Substrate specificity of Tulane virus protease

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ABSTRACT

Tulane virus (TV) is a cultivable calicivirus isolated from rhesus monkeys. In this study, we characterized the substrate specificity of TV protease in trans using recombinant proteases and TV polyprotein fragments containing the predicted proteolytic cleavage sites. Cleavage products have been obtained from 4 of the 5 fragments containing 573Q–S574 between the helicase and 3A-like protein, 712E–A713 between the 3A-like protein and Vpg, 802E–G803 between Vpg and the protease, and 976E–G977 between the protease and RdRp. We also characterized the enzymatic activities of the recombinant proteases of TV and Norwalk virus using synthetic fluorogenic peptide substrates. Under optimal conditions for enzymatic assays, partial cross-reactivities on reciprocal substrates were observed between TV and Norwalk virus proteases. The apparently shared substrate specificities between TV and Norwalk virus proteases suggested that the cultivable TV could be used as a model for in vivo evaluation of lead candidates of protease inhibitors for human norovirus.

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Introduction

Caliciviruses (CVs) are small, non-enveloped, positive-stranded RNA viruses. Members of the CV family can cause a wide variety of diseases in humans and animals, including respiratory infections, vesicular lesions, gastroenteritis, and hemorrhagic diseases. Norovirus (NoV) belongs to one of the five genera of CVs which mainly infects humans and causes acute gastroenteritis in all age groups (Glass et al., 2009). Currently, there are no effective treatments or vaccines available against NoVs. The lack of a cell culture system or animal model for NoVs has been a major hurdle for studies of NoV vaccines available against NoVs. The reason for NoVs’ need of a cell culture system is that the infection of human cells is very slow, and the cell culture conditions are not compatible with human cell physiology. The virus inactivated easily by conditions that are compatible with human cells (Oka et al., 2005). The CV non-structural polyprotein is known to be processed into six functional proteins by the viral 3C-like protease (Blackeney et al., 2003), which plays an essential role in maturation of functional proteins and virus replication, as well as pathogenesis. The crystallographic structures of several NoV proteases have been elucidated (Hussey et al., 2010; Nakamura et al., 2005; Zeitler et al., 2006). These data suggest that NoV proteases form dimers (Nakamura et al., 2005; Zeitler et al., 2006) and display a trypsin or chymotrypsin-like serine protease fold, involving a nucleophilic cysteine thiol (at amino acid residue 139) in the catalytic triad (Zeitler et al., 2006). Two other critical residues in the catalytic site, completing the catalytic triad, are a histidine at position 30 and a glutamic acid at position 54 (Zeitler et al., 2006). Alignments of amino acid sequences for TV, NoV, and other CV proteases revealed that the critical residues in the catalytic triad are well conserved.

The proteases of the CV family cleave substrates between Glu or Gln in the P1 position and various amino acid residues, such as Ala and Gly in the P1’ position (Belliot et al., 2003; Blackeney et al., 2003; Joubert et al., 2000; Liu et al., 1999; Oka et al., 2005; Robel et al., 2008; Sosnovtsev et al., 2006, 2002; Wirlich et al., 1995). Amino acid residues adjacent to P1–P1’ positions may also affect substrate specificities and cleavage efficiencies (Oka et al., 2009; Someya and Takeda, 2009). Sequence alignments of the
nonstructural polyproteins of TV and other CVs with known polyprotein cleavage maps revealed the presence of putative cleavage sites in the TV polyprotein (Farkas et al., 2008). However, the precise proteolytic cleavages of TV polyprotein have not been analyzed to date.

Here we report an investigation into the proteolytic processing of the TV nonstructural polyprotein in vitro using E. coli–expressed protease and polyprotein fragments. N-terminal sequencing of four cleaved products indicated that these cleavages occur precisely at the predicted sites, which suggests conservation of the substrate specificities between the TV protease and other CV proteases. We also characterized the enzymatic activities of the recombinant TV protease and NoV protease using recombinant polyprotein fragments and fluorescence resonance energy transfer (FRET) assays. Our results demonstrated that the fluorescence-based assay is sensitive and reliable for the evaluation of CV protease activities, and that the cultivable TV could be used as a model for in vivo validation of lead protease inhibitor candidates.

