Intracellular localization and effects of individually expressed human parechovirus 1 non-structural proteins

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Human parechovirus 1 (HPEV-1) has many unique features compared with other picornaviruses and it has been shown that the replication complex formed during HPEV-1 infection is different from that of other picornaviruses. Here, the intracellular localization and functional effects of individually expressed HPEV-1 non-structural proteins were studied. The 2A and 3D proteins were found diffusely in the cytoplasm and nucleus of the cell. The 3A and 3AB proteins were observed to co-localize with the markers for the Golgi apparatus, whereas 2B co-localized with markers for the endoplasmic reticulum and the 2C and 2BC proteins were observed mainly on the surface of lipid droplets. The 2C protein, which has been implicated in replication-complex formation in enterovirus-infected cells, was not able to induce vesicles similar to those seen in HPEV-1-infected cells when expressed individually. However, in superinfected cells, the fusion protein was able to relocate to the virus replication complexes. Similar to other picornaviruses, HPEV-1 was found to interfere with cellular secretion, but this function could not be ascribed to any of the individually expressed non-structural proteins.

INTRODUCTION

In cells infected with positive-stranded RNA viruses, the viral non-structural proteins associate with host-cell membrane compartments and modify these to establish a specialized complex for viral RNA synthesis (Carette et al., 2000; Chen & Ahlquist, 2000; Egger et al., 2000; Mackenzie et al., 1999; Schlegel et al., 1996; van der Meer et al., 1999). Among the intracellular changes that have been documented are membrane proliferation and modification of different cellular organelles, such as the endoplasmic reticulum (ER) (Carette et al., 2000), the Golgi apparatus (Mackenzie et al., 1999), endosomes (van der Meer et al., 1999) and mitochondria (Miller et al., 2001). Cells infected with Poliovirus (PV), the type species of the genus Enterovirus in the family Picornaviridae, accumulate large quantities of double-membranned vesicles. These vesicles have been shown to contain components of the COP II anterograde transport pathway (Rust et al., 2001); however, the process of cellular autophagy has also been implicated in the formation of the vesicles (Schlegel et al., 1996; Suhy et al., 2000). Recently, the ADP-ribosylation factor (ARF) family of small GTPases has been found to be involved in PV RNA replication. As the normal function of ARFs is prevented by the fungal metabolite brefeldin A (BFA), this finding may explain the sensitivity of PV replication to BFA (Belov et al., 2005).

Concomitantly with replication-complex formation, PV has been shown to induce a general blockage of cellular protein secretion (Doedens & Kirkegaard, 1995) that, together with the cellular-membrane modifications and the inhibition of host-cell protein synthesis, plays an essential role in the intracellular pathology of infection.

Parechoviruses are different in many biological properties from other picornaviruses (Stanway et al., 1994). Among their unique features are natural resistance to guanidine hydrochloride (Tamm & Eggers, 1962) and insensitivity to brefeldin A (Gazina et al., 2002). The biochemical properties of the human parechovirus 1 (HPEV-1) non-structural proteins also differ from those described for other picornaviruses (Samuilova et al., 2004, 2006; Schultheiss...
Moreover, we have shown that the replication complex of HPEV-1 is substantially different from its enteroviral counterpart (Krogerus et al., 2003). In HPEV-1-infected cells, virus replication takes place in several small, discrete foci in the cytoplasm, rather than in large accumulations of membranous vesicles. Also, the membrane vesicles seen harbouring HPEV-1 RNA are smaller in diameter than their enteroviral counterparts. Furthermore, whilst in PV-infected cells, the viral non-structural protein 2C is found exclusively associated with the virus replication complex (Bienz et al., 1994; Egger et al., 2000), the HPEV-1 2C protein is also found on modified ER membranes seemingly not involved directly in viral RNA replication (Krogerus et al., 2003).

To identify individual viral proteins that can cause membrane rearrangements, different regions encoding individual proteins of several positive-strand RNA viruses have been expressed in a variety of cell types. Expression of PV 2C protein, a 329 aa membrane-associated protein with ATPase activity (Mirzayan & Wimmer, 1994; Rodriguez & Carrasco, 1995), has been shown to target the ER and to cause both membrane vesiculation and formation of multilamellar structures (Aldabe & Carrasco, 1995; Cho et al., 1994). Upon expression of the 426 aa precursor protein 2BC, more extensive vesiculation, more consistent morphologically with the pattern seen in PV-infected cells, has been observed (Cho et al., 1994). In particular, the combined actions of the non-structural proteins 2BC and 3A have been found to mimic both the biochemical and ultrastructural alterations in PV-infected cells (Suhy et al., 2000).

