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NOTES

Infection of Naïve Target Cells with Virus-Like Particles: Implications for the Function of Ebola Virus VP24

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Infectious virus-like particle (iVLP) systems have recently been established for several negative-strand RNA viruses, including the highly pathogenic *Zaire ebolavirus* (ZEBOV), and allow study of the viral life cycle under biosafety level 2 conditions. However, current systems depend on the expression of viral helper nucleocapsid proteins in target cells, thus making it impossible to determine whether ribonucleoprotein complexes transferred by iVLPs are able to facilitate initial transcription, an indispensable step in natural infection. Here we describe a ZEBOV iVLP system which overcomes this limitation and show that VP24 is essential for the formation of a functional ribonucleoprotein complex.

Ebola virus (EBOV) causes severe hemorrhagic fever in humans, for which treatment is currently unavailable (6, 8). To study particle morphogenesis, genome packaging, and virion entry into target cells under biosafety level 2 conditions, an infectious virus-like particle (iVLP) system for *Zaire ebolavirus* (ZEBOV) was established (20). This system extends the original minigenome systems (4, 7, 14) by including VP24, VP40, and GP to produce iVLPs which resemble wild-type (WT) virions but contain minigenomes instead of full-length viral genomes. These iVLPs can deliver the minigenomes to target cells, where they are transcribed and replicated in the presence of helper ribonucleoprotein (RNP) components (the nucleoprotein [NP], VP35, VP30, and L components). Similar systems are available for other negative-sense RNA viruses, but RNP components must always be provided to target cells in *trans*, via either helper virus infection or expression plasmid transfection (12, 15–17, 19–21). Therefore, it is impossible to determine whether iVLPs contain fully functional RNP complexes or whether they contain minigenomes but are unable to facilitate initial transcription, as is necessary in virus infection.

EBOV VP24 has been described as a minor matrix protein, but its contribution to budding is controversial (10, 13). Electron microscopic studies imply a role in the formation of nucleocapsid-like structures (11), while another study suggests that VP24 is unnecessary for the packaging and delivery of minigenomes by iVLPs (20). To better understand the contribution of VP24 to morphogenesis, we established an iVLP system with naïve target cells to allow assessment of iVLP-associated RNP complex function. As a first step, an iVLP system based on that previously described (20) was established in our laboratory (Fig. 1A). 293T cells (p0) were transfected using FuGENE (Roche) with plasmids encoding each ZEBOV structural protein (125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, 1,000 ng pCAGGS-L, 250 ng pCAGGS-GP, 250 ng pCAGGS-VP40, and 60 ng pCAGGS-VP24) and a T7-driven minigenome encoding a *Renilla* luciferase reporter (250 ng), which is detectable in minute amounts (23). Cell supernatant containing released iVLPs was harvested 3 days posttransfection, cleared of cellular debris, and used to infect target 293T cells (p1) previously transfected with RNP components (125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, and 1,000 ng pCAGGS-L). Reporter activity in p0, as determined by dual-luciferase assay (Promega), was dependent on VP30 and L but not VP24 or VP40 (Fig. 1B). The infection of 293T cells in p1 resulted in reporter activity 3 days postinfection (p.i.) that was dependent on the presence of L in p1 and VP40 in p0 (Fig. 1C) as previously published (20). Omitting VP24 or VP30 in p0 did not reduce reporter activity in p1, showing that, under these conditions, neither VP24 nor VP30 was necessary for minigenome replication, iVLP budding, or minigenome delivery and that VP30 in p1 compensates for the absence of VP30 in p0.

