Involvement of Vacuolar Protein Sorting Pathway in Ebola Virus Release Independent of TSG101 Interaction

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Budding of Ebola virus (EBOV) particles from the plasma membrane of infected cells requires viral and host proteins. EBOV virus matrix protein VP40 recruits TSG101, an ESCRT-1 (host cell endosomal sorting complex required for transport–1) complex protein in the vacuolar protein sorting (vps) pathway, to the plasma membrane during budding. Involvement of other vps proteins in EBOV budding has not been established. Therefore, we used VP40 deletion analysis, virus-like particle–release assays, and confocal microscopy to investigate the potential role of ESCRT-1 proteins VPS4, VPS28, and VPS37B in EBOV budding. We found that VP40 could redirect each protein from endosomes to the cell surface independently of TSG101 interaction. A lack of VPS4 adenosine triphosphatase activity reduced budding by up to 80%. Inhibition of VPS4 gene expression by use of phosphorodiamidite morpholino antisense oligonucleotides protected mice from lethal EBOV infection. These data show that EBOV can use vps proteins independently of TSG101 for budding and reveal VPS4 as a potential target for filovirus therapeutics.

Ebola virus (EBOV) particles exit host cells by budding from the plasma membrane, a process that involves interaction between virus-encoded and cellular proteins [1, 2]. The viral matrix protein VP40 is a major component of EBOV virions and is necessary for the formation of the filamentous particles [3, 4]. Oligomerization of VP40 at the plasma membrane is required for assembly of particles and for virus budding [5–7]. There is increasing evidence that many different viruses, including the filoviruses and retroviruses, use host proteins normally involved in the vacuolar protein sorting (vps) pathway for the final steps of budding [8, 9]. This requires movement of the vps proteins from multivesicular bodies to the plasma membrane, an event mediated or performed by viral proteins.

The cellular protein TSG101 is involved in budding of both HIV and EBOV [5, 10, 11]. TSG101 functions in early steps of the vps pathway, in a complex of proteins termed “ESCRT-1” (host cell endosomal sorting complex required for transport–1) [8]. This complex, which also includes proteins VPS28, VPS37B, and VPS4, sorts ubiquitinated proteins to promote their incorporation into multivesicular bodies in a budding process. Recruitment of TSG101 to the plasma membrane away from its normal site of function in endosomes has been shown to occur via binding to EBOV VP40 [5, 10]. Interaction of VP40 with TSG101 requires a specific amino acid motif, or late domain (L domain) [9]. EBOV VP40 contains 2 overlapping L domains: PPXY and PT/SAP motifs arranged as
Figure 1. Analysis of multimerization and virus-like particle (VLP) release of VP40 deletion mutants. 

**A**, Mutagenic primers used to create VP40 deletion mutants. **B**, VP40 constructs transfected into 293T cells and tested by Western blot for multimerization. Samples were either heated (H) or unheated (U) before loading. VP40 was detected using a mixture of mouse anti-VP40 (AE11) antibody and rabbit anti-VP40 antibody. Oligomeric and monomeric forms of VP40 are indicated. **C**, VLP release measured in supernatants of transfected cells by ELISA specific to VP40. The amount of VP40 present in supernatants from cells transfected with wild-type VP40 was set at 100%.

7-PTAPPEY-13. Similar to the HIV-1 Gag protein, the VP40 L domain motif PTAP binds to TSG101 [10]. The PPEY motif binds to Nedd4 ubiquitin ligase [12]. L domain–containing viruses may recruit the entire ESCRT machinery to sites of virus budding through interactions with such molecules as TSG101 or Nedd4. In the final step of the vps pathway, the ATPase activity of VPS4 generates the energy needed for dissociation of the protein complex, allowing for subsequent rounds of sorting. A dominant-negative VPS4 mutant, lacking ATPase activity, can inhibit release of both HIV and EBOV [12, 13]. EBOV VP40 may redirect other ESCRT-1 proteins from endosomes to the plasma membrane, but these interactions have not been fully characterized.

