

Crystal Structure of the Low-pH Form of the Vesicular Stomatitis Virus Glycoprotein G

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The vesicular stomatitis virus has an atypical membrane fusion glycoprotein (G) exhibiting a pH-dependent equilibrium between two forms at the virus surface. Membrane fusion is triggered during the transition from the high- to low-pH form. The structure of G in its low-pH form shows the classic hairpin conformation observed in all other fusion proteins in their postfusion conformation, in spite of a novel fold combining features of fusion proteins from classes I and II. The structure provides a framework for understanding the reversibility of the G conformational change. Unexpectedly, G is homologous to gB of herpesviruses, which raises important questions on viral evolution.

Entry of enveloped viruses into host cells requires fusion of the viral envelope with a cellular membrane. This step is mediated by viral glycoproteins that undergo a dramatic fusogenic structural rearrangement induced by a specific trigger (e.g., low pH in the endosome or interactions with receptors).

Two classes of viral fusion proteins have been identified so far. The best characterized members of class I are the influenza virus hemagglutinin (HA) (1, 2) and the fusion protein (F) of the paramyxoviruses (3–5). Class I also includes fusion proteins from retroviruses (6), filoviruses (7), and coronaviruses (8). The active fusogenic form is obtained by proteolytic cleavage of a precursor into two fragments and bears a hydrophobic fusion peptide at or near the amino terminus generated by this cleavage. The proteins are organized as trimers and their postfusion conformation contains a trimeric coiled-coil core, beginning near the carboxy-terminal end of the fusion peptide, against which are packed, in an antiparallel manner, the segments abutting the transmembrane region. The protein shape is thus an elongated hairpinlike structure bringing together the fusion peptide and the C-terminal transmembrane domain.

Class II contains the E protein of flaviviruses (9, 10) and E1 of alphaviruses (11). They have an internal fusion peptide, located in a loop between two β strands, and are synthe-

sized within a polyprotein. Folding takes place as a complex with a second viral envelope protein that plays a chaperone role. Proteolytic cleavage of the chaperone primes the fusion protein to trigger membrane merger. In their native conformation, they form dimers that lie flat at the viral surface and are organized with icosahedral symmetry (11). On exposure to low pH, the dimers dissociate, and the protomers reassociate to form trimers. Similarly to class I proteins, this transition results in a hairpin structure with the fusion loops and the transmembrane domains at the same end of an elongated molecule that is then perpendicular to the membrane (12–14).

A number of viral fusion proteins, from rhabdoviruses and herpesviruses, for instance, do not appear to fall within either of these classes. Among them, glycoprotein G of the vesicular stomatitis virus (VSV), from the *Rhabdoviridae* family, has been the most studied. Rhabdoviruses are bullet-shaped and are widespread among a great variety of organisms (including plants, insects, fishes, mammals, reptiles, and crustaceans). Their genome is a single RNA molecule (about 12 kb) of negative polarity encoding five or six proteins in total, among which is a single-transmembrane glycoprotein (G) that is trimeric and forms the spikes that protrude from the viral surface. G is both responsible for viral attachment to specific receptors and for low pH-induced membrane fusion after endocytosis of the virion. Most of the mass of G (446 amino acids out of 495 for VSV Indiana strain) is located outside the viral membrane and constitutes the N-terminal ectodomain, which is the target of neutralizing antibodies. The two most studied genera of rhabdoviruses are the lyssaviruses [prototype virus: rabies virus (RV)] and the vesiculoviruses (prototype virus: VSV).

Fusion of rhabdoviruses is optimal around pH 6 (15–19). Preincubation of the virus at low

