Subcellular localization and live-cell imaging of the *Helicoverpa armigera* stunt virus replicase in mammalian and *Spodoptera frugiperda* cells

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Whilst their structure has been well studied, there is little information on the replication biology of tetraviruses because of the lack of suitable tissue-culture cell lines that support virus replication. In this study, the potential site of *Helicoverpa armigera* stunt virus replication was investigated by transient expression of the replicase protein fused to enhanced green fluorescent protein (EGFP) in mammalian and insect cells. When EGFP was present at the C terminus of the protein, fluorescence was located in punctate cytoplasmic structures that were distinct from the peripheral Golgi, endoplasmic reticulum, early endosomes, lysosomes and mitochondria, but overlapped partially with late endosomes. In experiments where targeting to endosomal compartments was examined further by using Cascade Blue–dextran in live cells, no overlap between the replicase and active endocytic organelles was apparent. Analysis of the punctate structures using time-lapse imaging in live cells revealed that they undergo fusion, fission and ‘kiss-and-run’ events. Whilst the source of the membranes used to form the punctate structures remains unclear, we propose that the replicase sequesters membranes from the late endosomes and actively excludes host proteins, either by normal recycling processes or by a replicase-dependent mechanism that may result in the destabilization of the associated membranes and a release of luminal contents into the cytosol. This is the first study describing the localization of a tetravirus.

INTRODUCTION

The Tetraviridae is a family of small RNA viruses that exclusively infect the larvae of lepidopteran insects. Members are characterized by a very narrow host range, sometimes infecting only a single species, and a high degree of tissue tropism limited to the midgut cells of larval hosts (Fauquet *et al.*, 2005). Tetravirus particles consist of one (genus Betatetravirus) or two (genus Omegatetravirus) positive-sense, single-stranded genomic RNAs encapsidated in a non-enveloped capsid of approximately 40 nm in diameter, with *T*=4 icosahedral symmetry. The genus Omegatetravirus contains the type virus *Nudaurelia capensis* co virus (NoCV), *Helicoverpa armigera* stunt virus (HaSV) and *Dendrolimus punctatus* virus (DpTV) (Hendry *et al.*, 1985; Hanzlik *et al.*, 1993; Yi *et al.*, 2005). Omegatetravirus capsids encapsidate two genomic RNAs (Fig. 1) that are approximately 5.3 kb (RNA1) and 2.5 kb (RNA2) in size (Fauquet *et al.*, 2005). Both RNAs are capped at their 5′ ends and encode distinctive tRNA-like structures at their 3′ ends (Gordon *et al.*, 1995; Hanzlik *et al.*, 1995; Yi *et al.*, 2005). The capsid protein precursor is encoded by RNA2 (Hanzlik *et al.*, 1995; Du Plessis *et al.*, 2005; Yi *et al.*, 2005). Autoproteolytic cleavage of the capsid protein precursor during capsid maturation yields the β and γ proteins found in the mature virus particle (Fauquet *et al.*, 2005). RNA2 also encodes a second open reading frame (ORF), p17, which overlaps with the 5′ coding sequence of the capsid protein precursor (Hanzlik *et al.*, 1995; Du Plessis *et al.*, 2005; Yi *et al.*, 2005). *In vitro* binding studies using recombinant DpTV p17 have shown that the protein may bind RNA (Zhou *et al.*, 2008). HaSV and DpTV RNA1 encodes the viral replicase, which contains the methyltransferase (MT), helicase (Hel) and polymerase (RdRp) domains that are conserved within the alpha-like virus supergroup of viral replicases (Koonin, 1991; Gordon *et al.*, 1995; Yi *et al.*, 2005). The RdRp domain of the DpTV replicase has been expressed in *Escherichia coli* and was shown to initiate primer-independent synthesis of viral RNA *in vitro* (Zhou *et al.*, 2006). Also encoded on RNA1 and overlapping with the 3′ coding sequence of the replicase, but in a different reading frame, are three putative small ORFs. Whether the products of these ORFs are expressed in infected cells and function in the virus life cycle is unknown (Gordon *et al.*, 1995; Yi *et al.*, 2005).
The replication of all RNA viruses studied to date is associated with host membranes. Immunofluorescence analysis shows that non-structural proteins (1a and 2a) of brome mosaic virus localize to the endoplasmic reticulum (ER) in infected barley protoplasts and yeast cells, and that 1a is capable of localization independent of other viral proteins (Restrepo-Hartwig & Ahlquist, 1996, 1999). Biochemical analysis of cells expressing the poliovirus 2C protein demonstrates that several host-cell membranes, including lysosomes, ER, Golgi apparatus and endosomes, contribute to virus-induced vesicles (Schlegel et al., 1996). When expressed alone in cells, foot-and-mouth disease virus non-structural proteins 2B and 2C are found in association with ER and Golgi membranes (Moffat et al., 2005). Flock House virus, which is related structurally to the tetraviruses, replicates its RNA in association with the outer mitochondrial membrane in infected Drosophila cells (Miller et al., 2001). This localization is independent of infection and viral RNA replication and can be redirected to the ER, resulting in a replicase that is functional both in vivo and in vitro (Miller & Ahlquist, 2002; Miller et al., 2003). The alphaviruses Semliki Forest virus and Sindbis virus replicate in association with modified lysosomes and endosomes, creating fibrous structures linked to the rough ER (Froshauer et al., 1988).

