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INITIAL SIZE AND DYNAMICS OF VIRAL FUSION PORES ARE A FUNCTION OF THE FUSION PROTEIN MEDIATING MEMBRANE FUSION

I. Plonsky[#], D. H. Kingsley^{\$}, A. Rashtian[@], P.S. Blank, and J. Zimmerberg

Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1855

Abstract

To investigate the role of the fusogenic protein in the initial size and dynamics of the pore that widens to finalize membrane fusion, two different fusion proteins expressed in the same cell line were investigated: the major glycoprotein of baculovirus *Autographa californica* (GP64) and the hemagglutinin of influenza X31 (HA). The host Sf9 cells expressing these viral proteins, irrespective of protein species, fused to human red blood cells (RBC) upon acidification of the medium. High time resolution electrophysiological study of fusion pore conductance revealed fundamental differences in a) the initial pore conductance (pores created by HA were smaller than those created by GP64), b) the ability of pores to flicker (only HA-mediated pores flickered), and c) the time required for pore formation (HA-mediated pores took much longer to form following acidification). Thus 1) HA and GP64 have divergent electrophysiological phenotypes even when they fuse identical membranes, and 2) fusion proteins play a crucial role in determining initial fusion pore characteristics. The structure of the initial fusion pore detected by electrical conductance measurements is sensitive to the nature of the fusion protein.

Keywords

viral fusion; baculovirus GP64; influenza hemagglutinin; time-resolved admittance measurements

INTRODUCTION

In biology, membrane fusion is the protein-mediated rearrangement of lipid structure that melds two surfaces into one surface; the process is crucial for intracellular membrane trafficking, fertilization, synaptic transmission, and enveloped virus infection. Enveloped viruses infect target cells by directly fusing to either the plasma membrane or, following endocytosis, the endosomal membrane. The latter process occurs after exposure of viral fusion proteins to the acidic endosomal environment that triggers their transition to fusogenic conformations (Weissenhorn et al., 1999). Since viral proteins are the best characterized fusogenic macromolecules, they are extensively used in studies on membrane fusion.

Address all correspondence to: Joshua Zimmerberg, Building 10, Rm. 10D14, 10 Center Drive MSC1855, Bethesda, MD 20892-1855, Tel: 301-496-6571, Fax: 301-594-0813, joshz@helix.nih.gov.

[#]current address: The Institute for Information Transmission Problems, Russian Academy of Science, 19 Bolshoy Karetny per., Moscow 101447, Russia.

^{\$}current address: Agricultural Research Service, USDA, Delaware State University, W. W. Baker Center, Dover, DE 19901

[@]current address: Afshin.X.Rashtian@kp.org

The envelope glycoproteins of baculovirus and influenza, GP64 and HA, respectively, are both necessary and sufficient for “low pH”-triggered membrane fusion (Blissard and Wenz, 1992; Monsma et al., 1996; White et al., 1982). Current models postulate that to enable fusion, homotrimers of both proteins aggregate in supramolecular structures of higher order (Blumenthal et al., 1996; Danieli et al., 1996; Plonsky and Zimmerberg, 1996; Chernomordik et al., 1998; Kozlov and Chernomordik, 1998; Markovic et al., 1998; Chernomordik and Kozlov, 2005). When a conformational change is triggered in HA, an N-terminal hydrophobic sequence (the fusion peptide) is exposed and inserted into merging membranes (Weber et al., 1994). GP64 is known to undergo a low pH conformational change and an internal hydrophobic segment of GP64 (a putative fusion peptide) has been identified and was found to be crucial for fusion (Monsma and Blissard, 1995). Despite substantial progress in biochemistry, molecular biology and crystallography of fusion proteins, the mechanism of membrane merger upon protein activation has not been elucidated. Further progress requires a combination of approaches.

Currently, electrophysiological investigation provides the highest temporal and signal-to-noise resolution of membrane fusion pores. Dynamic changes in the electrical admittance allow calculation of the capacitance of fusing entities, such as granules, vesicles, and cells (Neher and Marty, 1982). At early stages of membrane merger, two fusing objects are connected through a narrow aqueous pathway, defined as the fusion pore (Breckenridge and Almers, 1987; Zimmerberg et al., 1987). Fusion pore conductance also can be derived from admittance data. Analysis of fusion pore properties yields information about the ionic permeability of the initial pore intermediate and the kinetics and reversibility of its formation and growth. These properties are determined by many factors, including the conformational structure of the fusion protein in the membrane, the composition of the membrane, the forces between fusing membranes, and the membrane fusion pathway.

To begin to elucidate the contributions that each of these factors make in determining fusion pore “phenotype”, we have attempted to constrain the number of variables. Studying fusion of GP64-bearing Sf9 cells with either naive Sf9 cells or RBC, the target membrane was found not to dictate fusion pore characteristics (Plonsky et al., 1999). In the present study, the role of the host membrane is examined, as electrophysiological features of fusion pores created by acidification of either HA or GP64 were explored in one cellular system. Both glycoproteins were expressed in SF9 cells using the same promoter, and human red blood cells (RBC) were used as target cells. The initial fusion pores in the HA-mediated SF9/RBC system were similar in amplitude to those studied using HA-expressing mouse fibroblasts (Zimmerberg et al., 1994; Spruce et al., 1989), suggesting that the baculovirus expression system has little impact on initial fusion pore characteristics. More importantly, HA and GP64 induced markedly different initial fusion pore conductance and kinetics. These results support the hypothesis that the initial fusion pore conductance and kinetics are determined primarily by the fusion protein.

