RNA binding by human Norovirus 3C-like proteases inhibits protease activity

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A highly active, fluorescence-based, in vitro assay for human Norovirus protease from genogroup I and II viruses was optimized utilizing as little as 0.25 μM enzyme, pH 7.6, and substrate/enzyme of 50–100. Activity in Tris–HCl or sodium phosphate buffers was 2-fold less than HEPES, and 2-fold lower for buffer concentrations over 10 mM. Protease activity at pH 7.6 was 73% (GI) or 63% (GII) of activity at the optimal pH 9.0. Sodium inhibited activity 2–3 fold, while potassium, calcium, magnesium, and manganese inhibited 5–10 fold. Differences in efficiency due to pH, buffer, and cations were due to changes in $K_{cat}$ and not $K_m$. Norovirus protease bound short RNAs representing the 3’ or 5’ ends of the virus, inhibiting protease activity ($IC_{50}$ 3–5 μM) in a non-competitive manner. Previous reports indicated participation of the protease in the Norovirus replicase complex. The current studies provide initial support for a defined role for the viral protease in Norovirus replication.

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Introduction

Noroviruses are a group of related non-enveloped, single-stranded, positive sense RNA viruses that cause acute gastroenteritis in humans. Noroviruses are the most common cause of epidemic gastroenteritis, responsible for at least 50% of all gastroenteritis outbreaks worldwide, while 1 in 15 (21 million) residents in the United States acquire these infections annually (Hall et al., 2011; Scallan et al., 2011). An estimated 9.4 million episodes of foodborne illness occur in the United States every year and 5.5 million (58%) are caused by Noroviruses (Hall et al., 2011; Scallan et al., 2011). Noroviruses belong to the genus Norovirus of the family Caliciviridae (Kapikian et al., 1997; Zheng et al., 2006). At least five genogroups (GI–GV) of Noroviruses have been recognized based on the amino acid identity in the capsid protein, VP1, with the human Noroviruses belonging to groups GI, GII, and GIV (Kapikian et al., 1997; Zheng et al., 2006). GI viruses have emerged as the most prevalent cause of disease (Hall et al., 2011; Scallan et al., 2011).

The prototype human Calicivirus, Norwalk Virus, has a 7.7 kb positive sense single-stranded RNA genome that encodes three open reading frames (ORFs) (Kapikian et al., 1997). ORF 1 encodes a 200 KDa polyprotein, which is cleaved by the 3C-like cysteine protease of the virus into six non-structural proteins necessary for viral replication, one of which is the viral protease. The absolute requirement of the protease of Noroviruses (NoV pro), makes it an attractive target for antiviral intervention (Blakeney et al., 2003; Hardy et al., 2002; Tiew et al., 2011). A crystal structure of the Norwalk virus 3C protease has been solved and mutational analysis has identified the amino acids critical for activity (Nakamura et al., 2005; Someya et al., 2008; Someya and Takeda, 2011; Zeitler et al., 2006). A peptide mimic inhibitor of the Southport Norovirus 3C protease has been modeled into a crystal structure that provides further insight into functional residues (Zeitler et al., 2006).

We have developed a highly active fluorescence-based in vitro assay to quantitatively measure the activity of NoV pro for both the GI and GII viruses that is notably more robust than most previously reported assays (Belliot et al., 2003; Blakeney et al., 2003; Chang et al., 2012; Hardy et al., 2002; Hussey et al., 2011; Scheffler et al., 2007; Tiew et al., 2011; Someya et al., 2008; Someya and Takeda, 2011; Zeitler et al., 2006). This assay operates in a 96-well format with low enzyme concentrations, produces a high signal-to-background ratio, is highly reproducible, and was used to characterize buffer, ion, and pH requirements of the protease, which have not been systematically examined in previous reports. To provide a basis for the mechanisms involved in the observed effect on activity, we have determined that these components affect catalytic activity ($k_{cat}$), but not binding affinity for the peptide substrate ($K_m$).

