Vaccinia Virus Uses Retromer-Independent Cellular Retrograde Transport Pathways To Facilitate the Wrapping of Intracellular Mature Virions during Virus Morphogenesis

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ABSTRACT

Poxviruses, such as vaccinia virus (VACV), undertake a complex cytoplasmic replication cycle which involves morphogenesis through four distinct virion forms and includes a crucial wrapping step whereby intracellular mature virions (IMVs) are wrapped in two additional membranes to form intracellular enveloped virions (IEVs). To determine if cellular retrograde transport pathways are required for this wrapping step, we examined VACV morphogenesis in cells with reduced expression of the tetrameric tethering factor known as the GARP (Golgi-associated retrograde pathway), a central component of retrograde transport. VACV multistep replication was significantly impaired in cells transfected with small interfering RNA targeting the GARP complex and in cells with a mutated GARP complex. Detailed analysis revealed that depletion of the GARP complex resulted in a reduction in the number of IEVs, thereby linking retrograde transport with the wrapping of IMVs. In addition, foci of viral wrapping membrane proteins without an associated internal core accumulated in cells with a mutated GARP complex, suggesting that impaired retrograde transport uncouples nascent IMVs from the IEV membranes at the site of wrapping. Finally, small-molecule inhibitors of retrograde transport strongly suppressed VACV multistep growth in vitro and reduced weight loss and clinical signs in an in vivo murine model of systemic poxviral disease. This work links cellular retrograde transport pathways with the morphogenesis of poxviruses and identifies a panel of novel inhibitors of poxvirus replication.

IMPORTANCE

Cellular retrograde transport pathways traffic cargo from endosomes to the trans-Golgi network and are a key part of the intracellular membrane network. This work reveals that the prototypic poxvirus vaccinia virus (VACV) exploits cellular retrograde transport pathways to facilitate the wrapping of intracellular mature virions and therefore promote the production of extracellular virus. Inhibition of retrograde transport by small-molecule inhibitors reduced the replication of VACV in cell culture and alleviated disease in mice experimentally infected with VACV. This research provides fundamental new knowledge about the wrapping step of poxvirus morphogenesis, furthers our knowledge of the complex cellular retrograde pathways, and identifies a new group of antipoxvirus drugs.

Vaccinia virus (VACV) is the prototypic orthopoxvirus. It is a large double-stranded DNA virus with a complicated intracytoplasmic life cycle involving progression through four virion forms. The initial and most abundant virion type is the intracellular mature virion (IMV), which consists of a viral core surrounded by a single membrane. IMVs are fully infectious and can be released on cell lysis. Prior to lysis, a minority of the IMVs are wrapped in a double membrane to form intracellular enveloped virions (IEVs), which have three membranes (reviewed in reference 1). The origin of both the IMV and IEV membranes is much debated (2). Once it is wrapped, the IEV is transported to the cell periphery, where its outer membrane fuses with the plasma membrane to form a double-walled associated enveloped virion (CEV) on the surface of the cell. A selection of IEV-associated viral proteins becomes embedded in the plasma membrane of the cell as a result of this membrane fusion event. A proportion of the CEVs stimulates actin polymerization, resulting in the formation of actin tails (3,4). Once CEVs detach from the cell surface, they are known as extracellular enveloped virions (EEVs) (1). CEVs and EEVs are few in number (often only 1% of the number of IMVs present in the cell) but crucial for the virus since they mediate spread between neighboring cells and to more distant cells in vitro and in vivo (5–7).

Previous research has suggested that VACV may use retrograde transport pathways as part of its complex virion morphogenesis. The viral IEV membrane proteins A33, A36, B5, and F13 were found to accumulate at the plasma membrane following disruption of clathrin-mediated endocytosis (8–10), leading to the hypothesis that clathrin-mediated endocytosis is the first step in ret-
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One-step and multistep growth curves. One-step and multistep growth curves have been described previously (24, 25). Briefly, for one-step growth curves, cells were infected at a multiplicity of infection (MOI) of 5 PFU/cell for 1 h at 37°C and then washed with medium and incubated for the times indicated below. Supernatants were collected and centrifuged at low speed to remove the cell debris and then incubated with anti-I1 antibody (BEI Resources) for 1 h at 37°C in order to neutralize IMV particles. The titers of the virus present in the supernatants and cellular fraction were then determined on BS-C-1 cells. For the multistep growth curves, cells were infected at a low MOI (0.05 or 0.1 PFU/cell) for 1 h at 37°C. At the times indicated below, the supernatant and cellular fraction were combined by scraping the cells into the supernatant, and titers were determined by plaque assay on BS-C-1 cells.

