Oligomerization of Ebola virus VP30 is essential for viral transcription and can be inhibited by a synthetic peptide

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Running title:

Oligomerization of Ebola virus VP30

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Summary

Transcription of Ebola virus (EBOV)-specific mRNA is driven by the nucleocapsid proteins NP, VP35, and L. This process is further dependent on VP30, an essential EBOV-specific transcription factor. The present study addresses the self-assembly of VP30 and the functional significance of this process for viral transcription and propagation. Essential for oligomerization of VP30 is a region spanning amino acids 94-112. Within this region a cluster of four leucine residues is of critical importance. Mutation of only one of these leucine residues resulted in oligomerization-deficient VP30 molecules that were no longer able to support EBOV-specific transcription.

The essential role of homooligomerization for the function of VP30 was further corroborated by the finding that mixed VP30 oligomers consisting of VP30 and transcriptionally inactive VP30 mutants were impaired in their ability to support EBOV transcription. The dominant-negative effect of these VP30 mutants was dependent on their ability to bind to VP30. The oligomerization of VP30 could be dose-dependently inhibited by a 25mer peptide (E30pep-wt) derived from the presumed oligomerization domain (IC50: 1 µM). A control peptide (E30pep3LA), in which three leucines were changed to alanine, had no inhibitory effect. Thus, E30pep-wt seemed to bind efficiently to VP30 and consequently blocked the oligomerization of the protein. When E30pep-wt was transfected into EBOV-infected cells, the peptide inhibited viral replication suggesting that inhibition of VP30 oligomerization represents a target for EBOV antivirals.
Introduction

Ebola virus (EBOV), a filovirus, causes sporadic outbreaks of a fatal hemorrhagic fever in Africa (1-4). Since the natural host of the virus is not yet identified, the outbreaks are unpredictable and so are the risk factors for the population in the endemic areas in Central Africa. To date, neither a vaccine nor a treatment of the EBOV infection are available.

The enveloped 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 (5,6). 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 polymerase. VP30 represents an EBOV-specific transcription activation factor (7).

Most viruses of the order Mononegavirales contain three proteins, N (NP), P, and L, that drive the processes of replication (synthesis of genomic RNA) and transcription (synthesis of 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 (8).
An EBOV-specific 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 (7,9). The exact mechanism of how VP30 activates transcription is still unclear. Recent investigations have suggested that VP30 most probably is effective at a very early stage of transcription. Transcription of the EBOV minigenomes could take place in the absence of VP30 if a RNA secondary structure that includes the transcription start site of the first gene (NP) is destroyed (10). Thus, one function of VP30 during transcription might be to overcome this barrier. Transcription activation by VP30 is strongly regulated by the phosphorylation state of VP30. While the non or weakly phosphorylated protein activates transcription, the fully phosphorylated VP30 is strongly inhibited in this function. In contrast, the binding of VP30 to nucleocapsid-like structures that are formed by the major nucleocapsid protein NP is inversely regulated. The phosphorylated VP30 binds to the nucleocapsid-like structures whereas the nonphosphorylated protein does not (11). VP30 contains a nonconventional Zinc finger whose integrity is a prerequisite for the function of the protein (12).

In the present study we have investigated whether VP30, like many other transcription factors, is a homooligomer and if so, whether the oligomerization is essential for its function. It was found that VP30 is, indeed, present as an oligomer.
Relevant for oligomerization is a stretch of approximately 20 amino acids flanking a central tetra leucine cluster whose integrity is essential for VP30 self association. While oligomerization is not needed for the interaction of VP30 with the EBOV nucleocapsid, oligomerization of VP30 is essential for the transactivating function and for the propagation of EBOV in cell culture.
Experimental procedures

Viruses and Cell Lines

EBOV-Zaire strain Mayinga was grown and passaged under biosafety level 4 conditions as described elsewhere (13). The recombinant vaccinia virus MVA-T7 was grown and titred in chicken embryo fibroblasts (14). Monolayer cultures of HeLa cells were used for all experiments with the recombinant vaccinia virus MVA-T7 and cells were cultured as described by Mühlberger et al. (7). BSR T7/5 cells (a BHK-21 cell clone) which constitutively expressed T7 RNA polymerase, were cultured as described by Buchholz et al. (15). For transfection experiments, cells were grown in six-well plates (7cm²). HUH7 cells were grown in Dulbecco’s modified Eagles medium supplemented with 10% fetal calf serum.

