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Mechanisms of influenza viral membrane fusion

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Abstract

Influenza viral particles are enveloped by a lipid bilayer. A major step in infection is fusion of the viral and host cellular membranes, a process with large kinetic barriers. Influenza membrane fusion is catalyzed by hemagglutinin (HA), a class I viral fusion protein activated by low pH. The exact nature of the HA conformational changes that deliver the energy required for fusion remains poorly understood. This review summarizes our current knowledge of HA structure and dynamics, describes recent single-particle experiments and modeling studies, and discusses their role in understanding how multiple HAs mediate fusion. These approaches provide a mechanistic picture in which HAs independently and stochastically insert into the target membrane, forming a cluster of HAs that is collectively able to overcome the barrier to membrane fusion. The new experimental and modeling approaches described in this review hold promise for a more complete understanding of other viral fusion systems and the protein systems responsible for cellular fusion.

Keywords: influenza, hemagglutinin, membrane fusion, single-particle, structure, modelling

1. Introduction

Membrane fusion is a key step in many biological processes. Processes such as intracellular compartmentalization and trafficking, neuronal signaling, entry of enveloped viruses, exocytosis, muscle repair, and cell-to-cell fusion in development all depend on enzymes that catalyze the merging of two lipid bilayers [1-8]. In cellular infection by enveloped viruses, membrane fusion represents the final step before the viral genome is released into the cytosol of the target cell. The key molecular step underlying fusion involves viral proteins that insert hydrophobic sequences into the target membrane and refold to drive merging of the lipid bilayers. So far, three major classes of viral fusion proteins have been characterized [4,5]. The first class comprises the fusion proteins of viruses such as HIV-1, ebola, and influenza. Class I fusion proteins are trimeric proteins with central coiled coil motifs as the key structural scaffold that enables the conformational changes needed for fusion. Class II fusion proteins, found in viruses such as dengue, zika and chikungunya, generally possess extended beta-sheet structures and rearrange from a dimeric geometry in the prefusion state into a trimer in the postfusion form. Class I and II proteins need to undergo a proteolytic priming and triggering event. Class III fusion proteins, for example from vesicular stomatitis virus and herpes simplex virus, show combinations of these structural motifs and lack a major priming event. The reovirus small proteins that induce cell-cell, but not virus-cell fusion have been proposed to represent a fourth class of viral fusogens [8,9].

One could consider viruses as evolutionarily optimized nanodevices, primed to enter and take over a host to ensure their continued existence [10]. The different viral fusion systems encountered in nature each represent elegant solutions to a biophysically challenging problem: the catalysis of the kinetically highly unfavorable merging of two bilayers. This review will discuss our current knowledge of the mechanistic operating principles of the influenza fusion machinery, arguably the most intensively studied viral fusion system. In particular, we will observe the problem through a biophysical lens; we will review structural knowledge on the influenza fusion system and discuss recent approaches relying on molecular modeling and single-particle microscopy that describe the fusion process. These biophysical studies suggest an intricate orchestration of the activity of a large number of fusion proteins as a key requirement for membrane fusion, suggesting that full understanding of viral fusion will need to come from both a detailed knowledge of the structural and chemical properties of the fusion proteins and a more holistic treatment of the interactions between larger numbers of fusion proteins connecting the viral and target membrane.

Influenza virus is a canonical example of an enveloped virus that has caused world-wide pandemics [11]. Because it inhabits multiple hosts and readily mutates, the threat of a new pandemic is real. The fusion of the viral and host cell membranes is mediated by hemagglutinin, a class I trimeric fusion protein. Viral entry is initiated by the virus binding to host-cell receptors via an interaction with a subdomain of the hemagglutinin and followed by cellular uptake into an endosomal compartment [12]. The low-pH environment of the matured endosome initiates a conformational change in the hemagglutinin structure causing it to extend and insert a hydrophobic N-terminal peptide into the target membrane. A subsequent refolding of the protein results in the two membranes to be pulled together and fuse.

Membrane fusion generally is not a spontaneous process on a biological timescale: the merger of two lipid membranes is thermodynamically favorable but has several kinetic barriers [13]. The essential characteristic of a biological membrane is the combination of a polar, hydrophilic exterior formed by the lipid headgroups and an apolar, hydrophobic interior containing the lipid tails [14]. Major players in membrane arrangement are the hydrophobic effect, giving rise to a poorly understood but strong interaction, polar and polarizing (Van der Waals) forces, and the interaction with water (hydration force) [15,16]. The interplay between these forces and the geometries of the system, such as lipid shape and membrane curvature, are key to the energetics of the membrane fusion process.

