Detection of Cell-Cell Fusion Mediated by Ebola Virus Glycoproteins

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Ebola viruses (EboV) are enveloped RNA viruses infecting cells by a pH-dependent process mediated by viral glycoproteins (GP) involving endocytosis of virions and their routing into acidic endosomes. As with well-characterized pH-dependent viral entry proteins, in particular influenza virus hemagglutinin, it is thought that EboV GP require activation by low pH in order to mediate fusion of the viral envelope with the membrane of endosomes. However, it has not yet been possible to confirm the direct role of EboV GP in membrane fusion and the requirement for low-pH activation. It was in particular not possible to induce formation of syncytia by exposing cells expressing EboV GP to acidic medium. Here, we have used an assay based on the induction of a β -galactosidase (*lacZ*) reporter gene in target cells to detect cytoplasmic exchanges, indicating membrane fusion, with cells expressing EboV GP (Zaire species). Acidic activation of GP-expressing cells was required for efficient fusion with target cells. The direct role of EboV GP in this process is indicated by its inhibition by anti-GP antibodies and by the lack of activity of mutant GP normally expressed at the cell surface but defective for virus entry. Fusion was not observed when target cells underwent acidic treatment, for example, when they were placed in coculture with GP-expressing cells before the activation step. This unexpected feature, possibly related to the nature of the EboV receptor, could explain the impossibility of inducing formation of syncytia among GP-expressing cells.

Filoviruses, comprising the Marburg virus and Ebola virus (EboV), the latter subdivided into four species, are negative-stranded enveloped RNA viruses responsible for lethal hemorrhagic disease in humans and nonhuman primates (reviewed in references 13, 19, and 33). The nonsegmented genome comprises seven genes, with gene 4 encoding glycoproteins (GP) apparently responsible for the cell entry process. It is indeed possible to infect a wide variety of mammalian cells, with the notable exception of lymphocytes and derived cell lines, with GP-coated pseudotypes derived from vesicular stomatitis virus (VSV) or human immunodeficiency virus type 1 (HIV-1) (31, 39). The functional form of filovirus GP appears to be a complex of two subunits, GP1 and GP2, which are the amino- and carboxy-terminal cleavage products of an ~680-amino-acid precursor (14, 35). In the case of EboV, the precursor GP is translated from a subset of viral mRNAs edited in a polyuridine sequence to produce a –1 frameshift and is therefore less abundant than an ~360-amino-acid soluble glycoprotein of unknown function translated from unedited mRNAs (14). However, certain EboV strains from the Zaire species with an additional uridine in the frameshift region express predominantly the full-length GP (34).

The overall organization and processing of filovirus GP indicate their homology with other viral proteins mediating cell entry, such as the influenza virus hemagglutinin (HA) processed into HA1 and HA2 subunits and the precursor of the retroviral envelope proteins (Env) processed into surface and transmembrane (TM) subunits. This homology leads us to pro-

pose distinct roles for the filovirus GP subunits, with GP1, like HA1 and the surface subunit, responsible for cell adhesion and interaction with receptors (21) and GP2, like HA2 and the TM subunit, mediating the membrane fusion step required for delivery of the viral genetic material into the cytoplasm (reviewed in references 7 and 29). Structural elements characteristic of viral proteins mediating membrane fusion can indeed be identified in the filovirus GP2 subunit, in particular a cluster of hydrophobic residues close to the amino-terminal end, representing a putative fusion peptide inserting in the target cell membrane and two helix-forming domains intervened by a disulfide-bonded loop (14). In the case of EboV GP2, crystallographic studies revealed that the helices could associate to form a tight six-helix bundle structure previously identified in the TM of different retroviruses and apparently required for the membrane fusion process (20, 37).

