Delay of Influenza Hemagglutinin Refolding into a Fusion-Competent Conformation by Receptor Binding: A Hypothesis

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ABSTRACT Two subunits of influenza hemagglutinin (HA), HA1 and HA2, represent one of the best-characterized membrane fusion machines. While a low pH conformation of HA2 mediates the actual fusion, HA1 establishes a specific connection between the viral and cell membranes via binding to the sialic acid-containing receptors. Here we propose that HA1 may also be involved in modulating the kinetics of HA refolding. We hypothesized that binding of the HA1 subunit to its receptor restricts the major refolding of the low pH-activated HA to a fusion-competent conformation and, in the absence of fusion, to an HA-inactivated state. Dissociation of the HA1-receptor connection was considered to be a slow kinetic step. To verify this hypothesis, we first analyzed a simple kinetic scheme accounting for the stages of dissociation of the HA1/receptor bonds, inactivation and fusion, and formulated experimentally testable predictions. Second, we verified these predictions by measuring the extent of fusion between HA-expressing cells and red blood cells. Three experimental approaches based on 1) the temporal inhibition of fusion by lysophosphatidylcholine, 2) rapid dissociation of the HA1-receptor connections by neuraminidase treatment, and 3) substitution of membrane-anchored receptors by a water-soluble sialyllactose all provided support for the proposed role of the release of HA1-receptor connections. Possible biological implications of this stage in HA refolding and membrane fusion are being discussed.

INTRODUCTION

Influenza virus enters the host cell by receptor-mediated endocytosis followed by fusion of the viral envelope with the endosomal membrane. This fusion reaction, a paradigm of ubiquitous biological membrane fusion, is mediated by the homotrimeric envelope glycoprotein hemagglutinin (HA) (White, 1996). In the fusion-competent form each of the HA monomers contains two disulfide-bonded polypeptide subunits, HA1 and HA2. Acidification of the endosome triggers a major conformational change in HA, which is associated with membrane fusion. This refolding involves release of the amphiphilic amino-terminal peptide of HA2 ("fusion peptide") and its insertion into membranes (Gaudin et al., 1995). Low pH application also causes extension and reorientation of the central coiled coil core of HA2 (Bullough et al., 1994; Carr and Kim, 1993; Chen et al., 1999; Weissenhorn et al., 1997) and relocation of the globular HA1 subunits from their initial position at the top of the central fibrous stem of HA, which is composed largely of HA2 (Godley et al., 1992; Kemble et al., 1992; White and Wilson, 1987; Wiley and Skehel, 1987).

According to a common view, the two subunits of HA perform distinct tasks in the fusion process. The HA2 subunit is required and sufficient for fusion *per se* (Wiley and Skehel, 1987), whereas HA1 plays an accessory role providing virus binding to the host cell via sialic acid-containing receptors (Wiley and Skehel, 1987). In the present study we show that involvement of HA1 subunit in the fusion reaction goes beyond the binding mechanism.

This work was initially motivated by studies on HA inactivation with respect to fusion. Low pH application in the absence of an appropriate membrane contact (Puri et al., 1990) or in the presence of lysophosphatidylcholine (LPC) (Chernomordik et al., 1997) leads to the loss of HA's ability to mediate fusion. Moreover, such low pH pretreatment results in a profound inhibition of fusion observed after the application of an additional low pH pulse in conditions already favorable for fusion (Gutman et al., 1993; Junankar and Cherry, 1986; Puri et al., 1990; Ramalho-Santos et al., 1993; White et al., 1982). The presence of the target membrane during low pH application dramatically slows down this inactivation (Alford et al., 1994; Chernomordik et al., 1997, 1998; Ramalho-Santos et al., 1993; Schoch et al., 1992). We hypothesized that this inhibition of HA inactivation in the presence of the target membrane reflects the effect of the HA1-receptor interaction.

The current knowledge of the involvement of HA1 and its receptor in fusion remains fragmentary. It has been demonstrated that specific interaction of HA1 with sialic acid is not a prerequisite for fusion. HA-expressing membranes (HA membranes) readily fuse with bound membranes lacking receptors (Schoen et al., 1996; White et al., 1982), although the kinetics of fusion can differ from that in the presence of receptors (Alford et al., 1994; de Lima et al., 1995; Niles and Cohen, 1993; Stegmann et al., 1995). Furthermore, the HA1-sialic acid connection is not required

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for fusion completion at low pH-independent stages of fusion subsequent to low pH triggering (Chernomordik et al., 1997, 1998; Schoch et al., 1992).

While HA refolding leading to fusion could occur in the absence of HA1-sialic acid binding, this binding and the HA1 subunit itself could modulate the refolding. HA1 was hypothesized to lock HA2 in a metastable conformation before low pH activation (Carr et al., 1997; Carr and Kim, 1993; Chen et al., 1995; Kim et al., 1996). Tilting of the HA2 subunit at low pH, which is apparently involved in HA-mediated fusion (Tatulian et al., 1995), was recently found to require the presence of the HA1 subunit (Gray and Tamm, 1998). The HA1-sialic acid connection can promote refolding of HA toward its fusion-competent conformation (de Lima et al., 1995; Stegmann et al., 1995). On the other hand, based on the decrease in the fusion rates observed for higher surface density of receptors, Alford et al. (1994) hypothesized that HA molecules bound to sialic acids are incapable of mediating fusion (Alford et al., 1994). Interestingly, HA molecules bound to surrogate receptors (anti-HA antibodies) can mediate fusion (Millar et al., 1999).

In the present work we studied the effect of HA1-receptor binding on the fusion reaction downstream of establishing membrane contact. We hypothesized that the HA1-sialic acid connections slow down refolding of the low pH-activated HA. To test this hypothesis we first analyzed theoretically the kinetics of HA refolding and the related fusion reaction by considering dissociation of the HA1-receptor connections as a slow stage of the protein rearrangements leading to fusion. Second, the predictions of this hypothesis were verified experimentally for HA inactivation and fusion of HA-expressing cells (HA cells) to human red blood cells (RBCs).

THE MODEL

Amount of HA molecules bound to their receptors before low pH application

First, let us demonstrate that after establishing contact between an HA membrane and a target membrane containing the sialic acid receptors, most of the HA molecules are bound to receptors.

This binding can be described in the familiar terms of a dynamic equilibrium between association and dissociation of the HA-receptor complexes. Note that in our system two kinds of molecules undergoing binding are located in the opposing membranes, where they can move by lateral diffusion. Hence the HA-receptor binding can be seen as a chemical reaction in a two-dimensional solution of the reacting substances, where the lipid molecules constituting the membranes play the role of a solvent. Thus it is convenient to describe the system by referring to the surface concentrations of the reacting substances, denoted by $C_{HA\text{-}R}$, C_{HA}, and C_R, for the HA-receptor complexes, the unbound HA molecules, and the unbound receptors, respectively. The equation expressing the dynamic equilibrium is

$$C_{HA}C_R = KC_{HA\text{-}R} \tag{1}$$

where K is the dissociation constant of the HA-receptor complexes. Provided that the total surface concentrations of the HA and receptor molecules are $C_{HA,t}$ and $C_{R,t}$, respectively, Eq. 1 determines the concentration of the complexes:

$$(C_{HA,t} - C_{HA\text{-}R})(C_{R,t} - C_{HA\text{-}R}) = KC_{HA\text{-}R} \tag{2}$$

