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A novel point mutation (L70P) inactivates poliovirus 3C protease

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Summary. – Poliovirus (PV) contains a single-stranded positive-sense RNA genome, which is translated into a single polyprotein. Viral proteases process this polyprotein to produce several individual as well as fused proteins. The major viral protease 3C cleaves at nine of the eleven cleavage sites. During the process of expressing PV 3ABC protein in *Escherichia coli*, we identified a 3C mutant (L70P), which lost its protease activity. This loss of function was confirmed by generating recombinant adenoviruses expressing mutant and wild-type 3C. Further, infectious PV could not be recovered from PV full-length cDNA containing the L70P mutation. However, 3C L70P mutant cDNA could complement a PV cDNA containing a 1AB deletion, producing a viable virus population containing defective complementing genomes. Structural analysis of the mutant protein indicated that the L70P mutation resulted in the loss of a hydrogen bond between two residues located within a loop between two β-sheets, potentially leading to strain on the catalytic site. We conclude that L70P inactivates 3C protease because of its close proximity to the 3C catalytic site.

Keywords: poliovirus; 3C protease; inactivating mutant; prokaryotic expression; eukaryotic expression; infectious cDNA; virus rescue

Introduction

Polio is an acute viral disease of humans and is caused by poliovirus (PV), a single-stranded positive-sense RNA virus belonging to the genus *Enterovirus* of the family *Picornaviridae*. Three serotypes of PV, PV1, PV2 and PV3, are recognized, resulting from slightly different capsid structures. The PV genome of about 7,500 nucleotides encodes a single open reading frame flanked by a 5' non-translated region (NTR), which includes the internal ribosome entry site (IRES), and a 3'NTR, which contains a polyA tail. Translation of the viral genome results in the synthesis of a 247 kDa polyprotein, in the sequence representing the proteins VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D. The polyprotein is cleaved by viral proteases (Krausslich and Wimmer, 1988). The first cleavage occurs at Tyr-Gly junction by 2A protease, which releases the P1 fragment consisting of VP0, VP3, and VP1 proteins (Krausslich and Wimmer, 1988; Toyoda *et al*., 1986). The rest of the proteolytic cleavages are a result of the action of the major viral protease 3C (Palmenberg *et al*., 1979; Hanecak *et al*., 1982), which cleaves specifically at the Gln-Gly peptide bond (Kitamura *et al*., 1981; Hanecak *et al*., 1982). The final processing event is the cleavage of VP0 into VP4 and VP2, which occurs during the final stages of virion morphogenesis, probably following the association of capsid proteins and RNA, and has been suggested to be autocatalytic (Arnold *et al*., 1987; Wellink and van Kammen, 1988).

Poliovirus 3C is structurally similar to chymotrypsin-like serine proteases, and is comprised of two anti-parallel six-stranded β-barrel domains. The shallow active site cleft is located at the junction of the two β-barrel domains and contains a His40-Glu71-Cys147 catalytic triad (Mosimann *et al*., 1997). Five residues in 3C have so far been shown to be important for its proteolytic function. Substitutions at the putative catalytic triad (His40, Glu71 and Cys147) as well

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Abbreviations: Ad vector = adenovirus vector; CPE = cytopathic effect; HEK = human embryonic kidney; MOI = multiplicity of infection; PEI = polyethyleneimine; pNA = *p*-nitroaniline; PV = poliovirus; SMP = skimmed milk powder

as at a distant site (His161) inactivate the enzyme, whereas Gly51 is required for the production of infectious virus (Kean *et al.*, 1991).

Polio is on the verge of being eradicated from the globe, and this has resulted in restrictions on handling PV. We have been working on generating reagents in the form of antigens and antibodies (Uma *et al.*, 2016b), which can be used following polio eradication. During this process, we attempted to express the PV 3ABC protein in prokaryotic systems. Possibly due to the toxicity of 3C protease, we encountered either low or no expression of the protein (Uma *et al.*, 2016a). However, these experiments led to the serendipitous discovery of a novel mutation adjacent to the catalytic domain of 3C, inactivating its enzymatic activity, and resulting in abortive infection of susceptible cells by PV.

Materials and Methods

Plasmids, cells and genes. The plasmid pRSET B (Invitrogen, Bengaluru, India) was used for cloning and expression of genes. The vector contains a T7 ϕ10 promoter to drive protein expression in *Escherichia coli*, as well as an N-terminal His-tag for the detection of the recombinant proteins. For cloning and gene expression studies, the *E. coli* DH5α (Invitrogen) and *E. coli* BL21(DE3) (Invitrogen) strains were used, respectively. The HEK293 (Graham *et al.*, 1977) and HEK293IQ (Matthews *et al.*, 1999) cells were obtained from Microbix Biosystems Inc., Canada. The HEK293IQ cells were used to produce and propagate recombinant adenoviruses and HEK293 cells were used to study the protein expression. CV1 cells (ATCC CCL-70) (Hronovsky *et al.*, 1978) were used for the rescue of poliovirus from full-length cDNA plasmid. The PV full-length infectious plasmid pVS(1)IC-O(T) (Omata *et al.*, 1984; Kohara *et al.*, 1986) was a kind gift from Akio Nomoto, Tokyo University, Japan. All synthetic genes were obtained from GenScript, USA. Synthetic DNA fragment containing the L70P mutation between the naturally existing *Bgl*II and *Acc*I sites (657 bp) of Sabin PV1 genome (AY184219.1) was obtained to construct the L70P 3C mutant clone, whereas the *1AB* gene-deleted fragment 5'UTR-1C between the naturally existing *Pml*I and *Nhe*I sites (1,422 bp) was obtained to construct the PV1 1AB deletion clone.

