Kinetics of Influenza Hemagglutinin-Mediated Membrane Fusion as a Function of Technique

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Reliable techniques are required to evaluate the plausibility of proposed membrane fusion mechanisms. Here we have studied the kinetics of establishing the lipidic connection between hemagglutinin-expressing cells (HA-cells) and red blood cells (RBC) labeled with octadecylrhodamine, R18, using three different experimental approaches: (1) the most common approach of monitoring the rate of the R18 dequenching in a cuvette with a suspension of RBC/HA-cell complexes; (2) video fluorescence microscopy (VFM) to detect the waiting times before the onset of R18 redistribution, not dequenching, for each RBC attached to an adherent HA-cell; and (3) a new approach based on blockage of RBC fusion to an adherent HA-cell at different time points by lysophosphatidylcholine (LPC), so that only the cell pairs which, at the time of LPC application, had fused or were irreversibly committed to fusion contributed to the final extent of lipid mixing. The LPC blockage and VFM gave very similar estimates for the fusion kinetics, with LPC monitoring also those sites committed to the lipid mixing process. In contrast, R18 dequenching in the cuvette was much slower, i.e., it monitors a much later stage of dye redistribution.

Membrane fusion is a ubiquitous biological event and understanding its molecular mechanism is central to biology. Based on the current knowledge of crystal structures of fragments of some fusogenic proteins and similarities between the dependencies of diverse fusion processes on the composition of the membrane lipid bilayers, it is hypothesized that fusion processes catalyzed by different proteins share a basic molecular mechanism (1–3). Elucidation of these mechanisms requires coupling of membrane fusion kinetics, and its analysis, with different aspects of the proteins involved, like conformational and mutational studies.

HA-mediated fusion involves three distinct kinetically significant events, which are detected using different experimental approaches. Electrophysiological measurements detect the establishment of the earliest fusion pore, a local aqueous connection between fusing cells that allows the first transfer of ions. Lipid mixing between the merged membranes is monitored as fluorescent lipid dye redistribution between labeled and unlabeled membranes. Similarly, aqueous contents mixing between membrane compartments can be measured as a spread of aqueous fluorescent probes within the fusing cells.

Much of our knowledge on membrane fusion mechanisms is based on different lipid mixing assays because of their relative simplicity in the technique. There have been a number of studies on the reliability of different probes that can be utilized for these lipid mixing assays (4–8). However, the question of how to best assay redistribution of any given probe has not been solved (9–11).

Here, we studied low pH-triggered fusion of HA-expressing cells and red blood cells (RBCs) labeled by the same fluorescent lipid probe, octadecylrhodamine (R18) using three different lipid mixing assays. We note that R18, which is a self-quenching probe, is not always a reliable reporter of transmembrane lipid flow. While in some cases the analysis of the total dequenching signal as a measure of fusion is complicated by the slow transfer of the probe without fusion and by R18

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2 Abbreviations used: HA, hemagglutinin; RBC, red blood cell; VFM, video fluorescence microscopy; LPC, lysophosphatidylcholine; PBS, phosphate-buffered saline; FDQ, fluorescence dequenching.
interactions with hydrophobic regions of the membrane proteins (12–14), R18-based fusion assays remain widely used because of their convenience (7, 15–18). Thanks to its relative rapidness and internal controls (only trypsin-cleaved and low pH-treated HA mediate fusion) HA-mediated cell fusion appears to be less affected by the known limitations of the R18 probe. The basic aim of our study was to compare three R18 redistribution assays: conventional R18 dequenching assay (15, 18, 19); video fluorescence microscopy (VFM) (20–22); and a new assay, where the kinetics is characterized by measuring the percentage of cell-RBC pairs irreversibly committed to lipid mixing at different time points. In the latter assay, we used lysophosphatidylcholine (LPC), a reversible inhibitor of disparate fusion reactions (2), to prevent new membrane merger events afterward. The validity of this assay is independent of the specific mechanism of the LPC inhibition (either LPC-induced increase in the elastic energy of the early fusion intermediates or direct interaction of LPC with fusion proteins (23)). Our results enable us to identify the usefulness and limitations of each technique in focusing on particular stages of lipid mixing kinetics.