MATERIALS AND METHODS

Cells, Expression Plasmids, and Transfection

Sf9 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 28 °C in Grace’s insect cell medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 ug/ml streptomycin. Cells were used only until their 25th passage. The *Autographa Californica* multinuclear polyhedrosis virus (AcMNPV) GP64 expression plasmid pATH-1 was described previously by Kingsley et al., (1999). This plasmid utilized the *Orgyia pseudotsugata* multinuclear polyhedrosis virus (OpMNPV) GP64 early promoter to express the AcMNPV GP64 gene within Sf9 cells. In order to express HA in Sf9 cells, plasmid pHA-rhd was engineered by deletion of

the GP64 coding sequences and insertion of hemagglutinin (HA) sequences from the X31 strain of HA (Kingsley et al., 1999). Supercoiled plasmids were double purified by cesium chloride ultracentrifugation. Transfection of Sf9 cells was performed by the calcium phosphate method as described by Monsma and Blissard (1995). Forty μg of pHA-rhd or 10 μg pATH-1 were used to transfect 3×10^6 Sf9 cells in mid-log growth phase for 4 hrs followed by two washes with Grace's complete culture media and culture for 40 – 48 hrs in the same media. To render surface-expressed HA competent for fusion, HA-transfected cells were treated for 15 min with 20 $\mu\text{g}/\text{ml}$ trypsin (Fluka Inc, Ronkonkoma, NY) prior to use.

Electrophysiological Measurements

Fusion pore conductance and RBC capacitance were calculated using time-resolved admittance measurements as described previously (Ratinov et al., 1998; Plonsky et al., 1999), with modifications. A temperature that was permissive for fusion of cells mediated by both HA and GP64, $30 \pm 1^\circ\text{C}$, was determined and used in all experiments. A 2 KHz, 50 mV sine wave was superimposed on the holding potential (-20mV); the resulting signal (V_m) was applied to the patch-clamped Sf9 cell. The related current (I_m) was acquired and separated into “in-phase”, “out-of-phase”, and direct current components. The input conductance (G_{DC}), the real (Re), and the imaginary (Im) components of the cell-pipette admittance were calculated using a digital phase-sensitive detector (Figure 1A). The phase was adjusted using the automated Neher-Marty technique; fusion pore conductance (G_p) and RBC capacitance (C_{m2}) were calculated according to equations 55 and 56, respectively, derived by Ratinov and colleagues (1998). For whole-cell recordings, internal and external solutions had the following composition: 130 mM KGlu, 16 mM KCl, 2 mM MgCl_2 , 10 mM HEPES, 5 mM EGTA pCa 8, pH 7.0 (internal), and 55 mM NaCl, 10 mM KCl, 4 mM CaCl_2 , 5 mM MgCl_2 , 5 mM Glucose, 2 mM HEPES, 140 mM Sucrose, pH 7.0 (external). Fusion was triggered by an acidic solution: 50 mM NaCitrate, 50 mM NaCl, 10 mM KCl, 4 mM CaCl_2 , 5 mM MgCl_2 , 5 mM Glucose, 20 mM Sucrose (pH 5.0). This solution was ejected from a micropipette by a pressure pulse. The pipette was placed approximately one cell diameter from the transfected SF9 cell.

Fusion Pore Kinetics

The waiting time (t_w) was measured from the onset of the pressure pulse used to deliver the acidic solution until fusion pore opening. t_w points were displayed as a semi-log survival plot. For this purpose, the probability that a fusion pore has not yet appeared at time t (P_t) was defined as $1 - N_t/N_e$, where N_t is the number of pores that have formed by time t , and N_e is the total number of experiments (Melikyan et al., 1993). P_t was fit using the multi-element parallel model (MPM), described previously (Plonsky and Zimmerberg, 1996). Briefly, MPM suggests that fusion occurs when n elements of type **a** and one element of type **b**, independently enter their permissive states **a***, **b***:

where k_1 , k_{-1} , k_2 are rate constants. This kinetic scheme leads to the following theoretical probability function, $P(t)$:

$$P(t) = 1 - \left[k_1 (\exp(t(k_1 + k_{-1})) - 1) \cdot \frac{\exp(-t(k_1 + k_{-1}))}{k_1 + k_{-1}} \right]^n \cdot (1 - \exp(-k_2 t)).$$

Experimental data were fit using TableCurve 2D, v5.00 (SPSS Inc., Chicago, IL). Goodness of fit was evaluated using the F-statistic, parameter standard error, t-value, and residuals. Since GP64 dependent fusion was examined previously (Plonsky and Zimmerberg, 1996; Plonsky, et al., 1999), a limited data set was collected. The multi-element parameter, n , was set to 19, a value consistent with previous data; this parameter reduction was necessary to fit the reduced number of fusion events examined in this study. HA dependent fusion was modeled using integer n values (1 – 20). The $n = 3$ fit maximized the F-statistic and was used to characterize the model rate constants.

