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Association of the Caveola Vesicular System with Cellular Entry by Filoviruses

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The filoviruses Ebola Zaire virus and Marburg virus are believed to infect target cells through endocytic vesicles, but the details of this pathway are unknown. We used a pseudotyping strategy to investigate the cell biology of filovirus entry. We observed that specific inhibitors of the caveola system, including cholesterol-sequestering drugs and phorbol esters, inhibited the entry of filovirus pseudotypes into human cells. We also measured slower cell entry kinetics for both filovirus pseudotypes than for pseudotypes of vesicular stomatitis virus (VSV), which has been recognized to exploit the clathrin-mediated entry pathway. Finally, visualization by immunofluorescence and confocal microscopy revealed that the filovirus pseudotypes colocalized with the caveola protein marker caveolin-1 but that VSV pseudotypes did not. Collectively, these results provide evidence suggesting that filoviruses use caveolae to gain entry into cells.

Ebola virus (EBO) and *Marburg virus* (MBG) are enveloped, filamentous viruses containing negative-stranded RNA and are members of the family *Filoviridae* (23). Both viruses are causative agents of fatal hemorrhagic fevers in humans and, as such, are classified as biosafety level 4 agents (23). Recent EBO hemorrhagic fever outbreaks include the Uganda outbreak in 2000–2001 (6) and ongoing outbreaks in Congo and Gabon (World Health Organization [http://www.who.int/disease-outbreak-news/]). Elucidating the mechanism by which these pathogens enter cells might serve to identify the steps within the infection process that may be inhibited as a means of preventing or ameliorating viral spread and hemorrhagic fever in vivo.

Although very similar both structurally and functionally, envelope glycoproteins (GPs) of EBO and MBG have incomplete homology and differing GP transcription events and sensitivities to various glycosylation inhibitors, suggesting that there are distinctions between the biological characteristics of EBO and MBG (7, 8, 30, 31). Pseudotyping, which has been described previously (8, 13, 39, 46), allows researchers to work with filoviruses outside a biosafety level 4 setting. This strategy has proven to be a powerful tool in the study of the earlier events in the filovirus life cycle, i.e., target cell binding and entry. Indeed, such a strategy has been used to identify human folate receptor alpha (FR-α) as a cofactor for filovirus cell entry (7).

FR-α is a 38- to39-kDa glycosyl phosphatidylinositol (GPI)-linked cell surface protein that binds and internalizes extracellular folic acid via vesicles (3). Additionally, in vitro artificial-vesicle studies have shown that EBO GP-mediated fusion requires phosphatidylinositol within the membranes of target vesicles (29), suggesting a direct role for GPI in EBO fusion. As is known for other GPI-linked surface proteins, FR-α is thought to be endocytosed via caveolae (2), although some controversy remains about the complete nature of intracellular trafficking by this molecule (17, 25). Given the apparent role of a GPI-linked surface protein in filovirus entry, we hypothesized that these viruses utilize caveolae during infection. Briefly, caveolae are vesicles enriched with cholesterol and sphingolipids and have been shown to be involved in a wide range of biological events such as transmembrane signaling, cellular cholesterol homeostasis, and cellular entry by certain bacteria, natural ligands, toxins, and viruses (2, 15, 32–34, 37, 41, 42). We performed several studies to investigate the possible role of caveolae in filovirus cell entry.

