Structural view of the 2A protease from human rhinovirus C15

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The majority of outbreaks of the common cold are caused by rhinoviruses. The 2A protease (2A^{pro}) of human rhinoviruses (HRVs) is known to play important roles in the propagation of the virus and the modulation of host signal pathways to facilitate viral replication. The 2A^{pro} from human rhinovirus C15 (HRV-C15) has been expressed in *Escherichia coli* and purified by affinity chromatography, ion-exchange chromatography and gel-filtration chromatography. The crystals diffracted to 2.6 Å resolution. The structure was solved by molecular replacement using the structure of 2A^{pro} from coxsackievirus A16 (CVA16) as the search model. The structure contains a conserved His–Asp–Cys catalytic triad and a Zn²⁺-binding site. Comparison with other 2A^{pro} structures from enteroviruses reveals that the substrate-binding cleft of 2A^{pro} from HRV-C15 exhibits a more open conformation, which presumably favours substrate binding.

1. Introduction

Human rhinoviruses (HRVs) are positive-stranded RNA viruses belonging to the *Enterovirus* genus within the *Picornaviridae* family. Most patients suffering from the common cold test positive for rhinoviruses, suggesting that rhinoviral infections are the main cause of the common cold. Until a decade ago, only two groups of HRVs were known: human rhinovirus A (HRV-A) and human rhinovirus B (HRV-B). A novel group of HRVs was discovered in 2006 with a genetic difference from previously known rhinoviruses. Almost half of the HRV infections found in young children could be attributed to this new group of HRVs, named human rhinovirus C (HRV-C; Lamson *et al.*, 2006). HRV-C can cause more severe respiratory infections in infants and children compared with both HRV-A and HRV-B, and is closely associated with acute exacerbations of asthma (Bizzintino *et al.*, 2011; Drysdale *et al.*, 2014; Miller *et al.*, 2009).

Amongst the proteins encoded by the genomes of picornaviruses, the function of the 2A protein appears to be the most diverse. For instance, the 2A protein functions as a proteinase in enteroviruses, playing an important role in processing the viral polyprotein (Hsu *et al.*, 2007). In contrast to this role, the 2A protein of *Foot-and-mouth disease virus* (FMDV) appears to be a short peptide made up of ~20 amino-acid residues that plays a role in the regulation of gene expression (Donnelly *et al.*, 2001). Surprisingly, the role of the 2A protein from

Table 1

Macromolecule-production information.

The restriction-enzyme cleavage sites are underlined in the primers. The sequence of HRV-C15 2A^{pro} is underlined; the GST tag and PreScission protease site add an extra 234 residues to the N-terminus of this construct.

Source organism	Human rhinovirus C15
DNA source	Commercially synthesized
Forward primer	5'-CCGGAATTCGGACCGAGCATGTTT GT-3'
Reverse primer	5'-CCGCTCGAGTTGGTGTCTGACAAGCT TG-3'
Cloning vector	pGEX-6p-1
Expression vector	pGEX-6p-1
Expression host	<i>E. coli</i> BL21 (DE3) strain
Complete amino-acid sequence of the construct produced	MSPILGYWKIKGLVQPTRLLEYLEEKYEE HLYERDEGDKWRNKKFELGLEFPNLPYY IDGDVKLTQSMATIRYIADKHNMLGGCP KERAISMLEGAVLDIRYGVSRISYKSD FETLKVDFLSKLPEMLKMFEDRLCHKTY LNGDHVTHPDFMLYDALDVVLYMDPMCL DAFPKLVCFFKRIEAIPIQIDKYLKSSKY IAWPLQGWQATFGGGDHPKSDLEVLFO GPLGSPFEGPSDMFVHTRDAIYKCAHLT NPTDETILLALTADLQVDSTNVPGPDVI PCCDCTAGCYYSRSKDRYFPVECVSHDW YEIQESGYYPKHIQYNLLIGEGHCEPGD CGGKLLCKHGVIGMITAGGDNHVAFTDL RPYSSLSEHQ

Hepatitis A virus has been switched from its usual role as a nonstructural protein to that of a structural protein. The protein participates in the assembly of viral particles (Graff *et al.*, 1999; Wang *et al.*, 2015). Thus, elucidation of the structures of 2A proteins from different picornaviruses and their analyses could provide clues to the divergence of the 2A protein as well as to the evolution of picornaviruses.

