Phosphorylation of Ebola Virus VP30 Influences the Composition of the Viral Nucleocapsid Complex

IMPACT ON VIRAL TRANSCRIPTION AND REPLICATION*

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Nadine Biedenkopf, Bettina Hartlieb, Thomas Hoenen, and Stephan Becker

From the Institut für Virologie, Philipps-Universität Marburg, Hans-Meerwein-Str. 2, 35043 Marburg, Germany

Background: Ebola virus VP30 is an essential transcription factor dispensable for viral replication whose activity is regulated via phosphorylation.

Results: Phosphorylation of VP30 impacts viral transcription and replication by modulating interaction with the nucleocapsid proteins VP35 and NP.

Conclusion: VP30 phosphorylation influences the composition of the viral polymerase complex via phosphorylation-dependent interaction with VP35.

Significance: VP30 phosphorylation status modulates both viral transcription and replication.

Ebola virus is a non-segmented negative-sense RNA virus causing severe hemorrhagic fever with high fatality rates in humans and nonhuman primates. For transcription of the viral genome four viral proteins are essential: the nucleoprotein NP, the polymerase L, the polymerase cofactor VP35, and VP30. VP30 represents an essential Ebola virus-specific transcription factor whose activity is regulated via its phosphorylation state. In contrast to viral transcription, VP30 is not required for viral replication. Using a minigenome assay, we show that phosphorylation of VP30 inhibits viral transcription while viral replication is increased. Concurrently, phosphorylation of VP30 reciprocally regulates a newly described interaction of VP30 with VP35, and strengthens the interaction with NP. Our results indicate a critical role of VP30 phosphorylation for viral transcription and replication, suggesting a mechanism by which VP30 phosphorylation modulates the composition of the viral polymerase complex presumably forming a transcriptase in the presence of non-phosphorylated VP30 or a replicase in the presence of phosphorylated VP30.

Ebola virus (EBOV) together with Marburg virus constitutes the family Filoviridae within the order Mononegavirales. Filoviruses are characterized by the filamentous shape of their particles and contain a non-segmented negative-sense RNA genome (1). EBOV is classified as a BSL4 pathogen, and human infection results in a severe hemorrhagic fever with case fatality rates of up to 90% (2, 3). Currently, neither an approved vaccine nor antiviral therapy is available for humans (4, 5). The enveloped EBOV particle is composed of seven structural proteins, five of which form the helical nucleocapsid that represents the template for viral transcription and replication. The viral genome is encapsidated by the major nucleocapsid protein NP, and VP35, VP30, and VP24 interact with NP to form the mature nucleocapsid (6–8). The enzymatically active part of the viral polymerase L is bound to the nucleocapsid by interaction with the polymerase cofactor VP35 (9). The hexamer zinc-finger protein VP30 is an EBOV-specific viral transcription factor (10–12).

After entry into the host cell, the EBOV envelope fuses with host cell membranes to release the nucleocapsid into the cytoplasm where transcription and replication take place (9, 13). Initial transcription of the newly entered encapsidated RNA genome is entirely accomplished by the nucleocapsid proteins that are associated with the intruding virus (primary transcription). Transcription is regulated by conserved transcription start signals and stop signals at the viral gene borders (7). The gene start signals are part of RNA secondary structures, and it has been proposed that VP30 binds to the RNA at the first gene start signal to initiate transcription (14, 15). In addition, VP30 was shown to be important for transcription reinitiation of subsequent genes (16). After transcription, EBOV mRNA is then translated by the cellular translation machinery, and newly formed viral proteins accumulate in inclusion bodies. Subsequently, a positive-sense full-length genome is replicated, which is concomitantly encapsidated by newly synthesized NP molecules. The produced antigenomic intermediate serves as a template for the replication of new viral genomes that, in turn, are available for transcription. In contrast to its essential role in viral transcription, VP30 is dispensable for viral replication, which is accomplished by L and VP35 alone (9). How the viral polymerase complex switches between its transcription and replication activity is still elusive.

