Genomic RNA Editing and Its Impact on Ebola Virus Adaptation During Serial Passages in Cell Culture and Infection of Guinea Pigs

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Synthesis of the structural, surface glycoprotein (GP) of Ebola virus (EBOV) is dependent on transcriptional RNA editing phenomenon. Editing results in the insertion of an extra adenosine by viral polymerase at the editing site (7 consecutive template uridines) during transcription of GP gene of the wild-type virus (EBOV/7U). In this study, we demonstrate that passage of EBOV/7U in Vero E6 cells results in the appearance and rapid accumulation of a variant (EBOV/8U) containing an additional uridine at the editing site in the viral genome. EBOV/8U outgrows and eventually replaces the wild-type EBOV during 4–5 passages. On the contrary, infection of guinea pigs with EBOV/8U leads to the appearance and rapid predominance by EBOV/7U. These rapid conversions suggest that editing of the genomic RNA occurs at a higher frequency than previously thought. In addition, it indicates that the EBOV/7U phenotype has a selective advantage that is linked to controlled expression of GP and/or expression of secreted sGP, the primary gene product for wild-type EBOV. This study demonstrates the potential for insertion and deletion of uridines in the editing site of the EBOV genomic RNA, depending on environmental constraints.

Taxonomically, the genera Ebolavirus and Marburgvirus, together constitute the family Filoviridae, a member of the order Mononegavirales [1]. Infection of humans with Zaire ebolavirus (EBOV) results in a most severe, often fatal hemorrhagic fever. The majority of EBOV genes are transcribed into individual monocistronic, subgenomic messenger RNAs (mRNAs) encoding viral proteins [2, 3]. The GP gene is an exception to this rule, since at least 2 mRNA species are synthesized from this gene as a result of transcriptional editing [4, 5], which leads to the insertion by the viral polymerase of an extra adenosine at a specific-editing site near the middle of the coding region. This site consists of 7 consecutive uridines in the genomic sequence and editing is believed to result from a slippage or stuttering mechanism, similar to the polyadenylation that occurs at the 3’ end of filovirus mRNAs and those of other viruses [6, 7]. The only other known example of transcriptional editing is that described for the P gene of members of the Paramyxoviridae, another family in the order Mononegavirales [7, 8].

The EBOV polymerase transcribes the GP gene with fidelity ~80% of the time, and these unedited mRNAs program the expression of the predominant GP gene product, sGP, a nonstructural, secreted glycoprotein [5, 9]. Transcriptional editing of GP mRNA results in a frame shift that connects 2 partly overlapping reading frames in a longer coding frame, which when translated results in the expression of GP, the structural, membrane-anchored glycoprotein. Trimerization of GP leads to the formation of peplomers on the surface of virions, which are responsible for virus attachment to susceptible cells and membrane fusion leading to the
release of the viral nucleocapsid into the cytoplasm [10]. Both glycoproteins of EBOV are proteolytically processed during intracellular trafficking by furin, a cellular protease [9, 11, 12].

An extensive expression and heavy GP presence on the plasma membrane is known to interfere with functions of cell surface proteins, eventually causing death of the cells [13–16]. Recently it has been demonstrated that the cytotoxic properties of highly glycosylated GP are associated with the ability of this protein to cause steric hindrance and masking of cellular surface molecules preventing their normal function [16, 17].

In this study, we investigate the effect of EBOV passaging in cell culture and in guinea pigs on GP gene editing site sequence variation. We demonstrate that RNA editing during genome replication is taking place, which has important implications for virus host adaptation, and that replication of EBOV with the wild-type editing site is selected in the guinea pig model.

MATERIALS AND METHODS

Cells
Vero E6 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37°C under 5% CO2. BSR-T7/5 cells, a BHK-21 cell line stably expressing T7 RNA polymerase (a kind gift of K.-K. Conzelmann), were grown in Glasgow medium (Invitrogen) as described elsewhere [18].

Generation of the Recombinant EBOVs
Recombinant plasmids containing the full-length genomic sequence of Zaire Ebolavirus (EBOV) strain Mayinga were generated as described elsewhere [18, 19]. An additional adenosine was inserted into the GP gene editing site by site-directed mutagenesis using following primers: 5’-GGGAAACTAAAAAAAACCTCAC-TAG and 5’-CTAGTGAGTTTTTTATTGTTCC. Amino acid substitutions M71I, L147P, T187I were introduced into the VP24 gene to increase the pathogenicity in recombinant EBOVs for guinea pigs [19, 20]. Using a reverse genetic system for EBOV [18], 2 recombinant viruses designated EBOV/7U (wild-type editing site) and EBOV/8U were rescued in BHK T7 cells for EBOV [18], 2 recombinant viruses designated EBOV/7U (wild-type editing site) and EBOV/8U were rescued in BHK T7 cells. Both recombinant viruses were consecutively passaged in Vero E6 cells. Monolayers of cells at 40% confluence were inoculated with the viruses at an MOI of 0.01. Four days postinfection culture supernatants were harvested, clarified by low-speed centrifugation, diluted 1:1000, and used for the next round of infection. Five generations of consecutive passages were analyzed by virus titration and sequencing of the GP gene.