Cloning, transformation and expression. The *3ABC* gene was amplified by RT-PCR from RNA extracted from Sabin PV1 (obtained from Bharat Biotech International Limited, Hyderabad, India). The amplicon was purified and cloned into pRSET B vector. Recombinant clones were screened by restriction digestion according to standard protocols (Sambrook and Russell, 1989). Two positive clones were sequenced and analyzed by comparing with the nucleotide sequences of Sabin PV1 reference sequence (AY184219.1). The two positive clones were transformed into chemically competent BL21(DE3) *E. coli* cells (Hanahan *et al.*, 1991). Transformed cells were grown in one ml of LB, and after one hour of incubation at 37°C, 100 µl of the culture was spread on LB agar plates with 2% glucose and ampicillin (100 µg/ml) for selection. Colonies from each plate were inoculated into 5 ml of LB containing ampicillin (100 µg/ml) with 2% glucose, and incubated overnight in a 37°C shaker incubator. Overnight cultures were diluted 1:100 in LB with 2% glucose, and grown at 37°C in a shaker incubator until OD_{600} reached 0.5, and the cells were pelleted and resuspended in fresh LB broth containing 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction. Three hours after induction, the cells were pelleted and used for further analysis.

SDS-PAGE and western blotting. To analyze the bacterially expressed 3ABC protein, cell lysates were resuspended in reducing SDS-PAGE loading buffer, boiled at 95°C for 10 min, electrophoresed on 12% polyacrylamide gels, and the proteins were blotted to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% skim milk powder (SMP) in phosphate-buffered saline (PBS, pH 7.2) overnight at 4°C, incubated with anti-His monoclonal antibody (Sigma-Aldrich, Bengaluru, India) at a dilution of 1:15,000 in 3% SMP at 37°C for 1h, washed three times with PBS containing 0.05% Tween-20 (PBST), and probed with horseradish peroxidase (HRP)-conjugated anti-mouse IgG in 3% SMP at 37°C for 1h. The membrane was washed three times with PBST, and once with PBS, and the expressed proteins were detected using the Pierce ECL reagent (Thermo Fisher Scientific, Bengaluru, India). To confirm the expression of PV proteins in eukaryotic cells, rabbit anti-3AB (1:2,000 dilution) or anti-1AB (1:5,000 dilution) serum (Uma *et al.*, 2016b) was used as primary antibody along with HRP-conjugated anti-rabbit IgG antibody (1:5,000 dilution).

Generation of recombinant adenovirus and analysis of gene expression. The AdMax Hi-IQ system (Microbix Biosystems Inc., Canada) was used for the generation of recombinant adenoviruses (Matthews *et al.*, 1999). The *3ABCwt* and *3ABCmut* genes were cloned into *Eco*RI and *Hin*dIII sites of the pDC515(io) adenovirus shuttle vector. To generate recombinant adenovirus expressing wild-type 3ABC (rAd-3ABCwt), mutant 3ABC (rAd-3ABCmut) or control adenovirus (rAd-null), 1 µg of adenovirus shuttle vectors pDC515(io)-3ABCwt, pDC515(io)-3ABCmut or pDC515(io), respectively, and 7 µg of adenovirus genomic plasmid (pBHGfrtΔE1,3FLP) were mixed with 2 µg of polyethyleneimine (PEI), incubated at room temperature for 30 min, and transferred onto HEK293IQ cell monolayer. Following a 6 h incubation at 37°C, the medium was changed, and the cells were observed daily for the appearance of cytopathic effect (CPE). The resultant recombinant adenoviruses were titrated for tissue-culture infective dose-50 ($TCID_{50}$) on HEK293IQ cells. For gene expression, the HEK293 cells were infected with rAd-3ABCwt or rAd-3ABCmut viruses at a multiplicity of infection (MOI; $TCID_{50}$ per cell) of 5. The cells were harvested 24h post-infection, and lysed in Tris-saline (50 mmol/l Tris-HCl [pH 7.5], 100 mmol/l NaCl) containing 1% Nonidet P-40 (NP-40) and 1 mol/l phenylmethyl sulphonyl fluoride (PMSF), and analysed by western blotting using anti-PV3AB polyclonal antibodies (Uma *et al.*, 2016b).

Generation of mutant PV cDNA clone and rescue of virus. The plasmid pVS(1)IC-O(T) carries the full-length cDNA of wild-type

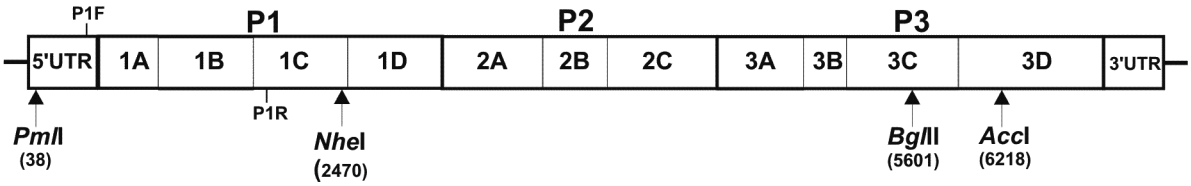

Fig. 1

Schematic representation of PV genome with naturally existing *Pml*I, *Nhe*I, *Bgl*II, and *Acc*I sites
The numbers indicate the position of each restriction site. P1F and P1R denote primer sites flanking, and used for the amplification of, the 1AB fragment.