We investigated the interactions of EBOV VP40 with the ESCRT proteins VPS4, VPS28, and VPS37B by examining the effect of VP40 L domain mutations on ESCRT protein localization and virus-like particle (VLP) release. Our results show that recruitment of VPS4, VPS28, and VPS37B to the plasma membrane was not dependent on VP40-TSG101 interaction. A lack of VPS4 ATPase activity did not affect its recruitment to the plasma membrane, but it did inhibit VLP release. Administration of phosphorodiamidite morpholino oligonucleotides (PMOs) to knock down VPS4 gene expression protected mice from lethal EBOV infection. The results presented here demonstrate that EBOV can use the vps pathway independently of TSG101 interaction and suggest a potential for VPS4 as a therapeutic target for the development of countermeasures against EBOV and, possibly, against other viruses.

**MATERIALS AND METHODS**

**Cells, plasmids, mutagenesis, and transfections.** 293T cells were maintained in Dulbecco's MEM (DMEM) containing 10% fetal bovine serum at 37°C with 5% CO₂. Cells were seeded 24 h before transfection. DNA was mixed with OptiMem (Invitrogen) and transfected using 1 μL of Lipofectamine2000 (Invitrogen), in accordance with the manufacturer's instructions. Tetracysteine-tagged VP40 has been described elsewhere [5]. Mutations were made by use of the QuickChange Mutagenesis Kit (Stratagene). Mutagenic primers are listed in figure 1A. Plasmids encoding VPS28, VPS37B, and a green fluorescence protein (GFP) fusion of VPS4A were kindly provided by W. Sundquist (University of Utah, Salt Lake City).

**VLP-release assay.** The assay was performed in 2–3 replicates, as described elsewhere [14]. Briefly, supernatants were centrifuged and adjusted to 1.5% Triton X-100. ELISA plates were coated with mouse anti-VP40 (AE11; 1:100) in PBS, blocked with 5% milk/PBST (PBS Tween; 0.02%) for 2 h, then washed with PBST. One hundred microliters of supernatant was added to each well and incubated at room temperature for 4 h. Rabbit anti-VP40 and HRP-conjugated goat anti-rabbit
antibody were used to detect VP40. 3,3',5,5'-Tetramethylbenzidine substrate was added, and absorption was read at 650 nm by use of a plate reader.

Confocal microscopy. 293T cells were transfected with the indicated plasmids in 2–3 replicates, incubated at 37°C for 24 h, then fixed with 4% buffered formalin. VP40 was detected with rabbit anti-VP40 antibody, and TSG101 and VPS37B were detected using anti-FLAG monoclonal antibody M1 (Sigma). VPS28 was detected using a rabbit polyclonal antibody. The VPS4 plasmid produces a GFP-VPS4 fusion protein for direct visualization. Secondary antibodies were Alexa 488 and Alexa 568 conjugated antibodies (Molecular Probes). Nuclei were labeled with Hoechst stain (Molecular Probes). Images were obtained with a Bio-Rad 2000MP confocal/multiphoton system attached to a Nikon TE300 inverted microscope.

Design of PMOs and EBOV challenge. PMOs were synthesized by AVI Biopharma. VPS4A PMO complements the region spanning the ATG start codon, a region 100% conserved between human and mouse. The control VPS4B PMO complements the region immediately following the ATG start codon of human VPS4B (positions +5 to +25), a region that is only 66% identical to the mouse sequence. The PMO sequences are as follows: VPS4A, 5'-GGAGGGTTGACGTTGTCATTTC-3'; and VPS4B, 5'-GGAGGTTGCGAAGTGGATG-3'. Groups of 10 C57BL/6 mice were injected intraperitoneally (ip) with 0.5 mg of PMO VPS4A or VPS4B or with PBS alone, 24 h before and 24 and 48 h after challenge with an ip dose of 1000 pfu of mouse-adapted Zaire EBOV. Mice were monitored for signs of disease for 28 days after infection.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals [15]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

RESULTS

Analysis of VP40 deletion mutants in VLP release and oligomerization. EBOV VP40 forms oligomers at the plasma membrane, an important step for virus budding [5–7]. To search for regions important in oligomerization and budding, VP40 mutants containing progressive 15-aa deletions were constructed. Mutants were tested for the ability to produce VP40 oligomers and release of VLPs from transfected cells. All of the mutants, except those with deletions within aa 76–105, were well expressed (figure 1B). Consistent with our previous observations [5], deletion of the C-terminal 15 aa resulted in a completely oligomerized protein (figure 1B). In contrast, deletion of the N-terminal 30 aa did not significantly influence the degree of oligomerization, whereas deletion of aa 30–60 significantly impaired oligomerization (figure 1B). As predicted by structural data [16–18], no deletions in the core folded region of the N-terminal domain (aa 61–195) were tolerated with respect to oligomerization.