## Results

### Expression and purification of recombinant proteases.
To obtain an adequate amount of proteases for enzymatic characterization, we expressed the TV and a NoV (the Norwalk virus) proteases as the GST or histidine (His) tagged fusion proteins, as suggested by Zeitler et al. (Zeitler et al., 2006). Both the GST– and His-tag proteins were soluble and readily purified using non-denaturing affinity purification methods. The purified enzymes appeared homogeneous by the SDS-polyacrylamide gel electrophoresis (PAGE) and FPLC analyses (Fig. 1). Purified proteases with the His-tag or after removal of the GST tag appeared as monomers in solution for both TV and Norwalk virus proteases by comparison with the chymotrypsinogen A standard (molecular weight of 20.4 kDa) in the FPLC analysis (data not shown).

### Proteolytic cleavage between the helicase and 3A-like protein.
To facilitate the proteolytic analysis of TV polyprotein, recombinant protein fragments containing different regions of the polyprotein were produced. To analyze the cleavage site between the helicase and 3A-like protein, the coding region for amino acid residues 495 to 795 was expressed as a MBP fusion protein (79.7 kDa; Fig. 2A, F2). Proteolytic cleavage of this protein by the TV protease yielded a 42 kDa band that was expected to be the MBP, a 32 kDa protein of the VpG–GST fusion protein, and an unknown band of ~19 kDa (Fig. 2C). The N-terminal sequencing of the 32 kDa protein revealed a sequence “AKGKT” that was the expected N-terminal sequences of the VpG-GST fusion protein.

### Proteolytic cleavage between the 3A-like protein and Vpg.
Due to a small size upon the cleavage by protease, the Vpg protein (10.8 kDa) is difficult to isolate from a PAGE gel for N-terminal sequencing analysis. Hence, a double fusion protein containing a protein fragment from amino acid residues 632 to 779 with the predicted cleavage site flanked by the MBP and GST at the N– and C-termini, respectively (Fig. 2A, F3) was expressed. Proteolytic cleavage of this protein by the TV protease yielded a 42 kDa band that was expected to be the MBP, a 32 kDa protein of the VpG-GST fusion protein, and an unknown band of ~19 kDa (Fig. 2C). The N-terminal sequencing of the 32 kDa protein revealed a sequence “AKGKT” that was the expected N-terminal sequences of the VpG-GST fusion protein.

### Proteolytic cleavage between the TV protease and RdRp.
The TV polyprotein fragment containing the cleavage site between Vpg and protease was fused to MBP at the N-terminus (Fig. 2A, F4). To prevent potential auto-cleavage of the protease, we replaced residue C134 with an A134 at the catalytic site of the protease. Since the cleaved protease fragment from F4 would be the same size as the active protease in trans, we fused GST to the C-terminus of the protease. Upon cleavage, two cleavage products, the 52.2 kDa MBP–Vpg C-terminus and the 44.7 kDa protease-GST, were produced. N-terminal sequencing analysis of the 44.7 kDa fragment resulted in a perfect match to all of three amino acid residues detected with the predicted N-terminal sequence of TV protease (Fig. 3A).

### Proteolytic cleavage between the protease and RdRp.
The TV polyprotein fragment containing the cleavage site between the protease and RdRp (Fig. 2A, F5) was fused to GST at the N-terminus. The cleavage of the 96.9 kDa F5 fragment was expected to generate at least five smaller fragments: the 52.9 kDa RdRp, the 18 kDa protease, the 70.9 kDa protease–RdRp, the 44 kDa GST–protease, and the 26 kDa GST. The N-terminus sequencing of the 52.9 kDa fragment (RdRp) showed a perfect match of the first five amino acid residues GKTYY (Fig. 3B) with that of the predicted RdRp.

