Orsay δ Protein Is Required for Nonlytic Viral Egress

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ABSTRACT Nonenveloped gastrointestinal viruses, such as human rotavirus, can exit infected cells from the apical surface without cell lysis. The mechanism of such nonlytic exit is poorly understood. The nonenveloped Orsay virus is an RNA virus infecting the intestine cells of the nematode *Caenorhabditis elegans*. Dye staining results suggested that Orsay virus exits from the intestine of infected worms in a nonlytic manner. Therefore, the Orsay virus-*C. elegans* system provides an excellent *in vivo* model to study viral exit. The Orsay virus genome encodes three proteins: RNA-dependent RNA polymerase, capsid protein (CP), and a nonstructural protein, δ . δ can also be expressed as a structural CP- δ fusion. We generated an ATG-to-CTG mutant virus that had a normal CP- δ fusion but could not produce free δ due to the lack of the start codon. This mutant virus showed a viral exit defect without obvious phenotypes in other steps of viral infection, suggesting that δ is involved in viral exit. Ectopically expressed free δ localized near the apical membrane of intestine cells in *C. elegans* and colocalized with ACT-5, an intestine-specific actin that is a component of the terminal web. Orsay virus infection rearranged ACT-5 apical localization. Reduction of the ACT-5 level via RNA interference (RNAi) significantly exacerbated the viral exit defect of the δ mutant virus, suggesting that δ and ACT-5 functionally interact to promote Orsay virus exit. Together, these data support a model in which the viral δ protein interacts with the actin network at the apical side of host intestine cells to mediate the polarized, nonlytic egress of Orsay virus.

IMPORTANCE An important step of the viral life cycle is how viruses exit from host cells to spread to other cells. Certain nonenveloped viruses can exit cultured cells in nonlytic ways; however, such nonlytic exit has not been demonstrated *in vivo*. In addition, it is not clear how such nonlytic exit is achieved mechanistically *in vivo*. Orsay virus is a nonenveloped RNA virus that infects the intestine cells of the nematode *C. elegans*. It is currently the only virus known to naturally infect *C. elegans*. Using this *in vivo* model, we show that the δ protein encoded by Orsay virus facilitates the nonlytic exit of the virus, possibly by interacting with host actin on the apical side of worm intestine cells.

KEYWORDS *Caenorhabditis elegans*, Orsay, viral egress

Viral egress is an important step of the viral life cycle. Two mechanisms are commonly employed by viruses to exit host cells, cell lysis and viral exocytosis (1). In cell lysis, viruses are released after the burst of host cells. In viral exocytosis, viruses bud through host cell membranes to reach the extracellular space. It is commonly believed that enveloped viruses egress via viral exocytosis and acquire their envelopes when traversing host cell membranes and that nonenveloped viruses rely on cell lysis to egress. However, several reports demonstrated that nonenveloped viruses could also exit cells via nonlytic pathways. Nonenveloped viruses, such as human hepatitis A virus (HAV) (2), poliovirus (3), simian virus 40 (SV40) (4), and rotavirus (5), can all exit polarized cells from the apical surface without cell lysis. The mechanism of this process is poorly understood.

One challenge in studying viral egress is that this process is affected by the host cell

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physiology. For example, it was reported previously that the nonlytic egress process of poliovirus may be observed only in polarized cells mimicking natural host cells and that lysis is required for the same virus to be released from nonpolarized cultured cells (3). Therefore, *in vivo* models of viral egress are important to study this host-virus interaction process under natural host cell physiology.

The nematode *Caenorhabditis elegans* has been a successful *in vivo* model for studies of host-pathogen interactions, including bacterial pathogens such as *Pseudomonas*, intracellular parasites such as *Nematocida parisii* (6), and, lately, the intestinal Orsay virus (7). Discovered in 2011, the nonenveloped Orsay virus is currently the only known virus that naturally infects the nematode *C. elegans* (7). Orsay virus infects *C. elegans* intestine cells and is transmitted horizontally (7). Orsay virus infection does not affect the animal life span or brood size but dramatically changes the morphology of intestine cells: the storage granules disappear, the cytoplasm loses viscosity and becomes fluid, and intermediate filaments become disorganized near the apical border (7). The *C. elegans* intestine consists of 20 large epithelial cells that resemble human intestinal epithelial cells, in that both types of cells are polarized and have structures such as microvilli and a terminal web (8). *C. elegans* intestine cells can be easily observed *in vivo* because of the transparent body of the worms. Therefore, the *C. elegans*-Orsay virus system provides an excellent model to study natural host-virus interactions in a live, intact animal.

