

Foot-and-mouth disease virus replication sites form next to the nucleus and close to the Golgi apparatus, but exclude marker proteins associated with host membrane compartments

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Picornavirus infection of cells generally results in the production of membranous vesicles containing the viral proteins necessary for viral RNA synthesis. To determine whether foot-and-mouth disease virus (FMDV) infection induced similar structures, and which cellular components were involved, the subcellular distribution of FMDV proteins was compared with protein markers of cellular membrane compartments. Using immunofluorescence analysis and digital deconvolution, it was shown that FMDV structural and non-structural proteins co-localize to punctate structures in juxtannuclear virus assembly sites close to the Golgi complex. Significantly, viral protein 2C did not co-localize with marker proteins of the *cis*- or *medial*-Golgi compartments or *trans*-Golgi network. Furthermore, incubation of infected cells with brefeldin A caused a redistribution of Golgi proteins to the endoplasmic reticulum, but did not affect the distribution of 2C and, by inference, the integrity of the virus assembly site. Taken with the observation that 2C was membrane-associated, but failed to fractionate with Golgi markers on density gradients, it was possible to conclude that Golgi membranes were not a source of structures containing 2C. Further immunofluorescence analysis showed that 2C was also separate from marker proteins of the endoplasmic reticulum, endoplasmic reticulum intermediate compartment, endosomes and lysosomes. The results suggest that the membranes generated at FMDV assembly sites are able to exclude organelle-specific marker proteins, or that FMDV uses an alternative source of membranes as a platform for assembly and replication.

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INTRODUCTION

A common feature of infection of cells by plus-strand viruses is the extensive rearrangement of host cell membranes, and the generation of cytoplasmic vesicles which are apparently required for replication. These membranous structures were described several years ago in cells infected by picornaviruses such as poliovirus (Bienz *et al.*, 1983; Caligiuri & Tamm, 1970; Dales *et al.*, 1965), echovirus (Skinner *et al.*, 1968) and coxsackievirus A (Jezequel & Steiner, 1966). Several studies suggest that these replication complexes are associated with membrane compartments of the host cell secretory pathway, and most of the work on picornaviruses is based on analysis of cells infected with poliovirus. Electron micrographs indicate that poliovirus-induced vesicles are initially formed from endoplasmic reticulum (ER) membranes (Bienz *et al.*, 1987), at sites

enriched for the COPII protein complex (Rust *et al.*, 2001) that is important for vesicle transport between the ER and Golgi, while at later times poliovirus proteins co-localize with Golgi marker proteins (Bolten *et al.*, 1998). Biochemical studies, on the other hand, suggest a less stringent location, since virus-induced membranes isolated from poliovirus-infected cells contain marker proteins of the ER, Golgi and lysosomes (Schlegel *et al.*, 1996). The use of several compartments of secretory pathway by poliovirus is consistent with the redistribution of Golgi compartments (Sandoval & Carrasco, 1997) and inhibition of cellular protein secretion (Doedens & Kirkegaard, 1995) observed in infected cells. An involvement of the secretory pathway in the life cycle of poliovirus is further indicated by the observation that brefeldin A (BFA), which leads to the dissociation of the COPI coat from the Golgi apparatus and blocks vesicular transport between the ER and Golgi complex (Lippincott-Schwartz *et al.*, 1989), inhibits the replication of poliovirus (Cuconati *et al.*, 1998; Irurzun

Supplementary material supplied in JGV Online.

et al., 1992; Maynell *et al.*, 1992). Interestingly, not all picornaviruses are affected by BFA and there seem to be differences in the involvement of COPI in the formation of the replication complex. For echovirus 11, which is sensitive to the drug, COPI coats are retained in the replication complex, but, interestingly, COPI coats are not located in replication complexes formed by encephalomyocarditis virus which is resistant to BFA (Gazina *et al.*, 2002).

FMDV, like other members of the family *Picornaviridae*, is a non-enveloped RNA virus which contains a single-stranded, positive-sense RNA molecule of approximately 8500 nt that serves as a genome (Forss *et al.*, 1984). This (+) RNA, functioning as mRNA, encodes a high molecular mass polyprotein that undergoes co-translational autoproteolysis to yield four structural polypeptides (VP1–4) which comprise the viral capsid, and nine non-structural proteins of the P2 and P3 regions that control the viral life cycle within host cells (Ryan *et al.*, 1989). In contrast to poliovirus, the replication complex of FMDV, and its possible association with cellular membranes, has not yet been described in detail. Our recent work using electron microscopy shows that the virus assembly site forms next to the nucleus and contains membranous structures (Monaghan *et al.*, 2004), yet the origins of the membranes are unknown. In this report, we use immunofluorescence analysis to demonstrate that cytoplasmic structures staining for viral proteins form at perinuclear sites close to a dispersed Golgi apparatus. The viral non-structural protein 2C co-localizes with VP1, 3A and 3D within these structures, but not with proteins associated with organelles of the host cell secretory pathway, including the ER, the ER to Golgi intermediate compartment (ERGIC), Golgi complex, *trans*-Golgi network (TGN) or lysosomes. The results suggest that these membranes of the secretory pathway are not used by the virus as a platform for replication and assembly, or that the FMDV-induced structures are formed by membrane rearrangements which involve exclusion of organelle-specific protein markers.

METHODS

Cells and viruses. Chinese hamster ovary (CHO-K1), African green monkey kidney (GMK) and Renal swine-2 (RS-2) cells are maintained at the Institute for Animal Health, Pirbright Laboratory, Surrey, UK. Baby hamster kidney (BHK-21) cells were purchased from Invitrogen. Cells were maintained in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) or, in the case of CHO-K1 cells, RPMI 1640 medium supplemented with 5% fetal calf serum, 100 U penicillin ml⁻¹ and 5 mg glutamine ml⁻¹ at 37 °C in 5% CO₂. FMDV type O1K was used to infect cells in all experiments.