Our examination of the published crystal structure of NoV pro revealed a potential ATP binding motif. Since NoV pro is hypothesized to directly participate in the viral replicase (Belliot et al., 2005, 2008; Cancio-Lonches et al., 2011; Machín et al., 2009; Wei et al., 2001), we investigated the interaction of RNA oligomers representing the 3’ and 5’ ends of the viral genome and NTPs with the viral protease. We provide the first evidence for direct RNA
binding by NoV pro and demonstrate that RNA binding efficiently inhibits protease activity. These observations provide initial evidence for a defined role of NoV pro in the viral replicase, possibly similar to that observed for Rhinovirus and Poliovirus (Andino et al., 1993; Hammerle et al., 1992; Leong et al., 1993; Matthews et al., 1994).

Results

Cations and buffer composition reduce NVpro activity

Nearly all previously published human Norovirus protease (NoV pro) assays have utilized 50–100 mM sodium phosphate buffer, pH 8.0 or greater, NaCl at 100–150 mM, enzyme concentrations of 2 μM or greater, and relatively low substrate:enzyme ratios (less than 10) (Belliot et al., 2003; Blakeney et al., 2003; Chang et al., 2012; Hardy et al., 2002; Hussey et al., 2011; Scheffler et al., 2007; Tiew et al., 2011; Someya et al., 2008; Someya and Takeda, 2011; Zeiter et al., 2006). However, there is little information on the relative effect of alternative assay conditions, or a systematic examination of reaction conditions and components. The standard buffer composition for the NoV pro activity assay in the current study (10 mM HEPES, pH 7.6, 0.1% CHAPS, 5 mM DTT, 30% glycerol) was based on empirical observations that examined the effect of pH, detergent, buffering agent, and commonly utilized cations.

Activity of NoV pro from both genogroup I (GI) and II (GII) viruses was examined and was found to be essentially identical overall. Protease activity was maximal at pH 8.5–9.0, but strong protease activity at more physiologically relevant levels was retained (Fig. 1A). At pH 7.6, the GI pro retained 73% and the GII pro retained 63% of the activity at the optimal pH (Fig. 1A). Protease activity in HEPES was at least 2-fold higher than in Tris–HCl or NaPO₄ across a range of pH values (Fig. 1B,C). Protease activity at buffer concentrations higher than 10 mM was reduced for all three buffering agents examined at pH 7.6 (Fig. 1C).

For the GI pro, NaCl inhibited activity 2-fold at approximately 90 mM, but higher concentrations up 300 mM had little additional effect (Fig. 2A). KCl, MgCl₂, MgSO₄, CaCl₂, and MnCl₂ were more inhibitory, inducing a 2-fold reductions at as little as 5–10 mM in some instances, and up to 10-fold at higher concentrations (Fig. 2A,B). Relative to the GI protease, the GII enzyme appeared to be slightly more sensitive to NaCl and MgCl₂ and slightly less sensitive to MgSO₄ and CaCl₂ (Fig. 2A,B). Data for zinc is not included as addition at any concentration induced precipitation.

While the addition of CHAPS and DTT only modestly enhanced GI pro activity (with a greater apparent effect on GI pro at lower concentrations) (Fig. 2C,D), DTT and CHAPS were included in the final reaction buffer to potentially aid solubility and stability of the enzyme. EDTA at concentrations up to 0.5 μM, and DMSO at concentrations of up to 5%, did not affect protease activity (data not shown).

In the standard assay buffer, NoV pro concentrations as low as 0.25 μM consistently produced signals at least 2-fold higher than background (no enzyme) control reactions (Fig. 3, top panel). Enzyme concentrations of 0.5 μM and 1.0 μM were much superior to lower concentrations. Maximal efficiency of the reaction was observed at a 100-fold excess of substrate relative to NoV pro (Fig. 3, bottom panel).