Immunofluorescence. Cells to be examined for immunofluorescence were seeded on 20-mm by 20-mm coverslips at a density of 1.5 × 10^5 cells for HeLa cells and 2 × 10^5 cells per well for MEFs, and the coverslips were placed into six-well plates. The cells were fixed with 2 μl of neutral buffered formalin for 30 min, washed in PBS, permeabilized in 0.2% Triton X-100 for 5 min at room temperature, and washed 3 times in PBS. The cells were incubated with primary antibody at room temperature (RT) for 1 h in a humidity chamber. The coverslips were washed three times in PBS–2% FBS before addition of secondary antibody and fluorescein labeled phallolidin (Molecular Probes) diluted in PBS–2% FBS for 1 h at RT in a humidity chamber. After washing three times in PBS and once in distilled water, the coverslips were mounted in ProLong Gold mounting media.
medium containing DAPI (4’,6-diamidino-2-phenylindole; Molecular Probes). The slides were examined on a Zeiss 710 confocal microscope. The primary antibodies used were sheep anti-TGN46 (catalog no. AHP500; AbD Serotec), mouse anti-B5 (catalog no. NR-556; BEI Resources), and rat anti-F13. Secondary antibodies were sourced from Life Technologies (Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin, Alexa Fluor 568-conjugated goat anti-rabbit immunoglobulin, Alexa Fluor 568-conjugated goat anti-mouse immunoglobulin, and Alex Fluor 594-conjugated donkey anti-sheep immunoglobulin).

**Imaris image analysis.** Three-dimensional (3D) image analysis was carried out using Imaris image analysis software (Imaris, version 8.2; Bitplane AG, Switzerland). Two-dimensional confocal z-stacks were acquired on a Zeiss 710 microscope and rendered into three dimensions. The ImarisCell module was then used to segment the nucleus, cell, and B5-labeled vesicles. Vesicles were exported to spots and filtered for enhanced green fluorescent protein (EGFP) colocalization to identify B5-labeled structures with a core center.

**Pharmacological inhibition of retrograde transport in vitro.** Retro-1, VP-184, Retro-2, and Retro-2.1 were synthesized in-house (26, 27) and resuspended in dimethyl sulfoxide (DMSO) before serial dilution to a range of concentrations in FBS-free, antibiotic-free DMEM. Appropriate dilutions were added to a 6-well plate seeded with HeLa cells, and the plates were incubated for 2 h at 37°C in 5% CO2 before infection with VACV-EGFP for the length of time indicated below. The drugs remained present throughout infection and postinfection incubation. VP-184 is a Retro-1 analogue which gives increased protection against Shiga toxin and *Stigmatis negevensis* (28), and Retro-2.1 is an N-methylhydriodiquinazolinone derivative of Retro-2 with enhanced efficacy against Shiga toxin (26).

**Retro-2 intraperitoneal administration.** Retro-2 was resuspended in DMSO to a concentration of 66.6 mg/ml (210 mM). Groups of 6 mice were injected intraperitoneally with 270 µl Retro-2 diluted in 20 µl PBS was inoculated into the nostril. The mice were monitored daily for weight loss and changed in fluorescence levels (14, 23). HeLa cells were mock transfected or transfected with siRNA targeting VPS52 or nontargeting control siRNA and, after 48 h, fixed and labeled with antibody raised against TGN46 as well as with fluorescent phalloidin to outline the cytoskeleton. In the mock-transfected and nontargeting siRNA-transfected cells, TGN46 displayed a classic perinuclear location, consistent with the location of the trans-Golgi network. However, in cells transfected with siRNA targeting VPS52, the localization of TGN46 was altered to a much more punctate, juxtanuclear distribution (Fig. 1C), consistent with the findings presented in previous reports (17). When multiple cells were scored for normal or collapsed TGN46 localization, a highly statistically significant disruption of TGN46 localization, indicative of the disruption of EGRTP function, was evident in response to the depletion of VPS52 (Fig. 1D).