To date, most studies on the effects of picornavirus non-structural proteins on intracellular morphology have been carried out by using enterovirus proteins. The cellular effects of the non-structural proteins of another picornavirus, Foot-and-mouth-disease virus (FMDV), have been shown to differ substantially from those of the enterovirus non-structural proteins (Moffat et al., 2005), suggesting that molecular mechanism of cellular pathology may differ significantly between different picornavirus groups.

The aim of our present work was to study whether the distinctive characteristics of the HPEV-1 replication complex and the cellular pathology observed during infection (Coller et al., 1990; Wigand & Sabin, 1961) could be explained by specific membrane alterations and other intracellular events induced by individual viral non-structural proteins. In cells expressing individual viral non-structural proteins [2A, 2B, 2C, 2BC, 3A, 3AB and 3D as haemagglutinin (HA)- and green fluorescent protein (GFP)-tagged fusion proteins], 2A and 3D could be seen diffusely in the cytoplasm and nucleus, whereas 2B, 2C, 2BC, 3A and 3AB were detected at precise locations in the cytoplasm. The 2B protein was found in the ER, 2C and 2BC were found surrounding lipid droplets and 3A and 3AB co-localized with a marker for the Golgi apparatus. The parechovirus non-structural proteins were found not to induce intracellular changes similar to those seen during viral infection when expressed in isolation, and co-expression of the proteins did not change their individual localizations. However, some of the 2C-positive structures in transfected cells were found to associate with viral RNA in superinfected cells. Similar to other picornaviruses, HPEV-1 was found to interfere with cellular protein secretion, but this function could not be ascribed to any of the individually expressed non-structural proteins.

**METHODS**

**Cells and virus.** A-549 cells (a human lung carcinoma cell line; ATCC) and HeLa cells were infected with HPEV-1 (Harris strain) or coxsackievirus B3 (CBV-3; Nancy strain) by adsorption at 37 °C for 1 h. The virus stocks were originally obtained by transfection of the cells with the infectious genomic RNA obtained by in vitro transcription from clone pHPEV-1 (kindly provided by G. Stanway, University of Essex, Colchester, UK) and clone pCBV-3 (kindly provided by R. Kandolf, University Hospital of Tübingen, Tübingen, Germany).

**Cloning and expression procedures.** The pEGFP-C1 vector (Clontech) was used for the expression of HPEV-1 non-structural proteins 2A, 2B, 2C, 2BC, 3A and 3AB as fusions to the C terminus of enhanced GFP (EGFP) (Fig. 1). The primers used for amplification of selected coding regions in pHPEV-1 cDNA are shown in Supplementary Table S1 (available in JGV Online). The pCI-neo mammalian expression vector (Promega) was used for constructing a plasmid that contains an N-terminal HA epitope tag (YPYDVPDYA) and a multiple cloning site, using the oligonucleotides shown in Supplementary Table S1, HA–2C was recloned from the pGex2C vector (Krogerus et al., 2003). HA–2B, HA–3AB, HA–CBV-3 2C and HA–CBV-3 2BC were constructed by amplification of the respective regions using primers that can be seen in Supplementary Table S1.

The plasmid pVSVG3-GFP (Toomre et al., 1999), which encodes the ts045 vesicular stomatitis virus G (VSVG) protein fused to GFP at its C terminus, was kindly provided by F. van Kuppeveld (Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands) with the kind permission of P. Keller (Max-Planck-Institute of...
Molecular Biology and Genetics, Dresden, Germany). Transfection with Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen) was carried out according to the manufacturers’ protocols.

**Antibodies (Abs).** The production of rabbit antisera against HPEV-1 non-structural protein 2C and CBV-3 2C has been described previously (Auvinen et al., 1993; Krogerus et al., 2003).