Together with two other nematode viruses, Santeuil and Le Blanc viruses, both of which infect *Caenorhabditis briggsae* (7, 9), Orsay virus represents a new class of viruses. These three viruses have a bipartite, positive-stranded RNA genome that is distantly related to the genome of nodaviruses (7, 9). Their genomes range in size from 6.3 to 6.5 kb, with only three open reading frames (ORFs). The RNA1 segment contains one ORF, encoding the RNA-dependent RNA polymerase (RdRP), and the RNA2 segment contains two ORFs, encoding capsid protein (CP) and δ (7, 9). δ is particularly intriguing as it shows no homology to any sequence in GenBank (7). δ can also be expressed as a CP- δ fusion protein through ribosomal frameshifting (10). CP- δ has been detected in purified viruses, but the expression of free δ has yet to be confirmed (10). It was reported previously that δ has no RNA interference (RNAi) suppression activity (11), unlike nonstructural proteins from related nodaviruses.

We have recently shown that δ forms a pentameric filament and that CP- δ is incorporated into the Orsay virus capsid with an essential role in viral entry (12). Here we show that Orsay virus exits the host intestine cells in a nonlytic fashion and that δ is required for efficient viral egress. δ accumulates near the apical surface of intestine cells and genetically interacts with host actin. Our data suggest that Orsay virus uses δ to rearrange host actin at the terminal web to facilitate nonlytic viral exit. These results bring insights into the mechanisms of how nonenveloped viruses achieve polarized nonlytic exit *in vivo*.

RESULTS

Nonlytic egress of Orsay virus. Several empirical observations suggested that Orsay virus may utilize nonlytic exit from *C. elegans* intestine cells. For example, we have never observed any infected animal with a gap of missing intestine cells that might indicate lytic exit. The infective animals are alive and have a life span similar to that of uninfected worms (7), suggesting that lytic exit is unlikely for Orsay virus, considering that the entire *C. elegans* intestine is composed of only 20 large epithelial cells (8). However, it is still possible that infected intestine cells have damaged cellular integrity while appearing intact at the gross-morphology level.

To investigate whether Orsay virus infection caused damage to the cellular integrity of intestine cells, we fed the animals propidium iodide (PI), a dye that cannot diffuse through intact cell membranes (13). In normal worms with intact intestine cells, PI stayed in the intestine lumen, and there was no intracellular PI staining (Fig. 1A). As a positive control, worms fed the *Bacillus thuringiensis* pore-forming toxin Cry5B (14) had extensive intracellular PI staining (Fig. 1B). Orsay virus-infected animals often had an

FIG 1 Orsay virus has nonlytic exit. Shown is propidium iodide staining of uninfected (A), Cry5B-treated (B), and Orsay virus-infected (C) animals. From left to right, the images show PI staining, merged PI staining, and Nomarski images, with merged PI staining and autofluorescence in the blue channel. *, intracellular regions that were not stained by PI. Bar, 10 μ m.

enlarged intestine lumen, possibly caused by constipation, yet PI staining was limited to the lumen and was not found in the cytosol (Fig. 1C). Therefore, Orsay virus infection did not appear to cause any damage to host cell integrity.

We quantified the PI staining results for intestine cells. One hundred percent (56/56) of uninfected animals had no intracellular PI staining, 100% (65/65) of Cry5B-treated animals showed intracellular PI staining, and 97% (60/62) of Orsay virus-infected animals showed no intracellular PI staining, while results for the remaining 3% (2/62) of worms were inconclusive due to an overenlarged and distorted intestine lumen. These data strongly suggested that Orsay virus exits host cells nonlytically.

δ ATG mutant virus has viral exit defects. To investigate the free δ function, we used a reverse-genetics system (15) (Fig. 2A) to generate a mutant virus that had an ATG-to-CTG mutation in the start codon of the δ ORF and thus in theory could not produce free δ due to the lack of the start codon. Upon infection by the ATG mutant virus, worms showed normal viral loads, as determined by reverse transcription-quantitative PCR (qRT-PCR) (Fig. 2B), and displayed normal viral infection symptoms (12), suggesting that free δ is not required for viral entry or replication. In contrast, the culture medium showed a significantly reduced viral load (Fig. 2B), suggesting a viral exit defect.

Considering that the ATG mutation also introduced an M $\rightarrow$ L amino acid change in CP- δ , we examined the effects of the ATG mutation on viral entry using a worm strain with the transcriptional reporter *F26F2.1p::GFP*. This strain expresses green fluorescent protein (GFP) upon Orsay virus exposure, presumably due to certain innate immune responses (16). Both the ATG mutant virus and the wild-type (WT) virus showed similar infection kinetics, with similar percentages of GFP-positive worms at each time point after viral exposure (Fig. 2C), suggesting that the ATG mutant virus had few or no entry defects.

