Protruding Domain of Capsid Protein Is Necessary and Sufficient To Determine Murine Norovirus Replication and Pathogenesis *In Vivo*

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Human noroviruses (HuNoVs) are the major cause of epidemic, nonbacterial gastroenteritis worldwide. Due to the lack of a tractable model system and the inability to grow HuNoVs in cell culture, factors required for the norovirus (NoV) life cycle and pathogenesis in the host remain largely unknown. The discovery of murine norovirus (MNV) and the development of reverse-genetics systems for this virus provide an opportunity to study these aspects of NoV infection *in vitro* and *in vivo*. Previous studies identified a single amino acid at residue 296 in the protruding (P) domain of the capsid protein that is responsible for determining the virulence of the MNV clone MNV1.CW1 in 12956/SvEv background STAT1-deficient (STAT1^{-/-}) mice. In this report, we identified and characterized another determinant of lethality in the P domain that is necessary and sufficient to determine the replication and pathogenesis of the MNV clones MNV1.CW3 and CR6.STL1 in C57BL/6 background STAT1^{-/-} mice. Furthermore, we describe how the role of residue 296 in MNV virulence differs between STAT1^{-/-} mouse strains. We also describe potential interactions between subdomains of the P domain, as well as between other virus elements, which facilitate recovery of MNV using a reverse-genetics system.

Human noroviruses (HuNoVs) are the primary cause of epidemic, nonbacterial gastroenteritis worldwide (17, 46). HuNoVs are genetically diverse, with >45% divergence in the amino acid sequence of the major capsid protein, or viral protein 1 (VP1) (65). Infection with HuNoVs can be asymptomatic or can cause clinical symptoms, including malaise, nausea, vomiting, and watery diarrhea (1, 2, 20, 47). Although human norovirus (HuNoV) infection is typically self-limiting, severe and long-term infection can occur in elderly or immunocompromised individuals (22, 44, 50, 60, 62). More recently, HuNoV infection has also been associated with necrotizing enterocolitis in infants and postinfectious irritable bowel syndrome in adults, as well as extraintestinal symptoms, such as benign infantile seizures and encephalopathy (12, 37, 40, 45, 54, 59).

Due to the inability to grow HuNoVs in cell culture and the lack of a small-animal model, our understanding of determinants of HuNoV replication and pathogenesis is limited. HuNoVs, as well as recombinant virus-like particles (VLPs) of HuNoVs, have differential interactions with ABH histo-blood group antigens (HBGAs) which vary over time (23, 27, 35). Moreover, HBGA influence susceptibility to HuNoV infection, as well as to symptomatic infection (29). However, the mechanism by which differential binding of VP1 to HBGA alters HuNoV infectivity and pathogenesis is unknown.

Murine noroviruses (MNVs) are related noroviruses (NoVs) that infect mice, can be grown in cell culture, and share many of the molecular and biological properties of HuNoVs (reviewed in references 24 and 64). MNVs have a positive-sense, single-stranded RNA genome that contains four open reading frames (ORFs) (31, 58). ORF1 encodes a polyprotein that is cleaved into six nonstructural proteins, while ORF2 encodes VP1, which contains antigenic and binding determinants (32, 33, 53, 56, 57). ORF3 encodes the small basic protein, or viral protein 2 (VP2), which presumably functions like HuNoV VP2 (6, 16). In addition, MNVs possess a conserved ORF4, which encodes a gene product recently named virulence factor 1 (VF1) (39, 58). VF1 is expressed

from an alternative reading frame which overlaps the VP1 coding region and may regulate innate immune responses during MNV infection (39).

Use of the murine norovirus (MNV) model system has demonstrated a role for both adaptive and innate immunity in control of MNV infection. CD4 and CD8 T cell subsets, as well as antibody produced by B cells, participate in MNV clearance and are required for effective vaccination (8, 9, 31). While immunocompetent mice are resistant to lethal MNV infection, mice lacking type I and type II interferon (IFN) receptors, as well as downstream signal transducer and activator of transcription 1 (STAT1), are highly susceptible (31). Furthermore, mice and cells lacking IFN receptors and STAT1 have elevated levels of MNV replication compared to those found in immunocompetent mice and cells (31, 42, 63).

In addition to identifying host determinants of NoV replication and pathogenesis, the MNV model has been used to identify virus determinants of replication and pathogenesis. A single amino acid substitution in the protruding (P) domain of VP1 is responsible for determining the virulence of the MNV strain MNV1 in 12956/SvEv-Stat1^{tm1Rds} (129/STAT1^{-/-}) mice (4, 63). A lysine (K)-to-glutamic acid (E) substitution at residue 296 of VP1, which arose during serial passage of the CW1 clone of MNV1 (MNV1.CW1, herein CW1), is associated with the attenuated virulence of CW1 passage 3 (CW1P3) in 129/STAT1^{-/-} mice. Reversion of this amino acid substitution in a cDNA clone of CW1P3 (11) is sufficient to restore virulence.

Numerous strains of MNV have been isolated and sequenced

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subsequent to the isolation of MNV1 in 2002 (5, 25, 52, 58). These strains differ both genetically and phenotypically from MNV1 (7, 26, 30, 58). As some of these strains possess E296 in VP1, which is responsible for the decreased virulence of CW1P3 (4, 63), we sought to determine whether residue 296 is a virulence determinant in other MNV strains. In addition, we sought to better define the role of virus-encoded factors in the pathogenesis of lethal MNV infection by using molecular clones of two previously isolated strains of MNV that differ in their biological properties as well as in date, location, and tissue of their origin (58). In this study, we report the cloning and characterization of the CW3 clone of MNV1 (MNV1.CW3, herein CW3) and a clone of a more recently isolated CR6 strain of MNV (CR6.STL1, herein CR6). Using the cDNA clones of these two MNVs, we identified and characterized another determinant of virulence in the P domain of VP1, which is necessary and sufficient to alter *in vivo* replication and pathogenesis of MNV in immunodeficient mice.

