Role of VP30 Phosphorylation in the Ebola Virus Replication Cycle

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Ebola virus (EBOV) transcription is dependent on the phosphoprotein VP30, a component of the viral nucleocapsid. VP30 is phosphorylated at 2 serine residue clusters located at the N-terminal part of the protein. In this report, we have investigated the role of VP30 phosphorylation in EBOV replication using a reverse genetics approach. In effect, recombinant EBOVs with the VP30 serine clusters substituted either by nonphosphorylatable alanines or phosphorylation-mimicking aspartates were generated and characterized. We show that in comparison to the wild-type EBOV the mutated viruses possess reduced infectivity. This difference is explained by alterations in the balance between the transcription and replication processes and appear to be associated with the state of VP30 phosphorylation. Here we propose a model in which dynamic phosphorylation of VP30 is an important mechanism to regulate the EBOV replication cycle.

Filovirus infections cause a severe hemorrhagic fever in both monkeys and man. The Filoviridae family consists of 2 genera, Ebola virus and Marburgvirus, which are enveloped negative-sense single-stranded RNA viruses [1, 2]. Among Ebola virus species, Zaire Ebolavirus (EBOV) is the most pathogenic for humans with a mortality rate reaching up to 90%. The genome of EBOV encodes 7 structural proteins and at least 2 nonstructural secreted glycoproteins, sGP and L-peptide [3–5]. EBOV genomic RNA is encapsidated by 4 viral proteins: the nucleoprotein (NP), viral proteins VP35 and VP30, and the polymerase L. This distinguishes filoviruses from the majority of the viruses in the order Mononegavirales, which have only 3 proteins (N, P, and L) to form the viral nucleocapsid and to also perform transcription and replication of the viral genome [6]. VP30 of EBOV is essential for the initiation and reinitiation of EBOV transcription but is dispensable for genome replication [7, 8]. To a certain degree, the function of VP30 mirrors that of M2-1 of respiratory syncitial virus (RSV), which also possesses an additional viral protein that is involved in transcription of the viral RNAs. M2-1 and VP30 share several common features: both proteins are phosphorylated, possess both an RNA and a zinc-binding domain, and interact with the nucleoprotein [9–14]. The mechanism of the proteins’ action, however, is different. While M2-1 acts as an elongation and antitermination factor [15], this activity was not observed for VP30 when investigated in a minigenome system [16].

VP30 of EBOV is phosphorylated at 2 serine clusters (aa 29–31 and 42–46), each containing 3 serine residues [17]. The implication of phosphorylation in VP30’s function has been studied using alanine and aspartate substitutions, which mimic nonphosphorylated and constantly phosphorylated serines, respectively. A VP30 with both serine clusters replaced by alanine residues (VP30-AA) was shown to be active in the transcription of an EBOV minigenome and was also capable of supporting the rescue of a recombinant EBOV in reverse genetics experiments [7, 12, 17]. In contrast, a VP30 with all serine residues replaced by aspartate residues
(VP30-DD) was defective in transcription of the minigenome and was unable to support the recovery of recombinant EBOV. A phosphorylated form of VP30 is present in EBOV virions [18], suggesting that VP30 phosphorylation is important for the function of the protein in EBOV replication and may be necessary for either an early or a late step in the viral life cycle.

In this study, using a reverse genetics approach we further investigate the role of VP30 phosphorylation in EBOV replication. We show that substitution of serines by alanine or aspartate residues alters the replication of recombinant EBOVs by affecting both the transcription and replication of viral RNAs. The data obtained allowed us to propose a model for the role of VP30 in the regulation of the EBOV replication cycle.

**MATERIALS AND METHODS**

**Cell Lines and Viruses**

BSR T7/5 cells (a BHK-21 cell line stably expressing T7 polymerase) were maintained in Glasgow medium (Gibco) supplemented with 10% newborn calf serum (NCS) and Vero E6 cells were maintained in Dulbecco modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS). The recombinant Zaire Ebolavirus strain Mayinga (EBOV) has been described previously [19] and is designated EBOV-WT in this work.