Transcriptional support activity of VP30 is modulated via phosphorylation at two N-terminal serine clusters, each con-
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sisting of three serine residues (S29-S30-S31, and S42-S44-S46) (Fig. 1A) (17). Mutation of serine residues to alanine mimicking nonphosphorylated serine or to aspartate mimicking phosphorylated serines indicated that non- or weakly phosphorylated VP30 supports initiation of viral transcription, while fully phosphorylated VP30 does not. The VP30-specific cellular kinase is unknown. VP30 can be dephosphorylated by the cellular phosphatases PP1 and PP2A, and inhibition of these enzymes by okadaic acid blocked viral transcription comparable to a VP30 mutant, which mimics the fully phosphorylated protein (17).

Recently, a positive influence of VP30 phosphorylation mutants on replication of EBOV was described by Martinez et al. (18). This result was of great interest because, so far, EBOV replication was thought to be independent of VP30 (9). Most of these experiments were performed by overexpressing VP30 phosphorylation mutants in EBOV-infected cells, where naturally wild type VP30 (VP30_wt) is present. Therefore, the question arose whether in the absence of VP30_wt the same results were to be expected. In addition, in the infected cell, it is difficult to differentiate effects on transcription and on replication since viral replication enhances the number of templates for transcription. Therefore, it remained unclear whether the observed influence of VP30 phosphorylation was the result of a direct effect on replication, or an indirect effect of down-regulation of viral transcription. As a consequence, the mechanism of how VP30 exerts its effect on viral transcription is still unknown.

In this study, we used previously characterized VP30 phosphorylation mutants mimicking either completely phosphorylated or completely non-phosphorylated VP30 to study their impact on viral replication and viral transcription (16–18). To this end we employed two different minigenome systems allowing investigation of viral genome replication and transcription in absence of infectious virus and, more importantly, differentiation of these two processes (9, 19, 20). Positive influence of VP30 phosphorylation on viral replication and a negative influence on transcription were confirmed, suggesting VP30 phosphorylation to balance viral transcription and replication. To understand the mechanism by which phosphorylation conveys VP30 influence on transcription and replication, we investigated the role of VP30 phosphorylation for interaction with other viral nucleocapsid proteins, which might influence the composition of the polymerase complex. We showed for the first time an interaction of VP30 with VP35, which was reciprocally regulated by VP30 phosphorylation. We suggest that phosphorylation leads to dissociation of VP30 from the VP35-L complex, thereby modulating transcription and replication activities of the polymerase. In addition, we could demonstrate that the interaction of VP30 and NP was positively influenced by phosphorylation of VP30. The VP30-NP complex was not required for transcription and replication, but was essential for the incorporation of VP30 into new virus particles and the prerequisite for primary viral transcription in target cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293 (human embryonic kidney) and HUH7 (human hepatoma) cells were cultivated with Dulbecco’s modified Eagle’s medium with penicillin and streptomycin, 5 mM glutamine, and 10% fetal calf serum at 37 °C and 5% CO2.

Plasmids—All plasmids coding for wild type EBOV proteins (pCAGGS NP, -VP35, -L, -VP24, -VP40, and -GP) as well as the EBOV-specific minigenome (pANDY 3E5E) and pCAGGS T7 polymerase have been described earlier (19). The replication-deficient minigenome was first described in Ref. 20. pCAGGS VP30_wt as well as VP30_AA and VP30_DD phosphorylation mutants contain a C-terminal FLAG epitope and have been described earlier (VP30_6A and VP30_6D, respectively). pCAGGS NP_myc was described in Ref. 21. Cloning of pCAGGS-VP30_E197AFLAG, pCAGGS-VP30_myc, pCAGGS-VP35HA, and pCAGGS-NP_myc was performed using standard cloning techniques. Detailed cloning strategy and primer sequences are available on request. All constructs have been verified by sequencing.