GP-mediated entry of EBO-Z and MBG into human cells is inhibited by two distinct classes of cholesterol inhibitors. To generate pseudotypes for our studies, a human immunodeficiency virus type 1 provirus, NL4-3, that lacks *envelope* but carries a luciferase reporter gene, pNL-Luc-E$^-$R$^-$ (9), was pseudotyped with either Ebola Zaire virus (EBO-Z) or MBG GPs as previously described (8). Vesicular stomatitis virus (VSV) and amphotropic (Ampho)-virus pseudotypes were generated in similar manners with VSVG protein and the Ampho-murine leukemia virus (MLV) envelope, respectively. With regard to their modes of entry, both wild-type VSV and VSV pseudotypes have been observed to enter cells via clathrin-coated pits while Ampho MLV directly fuses at the cell membrane (18, 38, 45). We used EBO-Z, MBG, VSV, and Ampho-virus pseudotypes to test the effects of cholesterol-sequestering drugs on infection to determine whether caveolae are involved in virus entry. The latter two pseudotypes represent control viruses that are known to enter cells independently of caveolae. Cholesterol is a necessary constituent of caveolar vesicles (2), and its depletion results in the blocking of caveola-mediated events (12, 19, 20, 28). Human 293T cells were pretreated with nystatin (25 μg/ml), methyl-β-cyclodextrin (MβCD) (500 μM), or no drug at 37°C for 30 min, and then the treated cells were cultured with the above-indicated pseudotypes for 6 h at 37°C. Subsequently, noninternalized virus was removed by treatment with trypsin (0.25%) at 37°C

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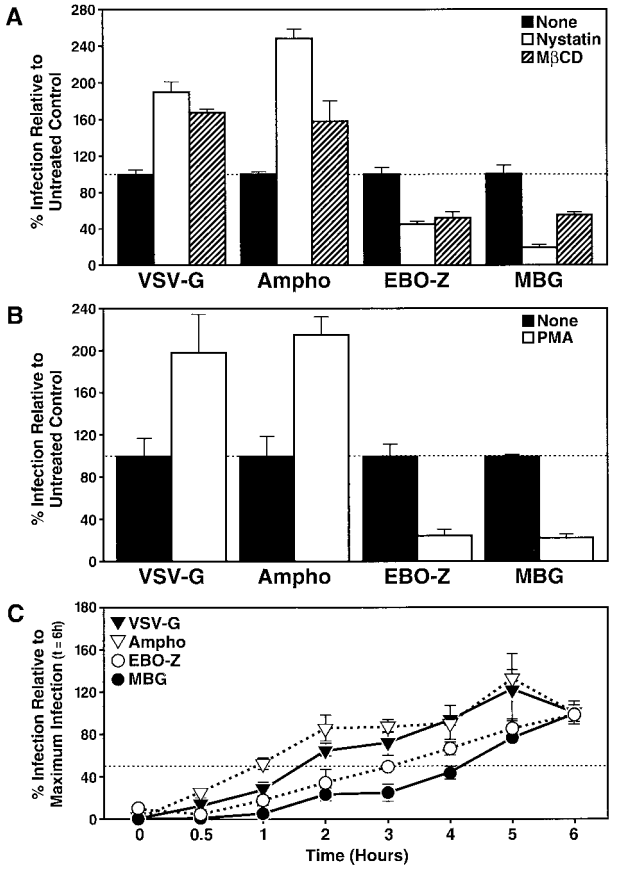

FIG. 1. Characterization of entry into human 293T cells by EBO-Z and MBG pseudotypes. Shown are the levels of infections in 293T cells treated with nystatin (25 μg/ml) or MβCD (500 μM) (A) and with PMA (10 μM) (B). Values are mean levels (± standard errors) of luciferase activity in cell lysates ($n = 3$). Dashed lines indicate infection of untreated control set at 100% infection. (C) Kinetics of pseudotype entry into 293T cells. The indicated pseudotypes were preadsorbed to 293T cells on ice for 30 min, and then the cells were shifted to 37°C. At 0, 0.5, 1, 2, 3, 4, 5, and 6 h following the shift, the cells were treated with trypsin (0.25%) to remove noninternalized virus. Infection was then measured 3 days postinfection by measuring luciferase activity in cell lysates. Values are mean levels (± standard errors) of luciferase activity in cell lysates ($n = 3$ for each time point). The horizontal dashed line indicates a 50% level of infection relative to the maximum level of infection at 6 h.