pH in the absence of a target membrane leads to inhibition of viral fusion. However, this inhibition is reversible, and readjusting the pH to above 7 leads to the complete recovery of the initial fusion activity (20). Low pH-induced conformational changes of G and their relations with the fusion activity have been studied by different biophysical and biochemical techniques (16, 21–26). G can adopt at least three different conformational states (15, 16): the native state detected at the viral surface above pH 7; the activated hydrophobic state, which interacts with the target membrane as a first step of the fusion process (24); and the fusion inactive, postfusion conformation that is antigenically distinct from both the native and activated states (27). There is a pH-dependent equilibrium between the different states of G that is shifted toward the inactive state at low pH (27). Thus, unlike fusogenic glycoproteins from other viral families, the native, prefusion conformation is not metastable. Furthermore, no α -helical coiled-coil motif characteristic of class I viral fusion proteins (28) is predicted from the amino acid sequence (29). Finally, although G contains an internal fusion domain (24), it is not cleaved from a polyprotein precursor or associated with a second envelope protein, as is the case for class II viral fusion proteins (9, 11). All these characteristics suggest that the structure of G is distinct from the structure of any fusion protein described so far.

Here we describe the structure of the VSV-G ectodomain (residues 1 to 410), generated by limited proteolysis with thermolysin (G_{th}), under its postfusion conformation at 2.4 Å resolution.

Molecular architecture. The structure of the G_{th} trimer is depicted in Fig. 1. The overall shape of the molecule resembles an inverted cone (Fig. 1B). The length of the molecule is 125 Å, and the diameter at the head is 60 Å. The crystals that allowed the structural determination were grown at pH 7.0, but the same conformation was found in crystals grown at pH 6.0 (see Materials and Methods). The dimensions of the molecule—identical to those measured for RV G ectodomain low-pH form by electron microscopy (16)—and its hairpinlike organization (see below) indicate that this structure corresponds to the low-pH, postfusion conformation (i.e., the fusion inactive conformation). Thus, during crystal growth at pH 7.0, the minor fraction of G in the low-pH conformation was sequestered in the crystals, displacing the equilibrium between the different states of G (27).

G_{th} has an altogether different structural organization from those of both class I and class II viral fusion proteins described so far. The polypeptide chain of G_{th} folds into four distinct domains (Fig. 1, A and D, and fig. S1). A β sheet-rich lateral domain at the top of the molecule (domain I), a central, mostly α -helical

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domain that is involved in the trimerization of the top of the molecule (domain II), a neck domain that has the characteristic fold of pleckstrin homology (PH) domains (domain III), and the elongated fusion domain that makes the trimeric stem of the molecule (domain IV). Three of these compact domains are made from noncontiguous segments of the polypeptide chain (Fig. 1D). The single segment making the fusion domain is inserted into the PH domain, and the PH domain is inserted into domain II. The C-terminal part of G_{th} (411 to 422) is not ordered in either crystal form, and the polypeptide chain can be drawn up to residue 410 on one protomer and only to residue 408 on the other two. Nevertheless, the orientation of the chain after the end of domain II [residues 406 to 410 in magenta on Fig. 1A (right) and 1B] indicates that it is pointing toward the tip of the fusion domain. Thus, as in the postfusion conformation of other fusion proteins, the transmembrane domain and the fusion domains are located at the same end of the molecule.

Alignment of five G protein sequences from animal rhabdoviruses belonging to different genera is shown in fig. S2. Although the overall amino acid identity is very low, it remains significant, and all of them are predicted to display the same fold except possibly the C-terminal part of ephemeroiruses G.

Surprisingly, the structural organization of G is the same as that of herpesvirus gB that is described in this issue (30). This similarity extends from the N-terminal part to at least the end of helix G of domain II. It includes both the PH domain and the fusion domain [Dali score, $Z = 5.2$ for 109 residues of the fusion domain (31)], as well as part of the trimerization domain (fig. S3), and reveals a clear and unexpected homology between the two proteins.

Description of the domains. The top lateral domain I (Fig. 2A) contains about 90 residues in two segments (1 to 17 and 310 to 383). It is made of three antiparallel β sheets (astu, rsa', and vwxy's) that are wrapped around the N-terminal β strands a to a'. β Strand s is also involved in the formation of β sheet rsa' with the glycosylation site at position 320 on loop rs that is located at the very top of the molecule. Four other loops of this domain (vw, xy, s't, and tu) are exposed at the surface of the molecule. It is noteworthy that an antigenic site has been reported in this domain, on loop s't on the native conformation (32).