The degree of binding is determined by the ratio between the total surface concentrations $C_{HA,t}$ and $C_{R,t}$ of the HA and receptor molecules, and the dissociation constant K. The total surface concentration of the HA molecules in an HA cell, $C_{HA,t}$, estimated for HAb2 cells, for instance, is $\sim$2500 μm^{-2} (Danieli et al., 1996). This is $\sim$10 times lower than the HA concentration in the envelope of the influenza virus. The number of sialic acid-containing receptors in one RBC is $\sim 10^6$, which corresponds to a surface density of $C_{R,t} \approx 8000\ \mu m^{-2}$. Liposomes containing 1 mol% of gangliosides can have an even higher surface density of receptors. To estimate the dissociation constant K, we assumed that the energy of interaction between the HA1 subunit of membrane-anchored HA and the membrane receptor is equal to the energy of interaction between the sialic acid-containing molecules and the soluble extracellular domains of HA in the bulk aqueous solution (Sauter et al., 1992b). The dissociation constant $K_d = 3$ mM of the sialic acid-HA ectodomain complexes measured for sialyllactose by Sauter et al. (1992b) can be related to the energy of formation of one complex, ϵ, by $K_d = W\exp(\epsilon/kT)$, where $W = 55$ M is the concentration of water molecules. The dissociation constant K determining the HA-receptor binding in the membrane system (Eqs. 1 and 2) can be represented as $K = C_l\exp(\epsilon/kT)$, where $C_l = 1.7 \times 10^6\ \mu m^{-2}$ is the surface concentration of the lipid molecules. Hence K is given by $K = K_dC_l/W$ and can be estimated as $K = 100\ \mu m^{-2}$. This value is more than 10 times lower than the total surface concentrations $C_{HA,t}$ and $C_{R,t}$ of the HA and receptor molecules. Solving Eq. 2 with the proposed values for K, $C_{HA,t}$, and $C_{R,t}$ gives $\sim$98% of all HA molecules in the contact region as being involved in HA-receptor complexes. Even if because of some steric hindrance only 1 of 10 receptors is accessible for an HA binding site, $\sim$60% of HA molecules in the contact region are expected to be receptor-bound.

Sequence of events downstream of low pH application

The above estimate shows that the majority of HA molecules in the contact region are bound to the receptors before low pH activation. We suggest that this binding constrains the low pH-triggered refolding of the HA2 subunits neces-

sary for fusion. Consequently, to undergo the refolding HA molecules have to first break the HA1-receptor connections.

Low pH-triggered conformational change of a receptor-bound HA molecule is hypothesized to proceed through the following states:

In the first state, the "*activated-locked*" conformation, the initial conformational change of the HA2 subunit (i.e., exposure of the fusion peptide), has already taken place. Nonetheless, the subsequent major refolding is still restricted by HA1-receptor interaction.

Next, the tendency to undergo further refolding produces an effective stress inside each HA molecule. This stress results in the breakdown of the HA1-receptor bond and consequent transition of the HA molecule into the second, "*activated-unlocked*" conformation. In this state HA mediates membrane fusion with a certain probability.

Final structural changes in the HA molecule proceed from the activated-unlocked state into an "*inactivated*" state that is incapable of inducing membrane fusion. This inactivation may correspond to the completion of the coiled coil extension and reorientation of parts of HA2 (Bullough et al., 1994) and related relaxation of the membrane stresses and smoothing out of the fusion dimple (Kozlov and Chernomordik, 1998). Alternatively, it may reflect aggregation of fusion peptides of adjacent low pH-activated HA molecules and the irreversible insertion of fusion peptides into HA membrane. For the present study the specific nature of the inactivated configuration is not crucial.

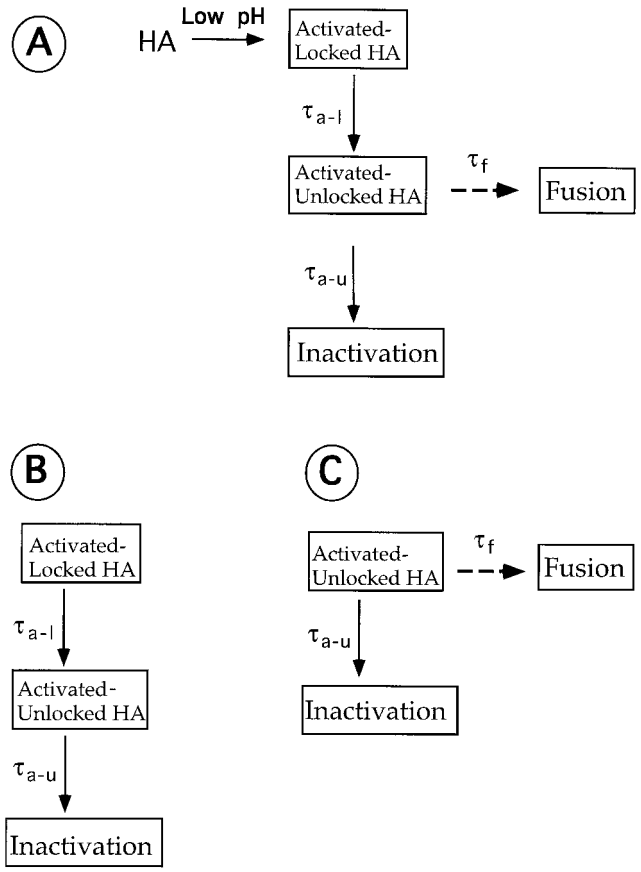

FIGURE 1 Hypothetical kinetic model for HA refolding after low pH activation leading to fusion or inactivation. (*A*) The normal pathway. (*B*) The pathway in the presence of the fusion inhibitor. (*C*) The pathway after dissociation of the HA1-receptor connection by neuraminidase. For more details see the text.

Kinetic scheme and equations

A sophisticated kinetic model of HA multimerization and activation was presented by Bentz (1999). For the qualitative purpose of the present work we analyzed a simplified kinetic scheme (illustrated in Fig. 1 *A*) that corresponds to the sequence of the above-discussed states. Three boxes depict 1) the *activated-locked*, 2) *activated-unlocked*, and 3) *inactivated* configurations of the HA molecules. The arrows between these configurations indicate the pathway of the HA evolution. The arrow pointing to the side from the *activated state* box represents fusion between HA-containing and target membranes induced by the HA in this conformation. Hence, although shown for illustration in the same scheme, this arrow indicates the transition of the whole cell from a nonfused to a fused state. All other arrows in Fig. 1 describe the evolution of the protein molecules, where all transitions are assumed to be irreversible.

Let us now consider a situation where HA membrane is prebound to the target membrane. At time $t = 0$ the low pH activation results in the transition of the number N^0_{a-l} of the HA molecules into the activated-locked state followed by transition into the activated-unlocked HA state and then the inactivated state. In parallel, the membranes can fuse at any moment t. The probability of this event depends on the number of the HA molecules in the activated-unlocked state. Below we describe all stages of the evolution of this system with the following simple equations.

HA transition from the activated-locked state to the activated-unlocked one is characterized by a time constant τ_{a-l} and is described by a kinetic equation determining the decrease in the number N_{a-l} of the HA molecules in the activated-locked state with time t,

$$\frac{dN_{a-l}}{dt} = -\frac{N_{a-l}}{\tau_{a-l}} \tag{3}$$

The same assumptions were made with respect to the HA transition from the activated-unlocked state to the inactivated one; the corresponding time constant will be denoted by τ_{a-u}. The number N_{a-u} of the protein molecules in the activated-unlocked state changes with time according to the equation

$$\frac{dN_{a-u}}{dt} = -\frac{N_{a-u}}{\tau_{a-u}} + \frac{N_{a-l}}{\tau_{a-l}} \tag{4}$$

where the first term on the right side accounts for the transition to the inactivated state, whereas the second one corresponds to the number of HA molecules leaving the activated-locked state, N_{a-u}.