MATERIALS AND METHODS

Cloning and production of recombinant MNV. MNV strain CR6 (GenBank accession no. [EU004676.1](#)) was plaque purified three times and amplified three times on RAW264.7 (RAW) cells as previously described for MNV1 (58, 63). The consensus sequence of CR6.STL1 (herein referred to as CR6) was determined as previously described for MNV1 clones (58, 63). CW3 (GenBank accession no. [EF014462.1](#)) and CR6 (GenBank accession no. [JQ237823](#)) were cloned into the pMNV* vector as previously described for CW1 (61). Briefly, total RNA was isolated from RAW cells inoculated with each strain, and cDNA was synthesized. PCR products were amplified using primers 5'-AAC TTG GGA TCC ACC GGT GTG AAA TGA GGA TGG CAA CGC-3' and 5'-ATG CGG CCG CT₍₅₀₎A AAA TGC ATC TAA-3' and cloned into the pCR2.1-TOPO vector (Invitrogen). The sequence was then moved into the pMNV* vector in fragments (CW3, AgeI-SacII and SacII-NsiI; CR6, AgeI-DraIII, DraIII-NsiI, and NsiI-NsiI). Each cDNA clone was sequenced as previously described (58). Deviations from the CW3 consensus sequence (GenBank accession no. [EF014462.1](#)) were repaired using a Quick Change II XL mutagenesis kit (Agilent Technologies). A synonymous C1001A nucleotide substitution was introduced into the CW3 cDNA clone by using the Quick Change II XL mutagenesis kit, resulting in a novel EcoRV restriction enzyme site to facilitate identification of plasmid-derived CW3 (pCW3). Comparison of the consensus sequences of the CR6 cDNA clone and CR6.STL1 (GenBank accession no. [JQ237823](#)) identified a synonymous T5530C nucleotide substitution in ORF2 that was not repaired. A synonymous C512G nucleotide substitution was introduced into the CR6 cDNA clone by using the Quick Change II XL mutagenesis kit, resulting in a novel SacII restriction enzyme site to facilitate identification of plasmid-derived CR6 (pCR6).

Cloning of MNV chimera constructs. Nucleotide sequences corresponding to each MNV protein or domain were amplified with *Pfu*Turbo (Stratagene) using the primer pairs listed in Table 1. PCR products were gel purified using a S.N.A.P. gel purification kit (Invitrogen). To produce chimeric plasmids, approximately 1 μ g of purified PCR product was used as the primer with the Quick Change II XL mutagenesis kit, using the following cycling program: MNV template and primer were incubated separately at 95°C for 1 min and then on ice for 5 min. All reaction components were combined and subjected to 17 additional cycles according to the following protocol: 50 s at 95°C, 20 s at 4°C, 50 s at 60°C, and 11 min at 68°C. The remaining cycling parameters were per the manufacturer's protocol. Each cDNA clone was sequenced as previously described (58).

Production of parental and plasmid-derived MNV. Stocks of CW3 and CR6 were generated in RAW cells using a multiplicity of infection (MOI) of 0.05 as previously described (63). Briefly, 48 h after inoculation, cells were lysed by freeze-thaw at -80°C , and the supernatant was clarified by centrifugation. For plasmid-derived stocks, HEK-293T (293T) cells were seeded at

5×10^5 cells per well in a 6-well culture dish. Cells were incubated for 16 to 24 h and transfected with plasmids containing MNV cDNA clones using FuGeneHD (Promega). Forty-eight hours after transfection, cells were lysed by freeze-thaw at -80°C , and the supernatant was clarified by centrifugation. pCW3, pCR6, and pCW3/pCR6 chimeras were passed in RAW cells 1 to 2 times at an MOI of ≤ 0.05 and virus stocks were generated as described above. The consensus sequences of pCW3 and pCR6 produced using this protocol were generated and analyzed as described above and were observed to have no deviations from the input plasmid sequence. At least two independently generated virus stocks were used for each experimental group, except for experiments using 3×10^7 PFU of pCR6; this virus was pooled and concentrated as previously described (9).

Cell culture and viral growth and quantification. 293T and RAW cells (ATCC, Manassas, VA) were grown as previously described (63). For MNV growth curves, 4×10^5 RAW cells were seeded in 24-well culture dishes and inoculated with MNV at an MOI of 5 or 0.05 as previously described (63), with the following modifications: MNV was diluted in 0.2 ml of medium per well, and samples underwent one freeze-thaw cycle at -80°C . Virus titer was determined by plaque assay on RAW cells as previously described (63).

Mouse inoculation, organ harvest, and tissue processing. C57BL/6-Stat1^{tm1Dlv} mice (15) were bred and housed at Washington University, St. Louis, MO, under specific-pathogen-free conditions as described previously (7), in accordance with federal and university guidelines. 129S6/SvEv-Stat1^{tm1Rds} mice (41) were purchased from Taconic (Germantown, NY). Seven- to 10-week-old mice were orally inoculated with 25 μ l of MNV diluted in phosphate-buffered saline (PBS). Mice were sacrificed 3 days after inoculation, and tissues were harvested for virus titer or histological analysis as previously described (9, 55). Pathology in liver sections was scored blinded.

Statistical analysis. All data were analyzed by using Prism 5 software (Graph-Pad Software, San Diego, CA). All differences not specifically stated to be significant were not significant ($P > 0.05$).

Nucleotide sequence accession number. The nucleotide sequence of CR6.STL1 has been deposited in GenBank under accession number [JQ237823](#).

RESULTS

MNV strain CR6 is attenuated *in vivo*. With the identification of new MNV strains (58), we sought to determine whether, like MNV1, these strains were virulent *in vivo*. C57BL/6-Stat1^{tm1Dlv} (B6/STAT1^{-/-}) mice were inoculated with 3×10^4 PFU of CW3 or CR6. Consistent with a previous study (58), all B6/STAT1^{-/-} mice succumbed after inoculation with CW3 (Fig. 1A). In contrast, all B6/STAT1^{-/-} mice survived after inoculation with CR6. These data show that CR6 is significantly attenuated ($P < 0.0001$) in B6/STAT1^{-/-} mice.

