Domain III from class II fusion proteins functions as a dominant-negative inhibitor of virus membrane fusion

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Alphaviruses and flaviviruses infect cells through low pH-dependent membrane fusion reactions mediated by their structurally similar viral fusion proteins. During fusion, these class II viral fusion proteins trimerize and refold to form hairpin-like structures, with the domain III and stem regions folded back toward the target membrane-inserted fusion peptides. We demonstrate that exogenous domain III can function as a dominant-negative inhibitor of alphavirus and flavivirus membrane fusion and infection. Domain III binds stably to the fusion protein, thus preventing the foldback reaction and blocking the lipid mixing step of fusion. Our data reveal the existence of a relatively long-lived core trimer intermediate with which domain III interacts to initiate membrane fusion. These novel inhibitors of the class II fusion proteins show cross-inhibition within the virus genus and suggest that the domain III–core trimer interaction can serve as a new target for the development of antiviral reagents.

Introduction

The alphaviruses and flaviviruses, which are members of the Togaviridae and Flaviviridae families, include several serious human and animal pathogens that are disseminated in nature by mosquito or tick vectors (for reviews see Lindenbach and Rice, 2001; Schlesinger and Schlesinger, 2001). The alphaviruses eastern equine encephalitis virus, western equine encephalitis virus, and Venezuelan equine encephalitis virus cause periodic epidemics of severe encephalitis in humans (Weaver and Barrett, 2004). Important flavivirus pathogens include Japanese encephalitis virus, tick-borne encephalitis virus (TBE), yellow fever virus, West Nile virus, and dengue virus (DV). Current estimates are that more than one third of the world's population lives in dengue fever endemic areas, with ~100 million cases of dengue infection and 500,000 cases of the more lethal complication, dengue hemorrhagic fever, per year (Clarke, 2002; Gubler, 2002). Given the known spread of mosquito vectors into new regions, several alphaviruses and flaviviruses are also potential emerging pathogens (for review see Mackenzie et al., 2004; Weaver and Barrett, 2004). There are no effective therapeutic drugs for these viruses, and vaccine development, although an important focus of research, is complicated by the potential for antibody enhancement of infection, as observed in the case of DV (Halstead, 1988; for review see Mackenzie et al., 2004).

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Abbreviations used in this paper: DV, dengue virus; Ex/M, excimer to monomer peak ratio; FIA, fusion-infection assay; HT, homotrimer; SFV, Semliki Forest virus; SIN, Sindbis virus; TBE, tick-borne encephalitis virus; TM, transmembrane; VSV, vesicular stomatitis virus.

Alphaviruses and flaviviruses are small, spherical viruses containing plus-strand RNA genomes packaged with a capsid protein. The nucleocapsid is enveloped by a lipid bilayer containing the virus membrane fusion protein (alphavirus E1 or flavivirus E). This transmembrane (TM) protein mediates the fusion of the virus membrane with the cell membrane, delivering the viral RNA into the cytoplasm and initiating virus infection. In mature virions, alphavirus E1 is associated as a heterodimer with the viral E2 protein, whereas the flavivirus E protein is found as an E–E homodimer. Infection by alphaviruses and flaviviruses occurs via an initial interaction of the virus with cell surface receptors, followed by internalization of the virus by endocytosis (for reviews see Kielian et al., 2000; Heinz and Allison, 2001). Virus membrane fusion is triggered by the mildly acidic pH within the endocytic pathway and is specifically blocked by inhibitors of endosome acidification. Low pH causes a dramatic rearrangement of the fusion protein, dissociating its dimeric interactions and producing a target membrane-inserted homotrimer (HT) that is believed to drive the membrane fusion reaction (Wahlberg and Garoff, 1992; Allison et al., 1995; Kielian et al., 1996).

Although the alphavirus and flavivirus fusion proteins do not have detectable amino acid sequence similarity, they have remarkably similar secondary and tertiary structures, indicating their evolutionary relationship and leading to their classification as the inaugural members of the class II virus fusion proteins (Lescar et al., 2001). The neutral pH structures of the fusion protein ectodomains have been determined for the alphavirus Semliki Forest virus (SFV; Lescar et al., 2001) and the flavi-

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viruses TBE, DV2, and DV3 (Rey et al., 1995; Modis et al., 2003, 2005; Zhang et al., 2004). The proteins are elongated molecules composed almost entirely of β strands and contain three domains: the centrally located domain I; domain II, which is located at one side of domain I and contains the target-membrane–interacting fusion peptide loop at its tip; and an Ig-like domain III, which is connected to the other side of domain I (Fig. 1 A). Although not present in the ectodomain structure, in the full-length proteins the stem region and TM anchor are found at the COOH terminus of domain III, at the opposite end of the protein from the fusion loop. The fusion proteins are arranged with icosahedral symmetry and lie tangential (almost parallel) to the virus membrane (Lescar et al., 2001; Kuhn et al., 2002; W. Zhang et al., 2002).

Treatment of the SFV fusion protein ectodomain, E1*, at low pH in the presence of target membranes converts the protein to a membrane-inserted HT (Klimjack et al., 1994; Gibbons et al., 2004a). The three-dimensional structure of the E1*HT reveals that during trimerization, domain III and the stem region of E1 move ~37 Å toward the fusion loop (Fig. 1 A; Gibbons et al., 2004b). This foldback reaction generates a hairpin-like conformation with the fusion loop and the TM domain at the same end of the E1 trimer. The structures of the DV and TBE HTs are remarkably similar to that of SFV, although the stem region of the protein is not present in these ectodomains (Bressanelli et al., 2004; Modis et al., 2004). Thus, the alphavirus and flavivirus membrane fusion proteins share common structural and functional features in both their prefusion and postfusion conformations.

Although the class I viral membrane fusion proteins are structurally very different from the class II proteins, the class I proteins are known to refold to a hairpin-like structure during fusion (for reviews see Skehel and Wiley, 2000; Jardetzky and Lamb, 2004; Earp et al., 2005). Class I proteins are exemplified by the influenza virus HA and the HIV-1 gp41. The class I proteins are trimeric both before and after fusion. In the postfusion conformation, the membrane-proximal COOH-terminal regions interact with a more NH_2-terminal trimeric α-helical coiled–coil domain to form a "trimer of hairpins" that brings the fusion peptides and TM domains together. Importantly, for several class I proteins, peptides containing sequences of these NH_2- or COOH-terminal interacting regions can bind to the viral fusion protein and inhibit fusion and infection by preventing refolding to the final hairpin conformation (for review see Moore and Doms, 2003). This dominant-negative approach is exemplified by the HIV peptide T20/Enfuvirtide, a licensed antiretroviral drug that corresponds to the COOH-terminal helix of gp41 (Wild et al., 1993).