After being brought into close proximity, both the viral and target membranes have to be stripped of the hydration layer, the water layer that is tightly interacting with the polar headgroups of the lipids. Fusion of the two membranes then proceeds through a hemifusion stalk, an intermediate in which the proximal leaflets have merged. The final steps of fusion are the opening of a pore, so that aqueous contents on both sides of the membrane are connected, and subsequent expansion of the pore [13,17,18]. The progression from two separate membranes into a single contiguous one may be supported by lowering of the transition barriers, i.e. through the interaction with a catalyst or enzyme, or by the input of additional free energy through work [17,18].

This review aims to highlight the recent insights into the action of the influenza hemagglutinin as a catalyst and workhorse of this intricate membrane fusion process, and the role played by the kinetic steps and spatial distribution of HA as elucidated by single-particle studies. We will first discuss our current knowledge of the structural states and conformational dynamics of HA acquired from structural, computational and biochemical studies. As a central component of this review, we will then provide a description of single-particle methodologies and the insight they have given us, and discuss how collaboration of multiple hemagglutinins overcomes the membrane fusion barrier.

2. Hemagglutinin structure and conformational rearrangement

2.1 Hemagglutinin-mediated membrane fusion

The influenza A hemagglutinin (HA) is intensively studied and has since long served as a model system for viral fusion proteins [19]. The HA glycoprotein is synthesized as an inactive precursor, designated HA0 [20]. Cleavage in the trans-Golgi network by a host-cell protease results in a metastable, disulfide-bonded complex of HA1 and HA2 [21,22]. The crystallization of both the prefusion [23] and postfusion [24,25] structures of HA2 has brought tremendous insight into the large conformational changes involved in the fusion process. Biochemical and computational work has helped to fill in many details, including the role of HA1, the fusion peptide and possible intermediate states.

The global rearrangements of the trimeric HA1/HA2 complex and their hypothesized relations to the different steps of membrane fusion are depicted in Figure 1. The virus particle engages the target membrane with receptor attachment mediated by HA1 (A), which in later steps gives way for HA2 to extend (B). Upon lowering of the pH, the hydrophobic N-terminal end of the hemagglutinin is liberated from a pocket in which it was sequestered (B). This fusion peptide inserts into the target membrane, driven by the formation of an extended coiled-coil structure bridging the two membranes (C). The globule (yellow in Figure 1) at the base of HA melts and subsequently zippers up along the formed coiled coil, fusing the outer leaflets of the two membranes (hemifusion) (D). A pore is formed (E) when fusion peptide and transmembrane domain come together and the distal leaflets merge. Expansion of the pore then allows the viral genome to enter the cell.

2.2 HA structural rearrangements

The crystallographic structure of HA at neutral pH, shown in Figure 2A, reveals that HA1 forms a globular head in a region that is distal from the viral membrane. This part of the protein bears the receptor-binding domain (shown as a green hash) and is located 135 Å from the viral membrane [26]. Both the C- and N-terminal ends of HA1 extend towards the viral membrane, where they form a hydrophobic pocket for the fusion peptide (red in Figure 2). A disulfide bond near the N terminus of HA1 connects it to HA2 (black star in Figure 2A2). The core of the protein complex is formed by an 80 Å-long triple-stranded coiled coil of alpha helices from each of the three HA2 subunits. A globular domain at the bottom of this coiled coil forms the base of the protein (yellow in Figure 2A) and is connected to the three transmembrane helices that anchor the HA in the viral membrane. From the top of the coiled coil an unstructured loop (B-loop, blue in Figure 2A) doubles back towards the viral membrane, terminating in the fusion peptide. The sequence of the fusion peptide is highly conserved amongst different virus strains [27] and plays an important role in both triggering the conformational change [28] and manipulating the target membrane (reviewed in [29] and [30]).

In the low-pH, postfusion structure (Figure 2E), the B-loop has undergone a loop-to-helix transition and extends the central coiled coil (blue), together with the alpha helix that was already present in the prefusion state (grey helices). The helical stretch originally at the bottom of the central coiled coil (purple) has partly undergone a helix-to-loop transition, forming the turn in the postfusion hairpin structure. To facilitate this transition, the small globular bottom of HA2 (yellow in Figure 2A) is required to (partially) unfold while breaking the threefold symmetry of the trimer. This domain then packs into the grooves between the helices that form the core of the postfusion conformation (visible as yellow in Figure 2E1). For the related Influenza B virus, similar structural rearrangements have been found [32]. The Influenza C virus hemagglutinin esterase in addition functions as the receptor-cleaving enzyme [33].

2.3 HA intermediate conformational stages

While the structures of the prefusion and postfusion states of HA are known, the exact nature of the conformational transition between these two states is poorly understood. In the prefusion structure, HA2 is held in a metastable state by the surrounding subunit HA1 and the tight binding of the fusion

peptide. Destabilization of HA at a pH between 5 and 6 [34,35] or at elevated temperatures [36] induces the release of the fusion peptides from their pockets and dissociation of the 'clamp' formed by the HA1 globular domains, enabling a cascade of refolding events. The resulting release of energy is used to pull the membranes together for fusion [37].