NVpro binds RNA which inhibits protease activity

Previous studies have provided evidence that Norovirus and other Calicivirus proteases participate in the viral replicase complex either as the mature cleaved protein (pro) or the un-cleaved precursor protein (propol) (Belliot et al., 2005, 2008; Cancio-Lonches et al., 2011; Machin et al., 2009; Wei et al., 2001) but no distinct role for protease in this complex has been demonstrated. Our examination of the amino acid sequence and three dimensional structure of NoV pro (SitePredict™, http://sitepredict.org) indicated the presence of a potential ATP binding pocket defined by the following amino acids: M107, I109, Q110, R112, V114, S118, L121, G133, T134, I135, P136, G137, D138, C139, H157, A158, A159, A160, T161, K162, S163, G164, N165, T166, V167, V168. The effects of NTPs on protease activity were subsequently examined. All four NTPs appeared to weakly inhibit GI pro activity (IC₅₀ 1.2–2.9 mM), but did not inhibit activity substantially further at increasing concentrations up to 10 mM.
RNA oligonucleotides (14-mer) representing both polarities of the 3' and 5' ends of the viral genome were also examined for interactions with NoV pro. In contrast to the NTPs, all RNA oligonucleotides efficiently inhibited the activity of the GI pro in a dose responsive manner at 1000-fold lower concentrations (IC_{50} 3.5–5.5 µM), reducing activity 10-fold or more at 20–25 µM (Table 1, Fig. 4A). The GI pro was similarly inhibited by the presence of the RNA oligomer representing the anti-genomic polarity 5' end (5'SRS) at the same concentration range (IC_{50} 6.9 ± 0.4 µM), demonstrating that this is likely a general feature of the human Norovirus proteases. The K_{i} for the 5'SRS oligomer with the GI pro was 5.2 ± 0.3 µM.

To determine if more natural RNAs inhibited the NoV protease, a 600nt RNA encoding for GI pro from pET32Nvpro3C-1 was utilized. This RNA inhibited GI pro activity substantially more efficiently, and at more physiologically relevant concentrations, than the 14-mer oligonucleotides (Table 1). To determine if this RNA represented evidence for sequence preference, a 660nt RNA initiated from the from the same T7 promoter in the pET-32a vector was used. This non-NoV related, plasmid RNA inhibited GI pro with equal efficiency (Table 1), indicating that length, not sequence, was responsible for the more potent inhibition. GI pro was also inhibited by the 658nt GI pro RNA at the same low concentration range (IC_{50} 22 ± 0.5 nM; IC_{50p} 62 ± 1.6 nM), as was GI pro (IC_{50p} 9.3 ± 1.0 nM; IC_{50p} 29.5 ± 2.1 nM).

**Discussion**

In this report, we present the first evidence of direct binding of RNA by human Norovirus proteases. Furthermore, the binding of RNA by these viral enzymes inhibits protease activity in a dose responsive manner. While NTPs also appeared to inhibit protease activity in a concentration range (IC_{50}, 2–22 nM), reducing activity 10-fold or more at 20–25 µM (Table 1, Fig. 4A). The GI pro was similarly inhibited by the presence of the RNA oligomer representing the anti-genomic polarity 5' end (5'SRS) at the same concentration range (IC_{50} 6.9 ± 0.4 µM), demonstrating that this is likely a general feature of the human Norovirus proteases. The K_{i} for the 5'SRS oligomer with the GI pro was 5.2 ± 0.3 µM.

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**NoV pro directly binds RNA**

Incubation of the GI pro with the ^{32}P-labelled 5'SRS RNA oligomer induced a migration shift in acrylamide gel electrophoresis (Fig. 5). This is the first evidence presented for RNA binding by a human Norovirus protease. RNA binding by other viral 3C proteases, such as Rhinovirus and Poliovirus, is well established (Andino et al., 1993; Hammerle et al., 1992; Leong et al., 1993; Matthews et al., 1994).