To describe the kinetics of the fusion process we have to determine the probability $Y(t)$ that the HA cell fuses with the target cell in the period of time from $t = 0$ to t. We assumed that the fusogenic action of the HA molecules in the activated-unlocked state is determined by their number $N_{a-u}(t)$ and cooperativity between them (Blumenthal et al., 1996; Danieli et al., 1996; Ellens et al., 1990). The probability $p(t)dt$ that one cell fuses with the target cell within a small time interval dt taken at an arbitrary moment t is determined by the equation

$$p(t)dt = (N_{a-u})^m \frac{dt}{\tau_f} \quad (5)$$

where m is the number determining the cooperativity in the action of HA molecules, and τ_f is the time constant accounting for all related processes, such as the time of formation of a multiprotein fusion machine (if needed) or the time of a possible local rupture of a membrane monolayer required for fusion (Leikin et al., 1987). The probability $P(t)dt$ that a cell will fuse within a small interval dt at a certain moment t (i.e., it does not fuse in the period from 0 to t and then fuses in the interval from t to $t + dt$) is determined, accounting for Eq. 5, by

$$P(t)dt = \left[1 - \int_0^t P(t)dt\right](N_{a-u})^m \frac{dt}{\tau_f} \quad (6)$$

Finally, the probability $Y(t)$ that the cell fuses between $t = 0$ and t can be expressed by $Y(t) = \int_0^t P(t)dt$. Taking into account Eq. 6, we obtain for this probability the following equation:

$$\frac{dY(t)}{dt} = [1 - Y(t)]\frac{N_{a-u}^m}{\tau_f} \quad (7)$$

Solving Eqs. 3, 4, and 7, we can describe the evolution of the system, including time-dependent changes in the number of the HA molecules in all three states represented in Fig. 1 *A*, and find the probability of membrane fusion $Y(t)$ as a function of the time t after the low pH application.

APPROACHES TO EXPERIMENTAL VERIFICATION OF THE MODEL

Fluorescence microscopy allows one to simultaneously observe a large number of HA cells bound to the target membranes and to determine the percentage of cells that underwent fusion by the time t after the low pH application at $t = 0$. This value is referred to as the extent of fusion. The extent of fusion corresponding to a long enough period of time after which no additional fusion events occur is referred to as the final extent of fusion.

Provided that all observed cells are similar, the extent of fusion, normalized to 1 rather than to 100%, is equal to the probability that one cell fuses, $Y(t)$. The final extent, Y_{fin}, corresponds to the probability of fusion during an infinite time of observation $Y_{fin} = Y(t = \infty)$. Hence our strategy for the experimental verification of the model relates to the final extent, Y_{fin}, which is readily measurable and can be determined theoretically. We suggest three approaches for experimental verification of the model based on three experimental ways of influencing the evolution of the system.

Time course of inactivation in the presence of the target membrane

Lysophosphatidylcholine (LPC) added exogenously to HA cells with bound target membranes (e.g., RBCs) reversibly blocks fusion at a stage that follows low pH activation of HA but precedes the actual fusion event (Chernomordik et al., 1997). Upon removal of LPC the fusion is allowed to proceed. In terms of the kinetic scheme (Fig. 1), LPC blocks the pathway leading to fusion, but not the conformational change of HA. Thus, in the presence of LPC, the HA molecules convert from the activated-locked to the activated-unlocked and, finally, to the inactivated state (Fig. 1 *B*). The kinetic scheme returns to the initial form (Fig. 1 *A*) upon LPC removal. We will use this property of LPC to modify the time course of the system in the controlled way.

To test the model, the HA molecules will be activated by a pulse of low pH in the presence of a fusion-inhibiting concentration of LPC. Then LPC will be removed at a different time t^* after the end of a low pH pulse and the resulting final extent of fusion, $Y_{fin}(t^*)$ will be measured. Our model gives a rather simple prediction for the character of the function $Y_{fin}(t^*)$. During the time t^*, when fusion is inhibited by LPC, some of the initially activated-unlocked HA molecules undergo transition to the inactivated state, leaving fewer HA molecules in the fusion-competent state. Hence the probability $Y_{fin}(t^*)$ that the cells fuse after LPC removal should decrease with increasing t^*.

The probability of fusion $Y_{fin}(t^*)$ predicted by our model can be determined by solving Eqs. 3, 4, and 7, taking into account that fusion is arrested during the period of time t^*. The details of these calculations are presented in the Appendix. The main assumption allowing an analytical expression for $Y_{fin}(t^*)$ is that the time constant τ_{a-l} of the HA transition from the activated-locked state to the activated-unlocked state exceeds the time constant τ_{a-u} of the protein inactivation, $\tau_{a-l} > \tau_{a-u}$. This assumption is supported by observations that the presence of the target membrane slows down the kinetics of HA refolding. Hence the characteristic time τ_{a-l} of the dissociation of the HA proteins from the target membrane has to be the longest in the hierarchy of

time constants. The resulting expression for the final extent of fusion is

$$Y_{\text{fin}}(t^*) = 1 - \exp\left[-\frac{\tau_{\text{a-l}}}{m \cdot \tau_{\text{f}}} \cdot \left(\frac{\tau_{\text{a-u}}}{\tau_{\text{a-l}} - \tau_{\text{a-u}}}\right)^{\text{m}} (N^0_{\text{a-l}})^{\text{m}} \cdot \exp\left(-\frac{m \cdot t^*}{\tau_{\text{a-l}}}\right)\right] \quad (8)$$

The predicted total extent of fusion decreases rather sharply with increasing time t^* (i.e., duration of LPC treatment), where the dependence (Eq. 8) is double-exponential. The major parameter determining the sharpness of this decrease is the time constant $\tau_{\text{a-l}}$. The experimental function $Y_{\text{fin}}(t^*)$ will be compared with that predicted theoretically (Eq. 8).

Unlocking of the activated-locked state

Neuraminidase cleaves sialic acids at the surface of the target membrane and disrupts the HA1-receptor connection (Drzeniek, 1972). According to our model, such treatment should transfer low pH-treated HA molecules directly into the activated-unlocked state. In the kinetic scheme (Fig. 1), the neuraminidase treatment is predicted to abolish the first box corresponding to the activated-locked state and the related steps in the system evolution (Fig. 1 *C*).

As in the previous experimental approach, we will use LPC to block the fusion of HA cells with bound RBCs. Immediately after the end of the low-pH application, still in the presence of LPC, cells will be treated with neuraminidase, followed by LPC removal at a different time t^* after the end of the low pH pulse. Then we will measure the resulting final extent of fusion, $Y_{\text{fin}}(t^*)$.

The theoretical dependence of the final extent of fusion (see Appendix) can be expressed as

$$Y_{\text{fin}}(t^*) = 1 - \exp\left[-\frac{\tau_{\text{a-u}}}{m \cdot \tau_{\text{f}}} \cdot (N^0_{\text{a-l}})^{\text{m}} \cdot \exp\left(-\frac{m \cdot t^*}{\tau_{\text{a-u}}}\right)\right] \quad (9)$$

As in the previous case, the longer t^* is, the lower is the final extent of fusion. However, neuraminidase treatment should alter the time course of this fusion decline (Eq. 9), as the kinetic stage characterized by the longest time constant $\tau_{\text{a-l}}$ is now excluded. As a result, the steepness of the function $Y_{\text{fin}}(t^*)$ is determined by the shorter characteristic time $\tau_{\text{a-u}}$. In other words, after neuraminidase treatment the total extent of fusion is expected to decrease as a function of time of the fusion arrest t^* faster than in the control experiment. The results of this experiment will be compared with the theoretical prediction (Eq. 9) to verify the model.

Substituting the target membrane with soluble receptors

As previously mentioned, some strains of HA (e.g., X:31 HA) rapidly inactivate at low pH in the absence of the target membrane. Our model postulates that HA1 binding to its receptor slows down the major refolding of the HA molecule. As a third approach in the model verification, we will test whether water-soluble sialosides inhibit HA inactivation in the absence of the target membrane.

EXPERIMENTAL METHODS

Preparing the cells for fusion experiments

HA300a cells, CHO-K1 cells expressing the X:31 strain of influenza virus HA, were grown as described by Kemble et al. (1993). Human RBCs, freshly isolated from whole blood, were labeled with fluorescent lipid, PKH26 (Sigma, St. Louis, MO) as done by Chernomordik et al. (1997).

HA cells were treated with 5 μg/ml trypsin (Fluka, Buchs, Switzerland) and 0.5 unit/ml neuraminidase type V *Clostridium perfringens* (Sigma) for 10 min at 37°C to cleave HA0 into its fusion-competent HA1-S-S-HA2 form and to improve RBC binding, respectively. Then HA cells with 0–2 bound RBC per cell were washed three times with phosphate-buffered saline (PBS) to remove unbound RBC and then used for fusion experiments. RBC binding to cells (i.e., the average number of RBCs bound to each HA cell) was assayed for a sample of 200 cells in several different areas of the dish. In some experiments RBC contacts with HA cells were stabilized by cross-linking with paraformaldehyde (4% in PBS w.o. Ca, Mg) for 20 min at room temperature.