2.3.1 Fusion peptide release mechanism

The release of the fusion peptides upon pH drop precedes the dissociation of HA1, as shown by antibody binding [38] and hydrogen-deuterium exchange experiments [39], and seems to be a reversible step [40,41]. This release is caused by protonation of specific residues in and around the peptide and its binding pocket [28]. Among others, His17 in HA1 and Asp109 and Asp112 in HA2 have been shown to influence the pH sensitivity, using mutants of HA that fuse at an elevated pH relative to the wild type [42-44]. However, protonation of one residue influences the protonation equilibrium of neighboring residues, which complicates the identification of single critical residues and makes it more likely that multiple residues can contribute to the destabilization of this region [44-46].

2.3.2 HA1 dissociation mechanism

Dissociation of HA1 is a necessary step for fusion, as shown by a chemical cross-linking of the globular domains inhibiting the fusogenic conformational changes and abolishing membrane fusion. [47-49]. At low pH, the HA1 subunits retain their structure and the ability to bind the sialic-acid cell receptor [50,51]. Key molecular switches that interrupt the association between the HA1 subunits have not been unambiguously determined. Fusion assays on HA mutants have revealed several salt bridges and hydrogen bonds at the subunit interfaces that are weakened upon protonation of one of the participating residues [28]. Among these are the highly conserved His184 at the HA1-HA1 interface [52] and His205 in a pandemic 2009 H1N1 strain [53]. Both the loss of specific stabilizing contacts and an increased net charge on the subunits could contribute to the dissociation of HA1 [46].

2.3.3 The extended intermediate

Before crystallization of the postfusion structure, the existence of the loop-to-helix transition had already been predicted by the discovery of a strong tendency for coiled coil formation in the initially unstructured B-loop [22]. The energy stored in this part of the prefusion trimer is released after removal of the clamp formed by the fusion peptide and HA1, inducing a 'spring-loaded' conformational change towards the state with lower energy [54]. Additionally, the fusion peptide, connected to the B-loop, has been shown to insert into the target membrane before fusion [55,56]. Together, these observations lead to the hypothesis of an extended intermediate that establishes the connection between the two membranes [22]. Indirect evidence for the existence of such an intermediate in fusion mediated by class I proteins stems mainly from the development of peptides that inhibit HIV entry by binding to an extended intermediate of the HIV fusion protein gp41 [57], especially when these peptides are anchored to the target membrane [58]. Time-of-addition

experiments with the peptides indicate that the gp41 extended intermediate exists for at least a few minutes [59]. Similar inhibitory peptides indicate the existence of the intermediate during refolding of influenza HA, although much higher peptide concentrations as well as cholesterol conjugation are needed for effective inhibition of influenza fusion [60]. Based on the average lag time between virion arrest and subsequent hemifusion, the lifetime of the extended intermediate of HA could be as much as one minute [61].

2.3.4 Refolding for hemifusion

The energy required to bring the membranes together is delivered by the unfolding of the globular bottom of HA2 and its packing into the groove between the helices of the extended intermediate (yellow in Figure 2) [62]. This leash-in-a-groove mechanism is inhibited by peptides derived from the amino-acid sequence in the leash, presumably by occupying the groove before HA refolding is complete [60]. Additionally, mutation of hydrophobic residues at the end of the leash decrease the efficiency of hemifusion. Further, additional residues beyond the leash, contacting the residues that cap the N-terminal end of the coiled coil, are likely to add a significant amount of energy by stabilizing the postfusion conformation [25]. It is still unclear whether the tight packing of these residues is necessary only for pore formation [63] or also for hemifusion [62]. If the fusion peptides fail to insert into the target membrane before hairpin formation, the HA protein can refold unproductively and end up in an inactivated state. This inactivation is demonstrated by an irreversible loss of fusion activity after pretreatment of the protein with low pH [64]. Moreover, in the absence of target membrane, the fusion peptides insert into the viral membrane, completing inactivation [65,66].

2.4 Membrane sculpting and pore formation

The HA transmembrane domains and fusion peptides play an active role in the fusion process rather than just providing a passive mechanical connection between the viral and the target membrane. For example, certain single amino-acid substitutions in the fusion peptide abolish fusion, while not hampering HA expression or conformational changes (reviewed in [30]). On the membrane surface, the 23 amino-acid fusion peptide forms a helical hairpin structure [67,68] with an inverted wedge shape that induces negative curvature in the membrane [69]. This membrane deformation is thought to have a stabilizing effect on the hemifusion stalk with its strong negative curvature. In addition to the active role of the fusion peptides, it has been shown that part of the transmembrane domain is necessary for pore formation and enlargement, but not for hemifusion [70-73]. The association of the fusion peptides with the transmembrane domain [25] is also implicated in membrane remodeling through an increase in membrane perturbation [74-76]. Hence, interactions between the fusion peptides, transmembrane domains and the lipid bilayers can lower the barriers involved in membrane fusion.