The replication biology of tetraviruses, particularly their interaction with host cells, is poorly understood because, with the exception of Providence virus (PrV), which persistently infects Helicoverpa zea midgut cells (Pringle et al., 2003), they are unable to replicate in established cell lines. Research into tetravirus biology has therefore been limited to the heterologous expression of viral proteins in E. coli (Zhou et al., 2006, 2008), Saccharomyces cerevisiae (Tomasicchio et al., 2007), insect tissue-culture cell lines (Agrawal & Johnson, 1995) or transfected plant protoplasts (Gordon et al., 2001).

As a first step towards developing an experimental system for studying the replication biology of tetraviruses, we investigated the subcellular localization of the HaSV replicase in the absence of other viral proteins. Replicase-enhanced green fluorescent protein (REP–EGFP) fusion proteins were expressed in mammalian and insect tissue-culture cells and their distribution was compared with those of the major cellular organelles, including the peripheral Golgi, ER, mitochondria, endosomes and lysosomes. The results show that the fusion protein is located in small punctate structures within the cytoplasm that are possibly derived from late endosomal compartments. In addition, live-cell imaging experiments reveal that these structures undergo fusion and fission events, as well as movement along defined pathways that may be microtubule-associated.

### RESULTS

**Localization of EGFP-tagged HaSV REP proteins in mammalian and insect cells**

In the absence of tissue-culture cell lines permissive for HaSV infection, we focused on developing a robust experimental system for studying the subcellular localization of the HaSV replicase in the absence of other viral proteins. First, the HaSV replicase with C- and N-terminal EGFP tags (REP1–1704–EGFP and EGFP–REP1–1704, respectively) was expressed and its distribution was analysed by confocal microscopy. REP1–1704–EGFP localized to small punctate structures in the cytoplasm of HeLa cells (Fig. 2b) and Sf9 cells (Fig. 2e). In contrast, EGFP displayed a typical distribution throughout the cytoplasm with accumulation in the nucleus (Fig. 2a). Punctate structures containing EGFP were not observed in cells expressing EGFP–REP1–1704 and the fusion protein was distributed evenly throughout the cytoplasm, but excluded from the nucleus (Fig. 2d). A similar cytoplasmic distribution was observed in cells expressing REP16–1704–EGFP (Fig. 2c), indicating that the fusion of EGFP to the REP C terminus was not responsible for the punctate distribution of the fusion protein. These data suggest that the N terminus of REP might be involved in targeting of REP–EGFP.

**Distribution of REP1–1704–EGFP in relation to organelle markers**

To investigate the origin of the punctate structures observed in cells, we examined the localization of REP1–1704–EGFP in relation to the peripheral Golgi, ER, early endosomes and late endosomes by immunofluorescence.
Due to the unavailability of suitable antibodies against organelle marker proteins, Sf9 cells could not be used. Therefore, HeLa cells expressing REP<sub>1−1704</sub>−EGFP were fixed and stained with antibodies recognizing ERP60, β-COP, EEA1 and CD63 before being analysed by confocal microscopy. Merged images showed no overlap between REP<sub>1−1704</sub>−EGFP fluorescence and that of the peripheral Golgi, ER or early endosomal marker proteins (Fig. 3a–i). However, when the distribution of REP<sub>1−1704</sub>−EGFP was compared with that of CD63, there was an overlap of up to 20% of the two signals in approximately 10% of cells at 16 h post-transfection (Fig. 3j–l). At later time points (>20 h), however, no overlap of the two signals was apparent (Fig. 3m–o).