Fusion was triggered by cell incubation with PBS titrated by citrate to acidic pH. After low pH treatment acidic solution was replaced by PBS. The final fusion extent was assayed by fluorescence microscopy more than 20 min after low pH application or removal of LPC as the ratio of dye-redistributed bound RBCs to the total number of bound RBCs. Longer incubations (up to 2 h) did not increase the extent of fusion.

In the experiments where RBC/HA cell complexes were treated by neuraminidase there was some loss of initially bound but not fused RBCs before the assay for fusion. This selective loss of non-fused RBCs, apparently caused by the dissociation of HA1-receptor binding, resulted in an overestimation of the fusion extent, F_{N}. To account for it, in the experiments presented in Fig. 2, *A* and *B*, we counted the percentage, P_{N}, of dye-redistributed HA cells among more than 1000 HA cells. Using the found P_{N} along with the fusion extent F_0 and the percentage, P_0, of labeled HA cells in the absence of neuraminidase, we calculated the fusion extent F_{N} according to the relationship $F_{\text{N}} = F_0 * P_{\text{N}}/P_0$. This expression is based on the fact that the initial binding and the numbers of RBCs and HA cells were equal in the experiments with and without neuraminidase.

Each set of experiments for each graph presented here was repeated on at least three occasions with similar results. Data were averaged from the same set of experiments.

Application of exogenous lipids and enzymatic treatments

As described by Chernomordik et al. (1997), a stock solution of lauroyl LPC (Avanti Polar Lipids, Birmingham, AL) was freshly prepared as a 0.5% (w/w) aqueous dispersion. PBS titrated by citrate to acidic pH and used to trigger cell fusion was also supplemented with LPC. The same concentration of LPC was added to the neutral pH medium (PBS, pH 7.4) used to end the low pH application.

In some experiments, HA cells with bound RBCs were treated with neuraminidase (0.5–1 unit/ml of PBS, 5 min at room temperature) or with thermolysin (Sigma, 0.1 mg/ml of PBS, 10 min at room temperature) immediately after the end of the low pH application. Washing cells twice with complete medium terminated enzymatic reactions.

In the typical experiment a pulse of low pH was applied in the presence of a fusion-inhibiting concentration of LPC. Then, still in the presence of

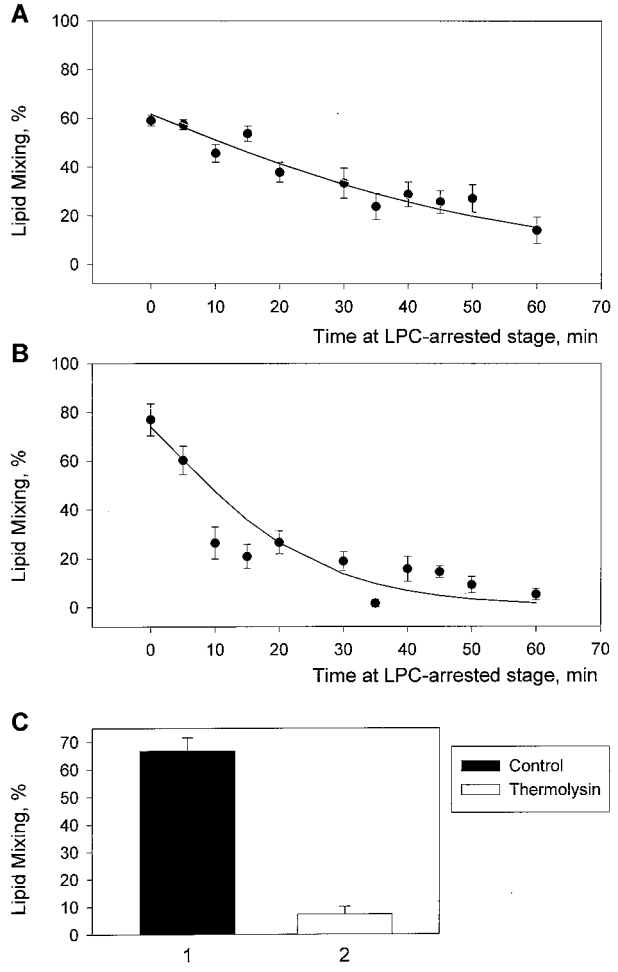

FIGURE 2 Neuraminidase applied after HA activation modulates the time course of the fusion inactivation at the LPC-arrested stage. (*A* and *B*) Neuraminidase effect on the decrease in the fusion extent observed upon LPC removal. HA cells with bound RBCs were treated by a 5-min pulse of pH 4.9 at 22°C in the presence of 230 μM lauroyl-LPC. Low pH medium was replaced with LPC-containing PBS (pH 7.4) supplemented (*B*) or not supplemented (*A*) with neuraminidase (0.5 unit/ml). Five minutes later the medium was again replaced by LPC-containing, neuraminidase-free PBS. After different time intervals, LPC was removed by washing cells with LPC-free PBS. Lipid mixing was measured as PKH26 redistribution from RBC to HA cells (see Experimental Methods) 20 min after LPC removal. Each point is a mean $\pm$ SEM over 9–22 independent experiments. Solid lines represent the theoretical curves determined by fitting the experimental data with Eqs. 8 and 9 with parameters and standard error of the fit as follows: $A1 = 34.17 \pm 4.27$, $B1 = 0.96 \pm 0.069$ (*A*) and $A2 = 13.5 \pm 2.85$, $B2 = 1.35 \pm 0.22$ (*B*). The norms of the residuals (square root of the sum of squares of the residuals) for the presented fits were 13.8 and 28.9 for *A* and *B*, respectively. The meaning of the parameters is discussed in the text. (*C*) Fusion inhibition by thermolysin applied at the onset of the LPC-arrested stage. HA cells with bound RBCs were treated with a 3-min pulse of pH 4.9 at 22°C in the presence of 230 μM lauroyl-LPC. Low pH medium was replaced with LPC-containing PBS (pH 7.4) supplemented (*bar 2*) or not supplemented (*bar 1*) with thermolysin (0.05 mg/ml). After 10 min of incubation, the medium was again replaced by LPC-free, thermolysin-free PBS. Fusion extent was assayed as the ratio of dye-redistributed bound RBCs to the total number of bound RBCs. The extent of fusion (13%) observed in the experiment with no thermolysin treatment where fusion was assayed, still in the presence of LPC, was subtracted from both bars. Each bar is a mean $\pm$ SEM, $n = 3$.

LPC, but already at neutral pH, cells were treated or not with neuraminidase, and then, at different time points after the end of a low pH pulse, LPC was washed out and fusion was assayed.

Functional assay for HA inactivation in the absence of the target membrane

HA cells, pretreated with trypsin and neuraminidase as described above, were incubated at low pH in the absence of the target membrane (referred to as "low pH pretreatment"). Then low pH medium was replaced with pH 7.4 PBS, RBCs were added, and, 15 min later, after unbound RBCs were washed out, a second pulse of low pH was applied to trigger fusion (referred to as the "fusion-testing pulse"). The greater the number of HA molecules that were inactivated during the low pH pretreatment, the lower was the final fusion extent observed after the fusion-testing pulse. In some experiments low pH pretreatment was preceded by a 5-min incubation of the HA cells with sialyllactose (SL, α-neu5ac-[2-3]- and -[2-6]-β-D-gal-[1-4]-D-Glc from human milk; Sigma). We also studied the effects of two other water-soluble glycosides, one of which, Neu5Ac alpha-benzyl glycoside (AG) (GlycoTech, Rockville, MD) binds to HA1, and the other of which, Neu5Ac beta-methyl glycoside, (BG) (GlycoTech), in contrast to alpha-anomers SL and AG, is a beta-anomer and does not bind to HA1 (Matrosovich et al., 1993; Pritchett et al., 1987; Sauter et al., 1992b). Glycosides were dissolved in PBS and the pH was adjusted as needed.

