2017

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The Norovirus NS3 Protein Is a Dynamic Lipid- and Microtubule-Associated Protein Involved in Viral RNA Replication

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ABSTRACT Norovirus (NoV) infections are a significant health burden to society, yet the lack of reliable tissue culture systems has hampered the development of appropriate antiviral therapies. Here we show that the NoV NS3 protein, derived from murine NoV (MNV), is intimately associated with the MNV replication complex and the viral replication intermediate double-stranded RNA (dsRNA). We observed that when expressed individually, MNV NS3 and NS3 encoded by human Norwalk virus (NV) induced the formation of distinct vesicle-like structures that did not colocalize with any particular protein markers to cellular organelles but localized to cellular membranes, in particular those with a high cholesterol content. Both proteins also showed some degree of colocalization with the cytoskeleton marker β-tubulin. Although the distribution of MNV and NV NS3s were similar, NV NS3 displayed a higher level of colocalization with the Golgi apparatus and the endoplasmic reticulum (ER). However, we observed that although both proteins colocalized in membranes counterstained with filipin, an indicator of cholesterol content, MNV NS3 displayed a greater association with flotillin and stomatin, proteins known to associate with sphingolipid- and cholesterol-rich microdomains. Utilizing time-lapse epifluorescence microscopy, we observed that the membrane-derived vesicular structures induced by MNV NS3 were highly motile and dynamic in nature, and their movement was dependent on intact microtubules. These results begin to interrogate the functions of NoV proteins during virus replication and highlight the conserved properties of the NoV NS3 proteins among the seven Norovirus genogroups.

IMPORTANCE Many mechanisms involved in the replication of norovirus still remain unclear, including the role for the NS3 protein, one of seven nonstructural viral proteins, which remains to be elucidated. This study reveals that murine norovirus (MNV) NS3 is intimately associated with the viral replication complex and dsRNA. We observed that the NS3 proteins of both MNV and Norwalk virus (NV) induce prominent vesicular structures and that this formation is dependent on microtubules and cellular cholesterol. Thus, this study contributes to our understanding of protein function within different Norovirus genogroups and expands a growing knowledge base on the interaction between positive-strand RNA [(+)]RNA viruses and cellular membranes that contribute to the biogenesis of virus-induced membrane organelles. This study contributes to our understanding of viral protein function and the ability of a viral protein to recruit specific cellular organelles and lipids that enable replication.

KEYWORDS norovirus, cytoskeleton, virus replication, cellular lipids
Noroviruses (NoVs) belong to the Caliciviridae family and are small, nonenveloped, positive-sense RNA viruses known to cause acute and chronic diseases in humans (1, 2) and other mammals (3). The clinical symptoms of humans infected with NoV include vomiting, abdominal cramps, and nonbloody loose or watery diarrhea that can lead to severe dehydration (4, 5). NoV infections result in an estimated 230,000 deaths per year, particularly in developing nations (6). This pathogen is highly infectious (7) and transmitted primarily from person to person within enclosed settings such as schools, cruise ships, hospitals, and aged-care and child care facilities (8, 9). NoV is the only virus associated with global pandemics of acute gastroenteritis that emerge every 2 to 3 years, affecting hundreds of millions of people (10, 11). NoV pandemics have occurred globally since the mid-1990s with increasing frequency (10–12). Consequently, NoV-associated gastroenteritis has become a major public health concern for which there is no available antiviral agent or preventative vaccine.

NoV host cell tropism appears to be exceptionally restricted (13); therefore, human NoVs are difficult to study due to the lack of a reliable cell culture system or any small-animal models. Recently, it was observed that B cells could be infected with human NoV but only in the presence of histo-blood group antigen-expressing enteric bacteria, and infection was observed to be inefficient (14). Subsequently, multiple human NoV strains have been more robustly cultivated in stem cell-derived enterocytes forming human intestinal enteroid monolayer cultures (15). Consequently, the molecular mechanisms of human NoV pathogenesis and immunity remain poorly defined (5).

In 2003, murine norovirus (MNV) was discovered and has been used to study the immunity and pathogenesis of NoV infection in a mouse model (16). Subsequently, the tropism of MNV for mononuclear cells was identified and led to the establishment of the first in vivo culture system to study NoV replication, a significant advancement in NoV biology (17).

NoV harbors an RNA genome of ~7,500 nucleotides that is protein linked at the 5’ end by VPg (encoded by the viral gene NS5), polyadenylated at the 3’ end, and divided into 3 open reading frames (ORFs) (4 for MNV). ORF1 encodes a large polyprotein that undergoes proteolytic cleavage by the virus-encoded protease (NS6) to produce the mature viral nonstructural proteins (18). The two structural proteins, VP1 (major capsid protein) and VP2 (minor capsid protein), are encoded by ORF2 and ORF3, respectively, and can be additionally translated from a viral subgenomic RNA (18). Importantly, the ORF1/ORF2 overlap has been shown to be the breakpoint for genetic recombination (19–22).