for 10 min, culture medium was replaced with trypsin-free medium, and infection was measured 3 days postinfection by quantitating luciferase activity in cellular lysates. We observed that both cholesterol-sequestering drugs specifically inhibited infections by EBO-Z and MBG pseudotypes (Fig. 1A). Nystatin reduced the levels of infections by EBO-Z and MBG pseudotypes by 55 and 81%, respectively. Similarly, MβCD reduced infections by EBO-Z and MBG pseudotypes as well (by 48 and 45%, respectively). In contrast, both drugs had stimulatory effects on infections by VSV and Ampho-virus pseudotypes. Neither of these treatments increased infection by core particles lacking envelope proteins above negligible background levels (data not shown). The differential effects of the drugs on the filovirus pseudotypes compared to the effects on the two control pseudotypes emphasize the dissimilarity among the pathways used by these virus types, suggesting that a cholesterol-dependent mechanism, such as caveolae, is involved in filovirus entry.

Entry by EBO-Z and MBG pseudotypes is inhibited by PMA. Phorbol-12-myristate-13-acetate (PMA) has also been shown to specifically inhibit virus entry via caveolae, possibly by suppressing caveola invagination from the intracellular face of the cell membrane (1, 21, 24, 27, 36). Consequently, we sought to determine whether filovirus entry was sensitive to PMA. Briefly, 293T cells were preincubated in PMA (10 μM) at 37°C for 30 min and then inoculated with the pseudotypes as in the earlier experiments. PMA treatment reduced infections by EBO-Z and MBG pseudotypes by 75 and 77%, respectively, but enhanced infections by VSV and Ampho-virus pseudotypes (Fig. 1B). PMA treatment did not affect the entry of envelope-lacking virions (data not shown). These results suggest that filovirus entry depends upon caveolae or another endocytic pathway that is sensitive to PMA.

Both EBO-Z and MBG pseudotypes have slower cell entry kinetics. While supportive of our hypothesis, the drug inhibition studies described above cannot rule out the possibility that other distinct entry pathways are involved or that the drugs inhibit entry independently of an effect on caveolae. We therefore proceeded to further characterize filovirus entry using other methods. Caveolae have been shown to endocytose specific ligands more slowly than do other equally well-characterized endocytic mechanisms (11, 40, 43). This distinctly slower cell entry kinetic was also observed for the caveola-utilizing polyomavirus simian virus 40 (SV40) (1, 24). We therefore

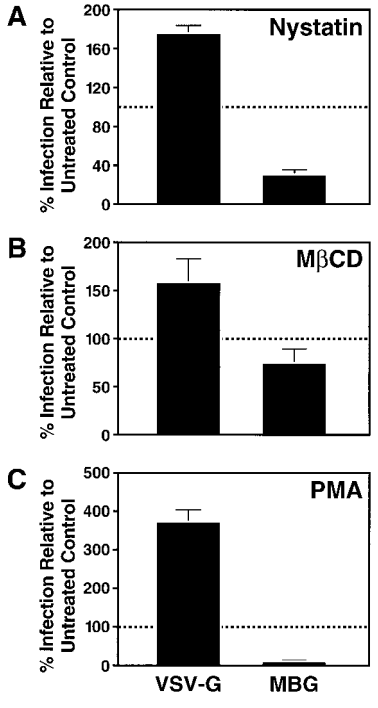

FIG. 2. Inhibition of MBG infection of human HeLa cells. Shown are the levels of infection of HeLa cells treated with nystatin (50 μg/ml) (A), MβCD (5 mM) (B), and PMA (10 μM) (C). Values are mean levels (± standard errors) of luciferase activity in cell lysates ($n = 3$). The dashed lines indicate infection of untreated control set at 100% infection.