MATERIALS AND METHODS

RBC Labeling, Cell Preparation, and Fusion

To label RBC membranes with R18 (18), we rapidly added 15 μl of R18 solution in ethanol (1 mg/ml) to 10 ml of a RBC suspension (1% hematocrit) in phosphate-buffered saline (PBS). Unbound R18 was removed by washing RBC with complete medium (15 min at room temperature) followed by 4 washings with PBS. Under these conditions, R18 incorporated into RBC membranes to a high enough concentration to cause significant self-quenching of fluorescence. HAb2 cells expressing HA of A/Fla strain (A/Japan/305/57) used for fusion experiments (19, 24) were grown to 10^6 erythrocytes per cell. The unbound RBC were removed by 3 washings with PBS. The HA-expressing cells with bound RBCs (0.05% hematocrit) were then used for experiments. Fusion of HAb2 cells with human red blood cells labeled by membrane dye R18 was triggered by application of the low pH medium (PBS titrated by citrate to acidic pH supplemented with 1 mM n-propylgallate), and assayed with fluorescence microscopy or spectrofluorometry as dye redistribution from RBC to HA-expressing cells. All experiments were performed at room temperature (20–22°C).

R18 Dequenching Assay

A SLM Aminco Luminescence Spectrometer (Urbana, IL) was used for all spectrofluorometric experiments. Excitation and emission wavelengths were 550 and 590 nm. Two milliliters of suspension of HA-expressing cells with prebound RBCs in PBS was placed into a fluorescence cuvette and stirred with a Teflon-coated flea. The pH in the cuvette was changed to the desired pH by injecting citric acid. The increase in fluorescence was normalized to a scale of 0–1 for the fraction of fluorescence due to lipid mixing at any given time, using the formula

\[
I(t) = \frac{[F(t) - F(0)]/[F(\infty) - F(0)]}{[F(\infty) - F(0)]},
\]

where, \(F(\infty)\) is the maximum possible probe redistribution due to fusion or lipid mixing only, i.e., not due to detergent lysis (25).

We have used a recently developed transformation for the dequenching data in a cuvette to waiting time data for proper comparison with the other assays (25). If the time required for the probe to redistribute is fast and not rate limiting compared to lipid mixing kinetics, this transformation is given by

\[
N(t) = 1 - (1 - I(t))^{1/2},
\]

where, \(N(t)\) denotes the fraction of cells with lipid mixing at time \(t\), and \(I(t)\) is the fluorescence intensity defined above. In Fig. 1, circles show \(I(t)\), the relative fluorescence due to lipid mixing. The corresponding fraction of cells with lipid mixing is shown by "x" sym-
bols using the above transformation. Smooth curves are shown to guide the eye through noise of the data.

**Video Fluorescence Microscopy Assay**

Fusion of HA-expressing cells with bound RBC was triggered by replacing PBS with an isoosmotic buffer titrated by citrate to acidic pH. Redistribution of R18 from RBC to HA-b2 cells was observed using a CCD camera and recorded on videotape. The videos were digitized using the WinTV-USB (Hauppauge Computer Works, Inc.) at a frame capture rate of 1 frame per second, and converted to Microsoft AVI format files using software AMCAP (Hauppauge Computer Works, Inc.). From the digitized video files, images were extracted at the resolution of 1 image per second. The images were then analyzed by eye to determine the time point showing the first indication for spread/redistribution of the fluorescent dye on the RBC (20–22). These images were also analyzed for obtaining the time point where no further spread of fluorescent dye from each of the above RBCs was observed, i.e., the time point beyond which no significant additional dye spread was observed. Image analysis, which involved only stacking of the digitized images to accelerate and enhance accurate second by second observation of images as compared to simple video playback, was done using Scion Image (National Institutes of Health, Bethesda, MD). Results are expressed as a cumulative distribution for fraction of cells showing lipid mixing at a given time. The cumulative distribution was obtained based on observing more than 10 fields at each pH, each field having at least 5 pairs of fusing cells. We ensured that HA-b2 cells with only one bound RBC were used to obtain the cumulative distribution.

**Lysophosphatidylcholine Blockage Assay (LPC Block)**

In these experiments, after low pH application, the medium bathing the plastic attached HA-expressing cells with prebound RBCs was replaced by neutral pH buffer supplemented with 270 μM LPC (lauroyl lysophosphatidylcholine) at different time points. The extents of lipid mixing were quantified using fluorescence microscopy as the ratio of dye-redistributed (in which there is observable spread of the dye) bound RBCs to the total number of the bound RBCs (14). Two hundred RBC-HA-b2 cell pairs were used to obtain the extents at each time point. Lipid mixing extents were always measured more than 20 min after low pH application. Longer incubations (up to 1 h) did not increase the extent of fusion.

