

The Importance of the NP: VP35 Ratio in Ebola Virus Nucleocapsid Formation

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Ebola virus VP35 is a cofactor of the viral RNA polymerase complex and, together with NP and VP24, is an essential component for nucleocapsid formation. In the present study, we examined the interactions between VP35 and NP and found that VP35 interacts with helical NP-RNA complexes through the C-terminus of NP. We also found that coexpression of excess VP35 with NP reduced the yields of NP-RNA complexes purified by CsCl gradient ultracentrifugation and inhibited the formation of the NP-induced inclusion bodies that typically form in Ebola virus–infected cells. These findings suggest that the NP to VP35 ratio is important in the Ebola virus replication cycle and advance our knowledge of nucleocapsid morphogenesis.

Ebola viruses, which comprise the family *Filoviridae* of the order *Mononegavirales* with Marburg viruses, have single-stranded, nonsegmented, negative-sense RNA genomes [1]. Their genome encodes 7 structural proteins, which form enveloped filamentous virions of ~80 nm in diameter. The glycoprotein (GP) and matrix protein (VP40) are membrane-associated and play central roles in the formation of filamentous virus particles [2–4]. Nucleoprotein (NP), VP30, VP35, and RNA-dependent RNA polymerase (L), together with viral genomic RNA, constitute the nucleocapsid—a principal unit of transcription and replication of the viral genome [5, 6]. The nucleocapsid is a helical structure of ~50 nm in diameter and resides along the central axis of the filamentous virion.

VP35 contains an N-terminal coiled-coil domain and a C-terminal RNA binding domain. It suppresses the production of type I interferon (IFN) by preventing the activation of IFN regulatory factor 3 [7–11]. Homooligomerization through its N-terminal domain facilitates the IFN antagonism exerted by the C-terminal domain [12]. VP35 also inhibits the activation of RNA-dependent protein kinase (PKR) [13, 14] and RNA silencing through its C-terminal domain [15]. Thus, VP35 plays an important role in the pathogenesis of Ebola virus infection. VP35 is also a cofactor of an RNA-dependent RNA polymerase complex. Both L and NP interact with VP35, but they do not interact directly with each other [16, 17], indicating that VP35 serves as a bridging molecule between them. Coexpression of NP, a minor matrix protein (VP24), and VP35 results in the formation of nucleocapsid-like structures that are morphologically indistinguishable from the nucleocapsids in the virions [18, 19]. When VP35 is coexpressed with NP in mammalian cells, it colocalizes with NP in NP-induced inclusion bodies and coprecipitates with NP [17, 18], suggesting that the interactions between VP35 and NP are important for nucleocapsid formation. Because it is unclear how VP35 interacts with NP during nucleocapsid formation, here, we examined the interactions between VP35 and NP both biochemically and morphologically.

Potential conflicts of interest: none reported.

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MATERIALS AND METHODS

Cells

Human embryonic kidney 293T cells and 293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin-streptomycin-amphotericin B. The cells were maintained at 37°C under 5% carbon dioxide.

Plasmids and Cell Transfection

The open reading frames of *Zaire ebolavirus* (Mayinga strain) NP and VP35 were cloned in the expression vector pCAGGS/MCS, as described elsewhere [20]. The resulting constructs were designated pCAGGS/NP and pCAGGS/VP35, respectively. 293T cells (~10⁶ cells/well) were transfected with the plasmids by using Trans IT 293 reagent (Mirus), according to the manufacturer's instructions.

Purification of NP-RNA Helices

Purification of the NP-cellular RNA complexes is described elsewhere [21]. In brief, cells expressing NP and VP35 were lysed in lysis buffer containing 1% Nonidet P-40 for 30 minutes at 4 °C and centrifuged at 13 000 rpm for 10 minutes at 4°C to separate the soluble supernatant (soluble fraction) from the insoluble component (insoluble fraction). The soluble fraction was loaded onto a discontinuous 25%–40% (w/w) CsCl gradient and ultracentrifuged. A visible band was collected and subjected to ultracentrifugation, and the resultant pellet was then resuspended in phosphate-buffered saline (PBS).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

Purified NP-RNA complexes were lysed in SDS-PAGE sample buffer, separated on a Tris-glycine gel, and visualized with a Silver stain Plus kit (Bio-Rad). For Western blot, blots were probed with rabbit anti-NP and anti-VP35 serum samples. Primary antibody binding was detected using a Vectastain ABC kit (Vector Laboratories).

