Membrane Fusion by Single Influenza Hemagglutinin Trimers

KENNEDY EVIDENCE FROM IMAGE ANALYSIS OF HEMAGGLUTININ-RECONSTITUTED VESICLES

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Influenza virus hemagglutinin (HA) is the best characterized among the fusogenic membrane glycoproteins. HA mediates a local point of viral fusion with endosomal membranes, causing viral nucleocapsids to be released into the cytoplasm. HA is a homotrimer in which each viral particle is covered with ~400 HA trimers copies. Several previous reports have proposed that multiple HA trimers are needed for fusion (10–16) and for viral entry into cells (17), and this view is generally accepted at this moment, whereas others have suggested that a single HA trimmer is sufficient for fusion (8, 18, 19). The minimum number of HA trimers needed for fusion should be determined unambiguously, because this issue is a prerequisite for clarifying the mechanism of HA-induced fusion and is also of primary importance for studies of intracellular membrane fusion.

HA has receptor-binding as well as membrane-fusion activity, both of which are indispensable for viral infection of host cells. Viral particles attached to cell surfaces through binding of HA to viral receptors are endocytosed and transported to endosomes (20–22, 55). The low pH inside endosomes triggers a conformational change of HA (23, 24) to induce viral fusion with endosomal membranes, causing viral nucleocapsids to be released into the cytoplasm. HA is a homotrimer in which each monomer consists of two disulfide-linked polypeptides, HA1 and HA2, generated by proteolytic cleavage of the primary translation product, HA0. This cleavage is an absolute requirement for low pH-induced fusion (25, 26), although both uncleaved and cleaved forms of HA have receptor-binding activity.

The aim of this study was to obtain conclusive evidence of whether HA-induced fusion occurs by a multiple-trimer or a single-trimer mechanism by the use of microscopic image analysis. The single-vesicle analysis performed was vastly superior to the conventional fusion assay using fluorescence lipid mixing measurement for obtaining precise kinetic data on HA-induced fusion. We used two approaches to manipulate the surface density of HA. One series of experiments involved co-reconstitution of fusogenic HA1,2 trimers and non-fusogenic HA0 trimers with various HA1,2:(HA1,2 + HA0) ratios and with a constant protein/lipid ratio to assess the effect of the surface density of the fusion-activity sites of HA without changes in the surface density of the receptor-binding sites. The other series of experiments involved reconstitution of HA1,2 trimers without HA0 trimers, in which the surface densities of the fusion-activity sites and the receptor-binding sites were changed in parallel. The former series of experiments allowed us to deduce the minimum number of HA trimers required for fusion, whereas the latter series, in comparison with the former, could be used to evaluate how much HA-receptor binding interfered with fusion.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Reagents—Madin-Darby canine kidney cells were grown in Dulbecco’s modified minimum essential medium with 10% fetal calf serum. Influenza virus A/PR/8/1934(H1N1) was prepared from the supernatant of infected Madin-Darby canine kidney cells at

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1 To whom correspondence should be addressed: Tel.: 81-29-861-6602; Fax: 81-29-861-6147; E-mail: kawasaki.k@aist.go.jp.
2 The abbreviations used are: HA, hemagglutinin; PBS, phosphate-buffered saline; PC, phosphatidylycholine; R18, octadecylrhodamine B; PIPES, 1,4-piperazinediethanesulfonic acid.
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12 h postinfection. The virus was purified by differential centrifugation and sucrose density gradient centrifugation. Viral protein was quantified by Lowry's method. Trypsin, benzamidine, aprotinin, Triton X-100, and cholesterol were purchased from Sigma. Soybean trypsin inhibitor was obtained from Wako (Osaka, Japan), egg yolk phosphatidylcholine (PC) from NOF (Tokyo, Japan), and octadecylrhodamine B chloride (R18) from Molecular Probes (Eugene, OR).