**Recombinant Plasmids and Generation of Recombinant EBOVs**

Plasmids containing overlapping complementary DNA (cDNA) fragments covering the full-length antigenomic sequence of EBOV were generated as described elsewhere [19]. Mutations in the coding region of the VP30 were introduced by site-directed mutagenesis of the intermediate plasmid pKSS25. Plasmids containing full-length cDNAs with mutations in the VP30 gene were generated and recombinant EBOVs were rescued in BSR T7/5 cells then passaged once and amplified in Vero E6 cells as described elsewhere [19]. Virus stocks were stored at −80°C. Mutations introduced into these viruses were confirmed by sequencing of genomic RNA isolated from the virus stocks. Virus titers were evaluated by TCID50 using Vero E6 cells then passaged once and amplified in BSR T7/5 cells (a BHK-21 cell line stably expressing T7 polymerase) were maintained in Glasgow medium (Gibco) supplemented with 10% newborn calf serum (NCS) and Vero E6 cells were maintained in Dulbecco modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS). The recombinant Zaire Ebolavirus strain Mayinga (EBOV) has been described previously [19] and is designated EBOV-WT in this work.

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To generate plasmids containing mutated VP30 under the control of either the T7- or CMV- promoter, the sequences were amplified by polymerase chain reaction (PCR) using corresponding plasmids containing full-length cDNA of EBOV and primers containing BamHI and PstI restriction sites. The resulting PCR fragments were cloned either in pTM1 or phCMV plasmid vectors. The sequences of all recombinant plasmids were confirmed by sequence analysis. The nomenclature used for each VP30 mutant is indicated in Figure 1.

**Virus Growth Kinetics and Relative Infectivity**

Subconfluent Vero E6 cells were infected at a multiplicity of infection (MOI) of 0.01. Culture supernatants were collected at day 3–7 postinfection, and virus titers were evaluated by TCID50. To assess the relative infectivity of the viruses, infectious virus titers were compared either with the amount of viral protein or the amount of genomic copies present in the samples. The values obtained for EBOV-WT were set to 100%, and the relative infectivity of the mutated viruses was expressed as a percentage of the EBOV-WT value.

**RNA Isolation, RT, and Quantitative PCR (qPCR)**

Total RNA from EBOV-infected cells was isolated following the RNaseasy protocol (Qiagen). RNA was denatured and annealed with primers (200 nmol/L final concentration) at 65°C for 5 minutes in the presence of 0.5 mmol/L dNTP and the RT reaction was performed for 50 minutes at 42°C using SuperScript II RT (Invitrogen). In total, 500 ng of oligo(dT)12–18 (Invitrogen) were used for cDNA synthesis of virus-specific messenger RNAs (mRNAs). The qPCR reactions were performed using the ABI PRISM 7000 Sequence Detection System (Applied). Briefly, the amplification was carried out in a 50 μL reaction volume containing 8 μL of 10 × diluted cDNA or plasmid DNA standards, 25 μL of Platinum SYBR Green qPCR SuperMix UDG with ROX and 17 μL of forward and reverse primers (400 nmol/L final concentration). Reactions were set up in duplicate. Each PCR included an initial heat-denaturing step at 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. At the end of the PCR, the melting temperature of the final double-strand DNA products was determined by heating gradually from 60°C to 95°C over 20 min. RT-qPCR results were analyzed using ABI PRISM 7000 SDS Software 1.1 (Applied). A standard curve was established using 10-fold dilutions of a plasmid containing the full-length cDNA of EBOV (pFL-EBOVe+, [19]) and was used to determine the cDNA amounts in the samples. Primers were designed to amplify regions corresponding to NP, GP and VP40 genes, as well as the intergenic and trailer regions of EBOV genome; NP forward: 5’-TAGATCGACCAAGGGTGGAC, NP

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>First cluster</th>
<th>Second cluster</th>
<th>Substitution</th>
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<tr>
<td>VP30-AS</td>
<td>AAA</td>
<td>SSS</td>
<td>S29,30,31 → A</td>
</tr>
<tr>
<td>VP30-SA</td>
<td>SSS</td>
<td>AAA</td>
<td>S42,44,46 → A</td>
</tr>
<tr>
<td>VP30-AA</td>
<td>AAA</td>
<td>AAA</td>
<td>S29,30,31 → A</td>
</tr>
<tr>
<td>VP30-AD</td>
<td>AAA</td>
<td>AAA</td>
<td>S42,44,46 → A</td>
</tr>
<tr>
<td>VP30-DS</td>
<td>DDD</td>
<td>SSS</td>
<td>S29,30,31 → D</td>
</tr>
<tr>
<td>VP30-DD</td>
<td>DDD</td>
<td>DDD</td>
<td>S29,30,31 → D</td>
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<td>S42,44,46 → D</td>
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**Figure 1.** VP30 phosphorylation sites and mutants nomenclature. VP30 of EBOV contains 2 clusters of serine residues that can be phosphorylated (29, 30, 31 and 42, 44, 46). Serine residues from the first or the second cluster were replaced by alanine or aspartate residues.
Influence of VP30-AA and VP30-DD on EBOV Transcription and Replication