Our group has been instrumental in identifying and interrogating the intracellular site of MNV replication (20, 21). We have shown that the MNV replication complex (RC) is a collection of irregular-sized vesicles tightly associated with the endoplasmic reticulum (ER), the trans-Golgi network, endosomes, and the centrosome (20, 22). In addition, we have identified the discrete intracellular localization patterns of the MNV ORF1 proteins and their relationship with cellular organelles and membranes utilized during MNV replication (23). We showed that MNV NS1-2 localizes to the ER, NS4 localizes to endosomes, and NS6 localizes to mitochondria (23). Interestingly, NS3 induced discrete vesicular structures that were unidentifiable by typical cell organelle markers but showed some association with microtubules (23, 24).

In this study, we aimed to further investigate the function of MNV NS3 during the replication cycle and the origin of the vesicular structures induced by MNV NS3 expression, comparing it to the expression pattern of human NoV (Norwalk virus [NV]) NS3. We show that MNV NS3 is intimately associated with double-stranded RNA (dsRNA) within the MNV RC and show that the localization pattern of transiently expressed human NV NS3 is similar to that of MNV NS3, although there are some subtle differences. Additionally, we show that the vesicular structures induced during NS3 expression contain lipids, and using time-lapse epifluorescence microscopy, we demonstrate that these structures are highly dynamic and undergo microtubule-dependent transport.
RESULTS

The MNV NS3 protein localizes to the cytoplasmic face of membranes comprising the MNV replication complex. In our previous studies, we showed by immunofluorescence (IF) analysis that MNV NS3 localized with dsRNA in the perinuclear region of infected cells over the course of infection (25). Thus, we aimed to utilize ultramicrotomy and immunogold labeling to address the specific localization of NS3 within MNV-infected cells. As shown in Fig. 1, membrane vesicles comprising the MNV

FIG 1 The MNV NS3 protein associates with the viral replication complex. Thawed cryosections of MNV-infected RAW264.7 cells were fixed at 12 h p.i. (A and B), 18 h p.i. (C and D), or 24 h p.i. (E and F) and were immunolabeled with anti-NS3 antibodies and 10-nm protein A gold. Bars, 200 nm in all cases.
RC were heavily decorated with anti-NS3 antibodies during the course of infection. At all time points investigated (namely, 12, 18, and 24 h postinfection [p.i.]), NS3 labeling was observed to be intimately associated with the membranes comprising the RC. In particular, the majority of the labeling of NS3 was confined to the RC, and we observed very little labeling in other areas of the cell. The localization of NS3 to the RC is not surprising, as it contains helicase and nucleoside triphosphatase (NTPase) motifs that are characteristic of an enzyme involved in positive-strand RNA virus replication (16).

To determine the precise localization of NS3 with dsRNA, we dually labeled thawed cryosections obtained from MNV-infected cells with antibodies to dsRNA (5-nm protein A gold) and NS3 (15-nm protein A gold). Arrowheads indicate areas enriched for both the 5-nm and 10-nm gold particles corresponding to immunolabeling for dsRNA and NS3. RC; replication complex. Bars, 200 nm in all cases.

**FIG 2** The MNV NS3 protein associates with dsRNA within the viral replication complex. Thawed cryosections of MNV-infected RAW264.7 cells fixed at 12 h p.i. (A and B) and 18 h p.i. (C and D) were dually labeled with antibodies to dsRNA (5-nm protein A gold) and NS3 (15-nm protein A gold). Arrowheads indicate areas enriched for both the 5-nm and 10-nm gold particles corresponding to immunolabeling for dsRNA and NS3. RC; replication complex. Bars, 200 nm in all cases.

RC were heavily decorated with anti-NS3 antibodies during the course of infection. At all time points investigated (namely, 12, 18, and 24 h postinfection [p.i.]), NS3 labeling was observed to be intimately associated with the membranes comprising the RC. In particular, the majority of the labeling of NS3 was confined to the RC, and we observed very little labeling in other areas of the cell. The localization of NS3 to the RC is not surprising, as it contains helicase and nucleoside triphosphatase (NTPase) motifs that are characteristic of an enzyme involved in positive-strand RNA virus replication (16).

To determine the precise localization of NS3 with dsRNA, we dually labeled thawed cryosections obtained from MNV-infected cells with antibodies to dsRNA and NS3 that were subsequently conjugated with 5-nm and 10-nm protein A-gold particles, respectively (Fig. 2). As can be observed, the smaller 5-nm gold particles, corresponding to labeling for dsRNA, were consistently associated with NS3 (larger 10-nm gold particles) within the vesicles comprising the MNV RC. Interestingly, a majority of the labeling for dsRNA was associated with cytoplasmic components morphologically similar to NS3, with some labeling also being observed on the luminal side of the RC membranes.

These results suggest that MNV NS3 is abundant in the RC at the site of active viral RNA replication.