Red Blood Cell Ghost Membranes—Human red blood cells (type A+) obtained from a single healthy donor were washed three times with PBS and diluted to 1% (v/v) in PBS. The red blood cells (0.2 ml) were allowed to attach to a poly-L-lysine-coated glass coverslip at 4 °C for 15 min, then lysed to ghost membranes by three washes with ice-cold 5 mM sodium phosphate (pH 8.0). The ghost membranes were resuspended in PBS containing 0.9 mM CaCl2 and 0.49 mM MgCl2 for 30 min at 37 °C, and kept in PIPES buffer (5 mM PIPES, 145 mM NaCl, pH 7.5) on ice until use.

Reconstituted Vesicles of HA—A/PR/8/1934 virus grown in Madin-Darby canine kidney cells contains HA as a precursor, HA0 (27). To cleave HA0, 5 μl of trypsin (10 mg/ml) was added to a virus suspension (12 mg of total viral protein in 1 ml of PIPES buffer), and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by addition of 10 μl of soybean trypsin inhibitor (10 mg/ml), 2 μl of benzamidine (62 mg/ml), and 1 ml of aprotinin (1 mg/ml) in PIPES buffer. The virus was then pelleted by centrifugation (55,000 × g for 30 min) and resuspended in PIPES buffer. HA1,2 and HA0 were purified from trypsin-treated virus and non-treated virus, respectively, by solubilization with Triton X-100 and sucrose density gradient centrifugation, as described previously (28). Neuraminidase was not removed by this procedure but remained at a reduced level, as shown under “Results.”

The isolated HA (1 mg in 1 ml of PIPES buffer) was resolubilized by addition of 40 μl of 20% (wt/wt) Triton X-100 and by incubation for 1 h at room temperature. Triton X-100 treatment at 4 °C is often used for studies of lipid rafts to obtain detergent-insoluble glycolipid-enriched complexes (29). The present procedure at room temperature (−25 °C) was distinct from that at 4 °C in that HA trimers were not aggregated after Triton X-100 treatment as examined by gel-filtration chromatography (Sephacryl S-300, Amersham Biosciences, Uppsala, Sweden; data not shown). A lipid mixture (2.5 mg) of egg PC/cholesterol/R18 (3.8:1:9:1.0, by weight) in chloroform/methanol (2:1) was dried and kept under vacuum overnight. The lipid film was suspended in 0.31 ml of PIPES buffer and mixed with 0.19 ml of 20% Triton X-100. For the first series of reconstitutions (the "variable [F] experiment"), HA1,2 and HA0 samples mixed at various HA1,2:(HA1,2 + HA0) ratios were added to the lipid solution at 1.4 mg of HA per 1 mg of lipid. To remove the detergent, the mixtures were dialyzed through Spectrapor membrane tubing 2 (25 mm, Spectrum Laboratories, Rancho Dominguez, CA) against 2.0 liters of PIPES buffer containing CaCl2 and MgCl2 at 2 mM each and 3 g of Bio-beads SM-2 (Bio-Rad) at room temperature for 4 h and then at 4 °C for 80 h (28, 30). The HA-reconstituted vesicles were purified by sucrose density gradient centrifugation. For the second series of reconstitutions (the "variable [F-B] experiment"), HA1,2 and lipid were mixed at various HA1,2 to lipid ratios. The vesicles were prepared in the same manner as described above.

Total lipid in HA vesicles was quantified by the amount of R18 in the vesicles, based on the fact that R18 accounts for 15% of the total weight of lipids in the vesicles. The vesicles were solubilized by adding 0.1% (final concentration) Triton X-100 in PBS, and the fluorescence of R18 (excitation at 560 nm and emission at 590 nm) was measured using an aliquot of the solution with a spectrofluorometer (RF5300-PC, Shimadzu, Kyoto, Japan), calibrated with R18 standard solutions containing 0.1% Triton X-100. The range of the HA:lipid ratio (wt/wt) was 3.2–4.5 for vesicle preparations in the variable [F] experiment, and 0.29–3.4 for those in the variable [F-B] experiment.

The degree of R18 self-quenching in each HA vesicle was examined by comparison of R18 fluorescence before and after solubilization of the vesicles with 0.1% Triton X-100 in PBS. R18 fluorescence was quenched to an almost equal extent independently of the vesicle composition, i.e. to a range of 3.3–3.7% in the HA vesicles of the variable [F] experiment and to a range of 3.0–4.3% in those of the variable [F-B] experiment.