PV1 along with replication and transcription signals of simian virus 40 (SV40) (Omata *et al*., 1984; Kohara *et al*., 1986). To generate the mutant cDNA clone (pVS(1)IC-O(T)/3Cmut), a synthetic *Bgl*II to *Acc*I fragment corresponding to the PV1 genome, but carrying the L70P mutation, was introduced into PV1 cDNA clone by replacing the corresponding fragment in the wild-type infectious clone (Fig. 1). Similarly, pVS(1)IC-O(T)Δ1AB was generated by replacing the *Pml*I to *Nhe*I fragment in the wild-type infectious clone with the *Pml*I to *Nhe*I synthetic fragment deleted of the complete 1AB region (Fig. 1). To rescue the virus, CV1 cells were transfected with 5 μg of pVS(1)IC-O(T) or pVS(1)IC-O(T)/3Cmut plasmids along with 2 μg of PEI reagent. The ability of rAd-3ABCwt to complement pVS(1) IC-O(T)/3Cmut to produce PV was tested by transfection of pVS(1) IC-O(T)/3Cmut plasmid followed by infection with rAd-PV3ABCwt 6h post-transfection. The ability of pVS(1)IC-O(T)/3Cmut and pVS(1) IC-O(T)Δ1AB plasmids to complement each other in producing viable PV was tested by co-transfecting CV1 cells with 3 μg each of pVS(1) IC-O(T)/3Cmut and pVS(1)IC-O(T)Δ1AB plasmids or transfecting individually with 5 μg of pVS(1)IC-O(T) or pVS(1)IC-O(T)/3Cmut or pVS(1)IC-O(T)Δ1AB along with 2 μg of PEI reagent. Cells were observed daily for the appearance of CPE, after which, cell lysates were tested for the expression of 1AB protein. The virus rescued from pVS(1) IC-O(T)/3Cmut and pVS(1)IC-O(T)Δ1AB co-transfection was further confirmed by evaluating the presence of RNA transcripts for *1AB* gene using flanking primers (P1F-GCCATCCGGTGAAAGTGAG, P1R-CGGGTATGTCAATAGGTGGG). RNA was isolated from the virus rescued samples using mono-phasic guanidinium isothiocyanate (MPGIT) (Gauthami, 2015) reagent, and the residual DNA contamination in RNA was removed using DNA-free DNA Removal Kit (Ambion-AM1906) according to the manufacturer's instructions. The treated RNA was used for RT-PCR and PCR to assess the presence or absence of *1AB* gene in the PV genomes.

Colorimetric protease assay. To test the protease activity of wild-type and mutant 3C, HEK293 cells were infected with 100 MOI of rAd-3ABCwt, rAd-3ABCmut or rAd-null viruses, or 1 MOI of PV1 virus (positive control). The cells were harvested 24 h post-infection, resuspended in PBS, and lysed by three cycles of freeze-thaw. The 3C protease activity present in the cell lysates was measured using the peptide assay, as described previously (Wang *et al*., 1997). Briefly, 50 μl of virus-infected cell extracts were incubated in a 200 μl reaction mix containing 2 mmol/l HEPES (pH 8.0), 150 mmol/l NaCl, 1 mmol/l EDTA, 6 mmol/l DTT, and 500 μmol/l pNA peptide substrate (Y-A-I-E-Q-pNA; GenScript, USA) at 30°C, and color production was monitored by reading absorbance at 405 nm in an ELISA reader. To test protease activity in the cells transfected with full-length cDNA plasmids with or without mutation, CV1 cells were transfected with 5 μg of each plasmid pVS(1)IC-O(T) or pVS(1)IC-O(T)/3Cmut, cell extracts were prepared on day 4, and protease activity was tested as described above.

Analysis of wild-type and mutant 3C structure. Coordinates for PV 3C protease (PDB ID: 1L1N) were obtained from RCSB-PDB (Berman *et al*., 2000). Homology modeling and energy minimization of the mutant 3C were performed using Schrödinger suite (Schrödinger, 2016), and structures were visualized using UCSF Chimera package (Pettersen *et al*., 2004). For comparison with other picornaviral 3C proteases, sequences were downloaded from NCBI database (Pruitt *et al*., 2007), and multiply aligned by STRAP (Gille *et al*., 2014).

Results

Cloning and expression of PV 3ABC in E. coli identifies a novel mutation

The PV1 *3ABC* gene was amplified by PCR, cloned into pRSET B plasmid, and positive clones were confirmed by restriction digestion. Two clones of pRSETB-PV3ABC were selected for expression studies. Transformation of BL21(DE3) cells with clone 1 resulted in small colonies compared to that with the control and clone 2 plasmids (Fig. S1).

The PV 3C protease is expected to cleave at 3A-3B and 3B-3C junctions, potentially resulting in two partially cleaved peptides (3AB and 3A). On the other hand, non-functional 3C is expected to result in uncleaved 3ABC. Expression of PV *3ABC* gene in clone 1 resulted in cleavage of 3ABC, producing two His-tagged protein bands i.e., 3AB and 3A. On the other hand, 3ABC was uncleaved in clone 2, indicating either loss of cleavage site or loss of 3C function (Fig. 2). To ascertain the reason, both clones were sequenced and a single non-synonymous nucleotide change T209C resulting in the L70P mutation was observed in clone 2, whereas clone 1 contained the wild-type L70 residue.