**Comparative analysis of the Norwalk virus and murine norovirus NS3 proteins.**

In our attempts to determine the potential function and distribution of NoV NS3, we aimed to compare the expressions and distributions of genogroup I (Norwalk) and
genogroup V (murine) NoV NS3 proteins. Initially, we compared the biochemical characteristics of each protein (Fig. 3). We aligned the amino acid sequence of each protein by ClustalW (Fig. 3A), with dark shading corresponding to identical amino acids and light shading corresponding to conserved amino acids. PROSITE scanning analysis reveals a conserved SF3_Helicase motif in both proteins. TMpred prediction of transmembrane regions of each protein indicates a potential transmembrane alpha-helix within the N termini of both proteins.
of the NV and MNV NS3 amino acid sequences additionally revealed a conserved superfamily 3 helicase (SF3_Helicase) motif in both proteins (Fig. 3B), and further TMpred prediction of transmembrane regions of each protein indicates a potential transmembrane alpha-helix within the N terminus of both proteins (Fig. 3C).

Overall, these analyses revealed that there is a high degree of conservation between these two proteins at the amino acid level, implying a potential conserved function during the replication cycles of both NV and MNV.

**Transient expression of either mouse or human NoV NS3 induces cytoplasmic vesicular structures that display colocalization with the Golgi apparatus and microtubules.** We previously transiently expressed MNV NS3 from a recombinant cDNA expression plasmid and determined its intracellular localization (23). In this study, we aimed to determine if MNV and human genogroup I NoV strain NV NS3 shared the same localization pattern and thus potential function in cells. Due to the lack of NV NS3-specific antibodies, we generated expression vectors encoding recombinant NV with a C-terminal c-Myc epitope tag and MNV NS3 with a C-terminal 6×His epitope tag (23) for comparative analysis, which were expressed in Vero cells and used to assess and compare their intracellular localizations by IF analysis. As shown in Fig. 4, both NS3 proteins were expressed well in transfected cells and were recognized by antibodies specific to their recombinant tag. The MNV and NV NS3 proteins displayed striking similarity in their localization patterns. Both proteins induced a vesicular staining pattern (Fig. 4, arrows), with some additional strong perinuclear staining for NV NS3, and cytoplasmic reticular staining was observed for both proteins. This staining pattern for NV NS3 was consistent with what we previously described for MNV NS3 (23).
cytoplasmic reticular staining for both proteins. This similarity of the cellular distribution patterns for the two NS3 proteins was consistent with a conserved function.

In our previous studies, we aimed to determine the intracellular localization of MNV NS3 and observed very little colocalization of MNV NS3 with markers for known organelles, namely, PDI (for the rER), GM130 (for the Golgi apparatus), EEA1 (for endosomes), and β-tubulin (for microtubules). MNV NS3, like NV NS3, showed little colocalization with markers for the ER and endosomes (a to d and i to l). However, we observed colocalization of NV NS3 with the Golgi apparatus and microtubules using β-tubulin (e to h and m to p). dapi, 4',6-diamidino-2-phenylindole.

**FIG 5** NV NS3 displays significant colocalization with PDI (n = 50), GM130 (n = 49), and β-tubulin (n = 33). Vero cells were transfected with an NV NS3-containing plasmid and fixed at 24 h.p.t., and localization was assessed by IF analysis. Shown is a comparison of the intracellular staining pattern of NV NS3 with those of cellular markers for specific organelles, namely, PDI (for the rER), GM130 (for the Golgi apparatus), EEA1 (for endosomes), and β-tubulin (for microtubules). MNV NS3, like NV NS3, showed little colocalization with markers for the ER and endosomes (a to d and i to l). However, we observed colocalization of NV NS3 with the Golgi apparatus and microtubules using β-tubulin (e to h and m to p). dapi, 4',6-diamidino-2-phenylindole.
and not dispersed, similarly to our previous observations with MNV NS3 (23). We also noted that although Pearson’s coefficient of colocalization between NS3 and H9252-tubulin was below the threshold value of 0.5, this discrepancy relates to \( R_r \) values calculated for localization in the entire cell rather than colocalization only in the perinuclear region. These results indicate that when expressed individually, both the MNV and NV NS3 proteins have similar localization patterns, and most likely properties, in transfected cells. These results suggest that, at least for NS3, the functions of this protein are similar between the two genogroups, although some differences were observed, particularly at the level of the localization of NV NS3 within the Golgi apparatus. These observations also support the notion that the events of intracellular replication of MNV and NV may be similar.