Molecular cloning of VP30 and VP30 mutants

For expression of VP30 and the VP30 mutants, the respective genes were cloned into the plasmid pTM1 under the control of the T7 RNA polymerase promoter (16). The cloning of pT-VP30f, pT-VP30 and pT-VP30ΔN2-68 has been previously described (11).

Cloning of the mutants pT-VP30fΔC114-288 and pT-VP30ΔC114-288 The coding region for amino acids 1-113 of the VP30 gene was amplified by PCR using pT-VP30 as template. For synthesis of pT-VP30fΔC the primers #410 (5’-ACC GGA
TCC ATG GAC TAC AAG GAC GAC GAT GAC AAG GAA GCT TCA TAT GAG AGA GGA CG-3’) and #711 (5’-AGA CTC GAG TTA TTC TAC TGA TCC ACA AGT CTT ACG G-3’) were used. #410, the forward primer, contained a BamHI restriction site (underlined) and the sequence encoding the Flag-epitope (abbreviated f, in italics).

The reverse primer #711 contained a XhoI restriction site (underlined). To construct pT-VP30ΔC, the forward primer #408 (5’-ACC GGA TCC ATG GAA GCT TCA TAT GAG AGA-3’) was used in combination with #711. The resulting DNA fragments were cloned into the plasmid pTM1.

Cloning of the mutants pT-VP30Δ72–112 and pT-VP30fΔ72–93 For the internal deletions of the mutants pT-VP30fΔ72–112 and pT-VP30Δ72–93 a second PshAI restriction site was inserted into the vector pT-VP30f at position 8721 (EBOV genome) in case of pT-VP30fΔ72–112 or at position 8786 for the construct pT-VP30Δ72–93 using the QuickChange site-directed mutagenesis kit (Stratagene). The resulting vector was digested with PshAI, the DNA fragments encoding either aa 72–112 or 72–93 were removed and the remaining fragments were religated.

Cloning of VP30 substitution mutants – Cloning of the VP30 substitution mutants was performed using the QuickChange site-directed mutagenesis kit (Stratagene), with either pT-VP30f, pT-VP30, pT-VP30fΔN or pT-VP30fΔC as template.

All mutants were verified by sequencing.
Ebola Virus transcription analysis

BSR T7/5 cells (5 x 10^5 in a 7 cm² well) were transfected with plasmids encoding the EBOV nucleocapsid proteins NP, VP35, VP30 (or/and VP30-mutants), L, and an EBOV-specific minigenome containing the CAT reporter gene (7), using FuGENE 6 (Roche) according to the suppliers prescription. Cells were incubated at 37°C for 8-12 h and then washed two times with DMEM and further incubated in DMEM containing 10% FCS for 12 – 36 h at 37°C. Subsequently, cells were harvested and CAT activity, as a readout for transcription activity, was determined.

CAT-Assay

CAT activity was determined using a standard protocol (17). Quantification of the radioactivity was done with a Fuji BAS-1000 Bio-Imaging analyzer (Fujifilm) by using the TINA software (Raytest).

Peptide synthesis

The peptides were synthesized by Dr. M. Krause at the Institute of Molecular Biology and Tumor Research (Philipps University of Marburg).

The peptides $E30_{pep-wt}$ (QLESRTDRELILLIARKTCGSVE-CONH₂) and $E30_{pep-3LA}$ (QLESRTDREAAAALIARKTCGSVE-CONH₂) were derived from the Ebola virus VP30 amino acid sequence 91-113, in $E30_{pep-3LA}$ the leucines at position 100-
102 were replaced by alanines.

**In vitro translation with peptide inhibition**

pT-VP30 and pT-VP30f were in vitro translated simultaneously and metabolically labeled with \[^{35}S\]Promix using the TNT T7 Quick Coupled Reticulocyte Lysate System (Promega) according to the supplier's prescription. Before starting the in vitro translation, peptides were added in different concentrations which are given in Fig. 6. Afterwards, 5 µl of each in vitro translate was diluted into 500 µl CoIP-buffer (20 mM Tris/HCl, 100 mM NaCl, 5mM EDTA, 1% NP40, 0.4% desoxycholate, 1mM DTT, 1mM PMSF). Coimmunoprecipitation with the monoclonal α-Flag antibody M2 (Sigma-Aldrich) was performed as described elsewhere (18).