Inhibitors of the class II fusion proteins would be very valuable tools in studying fusion mechanisms and developing antiviral agents for these important viruses. The structures of the class II fusion proteins suggest several features that might serve as targets for inhibitors of the fusion reaction. The pH 7.0 form of the DV E protein reveals a hydrophobic pocket within a flexible "hinge" region between domains I and II (Modis et al., 2003). Because the hinge changes its angle during the transition to the trimer form, molecules that bind to the hydrophobic pocket may inhibit hinge flexibility and block fusion (Modis et al., 2003). The structure of the SFV E1*HT reveals that the stem region of the protein interacts along the "core trimer," which is the central region of the trimer containing domains I and II (Gibbons et al., 2004b). Thus, the stem peptide and its HT interaction site are potential targets (Bressanelli et al., 2004; Modis et al., 2004). One of the most prominent features of the class II protein refolding reaction is the striking movement of

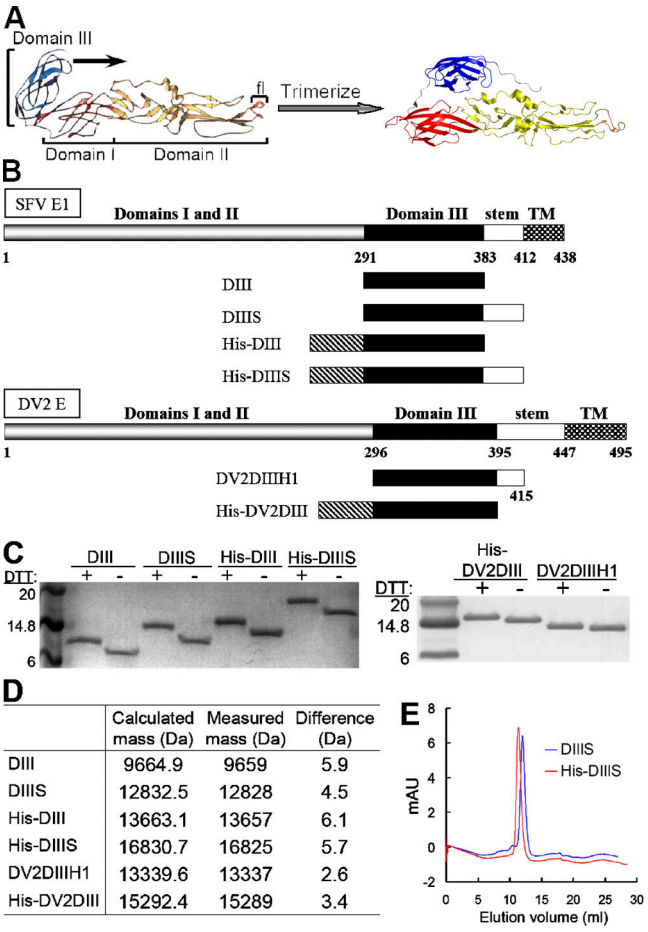

Figure 1. **Summary of domain III proteins.** (A) Structure of the SFV E1 ectodomain in the neutral pH monomer conformation (left; modified from Gibbons et al., 2004b) and in the low pH-induced trimer conformation (right), showing a single E1 protein of the trimer (drawn using PyMOL; DeLano, 2002). The colors indicate domains I (red), II (yellow), and III (blue), and the fusion loop (fl; orange) at the tip of domain II. The movement of domain III and the stem toward the fusion loop is indicated by the small black arrow. (B) Linear diagram of sequences of SFV E1 and DV2 E and domain III constructs, showing the boundaries of the domains, stem region, and TM anchor region. The SFV E1 domain III proteins are as follows: DIII (residues 291–383), DIIIS (291–412), His-DIII (His tag plus 291–383), and His-DIIIS (His tag plus 291–412). The DV2 E domain III proteins are as follows: DV2DIIIH1 (296–415) and His-DV2DIII (His tag plus 296–395). The His tag adds 36 residues at the NH_2 terminus; untagged proteins contain an added methionine at the NH_2 terminus. (C) 2 μg of each purified domain III protein was treated with or without 10 mM DTT, alkylated, and analyzed by SDS-PAGE. Marker proteins are shown on the left with their molecular masses listed in kilodaltons. (D) The molecular mass of each domain III protein was measured by mass spectrometry and compared with the mass calculated from the amino acid sequence. The predicted error rate is 0.01%. The mass for DV2DIIIH1was calculated without the added NH_2-terminal methionine because the measured mass indicated that this residue was not contained in the protein. (E) Elution profiles of 50 μM His-DIIIS and DIIIS on Superdex G-75 in 0.1 M Na Acetate, pH 5.5.

domain III and the stem toward the trimer tip (Fig. 1 A). Although domain III undergoes a dramatic reorientation during fusion, the significance of domain III in the fusion protein refolding reaction and in driving membrane fusion is unknown.

We demonstrate here that recombinant forms of domain III can inhibit the low pH-dependent fusion reactions of the alphaviruses and flaviviruses. Domain III proteins show cross-inhibition within each virus genus, suggesting the presence of critical interaction sites. Studies of SFV demonstrate that inhibition occurs by a dominant-negative mechanism in which exogenous domain III binds stably to an E1 trimer intermediate and blocks the initial mixing of the target and virus lipid bilayers. Our results suggest a new antiviral strategy that should be generally applicable to all class II viruses.

Results

Generation and characterization of domain III proteins

Several previous studies have demonstrated that flavivirus domain III can be produced in bacteria as fusion proteins (Bhardwaj et al., 2001; Volk et al., 2004), as epitope-tagged proteins (Wu et al., 2003; Hung et al., 2004), or by refolding of the molecule from inclusion bodies (Jaiswal et al., 2004). The structures of recombinant domain III from West Nile virus (Volk et al., 2004) and Japanese encephalitis virus (Wu et al., 2003) were determined by nuclear magnetic resonance and shown to be essentially identical to the structure of domain III in TBE E protein purified from virus (Rey et al., 1995). Domain III is contiguous in the linear sequence of SFV E1 or DV E protein and forms an Ig-like β-barrel structure that has three disulfide bonds in SFV E1 or one disulfide bond in DVE. We prepared four domain III constructs for SFV, containing domain III with or without the stem region and with or without an NH_2-terminal His tag (DIII, DIIIS, His-DIII, and His-DIIIS; Fig. 1 B). We also prepared two constructs of domain III from the DV2 serotype, containing DV2 domain III plus the helix 1 region of the stem (DV2DIIIH1) or DV2 domain III with an NH_2-terminal His tag (His-DV2DIII). The proteins were expressed in *Escherichia coli*, refolded using a fast dilution method successfully used to refold proteins containing Ig-like domains (X. Zhang et al., 2002), and purified by gel filtration chromatography. Tests of the purified SFV proteins demonstrated that all four eluted as a single peak at the predicted monomer position when chromatographed at pH 5.5 or 8.0 (Fig. 1 E and not depicted) and migrated as a single band on native gels (unpublished data). Thus, no evidence of aggregation was observed. All of the purified proteins migrated as a single band of the predicted size in SDS-PAGE and showed a mobility shift upon reduction, indicating the presence of disulfide bonds (Fig. 1 C). Analysis by mass spectrometry confirmed the predicted protein sizes and suggested that the SFV domain III proteins contain three disulfide bonds because their measured masses are approximately six units less than those predicted if all six cysteines are reduced (Fig. 1 D). Similarly, the dengue domain III constructs appear to contain the single predicted disulfide bond. The disperse location of the cysteines in SFV domain III suggests that they cannot form aberrant disulfides without radically changing the protein fold (Lescar et al., 2001). Thus, the presence of all three disulfide bonds, the proteins' high solubility (>10 mg/ml), and the biological activity described in this paper strongly suggest that all of the domain III proteins are correctly folded.