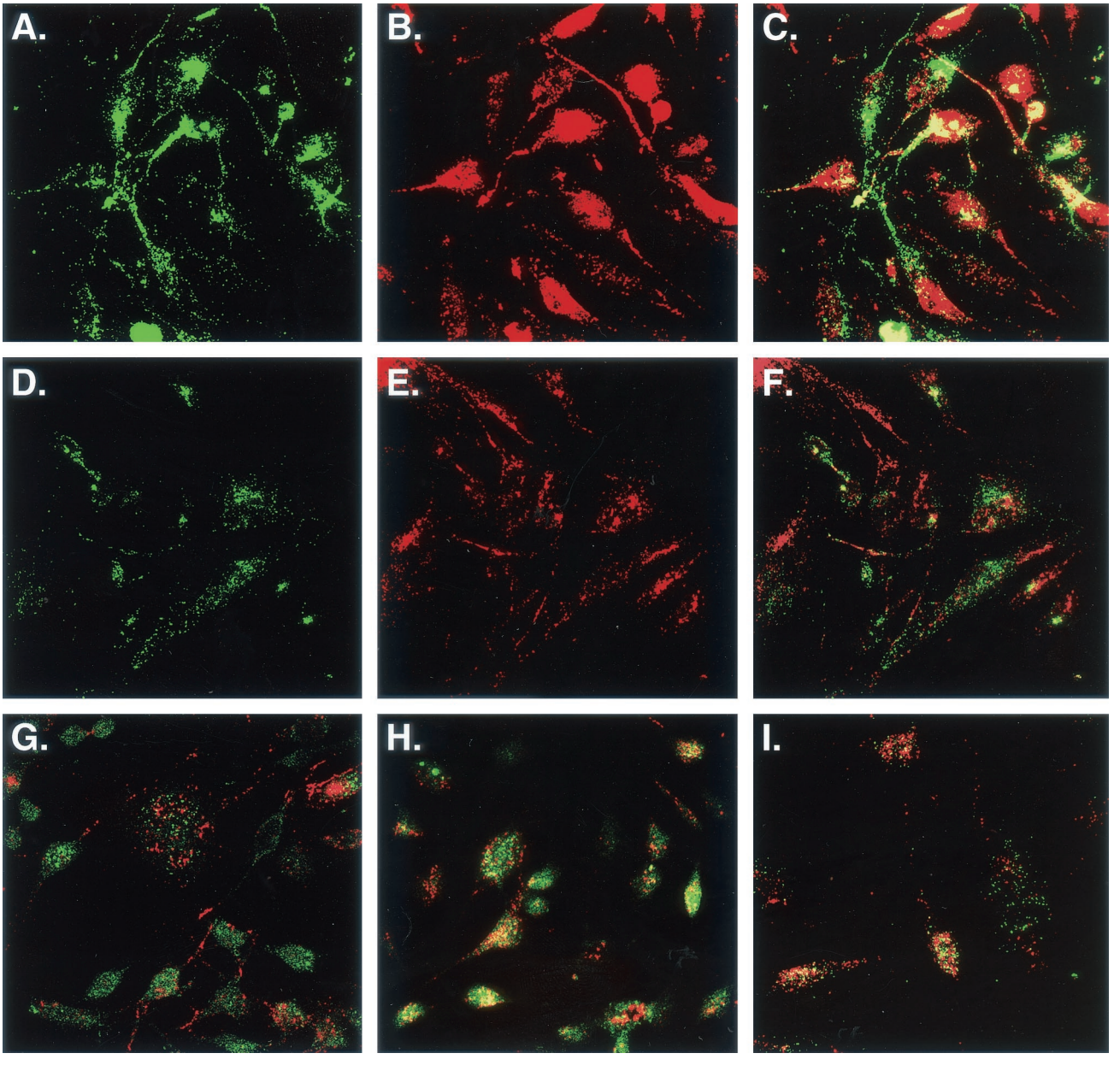

FIG. 3. Colocalization of EBO-Z and MBG pseudotypes with the caveola protein marker CAV-1 after infection of HeLa cells. (A to I) Images of cells with Ctxn B-FITC (A to C), tfn-FITC (D to F), and the VSV, EBO-Z, and MBG pseudotypes (G, H, and I, respectively); (C) Ctxn B-FITC and CAV-1 colocalization following incubation of cells with Ctxn B-FITC for 30 min at 37°C; (F) absence of colocalization of tfn-FITC and CAV-1 following incubation of cells with tfn-FITC for 30 min at 37°C; (G) absence of colocalization of VSV and CAV-1 following incubation of cells with VSV pseudotypes for 30 min at 37°C; (H and I) colocalization of EBO-Z and MBG with CAV-1 following a 90-min incubation of cells with EBO-Z (H) and MBG (I) pseudotypes at 37°C. Following the incubations, all samples were fixed in paraformaldehyde (2%), permeabilized in Triton X-100 (0.1%), and immunostained for p24 (to detect pseudotypes) and/or CAV-1 (to detect caveolae). p24 was detected with an FITC-conjugated mouse anti-p24 antibody (Coulter Corp.), and the FITC signal was amplified with Alexa sandwich antibodies (Alexa Fluor 488-conjugated goat polyclonal anti-FITC antibody and Alexa Fluor 488-conjugated donkey anti-goat IgG (H+L) conjugate (Molecular Probes, Inc.). CAV-1 was detected with a rabbit polyclonal antibody against CAV-1 (BD Transduction Laboratories) and a Texas Red-conjugated anti-rabbit IgG (H+L) $F(ab')_2$ fragment (Jackson ImmunoResearch Laboratories, Inc.). Cell images were taken under oil at a ×60 magnification with a Bio-Rad Radiance2100 laser scanning confocal microscope system operated via LaserSharp2000 software. For intracellular analyses of cells, the *z*-axis position was computer controlled and the basal and apical membranes were set as the start and stop points, respectively. All images were at least 2 μm from either vantage point. Immunostained cells were excited with krypton or argon lasers at 488 nm (Alexa Fluor 