Agents raising the pH of endosomes, such as chloroquine, could inhibit infection with VSV-EboV-GP pseudotypes (31, 39), which seems to indicate a pH-dependent cell entry process, including endocytosis of virions, as is the case for infection mediated by HA or by VSV G protein (VSV-G) (reviewed in references 7, 25, and 29). Like these viral entry proteins, EboV GP could require activation by acidic pH in order to reach its fusion-active conformation. But while the role of HA and VSV-G in membrane fusion and their requirement for activation by low pH can be readily observed in virus-cell or cell-cell fusion assays, it has not yet been possible to obtain such evidence for filovirus GP, which leads us to question the validity of current pH-dependent entry models in their case. In particular, exposing transfected or infected cells expressing high levels of EboV GP to acidic medium did not result in detectable syncytia formation (31 and V. Vochkov [INSERM U412, Lyon, France], personal communication). The lack of a

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direct membrane fusion assay also limits the scope of structure-function studies of filovirus glycoproteins, since it is possible to address the cell entry process only in its whole and not to dissect its individual steps.

In the absence of a known explanation for the failure of previous attempts to detect cell-cell fusion mediated by EboV GP, we sought to address this issue again by using a highly sensitive technique initially designed for functional studies of HIV-1 Env (12) and later used with other viral proteins (11, 12, 23). This type of assay, based on the activation of a reporter gene in target cells upon membrane fusion and cytoplasmic exchanges with cells expressing Env or other viral proteins, indeed allows us to detect fusion events involving two cells while morphology-based syncytium formation assays can score only relatively large multinucleated structures. By this technique, we could obtain evidence of membrane fusion between acid-treated cells expressing EboV GP and target cells under certain experimental conditions. This result allowed us to confirm the direct role of the EboV GP2 subunit in the membrane fusion process as well as a number of observations made from previous infection assays, such as the requirement for a proteinase-sensitive receptor on target cells (31). We also found that target cells became rapidly resistant to EboV GP-mediated fusion upon exposure to acidic medium, which could explain the impossibility of directly inducing formation of syncytia among GP-expressing cells.

MATERIALS AND METHODS

Cell lines. HeLa cells (ATCC no. CCL-2) were used for expression of all viral envelope proteins. HeLa and COS target cell lines stably transfected with the *Escherichia coli* β -galactosidase gene (*lacZ*) under transcriptional control of the HIV-1 long terminal repeat (LTR) are described elsewhere (12), as is the CD4⁺ HeLa-P4 cell line (6). All cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics (penicillin and streptomycin), and 2 mM glutamine.

Plasmids. The Zaire EboV GP were expressed from the cytomegalovirus immediate-early promoter using a pCDNA3-derived vector kindly provided by M.-P. Grange and F.-L. Cosset (INSERM U412, Lyon, France) or from the β -actin promoter for the mutant glycoproteins with the amino acid substitution F535R, G536A, or P537R in the putative fusion peptide of GP2 (15). EboV cDNA in these vectors allows expression of GP without RNA editing (35, 36). The human T-cell leukemia virus type 1 (HTLV-1) envelope proteins (9) and VSV-G protein (40) were also expressed from the cytomegalovirus promoter, while the HIV-1 envelope proteins (LAI strain) and Tat were expressed from the HIV-1 LTR (26).

Antibodies and other reagents. Anti-EboV immunoglobulin Gs (IgGs) prepared from a horse antiserum (17) were a gift from V. Volchkov (U412 INSERM, Lyon, France). The anti-GP1 mouse monoclonal antibody (MAb) 746/16.4 is a subclone of MAb 746/16.2 (32) raised against a synthetic peptide corresponding to residues 391 to 410 of the Zaire EboV GP. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was purchased from Dako (Denmark), dextran sulfate (M_r , 500,000) from Sigma Aldrich (France), and proteinase K from Boehringer (Mannheim, Germany). Cell Tracker Green (CTG; 5-chloromethyl-fluorescein-diacetate) and CMTMR (5-[and 6-]-[(4-chloromethyl)benzoyl]amino]tetramethylrhodamine) were purchased from Molecular Probes (Eugene, Oreg.).