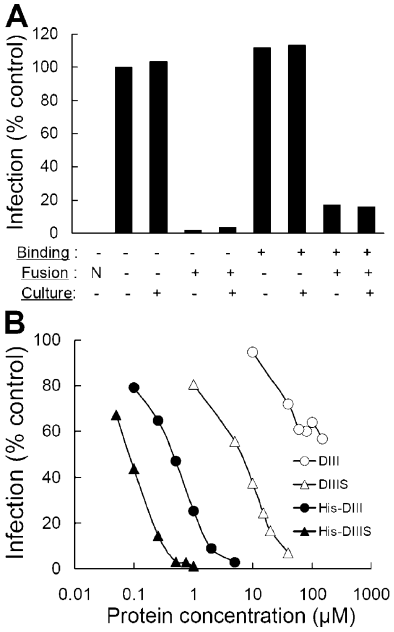

Figure 2. **SFV E1 domain III proteins inhibit SFV fusion with target cell membranes.** (A) Exogenous domain III specifically inhibits SFV fusion. SFV was added to BHK cells (multiplicity of infection ~0.002) for 90 min on ice (Binding). The cells were incubated at pH 7.4 (N) or pH 5.5 for 1 min at 37°C to induce fusion (Fusion) and cultured at 28°C overnight in medium containing 20 mM NH_4Cl (Culture). The presence or absence of 4 μM His-DIII in each step is indicated by + or −. Infected cells were quantitated by immunofluorescence. Results are shown as a percentage of control infection in the absence of His-DIII at any step. Representative example of two experiments. (B) The concentration dependence of inhibition by domain III proteins was determined using the assay in A and adding the indicated concentrations of domain III proteins only during the 1-min low pH treatment. Representative example of two experiments.

Inhibition of class II virus fusion and infection by domain III proteins

We screened the SFV DIII proteins for activity in a fusion-infection assay (FIA) that quantitates low pH-dependent SFV fusion with the plasma membrane (Vashishtha et al., 1998). Viruses were bound to cells on ice and treated for 1 min at 37°C at low pH to trigger the fusion of the virus with the plasma membrane of the cell. This fusion results in virus infection. The cells were cultured overnight in the presence of 20 mM NH_4Cl to prevent secondary infection, and the cells infected due to the low pH pulse were quantitated by immunofluorescence. Under these conditions, we could test the effects of domain III proteins specifically during the binding step, the fusion step, and the postfusion culture step. As shown in Fig. 2 A, 4 μM His-DIII almost completely inhibited SFV infection of BHK cells, but only when present during the low pH-induced fusion step. Similar results were obtained for His-DIIIS (unpublished data). In contrast, preincubation of the virus with domain III proteins at 37°C at neutral pH had no effect (unpublished data). In agree-

ment with studies showing that alphavirus receptor interaction is mediated by the E2 protein (for review see Schlesinger and Schlesinger, 2001), exogenous domain III proteins did not inhibit virus cell binding or release prebound virus from cells (Fig. 2 A and see Fig. 6). Inhibition by domain III protein was comparable when virus was prebound to cells at pH 6.5, 6.8, 7.4, or 8.0, or when the low pH pulse was at pH 5.5 or 6.0 (unpublished data). Comparison of the four SFV domain III proteins showed that the strongest inhibition was obtained with His-DIIIS (IC_{50} ~0.1 μM), followed by His-DIII (IC_{50} ~0.5 μM), DIIIS (IC_{50} ~6 μM), and DIII, which gave ~40% inhibition at a concentration of 80 μM (Fig. 2 B). Thus, the presence of both the stem region and the NH_2-terminal His tag resulted in increased effectiveness. Although enhancement by the stem region is suggested from the structure of the low pH-induced HT, the reason for the increase in inhibition observed with His-tagged forms of SFV domain III is not known. The tag at the domain III NH_2 terminus could act by stabilizing binding to E1, mimicking the important domain I–domain III linker region and/or enhancing its trimeric interactions, concentrating the protein at the membrane at low pH, preventing displacement of the exogenous DIII by the endogenous DIII, and/or preventing cooperative HT–HT interactions. High concentrations of His-tagged DV2 domain III protein did not affect SFV fusion (Fig. 3 B), indicating that there is no nonspecific effect of the His tag.

The specificity of domain III inhibition was addressed by comparing the effect of the SFV proteins on fusion of the alphavirus Sindbis virus (SIN) and the flavivirus DV2. The overall sequence of domain III is ~50% identical between SFV and SIN, and the surface of domain III that interacts with the core trimer contains several conserved residues (Gibbons et al., 2004b). In contrast, the DV2 E protein shows no detectable sequence conservation with the alphavirus fusion proteins. SFV, SIN, and DV2 all showed efficient fusion upon treatment at pH 5.5 and little fusion at pH 7.4 (Fig. 3 A). Inclusion of SFV His-DIII or His-DIIIS during the low pH pulse inhibited SIN fusion with comparable (or even slightly higher) efficiency to SFV fusion. The SFV domain III proteins did not cause any inhibition of DV2 fusion.