Immunofluorescence and Confocal Microscopy

293 cells grown on glass cover slips were transfected with pCAGGS/NP and pCAGGS/VP35. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde for 1 hour, permeabilized with PBS containing 0.1% Triton X-100, and incubated with mouse anti-NP and rabbit anti-VP35 antibodies for 1 hour at room temperature. After being washed with PBS, they were incubated with Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen/Molecular Probes) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen/Molecular Probes). Microscopic assessment was performed using an LSM510 microscope (Carl Zeiss).

Electronmicroscopy

Transmission electronmicroscopy of cultured cell thin sections was performed as described elsewhere [19]. In brief, 48 hours

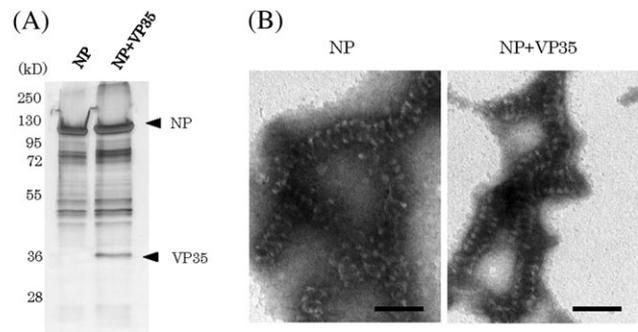


Figure 1. Copurification of VP35 with NP-RNA helices. *A*, SDS-PAGE and silver staining of the visible band isolated by using CsCl gradient ultracentrifugation. *B*, Electron micrographs of the negatively stained NP-RNA helices. Bars, 50 nm.

after transfection, plasmid-transfected cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer and postfixed with 2% osmium tetroxide in the same buffer. They were then dehydrated with a series of ethanol gradients, followed by propylene oxide, before being embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment). Thin sections were stained with 2% uranyl acetate and Reynold's lead and were examined under a Tecnai G2 F20 electron microscope (FEI) at 200 kV. For negative staining, purified NP-RNA helices were placed on a Formvar-coated copper grid, stained with 1% uranyl acetate, and examined with a Zeiss 109 electron microscope at 80 kV.

RESULTS

The NP-RNA Helix Is Associated With VP35

Recently, we found that, when NP is expressed alone in mammalian cells, it assembles into helices that associate with cellular RNA [21]. To examine whether this NP-RNA helices also associate with VP35, we purified NP-RNA helices from cells expressing both NP and VP35 with use of CsCl gradient

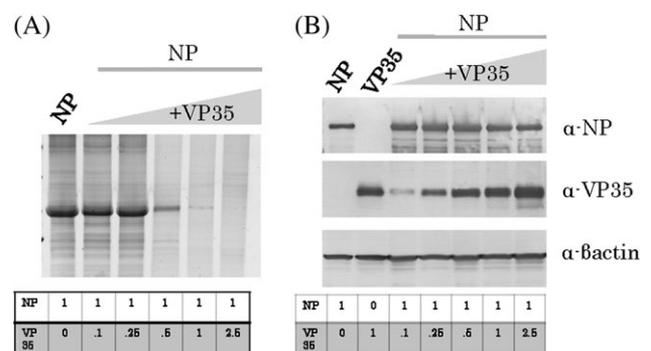


Figure 2. Yields of NP-RNA helices, isolated by using CsCl gradient ultracentrifugation, diminished in a pCAGGS/VP35-dose dependent manner. *A*, Yields of NP-RNA helices at different pCAGGS/NP to pCAGGS/VP35 ratios. *B*, Expression levels of NP in cells at different ratios of pCAGGS/NP to pCAGGS/VP35.

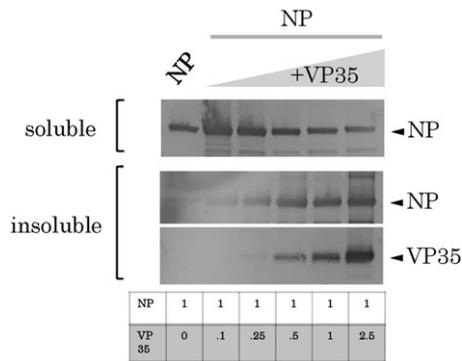


Figure 3. VP35 forms an insoluble complex with NP. The amount of insoluble NP increased in a pCAGGS/VP35-dose dependent manner.