**Other methods**

Infection and transfection of HeLa cells, metabolic labeling with \[^{35}S\]Promix, and Western blot analysis were carried out as described by Modrof et al. (18).
Results

VP30 forms oligomers

To investigate oligomerization of VP30, we took advantage of the fact that the fusion of a FLAG-epitope to the C-terminus of VP30 (VP30f) resulted in a SDS-PAGE migration velocity of the fusion protein that is distinguishable from VP30 (Fig.1A, lane 3). Upon single expression of VP30f and VP30 and subsequent immunoprecipitation, the used α-flag monoclonal antibody (MAB) recognized the epitope-tagged protein but not wild-type VP30 (Fig.1, lanes 1 and 2). However, upon coexpression, the α-flag antibody, sedimented both proteins indicating that VP30 interacted with VP30f and the mixed oligomers were precipitated (Fig.1, lane 3). An interaction between VP30f and VP30 was only detected when both proteins were coexpressed. Mixing of preformed VP30 and VP30f did not lead to significant amounts of VP30/VP30f complexes (data not shown). These results suggest that (i) VP30 oligomerizes cotranslationally or rapidly after synthesis and (ii) the formed oligomers are stable and do not dissociate easily (19).

Localization of the oligomerization site

To map the oligomerization site of VP30, N- and C-terminal deletion mutants as well as internal deletion mutants of VP30 were constructed (Fig. 2 A) and employed in a coimmunoprecipitation assay. VP30 and VP30 mutants were expressed in HeLa cells, and cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. Mutants lacking the N-terminal 68 (VP30ΔN) and the C-terminal 174 amino acids...
(VP30ΔC) were still able to interact with VP30 (Fig. 2 B, lanes 1 and 2). While an internal deletion of amino acid 72-93 left VP30 in an oligomerization competent state (lane 3, VP30Δ72-93), deletion of amino acids 72-112 abolished oligomerization completely (lane 4, VP30Δ72-112). Thus, amino acids 94112 are essential for oligomerization of VP30.

Oligomerization via a tetra-leucine motif

In silico analyses of the amino acid sequence of the determined region (20) predicted an α-helical structure between amino acid residues 97-106. Within this region, a cluster of four leucines was most remarkable (Fig. 3A). To investigate the significance of this cluster for the oligomerization of VP30, single leucine residues were substituted by alanines. Each mutant was also constructed as a FLAG-tagged twin. Pairs of tagged and untagged VP30 mutants were coexpressed in Hela cells, radioactively labeled and immunoprecipitated with an α-flag antibody (Fig. 3B). Substitutions in the tetra-leucine motif impaired oligomerization of VP30 drastically even if only one of the leucine residues was changed to alanine (Fig. 3B, lanes 4-7). Immuno precipitation of the cell lysates with an α-VP30 serum confirmed the presence of the non-interacting VP30 mutants (data not shown).

Oligomerization does not influence the interaction between VP30 and the NP-induced inclusions

It was then analyzed whether the oligomerization of VP30 is essential for the protein’s ability to interact with the nucleocapsid of EBOV. To this end, we took
advantage of the fact that upon coexpression with the major nucleocapsid protein NP the homogenously distributed VP30 relocalizes into NP-induced inclusions (Fig. 4 A, (11). When the oligomerization-deficient mutant VP30L100-102A was coexpressed with NP, both proteins were still detected in the NP-induced inclusions suggesting that the oligomerization of VP30 is not essential for its binding to NP inclusions (Fig. 4 B).

Oligomerization is essential for transcription activation by VP30

It was then of interest whether the oligomerization of VP30 influenced EBOV-specific transcription. This was investigated by using an EBOV-specific transcription system that is active only in the presence of VP30 (7). VP30 activates the transcription of an EBOV-specific minigenome that contains a CAT reporter gene. The resulting CAT activity is a readout for the EBOV-specific transcription (Fig 3 C, lane 1 and 2 (7). In this system, the wild-type VP30 was replaced by the VP30 L→A-substitution mutants (Fig. 3 A) and the effect of the replacement was determined by quantifying the CAT reporter gene activity. None of the substitution mutants was able to activate EBOV transcription (lanes 3-6), strongly suggesting that oligomerization of VP30 is required for EBOV transcription.

To confirm this result we investigated the following working hypothesis. If oligomerization of functional VP30 molecules is essential for transcription activation, mixed oligomers of wild-type VP30 and inactive VP30 mutants that still contain an intact oligomerization domain (VP30ΔN or VP30ΔC, Fig. 2 B, lanes 1-3) should be
impaired in their ability to activate transcription. In contrast, a combination of VP30 and inactive VP30 mutants, which should not bind to VP30 since the presumed oligomerization site contained leucine to alanine mutations (e.g. VP30ΔN L100A, VP30ΔC L100A), should leave the transcription activation function of wild-type VP30 unimpaired.