To address the general applicability of domain III inhibition to class II fusion, we tested the ability of His-DV2DIII and DV2DIIIH1 to inhibit fusion by the DV2 and DV1 serotypes. These two serotypes show ~60% overall sequence identity in domain III. Unlike alphaviruses, flavivirus receptor binding is directly mediated by the membrane fusion protein (Lindenbach and Rice, 2001). Prior studies of flavivirus domain III showed that it could block virus-cell binding (Bhardwaj et al., 2001; Hung et al., 2004). Therefore, we prebound DV1 and DV2 to cells in the cold and added domain III protein only during the 1-min pH pulse used to trigger fusion. As shown in Fig. 3 B, DV2DIIIH1 strongly inhibited both DV1 and DV2 fusion (~70% inhibition of DV2 at a concentration of 50 μM), but showed no activity against SFV. Interestingly, His-DV2DIII did not inhibit DV fusion, suggesting a possible role for helix 1, an NH_2-terminal region of the stem previously shown to promote E protein trimerization (Allison et al., 1999). We have less information on the domain III requirements for optimal DV inhibition and have not yet evaluated if constructs containing both helix 1 and the NH_2-terminal His tag would show increased activity. Treatment at 37°C for 1-min at neutral or low pH with DV2DIIIH1 did not release prebound virus from the cell membrane (Fig. 3 C), indicating that domain III inhibition was not due to effects on virus receptor interaction. Exogenous domain III can thus act as a specific inhibitor of the class II membrane fusion reaction. The observed cross-inhibition within the alphaviruses and flaviviruses suggests conservation of domain III contacts.

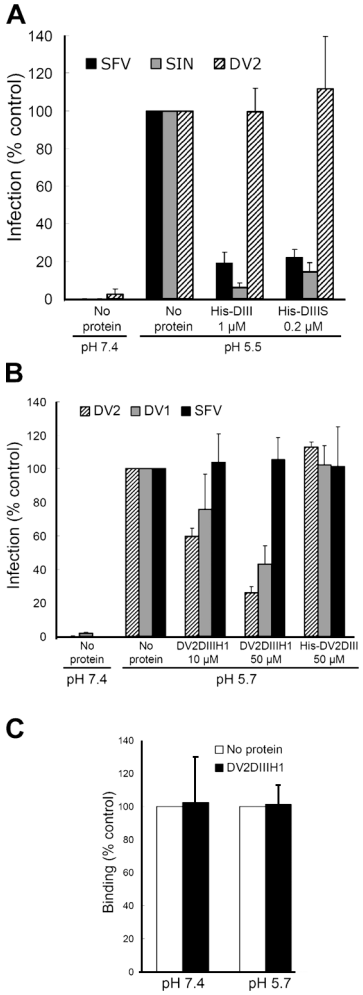

Figure 3. **Domain III proteins specifically inhibit alphavirus and flavivirus fusion.** (A) Inhibition by alphavirus domain III proteins. Viruses were bound to BHK cells for 90 min on ice and incubated at 37°C for 1 min under the indicated conditions. The cells were washed and cultured in medium containing NH_4Cl, and infected cells were quantitated by immunofluorescence. Results are shown as a percentage of control infection (pH 5.5, no protein). (B) Inhibition by flavivirus domain III proteins. Viruses were bound to BHK cells for 90 min on ice and incubated at 37°C for 1 min under the indicated conditions. Infected cells were quantitated as in A. (C) Domain III protein does not release DV2 from cells. DV2 was bound to BHK cells for 90 min on ice and incubated at 37°C for 1 min at the indicated pH in the presence or absence of 50 μM DV2DIIIH1. For samples treated at pH 7.4, cells were incubated for 2 h at 37°C and infected cells were quantitated by immunofluorescence as in A. For samples treated at pH 5.7, cell-associated radiolabeled virus capsid protein was quantitated by SDS-PAGE of cell lysates. Average of three experiments. Error bars are the mean ± SD. $n = 3$.

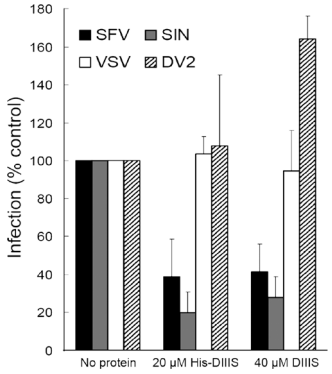

Figure 4. **SFV E1 domain III proteins inhibit alphavirus infection in the endocytic pathway.** SFV, SIN, VSV, and DV2 were diluted in medium of pH 7.2, containing the indicated concentrations of SFV domain III proteins and incubated with BHK cells for 1 h at 20°C to allow endocytic uptake. Infection was blocked by addition of medium containing NH_4Cl, and infected cells were quantitated by immunofluorescence. Data are shown as a percentage of control infection in the absence of domain III proteins. Error bars are the mean ± SD. $n = 3$.

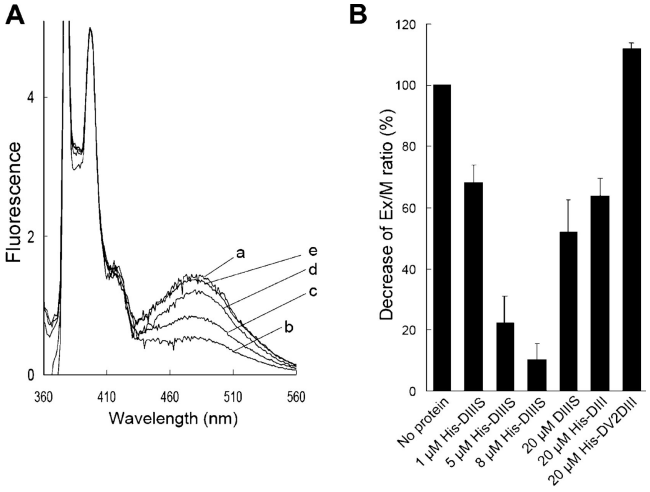

Figure 5. **SFV E1 domain III proteins inhibit the lipid mixing step of fusion.** (A) Fluorescence scan of pyrene-labeled SFV fused with BHK cells. Pyrene-labeled SFV was prebound to BHK cells and incubated at 37°C for 1 min in pH 7.4 medium without domain III protein (curve a), in pH 5.5 medium without domain III protein (curve b), or in pH 5.5 medium with 1 (curve c), 5 (curve d), or 8 μM (curve e) His-DIIIS. Background fluorescence from cells alone was subtracted and the fluorescence emission was normalized for each sample by setting the monomer peak at 397 nm to 5 (arbitrary units). Representative example of three experiments. (B) Comparison of inhibition of lipid mixing by domain III proteins. The fusion between pyrene-labeled SFV and BHK cells was assayed as in A, in the presence of the indicated concentrations of domain III proteins. The difference between the Ex/M at pH 7.4 and after treatment at pH 5.5 without domain III proteins was defined as 100% (control). The difference between the ratios of the pH 7.4 sample and each experimental sample was determined and expressed as a percentage of this control difference. Error bars are the mean ± SD. $n = 3$.