3. Collaboration between hemagglutinins as unraveled by single-particle experiments

3.1 Single-particle approaches to study influenza viral fusion

3.1.1 Kinetic studies of influenza viral fusion

The first methods to study the kinetics of fusion were developed in the 80s, with assays employing viruses fusing to liposomes in solution [77,78], viruses fusing to cells [79] and HA-mediated cell-cell fusion [80]. These and later studies revealed significant new mechanistic information. It was shown that fusion initiates by a pH-dependent step of HA2 [81]. The rates of HA inactivation and HA-mediated fusion were found to be correlated [82], and particle docking via receptor binding influenced the fusion rate [83]. The fusion rate correlates with the density of HAs expressed on cell surfaces [84-87], suggesting that fusion involves a step that relies on the participation of more than one HA trimer. However, whether this necessarily involves inter-HA interactions is hard to conclude from these types of experiments. A large number of studies have established that multiple HAs are needed for fusion, yet there is no consensus on the number of HAs involved [88-93]. The number found depends on the experimental technique used and the model applied. The inherent limitation of bulk fusion studies is the observation of only ensemble averages, obfuscating differences within the population that are likely to arise from stochastic molecular events. Furthermore, the advantage of using intact virions instead of HA-expressing cells is that HA is studied in the native context of a whole virus particle, and enables the extension of the system under study to include fusion inhibitors. Finally, the use of fast and synchronous triggering of the virus population has been difficult for bulk assays and observing with high data acquisition rates is paramount to resolving distributions within populations and short-lived intermediate states.

3.1.2 Single-particle assays provide access to hidden intermediates

In recent years, new experimental tools have been developed that enable the visualization of fusion events at the level of single viral particles. By monitoring distributions of properties of individual particles within a population rather than an ensemble average, information can be inferred about subpopulations. Furthermore, observation of the fusion process at the single-particle level allows for the visualization of short-lived states that otherwise would be averaged out due to the asynchronicity of the different kinetic transitions. The reader is referred to [94] for a review on single-particle methods to study fusion and to [95] for an overview of the various kinetic approaches to the study of influenza fusion in particular, both at the ensemble and single-particle level. Here, we will focus on new insights obtained by single-particle methods into the collaborative action of HA proteins on the surface of an influenza particle to mediate membrane fusion.

3.1.3 Experimental design of single-particle viral fusion assays

The main features and outcomes of a typical single-particle fusion assay are shown in Figure 3. The membrane and the aqueous interior of the virus particle are fluorescently labeled and shown in green and red, respectively, and their fluorescence is imaged using total internal reflection fluorescence microscopy (TIRF-M), a technique that allows the selective laser excitation of a very thin volume near the coverslip surface (Figure 3B). A planar target bilayer of controlled lipid composition is formed on a glass support and can be designed to incorporate lipid or proteinaceous receptors and a lipid-coupled pH-sensitive fluorescent probe. Synchronous acidification is achieved in a microfluidic channel by flowing in low-pH buffer [96], by light-induced liberation of caged protons [97] or by pre-mixing [98]. Using TIRF-M, low-background and high-contrast fluorescence signals are extracted to monitor particles rolling along the bilayer and to visualize arrest, hemifusion and opening of a pore (Figure 3A). The high concentration of dye in the viral membrane causes self-quenching of its fluorescence, allowing hemifusion to be detected as a dequenching and sudden increase in fluorescence when the dye escapes into the target membrane through the hemifusion stalk, followed by dissipation of the signal as the dye diffuses outwards into the supported bilayer (Figure 3C). Depending on the virion labeling procedure, the inner leaflet may hold dye that cannot escape, so abortive hemifusion events are not discriminated from successful ones. Disappearance of the content signal reports on the formation of a pore as the dye inside the particle escapes underneath the supported membrane (Figure 3D). Partway dissipation of the content dye shows either closure of the pore, or the presence of more than one particle in the spot. The times for individual particles to arrest, to hemifuse and to form a pore are collected and plotted in histograms such as the one shown in Figure 3E, F and G. Hence, by observing large numbers of single particles, the distribution of the population of fusing particles is observed, rather than an average of the whole population. These distributions allow us to draw conclusions about the molecular mechanisms underlying the fusogenic activity of HA (as discussed in more detail below).