To compare the distribution of REP<sub>1−1704</sub>−EGFP with those of lysosomes and mitochondria as possible sites of localization, live HeLa and Sf9 cells expressing REP<sub>1−1704</sub>−EGFP were incubated with MitoTracker and LysoTracker before being analysed by confocal microscopy. Merged images showed no overlap between REP<sub>1−1704</sub>−EGFP fluorescence and signals labelling lysosomes or mitochondria in either cell type (Fig. 4a, b, respectively). To further examine association between REP<sub>1−1704</sub>−EGFP and the endocytic compartments, cells were incubated in the presence of Cascade Blue-conjugated dextran, which moves through the classical clathrin-dependent endocytic pathway in Drosophila cells (Entchev et al., 2000; Sasamura et al., 2007), for up to 24 h. This allowed sufficient time for the accumulation of endocytosed dextran throughout the endocytic pathway, as confirmed by overlap between dextran and LysoTracker signals (data not shown). Once again, no overlap was observed between REP<sub>1−1704</sub>−EGFP fluorescence and Cascade Blue-conjugated dextran (Fig. 4c).

**Time-lapse imaging of HaSV REP<sub>1−1704</sub>−EGFP in live cells**

Endosomes are small and dynamic membrane-bound vesicles that can undergo fusion, fission and/or ‘kiss-and-run’ events and are subjected to cellular transport in cells (Gruenberg et al., 1989; Bright et al., 2005). To investigate the origin of the EGFP-labelled punctate structures and to compare them with other endocytic organelles, time-lapse experiments were performed in HeLa and Sf9 cells. Cells transiently expressing REP<sub>1−1704</sub>−EGFP were incubated under the appropriate conditions (28 °C for Sf9 cells, or 37 °C and 10% CO<sub>2</sub> for HeLa cells) and observed between 16 and 24 h post-transfection.

Analysis of image sequences obtained in both cell lines showed that EGFP-labelled punctate structures were of different sizes ranging between 0.2 and 4.0 μm. We also observed that they were highly dynamic in live cells. This appeared to be size-dependent, with smaller structures (<0.8 μm) being far more mobile than larger ones (>2 μm). Interestingly, although the majority of the

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**Fig. 2.** Distribution of EGFP and full-length HaSV REP<sub>1−1704</sub>−EGFP fusion proteins in fixed HeLa (a–d) and unfixed Sf9 (e) cells. Cells were transfected with plasmids expressing EGFP, full-length HaSV REP with EGFP fused to its C terminus (REP<sub>1−1704</sub>−EGFP) or N terminus (EGFP–REP<sub>1−1704</sub>), or an N-terminal deletion mutant (REP<sub>46−1704</sub>−EGFP) and imaged by confocal microscopy. Bars, 10 μm.
movements seemed random, we did observe what appeared to be transport along a defined pathway (Fig. 5) and instances where two or more structures were seen to move along similar pathways before dissociation followed by random movements (see Supplementary Video S1, available in JGV Online). Most interesting was the observation that the punctate structures were seen to undergo fusion and fission events. Fusion was observed most frequently between small structures (1 \mu m or less in diameter) (Fig. 6), culminating in the formation of large EGFP-labelled structures (2–5 \mu m) that made up the greater proportion of the population at later time points (20–24 h) (data not shown). Fission events involving both small and large structures were also observed, but at a lower frequency than for fusion (Fig. 7). It has been shown that sequential fusion–fission, or ‘kiss-and-run’, events occur between endosomal vesicles as a means of content mixing between compartments (Bright et al., 2005). A number of similar interactions were also observed between EGFP–REP-containing structures (data not shown). These data

Fig. 3. Comparison of REP\textsubscript{1–1704}–EGFP distribution with the peripheral Golgi (a–c), ER (d–f), early endosomes (g–i) and late endosomes after 16 h (j–l) and after 24 h (m–o). HeLa cells expressing REP\textsubscript{1–1074}–EGFP were fixed in 4% paraformaldehyde and incubated with antibodies recognizing \(\beta\)-COP, ERp60, EEA1 and CD63. Primary antibodies were detected with species-specific Alexa Fluor 546- or Texas red-conjugated secondary antibodies. Areas of partial overlap between EGFP fluorescence (green) and CD63 (red) are enlarged approximately 3-fold (l). Bars, 10 \mu m.
led us to conclude that the punctate structures observed in transfected HeLa and Sf9 cells were likely to be derived from the endocytic pathway. The mobility of these structures suggested that the possibility that they represented aggregated EGFP–REP protein was unlikely.