Domain II (Fig. 3A) is made of three segments (18 to 35, 259 to 309, and 384 to 405) and contains four helices (A, F, G, and H). In the trimer, the two longest helices F and H make a six-helix bundle, reminiscent of the structure found in class I fusion proteins in their postfusion conformation (28), in which helix F forms the trimerization region (Fig. 1C, Fig. 3C). As in class I proteins, the fusion domain is N-terminal to the central helix F, and the trans-

membrane domain is located at the C terminus of the antiparallel outer helix H. Helices F and H are zipped together by a small antiparallel β sheet formed by strands q and z. Helix F is linked by a disulfide bond (bridging cysteines C²⁴ and C²⁸⁴) (33) to the long extended N-terminal part of this domain, which is wound around the top of the molecule. The FG loop is exposed at the top of the domain. The segment delimited by P²⁹⁶ and P³¹⁰, which contains helix G, and the top of helix H (residues 384 to 391) are hydrophobically packed against domain I, interacting with strands s, r, v, w, and x.

Domain III is inserted within domain II. It is made of two segments (36 to 50 and 181 to 258) and has the fold of a PH domain (Fig. 2B). It contains two four-stranded β sheets (bjkl and pmno) and two helices D and E that are respectively located between strands j and k and strands o and p. The domain contains a disulfide bridge that stabilizes sheet pmno by bridging C²¹⁹ and C²⁵³. Numerous epitopes of the native prefusion conformation have been reported in this domain (32).

Domain IV (51 to 180) is inserted in a loop of the PH domain (Figs. 1D and 2B). It is an