**MNV and NV NS3 associate with cholesterol-rich membranes.** As we observed minimal colocalization of NS3 with any cellular organelles, except the Golgi apparatus and microtubules, we additionally aimed to determine if NS3 was associated with lipids. To investigate this, we initially stained NS3-transfected cells with the fungal metabolite filipin, which stains cholesterol in membranes (Fig. 6). As observed in mock-transfected

![NS3 (green) Filipin (blue) merge](image)

**FIG 6** Both MNV NS3 and NV NS3 vesicular structures colocalize with filipin cholesterol stain upon transfection. Vero cells were transfected with either MNV NS3- or NV NS3-containing plasmids, fixed at 24 h p.t., and stained with filipin. In mock-transfected cells, filipin staining was confined to the plasma membrane and also foci within the cytoplasm and perinuclear region, which are characteristic of the trans-Golgi network and endosomes. Transfection of cells with either NV NS3-c-myc or MNV NS3-His resulted in significant colocalization of NS3 proteins, with filipin surrounding the NS3 vesicles (f and i (\( n = 15 \) and \( n = 20 \), respectively)). In all cases, the anti-6His and anti-c-myc antibodies were detected with species-specific antibodies conjugated to Alexa Fluor 488, and the filipin stain naturally fluoresces blue. Pearson’s coefficient values are provided in the merged panels, with a value of >0.500 corresponding to colocalization.
cells, filipin staining (Fig. 6, blue) was confined to foci within the cytoplasm and the perinuclear region, most likely representing the site of the trans-Golgi network and endosomes. Interestingly, for cells transfected with MNV NS3, we observed a significant colocalization with filipin that appeared to surround the NS3 vesicles \((Rr = 0.75 \pm 0.10)\) (Fig. 6d to f). Additionally, we observed an accumulation of filipin staining within some foci, perhaps evidence that it was recruited there by the introduction of NS3, as the distribution differs from that in mock-transfected cells. In contrast, we observed a limited degree of colocalization of NV NS3-induced vesicular structures with the filipin stain \((Rr = 0.50 \pm 0.10)\) (Fig. 6g to i).

This may suggest that the MNV and NV NS3 proteins inherently associate with intracellular lipids in different manners, which may impact their underlying functions within the host cell during virus replication and RC establishment.

**MNV NS3 associates with stomatin and flotillin, resident host proteins within cholesterol microdomains.** To investigate the association of NoV NS3 with cholesterol further, we cotransfected Vero cells with cDNA plasmids encoding MNV or NV NS3 and green fluorescent protein (GFP)-tagged stomatin/prohibitin/flotillin/H/PC (SPFH) proteins. SPFH proteins are found to be enriched in lipid microdomains within different organelles (26). Thus, we utilized GFP–erlin-1/2, GFP-prohibitin, GFP-stomatin, and GFP-flotillin to detect lipid-rich microdomains in the ER, mitochondria, endosomes, and endosomes/plasma membrane, respectively. We did not observe any association between MNV or NV NS3 and GFP–erlin-1/2 or GFP prohibitin (data not shown). However, we observed significant colocalization of MNV NS3 with stomatin and flotillin \((Rr = 0.68 \pm 0.13 \text{ and } Rr = 0.48 \pm 0.15, \text{ respectively})\) (Fig. 7A to F). Interestingly, MNV NS3 predominantly associated with stomatin-GFP, particularly within the vesicular structures induced within the cytoplasm (Fig. 7D to F). In contrast, NV NS3 displayed less of an association with these proteins \((Rr = 0.39 \pm 0.14 \text{ and } Rr = 0.29 \pm 0.15 \text{ for flotillin-GFP and stomatin-GFP, respectively})\) (Fig. 7G to I), although some colocalization between NV NS3 and flotillin-GFP in the perinuclear region was also observed (Fig. 7G to I). These results suggest that MNV NS3 may have the propensity to locate to lipid-rich microdomains within the endocytic pathway and again support a difference in the localization of the MNV and NV NS3 proteins.

**Depletion of cellular cholesterol by lovastatin and not methyl-β-cyclodextrin alters NS3 vesicle formation.** To further investigate the relationship between lipids and NS3, cells were transfected with either MNV or NV NS3 and treated with lovastatin (10 \(\mu M\)) or methyl-β-cyclodextrin (MβCD) (10 \(\mu g/ml\)). Lovastatin inhibits the synthesis of new cholesterol within the cell via the inhibition of hydroxyl-methylglutaryl-coenzyme A reductase (HMGCR), whereas treatment with MβCD reduces cellular levels of cholesterol at the plasma membrane via dissolution of lipids through inclusion complexes. Treatment of MNV and NV NS3-transfected cells with lovastatin induced a disruption of the formation of the vesicle structures and dispersion from the perinuclear regions to a more scattered distribution within the cytoplasm (Fig. 8A to C and G to I). In addition, labeling of both the MNV and NV NS3 proteins was observed as smaller vesicles (or aggregations) following lovastatin treatment. In contrast, treatment of MNV and NV NS3-transfected cells with MβCD did not induce any significant visible changes in the NS3 distribution and vesicle structures (Fig. 8D to F and J to L), as the NS3-induced vesicular structures were still clearly visible in treated cells. However, we observed a reduction in the diffuse cytoplasmic NV NS3 labeling signal upon MβCD treatment (Fig. 8J to L).

This result suggests that the formation of NS3-induced vesicles requires active cholesterol synthesis rather than redistribution or sequestration from intracellular sites, including the plasma membrane.