**RNA and cations affected the rate of reaction but not affinity for the peptide substrate**

To provide a basis for potential mechanisms responsible for observed differences in protease activity in the presence of RNA and under various assay conditions, enzyme kinetics and parameters for NoV pro were determined. Overall, K_{m} and k_{cat} for the GI and GI pro were essentially identical under the standard assay conditions (Table 2, Fig. 6A). The presence of 5.0 µM RNA (5' sense oligonucleotide) did not significantly affect the relative affinity of the GI pro for the peptide substrate (K_{i}) (p > 0.1), but reduced the relative rate of reaction (k_{cat}) over 2-fold, consistent with a pattern of non-competitive inhibition (Table 2, Fig. 6D).

Differences in the relative activity of the GI pro under different assay conditions also appeared to be due to changes in k_{cat} and not alterations of K_{m} (Table 2, Fig. 6B). For the GI pro, k_{cat} was 3-fold greater in Tris buffer at pH 8.8 than at pH 7.6, resulting in a 3-fold increase in enzymatic efficiency. At pH 7.6, k_{cat} was 2-fold higher in HEPES relative to Tris–HCl, for the GI pro, resulting in more than a 2-fold increase in reaction efficiency. The reasons for these differences are currently unclear. The effects of pH may potentially be due to differences in overall charge of the GI and GI enzymes (GI pl = 7.87, GI pl = 8.74) and/or overall charge of the substrate (−3.2 at pH 7.6 vs. −4.0 at pH 8.8) (Protein Calculator™, v3.3, www.scripps.edu).

The effects of two cations at concentrations that reduced protease activity approximately 2-fold were also studied. In HEPES, pH 7.6, the presence of 100 mM NaCl or 10 mM MgCl_{2} did not affect K_{m} significantly, but reduced k_{cat} and enzymatic efficiency significantly (Table 2, Fig. 6C).
activity, the concentrations required were 1000-fold greater than that observed for several RNA oligomers, and the lack of dose response beyond 2-fold inhibition indicates a relatively weak, non-specific interaction overall.

RNA binding by other viral 3C proteases, such as the Poliovirus and Rhinovirus proteases, is well established (Andino et al., 1993; Hammerle et al., 1992; Leong et al., 1993; Matthews et al., 1994). Unlike that observed for the Picornavirus proteases, there currently appears to be little evidence for RNA sequence specificity for the Norovirus enzymes, although RNA length appears to play an important role in the interaction. These issues need to be further investigated. Norovirus and other Calicivirus proteases have been implicated as being a crucial part of the viral replicase (Belliot et al., 2005, 2008; Cancio-Lonches et al., 2011; Machin et al., 2009; Wei et al., 2001) but, no distinct role for the protease in this complex has been demonstrated. Our data provides initial evidence for a distinct role, possibly similar to that observed for the Poliovirus and Rhinovirus proteases in template recognition (Andino et al., 1993; Hammerle et al., 1992; Leong et al., 1993; Matthews et al., 1994).

Unlike that observed for the human Norovirus proteases in our studies, RNA binding by the Polioivirus and Rhinovirus proteases does not affect protease activity, presumably due to RNA binding sites that are distant and well-displaced from the catalytic site (Andino et al., 1993; Hammerle et al., 1992; Leong et al., 1993; Matthews et al., 1994). The amino acids involved with RNA binding by NoV pro have yet to be determined and are the subject of ongoing investigations in our laboratory. It is possible that, for Noroviruses, the RNA binding motif overlaps, or is adjacent to, the catalytic site, which would explain the observed inhibition. However, the non-competitive pattern of the inhibition of protease activity by RNA is more consistent with adjacent binding motifs. Previous studies have suggested that the un-cleaved precursor protein (propol) composed of the Norovirus protease and polymerase is active in the viral replicase since it possesses both protease and polymerase activity (Belliot et al., 2005, 2008; Cancio-Lonches et al., 2011; Machin et al., 2009; Wei et al., 2001). In this case, inhibition of viral protease activity to prevent the self-cleavage of propol under ionic conditions that favor Norovirus polymerase activity, such as the presence of magnesium, sodium, as well as the presence of RNA, would be favorable to the virus.

