Identification of amino acids within norovirus polymerase involved in RNA binding and viral replication

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Abstract

Until recently, molecular studies on human norovirus (HuNoV), a major causative agent of gastroenteritis, have been hampered by the lack of an efficient cell culture system. Murine norovirus-1 (MNV-1) has served as a surrogate model system for norovirus research, due to the availability of robust cell culture systems and reverse genetics. To identify amino acids involved in RNA synthesis by the viral RNA-dependent RNA polymerase (NS7), we constructed NS7 mutants in which basic amino acids surrounding the catalytic site were substituted with alanine. Electrophoretic mobility shift assay revealed that these residues are important for RNA binding, particularly R296. Furthermore, in vitro RNA synthesis and reverse genetics were used to identify conserved amino acids essential for RNA synthesis and viral replication. These results provide additional functional insights into highly conserved amino acids in NS7 and provide potential methods of rational attenuation of norovirus replication.

Human noroviruses (HuNoVs) are considered a major cause of nonbacterial acute gastroenteritis worldwide [1]. Studies on the molecular mechanisms of HuNoV replication and its pathogenic properties have been hampered by the lack of an efficient virus propagation system in cell culture and an animal model. The recent development of two culture systems for HuNoV provides additional experimental systems with which to dissect the molecular mechanisms involved in norovirus genome replication [2, 3]. Since 2003, however, murine norovirus-1 (MNV-1) has been used as the most robust experimental model due to the availability of a robust cell culture system, reverse genetics and small animal model [4, 5], and has served as a surrogate model system to study norovirus (NoV) replication and some aspects of viral pathogenicity [6].

The MNV-1 genome is a positive-sense single-stranded RNA of approximately 7.4 kb. A virus-encoded protein VPg is covalently attached to the 5’ end of the RNA [7], with a poly(A) tail at the 3’ end [4, 8]. Of the four ORFs within the MNV genome, ORF1 encodes a polyprotein that is cotranslationally processed into six functional proteins by a virally encoded protease (NS6): NS1, NS3, NS4, NS5 (VPg), NS6 (Pro) and NS7 (RdRp, Pol) [9]. In addition to full-length genomic RNA (gRNA), a 2.5-kb-long subgenomic RNA (sgRNA) carrying ORF2 and ORF3 is also produced in MNV-1–infected cells [5]. ORF2 and ORF3 encode the major capsid (VP1) and minor capsid (VP2) proteins, respectively. ORF4, encoding the VF1 protein, functions to antagonize the innate immune response to infection [10].

Upon infection with members of the family Caliciviridae family, the positive-sense full-length gRNA is expected to play a dual role. The RNA is used as a template for translation to produce nonstructural proteins and used as a template to generate negative-sense RNA, which will serve as a template to generate gRNA and sgRNA [11]. A virally encoded RNA-dependent RNA polymerase (RdRp) and its uncleaved proteinase-polymerase precursor are involved in the synthesis of viral RNAs [12–14]. Sequence comparison and structural analysis of the rabbit hemorrhagic disease virus (RHDV) RdRp identified six motifs within the palm domain, including the active site YGDD-containing motif C and motif A (Fig. 1a) [15, 16]. In addition, crystallographic studies showed that the overall structure of the RHDV [16], Norwalk virus (NV) [17], sapovirus (SV) [18], and MNV [19] RdRps are similar to those of poliovirus [20] and hepatitis C virus (HCV) [21], and consist of a cupped right hand with palm, fingers and thumb domains (Fig. 2a). The N-terminal domain of NV, RHDV, MNV and SV RdRp connects the fingers and thumb domains, whereas the C-terminal...
domain is located within the active site cleft and potentially interferes with primer and/or template RNA during viral RNA synthesis.

We have previously demonstrated that recombinant MNV-1 NS7 expressed and purified from *Escherichia coli* is functionally active, possesses RNA synthesis activity and retains a closed right hand structure, with palm, fingers and thumb domains [19, 22]. To identify specific amino acid(s) involved in RNA binding and RNA synthesis activities conserved across the family *Caliciviridae*, the sequences of RdRps from NV, RHDV and MNV-1 were aligned (Fig. 1a).

We assumed that the basic amino acids residing within or adjacent to the active site of NS7 are likely involved in interacting with template RNA. Amino acid sequence alignment revealed that MNV-1 residues K\(^{169}\), K\(^{210}\), K\(^{183}\), K\(^{184}\), R\(^{185}\), R\(^{395}\) and R\(^{396}\) are well-conserved between these viruses. Studies of the crystal structure of RHDV RdRp–primer–template triplex indicated that residues K\(^{173}\), K\(^{213}\), K\(^{403}\) and R\(^{404}\) are involved in the interaction with the RNA template, and that amino acid R\(^{188}\) interacts with the nucleotide [16].