The HA1,2:(HA1,2 + HA0) ratio reconstituted on the vesicles in the variable [F] experiment was determined by SDS-polyacrylamide (10%) gel electrophoresis. The profiles of silver-stained gels of each vesicle preparation were digitized (Pictography 3000, Fuji Photo Film, Tokyo, Japan) and were quantified with National Institutes of Health Image 1.61 software. It should be noted that the vesicles prepared with the non-trypsinized HA sample only contained a trace amount (~2%) of HA1,2.

Electron Microscopy—A droplet of HA vesicle sample was put on a 400-mesh copper grid (Veco, Eersbeck, Netherlands) coated with collodion and then negatively stained with 2% uranyl acetate. Transmission electron microscopic images were obtained using a model H-7000 (Hitachi, Tokyo, Japan) operated at 75 kV.

Image Analysis of Fusion Events of Single Vesicles—HA vesicles in 0.1 ml of PIPES buffer (0.05–0.15 μg of lipid/ml) were added to ghost membranes on coverslips mounted in a metal chamber (31). After 5 min at 20 °C, unbound vesicles were removed by three washes with PIPES buffer, and the chamber was mounted on a laser scanning microscope (LSM 410, Carl Zeiss, Jena, Germany) equipped with a Planapochromat 63/1.4 numerical aperture objective. Capture of time-lapse sequences of the fluorescence images at 1.5-s intervals was started, and then to trigger fusion activity of HA, the PIPES buffer was replaced with 0.5 ml of an acidic buffer (145 mM NaCl, 20 mM sodium citrate, pH 5.2). Fluorescence of R18 upon excitation with a 543 nm HeNe laser was imaged by using a 590 nm cut-off filter.

To monitor the fluorescence intensities of individual fusing membranes, two kinds of regions of interest were drawn (see Fig. 2, B and H): 1) outlines of punctate fluorescence from HA vesicles, and 2) roughly circular regions, enclosing the periphery of fluorescence redistributed to ghost membranes, as judged from the final fluorescence images of the time-lapse sequences. The changes in fluorescence intensity at individual regions were measured with National Institutes of Health Image 1.61 software. The response times before fusion of single vesicles were determined by the timing of fluorescence flashes at more than 300 vesicle regions for each vesicle preparation. Kinetics of vesicle fusion was obtained from the cumulative sums of the response time distributions. The fusion rate, V, was calculated in each case from the tangent drawn to the straight line of the time-dependence curve of vesicle fusion and used for log-log plot analysis.

Reconstitution of HA into lipid vesicles by detergent dialysis often leads to fractions of protein/detergent/lipid micelles, “HA rosettes” (28). Although the maximum level in the ratio of cholesterol to egg PC (cholesterol/egg PC = 1:2 by weight) used for reconstitution was effective to reduce the formation of HA rosettes, minor fractions of HA rosettes were still possible. HA rosettes, however, were not included in the fluorescence flash analysis, because HA rosettes could not be discerned as bright punctate fluorescence under the microscope due to the size much smaller than that of HA vesicles. The results described below on fusion activity were attributable only to HA vesicles with intense fluorescence but not to HA rosettes.

K. Kawasaki, unpublished result.
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RESULTS

Response Time before Fusion Events of Individual HA Reconstituted Vesicles—Morphology of the HA1,2-reconstituted vesicles was examined by negative staining electron microscopy (Fig. 1). The surfaces of vesicles were covered with spike structures, consistent with the well known characteristic morphology of HA trimers.

As a first step in kinetic analysis of fusion by single-vesicle imaging, fluorescence microscopic images during fusion between the HA1,2-reconstituted vesicles and ghost membranes were examined. Individual vesicles were discerned as punctate fluorescence at pH 7.5, whereas the ghost membranes bound to the vesicles were invisible (Fig. 2A). Low pH treatment induced diffusion of R18 from the HA vesicles to the ghost membranes (Fig. 2B–H), which agreed well with R18 redistribution due to membrane fusion (32).

