Identification of specific histidines as pH sensors in flavivirus membrane fusion

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The flavivirus membrane fusion machinery, like that of many other enveloped viruses, is triggered by the acidic pH in endosomes after virus uptake by receptor-mediated endocytosis. It has been hypothesized that conserved histidines in the class II fusion protein E of these viruses function as molecular switches and, by their protonation, control the fusion process. Using the mutational analysis of recombinant subviral particles of tick-borne encephalitis virus, we provide direct experimental evidence that the initiation of fusion is crucially dependent on the protonation of one of the conserved histidines (His323) at the interface between domains I and III of E, leading to the dissolution of domain interactions and to the exposure of the fusion peptide. Conserved histidines located outside this critical interface were found to be completely dispensable for triggering fusion.

Introduction

The entry of enveloped viruses into host cells involves a fusion step between the viral and a cellular membrane. This process is mediated by viral fusion proteins that are associated with the viral membrane and primed to undergo structural rearrangements that drive fusion (Kielland and Rey, 2006; Weissenhorn et al., 2007; Harrison, 2008; White et al., 2008). These conformational changes are activated by specific triggers, allowing fusion to occur at the right time and at the right place in the viral life cycle. Different trigger mechanisms (and combinations thereof) have been identified, including (a) interactions with cellular receptors, leading to fusion at the plasma membrane, and (b) protonation by the acidic pH in endosomes, leading to fusion from within endosomes after virus uptake by receptor-mediated endocytosis (White et al., 2008). In the latter case, like in other protein systems of intracellular pH sensors (Srivastava et al., 2007), histidines have been discussed to play a key role as molecular switches because their protonation state changes from uncharged to doubly positively charged at the slightly acidic pH found in endosomes (Carneiro et al., 2003; Bressanelli et al., 2004; Stevens et al., 2004; Kampmann et al., 2006; Kanai et al., 2006; Roussel et al., 2006; Mueller et al., 2008; Roche et al., 2008; Thoennes et al., 2008).

So far, two structurally unrelated classes of viral fusion proteins have been identified (class I in myxo-, paramyxoviruses, retro-, filo-, and coronaviruses and class II in α- and flaviviruses; Kielland and Rey, 2006), together with a third class that combines features of both class I and II (rhabdo- and herpesviruses; Weissenhorn et al., 2007; White et al., 2008). They all comprise representatives that are triggered by acidic pH. Despite the knowledge of atomic structures from all three protein classes (Weissenhorn et al., 2007; Harrison, 2008; White et al., 2008), it proved difficult, both in experimental and molecular simulation approaches, to conclusively answer the question of whether the initial trigger for destabilization and conformational changes is provided by the protonation of individual histidine residues, by combinations thereof, or by a cumulative effect through the increase of positive charge (Kampmann et al., 2006; Mueller et al., 2008; Thoennes et al., 2008). In class II fusion proteins, the molecular sensors for triggering fusion have not yet been identified. We therefore conducted a study in a prototypic class II fusion protein system (the flavivirus tick-borne encephalitis virus) and provide experimental evidence that the protonation of a specific histidine plays a key role for the destabilization of an intramolecular interface in the fusion protein and thus allows the initiation of the fusion process.

Flaviviruses (genus Flavivirus and family Flaviviridae) have an acidic pH–dependent fusion machinery (Stiasny and Heinz, 2006) and comprise several closely related important human pathogens, including yellow fever, dengue, Japanese...
envelopes, West Nile, and TBE viruses (Gubler et al., 2007). The surface of mature flaviviruses is made up of a herringbonelike assembly of 90 homodimers of the envelope glycoprotein E (Kuhn et al., 2002; Mukhopadhyay et al., 2003). The atomic structures of soluble forms of E (sE), lacking the membrane anchor and the so-called stem (Fig. 1 B), have been determined for different flaviviruses in pre- and postfusion conformations (Fig. 1, B and C; Rey et al., 1995; Modis et al., 2003, 2004, 2005; Bressanelli et al., 2004; Zhang et al., 2004; Kanai et al., 2006; Nybakken et al., 2006). In the prefusion conformation, the internal fusion peptide (FP) loop at the tip of domain II (DII) is buried through the interaction with a hydrophobic pocket provided by DI and III of the second partner in the homodimer (Fig. 1 B). Exposure to acidic pH leads to the initiation of the fusion process as depicted in Fig. 1 D.