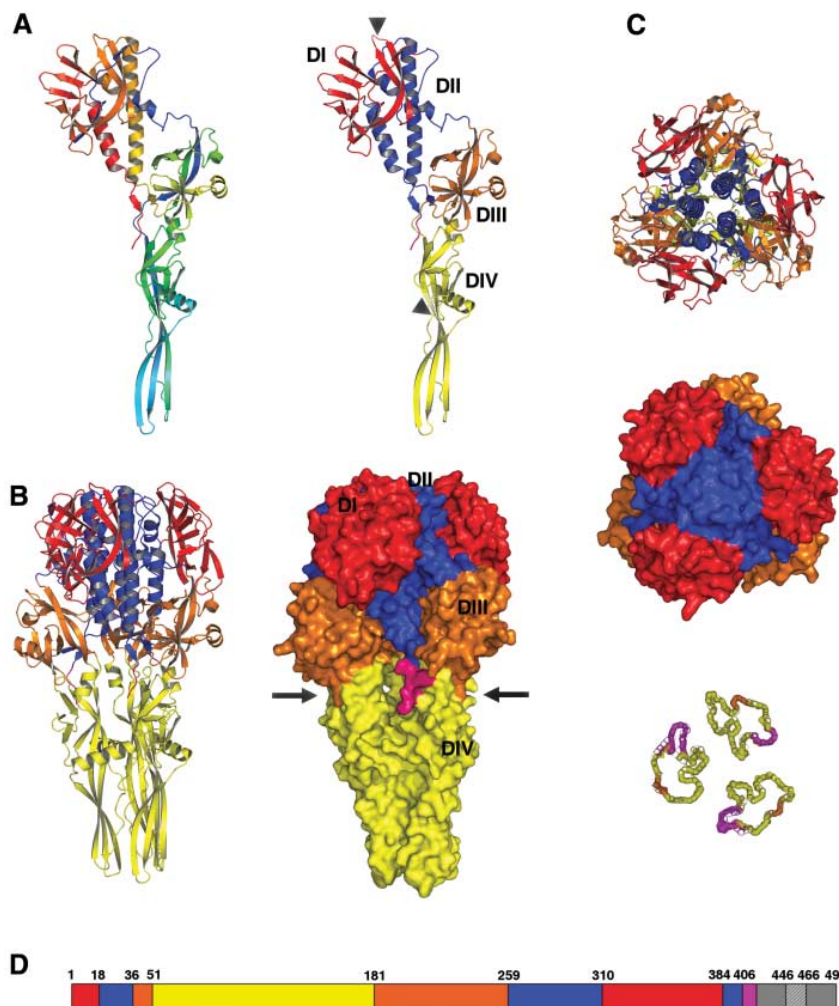


Fig. 1. Overall structure of glycoprotein G. (A) Ribbon diagram of the G protomer (residues 1 to 410). (Left) The chain is colored by residue number in a gradient from blue (N terminus) to red (C terminus); (right) the chain is colored by domain. The triangles indicate the glycosylation sites (on N¹⁶³ and N³²⁰). (B) (Left) Ribbon diagram of the G trimer, colored by domain; (right) surface representation of the G trimer, colored by domain. The arrows indicate the plane of the section shown in (C), bottom. (C) (Top) Top view of the trimer (ribbon diagram), colored by domain; (middle) top view of the trimer (surface representation); and (bottom) plane section of the G trimer, at the level of the C-terminal segment, showing the cavity inside the molecule. (D) Domain architecture of G. Domains observed in the crystal structure are colored as in (A), right. The C terminus, not observed in the structure, is in gray with the transmembrane segment hatched. All structural figures were generated with PYMOL (38).

extended β -sheet structure organized around two long antiparallel β strands [c and e in (Fig. 4A)] that contribute to the formation of a small six-stranded β barrel (cefggh) at one end, and at the other, to a three-stranded β sheet (dce) (Fig. 4A). The six-stranded β barrel exposes the glycosylation site at position 163 on loop hi. It is stabilized by the disulfide bridge linking C¹⁵³ and C¹⁵⁸. The segments of the protein making the β barrel are the most conserved elements of the G amino acid sequence (fig. S2). Just before the junction of domain IV and the second segment of domain III is α helix C, which is covalently linked by a disulfide bond (bridging C¹⁷⁷ and C²²⁶) to loop lm of domain III (Fig. 4A).

Separating the top β barrel (cefggh) from the bottom sheet (dce), the horizontal helix B packs against strands c and e via hydrophobic interactions. Helix B is linked to strand c by the disulfide bond between C⁵⁹ and C⁹². After helix B, the chain adopts an elongated conformation through a P¹⁰⁷GFPP¹¹¹ motif that has a polyproline helix conformation. The very tip of the protomer is made of two loops (cd and Pe) containing aromatic residues and kept together by the disulfide bridge between C⁶⁸ and C¹¹⁴ (Fig. 4A). As discussed below, these loops constitute the membrane-interacting motif of the G ectodomain.

The overall structural organization of this fusion domain, particularly the base of the stem, is strikingly similar to the one of class II fusion proteins (Fig. 4A). Nevertheless, these domains are not homologous: The topology of the strands making the sheet that exposes the fusion loops in VSV G is unrelated to the one in class II fusion proteins. Thus, this structural similarity appears as the result of convergent evolution.

The fusion loops. The tip of the trimeric stem has a bowl-like concave shape similar to that already described for E protein of flaviviruses (Fig. 4B). Nevertheless, unlike these fusion proteins, the fusogenic motif of VSV G

is made of both loops cd and Pe and, thus, is bipartite as already suggested for the viral hemorrhagic septicemia virus, another rhabdovirus (19). Indeed, four hydrophobic residues W⁷², Y⁷³ (both located on loop cd), Y¹¹⁶, and A¹¹⁷ (both located on loop Pe) are fully exposed at the tip of VSV G (Fig. 4, A and C). Not surprisingly, replacement of A¹¹⁷ by lysine abolishes G fusion properties (18).

This “fusion patch” cannot penetrate deep into the membrane: Even if the hydroxyl of Y¹¹⁶ participates in a hydrogen bond with the carbonyl of W⁷², the nitrogen of the indole ring of W⁷² and several carbonyls (e.g., those of Y⁷³ and A¹¹⁷) remain exposed and hinder penetration into the hydrophobic moiety of the membrane. R⁷¹ and D⁶⁹, located on the rim of the bowl, and K⁷⁶, which points toward the threefold axis, set an upper limit of about 8.5 Å for membrane insertion (Fig. 4C).

Sequence alignments reveal that all the rhabdoviral G proteins have at least one polar aromatic residue in their fusion loops (fig. S2). Tyrosines and tryptophans are residues typically found at the interface between the fatty acid chains and head-group layers of lipids (34). Such an interfacial interaction involving a large number of aromatic residues per trimer is probably sufficient to destabilize the membrane.

