Densovirus Crosses the Insect Midgut by Transcytosis and Disturbs the Epithelial Barrier Function


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Densovirus are paroviruses that can be lethal for insects of different orders at larval stages. Although the horizontal transmission mechanisms are poorly known, densoviral pathogenesis usually starts with the ingestion of contaminated food by the host. Depending on the virus, this leads to replication restricted to the midgut or excluding it. In both cases the success of infection depends on the virus capacity to enter the intestinal epithelium. Using the Junonia coenia densovirus (JcDNV) as the prototype virus and the lepidopteran host Spodoptera frugiperda as an interaction model, we focused on the early mechanisms of infection during which JcDNV crosses the intestinal epithelium to reach and replicate in underlying target tissues. We studied the kinetics of interaction of JcDNV with the midgut epithelium and the transport mechanisms involved. Using several approaches, in vivo, ex vivo, and in vitro, at molecular and cellular levels, we show that JcDNV is specifically internalized by endocytosis in absorptive cells and then crosses the epithelium by transcytosis. As a consequence, viral entry disturbs the midgut function. Finally, we showed that four mutations on the capsid of JcDNV affect specific recognition by the epithelial cells but not their binding.

Densovirus are small, nonenveloped, single-stranded DNA viruses that are pathogenic for arthropods; their genomic structures and organization place them in the Paroviridae family. Densovirus have been found in both aquatic and terrestrial ecosystems. Their distribution among arthropods from shrimp to mosquitoes and their presence in diverse ecological niches and distant geographic areas further argue that they are ubiquitous in the environment, where they may have a strong, although unexplored, impact on host populations. Contrasting with their wide distribution among arthropods, only 33 densovirus genomes have been sequenced so far; they display a wide variety of structures, sequences, and organizations, falling into four genera, Densovirus, Brevivirus, Iteravirus, and Pefudensovirus, according to the International Committee on Taxonomy of Viruses (ICTV) (1).

The Densovirus genus encompasses so far viruses restricted to insects from the Lepidoptera order. Although transmission is not well characterized, contamination occurs orally, through the fecal-to-oral route, but also by intraspecific predation due to the cannibalistic behavior of the caterpillars, a route that could potentially lead to the transmission of large virus populations (2) (D. Mutuel, unpublished data). This cannibalistic feature is shared by several insect species (3). The viral pathogenesis starts with the ingestion of contaminated food, reaching then the intestinal epithelial cells, where the fate of the viral particles may differ depending on the densovirus; some densovirus establish the infection only in midgut cells, while others are transported across the midgut without replicating to reach and replicate in the internal target tissues, which differ depending on the tropism of the virus. The pathogenesis of the prototypical densovirus of Junonia coenia (JcDNV) in an opportunistic lepidopteran host, the caterpillar of the highly phytophagous pest Spodoptera frugiperda, has been described previously (2). Briefly, following ingestion, the 26-nm particles of JcDNV escape the midgut without replicating to spread to subepithelial tissues by two routes: (i) to subepithelial respiratory cells (trachea) and visceral muscles by direct cell-to-cell infection and (ii) to hemocytes and the epidermis by systemic infection via the hemocoel.

In any case of densovirus infection, the first interaction with the gut barrier is a crucial process to initiate infection. We recently showed that JcDNV infection of S. frugiperda is mediated by a gut-specific receptor and determined by four amino acids in the 5-fold axis of the virus capsid (4). However, the very first cellular targets are unknown, and no receptor has been yet characterized for any densovirus.

Crossing a tight epithelial monolayer is crucial for many nonenveloped viruses, although our understanding of the mechanisms involved is limited to mammalian viruses infecting respiratory or intestinal tracts, e.g., adenoviruses, enteroviruses, reoviruses, and paroviruses (for a review see reference 5). Naked viruses developed several pathways to infect or cross monolayered epithelia, with no correlation between the mechanism and the family (or even the type) of virus. The virus entry into the epithelium usually starts by receptor-mediated endocytosis; viral particles are then transported either by intracellular trafficking through endosomes to their replication compartment (Reoviridae, Adenoviridae) or across the epithelial cell by rapid vesicular transport (transcytosis), a process used by adeno-associated virus (AAV) and poliovirus (6–10). The polarized nature of epithelial cells constitutes an additional level of complexity for
viral entry, which cannot easily be studied in tissue culture systems. Receptor molecules are not necessarily apically distributed. Rather, they can be sequestered within or beneath the tight junctions, as exemplified by the Reoviridae and Adenoviridae (9, 11, 12). Reaching these receptors requires highly dynamic reorganization of the cell triggered by the initial virus attachment to apical cell surface molecules (for a review see reference 13) This process often results in the disruption of the tight junctions, thus opening the paracellular pathway and increasing tissue permeability, although no virus passage has been shown to occur through this paracellular route.