**NS3 vesicular structures fuse together and are highly motile, and their movement is microtubule dependent.** To determine if the MNV and NV NS3 vesicle structures were dependent on microtubules for their localization and movement, we transfected Vero cells with recombinant MNV and NV NS3 cDNA plasmids. At 6 h posttransfection (p.t.), transfected cells were incubated in the presence of medium
containing 10 μM the microtubule-destabilizing agent nocodazole (NOZ) and fixed at 18 h p.t. for IF analysis. As shown in Fig. 9, the induction of vesicular structures and the distribution of the MNV and NV NS3 proteins appeared dispersed throughout the cytoplasm during NOZ treatment (Fig. 9D to F and J to L, respectively). The NS3 vesicles appeared smaller than the large aggregations of NS3 observed in the absence of NOZ.

FIG 7 Both MNV NS3 and NV NS3 vesicular structures colocalize with the lipid markers stomatin-GFP and flotillin-GFP. Vero cells were transfected with either MNV NS3- or NV NS3-containing plasmids, concurrently transfected with either stomatin-GFP or flotillin-GFP, and fixed at 24 h p.t. Colocalization of both the MNV and NV NS3 proteins with stomatin-GFP and flotillin-GFP was observed. MNV NS3 predominantly associated with stomatin-GFP, particularly within the vesicular structures induced in the cytoplasm (n = 29) (D to F), rather than flotillin-GFP (n = 29) (A to C). In contrast, NV NS3 appeared to associate more with flotillin-GFP in the perinuclear region (n = 20) (G to I), although some colocalization was also observed with stomatin-GFP (n = 24) (J to L).
Interestingly, remnants of the vesicular structures could still be observed, yet the majority of the NS3 staining was not confined to these foci, as previously observed. Also notable was that we observed significant colocalization of NV NS3 with H9252-tubulin during NOZ treatment (Rr of 0.50 ± 0.22, compared to an Rr of 0.39 ± 0.13 for MNV NS3) (Fig. 9D to F), suggesting a very tight association of NV NS3 with microtubules.
To further characterize the nature of the NS3 vesicular structures, we utilized a recombinant version of MNV NS3 that was fused to Aequorea coerulescens green fluorescent protein (AcGFP) at its C terminus (see Materials and Methods for vector construction). This allowed us to visualize the movement of the NS3 structures in real time using time-lapse epifluorescence microscopy and video capture. Vero cells were transfected with the MNV NS3-GFP plasmid, and at 12 h p.t., images were collected at intervals of 5 min until 24 h p.t. Over this time period, we observed that the MNV NS3
vesicular structures moved at a rapid pace, particularly in the x and y axes but also in the z axis (Fig. 10A; see also Movie S1 in the supplemental material). Additionally, we observed that the smaller NS3-GFP vesicles often appeared to fuse with one another to generate larger foci (Fig. 10A and Movie S1). Plotting of the movement of individual foci (25 to 225 min) (Fig. 10A) of these vesicles with Imaris (Bitplane) revealed that a majority of the vesicles remained motile throughout the viewing period. Data from the visualization and analysis of this movement are consistent with microtubule-dependent transport.

To further interrogate the role of microtubules in the formation and movement of the NS3 vesicular structures, we transfected Vero cells with the recombinant cDNA plasmid encoding MNV NS3-AcGFP, and at 7.5 h p.t., cells were subsequently incubated with or without NOZ and visualized every 5 min from 8 to 12 h p.t. The movement of the MNV NS3-GFP structures was imaged in real time by using time-lapse epifluorescence microscopy and video capture. As demonstrated in Fig. 10B and Movies S1 and S2 in the supplemental material, movement or trafficking of the MNV NS3-AcGFP vesicular structures was not evident in the NOZ-treated sample, nor was the formation and fusion of larger NS3-AcGFP vesicles observed in the NOZ-treated sample, which we presume to be caused by their lack of movement and also points to a microtubule-dependent trafficking mechanism (Fig. 10).

Microtubule disruption affects the localization of NS3 and dsRNA in MNV-infected cells. To further investigate the effects of NOZ treatment during infection, we performed IF analysis on the distribution of MNV NS3, β-tubulin, and dsRNA in MNV-infected RAW264.7 cells (Fig. 11). Cells were infected only and viewed for the distribution of MNV NS3 (Fig. 11, red) and β-tubulin (Fig. 11A to C, green) or dsRNA (Fig. 11G to I, green). MNV-infected cells were additionally treated with NOZ from 1 h p.i. and viewed for the distribution of MNV NS3 (Fig. 11, red) and β-tubulin (Fig. 11D to F, green) or dsRNA (Fig. 11J to L, green). Although the overall distributions of NS3 and dsRNA were affected by NOZ treatment, the association between both NS3 and β-tubulin ($R_r$ of 0.47 ± 0.14 [untreated] versus $R_r$ of 0.46 ± 0.15 [treated]) and NS3 and dsRNA ($R_r$ of 0.83 ± 0.08 [untreated] versus $R_r$ of 0.74 ± 0.11 [treated]) remained unaltered.