The systematic investigation of assay and enzyme storage conditions, which has not been previously reported for the human

Fig. 3. Effect of enzyme and substrate concentration on NoV pro activity. Protease assays were conducted in standard assay buffer as described in the methods section. Data for triplicate reactions at each time point are presented. Comparisons of different enzyme concentrations (top panel) were performed at a constant enzyme:substrate ratio of 1:100. Comparisons of enzyme:substrate ratios (bottom panel) were conducted using 1.0 µM enzyme.
Table 1

Potency of RNA oligomers and NTPs against NoV protease activity.

<table>
<thead>
<tr>
<th>Oligonucleotides (14-mer)</th>
<th>IC50 (µM)</th>
<th>IC90 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-End, sense</td>
<td>5.5 ± 0.1</td>
<td>23.3 ± 1.1</td>
</tr>
<tr>
<td>5′-End antisense</td>
<td>4.8 ± 0.5</td>
<td>18.6 ± 1.2</td>
</tr>
<tr>
<td>3′-End, sense</td>
<td>5.4 ± 0.1</td>
<td>21.5 ± 0.2</td>
</tr>
<tr>
<td>3′-End antisense</td>
<td>3.5 ± 0.2*</td>
<td>10.7 ± 0.9**</td>
</tr>
</tbody>
</table>

Nucleotides

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>ATP</th>
<th>UTP</th>
<th>CTP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1500 ± 91</td>
<td>1252 ± 40</td>
<td>2900 ± 416***</td>
<td>1752 ± 23</td>
</tr>
</tbody>
</table>

RNAs

<table>
<thead>
<tr>
<th>RNAs</th>
<th>IC50 (µM)</th>
<th>IC90 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NoV pro (642nt)</td>
<td>0.013 ± 0.001</td>
<td>0.046 ± 0.003</td>
</tr>
<tr>
<td>pET32 (589nt)</td>
<td>0.017 ± 0.002</td>
<td>0.062 ± 0.002</td>
</tr>
</tbody>
</table>

NTPs or RNAs were pre-incubated with GI pro in standard assay buffer for 15 min. Substrate was then added and the reaction incubated for an additional 30 min at 37 °C as described in the methods section. IC50 (2-fold reduction of NoV pro activity relative to control) and IC90 (10-fold reduction of NoV pro activity relative to control) values (± standard deviations) were calculated by linear regression analysis (MS Excel). For the longer length RNAs, ‘NoV pro’ specifies the RNA encoding the GI pro gene in pET32NVpro3C-1, and ‘pET32’ specifies non-NoV RNA from the parent vector, pET32.

* p < 0.01 (t test) vs. other nucleotides.
** p < 0.001 (t test) vs. other nucleotides.
*** p < 0.001 (t test) vs. other nucleotides.

** IC90 not calculated due to lack of sufficient inhibition (see Fig. 3).

Norovirus protease, has resulted in one of the most robust human Norovirus protease assays reported to date (Belliot et al., 2003; Blakney et al., 2003; Chang et al., 2012; Hardy et al., 2002; Hussey et al., 2011; Scheffler et al., 2007; Tiew et al., 2011; Someya et al., 2008; Someya and Takeda, 2011; Zeitler et al., 2006). Importantly, the optimization of assay conditions in this report allows for the efficient conduct of studies with this important viral enzyme to be conducted at a more physiologic pH (7.2–7.6) than those previously used (8.0–9.0).

Most previous NoV pro assays utilized conditions which we have demonstrated to be suboptimal or inhibitory. In previous assays, this has necessitated the use of 5- to 20-fold more enzyme per reaction, and much smaller substrate to enzyme ratios (0.1–10), the latter of which we have shown limits assay dynamic range. Most previous assays used reaction buffers containing 50 mM sodium phosphate (or Tris–HCl) with 100–150 mM NaCl, and enzyme stored in 10–30% glycerol in the presence of 100–150 mM NaCl in sodium phosphate buffer. We attribute the increased activity of the current assay to the (i) use of higher (50%) glycerol concentrations, HEPES, and the lack of cations for enzyme storage, (ii) the absence of cations in the reaction buffer, (iii) lower concentrations of buffering agent, and (iv) the use of HEPES instead of sodium phosphate or Tris–HCl as the buffering agent.