Syncytium formation assays. In most experiments, cell-cell fusion was detected by an in situ β -galactosidase assay after coculture of LTR*lacZ* target cells and HeLa cells expressing HIV-1 Tat and either EboV GP or other viral glycoproteins, essentially as described previously (11, 12). To this end, adherent HeLa cells ($\sim 2 \times 10^5$ per well; six-well trays) were cotransfected with expression vectors for HIV-1 Tat (1 μ g) and either EboV GP, HIV-1 Env, HTLV-1 Env, or VSV-G (3 μ g) by a standard calcium phosphate precipitation technique. Acid treatment, if any, was applied 24 h after transfection by replacing the culture medium with 3 ml of DMEM-HCl prepared immediately before use at the indicated pH. After 10 min at room temperature under a tissue culture hood,

DMEM-HCl was aspirated, and coculture was initiated by adding $\sim 4 \times 10^5$ target cells in 2 ml of culture medium. The pH was in the neutral range at the initiation of the coculture. After 24 h, adherent cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) and stained with the β -galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside). Blue-stained foci representing fusion events were scored under $\times 4$ magnification. Pictures were taken on a Nikon Microphot-FAX microscope. To address the effect of neutralizing antibodies on syncytia formation, anti-EboV IgGs were added at the same time as that of target cells and left during coculture. To investigate the effect of target cell treatment on cell-cell fusion, adherent HeLa-P4 target cells were digested with proteinase K for 1 h before being washed and added to effector cells or incubated 2 h with dextran sulfate and then washed and detached for coculture.

For the detection of cell-cell fusion by exchange of cytoplasmic fluorescent dyes, transfected HeLa cells expressing EboV GP or HIV-1 Env were labeled with the green fluorescent probe CTG and target HeLa cells with the red fluorescent probe CMTMR, as described previously (1). Briefly, transfected HeLa cells were left in contact with CTG (10 μ M in PBS) for 45 min at 37°C and then washed and returned to standard culture medium for 30 min before low-pH treatment (pH 5). Adherent HeLa cells were labeled by a 30-min contact with CMTMR (8 μ M in PBS) at 37°C, washed, detached with PBS and 1 mM EDTA, and added to adherent CTG-labeled cells for coculture. After 24 h, adherent cells were fixed in situ with 4% formaldehyde in PBS (10 min) and then washed and examined on a Leica DMIRE2 confocal microscope under $\times 63$ magnification. Images were pseudocolored according to their respective emission wavelengths and overlaid using Metamorph software (Universal Imaging Corporation, West Chester, Pa.).

Detection of EboV glycoproteins. Expression of wild-type (WT) and mutant EboV GP in HeLa cells was monitored by flow cytometry 24 h after transient transfection. Cells were stained with the M746/16.4 anti-GP1 MAb (1:1,000 in PBS with 2% fetal calf serum) for 1 h at 4°C, followed by FITC-conjugated anti-mouse IgGs (1:50; 45 min at 4°C). Cells were then fixed with PBS and 4% formaldehyde and analyzed on an Epics XL flow cytometer (Coultronics).

RESULTS

Detection of cell-cell fusion mediated by the Ebola virus glycoproteins. The ability of EboV GP to mediate cell-cell fusion was investigated with a content-mixing assay based on the ability of the HIV-1 transactivator Tat to control expression of a stably transfected reporter gene, such as the *E. coli* β -galactosidase *lacZ*, placed under transcriptional control of the HIV-1 LTR (12). It allows us to detect and quantitate cytoplasmic exchanges, indicating membrane fusion, occurring between LTR*lacZ* target cells and cells expressing Tat by a simple β -galactosidase assay performed either in cell extracts or in situ by staining fixed cells with a chromogenic substrate, such as X-Gal (blue).

Since HeLa cells are permissive to infection mediated by EboV GP (39), initial attempts to detect cell-cell fusion used the well-characterized HeLa-P4 cell line (CD4⁺), in which the LTR*lacZ* transgene is highly responsive to activation by Tat in spite of a particularly low basal level of β -galactosidase activity (6). EboV GP (Zaire species) were expressed by transient transfection of HeLa cells, which were found to be relatively resistant to the cytopathic effects of filovirus GP (3, 28) compared to other cell lines tested. The expression vector (a kind gift of M. P. Grange and F. L. Cosset, Lyon, France) was based on an EboV cDNA allowing translation of the full-length (membrane-associated) GP precursor without mRNA editing, which should maximize expression of functional forms of the GP relative to soluble GP. Adherent transfected HeLa cells were exposed to pH 5 medium for 10 min to take into account the expected requirement of EboV GP for activation by low pH and coculture initiated after removal of the acidic medium by adding LTR*lacZ* target cells in neutral-pH culture medium.