Overall, these results have shown that the vesicular structures induced by both the MNV and NV NS3 proteins share similar properties; they are highly motile, and their movement is microtubule dependent.

DISCUSSION

In this study, we have compared the intracellular localizations of the genogroup I NV and the genogroup V MNV NS3 proteins. Overall, we showed that MNV NS3 is an integral component of the MNV RC and closely associates with the replicative intermediate dsRNA throughout the course of infection (Fig. 1 and 2). We observed that both proteins share the ability to induce vesicular structures in the cytoplasm of transfected cells, an induction that appears to be dependent on both cholesterol and microtubules. Notably, both the NV and MNV NS3 proteins colocalize with microtubules and, to a lesser extent, with cholesterol, implying that their roles during their respective virus replication cycles are to recruit and/or utilize these host components to facilitate efficient virus replication. These observations are consistent with previous studies by our group showing a reduction in viral titers following nocodazole treatment (24). Interestingly, although we observed many similarities in the subcellular localizations of the NV and MNV NS3 proteins, we observed a greater proportion of NV NS3 colocalizing with the Golgi apparatus and microtubules than for MNV NS3, which exhibited more extensive localization with cholesterol-rich membranes. All of these aspects are extremely relevant to MNV replication, as we have previously shown that the MNV replication complex is housed within membranes derived from the endocytic pathway and is centered on or around the microtubule organizing center (MTOC) and adjacent to the Golgi apparatus. Furthermore, cholesterol has been observed to play a role in the replication and entry of NV and MNV, respectively (27–29). Thus, the recruitment and
FIG 10 (A) MNV NS3-GFP vesicular structures display regular movement upon transfection. Vero cells were transfected with an MNV NS3-GFP plasmid and viewed over a 4-h period at 8 to 12 h p.t. NS3-GFP-transfected cells show movement of NS3-GFP vesicles throughout the cell. (A) Still images show the movement of NS3-GFP over time (images shown were those collected every 25 min). The time-lapse merge panel shows tracking over an ∼4-h period at 8 to 12 h p.t., showing highly motile vesicle structures (with images being taken every 5 min) (see Movie S1 in the supplemental material). (B) MNV NS3-GFP vesicular structures show a lack of movement upon NOZ treatment. Vero cells were transfected with an MNV NS3-GFP recombinant cDNA plasmid, and at 7.5 h p.t., cells were subsequently incubated with medium containing 10 μM NOZ and visualized from 8 to 12 h p.t. Still images show the movement of NS3-GFP over time (images shown were those collected every 25 min). The time-lapse merge panel shows tracking over an ∼4-h period at 8 to 12 h p.t. and was analyzed by using IMARIS software, showing immotile GFP structures. NOZ-treated NS3-GFP-transfected cells show a lack of vesicle movement (with images being taken every 5 min) (Movie S2).
potential redistribution of cholesterol may be required to facilitate these events in the virus life cycle.

In addition, we observed that the vesicle structures induced during MNV NS3-GFP expression displayed rapid movement when viewed via time-lapse epifluorescence microscopy. Throughout the course of expression, smaller individual vesicles appeared to additionally aggregate into larger foci, implying that the vesicles are also fusogenic, with the potential high cholesterol content contributing to the observed membrane dynamics. Our previously reported observations identified a role for microtubules in the

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FIG 11 IF analysis showing the effect of NOZ treatment on the distribution of MNV NS3, β-tubulin, and dsRNA in infected RAW264.7 cells. Cells were infected only and viewed for the distribution of MNV NS3 (red) and β-tubulin (green) (A to C) or dsRNA (green) (G to I). MNV-infected cells were additionally treated with NOZ at 1 h p.i. and viewed for the distribution of MNV NS3 (red) and β-tubulin (green) (D to F) or dsRNA (green) (J to L). The distributions of NS3 and dsRNA are affected by NOZ treatment, and the associations between NS3 and β-tubulin (Rr of 0.47 ± 0.14 [untreated] [n = 21] versus Rr of 0.46 ± 0.15 [treated] [n = 23]) and between NS3 and dsRNA (Rr of 0.83 ± 0.08 [untreated] [n = 20] versus Rr of 0.74 ± 0.11 [treated] [n = 19]) are unaltered.
establishment and positioning of the MNV RC within cells (24), and this study indicates that NS3 may facilitate the transport of the vesicles along microtubules during infection. Our current research is focused on investigating the role of cholesterol and lipid transport during MNV replication, and these aspects are under investigation.