Antibodies and reagents. Monoclonal antibodies (mAb) against FMDV non-structural proteins 2C, 3A and 3D were a kind gift from E. Brocchi at The Instituto Zooprofilattico in Brescia, Italy. Mouse mAb B2 against FMDV structural protein VP1, and rabbit antiserum DM10 recognizing FMDV non-structural protein 2C were provided by World Reference Laboratory (IAH, Pirbright, UK). Polyclonal antibodies against α -mannosidase II (Moreman *et al.*, 1991) were purchased from the Dept of Biochemistry and Molecular Biology,

University of Georgia, USA. Antibodies recognizing ERp57 were raised in rabbits by using a peptide PIIQEEKPKKKKKAQEDL found at the C terminus of the protein. Rabbit polyclonal antibodies against the peripheral Golgi marker β -COP, and mAb G1/93 against ERGIC p53 were kind gifts from J. Lippincott-Schwartz and Hans-Peter Hauri, respectively (University of Basel, Switzerland). Antibodies recognizing TGN46 (Prescott *et al.*, 1997) were raised in sheep and kindly provided by V. Ponnambalam (University of Leeds, UK). An antibody specific for CD63 was provided by M. Marsh (University of London, UK). mAb H4B4 against human LAMP-2 was purchased from the Developmental Studies Hybridoma Bank, University of Iowa, USA. FITC- and Alexa dye conjugated secondary antibodies were purchased from Molecular Probes. BFA was purchased from Sigma.

Infections. Near-confluent cultures growing in 100 mm Petri dishes containing 13 mm glass coverslips were infected at 37 °C with FMDV (m.o.i approximately 5 p.f.u. per cell) in medium containing 5% fetal calf serum for 30 min. Fresh medium was then added and incubation continued for a further 2–6 h (depending on cell type) to allow expression of viral proteins. Cells were then either lysed for biochemical experiments or fixed for immunofluorescence analysis (see below).

Labelling of cells, preparation of lysate fractions and immunoprecipitation. FMDV-infected BHK-21 cells growing in 100 mm Petri dishes were incubated for 10 min at 37 °C in methionine/cysteine-free Eagle's medium and then pulse-labelled for 60 min with 2 MBq of ³⁵S-Promix (Amersham Life Science) per ml of the same medium. Cells were then trypsinized, washed twice in 0.25 M buffered sucrose (50 mM Tris/HCl, 1 mM EDTA, pH 7.4) and resuspended in 200 μ l of the same buffer before being homogenized by 15 passages through a 25 gauge needle. Whole cells and nuclei were removed by pelleting at 2000 r.p.m. for 1 min at 4 °C in an Eppendorf 5402 centrifuge and resuspended in immunoprecipitation buffer (10 mM Tris/HCl pH 7.8, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mg ml⁻¹ each of leupeptin, pepstatin, chymostatin and antipain) for further analysis by immunoprecipitation (see below). Membrane and cytosolic fractions were then obtained by centrifugation of the post-nuclear supernatant at 14 000 r.p.m. (in an Eppendorf 5415 centrifuge) for 15 min. The cytosolic fraction was retained for immunoprecipitation while the membrane fraction was solubilized in immunoprecipitation buffer and recentrifuged at 14 000 r.p.m. (in an Eppendorf 5415 centrifuge) for 15 min to separate protein aggregates and solubilized membrane protein fractions. Membrane fractions were also resuspended in 1 ml 30% Nycodenz and layered under Nycodenz step gradients prepared using 5% (2 ml), 10% (3 ml) and 17.5% (5 ml) Nycodenz in buffered sucrose. Gradients were centrifuged at 60 000 g for 90 min in a SW40 Ti rotor at 4 °C. Fractions were collected from the bottom and assayed for ER marker α -glucosidase by using 4-methylumbelliferyl- α -D-glucose, for the Golgi marker enzyme galactosyltransferase by monitoring transfer of UDP-6-[³H]galactose (New England Nuclear) to ovalbumin, and for the lysosomal enzyme β -hexosaminidase using 4-methylumbelliferyl-N-acetylglucosamine (Cobbold *et al.*, 1996). For immunoprecipitation of FMDV 2C from each fraction, polyclonal antibody DM10 bound to protein A-Sepharose beads (Pharmacia) was incubated overnight at 4 °C with precleared lysate. Beads were then washed four times in immunoprecipitation buffer, denatured in SDS gel loading buffer and separated by 12% SDS-PAGE.

Immunofluorescence. Infected cells growing on 13 mm coverslips were fixed for 30 min at room temperature in 4% paraformaldehyde adjusted to pH 7.5–8.0, washed three times in TBS (150 mM NaCl, 100 mM Tris/HCl, pH 7.5) and permeabilized by incubation in TBS containing 1% gelatin and 0.5% NP-40. Samples were incubated with antibodies against viral proteins together with antibodies

specific for host cell protein markers diluted in TBS containing 1% gelatin, 0.5% NP-40 and 30% goat serum. Samples were washed with PBS containing 0.1% Tween-20 and then incubated with secondary antibodies conjugated to Alexa dyes. Finally, washed samples were mounted in Fluoromount G (Southern Biotechnologies).

Digital imaging. Cell preparations were viewed at $\times 60/1.4$ NA with a Nikon E800 inverted microscope fitted with epifluorescent optics, and photographed with a Hamamatsu C-4746A charge coupled camera device (CCD). For each cell preparation, nine digital images were collected at $0.2\ \mu\text{m}$ intervals and deconvolved using an Openlab software package (Improvision). After being adjusted for contrast and intensity, images were compiled using Quark Xpress software.

Serial optical sectioning. In order to enhance resolution and reveal the precise positions of the proteins with respect to each other, several optical sections were taken at $0.2\ \mu\text{m}$ intervals through the cell and digitally deconvolved. To obtain double-label fluorescence images, corresponding deconvolved slices of two different colourized protein signals were superimposed. In such images, red and green pixels represent separate protein localizations, whereas yellow areas indicate co-localization of the two proteins.

