike proteins in X:47 virions, which suggested clustering at low pH (Junankar & Cherry, 1986).

The sequence of events upon incubation of cells expressing X:31 HA at low pH suggests that the first step is exposure of the hydrophobic fusion peptide and formation of the fusion-active state; in the absence of a target membrane, immobilization and inactivation follow. Thus, interactions among the exposed fusion peptides of neighboring HA trimers could be involved in further aggregation and inactivation of the presumed fusion complexes. Obviously, such interactions would reduce the amount of fusion peptides available for interaction with the target membrane, a phenomenon which is expected to reduce the fusion activity. Immobilization of HA and reduction in the amount or density of the available fusion peptides may therefore be interrelated and could act together to inhibit fusion.

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