The impact of VPS52 on VACV replication and spread was then examined using VACV-A5-EGFP, a VACV strain which has the A5 viral core protein tagged with EGFP, thereby enabling virus growth to be estimated by measuring fluorescence levels (14, 23). HeLa cells were mock transfected or transfected with siRNA targeting RAB1A (a positive control), which is required for efficient VACV replication (25) or the VPS52 siRNA SMARTpool. In addition, the VPS52 SMARTpool was deconvoluted to its four constituent siRNAs, and each was tested individually. After 48 h, the cells were infected with VACV-A5-EGFP at a low MOI of 0.1 PFU/cell, and fluorescence was measured at 12-h intervals for a further 48 h, allowing multiple rounds of virus replication to occur (Fig. 1E). Fluorescence levels increased over time, as expected, and were comparable in the negative-control samples (mock and VP16 transfected) but significantly reduced at 36 and 48 h postinfection (p.i.) in the wells treated with positive-control siRNA (RAB1A), the VPS52 SMARTpool, and all four of the deconvoluted VPS52 siRNAs.

In order to determine directly the effect of depletion of VPS52 on infectious virion production, the experiment was repeated and VACV replication was measured using virus titration (Fig. 1F). After 48 h there was an average of a 1-log10 reduction in the amount of virus produced in the positive-control samples depleted of RAB1A, consistent with previous findings (25). In cells depleted of VPS52, there was a smaller but reproducible and statistically significant ($P < 0.05$) reduction of 0.5 log10 in the amount of virus detected compared to the amount in the negative-control samples transfected with nontargeting siRNA. Overall,
these results substantiate previously reported evidence from the high-throughput siRNA screens that VPS52 is required for the efficient multicycle growth of VACV.

Genetically induced destabilization of GARP impairs VACV replication. Depletion of GARP using siRNA resulted in a consistent and significant reduction of VACV replication in HeLa cells, but one that was less marked than that seen in response to depletion of other proviral host factors previously investigated (14). A possible explanation for this was the limited efficacy of the VPS52 siRNA treatment. As seen in Fig. 1D, 25% of the population of cells transfected with VPS52-targeted siRNA showed normally distributed TGN46, suggesting that the protein knockdown was insufficient in all cells to fully ablate EGRTP function. In order to achieve a uniform reduction in EGRTP function and to validate
the siRNA knockdown findings using an alternative method, we
used a mouse genetic mutant with a hypomorphic mutation in the
VPS54 subunit of GARP (30). In this mouse, a single missense
mutation converting codon 967 of the vps54 gene from leucine to
glutamine results in an unstable VPS54 protein with a shortened
half-life (29). This results in less overall GARP and a partial loss of
function, as seen in slower transport and mislocalization of the
retrograde-dependent cholera toxin B subunit (31). Wild-type
(WT) and mutant (MU) primary mouse embryonic fibroblasts
(MEFs) were isolated and immortalized through serial passage. In
the following experiments, two independently derived WT cell
lines and two MU cell lines were included to reduce the likelihood
that off-target mutations produced during immortalization were
responsible for the phenotype seen.

To confirm that the hypomorphic mutation destabilizes the
GARP complex, cell lysates of WT and MU immortalized MEFs
were collected and the levels of VPS52 were compared using West-
ern blotting. As shown in Fig. 2A and B, the levels of VPS52 pro-
tein were significantly reduced in the mutant cells by an average of
52%. A VACV multistep growth curve was then carried out on the
two WT cell lines and the two MU cell lines, and the amount of
virus present was determined by titration (Fig. 2C). A clear differ-
ence in virus titer between the WT and MU cell lines was apparent
from 12 h p.i., with an approximately 1-log
\(_{10}\) reduction in the
amount of virus being present in both MU cell lines at 12, 24, and
36 h p.i., and the amount narrowed to 0.5 log
\(_{10}\) at 48 h p.i. as virus
production plateaued. This result confirms by an independent
method and in a second cell type that GARP is required for opti-
mal multistep VACV replication.