Our studies utilizing NOZ treatment also demonstrated that the trafficking of MNV NS3 is highly motile and facilitates the coalescence of the NS3 vesicular structures to form larger membrane-rich aggregates. The use of the microtubular network as a means of trafficking viral components is not uncommon for viruses. Several RNA viruses, such as hepatitis C virus (HCV), poliovirus, and rabies virus, have demonstrated the use of microtubules in different ways to facilitate their replication (30–32). In the case of MNV, we have demonstrated that the abrogation of microtubules via NOZ treatment leads to a complete loss of vesicular movement and also the loss of the ability to form larger aggregations of NS3 than those in untreated MNV NS3-GFP-transfected cells. The exact purpose of these vesicles still remains unclear, although their regular movement and vesicular shape would lend themselves to a trafficking or recruitment mechanism to aid viral replication. Previously, we observed that NOZ treatment of MNV-infected cells resulted in the dispersion of the MNV RC (24); thus, NS3 may be the viral tether that traffics and positions the MNV RC proximal to the MTOC.

The colocalization of NS3 with cholesterol and lipid host cell components also implicates a role for this protein in the recruitment of cellular membranes required for viral RC formation, possibly via facilitating membrane curvature. We also observed some colocalization of both the NV and MNV NS3 proteins with stomatin, although MNV NS3 displayed a greater association, which has a strong association with the phospholipid bilayer and actin within detergent-resistant membrane domains (33). This suggests that NS3 may promote or disable the scaffold of these ordered domains, a hypothesis that we are currently investigating.

We also observed an association of MNV NS3 and, to some extent, NV NS3 with flotillin, which is noted for forming microdomains in the plasma membrane and providing a scaffold for membrane rafts (34) and also for forming microdomains in the endocytic pathway, where it is known to localize to early/late endosomes (26). Although we did not detect a great deal (if any) NS3 colocalization with early endosomes, further work is focused on identifying and characterizing the role of other cellular components of the endocytic pathways during NoV replication, as these components are known to be utilized by many viruses to facilitate their replication. The observed association of NoV replicative proteins with lipid-rich domains is in line with observations of many other positive-strand RNA ([+]-RNA) viruses that enrobe their RC in cellular membranes to avoid host cell detection and create a microenvironment to help facilitate their own replication. Further studies with a plethora of endosomal and other cytoplasmic markers, and more robust cell culture methods, may help to elucidate other components involved with NS3 and NoV replication.

This study has shown that the previously uncharacterized NS3 protein is highly motile upon transfection; unfortunately, we are unable to study it in its “native” state due to the inability to generate a tagged NS3 protein with infectious clones thus far. We have also demonstrated its association with lipids and cellular components, particularly those of the Golgi apparatus and microtubules. There also appear to be subtle differences between the localization of MNV NS3 and that of NV NS3, although both proteins appear to be highly motile and dependent on intact microtubules to facilitate their movement. Our results suggest that NS3 may act as a tether on microtubules and recruit lipids (particularly cholesterol) to drive the biogenesis of the NoV RC.

MATERIALS AND METHODS

Viruses and cells. RAW264.7 and VeroC1008 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Australia) supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland), 2 mM GlutaMAX (Gibco BRL), and 100 U/ml penicillin–100 μg/ml streptomycin (Gibco BRL). RAW264.7 cells were infected with MNV strain CW1 (GenBank accession no. DQ285629.1) at a multiplicity of infection (MOI) of 5, as previously described (17, 25), and infected cells were maintained...
in DMEM containing 10% fetal calf serum, 2 mM GlutaMAX, and 100 U/ml penicillin–100 µg/ml streptomycin.

Reagents. MNV-specific guinea pig polyclonal antibodies were described previously (35). Anti-rabbit-, anti-guinea pig-, and anti-mouse-specific IgG Alexa Fluor 488, 549, and 680 were purchased from Molecular Probes (Invitrogen, Leiden, The Netherlands). Anti-dsRNA (clone J2) was purchased from English & Scientific Consulting BT (Hungary). Protein A-gold (5, 10, and 15 nm) was purchased from Protein Technologies (ProSciTech, Kirwan, Australia). Lovastatin, methyl-β-cyclodextrin, and nocodazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 reagent was purchased from Invitrogen. Recombinant CDNA plasmids expressing enhanced GFP (eGFP)-tagged erlin-1, erlin-2, prohibitin, stomatin, and flotillin were kindly provided by Stephen Robbins (University of Calgary, Canada) (36).

Construction of pcDNA3.1-MNV-NS3-GFP and pcDNA3.1-NV-NS3-cmyc. For the construction of pcDNA3.1-MNV-NS3-GFP, which expresses a C-terminally fused NS3-AcGFP protein, a DNA fragment corresponding to the NS3 gene was amplified by PCR from p20.3 DNA (37) using the Elongase amplification system (Invitrogen), forward primer frwNS3.GFP (5′-TATATATTTACTCGAGCCACCAGTGATGGGCTCTCCTGAGATTCTGAGATCTCTGGAGCGAATCATCATGGCGCTCATGGACAAGACCGACACCTC-3′), and reverse primer revNS3.GFP (5′-TAAATTGATTGCAGCTGTTGACCACC-3′). Primer frwNS3.GFP was designed to introduce an XhoI restriction site (underlined) and the first AUG codon (in boldface type) upstream of the NS3 sequence. Reverse primer revNS3.GFP contained a sequence complementary to the 3′ end of the NS3 gene and an engineered BamHI site (underlined). The gel-purified PCR fragment was cleaved with XhoI and BamHI and ligated into the compatible sites of the pAcGFP1-N1 vector (Clontech). The resulting plasmid contained the MNV-1 NS3 sequence fused at the C terminus to the AcGFP protein under the control of a cytomegalovirus (CMV) promoter.