RESULTS

FMDV structural and non-structural proteins locate to punctate structures in the perinuclear cytoplasm

To determine the location of the FMDV assembly site, cells were infected with the virus for 3–4 h, fixed, and probed with antibodies specific for the non-structural protein 2C, and structural protein VP1. Distributions of 2C and VP1 are compared in Fig. 1, where (a)–(c) show conventional immunofluorescence images, and (d)–(f) show digitally deconvolved images. In the conventional images, 2C and VP1 showed diffuse staining throughout the cell and a concentrated punctate/vesicular signal close to the nucleus. The diffuse signal may represent soluble protein in the cytoplasm, and this is supported later by membrane fractionation experiments where FMDV proteins were recovered from both crude cytosol and membrane fractions (see Fig. 3). Deconvolution removed the diffuse cytoplasmic signal and out-of-focus haze, leading to better resolution of

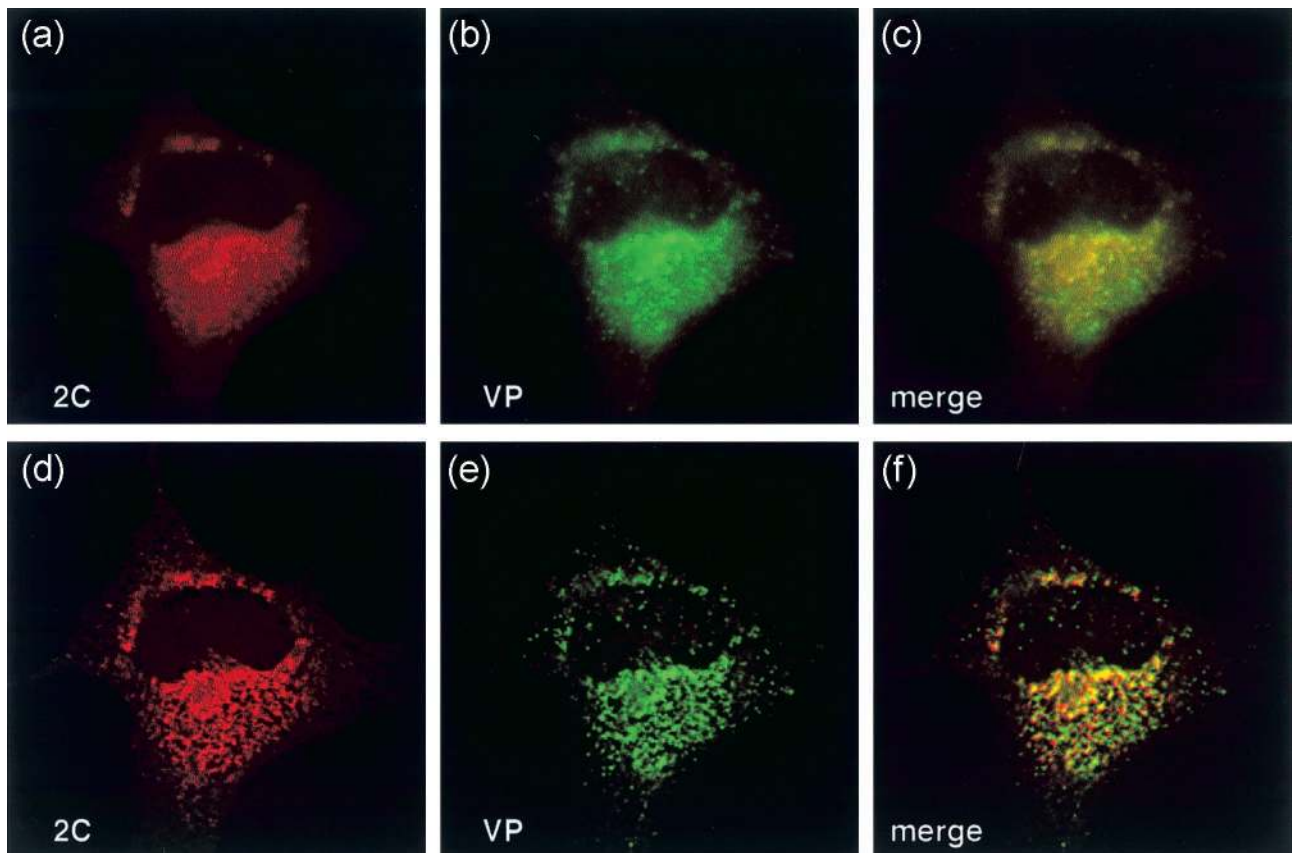


Fig. 1. Subcellular localization of FMDV protein VP1 and 2C. Four hours following infection with FMDV type O1K, CHO-K1 cells were fixed and processed for double-label immunofluorescence using rabbit antibody specific for 2C and mouse mAb specific for VP1. Antigens were visualized using Alexa 488-conjugated anti-mouse and Alexa 594-conjugated anti-rabbit IgG. Conventional images are depicted in panels (a–c). In panels (d–f) digital images of $0.2\ \mu\text{m}$ optical sections were deconvolved using Openlab software from Improvision. Panels (c) and (f) show merged images.

the juxtanuclear signal. Fig. 1(d) and (e) show that both proteins produced a bright punctate fluorescence signal, and the yellow signal in the digital merge in Fig. 1(f) showed that the bulk of 2C co-localized with VP1. There were, however, a few structures that remained separate as seen by single red and green pixels. Infected cells were next probed with antibodies specific for viral non-structural proteins 2C, 3A or 3D. Deconvolution, followed by a merge of corresponding slices showed that 2C largely co-localized with both 3A and 3D (not shown). The observation that the viral RNA polymerase, 3D, and non-structural protein 2C, which is also implicated in viral RNA replication (Barton & Flanagan, 1997; Li & Baltimore, 1988), were found in these structures, suggests that they represent the membrane-filled replication site described for all picornaviruses studied to date. We have also demonstrated that these structures incorporate bromouridine in the presence of inhibitors of cellular RNA production, and therefore contain synthesized viral RNA, and that the same regions are enriched with membrane vesicles when observed by electron microscopy (Monaghan *et al.*, 2004).

