The pH sensor for flavivirus membrane fusion

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Viruses that infect cells by uptake through endosomes have generally evolved to "sense" the local pH as part of the mechanism by which they penetrate into the cytosol. Even for the very well studied fusion proteins of enveloped viruses, identification of the specific pH sensor has been a challenge, one that has now been met successfully, for flaviviruses, by Fritz et al. (Fritz, R., K. Stiasny, and F.X. Heinz. 2008. *J. Cell Biol.* 183:353–361) in this issue. Thorough mutational analysis of conserved histidine residues in the envelope protein of tick-borne encephalitis virus led Fritz et al. (2008) to identify a histidine at a key domain interface as the critical pH sensor; its protonation triggers the large-scale conformational rearrangement that induces fusion of viral and endosomal membranes.

The acidic pH of endosomes is one of their simplest distinguishing characteristics. Most viruses that pass through these compartments en route to productive infection have evolved to "sense" the local proton concentration as part of their mechanism for crossing into the cytosol. For enveloped viruses, fusion of their lipid bilayer with the membrane of an endosome is generally the pH-dependent molecular step, catalyzed by a "fusion protein" on the viral surface (Harrison, 2008; White et al., 2008). Although these proteins have been studied in great detail for over 30 yr, it has not been easy in any of the well characterized examples to pin down the molecular identity of the pH sensor. Histidine residues are plausible candidates, as they titrate in the relevant range, but suitably poised carboxylate pairs can have a similar pK. The long history of working out the origins of the hemoglobin Bohr effect show how tricky such a search can be (e.g., see Riggs, 1988). Moreover, charge interactions, even those with conserved physiological functions, can move around on a protein relatively easily in the course of evolution. For example, a redundant charge pair can appear by mutation, with a similar pK as that of an existing one, allowing the initial charges to disappear in some subsequent evolutionary step, without drastic change in titration properties. Exquisite stereochemistry is often not required.

Fritz et al. (2008) (see p. 353 in this issue) have taken on the challenge of determining the pH sensor for flavivirus fusion by meticulous and exhaustive mutational analysis of conserved histidine residues in the fusion protein of tick-borne encephalitis virus (TBEV). Their work builds upon elegant analyses of TBEV fusion by Heinz and co-workers over many years, including their essential contributions to structure determinations of the protein, both at neutral pH and after acidification (Rey et al., 1995; Bressanelli et al., 2004). Flaviviruses are particularly compact structures, only ∼500 Å in diameter, tiled on their surface by 180 envelope protein (E) subunits in an icosahedral array (Zhang et al., 2003), as illustrated in Fig. 1 a. Within this outer layer is the viral membrane, a roughly spherical bilayer ∼410 Å in outer diameter. The viral positive-strand RNA genome encodes three structural proteins—an internal, RNA packaging "core" protein (C) and two membrane-anchored proteins, prM and E (Lindenbach et al., 2007). A C protein–RNA complex buds into the endoplasmic reticulum, acquiring a membrane with 180 prM-E heterodimers in the process. The prM protein is a specific chaperone for E (Fig. 1 b). In the trans-Golgi network (TGN), furin cleavage of prM to a membrane-anchored residual fragment (called M) allows E to settle into the regular array illustrated in Fig. 1 a and also allows it to undergo (when subsequently acidified) the low pH–induced, dimer-to-trimer reorganization shown in Fig. 2. Thus, when the mature virus particle secreted by one cell arrives in the acidic environment of an early endosome in a target cell, the large-scale molecular rearrangement of E facilitates fusion, first by exposing a hydrophobic "fusion loop," which inserts into the endosomal membrane, and then by drawing together the viral and target membranes as the conformational change proceeds.

In the 1990's, Heinz and co-workers (Allison et al., 1995; Schalich et al.,1996) showed that recombinant expression of TBEV prM and E in mammalian cells leads to secretion of recombinant subviral particles (RSPs), smaller than virions but still with an intact lipid bilayer. Later analysis showed that they contain just 60 copies of E (and, after passage through the TGN, the same number of copies of M), icosahedrally arrayed, and that the bilayer is only ∼210 Å in outer diameter (Ferlenghi et al., 2001). These particles nonetheless fuse with liposomes or other target membranes, at lowered pH, in a reaction that has precisely the same characteristics as virion fusion (Corver et al., 2000). For example, as described by Fritz et al. (2008), the reactivity of E with a panel of 22 monoclonal antibodies, with