EBOV-specific Transcription and Replication Competent Virus-like Particle (trVLP) Assay—The assay was performed as described in Refs. 19, 22. Transfection of plasmids was performed using TransIT (Mirus). Where indicated, the regular minigenome was replaced by a replication-deficient minigenome to analyze virus-specific transcription without the influence of replication (see Ref. 20). Additional transfection of pGL4.70 (Promega) encoding a firefly luciferase was performed for normalization of transfection efficiency. Reporter activity in producer cells was measured 72 h post-transfection (p.t.) using the Dual Luciferase assay (Promega). Released trVLPs were purified from the supernatant via ultracentrifugation over a 20% sucrose cushion and used for infection of target cells, which were either naïve or pretransfected with 500 ng of pCAGGS VP30_wtFLAG or VP30_E197AFLAG, respectively. An aliquot of trVLPs was analyzed regarding the incorporation of VP30 mutants using proteinase K digestion assay as described in Ref. 23. 60 h postinfection target cells were lysed and a Renilla reporter assay (Promega) was performed. Results obtained with VP30_wt were set to 100%.

Strand-specific Two-step Reverse Transcription and Quantitative Real-time PCR—Replication of the minigenome was analyzed using a strand-specific two-step RT quantitative real-time PCR (PeqLab). Briefly, producer cells (see EBOV-specific trVLP assay) were harvested 72 h p.t. and RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer’s protocol, including on-column digestion with the RNase-Free DNase Set (Qiagen) to avoid plasmid contamination. RNA samples were normalized to firefly luciferase levels, which correspond to transcription efficiency. First, RNA was reverse transcribed using a specific primer for replicated vRNA (Luc(+): 5’-GGC CTC TTC TTA TTT ATG GCG A-3’, see Ref. 20). Next, cDNA was subjected to quantitative real-time PCR running on an ABI StepOne™ PCR cycler using Primer Luc(+) and Luc(−) (5’-AGA ACC ATT ACC AGA TTT GCC TGA-3’). A probe with sequence specificity for the Renilla luciferase reporter (5’-CCA CAT ATT GAG CCA GTA GCG CCG-3’) containing 5’ FAM (6-FAM-Phosphoramidit) and 3’ DDQ-1 (Deep Dark Quencher-1, Biomers) was used for detection of amplificates. Serial 10-fold dilutions (106 to 109) of the minigenome plasmid were used as standards to determine the amount of minigenome copies in the samples. PCR conditions: Initial denaturation of cDNA was obtained at 95 °C for 5 min,
followed by 40 cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 95 °C for 15 s.

**Coimmunoprecipitation Analysis**—HEK293 cells were transfected using TransIT (Mirus) with the corresponding plasmids (1 μg each). DNA amounts were adjusted, if necessary, with empty vector. Cells were lysed at 24 or 48 h p.t. for 20 min at room temperature in ice-cold coimmunoprecipitation buffer (20 mM Tris-HCl, pH 7.5/100 mM NaCl/1% Nonidet P-40/17.5 mM EDTA) with 1× Complete protease inhibitor mixture (Roche) and 0.1% Triton X-100. Cell debris was removed by centrifugation (10 min at 8,000 × g, 4 °C). An aliquot was taken for expression control (input). The clarified supernatant was taken at a magnification of 63×. Precipitation was performed for 2 h at 4 °C. Precipitated protein complexes were washed three times with coimmunoprecipitation buffer without Triton X-100 via centrifugation (10 min at 8,000 × g, 4 °C). An aliquot was taken for expression control (input). The clarified supernatant was taken at a magnification of 63×.

**Electrophoresis and Western Blot Analysis**—Sodium dodecylsulfate (SDS) gel electrophoresis (12%) and subsequent Western blot analysis were performed as described in Ref. 24. Antibodies were diluted in PBS containing 0.1% Tween and 1% milk powder: mouse anti-FLAG® M2, biotinylated mouse anti-FLAG® BioM2, biotinylated mouse anti-HA-Bio (Sigma-Aldrich), and rabbit anti-c-Myc (Santa Cruz Biotechnology) antibodies 1:500, goat anti-GP/VP30/VP40 1:2000, and chicken anti-NP 1:10,000. Alexa Fluor® 680-conjugated secondary antibodies were purchased from Invitrogen (Molecular Probes), IRDye® 800-conjugated antibodies came from Rockland (all diluted 1:5000). Detection of antibodies was performed using the LiCor Odyssey® Infrared Imaging System (LI-COR, Lincoln, NE).

