Single Event Recording Shows That Docking onto Receptor Alters the Kinetics of Membrane Fusion Mediated by Influenza Hemagglutinin

Walter D. Niles and Fredric S. Cohen
Department of Physiology, Rush Medical College, Chicago, Illinois 60612 USA

ABSTRACT The initial steps of membrane fusion, receptor binding and membrane destabilization, are mediated by the envelope glycoprotein hemagglutinin of influenza virus. Interaction between these functions was determined from the time course of individual virion fusions to a planar membrane with and without receptor. With receptor, fusion was described by a Poisson process. In the absence of receptor, the time course was more complicated and could not be described with exponential rate constants. The conversion of a non-Markovian process into a simple Markov chain is direct evidence that receptor binding fundamentally alters the route of fusion.

INTRODUCTION

It is not known how proteins involved in the macromolecular event of membrane fusion interact to regulate each other's function. Functional fusion studies have been most quantitative at the nerve terminal because quantal exocytotic events can be detected and individually timed (1). The statistical distribution of inter-event time intervals or quantal latencies from depolarization of the nerve terminal yields a probabilistic description of the underlying fusion process (2). As proteins responsible for fusion become available (3–5), the macromolecular assemblies capable of fusion will be reconstituted, and the rate process will be the best characterization of fusion. We have developed a general procedure for detecting single fusion events and applied it to influenza virus fusion. The measured time intervals to fusion show that binding of hemagglutinin (HA) to its receptor alters the energetics of fusion.

We used HA because it is the best characterized fusion protein. Its structure is known to within 3 Å (6), binding to its receptor—sialic acids on both glycoproteins and glycolipids—is crystallographically and spectroscopically established (7–11), and its fusion function has been studied extensively (12, 13). Two sialic acid binding pockets have been found, the primary one located entirely within each monomeric HA1 subunit (9), and the secondary at the HA1-HA1 intermonomeric interface (11). Both sites are spatially separated from the hydrophobic fusion-initiating peptide, residing in the HA2 subunit, by 6–10 nm (6). Low pH, normally within endosomes, (14, 15) causes exposure of the fusion peptide which triggers fusion (16–20). We have found that the rate of fusion induced by HA is altered by the receptor in the target membrane.

MATERIALS AND METHODS

Single fusion events of influenza A/PR/8/34 virions with planar lipid membranes were detected as previously described (21, 22). Planar phospholipid membranes, bathed by a solution of the desired pH at 37°C, were observed with a video fluorescence microscope. Several microliters of virions, loaded with a self-quenching concentration of the lipophilic fluorescent probe octadecylrhodamine, R18 (23), and contained in a pH 7.4 solution in a micropipette, were ejected toward the planar membrane (22). This technique synchronized binding, as material ejected from the pipette became visible at the planar membrane within one video frame (1/30 s). The bathing solution was continuously stirred, so that virus not bound to the membrane was irretrievably lost to the bath. This ensured homogeneity of the measured time course of fusion, so that each virion introduced to the planar membrane entered the queue of reaction steps leading to fusion at relatively the same time as all other virions (24). When a virion fused and deposited its R18 into the planar membrane, concentration-quenching of fluorescence was relieved, producing a small localized region of transiently intense light emission or "flash" (21). The waiting time between the encounter of the ejected virions with the planar membrane and the onset of each flash was measured with single video frame accuracy using an image-processing program that recognized the dynamic pattern of the flash in sequences of video frames (25). Distributions of waiting times were used to calculate virion fusion rates based on rate process models.

RESULTS AND DISCUSSION

The kinetic process underlying influenza virus fusion was determined from the time course of individual fusion events with a planar lipid membrane. The waiting time (the period elapsing between the encounter of virus with the planar membrane and each fusion event) was measured. The number of fusion events with waiting times $>$ time t (the survivors) were plotted as a function of time, as shown in Fig. 1, so that all events remained at time $t = 0$, and the number of survivors decreased with time. Because unbound virus was quickly removed (21, 22), the kinetics of fusion were directly observed from the time course of single events without uncertainty in the initial time of interaction with the planar membrane and without contamination by repetitive unbinding and rebinding.