3.2 Mechanistic insight into HA activity from single-particle experiments

3.2.1 Kinetic insight from single-particle histograms

In single-particle assays, the main experimental readout is the distribution of times that individual particles take to reach a certain state (arrest, hemifusion or pore formation), as seen in Figure 3. The shapes of these distributions contain information about the number of rate-limiting kinetic transitions needed to reach the observable state [99]. A process that requires only a single rate-limiting step results in a distribution that can be described by an exponential decay function, with the decay constant equal to the rate constant of the single rate-limiting transition (Figure 4A). In the case of multiple, different steps that need to be taken in sequence, a delay is introduced: each step has to wait for the previous to complete. The latter scenario results in a so-called rise-and-decay distribution that, in contrast to a single-exponential distribution, has a rise and fall in the number of events over time. Importantly, when a final state can only be achieved by a number of identical processes that take place in parallel, a similar rise and decay is observed as in the sequential case (Figure 4B). After all, the system arrives in the final state not until all required parallel transitions have completed.

Single-particle experiments on influenza viral fusion showed that the waiting times between decrease of the pH and the cessation of rolling, and hemifusion and pore formation events showed rise-and-decay behavior, suggesting that these processes involve multiple steps [61,88,96,97,100]. The powerful combination of single-particle experiments and analytical [101] and numerical [61] modeling has resulted in a picture in which fusion is the result of a number of HAs acting in a parallel, stochastic fashion, whose proximity allows their stochastic activity to result in a level of coordination needed to overcome the large energetic barriers associated with fusion. In the remainder of this review, we will analyze in more detail the evidence for such a stochastic view of cooperativity.

3.2.2 Particle rolling and arrest as a proxy for HA fusion peptide insertion

Single-particle fusion experiments on influenza with the pH drop performed in the presence of a laminar flow, showed rolling of the particles along the planar target membrane for some time after acidification [61]. The distribution of times for the particles to stop rolling after acidification showed a rise-and-decay behavior (Figure 3E). The way in which this behavior is interpreted and modeled is shown in Figure 4. The section of the virus in contact with the target membrane, the contact patch (Figure 4C, D), is modelled on a hexagonal grid (Figure 4E). With the dense packing of HA on a virion, the number of HAs typically in a contact patch is estimated to be 50–150 [61]. For visualization purposes, a patch of only 19 HAs is shown. Upon acidification, each HA trimer in the contact patch activates at a rate described by a single rate constant, determined by the rate-limiting step in the conformational transitions leading to extension and insertion of the fusion peptide into the target membrane. When a critical number of HAs have formed a bridge between the viral and target membranes, the particle is arrested. This situation corresponds to the process depicted in Figure 4B: a critical number of HAs need to have extended and inserted their fusion peptides before the particle is halted. When the individual insertion times are exponentially distributed, the total time for a number of these reactions to be completed will be represented by a convolution of single-exponential distributions and will have a rise-and-decay shape (Figure 4B). Using the combination of single-particle experiments and modeling, the typical number of insertions required for arrest was found to be three [61,101].

3.2.3 Insertion of the fusion peptide as a single-barrier transition

A framework to model protein conformational changes with a kinetic barrier was previously developed and successfully applied to single-molecule pulling experiments [102]. This model was also applied to HA membrane insertion, using a rate constant that is a function of pH [101]. As noted in Section 2, the influenza HA undergoes a proteolytic priming step that renders its prefusion structure metastable: the postfusion, conformationally rearranged protein has a lower free energy of folding [33]. At neutral pH, the barrier for the HA to traverse to this lower-energy postfusion structure is too high to overcome by thermal fluctuations alone. The rate of HA rearrangement was inferred to be pH dependent [39,103]. Experiments performed at elevated temperature show that fusion can also proceed at physiological pH [36], indicating that there is a kinetic barrier for the HA to undergo the conformational rearrangement. Figure 4A (left) shows the resulting model: the

barrier for the HA to extend and insert its fusion peptide into the target membrane is lowered with decreasing pH, and the resulting transition rate is pH dependent. For certain strains, this rate-limiting step was found to correspond to the expulsion of the fusion peptide from the pocket in which it was sequestered [61]. Hence, the probability of insertion of a single HA trimer is modeled as a single-exponential decay (Figure 4A right).

3.2.4 Hemifusion is mediated by a cluster of independent HA insertions

The distribution of the hemifusion times (Figure 3F) also shows that there are multiple steps involved in forming this intermediate. It has been found that the barrier to membrane fusion is too large to be overcome by a single HA and that 2 to 6 trimers need to participate together as a fusogenic unit. For influenza, it has been proposed that multiple HA trimers act together in a small area and that the formation of a critical cluster of neighboring inserted HAs drives fusion (Figure 4E rightmost) [89]. Other viruses similarly require the involvement of multiple copies of the fusion protein [4,104-106].