RESULTS

Time-resolved admittance measurements were used to characterize fusion (Figure 1). Previously, the electrophysiology of GP64-mediated fusion to both naive Sf9 cells and to RBC were described in detail (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999). As demonstrated previously, the delivery of the acidic solution to GP64-expressing Sf9 cells with bound RBC induced characteristic changes in cell admittance (Fig. 2 A)¹. GP64-mediated Sf9/RBC fusion was efficient; pores developed in all experiments ($n=12$). Although fusion of cell pairs (Sf9 cell with a single erythrocyte) was typically studied, transfected Sf9 cells were able to fuse with several RBC (Fig. 2). GP64-induced pores formed rapidly; the waiting time between the delivery of the acidic solution and the formation of the pore, t_w , varied between 0.18 and 1.91 s with the median of 0.27 s. Initial pore conductance was calculated as shown in Figure 2B, inset; GP64-induced pores had a mean value of 1.4 ± 0.8 nS (range: 0.6 – 2.9 nS, median 1.2 nS). Flickering was not observed in any GP64-mediated fusion events. Pores always expanded beyond 10 nS (our arbitrary cutoff); the rapid increase in pore conductance, G_p , is depicted in Figure 2B. Thus, characteristics of GP64-induced pores matched those reported previously (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999).

HA-expressing Sf9 cells fused with RBC in 34 out of 47 experiments. Compared to GP64, the HA-induced pore required more time to open (see below). The initial pore conductance varied between 0.2 and 2.1 nS; the median value equaled 0.7 nS. Flickering was observed in 29% of experiments. Further widening of the HA pore was hampered; in typical recordings that lasted 5 minutes, only 4 out of 34 pores exceeded 10 nS. An example of a rare flickering pore that successfully expanded beyond this arbitrary threshold is shown in Figure 3. Abrupt increments of conductance other than the initial pore are seen in the G_p trace. These increments are consistent with openings of other pores in the cells' contact area. Multiple HA pores were observed previously (Zimmerberg et al., 1994); the formation of a "molecular sieve" in the contact area was proposed to explain a lack of cytoplasmic dye transfer at high G_p . In general, fusion pores, induced by HA in Sf9/RBC contacts, were similar to those measured between HAb2 cells and RBC (Spruce et al., 1989, 1991).

Initial pores induced by HA and GP64, were different (Fig. 4 A). The cumulative distributions (CDF) of initial pore conductance (G_p) were both described using a cumulative log normal distribution, $CDF = 0.5 * \text{erfc}(\ln(G_p/M)/(W * 2^{0.5}))$, with distribution midpoints (M) and width parameters (W), $M_{HA} = 0.67 \pm 0.01$, $M_{GP64} = 1.11 \pm 0.02$, and $W_{HA} = 0.60 \pm 0.02$, $W_{GP64} = 0.53 \pm 0.04$, $n_{HA} = 31$ and $n_{GP64} = 12$. HA-induced initial pores

¹Theoretically, widening of the fusion pore leads to the reversible alteration in Re and increase in Im to a plateau level. Indeed, Re and Im is calculated using components of membrane current, I_m , that are in-phase and out-of phase with the command potential, V_m , respectively. V_m is applied to G_p and C_{m2} in series in such a way that, if the pore is low conductive (narrow), V_m drops across G_p , and I_m is not phase-shifted (in-phase). If the pore is wide, V_m drops across C_{m2} . Because capacitors are phase-shifting elements, in this situation I_m is mostly out-of phase. Also, if membrane potentials of Sf9 cell and RBC are not equal, the amplifier generates a current spike to clamp the RBC membrane to the holding potential applied to Sf9 cell. This spike is displayed in G_{DC} trace (Breckenridge and Almers, 1987).

were significantly smaller than those induced by GP64 (M_{HA} compared to M_{GP64} , $p < 0.01$, Fig. 4B).

HA-induced fusion between Sf9 cells and RBC had a substantial lag; the median waiting time between triggering and pore formation was 11.3 s (range: 1.6 – 94.7 s). The distribution of waiting times, t_w , was plotted as survival probability, P_t (Fig. 5, see “Materials and Methods” for details). The P_t curve, reflecting the kinetics of pore formation, had a quasi-sigmoidal shape; this shape was observed in earlier studies (Spruce et al., 1989). P_t was fit to the theoretical probability function, $P(t)$, derived from the multielement parallel model, MPM, with the multi-element parameter $n = 3$. The results of the fitting are shown in Figure 5. The following parameter values were obtained ($\pm$ standard error of the estimate): $n = 3$ (fixed), $k_1 = 0.33 \pm 0.04 \text{ s}^{-1}$, $k_{-1} = 0.04 \pm 0.00 \text{ s}^{-1}$, $k_2 = 0.06 \pm 0.00 \text{ s}^{-1}$. Interestingly, the value $n = 3$ that best describes HA-mediated fusion, was much smaller than the value obtained for GP64-induced pores ($n \sim 19$) (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999). In this study, with $n = 19$ fixed, the following parameter values were obtained for GP64-induced fusion: $n = 19$ (fixed), $k_1 = 16.9 \pm 1.04 \text{ s}^{-1}$, $k_{-1} = 0.05 \pm 0.03 \text{ s}^{-1}$, $k_2 = 3.64 \pm 0.56 \text{ s}^{-1}$. The forward rate constants, k_1 and k_2 , for HA-induced fusion are significantly smaller than those observed for GP64-induced fusion.