### Proteolytic cleavage between the NTP and helicase proteins.
We have also attempted to express a protein fragment containing the protease cleavage site between the N-terminal protein and helicase protein using similar fusion expression strategies (Fig. 2A, F1). Unfortunately, despite tremendous efforts, we were

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**Table 1**

Primers used for the amplification of NoV protease, TV protease, and TV polyprotein fragment cDNAs.

| Primers | Sequence | Restriction enzyme site | Orientation | Amplifications |
|---------|----------|-------------------------|-------------|---------------|
| P1405b  | CCGCGGATCCGCTCCCCCAGACTATGGAGCGGAG | BamHI | Forward | NoV protease |
| P1406   | ATGGTGCACCTGTCACCTGAGTGGTTGGCCTGG | Sall | Reverse | TV protease |
| P1069c  | CCGCGGATCCGTGTGTTCTTTTGTCTTC | BamHI | Forward | TV protease |
| P1071b  | ATACTGTCAGTCACCTCCTCAAGGGTGAACTAGAG | Sall | Reverse | TV 3A1 |
| p1282b  | ATGTCCTGACCTCAAAACCAAGCGTAACCTGCACGAC | KpnI | Forward | Polyprotein F1 |
| p1283b  | ATGTCCTGACCTCAAAACCCCTAAGGGCAGTATAGTCGAC | Sall | Reverse | Polyprotein F2 |
| P1095   | ATGTCGACATGTCATGTCGTATATTACAGGAC | BamHI | Forward | Polyprotein F3 |
| P1077   | ATGTCGACATGTCATGTCGTATATTACAGGAC | Sall | Reverse | Polyprotein F4 |
| P1403   | TGGAAATCACCAGAACATCTGGAAAGAGGCAC | EcoRI | Forward | Polyprotein F5 |
| P1890   | TGGATGGATCCTGTCACCTGAACTGACTGGCGG | BamHI | Forward | Polyprotein F1 |
| P1891   | ACTGGTACCTCAGATGAATATCCAGAGC | Sall | Reverse | Polyprotein F2 |
| P1069   | CCGCGGATCCGCTCCCCCAGACTATGGAGCGGAG | BamHI | Forward | Polyprotein F3 |
| P1071c  | ATGTCGACATGTCATGTCGTATATTACAGGAC | Sall | Reverse | Polyprotein F4 |
| P1069   | CCGCGGATCCGCTCCCCCAGACTATGGAGCGGAG | BamHI | Forward | Polyprotein F5 |
| P1079   | ATGTCGACATGTCATGTCGTATATTACAGGAC | Sall | Reverse | Polyprotein F5 |
unable to express any protein fragment containing the C-terminal portion of the N-terminal protein in either GST- or MBP- fusion, or his-tagged forms.

**Characterization of the enzymatic activities of the TV and Norwalk virus proteases with fluorogenic peptide substrates.** As demonstrated in the above polyprotein cleavage analysis, E.coli-expressed proteases remain active upon purification, which makes possible to develop an in vitro antiviral screening system. To this end, we evaluated the Norwalk virus protease activities and the TV protease activities using synthetic fluorogenic peptide substrates NVS1 and TVS2 (Fig. 4A). In the presence of 2 nM NVS1, as shown in Fig. 4(B), a dose response of enzyme activities was observed in the reactions containing less than 5 μM of protease, which was followed by a plateau at protease concentrations between 5 and 20 μM. A similar fluorescence emission profile was also observed when the fluorogenic peptide substrate TVS2 was hydrolyzed by TV protease (Fig. 4C). These results indicate that Hylite Fluor 488/QXL520-labeled peptide substrates are ideal for measuring recombinant Norwalk virus and TV protease activities.