Plasmid-derived CR6 is attenuated *in vivo*. The full-length genomes of CW3 (GenBank accession no. [EF014462.1](#)) and CR6 (GenBank accession no. [JQ237823](#)) diverge by 13% at the nucleotide level. To map the determinant(s) of virulence that differs between these two MNV strains, the cDNA of CW3 and CR6 was cloned into a reverse-genetics system (61), and various genetic elements were exchanged between the viruses to determine which genetic element was responsible for the observed phenotypic differences. To determine whether pCW3 and pCR6 had the same virulence characteristics as each parental virus, B6/STAT1^{-/-} mice were inoculated with 3×10^4 PFU of pCW3 or pCR6. Similar to what was observed for the parental viruses (Fig. 1A), all B6/STAT1^{-/-} mice succumbed after inoculation with pCW3, while pCR6 was significantly attenuated ($P < 0.0001$) compared to pCW3 (Fig. 1B). We next sought to determine the magnitude of the difference in virulence between the two viruses. Even when inoculated with 30 PFU, all B6/STAT1^{-/-} mice succumbed after inoculation with pCW3 (data not shown). In contrast, even

TABLE 1 Primers used in this study

Virus	Substitution	Orientation	Primer sequence (5'–3')
CW3	VP1 K296E	Forward	GCGGCGGAGGCTGCCTATGAGTTCCAATC
		Reverse	GATTGGAATCATAGGCAGCCTCCGCCGC
	VP1	Forward	AGGAACGCTCAGCAGTCTTTGTGAATGAGGATGAGTGATGGCGCAG
		Reverse	GCACCAAAGAGTGCGCCAGCCATTATTGTTTGAGCATTCGGCCTGTTG
	S	Forward	TTGCCGCCCCGCCGCCGGGCAAATCAACCAAATTGACCCCTGGATC
		Reverse	TAGTTCTCTCGATAGGGGGGGTTAGGTAGACAAAATTGAAATCAGCCG
	P	Forward	ATCGAGAGAACTATTTACCGGATGGTCGACTTGCCCGTGATAC
		Reverse	CAGCCATTATTGTTTGTAGTGTTCGGCCTGTTGCCAAACTTC
	P2	Forward	AGTTGATGGGACTCTTCTTGGTACCACCCCAATCTCCGGTTC
		Reverse	CCTAGGGGTGGGGCAAGGGGAACCAAGTAGCCCATC
CR6	VP1 E296K	Forward	CTGAGGCTGCCTACAAAGTTCCAAGCTGGG
		Reverse	CCCAGCTTGGAAGTTGTAGGCAGCCTCAG
	Nterm	Forward	CAGAGCTCGTTTAGTGAACCGGTGTGAAATGAGGATGGCAACGCCATCTTC
		Reverse	CATGTCAAGAGCAAGGTCGAAGGGCCCTCAGCCTGCCACTCTCCAAAG
	NTPase	Forward	CTTCGGGAATGGCAGGCCGAAGGTCCCTTTGACCTCGCCCTGGATGTC
		Reverse	GCCGGCATCAAAGTCATAGACCTTGTTTGGAGGCCGAAGTCATCATG
	p18	Forward	CATGATGATTTTCGGCCTCCAGAGCAAGACCTATGACTTTGATGCTGGT
		Reverse	CTTGTTCTTGCCCTTCTTCTTCAGAGTGGTACCATCCGTCGT
	VPg	Forward	ATGATGGGTGGTACCACTCTGAGGGCAAGAAAGGCAGGAACAAG
		Reverse	GGACCAGATGGAGACTGGGGCCTCAAAGTTGATCTTCTCACCAT
	Pro	Forward	ACGGCGAGAAGATCAACTTTGAGGCTCCAGTCTCAATCTGGACC
		Reverse	GCGGGGAAGCATGGGGGGTCCCTGAACTCCAGGGCCTCAAGTGTG
	Pol	Forward	CACACTTGAGGCTCTGGAGTTCCAGGGTCCCCCATGCTCCCTCGC
		Reverse	GGCTTTTGGCGCTGCGCCATCACTCATCTCATTACAAAGACTGCTG
	VP1	Forward	AGGAACGCTCAGCAGTCTTTGTGAATGAGGATGAGTGATGGCGCAG
		Reverse	GCTCCAAAAGAGCACCAGCCATTATTGTTGAGTGTTCGGCCTGTTAC
	VP2	Forward	CAGGCCGAATGCTCAAACAATAATGGCTGGCGCACTCTTGGTGC
		Reverse	CACAAAAGGTTTCTCTTCCAACCTATGCCTTGCTACTCCCGATCTTTG
CR6	S	Forward	GCCGCCGGGCAAATTAACCAAATTGACCCCTGGATC
		Reverse	GATGGTCTCTCTATGGGGGGAGTCAGGTATACAAAGTTGAAATCAGGTG
	P	Forward	CATAGAGAGAACCATCTACCGGATGGTTGACTTGCCCGTGTTG
		Reverse	CACACGCATTATTGTTTGAGCATTGCGCCTGTTACCAAGCTTCCAC
	P2	Forward	CACGTTGATGGGACCCTGCTTGGTACACGCCTGTGTCTGGTTCATG
		Reverse	GAAAGGGACCAATTGGGGGGGCAAGGGGAACCAAAACACCATCATTATAC

when inoculated with 3×10^7 PFU, only 25% of B6/STAT1^{−/−} mice succumbed after inoculation with pCR6. Together, these data demonstrate that pCW3 and pCR6 differ significantly in virulence *in vivo*. As there was no significant difference in lethality observed between the parental strains and their plasmid-derived counterparts, all subsequent studies were performed using plasmid-derived viruses pCW3 and pCR6.