Firstly, activity of VP30 deletion mutants was tested with the result, that VP30ΔN and VP30ΔC turned out to be inactive (Fig. 5A panel I, lane 3; 5B panel I, lane 2). Then, a transcription assay was set up coexpressing VP30 and either VP30ΔN or VP30ΔC. CAT reporter gene activity was determined at 36 h p.t. The transcription activity that was detected using wild-type VP30 alone was set to 100% (Figs. 5 A and B, II). The addition of VP30ΔN or VP30ΔC led to a significant reduction of transcription (Fig. 5 A, lane 4, Fig. 5 B, lane 3). In contrast, the addition of VP30ΔNL100-102A or VP30ΔCL100-102A that both are inactive but cannot bind to VP30 did not modify significantly the activity of the transcription system (Fig. 5 A, lane 6 and Fig. 5 B, lane 5). The protein expression of VP30 and the VP30 mutants had been controlled by Western blotting (Fig. 5 A, B panels III). So far the results have been in accord with the above described hypothesis. Surprisingly, the mutants which had changed only one leucine to alanine (VP30ΔNL100A, VP30ΔCL100A) turned out to be dominant negative like VP30ΔN and VP30ΔC (Fig. 5 A, lane 5; Fig. 5 B, lane 4). Therefore we presumed that the mutants containing only a single leucine → alanine substitution were still able to bind to VP30 and thus induce the negative effect.
To test this presumption, several FLAG-tagged VP30ΔN mutants were coexpressed with VP30wt or VP30L100A and precipitated with an α-flag antibody (Fig. 5 C, lanes 1-4). The experiment revealed that, indeed, VP30ΔN_L100A was able to oligomerize with VP30 although binding was reduced (lane 2). The mutant VP30ΔN_L100-102A showed no interaction with VP30 (lane 3). Interestingly, no cosedimentation was observed if both, the full length VP30 protein and the ΔN mutant contained a mutation of one leucine residue to alanine (lane 4). The latter result confirmed the results of Fig. 3b. Taken together, the oligomerization of wild-type VP30 with an inactive VP30 mutant inhibited the transactivating function of VP30. Thus, it can be concluded that (i) the oligomerization of intact VP30 monomers is the prerequisite for the function of VP30 as a transcriptional activator. (ii) Inactive mutants of VP30 that are able to oligomerize display a dominant negative phenotype.

**Inhibition of VP30 oligomerization using synthetic peptides**

To further investigate the oligomerization of VP30, we employed peptides that were derived from the presumed oligomerization domain to check whether these could inhibit the self-assembly of VP30. One of the used peptides was identical to amino acids 91-113 (Fig. 6 A, E30pep-wt), the other contained three leucine to alanine substitutions (E30pep-3LA).

VP30 and Flag-tagged VP30 were in vitro translated in the presence of increasing concentrations of the peptides E30pep-wt or E30pep-3LA (0-10 µM). Then, the
FLAG-tagged VP30 was precipitated with an α-flag antibody, the immunoprecipitate was separated by SDS-PAGE, and the amount of precipitated VP30FLAG and cosedimented VP30 was quantified (Fig. 6 B and 6 C). The presence of E30pepw during the coexpression of VP30 and VP30f resulted in a dose-dependent inhibition of the oligomerization of VP30 (6 B and C, left panel). The IC(50) for the inhibition was approx. 1 µM. The presence of identical concentrations of the control peptide E30pep3LA had no effect on oligomerization (6 B and C, right panel). The peptides did not affect the expression of VP30 or VP30f (Fig 6 D).

To summarize these findings, peptides consisting of the amino acids 91-113 of VP30 were sufficient to compete with VP30 for its binding to VP30f. Essential for the ability of the peptide to bind VP30 was the presence of a leucine cluster at position 100–102. Thus, these 20 amino acids seemed to be sufficient to bind and block the oligomerization site of VP30.

Altogether our findings support the view that VP30 oligomerization is essential for the protein’s activity in viral transcription and that the oligomerization is mediated by amino acids 91-113 comprising a tetra leucine cluster whose presence is critical.