To determine the optimal reaction conditions for Norwalk virus protease activity assay, we also performed the assays at various pH, salt, glycerol and DMSO concentrations. At a constant ionic strength (50 mM NaCl), the optimal pH for proteolytic cleavage of Norwalk virus protease was found to be pH 8.5 (Fig. 5A), similar to that reported previously for NoV (Someya et al., 2005) and for the NS3 protease of Dengue-2 virus (Leung et al., 2001). The Norwalk virus protease activities decreased significantly as the NaCl concentration rose (from 50 mM to 300 mM, Fig. 5B). In contrast, glycerol in the reaction solution seemed to promote protease activities (Fig. 5C). Based on these observations, we used this buffer condition (50 mM Tris, pH 8.5, 20% glycerol, and 1 mM DTT) for all of our protease activity assays. DMSO at concentrations less than 10% did not affect either the Norwalk virus or TV protease activities.

**The TV and Norwalk virus proteases can partially cleavage heterologous substrates.** Although the TV and NoV proteases share less than 27% amino acid sequence identity, the catalytic triads are highly conserved (Fig. 6A), indicating that the TV and NoV proteases may be able to process each other’s polyprotein substrates. To test this hypothesis, we incubated the TV and Norwalk virus proteases with each other’s substrates, and found that both enzymes were able to cleave each other’s substrates, although the relative enzymatic activities were around 30–40%, compared with that on their native substrates (Fig. 6B and C).

**Inhibition of the Norwalk virus protease activities.** Since CV proteases are cysteine proteases that display a trypsin or chymotrypsin-like serine protease specificity, we tested whether common cysteine and serine protease inhibitors could inhibit the enzymatic activities of the Norwalk virus protease (Fig. 7A). Among five inhibitors tested: E64, leupeptin, phenylmethyl sulfonyl fluoride (PMSF), 1-Chloro-3-tosylamido-4-phenyl-2-butanone (TPCK), and 1-Chloro-3-tosylamido-7-amino-2-heptanone HCI (TLCK), four – leupeptin, TPCK, TLCK, and PMSF – revealed suppressive effects on NoV protease activities, with an IC50 of 18.8 μM for TPCK (Fig. 7B) and 175.1 μM for TLCK (Fig. 7C). These results are consistent with a report by Blakeney et al. (Blakeney et al., 2003).

**Discussion**

Many RNA viruses encode their functional proteins in a single open reading frame, where the proteins are translated as a polyprotein prior to being processed by a viral protease into individual functional proteins. Thus, the viral protease plays an essential role in virus replication and pathogenesis. Like that of picornaviruses, the CV proteases utilize a cysteine residue at its catalytic site as a nucleophile (Zeitler et al., 2006). Based on sequence alignments of several CV nonstructural proteins, we previously predicted the

![Fig. 1. Purification of NoV and TV proteases. (A) Lane 1&2, purified GST-NoV protease fusion protein expressed at room temperature and at 37 °C; Lane 3-6, NoV protease released by thrombin digestion under various conditions. Lane 7, GST protein bound on the beads. Lane 8, and 9, protein markers. Lane 10, purified GST-TV protease fusion protein. Lane 11, TV protease released by thrombin digestion. (B) FPLC profile of purified TV protease.](image-url)
location of the TV protease in the TV genome (Farkas et al., 2008).
In this study we have demonstrated that both the GST-fusion and
his-tagged TV proteases were functionally active using an in trans
assay on recombinant polyprotein fragments and synthetic
substrates. The precise boundaries of the protease were also mapped
by the N-terminal sequencing analysis.

In a parallel experiment, we also cloned and expressed the TV
protease with upstream sequences including the cleavage site for
the TV protease, and demonstrated that the TV protease is capable
of auto-cleavage and could release itself from the polyprotein. It is
also worthy to note that the expressed TV protease is able to
cleave the synthetic peptide containing nine amino acid residues,
suggesting that amino acid residues beyond P5 or P5' may be
dispensable for substrate processing by the protease. Successful
cleavage of a substrate containing seven amino acid residues by a
NoV protease has also been reported (Chang et al., 2012).