Residue 296 of MNV VP1 is not the sole determinant of virulence. Since pCW3 VP1 encodes K296 and pCR6 VP1 encodes E296, residues previously shown to determine the virulence of CW1 (4, 63), we sought to determine whether residue 296 was also responsible for the difference in virulence between pCW3 and pCR6. Accordingly, we made a mutation that resulted in a K296E substitution in pCW3 VP1 (pCW3 VP1^{K296E}) or an E296K substitution in pCR6 VP1 (pCR6 VP1^{E296K}). While we recovered pCW3 VP1^{K296E}, we were unable to recover pCR6 VP1^{E296K}. The inability to recover pCR6 VP1^{E296K} suggests that a K residue at residue 296 is incompatible with pCR6 VP1.

To determine whether residue 296 is responsible for virulence of pCW3, B6/STAT1^{−/−} mice were inoculated with 3×10^4 PFU of pCW3 VP1^{K296E}. While all B6/STAT1^{−/−} mice succumbed after inoculation with pCW3, only 83% of B6/STAT1^{−/−} mice succumbed

after inoculation with pCW3 VP1^{K296E} (Fig. 1B). Of note, pCW3 VP1^{K296E} was significantly less virulent than pCW3 ($P < 0.0001$) but significantly more virulent than pCR6 ($P < 0.0001$). These data demonstrated that although residue 296 may contribute, it is not the only virulence determinant between pCW3 and pCR6.

Since 129S6/SvEv-Stat1^{tm1Rds} mice (129/STAT1^{−/−} mice) were used previously to identify residue 296 as a virulence determinant for MNV1 (4, 63), we examined the virulence of pCW3, pCR6, and pCW3 VP1^{K296E} in these mice to compare our data to previously published findings. Similarly to what was observed in B6/STAT1^{−/−} mice, all 129/STAT1^{−/−} mice succumbed after inoculation with pCW3 but survived after inoculation with pCR6 (Fig. 1B). However, in contrast to what was observed in B6/STAT1^{−/−} mice, all 129/STAT1^{−/−} mice survived after inoculation with 3×10^4 PFU of pCW3 VP1^{K296E} (Fig. 1B). Furthermore, unlike what was previously demonstrated using 129/STAT1^{−/−} mice (58, 63), all B6/STAT1^{−/−} mice succumbed after inoculation with 3×10^4 PFU of CW1P3 (data not shown). These data demonstrate that the importance of residue 296 as a virulence determinant differs between 129/STAT1^{−/−} and B6/STAT1^{−/−} mice. Importantly, by using B6/STAT1^{−/−} mice, we identified a novel determinant of MNV virulence, as amino acid substitutions at

FIG 1 MNV strain CR6 is attenuated *in vivo*. Survival of mice inoculated with parental MNV strains (A) or plasmid-derived (p) MNV strains (B). The numbers of mice analyzed are indicated in parentheses. Differences in survival rates were analyzed by the log rank test.

residue 296 did not completely explain the difference in virulence observed between pCR6 and pCW3 in B6/STAT1^{-/-} mice (Fig. 1B). To identify this novel virulence determinant, B6/STAT1^{-/-} mice were used in all subsequent *in vivo* studies.

***In vitro* viral growth rates of pCR6 and pCW3 are similar.** A potential explanation for the difference in lethality between pCR6 and pCW3 is that they differ in ability to replicate and spread. To test this hypothesis, RAW cells were inoculated at high and low MOIs with pCR6 and pCW3. There was no significant difference in overall growth of pCR6 and pCW3 at either high or low MOI (Fig. 2). These data show that differences in virus replication *in vitro* do not explain the difference in lethality between pCR6 and pCW3 observed in STAT1^{-/-} mice (Fig. 1B).

MNV VP1 is necessary and sufficient to determine MNV lethality. To identify the novel determinant responsible for the

FIG 2 *In vitro* growth rates of pCR6 and pCW3 are similar. Growth of pCR6 and pCW3 in RAW cells. Titters shown represent the means $\pm$ standard errors of the means (SEM) of titers from three independent experiments. The limit of detection is indicated by the dashed line. No differences between the growth curves were detected by unpaired *t* test.

difference in virulence between pCR6 and pCW3, we generated pCW3 with single gene substitutions from pCR6. We were able to recover pCW3 with CR6 N-terminal protein (Nterm), NTPase, p18, VPg, polymerase (Pol), VP1, and VP2. We were unable to recover pCW3 with CR6 proteinase (Pro). The inability to recover pCW3 Pro^{CR6} suggests that CR6 Pro is incompatible with pCW3.

To determine whether a single MNV gene is responsible for the attenuation of pCR6, STAT1^{-/-} mice were inoculated with 3×10^4 PFU of each pCW3/pCR6 chimera recovered. While pCW3 VP1^{CR6} was significantly attenuated ($P < 0.0001$) in lethality compared to pCW3, all STAT1^{-/-} mice succumbed after inoculation with all other pCW3/pCR6 chimeras (Fig. 3). None of the other pCW3/pCR6 chimeras were significantly attenuated compared to pCW3 (Fig. 1B and 3). These data demonstrate that pCR6 VP1 is sufficient to confer attenuation *in vivo*. To determine whether pCW3 VP1 is sufficient to confer lethal infection, we generated pCR6 with pCW3 VP1 (pCR6 VP1^{CW3}) and inoculated STAT1^{-/-} mice with 3×10^4 PFU of this chimera. All STAT1^{-/-} mice succumbed after inoculation with pCR6 VP1^{CW3} (Fig. 3). Of note, there was no significant difference in lethality between pCR6 VP1^{CW3} and pCW3 (Fig. 1B and 3). Together, these data establish that VP1 is both necessary and sufficient to determine lethality of pCW3 and pCR6 in STAT1^{-/-} mice.

