Phosphorylation of VP30 Impairs Ebola Virus Transcription*

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Jens Modrof, Elke Mühlberger, Hans-Dieter Klenk, and Stephan Becker‡

From the Institut für Virologie der Philipps-Universität Marburg, Robert-Koch-Strasse 17, Marburg 35037, Germany

Transcription of the highly pathogenic Ebola virus (EBOV) is dependent on VP30, a constituent of the viral nucleocapsid complex. Here we present evidence that phosphorylation of VP30, which takes place at six N-terminal serine residues and one threonine residue, is of functional significance. Replacement of the phosphorylators by alanines resulted in an only slightly phosphorylated VP30 (VP30\textsubscript{AA}) that is still able to activate EBOV-specific transcription in a plasmid-based minigenome system. VP30\textsubscript{AA}, however, did not bind to inclusions that are induced by the major nucleocapsid protein NP. Three intracellular phosphatases (PP1, PP2A, and PP2C) have been determined to dephosphorylate VP30. The presence of okadaic acid (OA), an inhibitor of PP1 and PP2A, had the same negative effect on transcription activation by VP30 as the substitution of the six phosphorylators for aspartate residues. OA, however, did not impair transcription when VP30 was replaced by VP30\textsubscript{AA}. In EBOV-infected cells, OA blocked virus growth dose-dependently. The block was mediated by the extensive phosphorylation of VP30, which is evidenced by the result that expression of VP30\textsubscript{AA} in trans, led to the progression of EBOV infection in the presence of OA. In conclusion, phosphorylation of VP30 was shown to regulate negatively transcription activation and positively binding to the NP inclusions.

Ebola virus (EBOV), a filovirus, is notorious for its unpredictable sporadic outbreaks of a fatal hemorrhagic fever in Africa (1–4). To date, neither a vaccine nor a treatment of the EBOV infection is available.

The envelope EBOV particles are composed of seven structural proteins and the negative sense RNA genome. Four viral proteins NP, VP35, L, and VP30 are the constituents of the nucleocapsid. The main component of the nucleocapsid complex is NP, a heavily phosphorylated protein, that encapsidates the genomic RNA and forms intracellular inclusions upon recombinant expression (5–7). The NP inclusions are morphologically highly similar to the inclusions formed during EBOV infection of target cells. VP35 and L are the components of the viral nucleocapsid protein that encapsidates the viral genome. The nucleocapsid complex. N (or NP) represents the major viral mRNAs. These proteins also constitute the respective viral nucleocapsid complex. N (or NP) represents the major nucleocapsid protein that encapsidates the viral genome. The encapsidated genome serves as a template for the viral polymerase complex, which is constituted by the catalytic subunit L and the cofactor P (9).

A recently established minigenome-based reverse genetic system revealed that EBOV follows another strategy to synthesize the different RNA species. NP, VP35 (the P analogue), and L were sufficient for viral replication, similar to the other Mononegavirales. The fourth nucleocapsid protein VP30, although not influencing replication, dramatically activated the synthesis of the viral mRNAs (8). The mechanism, i.e. how the phosphoprotein VP30 activates transcription, is still unclear.

The phosphorylation state of a cellular or viral protein is determined by the coordinated action of kinases and phosphatases. Although the significance of several cellular kinases for the phosphorylation state of viral proteins of the order Mononegavirales is well established, only a few proteins are described whose function is influenced by phosphorylation (10, 11). Even less is known about the impact of cellular phosphatases on the viral replication cycle. However, for papovaviruses and adenoviruses as well as for the human immunodeficiency virus types 1 and 2, the activity of the ubiquitous phosphatase PP2A has been shown to be of functional significance (12, 13).

In this report, we have determined the phosphorylation sites of VP30 and found that phosphorylation of two N-terminal serine clusters positively regulated binding of VP30 to NP-induced inclusions and negatively regulated the transcription activation function of the protein. We further show that VP30 is a target for cellular protein phosphatases PP1 and PP2A. In a reconstituted minigenome system, EBOV-specific transcription was blocked by okadaic acid (OA) that is known to inhibit PP1 and PP2A. The effect of OA on the transcription could be attributed to an extensive phosphorylation of VP30. Moreover, the treatment of EBOV-infected cells with OA inhibited EBOV growth, which could be restored by the expression of a non-phosphorylatable VP30 in trans. Taken together, our results show for the first time that VP30 phosphorylation is a regulatory factor in the replication cycle of EBOV that might be a suitable target for the development of antiviral drugs.

