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A Single-Amino-Acid Change in Murine Norovirus NS1/2 Is Sufficient for Colonic Tropism and Persistence

Timothy J. Nice, David W. Strong, Broc T. McCune, Calvin S. Pohl, and Herbert W. Virgin

Human noroviruses (HuNoVs) are the leading cause of non-bacterial gastroenteritis and are a significant public health problem worldwide, causing both outbreaks and sporadic cases of acute gastrointestinal disease (reviewed in references 1, 2, and 3). There is no evidence for an animal reservoir of HuNoV, suggesting that asymptomatic shedding within the human population initiates symptomatic infection. Indeed, PCR-based detection has shown that HuNoVs are present in asymptomatic individuals within a variety of communities and ranges in prevalence from 3 to 13% (4–7). Furthermore, experimental infection of volunteers revealed that some individuals continue to shed virus for up to weeks after the resolution of symptoms (8), and there are examples where outbreaks likely originated with asymptomatic HuNoV shedders (9–11). Taken together, these studies demonstrate that asymptomatic and persistent infections are an important aspect of HuNoV biology. However, the molecular mechanisms of NoV persistence remain incompletely understood.

Noroviruses (NoVs) are positive-sense, single-stranded RNA viruses in the calicivirus family. They are classified into five genetic groups (genogroups), with HuNoVs in genogroups I, II, and IV (GI, GII, and GIV, respectively), bovine noroviruses in GIII, and murine noroviruses (MNV) in GV (12). The NoV genome is ~7.5 kb in length and encodes three open reading frames (ORFs) (13), with an additional ORF4 in MNV (14, 15). ORF1 encodes the NoV nonstructural polyprotein which is cleaved into six mature peptides (NS1/2 and NS3 through NS7) by the ORF1-encoded viral protease NS6 (16–18). ORF2 encodes the major structural protein (VP1), which self assembles into the icosahedral viral capsid (19), and ORF3 encodes a minor structural protein (VP2) (20). MNV ORF4 is an alternate reading frame within ORF2 and encodes virulence factor 1 (VFI) (14).

NS1/2 is the most N-terminal viral protein encoded by ORF1. The cleavage site between NS1/2 and NS3 has been confirmed by mutagenesis studies, and the position of this cleavage is conserved among all NoVs. All genera of caliciviruses other than NoVs have an additional viral protease cleavage site within the region homologous to NS1/2, resulting in two mature viral proteins derived from this region (21–23). It is possible that some NoVs have lost this cleavage event, but there is one report of additional cleavage of the human NS1/2 protein in cells transfected with HuNoV ORF1 (24), and MNV NS1/2 can be cleaved by caspase 3 (18). NS1/2 localizes to organelles in the secretory pathway (25, 26), and HuNoV NS1/2 disrupts protein secretion in transfected cells (25, 27). However, very little is known about the function of NS1/2 in NoV replication or pathogenesis.

Similar to HuNoV, MNV establishes persistent asymptomatic infection (15, 28) and therefore represents an ideal model system in which to study NoV persistence. While most strains of MNV persist, the strain MNV1 is cleared. MNV1 was identified as a cause of lethality in RAG/STAT1 knockout mice (29), and it was passaged intracranially in these mice until a culture system was established (30). While MNV1 rapidly kills STAT1 knockout mice, it persists avirulently in RAG knockout mice and is cleared from immune-sufficient mice (29, 31). Subsequently, many closely related strains of MNV isolated from stool samples were shown to persist in immune-sufficient mice (15, 28). One of these persistent stool isolates, CR6, has been shown to be avirulent in STAT1 knockout mice, demonstrating a second phenotypic distinction from MNV1 (31). A reverse genetics system for MNV exists (32), making phenotypic analyses of MNV1 and persistent strains possible (14, 31, 33, 34). The increased virulence of MNV1 has been linked to the protruding domain of the capsid gene (31, 33), but the gene responsible for MNV persistence has not been identified.

