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Subcellular localization of the MNV-1 ORF1 proteins and their potential roles in the formation of the MNV-1 replication complex

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ABSTRACT

Human noroviruses are the leading cause of nonbacterial gastroenteritis worldwide and are now recognised as a significant human pathogen. Whereas human noroviruses cannot be cultivated in the laboratory, mouse norovirus 1 (MNV-1) is easily cultivated and has a defined tropism for cells of a mononuclear origin. As such, MNV-1 provides an ideal opportunity to study many aspects of norovirus biology and replication. Previously, we have shown that MNV-1 RNA replication is associated with components of the early and late secretory pathway and that all six open reading frame 1 (ORF1) proteins are associated with the viral dsRNA within the replication complex (RC) during the course of infection. In this study, we further characterise the subcellular localisation of the MNV-1 ORF1 proteins when recombinantly expressed in cells. We show that two MNV-1 proteins, NS1-2 and NS4, associate with the endoplasmic reticulum and endosomes, respectively. Whereas NS6 (the viral protease) appeared to localize within the cytoplasm and to mitochondria, NS7 (the viral polymerase) was observed to localize diffusely within the cytoplasm and within the nucleus, and NS3 localized to discrete foci within the cytoplasm which were of unknown origin. Based on the localization patterns observed we propose a model by which NS1-2 and NS4 may recruit host membranes to the MNV-1 RC during replication.

Introduction

Caliciviruses are single-stranded positive sense RNA viruses which infect both humans and animals and cause a wide range of diseases. The *Caliciviridae* family is divided into four genera (Vesivirus, Lagovirus, Sapovirus, and Norovirus); two of which (Sapovirus and Norovirus) are more commonly associated with disease in humans. Human noroviruses (HuNoV) are the leading cause of nonbacterial gastroenteritis and are typically associated with the outbreak of acute disease in population dense settings. Relatively little is understood regarding human norovirus replication and biology and can be attributed to the inability to cultivate these viruses. In 2003, a novel mouse norovirus (MNV-1) was isolated from STAT1$^{-/-}$/RAG2$^{-/-}$ mice and subsequently shown to possess a tropism for monocytic cells (Karst et al., 2003; Wobus et al., 2004). The discovery of MNV-1 has consequently provided us with the first NoV tissue culture system and has allowed us to study aspects of NoV replication and biology that have previously been inaccessible.

MNV-1 possesses a single-stranded RNA genome approximately 7.3–7.8 kilo bases (kb) in length which is protein linked at the 5′ end of the genome and polyadenylated at the 3′ end (Karst et al., 2003). The MNV-1 genome consists of three ORFs (ORF1–3) which encode for the nonstructural proteins (ORF1) and structural proteins (ORF2 and 3) (Belliot et al., 2003; Sosnovtsev et al., 2006b). ORF1 encodes for six nonstructural proteins in the gene order 5′-NS1-2-NS3-NS4-NS5-NS6-NS7-3′. Like other positive sense RNA viruses, the ORF1 proteins are initially translated as a single polyprotein which is subsequently cleaved by a viral-encoded peptidase (NS6) to produce mature polyprotein subspecies (Sosnovtsev et al., 2006b). In contrast, the major (VP1) and minor (VP2) structural proteins are encoded by ORF2 and ORF3, respectively, and translated from a subgenomic RNA species generated during replication.

Relatively little is known regarding how replication is initiated and how the replication complex (RC) is formed, however, and an increasing number of studies have begun to shed light on this matter. Our recent observations have shown that all of the ORF1 proteins are associated with the MNV-1 viral RNA intermediate, dsRNA, during the course of infection (Hyde et al., 2009). We observed that like all positive sense RNA viruses, MNV-1 RNA replication was intimately associated virus-induced membrane rearrangements that comprised of a collection of cytoplasmic vesicles that ranged in size from 70 to 300 nm in size (Hyde et al., 2009; Wobus et al., 2004). Our subsequent investigations revealed that these vesicles were comprised of protein markers originating from major components of the endocytic and secretory pathway; typically, the endoplasmic reticulum, the Golgi apparatus, and endosomes (Hyde et al., 2009). Such observations are

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consistent with studies performed with the related *picornavirus* poliovirus, with the exception that complete dismantling of the Golgi apparatus was not observed for MNV-1.

While we are beginning to understand more about the mechanism of replication employed by NoVs, very little is known regarding the function of the ORF1 proteins during MNV-1 replication and how they contribute to the formation of the RC. Those NoV proteins which are best characterised are the NS5, NS6, and NS7 proteins which encode the cap analogue VpG, viral protease, and RNA-dependent RNA polymerase (RdRp), respectively. In contrast, very few data exist concerning the function of the N-terminal ORF1 proteins NS1-2, NS3, and NS4, and much of what is known has been inferred from studies of homologous proteins from related viruses. In the present study, we characterise the subcellular localisation of the individually expressed ORF1 proteins and propose a role for some of these proteins in the formation of the RC. We show that all ORF1 proteins exhibit distinct subcellular localisation profiles, yet are consistent to that previously observed in infected cells. Furthermore, we show the association of NS1-2 and NS4 with components of the endocytic and secretory pathway and propose a role for these proteins in the formation of the MNV-1 RC on cellular membranes.

Results

Localisation of individual MNV-1 nonstructural proteins in Vero cells reveals unique localisation patterns

Previously, we have characterised the subcellular localisation of the MNV-1 RC and demonstrated the association of all six ORF1 proteins with MNV-1 dsRNA in infected Raw264.7 cells (Hyde et al., 2009). To compare the localisation of the individual ORF1 proteins, each viral protein was fused with a hexa-histidine (His)-tag at the C-terminus and expressed in Vero cells. To determine whether each of the constructs produced the correct gene product, each construct was expressed *in vitro* via a coupled transcription/translation reaction, the products of which were analysed by Western blotting and anti-His antibodies (Fig. 1). With the exception of NS5, all individual ORF1 constructs produced detectable levels of protein when expressed and were observed to produce full length proteins of the correct size, namely 38 kDa for NS1-2, 39 kDa for NS3, 18 kDa for NS4, 19 kDa for NS6, and 57 kDa for NS7 (Fig. 1). Currently, we do not understand why NS5 was not expressed *in* vitro or in cells as sequencing of the construct revealed it was correct. There may be stability issues with the expression of NS5 in our hands, and we are currently investigating whether coexpression of the other MNV ORF1 proteins are required to achieve NS5 expression. To compare the localisation profiles of each viral protein, transfected cells were also compared by immunofluorescence analysis in which cells were fixed at 24 hpt and dual-labelled with antibodies against the His-tag and the respective ORF1 protein (Fig. 2).