To establish an iVLP system with naïve target cells, the above protocol was repeated with naïve VeroE6 as target cells in p1, since among cell lines tested, they showed the highest susceptibility to infection with a recombinant ZEBOV (data not shown). This virus expresses enhanced green fluorescent protein (GFP) from an additional open reading frame and shows growth comparable to that of wild-type ZEBOV in cell culture, similar to another recombinant GFP-ZEBOV recently

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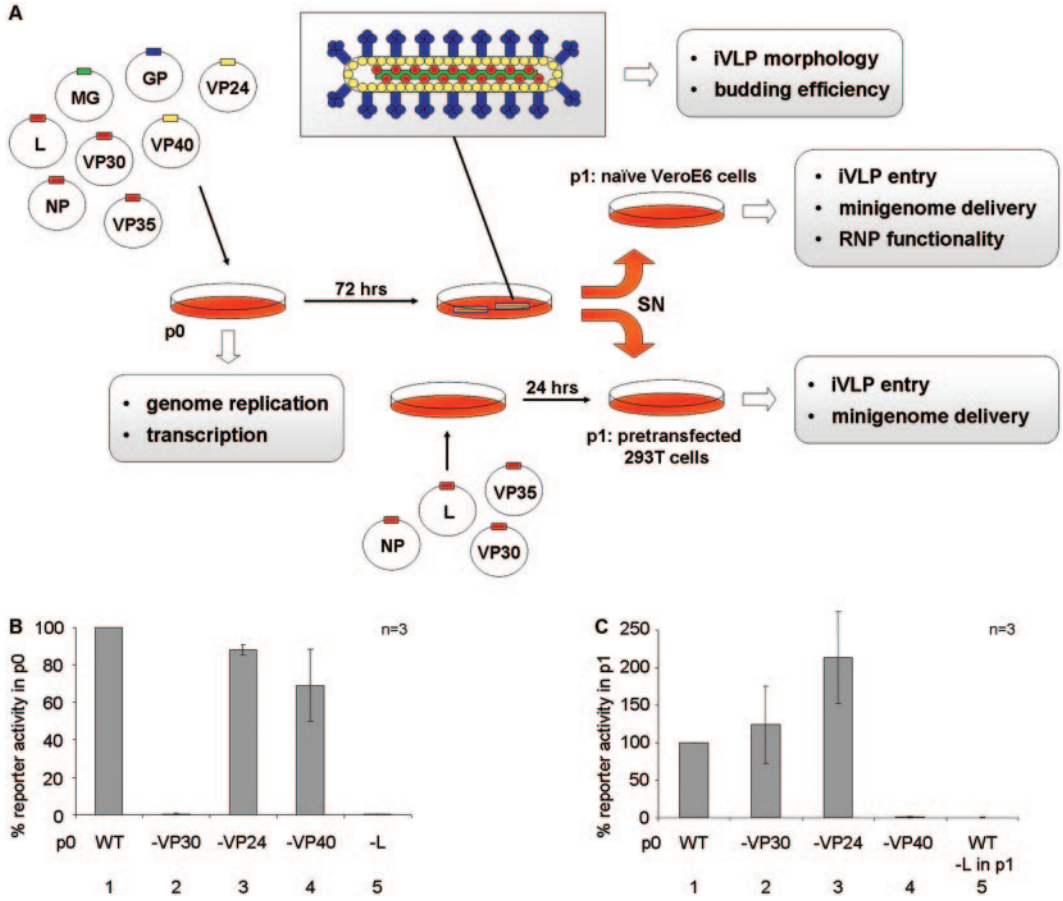