DISCUSSION

In the present study, we explored how two different viral envelope proteins fuse, on average, identical membranes. For this purpose, using the same promoter, the HA of the influenza virus and GP64 of the baculovirus were successfully expressed in the Sf9 system by transfecting cells with plasmids encoding these fusion proteins. Using admittance measurements, the admittance changes during membrane fusion mediated by HA of the influenza Japan strain (Spruce et al., 1989, 1991; Tse et al., 1993; Zimmerberg et al., 1994) and by GP64 of the AcMNPV baculovirus (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999) were recorded. HA-induced fusion pores had features closely resembling those previously described for the fusion of RBC to HA expressing fibroblasts, while GP64-induced pores had features closely resembling those described for the fusion of baculovirus infected Sf9 cells. Essential features of HA-induced fusion pores include small initial conductance (0.7 nS)², pore opening and closure before final expansion (flicker), and long waiting times (t_w) after triggering. GP64-induced pores reveal markedly distinct characteristics; their initial conductance is greater than 1 nS, they never flicker and, compared to HA, they have much shorter waiting t_w (Plonsky and Zimmerberg, 1996; Plonsky et al. 1999; this study). Prior to this study, conceivable reasons for the observed divergence in pore phenotypes may have been due to membrane composition and/or fusion protein since different membranes were fused in previous HA and GP64 studies. However, in this study, HA and GP64 induced membrane fusion revealed pores with clearly distinct characteristics while fusing identical membranes; indicating that the pore phenotype are functions of the protein mediating membrane fusion. GP64-induced pore had a high initial conductance ($M_{GP64} = 1.1 \text{ nS}$), never flickered, opened almost instantly (median $t_w = 0.27 \text{ s}$), and widened rapidly, whereas the initial conductance of the HA pore was smaller ($M_{HA} = 0.7 \text{ nS}$), and pores flickered, formed and dilated slowly or not at all. These data extend our earlier finding that the target membrane has little impact on the GP64 pore phenotype (Plonsky et al., 1999). Based on this study, we suggest that a) the molecular dimensions of the initial fusion site are determined by the fusion protein, and not by the fusing membranes; b) different fusion proteins induce pores with distinct characteristics; and c) the kinetics of HA pore formation can be described using the multielement parallel model, with $n \sim 3$

²These measurements were done with 1–3 ms time resolution. Faster acquisition yields HA pores having initial conductance of about 150 pS (Spruce et al., 1991).

elements. Thus, the properties of the initial fusion pore depend upon its initiator, the fusion protein. These findings rule out so-called proximity hypotheses which suggest that proteins bring membranes into contact and the ensuing fusion is a spontaneous reaction of contacting membranes, the same for all proteins capable of bringing membranes together. However, these findings do support studies showing that the transmembrane domain of fusogenic proteins and the amino acid sequence of the fusion peptide play an important role in determining fusion pore formation (Kemble et al., 1994; Han et al., 2004; Vaccaro et al., 2005). Thus, fusogenic proteins play a more intimate role throughout the pathway of protein-mediated biological fusion (Chernomordik et al., 1998; Kozlov and Chernomordik, 1998).

The size of the initial fusion pore is determined by fusion proteins

Electrophysiological methods provide valuable data on functional characteristics of the initial fusion site. These characteristics reflect a structure that is yet to be defined. To date, the initial fusion site is envisioned as a lipid-protein complex (Zimmerberg et al., 1991), rather than a pure protein aggregate (Almers and Tse, 1990; Lindau and Almers, 1995). Reported results support the first viewpoint (Kemble et al., 1994; Melikyan et al., 1995b; Chernomordik et al., 1998). Prior to fusion pore formation, membrane mechanics defines an energy barrier that must be overcome for successful fusion (Chernomordik et al., 1998; Kuzmin et al., 2001). Once the fusion pore is formed, its further dilation might be ensured by the purely mechanical properties of fusing membranes (Nanavati et al., 1992; Chizmadzhev et al., 1995). However, the major contributors to the system's free energy at the stage of the initial pore are unknown.

According to the lipid-protein model, the wall of the initial pore is coupled by supramolecular structures consisting of fusion proteins. The free energy of the initial pore is determined by interaction of two major forces provided by both the fusion proteins and highly bent membranes forming the pore walls. Lipid composition alters the elastic properties of membranes: lipids with positive spontaneous curvature (lysophosphatidilcholine) in the outer leaflet favor pore opening, whereas lipids with negative spontaneous curvature (oleic acid) would favor pore closure (Kozlov et al., 1989; Nanavati et al., 1992). Thus, the radius of the initial pore as well as the pore's tendency to close might be influenced by the lipid composition within the fusion "machine". In mast cells of the mutant (ruby-red eye) mouse, peculiarities of the exocytotic pore were interpreted in favor of a model where lipids are viewed as the major determinants of the pore free energy (Oberhouser and Fernandez, 1996). It is not known if this mutation affects the protein wall of the fusion site or alters the membrane lipid composition.

One clear difference between the membranes of insect and mammalian cells is their lipid composition. Yeh et al., (1997) indicate that insect Sf9 cell membranes contain a high proportion of diacyl phosphatidylethanolamine and fatty acid chains, with high proportions of 16:1 and 18:1 unsaturated lipids and low proportions of polyunsaturated fatty acids, making them considerably different than mammalian cell membranes which contain high proportions of phosphatidylethanolamine composed of both the diacyl and alkenylacyl forms. In addition, Sf9 cells are very low in cholesterol. These membrane compositional differences apparently did not affect greatly the observed electrophysiological characteristics of the initial pore in HA-mediated cell fusion, but may affect how pore enlargement evolves. This is investigated in another paper (Biswas et al., in preparation). Another compositional difference is that Sf9 insect cells generally do not produce complex glycoproteins containing penultimate galactose or terminal sialic acid (Hollister et al., 1998; Jarvis and Finn 1996). Studies with HA from an avian influenza A strain expressed in Sf9 cells has confirmed this altered glycosylation pattern (Wagner et al., 1996). Apparently terminal sugar modifications

of HA do not significantly effect the observed electrophysiological phenotypes either; HA expressed in mammalian and insect cells give congruent pore phenotypes.