Expression of each of the individual ORF1 proteins exhibited localisation profiles that were clearly different to the compact perinuclear labelling which we have observed in MNV-1-infected cells. In NS1-2-transfected cells, antibody labelling of the viral protein revealed a distinct ER-like reticular staining pattern, including apparent labelling of the nuclear envelope (Figs. 2a–c). In addition, NS1-2-transfected cells exhibited labelling of dense punctate foci that were dispersed throughout the cytoplasm. In NS3-transfected cells, immunolabelling showed the presence of distinct vesicle-like structures throughout the cytoplasm (Figs. 2d–f), in addition to some disperse cytoplasmic staining. Interestingly, these structures are not observed in infected cells, and furthermore, a small percentage of NS3-expressing cells did not show the presence of these vesicle-like structures, possibly indicating that the formation of these structures is dependent on the level of expression of NS3 within these cells. We observed the presence of dense cytoplasmic foci in NS4-transfected cells (Figs. 2g–i). In contrast to cells expressing NS1-2, however, no reticular labelling of NS4 was observed. Importantly, while the relative level of NS4 expression was markedly lower in the majority of cells when compared to NS1-2 expression, NS4-labelled foci appeared to aggregate more around the perinuclear region of transfected cells, in contrast to NS1-2 foci which appeared to be randomly distributed throughout the cytoplasm (Figs. 2a–c). In cells expressing NS6, a disperse network of cytoplasmic foci were also detected; however, the morphology of these structures differed greatly from those observed in either NS1-2-, NS3-, or NS4-expressing cells (Figs. 2j–l). In addition, we observed some nuclear labelling of NS6 in a small percentage of transfected cells (data not shown). This nuclear localisation may be attributed to a higher level of expression of NS6 in these cells, as the percentage of cells exhibiting nuclear localisation at 48 hpt was greater when compared to cells at 24 hpt (data not shown). In contrast to the other viral proteins, NS7 expression did not induce the formation of any distinct structures (Figs. 2m–o). Instead, NS7 labelling showed diffuse cytoplasmic labelling in addition to nuclear localisation, which is reminiscent of the localisation pattern of other RNA virus RdRps (Brockway et al., 2003; Hwang et al., 1997; Mackenzie et al., 2007; Sharma et al., 2004).

MNV-1 ORF1 proteins localise to distinct subcellular structures

Previously, we have shown that MNV-1 induces the formation of vesicle clusters which are derived primarily from components of the early and late secretory pathways (ER, Golgi, and endosomes (Hyde et al., 2009)). To determine whether the ORF1 proteins exhibit a similar association with these organelles when expressed individually in cells, we compared the localisation of each protein with various cell membrane markers in dual-labelling experiments. Vero cells were transfected with plasmid DNA, fixed at 24 hpt and subsequently dual-labelled with viral protein-specific antibodies and antibodies against the ER (Calnexin), Golgi apparatus (giantin and GM130), and endosomes (EEA1). Cells were then analysed by confocal immunofluorescence microscopy (Figs. 3–7).

NS1-2 was observed to colocalise with calnexin (Figs. 3a–c) but did not colocalise with markers for the Golgi (Figs. 3d–f, g–i) or

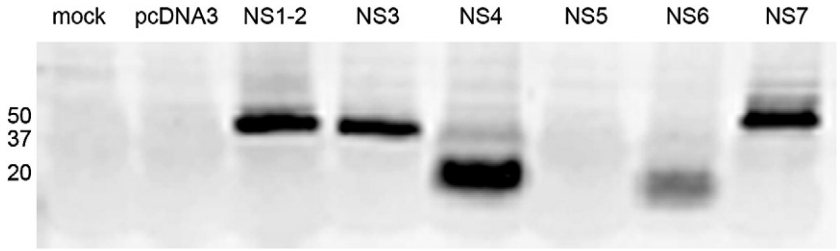

Fig. 1. Western blot of expression products of MNV-1 fusion proteins generated *in vitro*. MNV-1 fusion proteins were expressed *in vitro* using a couple transcription/translation system and analysed via Western blotting. Viral proteins were detected using an anti-His antibody that recognised the hexahistidine tag incorporated into the C-terminal end of each protein. With the exception of NS5, all transcription/translation reactions yielded products of the predicted size: 38 kDa for NS1-2, 39 kDa for NS3, 18 kDa for NS4, 19 kDa for NS6, and 57 kDa for NS7.

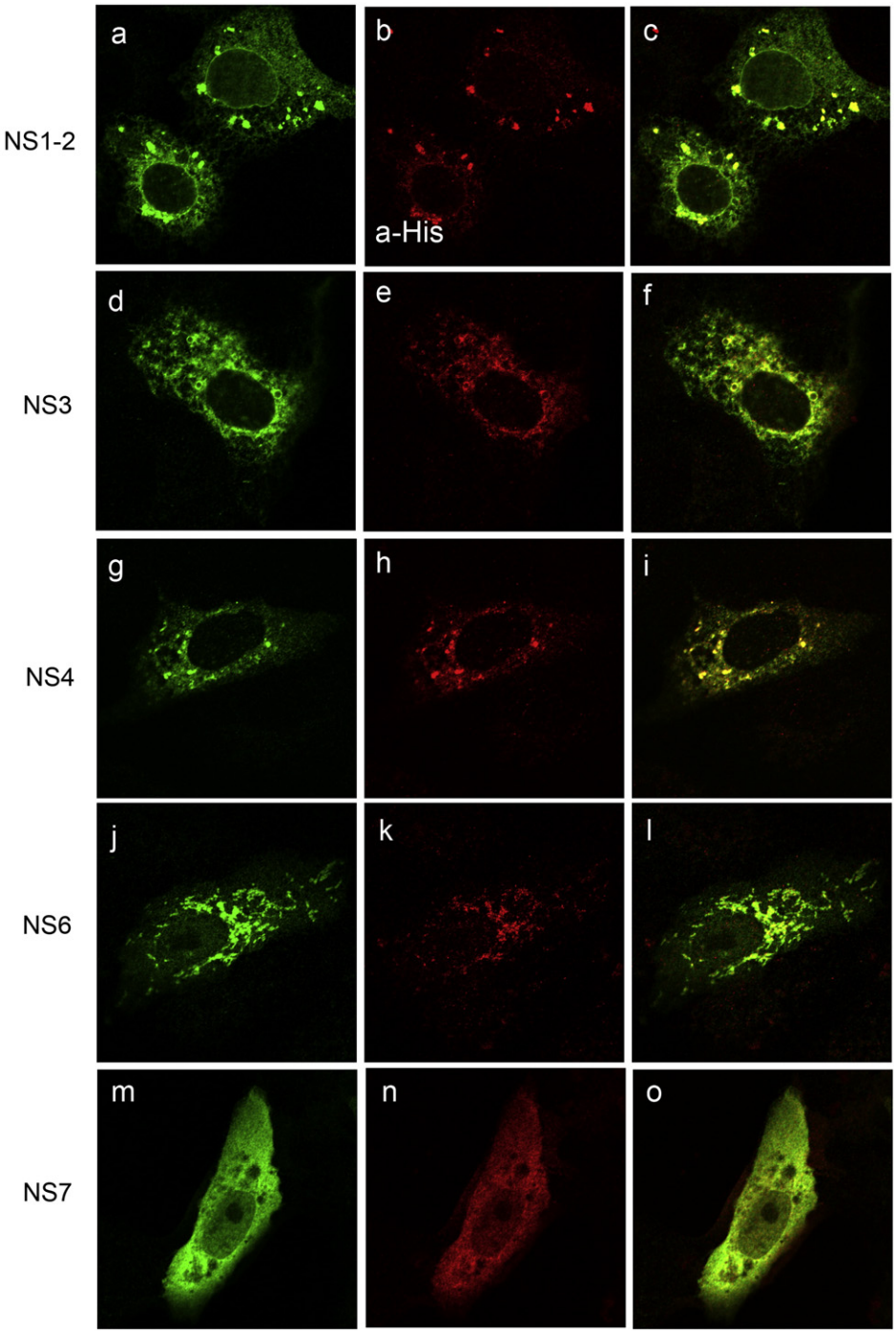

Fig. 2. Localisation profile of Vero cells individually transfected with MNV-1 fusion proteins. Vero cells were transfected with individual expression constructs, fixed at 24 hpt, dual-labelled with antibodies specific for each viral protein (panels a, d, g, j, and m), and an anti-His antibody (panels b, e, h, k, and n), and analysed via confocal immunofluorescence microscopy. Colocalization of the anti-His tag and the monospecific anti-MNV-1 ORF1 protein antisera is observed as a yellow hue only in panels c, f, i, l, and o.

endosomes (Figs. 3j–l). In addition, we observed a redistribution of calnexin labelling to punctate foci within the cytoplasm which was coincident with NS1-2-labelled foci. This is concurrent with our previous findings which demonstrated a redistribution of calnexin to the site of virus replication in infected cells and suggests that NS1-2 may mediate the recruitment of ER membranes to the RC. It is interesting to note that the NoV N-term protein (NS1-2 equivalent) is predicted to contain a membrane-spanning region, and its expression was observed to localize to the Golgi and induce disassembly (Fernandez-Vega et al., 2004). While we observed a significant change in the distribution of calnexin in transfected cells, NS1-2 did not appear to have any effect on the morphology and distribution of either the Golgi or the endosomes (Figs. 3d–l).