We also examined the stability of the ATG mutant virus by testing its titer over an extended storage period (Fig. 2D). The viral titer was determined by using serial

FIG 2 The ATG mutant virus has exit defects. (A) Schematic drawing of the strategy for the generation of recombinant virus. (B) qRT-PCR results showing viral loads in infected worms and in culture media. Data were obtained from three biological replicates, each with three technical replicates. Bars and error bars show means and standard errors (SE). **, $P < 0.01$ by Student's t test. (C) Viral infection kinetics based on *F26F2.1p::GFP* expression in infected worms ($n \geq 529$ worms for each time point). Data were compiled from three independent trials with six plates for each virus genotype in each trial. (D) Viral titers of the worm lysate over storage time. The titer was normalized so that the fresh lysate (day 1) had a relative titer of 1. (E) Schematic drawing of the single-worm infection assay. (F) Single-worm infection assay results comparing the ATG mutant virus and the wild type. Data were obtained from four independent trials, each with six plates for each time point. The graph displays means and SE.

dilutions of the worm lysate to test the ability of the viruses to turn on GFP in *F26F2.1p::GFP* worms. Our data showed that the stabilities of the mutant and wild-type viruses were comparable up to 10 days (Fig. 2D). Therefore, the observed viral load difference in the medium was not caused by a difference in viral stability. It is worth noting that all our other experiments used fresh worm lysates and were completed within 5 days.

To confirm the viral exit defect in the ATG mutant, we developed a single-worm infection assay (Fig. 2E). In this assay, a single infected adult worm was placed onto a clean plate with naïve young L1 larvae for a certain time before being removed. When the young L1 worms grew to day 3 adults, they were scored for the Orsay virus infection symptom of a transparent intestine, as previously described (12). If a plate of naïve worms had a high infection rate, with $>50\%$ of worms showing the symptom of a transparent intestine, we counted this plate as infected. In this assay, infection of naïve worms depended on the amount of virus shed from the single infected worm during the given time window.

The single-worm infection assay confirmed that the ATG mutant virus had an exit defect. For wild-type Orsay virus, the amount of virus shed in half an hour from an infected worm was sufficient to infect a plate of worms 67% of the time (Fig. 2F). In contrast, virus shed from a worm infected with the ATG mutant virus was sufficient to infect a plate only 13% of the time (Fig. 2F). Allowing the worms infected with ATG mutant viruses to shed virus for a longer time increased the efficacy of infection (Fig. 2F). Sequencing data confirmed that the ATG mutation in the virus was not reverted in

FIG 3 Free δ is localized near the apical membrane in intestine cells. (A to E) Nomarski and fluorescence images showing the subcellular localizations of GFP-tagged δ (A), CP- δ (B), CP (C), N-terminal aa 1 to 66 of δ (D), and C-terminal aa 67 to 346 of δ (E). Arrowheads indicate apical and basolateral membrane localizations. * indicates nuclear localization. Arrows indicate aggregates. (F) Colocalization of δ and ACT-5. The schematic drawing shows ACT-5 localization in an intestine cell. Bar, 10 μm .

any of the samples, suggesting that the mutant virus could still egress from the host although at a lower rate.

δ is located near the apical membrane of host intestine cells. To further investigate δ function, we made transgenic worms expressing GFP-tagged δ under the control of a heat shock promoter. Upon the heat shock-induced synthesis of δ , we observed that δ was predominantly located near the apical membrane in the intestine cells (Fig. 3A). Similarly, we investigated CP- δ . From our protein biochemical data (12), it is expected that the proper folding of CP- δ requires the coexpression of CP and CP- δ . Consistent with this, we found that CP- δ formed many aggregates in the absence of CP (Fig. 3B). However, a small soluble portion of CP- δ was located near the apical

membrane of the intestine cells, confirming the specificity of the δ subcellular localization. In contrast, CP displayed a more ubiquitous cellular localization: near both the apical and the basolateral membrane, in the cytosol, and in the nucleus (Fig. 3C). Therefore, the apical localization is likely specific to δ .

Similar to human epithelial cells, *C. elegans* intestine cells are polarized cells, with the apical membrane facing the intestinal lumen. The apical membrane is the most likely site where Orsay virus exits host cells to be spread to other worms. The apical localization of δ is thus highly consistent with its function in viral exit.

Next, we investigated which domain is essential for the apical localization of δ . The N-terminal δ fragment (amino acids [aa] 1 to 66) displayed an apical localization similar to that of full-length δ (Fig. 3D). In contrast, the C-terminal δ fragment (aa 67 to 346) was diffusively located in the cell (Fig. 3E), suggesting that the N-terminal 66 amino acids are necessary and sufficient to determine the δ apical localization.

δ colocalizes with the host actin ACT-5. Like human epithelial cells, *C. elegans* intestine cells have microvilli and a terminal web with an apical subcellular localization (8) (Fig. 3F). One well-known marker for apical subcellular localization in *C. elegans* intestine cells is the actin ACT-5. ACT-5 is a unique actin isoform that is exclusively expressed in microvillus-containing cells and is located in both microvilli and the terminal web in intestine cells (17). To pinpoint the localization of δ , we generated a worm strain that expresses both δ ::GFP and mCherry::ACT-5. δ was found to colocalize with ACT-5 (Fig. 3F).