Immunoblot Analysis
Western blot analysis was performed using monoclonal mouse anti-GP antibodies (1:100) and rabbit anti-VP24 antibodies (1:4000). Either rabbit anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:20,000) were used as secondary antibodies. Chemiluminescent reagents (Amersham ECL) were used for detection of specific protein bands.

EBOV Infection of Guinea Pigs
Guinea pigs, strain Hartley (3-week-old females), were infected intraperitoneally with 500 TCID50 of either recombinant virus. Animals were monitored for clinical manifestations and killed when they reached an ethical end point. Blood samples were collected 6 days postinfection and prior to euthanasia. The serum samples were purified using VenoSafe PET tubes (VenoSafe). Liver samples were collected at necropsy and were used for the next round of infection or analyzed by immunohistochemistry. Formalin-fixed tissues were embedded in paraffin, sectioned, deparaffinized, rehydrated, rinsed, and placed in PBS containing 3% bovine serum albumin (BSA) for 20 minutes. EBOV infection in tissues was detected by immunostaining using a mouse monoclonal anti-VP40 antibody 1:100.

Sequence Analysis
Viral RNA was isolated from clarified culture supernatants, viral stocks, or serum samples of infected guinea pigs using the RNAeasy kit (Qiagen). Reverse transcription–polymerase chain reaction (RT-PCR) amplification of GP gene was performed using the Titan-One-Tube RT-PCR kit (Roche Applied Biosciences) and the primers 5’-GCCGGTCAATGCAACGGAG and 5’-GTGGAAGGCTTGAGTCTCG. PCR fragments were purified and used to sequence the editing site region.
RESULTS AND DISCUSSION

It had been previously postulated that significant sequence variations in the GP gene editing site of wild-type EBOV (EBOV/7U) was not present [5, 23]. Nevertheless, variants containing 8 uridines at the GP gene editing site (EBOV/8U) have been reported [5, 23], suggesting that the insertion of an additional nucleotide may occur at the level of viral genome replication, possibly when positive-strand copies are synthesized. It seems reasonable that EBOV stocks could contain viruses that vary in their editing site sequence length, but the significance of this variation on virus population dynamics has not yet been investigated. In this study, we examine variations in the editing site sequence using a reverse genetics approach to produce 2 variants of EBOV: the wild-type EBOV/7U form and the edited EBOV/8U form. To ascertain that these recombinant viruses replicate in guinea pigs, 3 substitutions in the VP24 gene known to enhance EBOV virulence (and thus replication) in these animals were also introduced [20]. These recombinant viruses were rescued using a previously established reverse genetics system for EBOV [18] and amplified in Vero E6 cells in parallel to ensure that the recombinant viruses had the same passage history.

Recombinant EBOVs were first characterized using a plaque assay. Plaques produced by rEBOV/8U showed a markedly increased size compared with those formed by rEBOV/7U (Figure 1), suggesting that rEBOV/8U possesses a growth advantage over rEBOV/7U. To further explore the growth characteristics of these recombinant viruses, their rates of growth were determined in Vero E6 cells. Cell cultures were infected at an MOI of 0.01, and culture fluids sampled on 3, 4, 5, and 6 days postinfection, and TCID_{50} titers determined and plotted (Figure 1B), and results indicated that rEBOV/8U outgrows the rEBOV/7U early in the infection; 3 days postinfection, there was a log difference in titers that increased slightly thereafter. While the growth of EBOV/8U showed a tendency to slow down, the rate at which the EBOV/7U titer increased remained steady during
the 6 days incubation. Since the rate of rEBOV/8U release from infected cells later postinfection was likely affected by strong cytopathic effects caused by virus replication, the difference in titers between the 2 viruses lessened at the end of the experiment. The faster growth rate of rEBOV/8U correlates with its production of larger plaques and potentially could be due to higher level of GP expression. Previously, we have demonstrated that EBOV containing 8 uridines at the editing site expressed 80% of the GP gene mRNAs coding for GP, 10% for sGP, and 10% for ssGP [23], whereas wild-type EBOV/7U yielded only 20% of GP gene mRNAs coding for GP and 80% for sGP. Earlier we also described that expression of EBOV GP results in the release of membrane vesicles bearing surface GP peplomers [24], and others have shown that GP expression enhances VLP production mediated by the expression of the VP40 matrix protein [25, 26]. We thus speculate that the alteration in GP expression in rEBOV/8U-infected cells enhances the rate of virion release over that of rEBOV/7U.

To investigate the effect of passaging of EBOV on the GP gene editing site, 5 consecutive passages of each recombinant virus were performed in Vero E6 cells, and culture supernatants from each passage were harvested and editing sites sequenced. As shown in Figure 1C, there was no change in the editing site for rEBOV/8U during all 5 passages. In contrast, the editing site for rEBOV/7U started to show a change in sequence (towards that of rEBOV/8U) after only 2 passages and continued to increase until rEBOV/8U was the prominent form after 5 passages. Western blot analysis of culture supernatants using anti-GP and anti-VP24 antibodies confirmed the transformation of EBOV/7U into EBOV/8U during passaging, as seen by the alteration in relative amounts of sGP released into the medium (sGP/VP24). As expected, the relative amounts of sGP in case of EBOV/8U remained unchanged during passaging. The stability of the rEBOV/8U editing site is of interest, since other editing site variants could theoretically emerge due to editing activity of the viral polymerase. Apparently, such variants of EBOV possess no further selective advantage.