In contrast, we did not observe any colocalisation between NS3 and any of the membrane markers analysed (Fig. 4). Based on the morphology of the distinct vesicular structures observed in NS3-transfected cells, it is unsurprising that these NS3-associated structures do not originate from either the Golgi or the ER. The vesicular nature of these structures may suggest an association between NS3 and endosomes; however, the absence of any colocalisation between EEA1 and NS3 would suggest this is not the case. We have also extensively screened other endosomal markers

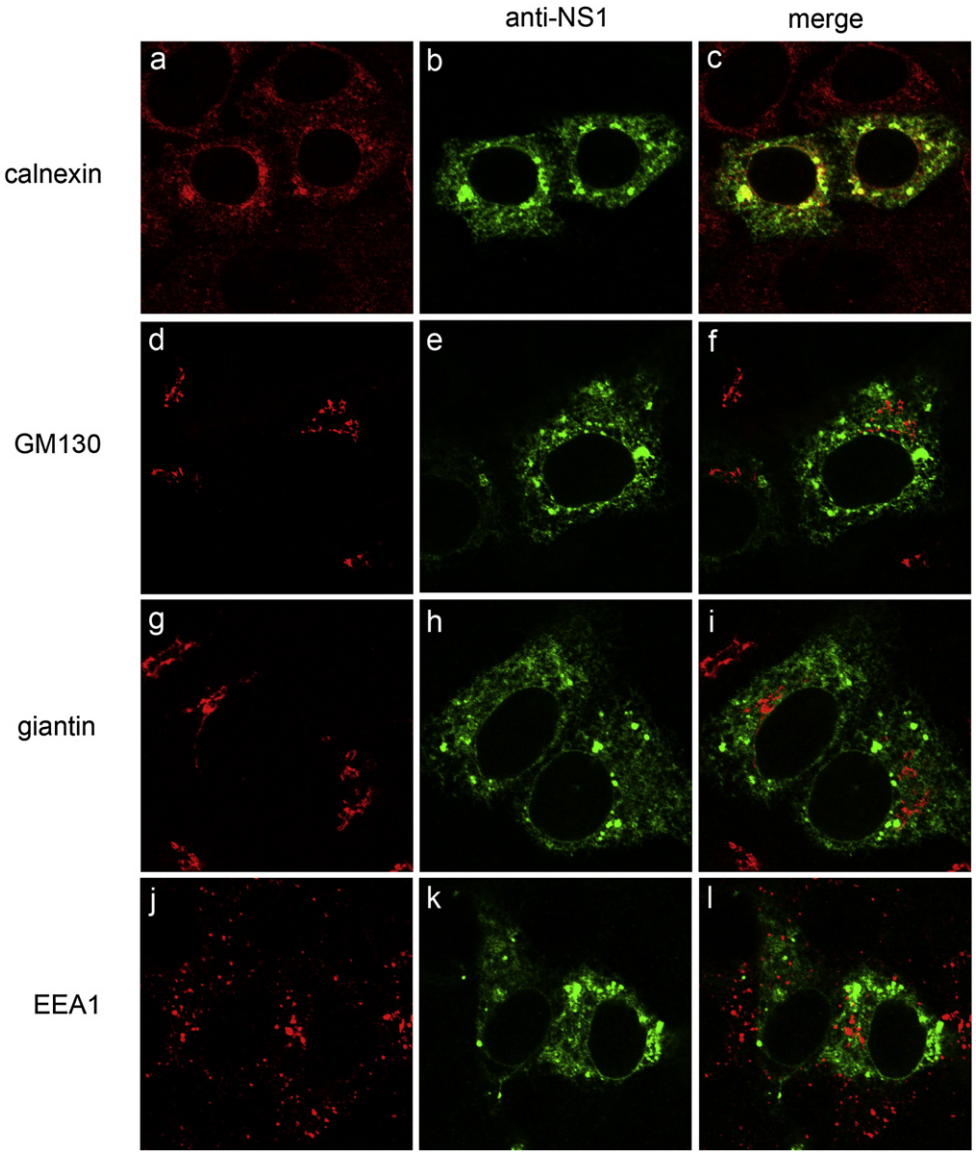

Fig. 3. MNV-1 NS1-2 localises with calnexin in membranes of the ER. Vero cells were transfected with NS1-2, fixed at 24 hpt and dual-labelled with antibodies specific for various cellular organelle markers (shown in red) as well as antibodies specific for NS1-2 (shown in green). Cells were subsequently analysed via confocal immunofluorescence microscopy. NS1-2 was observed to colocalise extensively with the ER (calnexin; panels a–c) and induce the rearrangement of the ER but was not observed to localise with the Golgi (giantin; panels d–f, or GM130; panels g–i) or endosomes (EEA1; panels j–l). In all cases, colocalisation is observed as a yellow hue in panels f, i, and l.

such as Rab5, Rab7, and Rab9, and no colocalization was observed with NS3 (data not shown). While there is the possibility of an association between NS3 and a subset of endosomes/lysosomes exclusive of EEA1 and other endosomal markers tested, it is also possible that NS3 localises to another cellular organelle that also exhibits this vesicular morphology, which, to date, we have been unable to identify.

In NS4-transfected cells, we observed colocalisation between the viral protein and multiple membrane markers: giantin, GM130, and EEA1 (Fig. 5). Given the relationship between the Golgi and endosomes in membrane and cargo trafficking, the simultaneous association between NS4 and these organelles is unsurprising and suggests an association between NS4 and a cellular component common to both these compartments. Interestingly, we also observed some alterations in Golgi morphology in NS4-transfected cells (Figs. 5d–f and g–i). This was particularly prominent in cells co-labelled with giantin in which Golgi labelling not only appeared more disperse but also less abundant (Figs. 5g–i), in contrast to the more compact, ribbon-like labelling seen in adjacent untransfected cells (compare in Fig. 3g). The partial dispersion of the Golgi in NS4-transfected cells could suggest that membrane trafficking may be hindered and implies a role for NS4 in the recruitment of membranes from the late secretory pathway in RC formation.

Our analysis of subcellular localisation of NS6 revealed no association between either the ER, Golgi, or endosomes, nor did there appear to be any significant alteration in the distribution or morphology of these organelles (Figs. 4 and 6). In attempts to further characterize the subcellular localization of NS3 and NS6, we included the visualization of mitochondria using MitoTracker. We also extended these analyses to include the other ORF1 proteins. Strikingly, NS6 was shown to colocalise extensively with these organelles (Figs. 7j–l) with some juxtaposed labelling also observed in the NS3-transfected cells. Interestingly, we have observed no association between dsRNA and the mitochondria in infected cells (unpublished data), suggesting that the MNV-1 RC does not recruit mitochondria during the replication cycle.