**EXPERIMENTAL PROCEDURES**

**Viruses and Cell Lines**

EBOV-Zaire strain Mayinga was grown and passaged as described elsewhere (14). MVA-T7 was grown and titered in chicken embryo fibroblasts (15). HeLa cells were cultured as described by Mühler et al. (8). Monolayer cultures of HeLa cells were used for all experiments with the recombinant vaccinia virus MVA-T7. BSR T7/5 cells (a BHK-21 cell clone), which constitutively expressed T7 RNA polymerase, were cultured as described by Buchholz et al. (16). For transfection experiments, cells were grown in six-well plates (7 cm\(^2\)).

**Molecular Cloning of VP30 and VP30 Mutants**

For expression of VP30 and VP30 mutants the respective genes were cloned into the plasmid pTM1 under the control of the T7 RNA polym-
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VP30 was expressed in HeLa cells using the vaccinia virus-T7 system, metabolically labeled with [32P]orthophosphate or 32S-Promix, immunoprecipitated, Western blot analysis, immunofluorescence analysis, and formic acid treatment were carried out as described by Modrof et al. (19).

RESULTS

Phosphorylation State Analysis of VP30 Substitution Mutants—Proteolytic digestion and phosphoamino acid analyses of [32P]P-labeled recombinant VP30 revealed that the phosphate acceptor sites are represented by serine and threonine residues within the 60 N-terminal amino acids (data not shown). Analysis of the amino acid sequence in this particular region revealed two serine clusters (amino acids 29–31 and 42–46) each containing three serine residues that seemed to represent suitable targets for cellular protein kinases (Fig. 1).

FIG. 1. Phosphorylation state analysis of VP30 and VP30 mutants. Top panel, amino acid sequence of position 26–55 of VP30. Serine and threonine residues, which were mutated, are printed in boldface letters. Middle panel, 5 × 10^5 HeLa cells were infected with MVA-T7 and subsequently transfected with 1 μg of DNA plasmids encoding either wild-type VP30 or mutants of VP30. Proteins were metabolically labeled with [32P]P and immunoprecipitated from the lysate using a monoclonal anti-FLAG antibody (dilution 1:500). Immune complexes were separated on SDS-PAGE, blotted onto polyvinylidene difluoride membranes and exposed to an Imaging Plate. Radioactive signals were quantified using the Raytest TINA software and normalized to the expression level of the respective mutant, which has been checked by Western blotting. As primary antibody a monoclonal anti-FLAG antibody (dilution 1:3,000) and as secondary antibody an POD-coupled sheep anti-mouse antibody (dilution 1:20,000) was used. Lower panel, quantification of the phosphorylation state analysis. Each mutant was tested in three independent experiments.

Other Methods

Infection and transfection of HeLa cells, metabollic labeling with [32P]Porthophosphate or [35S]-Promix, immunoprecipitation, Western blot analysis, immunofluorescence analysis, and formic acid treatment were carried out as described by Modrof et al. (19).

VP30 Dephosphorylation by Protein Phosphatases

VP30 was expressed in HeLa cells using the vaccinia virus-T7 system, metabolically labeled with [32P]P, and immunoprecipitated using an anti-FLAG monoclonal antibody M2 (Sigma, Deisenhofen, Germany) (19). Immune complexes were washed five times in the precipitation buffer without SDS, two times in saline and finally resuspended in five volumes buffer E. This suspension was used for incubation with the respective phosphatases. 300 ng of PPI, PP2A, PP2C, or alkaline phosphatase (AP) were mixed with 1.5 μl of 10% concentrated phosphatase buffer (PPI/PP2A: 500 mM Tris/HCl, pH 7.5, 10% glycerol, 1% mercaptoethanol, 50 μM MnCl2; PP2C: 250 mM Tris/HCl, pH 7.5, 10% glycerol, 1% mercaptoethanol, 100 μM MgCl2; alkaline phosphatase: 500 mM Tris/HCl, pH 7.5, 50 mM MgCl2), and 10 μl of VP30 suspension. Finally, the total volume was filled up with dH2O to 15 μl, and the samples were incubated for 30 min at 30 °C.
between amino acids 29 and 46 were substituted (VP30_{6A}). 16% of wild-type VP30 phosphorylation was detected (lane 5). The additional exchange of threonine 52 (VP30_{29–46T52A}) further decreased the phosphorylation signal to 6% in comparison with the phosphorylation of wild-type VP30 (lane 6). Exchange of single serine residues did not impair phosphorylation significantly (data not shown). To confirm that phosphorylation occurred mainly within the N terminus, the phosphorylation state of a VP30 deletion mutant was investigated that lacked the 68 N-terminal amino acids. This mutant was phosphorylated to the same extent as the substitution mutant VP30_{529–6T52A} (lanes 2 and 6), indicating that the major phosphate acceptor sites in VP30 have been identified. Moreover, these results point to the fact that most if not all of the serine residues in the amino acid region 29–46 and threonine 52 are targets for cellular kinases.

