Positive sense (+) ssRNA viruses target their replication complexes to diverse locations, including the mitochondria (nodaviruses) (Garzon et al., 1990; Miller et al., 2001), endocytic pathway (togaviruses) (Froshauer et al., 1988; Magliano et al., 1998; Matthews et al., 2009), endoplasmic reticulum (ER) (poliovirus and tomato ringspot virus) (Bienz et al., 1987; Han and Sanfaçon, 2003; Rust et al., 2001) and Golgi apparatus (poliovirus late in infection) (Bolten et al., 1998). The mechanisms employed by (+) ssRNA viruses to target their vRNA replication complexes to particular cellular organelles have been well characterized, but the factors that drive the selection of these organelles as replication sites have yet to be elucidated.

Providence virus (PrV), the sole member of the newly created Carmotetraviridae tetravirus family, was isolated from a persistently infected Helicoverpa zea MG8 midgut tissue culture cell line and is the only tetravirus to replicate in tissue culture. Previous studies showed that in persistently infected Helicoverpa zea MG8 cells, the PrV replicase associates with detergent-resistant membranes in punctate cytosolic structures, which is similar to the distribution of an alpha-like tetravirus replicase (Helicoverpa armigera stunt virus). Here, we demonstrate that the site of PrV vRNA replication coincides with the presence of PrV p40/p104 proteins in infected cells and that these replication proteins associate with the Golgi apparatus and secretory vesicles in transfected cells.

Providence virus (PrV) is the sole member of the family Carmotetraviridae (formerly Tetraviridae) sharing the characteristic $T=4$ capsid architecture with other tetravirus families. Despite significant structural similarities, PrV differs from other tetraviruses in terms of genome organization, non-structural protein sequence and regulation of gene expression. In addition, it is the only tetravirus that infects tissue culture cells. Previous studies showed that in persistently infected Helicoverpa zea MG8 cells, the PrV replicase associates with detergent-resistant membranes in punctate cytosolic structures, which is similar to the distribution of an alpha-like tetravirus replicase (Helicoverpa armigera stunt virus). Here, we demonstrate that the site of PrV vRNA replication coincides with the presence of PrV p40/p104 proteins in infected cells and that these replication proteins associate with the Golgi apparatus and secretory vesicles in transfected cells.
complexes *in vivo*. Previously, we conducted an investigation of the subcellular localization of HaSV replicase, showing that replicase–EGFP fusions were associated with detergent-resistant membranes (DRMs) and localized to punctate structures in the cytoplasm of transfected *Spodoptera frugiperda* cells (Short *et al.*, 2010; Short and Dorrington 2012). A preliminary study revealed a similar subcellular localization and strong association with DRMs for the PrV replicase in virus-infected cells raising the possibility of a common site of replication for both HaSV and PrV (Short and Dorrington, 2012).

We have examined more closely the subcellular localization of PrV replication proteins in relation to dsRNA in PrV-infected *H. zea* cells. To determine whether the putative PrV replication proteins (p40 and p104) co-localize with replicating vRNA (dsRNA), persistently infected MG8 cells were prepared for confocal microscopy as previously described in Short *et al.* (2010), using rabbit anti-p40 antiserum that would detect both p40 and p104 and mouse monoclonal anti-dsRNA antibodies (clone K1; English & Scientific Consulting). The distribution of both p40/p104 and dsRNA was observed to range from co-localizing punctate cytosolic structures (Fig. 1b, top) to larger structures with stronger signal intensities spread over larger areas of the cytoplasm (Fig. 1b, bottom). The small punctate structures could represent earlier stages of infection, while the larger structures appear later in the infectious cycle. Interestingly, in cells with larger p40/p104-containing structures, the strongest dsRNA signal was

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**Fig. 1.** PrV genome organization and co-localization of PrV p40/p104 with dsRNA in persistently infected *H. zea* MG8 cells. (a) The ORFs of the viral replicase (p40/p104), capsid protein precursor (VCAP) and a non-structural protein of unknown function (p130). Known 2A-like processing sites are indicated, as is the position of the read-through stop (RTS) codon that results in the generation of both p40 and p104 replication proteins from the p104 ORF. VCAP is translated from a subgenomic RNA (not shown). (b) Selected confocal images of infected MG8 cells stained by immunofluorescence using antibodies directed against PrV p40 (and p104) and dsRNA. Green staining (left) shows p40/p104, while the red (centre) shows dsRNA and the merged image (right) also includes nuclei stained with DAPI (blue). Inset image is magnified ×2 over the area in the white box. All images represent 1 μm optical slices. Bars, 10 μm.
often concentrated around the periphery of the p40/p104 signal, possibly in virus particles. Taken together, these findings strongly suggest that p40 and p104 are involved in the formation of replication complexes and the replication of vRNA, as has been previously implied by bioinformatic analysis (Walter et al., 2010).