Virus-induced structures form at juxtanuclear sites close to the Golgi apparatus

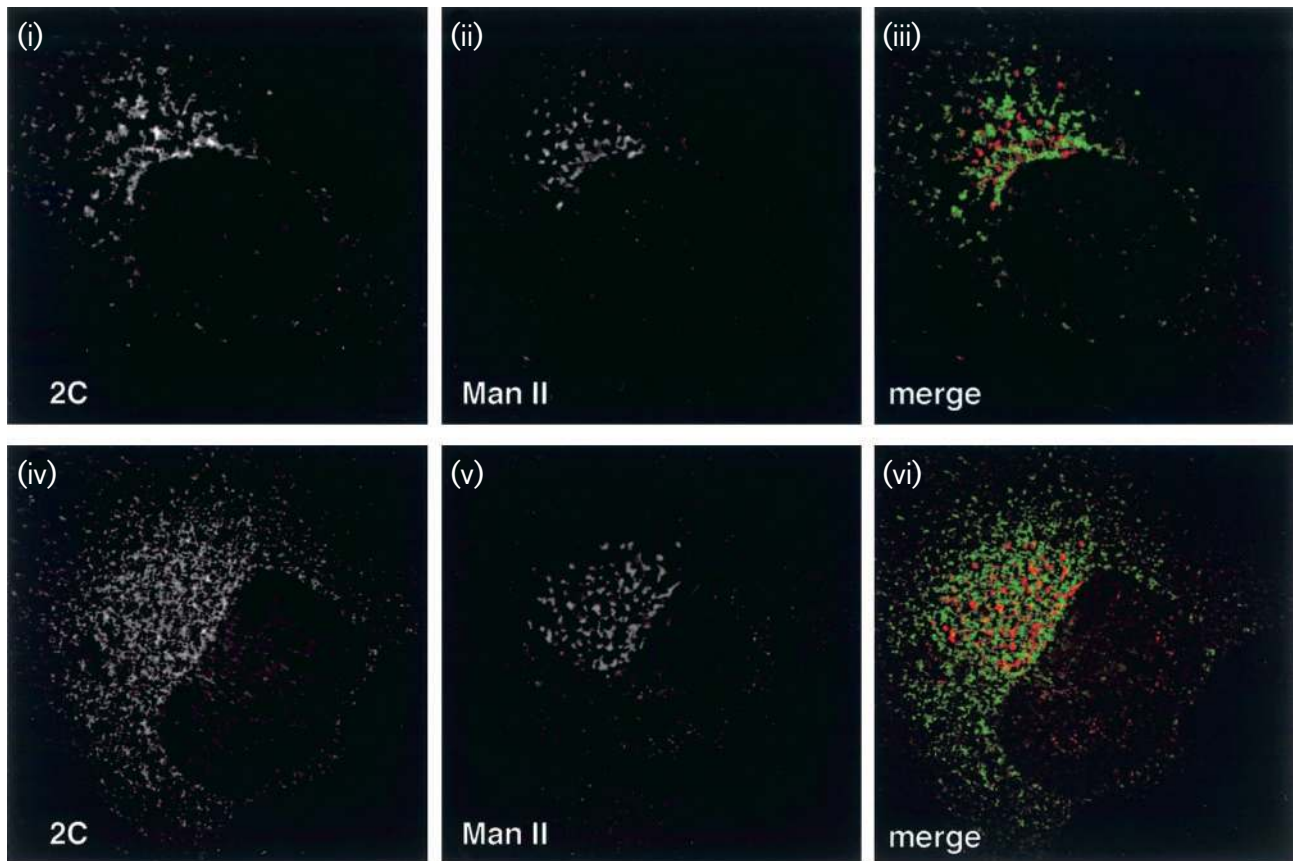
The images in Fig. 1, and images published previously (Monaghan *et al.*, 2004), show that FMDV proteins accumulate in perinuclear sites. Since this site is often occupied by the Golgi apparatus, the distribution of 2C and Golgi marker protein mannosidase II (Man II) was determined by immunofluorescence, and two sets of cells are shown in Fig. 2a. It appeared that the Man II and 2C signals largely overlapped, but surprisingly, when corresponding deconvolved images of 2C and Man II were superimposed (iii and vi), the two signals remained as separate green (2C) and red (Man II) pixels. These results suggested that the bulk of the FMDV 2C protein did not co-localize with Golgi membranes. To investigate the relationship between FMDV-induced complexes and Golgi membranes in another way, we made use of the ability of the fungal metabolite, BFA, to disrupt Golgi structure. BFA induces a rapid redistribution of *cis*- and *medial*-Golgi membranes and proteins into the ER, leaving no definable Golgi structure (Lippincott-Schwartz *et al.*, 1989). We reasoned that, if 2C and Man II were localized to the same subcellular structures, then the staining pattern of these proteins should be similar in cells after treatment with BFA, or that replication complexes would disperse. Fig. 2(b) shows images of 2C (i) and Man II (ii) in a cell treated with BFA. As expected, the staining pattern of Man II became noticeably dispersed, indicating a redistribution of Golgi membranes into the ER. In contrast, 2C remained distributed within the perinuclear virus assembly site. The experiments indicated that 2C and Man II occupy different subcellular locations. Although the two proteins are found in the same juxtanuclear part of the cell, it is not possible to conclude that Golgi membranes are a source of 2C-containing structures.

Membrane association of FMDV protein 2C

In the next experiments subcellular membrane fractionation was used to study further the association of FMDV proteins with cellular membrane compartments. The first experiment tested whether FMDV 2C was membrane-associated, as in the case of its poliovirus counterpart (Cho *et al.*, 1994; Schlegel *et al.*, 1996). BHK-21 cells infected with FMDV were radiolabelled for 60 min, homogenized and centrifuged to obtain nuclear pellet and post-nuclear supernatant fractions. The post-nuclear supernatant was then centrifuged to obtain crude cytosol and membrane fractions. The crude membrane fraction was solubilized in immunoprecipitation buffer and further centrifuged to obtain soluble and aggregate fractions. Fig. 3(a) shows that both 2C and its precursor 2BC were immunoprecipitated from infected cell lysates. Relatively small amounts were present in the nuclear pellet fraction (lane 1). The majority of the 2C and 2BC were equally distributed between cytosol and membrane fractions (lanes 2 and 4, respectively), while little if any protein was found in the pellet of insoluble protein aggregates (lane 3). In similar experiments, a significant proportion of other FMDV structural and non-structural proteins was also found in the membrane fraction and therefore considered to be, at least partially, membrane-associated (data not shown).