In our studies, cations, pH, and buffer composition affected the relative rate of reaction (kcat), but not affinity for the peptide substrate (Km). Similarly, the presence of RNA did not affect substrate binding substantially but reduced the rate of reaction, consistent with a pattern of non-competitive inhibition.

The basis for the increased activity in the current investigations may be attributed to the significant improvement in kcat/Km. For example, kcat values in our studies at pH 7.6 were comparable to those observed at pH 8.0 for a human GI pro in a recent report (Chang et al., 2012), but Km values in our studies were approximately 40-fold lower, resulting in a proportionately greater efficiency of reaction (kcat/Km). The kcat values in our studies at pH 7.6 were 2- to 6-fold less than those observed for a Southampton virus protease (a GI Norovirus) at pH 8.5 (Zeitler et al., 2006), but Km values in our studies were 16- to 80-fold lower.

Fig. 4. Effect of RNA and NTPs on NoV pro activity. Oligonucleotides and NTP’s were pre-incubated with GI pro in standard assay buffer for 15 min as described in the methods section. Data are presented as a percentage of the control (1% DMSO) reaction (set at 100%). Maximum net RLU (after background subtraction) for each panel were A, 2298; B, 1960.

Fig. 5. RNA binding by NoV pro. Products were electrophoresed on a 12% non-denaturing PAGE gel. Incubations with GI pro and 32P-labelled RNA (14-mer, 5′ end, genomic polarity) were in standard assay buffer at 37 °C for 15 min prior to electrophoresis. For each lane, 20 pM 32P-labelled RNA was used with the indicated amount of NoV pro. RNA was radio-labeled using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s instructions.
Conclusion

We present in this paper a well-characterized, highly active, assay for the analysis of protease activity of human Norovirus 3-C like proteases. Enzymatic efficiency of proteases from both a GI and GII virus were essentially identical. We have, for the first time, systematically characterized the buffer component requirements for these enzymes, and determined that these components affect catalytic activity ($k_{cat}$), but not binding affinity for the peptide substrate ($K_m$) which provides a basis for exploring the mechanisms related to these effects. We also present the first demonstration of RNA binding by a human Norovirus protease, providing a basis for a potential definitive role for its participation in the viral replicase, perhaps similar to that observed for the Rhinovirus and Poliovirus proteases. RNA binding inhibits Norovirus protease activity, distinguishing this interaction from that observed in Picornavirus 3C proteases.

Experimental procedures

Construction of the expression vectors

A clone, pETSumoNVpro (strain Hu/GI.1/8FIIa/1968/USA, accession number JX023285), obtained from K.O. Chang (Kansas State University, USA), was used as the source material for the GI protease. The NoV protease (NoV pro) gene was amplified from pETsumoNVpro using the forward primer NdeNVp (5'-TCAT-CAAGCTT CATTCTAGTGCGGTTTCGCCCTC-3') and the reverse primer HindNVp (5'-TCATCAAAGCTTCATT CTTAGTGCGGTTTCGCCCTC-3'). NdeNVp introduced an NdeI site and a hexahistidine tag into the 5' end of the amplicon. HindNVp introduced a stop codon after the NVprotease reading frame, a HindIII site, and six additional nucleotides to the 3' end of the amplicon. To facilitate efficient restriction digestion of the 5' end, an additional six nucleotides were added to the 3' end by re-amplifying the