GARP is required for production of IEVs and CEVs but not
IMVs. In order to examine the impact of GARP depletion on the
different virion forms produced during VACV replication, we car-
rried out a one-step growth curve on WT and MU MEFs, infecting
the cells at a high MOI (5 PFU/cell) and measuring virus produc-
tion over the following 24 h. The titers of virus present in the cells
and the supernatant were determined separately. Neutralizing an-
tibody to IMVs was added to the supernatant fraction prior to
titration to ensure that only the titers of EEVs that had been re-
leased from the cell were determined. No significant difference in
the amount of virus produced in the cellular fraction (which con-
ists almost entirely of IMVs) was detected at any time point (Fig.
2D), suggesting that initial stages of virus production were unaf-
fected by GARP depletion. However, a reduction in virus titer in
the supernatant fraction of up to 1 log
\(_{10}\) in the MU cells compared
with the titers in the WT cells was detected at 8, 12, and 24 h p.i. (Fig.
2E), indicating that fewer EEVs were produced from cells lacking
normal levels of GARP.

To identify the point at which EEV production was impacted
by reduced levels of GARP, we assessed and compared the number
of IEVs and CEVs present in the WT and MU MEFs using immu-
nolabeling and confocal fluorescence microscopy. MEFs were in-
fected with VACV-A5-EGFP at a high MOI of 5 PFU/cell and,
after 8 h, fixed, permeabilized, and labeled with antibody targeted to the IEV/CEV membrane protein B5 (32). Single optical slices taken through infected WT cells showed that the EGFP-tagged A5 protein was distributed in the expected pattern of large perinuclear viral assembly factories as well as in much smaller dispersed cytoplasmic puncta representing individual virus cores (Fig. 3A, top). Many, but not all, of these cores also stained positive for B5, thereby identifying them as IEVs/CEVs. However, this pattern altered in GARP mutant cells, as while there were still abundant peripherally dispersed viral cores (consistent with the unaltered titers of cell-associated virus), there were noticeably fewer puncta of B5 staining (Fig. 3A, bottom). Furthermore, high-magnification analysis of viral core and B5 localization showed that while most B5 puncta colocalized with the VACV-A5-EGFP fluorescence in WT cells (indicating that the foci were indeed IEV/CEVs), the MU cells contained many more B5 foci that lacked an associated core (Fig. 3B). Twenty-five WT and 24 MU cells from three independent experiments were analyzed by taking z-stacked images throughout the depth of the cells, reconstructing them as a 3D image, and analyzing the data using Imaris image analysis software to quantify the number of B5-labeled puncta. WT cells contained, on average, over 450 puncta per cell, but this was more than 2-fold lower in the MU cells (Fig. 4A). Analysis of the number of B5 puncta that coincided with EGFP fluorescence also confirmed a statistically significant drop in the proportion associated with cores, from over 90% in WT cells to about 60% in MU cells (Fig. 4B). However, when cells were costained for B5 and another IEV/CEV membrane protein, F13, there was no decrease in the level of colocalization in the MU cells, with a high proportion (over 90%) of vesicles being double positive for both IEV/CEV membrane proteins in WT and MU cells (Fig. 4C).

Further differences between B5-labeled structures in the cytoplasm of WT and MU MEFs were also evident, in that B5-labeled puncta in the MU cells showed more variation in size and had a small but statistically significant increase in their average diameter compared with IEVs/CEVs in the WT cells, from 0.96 μm to 1.08 μm. Frequency analysis of the data showed that this reflected an increase in the population of puncta with diameters of ≥1.6 μm in the MU cells (Fig. 4D). The two populations of B5 puncta in the MU cells were compared. The abnormal B5-labeled structures lacking an associated core were found to be significantly larger (125%) than the puncta associated with a core (IEVs/CEVs) (Fig. 4E). These results reveal that IMV wrapping is disrupted when the GARP complex is dysfunctional, resulting in accumulation in the cytoplasm of aberrant large B5-labeled puncta lacking an internal core.