**Indirect Immunofluorescence Analysis**—HUH7 cells were transfected with 500 ng of each plasmid using TransIT (Mirus). 16 h p.t. cells were fixed using 4% PFA in DMEM. Permeabilization of cells and blocking of nonspecific signals was performed as described in Ref. 24. Antibodies were diluted in blocking buffer: guinea pig anti-VP30, mouse anti-HA (Co-vance) both 1:100, mouse anti-FLAG® M2 (Sigma-Aldrich), and rabbit anti-c-Myc (Santa Cruz Biotechnology) 1:50. All secondary antibodies were diluted 1:100 (Invitrogen and Dianova), DAPI (4′,6-diamidino-2′-phenylindol) 1:10,000. Pictures were taken at a magnification of 63×.

**RESULTS**

**Influence of VP30 Phosphorylation on Viral Replication and Transcription**—Recent data revealed that VP30 is not only essential for viral transcription but influenced viral replication as well (18). EBOV replication was boosted upon simultaneous expression of a phosphomimetic VP30 mutant in trans (VP30_DD, Ref. 18). This was astonishing since the presence of VP30 is not required at all for replication (9, 20). With respect to these findings, we investigated the role of VP30 phosphorylation for viral replication. We used two different VP30 mutants mimicking either a completely non-phosphorylated VP30 (VP30_AAFLAG), or a completely phosphorylated VP30 (VP30_DDFLAG), which were described earlier (16, 17) (Fig. 1A). Using a transcription and replication competent virus-like particle (trVLP) assay that monitors viral transcription and/or replication under BSL2 conditions, the effects of the two mutants on replication were analyzed. In contrast to the experimental set up used by Martinez et al. (18), this assay is independent of the presence of VP30_wt allowing to investigate effects of completely phosphorylated or non-phosphorylated VP30 without interfering VP30_wt. The trVLP assay is based on the intracellular expression of an EBOV-specific minigenome containing a reporter gene (Renilla luciferase), which is flanked by sequences encoding the minimal required cis-acting transcription/replication and encapsidation signals of the viral genome (Fig. 1B). The minigenome is under the control of the T7 promoter allowing the transiently expressed T7 polymerase to synthesize the negative-sensed RNA minigenome, which is then encapsidated by NP and serves as a template for the EBOV polymerase complex. Simultaneous plasmid-based expression of VP24, VP40, and GP leads to assembly and release of trVLPs thus imitating an authentic infection cycle. (Fig. 1C, Ref. 19).

Influence of the different VP30 phosphomimetic mutants on the activity of the T7 promoter, was excluded by verifying that expression of the different VP30 mutants did not differentially impact the activity of a luciferase reporter gene under the control of the T7 promoter (data not shown).

First, we investigated the impact of VP30 phosphorylation on viral replication by using a negative-strand-specific two-step quantitative real-time PCR targeting specifically the replicated luciferase reporter gene (vRNA, see Ref. 20). Similar to the results gained by Martinez et al., expression of phosphomimetic VP30_DDFLAG stalled transcription and favored viral replication (180% compared with VP30_wtFLAG) (18). In contrast, expression of non-phosphorylated VP30_AAFLAG reduced the amount of replicated minigenomes to 73% (Fig. 1C).

Increase of viral replication positively impacts viral transcription because replicated minigenomes are again available as templates for viral transcription (20). With respect to the newly discovered influence of VP30 phosphorylation on viral replication, we reconsidered previous data concerning the transcriptional support activity of VP30 phosphorylation mutants (16, 18). To exclude the influence of viral replication for the activity of VP30 as transcription factor, we used a modified trVLP assay where the classical minigenome (template for transcription and replication) was substituted by a replication-deficient minigenome (Ref. 20), Fig. 1, B and D). Similar to previous results obtained with the classical minigenome (16, 17), VP30_AAFLAG supported transcription of the replication-deficient minigenome comparable to VP30_wtFLAG while VP30_DDFLAG was transcriptionally inactive (Fig. 1D). These results indicate that phosphorylation of VP30 influences transcription even in the absence of simultaneous replication.