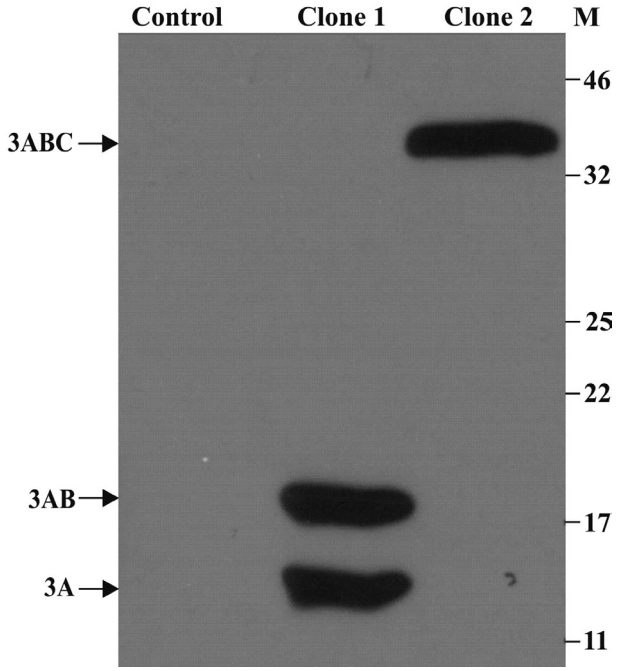

Fig. 2

Prokaryotic expression of 3ABC

BL21(DE3) cells were transformed with Clone 1 (small colony morphology) or 2 (large colony morphology) and pRSET B (Control) plasmids. Single colony from each transformed plate was cultured and protein expression was induced with IPTG. Induced cell lysates were subjected to SDS-PAGE, blotted, and the His-tagged proteins were detected using anti-His antibodies. M: molecular weight marker (kDa).

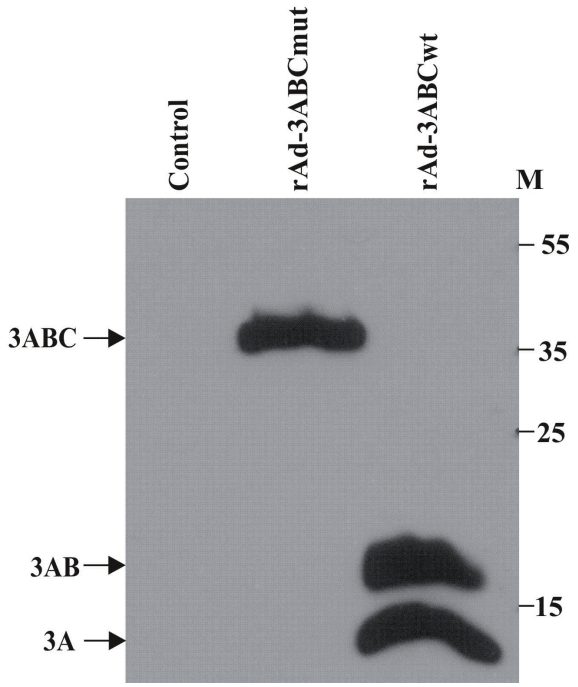

Fig. 3

Expression of 3ABC by the recombinant adenovirus

HEK293 cells were infected with rAd-null (control), rAd-3ABCwt or rAd-3ABCmut viruses. The cells were harvested 24 h post-infection and cell lysates were subjected to SDS-PAGE, blotted, and the expressed proteins were detected using rabbit anti-3AB polyclonal antibodies. M: molecular weight marker (kDa).

The loss of function of 3C is recapitulated when expressed through recombinant adenovirus

The loss of protease activity of 3C was further evaluated in a eukaryotic system. Recombinant adenoviruses expressing wild-type (rAd-3ABCwt), L70P mutant (rAd-3ABCmut) *3ABC* genes, or no insert (rAd-null) were used to infect HEK293 cells, and protease activity was analyzed by western blot using anti-3AB antibodies (Uma *et al.*, 2016b). Infection of rAd-3ABCwt virus resulted in cleavage of 3ABC, whereas infection with rAd-3ABCmut resulted in expression of uncleaved 3ABC (Fig. 3), confirming that the L70P mutation inactivates the 3C protease activity.

The single point mutation L70P disables rescue of infectious virus

Since cleavage of PV polyprotein is critical for infectious virus production, the effect of L70P mutation on PV production was then evaluated. When CV1 cells were transfected with either wild-type (pVS(1)IC-O(T)) or L70P mutant (pVS(1)IC-O(T)/3Cmut) plasmid DNA, characteristic CPE was observed only with cells transfected with pVS(1)IC-O(T), whereas transfections with pVS(1)IC-O(T)/3Cmut did not produce any CPE. Further, attempts to rescue the virus from cells transfected with pVS(1)IC-O(T)/3Cmut by complementing with recombinant adenovirus expressing wild-type 3ABC also failed (data not shown).