**Influence of the Phosphorylation of VP30 on the Association with NP Inclusions**—VP30 is a nucleocapsid-associated protein, which could be detected in EBOV-infected cells in intracytoplasmic inclusions together with the major nucleocapsid protein NP (7) (Fig. 2A). Ultrastructural analyses revealed that the inclusions contained viral nucleocapsids (20). Single expression of NP resulted in the formation of cytoplasmic inclusions similar to the inclusions in EBOV-infected cells (Fig. 2B).

To determine whether VP30 was able to associate with the NP inclusions, VP30 was coexpressed together with NP in HeLa cells, and cells were subjected to immunofluorescence analysis at 12 h post transfection. While solitarily expressed VP30 was homogeneously distributed (Fig. 2B, middle panel) coexpression with NP resulted in a redistribution of the protein into the NP-induced inclusion bodies (Fig. 2C). This result suggested that the connection of VP30 to the inclusions is directly or indirectly mediated by NP.

It was now of interest whether phosphorylation of VP30 has impact on the interaction of VP30 with the NP-induced inclusions. Immunofluorescence analyses of cells coexpressing VP30 mutants and NP revealed that VP30_{6A} was impaired in its ability to associate with the NP-induced inclusions. Although NP was still found in clusters, VP30_{6A} was homogeneously distributed (Fig. 2D). Substitution of either serines 29–31 or serines 42–46 to alanines changed the intracellular distribution of VP30 only slightly (Fig. 2, E and F). It was therefore concluded that phosphorylation of at least one of the serine clusters was essential for the interaction between NP inclusions and VP30.

To mimic the negative charges of the phosphate groups at serines 29–46, we replaced the six amino acids by aspartate residues (21, 22) and coexpressed the mutant (VP30_{6A}) with NP. Immunofluorescence analysis showed that VP30_{6D} was able to interact with the inclusions as the wild-type VP30 (Fig. 2G). This result indicated that the phosphorylation-induced negative charges at the serine residues rather than the serine residues itself were crucial for the interaction of VP30 with the NP-induced inclusions.

**Influence of Phosphorylation of VP30 on Transcription Activation**—To address the question of whether the phosphorylation of VP30 influenced the transcription activation function of VP30, the wild-type VP30 was replaced by phosphorylation-deficient VP30 mutants in an EBOV-specific transcription system (8). BSR T7/5 cells were transfected with plasmids encoding an artificial EBOV minigenome that contained the CAT reporter gene, and the EBOV nucleocapsid proteins NP, VP35, L, and VP30 or the VP30 substitution mutants, respectively (8).

As expected, EBOV-specific transcription was dramatically enhanced in the presence of VP30 (Fig. 3, lanes 1 and 2). Interestingly, the phosphorylation-deficient VP30 mutants were still able to support transcription, irrespectively, whether single or both serine clusters were exchanged for alanines (lanes 3–5). When one of the serine clusters was substituted with aspartates, VP30 was functional as well (lanes 6 and 7). However, substitution of all six phosphorylated serine residues with aspartates resulted in an inactive VP30 (lane 8). These results suggested that phosphorylation of VP30 plays a key role in regulating the activity of the EBOV transcription in a way that the critical serine residues are partly nonphosphorylated.