The HA receptor, sialic acid, structures the fusion process of PR8 into an organized rate scheme. When the planar membrane contains gangliosides, sialic acid-containing glycolipids, the waiting times are distributed exponentially at low pH (Fig. 1). This is consistent with virus fusion being organized into a Poisson jump process, in which the fusion reaction for

Received for publication 23 November 1992 and in final form 23 February 1993.

0006-3495/93/07/171/06 $2.00

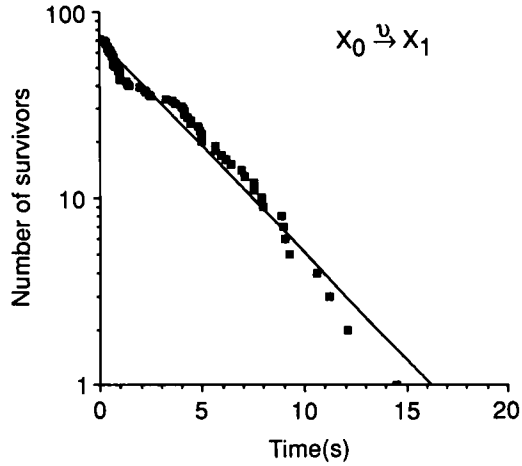

FIGURE 1 Exponential distribution of waiting times for receptor-containing membranes. The planar membrane was composed of asolectin: cholesterol (2:1) containing 10% gangliosides G_{D1a} + G_{T1b}, and the pH of the solution was 5.1. The 70 fusion events were obtained in 15 ejections of A/PR/8/34 strain on four planar membranes. The ordinate is logarithmic. The distribution is fitted with an exponential with rate constant 0.306 flashes/s (R^2 = 0.974). Inset, a transient two-state Markov chain.

each virion is described by a transient, continuous time parameter Markov process (24) with two distinct states and one forward rate constant ν (a Markov chain, see Fig. 1, inset). We interpret this scheme as follows: At time $t = 0$, the virions are brought into the vicinity of the planar membrane by pipette ejection, and any virion capable of fusion that attaches to the target membrane enters the initial state X_0. For any small interval of time (Δt) that the virion dwells in X_0, the probability that the virion fuses and enters the final, absorbing state X_1 is $\nu\Delta t$. Thus, the probability density function for a virion starting in the initial state at time 0 and fusing at time t is $\nu e^{-\nu t}$, and the distribution function for a waiting time $>t$ (the survivor distribution) is $\int_t^\infty \nu e^{-\nu t'} dt' = e^{-\nu t}$. In Fig. 1, the semilogarithmic distribution of survivors is linear (R^2 = 0.97), indicating that the fusion reaction for the ganglioside-containing planar membrane is reasonably well-described by the two-state Markov chain.

With the receptor-containing membranes, fusion is pH-dependent. Both the number of fusion events (21, 26) and ν increase with acidic pH (Table 1). This is consistent with the pH dependence of the protonation-induced conformational change in HA, which occurs near pH 5.5 for PR8 (27, 28). In Fig. 2, the waiting time distributions are plotted as probabilities, in which the number of survivors is normalized by the total number of fusion events in order to obviate the effect of pH on the number of events. The pH dependence is revealed as an increased slope of the probability distributions at lower pH.[1]

[1] Consistent with the fusion peptide's location at anywhere from 6 to 10 nm from the two sialate binding sites (6), we obtain similar pH dependencies with charged and zwitterionic membranes. Thus, the fusion-controlling moieties of HA attached to the planar membrane are exposed to the bulk pH of the bath rather than the ~0.5 unit lower pH within the space charge region (~1 nm) near the charged planar membranes.

TABLE 1 Gangliosides greatly augment the rate of virion fusion to planar membranes at low pH

pH	7.4	5.4	5.0
Number of membranes	10	10	3
Number of experiments	100	15	5
Number of fusion events	39	190	35
ν (mean ± SE) (s^{-1})	0.035 ± 0.003	0.071 ± 0.002	0.375 ± 0.011
R^2	0.90	0.92	0.98

Transition rate constants for the survivor probabilities shown in Fig. 2 *A*, obtained within a single batch of strain PR8 influenza virus. The increase in ν between pH 5.4 and 5.0 may reflect an increase in the rate of acidification of the virions after ejection from the pipette.