FIG. 1. Detection of cell-cell fusion by activation of a *lacZ* reporter gene in target cells (A) or by diffusion of cytoplasmic fluorescent probes (B). (A) X-Gal staining after a 24-hour coculture of HeLa-CD4-LTRlacZ target cells with HeLa cells previously transfected with expression vectors for HIV-1 Tat (a), Tat and HIV-1 Env (b), or Tat and EboV GP (c) and exposed for 10 min to pH 5 medium (see Materials and Methods). Blue foci indicate fusion of target and Tat-positive cells. Magnification, $\times 4$. (B) Confocal microscopy fields of cocultures of HeLa-CD4-LTRlacZ cells labeled with CMTMR (red fluorescence) and HeLa cells labeled with CTG (green fluorescence) after transfection with control vector (a), HIV-1 Env (b), or EboV GP (c) expression vectors. CTG-labeled cells were exposed to pH 5 medium for 10 min before a 24-h coculture in neutral medium with CMTMR-labeled cells. Pictures were taken at a $\times 63$ magnification.

Cells were fixed 24 h later, and staining with X-Gal revealed foci with high-level β -galactosidase activity (blue staining), indicating that cytoplasmic exchanges occurred between the two cell types. This seemed to indicate that EboV GP can mediate fusion with target cells, although the fusion process mediated by HIV-1 Env appeared much more efficient, given the respective number and size of stained foci in parallel experiments (Fig. 1A).

To rule out the possibility of an indirect activation of the reporter gene in target cells upon contact with GP-expressing cells, we sought to obtain direct evidence that cytoplasmic exchanges occurred between the two cell types. To this end, HeLa cells expressing either EboV GP or HIV-1 Env and HeLa-P4 target cells were labeled with green and red cytoplasmic fluorescent probes, respectively. Activation of EboV GP-expressing cells by acidic medium and coculture with target cells was performed as described previously. After a 24-hour coculture, confocal microscopy observation revealed multinucleated structures with distinctly stained nuclei and cytoplasm, which indicated fusion of the two cell types (Fig. 1B). Again, the fusion process mediated by HIV-1 Env appeared markedly more efficient, but this direct assay seemed to confirm the validity of the observations based on reporter gene activation.

Fusion requires low-pH activation. The ability of EboV GP to mediate cell-cell fusion could also be observed using CD4-negative HeLa LTRlacZ target cells, ruling out a possible role of CD4 in the fusion events detected with HeLa-P4 target cells or with COS LTRlacZ target cells (Fig. 2A). This experiment

also showed that exposing cells expressing EboV GP to acidic medium markedly increased the efficiency of fusion with target cells, although a number of blue-stained foci higher than background could be detected in experiments with untreated GP-expressing cells. Activation of EboV GP by a low-pH environment seems therefore necessary to achieve cell-cell fusion as well as virus infection. Fusion with HeLa-P4 target cells appeared optimal when cells expressing EboV GP were exposed to pH 4.5 medium, but the efficiencies were similar at pH 4 and pH 5 (Fig. 2B). Fusion with COS cells was optimal with GP-expressing cells activated at pH 5 and markedly decreased at the higher or lower pH tested (Fig. 2C). A 2-min exposure to low pH was sufficient to activate GP-expressing cells, but optimal cell-cell fusion efficiency required at least a 5-min exposure (data not shown). Note that low-pH treatment of cells expressing HIV-1 Env did not allow their fusion with CD4-negative target cells (Fig. 2A) and, therefore, does not seem to promote unspecific cell-cell fusion.