Initial fusion pore formation: cooperative vs. parallel kinetic models

The process of viral fusion is believed to be enabled by aggregates consisting of a few, fusion-active protein trimers (Bundo-Morita et al., 1988; Ellens et al., 1990; Bentz et al., 1990; Stegmann et al., 1990; Gauden et al., 1996; Plonsky and Zimmerberg, 1996; Markovic et al., 1998; Gibbons et al., 2004). In addition, the reaction lag phase dependence on fusion protein surface density suggested a cooperative component in the mechanism for HA-mediated fusion (Morris et al., 1989; Ellens et al., 1990). Assuming that a) all three fusion peptides in the HA trimer are activated together, and b) their successful insertion in the target membrane facilitates incorporation of additional trimers as components of the same fusion machine, Danieli and colleagues (1996) suggested that three to four HA trimers form the initial fusion site.

The sigmoidal distribution of times between small and partially open HA-induced pores, led Blumenthal and his colleagues (1996) to use another approach that yielded the stoichiometry of fusion aggregates. Since small pores are not permeable to cytoplasmic dyes, trimers in the aggregate must move apart before dye transfers through fusing membranes (from the labeled to the unlabeled cell). Assuming that all trimers move independently (i.e. in parallel), the authors fit their data to a model first introduced for ionic currents (Hodgkin and Huxley, 1952). This approach yields 6 trimers for the initial fusion site at the beginning of its disassembly. The apparent controversy between conclusions on HA stoichiometry (3 – 4 cooperative vs. 6 parallel acting trimers in the fusion machine) could indicate that additional trimers are recruited into the circumference of the fusion site while the fusion pore begins to widen.

Studying GP64-induced fusion, we have found that the Hodgkin-Huxley formalism describes the quasi-sigmoidal distribution of the time required for pore formation (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999; this study). Here, we suggest that for GP64-mediated fusion a) the time interval between acidification and pore formation reflects processes occurring after the formation of the aggregate, b) all GP64 molecules in the aggregate are activated independently, and c) yet another unknown process coincides with the peptides' volley for fusion to occur³. Converging data on pore kinetics and conductance suggests that 5 to 8 trimers of GP64 might assemble the initial fusion site (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999; this study). Compared to HA, the higher initial pore conductance of GP64-induced fusion may be due to the difference in the stoichiometry of the initial fusion site: the GP64 fusion machine appears to have more participating trimers than that of HA.

In the present study we have shown that a kinetic model implying multiplicity and independence of components can describe the distribution of waiting times for HA- and GP64-mediated fusion. For HA, the rate-limiting parallel stage (presumably, the aggregation of trimers) is assumed to occur upstream to the activation of the fusion complex⁴. If this assumption is correct, the assembly of the fusion-competent complex can be modeled by means used to describe the opposite process, the disassembly of such an aggregate

³This process is modeled as a transition of the element **b** with the rate constant k_2 (see "Materials and Methods"). It makes $P(t)$ curve deviate from pure sigmoidal shape by multiplying the term in the power of n to the last exponent. Earlier, we have suggested that this process reflects the lipid-dependent stage of fusion (Plonsky and Zimmerberg, 1996).

⁴According to our earlier suggestion, in GP64-mediated fusion the rate-limiting parallel stage lies downstream to the formation of the complex (i.e. the complex assembly takes less time than its subsequent activation; Plonsky and Zimmerberg, 1996). The lack of the sharp dependency of t_w on GP64 expression (J. Fan, personal communication) suggests that GP64 fusion machine is preassembled to some extent

(Blumenthal et al., 1996). The following events might underlie the kinetics, the initial conductance and the reversibility of HA-induced fusion pores. Approximately 3 HA trimers aggregate in fusion-competent complexes. Their aggregation occurs independently: the association of the first two trimers does not facilitate the subsequent assembly of the complex. Since the HA aggregate is smaller than that of GP64, the HA-induced initial pore has a smaller conductance. Compared to GP64, smaller complexes of HA apply a weaker bending force to the fusing membranes. Therefore, pore formation requires either a favorable thermal fluctuation(s) of the opposite membrane(s) or a local, permissive lipid composition where fusion peptides are inserted. These conditions are reflected in the k_2 value which is much lower than that found for GP64-mediated fusion (0.06 vs. 3.64 s⁻¹). Also, since the bending force provided by the HA aggregate is assumed to be weak, the fusion pore formation would be reversible (i.e. the pore flickers).