Here, we used recombinant cDNA clones of the nonpersistent strain MNV1.CW3 (subsequently referred to as CW3) and persistent strain CR6 to identify a genetic determinant of persistence. In contrast to virulence in STAT1 knockout mice (31), reciprocal
replacement of the capsid gene did not alter persistence. Instead, the NS1/2 gene of CR6 enabled persistence of CW3. Within NS1/2, we further narrowed the determinant of persistence to the 5′ domain and to a single amino acid within this domain. Furthermore, this same determinant increased growth of CW3 in the proximal colon, a major reservoir of CR6 persistence. This is the first demonstrated function for NS1/2 during infection and the first report of a protein other than the MNV capsid to alter viral tropism. This is also the first study identifying a genetic determinant for enteric viral persistence.

MATERIALS AND METHODS

Generation of recombinant MNV stocks. Construction of molecular clones of CW3 (EF014462.1) and CR6 (IQ237823) and the strategy for cloning of viral gene substitutions have been previously described (31). Primers used for the substitutions of NS1/2 5′ and 3′ domains were NS1/2 forward (5′-GTGAAATGAGGATGGCCGGCAAGCCTCCTCTTCTGC-3′), 5′ NS1/2 reverse (5′-GCGTCCTCCGATCGGAGGGCGC-3′), 3′ NS1/2 forward (5′-GGGGCCCTCCATGCGGAGGAC-3′), and CR6 3′ NS1/2 reverse (5′-AGGGCGGCGGCTTCCGCTGCA-3′).

Generation of viral stocks from plasmids carrying MNV was performed as described previously (31) with minor modifications. Briefly, plasmids were transfected into 293T cells with TransIT-LT1 (Mirus Bio, Madison, WI), and virus was recovered 48 h posttransfection by freezing and thawing. Clarified 293T supernatants were passaged one to two times serially passaged MNV isolates.

Mice and infections. C57BL/6 mice (stock number 000664) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at Washington University School of Medicine under specific-pathogen-free conditions (35) according to university guidelines. Cages of male or female mice were inoculated with virus at 6 to 8 weeks of age by the oral route in a volume of 25 to 35 μL. A dose of 3 × 10⁶ PFU was used for all experiments, with one exception (1 × 10⁶ PFU; see Fig. 5E).

Stool and tissues were harvested into 2-ml tubes (Sarstedt, Germany) with 1-mM diameter zirconia/silica beads (Biospec, Bartlesville, OK). Tissues were flash frozen in a batch of ethanol and dry ice and either processed on the same day or stored at −80°C.

Plaque assay. Culture of RAW264.7 cells and the plaque assay for determining the titer of virus stocks was performed as described previously (31). Growth curve plaque assays utilized suspension-adapted RAW264.7 cells maintained in minimal essential medium (MEM) supplemented with 10% bovine serum, 1% nonessential amino acids, 100 μg/ml penicillin-streptomycin, 2 mM t-glutamine, 10 mM HEPES, and 0.025% sodium bicarbonate. RAW264.7 cells were plated on 6-well plates at a density of 2 × 10⁵ cells per well. Sixteen to 24 h later, medium was removed and 10-fold serial dilutions of virus inoculum were added at 500 μL per well. Plates were incubated for 1 h at room temperature on a rocking platform before removing the inoculum and overlaying the cells with 2 ml per well of 1% methylcellulose in MEM supplemented with 10% fetal calf serum, 100 μg/ml penicillin-streptomycin, 2 mM t-glutamine, 10 mM HEPES. Plates were incubated at 37°C for 3 to 4 days, and plaques were visualized by staining with 0.2% crystal violet and 20% ethanol.