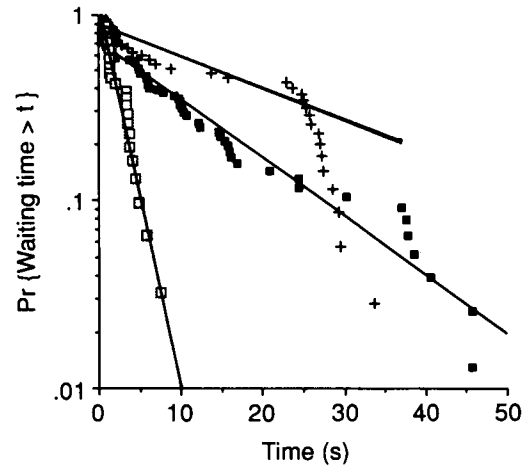

FIGURE 2 pH dependence of log-probability distributions with receptor. The waiting times were obtained at pHs 7.4 (+), 5.4 (■), and 5.0 (□). The ordinate indicates the fraction of virions (out of the total number of virions that fuse) with waiting times greater than the indicated time. The transition rate constant is pH-dependent. Virus was grown in several different batches of chicken eggs. Rates at different pH values were compared within the same batch of virus.

The survivor probabilities indicate that the nature of virion attachment to the planar membrane changes at low pH (Fig. 2). At pH 7.4, the earliest 70% of the waiting times are exponentially distributed but then the survivor distribution rapidly falls off at 25 s, whereas at pH 5.4 the distribution extends to 50 s. The fall-off observed at pH 7.4 indicates that the time course of the underlying process is truncated. This reveals that, at pH 7.4, there is an "inactivation" process removing virions from the initial state. This removal does not affect the virions until about 15–20 s after they have been attached to the planar membrane. Otherwise the distribution would remain a single exponential and the slope would be the sum of the transition rate constants for fusion (ν) and removal (k). The rapid fall-off after 20 s suggests that $k >> \nu$. If removal is a Poisson point process that does not become available until some time t' after attachment, then the

survivor function $S(t) = e^{-\nu t}$ for $t < t'$. For $t > t', S(t) = e^{-(\nu+k)t}$, which is dominated by the faster removal process. One interpretation is that the sialate-mediated interaction is weak and that the stirring of the bathing solution causes virion detachment at pH 7.4. In any case, the absence of truncation at pH 5.4 indicates that a more adherent mode of binding occurs at low pH, such as insertion of the acid-exposed hydrophobic fusion peptide into the planar membrane (14, 15, 29, 30). This insertion step would occur after the sialate-mediated attachment, because the waiting times are distributed over a longer period in the presence of receptor than in its absence (<15 s, Fig. 2). Alternatively, as the truncated distribution resembles those obtained with receptor-free films, it is possible that some virions are avoiding the gangliosides and interacting directly with the film.

With phospholipid membranes in the absence of HA receptor, the waiting time distribution does not have a simple form and curves in a direction opposite that expected for a Markov chain (Fig. 3 *A*). Waiting times obtained at pH 7.4 and at low pH (5.7–4.5) appear similar and, when pooled, yield a smooth, nonexponential distribution without components (Fig. 3 *B*). One rate process that accounts for some of these features is a non-Markovian chain, in which the transition rate "constant" is not actually constant over any small time interval of observation (say $[t_1, t_2]$) but changes during the period. As an example, let the transition rate constant increase linearly with the period of observation, so that during the small interval $[t_1, t_2]$, the transition rate "constant" changes from at_1 at the beginning to at_2 by the end of the small interval. The exit probability over the interval is $at_2(t_2 - t_1)$. For a long interval $[0, T]$, the probability density function for exit from the initial state at time T is $aTe^{-(1/2)aT^2}$ and the survivor distribution is $e^{-(1/2)aT^2}$. This model predicts the inverted parabolic shape of the survivor distribution in Fig. 3 *B*. Physically, the scheme might be realized with an energy barrier that decreases with time. The absence of receptor does not produce the biological configuration, and the biological, pH-dependent fusion is not reconstituted (26).