VACV replication is independent of the retromer cargo-sorting protein complex. In order to define the retrograde transport pathway components involved in VACV replication, we investigated the role of the retromer complex. Retromer is a well-characterized key component of endosome-to-Golgi retrograde pathways. It is located in the endosomal membrane and acts as a cargo
sorter, binding to cargo in the lumen that is destined for retrograde trafficking and facilitating the formation and budding off of retrograde transport vesicles (33). We separately targeted two subunits of retromer (VPS35 and VPS26) using siRNA transfected into HeLa cells. Western blotting indicated successful knockdown of protein expression to levels less than 50% of those detected in cells transfected with nontargeting siRNA (Fig. 5A to D). To confirm that retrograde transport was disrupted in these cells, the location of TGN46 was examined. Cells with reduced levels of VPS35 or VPS26 revealed an abnormal localization of TGN46, confirming functional disruption (Fig. 5E). The efficacy of multistep VACV growth in cells with a knockdown in expression of either the VPS26 or the VPS35 subunit of the retromer complex was then assessed by infecting the siRNA-transfected cells with VACV-A5-EGFP at a low MOI of 0.1 PFU/cell and measuring the fluorescence every 12 h as a marker of virus replication (Fig. 5G). The positive-control siRNA which targets RAB1A, a known pro-viral host factor, reduced the fluorescence levels by over 25% at 48 h p.i.; however, a reduction of either retromer subunit had no significant effect on VACV replication compared to that in mock-transfected cells or cells transfected with the control nontargeting siRNA. No significant difference in cell death was detected in the different treatment groups after 48 h of virus infection, proving that the siRNA treatments were not toxic (Fig. 5F). This result suggests that VACV uses a nonclassical, retromer-independent retrograde trafficking pathway.

VACV is susceptible to pharmacological inhibition of retrograde transport pathways. Toxins such as ricin, Shiga toxin, and cholera toxin use EGRTP to escape the endosomal compartment and travel through the cytosol of the cell (16). A high-throughput screen of a small-molecule library in a ricin cytotoxicity assay identified potent pharmacological inhibitors of EGRTP, including Retro-1 and Retro-2, two compounds with a heterocyclic structure incorporating a central benzodiazepine or imine moiety (34). The mechanisms of function of Retro-1 and Retro-2 are unknown; however, they both cause a rapid redistribution of the SNARE protein syntaxin 5 (22, 34). The effect of both drugs is highly specific to retrograde endosome-to-TGN pathways; neither drug interferes with anterograde transport along the biosynthetic/secretory pathway from the endoplasmic reticulum via the Golgi apparatus and TGN to the plasma membrane or with endocytosis, recycling from the early endosome back to the plasma membrane or trafficking to late endosomes and the lysosome (31, 34). This specificity of action makes Retro-1 and -2 powerful tools when investigating retrograde transport.