First, to further investigate the effect of VP30-DD and VP30-AA on EBOV replication we examined how these proteins affect the synthesis of virus-specific RNAs in EBOV-infected cells. For this purpose HEK 293T cells were transfected with plasmids expressing either VP30-WT, VP30-DD, or VP30-AA and subsequently infected with EBOV-WT (Figure 2A). Quantification of virus-specific RNAs by qPCR using primers targeting VP40 showed that in the presence of VP30-AA the synthesis of EBOV mRNA increased up to 200% compared with the values detected for VP30-WT–expressing cells. In contrast, expression of VP30-DD reduces the synthesis of EBOV mRNAs to the level of 40% from that of VP30-WT expressing cells (Figure 2B). Given that genomic RNA is the template for synthesis of both positive sense viral RNAs, the ratio antigeneic RNA plus mRNAs versus mRNAs allows us to estimate whether transcription or replication is indeed favored in the presence of mutated VP30s (Figure 2C). Data obtained suggest that replication (antigenome synthesis) is dominant in the presence of “phosphorylated” VP30-DD and vice versa, expression of VP30-AA favors transcription. In effect, these results support the notion that phosphorylation of VP30 is implicated in the switch from transcription to replication during EBOV replication. Previously, using EBOV minigenomes, it was shown that viral transcription depends on the presence of VP30, while this protein is not required for RNA replication [8]. Interestingly, replication was shown to be more efficient in the absence of phosphorylation of these proteins, however, remains obscure even if implication of P protein phosphorylation in the balance between transcription and replication has been proposed for some viruses [23, 24]. Virions of EBOV have been shown to contain phosphorylated forms of two proteins: NP and the transcription activation factor VP30 [18]. Previously, we and others have demonstrated that the phosphorylation state of VP30 is likely to play a role in transcription activation of the viral genes [7, 17]. The nonphosphorylatable VP30-AA facilitates transcription of the EBOV minigenome and is also capable of supporting the recovery of recombinant EBOV-WT [7]. On the contrary, VP30-DD, which mimics a permanently phosphorylated VP30, is inactive in both assays. Remarkably, recombinant EBOVs with the substitution of serines to either alanine or aspartate residues in both serine clusters could not be recovered [7, 17]. Taken together, these data indicate that mutations simulating either permanent phosphorylation states of VP30, namely, phosphorylated or nonphosphorylated, lead to a disability for supporting the complete replication cycle of EBOV. In this study, we further investigate the role of VP30 phosphorylation in EBOV replication and extend the set of VP30 mutants, which now also contain proteins with one serine cluster conserved and the other replaced by either alanine or aspartate residues (Figure 1).
VP30 than if it was expressed in the cells [25]. In this regard, phosphorylation of VP30 is likely to promote RNA replication by mimicking the absence of VP30’s transcription activating function.