Orsay virus infection rearranges host actin networks. It was observed that the microsporidian parasite *N. parisii* could rearrange ACT-5 to mediate its nonlytic release from *C. elegans* intestine cells (13). To understand the role of ACT-5 in Orsay virus infection, we asked whether Orsay virus infection could cause ACT-5 rearrangement. To test this, we obtained two marker strains that express mCherry::ACT-5 and YFP::ACT-5 (18, 19), respectively. In both marker strains, ACT-5 fluorescence was observed near the apical membrane of the intestine (18, 19). We put these worms on *rde-1* RNAi bacteria to make them sensitive to Orsay virus infection and observed ACT-5 localization after viral infection.

Abnormal YFP::ACT-5 localization was observed 50 h after Orsay virus infection, as ACT-5 fluorescence became weakened at the apical membrane (Fig. 4A). Image quantification revealed that 63% of infected animals had weakened fluorescence, a significant increase ($P < 0.0001$ by Fisher's exact test) from the 7% among uninfected animals (Fig. 4B). When worms were infected with the ATG mutant virus, only 23% of the animals had weakened fluorescence (Fig. 4B).

To detect whether this weakened fluorescence was caused by a decreased protein amount, we examined the amount of YFP::ACT-5 protein by Western blotting. The amount of YFP::ACT-5 protein remained largely the same after Orsay virus infection (Fig. 4C), suggesting that the weakened YFP::ACT-5 fluorescence in infected worms was likely caused by protein relocation.

Observation at a higher magnification revealed that the actin relocation appeared to follow the infection time course. ACT-5 relocation was observed 24 h after Orsay virus infection in anterior intestine cells as abnormal branches formed toward the basolateral side (Fig. 4D and E). Fifty hours after Orsay virus infection, ACT-5 branches were observed in intestine cells that were more posterior in the worms (Fig. 4F and G).

ACT-5 branches were difficult to be quantified using YFP::ACT-5 because fluorescence was weakened at 50 h postinfection. mCherry::ACT-5 fluorescence was brighter than YFP::ACT-5 fluorescence, possibly due to a different reporter or a different transgene copy number. The brighter mCherry::ACT-5 enabled us to better quantify the branching phenotype. Fifty hours after Orsay virus infection, mCherry::ACT-5 showed several abnormalities, including branches (Fig. 4H), gaps where fluorescence disappeared in one intestine cell (Fig. 4I), and visibly weakened fluorescence. A total of 26% of infected animals showed such abnormal mCherry::ACT-5 localizations, significantly ($P < 0.001$ by Fisher's exact test) higher than the 4% observed among uninfected animals (Fig. 4J).

FIG 4 Orsay virus infection rearranges the ACT-5 actin structure. (A) YFP::ACT-5 localization at the apical membrane is weakened 50 h after Orsay virus infection. Bar, 100 μ m. (B) Percentages of animals with weak YFP::ACT-5 in uninfected worms, worms infected with the wild-type virus, and worms infected with the ATG mutant virus. ***, $P < 0.001$ by Fisher's exact test ($n \geq 125$ animals for each group). (C) Western blotting. Tubulin was used as a loading control. The graph displays means and standard errors of data from two biological replicates, each with five technical replicates. N.S., not significant. (D to I) Orsay virus infection causes ACT-5 to have branches and gaps. (D and E) Twenty-four hours after Orsay virus infection, YFP::ACT-5 branches appear in anterior cells of infected (E) but not uninfected (D) worms. (F and G) Fifty hours after infection, YFP::ACT-5 branches appear in cells close to the midbody of infected (G) but not uninfected (F) worms. (H and I) Also 50 h after infection, branches (H) and gaps (I) can be observed in mCherry::ACT-5. Bar, 10 μ m. (J) Percentages of animals with abnormal mCherry::ACT-5 in uninfected and infected worms. **, $P < 0.01$ by Fisher's exact test ($n \geq 69$ animals for each group). For quantification purposes, if a worm showed multiple phenotypes, it was classified in the category of its most severe phenotype, following the phenotypic severity order of gap > branch > weak (from severe to weak).

Together, these data suggested that Orsay virus infection induces ACT-5 rearrangement.

δ genetically interacts with host actin. It was observed that in comparison with worms infected by the wild-type virus, a significantly reduced number of animals infected by the ATG mutant virus had weakened YFP::ACT-5 fluorescence (23% versus 63%; $P < 0.0001$ by Fisher's exact test) (Fig. 4B), suggesting that δ is required to rearrange host actin.