No cellular markers were observed to colocalise with the viral RdRp (NS7), nor did we detect any significant change in the

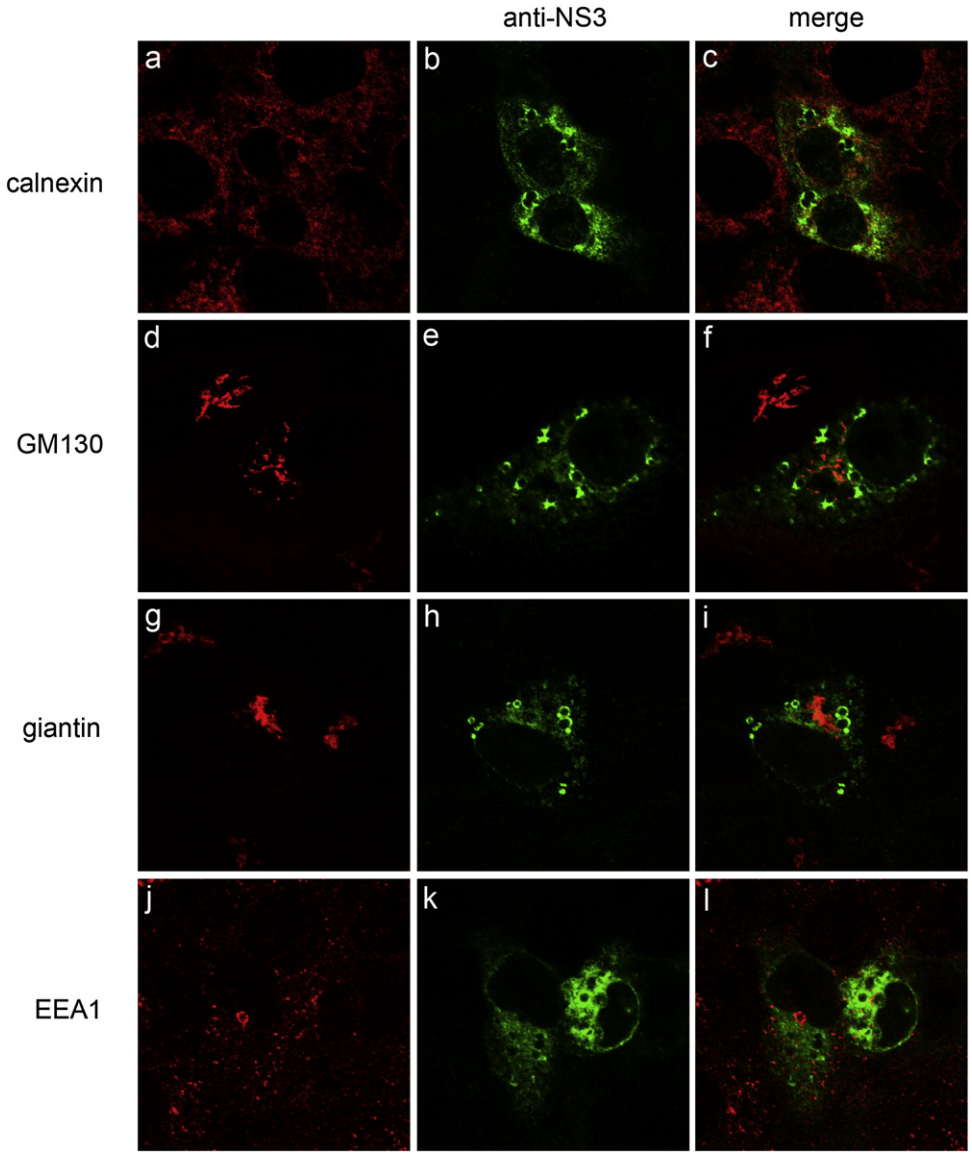

Fig. 4. Localisation of MNV-1 NS3 with cellular markers in transfected Vero cells. Vero cells were transfected with NS3, fixed at 24 hpt and dual-labelled with antibodies specific for various cellular organelle markers (shown in red; panels a, d, g, and j) as well as antibodies specific for NS3 (shown in green; panels b, e, h, and k). No colocalisation is observed with MNV-1 NS3 and the cellular markers utilized, as a yellow hue indicating colocalisation is not observed in the merged panels (panels c, f, i, and l).

morphology or distribution of these organelles in transfected cells (Fig. 8). NS7 did not appear to associate with any particular cellular compartment and did not exhibit a localisation pattern indicative of a particular cellular organelle but was observed to localize to the nucleus. As mentioned earlier, this localization pattern of NS7 is similar to that of other RNA virus RdRps. However, in our previous study, we did not observe any association of the nucleus with MNV1 replication and as such the role of NS7 within the nucleus remains unclear.

Discussion

The replication of arguably all positive-sense RNA viruses is associated with characteristic virus-induced clusters of vesicles (Mackenzie, 2005; Novoa et al., 2005). Although strikingly similar in their morphology, these vesicles appear to originate from various locations within the cell depending on the virus investigated (Bienz et al., 1987; Knox et al., 2005; Mackenzie, 2005; Martín-Acebes et al., 2008; Schlegel et al., 1996; Schwartz et al., 2004; Snijder et al., 2006). In most cases, these dramatic changes in membrane morphology can be attributed to a single viral gene product, suggesting that localization of the viral proteins to specific membrane locations is the driving force in establishing virus replication on these platforms. Previously, we have shown that MNV-1 replication is tightly associated with vesicle clusters that contain identifiable components of the endocytic and secretory pathway, namely the ER, Golgi, and endosomes (Hyde et al., 2009). In the present study, we have sought to characterize the subcellular localisation of the individual MNV-1 ORF1 proteins in transfected cells and to postulate the potential role of some of these proteins in the membrane recruitment and formation of the RC during replication. As we had previously shown that all of the ORF1 proteins localise with the replicative intermediate (dsRNA) within the MNV-1 RC in infected cells, we aimed to determine the contribution of each protein in localizing to (and thus potentially recruiting) cellular membranes that support MNV-1 RNA replication. To this end, we observed that when transfected individually into cells, the MNV-1 ORF1 proteins exhibited subcellular localisation profiles that were clearly distinct from one another and were specifically associated with different cellular organelles (Figs. 2–8). The localization patterns of the transfected ORF1 proteins displayed a more