VP1 P domain is necessary and sufficient to determine MNV lethality. MNV VP1 consists of an N-terminal arm, a shell (S) domain, and a protruding (P) domain that is further subdivided into P1 and P2 subdomains (32, 33). To define the VP1 domain that is necessary and sufficient to alter the lethality of pCW3 and pCR6, we generated chimeras that exchanged the shell or P domain of pCW3 and pCR6. Substitution of the shell domain alone (amino acids [aa] 49 to 218) did not significantly alter the lethality of the pCR6/pCW3 chimeras compared to that of their wild-type (WT) counterparts (Fig. 4A). In contrast, substitution of the P domain (aa 229 to 537) was suffi-

FIG 3 MNV VP1 is a major determinant of lethal infection. B6/STAT1^{-/-} mice were inoculated with chimeras consisting of a pCW3 or pCR6 backbone with a single pCR6 or pCW3 gene substitution. Nterm, N-terminal protein; Pol, polymerase.

cient to significantly increase ($P < 0.0001$) the lethality of pCR6 P^{CW3} and significantly decrease ($P < 0.0001$) the lethality of pCW3 P^{CR6} compared to that of pCR6 and pCW3, respectively (Fig. 4B). These data demonstrate that the P domain is both necessary and sufficient to determine the lethality of pCW3 and pCR6. Of note, pCR6 P^{CW3} was significantly less lethal ($P < 0.0002$) than pCW3 and pCR6 VP1^{CW3} (Fig. 1B, 3, and 4B). The difference in lethality conferred by pCW3 VP1 and P domains to pCR6 suggests that although determinants in the S domain are not sufficient to alter lethality, they may contribute to the virulence of pCW3 in STAT1^{-/-} mice.

VP1 P2 domain is sufficient for attenuation. To further define the VP1 domain responsible for the lethality of pCW3 and pCR6, we generated chimeras which exchanged the P1 (aa 229 to 277 and 416 to 537) or P2 (aa 278 to 415) subdomain of VP1. We were able to recover pCR6 P1^{CW3} and pCW3 P2^{CR6}, but we were unable to recover pCR6 P2^{CW3} and pCW3 P1^{CR6}. The inability to recover pCR6 P2^{CW3} and pCW3 P1^{CR6}, as well as pCR6 VP1^{E296K} (discussed above), provides further evidence that compatibility between elements of VP1 from distinct MNVs is required for production of live virus.

To determine whether the P1 and/or P2 subdomain contains a determinant that can alter the lethality of pCW3 or pCR6, STAT1^{-/-} mice were inoculated with 3×10^4 PFU of pCR6 P1^{CW3} or pCW3 P2^{CR6}. There was no significant difference in lethality of pCR6 P1^{CW3} compared to that of pCR6 or pCW3 P^{CR6} (Fig. 1B and 4B and C). In contrast, pCW3 P2^{CR6} was significantly more attenuated ($P < 0.0001$) than pCW3. These data demonstrate that the P2 domain is necessary for the lethality of pCW3 in STAT1^{-/-} mice.

VP1 P domain is necessary and sufficient to alter MNV replication *in vivo*. Increased MNV replication and dissemination, as well as pathogenesis in the spleen and liver, have previously been observed in STAT1^{-/-} mice compared to results seen with WT controls (31, 42). To determine whether the VP1 P domain is necessary and sufficient to alter the replication of MNV, STAT1^{-/-} mice were inoculated with 3×10^4 PFU of

FIG 4 VP1 P domain is necessary and sufficient to alter MNV lethality. Survival of B6/STAT1^{-/-} mice inoculated with chimeras consisting of a pCW3 or pCR6 backbone with S domain (A), P domain (B), or P1 or P2 subdomain (C) substitutions.

pCW3, pCW3 P^{CR6}, pCR6, and pCR6 P^{CW3}. Consistent with the differences in lethality observed in Fig. 1B, replication of pCW3 was significantly increased ($P < 0.05$) compared to that of pCR6 in all tissues examined except the ileum (Fig. 5). Furthermore, consistent with the differences in lethality observed in Fig. 1B and 4B, replication of pCR6 P^{CW3} was significantly increased compared to that of pCR6 in four out of six tissues examined, while replication of pCW3 P^{CR6} was significantly decreased compared to that of pCW3 in all tissues examined. These data demonstrate that the P domain is both necessary and sufficient to alter MNV replication in STAT1^{-/-} mice.

VP1 P domain is necessary and sufficient to alter MNV pathogenesis *in vivo*. To determine whether the VP1 P domain is necessary and sufficient to alter the pathogenesis of MNV, hematoxylin and eosin (H&E)-stained sections of spleen and liver from STAT1^{-/-} mice mock inoculated or inoculated with 3×10^4 PFU of pCW3, pCW3 P^{CR6}, pCR6, and pCR6 P^{CW3} were examined. In comparison with spleens of mock-inoculated controls, spleens of mice inoculated with pCW3 exhibited marked cell death and loss of architecture in the red and white pulp (Fig. 6A). High-magnification images of the white pulp show dying cells with condensed nuclei and ubiquitous cellular debris. In contrast, the only consistent finding in the spleens of mice inoculated with pCR6 was a mild expansion of the marginal zone (Fig. 6A). Spleens of mice inoculated with pCW3 P^{CR6} displayed mild marginal-zone expansion but lacked cell death and loss of splenic architecture. Spleens from

FIG 5 VP1 P domain is necessary and sufficient to alter MNV replication *in vivo*. Titers in tissues harvested from B6/STAT1^{-/-} mice 3 days after inoculation. MLN, mesenteric lymph node. Titers shown represent means $\pm$ SEM of titers from 12 mice from at least 2 independent experiments. Virus titers were analyzed with the nonparametric Mann-Whitney test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mice infected with pCR6 P^{CW3} displayed a range in pathology which is consistent with the intermediate lethality observed for this chimera. Approximately 50% of spleens from these mice had pathology similar to what was observed in spleens from mice inoculated with pCW3 (Fig. 6A). In the remainder of spleens from mice inoculated with pCR6 P^{CW3}, we observed cell death radiating from the T cell zone of the white pulp, as well as expansion of the marginal zone like that described for pCR6-infected mice (Fig. 6A).