Taking into account the organization of the alimentary canal of lepidopteran larvae, once ingested, the naked viral particles must overcome two successive and potentially restrictive barriers. The first is a highly glycosylated membrane, the peritrophic membrane (PM), protecting the midgut cells. Considering the dimension of the densovirus capsid, virions might theoretically diffuse freely through the pores of this relaxed chitinous matrix (14). The second is the midgut epithelium, a polarized barrier where apical and basolateral membrane domains are separated by septate junctions (SJs), which ensure gate and fence functions. The basal lamina secreted on the basal side of the epithelial cell layer may also provide physical barriers to viruses. Crossing this complex epithelial organization is crucial for the success of infection, and the processes involved have been poorly described for entomopathogenic viruses (for a review see reference 15).

In this study, we investigated the route by which JcDNV is transported across the intestinal barrier to spread to the internal target tissues of its host. Our results show that infection is initiated by a rapid transcytosis through columnar cells, which allows the viral particles to escape from the midgut and to reach the hemoepithelial compartment. The vascular transport of the virus across the monolayered epithelium rapidly alters the functional and the structural properties of the tissue, although these changes appear not sufficient in the early process to allow the direct permeation of the virus through the paracellular pathway. The rapid transcytosis process would allow the virus to escape elimination by the rapid induction of an antiviral response in the midgut.

MATERIALS AND METHODS

Insect rearing and in vivo infections. Larvae of Spodoptera frugiperda were reared under controlled conditions (25 ± 1°C, 65 to 70% relative humidity [RH], 16-h light, 8-h dark photoperiod) on a wheat germ-based artificial diet. Viral inoculums of wild-type JcDNV (wt-JcDNV) and mutant JcDNV (8m-JcDNV) carrying mutations on the 5-fold axis of the densovirus capsid, virions might theoretically diffuse freely through the pores of this relaxed chitinous matrix (14). The second is the midgut epithelium, a polarized barrier where apical and basolateral membrane domains are separated by septate junctions (SJs), which ensure gate and fence functions. The basal lamina secreted on the basal side of the epithelial cell layer may also provide physical barriers to viruses. Crossing this complex epithelial organization is crucial for the success of infection, and the processes involved have been poorly described for entomopathogenic viruses (for a review see reference 15).

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RESULTS
JcDNV particles reach the midgut cells within 15 min after ingestion and target columnar cells. We first wanted to track the fate of the viral particles shortly after the oral infection of the larvae. Following their ingestion with contaminated food, the viral particles move with the ingested diet along the gut lumen, helped by peristaltic movements. The midgut represents about 70% of the total gut length; it fulfills absorptive and digestive functions, by peristaltic movements. The midgut represents about 70% of the total gut length; it fulfills absorptive and digestive functions, and the epithelium is made up of at least four morphological cell types, (i) columnar cells, which are the most abundant, (ii) regenerative (stem) cells, (iii) goblet cells, and (iv) endocrine cells. Based on experience of feeding small second-instar larvae (L2, 4 mm long) with a methylene blue dye followed by transparency, we roughly estimated that about 5 to 30 min was required to fill the midgut lumen with virus-containing ingesta (data not shown). Thus, to track the viral particles once they reach the midgut, we fed L2 larvae with food containing 10^{10} viral equivalent genomes (veg) of JcDNV and sacrificed them 15 min after ingestion. Midguts along with the peritrophic membranes and the enclosed intestinal contents were collected for the subsequent analysis of virus localization. Figure 1A shows that JcDNV labeling is detected close to the peritrophic membrane. A faint signal was also observed at the microvillar level of columnar cells, characterized by a...
midgut is large enough to be studied ex vivo using Ussing chambers. Once mounted on this perfusion apparatus, the midgut separates two compartments, the luminal and the hemolymphatic ones. This apparatus has been shown to be a valuable tool to study insect transepithelial transport, including that of JcDNV (4, 28).