Quantitative reverse transcription-PCR. RNA from stool was isolated using either an RNaseasy Mini Kit (Qiagen, Valencia, CA) or Quick-RNA Mini Prep (ZymoResearch, Irvine, CA) kit. RNA from tissues was isolated using TRIzol (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Five μL of RNA from stool or 1 μL of RNA from tissue was used as a template for cDNA synthesis with the ImPromII reverse transcriptase system (Promega, Madison, WI). When evident in the melting curve analysis, DNA contamination was removed using the Dnafree kit (Life Technologies).
that shedding of CW3-NS1/2 was significantly greater than that of all other single-gene substitutions (Fig. 2C). To confirm and quantify the result of this screen, we generated two additional viral stocks of CW3-NS1/2 and tested all three stocks for persistence. All three biological replicates of CW3-NS1/2 were detected in stool at 35 days postinfection, and the quantity of MNV genomes was not significantly different from that of CR6 (Fig. 2D). The reciprocal insertion of NS1/2 from CW3 into CR6 did not generate virus and could not be tested for persistence. These findings indicate that the NS1/2 gene of CR6 is sufficient to enable CW3 persistence.

The NS1/2 gene encodes a 3’ domain conserved among NoVs (39) and a 5’ domain that is more divergent. To narrow the persistence determinant to a single domain, we generated CW3 with the 5’ domain of CR6 NS1/2 (CW3-5’ NS1/2) and CW3 with the 3’ domain of CR6 NS1/2 (CW3-3’ NS1/2) as depicted in Fig. 2A. While persistent CW3-3’ NS1/2 shedding was not detected, CW3-5’ NS1/2 shedding was indistinguishable from that of CR6 (Fig. 2E). To test whether the 5’ NS1/2 domain was also necessary for persistence, we generated CR6 with a CW3 substitution in the 5’ domain of the NS1/2 gene (CR6-5’ NS1/2). Persistent shedding of CR6-5’ NS1/2 was significantly lower than that of CR6 and not significantly different from that of CW3 (Fig. 2E). Taken together, these data demonstrate that the 5’ domain of the NS1/2 gene is necessary and sufficient for MNV persistence.

**A change of aspartic acid to glutamic acid at amino acid 94 of NS1/2 is sufficient for persistence.** CR6 is one of seven persistent strains identified to date (15, 28). To test whether CW3 has unique polymorphisms that correlate with its inability to persist, we analyzed the 5’ domain of NS1/2 from CW3 and the seven persistent MNV strains (Fig. 3A). Amino acid alignment showed that only a single residue was both unique to CW3 and conserved among the persistent strains of MNV. This difference was a conservative change at amino acid 94, with glutamic acid present in persistent strains and aspartic acid present in CW3 (Fig. 3A). To assess whether this difference was involved in determining persistence, we generated a mutant of CW3 that encoded the conserved glutamic acid at position 94 of NS1/2 (CW3E94G). Additionally, we made the reciprocal mutation in CR6, changing the conserved glutamic acid to an aspartic acid (CR6D94E). CR6 persistent shedding was not significantly changed by the E94D mutation (Fig. 3B), and sequencing demonstrated no reversion to E94 in vivo (data not shown). In contrast, CW3 shedding was significantly increased by the D94E mutation to levels that were not significantly different from those of CR6 (Fig. 3B). Furthermore, a D94E mutation in the nonpersistent CR6-5’ NS1/2 chimera rescued its ability to persist. This striking result demonstrates that glutamic acid at amino acid 94 of NS1/2 is sufficient, though not necessary, for persistence.

**Persistent and nonpersistent viruses have similar in vitro growth.** The association of NS1/2 with cellular membranes and persistent and nonpersistent NoV strains is sufficient to enable CW3 persistence.
the viral replication complex (40) suggested that it has a role in viral replication that is altered by the persistence determinant. To test this hypothesis, we compared *in vitro* growth of the persistent viruses CW3D94E and CR6 to that of the nonpersistent viruses CW3 and CR6-5’ NS1/2. Infection of RAW264.7 cells at an MOI of 5 revealed no significant overall difference in growth at any time postinfection between any pair of persistent and nonpersistent viruses (Fig. 4). At an MOI of 0.05 there were also no significant differences other than at 4 h postinfection between CR6 and CR6-5’ NS1/2 (P < 0.05) (Fig. 4). However, there were no differences between CW3 and CW3D94E at any time that would indicate a role for this early growth difference in determining the persistence phenotype. Together, these data show that the persistence determinant has little to no effect on *in vitro* growth in RAW264.7 cells.