ultracentrifugation, as described in the Materials and Methods. When NP was coexpressed with VP35, SDS-PAGE, followed by silver staining, revealed an additional band with a molecular mass corresponding to that of Ebola virus VP35 (~36 kD) (Figure 1); this band was confirmed to be VP35 by Western blot analysis using rabbit anti-VP35 serum (data not shown). When a C-terminal deletion mutant NP(Δ 451–739) that lacks

C-terminal amino acids 451–739 of NP [20] but still forms helical NP-RNA complexes [21] was coexpressed with VP35, VP35 did not copurify with the NP(Δ 451–739)-RNA helices (data not shown). Electron microscopy showed that the NP-RNA helices associated with VP35 were not significantly different morphologically from the products in the absence of VP35 (Figure 1B). These results suggest that the NP-RNA helices interact with VP35 through the C-terminal region of NP and that the association with VP35 does not appreciably affect the morphology of the NP-RNA helices.

The NP: VP35 Ratio Affects the Yield of Purified NP-RNA Helices

To investigate the effect of the NP to VP35 ratio on NP-RNA helix formation, 293 T cells were transfected with pCAGGS/NP and pCAGGS/VP35 at different ratios (with a fixed amount of pCAGGS/NP). Fewer NP-RNA helices were purified from cells expressing both NP and VP35 at ratios of >1:0.5 (Figure 2A). When cells were transfected with pCAGGS/NP and pCAGGS/VP35 at a ratio of 1:2.5, NP-RNA helices were barely recoverable by CsCl gradient centrifugation (Figure 2A). Western blotting revealed that the expression level of NP was constant in all cells, whereas that of VP35 increased in

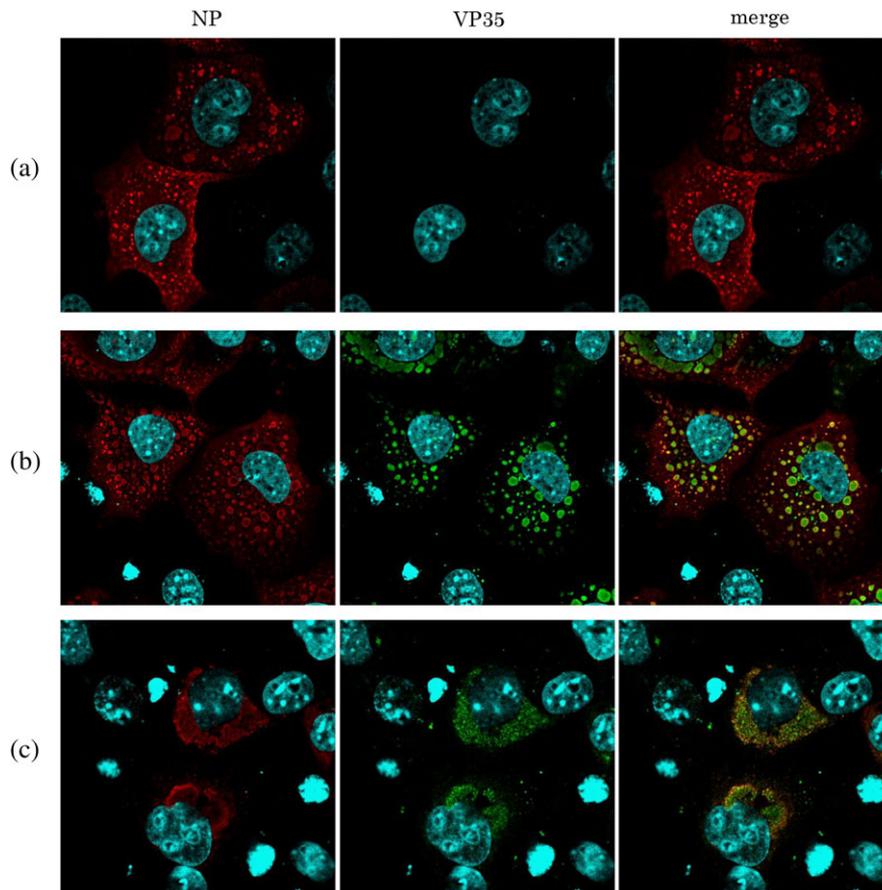


Figure 4. Intracellular localization of NP and VP35 at pCAGGS/NP to pCAGGS/VP35 ratios of 1:0 (A), 1:0.25 (B), and 1:2.5 (C). In the presence of excess VP35, NP did not form the spotted inclusion bodies that are typically observed in virus-infected cells.