The construction of pcDNA3.1-NV-NS3-cmyc was performed essentially as described above; however, a DNA fragment corresponding to the NV NS3 gene was amplified by PCR from phV-Neo (38) using Pfu polymerase (Promega), forward primer frwHuNS3 (5′-GCCACCATGGGACCCGAGGACTTG-3′), and reverse primer revHuNS3 (5′-TATATATTTACTCGAGCCACCAGTGATGGGCTCTCCTGAGATTCTGAGATCTCTGGAGCGAATCATCATGGCGCTCATGGACAAGACCGACACCTC-3′). Primer frwHuNS3 was designed to introduce an engineered BamHI site (underlined) and the first AUG codon (in boldface type) upstream of the NS3 sequence. Reverse primer revHuNS3 contained a sequence complementary to the 3′ end of the NS3 gene and an engineered BamHI site (underlined). The gel-purified PCR fragment was cleaved with XhoI and ligated into the pcDNA3.1+ vector (Life Technologies) digested with XhoI and EcoRV. The resulting plasmid contained the NV NS3 sequence fused at the C terminus to the c-myc protein under the control of a CMV promoter.

Immunofluorescence analysis. RAW264.7 cell monolayers on coverslips were infected with MNV at an MOI of 5 and incubated at 37°C for different time periods postinfection. VeroC1008 cell monolayers on coverslips were transfected with pcDNA3.1 fusion plasmids and Lipofectamine 2000 as specified by the manufacturer and incubated at 37°C for the specified periods posttransfection. The cells were subsequently washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (ProSciTech, Kirwan, Australia) for 10 min at 20°C, and permeabilized with 0.1% (vol/vol) Triton X-100 in 4% (vol/vol) paraformaldehyde for an additional 10 min at 20°C; the cells were then processed for IF analysis, as previously described (23). After a final wash with PBS, the coverslips were drained and mounted onto glass slides with quick-dry Ultramount mounting medium (United Biosciences, Brisbane, Australia) before visualization on a Zeiss LSM 710 confocal microscope. Images were collected from a single plane with a 60× objective and a numerical aperture of 1.4. The images were subsequently collated by using Zeiss Zen and Adobe Photoshop software, with all images being taken under identical acquisition settings. Colocalization was determined by Pearson’s coefficient using ImageJ JACoP plug-in software. A coefficient value exceeding 0.500 is considered colocalization.

Time-lapse epifluorescence microscopy. VeroC1008 cell monolayers on Fluorodish dishes (World Precision Instruments, USA) were transfected with the pcDNA3.1 fusion plasmids and Lipofectamine 2000, as specified by the manufacturer, and incubated at 37°C for the specified periods posttransfection. The cells were subsequently left untreated or treated with 10 µM nocodazole for 30 min at 37°C prior to viewing on a Zeiss LSM 700 confocal microscope for the specified time periods. Images were collected and collated by using Zeiss Zen, Bitplane Imaris, and Adobe Photoshop software.

Electron microscopy. Methods for cryofixation, preparation of cryosections, and immunolabeling were described previously (24, 27, 28). Briefly, MNV-infected cells were fixed with 4% paraformaldehyde–0.1% glutaraldehyde (ProSciTech, Kirwan, Australia) in PBS on ice for 60 min and subsequently embedded within a 10% gelatin block before postfixing with 1% paraformaldehyde in PBS. The sample blocks were infused with a mixture of sucrose and polyvinylpyrrolidone and mounted onto cryostubs (Leica) for cryosectioning. Ultrathin cryosections (∼55 nm) were cut with a Diatome Cryo-P diamond knife and retrieved from the cryochamber with a droplet of a 14:1 dilution of 2.3 M sucrose–2% methyl cellulose. The recovered sections were subsequently immunolabeled with the appropriate antibodies and protein A-gold and contrasted with 1.8 M methyl cellulose containing 0.4% uranyl acetate. The sections were then viewed on a JOEL 1010 transmission electron microscope, and images were captured with a MegaView III side-mounted charge-coupled-device (CCD) camera (Soft Imaging Systems, USA) and processed with Adobe Photoshop.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.02138-16.
ACKNOWLEDGMENTS

We thank Stephen Robbins (University of Calgary, Canada) for providing the stomin and flotillin expression plasmids. We acknowledge the facilities and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, University of Queensland, and the University of Melbourne’s Biological Optical Microscopy Platform (BOMP).

Project grants (no. 1010327 and 1083139) awarded to J.M.M. and P.A.W. from the National Health and Medical Council of Australia supported this research.

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