In virus-liposome fusion systems, pH dependence is observed in the absence of the receptor (31, 32); moreover, gangliosides produce quantitative but not qualitative differences (33, 34). Two notable differences between these systems are that virions have only a brief initial opportunity to attach to the planar membrane thus temporally synchronizing the fusion process for each virion and only quickly fusing virions are detected. In the virus-vesicle system, kinetic coupling between binding and fusion as observed in the planar membrane system is obscured by asynchronous initiation, repeated attachments and detachments of virions to vesicles at steady-state, and the inability to time single events. Asynchrony in the introduction of virions into the fusion rate process could mask the magnitude of the pH dependence of fusion. This would be especially severe if the entry step were pH-dependent (such as binding mediated by exposure of the hydrophobic fusion peptide). If, at each pH, the rate of entry were about four times slower than the transition rate constant

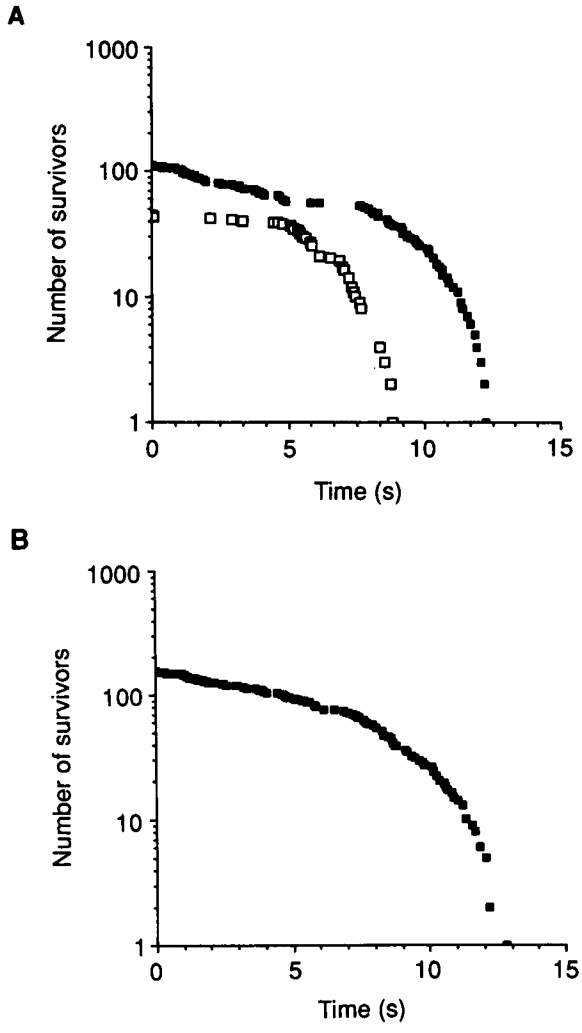

FIGURE 3 Log-survivor distributions of waiting times for influenza virion envelope fusion events with receptor-free planar membranes. (*A*) On five soybean lipid (asolectin):cholesterol (2:1 by mole fraction) membranes, fusion was first recorded at pH 7.4 (■) and then after addition of H_3PO_4 to lower the pH to the range of 5.3–4.7 (□). 111 events were detected in 23 ejections at pH 7.4 and 44 detected in 10 ejections at low pH. The log-survivor distributions are nonlinear and show clustering of events, indicating that the fusion process cannot be described by a two-state Markov chain. (*B*) Pooled log-survivor distribution of waiting times in *A*. The composite distribution forms a smooth curve that rapidly decreases at long times. Similar results were obtained with receptor-free membranes made from other phospholipids such as diphytanoyl phosphatidylcholine and phosphatidylethanolamine.

of fusion (ν), then the observed pH dependence in the macroscopic experiments would reside in entry to the "initial state" of the scheme. In fact, pH-dependent binding is observed to receptor-free liposomes at 0°C (34).