To visualize the Golgi apparatus, mAbs against giantin (a gift from Professor Hans-Peter Hauri, Biozentrum, University of Basel, Switzerland), as well as the cis-Golgi protein GM130 (Transduction Laboratories) and the trans-Golgi protein p230 (Transduction Laboratories), were used. The ER was detected with a mAb against BPAP31, kindly provided by E. Kuismanen, Viikki Biocenter, University of Helsinki, Finland. A mAb against HA (BabcO) was used to visualize the HA-tagged proteins and a rabbit Ab against the GFP tag (Molecular Probes) to detect the EGFP-fusion proteins by electron microscopy (EM) and to intensify the signal prior to hybridization with RNA probes.

Goat anti-rabbit and anti-mouse Alexa 488- and Alexa 546-labelled Abs (Molecular Probes) were used as secondary antibodies in immunofluorescence (IF). In immunoelectron microscopy (IEM), 1.4 nm diameter gold particle-conjugated Fab’ fragments, termed Nanogold (Nanoprobes), against rabbit or mouse IgG were used.

**IF and fluorescent in situ hybridization (FISH).** Cells grown on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma). Indirect IF was performed and the cells were mounted in glycerol containing 1% N-propyl gallate (Sigma). The subcellular localization of VSVG-GFP was determined by expressing either VSVG–GFP alone or VSVG–GFP together with a fusion protein at 40°C. After a temperature shift to 32°C, the cells were fixed, stained and analysed by confocal laser-scanning microscopy (CLSM).

Lipid droplets were visualized with Oil Red O (Sigma) solution (0.1% in 60% 2-propanol for 20 min).

For detection of viral genomic RNA, riboprobes of complementary polarity, covering the entire viral sequences, were prepared from plasmid pHPEV-1 and plasmid pCBV-3. In vitro transcription in the presence of Alexa 546-conjugated UTP (Molecular Probes) was performed with T3 RNA polymerase and T7 RNA polymerase, respectively. The probes were subjected to alkaline hydrolysis to generate fragments of approximately 100 nt in length and hybridized to the cells at 42°C overnight as described previously (Bolten et al., 1998; Egger et al., 1999; Krogerus et al., 2003). The specimens were mounted in glycerol containing 2.5% (w/v) DABCO (Sigma). Conventional light microscopy was performed by using a Zeiss Axiosplan 2 microscope, and confocal microscopy was carried out with a confocal laser-scanning microscope (Leica SP2 confocal microscope). For co-localization studies, pictures were recorded sequentially and mounted in glycerol containing 2.5% (w/v) DABCO (Sigma).

**RESULTS**

**Intracellular localization of the individually expressed HPEV-1 non-structural proteins**

As shown previously (Krogerus et al., 2003), the nascent viral RNA in HPEV-1-infected cells localizes to discrete spots in the cytoplasm of the cell [Fig. 2a(i)], whereas non-structural protein 2C can be seen in a stick-like pattern [Fig. 2a(ii)]. In cells infected with enteroviruses, on the other hand, both viral RNA and the 2C protein can be found in a perinuclear accumulation [Fig. 2a(iii–iv)].

To study the intracellular localization of individually expressed HPEV-1 non-structural proteins, fusion proteins were constructed and expressed in A-549 cells (Fig. 2b). 2A–EGFP was seen diffusely in the cytoplasm and nucleus starting 4 h post-transfection (p.t.), resembling the localization of the virus-encoded polypeptide (Samuïlova et al., 2004). No change in the localization pattern was observed during 48 h. The 2B–EGFP protein formed a cytoplasmic reticular pattern, whilst 2C–EGFP and 2BC–EGFP were both seen in accumulations of circular cytoplasmic formations and a faint diffuse dot-like or reticular cytoplasmic staining starting 4 h p.t. The 3A–EGFP and 3AB–EGFP proteins, which had an identical distribution, accumulated in the perinuclear area and, in some cells, additionally formed a reticular pattern in the cytoplasm. The 3D–EGFP protein was localized diffusely in the cytoplasm of the transfected cells, and the nuclei of the transfected cells, excluding the nucleoli, were also strongly fluorescent. The control plasmid, expressing only the fluorescent EGFP tag, was located diffusely in the cytoplasm and the nucleus.