To precisely examine the time course of fusion events at single HA vesicles, the time-lapse sequences of images of HA vesicle-ghost conjugates, such as those shown in Fig. 2, B–H, were analyzed quantitatively. Fluorescence intensities were measured in the region of the HA vesicle, in the region of the ghost membrane fusing with the vesicle, and in the area including both of them (Fig. 2I). The low pH treatment to induce fusion did not cause any instantaneous changes in the fluorescence intensity in any of the regions. After several seconds, however, an abrupt increase in fluorescence (fluorescence flash) and a subsequent decrease were observed in the vesicle region. We defined the interval between the low pH treatment and the fluorescence flash as the response time. The increase was ascribed to fluorescence dequenching of R18 due to lipid mixing between the vesicle and the ghost membrane upon fusion, similar to the previously observed increase in fluorescence of R18-labeled influenza virus fusing with ghost membranes (33, 34). The decrease, on the other hand, was attributable to lateral diffusion of R18 from the vesicle region out to the ghost region, consistent with the simultaneous appearance of fluorescence in the ghost region. As the sum of these changes in the vesicle and ghost regions, the fluorescence intensity in the total area increased in a simple saturating manner with response time. Fluorescence flashes at HA vesicles were not observed at neutral pH (see the next section).

Among several aspects of the changes in the fluorescence images of fusing membranes described above, we hereafter examined only the response time between the low pH trigger and the fluorescence flash at a single vesicle, rather than the later phases of the changes in fluorescence, to discriminate the initial fusion events from the following lipid redistribution steps. Thus, we defined the onset of the fluorescence flash, i.e. the onset of lipid mixing at a single vesicle, as the onset of single-vesicle fusion. The response times before the fluorescence flashes were determined at hundreds of HA vesicles for each preparation, and used for the following kinetic analysis.

Variable [F] Experiment: Fusion Rates of HA Vesicles with Varying Surface Density of HA1,2 but with a Constant Surface Density of Total HA—For the first series of reconstitutions, we prepared co-reconstituted vesicles of HA1,2 and HA0 at various HA1,2/(HA1,2 + HA0) ratios (from 0.02 to 1.0) and at a constant ratio of total HA to lipid (within the range 3.2:1 to 4.5:1 by weight). HA1,2/(HA1,2 + HA0) ratios reconstituted in each vesicle preparation was quantitatively examined by SDS-PAGE (Fig. 3). Because HA1,2 is fusogenic and HA0 is non-fusogenic, the surface density of fusion-activity sites of HA on the vesicle membranes should be proportional to the HA1,2 density.
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content. On the other hand, the surface density of receptor-binding sites of HA on the membranes should be constant, because both HA1,2 and HA0 have equal receptor-binding activities. The three-dimensional structures of HA1,2 and HA0 differ only at the proteolytic cleavage site; the other residues, including the receptor-binding pockets, are essentially superimposable (35). We refer to this series of HA1,2-HA0 co-reconstitutions as the "variable [F] experiment" (Fig. 4, top).

The distributions of the response times for single-vesicle fusion were obtained at various HA1,2:(HA1,2 + HA0) ratios (Fig. 5A). For the vesicle preparation with 100% HA1,2 (Fig. 5A, panel a), the vesicle fusion was the most synchronized and the frequency of fusion at the peak level was the highest among the samples tested. On the other hand, the histograms broadened as the HA1,2 content in the vesicles was reduced (Fig. 5A, panels b–g). Cumulative sums of the distributions of the response times in Fig. 5A gave the kinetics of vesicle fusion (Fig. 5B). In the samples with 61 and 100% HA1,2, fusion events were observed with >90% of the vesicles, demonstrating that the functional reconstitution of HA on lipid vesicles was highly successful. The efficient fusion was not due to the relatively high concentration of R18 in the vesicles, because the fusion kinetics was independent of R18 concentration within the range 2–15% (supplemental Fig. S1 and supplemental Table S1). The optimal value of fusion efficiency of HA vesicles and ghost membranes was 60 times higher than that of HA-expressing cells and liposomes (<1.56% (12)), indicating the remarkable suitability of HA reconstitution for the quantitative analysis of HA-induced fusion.