CR6 grows better than CW3 in the proximal colon. While no growth difference was seen in RAW264.7 cells, the significant difference in fecal shedding between CW3 and CR6 on day seven (Fig. 1) suggested that there was a difference in enteric growth during the early stages of infection *in vivo*. To identify the preferential enteric site(s) of CR6 replication, relative levels of persistent CR6 were measured in anatomically distinct regions along the gastrointestinal (GI) tract. CR6 was detected in the proximal colon at levels significantly greater than those of all other GI regions with the exception of the cecum (Fig. 5A). This observation was supported by recent publications which also predominantly detected persistent MNV strains in the cecum and/or proximal colon (34, 41).

Therefore, the proximal colon was included as the representative enteric tissue in a comparison between CR6 and CW3 growth during acute infection. Spleen and mesenteric lymph node (MLN) were also included in the comparison because MNV grows in macrophages and dendritic cells (DCs) and can spread to these tissues. The peak titer of both strains in all tissues was reached by day seven (Fig. 5B). In the proximal colon, CR6 grew significantly better than CW3 from day 3 through 14, and CW3 was only minimally detected at any time point (Fig. 5B, left). In the spleen, CW3 grew significantly better than CR6 between days 3 and 7 but was cleared by day 14 (Fig. 5B, middle). This difference in the spleen was in accordance with recent publications demonstrating the
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ability of CW3 and MNV1 to spread systemically (31, 42) and indicates that the failure of CW3 to persist in the intestine is not due to a failure to establish infection. Both strains were detected in the MLN with no significant difference before day 14 when CW3 was cleared (Fig. 5B, right). Taken together, these data demonstrate significant differences in tissue tropism between CW3 and CR6 in the spleen and proximal colon, the latter being a major site of persistence.

NS1/2 amino acid 94, rather than capsid, determines growth in the proximal colon. Other studies have mapped MNV1 tropism changes to VP1 (31, 34), so we used CW3-VP1 and CR6-VP1 to test the role of VP1 in determining the tropism differences revealed in Fig. 5B. Growth of CW3-VP1 in the proximal colon 7 days after infection was not significantly higher than that of CW3, and growth of CR6-VP1 was not significantly lower than that of CR6 (Fig. 5C, left). In the spleen, growth of CW3-VP1 was significantly lower than that of CW3 and not significantly different from that of CR6; CR6-VP1 growth was significantly higher than that of CR6 and not significantly different from that of CW3 (Fig. 5C, middle). Therefore, VP1 determines CW3 tropism for the spleen but not CR6 tropism for the proximal colon.

In parallel, we tested day seven growth of NS1/2 mutants to address the effect of the persistence determinant on tropism during acute infection. The persistent viruses CW3-NS1/2 and CW3<sup>D94E</sup> grew in both the spleen and proximal colon at levels significantly higher than that of CR6 (Fig. 5C, right). In the proximal colon, growth of CW3-NS1/2 was not significantly different from that of CW3 and CR6 (Fig. 5C, middle). Therefore, NS1/2 mitigates the tropism differences revealed.}

FIG 5 NS1/2 and amino acid 94 determine growth in the proximal colon, a major reservoir of persistent MNV. (A to E) Tissues were harvested from MNV-infected mice, and the genome copy number was measured by quantitative PCR. (A) Detection of CR6 in regions of the gastrointestinal tract 35 days postinfection. Data are combined from two experiments for a total of five mice. (B) A kinetic comparison of titers in spleen, proximal colon, and MLN following infection with either CW3 or CR6. Data are combined from three experiments for a total of nine mice per data point. (C and D) Detection of the indicated viruses in tissues at day seven postinfection. Data are combined from at least two experiments for a total of eight mice for each data point. CW3 and CR6 data from infection with either CW3 or CR6. Data are combined from three experiments for a total of nine mice per data point. (C and D) Detection of the indicated viruses in tissues at day seven postinfection. Data are combined from at least two experiments for a total of at least eight mice for each data point. (E) Detection of CR6-VP1 in the indicated tissues at 3 or 35 days postinfection with <sup>10</sup> PFU. Data are combined from two experiments for a total of six mice per data point.