Although the 2A proteins from HRVs are multifunctional enzymes, they all exhibit protease activity, which holds the key to the multiple functions that they exert. Consequently, 2A proteins from HRVs not only play a role in the maturation of the viral polypeptide, but also cleave host cellular factors, resulting in shutting down host protein production and the scuttling of immune responses so that the virus can divert resources to the production of its own viral proteins (Lu *et al.*, 2012; Molla *et al.*, 1993; Yu *et al.*, 1995). The 2A protease (2A^{pro}) specifically cleaves homologues of the human eukaryotic initiation factors eIF4GI and eIF4GII, which are required for cap-dependent mRNA translation by the ribosome (Lamphear *et al.*, 1993; Liebig *et al.*, 2002). eIF4G is part of the initiation-factor complex eIF4F, which comprises the unwinding protease eIF4A and the cap-binding protein eIF4E. The complex recruits capped cellular mRNA to the ribosome for translation. Cleavage of eIF4G by HRV 2A^{pro} impairs this process and shuts down cap-dependent translation of mRNA. Since the initiation of protein synthesis by picornaviruses is not cap-dependent, but requires the internal ribosome-entry site (IRES) present in the 5'-UTR of the picornavirus mRNA, shutting down the host cap-dependent translation machinery does not affect the synthesis of picornavirus proteins (Belsham & Sonenberg, 1996; Jackson *et al.*, 1990; Jang *et al.*, 1990).

Currently, the crystal structures of 2A^{pro} from HRV-A2 (Petersen *et al.*, 1999), enterovirus 71 (EV71; Mu *et al.*, 2013; Cai *et al.*, 2013) and CVA16 (Sun *et al.*, 2013) are known. The solution NMR structures of 2A^{pro} from coxsackievirus B4 (CVB4; Baxter *et al.*, 2006) and HRV-C2 have also been solved. However, quite surprisingly, polyclonal antibodies raised against CVA16 2A^{pro} could cross-react with HRV-C15 2A^{pro} but not with HRV-C2 2A^{pro}, suggesting structural differences between the two closely related enzymes from the same HRV-C group of viruses (Lee *et al.*, 2014). Here, using X-ray crystallography, we have determined the three-dimensional structure of HRV-C15 2A^{pro} at 2.6 Å resolution. The structure reveals a snapshot of a conserved active site captured in an open conformation, which is similar to that observed for 2A^{pro} from CVA16. Although a transduced HeLa cell line (HeLa-E8) stably expressing CDHR3-Y₅₂₉ has recently been reported to support HRV-C propagation *in vitro*, the infection efficiency of HRV-C in the HeLa-E8 cell line is much lower than those of HRV-A and HRV-B (Bochkov *et al.*, 2015), which may limit the development of HRV-C vaccines and infectivity assays for antiviral drug screening. Our structure of 2A^{pro} from HRV-C15 could therefore possibly guide the design of inhibitors targeting HRV-C 2A^{pro} to facilitate the development of antiviral drugs.

2. Materials and methods

2.1. Macromolecule production

The DNA construct encoding the full-length gene for HRV-C15 2A^{pro} was commercially synthesized and inserted into the pGEX-6p-1 vector between EcoRI and XhoI restriction sites. Competent *Escherichia coli* BL21 (DE3) cells were transformed with the recombinant plasmid. The bacterial cultures were grown at 37°C to an OD₆₀₀ of about 0.6, and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to induce 2A^{pro} production at 16°C for 18 h. The cells were harvested and suspended in lysis buffer consisting of 20 mM HEPES pH 7.0, 50 mM NaCl, 10% glycerol. After ultrasonication, the lysate was centrifuged at 30 700g to remove cell debris. The supernatant was loaded onto a Glutathione Sepharose 4B column (GE Healthcare) equilibrated with lysis buffer. After extensive washing with lysis buffer to remove nonspecifically bound proteins, the 2A^{pro} bound to the column was subjected to overnight PreScission protease treatment at 4°C to remove the GST tag. The 2A^{pro} samples after the affinity-chromatography step were further purified by ion-exchange chromatography on a HiTrap Q column (GE Healthcare) eluted with a linear gradient of NaCl in 20 mM HEPES pH 7.0, 10% glycerol. Fractions corresponding to the major peak were collected, pooled and concentrated before being loaded onto a Superdex 75 gel-filtration column (GE Healthcare) equilibrated with lysis buffer. The final purified samples were concentrated to approximately 10 mg ml⁻¹ before screening for crystallization. Macromolecule-production information is summarized in Table 1.