In comparison with livers of mock-inoculated controls, we observed focal areas of cell death and inflammation in the livers of mice inoculated with pCW3 (Fig. 6B). To determine whether the P domain was the determinant of this pathology, we counted the numbers of these foci in livers from mice inoculated with 3×10^4 PFU of pCW3, pCW3 P^{CR6}, pCR6, and pCR6 P^{CW3}. Mice inoculated with pCW3 developed foci at a rate of approximately 1 focus per 2 mm² of liver tissue (Fig. 6C). In contrast, none of the pCR6-inoculated mice developed liver inflammation. Mice inoculated with pCW3 P^{CR6} developed significantly fewer foci than mice inoculated with pCW3, while mice inoculated with pCR6 P^{CW3} developed significantly more foci than mice inoculated with pCR6 (Fig. 6C). Together, these data show that the P domain is both necessary and sufficient to determine the pathogenesis of MNV in the liver and spleen of STAT1^{-/-} mice.

DISCUSSION

The pathogenesis of HuNoV infection is still not well understood. The discovery of MNV, along with its capacity to grow in a small-animal model, provides an opportunity to investigate NoV pathogenesis *in vivo* in greater detail. Previous studies have used the MNV model to identify and characterize viral and host determinants of MNV infection (3, 10, 13, 18, 31, 38, 42, 58, 63). Some of these studies reveal a profound role for STAT1-dependent host responses as a major determinant of MNV replication and dissemination *in vivo*, as well as pathology and mortality following MNV infection. Previously, in studying two clones of MNV1 which differ at only 2 residues, we identified a single amino acid determinant of MNV lethality in 129/STAT1^{-/-} mice (4, 63). Here, we show that this site plays a minor role in determining the virulence of MNV *in vivo* in B6/STAT1^{-/-} mice (Fig. 1B) and identify another virulence determinant in the P domain of VP1. Moreover, we demonstrate that the P domain, which contains both determinants, is necessary and sufficient to alter (i) replication of MNV *in vivo* and (ii) pathology in the spleen and liver following MNV infection.

The nucleotide sequences of pCR6 and pCW3 VP1 differ at 197 residues, 33 of which are nonsynonymous. Alignment of pCR6 and pCW3 VP1 revealed an alanine (A)-to-threonine substitution at residue 24 in the N-terminal arm and an A-to-proline substitution at residue 211 in the S domain, as well as 17 and 14 amino acid substitutions in the P1 and P2 domains, respectively (Fig. 7). While our data implicate the P domain as a major determinant of virulence, determinants in the N-terminal arm or S domain may also contribute to virulence. Substitution of a lethal VP1 completely reconstituted virulence of pCW3, while substitution of a lethal P domain only partially reconstituted virulence (Fig. 4B). It is interesting to speculate that VF1, which has recently been shown to play a role in the virulence of MNV1 in 129/STAT1^{-/-} mice (39), may also participate in the difference in virulence observed between pCR6 and pCW3 in B6/STAT1^{-/-} mice, as 20 synonymous substitutions in the N-terminal arm and the S domain coding region of ORF2 are nonsynonymous substitutions in ORF4. Alternatively, virulence determinants that differ between pCR6 and pCW3 may function at the level of the nucleotide sequence or RNA secondary structure, as demonstrated for other sites in MNV (3, 51).

Extensive analysis of HuNoVs has demonstrated that the P2 subdomain contains evolving antigenic and receptor binding sites (reviewed in reference 14). Here, we confirm that a major determinant of MNV virulence lies within the P2 subdomain (Fig. 4C) (4, 63). Recently, structural and functional analysis of MNV showed that the A'-B' and E'-F' polypeptide loops contain binding sites for a neutralizing antibody, as well as the previously identified MNV virulence determinant residue 296, and a previously identified antibody escape mutation (Fig. 7). Antigenic variation in the VP2 capsid protein of some parvoviruses is associated with changes in cellular receptor binding, tissue tropism, virulence, and species specificity (19, 28, 34, 36, 43, 48). Recently, sialic acid has been shown to mediate attachment of MNV and some HuNoVs (49, 56). It is interesting to speculate that the evolution of the NoV capsid due to antigenic pressure may also alter binding to sialic acid or other attachment receptors, such as HBGAs. Understanding the structural and molecular mechanisms by which the surface-exposed

FIG 6 VP1 P domain is necessary and sufficient to alter MNV pathogenesis *in vivo*. Pathology in spleen (A) and liver (B and C) from B6/STAT1^{-/-} mice 3 days after inoculation. Pictures shown represent pathology observed in sections from at least 7 mice from at least 2 independent experiments. Hematoxylin and eosin staining of formaldehyde-fixed, paraffin-embedded tissues. Data shown are means $\pm$ SEM. Data were analyzed by the unpaired *t* test. *, *P* < 0.05.

loops of MNV VP1 participate in antigenic variation, binding to cellular receptors, and virulence may increase our ability to predict changes in virulence and host tropism of NoVs as a consequence of antigenic variation.

One striking finding reported here is that the impact of the virulence determinant residue 296 in VP1 differs between 129/STAT1^{-/-} and B6/STAT1^{-/-} mice (Fig. 1B; data not shown). Whether this difference is due to the genetic background of the mice is not directly addressed by the experiments presented here, since both the genetic background and the strategy used to target *Stat1* differ between the 129/STAT1^{-/-} and B6/STAT1^{-/-} mice used in this study (15, 41). However, since both 129/STAT1^{-/-} and B6/STAT1^{-/-} mice are profoundly susceptible to lethal pCW3 infection (Fig. 1B), we believe that the genetic background, rather than the nature of the *Stat1* mutation, is the most likely explanation for the differences in

virulence observed here. Furthermore, other studies have reported striking differences in susceptibility of different inbred mouse strains to infection with a variety of viruses (reviewed in reference 21). We believe that our observation raises a substantial caution for the use of mutant mice with mixed 129/B6 genetic background in studies of MNV pathogenesis.