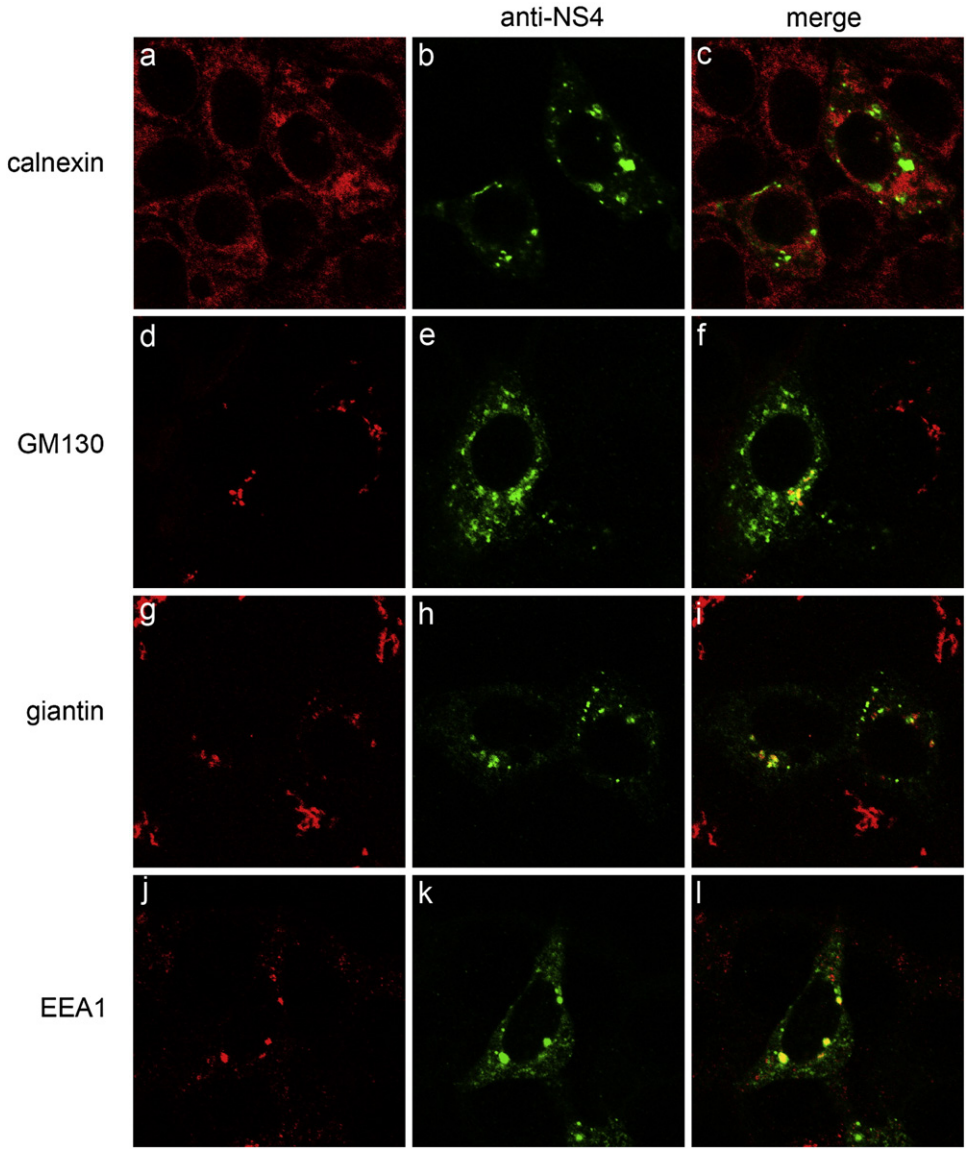

Fig. 5. MNV-1 NS4 localises with protein markers for the Golgi apparatus and endosomes. Vero cells were transfected with cDNA expression plasmids encoding the MNV-1 NS4 protein, fixed at 24 hpt and dual-labelled with antibodies specific for various cellular organelle markers (shown in red; panels a, d, g, and j) as well as antibodies specific for NS4 (shown in green; panels b, e, h, and k). A yellow hue in the merged panels indicates that MNV-1 NS4 colocalised with both the Golgi (giantin; panels d–f, or GM130; panels g–i) or endosomes (EEA1; panels j–l) but not the ER (calnexin; panels a–c).

diverse staining compared to the dense perinuclear foci observed during infection (Hyde et al., 2009). These observations imply that each of the ORF1 proteins may play a distinct role in replication and that the establishment and formation of the RC, characteristically seen in MNV-1-infected cells, is mediated via the interaction of multiple viral proteins, possibly also in conjunction with as yet unidentified host proteins/membranes.

Our analysis of the ORF1 proteins via dual-labelling immunofluorescence with cellular markers further demonstrated that the NS1-2, NS4, NS6, and NS7 proteins localized to the ER, endosomes/Golgi, mitochondria, and the cytoplasm/nucleus, respectively. On the other hand, NS3 was observed to localize to discrete vesicular structures of unknown origin. It is possible that this localization pattern is due to aggregation of overexpressed protein, particularly as these vesicular structures are not observed during MNV1 infection. However, this is a very unique and specific localization pattern that is additionally observed when expressing the Norwalk virus NS3 protein (data not shown). Thus, we believe it is a specific localization of this protein in transfected cells, and we are currently investigating this in more detail. Unfortunately, we were unable to express the NS5 protein under the conditions utilized during this study, and we are currently investigating whether coexpression of additional MNV1 ORF1 proteins are required for expression of this protein. The clear association of MNV-1 NS1-2 with the ER marker calnexin is especially significant as Bailey et al. (2009) recently showed that the feline calicivirus (FCV) p32 protein (the FCV equivalent of NS1-2) also localizes to the ER. Interestingly, NS1-2 was the only ORF1 protein that was observed to associate with the ER and induce significant alterations in the distribution and morphology of this organelle, whereas the FCV p32, p39, and p30 proteins (equivalent to MNV-1 NS1-2, NS3, and NS4) were all observed to localize to the ER (Bailey et al., 2009). In contrast, the human Norwalk virus NS1-2 homologue p48 was observed to localise to the Golgi apparatus and induced its dispersion (Fernandez-Vega et al., 2004). This is in stark contrast to our current findings which show no association between NS1-2 and the Golgi or any significant alteration in the morphology or distribution of this organelle in NS1-2-expressing cells (Figs. 3d–i).