Role of the Ebola virus glycoproteins. To ascertain the role of EboV GP in the formation of syncytia, cocultures of GP-expressing cells (activated at pH 5) with LTRlacZ target cells were performed in the presence of purified anti-GP immunoglobulins (kindly provided by V. Volchkov) prepared from an equine antiserum reacting with the GP1 and GP2 subunits (10) and shown to prevent infection of guinea pigs by Zaire EboV (16). The number of fusion events detected between cells expressing EboV GP and HeLa-P4 target cells was markedly reduced in the presence of anti-GP IgGs at a 1:500 dilution,

FIG. 2. Quantification of cell-cell fusion mediated by EboV GP or HIV-1 Env. (A) Fusion with HeLa-CD4, HeLa, or COS target cell lines was detected as described in the legend to Fig. 1A by scoring blue foci after they were stained with X-Gal. Bars represent number of blue foci per well (six-well plates) in one of three experiments. ND, not done. (B) Effect of treatment of cells expressing EboV GP with acidic medium (pH 3 to 7) on fusion with HeLa-CD4-LTRlacZ target cells. The experiment was conducted as described in panel A, and bars represent means from three independent experiments. (C) Same experiment as that described in panel B, with COS-LTRlacZ target cells. One of two experiments with similar results is shown.

and fusion was almost blocked at a 1:200 dilution, while no effect on fusion mediated by HTLV-1 Env at either dilution was observed (Fig. 3A).

To further establish the active role of EboV GP in the detected cell-cell fusion events, we sought to perform experiments with defective GP resulting from mutations expected to affect the fusion process but not the adhesion steps likely mediated by the GP1 subunit. Different mutations in the putative fusion peptide of GP2 were reported to prevent VSV pseudotype infection mediated by EboV GP while they had no apparent effect on the expression of the GP precursor and its processing into GP1 and GP2 subunits (15). The latter point was checked by flow cytometry analysis of HeLa cells transfected with WT EboV GP or three GP2 mutants, F535R, G536A, and P537R, revealing similar levels of GP1 at the cell

FIG. 3. Inhibition of EboV GP-mediated cell-cell fusion by neutralizing antibodies (A) or mutations in the GP2 subunit (B and C). (A) Cocultures of HeLa-CD4-LTRlacZ cells with HeLa cells transfected with expression vectors for Tat and either EboV GP or HTLV-1 Env were performed as described in the legend to Fig. 2A, except for the presence of purified horse anti-GP IgGs at 1:500 or 1:200 dilution. Bars represent means of three replicates. (B) Effect of mutations in the putative fusion peptide of GP2 on the expression of EboV GP at the surface of transfected HeLa cells measured by flow cytometry after staining with a mouse anti-GP MAb and a secondary FITC-coupled antibody. Results are shown as percentages of GP-expressing cells relative to cells transfected with a WT GP expression vector. Data are from one of two representative experiments. (C) Effect of GP2 mutations on fusion with HeLa-CD4-LTRlacZ target cells. The experiment was performed as described in the legend to Fig. 1B. Results are the means from three representative experiments.

surface (Fig. 3B). Each of these GP2 mutations was shown to abrogate fusion with HeLa-P4 target cells (Fig. 3C), in agreement with their effects in pseudotype infection assays. This result is therefore consistent with the fusion peptide function of this region of GP2 and indicates the active role of EboV GP in the formation of syncytia detected in our experiments. It also confirms that the activation of the lacZ reporter gene in target cells requires cytoplasmic exchanges with cells expressing EboV GP and not their mere contact (signaling effect).

Effects of target cell treatment. The efficiency of VSV pseudotype infection mediated by EboV GP was markedly reduced by limited digestion of target cells with proteolytic enzymes (31). To address the relevance of the cell-cell fusion process detected in our experiments to EboV entry, HeLa-P4 target cells were incubated with proteinase K (50 or 100 $\mu\text{g/ml}$) for 60 min before contact with acid-activated HeLa cells expressing either EboV GP or VSV-G. At both concentrations of proteinase K, we observed a marked decrease in the number of fusion events mediated by EboV GP but no apparent effect on fusion mediated by VSV-G (Fig. 4A). The latter result is consistent with the probable nonprotein nature of the VSV receptor (8, 25) and shows that the proteolytic treatment did not impair the viability of target cells during the time course of the assay. Similar numbers of fusion events were detected when EboV GP-expressing cells were cocultured with untreated HeLa-P4 cells or with a 1:1 mixture of proteinase-digested and untreated cells (data not shown), ruling out the possibility of EboV GP degradation by proteinase K carried over. These experiments indicate that proteins on the target cell surface are required both for cell-cell fusion and for pseudotype infection mediated by EboV GP. Similarly, incubation of HeLa-P4 target cells with dextran sulfate (100 or 500 $\mu\text{g/ml}$; 120 min), a glucidic macromolecule interacting with glycosaminoglycans and inhibiting infection by various types of viruses (18, 22), resulted in decreased efficiency of fusion with cells expressing EboV GP (Fig. 4A).