To study the functional interaction of δ with host actin in intestine cells, we performed *act-5* RNAi by feeding and studied its impact on Orsay virus infection. As *act-5* RNAi is lethal to larvae, we diluted the *act-5* RNAi bacteria with control bacteria at a 1:25 ratio. Under these conditions, *act-5*(RNAi) worms can grow to adults with morphologically normal intestine cells. We infected these worms with wild-type and ATG mutant viruses and performed a single-worm infection assay to examine the amount of virus shed from these animals in 24 h. Upon infection by wild-type Orsay

FIG 5 Free δ has biological functions. (A) δ genetically interacts with *act-5*. Single-worm infection assay results show the effects of the δ ATG mutation in the virus and *act-5(RNAi)* on hosts. Data are from four independent trials, each with six plates for each genotype. The graph displays means and SE. N.S., not significant. *, $P < 0.05$ by Student's t test. (B) Nomarski images showing distal tip cell (DTC) migration defects in some worms upon the heat shock-induced overexpression of δ ::GFP. Bar, 10 μ m. Schematic line drawings under the images show the DTC migration path. (C) Single-worm infection assay results showing that the effects of expressing δ ::GFP in host cells rescued the viral exit defects of the δ ATG mutant virus. Data are from five independent trials, with 6 or 12 plates for each genotype in each trial. The graph displays means and SE. *, $P < 0.05$ by Student's t test.

virus, *act-5(RNAi)* showed no significant impact on viral egress ($P = 0.15$ by Student's t test) (Fig. 5A). However, upon infection by the ATG mutant virus, *act-5(RNAi)* significantly reduced the viral egress efficiency ($P = 0.02$ by Student's t test) (Fig. 5A). The combination of *act-5(RNAi)* and the δ ATG mutation resulted in a 16.7% infection rate in this assay, which was significantly lower than the 43.8% expected from a quantitative epistasis model (20) assuming no genetic interactions between *act-5(RNAi)* and the δ ATG mutation ($P = 0.04$ by Student's t test) (Fig. 5A). The synergistic effects of *act-5(RNAi)* and the δ ATG mutation suggested that δ interacts with ACT-5 functionally to promote efficient viral exit.

Free δ expressed in host cells has biological functions. Free δ was not detected in Western blots (10). One hypothesis is that free δ is produced at a level too low to be detected by the current δ antibody. An alternative hypothesis is that free δ is not produced and that the viral exit phenotype of the δ ATG mutant virus was caused by defective CP- δ due to the M→L mutation. We have not generated a sensitive antibody to detect free δ ; thus, we could not rule out the possibility of the second hypothesis. However, several lines of evidence suggested that the second hypothesis is less likely. First, amino acid residues M and L both have hydrophobic side chains that are similar in size, and therefore, the mutation is unlikely to disrupt CP- δ folding. Second, mutant Orsay virus with CP- δ fibers containing the point mutation exhibits stability and infection kinetics similar to those of the WT virus (Fig. 2C and D). Third, the start codon ATG in the δ ORF is conserved in all three nematode-infecting viruses, Orsay virus, Santeuil virus (7), and Le Blanc virus (9), suggesting a functional requirement for the start codon to express free δ . Finally, the apical localization of free δ in *C. elegans* intestine cells (Fig. 3) suggested that this protein has specific biological functions.

To determine whether free δ can modulate host cell functions on its own, we further examined our transgenic *C. elegans* strain that ectopically expressed GFP-tagged free δ . In these animals, δ ::GFP was expressed under the control of a heat shock promoter. A few transgenic worms displayed a distal tip cell (DTC) migration defect when heat shocked at the L3 larval stage during DTC migration (Fig. 5B). While Orsay virus infects only intestine cells, the heat shock promoter can drive expression in multiple cell types, including DTCs. The DTC migration phenotype suggested that the overexpression of free δ caused a cytoskeleton rearrangement in the DTCs, consistent with our model in which δ interacts with actin.

FIG 6 Proposed model of δ functions. δ may function in both viral entry and exit. In the CP- δ fusion, δ may mediate viral entry, possibly by interacting with cell surface receptors. The free δ may interact with ACT-5 to promote polarized viral exit. Proteins are color-coded: orange represents the pentameric δ fiber, green highlights the Orsay virus capsid, and red indicates ACT-5.

If the lack of free δ caused the viral exit defect of the δ ATG mutant virus, then providing free δ would rescue such a defect. To test this, we exposed both wild-type and transgenic δ ::GFP worms to the δ ATG mutant virus. The worms were fed *rde-1* RNAi bacteria to make them sensitive to viral infection and heat shocked to induce transgene expression. When these worms were tested in the single-worm infection assay, the virus shed from a wild-type worm in 30 min was unable to infect any plate due to the exit defect of the δ ATG mutant virus (Fig. 5C). In contrast, transgenic worms that expressed free δ were able to shed enough virus to infect a plate of naive worms 23% of the time (Fig. 5C). These data demonstrated that GFP-tagged free δ is functional, and thus, its apical localization is of biological relevance. More importantly, the fact that free δ can rescue the viral exit defect of the δ ATG mutant virus suggested that the function of free δ is responsible for the viral exit defect.