VLP release by each mutant was analyzed by ELISA specific for VP40 [14] (figure 1C). A reduced level of VLP release (40%
VPS Pathway in Ebola Virus Release

Figure 4. Image of 293T cells expressing VPS28 or VPS37B plus VP40 late domain mutants. Cells were transfected with plasmids expressing the indicated VP40 constructs and either VPS28 (A) or VPS37B (B). VPS28 and VPS37B are stained green, and VP40 is red. Nuclei are stained with Hoechst’s (blue). PTAL, 7-PTALPEY-13 mutant; wt, wild type.

of wild type) was seen in the aa 2–15 mutant, in which the 7-PTAPPEY-13 L domain motifs were deleted (figure 1B). In contrast, deletions within aa 15–45 did not alter the ability of VP40 to bud from the cells (figure 1C). This is consistent with a proposed role for the vps pathway in VP40 vesicular release. However, the data also suggest that VP40 can use alternative pathways for budding in the absence of L domain.

Deletions in the rest of the N-terminal domain and almost the entire C-terminal domain greatly inhibited VLP release (figure 1C). Previous results with the aa 91–105 mutant suggested that these amino acids constitute at least a portion of the AE11 epitope (data not shown), making interpretation of the results for this mutant problematic. Overall data from deletions in the N-terminal domain show a strong correlation between oligomerization and the ability of VP40 to bud from the cells (compare figure 1B and 1C). It is apparent that C-terminal deletions reduce budding without a noticeable effect on oligomerization (figure 1B and 1C), most likely as a result of disruption of membrane binding, as has been suggested elsewhere [5, 6].

Reduction of VP40–glycoprotein (GP) VLP release by mutations in the L domains. Release of VLPs is enhanced by coexpression of GP. To further analyze the role of the L domains, constructs bearing L domain deletions and point mutations were tested for the release of VP40-GP VLPs. 293T cells were transfected with a VP40 construct and a GP expression plasmid and were analyzed for VLP release by ELISA. The ratios of VLPs in the media to cell-associated VP40 levels were determined and expressed as percentages of VLP release relative to wild-type VP40 plus GP. Figure 2 shows that deletion of 7-PTAPPEY-13 caused an ~40% reduction in the amount of VLPs released (aa 2–13 mutant). The mutants 7-PTALPEY-13 (PTAL), in which both L domains are mutated, and 7-PTEA-13 (PPEA) slightly reduced the amount of released VLPs, by ~10%–20%, compared with the control (figure 2). Thus, although some reduction in VLP release was achieved by single-amino-acid mutations within the L domain motifs, none of the mutations completely inhibited the release of VLPs. These data suggest that the presence of GP moderately reduces the dependence of VLP release on the L domain.

Necessity of VP40 L domains for localization of TSG101 to the cellular membrane. EBOV VP40 recruits TSG101 to the plasma membrane and lipid rafts [5]. Because the VP40 aa 2–13 mutant had only a 40% reduction in VLP release, compared with wild-type VP40 (figure 2), we examined whether this mutant was able to recruit TSG101 to the cell surface. Cells were transfected with each VP40 plasmid and a plasmid encoding TSG101, stained, and analyzed by confocal microscopy. When TSG101 was expressed alone, it was found compartmentalized within the cytoplasm of the cell (figure 3). TSG101 colocalized with full-length VP40 at the cell surface, whereas, in the presence of the VP40 aa 2–13 mutant, TSG101 remained concentrated within endosomes (figure 3). These data show that the
movement of TSG101 to the plasma membrane requires the L domains and suggests that the 60% release of VLPs achieved with the aa 2–13 mutant (figure 2) was via a TSG101-independent pathway.