In summary, we suggest that the lengthy lag phase, characteristic for HA fusion, is due to the two following processes having relatively low probabilities for completion. The first process is a non-cooperative aggregation of HA trimers. The second one is the successful bending of membranes to create the fusion pore. Although we have come to a similar conclusion for fusion complex stoichiometry, our approach differs from the cooperative model considered earlier (Danieli et al., 1996). It is important to note that the analysis performed by Danieli and colleagues did not preclude non-cooperative aggregation of HA trimers as a rate limiting stage of fusion. Since the present work lacks manipulation of HA surface density, cooperativity was not tested. However, we assume that the dependence of the lag phase, as well as the slope and the extent of fusion on HA surface density can be explained in terms of the parallel model. In studies on excitable cells, the more ion channels that are activated upon application of the triggering potential, the faster the current appears, the steeper is its slope, and the higher is its amplitude. The Hodgkin-Huxley formalism describes the time course of the current at any membrane potential above the threshold level (i.e. no matter how many ion channels are activated). Here the rate constants are dependent upon the membrane potential used in a trial while the n value, reflecting the number of permissive elements within the channel, remains the same. The kinetics of the HA and GP64 mediated fusion reaction resembles this type of behavior where the number of permissive elements in the fusion machine is dependent upon the fusion protein.

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References

- Almers W, Tse FW. Transmitter release from synapses: does a preassembled fusion pore initiate exocytosis? *Neuron*. 1990; 4:813–818. [PubMed: 1972885]
- Bentz J, Ellens H, Alford D. An architecture for the fusion site of influenza hemagglutinin. *FEBS Lett*. 1990; 276:1–5. [PubMed: 2265687]
- Blissard GW, Wenz JR. Baculovirus GP64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *Journal of Virology*. 1992; 66:6829–6835. [PubMed: 1404622]
- Blumenthal R, Sarkar DP, Durell SW, Howard DE, Morris SJ. Dilation of the influenza hemagglutinin fusion pore revealed by the kinetics of individual cell-cell fusion events. *Journal of Cell Biology*. 1996; 135:63–71. [PubMed: 8858163]
- Breckenridge LJ, Almers W. Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature*. 1987; 328:814–817. [PubMed: 2442614]
- Bundo-Morita K, Gibson S, Lenard J. Radiation inactivation analysis of fusion and hemolysis by vesicular stomatitis virus. *Virology*. 1988; 163:622–624. [PubMed: 2833025]

- Chernomordik LV, Frolov VA, Leikina E, Bronk P, Zimmerberg J. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. *The Journal of Cell Biology*. 1998; 140:1369–1382. [PubMed: 9508770]
- Chernomordik LV, Kozlov MM. Membrane hemifusion: crossing a chasm in two leaps. *Cell*. 2005; 123:375–382. [PubMed: 16269330]
- Chizmadzhev YA, Cohen FS, Shcherbakov A, Zimmerberg J. Membrane mechanics can account for fusion pore dilation in stages. *Biophysical Journal*. 1995; 69:2489–2500. [PubMed: 8599655]
- Clague MJ, Schoch C, Blumenthal R. Delay time for influenza virus hemagglutinin-induced membrane fusion depends on hemagglutinin surface density. *Journal of Virology*. 1991; 65:2402–2407. [PubMed: 1850019]
- Danieli T, Pelletier SL, Henis YI, White JM. Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. *Journal of Cell Biology*. 1996; 133:559–569. [PubMed: 8636231]
- Ellens H, Bentz J, Mason D, Zhang F, White JM. Fusion of influenza hemagglutinin-expressing fibroblasts with glycophorin-bearing liposomes: role of hemagglutinin surface density. *Biochemistry*. 1990; 29:9697–9707. [PubMed: 2271610]
- Gauden Y, Raux H, Flamand A, Ruigrok RWH. Identification of amino acids controlling the low-pH-induced conformational change of rabies virus glycoprotein. *Journal of Virology*. 1996; 70:7371–7378. [PubMed: 8892855]
- Gibbons DL, Vaney M-C, Roussel A, Vigouroux A, Reilly B, Lepault J, Kielian M, Rey FA. Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. *Nature*. 2004; 427:320–325. [PubMed: 14737160]
- Han X, Wang C, Bai J, Chapman ER, Jackson MB. Transmembrane segments of syntaxin line the fusion pore of Ca²⁺-triggered exocytosis. *Science*. 2004; 304:289–292. [PubMed: 15016962]
- Helfrich W. Elastic properties of lipid bilayers: theory and possible experiments. *Z Naturforsch*. 1973; 28:693–703.
- Hernandez LD, Hoffman LR, Wolfsberg TG, White JM. Virus-cell and cell-cell fusion. *Annu Rev Cell Biol*. 1996; 12:627–661.
- Hodgkin AL, Huxley AF. A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology (Lond)*. 1952; 117:500–544. [PubMed: 12991237]
- Hollister JR, Shaper JH, Jarvis DL. Stable expression of mammalian beta 1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. *Glycobiology*. 1998; 8:473–480. [PubMed: 9597545]
- Jarvis DL, Finn EE. Modifying the insect cell N-glycosylation pathway with immediate early baculovirus expression vectors. *Nat Biotechnol*. 1996; 14:1288–1292. [PubMed: 9631095]
- Kemble GW, Danieli T, White JW. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell*. 1994; 76:383–391. [PubMed: 8293471]
- Kingsley DH, Behbahani A, Rashtian A, Blissard GW, Zimmerberg J. A discrete stage of baculovirus GP64-mediated membrane fusion. *Mol Biol Cell*. 1999; 10:4191–4200. [PubMed: 10588652]
- Kozlov MM, Leikin SL, Chernomordik LV, Markin VS, Chizmadzhev YA. Stalk mechanism of membrane fusion. *European Biophysical Journal*. 1989; 17:121–129.
- Kozlov MM, Chernomordik LV. A mechanism of protein-mediated fusion: coupling between refolding of the influenza hemagglutinin and lipid rearrangements. *Biophys J*. 1998; 75:1384–1396. [PubMed: 9726939]
- Kuzmin PI, Zimmerberg J, Chizmadzhev YA, Cohen FS. A quantitative model for membrane fusion based on low-energy intermediates. *Proc Natl Acad Sci USA*. 2001; 98:7235–7240. [PubMed: 11404463]
- Lanzrein M, Kasermann N, Weingart R, Kempf C. Early events of Semliki Forest virus-induced cell-cell fusion. *Virology*. 1993; 196:541–547. [PubMed: 8372433]
- Leikina E, Onaran HO, Zimmerberg J. Acid pH induces fusion of cells infected with baculovirus to form syncytia. *FEBS Letters*. 1992; 304:221–224. [PubMed: 1618326]
- Lindau M, Almers W. Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr Opin Cell Biol*. 1995; 7:509–517. [PubMed: 7495570]