The collaboration of multiple HAs is modeled as follows. After the insertion of a critical number of HAs into the target membrane has firmly docked and immobilized the viral particle, visualized by the arrest of rolling, HAs continue to insert into the target lipid bilayer. With the HA trimers transitioning to the collapsed postfusion state, the two membranes will be pulled closer together at points where a trimer has successfully inserted its fusion peptides. When a group of HA trimers close enough in space and time (Figure 4E rightmost, example of 3 shown) convert to the collapsed conformation, the fusion barrier that is too large for a single HA can be overcome. Once such a cluster is formed, the collaborative refolding appears to proceed rapidly [61]. The rise and decay that is measured for hemifusion hence arises from the multiple steps involved, and has a different shape than the arrest distribution because of the geometrical requirement (Figure 4 right). Single-particle estimates for the cluster size vary somewhat between influenza strains, but lie around 2 to 3 [100,101].

3.2.5 Pore formation and pore expansion: multiple players

Pore formation is measured as a disappearance of the content dye signal from the particle. Figure 3G shows a typical experimentally determined distribution of times to pore formation. A particle-by-particle analysis of the times elapsed between hemifusion and pore formation reveals a single-exponential distribution and thus shows that the pore-formation step involves a single rate-limiting step. It is weakly pH dependent, but remains a one-step transition over the pH range probed [96]. The presence of a single rate-limiting step seems to suggest a mechanism that is not dominated by the stochastic action of individual HAs, as was observed for arrest and hemifusion. However, studies using HA expressing cells indicate that there is still involvement of the HAs [107] and the fusion peptide [108] in this step. Pressure from an expanding interior coat of matrix proteins has also been proposed to drive pore opening in a pH-dependent manner [109]. Moreover, the influence of properties of the membrane is still unclear. It is apparent that there is an intricate interplay of multiple factors driving pore opening and pore enlargement, the latter being the energetically most challenging step in the fusion process [13].

3.2.6 Hemifusion is abrogated at sub-stoichiometric levels of bound fusion inhibitor

The fusion step in the viral life cycle is a key target in the search for broad-spectrum antivirals, since the fusion mechanism is conserved across different strains or even across different viruses. Such drugs potentially provide longer-lasting efficacy than drugs targeting virus attachment or release (neuraminidase inhibitors), steps that are associated with pathways subject to much larger genetic variation [110-112]. Drugs targeting the conserved HA stem or extended intermediate seem particularly promising in finding a universal influenza therapy. A single-particle assay using antibodies and antibody fragments that bind the stem of the HA trimer in the prefusion state and prevent the low-pH conformational change showed the effect of inhibiting fusion-competent HAs one by one [113]. By counting the number of antibodies bound per individual virion and observing the resulting fusion yield, it was found that not all HA trimers need to be inhibitor-bound to fully abrogate fusogenicity, supporting the idea that hemifusion requires a network of HAs that can form a cluster (Figure 4B left). A virus particle decorated with a large number of inhibitors will take a longer time to achieve hemifusion, since it is less probable to form a cluster in the contact patch. When the number of inhibited HAs is sufficiently large to prevent the formation of a fusogenic cluster, the particle will fail to achieve hemifusion altogether (Figure 4G right and H rightmost). The presence of such time delay and concomitant reduction in fusion yield at intermediate inhibition levels was experimentally demonstrated [113].

3.2.7 The role of unproductive HAs

The modeling described above assumes a homogeneous coverage of fusogenic HAs on the viral particle. However, there are several reasons that such assumption is not correct. For instance, other proteins (neuraminidase, the M2 proton channel) may occupy space in the viral envelope otherwise taken by HA. Moreover, some HA trimers might fail to insert into the target membrane and inactivate. We will refer collectively to these as “unproductive” HAs. A parameter that sets the fraction of unproductive HAs in the population can be added to the model described above. In order to be able to quantitatively explain the inhibition levels measured in [113], a model including such unproductive HAs predicts that a large fraction of the HAs inherently is unproductive [114]. There are two major caveats, the first of which is that the contact patch size has not been experimentally measured making the relationship between experimental fusion data and modeling efforts somewhat uncertain [101,114]. Secondly, it is unknown whether all three epitopes on an HA trimer need to be inhibitor bound to render it nonfusogenic, or that one or two suffice. However, it seems clear that unproductive HA refolding occurs and that there might be evolutionary pressures that balance the efficiency of individual HA insertion with the ability to transition rapidly.

3.3 Open questions and future experiments

HA activation was found to correlate with the surface density of HA [84-87], consistent with the presence of interactions between trimers that facilitate fusion. Such inter-trimer interactions have been proposed, as well as the formation of a fusogenic ring of HAs. Nevertheless, single-particle

experiments show that such hypothesized inter-trimer interactions are not necessary to explain hemifusion kinetics. Instead, as argued above, a model of stochastically firing HAs suffices to explain all reported kinetics. It remains unclear, however, whether HA assembly into a cluster is rate-limited by the diffusion of the HAs in the membrane, given the low surface density of HA in cell-based assays. Also, HA activation is reduced in the absence of a target membrane [114-117], and there are reversible conformational changes in the activation pathway [39-41]. These are all phenomena that are not incorporated into the stochastic models due to the lack of single-particle evidence. Likewise, the treatment of the HA conformational transition as a single activation barrier, and hence as a single-rate transition, is likely to need amendment once more detailed data regarding this transition become available, for example from strains exhibiting a high pH threshold. Furthermore, whether HA can unproductively refold after successful target membrane insertion is still unknown.