The trimeric interface. The buried interface between two subunits in the trimer is roughly 3860 Å² per protomer; the main part of it (2620 Å²) is located in the top of the molecule (domain II) and the rest in the stem (domain IV). The core of the trimer in domain II is the six-helix bundle (Fig. 3C). The stabilizing interactions are mostly hydrophobic but also involve a salt bridge between E²⁸⁶ from one protomer and K²⁹⁰ from another. In domain IV, the trimer is stabilized by lateral interactions between the polyproline segment and strand d of the neighboring protomer. These interactions involve conserved residues P¹¹¹, I⁷⁸, and I⁸² (Fig. 4D), which keep together the fusion loops

of the three protomers and thus ensure their correct positioning at the tip of the molecule. The other contacts between domains IV in the trimer involve the C terminus of helix B, which makes two hydrogen bonds (through the carboxyl group of K¹⁰⁰ and the side chain of Q¹⁰¹, respectively) with the relatively conserved H¹³² (on strand e) and H¹⁶² (on loop hi) of the neighboring protomer (Fig. 4D).

Inside the trimeric structure, there is a cavity (Fig. 1C, bottom) that is limited at the base of the stem by Q¹¹². The bottom of the cavity is a narrow channel flanked by the polyproline motif. It enlarges at the level of L¹⁰⁶ into a chamber 5 nm long and up to 2 nm wide that ends at the base of helix F in domain II. This chamber has three large apertures delimited by the tops of domain IV of two protomers. These openings may be occluded by the C-terminal part of the ectodomain in the full-length protein.

This cavity, a relatively rare feature in proteins, is also found in the postfusion conformation of the flavivirus protein E (12, 14). For both E and G, it certainly limits the stability of the trimeric organization of the fusion domains. Only a few bonds have to be broken (Fig. 4D) to allow the structure to adopt an open conformation such as the one that has been crystallized in the case of the low-pH form of the Semliki Forest Virus E1 protein (13). The versatility of the association of the fusion domains is probably necessary during the fusion process. Indeed, mutations in the polyproline motif (at positions 108, 109, and 111) result in a decrease of the fusion efficiency or a drastic shift of the pH threshold for fusion toward lower values (17, 18).

Molecular basis for conformational change reversibility. It has been proposed that the reversibility of the low pH-induced conformational change is essential to allow G to be transported through the acidic compartments of the Golgi apparatus and to recover its native structure at the viral surface (35). The structure gives the clues to the molecular basis of this unusual property. Indeed, although one crystal form was grown at pH 7.0, it is clear that the structure of G_{th} that we have determined cannot be stable at high pH in solution.

Indeed, a large number of acidic amino acids are brought close together in the six-helix bundle (Fig. 3, A and D). These residues are clearly protonated and form hydrogen bonds (Fig. 3D), and therefore, the negative logarithm of acid constant (pK_a) for them is abnormally high. Their deprotonation at higher pH will induce strong repulsive forces that destabilize the trimer; this step initiates the transition back toward the prefusion form. The regions that will thus be pushed apart include the bottom part of helix F from each protomer (through D²⁶⁸), helices H and F from neighboring protomers (through D²⁷⁴ and D³⁹⁵), and helices H and F within the same protomer (through