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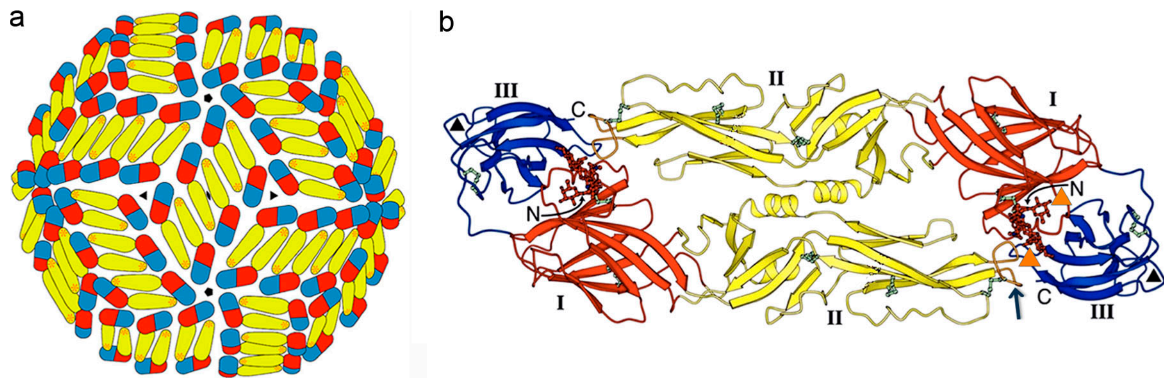

Figure 1. **Flavivirus structure.** (a) Diagram of the packing of 180 E subunits in the surface of a virion. The proteins are clustered as dimers. Each is represented by a symbol, colored to correspond to the domain representation in b. (b) The ectodomain of the E dimer, viewed as if looking toward the surface of the virion. Domains I, II, and III are labeled and colored in red, yellow, and blue, respectively. An arrow points to the fusion loop on one subunit. The locations of two histidines at the domain I–domain III interface are shown by orange triangles. His 146 is on domain I; His 323, close to the fusion peptide of the partner subunit, is on domain III. Black triangles mark a potential receptor-binding loop.

quite differently positioned epitopes, changes in just the same way during viral fusion and RSP fusion. The pH dependence and the kinetics of the process are likewise the same. As RSPs can be produced by transient transfection, mutagenesis is far more straightforward than it would be with virions. Fritz et al. (2008) could therefore pursue their hypothesis that histidines in E conserved among all flaviviruses are the likely pH sensors by an essentially complete analysis of the five such residues in TBEV. They monitored fusion by labeling the RSP membranes with 1-pyrenehexadecanoic acid, which has substantially altered fluorescence properties when diluted into the target membrane after merger of the bilayers.

Two of the five conserved histidines (H146 and H323) are at a particularly "interesting" interface between domains I and III of the E protein (see Fig. 1 b), and several previous papers (Bressanelli et al., 2004; Kampmann et al., 2006) had called attention to them. This interface rearranges completely when the protein undergoes its fusion-inducing change from dimer to trimer (Fig. 2), and protonation might indeed be expected to destabilize the dimer conformation. Two of the other conserved histidines (H248 and H287) are on the protein surface; the fifth (H438) is in the so-called stem that links domain III to the transmembrane anchor. Fritz et al. (2008) show that any of these last three can be mutated individually, without effect on the fusion properties of the corresponding RSPs, whereas mutation of H323 to alanine eliminates fusion, even at pH 5.0. Mutation of H146 to any of the other 19 naturally occurring amino acid residues prevents stable expression of E and hence prevents any formation of RSPs. H323 is not only buried at a key domain interface, it is also part of the pocket that protects the fusion loop of the dimer partner. Fritz et al. (2008) convincingly conclude that H323 is the critical pH sensor, with a possible additional contribution from H146.