Fig. 4. Comparison of REP1-1704–EGFP distribution with mitochondria, lysosomes and the endocytic pathway in HeLa and Sf9 cells. Live cells expressing REP1-1704–EGFP were stained with MitoTracker (a), LysoTracker (b) and Cascade Blue-conjugated dextran (10 000 Da) (c). Cells stained with Cascade Blue were incubated for 18 h after labelling prior to imaging. Bars, 10 μm.
DISCUSSION

In the absence of tissue-culture cell lines permissive to omegatetravirus infection, this study sought to identify the site of virus replication by localizing the viral replicase in HeLa and Sf9 cells. Our results show that the HaSV replicase with EGFP fused at its C terminus (REP1–1704–EGFP) accumulates in punctate structures in the cytoplasm of both cell types. These structures are similar to those observed for the PrV replicase protein p40 in persistently infected H. zea midgut cells (Walter, 2008). In contrast, when EGFP is present at its N terminus (EGFP–REP1–1704), fluorescence is distributed evenly throughout the cytoplasm, but excluded from the nucleus. However, replacing the N-terminal EGFP with a 6×His tag restores the punctate distribution in the majority of cells (J. R. Short & R. A. Dorrington, unpublished data), suggesting that the presence of EGFP at the replicase N terminus interferes with targeting of the protein. The abrogation of the punctate distribution by deletion of the replicase N-terminal amino acid residues up to the start of the methyltransferase domain provides further evidence that the N terminus of replicase is involved in targeting the protein to the punctate structures. We have found that a 6×His- and V5 epitope-tagged HaSV replicase expressed in Sf9 cells pellets with membrane fractions and that this is also the case with the PrV replicase (Walter, 2008; J. R. Short & R. A. Dorrington, unpublished data). We therefore propose that the replicase is targeted to a cellular compartment and that a localization signal is present within the N-terminal region of the protein.

We next investigated the origin of the punctate structures by examining the distribution of REP1–1704–EGFP in relation to the ER, peripheral Golgi, early endosomes, lysosomes and mitochondria. Immunofluorescence ana-
ysis of cells using antibodies against organelle-specific markers showed that there was no overlap between EGFP and the ER, peripheral Golgi, early endosomes or lysosomes, indicating that the punctate structures are probably not derived from these compartments. However, overlap was observed between EGFP fluorescence and the late endosomal marker CD63 in some cells at 16 h (but not at 24 h) post-transfection. Although no colocalization was observed with the early endosomes or lysosomes, and the overlap of the HaSV REP with the late endosomes was partial, the punctate structures displayed a number of similarities to endosomal compartments. For example, early endosomes are small, membrane-bound vesicles that undergo homotypic fusion (Dunn & Maxfield, 1992). Moreover, the delivery of endocytosed material from late endosomes to lysosomes occurs as the result of concurrent fusion, fission and ‘kiss-and-run’ events (Bright et al., 2005). Finally, delivery of endocytosed material to the late endosomes occurs in association with microtubules, as opposed to random movements (Gruenberg et al., 1989).

Our live-cell imaging data demonstrate that the punctate structures observed are also highly dynamic and capable of fusion, fission and ‘kiss-and-run’ events, as well as movement along defined pathways that may be associated with cytoskeletal elements. Surprisingly, though, the EGFP-labelled structures did not colocalize with endocytosed dextran in live cells, even though the dextran had been transported along the endocytic pathway to the extent that overlap was observed with the lysosomes. At this time point, it was expected that the dextran would have labelled all of the CD63-positive organelles and would therefore show partial overlap with REP1–1704–EGFP-associated fluorescence. Our results showing that there is no overlap between EGFP-labelled structures and Cascade Blue-conjugated dextran are unexpected, given that we observed partial overlap between CD63-positive structures and REP1–1704–EGFP. These results require further investigation.