The cleavage maps for the nonstructural proteins of several
CVs revealed that the substrate cleavage sites of CV proteases are
well conserved in this virus family, in which a Glu or Gln is
usually present in the P1 position, whereas an Ala or Gly occupies
the P1’ position. Based on this conservation of substrate cleavage
sites, we proposed a cleavage map for the TV nonstructural
polyprotein (Farkas et al., 2008). In this study, four of the five
predicted cleavage sites of the TV polyprotein have been experi-
mentally validated using recombinant polyprotein fragments by
in trans cleavage followed by N-terminal sequencing. The clea-
vage sites between Vpg and protease, and between protease and
RdRp share the same dipeptide residues of E/G. The amino acid
residues at the other two sites seemed variable with Q/S between
the helicase and 3A-like protein and E/A between 3A-like protein
and Vpg. The cleavage at Q/S or E/A sites seems to be more
efficient than at the E/G sites, according to the band intensities of
the cleaved products (data not shown). Although the cleavage site
between N-terminus protein and NTPase could not be validated
by the N-terminal sequence analysis, the Q/S scissile bond at
amino acid residues 233–234 remains to be the primary can-
didate for the cleavage, because this was the only one could
potentially be cleaved by TV protease within the adjacent 133
amino acid residues. Despite a lack of example for other calici-
virus proteases, our result on the cleavage between helicase and
3A-like protein suggested that the Q/S scissile bond could be an
efficient cleavage site for CV protease (Fig. 2).

In addition to the differences in scissile bonds at various cleavage
sites, amino acid residues at P2-5 and P2’-5’ also influence the
cleavage efficiencies of proteases (Nakamura et al., 2005; Oka et al.,
2009). As shown in Table 2, the protease cleavage sites of poly-
proteins from the CV family are well conserved. It is interesting to
note that a Q at the P1 position appears more often in between the
N-terminal protein and helicase, and between the helicase and 3A-
like protein; whereas an E at the P1 position appears more often
between Vpg and the protease and between the protease and RdRp.
Vpg, protease, and RdRp have been known to co-exist as precursor
protein fragments during the early stage of polyprotein processing
(Belliot et al., 2003). The differential preference for specific amino acid
residues at the P1 position could be one of the regulatory mechan-
isms in the control of polyprotein processing.

![Fig. 2. Expression of TV polyprotein fragments for protease cleavage site mapping. (A) Predicted TV non-structural polyprotein and cloning strategy for mapping analysis. Each fusion protein contains at least one predicted protease cleavage site, as indicated by an arrow. Open boxes represent TV polyprotein fragments; Black boxes represent GST; Grey boxes represent MBP. (B) Cleavage of fragment 2 (helicase-3Alike). Lane 1, protein markers. Lane 2 & 4, undigested fragment 2. Land 3 & 5, digested fragment 2. Lane 4 & 5 were detected with rabbit anti-3A like antibodies. The arrow indicates the protein fragment isolated for N-terminal sequencing analysis. The results of the N-terminal sequencing were shown in the bracket. (C) Cleavage of fragment 3 (3A like-VpG). Lane 1, protein markers. Lane 2, undigested fragment 3. Land 3, digested fragment 3, bound fraction. Lane 4, digested fragment 3, supernatant fraction. The arrow indicates the protein fragment isolated for N-terminal sequencing analysis. The results of the N-terminal sequencing were shown in the bracket.](image-url)
In this study, we also characterized the enzymatic activities of recombinant TV protease and a NoV protease using FRET substrates. FRET protease assays have been used in characterizations of several viral proteases, such as those of the SARS-Coronavirus (Blanchard et al., 2004; Grum-Tokars et al., 2008), hepatitis C virus (Mao et al., 2008; Santos et al., 2009), yellow fever virus (Kondo et al., 2011), and foot-and-mouth disease virus (Jaunlet et al., 2007). During the preparation of the manuscript, Chang et al. also reported the characterization and inhibition of proteases of two norovirus genogroups using FRET assays (Chang et al., 2012). Unlike other reports on FRET protease activity assays, the Hylite Fluor488 dye and QXL520 quencher were used to label the peptide substrates in our study. Hylite Fluor488 is a new type of fluorophore that provides superior fluorescence quantum yield and a longer emission wavelength, whereas QXL520 has been proven to be an efficient quencher for Hylite Fluor488. The Hylite Fluor 488/QXL520-based FRET peptide shows less interference from autofluorescence of test compounds, thus providing a better assay sensitivity. Our results showed that this newly developed fluorescent dye and quencher pair was effective for the assay. Optimization experiments with the NoV protease showed that the protease was mostly active at a pH between 8 and 8.5, without the need for additional ionic salts. This optimal pH is very similar to that reported for the Chiba virus in NoV genogroup II (Someya et al., 2005). While the presence of glycerol enhanced the protease activities, a low concentration of DMSO (up to 10%) did not affect the enzyme activities. This is important for our future studies in the screening for inhibitors against the NoV protease, because most synthetic compounds are dissolved in DMSO. We also found that the TV and NoV proteases can cleave each other’s substrate in the FRET assays. Since NoVs remain uncultivable in vitro, the observed reciprocal enzymatic activities between the NoV/TV proteases suggest that TV could serve as a surrogate for NoVs in future antiviral designs targeting at the protease.