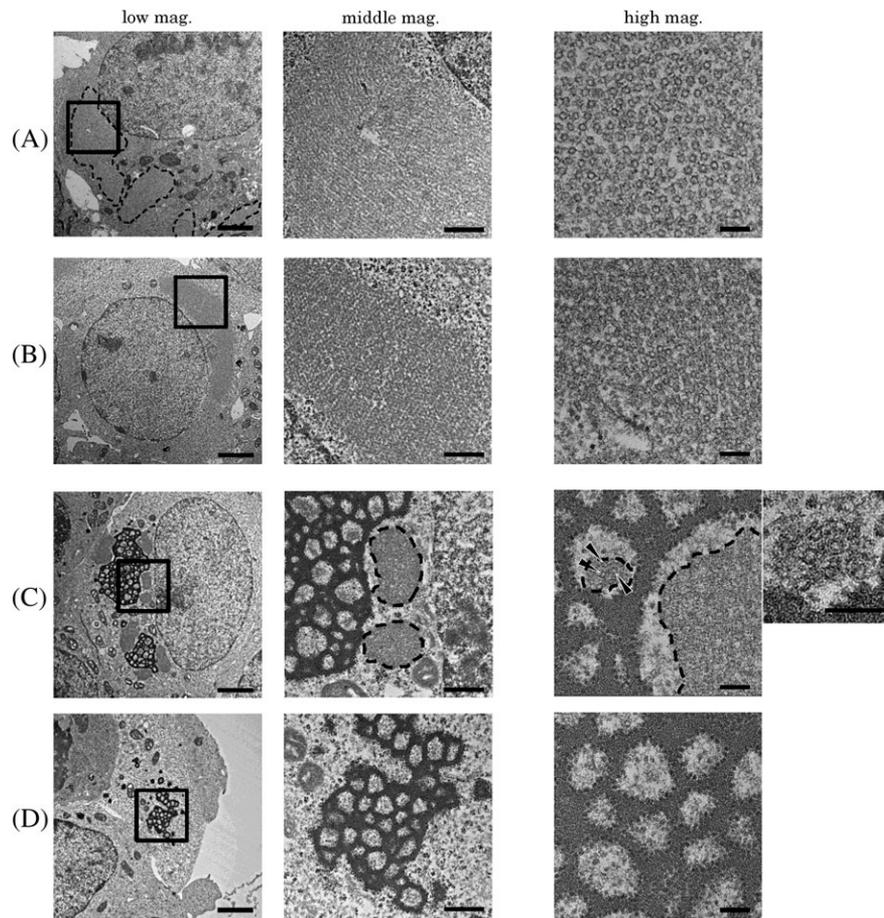


Figure 5. Electron micrographs of cells expressing NP and VP35 at pCAGGS/NP to pCAGGS/VP35 ratios of 1:0 (A), 1:0.25 (B), and 1:2.5 (C). D, Electron micrographs of cells expressing VP35 alone. Middle mag represents an enlargement of the boxed area at low mag. Dotted lines (a, low mag.; c, middle and high mags.) show bundles of NP-RNA helices. Arrowheads (c, high mag.) indicate vertically sectioned NP-RNA complexes within VP35-induced aggregates. The c, high mag, inset shows an enlargement of the vertically sectioned NP-RNA complexes (c, high mag). bars; 2 μ m (low mag), 500 nm (middle mag), 100 nm (high mag).

a pCAGGS/VP35 dose-dependent manner (Figure 2B). These results raise the possibility that excess VP35 inhibits the formation of NP-RNA helices.

VP35 Does Not Form a Soluble Complex With NP

The phosphoprotein (P protein) of rhabdoviruses binds to form a soluble complex with the viral nucleoprotein (N protein), which then prevents the newly synthesized N protein from binding nonspecifically to cellular RNA [22–24]. Thus, the P protein acts as a chaperone protein, conferring specificity to the N protein for the encapsidation of viral RNA during replication. Because Ebola virus VP35 and the P protein of rhabdoviruses share some functions, such as IFN antagonism, bridging between nucleoprotein and viral RNA polymerase, and binding to dynein light chain 8 [25–33], we hypothesized that Ebola virus VP35 also forms a soluble complex with NP, resulting in a decreased yield of NP-RNA helices purified by CsCl gradient ultracentrifugation.