Because of their distinct localization and to exclude the possibility that a large GFP tag could interfere with the protein localization, the 2B, 2C and 3AB proteins were studied further through the construction of a fusion protein containing the small (9 aa) HA tag. As expected, the HA–2B protein formed a similar pattern to 2B–EGFP, the HA–2C protein was seen in similar spherular structures to 2C–EGFP and the HA–3AB protein accumulated in the perinuclear area like 3AB–EGFP. Double transfection of EGFP- and HA-fusion proteins showed perfect co-localization (data not shown) and similar staining patterns were seen in transfected A-549 and HeLa cells (data not shown).

**The 2B protein co-localizes with the ER and the 3AB protein with the Golgi apparatus**

To study the localization of the fusion proteins more precisely, confocal microscopy using cellular markers and IEM were performed. The 2B proteins were found to co-localize with BAP31, a marker for the ER (Fig. 3a–c), and IEM confirmed the localization of the protein in the ER (Fig. 4c). The ultrastructures of the ER, the Golgi and other parts of the 2B-transfected cells were not altered compared with control cells (Fig. 4a).
The 3AB-fusion proteins were found to co-localize with the trans-Golgi-network marker p230 (Fig. 3d–f), but not with the cis-Golgi marker GM130 (data not shown), and the localization of the protein to the Golgi apparatus was verified by IEM (Fig. 4f). Again, the morphology of both the Golgi and the ER was found to be intact.

The 2C protein is found mainly on lipid droplets, but also on diverse intracellular membranes

Interestingly, the 2C protein exhibited a spherular staining pattern in transfected cells. These spherules were usually found in close vicinity to trans-Golgi structures (data not shown). IEM revealed that the majority of the protein was situated on the surface of lipid droplets (Fig. 4d, e). Similar lipid droplets were also found in untransfected control cells (Fig. 4a). The protein also partially localized on Golgi and ER membranes; however, no obvious change in the morphology of the Golgi or the ER could be observed. The association of the 2C protein with lipid vacuoles was further confirmed by Oil Red O staining (Fig. 3g–i). Because of the central role that has been ascribed to the 2C and 2BC proteins in enterovirus replication-complex formation, CBV-3 HA–2C and HA–2BC fusion proteins were constructed for comparison. The CBV-3 2C protein was found to form a reticular pattern with a perinuclear accumulation (Fig. 3j). This is consistent with earlier findings suggesting that the main target of the enterovirus 2C protein is the ER. The CBV-3 2BC protein could be

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**Fig. 2.** (a) Localization of viral RNA and 2C protein in HPEV-1- and CBV-3-infected A-549 cells 5 h post-infection. (i) Viral RNA in an HPEV-1-infected cell. (ii) 2C in an HPEV-1-infected cell. (iii) Viral RNA in a CBV-3-infected cell. (iv) 2C in a CBV-3-infected cell. (b) Individual expression of HPEV-1 non-structural proteins in A-549 cells 24 h p.t.
found in a quite similar pattern to 2C; however, the perinuclear accumulation was generally more extensive (Fig. 3k).

It has been reported for several different viruses that it is the combined action of several non-structural proteins, rather than individual proteins, that mediates the ultrastructural changes seen in the infected cell (Salonen et al., 2003; Suhy et al., 2000). We therefore co-expressed 2B–EGFP with HA–3AB (Fig. 5a), 2C–EGFP with HA–2B (Fig. 5b) and 2C–EGFP with HA–3AB (Fig. 5c). The localization of the individual proteins did not change significantly in co-transfected cells; however, 2B and 3AB were found to co-localize partially in the perinuclear region (Fig. 5a).

**Individually expressed 2C protein can co-localize with viral RNA in infected cells**

In view of the above findings, we next wanted to investigate whether the expressed non-structural proteins could be involved in the formation of the virus replication complex. Transfected cells were subsequently infected with HPEV-1. Six hours post-infection (p.i.), infected cells were identified by the presence of viral RNA or the viral 2C protein in the cell. The transfected cells were infected at the same efficiency as non-transfected cells. The typical punctate pattern of viral RNA localization and the 2C-positive branching structure (Krogerus et al., 2003) were observed in the transfected, superinfected cells. The pattern of transfected 2A–EGFP (Fig. 5d), 2B–EGFP (Fig. 5e) or 3D–EGFP (Fig. 5h) did not change upon infection, and no specific co-localization between the transfected proteins and the viral 2C protein was observed. Interestingly, the viral 2C protein, which has been shown to reside on modified ER membranes (Krogerus et al., 2003), did not co-localize with the 2B–EGFP construct, which could also be found in the ER (Fig. 5a–c). This finding suggests that the 2C-positive structures seen in HPEV-1-infected cells are indeed specialized membrane compartments lacking resident ER proteins.