In summary, we have four out of the five cleavage sites of the TV polyprotein confirmed by protein N-terminal sequencing analysis. We also established a convenient FRET assay system, which will allow us to screen compound libraries for antivirals.

Fig. 3. Mapping of VpG-Pro and Pro-RdRp cleavage sites. (A) Cleavage of fragment 4 (VpG-protease). Lane 1, protein markers. Lane 2, undigested fragment 4. Lane 3, digested fragment 4, supernatant fraction. Lane 4, partially digested fragment 4, bound fraction. The arrow indicates the protein fragment isolated for N-terminal sequencing analysis. The results of the N-terminal sequencing were shown in the bracket. (B) Cleavage of fragment 5 (pro-RdRp). Lane 1, protein markers. Lane 2 & 3, digested fragment 5. Lane 4, undigested fragment 5. The arrow indicates the protein fragment isolated for N-terminal sequencing analysis. The results of the N-terminal sequencing were shown in the bracket.

Fig. 4. Determination of enzymatic activities of NoV and TV proteases. (A) Fluorogenic peptide substrates NVS1 (HyliteFlour488–EPDFHLQLQGPEEDLAKE–K (QXL520)–NH2), derived from the cleavage site between N-terminal protein and helicase of NoV polyprotein; and TVS2 (Hylite Flours–VWKMQSDEY–K(QXL520)–NH2), derived from the cleavage site between helicase and 3A-like of TV polyprotein. (B) Enzymatic activities of NoV protease, and (C) Enzymatic activities of TV protease.
targeting the proteases of NoV/TV. In addition, we have demonstrated specific inhibition of NoV/TV proteases by conventional cysteine protease inhibitors. Furthermore, we have demonstrated the reciprocal substrate cleavage between TV and NoV proteases. Thus, we are in an excellent position to utilize the TV as a model for in vivo screening and evaluation of candidates of protease inhibitors for the uncultivable human NoVs.

Methods

Fluorogenic substrates. Fluorogenic peptide substrates—NVS1 (Hylite Fluor488-EPDFHLQGPEDLAKE-K(QXL520)-NH2) and TVS2 (Hylite Fluor488-VWKMQSDEY-K(QXL520)-NH2) – were designed based on the cleavage site between the N-terminal protein and helicase of the NoV polyprotein (Belliot et al.