Taken together, employing VP30 mutants mimicking either the completely phosphorylated or non-phosphorylated protein in a trVLP assay revealed a balancing influence of VP30 phosphorylation on viral transcription and replication. In presence of a non-phosphorylated VP30 (VP30_AAFLAG), viral transcription was enhanced and viral replication decreased, while in the presence of a phosphorylated VP30 (VP30_DDFLAG) viral
transcription was stalled and viral replication increased. These results suggest that either VP30 directly influences replication, or the phosphorylation state of VP30 regulates only the transcriptional process, and effects on replication are the result of transcriptional regulation. This could be the case if the transcription complex comprising L, VP35, NP, and VP30 is transformed into a replication complex by phosphorylation of VP30.

Interaction of VP30 Phosphorylation Mutants with VP30 and NP—We therefore examined whether the phosphorylation of VP30 has an impact on the interaction with nucleocapsid proteins, which can regulate the composition of the viral polymerase complex.

First, we focused on self-assembly of VP30, which is a hexameric protein composed of three VP30 dimers (10, 11). Only VP30 hexamers support viral transcription. While hexamerization of VP30 is readily detected by coimmunoprecipitation, dimerization can only be detected after chemical crosslinking (10, 11). We tested whether a permanent negative charge at the phosphorylation site influenced the oligomerization of VP30, which would then explain why VP30_DD is unable to support viral transcription (11). We used FLAG-tagged VP30 phosphorylation mutants (Fig. 1A) and a Myc-tagged VP30_wt to perform coimmunoprecipitation analyses. VP30_AAFLAG, VP30_DDFLAG, or VP30_wtFLAG were able to coprecipitate the
same amount of VP30_wt_myc, indicating that oligomerization with VP30_wt is not affected by the phosphorylation state of the protein (Fig. 2A, lanes 3–5). Coimmunoprecipitation experiments with Myc- and FLAG-tagged VP30_AA or Myc- and FLAG-tagged VP30_DD, were able to homooligomerize (Fig. 2B, lanes 3 and 6). Further, VP30_AA and VP30_DD were able to interact with each other supporting the conclusion that the charge at the VP30 phosphorylation sites does not influence the oligomerization of the protein (Fig. 2B, lane 7).

Next, we investigated the interaction between VP30 and NP. Previous work showed that in the absence of NP the non-phosphorylated VP30 was diffusely distributed in the cytoplasm, while phosphorylation leads to accumulation of VP30 in NP-induced inclusion bodies (17). Nevertheless, incorporation of VP30 into trVLPs was shown to be independent of the phos-
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In this study, we wanted to quantify the interaction of VP30 with NP by coimmunoprecipitation analysis (Fig. 2C). We observed that VP30_DDFLAG, mimicking the fully phosphorylated VP30, precipitated NP in significantly higher amounts compared with VP30_AAFLAG or VP30_wtFLAG (Fig. 2C, lanes 3–5) suggesting that the interaction of VP30_DD and NP is stronger. These data underline the previous results indicating that phosphorylation of VP30 modulates the interaction with NP (17).