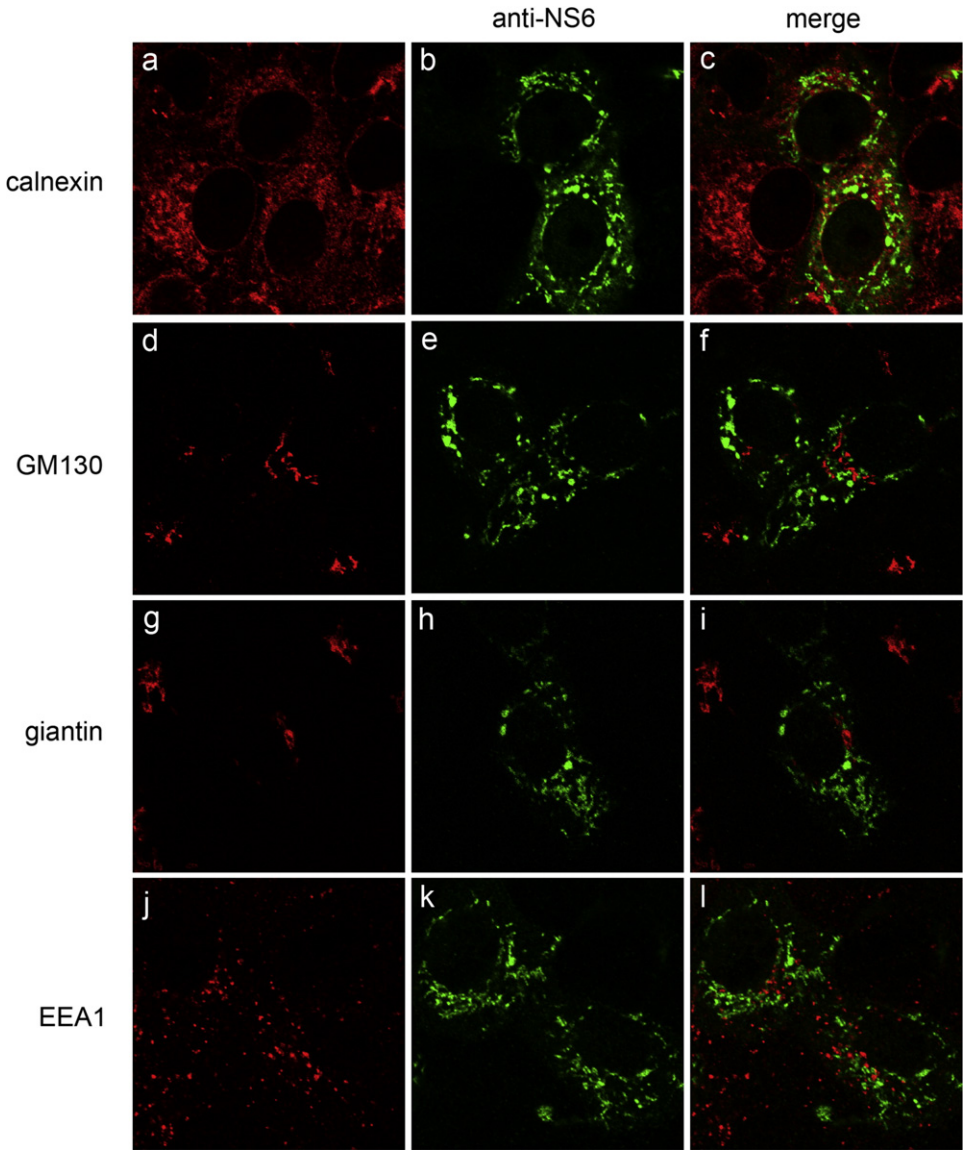

Fig. 6. The MNV-1 NS6 protein does not localise to organelles of the secretory pathway. Vero cells were transfected with cDNA expression plasmids encoding the MNV-1 NS6 protein, fixed at 24 hpt and dual-labelled with antibodies specific for various cellular organelle markers (shown in red; panels a, d, g, and j) as well as antibodies specific for NS6 (shown in green; panels b, e, h, and k). No colocalisation is observed with MNV-1 NS6 and the cellular markers utilized, as a yellow hue not observed in the merged panels (panels c, f, i, and l).

In contrast to our observations of NS1-2, NS4 was seen to colocalise with a broader range of membrane components including the Golgi apparatus and endosomes (Figs. 5d–f, g–i, j–l), which we have previously demonstrated to also localise with the MNV-1 RC during replication (Hyde et al., 2009). NS4 was the only ORF1 protein that was observed to localise with endosomes and again seems to suggest that this protein may be largely responsible for the recruitment of these membranes to the RC. Interestingly, while we have previously observed no significant change in Golgi morphology or distribution in MNV-1-infected cells, in the current study, we have shown that NS4 expression was associated with a partial dispersion of the Golgi (Figs. 5d–i). Importantly, the dispersion of the Golgi apparatus is characteristic of infection of cells with poliovirus (PV), a picornavirus. The dispersion of the Golgi apparatus has been attributed to the PV 3A protein (equivalent to MNV-1 NS4) via a mechanism that inhibits ADP-ribosylation factor 1 (Arf1)-dependent COPI recruitment to membranes (Choe et al., 2005; Doedens et al., 1997; Wessels et al., 2006). This inhibition of COPI recruitment restricts ER to Golgi trafficking and is postulated to be a means by which picornaviruses prevents immune recognition and stimulation (Cornell et al., 2007; Dodd et al., 2001; Sanz-Parra et al., 1998). However, as we have previously shown that MNV-1 replicates in a COPI/COPII-independent manner, it is unlikely that MNV-1 NS4 has the same impact on cellular membrane trafficking. Although we are currently investigating the potential involvement of the MNV-1 NS4 protein in restricting protein and membrane trafficking and secretion to further delineate its role.

While we observed no association between NS6 and components of the endocytic and secretory apparatus (Fig. 6), we did observe extensive colocalisation with mitochondria (Fig. 7). Interestingly, this is in contrast to our previous observations of MNV-1-infected cells in which no association between mitochondria and the RC was observed (unpublished data). This would seem to suggest that the function of NS6 during replication extends beyond its role in the posttranslational processing of the viral polyprotein. The interaction between viral proteases and the mitochondria has been well documented, and these proteins have been shown to be involved in modulating the host immune response as well as apoptosis (Barral et al., 2009; Boya et al., 2004; Drahos and Racaniello, 2009; Liu et al., 2009; Williamson and Berg-Poley, 2009; Yang et al., 2007). More significantly, Bok et al.

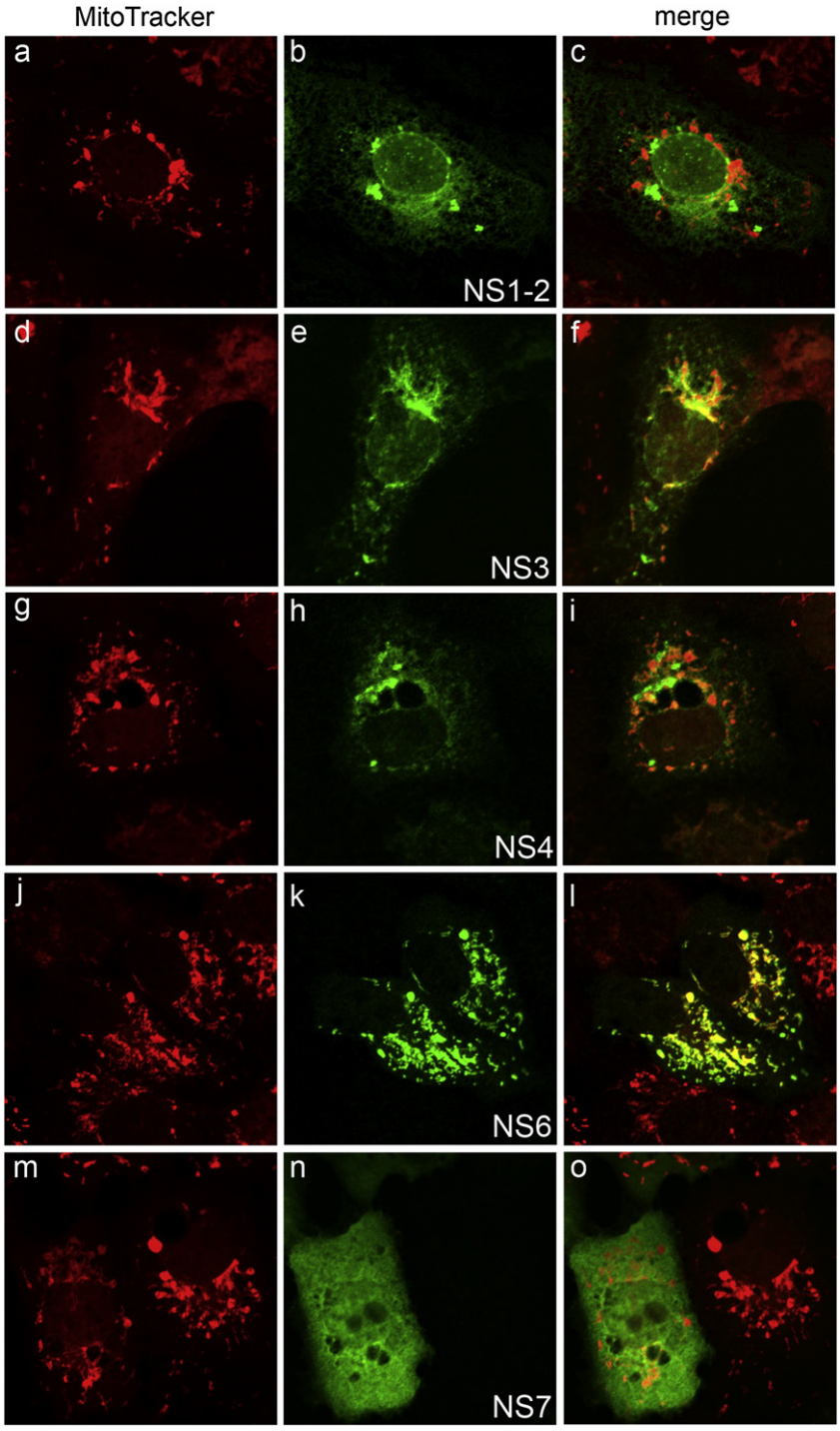

Fig. 7. The MNV-1 NS3 and NS6 proteins are localised to mitochondria when expressed individually. Vero cells were individually transfected with cDNA expression plasmids encoding the MNV-1 ORF1 proteins, fixed at 24 hpt and dual-labelled with MitoTracker (shown in red; panels a, d, g, and j) and antibodies specific for each ORF1 proteins (shown in green; panels b, e, h, and k). NS6 was observed to colocalise extensively with mitochondria (panels j–l), while NS3 also exhibited some juxtapose labelling (panels d–f). In contrast NS1-2 (panels a–c), NS4 (panels g–i), and NS7 (panels m–o) showed no colocalisation with mitochondria.