**RESULTS AND DISCUSSION**

In the present study R18 redistribution from RBC to HA-cell was followed by three different assays:

1. **Fluorescence dequenching (FDQ)** of R18 upon its dilution in unlabeled HA-membrane. Increase in the fluorescence is measured in real time in a cuvette, containing a population of HA-expressing cells with bound labeled RBCs. Results are expressed in relative fluorescence units or FDQ% at a given time (7, 14, 15, 19, 26, 27). These data are then transformed to the simplest waiting time distribution (25).

2. **Video fluorescence microscopy detection of the onset of R18 redistribution**, i.e., onset of dye spreading, from a single RBC to an adherent HA-expressing cell (Fig. 2). Results are expressed as a distribution of waiting times for the first indication of spread of lipid dye (21, 22).

3. A new assay based on adding LPC at different times to block subsequent lipid mixing. That is, LPC blocks further fusion between RBCs and HA-expressing cells allowing us to find the fraction of cells already fused or irreversibly committed to fusion by the time of LPC application. This fraction can be measured as the final fusion extent (i.e., maximal percentage of dye-redistributed cell-RBC pairs) observed under given conditions. Fusion kinetics is then detected as a gradual increase in final fusion extent upon extension the time interval between low pH and LPC applications. Figure 3 shows a simple schematic for the LPC-blockage assay.

Among these assays, the first one detects lipid mixing at a stage when enough of the R18 is released from RBC to allow measurable dequenching of the dye (7). The second technique aims at detection of the very onset of the dye redistribution (21, 22). Finally, the third approach in essence allows each irreversible membrane merger event enough time to reveal itself as dye redistribution event (14).

The basic aim of our study was to draw a comparison between these three techniques that would enable us to identify the usefulness and limitations of each technique in focusing on particular stages of lipid mixing kinetics. For proper comparison the data of different kinetic measurements had to be plotted in a similar scale. Therefore the data on the changes in the extent of dequenching in a cuvette with time were recalculated into the waiting time data as described previously (25).

Figure 4 shows lipid mixing kinetics as a function of pH using VFM. This is a very rigorous technique that enables us to track fusion kinetics of individual cells up to a resolution of 1/30 s (currently, the maximum achievable frame acquiring resolution, see Materials and Methods). We used a resolution of 1 s here. At pH 4.8, kinetics of establishing the lipidic connection between HA-expressing cells and RBCs are the fastest, as shown by the triangles. At pH 5.2, the kinetics of establishing lipidic connection is much slower than at
4.8 (shown by squares) and, at pH 5.3 the kinetics is even slower (shown by diamonds). Thus, as expected, faster lipid mixing was detected when more HA molecules were activated by low pH.

Figures 5a and 5b show comparisons in lipid mixing kinetics as a function of technique at pH 4.8 and 5.3. At pH 4.8, Fig. 5a, when all or most of the HA molecules are activated, R18 dequenching in the cuvette (shown by "x") is much slower than the kinetics detected by either VFM or LPC-block approaches. Underestimation of the fusion rate by R18 dequenching relative to VFM is consistent with earlier findings (22). Importantly, at a higher pH of 5.3, as shown in Fig. 5b, all three techniques show similar kinetics. These findings suggest that the R18 cuvette dequenching assay detects late stages in lipid mixing event. Indeed, as shown in Fig. 6, R18 dequenching kinetics at pH 4.8 closely correlated with characteristic times of the completion rather than onset of dye redistribution as evaluated by VFM. These results indicate that at optimal pH, when fusion is relatively fast the rate of R18 dequenching is determined not by actual membrane merger events but by slower processes of dye redistribution through already formed fusion sites. At suboptimal pH, such as pH 5.3, the kinetics of fusion is slow enough to allow completion of lipid mixing events for each cell pair to be essentially completed before the next one starts. Therefore, in this case, assays detecting different stages of the lipid mixing report similar kinetics.
Interestingly, while the results obtained with the LPC-block technique were consistent with those obtained with the more rigorous VFM, on closer inspection we observe two contrasting trends from low to high pH values. At lower pH, kinetics measured using the LPC-block assay were slightly faster than those measured by VFM at the earlier parts of the lipid mixing curves (Fig. 5a), suggesting that at the early times some cell pairs irreversibly committed to fusion do not yet demonstrate lipid mixing. Both assays seem to be similar toward the latter part of the lipid mixing curves, indicating that fusion, rather rapid at optimal pH, reaches a final extent by the end of the VFM recording. In contrast, at higher pH, when fusion is much slower, both assays show similar kinetics at the beginning of the lipid mixing curve but start separating at the latter part, with kinetics from LPC-block assay being faster than those from VFM (Fig. 5b). This finding and similar results observed at pH 5.2 (data not shown) suggest that at suboptimal pH, when the number of activated HA molecules is lowered, some committed fusion intermediates take longer times to allow detectable lipid mixing, then at optimal pH.