The virus was added to the luminal compartment, its transport was quantified by qPCR, and its localization within the epithelium was determined by immunolabeling. Ten minutes after the addition of purified JcDNV (10^6 veg) to the apical side of the midgut, about 10^6 veg were recovered in the hemolymphatic compartment, giving a crossing efficiency at this time of 1:10^4 (Fig. 2A).

Extending the infection to 30 min, the amount of JcDNV transported across the epithelium slightly increased (about 1 log veg), suggesting that the majority of virus particles cross the epithelium very rapidly.

To determine the virus internalization mechanism, we prepared ultrathin sections at 10 min p.i. and analyzed them by transmission electron microscopy (TEM). Ultrastructure analyses of the virus internalization have been made difficult by the size of the viral particles (similar to ribosomes), and no virus or virus-containing vesicles were observed at this time by this technique, suggesting a discrete and/or rapid uptake in the columnar cells. We thus analyzed virus localization by immunolabeling and confocal analysis. As showed in Fig. 2B, different cellular structures could be observed in one confocal section due to the natural folding of the midgut tissue. Nevertheless at 5 min p.i. the virus labeling was observable in the apical part of the cell, where microvilli are clearly visible, and at 10 min p.i. it was intracellular (Fig. 2B; see the supplemental material). Virus labeling was observed as large dots, sometimes organized as ringlike structures (Fig. 2B, a and b) near the cortical actin filaments located under the basolateral membranes. We confirmed the intracellular localization with the Rab 5 antibody (Fig. 2C), although overlay images showed that colocalization was partial.

These data suggest that, shortly after ingestion, the virus is transported across the midgut epithelium by transcytosis. Viral particles are taken up apically, traffic through the absorptive cells, and are released in the hemolymphatic compartment.

**JcDNV affects midgut barrier permeability.** During transcytosis, the initial virus endocytosis at the apical membrane of a polarized cell is followed by intracellular trafficking and release by exocytosis at the basolateral membrane. We showed previously that JcDNV entry depends on a yet-unknown midgut-specific receptor(s) (4). Depending on the distribution of the receptor molecule(s), viral entry might disrupt the cellular junctions, thus opening the paracellular route. To better assess the JcDNV entry mechanism, we first evaluated the ability of the virus to affect paracellular permeability. To evaluate the effect of JcDNV on paracellular permeability, registration of the paracellular electrical resistance, or shunt resistance (\( R_{sb} \)), was performed in midguts mounted in Ussing chambers. Figure 3A shows that shortly after JcDNV addition to the apical compartment a significant decrease of \( R_{sb} \) was recorded and that \( R_{sb} \) further declined with longer times of exposure. The decrease of \( R_{sb} \) indicates an increase of sepiate junction permeability, so ions move more rapidly through the aqueous channels formed by the intercellular junctions. This result suggests that JcDNV entry within columnar cells affects the gate function of the epithelium. To confirm this evidence and test whether the increased permeability of the paracellular pathway allows the viral particles to cross the midgut through this route, we...
measured the fluxes of labeled dextrans of different molecular weights. Fluorescent markers of two sizes, 4-kDa and 40-kDa FITC-dextrans (FD4 and FD40, respectively), were added to the luminal compartment in the absence and in the presence of JcDNV, and the fluorescence recovered in the hemolymphatic one was measured as an indicator of paracellular permeability. Following infection, a significant increase of the diffusion of 4-kDa, but not 40-kDa, dextran was observed (Fig. 3B).

These results show that the interactions of JcDNV with the apical membrane of absorptive cells perturb the gate function of the epithelium. However, taking into account that 40-kDa FITC-dextran with an estimated Stokes radius of <5 nm is excluded from the paracellular route, it is unlikely that the 26-nm viral particles could diffuse through this pathway early in infection.