**Identification and Inhibition of VP30-dephosphorylating Phosphatases**—To confirm that phosphorylation is critical for VP30 function, it was checked whether VP30-specific phos-
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Fig. 3. Transcription activation by EBOV VP30 and VP30 substitution mutants. Approximately 5 \times 10^5 BSR T7/5 cells were transfected with DNA plasmids encoding EBOV nucleocapsid proteins NP, VP24, L, and VP30 or VP30 mutants, respectively, together with a DNA plasmid encoding the EBOV-specific artificial minigenome 3E-5E, which contained the leader and trailer regions of the EBOV genome flanking the CAT reporter gene. At 2 days post transfection, cells were lysed and CAT activity was determined. Lane 1, control without VP30; lane 2, wild-type VP30; lanes 3–8, VP30 substitution mutants (the respective mutant is given at the top of the panel).

Phosphatases could be identified. Inhibition of such phosphatases would lead to an extensively phosphorylated VP30, which, like VP30a, is not able to mediate transcription.

VP30 was expressed using the vaccinia virus-T7 system, metabolically labeled with 32P, and purified by immunoprecipitation. The precipitate was then incubated with the catalytic subunits of protein phosphatases PP1, PP2A, PP2C, and alkaline phosphatase, and the phosphorylation of VP30 was checked by SDS-PAGE and autoradiography. It is shown in Fig. 4A that PP1 and PP2A almost completely dephosphorylated VP30 (Fig. 4, lanes 4 and 5). PP2C dephosphorylated VP30 as well, but to a lesser extent (Fig. 4, lane 3). Alkaline phosphatase did not recognize VP30 as a substrate (Fig. 4, lane 1). This result was confirmed by the finding that the VP30-dephosphorylating activity of HeLa cell lysates could be inhibited by OA, which is known to inhibit PP1 and PP2A (Refs. 23 and 24; data not shown). We concluded that VP30 was dephosphorylated in vitro and in vivo by OA-sensitive phosphatases, most likely PP1 and/or PP2A.

Using the above-described minigenome-based transcription/repllication system (8), the influence of OA on EBOV-specific transcription was investigated. To control possible side effects of OA on the cellular protein synthesis (25), we used VP30aΔα, whose transcription activation function is independent of phosphorylation (see above). An inhibitory effect of OA on transcription in the presence of VP30 wild-type and no effect in the presence of VP30aΔα would indicate that inhibition of PP1 and/or PP2A specifically interferes with EBOV-specific transcription by inducing a highly phosphorylated VP30. A transcription assay was set up in BSR T7/5 cells as described above using either VP30 wild-type or VP30aΔα. OA was added in different concentrations after transfection. Transcription activity was determined, and the values gained for VP30aΔα at the respective concentrations of OA were set to 100%. Fig. 4B shows that increasing amounts of OA concomitantly inhibited the ability of VP30 wild-type to activate transcription in a saturable manner reaching a plateau at ~200 nM OA. The IC50 was determined to be 130 nM OA. These results underlined that transcription activation function of VP30 is inhibited by extensive phosphorylation of the N-terminal phosphate acceptor sites.

Block of Ebola Virus Growth by Okadaic Acid Is Released by the Nonphosphorylatable VP30aΔα—Because OA strongly inhibited EBOV-specific transcription in the artificial minigenome system, it was investigated whether EBOV reproduction in target cells could be inhibited by OA, as well. BSR T7/5 cells were infected with EBOV and treated with different concentrations of OA. We found that the number of infected cells at 24 h p.i. was significantly decreased concomitantly with increasing amounts of the inhibitor (Fig. 5, gray columns). To confirm that this effect was due to a specific inactivation of VP30 by hyperphosphorylation, VP30aΔα was provided by plasmid-based expression in the EBOV-infected cells in the presence of OA. Under these conditions, OA had only minute effects on EBOV infection (Fig. 5, black columns). This result indicated that the OA-induced inhibition of EBOV growth reflects, indeed, the

Fig. 4. Identification and inhibition of VP30 dephosphorylating protein phosphatases. A, VP30 is recognized by PP1, PP2A, and PP2C. VP30 was expressed in HeLa cells, labeled with 32P, and immunoprecipitated as described under Fig. 1. Immune complexes were incubated for 30 min at 30 °C with the catalytic subunits of PP1, PP2A, PP2C, and alkaline phosphatase (AP). The immune complexes were then separated by SDS-PAGE, and the gel was exposed to an Imaging Plate. Radioactive signals were quantified using the Raytest TINA software. B, OA inhibits VP30-mediated EBOV-specific transcription. The transcription assay was performed as described under Fig. 3 with wild-type VP30 or VP30aΔα. OA was added in increasing concentrations at 8 h post transfection. Cells were lysed at 24–36 h post transfection, and CAT activity was determined. The inhibition of transcription activation mediated by wild-type VP30 is given in a percentage of the transcription activation by VP30aΔα. Inset, CAT assay representing selected points of the curve: Absence of OA (lanes 1 and 2), presence of 300 nM OA (lanes 3 and 4). Lanes 1 and 3, wild-type VP30; lanes 2 and 4, VP30aΔα.
phosphorylation-dependent down-regulation of VP30-mediated viral transcription.