A number of earlier attempts at deducing mechanistic information about the membrane fusion event have been based on population measurements in a cuvette. This analysis is clearly complicated by the fact that different mechanistic events in multiple contact zones take place at the same time (11, 37). Our data indicate that interpretation of R18 dequenching assay for measuring kinetics of HA-mediated lipid mixing might critically depend on the ratio between the waiting time of establishing lipid connection and the time required for redistribution of a significant portion of the dye. The contribution of the latter is relatively small if fusion is slow (for instance, at pH 5.3) or if lipid redistribution between fused membranes is very fast (for instance, most probably, for small labeled viral particle fusing with RBC or liposome vs RBC fusing with HA-expressing cell). In contrast to R18 dequenching assay, VFM determines fusion as an onset of dye redistribution and thus comes closer to being an assay for the first event.

In the LPC-block assay every cell pair committed to fusion at the given time is then given sufficient time to exchange lipid dye prior to the counting. This approach allows detection of the numbers of cells irreversibly committed to fusion at the time when lipid mixing is not yet observable. Thus, both VFM and LPC-block
assays are less dependent than R18 dequenching assay on the rate of actual redistribution of membrane dye.

At the same time, it must be emphasized that careful and proper analysis of the dequenching kinetics in a cuvette, as a part of the overall fusion process, provides useful mechanistic information about specific aspects of fusion reaction. Recently, kinetic data from HA-expressing cell lines, with different surface densities, fusing with RBCs were analyzed using a mass-action-based model for fusion (25). While kinetics of individual cell lines showed slower kinetic constants for cuvette dequenching experiments as compared to VFM, as supported by our findings here, the ratios between the key model parameters for different cell lines (i.e., relative to each other) were found to be insensitive to the experimental technique. Rigorous kinetic analysis of data from different experimental techniques yielded the same minimal fusion unit for HA-mediated fusion. It might be either that the results obtained were model-specific or that the model is adequate enough to extract HA-mediated fusion site architecture; however, the results emphasize a need for proper analytical treatment of the data obtained from a specific technique (25).

In a recent study, lipid mixing kinetics between RBCs and cells expressing different HA mutants were compared (28). While the assay used was VFM, single cell events were not monitored. Instead, total increase in fluorescence due to R18 dequenching in different fields was calculated. As a result, the kinetics obtained using VFM resemble those from cuvette measurements. Such a calculation is subject to arbitrary cut-off choices for threshold pixel values selected during image analysis, making the measurements dependent on these choices. Labeling inhomogeneity between individual RBCs further complicates the analysis since highly labeled cells dominate the measured kinetics for the population. In spite of the above important limitations of this experimental approach for quantitative analysis of lipid mixing, the approach clearly works for qualitative comparisons (as in 28).

In contrast to R18 dequenching in cuvette, VFM and LPC-blockage assays detect single events, which is always preferable (22). These techniques measure the kinetics of fusion rather than the overall rate of dye redistribution after fusion. Importantly, while VFM detects the fusion event only after lipid mixing between fused cells is allowed, LPC-blockage assay reports all cells which at the given time are irreversibly commit-
ted to allow lipid mixing eventually. Thus, future systematic comparison between the results obtained with these two assays under different conditions will be important for characterization of the elusive early fusion intermediates, where lipid flow through fusion sites is restricted (3, 23, 25, 29–36).

Note that possible application of VFM and LPC-blockage assays is not limited to using a particular lipid probe. We assume that any membrane or aqueous dye, which allows one to detect membrane fusion in any system (not necessarily viral fusion) with fluorescence microscopy, can be used to study fusion kinetics by VFM and LPC-blockage assays. Moreover, since LPC inhibits disparate fusion reactions (2), LPC blockage assay can be used to evaluate kinetics of these fusion reactions regardless of the specific assay used to determine final fusion extents. The successful application of the assay depends on finding the molecular species and concentration of LPC giving complete or almost complete LPC inhibition. In terms of time resolution VFM assay is limited by the video frame acquisition rate (e.g., 30 frames/s). LPC-blockage assay is limited by the rate with which exogenous LPC partitions into the membranes by \( \sim 10 \text{s}^{-1} \) (38). However, we note that these time scales are much faster as compared to our current understanding of lipid mixing kinetics for HA-mediated fusion. We believe that combining the strength of different kinetic measurements with known limitations will bring new clarity into the architecture of a membrane fusion site and molecular mechanisms of membrane fusion.

REFERENCES