Table 2

Kinetic analysis of NoV pro activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ ($s^{-1} \cdot 10^{-3}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1} \cdot s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI</td>
<td>18.37 ± 0.82</td>
<td>5.2 ± 0.10</td>
<td>283</td>
</tr>
<tr>
<td>GI</td>
<td>23.11 ± 2.35**</td>
<td>5.1 ± 0.20</td>
<td>224</td>
</tr>
<tr>
<td>GI</td>
<td>20.33 ± 2.71</td>
<td>2.3 ± 0.10*</td>
<td>113</td>
</tr>
<tr>
<td>GI</td>
<td>31.05 ± 2.09</td>
<td>6.5 ± 0.11***</td>
<td>209</td>
</tr>
<tr>
<td>GI</td>
<td>22.74 ± 1.71</td>
<td>0.9 ± 0.01*</td>
<td>40</td>
</tr>
<tr>
<td>GI</td>
<td>26.65 ± 2.95</td>
<td>2.0 ± 0.10*</td>
<td>75</td>
</tr>
<tr>
<td>GI</td>
<td>20.66 ± 2.86</td>
<td>2.7 ± 0.10*</td>
<td>101</td>
</tr>
</tbody>
</table>

Analysis was performed in triplicate.

* $p < 0.001$ (two-tailed t test, GraphPad™, GraphPad Software, Inc.) vs. 10 mM HEPES, pH 7.6.

** $p < 0.001$ (two-tailed t test vs. 10 mM Tris–HCl, pH 7.6). $K_m$ values were not statistically different ($p > 0.05$).

Fig. 6. Kinetics of NoV pro reaction. Reactions (average values from triplicate analyses) are displayed for GI pro comparing (Panel A) GI pro and GII pro in standard assay buffer (panel B) HEPES vs. Tris–HCl at pH 7.6, (panel C) effect of NaCl and MgCl$_2$. Panel D: Lineweaver-Burk analysis of the effect of RNA (14-mer, 5' end, genomic polarity) on GI pro activity. All graphs were created with GraphPad™.
amplicon with the primers NdeNVp2 (5′-CATCAACATATGCACTCATCAACATCACC-3′) and HindNvp. Both the pET-32a vector and the full length NVpro amplicon were digested with NdeI and HindIII, purified, and ligated to form the pET32NVpro3C-1 expression construct.

The GII NoV pro gene was cloned from the cDNA clone pGEMT-Easy-NV41 (strain Hu/GIL/4/ Ast6139/01/Sp, accession number AJ583672), provided by F. Parra (Universidad de Oviedo, Spain) using the forward primer NV3C-F (5′-TGCATACATCACCATCACCATACCGCCCAACAAAGACATGGTC-3′) and the reverse primer NV3C-R (5′-GCAGAATTCCTCATTAGTGGACCTCCCCCT-3′). The forward primer contained a hexadistidine tag along with a 5′-TG di-nucleotide that formed a start codon after ligation into the end-filled NdeI site in the vector. The pET32a vector was digested with NdeI and filled in with T4 DNA polymerase to form a blunt end, and subsequently digested with EcoRI. The cloned NVpro fragment was digested with EcoRI and ligated into the pET32a vector to form the pET32NVpro3C-2 expression construct. For both NVpro constructs, DH5α Escherichia coli were transformed and selected for with 100 μg/ml ampicillin.

The GII NoV propol gene was cloned from the same plasmid (provided by F. Parra) into the same vector, and using the same strategy used for the GII protease gene using the forward primer NV3C-F (5′-TGCATACATCACCATCACCATACCGCCCAACAAAGACATGGTC-3′) and the reverse primer NVPrp-R (5′-GCAGAATTCCTCATTCCGACCCATCTCATCCTCA-3′) to form the pET32NVpropol3C-2 expression construct. Clones from resistant colonies for all constructs were sequenced to ensure successful insertion and orientation.

Protein expression and purification

Expression and purification of NoV proteases was performed following previously described procedures (Yon et al., 2011). Briefly, E. coli BL21 Star (DE3) cells transformed with the individual protease expression constructs were grown at 37°C in 750 ml of Luria broth containing 100 μg/ml ampicillin. Protein expression was induced with IPTG. Cells were harvested, lysed by sonication, centrifuged, and the supernatant incubated with Talon metal affinity resin (Clontech Laboratories, Inc.). The resin was then washed, and the fusion protein was eluted with imidazole. Eluted fractions were checked for purity by SDS-PAGE and quantified by spectrophotometry.