Given that approximately half of 2C was membrane-associated, subcellular membrane fractionation was used to test whether 2C associated with membranes of the secretory pathway. The membranes within a post-nuclear membrane fraction prepared from cells infected with FMDV were analysed using Nycodenz gradients. The locations of ER and Golgi membrane markers are shown in Fig. 3(b), and the migration of 2C was determined by immunoprecipitation of gradient fractions. The gradient gave good separation of the ER and Golgi; however, 2C did not co-sediment with the markers tested, and was broadly distributed between fractions 1–6, suggesting association with membranes of variable density. Even so, the highest levels of 2C were found in fractions slightly heavier than the ER membranes. Since approximately half the 2C signal was found in fractions 3–5 that also contained ER and Golgi enzymes, these fractions were pooled and separated on a second shallow Nycodenz gradient to resolve ER and Golgi membranes further. The ER marker migrated towards the bottom of the gradient (Supplementary material in JGV Online) and the Golgi marker was found in two peaks. Under these conditions a proportion of the membrane-associated 2C signal co-fractionated with the light Golgi membrane peak, but not ER or heavy Golgi fractions. In summary, the bulk of the 2C signal was recovered from membranes that did not co-sediment with ER or Golgi marker enzymes, suggesting a lack of co-localization with these organelles. Approximately 25% of membrane-associated 2C did, however, co-fractionate with a light membrane fraction containing the Golgi sugar processing enzyme UDP-galactosyltransferase.

(a)



(b)

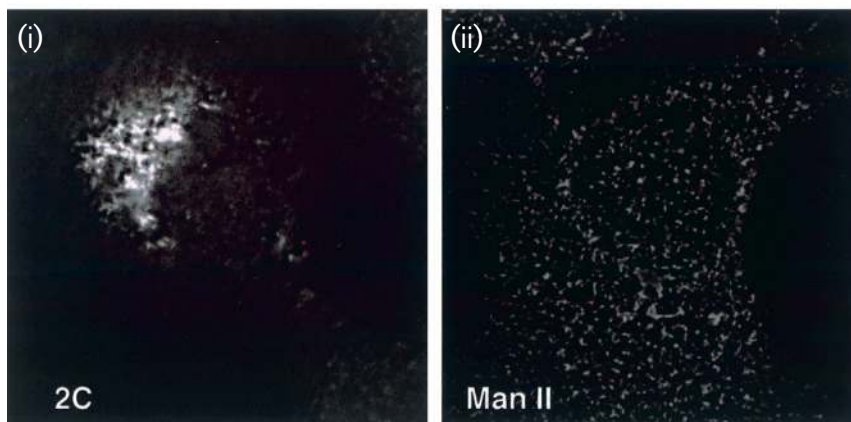


Fig. 2. Subcellular localization of Man II and FMDV 2C. (a) Infected cells. Four hours after infection with FMDV type O1K, CHO-K1 cells were processed for double-label immunofluorescence using mAb specific for FMDV 2C and rabbit antibody recognizing Man II. FMDV 2C and Man II distribution was visualized using Alexa 488 or 594 dyes conjugated to anti-mouse or anti-rabbit IgG, respectively. Digital images of $0.2\ \mu\text{m}$ optical sections were deconvolved using Openlab software from Improvision. Two different cells are shown in panels (i–iii) and (iv–vi), respectively. Panels (iii) and (vi) show digital merges of 2C (green) and Man II (red). (b) Infected cells incubated with BFA. Four hours after infection of CHO-K1 cells with FMDV type O1K, cells were incubated for 30 min with BFA ($10\ \mu\text{g ml}^{-1}$) and processed for double-label immunofluorescence using mAb specific for FMDV 2C and rabbit antibody recognizing Man II. FMDV 2C distribution (i) and Man II distribution (ii) were visualized using Alexa 488 or 594 dyes conjugated to anti-mouse or anti-rabbit IgG, respectively, as described.

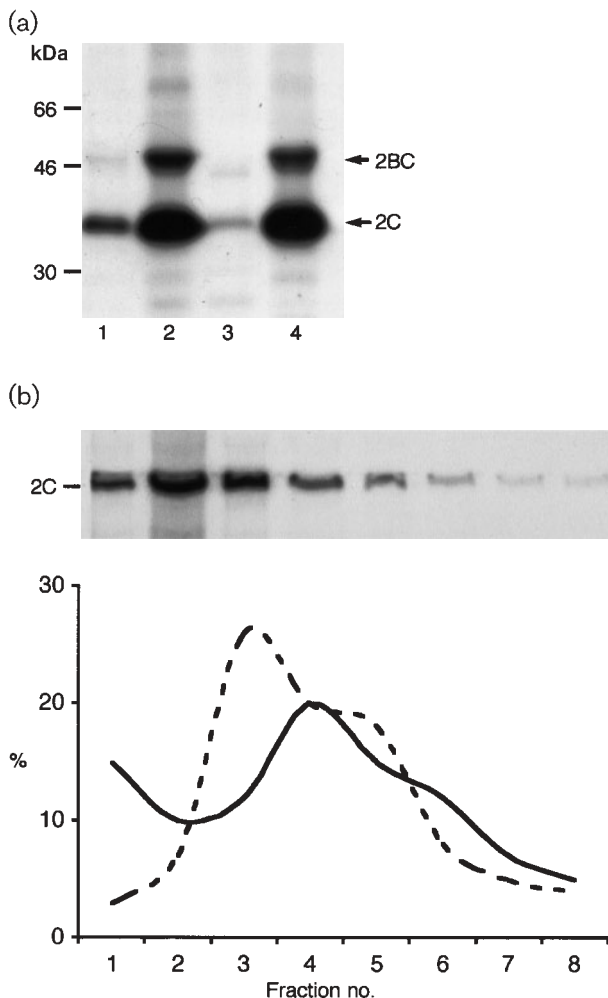


Fig. 3. Association of FMDV 2C with cellular membranes. (a) Crude subcellular membrane fractionation. Four hours after infection with FMDV type O1K, BHK-21 cells were labelled metabolically for 60 min with ^{35}S -methionine and cysteine, and then homogenized in buffered sucrose. Fractions containing whole cells and nuclei, post-nuclear membranes and cytosol were recovered by centrifugation and lysed in immunoprecipitation buffer. The membrane fraction was recentrifuged at 14 000 r.p.m. (rotor) for 15 min to produce a pellet or aggregated protein, and a supernatant of solubilized membrane. The fractions were immunoprecipitated with rabbit antibody specific for FMDV 2C and proteins resolved by SDS-PAGE followed by autoradiography. Lanes 1, whole cells and nuclear pellet; 2, cytosolic fraction; 3, protein aggregate; 4, solubilized membrane fraction. (b) Subcellular membrane fractionation on Nycodenz gradients. Membrane fractions collected as described in (a) were suspended in 30% Nycodenz and layered below a discontinuous Nycodenz gradient. Fractions were assayed as indicated for ER marker α -glucosidase (dashed line) or Golgi marker galactosyltransferase (solid line). The distribution of FMDV 2C was determined by immunoprecipitation of membrane fractions.