However, co-transfection with pVS(1)IC-O(T)/3Cmut and pVS(1)IC-O(T)Δ1AB resulted in characteristic CPE similar to cells transfected with wild-type plasmid, whereas transfection with either pVS(1)IC-O(T)/3Cmut or pVS(1) IC-O(T)Δ1AB separately did not produce any CPE. Further, PV 1AB protein expression was observed in the cells transfected with pVS(1)IC-O(T) or co-transfection with (pVS(1)IC-O(T)/3Cmut) and pVS(1)IC-O(T)Δ1AB plasmids but not with either of the individual plasmids containing mutant genome (Fig. 4). The rescued virus could be passaged three times in CV1 cells, and PCR amplification of the cDNA with primers flanking *1AB* resulted in two different amplicons corresponding to wild-type as well as 1AB deleted versions of PV (Fig. 5), suggesting co-existence of both the genomes.

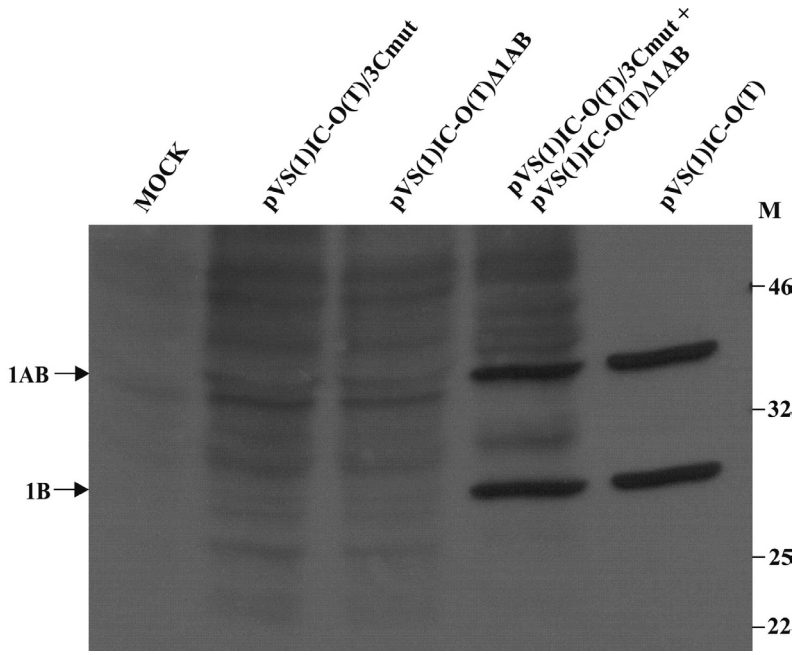

Fig. 4

Analysis of PV expression from transfected samples

CV1 cells were transfected with pVS(1)IC-O(T), (pVS(1)IC-O(T)/3Cmut) or pVS(1)IC-O(T)Δ1AB, or co-transfected with (pVS(1)IC-O(T)/3Cmut) and pVS(1)IC-O(T)Δ1AB plasmids. The cells were harvested after observation of CPE, lysates were subjected to SDS-PAGE, blotted, and the expressed proteins were detected using rabbit anti-1AB polyclonal antibodies. M: molecular weight marker (kDa).

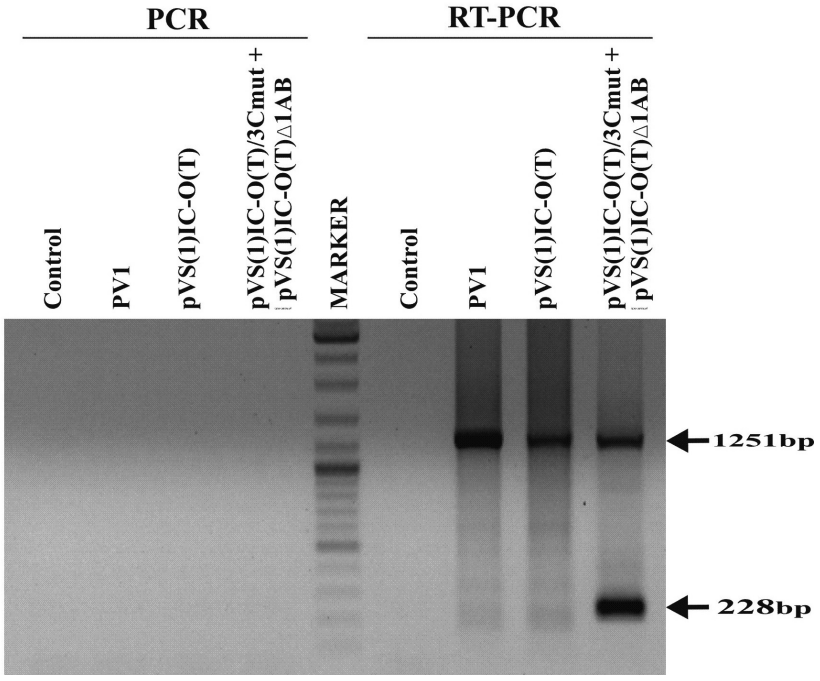

Fig. 5

RT-PCR for *1AB* gene in transfected cells

CV1 cells were transfected with either pVS(1)IC-O(T) or pVS(1)IC-O(T)/3Cmut and pVS(1)IC-O(T)Δ1AB plasmids, and the resultant virus was passaged in CV1 cells. RNA was isolated after 3 passages, treated with DNase, and used for PCR or RT-PCR. PV1-infected or uninfected cell lysates were used as positive and negative controls, respectively.