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**Fig. 3.** Intracellular localization of expressed HPEV-1 and CBV-3 non-structural proteins in A-549 cells, 24 h.p.t. (a) The ER (red) stained with a mAb against BAP31. (b) 2B–EGFP (green). (c) Merge of (a) and (b). (d) The Golgi (red) stained with an Ab against the trans-Golgi marker p230. (e) 3AB–EGFP (green). (f) Merge of (d) and (e). (g) Lipid staining using Oil Red O (red). (h) 2C–EGFP (green). (i) Merge of (d) and (e). (j) Cell expressing CBV-3 2C–EGFP. (k) Cell expressing CBV-3 2BC–EGFP. Bar, 10 μm (inset, 1 μm).
As HPEV-1 infection results in a disintegration of the Golgi apparatus, it was not surprising that the staining pattern of the expressed 3AB protein changed in the infected cells. The protein formed a diffuse reticular structure and additional vesicular structures appeared (Fig. 5f–g). No specific co-localization with either viral 2C protein (Fig. 5f) or with viral RNA could be seen (Fig. 5g). Expressed 3AB protein behaved in a similar manner to the 3A protein (data not shown).

Interestingly, in 2C-transfected, superinfected cells, the structures containing 2C–EGFP partially co-localized with viral RNA (Fig. 5j–l). The co-localization did not seem to involve the lipid droplets, but rather dot-like structures in the perinuclear area, suggesting that the portion of the protein residing on ER and Golgi membranes could relocate to sites of viral RNA synthesis.

**Effect of HPEV-1 infection and non-structural proteins on the secretory pathway**

It has been shown that several picornaviruses, as well as individually expressed viral non-structural proteins, can inhibit ER-to-Golgi transport (Doedens & Kirkegaard, 1995; Moffat *et al.*, 2005). We studied the effect of HPEV-1 infection and the individual non-structural proteins on the movement of membrane proteins from the ER into the secretory pathway by using a GFP-tagged ts045-VSVG protein, a well-known membrane-bound secretory marker (Toomre *et al.*, 1999). At the non-permissive temperature...
(40 °C), VSVG–GFP was retained in the ER (Fig. 6a), which was confirmed by using antibodies against the ER protein BAP31 (data not shown). Upon shifting to the permissive temperature (32 °C), the protein was transported out of the ER and, after 45 min, the majority of the protein was found in the Golgi complex (Fig. 6b), co-localizing with the Golgi protein giantin (data not shown). After 2 h, VSVG–GFP was localized mostly on the plasma membrane (Fig. 6c). To investigate whether HPEV-1 infection inhibits vesicular trafficking, A-549 cells were transfected with VSVG–GFP, incubated at 40 °C for 24 h and then infected with HPEV-1. The infected cells were further incubated at 40 °C for 4 h and then shifted to 32 °C for 2 h. The cells were fixed and stained with an antibody against GFP before being subjected to FISH, and VSVG–GFP localization was analysed by CLSM. In HPEV-1-infected cells, VSVG–GFP was retained in the ER (Fig. 6d–f), suggesting that protein transport was blocked between the ER and the Golgi. However, cells co-transfected with VSVG–GFP and 2B–HA (Fig. 6g–i), 2C–HA (Fig. 6j–l) or 3AB–HA (Fig. 6m–o) and incubated at 40 °C for 24 h before being shifted to 32 °C for 2 h did not exhibit inhibition of protein transport.

**DISCUSSION**

Already, during their original characterization, parechoviruses were found to exhibit distinct growth properties and cytopathogenicity compared with viruses in the enterovirus group (Coller et al., 1990; Stanway & Hyypia, 1999; Wigand & Sabin, 1961). Other exceptional biological features, such as natural resistance of HPEV-1 to guanidine hydrochloride (Tamm & Eggers, 1962) and insensitivity to brefeldine A (Gazina et al., 2002), have also been described. We have shown that the intracellular-membrane changes seen during HPEV-1 infection are different from those induced by enteroviruses and that, unlike the PV 2C protein, the HPEV-1 2C protein is not associated exclusively with the virus replication complex (Krogerus et al., 2003). Moreover, the
biochemical properties of the HPEV-1 non-structural proteins differ from those described for other picornaviruses (Samuilova et al., 2004, 2006).