The effect of Retro-1 and Retro-2 on VACV multistep replication was investigated. In addition, two related drugs, VP-184 and Retro-2.1, were also tested. VP-184 is closely related to Retro-1 (28), while Retro-2.1 is a novel, highly potent derivative of Retro-2 (26). HeLa cells were pretreated for 1 h with various concentrations of drug and then infected at a low MOI (0.05 PFU/cell) with VACV-A5-EGFP, and fluorescence was measured over the following 48 h. At 48 h p.i., cell viability was measured using CellTiter-Blue (Promega) and compared to the viability of cells infected and treated with medium alone. Increased levels of cell death were detected only at the highest concentration (100 μM) of Retro-1, VP-184, and Retro-2.1 tested (Fig. 6A). No cell death was present even at a 100 μM Retro-2 concentration, indicating a very low
FIG 5 VACV morphogenesis is independent of the retromer complex. HeLa cells were mock transfected or transfected with nontargeting siRNA (NT) or siRNA targeting VPS35 (A) or VPS26 (B). After 48 h, cells were harvested and proteins were separated using SDS-PAGE, probed with antibody raised against VPS35 or actin, and visualized using direct infrared fluorescence (LI-COR) in an Odyssey scanner. The levels of VPS35 (C) and VPS26 (D) relative to those of actin were quantified. (E) HeLa cells were mock transfected or transfected with nontargeting siRNA or siRNA targeting VPS26 or VPS35. After 48 h, cells were fixed and labeled with antibody raised against TGN46 and phalloidin. The percentage of cells with a normal punctate TGN46 distribution was analyzed. (F and G) HeLa cells were mock transfected or transfected with nontargeting siRNA or siRNA targeting VPS35, VPS26, or RAB1A (positive control). (G) After 48 h, cells were infected with VACV-EGFP at 0.1 PFU/cell and fluorescence was measured at the indicated times. (F) At 48 h p.i., cell death was measured using CellTiter-Blue and is presented as arbitrary fluorescence units. The data represent those from six technical replicates, and error bars represent SEMs. Results were analyzed by Student’s t test. ***, P < 0.001, kd, knockdown.
FIG 6 Inhibition of retrograde transport with small-molecule inhibitors reduces IEV production. (A) HeLa cells were treated with various concentrations of drug, as indicated, and infected with VACV-EGFP at 0.1 PFU/cell. After 48 h, cell death was measured using CellTiter-Blue. Cell death is expressed as a percentage of the survival of infected cells treated with medium alone. Data are averages and SEMs from six technical replicates. (B to E) HeLa cells were treated with various concentrations of the indicated drug and infected with VACV-EGFP at 0.1 PFU/cell. Fluorescence was measured at the indicated time points. Data are average and SEMs from six technical replicates and were analyzed using a one-way analysis of variance with multiple comparisons of the data at 48 h postinfection. ***, P < 0.001. (G and H) One-step growth curve. HeLa cells were treated with the compounds indicated and infected with VACV-WR at 5 PFU/cell. At 0 and 24 h p.i., cells (F) and supernatants (G) were collected and virus titers were determined by plaque assay on BS-C-1 cells. ***, P < 0.001, one-way analysis of variance with multiple comparisons of the data at 48 h postinfection.
toxic impact of the drug. EGFP fluorescence measurement revealed a strong inhibitory effect of Retro-1, VP-184, Retro-2, and Retro-2.1 on VACV growth (Fig. 6B to E) compared to the growth of the controls treated with medium or DMSO. This suggests that endosome-to-Golgi apparatus trafficking is required for VACV multistep replication.

The concentrations of Retro-1 (1 μM and 10 μM) and Retro-2 (10 μM and 100 μM) that showed efficacy against VACV replication are comparable to the concentration (25 μM) used to block the activity of ricin and Shiga toxin (34). When comparing the effective concentrations of Retro-2 and Retro-2.1, we found that the more potent derivative drug, Retro-2.1, displayed a 100-fold increase in efficacy against VACV, with over 90% inhibition of virus replication being found at a concentration of just 100 nM. This finding is similar to that of previously reported work which found Retro-2.1 to be 100-fold more potent at preventing AAV transduction (22) and 500- to 1,000-fold more potent at blocking Shiga toxin and ricin toxicity (27).

To determine the stage of the VACV life cycle that is targeted by the four retrograde transport inhibitors, a one-step growth curve was carried out in the presence of drug, DMSO, or medium alone, and the cell-associated virus titer was determined separately from the titer of virus present in the supernatant after 24 h of infection. There was no detectable difference in the titer of cell-associated virus, with a greater than 2-log10 increase in the amount of virus present in all samples being found after 24 h. In contrast, the amount of EEV in the supernatant increased by over 1.5 log10 in the control samples treated with medium alone or carrier (DMSO) but less than 1 log10 in the samples treated with any of the four drugs (Fig. 6F and G). These results are strikingly similar to those obtained from one-step growth curves of MEFs expressing a GARP mutant (Fig. 2D and E) and provide compelling evidence that VACV uses EGTP to facilitate virion morphogenesis.