To test this hypothesis, we cotransfected 293 T cells with different amounts of pCAGGS/VP35 and a fixed amount of

pCAGGS/NP. At 2 days after transfection, the cells were lysed and supernatants (soluble fractions) and pellets (insoluble fractions) were obtained by centrifugation, as described in Materials and Methods. At pCAGGS/NP to pCAGGS/VP35 ratios <1:0.25, most of the NP was found in the soluble fraction (Figure 3). However, the amount of NP in the insoluble fraction increased at pCAGGS/NP to pCAGGS/VP35 ratios >1:0.25 in a pCAGGS/VP35 dose-dependent manner (Figure 3). These results suggest that, unlike the P proteins of rhabdoviruses, VP35 does not form a soluble NP-VP35 complex. Instead, it forms an insoluble complex or aggregates with NP.

Intracellular Localization of NP in the Presence of Excess VP35

To assess the impact of excess VP35 on the intracellular localization of NP, 293 cells expressing NP and VP35 at pCAGGS/NP to pCAGGS/VP35 ratios of 1:0, 1:0.25, and 1:2.5 were analyzed using indirect immunofluorescence microscopy. Expression of NP alone resulted in the formation of spotted inclusion bodies, as reported elsewhere [17, 34] (Figure 4A). In cells expressing

both NP and VP35 at a pCAGGS/NP to pCAGGS/VP35 ratio of 1:0.25, NP formed spotted inclusions similar to those observed in the absence of VP35, by which VP35 colocalized with NP (Figure 4). However, in the presence of excess VP35 (at a pCAGGS/NP to pCAGGS/VP35 ratio of 1:2.5), NP did not form typical (ie, spotted) inclusion bodies and colocalized with VP35, which exhibited a pinpoint pattern in the cytoplasm (Figure 4).

Electron microscopy of thin-sectioned cells expressing NP alone showed that a large numbers of NP-RNA helices of ~25 nm in diameter were arranged in bundles (Figure 5), which is consistent with the result shown in Figure 4A. When VP35 was coexpressed with NP at the ratio of 1:0.25, numerous NP-RNA helices were similarly bundled (Figure 5B) and appeared to be arranged more closely than those in the absence of VP35 (Figure 5). In cells expressing NP and excess VP35, perforated and pleomorphic structures (Figure 5C), which are composed of VP35 (Figure 5), appeared in the cytoplasm. The VP35-induced aggregates were closely associated with the bundles of NP-RNA helices at the periphery (Figure 5C). In addition, in the perforated and pleomorphic VP35 aggregates, there were NP-RNA complexes, whose helical structures appeared to be somewhat disordered (Figure 5C). Taken together, these results suggest that coexpression of excess VP35 with NP inhibits the formation of the NP-induced spotted inclusion bodies that are typically observed in Ebola virus-infected cells.

DISCUSSION

Ebola virus VP35, a cofactor of the viral RNA polymerase complex, is essential for nucleocapsid formation, together with NP and VP24. In our study, we investigated the interaction of VP35 with NP-RNA helices and demonstrated the importance of the NP to VP35 ratio.

We first examined the mechanism by which coexpression of excess VP35 with NP reduced the quantity of NP-RNA helices that could be purified by CsCl gradient ultracentrifugation (Figure 2). Contrary to our hypothesis that, similar to the P proteins of rhabdoviruses, VP35 forms soluble complexes with NP and inhibits the formation of large NP-cellular RNA complexes, excess VP35 appeared to form insoluble complexes with NP (Figure 3). In the presence of excess VP35, the NP-RNA helices were not only associated at the periphery of perforated and pleomorphic VP35 aggregates but also were present in the aggregates (Figure 5C). These observations suggest that the association of the NP-RNA helices with the insoluble VP35 aggregates makes the helices also insoluble, resulting in reduced yields of NP-RNA complexes that could be purified by using CsCl gradient centrifugation (Figure 2A).

Of interest, in cells coexpressing NP and excess VP35, the spotted inclusion bodies (Figures 4 and 5) that are typically found in Ebola virus-infected cells [35] did not form, suggesting

that the nucleocapsids did not form correctly. In fact, an earlier study showed that, when VP35 is overexpressed in an artificial replication system, the reporter gene activity decreases [5], which also suggests the importance of the NP to VP35 ratio.

In conclusion, VP35 interacts with the NP-RNA complex through the C-terminal region of NP. However, when the levels of VP35 are too high, the formation of functional nucleocapsids is prohibited. Thus, the expression ratio of NP to VP35 in virus-infected cells appears to be important for efficient virus replication, although transcriptional attenuation, which is a common feature of mononegavirus gene expression, unlikely occurs [1, 36], Ebola virus must rigorously regulate the expression levels of both NP and VP35 when it replicates in cells.

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