Distribution of pre-Golgi marker proteins in cells infected with FMDV

The above observation led us to speculate that host cell membranes other than those of the Golgi may be the source of membranes in replication complexes. To examine the possibility that pre-Golgi compartments were involved we compared the distribution of 2C with that of the ER-resident protein ERp57. As expected, ERp57 produced a fine reticular staining pattern characteristic of the ER (Fig. 4b). FMDV 2C again localized in a juxtanuclear position (Fig. 4a). The ERp57 signal was reduced in this region but the reticular pattern was retained in the infected cell. When corresponding deconvolved images were superimposed, the two protein signals remained separate, and yellow areas were not observed, indicating that 2C is not associated with ERp57-positive structures (Fig. 4c). We next compared the distribution of 2C with a marker protein of the intermediate membrane compartment between the ER and Golgi. This tubulo-vesicular structure is identified by an integral membrane protein, ERGIC53 (Schweizer *et al.*, 1988), which cycles between the *cis*-Golgi and the ER. Since staining with the antibody available for ERGIC53 was found to be negative in CHO-K1 cells, we compared the distributions of 2C and ERGIC53 in porcine kidney RS-2 cells, which gave a positive signal for ERGIC53 and were susceptible to FMDV infection. In uninfected cells ERGIC53 was located to a crescent-shaped juxtanuclear area similar to the staining seen for Man II (data not shown). In infected cells, however, ERGIC53 was redistributed into weakly stained spots scattered throughout the cytoplasm (Fig. 4e). When corresponding deconvolved images of 2C and ERGIC53 were merged, the two protein signals remained completely separate as red and green pixels (Fig. 4f). These results indicate that, although FMDV caused disruption of ERGIC53 distribution, 2C was not associated with membranes of the ERGIC.

Distribution of post-Golgi marker proteins in cells infected with FMDV

We next compared the distribution of 2C with the *trans*-Golgi network (TGN), endosomes and lysosomes as examples of post-Golgi compartments. The experiments were carried out in GMK cells, a monkey kidney cell line, which gave a strong immunofluorescence signal with the antibodies available and could be infected by FMDV. Cells stained with antibodies against 2C and TGN46 are shown in Fig. 5. TGN46 was distributed in ribbon-like structures located in a juxtanuclear position (Fig. 5b), and there was no obvious variation in the staining pattern of this protein between the infected cell (top left) and uninfected cells. As seen for Man II, the distribution of 2C was close to that of TGN46 (Fig. 5a and b). However, when corresponding deconvolved images were superimposed, the protein signals were found to be completely separate (Fig. 5c). Fig. 5 also shows the distribution of the endosomal marker CD63 (Fig. 5d–f) and lysosomes labelled with antibodies specific for LAMP-2 in cells infected with FMDV (Fig. 5g–i). CD63