The transcytosis process of JcDNV across the midgut epithelium. Since the virus was localized within absorptive cells and a direct diffusion of the virus through the paracellular route is unlikely at this time p.i., we focused on the transcytosis process. We studied the three steps of this transcellular transport, which
drive the viral particles from the luminal to the hemolymphatic compartment, i.e., endocytosis, intracellular trafficking, and exocytosis.

We showed previously that JcDNV entry depends on a yet-unknown midgut-specific receptor (4). We investigated the mechanisms of endocytosis and trafficking in an isolated midgut ex vivo using specific drugs and molecular markers. Regarding the entry process, our previous work shows that JcDNV endocytosis in the permissive lepidopteran Ld652 cells is dynamin and clathrin dependent (23). Therefore, we first tested the role of dynamin, treating the midgut epithelium with different concentrations of dynasore (DYN), a highly specific inhibitor of dynamin’s GTPase activity (29). Midguts were mounted in Ussing chambers and treated with different concentrations of DYN (80, 400, and 800 μM), and virus transepithelial transport was assayed by qPCR. As shown on Fig. 4A, dynasore treatment negatively affected the transport of JcDNV to the hemolymphatic compartment in a dose-dependent manner, suggesting that the viral particle entry process depends on dynamin. We similarly tested the clathrin-dependent pathway by blocking it with a specific drug, chlorpromazine (CPZ). Midguts were treated with different concentrations of CPZ (100, 200, and 400 μM), and virus transport was assayed by qPCR. No effect on the virus transport was observed at any of the concentrations tested, making unlikely a clathrin-dependent entry mechanism (Fig. 4B).

To further investigate a dynamin-dependent and clathrin-independent endocytic mechanism, we next examined the role of caveola/lipid raft microdomains in virus uptake. We first used a specific drug to disturb the lipid distribution in the midgut cell membrane. Methyl-beta-cyclodextrin (MβCD) is a specific cholesterol-sequestering drug usually used to disrupt lipid rafts in mammalian cell systems. Cholesterol is a major constituent of eukaryotic cell membranes, and its depletion has been shown to impair the entry of several viruses, including nonenveloped ones (30). Isolated midguts in Ussing chambers were treated with increasing doses of MβCD, and JcDNV transepithelial transport after 10 min of incubation was quantified by qPCR as above. Surprisingly, a significant positive effect on the amount of virus transported to the hemolymphatic compartment and also within the epithelium was observed, although this effect was not dose dependent at the concentrations tested (Fig. 4C). A similar effect was obtained with filipin, another cholesterol-depleting drug (data not shown), suggesting that these drugs might affect nonspecifically the membrane organization under the conditions tested and would not be useful for understanding the uptake mechanism.

To investigate further the role of lipid rafts in JcDNV endocytosis, we took advantage of the conservation of the caveolin-1 protein in Lepidoptera and we used the fluorescent cholera toxin subunit B (FITC-CTxB) as a marker of these microdomains (24, 30). Our results indicate that, 10 min after its addition to the luminal side of the epithelium, FITC-CTxB colocalized, although partially, with caveolin-1-positive dots, validating both markers for studying this pathway (see Fig. 7B, top). The addition of JcDNV to the luminal compartment showed that, 10 min after its addition, virus labeling similarly colocalized with caveolin-1, suggesting that JcDNV endocytosis processes through caveola/lipid raft microdomains (Fig. 4D, bottom).

Last, we investigated the mechanism of virion delivery to the hemolymphatic compartment. Tannic acid (TA) is a cell-impermeable compound that selectively fixes the plasma membrane by cross-linking cell surface carbohydrate groups and inhibits plasma membrane fusion, preventing endocytosis and exocytosis (31, 32). TA has been shown to block the basolateral exocytosis of AAV-2 when added to the basal side of epithelial cells (6). We thus tested the ability of TA to block JcDNV transepithelial transport through the larval midgut, treating the apical or basolateral surfaces of the epithelium and measuring the virus accumulation within the cells by qPCR. When TA was added to the luminal compartment of Ussing chambers, complete inhibition of the virus entry was observed and no virus was recovered in the hemolymphatic compartment (data not shown). By contrast, when TA was added to the hemolymphatic compartment, the transepithelial transport of JcDNV added to the luminal one was blocked and the virus accumulated within the epithelium (Fig. 5A). Immunolabeling and confocal vertical sections showed that JcDNV accumulates in a plane surrounding the cells (Fig. 5B). These observations suggest that TA blocks exocytosis, causing viral particle accumulation within subcellular compartments; they further show that transcytosis is the mechanism involved in JcDNV transport across the midgut epithelium. It is noteworthy that midgut tissues incubated with JcDNV and TA showed wide intercellular spaces, with septa appearing as “opened zippers” not observed in controls that received only one treatment, either TA or JcDNV (Fig. 5B, compare CTL-TA and JcDNV-TA). The results above show that JcDNV binding to the apical membrane of columnar cells and/or its internalization alters the septate junction; this alteration was visualized by TEM only when TA was added together with the virus, which further suggests that their effects on junction organization are additive. TA blocks the membrane dynamics, which might enhance and/or fix changes in junctional complexes induced by virus internalization. The passage of JcDNV across the epithelium is probably highly dynamic, involving changes in junctional protein localization that may facilitate virus recognition and/or transport. Whether infection affects the fence function (apical polarity) of the epithelium remains to be assessed.