FIG. 1. iVLP assay with pretransfected target cells. (A) Schematic outline of iVLP assays. 293T cells (p0) are transfected with 125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, 60 ng pCAGGS-VP24, 1,000 ng pCAGGS-L, 250 ng pCAGGS-VP40, 250 ng pCAGGS-GP, 250 ng minigenome (MG), and 250 ng pCAGGS-T7. These plasmids encode the nucleocapsid proteins (red), matrix proteins (yellow), and the glycoprotein (blue) of ZEBOV, a ZEBOV-specific minigenome (green) with a *Renilla* luciferase reporter, and the T7 polymerase, respectively. Reporter activity in p0 reflects minigenome replication and transcription. Produced iVLPs resemble WT virions but contain a minigenome instead of a full-length viral genome. Each iVLP can be analyzed for its morphology and its budding efficiency. Target cells (p1) are either pretransfected with expression plasmids for NP, VP35, VP30, and L (iVLP assay with pretransfected target cells, lower half) or left untreated (iVLP assay with naïve target cells, upper half). After transfer of the supernatant from p0 to p1, these target cells become infected by the iVLPs. The reporter activity in p1 reflects iVLP entry and delivery and, in the case of naïve target cells, also initial transcription of the minigenome solely by the viral proteins transported in the iVLPs and, thus, RNP complex functionality. (B) 293T cells (p0) were transfected with all of the plasmids necessary to produce iVLPs (WT, bar 1) or with all of these plasmids except those indicated (bars 2 to 5). Seventy-two hours posttransfection, the cells were harvested and luciferase activity, reflecting minigenome replication and transcription, was determined. The supernatant of these cells was used to infect target cells. (C) Pretransfected 293T cells serving as target cells (p1) were infected with WT iVLPs (bars 1 and 5) or single-protein-deficient iVLPs (bars 2 to 4). Three days p.i., the luciferase activity, reflecting iVLP entry and minigenome delivery, was determined. Target cells transfected with all of the RNP components except L served as a negative control (bar 5). Error bars indicate standard deviations.

described (18). Reporter activity in iVLP-infected target VeroE6 cells was determined 1 to 3 days p.i. and showed a maximum on day 2 (Fig. 2A), consistent with the need for the transcription/translation of reporter protein prior to detection.

Notably, reporter activity in naïve target cells was ~100-fold lower than that in pretransfected cells (data not shown). This difference may be due to limited amounts of RNP components being available during the infection of naïve target cells, which makes minigenome replication unlikely (22). Together, limited amounts of both viral proteins and minigenome contribute to a low signal strength, which may explain why, in previously published iVLP systems which used chloramphenicol acetyltransferase or GFP reporters, infection of naïve target cells was undetectable.

To confirm that activity in p1 was not due to unspecific reporter protein transfer, a plasmid encoding firefly luciferase under the control of an SV40 promoter (250 ng pGL2-Control; Promega) was cotransfected with the other plasmids in p0. The ratio of firefly luciferase activity (control) to minigenome-encoded *Renilla* luciferase activity was then determined in p0 and in p1 (Fig. 2B). The ratio decreased ~10-fold, with firefly luciferase activity (4×10^2 relative light units/well/s) in p1 being in the range of the luminometer background (1×10^2 relative light units/well/s). This confirmed specific minigenome transfer, since unspecific packaging of *Renilla* and firefly luciferase should occur equally.

To further demonstrate that reporter activity in p1 was due to transcription by the viral RNP complex, VeroE6 target cells