Interaction of VP30 and NP Is Essential for the Viral Life Cycle but Dispensable for Viral Transcription and Replication—To investigate whether the strengthened interaction between VP30 and NP contributed to the switch from viral transcription to replication, we wanted to analyze the significance of the NP-VP30 interaction for the viral life cycle. Based on the available crystal structure of the VP30 C terminus we therefore created a VP30 mutant without NP binding activity, which would then be employed in the minigenome assay to test its ability to support viral transcription (11). We generated a VP30 construct carrying a point mutation of glutamate 197 to alanine and analyzed the interaction of VP30_E197AFLAG with VP30. Phenotypical characterization using immunofluorescence analysis revealed a diffuse intracellular distribution of VP30_E197AFLAG comparable to VP30_wtFLAG upon single expression (Fig. 4B). Upon coexpression with a Myc-tagged NP, VP30_E197AFLAG was not concentrated into NP-induced inclusion bodies in contrast to VP30_wt, indicating that the interaction between the proteins is impaired (Fig. 3A). The obtained results were confirmed by coimmunoprecipitation analysis. We observed that interaction between NP and VP30 was significantly inhibited upon mutation of glutamate 197 to alanine, indicating that E197 is necessary for the interaction of VP30 with NP (Fig. 3B). Next, we analyzed the significance of NP-VP30 interaction using the trVLP assay by replacing VP30_wt with VP30_E197AFLAG. Interestingly, VP30_E197AFLAG was able to support transcription and replication similar to VP30_wtFLAG indicating that interaction of VP30 with NP is not required for these processes (Fig. 3C). To elucidate the role of the NP-VP30 interaction for other steps of the viral life cycle, we used purified trVLPs from the supernatant of producer cells to infect target cells (Fig. 3D, Ref. 19). Infection with trVLPs produced by cells expressing VP30_E197AFLAG instead of VP30_wt did not result in reporter gene activity in target cells, similar to trVLPs containing no VP30 (2VP30). To confirm that this result was not due to the absence or inactivity of the minigenome, we provided the target cells with VP30_wtFLAG in trans, which were then infected with the generated trVLPs. Under these conditions, all trVLPs used for infection-induced reporter gene activity (Fig. 3D, + VP30_wtFLAG). In target cells complemented in trans with VP30_wtFLAG, trVLPs from cells expressing VP30_E197AFLAG induced approximately the same level of reporter gene activity as trVLPs containing no VP30 or VP30_wtFLAG. We therefore concluded that the amount of minigenomes in the different trVLPs was comparable, further showing that replication of minigenomes in producer cells was not affected by the missing interaction of VP30_E197AFLAG and NP. Similarly, when target cells were provided with VP30_E197AFLAG in trans, all tested trVLPs were able to induce reporter activity (Fig. 3D, + VP30_E197AFLAG). To understand why trVLPs purified from cells expressing VP30_E197AFLAG were unable to support primary transcription in target cells, we examined their protein composition. Western blot analysis revealed that only traces of VP30_E197AFLAG were recruited into trVLPs (Fig. 3E, lane 5). These data indicate that interaction of VP30 with NP is dispensable for activation of viral transcription and replication, but essential for incorporation of VP30 into trVLPs. Further, interaction of VP30 with NP does not seem to be necessary for the template function of the minigenome, since functional minigenomes were delivered to target cells in the absence of NP-VP30 interaction.

VP30 Does Interact with VP35 in a Phosphorylation-dependent Manner—Although phosphorylation of VP30 influenced its binding to NP, this interaction did not explain the phosphorylation-dependent effects of VP30 on transcription nor on replication. Nevertheless, it is conceivable that VP30 interacts with the polymerase complex for its direct impact on viral transcription. We, therefore, investigated whether VP30 interacted with the polymerase cofactor VP35. Coimmunoprecipitation analysis of cell lysates expressing VP30_wtFLAG and VP35_HA revealed that, indeed, these proteins do interact with each other (Fig. 4A, lane 6). When trying to confirm this result by immunofluorescence analysis we were faced with the problem that both VP30 and VP35 are diffusely distributed in the cytoplasm preventing colocalization analyses (Figs. 4, B+C). We therefore took advantage of the ability of NP to recruit VP35 as well as VP30_wt into inclusion bodies (Fig. 4, E+F, see also Fig. 3A and Ref. 25). We coexpressed NP and VP35 together with VP30_E197AFLAG which, due to its missing interaction with NP, was not concentrated in inclusions formed by NP alone (Fig. 4G, see also 3A). If VP30_E197AFLAG was recruited into NP-induced inclusions in the presence of VP35, this should be mediated by interaction with VP35. Immunofluorescence analyses revealed colocalization of VP30_E197AFLAG and VP35_HA in the NP-induced inclusions, confirming the interaction between VP30 and VP35 (Fig. 4G).

After having identified a complex between VP30 and VP35, it was interesting to test whether phosphorylation of VP30 might interfere with this interaction. Coimmunoprecipitation analyses of VP35_HA with VP30_AAFLAG and VP30_DDFLAG showed that VP35_HA readily coprecipitated VP30_wtFLAG and VP30_AAFLAG but only weakly VP30_DDFLAG (Fig. 5A, lanes 3–5). Quantification revealed that interaction between VP35_HA and VP30_AAFLAG was strengthened (155% compared with VP30_wt), while interaction with VP30_DDFLAG was decreased (40% compared with VP30_wt).

Taken together, these results demonstrate that VP30 interacts with VP35, an essential component of the viral polymerase complex. The interaction between the two proteins is positively influenced when the phosphorylation sites of VP30 contain no charged amino acids, while a negatively charged phosphorylation site mimicking a fully phosphorylated VP30 inhibited interaction with VP35.