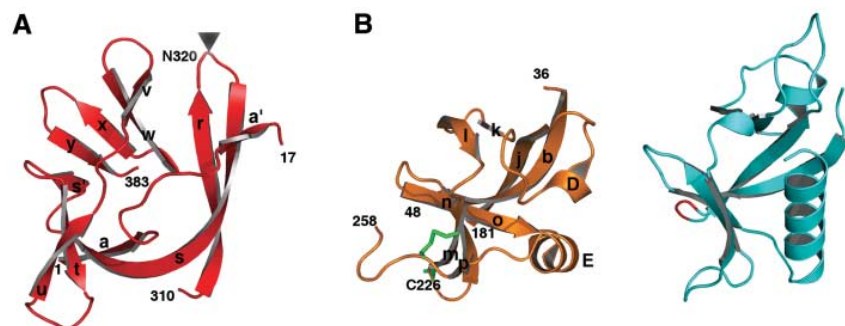


Fig. 2. Structural organization of domains I and III (PH domain). **(A)** Structure of domain I. The triangle indicates the glycosylation site. **(B)** Structure of domain III (left) and structure of the PH domain of the insulin receptor substrate 1, which is the best homolog found using Dali [Dali score $Z = 4.8$ (31)]. The loop of the PH domain in which G fusion domain is inserted is colored red. The disulfide bridge is indicated in green. Also shown is C²²⁶, which makes a disulfide bond with C¹⁷⁷ of domain IV.

E²⁷⁶ and D³⁹³) (Fig. 3D). Although these amino acids are not conserved among the rhabdovirus family, helices F and H of all rhabdoviruses G contain numerous acidic residues (fig. S2). These residues are certainly involved in the destabilization of the low-pH form above pH 7.

The trimeric state of domain IV, as it is in the G_{th} low-pH structure, should be also affected at pH above 7, because, in its deprotonated form, H¹³² cannot maintain its interactions with both the carboxyl group of K¹⁰⁰ of the neighboring protomer and the side chain of D¹⁴⁵ (Fig. 4D).

Finally, D¹³⁷, Y¹³⁹, and the dipeptide H⁴⁰⁷P⁴⁰⁸, which are conserved among the rhabdovirus family, cluster together to interact with the tip of domain II qz zipper. They direct the C-terminal part of the ectodomain toward the base of the molecule (Fig. 3B). The salt bridge between D¹³⁷ and H⁴⁰⁷ cannot exist after deprotonation of the histidine residue. Thus, a pH increase should also have a destabilizing effect on this small structural motif.

It is worth noting that the large number of protonated residues involved in the stability of the postfusion conformation explains the high cooperativity of the structural back transition

upon deprotonation (27): An initial destabilization of the low-pH form at the qz zipper results in an increased solvent accessibility of acidic residues in domain II, a drop in their pK_a, and their concomitant deprotonation.

Mutations affecting the structural transition of G. For both VSV and rabies virus, mutant viruses have been selected for their ability to escape neutralization by antibodies directed against G in its low-pH conformation (36) or for their ability to infect cells at low pH (37). Seven mutations have been described that result in stabilization of the prefusion conformation (either kinetically or thermodynamically). Two of them (Q²⁸⁵ → R for VSV and E²⁸² → K for RV) take place in the long helix F. Two others (V³⁹² → G and M³⁹⁶ → T for RV) take place in the neighborhood of the small qz sheet and the conserved dipeptide H³⁹⁷P³⁹⁸ (residue numbering of RV G, fig. S2). These observations confirm that helix F and the cluster made by D¹³⁷, Y¹³⁹, and the dipeptide H⁴⁰⁷P⁴⁰⁸ are involved in the structural rearrangements of G. The phenotype linked to the last three mutations (M⁴⁴ → V/I for RV and F² → L for VSV) cannot be explained and may affect only other conformations of G.

Final remarks. In spite of having a novel fold, the low pH of G displays the classic hairpin conformation expected for the postfusion form of a fusogenic protein. It combines features of both class I and class II proteins. Together with gB of herpesviruses, it defines a new family of fusion proteins having a new fusion module: an elongated β structure inserted in a PH domain and carrying two fusion loops. This homology between G and gB invites us to reconsider the evolution of the *Mononegavirales* order: It suggests that *Mononegavirales* are able to steal genes, probably from their cellular host, likely by copying exogenous mRNA during genome synthesis.