(2009) have illustrated that MNV-1 induces apoptosis by activating caspase-9 which is involved in the intrinsic (mitochondrial-mediated) apoptotic pathway. The association of NS6 with mitochondria that we have observed here may suggest that this protein may in fact be either directly or indirectly involved in the up-regulation of apoptosis, perhaps via cleavage of a mitochondrial-associated regulatory component, as has been shown to be the case for some picornaviruses (Drahos and Racaniello, 2009; Yang et al., 2007). In addition, both the hepatitis C virus and hepatitis A virus proteases have been observed to localise to mitochondria where they cleave the mitochondrial antiviral signalling protein, MAVS, to prevent activation of NF-κB and IFN regulatory factor 3 (via MDA-5) to induce IFN (Li et al., 2005; Yang et al., 2007). McCartney et al. (2008) have shown that MNV-1 replication is restricted via detection by MDA-5 and thus MNV-1 NS6 may play an additional role to prevent immune signalling via the degradation of MAVS. We are currently investigating the additional roles of NS6 in virus replication and pathogenesis.

In summary, we have shown that both NS1-2 and NS4 associate with distinct components of the endocytic and secretory pathway that we have previously observed to be associated with the MNV-1 RC

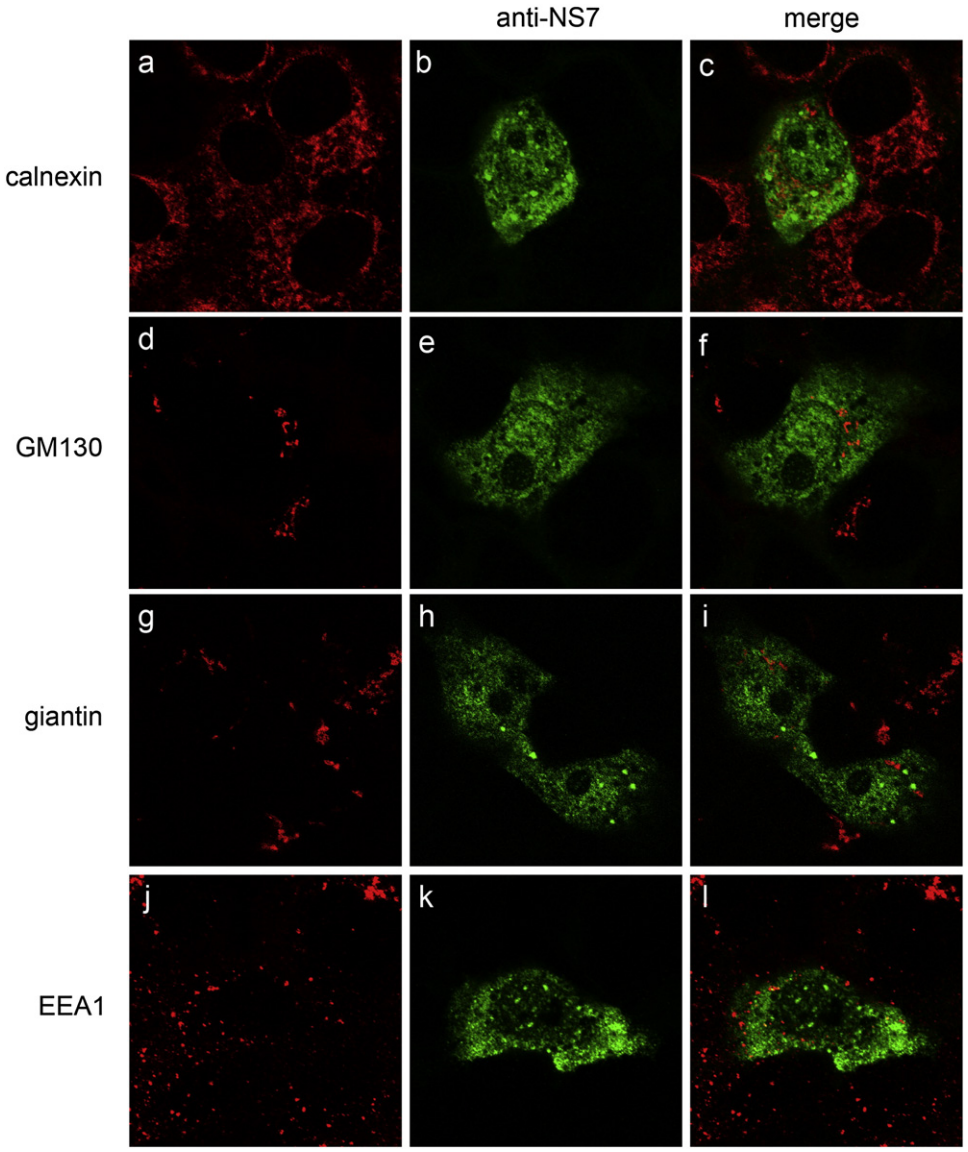

Fig. 8. The MNV-1 NS7 protein displays and diffuse cytoplasmic labelling pattern with some nuclear staining. Vero cells were transfected with cDNA expression plasmids encoding the MNV-1 NS7 protein, fixed at 24 hpt and dual-labelled with antibodies specific for various cellular organelle markers (shown in red; panels a, d, g, and j) as well as antibodies specific for NS7 (shown in green; panels b, e, h, and k). No colocalisation is observed with MNV-1 NS6 and the cellular markers utilized, as a yellow hue not observed in the merged panels (panels c, f, i, and l); however, diffuse nuclear staining is observed in panels b, e, h, and k.

during replication. These results imply a role for both these proteins in membrane recruitment to the RC and suggest that both these proteins may also serve to additionally block anterograde membrane trafficking, although this is yet to be proven. To collate our previous (Hyde et al., 2009) and current findings, we have proposed a model of MNV-1 replication whereby NS1-2 and NS4 function together to induce the formation of MNV-1-associated membrane vesicles (Fig. 9). In this model, we propose that, after translation and cleavage of the ORF1 polyprotein, both NS1-2 and NS4 localise to the ER and Golgi/endosomes, respectively, and recruit these cellular membranes to the dense perinuclear foci comprising the MNV-1 RC. The driving force to initiate the biogenesis of the vesicle clusters remains unknown, as it does not correlate with an inhibition of binding of coatamer proteins to transport vesicles resulting in vesicle accumulation (cf, PV and related picornaviruses). It is also important to consider the differences observed in the localisation of viral polyproteins versus individual nonstructural proteins. While we have examined the localisation of individual ORF1 proteins in this study, it is possible that the localisation, and even the function, of these proteins may alter when coexpressed with other ORF1 proteins. While the exact function of the MNV-1 ORF1 proteins still remains to be determined, it is clear that these proteins play distinct roles in the replication of MNV-1 and exhibit characteristics different to homologous proteins of other related viruses including members of the *Picornaviridae* and other members of the *Caliciviridae*.

Materials and methods

Cells

VeroC1003 cells were grown and maintained in DMEM medium (Invitrogen, Australia) supplemented with 5% fetal calf serum (Lonza, Basel, Switzerland), 2 mM glutamax (Gibco BRL), and 100 U/mL/100 μg/mL penicillin/streptomycin (Gibco BRL).