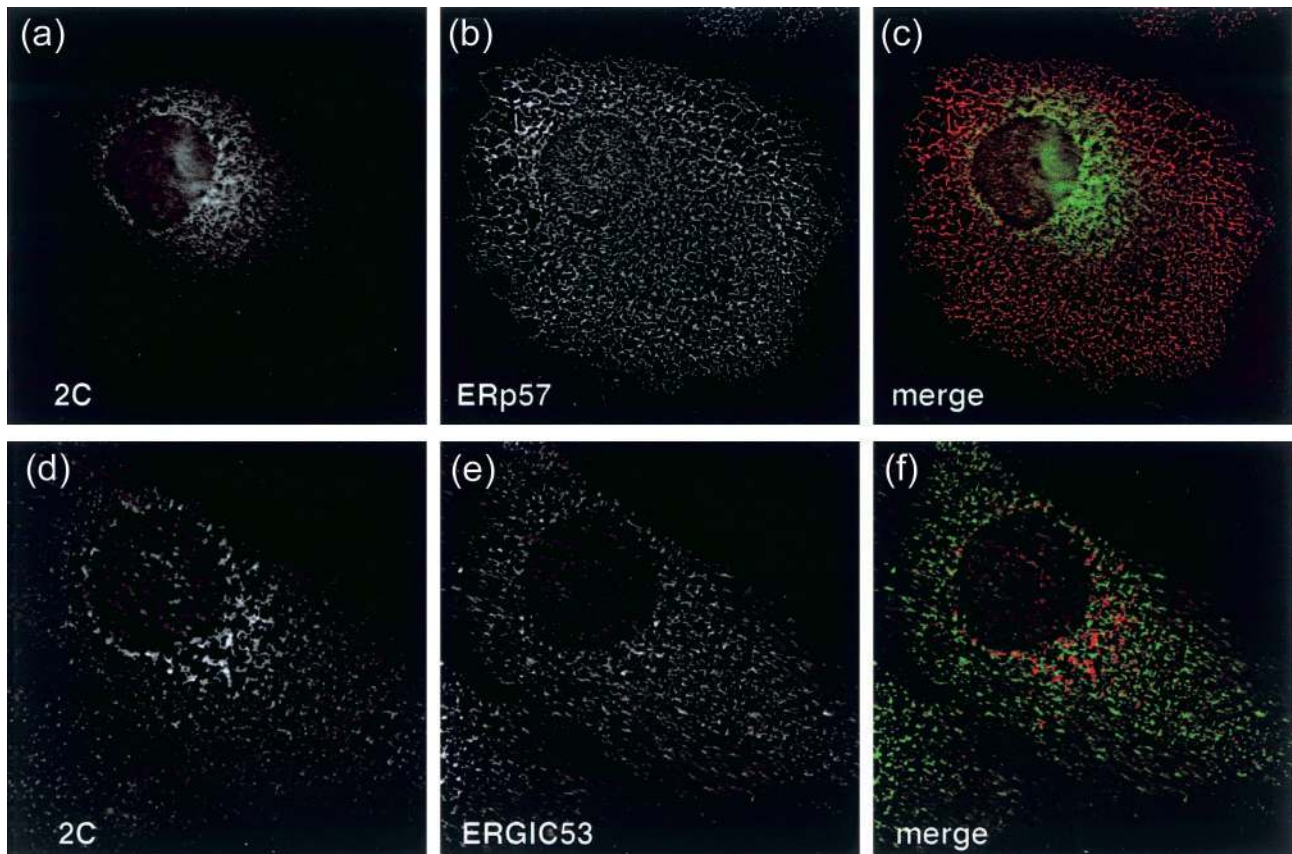


Fig. 4. Subcellular localization of FMDV 2C and pre-Golgi membrane compartments. Four hours after infection of CHO-K1 cells with FMDV type O1K, cells were processed for double-label immunofluorescence using antibody specific for FMDV 2C and antibodies recognizing pre-Golgi membrane compartments. (a–c) Location of 2C and ER labelled using antibody specific for ERp57. (d–f) Location of FMDV 2C and ER Golgi intermediate compartment labelled using antibody specific for ERGIC53. FMDV 2C and pre-Golgi marker distribution was visualized using Alexa 488 or 594 dyes conjugated to appropriate secondary antibodies. Digital images of 0.2 μ m optical sections were deconvolved using Openlab software from Improvision. Panels (c) and (f) show digital merges of signals from FMDV 2C and pre-Golgi markers.

and LAMP-2 localized to vesicular structures throughout the cytoplasm of cells. A merge of these images (Fig. 5f and i, respectively) revealed that the signals for 2C and CD63 or LAMP-2 remained as distinct red and green pixels indicating that, as in the case of TGN46, CD63 and LAMP-2 do not co-localize with 2C. From these results we conclude that FMDV replication complexes do not originate from post-Golgi membranes containing markers for the TGN, endosomes or lysosomes.

DISCUSSION

In this report we show that viral proteins concentrate in juxtanuclear structures in cells infected with FMDV. The initial experiments compared the subcellular distribution of both structural and non-structural viral proteins by immunofluorescence. In each case the cytoplasmic structures contained non-structural proteins 2C, 3A and 3D as well as structural protein VP1 (not shown). We have shown previously by electron microscopy (Monaghan *et al.*, 2004)

that the same perinuclear area of the cell contains membrane vesicles, and it is tempting to suggest that these structures represent clusters of membrane vesicles required for replication and assembly, similar to those identified in cells infected with other picornaviruses. This is supported by the observation that viral protein 2C was membrane-associated in infected cells, and that the cytoplasmic structures also contained the viral polymerase 3D and protein 2C, which are implicated in picornavirus RNA synthesis (Barton & Flanagan, 1997; Li & Baltimore, 1988).

The juxtanuclear location of FMDV replication complexes suggested an association with one or more Golgi compartments. In fact, when cells were double-stained with antibodies against 2C and Man II, which is found in the *medial*-Golgi, both proteins were found in the juxtanuclear region of the cell. The compact crescent-shaped distribution of Man II found in uninfected cells did, however, change during infection, and the protein became dispersed within structures positive for 2C. From these results we reasoned