**Retro-2 treatment alleviates systemic disease in mice infected with VACV.** Inhibition of EGRTP in vitro strongly inhibited the production of EEVs. *In vivo* EEVs are the virion form responsible for the systemic spread of VACV around the body (5, 7). We therefore investigated whether pharmacological inhibition of EGRTP influenced disease in mice experimentally infected intranasally with VACV. Mice were injected intraperitoneally with 100 mg of Retro-2 per kg of body weight on two occasions. One group received the drug 24 h before and 24 h after infection with 1 × 10^6 PFU VACV, while a second group received the drug 24 h and 48 h after infection with VACV. Control groups received no drug and no virus, drug alone, or virus inoculation plus intraperitoneal (i.p.) injection of vehicle alone (DMSO). Mice given i.p. drug but no virus consistently gained weight across the experiment and were indistinguishable from the negative-control group of mice given no drug or virus (Fig. 7A; compare groups 1, 5, and 6). This confirms previous reports of the excellent safety profile of Retro-2 after i.p. injection in mice (34–36). As expected, mice infected with VACV and treated with DMSO lost weight rapidly between day 4 and day 7 of the experiment and exhibited ruffled fur, a hunched posture, and reduced activity. Three out of the 6 mice in this group were euthanized due to the severity of clinical signs. Mice infected with VACV and treated with Retro-2 at 24 and
48 h postinfection lost weight just as rapidly as the untreated infected mice, and one mouse was euthanized. However, mice treated with Retro-2 24 h prior to and 24 h after VACV infection lost less weight ($P = 0.05$) and exhibited significantly less severe clinical signs of disease ($P < 0.01$) (Fig. 7A and B; compare group 3 with groups 2 and 4). None of the mice in this group reached the humane endpoint requiring euthanasia, revealing a protective effect of Retro-2 on VACV-induced disease in mice. The amount of virus in the lungs of each mouse in groups 2, 3, and 4 was determined (Fig. 7C). The average number of PFU of VACV in the lungs of mice in group 3 was lower than that in the lungs of mice in groups 2 and 4; however, this reduction did not reach the level of statistical significance (group 2 versus group 3, $P = 0.07$, Student’s $t$ test).

**DISCUSSION**

This work outlines the reliance of VACV on cellular endosome-to-Golgi retrograde transport pathways in order to produce the wrapped IEV form. Retrograde pathways were inhibited by three different mechanisms (siRNA knockdown, a genetic hypomorphic mutation, and pharmacological inhibition), all of which resulted in a significant reduction in VACV multistep replication. Closer analysis revealed that the levels of IMV were unaffected by a reduction of the level of expression of the GARP complex; however, there was a drop in the number of IEVs/CEVs accompanied by aberrant accumulations of B5, indicating that the wrapping of IMVs had been disrupted. Wrapping of IMVs in an additional two layers of membrane is a poorly understood but crucial process in the life cycle of VACV and other poxviruses (2). The site of wrapping to occur, but the exact function of each of these proteins in the process is unclear (37–39). The identification of retrograde pathways and, specifically, the GARP complex as a crucial component of this process marks a decisive step forward in our understanding of this unique stage of poxvirus morphogenesis.

The GARP complex was targeted in our studies on the basis of results from two siRNA screens. A screen from our laboratory identified the VPS52 subunit to be a strong proviral host factor (13). GARP is a member of the CATCHR (complexes associated with tethering containing helical rods) family of multisubunit tethering complexes, which are large proteins or protein complexes that establish long-range interactions between transport vesicles and their target compartment. GARP is localized to the TGN, where it facilitates tethering and, ultimately, the fusion of transport vesicles traveling in a retrograde direction from endosomes to the TGN (40). Cargoes which require GARP for transport to the TGN include receptors for lysosomal hydrolase precursors, such as the mannose 6-phosphate receptors, the TGN-resident protein TGN46 (17), and sphingolipids (18). Our work supports a model whereby the GARP complex tethers transport vesicles containing viral membrane proteins as they are recycled back from the plasma membrane to the TGN via retrograde transport pathways for reuse. VACV proteins embedded in the outer membrane of IEVs, including B5 and F13, remain on the surface of the cell after the IEV fuses with the plasma membrane during virion exit from the cell (1), and green fluorescent protein-tagged overexpressed B5 or F13 chimeras have been shown to relocate from the cell surface to a juxtanuclear location (8–10), suggesting retrograde transport of these crucial membrane proteins. In this model, the abnormal B5 puncta that lack an internal core that were identified in the MEFs with a mutated GARP complex would represent returning transport intermediates which were unable to dock with the site of IMV wrapping and IEV production. This model would support the hypothesis that the wrapping membranes originate from the TGN. However, we cannot rule out an alternative model whereby the GARP complex is needed for the recycling and correct localization of an as-yet-unidentified host protein(s) which is required for the wrapping process. In this model, the abnormal B5 accumulations would represent IEV membranes which failed to be successfully wrapped around the IMV, somewhat analogous to HSV-1 L particles (41).