There is evolutionary pressure that balances the number of trimers required for fusion, the rate at which the trimers activate, and the specific pH optimum of activation [100]. These reflect a balance between transmission stability in acidic environments, which contributes to potency to cause pandemics [118], and the location of viral RNA release in the infected cell. This location can be an early endosome, far from the nuclear target, or close to the target in a late endosome, with a higher risk of HA degradation by enzymes [100]. Variation in preferred entry pathway has been found for HIV-1 as well as for influenza [100,104] and future research into the connection between these factors may contribute to a better understanding and prevention of flu pandemics.

Summarizing, an emerging molecular view of influenza fusion is that of a tug-of-war that starts after a field of HA trimers have activated stochastically. With a section of the virus in contact with the target membrane (Figure 4A, flattened view shown in Figure 5), several HAs potentially can collaborate in fusion. After acidification, each HA in this contact patch individually has to overcome the barrier to extension and insertion (Figure 5A), and HAs may accumulate in different stages of refolding, apposing both membranes (Figure 5B). Once a critical cluster of neighboring HAs has inserted and folds back (Figure 5C), hemifusion ensues rapidly. Finally, formation and enlargement of a pore (Figure 5D) connects the cellular and viral compartments, initiating viral replication as the viral genome invades the cell.

4. Towards an understanding of viral and cellular fusion

Thus far, only a subset of the protein machineries mediating cellular fusion have been identified and their mechanisms of action characterized. Nonetheless, the proteins involved seem to be related to those of viruses (reviewed in [8]). Bearing semblance to the different classes of viral fusion proteins, cellular fusogens have been classified into class I and II: class I containing large alpha-helical domains, and class II defined by beta-sheet structures. The class II cellular fusogens share a common ancestral gene [119], later in evolution hijacked by different viruses [120]. Class I viral fusogens derive from retroviral genes [8]. Similarities between the mode of action of the viral fusion proteins and their cellular counterparts suggest that the advances in the field of viral fusion may help to guide understanding of the proteins involved in cellular and developmental fusion.

As recent work in viral fusion has demonstrated, significant advances in mechanistic understanding of the fusion process can be made by combining structural insight with the combination of single-particle experiments and modeling approaches. The strength in this philosophy is that each approach provides new hypotheses testable in the other. The ability to isolate systems *in vitro*, so that an incremental understanding can be obtained as the system is gradually made more complex, appears to be key. Atomistic and coarse-grained molecular modeling may provide the next step in bridging all relevant length and time scales and finally may provide a full dynamic, atomistic model of fusion. As more and more similarities across systems are revealed and experimental approaches become applicable to a wide range of viral and cellular fusion systems, the biophysical and biochemical foundations will be laid for the field of 'fusionology'.

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6. Author contributions

JB and SB wrote a draft of the manuscript and produced Figures 1 through 4. All authors contributed in revising the draft and finalizing the manuscript.

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Figure captions

Figure 1. The influenza hemagglutinin-mediated membrane fusion pathway. (A) The HA1 subunit (orange) binds sialic-acid moieties on target-cell receptors (dark brown). (B) After acidification, the HA1 subunits give way and the fusion peptide (red) is liberated from its sequestered position, to insert into the target membrane (C), allowing the HA to bridge the two membranes. The HA1 subunits are not shown from panel (C) onward. Subsequently, the trimeric HA2 then zippers up along itself, bringing both membranes in close proximity and leading to hemifusion (D) and the opening of a full fusion pore (E). Known structures are represented in (A) and (E), others are inferred. For clarity, only two subunits of the trimeric HA are shown.

Figure 2. Crystal structures of HA from the neutral pH prefusion state (A in Figure 1, PDB: 1HGF [31]) to the postfusion state (E in Figure 1, PDB: 1QU1 [25]) at low pH. Color coding and stage labelling as in Figure 1. The membrane (green), fusion peptide (red) and transmembrane domain (grey) are shown schematically, together with the linkers connecting them to the protein. (A1 and E1) Surface representation of the HA trimer. (A2 and E2) HA2 trimer in cartoon representation. In A2, HA2 is covered by HA1 in transparent blue, the disulfide bond linking HA1 to HA2 is indicated with a black star and one of the receptor binding sites with a green hash. (A3 and E3) HA2 monomer cartoons.