Four mutations on the capsid 5-fold axis send the viral particles to a degradation pathway. We recently showed that a chimeric JcDNV harboring 4 residues of the related Galleria mellonella DNV (GmDNV) on raised regions of the 5-fold axis of the capsid specifically affects midgut cell tropism (4, 33); this “Gm-ized” JcDNV has been called 8m-JcDNV, as opposed to the wild-type JcDNV (wt-JcDNV). First, we measured by qPCR the uptake (veg in the midgut) and transepithelial transport (veg in the hemolymphatic compartment) of wt- and 8m-JcDNV. As previously shown (4), a smaller amount of 8m-JcDNV was recovered in the hemolymphatic compartment (Fig. 6A). Similar amounts of 8m- and wt-JcDNV were recovered within the epithelium, and 8m-JcDNV labeling was observed within the cells (data not shown), suggesting that internalization was not the step limiting infection. Surprisingly, TEM analysis showed that viral particles accumulated within different-size vesicles in the apical cytoplasm, some of the vesicles being very close to the plasma membrane, although no typical features decorate their membrane. The large size of these vesicles (≤0.2 μm) and the large amount of mutant viral particles they contained suggested the uptake of virus clusters, probably by a macropinocytosis mechanism that remains to be clarified (Fig. 6B). Diverse sizes of vesicles (some of them were fusing) and very large vacuoles were also observed, with degraded material inside containing ghosts of virus-containing vesicles; localized close to...
endoplasmic reticulum (ER) structures, these vacuoles resemble enlarged cisternae and might reveal an ER stress.

Altogether these results showed that the mutant viral particles are taken up by a different mechanism than that used by wt-JcDNV, the subsequent trafficking of mutant virus-containing vesicles is blocked at an early step of internalization. The four residues at the surface of the capsid send the viral particles to a dead end pathway, further implicating elements around the 5-fold axis of symmetry of the capsid in specific receptor recognition at the surface of the midgut cells. Alternatively, virus-specific receptor recognition can occur during vesicular trafficking toward basolateral domains.

Mutant viral particles do not directly affect tissue permeability. Since the results reported above showed that wt-JcDNV disturbs midgut permeability, we analyzed the effects of 8m-JcDNV on this property by measuring the diffusion of the 4-kDa FITC-
dextran and we tested as well the passage of the 12-kDa FITC-CTxB. An increased diffusion of FD4 was observed following infections with both viruses, although to a lesser extent with 8m-JcDNV (Fig. 7A, left). Similar results were observed with FITC-CTxB; however, this experiment could not discriminate whether infection increased potential toxin transcytosis and/or its paracellular diffusion due to its small size (Fig. 7A, right). To verify this point, we next assessed the localization of the fluorescent CTxB together with caveolin-1 following infections with the two viruses (Fig. 7B). When midguts were infected with wt-JcDNV, both caveolin-1 and FITC-CTxB were found within large intracellular dots as in mock-infected cells, while FITC-CTxB was also found surrounding the cells, suggesting its paracellular accumulation following infection (Fig. 7B, CTL and + wt-JcDNV panels). By contrast, when midguts were infected with 8m-JcDNV, no fluorescence corresponding to CTxB was found around the cells, only