The membrane fusion activity of pH-dependent viral entry proteins, such as HA and VSV-G, is usually assayed by transiently exposing infected or transfected cells to low pH. In that situation, the viral proteins can interact with their receptors on adjacent cells before undergoing activation which must facilitate access of the fusion peptide to the target cell membrane and subsequent lipid exchanges. Our experimental setting could appear much less favorable since it can detect fusion events involving only LTRlacZ target cells, which were not immediately available to the activated EboV GP. We therefore sought to improve the efficiency of syncytium formation by allowing contact between cells expressing EboV GP or VSV-G and HeLa-P4 target cells before exposure of the coculture to pH 5 medium. While this experimental setting resulted in the formation of massive syncytia in the case of VSV-G, there was no detectable fusion mediated by EboV GP (Fig. 4B). This negative result seemed explainable only by a detrimental effect of the low-pH medium on target cells. This could be demonstrated by the absence of fusion events when HeLa-P4 target cells were exposed 10 min to pH 5 medium and returned in neutral-pH medium before contact with acid-activated EboV GP-expressing cells (Fig. 4B).

Cell-cell fusion mediated by EboV GP but not VSV-G therefore seems to require a low-pH-sensitive structure at the surfaces of target cells. Target cells recovered their ability to undergo GP-mediated fusion when they were in neutral-pH medium after the 10-min acid treatment. But although fusion events could be detected after 15 min, target cells must be left for 30 to 45 min at neutral pH for optimal fusion efficiency (Fig. 4C), which seems consistent with the de novo expression of the low-pH-sensitive structure required for GP-mediated fusion.

FIG. 4. Effects of target cell treatment on EboV GP-mediated cell-cell fusion. (A) Cocultures of HeLa-CD4-LTRlacZ cells treated with proteinase K (1 h) or dextran sulfate (2 h) with HeLa cells expressing Tat and either EboV GP or VSV-G were performed and fusion events scored as described in the legend to Fig. 2A. (B) Effect of low-pH treatment (pH 5; 10 min) applied either to HeLa cells expressing EboV GP or VSV-G (effector cells) before coculture, to both effector and target cells before coculture, or after a 60-min contact of effector and target cells. In all cases, exposure to acidic medium was ended by a wash in neutral-pH medium. Bars represent numbers of blue foci per well after overnight coculture and staining with X-Gal. Two independent experiments (exp 1 and exp 2) are represented for EboV GP and one for VSV-G. The asterisk indicates massive fusion with large multinucleated syncytia (>10 nuclei). Scoring the number of blue foci underestimated fusion efficiency in this situation. (C) Efficiency of fusion after return of acid-treated target cells into neutral-pH medium. HeLa-CD4-LTRlacZ cells were detached from the plate, resuspended in pH 5 medium for 10 min at room temperature (RT), and then returned to culture medium at 37°C. At different times, a fraction of these cells was added to acid-treated HeLa cells expressing either EboV GP or VSV-G, and fusion was monitored as described in the legend to Fig. 2A. Numbers of fusion events per well are shown as percentages relative to untreated target cells (means from three independent experiments).

FIG. 5. Kinetics of cell fusion efficiency after low-pH activation of EboV GP and VSV-G. The experiment was conducted as described in the legend to Fig. 2A except that HeLa cells expressing Tat and EboV GP or VSV-G were left in neutral-pH medium from 15 to 120 min after the acid activation step and before coculture in neutral medium with HeLa-CD4-LTR/lacZ cells. Numbers of fusion events per well are shown as percentages relative to cocultures initiated immediately after low-pH exposure (means from three independent experiments).