**Influence of VP30 oligomerization on EBOV propagation**

Finally, we checked whether VP30 oligomerization was also essential for the propagation of EBOV in cell culture. Thus, Vero cells were transfected with the peptide E30pepw, or E30pep3LA and subsequently infected with EBOV. At 24 h post infection, the number of infected cells was determined by the presence of the
typical NP-inclusions. We found that the presence of $E30_{\text{pep} \text{wt}}$, but not $E30_{\text{pep} \text{3LA}}$, inhibited the EBOV infection indicating that oligomerization of VP30 plays an essential role for the replication cycle of EBOV (Fig. 7). Experiments with EBOV-infected HUH7 cells essentially gave the same results (data not shown).
DISCUSSION

Because of the limited coding capacity of the EBOV genome the virus exploits extensively the available genomic information leading to the phenomenon that EBOV proteins often have several functions. This has been shown e.g. for the matrix protein VP40 that triggers on the one hand budding of progeny viruses and is on the other hand an important factor for viral morphology (21-23). Another example is VP35, the cofactor of the viral polymerase L. VP35 is essential for viral RNA synthesis and simultaneously shuts down the host cell’s interferon response (7,24). It is poorly understood, if and how the different functions of the two proteins are regulated. Some more information is available on the regulation of the two functions of VP30. It has been shown that the protein is not only an activator of viral transcription but is also involved in the formation of the viral nucleocapsid. The switch between the two functions of VP30 is presumed to be modulated by phosphorylation of six serine residues in the N-terminus of the molecule (11). In the present study, we focused on the function of another co- or posttranslational modification of VP30, the homooligomerization of the protein. We investigated whether the homooligomerization of VP30 is important for binding to the nucleocapsid, the transcription activation, or both. To this end, we firstly mapped the homooligomerization domain of EBOV VP30 and found that amino acids 94-112 are essential for binding. Of critical importance was a cluster of 4 leucine residues inside the detected region. Even the replacement of one of the leucines was sufficient to inhibit significantly the self assembly of VP30 molecules. In silico analyses based on
the algorithm of Rost & Sander (20) predicted an α-helical structure that encompasses amino acid residues 97-106. It is presumed that oligomerization of VP30 is dependent on the formation of the predicted alpha helix. The stoichiometry of the complex has yet to be determined. Examples of viral proteins whose oligomerization is also dependent on hydrophobic residues inside an α-helical structure are the 2B protein of coxsackievirus or the E4 Protein of human papillomavirus (25,26). However, within these proteins the hydrophobic residues essential for oligomerization are facing the same direction. The three leucines that are relevant for VP30 oligomerization are adjacent to each other and therefore face different directions within the α-helical structure. It is likely that the leucines are essential to support the formation of the α-helix that in turn is necessary for VP30 homooligomerization. The fact that the leucine cluster is highly conserved in VP30 of the closely related Marburg virus further pointed to an important biological role of this particular region. Further experiments showed that Marburg virus VP30 is also present as an oligomer (data not shown).

When the oligomerization-incompetent VP30 mutants were checked for their capability to interact with NP inclusions that represent accumulations of nucleocapsid-like structures, it was found that oligomerization was not necessary for binding to the NP inclusions. This result suggested that the nucleocapsid-binding domain of VP30 is not influenced by the oligomerization of the protein. A similar result has been observed for the protein of human parainfluenza virus type 3. Formation of the complex between P protein and the nucleocapsid protein N and
interaction with the N-RNA template were independent of P protein oligomerization (27).

In a reconstituted plasmid-based minigenome system, VP30 has been shown to activate efficiently viral transcription (7). When oligomerization-incompetent VP30 mutants were employed in the EBOV-specific replication/transcription system, it turned out that the transcription activation function of the protein was completely inhibited. This result suggested that the transactive form of VP30 is a complex of several VP30 molecules. This was underlined by the finding that inactive but oligomerization-competent VP30 mutants displayed a dominant-negative effect on VP30-mediated transcription. In contrast, inactive and oligomerization-deficient VP30 mutants did not impair the activity of wild-type VP30. It is known from several viral and cellular transcription factors that these are functional as homooligomers. Thus, the human T-lymphotropic virus type 1 Tax protein e.g., represents a transcriptional activator of viral and cellular genes and functions optimally as a homodimer (28). The active conformation of the tumour suppressor gene p53 is tetrameric, and the oligomerization is permitted by a tetramerization domain whose integrity is essential for DNA-binding (29). Moreover, many other DNA- or RNA-binding proteins like the Rev protein and the integrase of HIV-I or the Baculovirus IE1 are active as oligomers (30-32). Although it is not clear yet whether VP30 binds RNA directly, VP30 is essential for a very early step of transcription which is modulated by a RNA secondary structure that was found to be present in the EBOV genome and in the transcript of the first gene (NP). Possibly, VP30 functions as a
helicase that helps the polymerase complex to overcome the secondary structure. This can be concluded from the finding that after deletion of the secondary structure, EBOV transcription becomes independent of VP30 (10). Analysis of the VP30 amino acid sequence revealed a QxxR motif at aa 228-231 that is specific for the DEA(D/H)-box of RNA helicases (33). The arginine in this motif has been postulated to interact with RNA (34). Whether VP30 indeed acts as a helicase remains to be shown.