![Graph showing effects of tannic acid (TA) on JcDNV transepithelial transport](image)

**FIG 5** Effect of tannic acid (TA) on JcDNV transepithelial transport. TA (0.5%) was added (+ TA) or not (− TA) to the hemolymphatic compartment in the Ussing chamber for 30 min, and then JcDNV was added to the apical one for 10 min. (A) TA blocks the transepithelial transport of JcDNV. Viral equivalent genomes (veg) were quantified by qPCR in the 3 compartments of the Ussing chamber. The box plot represents the normalized amounts of veg (in log_{10}) transported to the hemolymphatic compartment (HemoL) and the amounts present in the midgut from at least 10 experiments (Wilcoxon test; **, P < 0.001). (B) TA induces JcDNV accumulation within subcellular compartments. JcDNV trafficking was assayed in TA-treated cells by immunolabeling and confocal analysis using JcDNV (red) and Rab 5 (green) antibodies. Nuclei are labeled with Hoechst (blue). (C) JcDNV infection induces changes in the junctions of TA-treated epithelia within 10 min. TEM analysis of intercellular midgut cell junctions realized from ultrathin transversal sections of midguts treated with TA and infected with JcDNV for 10 min (JcDNV-TA) or not infected (CTL-TA) was performed. The arrowhead shows the intercellular junctions as an open zipper. Bars, 100 nm.
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have provided evidence that a successful infection starts with the

**DISCUSSION**

We analyze the route and the mechanisms by which an insect parovirus is transported across the intestinal barrier of its cater-
pilar host, a crucial step in the Densovirus natural biology. We
have provided evidence that a successful infection starts with the
transcytosis of viral particles across the midgut epithelium alter-
ing, as a consequence, the gate functions of the midgut. To our
knowledge this study is the first analysis of the mechanism leading
to the transepithelial transport of a nonenveloped virus in an in-
sect model.

To decipher the interactions of the viral particles with midgut
cells, we developed several complementary approaches. From \textit{in vitro} studies on midgut cells in culture, we learned that viral particles have a strong specificity, but probably weak interactions with cell surface molecules that are located on both apical and basolateral domains of columnar cells. Such interactions of the viral particles are reminiscent of what is observed \textit{in vivo} with the peritrophic membrane, which the virus must first bind and then pass through. The labeling of the PM obtained with JcDNV antibody is similar to what has been described with peritrophin and chitin antibodies, suggesting an affinity of the viral capsids for
some of the PM proteoglycan components (34). A lectin-like af-
finitiy of the densovirus particles might be important in determin-
ing the virus tropism, as already shown for the binding and/or the infectivity of a number of vertebrate paroviruses, including the transcytosis of adeno-associated viruses, the only paroviruses shown to transcytose epithelial barriers among this group (7; re-
viewed in references 35 and 36).

After binding to cell surface molecules, viral particles are rap-
idly internalized by dynamin- and caveola-dependent endocyto-
sis; vesicles then traffic in the cytosol and are shuttled toward basolateral membranes to spread infection to subepithelial target
tissues. Whether this sequential process is mediated by a single
receptor or by multiple receptor molecules that cooperate remains
to be determined. However, the internalization of the “Gm-ized”
chimeric virus suggests that different molecules probably process
the binding and specific recognition of the viral particles. This
mutant virus binds the surface of the enterocytes but may be not
recognized by a specific receptor. We speculate that its accumula-
tion at the surface of the enterocyte eventually triggers a nonspe-
cific internalization by macropinocytosis. These results highlight
the finely regulated specificity of a densovirus infection in a non-
conventional insect model.

However, we cannot exclude the possibility that multiple path-
ways can mediate JcDNV entry, as shown previously only for the
porcine parovirus (37). More molecular markers and an RNA
interference approach need to be developed in the future to fully
understand the JcDNV entry process \textit{in vivo}. We cannot exclude
the possibility that alternative dynamin-independent entry routes
might be used, such as macropinocytosis, a bulk fluid phase endo-
cytic mechanism (37, 38), and/or pleiomorphic CLIC/GEEC
(clathrin-independent carriers/glycosylphosphatidylinositol [GPI]-
anchored-protein-enriched endosomal compartment)-mediated
entry, as shown for AAV-2 (39). Indeed, viruses are able to take
advantage of every available cellular internalization mechanism
and frequently use more than one route.