DISCUSSION

We showed that Orsay virus uses a nonlytic pathway for viral release. The δ ATG mutant virus with a point mutation eliminating the start codon of δ had viral exit defects, demonstrating that δ function is required for efficient viral exit. The ATG mutant virus can still exit host cells although at a lower efficiency (Fig. 2D). It is possible that there may be leaky protein translation through the CTG initiation codon. An annotation of 10 different *Escherichia coli* strains found that 82.5% of the start codons were ATG, 12.3% were GTG, and 5% were TTG, with CTG, ATT, and ATC being used at lower frequencies (21). While no such studies with *C. elegans* have been reported, genetic studies showed that mutation of a single gene, *iftb-1*, in *C. elegans* allowed a GFP reporter with a GTG start codon to be highly expressed (22), suggesting that non-ATG initiation is possible in *C. elegans*. It is also possible that there are alternative exit mechanisms that are independent of free δ .

While we found that free δ likely mediates viral exit in this study, we previously reported that δ functions in the fusion protein CP- δ to mediate viral entry, possibly by binding to receptors (12). Therefore, δ appears to have at least two distinct functions in the life cycle of Orsay virus: viral entry mediated by CP- δ and viral exit mediated by free δ (Fig. 6). While it is unclear whether CP- δ is also involved in viral exit, the ATG mutant virus caused infection symptoms similar to those caused by wild-type viruses (Fig. 2), suggesting that free δ is not required for viral entry or replication but functions in viral exit only. The two distinct functions, viral entry and viral exit, of δ may be carried out by different domains. In CP- δ , δ forms fibers protruding from the viral particle, with its N terminus being fused with CP and its C terminus forming a globular head at the distal end (12). This structure suggested that the δ C terminus is likely to mediate viral entry, possibly by host attachment. Indeed, we have shown that the δ N terminus is unlikely to be involved in host attachment, because the addition of this protein to the culture medium had no impact on viral infection (12). However, the expression of the

δ N terminus, but not the C terminus, is sufficient to carry out apical localization (Fig. 3D and E), suggesting that the N terminus is involved in viral exit. Overall, these results suggested a model of the δ N terminus functioning in viral exit and the C terminus functioning in viral entry.

Several results suggested that δ functionally interacts with host ACT-5 to mediate viral exit: (i) δ colocalizes with ACT-5, (ii) the δ ATG mutant virus failed to rearrange ACT-5 upon infection, and (iii) reducing ACT-5 levels by RNAi exacerbated the viral exit defects of δ ATG mutants. The actin cytoskeleton has been found to play various important roles during the replication life cycles of many viruses (23). Interestingly, instead of being a physical barrier preventing viral exit, ACT-5, a major component of the terminal web, appeared to be promoting Orsay virus exit (Fig. 5A). It should be noted that our assay exclusively tested viral exit; therefore, it is possible that ACT-5 may still function as a barrier to block other infection steps, such as viral entry.

Actin-facilitated viral exit is not unique to Orsay virus. A similar phenomenon has been observed for rotavirus, where actin treadmilling promotes apical viral exit (24), suggesting that actin-based exit may be utilized by multiple nonenveloped viruses. In another example, the enveloped vaccinia virus induces actin polymerization to facilitate its egress (25). The nonlytic, actin-based exit of Orsay virus is also similar to that of the microsporidian parasite *N. parisii* (13), despite the large difference in size between the two organisms and the lack of a δ -like protein in *N. parisii*. Notably, both Orsay virus and *N. parisii* are natural intracellular pathogens of *C. elegans* intestinal cells. More studies are needed to reveal the details of such actin functions, for example, how actin facilitates Orsay virus transportation or membrane remodeling. Future identification of host proteins that physically interact with Orsay virus δ will provide further insight into the molecular mechanism of how δ facilitates the nonlytic exit of Orsay virus.

MATERIALS AND METHODS

Strains. *C. elegans* was maintained on standard nematode growth medium (NGM) seeded with *E. coli* OP50, as described previously (26). The wild-type N2 strain was obtained from the Caenorhabditis Genetics Center (CGC). The transgenic strain used to generate wild-type recombinant virus, *Ex[hsp16-41p::Orsay RNA1+hsp16-41p::Orsay RNA2+pRF4]*, was kindly provided by David Wang (15). *JM125 cals[ges-1p::YFP::ACT-5]III* was kindly provided by James McGhee (17). *ERT104 jyls17[vha-6p::mCherry::ACT-5+txx-3p::RFP]IV* and *ERT71 jyls14[F26F2.1p::GFP; myo-2::mCherry]* were kindly provided by Emily Troemel (16, 19).

While strain N2 is resistant to Orsay virus infection, inactivation of the *rde-1* or *drh-1* gene on the N2 background can sensitize the worms to Orsay virus infection (7, 27). Therefore, all our experiments were conducted on the *rde-1(RNAi)*, *rde-1(ne219)*, or *drh-1(ok3495)* background. In addition, default naive worms also had the mutation *glp-4(bn2ts)*. *glp-4(bn2ts)* worms are sterile at 20°C (28), which allowed us to easily score day 3 adults without interference from progeny.