Fig. 5. Effects of pH, NaCl, glycerol, and DMSO on enzymatic activities of NoV protease. (A) Effect of pH. % of protease activity was determined by comparing relative fluorescence units at various reaction conditions to the one with the highest activities (100%). (B) Effect of ionic strength. Same conditions as A at pH 8.5 with varying ionic strength (NaCl: 50 mM, 65.6 mM, 81.3 mM, 112.5 mM, 175 mM, and 300 mM). (C) Effect of glycerol concentration. Same conditions as A at pH 8.5 with addition of glycerol at 0, 3.75, 7.5, 10, 15, and 20%. (D) Effect of DMSO. Same conditions as A at pH 8.5 with addition of DMSO at 0, 0.5, 1, 2, 4, 6, 8, and 10%.

Fig. 6. Predicted secondary structures and active site residues in TV protease, and the reciprocal substrate cleavages of the TV and NoV proteases. (A) Predicted secondary structures and active site residues in TV protease. The predicted beta strands are represented by green arrows, whereas predicted loops and helical turns are shown in blue and red, respectively. Residues conserved or conservatively substituted (while conserving physico-chemical properties) between NoV and TV proteases are highlighted using yellow background. Active site residues are highlighted in red, and residues in contact with a peptide inhibitor in 2IPH (and thus defining the overall substrate binding cleft) are shown in blue. Notice that the active site and substrate binding cleft are within regions conserved between NoV and TV proteases (yellow patches). (B) The relative enzymatic activities of NoV protease on TV protease substrate TVS2, in comparison to on its native substrate NVS1. (C) The relative enzymatic activities of TV protease on NoV protease substrate NVS1, in comparison to on its native substrate TVS2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
et al., 2003) or the cleavage site between the helicase and 3A-like protein of the TV polyprotein (Farkas et al., 2008), respectively. Both substrates were synthesized and labeled by Anaspec (CA). The two fluorophores on each substrate form a quenching pair and exhibit FRET within the intact peptide. Upon the cleavage between Q and G residues on NVS1 by NoV protease, or between Q and S residues on TVS2 by TV protease (Fig. 4A), the donor fluorophore (Hylite Fluor488) separates from the quenching fluorophore (QXL520), allowing activities of protease to be measured. Stock solution of the fluorogenic substrate was prepared in dimethyl sulfoxide (DMSO), and subsequent dilution was prepared with the assay buffer (50 mM Tris (pH 8.5), 20% glycerol, and 1 mM DTT).

Generation of protease expression constructs. To express the glutathione S-transferase (GST)-protease fusion proteins in E. coli, protease coding sequences were amplified from NoV cDNA (Genbank#: NC_001959) with primers p1405B and p1406 (Table 1), and TV cDNA (Genbank#: EU391643) with primers p1069c and p1071b (Table 1), respectively, and were cloned into vector pGEX4T1 (GE Health-care, PA) at BamHI/SalI sites. To express N-terminal histidine (his)-tagged proteases, the same inserts were cloned into vector pQE30 (Qiagen, CA) at the identical cloning sites.

Generation of recombinant TV polyprotein fragment expression constructs. To express TV polyprotein fragments containing predicted protease cleavage sites in the forms of maltose binding protein (MBP) or GST-fusion proteins, the corresponding TV cDNAs (Genbank#: EU391643) were amplified with specific primers (Table 1), and cloned into vector pMAL-c2X (New England Biolab, MA) or vector pGEX4T1 (GE Health-care, PA) (Fig. 2A).

Protein expression and purification. GST-protease fusion proteins, GST-polyprotein fragment fusion proteins, and MBP-polyprotein fragment fusion proteins were expressed in E.coli BL21 (DE3) plysS (EMD Millipore, MA) under the induction of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at room temperature. The his-tagged proteases were expressed in E. coli M15 (Qiagen, CA) under the identical induction and culture condition. The cells were harvested by centrifugation at 5000 × g for 15 min.