Generation and Analysis of Recombinant EBOV Expressing Mutated VP30

Since we were unable to rescue EBOVs encoding either VP30-AA or VP30-DD [7], we tried to generate recombinant viruses expressing VP30 with substitutions of serine residues in one of the clusters while preserving the serines in the other. Indeed, previously we demonstrated that a combination of alanine and aspartate residues in the VP30 phosphorylation domain (VP30-AD and VP30-DA) resulted in VP30 proteins capable of initiating transcription of an EBOV-specific monocistronic minigenome. However, both proteins were unable to reinitiate transcription of downstream genes and were thus defective in their ability to support replication of EBOV [7]. However, as these data suggested that a cluster of negative charges mimicking phosphorylation could inhibit VP30’s transcription reinitiation function, we also considered that the conservation of one of the serine clusters might allow proteins to be obtained with at least some degree of functionality. First, we tested if the plasmids expressing mutated VP30 were capable of supporting the recovery of EBOV-WT. For this purpose we used the virus recovery protocol described earlier [7, 19] and plasmids encoding VP30 mutants, VP30-AS, VP30-DS, VP30-SA, and VP30-SD, which replaced the plasmid pTM1-VP30/WT expressing VP30-WT. In effect, we demonstrate that all of these mutated VP30 proteins support the recovery of EBOV-WT (Figure 3A, left column). These results indicate that mutated VP30s, including those possessing a cluster of negatively charged aspartates (VP30-SD and VP30-DS), maintain their transcription reinitiation function and are capable of supporting the transcription of all viral genes including VP30-WT. Next, in an attempt to generate recombinant EBOV mutants we successfully recovered 3 viruses: EBOV-AS, EBOV-DS, and EBOV-SA (Figure 3A, right column). EBOV-SD was never recovered even after multiple attempts, suggesting that the presence of either serines or at least nonphosphorylatable alanine residues in the second cluster is somehow important for EBOV replication. The growth characteristics of recovered viruses were compared to that of the EBOV-WT. Vero E6 cells were infected with viruses at the same MOI and samples of culture medium were harvested at different intervals (days 3, 4, 5, 6, and 7 postinfection). TCID₅₀ titers for the culture supernatants were determined (Figure 3B, left panel), and results indicated that wild-type EBOV outgrows all 3 mutants very early in the infection by about a 1.5 log in titers. Culture supernatants were also analyzed for virus release by Western blot using anti-VP24 antibody (Figure 3B, right panel). Quantitation of the protein bands showed that all mutants released fewer virus than EBOV-WT. EBOV-SA appears to be the most affected by the introduced mutations. It had previously been postulated that the phosphorylation state of VP30 affects its intracellular association with NP inclusions [17]. Theoretically, this could affect nucleocapsid formation and thus influence virus release. To test this hypothesis, Vero E6 cells were infected with recombinant EBOVs, and immunofluorescence analysis of the intracellular localization of VP30 and NP was performed using anti-VP30 and anti-NP antibodies (Figure 3C). For all recombinant viruses variable patterns of the VP30 intracellular distribution were observed, which could be explained by different phases of infection in the individual cells. However, the VP30 of all recombinant viruses was colocalized with the NP-inclusions, which contradicts the postulation that the introduced mutations affect this association.

Comparison of the viruses with respect to amounts of infectious units versus amounts of viral proteins released into the culture supernatants revealed a certain degree of incoherency (Figure 3). EBOV-DS displayed lower titers than EBOV-WT, although these 2 were rather similar for virus release (Western blot). EBOV-SA and EBOV-AS differed in virus release but contained approximately the same infectious titers in culture supernatants. To assess the relative infectivity of the viruses, infectious virus titers of each virus were compared either with
amounts of the structural protein VP24 (quantified using Western blot and protein band quantification on a Typhoon 8600 Imager using ImageQuant TL software; Amersham) or with the amount of genomic copies quantified by qPCR using primers specific for the GP gene (Figures 4A and 4B). EBOV-DS showed the lowest infectivity values followed by 2 other mutants, EBOV-AS and EBOV-SA, but they all possessed remarkably lower infectivity compared with EBOV-WT.

Viruses from the order Mononegavirales are prone to generate defective interfering particles carrying genomes with massive internal deletions. For EBOV, such particles have been shown to contain 5’ and/or 3’ terminal sequences but not central regions of the genome [26]. In this regard the difference in infectivity can be explained if the mutated viruses generate increased amounts of defective interfering particles. To answer this question we performed a quantification of the genomic copies in the virus stocks using sets of primers directed at different regions of the viral genome: leader (Le) and trailer (Tr), the GP gene (GP), and the intergenic region between the VP30 and VP24 genes (IR). The values obtained for the intergenic region were set to 100% for each virus and the values for other regions were expressed as a percentage of the IR values.

Interestingly, the divergence in the viruses’ infectivity means that different amounts of viral particles have to be used to reach
the same MOI. For example, ~10-fold more viral particles of EBOV-DS than EBOV-WT were used in the experiment presented in Figure 3B. This divergence could be attributed to the capacity of the viruses to establish successive cycles of viral replication. Mutations affecting the primary step in the replication of the virus such as transcription likely can result in an abortive infection and thus reduce the infectivity. By loading higher amounts of viral particles we seemed to be able to compensate, at least to a certain degree, the defects caused by the mutations. In this regard, the efficient virus release of EBOV-DS seen in Figure 3B is likely to be due to high viral load rather than only efficient replication.