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increased growth in the colon without a significant change in the spleen (Fig. 5D, CR6-5’ NS1/2D94E). Therefore, D94E is sufficient for growth in the proximal colon, but not in the spleen, during acute infection.

MNV persistence is restricted to the intestine. The correlation between persistent fecal shedding and growth in the proximal colon during acute infection (Fig. 2E, 3B, and 5D) raised the possibility that MNV persistence is an intestine-specific phenotype. CR6 did not persist outside the intestine and associated lymphoid tissue, but its replication during acute infection was also restricted to these same sites (Fig. 5B and data not shown). Therefore, we took advantage of the expanded tropism of CR6-VP1 during acute infection to test this hypothesis. On day three postinfection with 1 × 10⁷ PFU, CR6-VP1 had grown in both enteric tissues (Fig. 5E, proximal colon and MLN) as well as systemic tissues (Fig. 5E, spleen and liver). However, while CR6-VP1 was still present in the intestine on day 35, it was reduced to the limit of detection in the spleen and liver (Fig. 5E). This result shows that MNV persistence is specific to the intestine and provides a possible explanation for the correlation between enteric growth during acute infection and persistence.

DISCUSSION

The findings presented here demonstrate that the 5’ domain of NS1/2 is necessary and sufficient for MNV persistence. Furthermore, a single conservative change from aspartic acid to glutamic acid at amino acid 94 within this domain is sufficient for CW3 to persist. This D94E change also determines tropism for the proximal colon but not the spleen during acute infection. Notably, the proximal colon is a major site of MNV persistence, while the spleen does not support persistent replication.

MNV as a model of persistent enteric viral infection. There is no clear animal reservoir for HuNoV, suggesting that it has other adaptations for viral maintenance between outbreaks. Asymptomatic and persistent shedding is likely to be one way that HuNoVs are maintained (4–7, 11), yet many questions remain about the mechanism of NoV persistence. Furthermore, while asymptomatic shedding has been demonstrated for many enteric viruses (43–45), a tractable model has not been established in which to study enteric persistence. We have characterized a novel model of persistence and identified the first genetic determinant of enteric persistence. Future study using the MNV model system has the exciting potential to reveal general principles of enteric persistence and host-pathogen interactions in the intestine.

Relationship between enteric growth and persistence. We have shown a correlation of persistent shedding with growth in the colon, but not the spleen, during acute infection (Fig. 2E, 3B, and 5D). This correlation suggests that efficient colonic replication is required for persistence. Support for this idea comes from the fact that CR6-VP1, which has broad early tissue tropism, persists in the colon and MLN but not systemic sites (Fig. 5E). Therefore, efficient early replication in colon may correlate with persistence, because MNV persistence is restricted to enteric tissues. The importance of enteric growth may reflect a requirement for fecal shedding and reexposure, replication in enteric cell types, or induction of enteric immune responses.

CR6 infection results in increased fecal shedding compared to CW3 (Fig. 1). Consequently, there are likely an increased number of viros in the environment and more frequent reexposure of the intestinal epithelium. Antigenic drift within a cage may increase exposure to viral mutations that outpace immune elimination and result in persistence. Sequencing of CW3D94E from persistently infected mice reveals that mutations in the protruding domain of the capsid are in fact present (T. J. Nice, data not shown). Furthermore, there is evidence that this type of selection takes place in human populations and is important for persistence of HuNoV (46). It is notable that infection with 3 × 10⁷ PFU of CW3, rather than the 3 × 10⁴ PFU used here, results in appreciable shedding in the stool during acute infection and yet is ultimately cleared (35). Therefore, while it is still possible that CR6 and other persistent strains have a unique ability to promote antigenic drift, reexposure to shed MNV per se is not likely to be sufficient to explain the capacity to persist.