Because alphavirus receptor binding is not mediated by the E1 protein, we used this system to test the ability of domain III proteins to inhibit virus fusion from within the endosome, which is the physiological route of virus infection. We infected BHK cells with either SFV, SIN, vesicular stomatitis virus (VSV), or DV2, in the presence or absence of 20 μM His-DIIIS or 40 μM DIIIS. VSV, an unrelated rhabdovirus, and DV2 are important controls because these viruses also infect cells by endocytosis and low pH-triggered fusion (Matlin et al., 1982; Heinz and Allison, 2001). After a 1-h endocytic uptake period, NH_4Cl was added to prevent further infection, and the primary infected cells were quantitated by immunofluorescence. Infection by both alphaviruses was significantly inhibited by the inclusion of either His-DIIIS or DIIIS (Fig. 4). In contrast, VSV and DV2 infection was not inhibited. Compared with the FIA, inhibition of alphavirus endocytic infection required a higher concentration of His-DIIIS and also showed lower efficacy versus untagged DIIIS. This could reflect relatively inefficient endocytic uptake of His-DIIIS by the cells or differential routing of virus and domain III within the endocytic pathway. Although its targeting to the endosomal site of virus fusion is probably not optimized, it is already clear that domain III protein can block fusion and infection under physiological virus entry conditions.

Exogenous domain III blocks the initial mixing of the virus and cell membranes

Class II virus fusion initiates through the interaction of the fusion loop with the target membrane and progresses through an initial lipid mixing stage termed hemifusion in which the outer leaflets of the virus and target membranes mix (Zaitseva et al., 2005). This stage is followed by the opening of a fusion pore, which widens to give complete fusion and content mixing, the end stage of fusion monitored by the FIA. To test for the effects of domain III proteins on initial lipid mixing and hemifusion, we followed the loss of the pyrene excimer peak upon fusion of pyrene-labeled SFV with unlabeled target cells (Chatterjee et al., 2002). Pyrene-labeled SFV was bound to cells in the cold and pulsed at low pH in the presence or absence of exogenous domain III. We determined the fluorescence emission spectrum of each virus cell mixture and compared the excimer to monomer peak ratio (Ex/M). Untreated virus (unpublished data) or virus treated at pH 7.4 showed a strong excimer peak, with an Ex/M of ~0.28 (Fig. 5 A, curve a). Virus treated at pH 5.5 showed efficient fusion with the cell plasma membrane, as reflected in the decrease of the excimer peak and an Ex/M of ~0.10 (Fig. 5 A, curve b). The presence of His-DIIIS caused a concentration-dependent inhibition of the lipid mixing step (Fig. 5 A, curves c–e). No effect was observed when His-DIIIS was added to the sample after low pH treatment (unpublished data). As observed in the FIA, His-DIIIS showed the highest activity, with ~90% inhibition of fusion at 8 μM (Fig. 5 B). Both His-DIII and DIIIS produced significant inhibition at 20 μM, whereas the DV domain III protein gave no inhibition at 20 μM. A higher concentration of His-DIIIS was required to completely inhibit pyrene virus fusion compared with the FIA, which could reflect an intrinsic difference in the inhibitor sensitivity of lipid mixing versus content mixing or the higher concentration of virus used in the pyrene versus FIA experiments.

Exogenous domain III binds to viral E1 during fusion

If domain III protein is inhibiting virus fusion by preventing the foldback of the full-length viral E1, it may interact stably

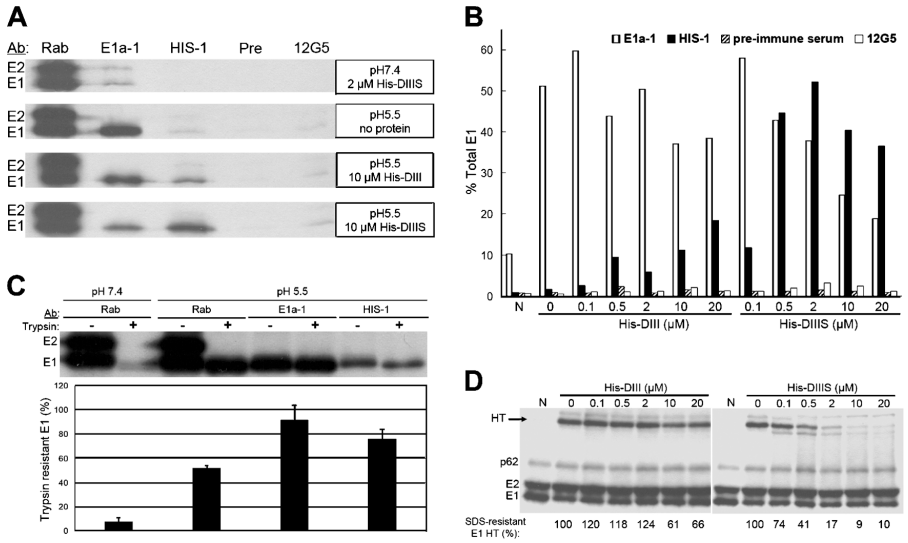

Figure 6. **SFV domain III proteins bind to trimeric E1 during the fusion reaction.** (A) Domain III proteins bind to E1 during fusion. ^{35}S-labeled SFV was bound to BHK cells on ice and treated at pH 7.4 or 5.5 at 37°C for 1 min in the presence of the indicated domain III proteins. Cells were washed, lysed, and immunoprecipitated with a rabbit polyclonal antibody against the SFV E1 and E2 protein (Rab), a mAb against the low pH conformation of E1 (E1a-1), a mAb against the His tag (HIS-1), a rabbit preimmune serum (Pre), or an isotype-matched irrelevant mAb (12G5). Samples were analyzed by SDS-PAGE and fluorography. (B) Quantitation of samples prepared as in A using the indicated concentrations of His-DIII or His-DIIIS. N indicates 1 min treatment at pH 7.4 with 2 μM His-DIIIS. The total E1 in each sample was defined as the amount of E1 immunoprecipitated by Rab. Representative example of two experiments. (C) Domain III selectively interacts with a trimeric form of E1. Fusion reactions were triggered at pH 7.4 or 5.5 in the presence of 10 μM His-DIII as in A. Samples were immunoprecipitated with the indicated antibodies, digested with trypsin where indicated, and analyzed by SDS-PAGE. The amount of trypsin-resistant E1 was quantitated and expressed as a percentage of the nontrypsinized E1 for each sample. Error bars are the mean ± SD. $n = 3$. (D) Exogenous domain III proteins affect the SDS-resistant conformation of the E1 HT. Samples were prepared as in B. An aliquot of the cell lysate was treated with SDS-sample buffer at 30°C and analyzed by SDS-PAGE and fluorography. For each sample, the SDS-resistant E1HT band (arrow) was quantitated and expressed as a percentage of the E1HT in the absence of domain III proteins. Representative example of two experiments.