**Effect of VP40 on cellular localization of ESCRT proteins VPS28 and VPS37B.** VPS28 and VPS37B bind to TSG101 within the ESCRT-I complex and are involved in the sorting of proteins on endosomal compartments [19]. To determine whether VP40 L domains mediate redistribution of VPS28 and VPS37B from endosomes to the cell surface, localization of VPS28 or VPS37B was analyzed in the presence and absence of VP40. In the absence of VP40, VPS28 was distributed throughout the cell, but it also appeared to be concentrated in some regions on the plasma membrane (figure 4A). In the presence of wild-type VP40 and the aa 2–13 mutant, VPS28 was slightly more concentrated at the surface than was observed when VPS28 was expressed alone. Membrane-bound VPS28 appeared to colocalize with VP40. The VP40 mutant PTAL was less effective in recruiting VPS28 to the plasma membrane than wild-type VP40 or the aa 2–13 mutant.

VPS37B, when expressed alone, had a punctuate appearance within the cytoplasm and, to a lesser degree, was seen in an uneven distribution pattern at the plasma membrane (figure 4B). When expressed with wild-type VP40, the majority of the VPS37B was found at the membrane and was more evenly distributed. However, VPS37B and VP40 were not clearly colocalized. The VP40 aa 2–13 mutant and PTAL also caused VPS37B to localize to the plasma membrane, with no significant colocalization of the proteins, similar to wild-type VP40. These findings suggest that VPS28 and VPS37B can be redirected to the plasma membrane by VP40 in the absence of TSG101 recruitment.

**Redistribution of VPS4A to the plasma membrane caused by VP40.** Localization of VPS4A in presence and absence of VP40 was analyzed by confocal microscopy. VPS4A, when expressed alone, had an intracellular globular pattern that most likely represented VPS4A associated with endosomes (figure 5). In the presence of wild-type VP40, however, a portion of VPS4A was redistributed to the plasma membrane, where VPS4A and VP40 colocalized. VPS4A that remained in the cytoplasm still appeared to be associated with endosomes. This distribution pattern of VPS4A was also observed in the presence of the VP40 aa 2–13 mutant. When coexpressed with mutants PTAL and PPEA, VPS4A was still distributed between the surface and endosomes, with a subset diffusely distributed throughout the cytoplasm. These data suggest that, similar to VPS28 and VPS37B, VPS4A can be recruited to plasma membrane independently of TSG101.

Because VPS4A is an ATPase, we sought to determine whether this enzymatic activity is required for VP40-induced redistribution of VPS4A using K173Q, a dominant-negative ATPase mutant of VPS4A. When expressed without VP40, VPS4A K173Q appeared as large globular structures within the
Figure 7. VPS4 gene knockdown protects mice against Ebola virus (EBOV) challenge. A, Western blot of lysates from human myeloid dendritic cells treated with a phosphorodiamidite morpholino oligonucleotide (PMO) against VPS4A. The position of the endogenous full-length VPS4A and the product of the VPS4A alternate reading frame (Alt. ORF) are indicated. VPS4A was detected using rabbit anti-VPS4. B, Protection of mice from lethal EBOV challenge with PMO treatment. Mice were treated with the indicated PMOs 24 h before infection with a lethal dose of mouse-adapted Zaire EBOV and again at 24 h and 48 h after challenge. Control mice received PBS.

cytoplasm (figure 5). Coexpression of wild-type VP40 caused clear accumulation of VPS4A K173Q at the plasma membrane (figure 5). These results show that the enzymatic activity of VPS4A is not required for redirecting VPS4A to the plasma membrane by VP40.

Requirement of enzymatic activity of VPS4A for efficient VLP release. Although our results show that the membrane recruitment of VPS4 by VP40 is independent of its enzymatic activity, a previous report suggested that the VPS4 enzymatic activity plays a role in the release of virions [12]. We examined this question in our VLP-release assay by transfecting cells with constructs encoding VP40 and GP plus wild-type VPS4A or enzymatically inactive VPS4A mutants K173Q and E228Q, [13]. The data are expressed as a ratio of the amount of VP40 detected in the media supernatant to the amount of VP40 contained in the cell lysates. The level of VLP release obtained by transfecting VP40 and GP was set at 100%. Figure 6 shows that expression of exogenous wild-type VPS4A did not have an impact on VLP release. However, mutants K173Q and E228Q reduced the amount of released VLPs by 60% to 80%, respectively, indicating that the ATPase activity of VPS4A is needed for efficient VLP release with an impact greater than that caused by deletion of the L domains.