A separate, but not mutually exclusive, hypothesis to explain the importance of enteric growth is that characteristics of the immune system unique to this anatomical location are critical for persistence. The mammalian mucosal immune system has evolved in proximity to abundant commensal microbes and has a unique requirement for regulatory mechanisms that prevent excessive inflammatory responses to these microbes (47–51). These mechanisms include both intestine-specific differentiation programs for innate leukocytes (52, 53) and the related ability to generate regulatory T cell responses (54). An analogous regulatory immune response may be required for tolerance of persistent MNV in the intestine. Alternatively, because MNV is known to replicate in macrophages and dendritic cells (30), the unique differentiation states of these cell types in the intestine may provide cell-intrinsic growth requirements for persistent replication. Clarification of the anatomical requirements for MNV persistence will provide complementary insights into molecular studies of NS1/2 in establishing the mechanism of persistence in this novel model system.

Potential mechanisms of NS1/2 in persistence. Studies implicating NS1/2 in membrane reorganization and formation of the replication complex (27, 40) raise the possibility that NS1/2 determines persistence through changes in MNV replication. We found no evidence for an in vitro replication function of the NS1/2 persistence determinant in RAW264.7 cells (Fig. 4). However, its role in determining growth in the proximal colon during acute infection (Fig. 5D) means that NS1/2 may have functions specific to a colonic cell type. Therefore, a role for NS1/2 in cell type-specific viral replication remains a possibility, and identification of the colonic cell types in which MNV persists will be necessary to address this possibility experimentally.

While there are multiple potential mechanisms for persistence, all persistent viruses share the capacity to prevent clearance by the immune system. Strategies for preventing immune elimination include inhibition of antigen presentation or innate immune signaling, evasion of adaptive immunity through mutation of immune-dominant epitopes, and infection of an immune-privileged site (55–57). Therefore, potential immune-modulatory functions of NS1/2 must also be considered in addition to basic replicative functions. Humoral immunity, cellular immunity, and innate immunity cooperate to clear infection with MNV1 (29, 37, 38), so NS1/2-mediated evasion of any of these immune components could account for its role in persistence. Identification of NS1/2 structures and interacting partners will be required to generate more detailed hypotheses about a role for NS1/2 in immune modulation.

Domains of NS1/2. The NS1/2 caspase cleavage sites (18) have
been used by us and others (58) to define NS1/2 domains. The region 5′ of the caspase cleavage site, which contains the amino acid 94 persistence determinant, is poorly conserved among NoVs and is also poorly conserved within genogroups. The region 3′ of the caspase cleavage site, however, is conserved among NoVs and is bioinformatically predicted to fold into a permuted papain-like fold (39). Characteristic of this fold, there are conserved cysteine, histidine, and histidine/asparagine residues predicted to form a catalytic triad. Supportive evidence for a catalytic function comes from a study of a picornaviral ortholog, where mutation of the conserved cysteine was found to block positive-strand RNA synthesis in an in vitro replication system (59). Similarly, mutation of the conserved cysteine or histidine in MNV prevents recovery of plaquelike virus from the reverse genetics system (Nice, unpublished). Whether the persistence determinant in the 5′ region is related to a hypothetical catalytic fold of the 3′ domain requires further biochemical and functional studies.

We find that the 5′ NS1/2 domain determines persistence and colonic growth of both CW3 and CR6 (Fig. 2E and 5D), but amino acid 94 determines persistence of CW3 only (Fig. 3B). This suggests that amino acid 94 interacts with CW3-specific sequences. The fact that the D94E mutation can increase colonic growth of both CW3 and CR6-5′ NS1/2 (CW3D94E and CR6-5′ NS1/2D94E in Fig. 5D) suggests that the CW3-specific interaction of amino acid 94 is within the 5′ domain. While these data do not preclude an additional role for strain-independent intergenic interections of the entire 5′ NS1/2 domain, they support the hypothesis that discrete domains exist within NS1/2. The separation of these domains by caspase 3 cleavage and the demonstrated induction of apoptosis during MNV infection (18, 60) raises the intriguing possibility that the MNV NS1/2 5′ domain acts independently of the 3′ domain to determine persistence and colonic tropism.

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