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM HEPES pH 7.0, 50 mM NaCl, 10% glycerol
Composition of reservoir solution	0.2 M potassium chloride, 0.1 M magnesium acetate tetrahydrate, 0.05 M sodium cacodylate, 10% PEG 8000
Volume and ratio of drop	200 nl protein solution mixed with 200 nl reservoir solution
Volume of reservoir (μl)	100

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL-17A, PF
Wavelength (Å)	0.979
Temperature (K)	100
Detector	ADSC Quantum 315
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	0.2
Space group	C222 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	80.99, 85.37, 208.91
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.45
Resolution range (Å)	50.00–2.60 (2.69–2.60)
Total No. of reflections	200681
No. of unique reflections	22666
Completeness (%)	99.9 (99.8)
Multiplicity	8.9 (8.1)
$\langle I/\sigma(I) \rangle$	12.01 (3.89)
<i>R</i> _{merge}	0.153 (0.636)
Overall <i>B</i> factor from Wilson plot (Å ²)	37.27

2.2. Crystallization

An initial crystallization screen was performed using the hanging-drop vapour-diffusion method. Crystals of HRV-C15 2A^{pro} were grown at 16°C over a reservoir solution consisting of 0.2 M potassium chloride, 0.1 M magnesium acetate tetrahydrate, 0.05 M sodium cacodylate, 10% PEG 8000. Cryo-protection was carried out by soaking the crystals in a reservoir solution containing 20% glycerol prior to flash-cooling with liquid nitrogen. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

All diffraction data sets were collected on beamline BL-17A at the Photon Factory (PF) synchrotron facility in Japan using a CCD detector. The highest resolution attainable for the diffraction data was ~2.6 Å. The space group of the HRV-C15 2A^{pro} crystals was C222₁. Data-collection and processing statistics are given in Table 3.

2.4. Structure solution and refinement

There are four protein molecules in the asymmetric unit. The initial phases were obtained by the molecular-replacement method using *Phaser* v.2.1 (McCoy *et al.*, 2007). The crystal

Table 4

Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	50.00–2.60 (2.69–2.60)
Completeness (%)	99.8 (99.8)
No. of reflections, working set	21461
No. of reflections, test set	1156
Final <i>R</i> _{cryst}	0.165
Final <i>R</i> _{free}	0.215
No. of non-H atoms	
Protein	4225
Ligand/ion	4
Water	122
Total	4351
R.m.s. deviations	
Bonds (Å)	0.006
Angles (°)	1.096
Average <i>B</i> factors (Å ²)	
Protein	37.27
Ion	69.73
Ramachandran plot	
Favoured regions (%)	95.9
Additionally allowed (%)	4.1
Outliers (%)	0

Table 5

Combinations of superimpositions.

Superimposed proteins		Sequence identity (%)	R.m.s.d. (Å)
HRV-C15 2A ^{pro}	HRV-A2 2A ^{pro}	64	1.06
HRV-C15 2A ^{pro}	HRV-C2 2A ^{pro}	75	1.81
HRV-C15 2A ^{pro}	CVA16 2A ^{pro}	42	1.11

structure of CVA16 2A^{pro} (PDB entry 4mg3; Sun *et al.*, 2013) served as the search model. Manual model building and refinement were performed with *Coot* (Emsley *et al.*, 2010) and *phenix.refine* (Afonine *et al.*, 2012), including rigid-body refinement, TLS parameters and individual *B*-factor refinement. Solvent molecules were located from stereochemically reasonable peaks in the σ_A -weighted $2F_o - F_c$ difference electron-density map. The quality of the final model, which was refined to 2.6 Å resolution with an *R*_{work} value of 0.16 (*R*_{free} = 0.21), was verified using *PROCHECK* (Laskowski *et al.*, 1993). The final refinement statistics are summarized in Table 4. Structural figures were prepared with *PyMOL* (DeLano, 2002).

3. Results and discussion

3.1. Overall structure of HRV-C15 2A^{pro}

The crystal structure of HRV-C15 2A^{pro} was solved by molecular replacement with *Phaser*. We initially tried both the crystal structure of CVA16 2A^{pro} and the NMR structure of HRV-C2 2A^{pro} as search models for molecular replacement, and the CVA16 2A^{pro} structure finally gave a better solution, although the sequence identity between HRV-C15 2A^{pro} and HRV-C2 2A^{pro} is higher than that between HRV-C15 2A^{pro} and CVA16 2A^{pro} (Table 5). There are four protein molecules (designated *A*, *B*, *C* and *D*) in the asymmetric unit. The overall structure of all four molecules is very similar (r.m.s.d. < 0.25 Å). 137 residues of molecules *B* and *C* were finally