In order to characterize the abnormal B5 accumulations, we compared the location of B5 and another IEV/CEV protein, F13, in WT and MU MEFs. F13 colocalized with B5 in both cell types, suggesting that the abnormal B5 puncta in the MU MEFs are likely related to IEV wrapping membranes rather than accumulations of individual mislocalized viral proteins.

We have uncovered evidence that VACV uses a nonclassical route of retrograde transport that is independent of the protein complex retromer. Previous work characterized two main retrograde pathways, with the first one being from early endosomes to the TGN (sometimes via recycling endosomes) and the second once being from late endosomes to the TGN. Each pathway involves a specific set of vSNAREs, tSNAREs, tethers, and GTPases; however, both rely on retromer for initial sorting of the endosomal cargo (16, 42). Retromer therefore plays a key role in both these pathways and is required for retrograde transport of many endogenous proteins as well as the correct localization of the HIV Env protein (20), the entry of HPV16 (21), and the transport of Shiga toxin (43, 44). Retromer is not, however, required for the retrograde pathways involved in AAV transduction (22) or VACV IEV formation (this work). Our results therefore confirm the existence of a retromer–independent retrograde transport pathway distinct from the two classical pathways previously described and highlights the complexity of cellular retrograde pathways. This complexity makes retrograde transport an attractive therapeutic option, since the precise inhibition of one component of a particular pathway can result in a very specific impact on a process, such as virion wrapping, but leave other retrograde pathways functional and therefore minimize indirect effects on the cell.

Our work shows that GARP is required for at least one of the retrograde transport pathways used by VACV but does not rule out the possibility that VACV uses multiple retrograde transport pathways, some of which are independent of GARP.

The highly effective inhibition of VACV multistep replication and EEV production by the retrograde transport inhibitors Retro-1, Retro-2, and related compounds highlighted the importance of retrograde transport to poxviruses. These drugs have been shown to inhibit processes dependent on retrograde transport, such as the activity of ricin, Shiga toxin, and Shiga-like toxins 1 and 2 (26, 34), and the entry of AAV (22). The wrapping process of VACV is targeted by the two well-characterized antioptoxovirus drugs IMCBH [N(1)-isonicotinoyl-N(2)-3-methyl-4-chlorobenzoylhydrazine] and ST-246 [4-trifluoromethyl-N-[3,3a,4,4a,5,5a,6,6a-octahydro-1,3-di oxo-4,6-ethenocycloprenyl][f]isoindol-2(1H)-yl]-benzamide]. IMCBH is a selective inhibitor of VACV multiplication (45). It targets the viral protein F13, which is essen-
tional for wrapping but has no activity in vivo and is therefore not a candidate for therapeutic use (46). The more recently characterized drug, ST-246, also targets F13 (47, 48). Retro-1 and -2 target the same process (wrapping) as these two drugs in vitro and show the same virus replication profiles as ST-246–treated cells, namely, a 5–10-fold reduction in EEV production in a one-step growth curve, which equates to an almost complete reduction in multistep growth. However, Retro-1 and -2 exert their effect via an as yet unknown host protein rather than a viral protein and are therefore less susceptible to viral escape via mutations in the F13 gene, as seen in VACV strains which develop resistance to IMCBH and ST-246 (46, 48).

Retro-1 and Retro-2 have been shown to have in vivo efficacy against ricin (34), Shiga toxin produced by enterohemorrhagic Escherichia coli O104:H4 (36), and Leishmania (35) in mice. Since the drugs showed strong inhibition of VACV replication in vitro, we tested the efficacy of Retro-2 in vivo using an intranasal murine model of poxvirus disease. We compared two treatment regimes of 100 mg/kg given twice, either once before and once after infection (days −1 and +1) or twice after infection (days +1 and +2). Mice treated with Retro-2 prior to infection lost less weight and displayed less severe disease, and all mice survived the challenge. Pretreatment with Retro-2 therefore provided protection against poxviral disease, indicating that this family of small molecules may be developed as novel antipoxviral drugs for diseases such as cowpox and monkeypox and to supplement vaccination in re-

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