Recently, EBOV GP was shown to antagonize the antiviral effect of tetherin [27], a type II transmembrane glycoprotein that inhibits the release of HIV-1 and Lassa virus from the plasma membrane [28, 29] and blocks the release of VP40-mediated VLPs [30]. Tetherin was also shown to interact exclusively with immature EBOV GP in the endoplasmic reticulum (ER), suggesting that their interaction may physically disrupt tetherin's ability to retain virions bound to the plasma membrane [27]. In this regard, antagonism of tetherin by EBOV GP is likely to be enhanced in cells infected with EBOV/8U and may explain the early release of virions and the apparent EBOV/8U growth advantage over the EBOV/7U.

Our next experiment examined the effect of passaging in guinea pigs on the number of uridines in the rEBOV/7U and rEBOV/8U editing sites, and also to determine if these viruses differed in pathogenicity. Two groups of guinea pigs were inoculated intraperitoneally with 500 infectious units (TCID50) of either rEBOV/7U or rEBOV/8U and were monitored for clinical manifestations until the animals reached the ethical end point and were killed. Viral genomic RNA was isolated from the serum samples collected on day 6 postinfection. The animals infected with rEBOV/7U showed an increase in temperature starting by day 3 postinfection. All rEBOV/7U-infected animals died within 7–9 days postinfection (mean time of death, 7.08 days). The disease observed in guinea pigs infected with rEBOV/8U was essentially indistinguishable from that caused by rEBOV/7U, except a small delay in the death of animals (mean, 7.75 days). Western blot analysis of serum samples showed that both infections caused efficient release of the virus into the blood (data not shown). Immunohistochemical staining of tissue samples from the virus-infected animals showed that both the wild-type and variant forms of EBOV produced extensive pathology in the livers of guinea pigs (Figure 2A). In the case of rEBOV/7U
infections, it appears that the foci of infection in livers were slightly more developed and uniform compared with those of rEBOV/8U. RT-PCR amplification of the GP gene region containing the editing site was performed using viral RNA isolated from the serum samples of each infected guinea pig. Unexpectedly, serum samples from all infected animals showed 7 uridines at the editing site, regardless of whether wild-type or variant EBOV was used for infection (Figure 2B). In the case of the rEBOV/8U infection, the wild-type editing site apparently emerged rapidly and became the predominant form after a single passage in guinea pigs. Further passaging of the viruses in guinea pigs did not reveal any variations in the number of uridines at the editing site, indicating that efficient replication of EBOV in guinea pigs required a wild-type editing site.

We have previously shown that the efficacy and accuracy of transcriptional RNA editing substantially changed with EBOV/8U, resulting in the appearance of GP-specific mRNAs carrying 6–15 adenosines at the editing site [23]; adenosine deletions were detected in ~10% of GP-gene specific mRNAs. The rapid conversion of EBOV/8U to the wild-type EBOV/7U in guinea pigs indicates that a deletion of one residue from the editing site also occurs during genome replication. This finding demonstrates that the EBOV polymerase is not only capable of inserting an adenosine at the editing site but also deleting one. Removal of a single residue from the editing site is likely to occur at a high frequency, since only one passage in guinea pigs was required for the EBOV/8U variant to be completely replaced by the EBOV/7U form. This finding suggests that the rEBOV/8U variant was incapable of replicating efficiently in the guinea pig model, and we speculate this restriction is a result of its increased capacity to express GP and/or decreased expression of sGP. Remarkably, we have recently demonstrated that replication of a recombinant “no-editing” EBOV (nonfunctional editing site) that expresses even more surface GP than EBOV/8U and has augmented cytotoxicity and a reduced plaque size [18] is severely attenuated in guinea pigs (V.Volchkova, unpublished observations). An increased cytotoxicity caused by GP overexpression may result in reduced EBOV shedding in guinea pigs. A decrease in sGP expression may also make the rEBOV/8U variant more susceptible to host clearance, since it has been postulated that sGP has a decoy function that misdirects the immune response away from virus-infected cells [12, 31, 32]. The finding that the wild-type editing site is rapidly selected for in the guinea pig model suggests that control of GP expression is important in the efficient production of infectious EBOV particles and that transcriptional editing may be a controlling element.

In conclusion, our study presents important insights into EBOV GP gene editing during transcription and replication. We have shown that a variant EBOV/8U can be selected for in cell culture, but that the wild-type EBOV/7U form is more capable of replicating in a guinea pig model. The maintenance of the wild-type editing site may indicate that it is an essential element in the replication and spread of EBOV in host animals and may function through its regulation of GP expression and/or the expression of sGP.

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**References**