Stability of acid-treated Ebola virus glycoproteins. In our experiments, EboV GP required activation by low pH in order to efficiently mediate fusion but were placed in a neutral-pH environment at the time of contact with target cells. It is therefore supposed that the viral proteins remain in a fusion-active conformation, probably for several minutes given the time necessary for GP-expressing cells to engage contact with target cells added in suspension. VSV-G could also mediate fusion in this experimental setting, although with a markedly reduced efficiency compared to the standard protocol, i.e., acid treatment of a coculture of VSV-G-expressing cells and target cells (Fig. 4B). We sought to compare the respective stability of the activated forms of these viral proteins by assaying fusion with HeLa-P4 target cells at different times after their exposure to acidic medium was ended. The number of fusion events decreased with time for both viral proteins, as could be expected, but with markedly different slopes. In the case of cells expressing VSV-G, the number of fusion events detected 15 min after the end of the acid treatment (time zero) was indeed less than 10% of the number at time zero while it was more than 80% for cells expressing EboV GP (Fig. 5). This experiment confirmed the relative stability of EboV GP in their fusion-active conformation.

DISCUSSION

By using an assay based on reporter gene activation in target cells, we have detected cell-cell fusion mediated by the GP of a Zaire species EboV. Fusion events were seen only under particular experimental conditions, i.e., activation of GP-expressing cells by low pH before contact with target cells, and formed with apparently limited efficiency compared to those between other viral proteins. However, the role of EboV GP was ascertained by the inhibition of syncytium formation by anti-GP neutralizing antibodies and by the lack of detectable fusion in experiments with defective mutant GP unable to mediate entry.

Among elements suggesting that the cell-cell fusion detected in our experiments bears relevance to virus entry was that both

processes apparently required activation of EboV GP by exposure to a low-pH environment. Infection with VSV-EboV-GP pseudotypes, as well as infection mediated by influenza virus HA and other pH-dependent proteins, is indeed blocked by agents raising the endosomal pH, such as chloroquine (31, 39). It is therefore thought that EboV entry involves internalization of virions and their routing to acidic endosomes where the GP can reach a fusion-active conformation. In the case of HA, exposure to low pH triggers a rearrangement of the membrane-associated subunit (HA2) which unmasks the hydrophobic amino-terminal end (fusion peptide), allowing its access to the target membrane and the initiation of lipid exchanges (reviewed in references 7, 29, and 30). The homology of filovirus GP with HA leads us to envision a similar mechanism of activation, with low pH triggering conformation changes in the membrane-associated GP2, in particular the exposure of the putative fusion peptide. Our experiments indeed showed that the integrity of this domain is required for cell-cell fusion as well as for infection by VSV-EboV-GP pseudotypes (15). An effect of low pH on the conformation of GP2 may seem in disagreement with the recent finding that pseudotype infection mediated by EboV GP was blocked by inhibitors of endosomal acid-dependent cysteine proteases (cathepsins) apparently able to cleave the GP1 subunit (4). This could indicate a different mechanism of activation by low pH for EboV GP, compared to previously characterized pH-dependent viral proteins, in particular HA. The cleavage of GP1 seems unlikely to take place in experimentally induced cell-cell fusion, unless a cathepsin-like activity is present at the plasma membrane, which could also represent a difference with the virus entry process. However, it is also envisioned that the cleavage of GP1 in endosomes is not alone sufficient for the GP complex to reach a fusion-active conformation and rather facilitates low-pH-induced rearrangements in the GP2 subunit, for example, by allowing partial or complete dissociation of the covalent GP1/GP2 complex (14). If this is the case, the absence of a cathepsin-like activity at the cell surface could contribute to the relatively low efficiency of cell-cell fusion.