Measuring HA activation by SDS-PAGE and quantitative Western blot analysis

To measure the percentage of low pH-activated HA we used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. HA300a cells, pretreated with trypsin as described above, were acidified by pH 4.9 medium for 5 min at room temperature. Next 20 mM dithiothreitol (DTT) was added to the cells to reduce disulfide bonds between HA1 and HA2 subunits. This bond becomes accessible to DTT only in the low pH-activated HA (Graves et al., 1983). Reducing this bond releases water-soluble HA1 from membrane-anchored HA2. Cells were lysed in 500 μl of nonreducing SDS-PAGE lysis buffer (68.5 mM Tris-HCl (pH 7.5), 2% SDS, 50 mM iodoacetamide, 5 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 100 μM leupeptin, 100 μM 3,4-dichloroisocoumarin, 10% glycerol, 0.01% bromphenol blue). Immediately after lysis, samples were transferred to ice, heated to 100°C for 5 min, centrifuged at 30,000 rpm in a TLA 100.1 rotor for 30 min, and stored at −25°C until use. Prepared lysate was analyzed by 4–12% gradient SDS-PAGE at 10 or 15 μg of total cellular protein per gel lane. Gels were run at a constant voltage (i.e., 120 V) until the bromphenol blue front reached the end of the gel. After proteins were blotted to Immobilon-P filters, blots were blocked with 6% bovine serum albumin (w/v) in T-PBS (PBS supplemented with 0.05% (v/v) Tween-20). Blots were incubated in rabbit polyclonal serum (1:500 or 1:2000), followed by goat anti-rabbit (1:14,000) IgG conjugated with alkaline phosphatase. Dried blots were photographed and scanned on a Molecular Dynamics scanner, using enhanced chemifluorescence mode. Analysis of SDS-PAGE images and quantification of individual bands were carried out with an ImageQuant software package (Molecular Dynamics). HA activation was presented as a ratio of the low pH HA0 band to the pH 7.4 band.

RESULTS

Time course of inactivation in the presence of target membrane

Under the conditions of our experiments, RBC binding to HA cells is dependent on HA1 binding to sialic acids on the

RBC surface as demonstrated by the following findings: 1) binding is abolished for neuraminidase-treated RBCs; 2) neuraminidase application to HA cells with bound RBCs releases RBCs (see also Chernomordik et al., 1997), and 3) binding is inhibited by either 20 mM SL or 5 mM AG (data not shown).

In agreement with earlier publications (Chernomordik et al., 1997; Melikyan et al., 1995b; Sarkar et al., 1989), low pH application to HA cells with bound RBCs resulted in a fast redistribution of the membrane dye PKH26 from RBCs to HA cells. This lipid mixing (referred to below as fusion) was observed only for HA cells, where the initial HA0 form of HA was cleaved by trypsin into the HA1-HA2 form (not shown).

To investigate the time course of HA inactivation in the presence of the target membrane, one needs to block fusion. In these experiments fusion was triggered by low pH application in the presence of the reversible fusion inhibitor LPC. Washing out LPC allowed fusion to ensue. The longer the time interval between the low pH pulse and LPC wash out, the lower the extent of fusion observed upon removal of the fusion inhibitor (Fig. 2 *A*). This fusion inactivation at the LPC-arrested stage, i.e., the transition from fusion-competent to fusion-incompetent state of HA molecules, was rather slow. The fusion extent observed upon LPC removal decreased from ~60% to ~20% within 45 min after the low pH pulse.

Exposure of the fusion peptide of HA2, an early indication of a low pH-induced refolding of HA (White, 1996; White and Wilson, 1987), apparently takes place at the very onset of the LPC-arrested stage. A low pH pulse applied in the presence of LPC was immediately followed by thermolysin, the enzyme that cleaves the fusion peptide of HA in the low pH conformation. Fusion inhibition observed upon subsequent removal of LPC (Fig. 2 *C*) indicated that fusion peptide exposure by low pH-activated HA molecules precedes slow inactivation of HA at the LPC-arrested stage. Thus, if slow inactivation of fusion at the LPC-arrested stage reflects unlocking of the activated-locked state as suggested by our model, this state follows fusion peptide exposure to thermolysin-accessible conformation.

Dissociation of HA1-receptor binding by neuraminidase as an attempt to unlock the activated-locked state of HA

Neuraminidase treatment of the cells at the LPC-arrested stage caused a notable change in the pattern of the inactivation (Fig. 2 *B*). In these experiments HA cells were incubated with neuraminidase for 5 min immediately after the end of low pH pulse in the LPC-containing medium. As HA cells were already pretreated with neuraminidase before the addition of RBCs (see Experimental Methods), the enzyme application at the LPC-arrested stage is mainly intended to remove the sialic acids from the surface of bound RBCs. Note that RBCs remain bound to HA cells even after neuraminidase treatment because of an additional low-pH-dependent binding apparently mediated by fusion peptide insertion into the RBC membranes (Chernomordik et al., 1997; Tsurudome et al., 1992). In the experiment presented in Fig. 2 *B*, neuraminidase treatment of the cells at the LPC-arrested stage was followed by LPC removal at different time points and then assaying the fusion extent. Washing LPC out immediately after neuraminidase treatment results in a higher fusion extent than that in the experiment with no neuraminidase treatment (see Fig. 2, *A* and *B*). A similar rise in the fusion extent (1.3 ± 0.1 (mean $\pm$ SD, $n = 19$)) was observed in the experiments where low pH pulse (pH 5, 5 min, 22°C) was applied in the absence of LPC and was immediately followed by neuraminidase treatment. Promotion of fusion by neuraminidase treatment right after the low pH application is consistent with our hypothesis. However, alternative interpretations such as cleaning of the membrane surfaces by enzymes cannot be excluded (Melikyan et al., 1995a).

In all of these experiments, the concentration of LPC that almost completely suppressed fusion was chosen. On the other hand, treatment with neuraminidase in the presence of the same LPC concentration resulted in significant fusion. To suppress fusion in these conditions we had to further increase the concentration of LPC. For instance, in a preliminary experiment the extent of fusion in the presence of 170 μM LPC was 58% and 6.7% with and without neuraminidase treatment, respectively. At an increased LPC concentration of 230 μM, the fusion extent observed with and without neuraminidase treatment was 3.4% and 0%, respectively. This is an additional indication of the fusion promotion by the neuraminidase.

Neuraminidase-induced promotion of HA-mediated fusion was transient. The decrease in fusion with time at the LPC-arrested stage was significantly faster after the neuraminidase treatment than in the control conditions (Fig. 2 *A*). These findings are consistent with our model suggesting that HA1-receptor interactions inhibit the inactivation of HA. Alternatively, these interactions can be important for directing HA refolding to a fusion-competent conformation rather than for slowing down subsequent inactivation of HA.

While RBCs remain bound to HA cells after neuraminidase treatment, fast fusion inactivation can be explained by the loss in "quality" of cell contacts. For instance, breaking the HA1-sialic acid connection between cells might inhibit fusion by weakening and decreasing the area of the contact. To test this possibility, we stabilized the contact between HA cells and RBCs by mild cross-linking with paraformaldehyde. Paraformaldehyde was applied to HA cells with bound RBCs before low pH application. After paraformaldehyde cross-linking, cell binding became independent of the HA1-sialic acid connection, as evidenced by the lack of RBC release upon neuraminidase treatment of the cells.

Importantly, the cross-linking did not decrease the final extent of low pH-triggered fusion mediated by HA. HA cells with bound and cross-linked RBCs were treated with a low pH pulse followed by neuraminidase in the presence of LPC. The rate of fusion inactivation for cross-linked cells was significantly decreased, and we were not able to detect any difference between the rates of inactivation for HA cell/RBC complexes treated versus untreated with neuraminidase. We suggest that under these conditions the kinetics of HA inactivation is not limited by the release of HA1-receptor connections. However, as for non-cross-linked cells, the neuraminidase treatment of cross-linked cells after low pH application promoted fusion (data not shown). These results argue against the role of cell binding changes in the inactivation of fusion.