The strength of sialate binding to the primary site in HA1 affects the Markovian organization of the rate process. Terminal sialate is linked to carbohydrate chains via various anomeric linkages. The sialate binding site in HA exhibits preferences for $\alpha 2 \rightarrow 3$ or $\alpha 2 \rightarrow 6$ anomeric conformations depending on the strain of virus. The basis of these preferences is appreciated in atomic detail (7–10, 35, 36). Sialic acids in gangliosides, however, are linked in $\alpha 2 \rightarrow 3$ but not in $\alpha 2 \rightarrow 6$

conformations. The PR8 strain exhibits a 2:1 selectivity for $\alpha 2 \rightarrow 3$ over $\alpha 2 \rightarrow 6$ anomeric links on red cells resialylated with anomeric-selective sialyltransferases (36), and its fusions have an exponential waiting time distribution (Figs. 1 and 2). The waiting time distribution for the A/Ukraine (duck)/1/63 strain, which exhibits a 32-fold preference for the $\alpha 2 \rightarrow 3$ anomer (36), also is fitted well by the exponential distribution with $\nu = 0.409\ s^{-1}$ ($R^2 > 0.99$) for ganglioside-containing membranes at pH 5.0 (Fig. 4 *A*). With receptor-free membrane, the waiting times are not exponentially distributed, consistent with the results from PR8. In contrast, with the X-31 strain, which prefers $\alpha 2 \rightarrow 6$ links by 256-fold (36), the survivor distributions obtained with ganglioside-containing planar membranes are not as well-fitted by exponential distributions (Fig. 4 *B*, *closed symbols*), ($R^2 < 0.9$). Without receptor, the survivor distribution resembles that of PR8 and Ukraine duck strains with their characteristic non-exponential distributions (Fig. 4 *B*, *open symbols*).