The early steps of viral capsid recognition are crucial for the
success of infection: by determining the trafficking pathway, they
determine whether the virus escapes or does not escape degrada-
tion. The subsequent accumulation of mistargeted viral particles
in subcellular compartments probably activates an innate defense
mechanism independent of viral genome replication. This mech-
anism is currently under study, in particular to determine the role
of the actin cytoskeleton in the trafficking of virus-containing ves-
icles. Disrupting the midgut integrity by triggering cell death or
sloughing might open gaps through which the virus can directly
reach subepithelial tissues, where we showed that it replicates as
well as the wild type when the midgut is bypassed (4). This direct

**FIG 6** Role of 4 amino acids on the capsid 5-fold axis in virus transcytosis. (A) 8m-JcDNV has a decreased capacity to cross the midgut compared to that of wt-JcDNV. Uptake (veg in the midgut, in log_{10}) and transepithelial transport (veg in the hemolymphatic compartment, in log_{10}) of wt- and 8m-JcDNV viral genomes were quantified by qPCR 10 min after addition of the viruses to the luminal compartment of an Ussing chamber. (B) Transmission electron micrographs of the ex vivo midgut 10 min after addition of 8m-JcDNV. The apical cytoplasm of a columnar cell shows endocytic structures containing virus-like material. Arrowheads indicate virus-like material-containing vesicles near the apical plasma membrane; the arrow indicates a large intracytoplasmic vacuole. The left panel shows a high magnification of a large virus-containing vesicle. Bars, 100 nm.
passage of mutant viral particles through breaches opened by cell death might also explain why fluorescence is recovered in the basal side and why high doses of this mutant virus are lethal for larvae, although with a delayed time to death (4).

Our results show that, although disturbed, the gate function of the midgut still remains size selective, but we need to settle whether or not the opening of the paracellular route increases with time in response to virus entry. Such an effect on insect midgut permeability would be of great interest for controlling insect pests, allowing combining, and potentially synergizing, the specificity of an intrahemocoelic delivery with fast-killing molecules, thus improving the relatively long-lasting effect of an entomopathogenic virus.

One intriguing observation is the positive effect of cholesterol depletion on virus transport. Insects are cholesterol auxotrophs, and cholesterol is not the main component of insect cell membranes; some insect cell lines display resistance to cholesterol depletion (40–42). Although we cannot exclude a nonspecific effect of the drugs, the caterpillar midgut epithelium might be more sensitive to cholesterol depletion than vertebrate epithelial systems (43). A few studies demonstrate that cholesterol depletion affects the barrier function in epithelial monolayers although the nature of the association is not clear (44).

These results highlight the importance of considering the epithelial organization to understand the complexity of the virus vectorial transport process. It will be important to define the epithelial-cell-specific proteins mediating densovirus entry.

We could not titrate the virus hemolymphatic suspension using the quantitative cell culture assay performed in the permissive Ld652 cells, probably due to the low virus concentration recov-
ered in the hemolymphatic chamber. However, we observed that the time to death of the Ld652 cells infected with the virus that crossed the midgut was considerably delayed (more than 5 days) compared to that for control infections performed with the same amount of virus from the initial inoculum. The lower infectivity of the transcytosed virions suggests that modifications of the viral particles occur during their transepithelial transport, which might change their tropism; this hypothesis needs to be tested.

JcDNV is a “generalist” capable of infecting several lepidopteran hosts, suggesting that receptor molecules are conserved. To test this point, we performed preliminary experiments on the midguts of four lepidopteran species belonging to different families, challenged with wt- or 8m-JcDNV. Similar results were obtained, suggesting that the transcytosis across the midgut epithelium is a conserved mechanism (Y. Wang, unpublished data). The next issue will be to characterize the midgut-specific receptor molecule(s). Interestingly, in the silkworm Bombyx mori, two genes conferring resistance to BmDNV-1 have been identified; one, affecting the early recognition step, is a promising midgut receptor candidate gene that remains to be characterized (45). Although Jc- and BmDNV-1 differ in their structures, tissue tropisms, and replication strategies, characterizing both entry pathways in the midgut would be of great interest.

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