Five histidine residues, located in DI, II, and III as well as in the stem region (Fig. 1 B and C), are conserved among all flavivirus E proteins and their function as pH sensors in flavivirus fusion has been discussed (Bressanelli et al., 2004; Kampmann et al., 2006; Kanai et al., 2006; Nybakken et al., 2006; Mueller et al., 2008). To support this hypothesis experimentally, we used recombinant subviral particles (RSPs) of TBEV as a model and targeted the conserved histidines in a mutational approach. As shown previously, RSPs are excellent tools for studying flavivirus fusion because they contain a lipid membrane, carry the E protein in a conformation that is indistinguishable from that on the virus, and, most importantly, display fusion characteristics similar to those of infectious virions (Schalich et al., 1996; Corver et al., 2000). This system allowed us to study the effect of histidine replacements on the different steps of fusion in the absence of resuscitating mutations that would occur during virus replication.

In our work, we demonstrate that His323, located at the interface of DI and III in the prefusion conformation of E, has a crucial function as a pH sensor for initiating fusion. Surprisingly, three of the five conserved histidines in E were shown to be completely dispensable for the early phases of fusion but two of these residues appeared to contribute to the overall stability of the postfusion trimer.

**Results**

**Generation of RSPs with mutated His residues**

The RSPs of TBEV used in this study display similar fusion characteristics as infectious virions and are assembled in eukaryotic cells after transfection with plasmids that coexpress prM and E. Their maturation and secretion pathway follows that of whole virions, including the cleavage of prM in the trans-Golgi network to yield mature and fusion-active particles (Schalich et al., 1996). To obtain specific information on the molecular pH sensors of fusion, we replaced each of the five absolutely
The fluorescence (caused by dilution of the probe in the target membrane) was continuously monitored. Consistent with previous results (Corver et al., 2000), WT RSPs fused rapidly at pH 5.4 within the first seconds after acidification (Fig. 2A), and the fusion activities of mutants lacking a histidine residue at position 248, 287, or 438 were identical to that of the WT (Fig. 2B). The replacement of His323, however, resulted in the loss of fusion activity (Fig. 2, A and B), even at pH 5.0 (not depicted).

We also analyzed combinations of mutations that alone did not affect fusion, i.e., H248N+H287A, H248N+H438N, and H287A+H438N. Unexpectedly, one of these combinations (H248N+H287A) impaired fusion to the same extent as did the replacement of His323 (Fig. 2, A and B). These experiments revealed the importance of individual conserved histidines for E-mediated fusion, but it remained unclear at which stage or how certain residues were involved in this multistep process.

Effect of His mutations on FP exposure

It was the primary hypothesis of our work that histidines play a role as pH sensors and that their protonation would be the initial trigger for fusion. To analyze the very first step of the fusion process, we made use of an FP-specific mAb (A1) and developed an assay that allowed us to measure the acidic pH-induced fluorescence (caused by dilution of the probe in the target membrane) was continuously monitored. Consistent with previous results (Corver et al., 2000), WT RSPs fused rapidly at pH 5.4 within the first seconds after acidification (Fig. 2A), and the fusion activities of mutants lacking a histidine residue at position 248, 287, or 438 were identical to that of the WT (Fig. 2B). The replacement of His323, however, resulted in the loss of fusion activity (Fig. 2, A and B), even at pH 5.0 (not depicted).