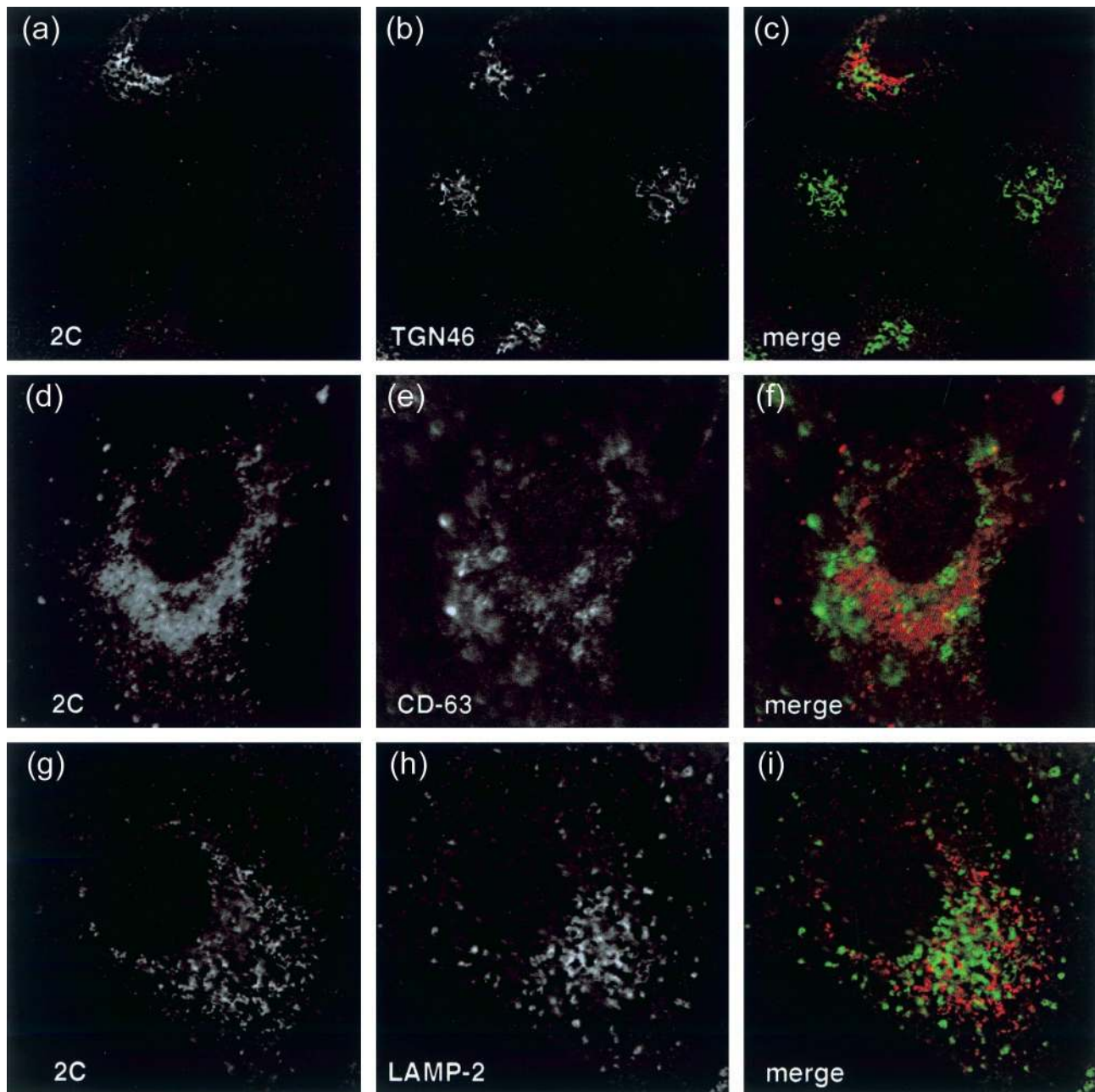


Fig. 5. Subcellular localization of FMDV 2C and post-Golgi membrane compartments. Six hours after infection of monkey GMK cells with FMDV, type O1K cells were processed for double-label immunofluorescence using mAb specific for FMDV 2C (a, d and g) and antibodies recognizing post-Golgi membrane compartments (b, e and h). (a–c) Location of 2C and the *trans*-Golgi network labelled by a sheep antibody recognizing TGN46. (d–f) Location of 2C and endosomes labelled with an antibody specific for CD63. (g–i) Location of 2C and lysosomes labelled with a mAb recognizing LAMP2. FMDV 2C and post-Golgi marker protein distributions were visualized using Alexa 488 or 594 dyes conjugated to anti-mouse or anti-rabbit IgG, respectively. Digital images of 0.2 μ m optical sections were deconvolved using Openlab software from Improvision. Panels (c), (f) and (i) show digital merges of signals from FMDV 2C (red) and post-Golgi marker (green).

that *medial*-Golgi membranes may contribute to the formation of replication complexes, or be incorporated into them. Surprisingly, three observations argued against this. Firstly, 2C and Man II remained as separate immunofluorescence

signals in merged, deconvolved images of infected cells (Fig. 2), and in the same cells examined by confocal microscopy (data not shown). Secondly, in cells treated with BFA, Man II redistributed to the ER, while 2C retained its

juxtannuclear localization, suggesting that the two proteins are associated with different subcellular structures. Thirdly, the bulk of the membrane-associated 2C produced in infected cells failed to co-fractionate with Golgi membranes on density gradients.

The above experiments established that *medial*-Golgi proteins did not co-localize with viral proteins present in FMDV assembly sites. This stimulated a study of other proteins known to locate to different compartments of the secretory pathway. Recent analysis of cells infected with poliovirus have implicated the ER, particularly COPII coated vesicles, as the major source of virus-induced vesicles (Rust *et al.*, 2001). In our study, however, we were unable to see co-localization of FMDV 2C with ER markers. Similar results were obtained for the ERGIC marker ERGIC53. The staining pattern of ERGIC53 was nonetheless found to be markedly altered in infected cells, with the protein dispersing from a juxtannuclear location into the cytoplasm. The next experiment investigated whether FMDV-induced structures were derived from membranes of post-Golgi compartments. The 2C signal was distributed within TGN46-containing membranes; however, deconvolved images again showed that the two proteins were in separate structures. Likewise, 2C was absent from endocytic vesicles and lysosomes.

In summary, our experiments show that membranes containing FMDV proteins thought to be important for virus replication form at a juxtannuclear sites close to a dispersed Golgi apparatus. Importantly, these membranes do not contain resident Golgi proteins, or protein markers associated with proximal and distal Golgi compartments. The Golgi is not therefore the source of the membranes found at virus assembly sites. This also means that the juxtannuclear location of the assembly site is not maintained by association with the Golgi apparatus, and probably involves an alternative mechanism. This could involve retrograde microtubule transport, as has been reported for cytoplasmic assembly sites formed by other viruses (Heath *et al.*, 2001; Ploubidou *et al.*, 2000; Sfakianos *et al.*, 2003). The inability to detect membrane markers in FMDV replication complexes makes it difficult to predict how these structures are formed in cells. One possibility is that they are formed *de novo* through the action of viral proteins on lipid biosynthesis pathways. A more likely mechanism, given the wealth of evidence from other picornaviruses, is that FMDV gains membranes from the secretory pathway, but somehow excludes host cell membrane proteins. A trivial explanation for our results could be that host proteins are present in replication complexes, but FMDV proteins mask epitopes. A coat of viral proteins cross-linked to the outside of vesicles during fixation could certainly prevent antibodies from binding epitopes present in the lumen of vesicles. Preliminary experiments suggest that epitope masking is unlikely because observation of cells expressing GFP-tagged ER or Golgi markers also show separation of these fluorescence signals from 2C (data not shown). Under these

circumstances the natural fluorescence of GFP cannot be masked by viral proteins coating the external surface of the organelle.

A second, and more interesting, explanation is that host proteins are indeed absent because they are removed during the delivery of membranes from the secretory pathway to virus assembly sites. Proteins are sorted to individual compartments of the secretory pathway through the use of coat proteins and membrane vesicles (Guo *et al.*, 2000). COPI and COPII coats, for example, sort proteins within the ER and Golgi apparatus, and clathrin coats regulate the formation of vesicles within the endocytic pathway. In each case the protein coat aids the separation of resident proteins from cargo proteins destined for other membrane compartments. It is possible that picornavirus proteins disrupt coat proteins and upset the normal sorting of membrane proteins in cells. Interestingly, Gazina *et al.* (2002) show that in cells infected with parechovirus 1 the Golgi COPI coat component, β -COP, is dispersed in the cytoplasm. This demonstrates that parechovirus 1 can displace coat proteins important for protein sorting, and this may be a mechanism used to prevent Golgi proteins from reaching, or remaining in, virus-induced membranes. It would be interesting to see if, as observed here for FMDV, other membrane markers are absent from replication sites formed by parechovirus 1. An inhibition of protein sorting has also been proposed for poliovirus by Rust *et al.* (2001) where the poliovirus 2BC protein is thought to be incorporated into COPII coated vesicles formed at the ER. The 2BC protein may prevent fusion of the ER-derived transport vesicles with the Golgi apparatus, thereby providing a source of vesicles for use in virus replication, which again exclude Golgi markers. In our study we could not find co-localization of FMDV proteins with COPII coats labelled with msec13 (data not shown). This means that FMDV either does not use COPII coats, or has a means of removing them before formation of the replication site. Recent experiments by Suhy *et al.* (2000) looked at the exclusion of host proteins from poliovirus replication complexes in some detail. They proposed a complex mechanism where poliovirus proteins 2BC and 3A induce the ER to produce vesicles containing two membranes, in a manner similar to autophagy. Again, the coating of vesicles by viral proteins is thought to exclude host proteins during vesicle maturation.

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