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Functional consequences of mutational analysis of norovirus protease

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ABSTRACT

Norovirus protease has been subjected to an extensive mutagenesis study. Ala-scanning mutation at 13 different positions (Trp6, Trp19, Thr27, Leu86, Leu95, Leu97, Met101, Gln117, Leu121, Thr134, Tyr143, Val144, and Val167) led to loss of function and/or stability. Considering the crystal structure of the protease, it was revealed that a hydroxyl group of Thr134 and an aromatic ring of Tyr143 were important for substrate recognition along with His157. It was notable that several of the residues identified were in close proximity to each other, suggesting their importance for the integrity and stability of the protease.

1. Introduction

Noroviruses are a major cause of non-bacterial acute gastroenteritis in humans, and genetically and antigenically diverse strains have been isolated worldwide [1,2]. Norovirus, a member of the family *Caliciviridae,* has a positive-sense single-stranded RNA genome which is ~7.7 kb in length with a poly(A) tail at its 3′-end. Genome-linked viral protein (VPg) is likely to be bound to the 5′-end in place of the cap structure [3].

The genome encodes three open-reading frames (ORFs) [1,2]. The ORF1 product is a polyprotein and is cleaved by its viral 3C-like protease activity into six non-structural proteins including 2C-like nucloside triphosphatase, 3B VPg and 3D RNA-dependent RNA polymerase in addition to 3C-like protease [1,2]. The ORF2 and ORF3 products are a major and a minor structural protein (VP1 and VP2), respectively. The norovirus virion consists of 180 virion structural protein 1 (VP1) molecules with the VPg-linked RNA genome and VP2 molecules inside. The sequence diversity of the P2 domain of VP1 is correlated with the wide variety of antigenicity among noroviruses [4,5] and potentially provides different patterns of binding to histo-blood group antigens [6].

Norovirus 3C-like protease is a central enzyme that is solely responsible for the maturation of ORF1 polyprotein [7,8] and has significant similarity with proteases of other caliciviruses and picornavirus 3C proteases. Norovirus protease is a potent target of medication for the treatment of norovirus gastroenteritis.

Our extensive mutagenesis study of 3C-like protease of the Chiba strain revealed that His30 and Cys139 were responsible for proteolysis [9]. The X-ray crystal structure revealed that the norovirus 3C-like protease had a chymotrypsin-like fold and that Glu54 was possibly involved in the catalysis [10,11]. A previous mutagenesis study showed that Arg8, Lys88, Arg89, Asp138, and His157 were sensitive to Ala mutation [9]. Arg89 and Asp138 were salt-bridged with each other, and His157 played a critical role in substrate recognition [9,10]. In order to obtain information on an enzyme–substrate interaction and the mechanism of proteolysis, we here complete our mutagenesis study that includes Ala-scanning mutagenesis of 106 of 181 residues constituting the protease (Table 1). Thirteen residues are identified as important, and their significance is discussed.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli JM109 and DH5α strains (Nippon Gene Co., Ltd., Tokyo, Japan) were used for plasmid construction. The BL21-CodonPlus(DE3)-RIPL strain (Stratagene, La Jolla, CA) was used for the expression of the recombinant proteins.

Abbreviations: VPg, genome-linked viral protein; VP, virion structural protein; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-galactopyranoside; PBS, phosphate-buffered saline

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To assess the effects of mutations on proteolytic activity in *E. coli* cells, an expression plasmid pGEX-2TK-3aBC (Fig. 1) [12] was used. All mutations were introduced into pGEX-2TK-3aBC using mutagenic oligonucleotides (Supplementary Tables S1 and S2). All mutations were first detected based on the appearance or disappearance of restriction sites, and then verified by DNA sequencing.

2.2. Expression of the recombinant proteins in E. coli cells and SDS–PAGE analysis

E. coli BL21-CodonPlus(DE3)-RIPL cells harboring each of the pGEX-2TK-3aBC plasmids were grown on 4 ml of MagicMedia *E. coli* Expression Medium (Invitrogen, Carlsbad, CA) at 37 °C for ~16 h, harvested and resuspended in 600 μl of phosphate buffered saline (PBS) containing 1.0% (v/v) Triton X-100. Cells were disrupted by vigorous vortexing in the presence of glass beads (0.1 mm diameter). After centrifugation at 15,000×*g* for 10 min, the supernatant was transferred to a new tube. Proteins were determined with a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) using BSA as a standard.

Extracted proteins were separated by SDS–PAGE, followed by Coomassie Brilliant Blue staining or Western blotting with rabbit anti-glutathione *S*-transferase (anti-GST) antibody (Abcam, Cambridge, MA), rat anti-VPg or mouse anti-protease antisera [13].

For several mutants, transformed *E. coli* cells were also grown on 4 ml of 2× YT broth at 37 °C until OD at 610 nm reached around 0.5. After the addition of isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM, protein expression was induced for 2 h. Cells were resuspended in 100 μl of PBS containing 1.0% (w/v) Triton X-100. Proteins were extracted in the same manner as described above.