Reagents

Antibodies

MNV specific guinea pig polyclonal antibodies have been described previously (Sosnovtsev et al., 2006a). Anti-hexahistidine antibody

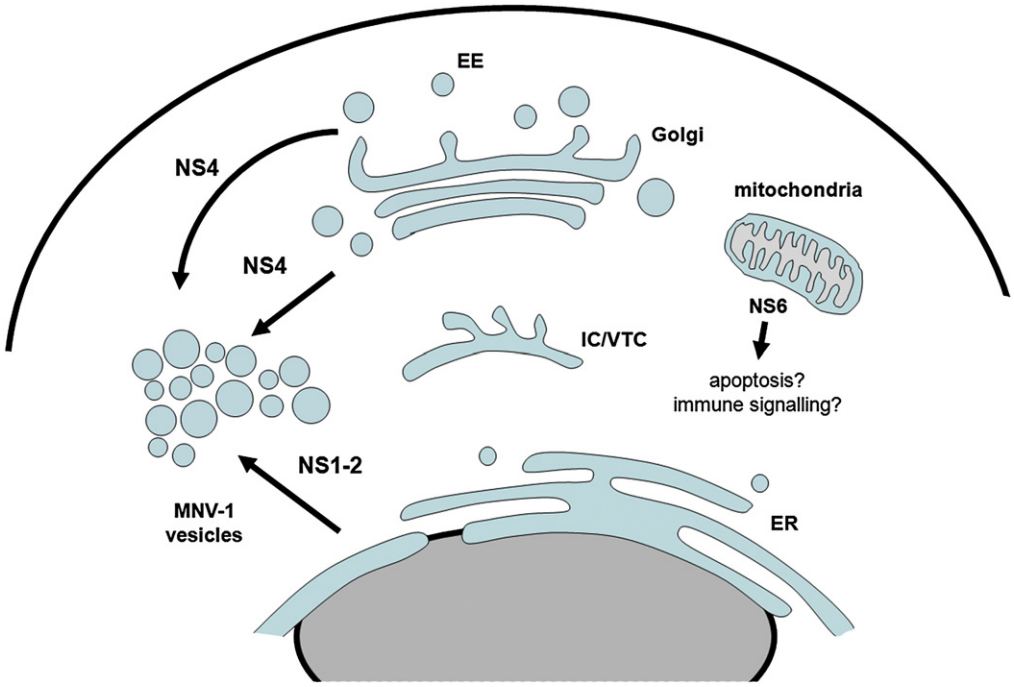

Fig. 9. Model of membrane recruitment by MNV-1 ORF1 proteins during replication. Based on our previous observations and current findings, we propose a model via which cellular membranes are recruited to the site of virus replication by ORF1 proteins. In this model, NS1-2 and NS4 localise to the ER and Golgi/endosomes, respectively, and recruit these membranes to the RC which resides adjacent to the nucleus. We also propose that NS6 localises to the mitochondria to potentially induce apoptosis or to perturb host immune function.

was purchased from Novagen (Merck). Anti-rabbit, anti-guinea pig, and anti-mouse specific IgG AlexaFluor 488, 549, and 647 were purchased from Molecular Probes (Invitrogen, Leiden, The Netherlands). Anti-calnexin, anti-giantin, and anti-GM130 were purchased from Merck-Calbiochem (Germany), and anti-EEA1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Chemicals

MitoTracker Red CMXRos was purchased from Molecular Probes (Invitrogen), and Lipfectamine 2000 transfection reagent was purchased from Invitrogen.

Construction of plasmids

The individual ORF1 proteins were cloned into the CMV promoter-driven expression vector pcDNA3.1(+) (Invitrogen) using primers in which a hexahistidine tag was incorporated into the reverse primer to produce a C-terminally tagged protein. Primers were additionally designed to incorporate an *Eco*RV restriction site in the forward primer and a *Spe*I restriction site in the reverse primer. Primer pairs for each construct were purchased from Geneworks (Australia), the sequences of which are outlined in Table 1. PCR fragments were generated using *Pfu* DNA polymerase (Promega) with conditions for each primer pair being optimised experimentally using the manufacturer's guides. Individual clones were then verified by sequencing.

In vitro transcription/translation

Translation products were generated from each clone using TnT® Quick Coupled Transcription/Translation System (Promega) as specified by the manufacturer's instructions. In brief, 20 μL of TnT® Quick master mix was combined with 0.5 μg of plasmid DNA and the total reaction volume adjusted to 25 μL by the addition of nuclease-free water. The reaction was then incubated at 30 °C for 90 minutes, after which time the reaction products were analysed by Western blotting.

Western blotting

Protein products generated using the transcription/translation reaction were separated on 4%–12% precast Tris–Bis polyacrylamide gels (Invitrogen). Proteins were subsequently transferred to a Hybond™–ECL™ nitrocellulose membrane (Amersham Biosciences Corp, Piscataway, NJ, USA) using the Biorad wet-transfer blotting module. The membrane was subsequently blocked with 5% Skim Milk Powder (Diploma) in PBS containing 0.1% Tween-20 at room temperature. Incubation with anti-hexahistidine antibodies was performed in blocking buffer on a rotating wheel overnight at 4 °C. The bound antibody was subsequently visualized with species-specific IgG conjugated to Alexa Flour 647 (Invitrogen) and the Amersham Storm scanner. The resulting images were digitally scanned and processed in Adobe Photoshop™ for publication.

Immunofluorescence (IF) analysis

VeroC1008 cell monolayers on coverslips were transfected with pcDNA3.1 fusion plasmids as specified by the manufacturer and incubated at 37 °C for the specified periods after transfection. The cells

Table 1
Sequences of primer pairs for generation of fusion peptides.

Construct	Primer pair sequence (5′–3′)
pcDNA3-NS1-2	F: gatatcaccaccatgaggatggcaacgcca, R: actagttcaatgatgatgatgatgttccgcctgccattc
pcDNA3-NS3	F: gatatcaccaccatgggacccttcgacctt, R: actagttcaatgatgatgatgatgctggaggccgaaatc
pcDNA3-NS4	F: gatatcaccaccatgaacaaggtctatgac, R: actagttcaatgatgatgatgaatgatgctcagagtggtacca
pcDNA3-NS5	F: gatatcaccaccatgggaaagaagggcaag, R: actagttcaatgatgatgatgatgctcaaagttgatctt
pcDNA3-NS6	F: gatatcaccaccatggcaccagtctccatc, R: actagttcaatgatgatgatgatgctggaactccagagc
pcDNA3-NS7	F: gatatcaccaccatgggacccccctatgctt, R: actagttcaatgatgatgatgatgctcatcctcattcac

were subsequently washed with PBS and fixed with 4% paraformaldehyde (ProSciTech, Kirwan, Australia) for 10 min at 20 °C and permeabilized with 0.1% Triton X-100 in 4% paraformaldehyde for an additional 10 min at 20 °C; the cells were washed with PBS, and aldehyde groups were quenched with 0.2 M glycine in PBS and additionally washed twice with PBS before incubation with antibodies. Alternatively transfected cells were fixed with 1:1 mixture of acetone and methanol and incubated at −20 °C for 10 min before washing with PBS. Primary and secondary antibodies were incubated with blocking buffer (PBS containing 1% BSA) and washed with PBS containing 0.1% BSA/0.1% Tween-20 between incubation steps. After a final wash with PBS, coverslips were drained and mounted onto glass slides with a quick dry mounting medium (United Biosciences, Brisbane, Australia) before visualizing on either a Zeiss LSM 510 META Confocal Microscope or a Leica confocal microscope. Images were collected and collated for publication using Adobe Photoshop™ software.

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