**DISCUSSION**

We have mapped the phosphorylation sites of VP30 to two N-terminal serine clusters and threonine 52. This pattern of phosphorylation is similar to that of Marburg virus VP30 (19).

When functional significance of VP30 phosphorylation was investigated it was found that phosphorylated VP30 was located inside NP inclusions; nonphosphorylated VP30, however, was evenly distributed throughout the cytoplasm. It is reasonable to presume that the NP inclusions, like their Marburg virus counterparts, consist of NP-induced helical structures representing the core structures of the nucleocapsid (26). The colocalization of NP and VP30 inside the inclusions is either mediated by a direct interaction between the NP helices and VP30 or by another component, e.g. RNA, as it has been shown recently for M2-1 and N of human respiratory syncytial virus (27).

In contrast to the positive effect on the interaction with NP inclusions, phosphorylation of VP30 negatively regulated its transcription activation function. Although nonphosphorylated VP30 activated transcription as the wild-type, a completely phosphorylated VP30 was inactive. These results were supported by the negative effect of the phosphatase inhibitor OA on viral transcription. OA inhibits PP1 and PP2A (23, 28) so that both are not able to dephosphorylate VP30. It is presumed that inhibition of the phosphatases leads to an increase of VP30 phosphorylation and consequently to an inactive protein as in the case of the pseudophosphorylated VP30(6D). This presumption is strongly supported by the result that the effect of OA on EBOV-specific transcription was overcome when VP30 was replaced by the nonphosphorylatable mutant VP30(6A).

Taken together, phosphorylation inversely influenced the two known functions of VP30. A completely phosphorylated form of VP30 was capable of interacting with NP inclusions, but was restricted in mediating viral transcription. In contrast, a phosphorylation-deficient VP30 was inhibited in its interaction with the NP inclusions but supported EBOV-specific transcription. An intermediately phosphorylated VP30 enabled both viral transcription and assembly. Phosphorylation of VP30 is therefore presumed to represent a molecular switch for the different functions of the protein. It is hypothesized that, depending on the respective demands, VP30 enables either viral transcription or assembly or both. Possibly, the non- or weakly phosphorylated VP30 supports transcription until it is removed from the general pool by the phosphorylation-induced binding to the nucleocapsid.

The only other viruses in the order Mononegavirales containing a structural protein of similar characteristics as filoviral VP30 belong to the pneumovirinae subfamily of Paramyxoviridae. Here, M2-1 has been shown to be phosphorylated in its N terminus and to interact with the nucleoprotein of human respiratory syncytial virus in intracellular inclusions (29–31). M2-1 is essential for respiratory syncytial virus transcription elongation and antitermination (32–34). The phosphorylation of M2-1 was shown to influence the RNA binding specificity (29) and to be essential for the transcription antitermination activity (27). Thus, phosphorylation of M2-1 is of functional significance, but the detected functions differ from that of VP30 phosphorylation.

Interestingly, our experiments revealed that OA was able to specifically inhibit the multiplication of EBOV in target cells. Because virus growth was only slightly affected by OA when VP30(6A) was expressed in trans, it is concluded that inhibition was, indeed, caused by an inactive, hence hyperphosphorylated, VP30.

It is reasonable to consider whether antiviral drugs could be developed, based on the detected phosphorylation dependence of VP30-mediated EBOV transcription. The severe side effects of OA, i.e. promotion of tumor growth and genetic instability (35, 36), prohibit its employment as a drug to block EBOV infection. For future antivirals it is necessary to separate the cytopathic and the VP30-directed effects of phosphatase inhibition. Because the specificities of most known phosphatases are determined by their subunit composition, i.e. regulatory and scaffolding units (37–39), it can be expected that the identification and subsequent inhibition of the VP30-specific regulatory subunit(s) of PP1 and PP2A could lead to promising tools for antagonizing EBOV infection.

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