- Markovic I, Pulyaeva E, Sokoloff A, Chernomordik LV. Membrane fusion mediated by baculovirus GP64 involves assembly of stable GP64 trimers into multiprotein aggregates. *Journal of Cell Biology*. 1998; 143:1155–1166. [PubMed: 9832546]
- Melikyan GB, Niles WD, Cohen FS. The fusion kinetics of influenza hemagglutinin expressing cells to planar bilayer membranes is affected by HA density and host cell surface. *The Journal of General Physiology*. 1995a; 106:783–803. [PubMed: 8648292]
- Melikyan GB, White JM, Cohen FS. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *Journal of Cell Biology*. 1995b; 131:679–691. [PubMed: 7593189]
- Melikyan GB, Niles WD, Cohen FS. Influenza virus hemagglutinin-induced cell-planar bilayer fusion: quantitative dissection of fusion pore kinetics into stages. *Journal of General Physiology*. 1993; 102:1151–1170. [PubMed: 8133243]
- Monsma SA, Oomens AGP, Blissard GW. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *Journal of Virology*. 1996; 70:4607–4616. [PubMed: 8676487]
- Monsma SA, Blissard GW. Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. *Journal of Virology*. 1995; 69:2583–2595. [PubMed: 7533858]
- Morris SD, Sarkar D, White J, Blumenthal R. Kinetics of pH-dependant fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells. Measurements by dequenching of fluorescence. *J Biol Chem*. 1989; 264:3972–3978. [PubMed: 2917985]
- Navavati C, Markin VS, Oberhauser AF, Fernandez JM. The exocytotic fusion pore modeled as a lipidic pore. *Biophysical Journal*. 1992; 63:1118–1132. [PubMed: 1420930]
- Neher E, Marty A. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Science, USA*. 1982; 79:6712–6716.
- Oberhauser AF, Fernandez JM. A fusion pore phenotype in mast cells of ruby-eye mouse. *Proceedings of the National Academy of Science, USA*. 1996; 93:14349–14354.
- Plonsky I, Oomens AGP, Blissard GW, Zimmerberg J. The target membrane determines neither the initial conductance nor the reversibility of the GP64-induced fusion pore. *Virology*. 1999 Accepted.
- Plonsky I, Zimmerberg J. The initial fusion pore induced by baculovirus GP64 is large and forms quickly. *Journal of Cell Biology*. 1996; 135:1831–1839. [PubMed: 8991094]
- Ratinov V, Plonsky I, Zimmerberg J. Fusion pore conductance: experimental approaches and theoretical algorithms. *Biophysical Journal*. 1998; 74:2374–2387. [PubMed: 9591664]
- Spruce AE, Iwata A, Almers W. The first millisecond of the pore formed by a fusogenic viral envelope protein during membrane fusion. *Proceedings of the National Academy of Sciences*. 1991; 88:3623–3627.
- Spruce AE, Iwata A, White JM, Almers W. Patch clamp studies of single cell-fusion events mediated by a viral protein. *Nature*. 1989; 342:555–558. [PubMed: 2586627]
- Stegmann T, White JM, Helenius A. Intermediates in influenza-induced membrane fusion. *EMBO J*. 1990; 9:4231–4241. [PubMed: 2265606]
- Tse FW, Iwata A, Almers W. Membrane flux through the pore formed by a fusogenic viral envelope protein during cell fusion. *Journal of Cell Biology*. 1993; 121:543–552. [PubMed: 8486735]
- Vaccaro L, Cross KJ, Kleinjung J, Straus SK, Thomas DJ, Wharton SA, Skehel JJ, Fraternali F. *Biophys J*. 2005; 88:25–36. [PubMed: 15475582]
- Wagner R, Geyer H, Geyer R, Klenk HD. N-acetyl-beta-glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. *J Virol*. 1996; 70:4103–4109. [PubMed: 8648750]
- Weber T, Paesold G, Galli C, Mischler R, Semenza G, Brunner J. Evidence for H(+)-induced insertion of influenza hemagglutinin HA2 N-terminal segment into viral membrane. *J Biol Chem*. 1994; 269:18353–18358. [PubMed: 8034580]
- Weissenhorn W, Dessen A, Calder LJ, Harrison SC, Skehel JJ, Wiley DC. Structural basis for membrane fusion by enveloped viruses. *Mol Membr Biol*. 1999; 16:3–9. [PubMed: 10332732]