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displayed the same acidic pH–induced coflotation behavior as the WT. Because the double mutant H248N-H287A was completely fusion negative (Fig. 2), we made sure that its unimpaired coflotation was indeed only caused by binding to the liposomes, in the absence of fusion. For that purpose, coflotation assays were performed with pyrene-labeled samples of the mutant and WT RSPs, and fluorescence emission spectra were recorded with the coflotated fractions. In the case of RSP H248N-H287A, a strong pyrene excimer peak (which equals no dilution of the fluorescence probe) was observed, whereas it was strongly reduced with coflotated WT RSPs (Fig. 4 C, inset). These results thus allowed the unambiguous conclusion that the coflotation with the double mutant, in contrast to that of the WT, was caused by an interaction with liposomes in the absence of fusion activity.

**Loss of membrane interactions by His mutations**

According to the proposed scheme of flavivirus membrane fusion (Fig. 1 D), the exposure of the FP allows its interaction with target membranes and thus mechanistically initiates the fusion process. To assess whether the observed FP exposure indeed correlated with the proposed functional activity, we performed liposome coflotation experiments for measuring membrane binding. Preparations of WT and His mutant RSPs were mixed with liposomes, acidified, and applied to sucrose step gradients as described in Materials and methods. Coflotation of E to the top of the gradients indicates either binding to or fusion with liposomes. The results of these experiments as depicted in Fig. 4 precisely matched those of the FP exposure assay (Fig. 3). With the exception of the severely impaired mutant H323A, all of the other single and combination mutants displayed the same acidic pH–induced coflotation behavior as the WT. Because the double mutant H248N-H287A was completely fusion negative (Fig. 2), we made sure that its unimpaired coflotation was indeed only caused by binding to the liposomes, in the absence of fusion. For that purpose, coflotation assays were performed with pyrene-labeled samples of the mutant and WT RSPs, and fluorescence emission spectra were recorded with the coflotated fractions. In the case of RSP H248N-H287A, a strong pyrene excimer peak (which equals no dilution of the fluorescence probe) was observed, whereas it was strongly reduced with coflotated WT RSPs (Fig. 4 C, inset). These results thus allowed the unambiguous conclusion that the coflotation with the double mutant, in contrast to that of the WT, was caused by an interaction with liposomes in the absence of fusion activity.

**Impairment of the formation and stability of E trimers**

The results obtained so far indicated that only His323, but none of the other histidines investigated, was involved in the disengagement of the FP from its protecting interactions in the E dimer and its exposure to the environment. In previous experiments, we had found that during conversion of E from the pre-fusion dimer to the postfusion trimer the accessibility of the FP for mAb A1 was transiently increased but lost upon conversion into the final trimeric conformation (Stiasny et al., 2007). We therefore measured this transient FP exposure using an enzyme immunoassay in which the antibody was already present in the RSP samples at the time of their acidification (see Materials and methods). The results of these experiments are shown in Fig. 3. With the exception of mutant H323A, none of the three other single His mutants differed significantly from the WT, neither with respect to the extent nor to the pH threshold of FP exposure (unpublished data). The same holds true for the double and triple mutants, which all displayed a WT-like pattern (Fig. 3). In contrast, FP exposure was severely impaired in the case of the H323A mutant, but the residual activity was induced at the same pH threshold as with the WT, i.e., around pH 6.6 (Fig. 3). These results allow us to conclude that of the four histidines analyzed only His323 plays an important role as an initial fusion trigger and that the lack of fusion activity observed with the double mutant H248N-H287A was caused by an impairment of later steps of fusion.
Because the mutations introduced into E may not only influence the formation of trimers but also their stability, we performed thermal denaturation experiments as follows. RSPs were acidified, back-neutralized, solubilized, incubated at 37 or 70 °C, and subjected to rate zonal sucrose density gradient centrifugation to determine the oligomeric state of E (Fig. 7). E trimers of the single mutants H248N, H287A, and H438N were as stable as WT trimers (Fig. 7 and not depicted). In contrast, not only was trimer formation with H323A and H248N-H287A less efficient (Fig. 5, C and D) but also these trimers were sensitive to incubation at 70 °C, as revealed by a strong reduction of the E trimer peak and an accumulation of presumably denatured and aggregated material in the pellet (Fig. 7). As expected, similar results were obtained when trimer formation was allowed to proceed in the presence of liposomes (unpublished data).