Residues K\(^{169}\), R\(^{185}\), K\(^{210}\), K\(^{395}\) and R\(^{396}\) of the MNV-1 NS7 correspond to K\(^{173}\), R\(^{188}\), K\(^{213}\), K\(^{403}\) and R\(^{404}\) of RHDV RdRp. In addition to these amino acids, a number of basic amino acids (K\(^{183}\), K\(^{184}\), R\(^{185}\) and K\(^{422}\)) at the active site were found to be located near the RNA template when we superimposed our crystal structure of the MNV-1 NS7 on the structure of the polymerase–RNA complex of NV (PDB ID 3BSO) (Fig. 2). These results suggested that these amino acid residues could be involved in the MNV-1 NS7–RNA interface.

To examine the functional roles of the residues K\(^{169}\), K\(^{183}\), K\(^{184}\), R\(^{185}\), K\(^{210}\), R\(^{395}\) and K\(^{422}\), seven alanine substituted mutants, K169A, K210A, K422A, KKRAAA (K183A/K184A/R185A), RRAA (R395A/R396A), R395A and R396A NS7s were constructed by site-directed mutagenesis. Wild-type and alanine-substituted mutants of NS7 were expressed in *E. coli* as N-terminal His-tagged proteins, which were purified by affinity chromatography as described previously [22]. Analysis of the recombinant proteins by SDS-PAGE indicated that the NS7s were purified to greater than 90 % homogeneity (Fig. 1b).

To examine the impact of each mutation on the ability of NS7 to interact with RNA, electrophoretic mobility shift assays (EMSAs) were conducted with the Ala substitution mutant NS7s. A \(^{32}\)P-labelled viral RNA probe was synthesized by in vitro transcription using the RibomAX RNA production system (Promega) followed by purification with the RNeasy Mini Kit (Qiagen) according to the manufacturers’ instructions. EMSA reactions contained 70 nM \(^{32}\)P-RNA.
labelled RNA probe, 1, 2, 4 and 6 µM of wild-type or mutant NS7, 0.67 mg ml⁻¹ yeast tRNA (Ambion), 50 mM HEPES (pH 7.4), 25 mM KCl, 2.5 mM MgCl₂, 1 mM DTT and 4 % (v/v) glycerol. Reactions were incubated for 10 min at 30 °C prior to separation on 4 % acrylamide gels containing 0.5 % TBE and 5 % (v/v) glycerol. Gels were dried and prior to separation on 4 % acrylamide gels containing 0.5 % TBE and 5 % (v/v) glycerol. Gels were dried and ³²P-labelled RNA was visualized using a BAS-1500 phosphorimager (Fuji Film).

Compared to wild-type NS7, all mutants exhibited decreased RNA-binding capability to varying degrees (Fig. 3a). The mutants KKRAAA, RRAA and R396A exhibited the greatest reduction in RNA–protein complex formation, implying that these amino acid residues might play a functional role in RNA binding. The mutants K210A and K422A were also significantly impaired, while the mutants R395A and K169A exhibited less detrimental effects in binding. The single amino acid alteration R396A mutant exhibited a similar level of RNA–protein complex formation as with KKRAAA or RRAA, whereas mutation of the adjacent residue, R395, had a much-reduced impact on RNA-binding capability. Further analysis of positions K₁⁸³, K₁⁸⁴ and R₁⁸⁵ by modelling of the MNV structure on the RNA polymerase–RNA complex indicated that R₁⁸⁵ most likely interacts with nucleotide instead of RNA (Fig. 2), similarly to R₁⁸⁸ in the RHDV RdRp [16]. Taken together, these data indicated that the amino acids R₉₉₆, K₁⁸³, K₁⁸⁴ and R₁⁸⁵ of NS7 play a potential role in the interaction with RNA. The observation that amino acid R₄⁰⁴ of RHDV RdRp, equivalent to R₉₉₆ in the MNV NS7, interacts with the primer–template duplex also supports this hypothesis.