It should be borne in mind that the increase in R18 fluorescence can sometimes be induced by lipid exchange between closely apposed membranes, as well as by fusion of the membranes (36). As shown in Fig. 5 (A and B), fluorescence flashes were not recorded with HA1,2 vesicles bound to ghost membranes at pH 7.5, indicating that the single-vesicle image analysis was not affected by possible R18 exchange without fusion. Under certain conditions of fusion, lipid mixing can proceed without any mixing of the aqueous content, a phenomenon known as "hemifusion," i.e. fusion that occurs only at the outer leaflets of lipid bilayers and not at the inner leaflets (37). Using single-vesicle analysis, we therefore examined whether or not vesicle-ghost fusion terminated at a hemifusion state. Microscopic analysis using HA vesicles double-labeled with R18 and calcein, a low-molecular-weight aqueous fluorescent probe, confirmed that the HA vesicles induced complete fusion with ghost membranes, without any indication of hemifusion (supplemental Figs. S2–S4 and supplemental Table S2).

The fusion rate (V) was obtained from the steepest slope of the curve of the plot in Fig. 5B, and the values were plotted against the surface density of HA1,2 (Fig. 5C). We hypothesized that the number of HA1,2 trimers required for fusion was n, and that assembly of n units of HA1,2 trimers was rate-limiting for induction of fusion. Then, V would be proportional to the nth power of [HA1,2], the surface density of HA1,2:

\[ V = k[HA_{1,2}]^n, \]

where \( k \) and \( C \) are constants. A log-log plot of \( V \) versus HA1,2 resulted in a straight line with a slope of 0.85 (Fig. 5D), evidently ruling out possible cooperativity of multiple
HA trimers for fusion. At the same time, the result strongly supported the other possible model that a single HA trimer is sufficient for fusion.

Variable [F-B] Experiment: Fusion Rates of HA Vesicles with Varying Surface Density of HA_{1,2} without HA_{0}. Next, we prepared vesicles with only HA_{1,2}, but without HA_{0}, at various protein-to-lipid ratios from 0.29:1 to 3.4:1 by weight. In this series of vesicles, the surface densities of fusion-activity sites and receptor-binding sites of HA on the membranes should change in parallel (Fig. 4, bottom). It was predicted that the kinetic data for fusion in this control experiment would be affected by the changes in receptor-binding ability.

Fig. 6A shows the distributions of the response times before single-vesicle fusion. Although the range of surface density of HA_{1,2} examined in the variable [F-B] experiments (HA_{1,2}/lipid ratio, 0.29–3.4) was similar to that in the variable [F] experiments (0.099–3.4), the change in the distribution of response time and fusion kinetics as a function of the surface density of HA_{1,2} appeared more profound in the variable [F-B] experiment (Fig. 6, A and B) than in the variable [F] experiment (Fig. 5, A and B). The highest rate of fusion plotted against the surface density of HA_{1,2} showed a sigmoidal dependence (Fig. 6C), in contrast to the result in Fig. 5C. A log-log plot of V versus [HA_{1,2}] gave a straight line with a slope of 2.2 (Fig. 6D), clearly distinct from the result in Fig. 5D. The difference between the results of the variable [F] and variable [F-B] experiments could be interpreted in terms of the difference in the condition of HA-receptor binding, as will be discussed in the next section.

**DISCUSSION**

Scheme of Fusion Induced by Single HA Trimmers—HA-induced fusion is known to consist of multiple steps (Fig. 7). First, binding of HA to viral receptors mediates close contact of the two membranes. Then, low pH triggers formation of long triplet α-helical coiled-coils on the vertical axis of the HA trimer, and this change relocates the fusion peptides at the amino terminus of HA_{0}, originally located ∼10 nm from the tip of the HA trimer, to the tip of the molecule (23, 24). The fusion peptides are inserted into the target membrane and is not bound to the receptors, the fusion peptides are inserted into the target membrane (39, 40). Additionally, the bottom half of the long α-helix, the portion near the carboxyl terminus of HA_{0}, reverses chain direction, which induces approach of the two membranes (2, 3, 24). The lipid mixing is preceded by opening and closing of the initial fusion pore (electrophysiologically recorded as “flickering”) and is then followed by pore dilation (6, 7, 14, 41).
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Despite various intermediates proposed for the mechanism of HA-induced fusion, the number and order of steps actually involved in the process have not been clarified, and therefore a consensus on the detailed kinetic scheme of HA-induced fusion has not so far been established (16, 42). Thus, we have employed a simple and practical approach, hypothesizing that assembly of multiple HA trimers on the membranes would be the time-limiting factor in the fusion. The result of kinetic analysis in the variable [F] experiment (Fig. 5D), however, evidently supported the mechanism of fusion by a single HA trimmer, as illustrated schematically in Fig. 7.