2.3. GST binding assay

Cell extract containing 500 μg of protein was applied to a Glutathione Sepharose 4B MicroSpin column (GE Healthcare, Piscataway, NJ). Glutathione-resin-bound proteins were eluted with 50 μl of 50 mM Tris–HCl (pH 8.0) containing 10 mM reduced glutathione. A portion (5 μl) of the eluates was analyzed by SDS–PAGE.

Table 1
Target amino acid residues in the 3C-like protease of the Chiba strain for Ala-scanning mutagenesis.

Amino acids	Completely conserved[a]	Highly conserved[b]	Less conserved[c]
Pro	2, 33, 136, 142	3, 66, 96	22, 43, 78
Val	20, 31, 82, 153, 156, **167**	9, 24, 72, 81, 99, 152, 168	10, 36, 64, 114, **144**, 171
Leu	73, **86**, **97**, **121**, 122, 132, 180	94	5, 68, **95**, 113, 135
Ile	26	49, 87, 109	32, 44, 47, 85, 104
Phe	40, 55, 60	12, 18, 25	39, 58
Tyr	**143**		145
Trp	**6**, 16, **19**		151
Ser	7, 21	14, 84, 91	46, 61, 106, 118, 163
Thr	**27**, 123, **134**, 166	161	4, 23, 28, 29, 34, 56, 69, 178, 179
Cys	**139**[d]	77, 169	83, 154
Met	**101**, 120	71, 130	107
Asn	126, 165	149	148
Gln	**117**	110	57, 172

Numbers indicate positions in the amino acid sequence of the 3C-like protease of the Chiba strain. The positions whose Ala mutation led to a loss of activity and/or a decreased level of expression are indicated by *bold letters*.

[a] Amino acid residues conserved in all noroviruses from genogroup I (human), II (human), III (bovine) and V (murine), whose nucleotide sequences are available in public databases.

[b] One of two kinds of amino acid residues is located at the positions indicated.

[c] Over three kinds of amino acid residues are found.

[d] The Cys139 residue has previously been shown to be essential for proteolysis [9].

3. Results

3.1. Ala-scanning mutagenesis

In this study, 106 of 181 amino acid residues constituting the 3C-like protease of the Chiba strain were subjected to Ala-scanning mutagenesis as targets (Table 1). Initially, transformed *E. coli* cells were grown in MagicMedia. As a result, Ala mutation at six positions (Trp6, Leu95, Leu97, Leu121, Thr134 and Val167) affected the protease activity (Fig. 2). The W6A, T134A and V167A mutants had absolutely no activity, whereas the L95A, L97A and L121A mutants retained very slight activity since very faint amounts of GST-3a and GST-3aB could be observed (Fig. 2). The amount of the V167A mutant fusion was extremely low, and the mutation might make the protease unstable. In Fig. 2 (and also Fig. 3), some very faint bands could be discerned in some samples. The bands of about 48 kDa were identified as the GST-3aB intermediate, but other faint bands were not detected by Western blotting with anti-GST antibody (data not shown). Therefore, these might be non-specifically eluted bacterial proteins and were not subjected to further analysis.

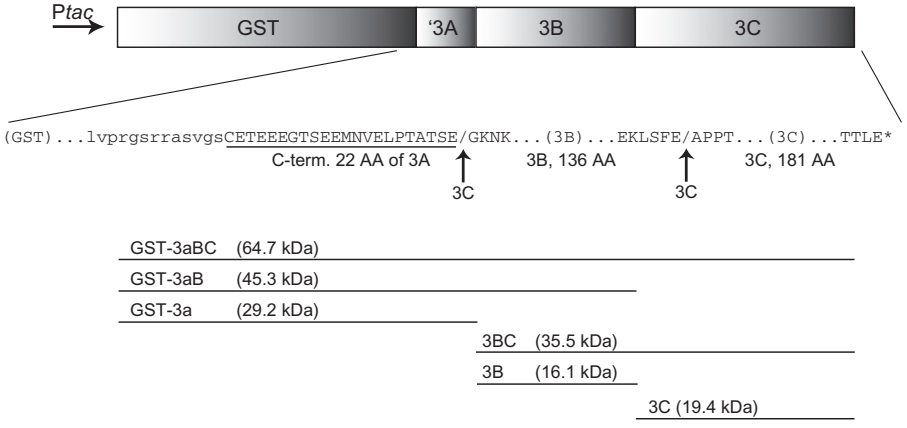

Fig. 1. Plasmid construction of pGEX-2TK-3aBC. The gene fragments encoding the 22 C-terminal amino acid residues of 3A and the entire 3B VPg and 3C-like protease were fused in-frame to the GST gene of the pGEX-2TK vector. The fusion protein, GST-3aBC, has two protease recognition sites indicated by *arrows*. The calculated molecular weight of the parental protein and possible intermediates along with the final products are shown.