SS104 *glp-4(bn2ts)I* (28) was kindly provided by Natasha Kirienko. RB2519 *drh-1(ok3495)IV* was obtained from the CGC, outcrossed six times, and crossed to SS104 to generate *glp-4(bn2ts); drh-1(ok3495)IV*. The *drh-1* deletion was confirmed by PCR.

Molecular cloning. Plasmids pHIP_RNA1 (*hsp16-41p::Orsay RNA1*) and pHIP_RNA2 (*hsp16-41p::Orsay RNA2*) were kindly provided by David Wang (15). These plasmids were used as the templates to obtain cDNA for each Orsay virus protein. Amplified viral genes were inserted into the vector pPD118.26 (Fire Lab *C. elegans* vector kit; Addgene) between NotI and KpnI. All constructs were confirmed by sequencing. cDNA for the ATG mutant virus was cloned by mutation PCR using primers to introduce a site mutation into plasmid pHIP_RNA2.

A truncated Orsay virus CP (capsid positions 42 to 391) was used in the reporter constructs to remove a nuclear localization signal (NLS)-like sequence, 10-RKGKPVKQPSS-20, as predicted by the program NLStradamus (29). The Orsay virus CP structure indicated that the first 41 residues of CP are located inside the virion and structurally disordered (30). We have previously shown that truncated CP and full-length CP had almost identical structures and assemblies (30). Therefore, the truncated version is likely to reflect CP localization.

Construction of transgenic animals. Microinjections on N2 young adults were conducted according to standard procedures (31). The GFP reporter lines were generated by injecting a mixture of 2 to 5 ng/ μ l the reporter construct, 5 ng/ μ l the injection marker *myo-2p::DsRed*, and the filler DNA pBlueScript to a total DNA concentration of 200 ng/ μ l. The recombinant virus lines were generated by injecting a mixture containing 50 ng/ μ l mutant pHIP_RNA2, 50 ng/ μ l pHIP_RNA1, and 100 ng/ μ l pRF4. Transgenic animals were cultured at 15°C to minimize the activation of the heat shock promoter. All transgenes were confirmed by worm PCR and sequencing. The ATG mutant virus was also confirmed by sequencing of the RT-PCR product from infected worms. Two independent ATG mutant lines were tested for viral exit defects. Multiple independent lines were tested to confirm the δ localization patterns.

Generation of recombinant virus. A previously reported procedure (15) was followed to generate recombinant viruses. Thirty roller L4 animals (5 worms/well) were placed onto a 6-well RNAi plate that contained NGM, 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG), and 50 ng/ μ l carbenicillin and that was seeded with *rde-1* RNAi bacteria. The worms were cultured for 5 days at 20°C, heat shocked at 33°C for 2 h, and maintained at 25°C for 2 days. Worms were fed IPTG-induced *rde-1* RNAi bacteria throughout the procedure to prevent starvation. The worms were then washed off the plates by using S basal buffer (26). A mixture of an equal volume of worms and buffer was sonicated on ice. The crude lysate was centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatant was passed through a 0.22- μ m filter and kept at 4°C until use.

Viral infection. Unless otherwise specified, synchronized L1 larvae were exposed to the virus and cultured at 20°C for 5 days until they were day 3 adults, when they were scored. For qRT-PCR, ~300 L1 larvae were dropped into 2 ml of S medium (26). A total of 200 μ l worm lysate containing recombinant viruses was added to these worms.

Viral RNA quantification. Worms and media were collected and processed separately. Worms were washed four times with 10 ml S basal medium. Culture media were centrifuged at $10,000 \times g$ at 4°C to remove the remaining bacteria. The liquid was passed through a 0.22- μ m syringe filter and concentrated with an Amicon Ultra 4 10K filter (Millipore, USA) to 100 μ l. Total RNA from worms or concentrated media was extracted by using TRIzol (Invitrogen). RNA was digested with DNase (Invitrogen) and reverse transcribed to cDNA by using RETROscript (Thermo). qRT-PCR was performed by using PerfeCTa SYBR green SuperMix (Quantabio). Primer pairs GW194/GW195 and AMA-1F/AMA-1R were used to target Orsay virus RNA1 and the internal reference gene *ama-1*, respectively (7). Viral quantification cycle (C_q) values were normalized to *ama-1* values. The viral load of the ATG mutant virus was then normalized to that of the wild-type virus. Three technical replicates were tested in each trial.