In the present analysis, HA vesicles pre-bound to ghost membranes were stimulated by low pH, and the onset of fusion was determined as the R18 fluorescence flash; the durations of the response times at individual vesicle-ghost conjugates were distributed over several seconds (Figs. 5A and 6A). This phase may still have consisted of multiple steps of membrane reorganization, such as the conformational change of HA, the fusion peptide insertion, the initial fusion pore formation, and the onset of lipid mixing. Thus, the kinetic evidence in Fig. 5D supports the hypothesis that assembly of multiple HA trimers is not required for the steps between the conformational change of HA and the onset of lipid mixing.

Several reports have argued that the lag phase (delay time) before lipid mixing indicates cooperativity of HA trimers for the fusion (11, 15, 43, 44). In general, however, a lag phase in kinetics would be an indication of multiple steps in the process, without necessarily implying cooperative involvement of multiple units (18, 45). Therefore, our model of fusion by single HA trimers (Fig. 7) is not inconsistent with the presence of lag before the onset of fusion.

Implications of the Restriction of Fusion by HA-Receptor Binding—The results of the variable [F-B] experiment were different from those of the variable [F] experiment. The sigmoidal curve in Fig. 6C would indicate a certain cooperativity of HA in the fusion mechanism, and the best-fit to the log-log plot in Fig. 6D apparently suggested that two or three HA trimers were required for fusion. It would not, however, be appropriate to deduce the number of HA trimers needed for fusion based on the variable [F-B] experiment, because this experiment involved a variation in the receptor-binding ability. Because such inappropriate experimental conditions would interfere with the fusion kinetics, we should not interpret the results in Fig. 6 (C and D) as being indicative of a fusion mechanism involving multiple HA trimers.

Alternatively, the results of the variable [F-B] experiment could be explained by the previously proposed hypothesis that fusion is induced by HA trimers not bound to sialate-containing receptors, rather than by those that are bound (12, 46–48). Binding of HA to viral receptors containing sialates enhances influenza viral fusion by stabilizing close contact between viral and target membranes (11, 46, 49, 50). On the other hand, viral fusion is rather reduced by a high surface density of receptors on target liposome membranes, suggesting that the subpopulation of HA trimers bound to the receptors is incapable of mediating fusion (46). It has also been reported that binding of HA trimers to sialate-containing receptors restricts the low pH-dependent refolding of bound HA trimers to a fusion-competent conformation (47).

These previous reports (46, 47) suggest that a proportion of HA trimers sandwiched between the membranes of HA vesicles and ghost membranes would be incapable of participating in fusion due to binding with the receptors (as drawn schematically in Fig. 7). Thus, in the variable [F-B] experiment, pre-binding of the HA₁₂ vesicles with ghost membranes would suppress the fusion activity of the vesicles by an extent dependent on the surface density of the receptors on the ghost membranes. The suppression would be more noticeable in the lower range of HA surface density, as depicted by the minimal fusion rates obtained with HA₁₂-lipid ratios below 2.0 (wt/wt) in Fig. 6C. The sigmoidal feature of the plot in Fig. 6C might therefore be attributable to the variation in the restriction of fusion activity by HA-receptor binding, as might be the steeper slope of the log-log plot in Fig. 6D (n = 2.2) compared with that in Fig. 5D (n = 0.85). Additionally, pre-binding of an HA vesicle to a ghost membrane might induce clustering of HA trimers in the contact region of the two membranes (48). The clustering of HA would increase the number of receptor-bound (fusion-restricted) HAs, which would also reduce the fusion activity of the vesicles especially in the lower range of HA surface density.