In summary, phosphorylation of VP30 modulates viral transcription and replication as well as interaction of VP30 with the polymerase complex protein VP35 and NP. It is suggested that effects of VP30 phosphorylation on viral RNA synthesis are
triggered by modulation of the VP30-VP35 interaction, which
changes the composition of the polymerase complex.

DISCUSSION

Phosphorylation of cellular proteins is a versatile and widely
used tool to regulate their function and it is therefore not sur-
prising that viruses use phosphorylation to modify the function
of their proteins, as well. For example, the P proteins within
the order Mononegavirales are phosphorylated and play an essen-
tial role in viral transcription and replication by acting as
polymerase cofactors, analogous to EBOV VP35. Phosphor-
ylation of Rinderpestvirus P protein, e.g. serves as a molecular
switch balancing viral transcription and replication (26). Filo-
viruses represent an exception within the order Mononega-
virales by having a non- or very weakly phosphorylated P-analog,
VP35, and an additional fourth nucleocapsid protein involved
in RNA synthesis, VP30, which is phosphorylated (12, 17). The
only protein among viruses in the order Mononegavirales with a
similar function as VP30 is M2–1 of respiratory syncytial virus
(RSV). M2–1 also acts as an essential phosphorylation-depen-
dent viral transcription factor. However, in contrast to VP30
which is active in supporting transcription in its non-phosphor-
ylated state, M2–1 supports transcription elongation and anti-
termination in its phosphorylated state (27, 28). For RSV, the

![FIGURE 3. Interaction of VP30 and NP is dispensable for viral transcription and replication.](image)

A, coimmunofluorescence analysis of VP30_wtFLAG and
VP30_E197AFLAG with NP_myc. HUH7 cells on glass cover slides transiently expressing FLAG-tagged mutants of VP30 and Myc-tagged NP were fixed at 16 h p.t. VP30 staining was performed using a mouse anti-FLAG® M2 antibody and Rhodamin-conjugated goat anti-mouse. NP_myc was stained using a rabbit anti-c-Myc and FITC-conjugated goat anti-rabbit antibody. B, coimmunoprecipitation analysis of VP30_wtFLAG and VP30_E197AFLAG with NP_myc. HEK293 cells transiently expressing FLAG-tagged VP30_wtFLAG or VP30_E197AFLAG were lysed at 24 h p.t. and coimmunoprecipitation was performed for 4 h using mouse anti-FLAG® M2 agarose. Samples were subjected to SDS-PAGE and Western blotting. VP30 staining was performed using a biotinylated mouse anti-FLAG® BioM2 antibody and Alexa Fluor® 680-conjugated streptavidin. NP_myc was stained using a rabbit anti-c-Myc and Alexa Fluor® 680-conjugated goat anti-rabbit antibody. Detection of proteins was obtained with LiCor Odyssey® Imaging Systems. C, reporter gene activity in producer cells. Cells have been transfected as described under Fig. 1B. Reporter gene activity was measured 72 h p.t. using the Dual Luciferase assay (Promega). Results obtained with VP30_wtFLAG were set to 100%. D, reporter gene activity in target cells. trVLPs generated from producer cells (described under C) were purified from supernatants and used for infection of (i) naive target cells, (ii) cells that express VP30_wtFLAG (ii), or VP30_E197AFLAG (iii). Reporter gene activity obtained upon infection with trVLPs containing VP30_wtFLAG was set for each setup to 100%. Ø: without VP30. E, expression and incorporation of VP30_E197A into trVLPs. trVLPs were treated with proteinase K to analyze specific incorporation of VP30 into trVLPs. Western blotting of cell lysate and purified trVLPs was performed using a mouse anti-FLAG® M2 antibody and Alexa Fluor® 680 goat anti-mouse antibody for VP30 staining, GP and NP were detected using a goat anti-GP/NP antibody and Alexa Fluor® 680-conjugated donkey anti-goat antibody. Detection was obtained using LiCor Odyssey® Imaging Systems.
activity of the transcription complex consisting of M2–1, P, and L has been proposed to be influenced by phosphorylation of the P protein which weakens its interaction with M2–1 resulting in a release of M2–1 from the polymerase complex to facilitate viral replication (29).