488 and FITC) and 568 nm (Texas Red), and fluorescence signals were collected with barrier filters for Alexa Fluor 488 and FITC and for Texas Red. Regions of colocalization were generated by digital overlay with Photoshop 6 (Adobe Systems) and appear yellow (C and F to I). For comparison purposes, images collected with green and red barrier filters were digitally colorized to green (A and D) and red (B and E), respectively, also with Photoshop 6.

measured EBO-Z and MBG pseudotype cell entry kinetics to determine whether these viruses share this property. The appropriate pseudotypes were allowed to bind to 293T cells on ice for 30 min, and then synchronous cell entry was initiated by shifting the cells to 37°C. At various time points postshift, noninternalized pseudotypes were removed with trypsin, and infection was measured at day 3 postinfection as before. For comparison purposes, the level of infection measured after 6 h of pseudotype adsorption was designated the maximum infection level for each particular virus type and used as a reference point for measuring kinetics. Both VSV (clathrin-mediated entry) and Ampho-virus (direct fusion entry) pseudotypes entered significantly more rapidly than did the EBO-Z and MBG pseudotypes, with time. The postadsorption entry times needed to achieve 50% of the maximum infection were 1 to 1.5 h for the control pseudotypes and 3 to 4 h for the filovirus pseudotypes (Fig. 1C). Moreover, these 50% entry times for the filovirus pseudotypes may well represent underestimates, since infections by these pseudotypes exhibited more-linear kinetics than did those by the two control pseudotypes and may not have reached a maximum by 6 h. These differences are highly suggestive of distinct entry pathways for these two sets of viruses. Finally, the slower cell entry kinetics that we observed for the EBO-Z and MBG pseudotypes are consistent with the results of published studies of the caveola-utilizing virus SV40 (1, 24).