Our hypothesis that the HA1-receptor connection has to be disrupted before a major conformational change in HA was indirectly substantiated by measuring the effect of low pH application on RBC binding to HA cells. RBC binding to HA300a cells pretreated with pH 4.9 before the addition of RBCs was significantly lower than that in the absence of the low pH pretreatment (Fig. 3). Thus at later stages of low pH-induced conformational change HA loses its ability to bind to membrane-anchored receptor, suggesting that the energy released during HA refolding should compensate for the lost energy of binding between HA1 and receptor. These data indicate the irreversibility of the transition between the receptor-bound initial state of HA and its final inactivated unbound state. Thus this transition has to be described by an irreversible, one-way kinetic scheme, such as the one suggested in Fig. 1, rather than by an equilibrium partitioning of HA between different states.

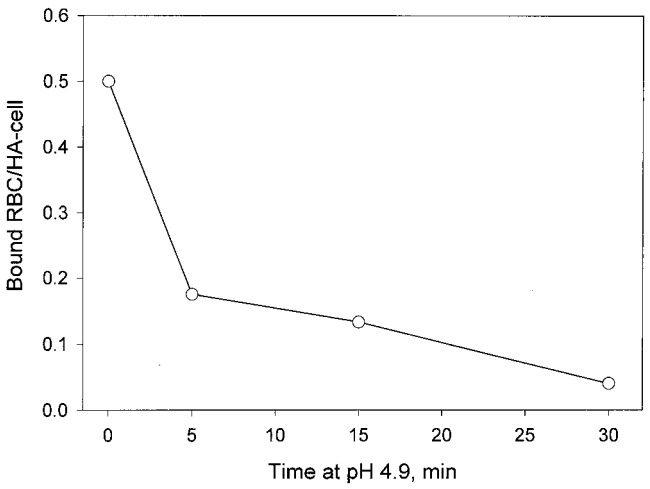

FIGURE 3 Pretreatment of HA cells with pH 4.9 medium inhibits subsequent binding of RBCs. In this representative experiment, HA cells were incubated at pH 4.9 (22°C) for different time intervals, then low pH medium was replaced with PBS (pH 7.4) supplemented with RBCs. After 15 min of incubation, followed by the removal of unbound RBC with five washings with PBS, binding was assayed by counting RBCs bound to more than 200 HA cells.

Water-soluble sialosides inhibit HA inactivation in the absence of the target membrane

As reported earlier for X:31 HA (Puri et al., 1990), short-term acidification of the X:31 HA-expressing HA300a cells in the absence of RBCs (referred to as low pH pretreatment) caused profound inactivation of HA (Fig. 4 *A*). After a 5-min-long pretreatment with pH 4.9, HA lost most of its ability to mediate fusion with the subsequent addition of RBCs, followed by a second, fusion-testing low pH pulse (see Experimental Methods). This inactivation of HA in the absence of the target membrane was inhibited by SL (Fig. 4 *A*). In these experiments, HA300a cells were incubated with 20 mM SL for 10 min and then treated with pH 4.9 for 5 min. After replacing acidic medium with SL-free PBS (pH 7.4), we added RBCs, applied the fusion-testing pulse, and assayed for fusion. As the final extent of fusion observed in this experiment was higher than that in the control (no SL), we concluded that SL lowers the rate of inactivation in the absence of the target membrane. Another soluble sialoside, AG, which binds to HA1 with even higher affinity than SL, also protects HA against inactivation in the absence of the target membrane (Fig. 4 *B*). Note that AG inhibited inactivation at a significantly lower concentration than SL. This difference can be explained by the known ~5–10-fold increase in the affinity of this sialoside for HA1 upon replacement of the methylic aglycon with the benzylic one (Matrosovich et al., 1993). In addition, SL from human milk used in this study contains mostly 2–6 isomer of SL, which has a lower affinity for X31 HA than 2–3 isomer (Matrosovich et al., 1993; Sauter et al., 1992b). We compared the effects of SL and AG with that of BG, beta-anomer methylglycoside, which does not bind to HA1 (Matrosovich et al., 1993; Pritchett et al., 1987; Sauter et al., 1992b). As expected, BG did not affect the rate of HA inactivation (Fig. 4 *C*) and RBC binding to HA cells (data not shown). In contrast to SL and AG, BG did not affect HA inactivation, suggesting that inhibition of HA inactivation by SL and AG involves their binding to HA1. Thus sialic acid-containing molecules inhibited inactivation of HA when present either at the surface of the target membrane (Fig. 2, *A* and *B*) or in a water-soluble form (Fig. 4).

One may hypothesize that soluble receptors inhibit the activation of HA rather than its further refolding, which was assayed above as HA inactivation. To exclude this possibility, we evaluated the percentage of activated HA as the percentage of HA molecules with the DTT-accessible disulfide bond stabilizing the HA1-HA2 complex. Because release of HA1 in the presence of DTT is an irreversible event, all low pH-activated HA are assumed to lose their HA1 subunit during a 20-min incubation with DTT. This low pH-triggered and DTT-induced loss of HA1 can be readily detected by immunoblotting with antibodies against HA1 (Fig. 4 *D*). As reported earlier (Puri et al., 1990), a 5-min application of pH 4.9 to HA cells in the absence of