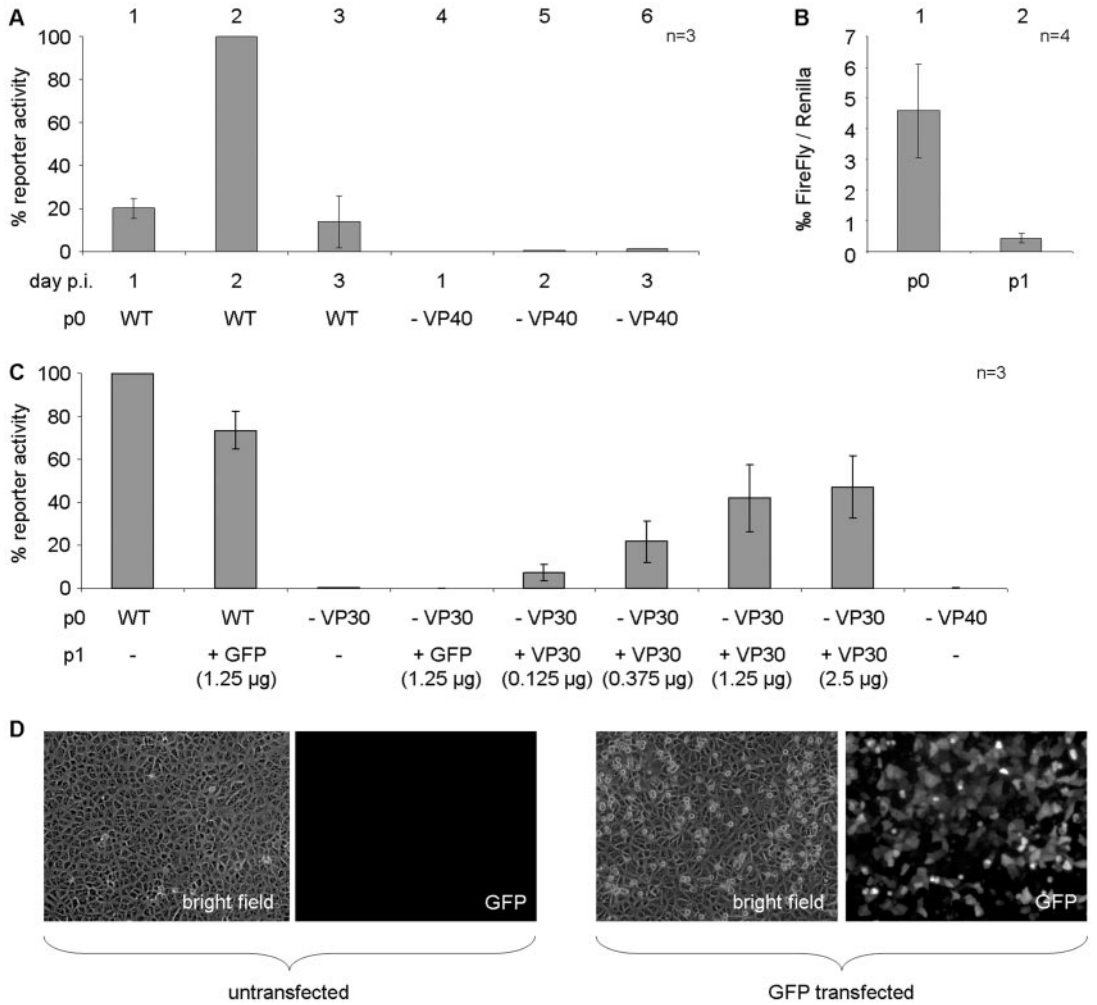

FIG. 2. iVLP assay with naïve VeroE6 target cells. (A) Time course of reporter activity in p1. VeroE6 cells were infected with iVLPs, and the reporter activity was determined at days 1 to 3 p.i. (bars 1 to 3). As a negative control, VeroE6 cells were infected with supernatant of iVLP-producing cells that were transfected with all of the plasmids necessary to produce iVLPs with the exception of VP40 (bars 4 to 6). (B) The ratio of firefly luciferase, expressed from a control plasmid that was cotransfected in p0, to *Renilla* luciferase, expressed from the minigenome, was determined in both p0 (bar 1) and p1 (bar 2), showing that luciferase is not unspecifically transferred. (C) Recovery of VP30-deficient iVLPs by pretransfecting VP30 in p1. WT or VP30-deficient iVLPs were produced and used to infect VeroE6 cells that had been pretransfected by electroporation with the indicated amounts of pCAGGS-VP30 or pCAGGS-GFP. Reporter activity was determined 2 days p.i. (D) Transfection efficiency of electroporation. VeroE6 cells were transfected with 1.25 μg of pCAGGS-GFP. GFP-expressing cells were visualized 24 h posttransfection by fluorescence microscopy. Error bars indicate standard deviations.

were infected with VP30-deficient iVLPs and VP30 was provided in *trans* by electroporation (2) of 0.125 to 2.5 μg pCAGGS-VP30/well or 1.25 μg pCAGGS-GFP/well (control) 1 day before infection. When VP30 was not provided in *trans*, reporter activity was undetectable in p1 (Fig. 2C). However, when VP30-pretransfected cells were infected, we observed recovery of reporter activity (Fig. 2C) correlating with transfection efficiency (Fig. 2D). This indicates that viral RNP components are responsible for transcription in p1 and demonstrates the possibility of providing VP30 to target cells in *trans*.