Other indications of the relevance of cell-cell fusion mediated by EboV GP to virus entry came from the study of target cells and their requirements. Experiments with pseudotypes have shown that EboV GP could mediate infection of all types of mammalian cells, with the exception of lymphocytes and derived cell lines (39). By using a sensitive flow cytometry assay (1), we did not detect exchanges of cytoplasmic fluorescent probes between cells expressing EboV GP and primary human lymphocytes or of CEM cells (T-cell line) labeled with distinct fluorescent probes, while fusion with HeLa target cells was detected by this technique, as was fusion with CEM cells mediated by HIV-1 Env (data not shown). It seems therefore that cells resistant to GP-mediated infection are also resistant to fusion with GP-expressing cells. Further studies are obviously needed to confirm these preliminary results. Digestion of HeLa target cells with proteinase K markedly reduced their ability to fuse with cells expressing EboV GP, in agreement with the reported inhibition of GP-mediated infection (31). As with other viral models, it seems likely that the cell surface proteins required for the cell-cell fusion process are identical to the as-yet-unidentified EboV receptor(s) (2, 27) interacting with the GP1 subunit (21). However, it cannot be ruled out

that the structure recognized by GP1 is a motif carried in a nonspecific way by cell surface proteins, such as sialic acid in the case of HA.

The observation of cell-cell fusion mediated by a filovirus GP is unprecedented to our knowledge. The apparently limited efficiency of the fusion process, as well as issues related to the cytotoxicity of these viral proteins or to the predominant expression by most EboV species of soluble nonfunctional GP (14), could have a role in the failure of previous attempts to detect the process (3, 28). But the principal reason could be the inadequacy of protocols currently used to detect fusion mediated by pH-dependent viral entry proteins, such as HA and VSV-G. Syncytia are indeed readily observed minutes following the exposure of cells expressing these viral proteins to acidic medium. In that situation, the viral proteins can interact with their ubiquitously expressed receptors on adjacent cells prior to the activation step, which must facilitate access of the low-pH-activated form to the target membrane and subsequent lipid exchanges. In agreement with previous observations (31), we found that this experimental setting did not allow us to detect cell-cell fusion mediated by EboV GP. By exploring this discrepancy between assays performed with the same materials, we found that exposure of target cells to acidic pH induced their resistance to GP-mediated fusion. This inhibitory effect could be observed minutes following exposure to acidic pH, while it took 30 to 45 min for cells to recover their ability to undergo GP-mediated fusion. Interactions of EboV GP with a cell surface receptor(s) seem therefore possible only under neutral-pH conditions and are disrupted by low pH before the viral proteins have reached their fusion-active conformation. This major difference between EboV GP and other well-characterized low-pH-dependent viral entry proteins, such as HA and VSV-G, could be related to the different natures of their receptors, which are likely to be proteins, in the case of EboV and other filoviruses while cell adhesion depends on simpler ubiquitous structures, such as sugars (sialic acid) in the case of HA (reviewed in reference 29) and probably membrane lipids for VSV (8, 25). These structures are unlikely to be affected by low pH, and their abundance could allow sufficient binding of viral proteins even under suboptimal conditions.

The inhibitory effect of low pH on GP-mediated membrane fusion obviously does not apply in the context of late endosomes during the virus entry process. A possible explanation for this apparent discrepancy is that the interaction disrupted by low pH is required only for the cell adhesion step and is no longer necessary once viral particles have undergone internalization into the endosomes. Intracellular vesicles could indeed offer easier access for the acid-activated GP to the membrane compared to a cell-cell contact situation. It will be of interest to address the ability of GP to interact with acid-treated cells and mediate pseudotype internalization.

The cellular context in which EboV GP were expressed was found to exert an influence on the ability to mediate cell-cell fusion. While negative results obtained with cell lines such as HEK could be due to their high sensitivity to the cytotoxicity of EboV GP (3, 28), we observed important variations in fusion efficiency between HeLa-derived cell lines that were all resistant to the cytopathic effects of GP, at least during the time course of the assay. The level of cell-cell fusion could not be correlated to transfection efficiency or to the level of GP ex-

pression. Given the morphological diversity of these cell lines, it seems possible that they do not offer the same membrane environment to EboV GP, which could affect parameters, such as the local density of the viral proteins, known to be critical for the efficiency of membrane fusion in the case of HA (5, 24). Differences in membrane lipid composition could also affect the stability of EboV GP after pH activation, for example, by favoring "self-fusion" (i.e., interaction of the fusion peptide with the viral membrane) to the detriment of cell-cell fusion (38). Although a number of questions remain open, our results indicate that it is possible to detect and quantify cell-cell fusion mediated by EboV GP, which can be particularly useful for structure-function studies of these viral glycoproteins.

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