For GST-fusion protein purification, the cell pellets were resuspended in 1X PBS with 1 mM phenylmethylsulfonyl fluoride (PMSF). For MBP-fusion protein purification, the cell pellets were resuspended in column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) with 1 mM PMSF. For his-tagged protease purification, the cell pellets were resuspended in cell resuspension buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole) with 1 mM PMSF. The cell resuspensions were subjected to sonication, then cell lysates were centrifuged at 12,000 × g for 60 min. MBP-, GST-fusion and his-tagged proteases were purified by affinity chromatography using amylose resins (New England Biolabs, MA), Glutathione Sepharose 4B resins (GE Healthcare, PA), or Talon cell trus affinity resins (Clontech, CA).

Purified GST-fusion proteases were further digested with thrombin and subsequently purified with gel permeation chromatography using a Superdex 200 gel filtration column (GE Healthcare, PA). The elution profile was monitored by measuring UV absorbance at 280 nm and the elution fractions were collected and dialyzed with protease buffer (50 mM Tris–HCl, pH7.6, 1 mM EDTA, 50 mM NaCl, 1 mM DTT) (Blakeney et al., 2003), while his-tagged proteases were dialyzed with buffer containing 50 mM NaH2SO4, pH4.5, 50 mM NaCl, 10 mM mercaptoethanol (Zeitler et al., 2006). Proteases were then stored at −80 °C in small aliquots. The purity of the proteins was verified with SDS PAGE and protein concentrations were determined using the Bradford protein concentration assay (BioRad, CA).

Protease activity analysis using fluorogenic peptide substrates. For protease activity assays, 2 μM of each protease was mixed with 10 nM of fluorogenic peptide substrate NVS1 or TVS2 in protease buffer, and were transferred to black 96-well non-treated microtiter plates (Corning, NY), with final reaction
volume of 50 μl/well. The reaction mixtures were incubated for 60 min at 37 °C. The fluorescence was detected using Multi-mode detector 880 (Beckman Coulter, CA) with excitation at 480 nm and emission at 520 nm, respectively. The protease activities were determined from averages of duplicate or triplicate tests.

Optimization of reaction conditions. To determine the effects of pH, ionic strength, glycerol, and dimethyl sulfoxide (DMSO) on the enzymatic activities of the NoV protease, the concentrations of individual buffer components in the standard protease activity assay conditions described above were adjusted. The pH profile was determined at constant ionic strength with a pH range from 5.0 to 9.0, using a variety of buffers suitable for various pHs: 50 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM EDTA, 50 mM 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 10 mM imidazole) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell resuspensions were then subjected to sonication, and the cell lysates were then centrifuged at 12,000 × g for 60 min. The recombinant protein was purified by affinity chromatography using TALON His-Tag Purification Resin (Clontech). Mouse anti-TV3AI polyclonal antibodies were generated follow a conventional antibody generation procedure.

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Table 2

| Virus          | N-terminal protein/NTPase | NTPase/3A-like protein | 3A-like protein/Vpg | Vpg/protease | Protease/RdRp | Reference               |
|---------------|--------------------------|------------------------|---------------------|--------------|---------------|------------------------|
| hNoV (GI-Southampton) | LQ/GP                   | LQ/GK                  | ME/GK              | FE/AP        | LE/GG         | Liu et al. (1999)       |
| hNoV (GII-MD145)   | LQ/GP                   | LQ/GP                  | TE/GK              | FE/AP        | LE/CD         | Belliot et al. (2003)   |
| MNV             | AE/GP                   | LO/NK                  | SE/GK              | FE/AP        | FQ/GP         | Sosnovtsev et al. (2006) |
| TV              | (PQ/SP)                 | MQ/SD                  | FE/AK              | DE/GY        | LE/GK         | Farkas et al. (2008)    |
| FCV             | SE/DV                   | AE/NG                  | SE/AK              | EE/SG        | /             | & this paper            |
| Sapovirus       | AQ/GP                   | EQ/AG                  | EE/EA              | EE/AP        | /             | Oka et al. (2005, 2009)  |
| RHDV            | VE/GV                   | FE/CA                  | DQ/GV              | YE/GL        | ME/GK         | Joubert et al. (2000), Wirblisch et al. (1995) |
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