Fractions of highest concentrations were pooled and dialyzed against storage buffer (10 mM HEPES, pH 7.6, 50% glycerol, 5 mM DTT), and aliquots were stored at either 4°C or -20°C. Enzyme yield was at least 20 mg/750 ml culture and was pure (>95%) of other detectable proteins following analysis in SDS-PAGE. Studies on the storage of NoV pro demonstrated that the presence of 50% glycerol is required for long term stability (data not shown). Currently, enzyme activity upon storage is stable for at least 4 weeks at 4°C and at least 12 months at -20°C or -70°C.

NoVpro expression assay

The protease assay depends on the sequence-specific cleavage of the 14 amino acid FRET peptide substrate, Edans-EPDHLQG-PEDLAK-Dabcyl, between Q and G (Hardy et al., 2002) by active NoV protease and the subsequent detection of fluorescence. The substrate was custom synthesized commercially (New England peptide, Inc.). The standard assay reaction consists of 10 mM HEPES (pH 7.6), 0.1% CHAPS, 10 mM DTT, 30% glycerol, 0.5–1.0 μM NoVpro enzyme and 25–100 μM substrate peptide (50–100:1 relative to NVpro). On addition of the substrate, the reaction was incubated at 37°C, and fluorescence was measured at excitation and emission maxima of 360 and 460 nm respectively in a SPECTRAMax® GEMINI-EM Fluorescence microplate reader ( Molecular Devices Inc.).

Analysis of enzyme parameters

Kinetic analysis of the enzyme was performed in 1X assay buffer (10 mM HEPES, pH 7.6, 0.1% CHAPS, 10 mM DTT, 30% glycerol) using 1.0 μM enzyme and 3.9–125 μM substrate peptide. Fluorescence formed over 20 min was monitored at 1.0 min intervals at 37°C. Measured fluorescence was converted to products formed in μM, using a standard curve of free EDANS. kcat and Km were determined by non-linear regression analysis using Prism software (v5.0a, Graphpad Software Inc.).

For this report, RNA was considered to be an inhibitor of NoV proteases, not a substrate. To determine the dissociation constant (Kd) of RNA oligonucleotides, Km and kcat analysis of NVpro was carried out in presence of 0, 1.25, 2.5 and 5.0 μM 5′RS RNA. Kd was determined by non-linear curve fit into non-competitive mode of inhibition using Prism software. Changes in kinetic parameters of the enzyme under differing pH and buffer conditions were analyzed in 10 mM Tris–HCl, 0.1% CHAPS, 10 mM DTT, and 30% Glycerol at pH 7.6 and 8.8. Statistical analysis of enzyme parameters was assessed by two-tailed t test (GraphPadTM, GraphPad Software, Inc).

Transcription and purification of RNAs

The HPLC-purified, 14-mer RNA oligonucleotides used for this study were manufactured by IDT, Inc. A 642nt RNA encoding the GI pro gene was transcribed from the T7 promoter of pET32NVpro3C-1 (linearized with HindIII). A non-NoV related, 589nt RNA was transcribed from the T7 promoter of the parent vector, pET32 (linearized with HindIII). Both RNAs were produced using the MEGAscript T7 kit (Life Technologies, Inc.) following the manufacturer’s instructions. Transcription reaction products were extracted twice by phenol:chloroform:isoamyl alcohol (1:1:1), precipitated with 0.3 mM sodium acetate, pH 5.5 and ethanol, the pellets washed twice with 70% ethanol to remove salts, re-suspended in RNase-free water (Ambion, Inc.), and stored at -70°C.

Gel shift RNA binding studies

RNA (14-mer, 5′ end, genomic polarity) was 32P-labeled using T4 polynucleotide kinase (New England Biolabs, Inc.) according to the manufacturer’s instructions. For each reaction, 20 μM 32P-labelled RNA was incubated with different amounts of NoV GI pro in standard assay buffer at 37°C for 15 min prior to electrophoresis. The reactions were then immediately subjected to electrophoresis on a 12% non-denaturing PAGE gel in TBE buffer.

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References