Since binding of template RNA to the active site of the NS7 is required for RNA synthesis, we measured the impact of the NS7 mutations on the ability of recombinant polymerase to use in vitro-transcribed MNV-1 sgRNA as a template for RNA synthesis. The assay was performed as described earlier [22] with minor modifications. Twenty microlitres of reaction mixture containing 50 mM HEPES (pH 7.4), 5 mM MgCl₂, 10 mM DTT, 1 µg sgRNA, 25 µM each NTP, 2.5 µCi [α⁻³²P]GTP (3000 Ci mmol⁻¹, 10 mCi ml⁻¹; 1 Ci=37 GBq), and 0.9 µg wild-type or mutant NS7 was incubated at 37 °C for 30 min. The reaction was stopped by adding an equal volume of 200 mM EDTA (pH 8.0), then 8 µl reaction mixture was spotted onto DE81 filter paper (Fisher Scientific). The filter paper was dried at room temperature for 10 min, washed three times with 2 ml 2× SSC solution for 10 min, dehydrated with 2 ml absolute ethanol, and dried at 80 °C. The radioactivity of incorporated [α⁻³²P]GMP was measured with a liquid Wallac 1407 scintillation counter (Wallac). As expected, all NS7 mutants exhibited decreased RNA synthesis activities. KKRAAA, RRAA and R396A, which showed the least affinity to RNA, also showed 6, 7 and 12 % of wild-type RNA polymerase activity, and K210A, K422A, R395A and K169A showed 42, 25, 35 and 20 %, respectively (Fig. 3b). The extent to which RNA synthesis was impaired correlated well with the RNA binding capability determined by EMSA, with the exception of K169A.

To examine whether the observed effects of NS7 mutations on RNA binding also impact MNV replication in cell culture, we introduced mutations into the MNV-1 cDNA clone, and the effect of the mutations on virus viability was examined by reverse genetics as previously described [23]. The NS7 mutations KKRAAA, RRAA, R396A and K169A were non-viable, while the recovery of the R395A and K210A mutants was decreased by ~3 and 0.5 log₁₀, respectively (Fig. 3c). The results of the three assays correlated well for most mutations, although it is worth noting that the magnitude to which each mutation affected each activity varied (Fig. 3d). This variability is likely due to the inherent properties of each assay. There are a number of notable exceptions, including the mutants K210A and K422A, which appeared to have a minimal impact on virus viability. It is also worth noting that the reverse genetics assay used is

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**Fig. 2.** Superposition of MNV-1 NS7 and NV polymerase–RNA complex structures. (a) Overall view of superimposed structures of MNV-1 NS7 (PDB ID 3QID) and NV polymerase–RNA complex (PDB ID 3BSO): front view (left) and side view (right) where a box is indicated to present important basic amino acids. Double-stranded RNA and CTP from NV polymerase–RNA complex are shown in grey. Fingers, palm and thumb domains are shown in blue, yellow and green, respectively, as shown in Fig. 1a). Basic amino acids are shown as stick model in domain colours and the GDD motif of MNV-1 NS7 is shown in cyan. (b) Close-up view of the box in (a), showing double-stranded RNA, CTP (dark grey) and basic amino acids.
an endpoint assay and therefore unable to identify mutations that simply slow replication. It is therefore possible that the mutants K210A and K422A may have more subtle phenotypes that may only be apparent in more detailed analysis including \textit{in vivo} virulence studies.

The alanine substitution mutants of arginine residues within motif E (R\textsuperscript{395} and R\textsuperscript{396}) showed decreased levels of RNA binding as well as \textit{de novo} RNA synthesis and viral replication (Fig. 3a, b). Collectively, these data suggest a clear role of these residues in viral RNA binding.
The NTP binding activity of motif F, at the fingers–thumb inter-domain, has been described for the HCV RdRp [24]. Mutation of the equivalent amino acids around that motif in MNV-1 K\textsuperscript{169} resulted in a minor effect on RNA binding, but showed a more significant effect on \textit{in vitro} RNA synthesis and viral recovery by reverse genetics (Fig. 3). These data suggest that this residue may influence RNA synthesis by a mechanism other than RNA binding. When we superimposed our NS7 structure with that of NV polymerase–RNA complex, K\textsuperscript{169} was found to interact with R\textsuperscript{185}, which is bound to CTP. Thus, we would propose that K\textsuperscript{169} may function to stabilize NS7–RNA–NTP binding.

In conclusion, we have identified a number of basic amino acids surrounding the norovirus RdRp active site that contribute to viral RNA binding, RNA synthesis and virus viability. Additional studies will be required to examine the role of the identified amino acids in other functions of the viral polymerase such as Vpg guanylylation or the interaction with the viral capsid protein VP1 [25]. Furthermore, the potential impact of some of the mutations on viral pathogenesis \textit{in vivo} remains to be determined, but this work provides a framework to potentially design rationally attenuated noroviruses.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**References**