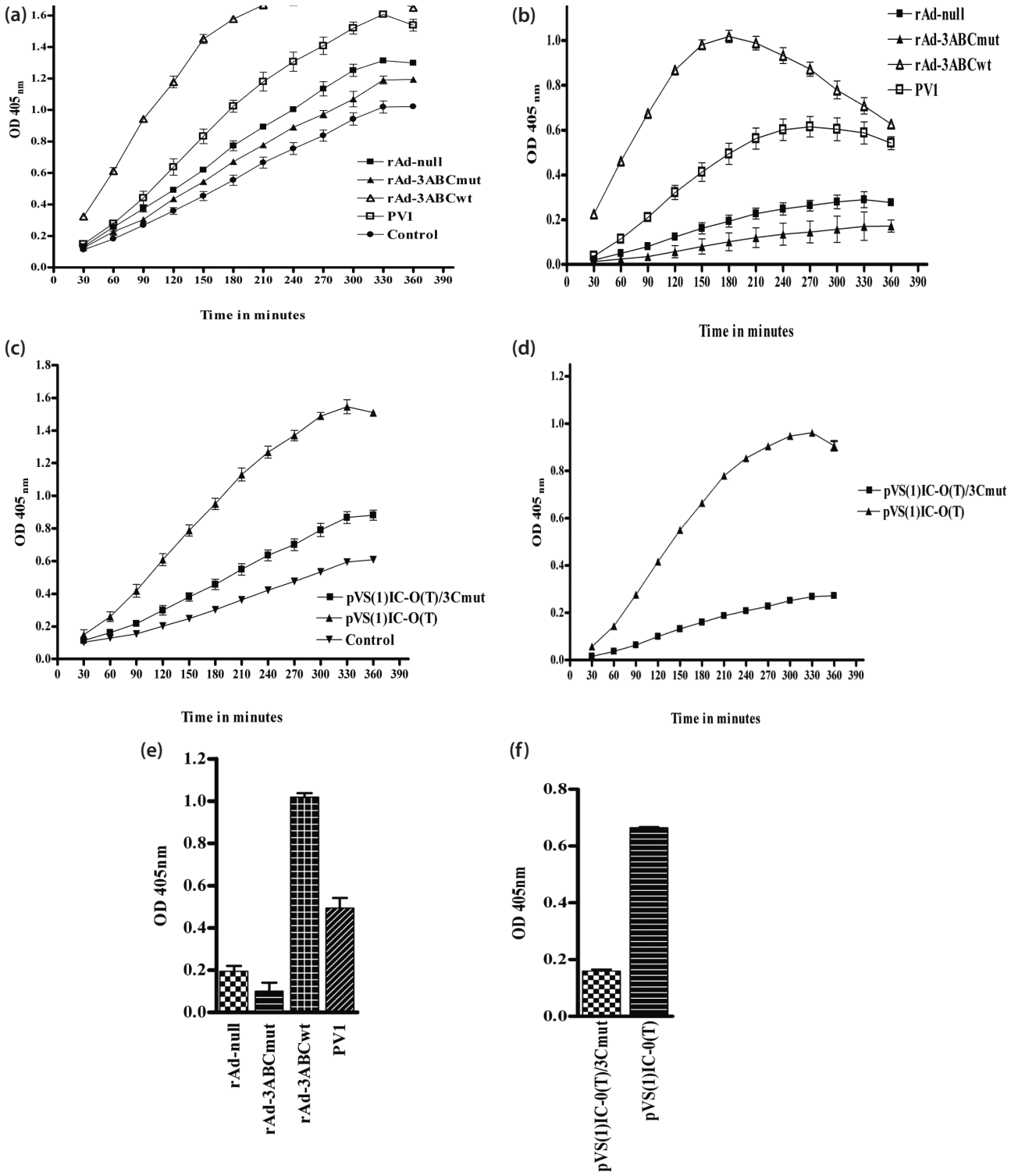

Fig. 6

Detection of 3C protease activity in infected cell lysates

Proteolytic activity was assessed by mixing lysates containing wild-type or mutant 3C with the chromogenic peptide. Absorbance at 405 nm was measured at different time-points as shown. **(a)** and **(b)** HEK293 cells infected with rAd-null, rAd-3ABCmut, rAd-3ABCwt, PV1 viruses, or uninfected cells (control). **(c)** and **(d)** pVS(1)IC-O(T) and pVS(1)IC-O(T)/3Cmut transfected HEK293 cell lysates and mock transfected HEK293 cells (control). Panels a and c show data with background, and panels b and d show the same data with the background subtracted. **(e)** and **(f)**. Bar diagram showing the data from panels b and d, respectively, at the 180 min time-point. The error bars show the standard deviation of duplicate samples.

The L70P mutation leads to loss of enzymatic activity of 3C

In order to biochemically assess the loss of function of 3C as a result of the L70P mutation, we subjected extracts of cells containing wild-type or mutant 3C to an enzymatic assay *in vitro*. For this, the chromogenic peptide substrate Y-A-I-E-Q-pNA (GenScript, USA) was used. Cleavage of this substrate between Gln and pNA by 3C releases the yellow colored free pNA, whose absorbance can be measured at 405 nm. Protease activity of 3C was compared between extracts of HEK293 cells infected with PV1, rAd-3ABCwt, rAd-3ABCmut or rAd-null, or uninfected control cells. As shown in Fig. 6, pNA hydrolysis was higher with cell extracts of rAd-3ABCwt and PV1 as compared to that of rAd-null or rAd-3ABCmut, demonstrating that 3C protease activity was markedly lower for the mutant (Fig. 6a and 6b). Similarly, pVS(1)IC-O(T) showed more pNA hydrolysis compared to pVS(1)IC-O(T)/3Cmut from the HEK293-transfected cell extracts (Fig. 6c and 6d).