Protection of mice from EBOV infection by knockdown of VPS4A gene expression. We have recently demonstrated that antisense PMOs targeted against several EBOV genes can inhibit viral replication and protect mice and monkeys from lethal infection [20]. To examine the role of VPS4A in viral replication in vivo, we constructed a PMO against a 100% conserved amino acid region that spans the start codon of human and mouse VPS4. We first examined the ability of this PMO to inhibit the expression of VPS4A in 293T, Vero E6, and dendritic cells. Treatment of human myeloid dendritic cells with this PMO for 24 h completely blocked the expression of VPS4A (figure 7A), and this blockage was sustained for at least 72 h (data not shown). Interestingly, blockage of the ATG start codon forced the expression of a truncated form of VPS4A, presumably resulting from initiation of translation at an alternative downstream ATG codon, most likely at position 595 (“Alt. ORF” in figure 7A). Similar results were also obtained in 293T cells and Vero E6 cells (data not shown). These data demonstrated that PMOs could potently inhibit the expression of VPS4 in primary cells.

We then sought to examine the effect of this PMO in a mouse lethal challenge model. We tested PMOs for 2 homologs of VPS4 found in both mice and humans: VPS4A and VPS4B. The mice that received PBS began to die of infection on the seventh day after challenge. More than half were dead by the eighth day, and only 20% survived. In contrast, mice that received the mouse/human VPS4A PMO had a delayed time to death at day 8, and 70% of the mice survived the challenge. Even those mice that were given the human VPS4B PMO (designed for a region of human VPS4B with just 66% homology to mouse sequence) had a 50% survival rate. Scrambled-sequence PMOs did not protect mice against lethal EBOV infection ([20] and data not shown). These data show that PMO inhibitors against VPS4 can be protective against lethal EBOV infection, indicating the importance of this molecule in regulation of EBOV replication in vivo.

DISCUSSION

In the present study, we demonstrated the ability of VP40 to utilize the vps machinery to support its budding at the plasma membrane in a manner independent of TSG101. VP40 mutants incapable of recruiting TSG101 to the plasma membrane were able to redirect other components of the vps pathway, such as VPS4, VPS28, and VPS37B, to the site of budding and to support VLP release. Our studies also demonstrate that the ATPase activity of VPS4 can be used as a potential therapeutic target against EBOV and, possibly, against other vps-using viruses.

Previous studies have suggested a key role for TSG101 in
linkage between the vps pathway and EBOV VP40 [5, 10] as a result of TSG101 binding to the PTAP motif of VP40. However, our findings demonstrate that this linkage is not entirely dependent on TSG101. In fact, the significant residual VLP release by L domain–deficient mutants was shown to still be dependent on the VPS pathway. In the absence of TSG101 recruitment to the plasma membrane, VP40 was able to redirect other vps proteins to the plasma membrane. When coexpressed, VP40 translocated VPS28, VPS37B, and VPS4 to the site of budding at the membrane. This effect was most pronounced with VPS4, which was entirely cytoplasmic when expressed alone but colocalized with VP40 at the plasma membrane when coexpressed with VP40. This effect was completely independent of both L domains of VP40. Although the VP40-dependent translocation of VPS4 to the plasma membrane did not require the enzymatic activity of VPS4, this enzymatic activity was required for VLP release.

The profound effect on VLP release caused by expression of dominant-negative VPS4, as well as previous reports showing a similar effect on replication of EBOV [12], prompted us to examine the potential of this enzyme as a therapeutic target in a lethal mouse model of EBOV infection. Specific knockdown of VPS4A and partial knockdown of VPS4B genes in mice resulted in partial protection against EBOV challenge. These data validate VPS4 as a promising target for filovirus therapeutics.

These data show, for the first time, that VP40 can use mechanisms other than those requiring the known L domains to hijack the vps pathway and use it for efficient budding. However, the molecular mechanism by which L domain mutants of VP40 interact with VPS4 remains to be determined. In summary, our findings suggest the existence of a novel pathway for recruitment of the cellular protein sorting machinery by EBOV for efficient budding. It remains to be seen whether such a pathway is also operational in other viruses. If so, targeting the VPS4 enzymatic activity by specific inhibitors or gene knockdown by antisense or RNA interference may lead to the development of broad-spectrum antivirals.

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