VP30-oligomerization could be inhibited by a synthetic peptide (E30pepwt), consisting of 25 amino acids that were homologous to the presumed oligomerization domain of VP30 (containing the leucine cluster). In contrast, oligomerization was not inhibited by a peptide (E30pep3LA) in which three of the four leucines were replaced by alanines. Inhibition of protein-oligomerization with synthetic peptides is a new but widely-used method. For example, Maroun et al. used peptides to inhibit the dimerization of the HIV-I-Integrase (35), and Shangary and Johnson showed inhibition of the heterodimerization of BAX/Bcl-2 and BAX/Bcl-xL by synthetic peptides (36). In both cases, the sequence of the used peptides was derived from proposed interaction domains of the respective protein targets. While the IC50 for inhibition of the heterodimerization of BAX/Bcl-2 was 15 µM, the IC50 tested by integration of HIV into the genome was 80 nM. The detected IC50 of approx 1 µM for the inhibition of VP30 oligomerization by VP30pepwt reflects that the peptide efficiently disturbed the VP30 self-interaction.
To address the role of VP30 oligomerization in the context of the viral life cycle, EBOV-infected cells were transfected with E30\textsubscript{pep}wt. The resulting inhibition of EBOV growth was specific since the control peptide E30\textsubscript{pep}3LA did not show a substantial inhibitory effect. These data provide evidence that oligomerization of VP30 is essential for EBOV propagation. Similar results concerning the function of homooligomerization have been reported for the ICP0-protein of Herpes simplex virus type I, a viral transactivator (37). Upon coexpression of active and inactive forms of ICP0, a mixed oligomer is formed resulting in an inactive ICP0. The concentration of E30\textsubscript{pep}wt that was necessary to inhibit the EBOV infection was considerably higher than the concentration used in the in vitro system. This is on one hand due to the necessity to transflect the peptide into the cell. On the other hand, E30\textsubscript{pep}wt had to compete with the already oligomerized VP30 that is introduced into the cell by the input virus.

In summary, the presented data showed that VP30 oligomerization is dependent on a cluster of hydrophobic amino acids with four central leucine residues. Mutation of the leucine residues inactivated the VP30-dependent EBOV transcription. Moreover, EBOV propagation was impaired upon inhibition of VP30 oligomerization by synthetic peptides that were identical to the presumed oligomerization domain.
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Legends to the Figures

**Fig. 1: Oligomerization of EBOV VP30** 5 x 10^5 HeLa-cells were infected with MVA-T7 and subsequently cotransfected with plasmids encoding VP30 and VP30 Flag (see table at the top). At 12 h after infection, cells were metabolically labeled with [35S]Promix. The proteins were immunoprecipitated with an α-flag monoclonal antibody (dilution 1:500) and protein A-sepharose, separated by SDS-PAGE, visualized by Bio-Imager analysis and quantified with the software package TINA2.0.

**Fig. 2: Mapping the oligomerization domain using VP30 deletion mutants.** (A) Schematic representation of the employed VP30 mutants. The numbers in subscript refer to the amino acids of VP30 deleted in the respective mutant. (B) Coimmunoprecipitation of VP30 and VP30 mutants. 5 x 10^5 HeLa-cells were infected with MVA-T7, cotransfected with plasmids encoding VP30 and the respective mutants with or without FLAG-tag. Cells were metabolically labeled, and immunoprecipitated as described under Fig. 1. Immunocomplexes were separated on SDS-PAGE and exposed to a Bioimager plate. The position of the FLAG-tagged VP30, the VP30 mutants and the cosedimented non-tagged proteins are shown by arrows. Asterisk: unidentified protein.
Fig. 3: Substitution analysis of the leucine-cluster in EBOV-VP30. (A) Schematic representation of the VP30 substitution mutants. The amino acids 94-112 of VP30 are displayed. The VP30 mutants have alanine substitutions (bold A) in the leucine-cluster (bold L) at aa 100-102. (B) HeLa cells were infected with MVA-T7 and transfected with plasmids encoding Flag-tagged VP30 or Flag-tagged VP30 mutants together with plasmids encoding untagged VP30 or VP30 mutants as indicated. Cells were labeled with [35S]Promix, and proteins were immunoprecipitated using a FLAG-specific (lanes 1-7) antibody, separated by SDS PAGE and visualized as described under Fig. 1. (C) Transcription activation by VP30 and VP30 substitution mutants. Approximately 5 x 10^5 BSR T7/5 cells were transfected with plasmids encoding the EBOV nucleocapsid proteins NP, VP35, L, and VP30 or VP30 mutants, respectively. Additionally, a plasmid was transfected 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 using [14C]chloroamphenicol and acetyl-CoA as substrate. The acetylated chloroamphenicol was separated via thin layer chromatography. Since chloroamphenicol has two acetylation sites and the respective products have a different migration velocity in thin layer chromatography, two products can be distinguished beside the slow-migrating non-acetylated chloramphenicol when CAT activity is high. *Lane 1*, control without VP30; *lane 2*, wild-type VP30; *lanes 3-6*, VP30 substitution mutations (the respective mutant is
Fig. 4: Influence of the leucine residues 100-102 on colocalization of VP30 and NP inclusions. Hela cells were infected with MVA-T7 and cotransfected with plasmids encoding VP30 wild-type (A) or the VP30<sub>L100-102A</sub> (B) together with pT-NP, encoding the NP of EBOV. At 8 h p.i., cells were fixed with 3% PFA and permeabilized using 0.2% Triton X-100. Subsequently, cells were subjected to immunofluorescence analysis using a mouse monoclonal anti-NP antibody (dilution 1:20) and an α-VP30 IgM antibody (dilution 1:10). Bound antibodies were detected with a rhodamine-labeled goat anti-mouse serum (Dianova, dilution 1:100) and a FITC-labeled goat anti-mouse IgM antibody (Dianova, dilution 1:100).