In certain fusion experiments using surrogate receptors such as anti-HA monoclonal antibodies (51) or streptavidin/biotin (52), instead of using the interaction of HA with sialate-containing receptors, receptor-HA binding did not exhibit any inhibitory effect on fusion. This type of fusion model would be potentially useful for performing the variable [F] experiment free from any interference with the fusion process by HA-receptor binding.

Variable [F] Experiments versus Variable [F-B] Experiments—In the present study, it was possible to directly compare the two types of fusion experiments with the same kinds of membranes, i.e. reconstituted vesicles of HA and ghost membranes. Distinct results were obtained in our variable [F] and variable [F-B] experiments, and as discussed above, the latter type of fusion experiment was not suitable for deducing the minimum number of HA trimers necessary for fusion. We should also reconsider previous kinetic analyses of HA-induced fusion according to this classification.

For example, Danieli et al. (15) utilized HA-expressing cells with various surface densities of HA. Because all the HA trimers were cleaved to be fusion-active, the experiment can be categorized as a kind of variable [F-B] experiment such as that shown in Fig. 4 (bottom). Therefore, their analysis of fluorescence lipid mixing and their conclusion that three to four HA trimers are needed for fusion should be carefully re-examined by taking the restriction of fusion due to HA-receptor binding into account, in the same manner as our variable [F-B] experiment.

The kinetic study by Günther-Ausborn et al. (19) used co-reconstitution of HAs from two strains of virus with different pH threshold values for the fusion. Their experiment and our variable [F] experiment were similar in that the surface density of fusion-activity sites was changed independently of that of receptor-binding sites (Fig. 4, top). Their results and ours led to a similar conclusion that a single HA trimer is sufficient for fusion. Their kinetic analysis, however, included ambiguity due to the use of bulk lipid mixing assay and of duration of the lag which will be discussed below. Our analysis by single-vesicle imaging without such factors could give more convincing results that were useful to resolve the long-standing problem of the minimum number of HA trimers required for fusion.

Rates of HA-induced Fusion Derived from Single-vesicle Imaging—The advantages of the analytical method described in the present report are discussed from two aspects.

First, the onsets of vesicle fusion were determined unambiguously by the fluorescence flashes at individual vesicles in the microscopic images. The fusion kinetics we obtained was not contaminated by lipid redistribution after fusion (Fig. 2I). In most cases, lipid redistribution between an HA vesicle and ghost membrane lasted for 20–40 s after the onset of fusion, irrespective of the surface density of HA₁₂. Similarly, the conventional bulk lipid mixing data would involve signals arising from both events averaged over a large number of membranes, as has already been pointed out (14, 53). The lipid mixing assay would therefore be unsuitable for the type of kinetic analysis employed in this study, because it would be difficult to obtain accurate fusion rates, even though the lipid
mixing assay is convenient for indicating the fusion activity of biological and artificial membranes.

Second, as a parameter for the kinetic analysis of fusion, the fusion rates should, in principle, be more appropriate than the lag time of lipid mixing in ensemble, which has sometimes been used in previous kinetic analyses (14, 42). The fusion rates estimated from the kinetic data in Figs. 5B and 6B reflected the overall distributions of the response times for vesicle fusion in Figs. 5A and 6A, including the population of non-fused vesicles. On the other hand, the lag time before lipid mixing in cuvette experiments would give information only about the small proportion of membranes that had fused by the earliest timing point (53).

Membrane Fusion Model Involving a Single Coiled-coil Motif Unit—In conclusion, our kinetic analysis based on the rate of membrane fusion strongly suggests that assembly of multiple HA trimers is not required for fusion. A single HA trimer appears to be sufficient to mediate the process between the low pH-induced conformational change of HA (including formation of a single unit of triplet coiled-coil) and the onset of lipid mixing (Fig. 7). The fusion by single influenza HA trimers leads us to speculate that intracellular fusion events might also be induced by a single coiled-coil motif unit consisting of intracellular fusogenic proteins. This simple mechanism might be suitable for certain fusion events that are much faster than HA-induced fusion and that take place on membrane vesicles smaller than influenza viral particles, such as the synaptic vesicle fusion induced by the α-helical coiled-coil motif of SNARE proteins (54).

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