Fritz et al. (2008) also examine a series of double and triple mutations. One of the double mutants, H248A-H287A, gives

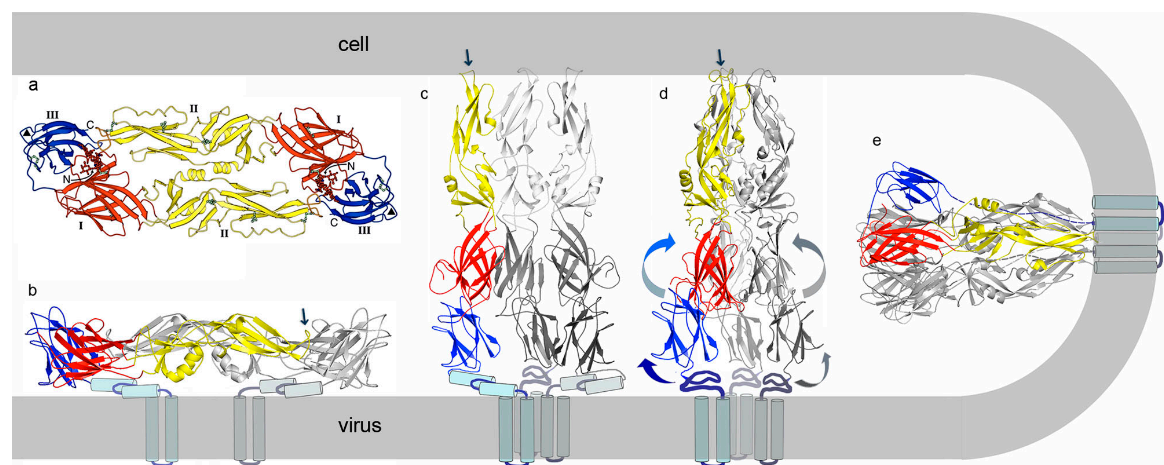

Figure 2. **Sequence of events during low pH–triggered, fusion-inducing conformational rearrangement of flavivirus E proteins.** (a) E ectodomain dimer, viewed as in Fig. 1 b. (b) Side view of the E dimer, illustrating how it is anchored in the viral membrane. A segment known as the stem connects the C terminus of domain III to the transmembrane anchor (a helical hairpin that traverses the bilayer once in each direction). (c) Low pH induces dissociation of the dimer interface and rotation outward of domains I and II, exposing the fusion loop (black arrows), which interacts with the endosomal target membrane. (d) The extended intermediate trimerizes and starts to collapse (curved arrows), so that domain III rotates back to dock against domains I and II and the stem zips up alongside the trimer-clustered domain II. (e) When the transition is complete, the two membranes have been brought together and induced to fuse. Several trimers probably participate cooperatively in this process, but only one is shown here. (This figure has been modified from Harrison, 2008.)

rise to stable RSPs but prevents fusion. In earlier work, Stiasny et al. (2007) found, by using a monoclonal antibody directed against the fusion loop, that its epitope, buried in the prefusion E dimer, is transiently exposed during the fusion process and becomes again protected in the postfusion trimer. As might be expected from its location, the H323A mutation prevents even the process (presumably dissociation of the dimer interface) that makes this epitope transiently accessible after acidification. The same is not the case for the H248A-H287A double mutant, however; lowered pH allows binding of the fusion loop monoclonal, just as to wild-type RSPs. The likely interpretation is that the double mutation impairs a later step in the fusion reaction. A good candidate would be the transition from the extended, intermediate structure (Fig. 2 c) to the folded back trimer (Fig. 2 e). The trapped intermediate is probably still monomeric, as suggested by sedimentation analysis of solubilized E protein from the various RSPs. Both H248 and H287 are in locations compatible with a contribution to trimer stability. The double mutant also binds liposomes at lowered pH, confirming exposure of its hydrophobic fusion loops.

Animating molecular structures and probing the processes in which they participate is still an arduous business. Directed mutagenesis, however well informed by structural information, is often a relatively blunt instrument. By building on nearly two decades of careful work on TBEV and its surface proteins, Fritz et al. (2008) have done more than simply identify the pH sensor that triggers E protein rearrangement, challenging as even that task has been. Together with the work of Liao and Kielian (2005) on the related alphavirus fusion proteins (and on dengue, another flavivirus), the experiments described in their paper fill in steps of the mechanism, illustrated in Fig. 2, for which we have had until now mainly the logical deductions from structures of the two end states. Methods to track the fusion of individual virus particles, by extension of the type of fluorescence assay used by Fritz et al. (2008), are likely soon to add further details and a proper time dimension. One should also recall that motivation to understand the details of this process goes beyond its considerable inherent cell biological interest. Blockade of fusion is among the mechanisms by which neutralizing antibodies prevent infection, and inhibiting viral fusion is a validated antiviral strategy, with at least one fusion inhibitor (the HIV entry inhibitor, enfuvirtide) now a licensed drug.

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