The L70P mutation results in the alteration in the structure of 3C protease

In order to examine how L70P substitution affected protein structure, homology modeling was performed for

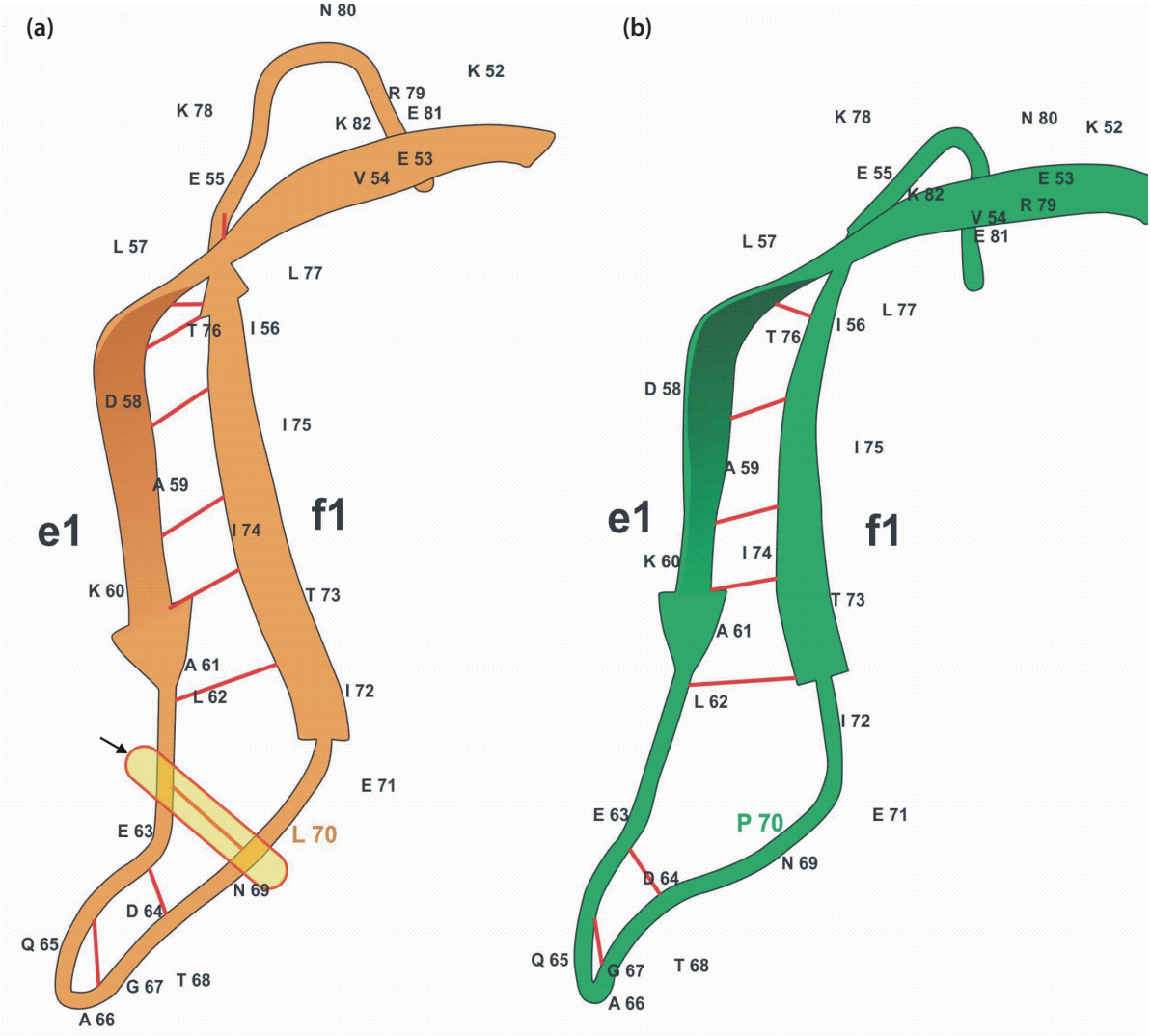

Fig. 7

Structural analysis of wild-type and mutant 3C

Intra-molecular interactions in the wild-type **(a)** and mutant **(b)** protein are shown. Arrow represents the hydrogen bond between amide group of L70 and carbonyl group of L62 in the wild-type. Designation of the chain and strands are as described previously (Mosimann *et al.*, 1997).