For EBOV, the mechanism that regulates the switch from transcription to replication is currently not understood. Recent experiments demonstrated an increase in EBOV replication upon expression of phosphomimetic VP30_DD in trans (18). The same mutant has been shown earlier to be unable to support viral transcription (17). These results led us to presume that EBOV transcription and replication might be interconnected. When transcription was switched on in presence of VP30_wt or VP30_AA, mimicking the nonphosphorylated VP30, replication was simultaneously reduced. Vice versa, viral transcription was not activated in presence of VP30_DD, while replication activity was enhanced (Fig. 1; Ref. 18). The mechanism of how the phosphorylation state of VP30 is conveyed to
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**FIGURE 5. Interaction of VP30 and VP35 is dependent on VP30 phosphorylation.** A coimmunoprecipitation of VP30 phosphorylation mutants with VP35HA. HEK293 cells expressing FLAG-tagged VP30 constructs and HA-tagged VP35 were lysed 48 h.p.t., and protein complexes were precipitated for 2 h, 4 °C using mouse anti-HA agarose. An aliquot for expression control (input) was collected from the cell lysate before precipitation. Western blot analysis was performed using a biotinylated mouse anti-HA-Bio antibody and Alexa Fluor® 680-conjugated streptavidin (shown in red). FLAG-tagged VP30 constructs were stained by a rabbit anti-FLAG antibody and IRDye® 800-conjugated goat anti-rabbit (shown in green). Detection of proteins was obtained with LiCor Odyssey® Imaging Systems. Precipitation of VP30_wtFLAG by VP35HA was set to 100%. Quantification of at least three independent experiments is shown to the left. Statistical significance: **, p < 0.01.

Phosphorylation of Ebola Virus VP30 might be involved in putative transcription or replication complexes have not yet been identified.

Phosphorylation of VP30 might also have effects on its binding to viral RNA. It has been published that VP30 is able to bind specifically EBOV RNA, which is assumed to be essential for its function in transcription activation (15). Since the phosphorylation sites of VP30 overlap with the putative RNA binding site, it is conceivable that phosphorylation of VP30 impairs its interaction with RNA due to electrostatic repulsion. Thus, phosphorylation of VP30 and subsequent dissociation of VP30 from the RNA template might additionally contribute to a release of VP30 from the transcription complex. This assumption needs further investigation.

Our experiments revealed that phosphorylated VP30 was better precipitated by NP than non-phosphorylated VP30 or VP30_wt suggesting the interaction of VP30 with NP is also influenced by VP30 phosphorylation. This result complements earlier findings that showed VP30_DD to be concentrated in the NP-induced inclusions harboring freshly formed nucleocapsids. In contrast, VP30_AA was homogenously distributed throughout the cell (17, 33). This leads to the assumption that the phosphorylated VP30 is predominantly associated with nucleocapsids while the non-phosphorylated VP30 interacts with soluble NP.

The study demonstrated that interaction of NP and VP30 is not necessary for viral transcription, but essential for the viral life cycle. This is because of the recruitment of VP30 into trVLPs is necessary to have VP30 at hand supporting primary transcription in the target cell.

Based on data obtained within this study, we propose the following model for VP30 phosphorylation status-dependent transition from transcription to replication (Fig. 6). Non-phosphorylated VP30 helps to initiate viral transcription, and the polymerase complex elongates the nascent RNA to produce capped and polyadenylated mRNAs (17, 34). Upon phosphorylation by a yet unidentified cellular kinase VP30 dissociates from the polymerase complex, due to the impaired interaction with VP35 and possibly the viral RNA. Released phosphor-
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Phosphorylation of VP30 remains bound to the nucleocapsid via a strengthened interaction with NP (17). If VP30 becomes dephosphorylated by the cellular phosphatases PP1 or PP2A, the non-phosphorylated VP30 is again recruited to the polymerase complex to switch on the transcription mode.

In summary, our data suggest that VP30 phosphorylation regulates the transcription and replication activities of the polymerase complex by binding to the polymerase complex via interaction with the polymerase cofactor VP35.

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