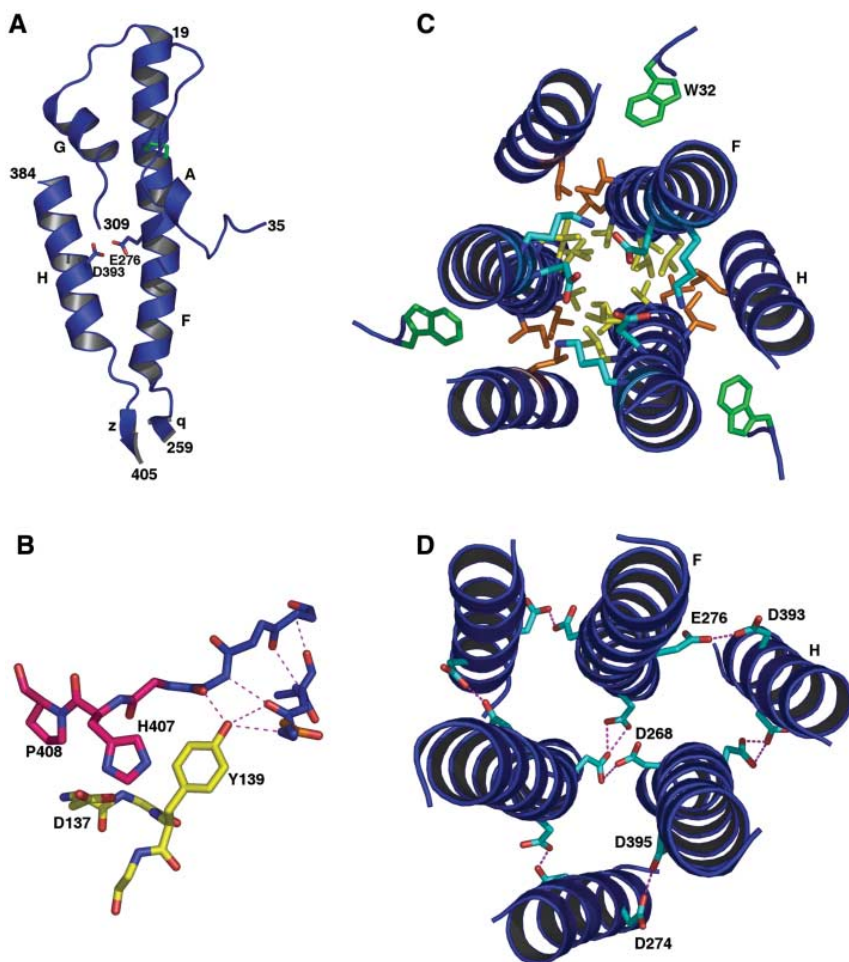


Fig. 3. Structural organization of domain II (trimerization domain). (A) Ribbon diagram of domain II. The disulfide bridge is indicated in green. Acidic residues E²⁷⁶ and D³⁹³, which destabilize the domain at pH above 7, are also represented. (B) Close-up view of the qz sheet (in blue) and how it is stabilized by residues D¹³⁷ and Y¹³⁹ of domain IV and by the dipeptide H⁴⁰⁷P⁴⁰⁸. Hydrogen bonds are indicated as dotted lines in magenta. D¹³⁷ makes a salt bridge with the imidazole ring of H⁴⁰⁷, which is stacked against the aromatic group of Y¹³⁹. (C) Top view of the six-helix bundle (the helices labeled H and F and the residue labeled W³², involved in lateral interactions, are from the same protomer). Aliphatic residues involved in interactions between helices F (I²⁷², V²⁷⁵, L²⁷⁹, L²⁸³) are indicated in yellow; those involved in lateral interactions between two protomers (L²⁷¹ and L²⁷⁸ in helix F and L³⁹² in helix H) are indicated in orange; and E²⁸⁶ and K²⁹⁰, which make a salt bridge, are indicated in cyan. (D) Top view of the six-helix bundle showing the cluster of acidic residues. Hydrogen bonds are indicated as dotted lines in magenta.