with the E1 protein during inhibition. To assay for such interaction, we used radiolabeled SFV and His-DIII or His-DIIIS in the FIA. After the low pH-treatment step, the cells were lysed in the nonionic detergent octylglucoside, which we have shown fully solubilizes membrane-inserted E1 and disrupts intertrimer interactions, but maintains trimer structure (Gibbons et al., 2004a). Aliquots of the samples were immunoprecipitated using a polyclonal antibody to quantitate total E1; mAb E1a-1, which specifically recognizes the low pH-induced conformation of E1 (Ahn et al., 1999); mAb HIS-1, which recognizes the His tag on domain III; and two control antibodies; and were analyzed by SDS-PAGE (Fig. 6 A). Equivalent amounts of radiolabeled virus proteins were present in cells treated at neutral or low pH with or without domain III proteins, confirming that bound virus was not released from the cell. Upon acid treatment the E1 protein was efficiently recognized by mAb E1a-1. Inclusion of either His-DIII or His-DIIIS during low pH treatment resulted in coimmunoprecipitation of the E1 protein by the HIS-1 antibody. Quantitative analysis showed that the amount of E1 retrieved by HIS-1 increased when increasing amounts of domain III proteins were present during the low pH step (Fig. 6 B). His-DIII retrieved ~18% of the total E1 at a concentration of 20 μM. Retrieval by His-DIIIS was maximal at 2 μM and ~50% of the total E1, similar to the amount of E1 that converted to reactivity with mAb E1a-1. His-DIIIS was thus more efficient for both coimmunoprecipitation and fusion inhibition. Similar to their effects on fusion activity, domain III proteins only interacted with viral E1 when present during the low pH treatment step and not at neutral pH (Fig. 6, A and B).

The target for exogenous domain III binding during fusion could be either the E1 monomer before trimerization or a trimeric form of E1. A general property of trimeric E1 is its relative resistance to trypsin digestion (Chatterjee et al., 2002). We treated cell-bound radiolabeled SFV at pH 7.4 or 5.5 in the presence of 10 μM His-DIII and quantitated the trypsin resistance of the E1 retrieved by the indicated antibodies (Fig. 6 C). The pH 7.4 treated monomeric E1 was almost completely digested by trypsin (7% resistant). After the low pH pulse, ~50% of the total E1 was in a trypsin-resistant trimer conformation, which is in keeping with the usual efficiency of HT formation (Gibbons and Kielian, 2002). The E1 population retrieved by either mAb E1a-1 or the antibody to the His tag was strongly enriched in trypsin-resistant E1. In addition, experiments with the monomeric E1* ectodomain revealed that exogenous SFV domain III did not bind E1* at either neutral or low pH, whereas binding was observed when E1* was triggered to trimerize (Klimjack et al., 1994) by treatment with low pH and target membranes (unpublished data). Together, these data suggest that the trimerization of E1 produces a binding site that interacts with exogenous domain III.

Dominant-negative binding of exogenous domain III would be predicted to alter the conformation of the E1 HT by preventing the folding back of the viral domain III, and consequently could decrease trimer stability. Exposure of the acid-conformation–specific mAb E1a-1 epitope on domain I closely correlates with HT formation, although the epitope is not formed by trimerization per se (Ahn et al., 1999). Interestingly, concentrations of His-DIIIS above 2 μM led to a gradual decrease in the retrieval of E1 by both the anti-His antibody and mAb E1a-1 (Fig. 6 B). This suggests that the binding of exogenous domain III is directly affecting the conformation of the E1HT. Destabilization of the trimer structure by domain III could also explain why somewhat less trypsin-resistant trimer was recovered after retrieval with domain III, as compared with the acid-specific mAb (Fig. 6 C). We directly evaluated HT stability by following the resistance of the SFV HT to dissocia-

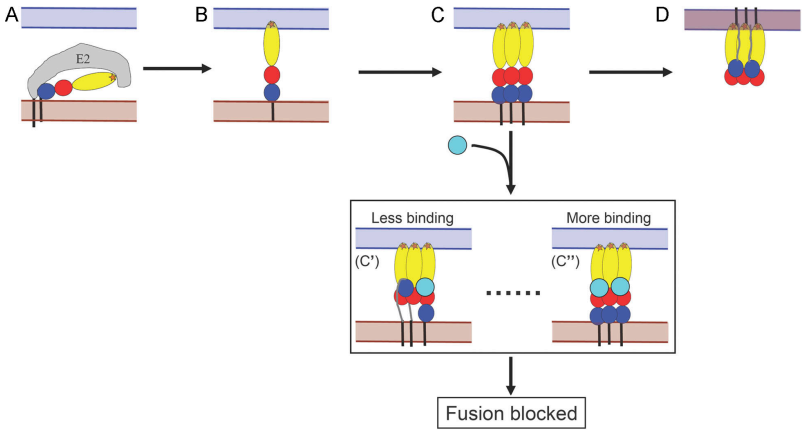

Figure 7. **Model for SFV E1 conformational changes during fusion and the action of exogenous domain III.** (A) Prefusion form of E1 on the virus surface, with the E2 protein shown in gray, the E1 domains colored as in Fig. 1 A, and the fusion loop indicated by an orange star. The virus membrane is shown in brown and the target membrane is shown in blue. At this stage E1 is mAb E1a-1negative, trypsin sensitive, and shows no SDS-resistant trimer band (Kielian et al., 2000). (B) Low pH triggers the dissociation of E2/E1 dimer and the initial interaction of monomeric E1 with the target membrane. (C) Proposed membrane-inserted E1 trimer, suggested as a relatively long-lived intermediate. Subsequent folding back of domain III and the stem region would drive membrane fusion. (D) Postfusion HT form of E1 with domain III and the stem region (gray) fully folded back. In this conformation, the E1 trimer is mAb E1a-1 positive, trypsin resistant, and SDS resistant (Kielian et al., 2000). (C′/C′′) This panel illustrates the interaction of exogenous domain III (turquoise circle) with the proposed E1 trimer intermediate shown in C. This interaction produces a mixed population of domain III–bound trimers as illustrated by the two states, C′ and C′′, and the dotted line connecting them. All the states in the mixed population would be blocked from fusing and would differ in their conformation and biochemical properties. In the C′ state, with low concentration and/or low affinity of domain III proteins, some E1 trimers would undergo partial foldback and binding of one exogenous domain III. In the C′′ state, with high concentration and/or high affinity of domain III proteins, trimers would bind three exogenous domain III proteins and would be completely blocked in foldback. We predict that the C′ state would be mostly SDS resistant and mAb E1a-1 positive, whereas the C′′ state would be SDS sensitive and E1a-1 negative. This model is simplified and does not illustrate the stages of membrane curvature, the potential roles of cooperative trimer interactions, or the initial lipid mixing and pore formation.

tion by SDS sample buffer at 30°C (Fig. 6 D). Increasing amounts of domain III proteins lead to the loss of the SDS-resistant HT conformation, with only 10% of the control HT observed in the presence of 10 μM His-DIIIS. Interestingly, bands migrating above and below the position of the E1HT were clearly observed with His-DIIIS, suggesting the presence of alternative E1 complexes. A decrease in the SDS-resistant E1HT was also observed in the presence of increasing amounts of His-DIII (60% of control HT at 10 μM His-DIII). Together, these results support a model (Fig. 7) in which exogenous domain III binds to an intermediate trimeric conformation of E1 and prevents final hairpin formation and fusion.