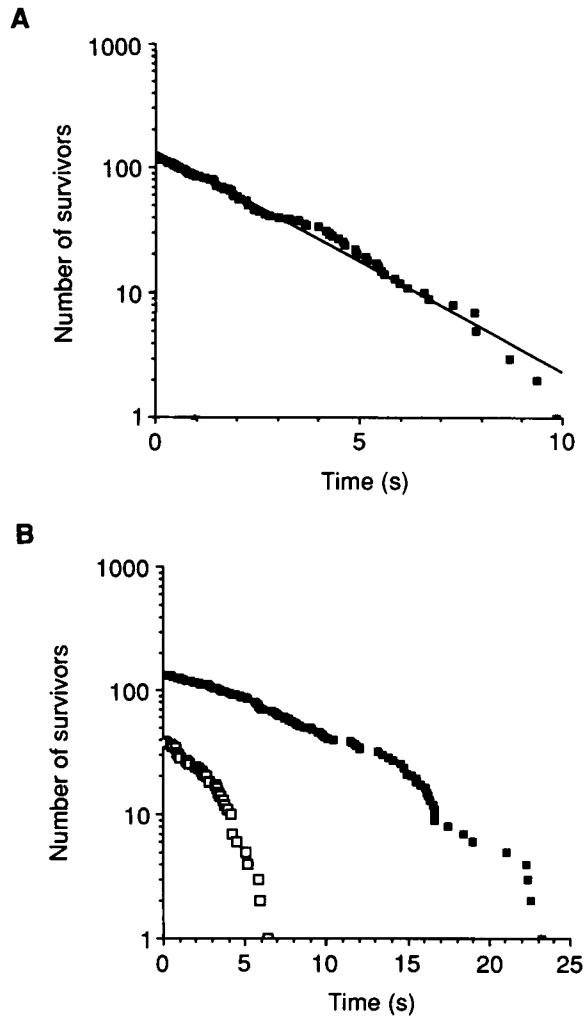

FIGURE 4 (*A*) Log-survivor distribution obtained with the $\alpha 2 \rightarrow 3$ linkage preferring influenza virus A/Ukraine (duck)/1/63 on ganglioside-containing planar membranes at pH 5 (pH 5.3–4.7). The fitted exponential distribution ($R^2 = 0.997$) has a rate constant $\nu = 0.406\ s^{-1}$. Membrane composition same as Fig. 2. (*B*) Log-survivor distribution of fusion waiting times obtained with $\alpha 2 \rightarrow 6$ linkage preferring strain X-31 on ganglioside-containing asolectin planar membranes at pH 4.7 (*closed symbols*) and ganglioside-free asolectin membranes at pH 4.7 (*open symbols*). The waiting time distributions are not well-described by exponentials ($R^2 = 0.77$ with gangliosides, $R^2 = 0.23$ without).

Interactions between virion binding and fusion have been implicated for several types of lipid-enveloped viruses. Expression and complementation studies of the binding, HN, and fusion, F, proteins of paramyxoviruses argue that these proteins functionally interact (37–39). With the env glycoprotein of some isolates of HIV-1, the CD4-binding subunit, gp120, dissociates from gp41 upon binding and generates a fusigenic intermediate (40, 41). The role of sialic acid as receptor for bringing influenza in close proximity to its host membrane has long been recognized (42). In influenza virus-liposome fusion, gangliosides increase the rate of fusion (43), although the effect is assigned minor importance (32, 33). Only by timing single events, however, does the receptor effect on the rate process of fusion become fully apparent.

Here we have demonstrated that the receptor alters the time course of fusion by "structuring" the reaction into a chain of Markovian-type states. The significance of the Markov process for the virion is that the jump frequency (the transition rate constant) between two states (i.e., the time that the virion remains in the initial state prior to fusion) is single-valued and independent of time. This suggests that the transition rate constant describes the jump frequency across a single, time-invariant energy barrier that is decreased in magnitude at low pH. This is consistent with the barrier being related to the energy of the conformational change and nucleation of fusion by the low pH form of HA. We propose that the energy of sialate-HA binding is transmitted to regions of HA involved in the pH-induced conformational change and stabilizes the energy barrier for fusion. In the absence of receptor, the time course indicates that the entire process is different. The initial and final states are different and the jump frequency varies with time but not pH. A possible interpretation is that the energy barrier for fusion is biphasic, briefly decreasing in size and then increasing.

The receptor effect on the rate process is unrelated mechanistically to a reduction in degrees of freedom (such as the number of HA configurations) or dimensionality upon binding. It is not the result of simply decreasing the number of states, changing the transition rate constants for the pathways, or adopting a diffusion-limited kinetic scheme. If we consider a set of states connected by trajectories or reaction paths, then a reduction in the degrees of freedom only removes particular states (and the corresponding reaction paths), but it does not change the process underlying the temporal behavior in the remaining states. Thus, the trajectories through the remaining states would be unaltered, and the time course would be dominated by their kinetics. Moreover, these paths would have been apparent prior to their selection, and their trajectories would have been present as a component in the time course prior to selection. (Diffusion-limited kinetics predict a $t^{-1/2}$ dependence at short times and then a long tail; our observations are counter to this prediction.) In our system of R18-labeled influenza virions fusing

with a planar membrane, time-homogeneous states, and trajectories defined between them exist only in the presence of sialate. The alteration is dramatic: formerly non-Markovian transitions are eliminated by binding, the reaction space is restructured into all new states, and the new transition is Markovian.

This restructuring of the fusion process by receptor interaction could extend to other fusion systems. In systems such as intracellular membrane trafficking and exocytosis, one major problem is the recognition of the transfer or secretory vesicle by the "correct" region of the target membrane. Undoubtedly, specific docking proteins perform this task, but an intriguing possibility emerges if docking and fusion functions were combined in a single protein (such as HA). The energy of binding the fusion protein to its specific receptor could favor a new set of allowed conformations and establish the magnitudes of the energy barriers between them and, thus, facilitate the conformational change. In this way, the specificity of the binding site would not only direct the fusion protein to the correct target but also provide the macromolecule with sufficient energy to trigger fusion only on encountering the correct membrane.