modelled (residues 1–137) and 136 residues of molecules *A* and *D* (residues 1–136). The structural analysis is therefore limited to molecule *B* because it has the most residues and is representative of all of the molecules present in the asymmetric unit. HRV-C15 2A^{pro} consists of two domains: an N-terminal domain comprising of a four-stranded sheet (bI2, cI, eI2 and fI) and a C-terminal domain made up of a six-stranded β -barrel (aII, bII1, cII2, dII, eII and fII) (Fig. 1*a*). The N-terminal domain is linked to the C-terminal domain by

a long inter-domain loop (residues 40–56). Within the N-terminal domain, a helical turn (Ala17–Leu19) connects cI to eI2. In the C-terminal domain, an antiparallel β -hairpin, formed by the bII2 and cII1 strands, is located next to the six-stranded β -barrel. Furthermore, this β -hairpin also makes close contacts with residues from the N-terminal domain. Three highly conserved residues, His18, Asp34 and Cys105, found in 2A^{pro} from all enteroviruses form the active site of HRV-C15 2A^{pro} (Figs. 1*b* and 1*d*). In addition, four conserved

Figure 1
Structure of 2A^{pro} from HRV-C15. (a) Overall structure of HRV-C15 2A^{pro} chain *B*. The N-terminal domain and the C-terminal domain of HRV-C15 2A^{pro} are shown in red and cyan, respectively. The zinc ion is represented as a magenta-coloured sphere. (b) The catalytic triad of HRV-C15 2A^{pro} is located beneath the bII2–cII1 loop. The active-site residues are shown as sticks. (c) A tetrahedral coordination site for the zinc ion in the HRV-C15 2A^{pro} structure. (d) The amino-acid sequences of 2A^{pro} from human rhinovirus C15 (HRV-C15), human rhinovirus C2 (HRV-C2), human rhinovirus A2 (HRV-A2), coxsackievirus A16 (CVA16), human enterovirus 71 (EV71) and poliovirus 1 (Polio1) were aligned using *ClustalW2* (Larkin *et al.*, 2007), and the result of the alignment was graphically displayed using *EScript* (Gouet *et al.*, 1999). Secondary-structure elements are indicated according to the structure of 2A^{pro} from HRV-C15. The red arrows show the conserved active site and the grey arrows show the Zn²⁺-binding site.

residues, Cys51, Cys53, Cys111 and His113, make up the zinc ion-binding site (Figs. 1c and 1d).

3.2. Comparison with other enterovirus 2A^{pro} structures

Exhibiting primary-sequence identities of about 64% to HRV-A2 2A^{pro}, 75% to HRV-C2 2A^{pro} and 42% to CVA16 2A^{pro}, the overall structure of HRV-C15 2A^{pro} shares a high degree of similarity with the 2A^{pro} proteins from all of these viruses. The structure of HRV-C15 2A^{pro} could be superimposed onto the structure of HRV-A2 2A^{pro} with an overall r.m.s.d. of 1.06 Å. Similarly, an overall r.m.s.d. of 1.11 Å was observed when the structure of HRV-C15 2A^{pro} was overlaid onto the structure of 2A^{pro} from CVA16, which causes human hand, foot and mouth disease (Fig. 2a). The N-terminal domain of HRV-C15 2A^{pro} is very similar to that observed in HRV-A2 2A^{pro}, except for some minor differences in the

orientations of the flexible loops. Compared with HRV-A2 2A^{pro}, HRV-C15 2A^{pro} possesses a shorter aII β -strand and a longer eII β -strand (Fig. 2b). The active site and the Zn²⁺-binding site are highly conserved between the two structures.

When compared with the CVA16 2A^{pro} sequence, the N-terminal domain of CVA16 2A^{pro} possesses two additional amino-acid residues (His25 and Asn26; Fig. 1d). This region forms an α -helical structure (AI) in CVA16 (Fig. 2c). The equivalent region in HRV-C15 2A^{pro} is occupied by a loop. The C-terminal domain of HRV-C15 2A^{pro} differs more than the N-terminal domain when compared with the structure of CVA16 2A^{pro}. Notably, HRV-C15 2A^{pro} has a longer bIII β -strand but a shorter bII2 β -strand, connected by a short bIII–bII2 loop. Furthermore, in the structure of CVA16 2A^{pro} the cII β -strand is very long and not only participates in the formation of the β -barrel in the C-terminal domain but also forms an antiparallel β -hairpin structure with the bII2

Figure 2

Comparison of the structure of HRV-C15 2A^{pro} with homologous structures. (a) Superimposition of 2A^{pro} homologues. Structures are shown as ribbons. (b) Superimposition of the structure of HRV-C15 2A^{pro} (shown in blue) with the structure of HRV-A2 2A^{pro} (shown in cyan). (c) Superimposition of the structure of HRV-C15 2A^{pro} with the structure of CVA16 2A^{pro} (shown in yellow). (d) Superimposition of the structure of HRV-C15 2A^{pro} with the structure of HRV-C2 2A^{pro} (shown in pink).