EBO-Z and MBG pseudotypes colocalize with caveola marker CAV-1. To obtain direct visual evidence of association between caveolae and the filovirus pseudotypes, we immunostained infected cells for the human immunodeficiency virus core protein p24 as a marker of the pseudotype virus particle and for CAV-1 as a marker of caveolae. Due to technical difficulties in working with 293T cells, we opted to utilize human HeLa cells, which are also susceptible to filovirus pseudotype infections, for this study (8, 46). We confirmed that filovirus pseudotype infection of HeLa cells was similarly inhibited by the panel of inhibitors we had used in the abovementioned studies with 293T cells. Indeed, nystatin (50 μg/ml), MβCD (5 mM), and PMA (10 μM) caused specific decreases in MBG pseudotype infection of HeLa cells while causing no effect on, or even enhancement of, VSV pseudotype infection (Fig. 2), suggesting that caveolae may also be involved in filovirus infection of HeLa cells. For microscopy studies, HeLa cells grown on chamber slides were infected with the appropriate pseudotype for 90 min at 37°C, fixed, and permeabilized. The cells were then stained for intracellular p24 with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-p24 antibody (Coulter Corp.), and the FITC signal was amplified with Alexa Fluor 488 sandwich antibodies (Molecular Probes, Inc.). Cells were also stained with a rabbit polyclonal antibody against CAV-1 (BD Transduction Laboratories) and a Texas Red-conjugated anti-rabbit immunoglobulin G (IgG) (heavy-plus-light-chain [H+L]) $F(ab')_2$ fragment (Jackson ImmunoResearch Laboratories, Inc.). The samples were then visualized by confocal microscopy. Cholera toxin B-FITC (Ctxn B-FITC), which endocytoses via caveolae (20, 21), served as a positive control for caveola entry, and we observed a significant degree of colocalization between Ctxn B-FITC and CAV-1 (Fig. 3A to C). In contrast, transferrin (tfn)-FITC, a positive control for clathrin-mediated endocytosis, did not colocalize with CAV-1 to a significant degree (Fig. 3D to F). Importantly, VSV pseudotypes, which enter cells by clathrin-mediated endocytosis (38), did not measurably colocalize with CAV-1 (Fig. 3G). In stark contrast, EBO-Z and MBG pseudotypes (Fig. 3H and I, respectively) were observed to colocalize substantially with CAV-1. Virions lacking envelope did not colocalize with CAV-1 to a significant degree (data not shown). Distinctions in their biological characteristics may explain the differences in the colocalization levels that were observed for Ctxn B-FITC and the filovirus pseudotypes. Finally, cells with detectable p24 and CAV-1 but no colocalization may be due to entry of envelope-lacking virions that are brought about by GP shedding. Such virions, as mentioned earlier, do not colocalize with CAV-1.

The clathrin-mediated endocytic pathway has typically been cited as part of the entry processes of most viruses that enter target cells via receptor-mediated endocytosis. However, a growing body of evidence suggests that clathrin-independent endocytosis of viruses also exists in nature (4, 10, 16). In particular, caveolae have been implicated in several virus entry mechanisms, such as those of SV40 and the respiratory syncytial virus (1, 4, 24, 44). SV40 has been shown to enter via caveolae by its sensitivity to cholesterol inhibitors and PMA, its characteristically slow cell entry kinetic, and its colocalization with CAV-1 (1, 22, 24). In the present study, we report that filovirus entry appears to involve caveolae as well, since EBO-Z and MBGpseudotypes (i) are sensitive to cholesterol inhibitors, (ii) are sensitive to PMA, and (iii) have distinctly slower cell entry kinetics than do pseudotypes of control viruses. More-direct evidence was derived from confocal microscopy studies that revealed that EBO-Z and MBG pseudotypes colocalize with the caveola protein marker CAV-1. A logical proposal is that lipid rafts are involved directly or indirectly in the filovirus entry process as well, since these membrane domains are precursors to caveolae. Although the specific demarcation of lipid rafts and caveolae remains somewhat controversial, one model proposes that caveolae are formed from lipid rafts by the active polymerization of caveolins (19, 35, 37).

Our results are consistent with the earlier finding that FR-α is a cofactor for filovirus entry (7), since FR-α endocytosis is believed to be dependent on caveolae (28). Also, caveolae have been reported to be present in human cell types that are known to be major targets for wild-type EBO-Z and MBG, namely, endothelial cells, hepatocytes, and macrophages (5, 14, 26), which further supports the proposal that caveolae are involved in wild-type filovirus infections. Eventually, it will be crucial to confirm the involvement of caveolae in filovirus entry by use of live EBOs and MBGs. Further work should identify the points in filovirus entry at which the process could be interrupted by an appropriate inhibitor(s), thereby rendering the virus inactive and nonpathogenic.

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