Finally, we have demonstrated the utility of our MNV reverse-genetics system (61) for elucidating aspects of MNV biology. We cloned two MNV strains into this system and identified determinants of pathogenesis in MNV by transferring whole viral proteins or protein subdomains. We were unable to recover live virus from several cDNA clones expressing K296E in pCR6, a P domain composed of pCR6 P1 and pCW3 P2 subdomains in either the pCW3 or the pCR6 background, and pCW3 with CR6 Pro. The inability to recover these viruses suggests that interactions between the P1 and P2 subdomains of VP1, as well as between other viral proteins,

FIG 7 Alignment of pCR6 and pCW3 VP1. Alignment of VP1 of pCR6 and pCW3 using Geneious v5.0. Lack of consensus is indicated by a vertical black line or a one-letter amino acid code. Consensus is indicated by a gray background or a horizontal dash. Previously identified virulence and neutralizing antibody escape determinants, neutralizing antibody binding sites, and surface-exposed loops are indicated with red, black, and green bars, respectively. N, N-terminal arm; S, shell domain; H, hinge region; P1, P1 domain; P2, P2 domain.

are essential for production of infectious virus. We believe that the potential cooperative interactions between viral elements observed here is an important consideration for future studies of NoV genetics.

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REFERENCES

- Atmar RL, Estes MK. 2006. The epidemiologic and clinical importance of norovirus infection. *Gastroenterol. Clin. North Am.* 35:275–290.
- Atmar RL, et al. 2008. Norwalk virus shedding after experimental human infection. *Emerg. Infect. Dis.* 14:1553–1557.
- Bailey D, et al. 2010. Functional analysis of RNA structures present at the 3' extremity of the murine norovirus genome: the variable polypyrimidine tract plays a role in viral virulence. *J. Virol.* 84:2859–2870.
- Bailey D, Thackray LB, Goodfellow IG. 2008. A single amino acid substitution in the murine norovirus capsid protein is sufficient for attenuation in vivo. *J. Virol.* 82:7725–7728.
- Barron EL, et al. 2011. Diversity of murine norovirus strains isolated from asymptomatic mice of different genetic backgrounds within a single U.S. research institute. *PLoS One* 6:e21435.
- Bertolotti-Ciarlet A, White LJ, Chen R, Prasad BV, Estes MK. 2002. Structural requirements for the assembly of Norwalk virus-like particles. *J. Virol.* 76:4044–4055.
- Cadwell K, et al. 2010. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell* 141:1135–1145.
- Chachu KA, LoBue AD, Strong DW, Baric RS, Virgin HW. 2008. Immune mechanisms responsible for vaccination against and clearance of mucosal and lymphatic norovirus infection. *PLoS Pathog.* 4:e1000236.
- Chachu KA, et al. 2008. Antibody is critical for the clearance of murine norovirus infection. *J. Virol.* 82:6610–6617.
- Changotra H, et al. 2009. Type I and type II interferons inhibit the translation of murine norovirus proteins. *J. Virol.* 83:5683–5692.
- Chaudhry Y, Skinner MA, Goodfellow IG. 2007. Recovery of genetically defined murine norovirus in tissue culture by using a fowlpox virus expressing T7 RNA polymerase. *J. Gen. Virol.* 88:2091–2100.
- Chen SY, et al. 2009. Norovirus infection as a cause of diarrhea-associated benign infantile seizures. *Clin. Infect. Dis.* 48:849–855.
- Daughenbaugh KF, Wobus CE, Hardy ME. 2006. VPg of murine norovirus binds translation initiation factors in infected cells. *Virology* 3:1–7.
- Donaldson EF, Lindesmith LC, LoBue AD, Baric RS. 2010. Viral shape-shifting: norovirus evasion of the human immune system. *Nat. Rev. Microbiol.* 8:231–241.
- Durbin JE, Hackenmiller R, Simon MC, Levy DE. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84:443–450.
- Glass PJ, et al. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. *J. Virol.* 74:6581–6591.
- Glass RI, Parashar UD, Estes MK. 2009. Norovirus gastroenteritis. *N. Engl. J. Med.* 361:1776–1785.
- Goodfellow I, et al. 2005. Calicivirus translation initiation requires an interaction between VPg and eIF 4 E. *EMBO Rep.* 6:968–972.
- Govindasamy L, Hueffer K, Parrish CR, Agbandje-McKenna M. 2003. Structures of host range-controlling regions of the capsids of canine and feline parvoviruses and mutants. *J. Virol.* 77:12211–12221.
- Graham DY, et al. 1994. Norwalk virus infection of volunteers: new insights based on improved assays. *J. Infect. Dis.* 170:34–43.
- Guenet JL. 2005. Assessing the genetic component of the susceptibility of mice to viral infections. *Brief. Funct. Genomic. Proteomic.* 4:225–240.
- Gustavsson L, Andersson LM, Lindh M, Westin J. 2011. Excess mortality following community-onset norovirus enteritis in the elderly. *J. Hosp. Infect.* 79:27–31.
- Harrington PR, Vinje J, Moe CL, Baric RS. 2004. Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. *J. Virol.* 78:3035–3045.
- Henderson KS. 2008. Murine norovirus, a recently discovered and highly prevalent viral agent of mice. *Lab. Anim.* 37:314–320.
- Hsu CC, Riley LK, Livingston RS. 2007. Molecular characterization of three novel murine noroviruses. *Virus Genes* 34:147–155.
- Hsu CC, Riley LK, Wills HM, Livingston RS. 2006. Persistent infection with and serologic cross-reactivity of three novel murine noroviruses. *Comp. Med.* 56:247–251.
- Huang P, et al. 2003. Noroviruses bind to human ABO, Lewis, and

- secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. *J. Infect. Dis.* 188:19–31.
28. Hueffer K, et al. 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. *J. Virol.* 77:1718–1726.
  29. Hutson AM, Atmar RL, Graham DY, Estes MK. 2002. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J. Infect. Dis.* 185:1335–1337.
  30. Kahan SM, et al. 2011. Comparative murine norovirus studies reveal a lack of correlation between intestinal virus titers and enteric pathology. *Virology* 421:202–210.
  31. Karst SM, Wobus CE, Lay M, Davidson J, Virgin HW. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. *Science* 299:1575–1578.
  32. Katpally U, et al. 2010. High-resolution cryo-electron microscopy structures of murine norovirus 1 and rabbit hemorrhagic disease virus reveal marked flexibility in the receptor binding domains. *J. Virol.* 84:5836–5841.
  33. Katpally U, Wobus CE, Dryden K, Virgin HW, Smith TJ. 2008. The structure of antibody neutralized murine norovirus and unexpected differences to virus like particles. *J. Virol.* 82:2079–2088.
  34. Kontou M, et al. 2005. Structural determinants of tissue tropism and in vivo pathogenicity for the parvovirus minute virus of mice. *J. Virol.* 79:10931–10943.
  35. Lindesmith LC, et al. 2008. Mechanisms of GII 4 norovirus persistence in human populations. *PLoS Med.* 5:e31.
  36. Lopez-Bueno A, et al. 2008. Evolution to pathogenicity of the parvovirus minute virus of mice in immunodeficient mice involves genetic heterogeneity at the capsid domain that determines tropism. *J. Virol.* 82:1195–1203.
  37. Marshall JK, Thabane M, Borgaonkar MR, James C. 2007. Postinfectious irritable bowel syndrome after a food-borne outbreak of acute gastroenteritis attributed to a viral pathogen. *Clin. Gastroenterol. Hepatol.* 5:457–460.
  38. McCartney SA, et al. 2008. MDA-5 recognition of a murine norovirus. *PLoS Pathog.* 4:e1000108.
  39. McFadden N, et al. 2011. Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4. *PLoS Pathog.* 7:e1002413.
  40. Medici MC, Abelli LA, Dodi I, Dettori G, Chezzi C. 2010. Norovirus RNA in the blood of a child with gastroenteritis and convulsions—a case report. *J. Clin. Virol.* 48:147–149.
  41. Meraz MA, et al. 1996. Targeted disruption of the Stat 1 gene in mice reveals unexpected physiologic specificity of the JAK-STAT signalling pathway. *Cell* 84:431–442.
  42. Mumphy SM, et al. 2007. Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. *J. Virol.* 81:3251–3263.
  43. Nelson CD, Palermo LM, Hafenstein SL, Parrish CR. 2007. Different mechanisms of antibody-mediated neutralization of parvoviruses revealed using the Fab fragments of monoclonal antibodies. *Virology* 361:283–293.
  44. Nilsson M, et al. 2003. Evolution of human calicivirus RNA in vivo: accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. *J. Virol.* 77:13117–13124.
  45. Obinata K, et al. 2010. Norovirus encephalopathy in a previously healthy child. *Pediatr. Infect. Dis. J.* 29:1057–1059.
  46. Patel MM, Hall AJ, Vinje J, Parashar UD. 2009. Noroviruses: a comprehensive review. *J. Clin. Virol.* 44:1–8.
  47. Phillips G, Tam CC, Rodrigues LC, Lopman B. 2010. Prevalence and characteristics of asymptomatic norovirus infection in the community in England. *Epidemiol. Infect.* 138:1454–1458.
  48. Rubio MP, Lopez-Bueno A, Almendral JM. 2005. Virulent variants emerging in mice infected with the apathogenic prototype strain of the parvovirus minute virus of mice exhibit a capsid with low avidity for a primary receptor. *J. Virol.* 79:11280–11290.
  49. Rydell GE, et al. 2009. Human noroviruses recognize sialyl Lewis x neoglycoprotein. *Glycobiology* 19:309–320.
  50. Schwartz S, et al. 2011. Norovirus gastroenteritis causes severe and lethal complications after chemotherapy and hematopoietic stem cell transplantation. *Blood* 117:5850–5856.
  51. Simmonds P, et al. 2008. Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses. *Nucleic. Acids Res.* 36:2530–2546.
  52. Smith D, McFadden N, Blundell RJ, Meredith A, Simmonds P. 2012. Diversity of murine norovirus in wild-rodent populations: species-specific associations suggest an ancient divergence. *J. Gen. Virol.* 93:259–266.
  53. Sosnovtsev SV, et al. 2006. Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. *J. Virol.* 80:7816–7831.
  54. Stuart RL, et al. 2010. An outbreak of necrotizing enterocolitis associated with norovirus genotype GII 3. *Pediatr. Infect. Dis. J.* 29:644–647.
  55. Tarakanova VL, et al. 2005. Murine gammaherpesvirus 68 infection is associated with lymphoproliferative disease and lymphoma in BALB beta2 microglobulin-deficient mice. *J. Virol.* 79:14668–14679.
  56. Taube S, et al. 2009. Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. *J. Virol.* 83:4092–4101.
  57. Taube S, et al. 2010. High-resolution x-ray structure and functional analysis of the murine norovirus 1 capsid protein protruding domain. *J. Virol.* 84:5695–5705.
  58. Thackray LB, et al. 2007. Murine noroviruses comprising a single genotype exhibit biological diversity despite limited sequence divergence. *J. Virol.* 81:10460–10473.
  59. Turcios-Ruiz RM, et al. 2008. Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. *J. Pediatr.* 153:339–344.
  60. van Asten L, et al. 2011. Unspecified gastroenteritis illness and deaths in the elderly associated with norovirus epidemics. *Epidemiology* 22:336–343.
  61. Ward VK, et al. 2007. Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. *Proc. Natl. Acad. Sci. U. S. A.* 104:11050–11055.
  62. Westhoff TH, et al. 2009. Chronic norovirus infection in renal transplant recipients. *Nephrol. Dial. Transplant.* 24:1051–1053.
  63. Wobus CE, et al. 2004. Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol.* 2:e432.
  64. Wobus CE, Thackray LB, Virgin HW. 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. *J. Virol.* 80:5104–5112.
  65. Zheng DP, et al. 2006. Norovirus classification and proposed strain nomenclature. *Virol.* 346:312–323.