We thank Dr. Judith M. White for a generous gift of X-31 strain influenza virus, Dr. Robert G. Webster for providing us quantities of the avian Ukraine influenza strain on several occasions, and Drs. Robert Eisenberg, Mark Peeples, and Eduardo Rios for critical reviews of the manuscript.
This work was supported by National Institutes of Health grant GM27367.

REFERENCES

1. Katz, B. 1969. *The Release of Neural Transmitter Substances.* Liverpool University Press, Liverpool. 60 pp.
2. Katz, B., and R. Miledi. 1965. The measurement of the synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc. Lond. Ser. B. Biol. Sci.* 161:483–495.
3. Südhof, T. C., and F. Jahn. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron.* 6:665–671.
4. Trimble, W. S., M. Linial, and R. H. Scheller. 1991. Cellular and molecular biology of the presynaptic nerve terminal. *Ann. Rev. Neurosci.* 14:93–122.
5. Vogel, S. S., and J. Zimmerberg. 1992. Proteins on exocytic vesicles mediate calcium-triggered fusion. *Proc. Natl. Acad. Sci. USA.* 89:4749–4753.
6. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å. *Nature.* 289:366–373.
7. J. C. Paulson. 1985. Interactions of animal viruses with cell surface receptors. In *The Receptors.* P. M. Conn, editor. Academic Press, Orlando. 131–219.
8. Suzuki, Y., Y. Nagao, H. Kato, M. Matsumoto, K. Nerome, K. Nakajima, and E. Nobusawa. 1986. Human influenza A virus hemagglutinin distinguishes sialyloligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. *J. Biol. Chem.* 261:17057–17061.
9. Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature.* 333:426–431.
10. Sauter, N. K., M. D. Bednarski, B. A. Wurzburg, J. E. Hanson, G. M. Whitesides, J. J. Skehel, and D. C. Wiley. 1989. Hemagglutinins from two influenza virus variants bind to sialic acid derivatives with millimolar dissociation constants: a 500-MHz proton nuclear magnetic resonance study. *Biochemistry.* 28:8388–8396.
11. Sauter, N. K., G. D. Glick, R. L. Crowther, S.-J. Park, M. B. Eisen, J. J. Skehel, J. R. Knowles, and D. C. Wiley. 1992. Crystallographic identification of a second ligand binding site in influenza virus hemagglutinin. *Proc. Nat. Acad. Sci. USA.* 89:324–328.
12. Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56:365–394.
13. J. M. White. 1990. Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* 52:675–697.
14. Yoshimura, A., K. Kuroda, K. Kawasaki, S. Yamanishi, T. Maeda, and S.-I. Ohnishi. 1982. Infectious cell entry mechanism of influenza virus. *J. Virol.* 43:284–293.
15. White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped viruses. *Quart. Rev. Biophys.* 16:151–195.
16. Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley. 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci. USA.* 79:968–972.
17. Doms, R. W., A. Helenius, and J. White. 1985. Membrane fusion activity of the influenza virus hemagglutinin. *J. Biol. Chem.* 260:2973–2981.
18. White, J. M., and I. A. Wilson. 1987. Anti-peptide antibodies detect steps in a protein conformational change: low pH activation of the influenza virus hemagglutinin. *J. Cell Biol.* 105:2887–2896.
19. Godley, L., J. Pfeifer, D. Steinhauer, B. Ely, G. Shaw, R. Kaufman, E. Suchanek, C. Pabo, J. J. Skehel, D. C. Wiley, and S. Wharton. 1992. Introduction of intersubunit disulfide bonds in the membrane-distal region of influenza hemagglutinin abolishes membrane fusion activity. *Cell.* 68:635–645.
20. Kemble, G. W., D. L. Bodian, J. Rosé, I. A. Wilson, and J. M. White. 1992. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. *J. Virol.* 66:4940–4950.
21. Niles, W. D., and F. S. Cohen. 1991. Fusion of influenza virions with a planar lipid membrane detected by video fluorescence microscopy. *J. Gen. Physiol.* 97:1101–1119.
22. Niles, W. D., and F. S. Cohen. 1987. Video fluorescence microscopy studies of phospholipid vesicle fusion with a planar phospholipid membrane. Nature of membrane-membrane interactions and detection of release of contents. *J. Gen. Physiol.* 90:703–735.
23. Hoekstra, D., T. de Boer, K. Klappe, and J. Wilschut. 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. *Biochemistry* 23:5675–5681.
24. Parzen, E. 1967. *Stochastic Processes.* Holden-Day, Inc., San Francisco. pp. 30–32, 187.
25. Niles, W. D., Q. Li, and F. S. Cohen. 1992. Computer detection of the rapid diffusion of fluorescent membrane fusion markers in images observed with video microscopy. *Biophysical J.* 63:710–722.
26. Niles, W. D., and F. S. Cohen. 1991. The role of *N*-acetylneuraminic (sialic) acid in the pH dependence of influenza virion fusion with planar phospholipid membranes. *J. Gen. Physiol.* 97:1121–1140.
27. Huang, R. T. C., R. Rott, and H.-D. Klenk. 1980. Influenza viruses cause hemolysis and fusion of cells. *Virology.* 110:243–247.
28. Yewdell, J. W., W. Gerhard, and T. Bachi. 1983. Monoclonal anti-hemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/834-mediated hemolysis. *J. Virol.* 48:239–248.
29. Brunner, J., C. Zugliani, and R. Mischler. 1991. Fusion activity of influenza virus PR8/34 correlates with a temperature-induced conformational change within the hemagglutinin ectodomain detected by photochemical labelling. *Biochemistry.* 30:2432–2438.
30. Stegmann, T., J. M. Delfino, F. M. Richards, and A. Helenius. 1991. The HA2 subunit of influenza hemagglutinin inserts into the target membrane prior to fusion. *J. Biol. Chem.* 266:18404–18410.
31. White, J., J. Kartenbeck, and A. Helenius. 1982. Membrane fusion activity of influenza virus. *EMBO J.* 1:217–222.
32. Wharton, S. A., J. J. Skehel, and D. C. Wiley. 1986. Studies of influenza haemagglutinin-mediated membrane fusion. *Virology.* 149:27–35.
33. Stegmann, T., S. Nir, and J. Wilschut. 1989. Membrane fusion activity of influenza virus. Effects of gangliosides and negatively charged phospholipids in target liposomes. *Biochemistry.* 28:1698–1704.
34. Stegmann, T., J. M. White, and A. Helenius. 1990. Intermediates in