The purpose of this study was to examine the possible involvement of the 2C protein and other non-structural proteins in replication-complex formation and to study their individual localization and possible membrane modifications. The 2A and 3D proteins were found diffusely in the cytoplasm and, particularly, the 3D protein was found concentrated in the nucleus. The 2B, 2C, 2BC, 3A and 3AB proteins were found at precise locations in the transfected cells: 2B on the ER, 2C and 2BC mainly on lipid droplets, but also on Golgi and ER membranes, and 3A and 3AB on Golgi membranes. None of the proteins studied was able, alone or in combination, to induce changes in the intracellular morphology similar to those seen in HPEV-1-infected cells. However, 2C–EGFP was partially redirected to the virus replication complexes in transfected, HPEV-1-infected cells, suggesting that a subset of the 2C protein is able to relocate to the sites of viral RNA synthesis.

Recently, new data have been obtained about the intracellular alterations induced by different picornaviruses, as well as by individual picornaviral proteins (Knox et al., 2005; Krogerus et al., 2003; Monaghan et al., 2004). Among the picornaviruses, RNA replication complexes from enterovirus-infected HeLa cells have been the best studied; however, the precise mechanism of replication-complex formation remains elusive. COP II-mediated vesicle budding from the ER has been suggested to occur during formation of the replication vesicles at the beginning of infection (Rust et al., 2001), and data implicating the cellular autophagic pathway have also been presented (Schlegel et al., 1996; Suhy et al., 2000). Very recently, the ARF family of small GTPases has been found to be involved in PV RNA replication, a finding that may explain the sensitivity of enterovirus replication to BFA (Belov et al., 2005). When expressed in isolation, the enterovirus 2C and 2BC proteins have been shown to induce extensive rearrangements of intracellular membranes and vesicles resembling those seen during viral infection (Aldabe & Carrasco, 1995; Cho et al., 1994). The 3A protein has also been implied in the formation of the enterovirus replication complex (Suhy et al., 2000), most recently by the finding that individual expression of the protein can translocate different ARFs to membranes (Belov et al., 2005). The 2C and 2BC proteins of Hepatitis A virus have also been shown to cause membrane rearrangements (Teterina et al., 1997); the relationship of these to the formation of the virus replication complex is, however, not clear.

Individually expressed HPEV-1 2C and 2BC proteins were found to associate with lipid droplets, but also with ER and Golgi membranes. No apparent rearrangements of intracellular membranes could be seen. This suggests that 2C alone is not sufficient to generate the membranous changes seen during HPEV-1 infection, although the finding that transfected 2C can associate with viral RNA in superinfected cells suggests that the protein may interact with cellular and/or viral factors present in the replication complex. Pairwise co-transfection of the HPEV-1 non-structural proteins did not change the individual staining patterns. It has been shown for other viruses, however, that several or all of the replicative proteins might be needed for correct localization of the replicative machinery (Becker et al., 2003; Salonen et al., 2003). However, the inability of the expressed proteins in the cytoplasm and, particularly, the 3D protein was found concentrated in the nucleus. The 2B, 2C, 2BC, 3A and 3AB proteins were found at precise locations in the transfected cells: 2B on the ER, 2C and 2BC mainly on lipid droplets, but also on Golgi and ER membranes, and 3A and 3AB on Golgi membranes. None of the proteins studied was able, alone or in combination, to induce changes in the intracellular morphology similar to those seen in HPEV-1-infected cells. However, 2C–EGFP was partially redirected to the virus replication complexes in transfected, HPEV-1-infected cells, suggesting that a subset of the 2C protein is able to relocate to the sites of viral RNA synthesis.