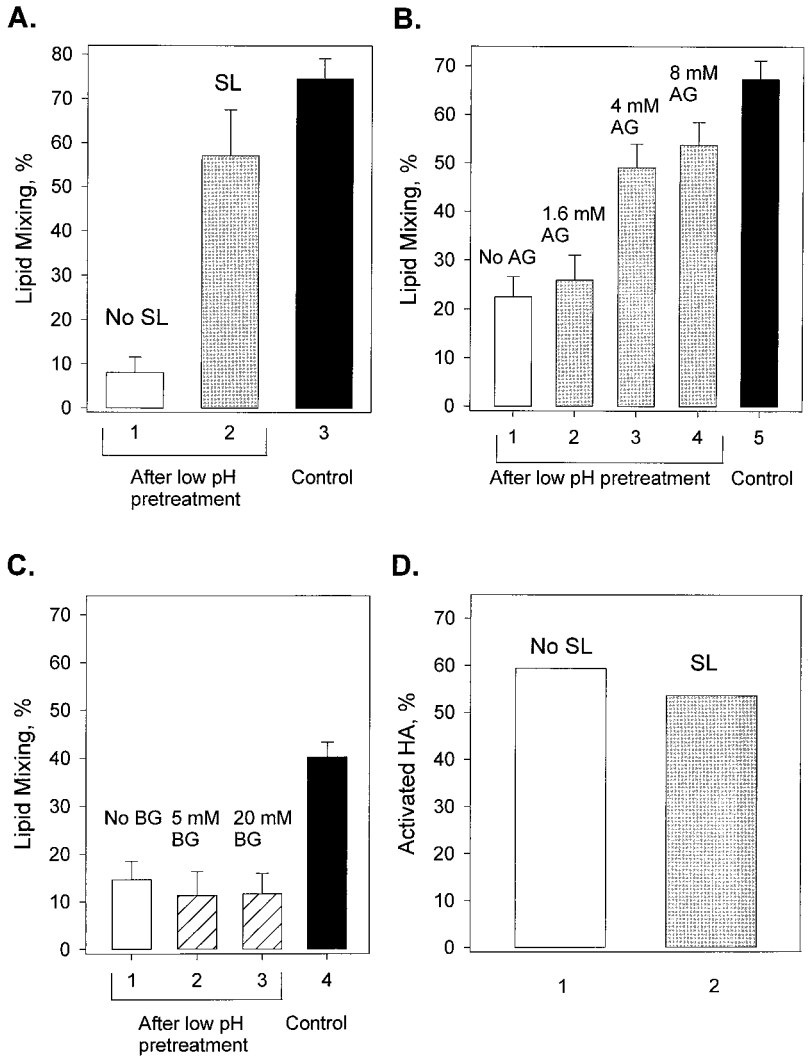

FIGURE 4 Fusion inactivation (*A* and *B*) and HA activation (*C*) in the presence of soluble glycosides. (*A*) HA cells were incubated at pH 4.9 for 5 min at 37°C with (*3*) or without (*2*) 20 mM SL. Then low pH medium was replaced with PBS (pH 7.4) supplemented with RBCs. After 15 min of incubation, followed by wash-out of unbound RBCs, a second low pH pulse (pH 5.5, 5 min, 37°C) was applied. The fusion extent was assayed as the ratio of dye-redistributed bound RBCs to the total number of bound RBCs. The fusion extent observed in the experiment, where the first low pH pulse (pretreatment of HA cells; Puri et al., 1990) was skipped, is represented by bar 3. Each bar is a mean ± SEM, $n = 3$. (*B* and *C*) HA cells were treated by pH 4.9 for 2 min at 22°C in the presence of 0, 1.6, 4, or 8 mM AG (*B*, *bars 1–4*, respectively), or 0, 5, or 20 mM BG (*C*, *bars 1, 2, 3*, respectively). Then low pH medium was replaced with glycoside-free PBS (pH 7.4) supplemented with RBCs. After 15 min of incubation, followed by washing out of unbound RBCs, a second low pH pulse (pH 4.9, 1 min, 22°C) was applied. The fusion extent was assayed as the ratio of dye-redistributed bound RBCs to the total number of bound RBCs. The fusion extent observed in the experiment, where low pH pretreatment of HA cells in the absence of the target membrane was skipped, is represented by bars 5 (*B*) and 4 (*C*) for the experiments shown in *B* and *C*, respectively. Each bar is a mean ± SEM, $n = 3$. (*D*) HA cells were incubated at pH 4.9 for 5 min at 37°C with (*3*) or without (*2*) 20 mM SL. Then cells were lysed, and the percentage of the activated HA (defined as HA molecules with DTT-accessible S-S bond between HA1 and HA2 subunits; Graves et al., 1983) was measured as described in Experimental Methods.

RBC activated a significant part of HA molecules. The percentage of activated HA was not affected by SL. This finding, along with the functional data in Fig. 4, *A–C*, suggested that a soluble receptor of HA1 slows down the refolding of already activated HA.

DISCUSSION

Influenza virus entry into a host cell starts with specific binding between HA1 at the viral envelope and sialic acids at the cell surface. In the present work we tested the possibility that this binding restricts low pH-induced conformational changes in HA and resulting membrane fusion. The dissociation of the HA-receptor bonds was hypothesized to constitute a slow stage in HA refolding, leading to the fusion-competent conformation. This hypothesis explains the earlier finding that inactivation of HA-expressing membrane with respect to fusion is slower in the presence of the target membrane.

To verify this hypothesis we first formulated a simple kinetic scheme that allowed us to analyze the probability that an HA cell fuses with its target membrane. This probability is equal to the fusion extent measured experimentally. HA evolution in our scheme starts with a low pH activation. This early conformational change in HA can be detected as an exposure of the fusion peptide by specific antibodies (White and Wilson, 1987) and enzymes such as thermolysin (Wiley and Skehel, 1987). For receptor-bound HA, the activated-locked state and then the activated-unlocked state follow this early conformational change. In the absence of the receptors, however, the activated-locked state is omitted.

The predictions of the hypothesis on the role of the HA1-receptor connections were verified by measuring fusion inactivation in time at the LPC-arrested stage. The corresponding theoretical dependence of the final extent of fusion Y_{fin} on the time interval t^* between low pH application and LPC removal is given by Eq. 8. We also studied the

time course of the fusion inactivation after neuraminidase application at the LPC-arrested stage. Neuraminidase releases water-soluble sialic acid from the HA receptors at the surface of RBC and thus causes rapid dissociation of the HA1-receptor bonds. These experimental conditions are described by the theoretical dependence in Eq. 9.

Our experimental findings are consistent with the predictions of the hypothesis. Breaking the HA1-receptor bonds by neuraminidase appears to temporarily boost the number of fusion-competent HAs, as indicated by an increase in fusion in the experiments where LPC block was lifted immediately after neuraminidase treatment. As predicted, this increase is only transient, and the rate of fusion inactivation after neuraminidase treatment is significantly higher than in the control (Fig. 2, *A* and *B*). We fitted two data sets obtained in the representative experiment (with and without neuraminidase) with the theoretical equations (Eqs. 8 and 9), which can be presented in a common form, $Y(t) = 1 - \exp(-B^*\exp(-t/A))$. We took into account that the fitting parameters,

$$A_1 = \frac{\tau_{a-l}}{m}, \quad A_2 = \frac{\tau_{a-u}}{m},$$

$$B_1 = \frac{\tau_{a-l}}{m \cdot \tau_f} \cdot \left(\frac{\tau_{a-u}}{\tau_{a-l} - \tau_{a-u}}\right)^m (N^0_{a-l})^m, \quad \text{and} \quad B_2 = \frac{\tau_{a-u}}{m \cdot \tau_f} \cdot (N^0_{a-l})^m$$

are related by

$$\frac{B_1}{B_2} = \frac{A_1/A_2}{(A_1/A_2 - 1)^m}.$$

To decrease the number of fitting parameters we assumed that the number m characterizing the cooperativity of fusogenic action of the activated HA molecules was equal to $m = 3$ (Danieli et al., 1996). We have chosen this estimate rather than the estimate of $m = 6$ suggested by Blumenthal et al. (1996), who based their analysis on fusion pore formation, whereas we and Danieli et al. (1996) assayed fusion as lipid mixing. Lipid mixing could involve fewer HA molecules than necessary for fusion pore opening (Chernomordik et al., 1998). Note that the choice of the specific value of m does not change the quality of the fit but does affect the obtained characteristic times.

To fit Eqs. 8 and 9 to the experimental data, taking into account the relationship between B_1/B_2 and A_1/A_2 (see above), we used the standard SigmaPlot software minimizing the mean square deviation with respect to two independent fitting parameters, A and B. While the constraints drastically reduced the number of fitting solutions and, thus, did not allow us to reach a better fitting, the model does provide a reasonable qualitative description of the experimental points. The kinetic parameters found from the fitting are $A_1 = 34$ min, $A_2 = 13.5$ min, $B_1 = 0.96$, and $B_2 = 1.35$. The time constants of the HA transition from the activated-locked state to the activated-unlocked state τ_{a-l} and from the activated-unlocked state to the inactivated state τ_{a-u} are $\tau_{a-l} = 102$ min and $\tau_{a-u} = 41$ min, respectively. The found values of the characteristic times satisfied the assumption of the model that $\tau_{a-l}/\tau_{a-u} > 1$.

The estimated value of τ_{a-u} is significantly longer than the duration of low pH pretreatment of X:31 HA-expressing membrane, which results in complete loss of its ability to fuse (~10 min; see above and Puri et al., 1990). We suggest that this difference reflects the cooperativity of HA-mediated fusion: the probability of fusion is a nonlinear function of the membrane concentration of fusion-competent HA, and, therefore, fusion becomes improbable already at early stages of HA inactivation. The time constant of the transition from the activated-unlocked state to the inactivated state $\tau_{a-u} \approx 41$ min is close to the half-time of exposure of the interface between HA1 monomers in a trimer (≈ 50 min) observed at 25°C for HA ectodomain in the absence of any membranes and receptors (White and Wilson, 1987). One may hypothesize that HA inactivation beyond the activated-unlocked state involves dissociation of the globular head HA1 domains.