Figure 3

Features relevant to the cleft for substrate binding. (a) Surface rendering of HRV-C15 2A^{pro} shows an open cleft, with the active site being accessible. The width of the cleft is about 12 Å and is mainly estimated from the distance between Gln80 and Asp124 and the distance between Tyr84 and Pro102. (b) Surface rendering of HRV-A2 2A^{pro} presents a closed channel owing to formation of the ‘EP bridge’, and therefore the active-site residues are inaccessible in this conformation. The width of the cleft is about 8 Å and is mainly estimated from the distance between Gln81 and Asp125 and the distance between Glu84 and Pro103. (c) Surface rendering of CVA16 2A^{pro} shows that Tyr89 is much closer to the conserved proline (Pro107) when compared with the structure of HRV-C15 2A^{pro}. The width of the cleft is about 10 Å and is mainly estimated from the distance between Glu85 and Asn129 and the distance between Tyr89 and Pro107. Residues located at the top of the rim are coloured red and those at the bottom of the rim are coloured blue. Active-site residues are coloured green.

β-strand. In contrast, in the HRV-C15 2A^{pro} structure the cII β-strand is divided into two short β-strands, cII1 and cII2, which are involved in the composition of the β-barrel and β-hairpin structures, respectively (Fig. 2c).

The overall r.m.s.d. between the crystal structure of 2A^{pro} from HRV-C15 and the solution structure of 2A^{pro} from HRV-C2 is 1.81 Å, which is higher than the r.m.s.d. between the crystal structures of HRV-C15 2A^{pro} and CVA16 2A^{pro} (Table 5). The more pronounced differences in overall structure between HRV-C15 2A^{pro} and HRV-C2 2A^{pro} may structurally explain why polyclonal antibodies raised against CVA16 2A^{pro} could cross-react with HRV-C15 2A^{pro} but do not cross-react with HRV-C2 2A^{pro}, although we do not know the epitopes of the antibodies to analyze the structural differences directly. In the structure of 2A^{pro} from HRV-C2, the antiparallel β-hairpin observed in HRV-C15 is not obvious because of the shorter lengths of bII2 and cII1 in 2A^{pro} from HRV-C2. In addition, the shorter aII and longer dII β-strands in 2A^{pro} from HRV-C15 lead to a different shape of the C-terminal region. The loops connecting the β-strands as well as the loops located at the termini seem to vary the most in position between the two structures (Fig. 2d).

3.3. Open conformation of HRV-C15 2A^{pro}

Surface rendering of HRV-C15 2A^{pro} reveals an open cleft, with the active site being accessible. The width of the cleft is about 12 Å (Fig. 3a). In contrast, surface rendering of HRV-A2 2A^{pro} reveals a closed channel owing to formation of the ‘EP bridge’ (by Glu84 and Pro103), and the width of the cleft decreases to about 8 Å (Fig. 3b). Furthermore, blocking of the channel by Tyr85 makes the active-site residues inaccessible. The conformation of the active site of HRV-C15 2A^{pro} is more

similar to the open conformation of 2A^{pro} observed in the structure of CVA16, in which the width of the cleft is about 10 Å (Fig. 3c). Owing to the replacement of Glu84 in HRV-A2 by Gly83 in HRV-C15 and the switch of Tyr84, the substrate-binding site of HRV-C15 2A^{pro} is more open than that of CVA16 2A^{pro}. Hence, the active site of HRV-C15 2A^{pro} reveals a more open conformation when compared with other known 2A^{pro} structures. These differences reflect the dynamic nature of the active site, presumably for binding different substrates. This is further supported by the high *B* factors that are observed for residues in this region. Additionally, the *B* factors for the ions are also very high. Although we do not know the reason, we assume that the Zn²⁺-binding domain in the structure is relatively more flexible than the other regions.

4. Conclusion

In conclusion, the 2.6 Å resolution crystal structure of 2A^{pro} from HRV-C15 reveals the presence of a conserved His–Asp–Cys catalytic triad and a Zn²⁺-binding site. Comparison with structures of 2A^{pro} from other enteroviruses reveals that the substrate-binding cleft of 2A^{pro} from HRV-C15 shows a more open conformation, which presumably favours substrate binding. We believe that our structural study of HRV-C15 2A^{pro} will help to rationalize the design of efficient peptide inhibitors or antiviral compounds targeting 2A^{pro} to block the replication of HRVs.

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