Collectively, these results allow two important conclusions: (1) the lack of fusion activity of mutant H248N-H287A is likely to be caused by an impairment of trimer formation combined with a reduction in trimer stability, and (2) H323 apparently has a double role in the fusion process and not only functions as a pH sensor for initiating fusion but also contributes to the stability of the postfusion trimer.

**Discussion**

Although histidines have been speculated to play an important role as acidic pH sensors in class II viral fusion proteins (Bressanelli et al., 2004; Kampmann et al., 2006; Kanai et al., 2006; Nybakken et al., 2006; Roussel et al., 2006; Mueller et al., 2008), experimental evidence for such a role is still lacking. The mutational analysis presented in this work suggests that one of the five conserved histidines in the TBEV E protein (His323) plays a dominant role in the initiation of the multistep fusion process. This amino acid residue forms part of an intricate intramolecular network of interactions between DI and III of the E monomer in its prefusion conformation, consisting of hydrogen bonds, van der Waals contacts, and a salt bridge between Arg9 in DI and Glu373 in DIII (Fig. 8 A; Bressanelli et al., 2004). Both of these amino acids as well as several additional residues of this contact...
Figure 7. Analysis of the stability of acidic pH-induced E trimers of WT and selected mutant RSPs. Acidic pH-induced trimers of WT (red) and mutant RSPs (green, H248A; orange, H323A; blue, H248A-H287N) were exposed to 70°C and subjected to rate zonal sucrose density gradient centrifugation. The sedimentation direction is from left to right. The pellet (P) was resuspended in 0.6 ml corresponding to the volume of a single fraction.

The DI–DIII interface contains another absolutely conserved histidine at position 146 (Fig. 8 A), which we were not able to investigate directly because its replacement by any of the other 19 amino acids abolished the formation of native RSPs by the quality criteria applied. Although the mutation H323A dramatically reduced the acidic pH–induced exposure of the FP, there was some residual activity that occurred at the same pH threshold of 6.6. Because all of the other His mutations as well as their combinations did not impair FP exposure, the residual activity is probably related to the protonation of His146 and suggests a possible accessory role of this residue in the destabilization of the DI–DIII interface. Surprisingly, and in contrast to what has been observed for the influenza virus class I fusion protein hemagglutinin (Thoennes et al., 2008), none of the histidine mutations in this work affected the pH threshold of fusion-related processes. Flavivirus variants that exhibited a pH shift for membrane fusion were described to contain mutations in the DI–DII hinge region (Rey et al., 1995; Harrison, 2008), but the mechanism causing this behavior remains to be elucidated. It has been suggested, however, that these mutations influence the stability of E (Modis et al., 2003) and thus may modulate the pH required to induce those conformational changes that drive fusion. Such “stability effects,” however, appear to be distinct from the actual pH-sensing machinery, which apparently involves His323 and probably His146.

The conversion of the E dimer to the postfusion trimer not only requires the relocation of DIII but also a change in the orientation of DII relative to DI (Bressanelli et al., 2004; Modis et al., 2004). This is made possible by the structural flexibility of the junction between the two domains (Modis et al., 2003), which is also necessary for conformational transitions during other phases of the viral life cycle, such as virus assembly and maturation (Zhang et al., 2004; Li et al., 2008). In the context of fusion, this hinge motion may occur spontaneously upon release from dimer constraints, caused by the dissolution of the DI–DIII interface, but could also require an independent pH-sensing step (Kanai et al., 2006). With respect to the conserved histidine at the DI–DII junction (His287), we did not, however, find evidence for such a pH sensor function because its replacement did not have any measurable effect, neither on early nor late stages of fusion.