Next, to better understand the nature of the difference in infectivity between recombinant viruses, we performed quantitative analysis of virus-specific RNAs present in the virus-infected cells. For this purpose Vero E6 cells were infected with the same amounts of viral particles quantified by qPCR (200 genomic copies/cell) and analyzed at different intervals post-infection (Figure 5). An estimate of the NP mRNA levels by qPCR at 6 hours postinfection showed (Figure 5A) that transcription, in the case of EBOV-DS, is significantly reduced compared to EBOV-WT (35% of WT values), whereas EBOV-AS, on the contrary, showed an up-regulation of transcription (166% of WT). Both EBOV-SA and EBOV-WT possessed approximately the same level of transcription at this time interval postinfection. Next, to estimate the influence of mutations in VP30 on replication we quantified viral RNAs present in the cells at 24 hours postinfection (mRNAs, genomic and antigenomic RNAs) (Figure 5B). In comparison with EBOV-WT, EBOV-DS showed significantly lower amounts of all virus-specific RNAs. This reduction is explained by a reduction in transcription, which apparently affected the complete replication cycle of the virus. Both EBOV-SA and EBOV-AS revealed higher amounts of all viral RNAs in comparison to EBOV-DS and lower amounts compared with EBOV-WT, which correlates with the data presented in Figures 4A and 4B. Remarkably, an enhanced transcription observed for EBOV-AS at 6 hours postinfection does not result in better replication of this virus later post-infection. In effect, both an increase and decrease in transcription had a negative effect on the replication of EBOV. A decrease in infectivity observed with EBOV mutants generated in this study is likely to be associated with a reduced capacity to initiate primary transcription as in the case of EBOV-DS and to switch the viral polymerase complex to the replication mode as in the case of EBOV-AS and EBOV-SA.

Several cellular protein kinases (eg, PKA, CKII, and cAMKII) are predicted to recognize VP30 phosphorylation sites [7, 17]. Moreover, it has been reported that protein phosphatases PP1A and PP2A can dephosphorylate VP30-WT [17]. Inhibition of these phosphatases by okadaic acid blocks virus growth, most likely by inducing an accumulation of a phosphorylated form of VP30. Importantly, the effect of okadaic acid could be abrogated by the expression of VP30-AA mimicking permanently nonphosphorylated VP30 [17]. The results obtained in our study clearly demonstrate that reduction in the flexibility of VP30 phosphorylation diminishes EBOV fitness and furthermore suggest that phosphorylation of VP30 is a dynamic process essential for the function of this protein in the EBOV replication cycle.

Based on data presented in this study, a model for the role of EBOV VP30 in the regulation of viral RNA synthesis can be proposed (Figure 6). In this model both phosphorylated and nonphosphorylated VP30s are necessary to maintain finetuning of the transcription and replication of viral RNAs. Transcription of EBOV genes is supported by low- or nonphosphorylated forms of VP30. The appearance of phosphorylated VP30, which is transcriptionally inactive, is required to switch off transcription and to favor the replication of viral RNAs. Indeed, based on our and previously published data, we envision a dynamic, 2-way process of the regulation of virus replication through phosphorylation of VP30.

In conclusion, while other viral proteins—for example, NP—could also contribute to the regulation of a switch between transcription and replication processes, the data obtained in this study indicate that EBOV VP30 and, in particular, phosphorylation of this protein, plays an important role in the regulation of viral RNA synthesis.
Figure 6. Model for the role of VP30 phosphorylation in the replication cycle of EBOV. Different phosphorylation forms of VP30 are involved in the regulation of EBOV replication. Non- or weakly phosphorylated forms of VP30 support transcription of the viral genes and thereby facilitate protein synthesis. Phosphorylated VP30 impairs transcription and thus favors genome replication—which is indeed independent of VP30—and assembly and budding of the viral particles. Changes in the phosphorylation state of VP30 are driven by the action of cellular kinases and phosphatases. The proposed model suggests that dynamic phosphorylation of VP30 is playing a regulatory role during the EBOV replication cycle.

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