- White JM. Membrane Fusion. *Science*. 1992; 258:917–924. [PubMed: 1439803]
- White JM, Helenius A, Gething M-J. The hemagglutinin of influenza expressed from a cloned gene promotes membrane fusion. *Nature*. 1982; 300:658–659. [PubMed: 6815542]
- Yeh LP, Bajpai RK, Sun GY. Membrane lipid metabolism and phospholipase activity in insect *Spodoptera frugiperda* 9 ovarian cells. *Lipids*. 1997; 32:481–488. [PubMed: 9168454]
- Zimmerberg J, Blumenthal R, Sarkar DP, Curran M, Morris SJ. Restricted movement of lipid and aqueous dyes through pores formed by influenza hemagglutinin during cell fusion. *Journal of Cell Biology*. 1994; 127:1885–1894. [PubMed: 7806567]
- Zimmerberg J, Curran M, Cohen FS. A lipid/protein complex hypothesis for exocytotic fusion pore formation. *Annals of the New York Academy of Sciences*. 1991; 635:307–17. [PubMed: 1741589]
- Zimmerberg J, Curran M, Cohen FS, Brodwick M. Simultaneous electrical and optical measurement show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cell. *Proceedings of the National Academy of Sciences*. 1987; 84:1585–1589.

Fig. 1.

Principles of time-resolved admittance measurements. A. A sketch of the Sf9/RBC aggregate with the low pH buffer-containing delivery pipette and the recording pipette, connecting cells to the patch-clamp amplifier. The amplifier applies the command sine wave potential, V_m , to the cells; the resulting current, I_m , is transferred to the phase-sensitive detector. The latter device computes the cell(s)-pipette admittance vector characterized by its coordinates in orthogonal axes, Re and Im (real and imaginary, respectively). B. Electrical equivalent circuit of fusing cells. Before fusion (switch is off), the circuit consists of Sf9 cell elements: G_{m1} (membrane conductance), C_{m1} (membrane capacitance), and E_r (resting potential). After fusion has occurred (switch is on), the RBC sub-circuitry with the elements G_{m2} and C_{m2} (RBC membrane conductance and capacitance, respectively) becomes connected to the voltage-clamped Sf9 cell through the fusion pore. The related alterations in pipette-cells admittance allow to calculate the pore conductance, G_p , and C_{m2} and the direct current conductance, G_{DC} .

Fig. 2. GP64-induced fusion of transfected Sf9 cell with two RBC. A. Pressure pulse delivery of the low pH buffer (arrows) induced fusion pore formation with a short lag (~ 0.2 s). Fusion was revealed as characteristic changes in real and imaginary components of the pipette-cells admittance. Transients in G_{DC} are indicative of Sf9 cell fusion with two RBC. Note that during the experiment G_{DC} did not change drastically, signifying that the recording was “tight” (i.e. no corruptive changes in pipette-cells conductance occurred). B. Alterations in the cells-pipette admittance, shown in A, were converted to G_p and C_{m2} . G_p has risen beyond 10 nS in 0.04 s. The background noise in C_{m2} trace was defined as zero. The total increment of the Sf9 cell capacitance was equal to 2.1 pF, as expected for 2 RBCs; calculations of C_{m2} became more reliable with pore growing. (*Inset*) The conductance of the first fusion pore (red circles) is displayed in a milliseconds time scale. The abrupt opening of the pore was followed by its slower widening. Here and elsewhere, the initial conductance (2.8 nS) was calculated using linear regression of the recording segment adjacent to the abrupt pore opening.

Fig. 3.

HA-induced fusion of Sf9 cell with a bound erythrocyte. Fusion was triggered 4.5 s before the pore opening (not shown). G_p has risen to 10 nS in 0.9 s. C_{m2} was equal to 1.0 pF. (Inset) The flickering initial pore (red circles) with concurrent changes in G_{DC} (solid line) is shown in greater detail. Background G_{DC} was subtracted. Pore closure (arrows) interrupted the electrical connection between fusing cell, making the G_{DC} trace return to baseline. The conductance of the erythrocyte exceeded the noise level and equaled 0.1 nS. The initial conductance of the pore was 0.8 nS.

Fig. 4. Initial fusion pores induced by different viral fusion proteins in Sf9/RBC experimental system. A. GP64 induced non-flickering, highly conductive initial pores (panels 1 - 4), whereas HA pores often flickered and had lower initial conductance (panels 5 - 7). G_p (red circles) is co-plotted with G_{DC} (solid lines) for reasons explained in Figure 2, legend. A lack of G_{DC} transients in panels 2 and 6 signifies that in these experiments Sf9 cells fused with RBC having resting potentials about -20 mV. Gradual closure and reopening of an HA pore is shown in panel 5. Repetitive opening of an HA pore with a small conductance (0.4 nS) is shown in panel 8. B. Cumulative distribution functions of the initial conductance of GP64- (white circles) and HA- (black circles) induced fusion pores and the cumulative log-normal fits to the data. The median conductance values were 1.2 and 0.7 nS for GP64 and HA, respectively.

Fig. 5.

Waiting times between triggering of fusion and initial pore formation are displayed as a survival probability semi-log plot (see “Materials and methods” for details on data handling). Compared to HA (open squares), the waiting times for GP64-induced fusion (black circles) was drastically shorter. Both data sets were fit to the multielement parallel model (solid lines) with the number of elements, n , fixed at 19 and 3 for GP64 and HA, respectively.