Discussion

In this study, we demonstrated that exogenously added domain III could inhibit the alphavirus and flavivirus membrane fusion reactions. Exogenous domain III blocked low pH-induced virus fusion at the cell surface and within the normal endosomal entry pathway and prevented both complete fusion and lipid mixing. Domain III inhibition thus provides proof of principle of a dominant-negative inhibitor strategy for the class II fusion reaction and demonstrates the key role of the domain III–core trimer interaction in virus fusion and infection.

Our studies with SFV demonstrate that exogenous domain III stably interacted with a trimeric form of E1. Domain III inhibition thus identifies an important intermediate in the fusion reaction, defined by the presence of a relatively long-lived "target" core trimer (Fig. 7 C). The HT structure suggests that the binding site for exogenous domain III would be located within the groove formed by two E1 subunits in the central domain I/domain II portion of the class II hairpin (Gibbons et al., 2004b). This model agrees well with our finding that the monomer did not bind domain III because initial oligomerization would be required to form the critical binding site. The binding of exogenous domain III to the core trimer was very stable, resisting repeated detergent washes during coimmunoprecipitation. Binding affected the conformation of the HT to varying extents by preventing the normal folding back of one or more E1 subunits (Fig. 7). Inhibition showed cross reactivity among related viruses, in keeping with the presence of conserved residues in the domain III–core trimer interface. Although our data do not yet indicate which residues are most critical to the domain III interaction, they clearly suggest conservation of key protein contacts. The SFV E1HT structure indicates that the AB loop and C′-strand may be important in this interaction (Gibbons et al., 2004b). Together, the properties of inhibition indicate that domain III–core trimer binding is an important step in fusion protein refolding, with the potential to provide significant driving force in fusion.

Our studies also revealed a strong enhancement of inhibition by the presence of the fusion protein stem region. We have tested several stem peptides for their ability to inhibit SFV HT formation and/or membrane fusion (unpublished data). To date we have not observed inhibition by the stem region alone, and thus we hypothesize that domain III acts to orient the stem for its interaction with the core trimer (but see flavivirus results in Hrobowski et al., 2005). It may also be that the binding site for domain III is kinetically favored compared with that of the stem, which may occur later in the fusion reaction and be relatively short-lived. Our data are consistent with the exogenous domain III–core trimer interaction acting as a key first step in inhibition, with subsequent stem binding along the body of the trimer providing further stabilization of the inhibitory interaction.

An alternative model for inhibition by domain III is that it prevents cooperative HT–HT interactions during fusion. Studies of the membrane insertion of class II fusion protein ectodomains indicate that insertion is highly cooperative (Gibbons et al., 2003; Stiasny et al., 2004). In the case of SFV, ectodomain insertion produces rings of five to six trimers, re-

flecting the physical associations of adjacent HTs through interactions of their fusion loops and of their domain III regions (Gibbons et al., 2003, 2004b). These cooperative interactions produce a volcano-like assembly of E1HTs that may help to induce membrane curvature at the fusion site (Gibbons et al., 2004b). Although we hypothesize that these intertrimer interactions are important for fusion, we feel that the strongest model for the action of domain III is that it acts not to prevent interactions between adjacent HTs, but to inhibit the foldback reaction within one E1 molecule. This agrees well with the resistance of domain III–E1 binding to octylglucoside, a detergent that we previously found disrupted HT–HT interactions (Gibbons et al., 2003, 2004a). It also agrees with the increase in inhibition and binding that is observed when the stem is present on domain III because no role for the stem in HT–HT interactions was observed in the previous studies. However, domain III could also be acting by some combination of these two models. For example, prevention of E1 refolding by binding of exogenous domain III could inhibit the ability of the viral domain III to interact with an adjacent trimer.

Both the alphavirus and flavivirus fusion reactions are very rapidly triggered by low pH, with maximal fusion observed within seconds of low pH treatment at 37°C (Kielian et al., 2000; Heinz and Allison, 2001). Given the speed of the SFV fusion reaction, it is perhaps surprising that exogenous domain III can compete with the endogenous domain III for binding to the core HT. Such an intermolecular interaction of domain III would seem to be at a disadvantage compared with the intramolecular interaction of the viral domain III. Several factors may help to explain this paradox. The movement of domain III in the full-length E1 may be constrained by its attachment to the virus membrane through the stem/TM domains. Indeed, we found that binding of exogenous domain III to the E1* ectodomain trimer was not as efficient as binding to the full-length trimer (unpublished data), in keeping with the possibility of a more rapid foldback of E1* domain III due to the loss of its membrane anchor constraint. The structure of the E1 HT also reveals that the linker region between domain I and III becomes highly extended during the movement of domain III toward the fusion loop (Gibbons et al., 2004b). This could provide an additional constraint to viral domain III movement, favoring the interaction of exogenous domain III with the core trimer.

Previous studies of flavivirus domain III proteins have focused on their interactions with virus receptors on the cell surface. Our data identify a novel inhibitory effect of exogenous domain III in the fusion reactions of alphaviruses and flaviviruses. Such domain III proteins can serve as useful basic research tools to study alphavirus and flavivirus fusion. Although unlikely to be directly applicable as antivirals, their inhibitory activity has important implications for the development of clinically useful inhibitors of the class II fusion reaction. Because exogenous SFV domain III showed stable binding to a trimeric E1 target, this interaction could be used to screen for peptides or small molecules that would block critical domain III–trimer contacts. Given the cross-inhibition we have observed with domain III, such screens have the potential to identify broad-specificity inhibitors of class II fusion proteins.

Materials and methods

Cells and viruses

BHK-21 cells and C6/36 mosquito cells were cultured as previously described (Vashishtha et al., 1998). SFV was a well-characterized, plaque-purified isolate (Vashishtha et al., 1998) and SIN was derived from the infectious clone of Toto 1101 (Rice et al., 1987). VSV expressing GFP (Boritz et al., 1999) was obtained from J.K. Rose (Yale University, New Haven, CT), DV2 (strain New Guinea C) from J. Roehrig (Center for Disease Control, Fort Collins, CO), and DV1 (strain Western Pacific) from R. Stockert (Albert Einstein College of Medicine, Bronx, NY). SFV, SIN, and VSV were propagated in BHK-21 cells, and DVs were propagated in C6/36 cells in DME containing 2% heat-inactivated FCS and 10 mM Hepes, pH 8.0. ^{35}S-labeled SFV was prepared as previously described (Vashishtha et al., 1998), and ^{35}S-labeled DV2 was prepared in C6/36 cells (Hilgard and Stockert, 2000) and pelleted through a sucrose cushion (Kielian et al., 1996).