Fig. 5: Analysis of EBOV transcription activation by mixed oligomers of VP30 and VP30 truncation mutants. (A) and (B) 5 x 10<sup>5</sup> BSR T7/5 cells were transfected with the same plasmids as described under Fig. 3c and additionally with plasmids encoding VP30ΔN (A) or VP30ΔC (B) as indicated above the panels. At 2 days post transfection, cells were lysed and CAT activity was determined using chloroamphenicol and [14C]-labeled acetyl-CoA as substrate. The acetylated chloroamphenicol was separated via thin layer chromatography. (I). (II) Quantification of CAT-activity was done using the Raytest TINA sofware. The transcription activation by wild-type VP30 was set to 100%. (III) The expression level of the
respective mutants was checked by Western blotting and CAT signals were normalized accordingly. As primary antibody a monoclonal anti-Flag antibody (dilution 1:3000) and as secondary antibody, an POD-coupled sheep anti-mouse antibody (dilution 1:20000) was used.

(C) Coprecipitation of VP30 and VP30ΔN mutants. 200 ng of plasmids encoding VP30 or different VP30ΔN-mutants were coexpressed by in vitro translation using the TNT T7 Quick Coupled Reticulocyte Lysate System. The proteins were precipitated with an α-flag MAB (dilution 1:500). Immune complexes were separated by SDS-PAGE and visualized by Bio-Imager analysis. Lanes 1-4 show the coprecipitated proteins and lanes 5-8 show the expression controls of the in vitro translation. Asterisk: unidentified protein.

Fig. 6: Coprecipitation of VP30 and VP30f in the presence of peptides. (A) Amino acid sequence of the used peptides. (B) 200 ng of plasmids encoding VP30 and 20 ng plasmids encoding Flag-tagged VP30 were employed to coexpress the two proteins using the TNT T7 Quick Coupled Reticulocyte Lysate System. The peptides E30pep-wt (lanes 2-6) or E30pep-3LA (lanes 7-11) were added in different concentrations as indicated below the panels. Translation was followed by coprecipitation with an α-flag MAB (dilution 1:500). Immune complexes were separated by SDS-PAGE and visualized by Bio-Imager analysis. Panel (C) shows the quantification of the radioactive signals, which was done with the Raytest TINA.
software. The bars demonstrate the amount of VP30 that was coprecipitated with VP30f. The value in the absence of peptides was set to 100% (lane 1). In panel (D) the expression control of the in vitro translation is shown.

**Fig. 7: Influence of VP30 oligomerization on EBOV propagation.** Vero cells were transfected with 50, 100 and 200 µM of peptide E30_{pep}wt or E30_{pep}3LA using Chariot-reagent and subsequently infected with EBOV. 24 h p.i. cells were fixed with 3% PFA and permeabilized using 0.2% Triton X-100. Afterwards, cells were subjected to immunofluorescence analysis (A) using a mouse monoclonal anti-NP antibody (dilution 1:20). Bound antibodies were detected with a rhodamine-labeled goat anti-mouse serum (Dianova, dilution 1:100). (B) EBOV-infected cells were counted and the number of infected cells without peptide treatment was set to 100%.
Fig. 1

![Graphical representation of protein bands with molecular weight markers (kDa) and protein concentrations (µg).]