Propidium iodide staining. PI (catalog number P4170; Sigma) staining was conducted as described previously (14, 19). Synchronized *glp-4(bn2ts); rde-1(ne219)* L1 worms were dropped onto NGM seeded with OP50 bacteria and cultured at 20°C until they were day 3 adults. The Cry5B-treated worms were washed off the plates by using M9 buffer, transferred to RNAi plates (NGM with 1 mM IPTG and 50 ng/ μ l carbenicillin) seeded with Cry5B-OP50 bacteria, and cultured at 25°C for 45 min. Cry5B-OP50 bacteria were kindly provided by Raffi Aroian. Worms were then washed with M9 buffer and transferred to 96-well plates that contained 50 μ l 5 mg/ml serotonin (catalog number H9523; Sigma) in M9 medium in each well to force-feed the worms. The plates were placed on a 20°C shaker for 15 min. Two microliters 0.5 mg/ml PI was then added to each well to reach a final PI concentration of 20 μ g/ml. The worms were incubated on the 20°C shaker for an additional 30 min before being washed by M9 medium and then scored.

Single-worm infection assay. Synchronized L1 worms were infected with viruses and cultured at 20°C until they were day 3 adults. For Fig. 5C, L1 worms were exposed to the virus, cultured at 20°C for 24 h, heat shocked at 33°C for 2 h, and cultured at 25°C until they were day 3 adults. Infected day 3 adults with the transparent-intestine phenotype were picked into a test tube containing 10 ml S basal medium. Worms were washed twice with S basal medium and dropped onto an unseeded NGM plate. Each animal was then picked onto a new NGM plate that contained ~100 L1 *glp-4(bn2); rde-1(ne219)* worms and removed after a certain time. For Fig. 5A, these new plates were RNAi plates seeded with *act-5* RNAi bacteria (1:25 diluted with control bacteria) to maintain *act-5* RNAi effects on the original infective worm. As the *rde-1(ne219)* strain is resistant to RNAi, *act-5* RNAi had no impact on these naive worms. L1 naive worms were cultured at 20°C until they were day 3 adults. A plate with >50% of worms showing the transparent-intestine phenotype was scored as infected. Biological replicates were obtained by using naive L1 worms collected from different batches of parental animals, and recombinant viruses were generated from different batches of transgenic worms.

Determination of viral infection kinetics. About 90 synchronized L1 larvae of *jyIs14* worms were cultured on a 3-cm NGM plate seeded with OP50 at 20°C for 24 h. A mixture of 10 μ l water and 10 μ l crude worm lysate containing recombinant viruses was added to the plate. GFP-positive worms were counted and removed every 2 h. In this assay and the titer assay examining viral stability described below, a control group of uninfected worms was always tested to ensure that no GFP was observed there.

Determination of viral stability. Aliquots of the virus-containing worm lysate were tested for relative titers on different dates. To test relative titers, the lysate was diluted in water to 5%, 2.5%, 1.25%, 0.63%, 0.31%, 0.16%, 0.08%, and 0.04%. About 90 synchronized L1 larvae of *jyIs14* worms were placed onto a 3-cm NGM plate and exposed to 20 μ l of diluted virus. Four worm plates were tested for each viral dilution. The worms were observed after being cultured at 20°C for 3 days, when they were adults. A plate with at least five GFP-positive worms was counted as infected. The most diluted viral concentration that had at least two infected plates was used. The viral titer was divided by the day 1 titer to obtain the relative titer.

Western blotting. JM125 worms were fed IPTG-induced *rde-1* RNAi bacteria and grown in a 50-ml liquid culture, as described previously (26). For the infected group, 500 μ l of the viral filtrate (7) was added at the beginning of the liquid culture. Infected and uninfected worms were cultured at 20°C for 6 days before being pelleted and sonicated in lysis buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 10% (vol/vol) glycerol, 5 mM 2-mercaptoethanol (2-ME), 1 mM NaN_3 , and 1 mM phenylmethylsulfonyl fluoride (PMSF). Total cell lysates were resolved on a 15% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% milk in Tris-buffered saline (TBS) (pH 7.4) containing 0.1% Tween 20 (TBST), membranes were probed with antibodies for yellow fluorescent protein (YFP) (catalog number G1544; Sigma) and tubulin (loading control) (catalog number T9026;

Sigma) overnight at 4°C. Membranes were then washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at 4°C. After washing three times with TBST, immune-reactive bands were detected by using the SigmaFast BCIP/NBT alkaline phosphatase substrate (catalog number B5655; Sigma). The intensities of various analyte proteins and their respective loading controls from the same blot were measured by using the program Fiji (32). The amount of ACT-5 in each sample was quantitated by dividing the intensity of the ACT-5 band by that of the loading control.

Microscopy. Epifluorescent images were taken by using a Zeiss AxioImager M2m microscope equipped with a Zeiss AxioCam MRm camera. AxioVision software 4.8 was used for camera control. To quantify the YFP::ACT-5 expression level, images were taken by using the same exposure time. Pixel brightness and the number of bright pixels were measured by using PhenoCapture 3.3 software (<http://PhenoCapture.com/>). For the δ localization experiment, to induce the heat shock promoter in these GFP reporter lines, transgenic worms were placed in a 33°C water bath for 2 h and then recovered at 20°C for 90 min before being observed. *jyIs14* GFP was observed under a Zeiss SteReo Discovery V20 stereoscope.

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