In order to better understand the dynamic processes involved in the different distributions observed, more than 2000 cells were imaged and analysed for the presence and distribution of p40/p104 and dsRNA. Of these, 246 cells (~11%) scored positive for p40/p104 expression and/or the presence of dsRNA as detected by immunofluorescence. The overlap and relative mean intensity of the signals were then analysed for each infected cell. Most of the cells (~71.5%) displayed roughly equivalent relative fluorescence intensities and distribution for p40/p104 and dsRNA. The remainder of the cells exhibited a higher intensity and a greater distribution of p40/p104 (~5%) or dsRNA (~20%), while a small proportion (~3%) were positive for only one of the immunofluorescence stains.

The mechanism by which the apparently persistent infection of MG8 cells by PrV is maintained is as yet unknown. The most likely possibilities are either: (i) the MG8 cells are only mildly susceptible to PrV infection and PrV multiplies within the population through cell division or cell-to-cell movement; or (ii) PrV infection of MG8 cells is inefficient. Consequently, the images of infected and uninfected cells were also analysed for signs of cell division (DNA metaphase or later) and cell death (chromosomal condensation and cell granularity). Of the cells imaged, 13 uninfected cells (0.66%) showed signs of cell division. While no dividing cells were observed among the infected cells, a significantly larger population of cells would need to be scored for a statistically significant conclusion to be drawn regarding the movement of PrV between cells via cell division. However, an increased rate of chromosomal condensation and vesiculation in infected cells (41% vs 5% in uninfected cells) suggests that PrV replication ultimately results in cell death, which therefore implies a lytic life cycle. This increase in granularity could, however, be due to the formation of spherules in organelle membranes or a stimulation of membrane production as opposed to apoptosis or necrosis and further investigation would be necessary to determine if that is the case. Additional evidence of a lytic PrV infection lies in the observation of cells that were positive for dsRNA or p40/p104 only, indicating that replication progresses in infected cells and is not simply continuous at low levels. Finally, our preliminary attempts at superinfection of MG8 cells with purified PrV results in an increase in cell death, albeit with no significant increase in total PrV production (C. Adams and R.A. Dorrington, personal communication). Taken together, these data suggest that the persistence of PrV infection in the MG8 cell line is probably due to inefficiencies at establishing PrV infection, due to a combination of poor infectivity of progeny virions, a high level of immunomodulation within infected insect host cells and low levels of PrV virus production.

Direct demonstration of the co-localization of the vRNA replication complexes with cellular organelles was not possible due to a lack of suitable antibodies directed against organelle markers in MG8 cells. As a result two constructs for the expression of EGFP-tagged PrV replication proteins from an hr/ACMNPV hybrid promoter in S. frugiperda Sf9 cells were generated. The constructs contained the entire p104 ORF fused to the C terminus of EGFP and generated EGFP–p104 (wt) (EGFP–p40 and EGFP–p104 expression) or EGFP–p104 (Leu) (producing only EGFP–p104 with the read-through ‘UAG’ stop codon mutated to ‘CUU’ encoding a leucine residue). Expression of only p104 from the p104 (Leu) ORF was confirmed using coupled transcription and translation reactions in insect cell extracts (data not shown).

First, we assessed the effect of the EGFP-tag on the distribution of p40 and p104 in the absence of other PrV proteins. S. frugiperda Sf9 cells expressing each of the EGFP-tagged fusion proteins were fixed with paraformaldehyde and examined by confocal fluorescence microscopy (Fig. 2). EGFP-associated fluorescence in cells expressing EGFP-tagged p104 or p40/p104 produced a small number (usually fewer than 10) of spherical structures distributed throughout the cytoplasm that typically appeared to be between 1.5 μm and 250 nm in diameter, reminiscent of the p40/p104 distribution in infected cells (compare Fig. 1b, top, to Fig. 2).