Figure 3. Single-particle assay features. (A) Fluorescently labeled viral particles are imaged as they interact with a planar supported lipid bilayer, and their dynamics (rolling, arrest, hemifusion and pore formation) are visualized. Viral membrane is labelled green, aqueous contents are red, and hemifusion and pore formation are detected as the escape of each respectively. (B) A thin layer (~100 nm) is imaged using total internal reflection fluorescence microscopy (TIRF-M), selectively exciting and detecting the weak fluorescence from individual particles that are associated with the membrane. (C) Hemifusion is detected as an increase of green fluorescence upon lipid mixing due to relief of dye quenching within the viral membrane. (D) Pore formation is detected as dissipation of the red signal as the content dye can leave the particle. (E) A histogram of viral arrest times shows a rise and decay, suggesting the presence of multiple kinetic transitions. (F) Hemifusion times also show a rise and decay. Both rise and decays are explained in Figure 4. (G) The time from hemifusion to forming a pore is exponentially distributed, suggesting the presence of only one rate-limiting transition; the black line shows a single-exponential fit. C and D adapted from [94]; E, F and G data from [61,96].

Figure 4. Particle arrest and hemifusion mediated by multiple HAs within a contact patch. (A) At neutral pH, the transition of HA from prefusion (blue) to inserted (red) form is dictated by a large energy barrier (dotted line). At low pH, the barrier is lowered (thick line) and the HAs more readily cross the barrier with a single rate k , yielding a single-exponential distribution for individual insertion times (shown right). (B) HAs in the contact patch, synchronously acidified, insert stochastically and in parallel with single-exponential probability. The arrest distribution is then found as a rise and decay from the convolution of multiple exponentially distributed insertions. (C) The contact patch (blue) is defined as the region of the viral coat (grey) in contact with the target membrane (green). (D) A network of HAs (blue) that can participate in mediating fusion fills the contact patch. (E) The network of HAs is modeled on a regular grid. The virus particle rolls along the membrane in the flow, forming and breaking interactions with the sialic-acid receptor on the target membrane. After synchronous

acidification, HAs independently and stochastically extend and insert their fusion peptide into the target membrane (red). A critical number of insertions (circles) arrests rolling of the particle. Formation of a critical, local cluster of inserted HAs (circle) induces hemifusion. (F, G) Inhibitors (black; for example antibodies) interfere with the network of HAs. (H) Distributions of times to arrest (left) and hemifusion (middle) show a rise and decay because of the multiple intermediate steps involved. For hemifusion, the shape of the distribution is a combination of the independent insertions and the requirement to form a local cluster. For virions with a certain percentage of their HAs blocked by inhibitors, hemifusion, i.e. formation of a fusogenic cluster, is delayed, or abolished (right). Example contact patch of 19 HAs shown; realistic estimates are 50-150 HAs. Critical number of insertions to arrest and cluster size for hemifusion may vary across strains; the example shown assumes critical numbers of 3.

Figure 5. The interplay of multiple HAs to collectively overcome the barrier to membrane fusion. A flattened artist's impression of the viral surface (bottom layer) and parts of the target cell membrane (top membranes) is shown to convey the three-dimensional geometry of the process. Metastable HA in the prefusion conformation cover the virus surface (blue bundles). (A) An extended intermediate is formed after low-pH triggering. (B) HA refolding brings the membranes closer together, forming dimples in the viral and cell membranes. (C) Once a critical, minimal cluster of inserted HAs has formed, hemifusion ensues. (D) A fusion pore is formed, connecting viral and cellular interiors to allow transfer of the viral genome.

Neutral pH: High barrier

Low pH: Lowered barrier

Rate k

Insertion probability

Frequency of insertion

Insertion time

Rate k

The diagram illustrates the relationship between time to insertion, time to arrest, and the resulting arrest distribution. It shows three rows of plots, each representing a different scenario. The first row shows a plot of Time to insertion (blue curve) and a plot of Time to arrest (red curve). The second row shows a plot of Time to insertion (blue curve) and a plot of Time to arrest (red curve). The third row shows a plot of Time to insertion (blue curve) and a plot of Time to arrest (red curve). The bottom row shows the convolution of the Time to insertion plots, resulting in a plot of Time to arrest (red curve). The label 'Arrest distribution' is placed above the final plot.

C membrane
contact patch
virus

D

E Docked & acidified → Insertion₁ → Insertion₂ → Insertion₃: Arrest → ... → Cluster: Hemifusion

F

G

H Arrest distribution Hemifusion distribution
Time to arrest Time to hemifusion
Inhibitor: delay or abolition

● = hemagglutinin
● = inactivated hemagglutinin
● = inserted hemagglutinin
Y = inhibitor (antibody)
--- = potential connection
○ = critical insertions/cluster