influenza induced membrane fusion. *EMBO J.* 9:4231–4239.

35. Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley. 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature.* 304:76–78.
36. Rogers, G. N., and J. C. Paulson. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology.* 127: 361–373.
37. Morrison, T., C. McQuain, and L. McGinnes. 1991. Complementation between avirulent Newcastle Disease Virus and fusion protein gene expressed from a retrovirus vector: requirements for membrane fusion. *J. Virol.* 65:813–822.
38. Ebata, S. H., M-J. Côté, C. Y. Yang, and K. Dimock. 1991. The fusion and hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus 3 are both required for fusion. *Virology.* 183:437–441.
39. Hu, X., R. Ray, and R. W. Compans. 1992. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza virus. *J. Virol.* 66:1528–1534.
40. Moore, J. P., J. A. McKeating, R. A. Weiss, and Q. J. Sattentau. 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science (Wash. DC).* 250:1139–1142.
41. Allan, J. S. 1991. Receptor-mediated activation of immunodeficiency viruses in viral fusion. *Science (Wash. DC).* 252:1322.
42. Hirst, G. K. 1948. The nature of the virus receptor of red cells. 1. Evidence on the chemical nature of the virus receptors of red cells and of the existence of a closely analogous substance in normal serum. *J. Exp. Med.* 87:301–314.
43. Wunderli-Allenspach, H., and S. Ott. 1990. Kinetics of fusion and lipid transfer between virus receptor containing liposomes and influenza viruses as measured with the octadecylrhodamine B assay. *Biochemistry.* 29:1990–1997.