The time constant of transition of HA molecules from the activated-locked to the activated-unlocked state, τ_{a-l}, is estimated as 102 min. At present, we have no independent way to verify how reasonable this value is. We hope that future experiments with conformation-specific antibodies will allow a direct measurement of the time course of structural changes of HA from the hypothetical activated-locked state to inactivated state. Equations A1 and A5 in the Appendix give a prediction for this evolution. One may ask whether this result is consistent with the rather fast kinetics of HA-mediated fusion. In our model the release of HA from the activated-locked state has a continuous character: the probability that each HA is unlocked increases exponentially with time. Therefore, for a sufficiently high total number of activated HAs in each cell, even in the first minute there are enough unlocked HA molecules to mediate fusion in some of the cells observed. According to Danieli et al. (1996), fusion of HAb2 cells with RBC reaches ~9% of the maximum extent within 1 min after low pH application at 29°C. The estimate based on our model with the parameters presented above gives ~3% of fusion within 1 min. We consider this agreement to be satisfactory, taking into account the difference in cell lines (HAb2 vs. HA300a) and temperatures (29°C versus 22°C) and other differences in the experimental protocols.

One may think that the HA1-receptor connection slows down the HA refolding because of mechanical constraints. Fusion requires sideways relocation of the globular subunits of HA1 from the top of HA molecule (Godley et al., 1992; Kemble et al., 1992). In the case of HA1 bound to membrane-anchored receptor, such a relocation should be hindered by the required deformation of either membranes or proteins. Alternatively, the HA-receptor connection may inhibit the lateral mobility of HA molecules required for

their assembly into functional or inactivated complexes (Alford et al., 1994; Gutman et al., 1993). However, water-soluble receptors, SL and AG, also slowed down HA inactivation. This finding suggests that just the presence of the receptor in the binding site of HA1 inhibits the completion of HA conformational change. Strikingly similar phenomena were reported for other proteins. Binding of the B subunit of cholera toxin to sialic acid in a ganglioside receptor does not prevent a conformational change upon low pH application, but stabilizes the structure of the B subunit against denaturation and collapse at low pH (McCann et al., 1997). Transition of an enveloped glycoprotein from Rous sarcoma virus to a membrane-binding conformation is associated with the release of a cellular receptor of the protein (Damico et al., 1998).

One of the insights from our work is that while the dissociation constant for the interaction of soluble HA1 and sialic acid appears to be rather high, all HAs that are close enough to the receptor-expressing target membrane should be bound because of the very high membrane concentration of the proteins under these conditions. Note that if one of the interacting molecules is present in a water-soluble form, as in our experiment with SL, its concentration required to achieve considerable binding should be above the dissociation constant $K_d \approx 3$ mM (Sauter et al., 1992b). At a lower concentration of SL, such as the one used by Stegmann et al. (1995) (less than 0.1 mM, as estimated from the data presented there), the percentage of bound HA is expected to be very low.

Our model is clearly oversimplified and neglects a number of known and important features of the system, such as any effects of receptors at the earlier stages of HA refolding (de Lima et al., 1995; Stegmann et al., 1995) and the existence on HA1 of two different types of receptor-binding sites (Sauter et al., 1992a). In addition, our kinetic scheme does not account for known substeps of the fusion reaction and does not suggest a specific structure for any of the states.

Both conformational change in HA and membrane fusion are known to involve a number of distinct stages (Blumenthal et al., 1991; Chernomordik et al., 1998; Melikyan et al., 1997; White, 1996; Zimmerberg et al., 1994). This work proposes a new stage: unlocking of the HA1-receptor connection, with a yet undefined place within the fusion pathway. We know that this stage follows an early low pH-induced activation of HA, such as exposure of the HA fusion peptide, and precedes a major refolding of HA leading to fusion or inactivation. We hypothesize that activated HA molecules can build up functional fusion complexes while still being locked. This would prevent the premature inactivation of HA and thus increase fusion efficiency. If this is correct, the unlocking stage has a clear biological relevance. Alternatively, slowing down the major refolding of HA by the HA1-receptor connection may allow more time for the fusion peptide insertion into the right membrane and in the right orientation. The well-timed release of connections between HA1 and receptor molecules would also facilitate the final expansion of a fusion pore. The hypothetical contribution of unlocking of the HA1-receptor connection in the time course of the fusion reaction suggests a new role for viral neuraminidase. Cleaving of the viral receptors after acidification of the endosome content can facilitate a major conformational change in unlocked HA molecules. Interactions between some fusion proteins (e.g., HIV gp120) and their membrane receptors play an important role in fusion protein activation (Dimitrov, 1997). This study proposes an additional mechanism by which a receptor can control the time course of protein refolding to a fusion-competent conformation.

APPENDIX: SOLUTION OF THE KINETIC EQUATIONS

We should first consider the conditions corresponding to our experimental protocol, where a low pH pulse is applied at the moment $t = 0$ in the presence of LPC. Then, after a time interval t^*, LPC is removed and fusion is allowed to proceed. The time evolution of the number N_{a-u} of HA molecules in the activated-unlocked state as determined by solution of Eqs. 3 and 4 is given by

$$N_{a-u} = \frac{\tau_{a-u}}{\tau_{a-l} - \tau_{a-u}} \cdot N^0_{a-l} \cdot \left[\exp\left(-\frac{t}{\tau_{a-l}} \right) - \exp\left(-\frac{t}{\tau_{a-u}} \right) \right] \tag{A1}$$

We insert Eq. A1 into Eq. 7, assuming that $\tau_{a-l} > \tau_{a-u}$ (see the text) and, therefore, neglecting the second contribution in Eq. A1. Solution of Eq. 7 provides us with an expression for the time dependence of the probability of fusion,

$$Y(t) = 1 - B \cdot \exp\left[\frac{\tau_{a-l}}{m \cdot \tau_f} \cdot \left(\frac{\tau_{a-u}}{\tau_{a-l} - \tau_{a-u}} \right)^m \cdot (N^0_{a-l})^m \cdot \exp\left(-\frac{m \cdot t}{\tau_{a-l}} \right) \right] \tag{A2}$$

where B is an integration constant. According to the experimental setup, no fusion occurs before t^*. This means that the probability of fusion $Y(t)$ is meaningful only for $t > t^*$ and satisfies the condition $Y(t^*) = 0$. Using this condition, we find B, and the resulting probability of fusion is presented by

$$Y(t) = 1 - \exp\left\{ \frac{\tau_{a-l}}{m \cdot \tau_f} \cdot \left(\frac{\tau_{a-u}}{\tau_{a-l} - \tau_{a-u}} \right)^m \cdot (N^0_{a-l})^m \left[\exp\left(-\frac{m \cdot t}{\tau_{a-l}} \right) - \exp\left(-\frac{m \cdot t^*}{\tau_{a-l}} \right) \right] \right\} \tag{A3}$$

Equation A3 shows that the probability of fusion increases with the time of observation t. The maximum probability of fusion corresponding to the final extent of fusion measured experimentally is reached for a sufficiently long time $t \to \infty$ and is presented by Eq. 8.

Now let us consider the conditions of the experimental protocol, where low pH pulse activating the HA molecules in the presence of LPC was followed, still in the presence of LPC, by neuraminidase treatment. We assumed that N^0_{a-l} of the HA molecules are transferred directly to the activated state and no HA resides in the activated-locked state. In this case

the time evolution of the number N_{a-u} of the activated HA molecules is determined by the equation

$$\frac{dN_{a-u}}{dt} = -\frac{N_{a-u}}{\tau_{a-u}} \quad \text{(A4)}$$

and is equal to

$$N_{a-u} = N^0_{a-l} \cdot \exp\left(-\frac{t}{\tau_{a-u}}\right) \quad \text{(A5)}$$

By inserting Eq. A5 into Eq. 7, we can solve the latter equation, accounting, as above, for the boundary condition $Y(t^*) = 0$. The resulting expression for the time dependence of the probability of fusion beginning from the time t^* is given by

$$Y(t) = 1 - \exp\left\{\frac{\tau_{a-u}}{m \cdot \tau_f} \cdot (N^0_{a-l})^m \left[\exp\left(-\frac{m \cdot t}{\tau_{a-u}}\right) - \exp\left(-\frac{m \cdot t^*}{\tau_{a-u}}\right)\right]\right\} \quad \text{(A6)}$$

The probability of fusion corresponding to a long enough experimental time, $t \to \infty$, and representing the total extent of fusion is given by Eq. 9.

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