The subcellular location of the punctate structures observed for the EGFP-tagged PrV replication proteins was compared to that of fluorescent protein-tagged organelle marker proteins (Table S1, available in JGV Online). Comparison of the EGFP-tagged PrV replication proteins with co-expressed actin–YFP (Fig. 3a) revealed small amounts of actin accumulation at the same structures populated with EGFP–p104 (Leu) (Fig. 3a, top). Although no such accumulation was observed for EGFP–p104 (wt) (Fig. 3a, top).
bottom row), these data suggested that actin might be involved in the formation of PrV replication complexes. To assess the role of actin in the initial formation of the punctate structures, cells expressing EGFP–p104 (wt) were treated with 5 or 10 \( \mu \)g ml\(^{-1} \) cytochalasin D for 15 h. Control cells expressing actin–YFP showed a collapse of the

**Fig. 3.** Co-localization of EGFP-tagged p40 and p104 in *S. frugiperda* Sf9 cells co-transfected with fluorescent organelle marker proteins. Confocal fluorescence images of cells expressing EGFP-tagged PrV replication proteins and actin–YFP (a) or Golgi-targeted YFP (b) and/or CFP targeted to secretory vesicles (c). Insets represent a \( \times 2 \) magnification of the white box. All images represent 1 \( \mu \)m thick optical slices. Bars, 10 \( \mu \)m.
actin cytoskeletal network at both concentrations, while the cellular distribution of EGFP-p40/p104 remained unchanged (data not shown). Therefore, if actin is involved in the replication of vRNA, it is likely to be involved in the condensation of membranes containing the viral replicase later in infection (Fig. 1b, bottom) and not in the initial formation of the punctate structures.

No overlap was observed between EGFP-tagged p40/p104 or p104 alone and the ER, peroxisomes or lysosomes (Fig. S1, data not shown). Since the lysosome-targeted RFP did not resolve the lysosomes satisfactorily in any of our experiments, this result was confirmed using LysoTracker (Fig. S2). In contrast, significant overlap between fluorescent proteins targeted to the Golgi apparatus (Fig. 3b) and secretory vesicles (Fig. 3c) and all of the EGFP-tagged p40/p104 variants was observed in more than 75 % of the co-transfected cells. The somewhat altered distribution of the PrV proteins in the presence of the Golgi-directed YFP suggests that some of the overlap observed in this instance may be due to the overloading of common protein transport machinery, resulting in the stalling of transport to the secretory pathway, which is more likely to be the ultimate destination for the replicase.

The subcellular trafficking of the PrV replicase proteins to the secretory pathway is unexpected. This is because the distribution and membrane association of p40/104 is similar to that of the HaSV replicase, which has been suggested to associate with membranes derived from an alternate endocytic pathway due to a partial overlap with the late endosomal marker, CD63, in transfected mammalian cells (Short et al., 2010). Both replicases have been shown to associate strongly with cellular DRMs and the distribution of p40/104 and the HaSV replicase in insect cells appears virtually indistinguishable (Short and Dorrington, 2012). However, the data obtained with the HaSV replicase demonstrated only partial overlap with late endosomes, which was thought to be the result of endocytic organelle associations based upon an hypothesis that virus replication may be associated with acidic subcellular compartments (Tomasicchio et al., 2007). This partial association with late endosomes could also be the result of the fusion of secretory vesicles containing the replicase with the late endosomes as part of normal vesicular trafficking. Further studies to confirm the targeting of the HaSV replicase to the secretory pathway are currently under way.

In conclusion, this study provides the first insight into the in vivo formation of tetravirus replication complexes and demonstrates that PrV replicates vRNA in association with secretory pathway vesicles. The data also shed light on the nature of the PrV infectious life cycle in H. zea MG8 cells, providing evidence for a low level lytic infection rather than a persistent infection. Our preliminary data indicate that the HaSV replicase also localizes to the secretory pathway (J.R. Short and R.A. Dorrington, unpublished results). The similarities between the distribution of the PrV and HaSV replicases suggest that, although there are significant differences in the sequence and expression strategies of these viral replicases, there may be important biological and structural determinants that require replication to occur at the same location.

References