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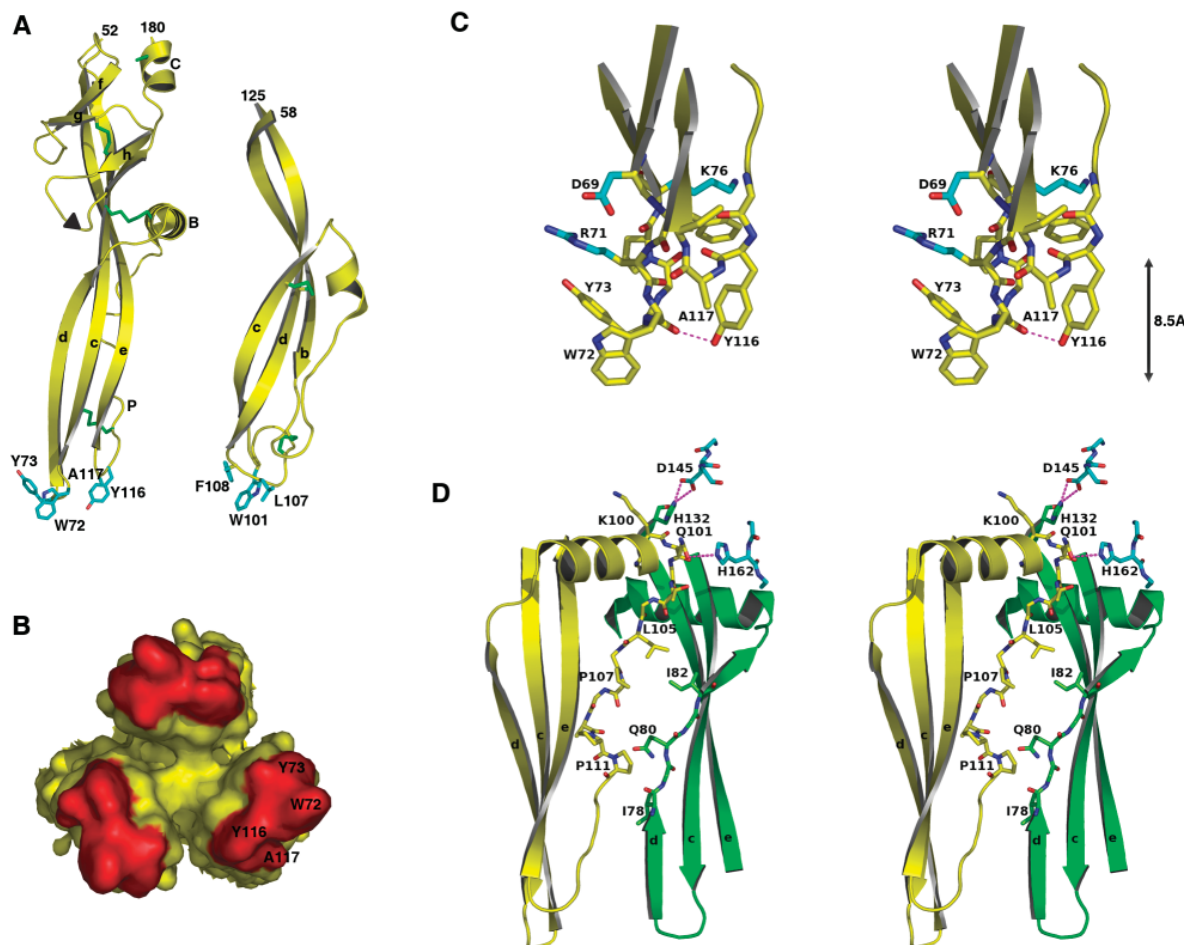


Fig. 4. Structural organization of domain IV (fusion domain). **(A)** Ribbon diagram of VSV G (left) and Dengue virus E (right) fusion domains showing their structural similarity. The disulfide bridges are indicated in green. Also shown is C¹⁷⁷, which makes a disulfide bond with C²²⁶ of domain III. The hydrophobic residues in the fusion loops are shown in cyan. The black triangle indicates the glycosylation site. **(B)** Surface representation of the tip of VSV G fusion domain. Hydrophobic residues

inside the loops are shown in red. **(C)** Stereo view of the fusion loops of one protomer showing how charged residues (in cyan) limit the penetration of the fusion domain inside the membrane. **(D)** Stereo view showing the interactions between two protomers (one in yellow, the other in green) in the fusion domain. For clarity, only the side chains of residues playing a role in the stabilization have been drawn, and the polypyrroline segment of the green protomer has been omitted.

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33. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Materials and Methods

SOM Text

Figs. S1 to S3

References and Notes

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