It is a further finding of our work that His323 apparently has a dual role in the fusion process and contributes both to the initial pH trigger and to the stability of the postfusion trimer. The lower stability of this mutant trimer could be a result of the lack of the salt bridge between His323 and Glu373 in the postfusion structure of DIII (Fig. 8 B) and a concomitant weakening of DIII-mediated trimer contacts and/or the proper positioning of the stem for zippering along the grooves provided by DII. None of the other conserved histidines (except His146, which was irreplaceable and therefore not amenable to analysis) seems to have a similar impact on E trimerization as His323 because their single replacements neither affected trimer formation nor trimer stability to a measurable extent (Fig. 7). An additive effect, however, was observed in the double mutant H248N-H287A, which was fusion negative and was impaired in trimer formation and trimer stability as was the mutant H323A. His287 forms part of the trimer interfaces (Fig. 1 C) and His248 is located at a position at the groove, close to the FP (Fig. 1 C), that is likely to accommodate the stem during the “zipping up” process (Fig. 1 D). Theoretically, the replacement of each of these residues alone could interfere with late stages of the fusion process, leading to the opening of the fusion pore. More specific interpretations of these results, however, will depend on the elucidation of the atomic structure of the stem in the postfusion structure of E and assays that distinguish between lipid and contents mixing.
Although three of the conserved histidines in TBEV E (His248, His287, and His438) were ruled out as pH sensors for initiating fusion, they could be involved in other pH-dependent mechanisms, such as the conversion of immature (containing prM-E) into mature particles (Yu et al., 2008). The structure of the prM-E heterodimer of dengue 2 virus has recently been determined by x-ray crystallography and it is of special interest that His244 (corresponding to His248 in TBEV-E) is situated opposite a conserved Asp63 in prM (Li et al., 2008). It has therefore been speculated that the loss of protonation of His244 could be required for the release of the pr-peptide at neutral pH (Li et al., 2008; Yu et al., 2008). In our TBEV system, the replacement of the homologous His248, however, had no apparent effect on virus maturation and further experiments will be necessary to clarify this issue.

Despite the structural similarity of the class II fusion proteins of alphaviruses (E1) and flaviviruses (E; Kielian, 2006), it is likely that the pH-sensing machineries for initiating fusion are significantly different in the two virus systems. Indeed, there is only one conserved histidine at a strictly homologous position in E and E1. This residue (His248 in TBEV and His230 in Semliki Forest virus, respectively) has been ruled out as a pH sensor for both viruses and was shown to affect only a late stage of fusion (Chanel-Vos and Kielian, 2004, 2006; this study). The major difference lies in the structural details of FP protection in the prefusion conformations of E and E1. In flaviviruses, the FP in E is buried through homodimeric interactions, whereas in alphaviruses, the FP in E1 is protected by a heterodimeric interaction with a second, overlying protein (E2; Kielian, 2006). Alphavirus fusion could therefore be triggered by the protonation of as yet unidentified residues in both E1 and E2.

In conclusion, our study provides experimental information on the pH trigger of flavivirus membrane fusion and the critical role of the DI–DIII interface in this process. Although several of the absolutely conserved histidines in the viral fusion protein were shown to be completely dispensable for fusion initiation, our study does not rule out possible roles of these residues in other pH-dependent processes of the viral life cycle. These new insights can also contribute to the design of antiviral approaches that target the structural transitions of flaviviruses during entry and morphogenesis.

Materials and methods

Mutagenesis of RSPs

Using the site-directed mutagenesis kit Gene Tailor (Invitrogen), mutations were introduced into the recombinant plasmid SYVE WT (Allison et al., 1994), which contained the TBEV prM and E genes under the control of an SV40 early promoter, at the codon positions 146, 248, 287, 323, and 438 of the E gene. The WT and mutant plasmids were sequenced throughout the prM and E regions to confirm that only the desired mutations were present.