It is tempting to speculate that late endosomes may be the initial site to which the replicase is targeted. We suggest that, at this early stage, the replicase begins to sequester membranes, forming CD63-negative structures by excluding cellular proteins. With further accumulation of the replicase, destabilization or disruption of the membranes occurs with release of luminal contents into the cytoplasm, which would account for the lack of overlap between EGFP fluorescence and dextran-labelled endocytic vesicles. Alternatively, the replicase could be associating with membranes derived from a clathrin-independent endocytic pathway that intersects with the clathrin-dependent endocytic pathway through the late endosomes. A number of alternative endocytic compartments are used by viruses as a means of gaining entry into host cells (reviewed by Pelkmans & Helenius, 2003) and these unconventional pathways may interact with the classical endocytic pathway (Pelkmans et al., 2004). The specificity exhibited by some of these clathrin-independent endocytic pathways may result in the exclusion of dextran, accounting for the lack of overlap with fluorescent label-conjugated dextran observed in this study. Thus, the partial overlap observed between REP1–1704–EGFP-associated fluorescence and the late endosomal marker may be a result of the merging of the two pathways, with the accumulation of REP1–1704–EGFP again resulting in the inhibition of natural transport and/or fusion potential. Finally, the possibility that tetravirus replication may occur in association with membranes derived from the endocytic pathway is not unexpected as tetraviruses infect larval midgut cells, which would have a well-developed endocytic pathway given their function in the absorption of nutrients.

Interestingly, previous studies have highlighted a link between maturation of virus-like particles (VLPs) and apoptosis, which is associated with lysosomal and mitochondrial destabilization and cytosolic acidification (Matsuyama & Reed, 2000; Nilsson et al., 2006). For...
example, it has been shown that NoV VLPs undergo spontaneous maturation at acidic pH (Canady et al., 2000, 2001). Moreover, in HaSV-infected midgut cells, there appears to be an accumulation of lysosomes and a high incidence of apoptosis (Brooks et al., 2002). The observation that HaSV VLPs undergo maturation upon the induction of apoptosis in yeast cells led Tomasicchio et al. (2007) to the hypothesis that virus replication might promote apoptosis in infected cells, and that cytosolic acidification is required for the maturation of virus particles.

This study represents the first step towards understanding the replication biology of members of the Tetraviridae. Their unusually narrow host range and tissue tropism raise interesting questions about the molecular mechanisms of infection and the site of replication in infected cells. It will be interesting to determine whether tetraviruses behave similarly to other members of the alpha-like virus superfamily that replicate in association with lysosomes and endosomes.

METHODS

Tissue-culture cell lines and maintenance. HeLa cells were maintained in buffered Dulbecco’s modified Eagles’ medium (DMEM; Lonza) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 U penicillin ml⁻¹, 10 mg streptomycin ml⁻¹ and 25 μg Fungizone ml⁻¹ (Lonza) at 37 °C in 10% CO₂. Spodoptera frugiperda pupal ovarian (Sf9) cells were maintained in TC100 insect medium (Lonza) supplemented with 10% fetal calf serum, 100 U penicillin ml⁻¹ and 10 mg streptomycin ml⁻¹ (Lonza) at 28 °C without CO₂.

Recombinant plasmids. The HaSV replicase coding sequence was obtained by PCR amplification from the full-length HaSV RNA1 cDNA (GenBank accession no. EU345431) obtained from Terry Hanzlik and Karl Gordon (CSIRO, Division of Entomology, Australia). All recombinant plasmids constructed in this study and the coordinates of the HaSV replicase coding sequences that they contain are summarized in Table 1.

Full-length replicase (REP₁–1704) was expressed in mammalian cells with N- and C-terminal EGFP tags (EGFP–REP₁–1704 and REP₁–1704–EGFP, respectively) using the expression vectors pEGFP-N1 and pEGFP-C1 (Clontech), whilst the construct pJRS7N46A expresses the REP minus its first 46 aa, fused at its C terminus to EGFP (Table 1). The insect expression vector pCW56 (Walter, 2008) is equivalent to pEGFP-N1 with the exception that the mammalian promoter has been replaced with an insect-specific hr/AcNPV hybrid promoter containing the Autographa californica nuclear polyhedrosis virus hr5 enhancer fused to the immediate-early promoter, also from Autographa californica nuclear polyhedrosis virus (obtained from Vernon Ward, Department of Microbiology, University of Otago, New Zealand).

DNA transfection. HeLa and Sf9 cells were grown to 70–80% confluence in 24-well plates on 13 mm coverslips and transfected with 150–200 ng purified plasmid DNA using Fugene HD (Roche Applied Science) according to the manufacturer’s instructions. Cells were incubated in 1 ml complete medium (DMEM for mammalian cells and TC100 for Sf9 cells) for between 16 and 24 h before further analysis.