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**Fig. 6.** Effects of HPEV-1 infection and individual non-structural proteins on cellular secretion. (a) Control cells; at 40 °C, VSVG is retained in the ER. (b) VSVG observed in the Golgi complex after 45 min incubation at 32 °C. (c) After 2 h at 32 °C, the protein is seen on the plasma membrane. (d–f) In cells infected with HPEV-1 for 4 h at 40 °C and then shifted to 32 °C, the VSVG protein is retained in the ER. The viral RNA is visualized by FISH (red) and the VSVG protein is seen in green. (g–i) Cells expressing HA–2B (red) and VSVG–GFP (green). (j–l) Cells expressing HA–2C (red) and VSVG–GFP (green). (m–o) Cells expressing HA–3AB (red) and VSVG–GFP (green). Bars, 10 μm.
to induce membrane changes similar to those seen during infection could also be a result of misfolding or malfunction of the proteins, a possibility that cannot be excluded by the experiments presented here.

Recently, more information has been obtained on the nature of the proteins, a possibility that cannot be excluded by the experiments presented here. A hepatitis C virus (HCV) non-structural protein, NS5A, has been shown to associate with lipid droplets, as well as Golgi and ER membranes, when expressed in isolation (Shi et al., 2002). NS5A has several features in common with picornaviruses, and the NS5A protein is similar to the picornaviral 2C protein in several features in common with picornaviruses, and the NS5A protein is similar to the picornaviral 2C protein in that it has an amphipathic α-helix in the N-terminal region (Teterina et al., 2006). The helix is thought to represent an individual module that is responsible for the protein's association with membranes in the virus replication complex (Teterina et al., 2006). However, in cells containing HCV subgenomic replicons, the protein is not associated with lipid droplets, but rather with a structure of modified membranes termed the 'membranous web', presumably derived from ER membranes (Egger et al., 2002; Mottola et al., 2002). Another study has associated the protein with lipid rafts and caveolin-2, a protein that is found also on lipid droplets (Shi et al., 2003). In HPEV-1-infected cells, the 2C protein co-localizes both with a trans-Golgi marker and with modified ER membranes (Krogerus et al., 2003). The relationship of the 2C protein with lipid rafts has not been investigated. It is, however, tempting to speculate that, during HPEV-1 replication-complex formation, the 2C protein might interact with certain ER proteins or a subregion of the ER, as well as with other viral components, to form a specialized membrane compartment.

The enterovirus 2B protein is an integral membrane protein that is localized predominantly at the Golgi complex upon individual expression (de Jong et al., 2003). Expression of the protein releases calcium from intracellular stores and increases plasma-membrane permeability to calcium and low-molecular-mass compounds (Aldabe et al., 1996; Doedens & Kirkegaard, 1995; Lama & Carrasco, 1992; van Kuppeveld et al., 1997a, b). It also interferes with the anterograde membrane-transport pathway (Doedens & Kirkegaard, 1995). Individually expressed FMDV 2B protein has been localized to the ER (Moffat et al., 2005). In this study, HPEV-1 2B fusion proteins localized to the ER and the morphology of the ER and Golgi was similar to that in control cells. At very high levels of expression, the 2B protein caused rearrangement of the ER membranes into large, vacuolar structures (data not shown). It is known, however, that such aberrant structures can form in response to the overexpression of ER-localized proteins (Snapp et al., 2003). Whether the permeability of the secretory membranes is increased in HPEV-1-infected cells or cells expressing HPEV-1 2B protein is currently not known and warrants further studies.

The 3A proteins of both enteroviruses and FMDV have been shown to reside in the ER when expressed individually and have been found to interfere with ER-to-Golgi transport (Doedens et al., 1997; O’Donnell et al., 2001; Wessels et al., 2005). The expressed HPEV-1 3A-fusion proteins were found to co-localize with the Golgi without causing any gross changes in the intracellular morphology. We show here that, similarly to other picornaviruses studied, HPEV-1 inhibits cellular secretion. However, the individual protein responsible for this feature seems to be neither 3A, like in enteroviruses (Doedens et al., 1997), nor 2BC, like in FMDV (Moffat et al., 2005). At present, we cannot rule out the possibility that other precursor proteins, unique to HPEV-1, and/or the concomitant translation of the viral polyprotein are responsible for inhibiting cellular secretion in HPEV-1-infected cells.

Despite overall similarities, picornaviruses employ remarkably different mechanisms in replication-complex formation. It also seems that the functions of individual proteins in the virus replication cycle and the induction of intracellular pathology cannot be derived directly from the properties of analogous proteins of other picornaviruses.

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