Production of RSPs

For the production of RSPs, COS-1 cells were transfected with recombinant plasmids by electroporation as described previously (Schalich et al., 1996). RSPs were harvested 48 h after transfection from cell culture supernatants, pelleted by ultracentrifugation, and purified by sucrose gradient centrifugation (Schalich et al., 1996; Allison et al., 2001). For membrane fusion (lipid mixing) assays, the RSPs were metabolically labeled with 1-pyrenehexadecanoic acid (Invitrogen) as described previously (Corver et al., 2000; Allison et al., 2001).

Quality controls of RSPs

The amount of RSPs secreted from transfected cells was quantified by a four-layer ELISA after solubilization with 0.4% sodium dodecyl sulfate at 65°C for 30 min (Heinz et al., 1994). The conformation of E was probed in comparison to that of the WT by epitope mapping with 22 E protein-specific mAbs (Allison et al., 1995, 2001; Heinz et al., 1994; Stiasny et al., 2005) and their maturation state (presence of prM) by Western blotting (Allison et al., 2003).

pH treatment of RSPs

Acidic pH incubations were performed at 37°C and the different acidic pHs were adjusted by the addition of morpholinoethanesulfonic acid (MES).
Lipid mixing fusion assay
Pyrene-labeled RSPs were mixed with large unilamellar liposomes (0.3-mM total lipid) consisting of phosphatidycholine, phosphatidylethanolamine, and cholesterol (molar ratio 1:1:2) in a continuously stirred fluorimeter cuvette at 37°C ([Allison et al., 2001]; Stiasny et al., 2003). Fluorescence was monitored continuously using a fluorescence spectrophotometer (LS-50B; Perkin-Elmer). Lipid mixing was initiated by the addition of MES to yield a final pH of 5.4. The extent of fusion was calculated by using the initial ex- citer fluorescence as 0% fusion and the fluorescence after solubilization of the RSP-liposome mixture by the detergent octa(ethylene glycol)-n-dodecyl monoether (Sigma-Aldrich) as 100% fusion.

Cotranslation assay
The exposure of the FP was measured by the decrease of the octylglycoside- detergent-extracted fraction of the FP to yield the amount of FP present in the RSP-liposome mixture by the detergent octa(ethylene glycol)-n-dodecyl monoether (Sigma-Aldrich) as 100% fusion.

Cotranslation assay
To investigate the thermostability of the postfusion E protein, acidic pH– induced E dimer-to-trimer transition was measured by sedimentation analysis (Stiasny et al., 2005).

E trimer formation and stability
The acidic pH–induced E dimer-to-trimer transition was measured by sedimentation analysis as described previously (Allison et al., 1995, 2001). 3 mg RSPs in TAN buffer, pH 8.0, were adjusted to different acidic pHs by the addition of MES (see pH treatment of RSPs) and incubated at 37°C for 10 min. Samples were back-neutralized with 150 mM triethanolamine, solubilized for 1 h at room temperature with 1% Triton X-100, and applied to a 1 ml cushion of 50% sucrose in TAN buffer, pH 8.0, and overlaid with 1.4 ml of 15% sucrose (Allison et al., 2001; Stiasny et al., 2002). The step gradients were centrifuged for 2 h at 4°C (rotor SW 55; Beckman Coulter) at 50,000 rpm, and 0.2-ml fractions were collected by upward displacement using a Piston Gradient Fractionator (BioComp Instruments Inc.). E protein was quantified by fourierier ELISA after solubilization with 0.4% sodium dodecyl sulfate at 65°C for 30 min (Heinz et al., 1994).

FP exposure assay
Native RSPs (at a concentration of 0.5 μg/ml of E protein) in phosphate-buffered saline, pH 7.4, containing 2% lamb serum were captured by polyclonal anti-TBEV immunoglobulin G for 1 h at 37°C as described previ- ously (Stiasny et al., 2007). The exposure of the FP was measured by the addition of biotinylated mAb A1 (FP-specific mAb) in MES buffer (50 mM MES and 100 mM NaCl), titrated to the appropriate pH by titration with 1 N NaOH. After incubation for 1 h at 37°C, the bound antibody A1 was detected by using streptavidin-peroxidase (Sigma-Aldrich).

References


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