We then reevaluated the role of ZEBOV VP24 using this newly established system by omitting VP24 from the transfection of p0. Surprisingly, in contrast to the results with pretransfected target cells, reporter signals were decreased in p1 by 90% after infection with VP24-deficient iVLPs (Fig. 3A). When target VeroE6 cells were pretransfected with all RNP components, reporter activity was independent of VP24 in iVLPs (data not shown). However, neither pretransfection of target cells with any single RNP component nor that with VP24 resulted in the recovery of reporter activity (Fig. 3B). Also, combinations of RNP components (VP35/L and NP/VP35/VP30) could not restore reporter activity (data not shown). This suggests that VP24 does not simply recruit a single RNP component into particles and makes a direct role of VP24 in target cells (e.g., interaction with host factors) unlikely. However, since VeroE6 cells do not produce type I interferon (5), any effects of VP24 on the interferon cascade (3, 9) may not be detectable.

Electron microscopic analysis of negative-stained wild-type and VP24-deficient iVLPs revealed no obvious size differences, and RNP-like structures were found in both types of particles (Fig. 3C). We further analyzed iVLPs for their protein compositions by purifying them by ultracentrifugation over a 20%

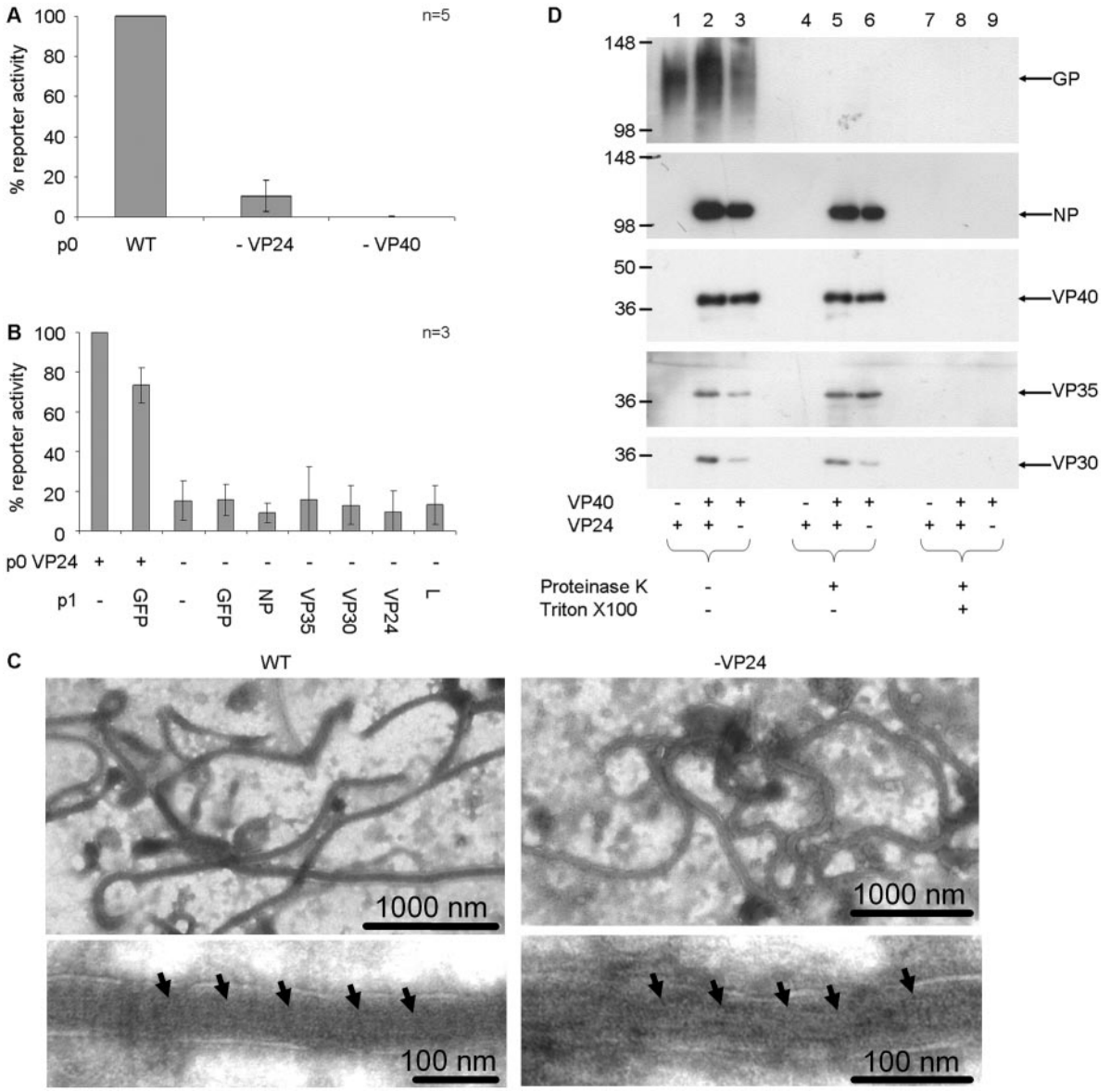

FIG. 3. iVLP assay with VP24-deficient particles. (A) VeroE6 cells were infected with WT or VP24-deficient iVLPs. The reporter gene activity was determined 2 days p.i. As a negative control, the plasmid encoding VP40 was omitted in p0. Error bars indicate standard deviations. (B) Recovery of infectivity of VP24-deficient iVLPs by pretransfection of p1. VeroE6 cells were pretransfected by electroporation with 1.25 μg of plasmid encoding the indicated protein. One day posttransfection, the cells were infected with WT or VP24-deficient