Construction of domain III protein expression plasmids

DNA sequences of SFV E1 domain III (with or without stem region) were amplified from DNA derived from the infectious SFV clone of Toto1101 (Chatterjee et al., 2002), and the DNA sequences of DV2 E domain III were obtained by RT-PCR using viral RNA extracted from DV2-infected C6/36 cells. These sequences were subcloned into the protein expression plasmid pET-14b (Novogen) to express domain III proteins with an added NH_2-terminal methionine, or the pRSET A plasmid (Invitrogen) to express NH_2-terminal 6× Histidine-tagged domain III proteins with an added NH_2-terminal 36 amino acids, and confirmed by DNA sequencing.

Protein expression, refolding, and purification

Domain III proteins were expressed and refolded essentially as described for other Ig-like domain proteins (X. Zhang et al., 2002). In brief, proteins were expressed in *E. coli* strain BL21(DE3), solubilized from inclusion bodies in buffer containing 6 M guanidine-HCl, refolded by the fast dilution method, and purified by fast protein liquid chromatography on a Superdex G-75 gel filtration column (GE Healthcare). The concentration of purified domain III proteins was determined by absorption at 205 nm (Scopes, 1974).

Protein analysis

The mass of domain III proteins was measured by ESI mass spectrometry using a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corporation). Analysis by SDS-PAGE was performed using a standard Tris–glycine system and 11% acrylamide gels, except in Fig. 1 C, where a Tris–tricine buffer system and 16.5% acrylamide gels were used (Gibbons and Kielian, 2002). ^{35}S-labeled proteins were quantitated by PhosphorImager analysis with Image Quant version 1.2 software (Molecular Dynamics).

FIA

Fusion of viruses with the plasma membrane of BHK cells was assayed using a variation of a previously described SFV FIA (Vashishtha et al., 1998). BHK cells grown on 12-mm coverslips in 24-well plates were washed twice with ice cold binding medium (RPMI without bicarbonate, plus 0.2% BSA, 10 mM Hepes, and 20 mM NH_4Cl) at the indicated pH. Cells were incubated on ice for 90 to 120 min with gentle shaking with SFV and SIN in binding medium plus 20 mM MES, pH 6.8, or with DVs in binding medium, pH 7.9. Cells were washed twice with binding medium to remove unbound virus and pulsed for 1 min at 37°C in 200 μl pH medium (RPMI/BSA/Hepes plus 30 mM MES for pH 7.4 or RPMI/BSA/Hepes plus 30 mM sodium succinate for pH 6.0 or lower). SFV- and SIN-infected cells were incubated at 28°C overnight in BHK growth medium plus 20 mM NH_4Cl; DV-infected cells were incubated in MEM plus 2% FCS and 50 mM NH_4Cl for 3 h at 37°C, and then at 37°C for 2 d in the presence of 20 mM NH_4Cl. Domain III proteins were added at specific steps as indicated. The threshold for DV fusion (approximately pH 7.0) was higher than that of SFV, with maximal DV fusion observed at approximately pH 6.2 and below, resulting in infection by ~30% of the bound infectious DV.

Immunofluorescence microscopy

VSV-infected cells were fixed in 3% formaldehyde at RT for 20 min and GFP-expressing cells were quantitated by fluorescence microscopy. Cells infected by all other viruses were fixed in ice-cold methanol for 10 min. SFV- and SIN-infected cells were stained with rabbit polyclonal antisera against SFV or SIN envelope proteins and fluorescein-conjugated secondary antibody (Vashishtha et al., 1998). DV2- and DV1-infected cells were stained

with a mouse polyclonal hyperimmune ascitic fluid against DV2 (obtained from R.B. Tesh, University of Texas Medical Branch, Galveston, TX), followed by an Alexafluor 488–conjugated secondary antibody (Molecular Probes). For each sample, duplicate coverslips were evaluated at an infection level of >200 positive cells/coverslip in the absence of inhibitor.

Assays of the SFV E1 HT

To assess the conformational change of SFV E1 protein during fusion in the presence of domain III proteins, purified ^{35}S-labeled SFV was bound to BHK cells on ice and pulsed at low pH, as in the FIA. The cells were washed to remove exogenous domain III and solubilized in lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1.5% octylglucoside, 1 mM EDTA, plus 1 μg/ml pepstatin, 50 μg/ml leupeptin, 0.1% BSA, 100 μg/ml aprotinin, and 1 mM PMSF). To quantitate the SDS-resistant E1 HT, an aliquot of each lysate was added to SDS sample buffer and heated to 30°C for 3 min before SDS-PAGE. Another aliquot of cell lysate was subjected to immunoprecipitation (Kielian et al., 1996) using the indicated antibodies and zysorbin as immunosorbant, and analyzed by SDS-PAGE.

To test trypsin resistance, the zysorbin with retrieved immunoprecipitate was pelleted, resuspended in PBS containing 1% Triton X-100, and digested with 125 μg/ml trypsin at 37°C for 1 h. The digestion was stopped by adding 5 mM PMSF. The zysorbin was eluted by treatment with 2% SDS and three cycles of heating to 95°C for 3 min. It was pelleted and the supernatants were concentrated by acid precipitation and analyzed by SDS-PAGE.

Pyrene-labeled SFV fusion with cells

Pyrene-labeled SFV was prepared by propagation of virus in BHK cells metabolically labeled with C_{16}-pyrene (Chatterjee et al., 2002) and tested using a protocol similar to the FIA. Virus was bound on ice for 120 min to BHK cells on 35-mm plates at a multiplicity of ~2,000 pfu/cell. Virus fusion was induced at 37°C for 1 min in pH 7.4 or 5.5 medium. Cells were put back on ice and washed once with binding medium at pH 6.8 and once with H-H solution (HBSS containing 10 mM Hepes, pH 7.4, and 20 mM NH_4Cl). The cells were scraped in H-H solution, transferred to a quartz cuvette, and the emission spectrum determined using an AB-2 fluorometer (Thermo Electron Corporation) at 37°C with an excitation wavelength of 343 nm (Chatterjee et al., 2002). The fluorescence emission from 360 to 560 nm was recorded as the average of two serial scans, and the background fluorescence from cells alone was subtracted from each sample. The excimer and monomer peaks were determined at emission wavelengths of 475 and 397 nm, respectively.

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