**Table:**

| pT-VP30f | 2.5 | 2.5 | 2.5 |
|----------|-----|-----|-----|
| pT-VP30  | -   | -   | 2.5 |

**Molecular Weight (kDa):**

- 46
- 30
- 21

**Proteins:**

- VP30f
- VP30

**Samples:**

1 2 3 4
Fig. 2 A

| Protein | Description | Multimer |
|---------|-------------|----------|
| VP30   | N           | +        |
| VP30ΔN2-68 |             | +        |
| VP30ΔC114-288 |             | +        |
| VP30Δ72-93  |             | +        |
| VP30Δ72-112 |             | -        |

Fig. 2 B

[Image of gel electrophoresis with bands labeled VP30f, VP30, VP30ΔN2-68, VP30Δ72-93, VP30Δ72-112, VP30ΔC, and VP30ΔfN.]
Fig. 3 A

SLTDRE LLLLIAIRKTCGSV  VP30
SLTDRE ALLLIARKTCGSV  VP30\text{L}_{100}\text{A}
SLTDRE ALLLIARKTCGSV  VP30\text{L}_{102}\text{A}
SLTDRE ALLLIARKTCGSV  VP30\text{L}_{100/102}\text{A}
SLTDRE ALLLIARKTCGSV  VP30\text{L}_{100-102}\text{A}

Fig. 3 B

Fig. 3 C
Fig. 4

|      | α-NP   | α-VP30 | merge               |
|------|--------|--------|---------------------|
| **A** | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| **B** | ![Image](image4) | ![Image](image5) | ![Image](image6) |

- NP + VP30
- NP + VP30
- NP + VP30<sub>L100-102A</sub>
Fig. 5 A

I

II

III

[kDa]

Fig. 5 B

I

II

III

[kDa]

Fig. 5 C

[kDa]

α-flag

i.v.t.
Fig. 6

A

\[ \text{E30}_{\text{pep-wt}}: \quad H_2N-\text{QLES LTDEL LLI ARKTCGSVE- COOH} \]

\[ \text{E30}_{\text{pep-3LA}}: \quad H_2N-\text{QLES LTDEA ALI ARKTCGSVE- COOH} \]

B

\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\text{[ peptide ]} & 0 & 0.1 & 0.5 & 1 & 5 & 10 \text{µM} \\
\hline
\text{VP30f} & 120 & 110 & 100 & 90 & 80 & 70 \\
\text{VP30} & 100 & 90 & 80 & 70 & 60 & 50 \\
\hline
\end{tabular}

\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\text{VP30 / VP30f [%]} & 100 & 110 & 100 & 90 & 80 & 70 \\
\hline
\text{[ peptide ]} & 0,1 & 0,5 & 1 & 5 & 10 \text{µM} \\
\hline
\text{[ peptide ]} & 0,1 & 0,5 & 1 & 5 & 10 \text{µM} \\
\hline
\end{tabular}

C

\begin{tabular}{|c|c|c|c|c|c|}
\hline
\text{VP30 / VP30f [%]} & 100 & 90 & 80 & 70 & 60 \\
\hline
\text{[ peptide ]} & 0,1 & 0,5 & 1 & 5 & 10 \text{µM} \\
\hline
\end{tabular}

D

\begin{tabular}{|c|c|c|c|c|c|}
\hline
\text{VP30f} & 120 & 110 & 100 & 90 & 80 \\
\text{VP30} & 100 & 90 & 80 & 70 & 60 \\
\hline
\end{tabular}
Fig. 7A

| α-NP | DAPI |
|------|------|
| ![Image](EBOV_wt.jpg) | ![Image](DAPI_wt.jpg) |
| ![Image](EBOV_E30pep_wt.jpg) | ![Image](DAPI_EBOV_E30pep_wt.jpg) |
| ![Image](EBOV_E30pep3LA.jpg) | ![Image](DAPI_EBOV_E30pep3LA.jpg) |

Fig. 7B

![Graph](Graph.jpg)

- **EBOV**
- **EBOV + E30pep\text{wt}**
- **EBOV + E30pep_{3LA}**

The graph shows the percentage of infected cells at different concentrations of peptide. The concentration levels are 50, 100, and 200 [μM peptide].
Oligomerization of Ebola virus VP30 is essential for viral transcription and can be inhibited by a synthetic peptide
Bettina Hartlieb, Jens Modrof, Elke Mühlberger, Hans-Dieter Klenk and Stephan Becker

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