iVLPs, and 2 days p.i., the reporter activity was measured. Error bars indicate standard deviations. (C) Electron microscopy analysis of iVLPs. WT and VP24-deficient iVLPs were produced and purified by ultracentrifugation over a 20% sucrose cushion. Particles were visualized using electron microscopy after negative staining. Arrows show nucleocapsid-like structures. (D) Analysis of iVLP composition. WT (lanes 2, 5, and 8) or VP24-deficient (lanes 3, 6, and 9) iVLPs were produced and purified over a 20% sucrose cushion. As a control, the plasmid encoding VP40 was omitted in p0 (lanes 1, 4, and 7). The purified iVLPs were either mock treated (lanes 1 to 3), treated with proteinase K (lane 4 to 6), or treated with proteinase K and Triton X-100 (lanes 7 to 9). The samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by Western blotting using specific antibodies. Proteins inside the iVLPs are visible in lanes 4 to 6.

sucrose cushion and performing proteinase K digestion and Western blotting (1). Most obvious, VP30 levels were diminished in VP24-deficient particles (Fig. 3D, lanes 5 and 6), indicating changes in the composition of the RNP complexes.

A possible explanation for the observed results is that VP24 is necessary for the correct assembly of fully functional nucleocapsids. According to this hypothesis, minigenomes are not properly encapsidated in VP24-deficient cells, preventing correct processing by the polymerase complex in target cells. With excessive amounts of all nucleocapsid proteins present in p1, due to pretransfection, the processing of transferred minigenome may be possible because it can be replicated and the resulting progeny serves as a template for transcription. Based on the obtained data, we therefore propose that one function of VP24 is to facilitate the assembly of fully functional nucleocapsids.

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