Antibodies and organelle stains. Rabbit polyclonal antibodies to N-β-COP (recognizing the peripheral Golgi marker, β-COP) and ERp60 were a gift from Tom Wileman (University of East Anglia, UK). Antibodies specific for CD63, a late endosomal marker, were obtained from Mark Marsh (University College London, UK) or purchased from Invitrogen. Anti-EEA1 antibodies were purchased from Santa Cruz Biotechnology. Alexa Fluor 546-conjugated goat anti-rabbit, donkey anti-goat and goat anti-mouse antibodies, as well as Texas red-conjugated goat anti-mouse antibodies, were purchased from Molecular Probes (distributed by Invitrogen), as were MitoTracker Red 580, LysoTracker Red DND-99 and Cascade Blue-conjugated dextran (10000 Da).

Indirect immunofluorescence, live-cell imaging and confocal microscopy. For indirect immunofluorescence using antibodies to β-COP and ERp60, transfected cells were fixed at room temperature with 4% paraformaldehyde (pH 7.5–8.0) for 15 min. Cells were then incubated in permeabilization buffer [PBS (pH 7.6), 1% Triton X-100, 10% sucrose, 5% normal goat serum] for a further 20 min. Primary antibodies were diluted (1:400 for β-COP or 1:8000 for ERp60) in permeabilization buffer and incubated with coverslips at room temperature for 60 min. Coverslips were then washed in PBS–Tween [PBS (pH 7.6), 1% Tween 20] and incubated with Alexa Fluor 546-conjugated goat anti-rabbit or Texas red-conjugated goat anti-mouse antibodies diluted 1:750 in permeabilization buffer. Anti-EEA1 (1:200) and anti-CD63 (1:35) antibodies were diluted in, and cells were permeabilized with, PBS containing 0.2% BSA and 0.05% saponin. Coverslips were mounted for imaging in Fluorescent Mounting Medium (DakoCytomation).

For live-cell imaging using fluorescent dyes, HeLa and Sf9 cells were transfected as described above in glass-bottomed 35 mm Petri dishes (Asahi Techno Glass Corporation) and stained 24 h after transfection by incubation in appropriate medium containing MitoTracker Red

Table 1. Plasmids used for the expression of HaSV REP–EGFP fusion proteins

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter*</th>
<th>EGFP fusion</th>
<th>Expressed protein</th>
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<tbody>
<tr>
<td>pEGFP-N1</td>
<td>CMV</td>
<td>–</td>
<td>EGFP</td>
</tr>
<tr>
<td>pJRS7</td>
<td>CMV</td>
<td>REP C terminus</td>
<td>REP₁–1704–EGFP</td>
</tr>
<tr>
<td>pJRS8</td>
<td>CMV</td>
<td>REP N terminus</td>
<td>EGFP–REP₁–1704</td>
</tr>
<tr>
<td>pJRS7N46A</td>
<td>CMV</td>
<td>REP C terminus</td>
<td>REP₄₆–1704–EGFP</td>
</tr>
<tr>
<td>pCW56</td>
<td>hr/AcNPV (insect)</td>
<td>–</td>
<td>EGFP</td>
</tr>
<tr>
<td>pJRS26</td>
<td>hr/AcNPV (insect)</td>
<td>REP C terminus</td>
<td>REP₁–1704–EGFP</td>
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*CMV, Cytomegalovirus (mammalian).
580 or LysoTracker Red DND-99 for 30 min. These stains have been used in previous studies to identify lysosomes in Sf9 cells (Kawar & Jarvis, 2001; Kawar et al., 2001; Aumiller et al., 2006) and mitochondria in Sf9 (Chow et al., 2009) and Sf21 (Kurisu et al., 2003) cells. Cascade Blue-conjugated dextran was added to the medium at the time of transfection and incubated with cells for up to 24 h according to the manufacturer's instructions. Mammalian cells were rinsed and incubated in phenol red-free DMEM prior to imaging. Time-lapse imaging was performed on cells from 16 h after transfection for 6–8 h and the image series was analysed by using ImageJ 1.42o (http://rsb.info.nih.gov/ij/) and a manual particle-tracking plug-in (Sbalzarini & Koumoutsakos, 2005).

Confocal laser-scanning microscopy was conducted by using a Zeiss LSM 510 META confocal microscope with live-cell imaging facility. Additional images of CD63 immunofluorescence were obtained by using a Zeiss LSM 5 Pa. Images were taken using 488 nm Ar and 543 nm HeNe laser lines, standard filter sets and ×63 objective lenses and represent 0.8 μm optical slices, with the exception of live cells imaged over time (1 μm optical slices). All images were processed and annotated by using GIMP 2.4.6 or 2.6.6 (http://www.gimp.org), ImageJ 1.42o and/or Zen 2008 Light Edition (Carl Zeiss).

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