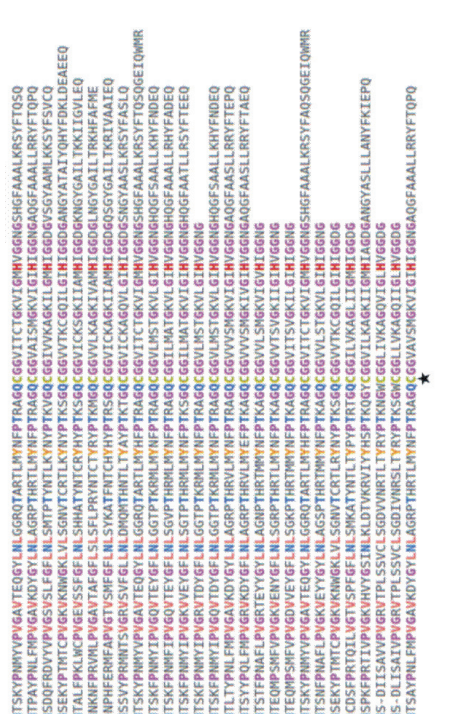

Fig. 8

Sequence alignment of 3C proteases

Different picornaviral 3C protease sequences were multiply aligned by STRAP (Gille *et al.*, 2014). Arrow denotes the conservation of Leu, which is proximal to Glu of the catalytic triad. Residues forming the proteolytic active site are marked with asterisk. Other conserved residues are highlighted. The various viruses and their GenBank accession numbers are as follows: EV-C (NP_740476), EV-F (YP_009020983), SSV-1 (NP_937978), RaBoV-A (YP_009118289), CanPV (YP_005352654), FelPV (YP_004934029), BatPV (YP_004782554), ASV-1 (YP_164830), CV-A24 (AGG78612), EV-B (NP_740546), SA5/EV-B (AF326751), EV-A (AF326750), SVDV/EV-B (AAF81252), Ev(E6)/EV-B (AAA65044), DcEV(AKA64452), BEV/EV-E (AAF05694), EV-G (AFK66746), WUHARV (AFV48063), SV46/EV-A (AAL69636), CA24/EV-C (AGG78621), PEV-9/EV-G (CAA74807), Rat/PV (AIU36166), Csl SapV-1 (AEM37584), Pigeon PV B (YP_004564618), Phacovirus (A1R74732), QPV1 (YP_009020971), EV-F (YP_009020971).

the mutant protein using wild-type protein structure as the template (Mosimann *et al.*, 1997). The modeled structure was energy minimised and differences in the intra-molecular interactions were analyzed. In the wild-type, the nitrogen of amide group of L70 interacts with the oxygen of carbonyl group of L62 through a hydrogen bond, whereas this bond cannot be formed with nitrogen of the pyrrolidine ring in the substituted Pro residue in the mutant protein. As a consequence, e1 and f1 strands move away at this position in the mutant protein structure (Fig. 7), suggesting that the loss in hydrogen bonding might affect the overall stability of the β-barrel structure near the catalytic triad of the protein. Multiple sequence alignment of different 3C proteases revealed that L70 is one of the conserved residues among different members of the family *Picornaviridae* (Fig. 8).

Discussion

Picornaviruses produce a single polyprotein, which is cleaved by proteases into individual structural and non-structural proteins. The major protease involved in the cleavage process is the viral endopeptidase 3C, a cysteine protease with chymotrypsin-like activity. Cleavage of the polyprotein is an important step in picornaviral replication, and inactivation of the protease leads to abortive infection.

The PV 3C is a 20 kDa protein (183 amino acids), consisting of two β-barrel domains (I and II) connected by four helices with several turns. Each β-barrel domain is made up of six anti-parallel strands (aI to fI and aII to fII). Extensive contacts between the two β-barrels leave a shallow groove that forms the proteolytic active site. The active site consists of a catalytical triad (Cys147, His40 and Glu71) and an electrophilic oxyanion hole (Gly145, Gln146 and Cys147). Other residues surrounding the groove may be involved in stabilizing the site, as well as in interactions with the substrate (Mosimann *et al.*, 1997).

While expressing PV 3ABC in *E. coli*, we observed two morphologically distinct colonies. Cells from the small colony morphology showed expression of processed 3ABC (autocatalysis) into individual proteins but cells from large colony morphology expressed 3ABC in uncleaved form. Small colony morphology was suspected to be due to leaky expression of PV 3C and its toxicity to BL21(DE3) cells. Upon DNA sequencing, an L70P mutation was observed with the large colony variants. The L70P mutation resulted in the loss of the autocatalytic processing of 3ABC polyprotein expressed in *E. coli*, probably leading to the loss of toxicity of 3C protease, and hence allowing the cells to grow normally. Loss of 3C protease activity was also observed in eukaryotic cells infected with recombinant adenoviruses expressing the L70P mutant. An *in-vitro* peptide-based assay also confirmed the loss of activity of the L70P mutant. It is

important to note that prolonged incubation with control cell lysates produced cumulatively increased signal in the peptide assay, suggesting that there was a slow non-specific background cleavage similar to 3C. This increase was higher beyond 180 min of incubation, thus leading to a slow decline in the curve for 3C. However, at time points up to 360 min, there was a marked difference in the cleavage between wt and mutant 3C, clearly demonstrating the effect of the L70 mutation on the enzyme activity of 3C.

Cleavage of the viral polyprotein by 3C is indispensable for virus protein maturation and virus replication. Transfection of susceptible cells with plasmid carrying full-length PV cDNA clone with 3C L70P mutation could not produce infectious virus particles, indicating that the inactivation of 3C protease could abolish virus rescue. Attempts to rescue 3C L70P mutant PV by trans-complementation with recombinant adenovirus expressing wild-type 3C also failed. This is not surprising as earlier work has also indicated that trans-complementation of defective PV with proteins supplemented from heterologous systems is unproductive (Towner *et al.*, 1998; Cornell *et al.*, 2004) and it appears that the catalytic activity of 3C in processing the P1 structural region is dependent on it being expressed as 3CD rather than 3C alone, both *in vitro* and *in vivo* (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988). However, 3C L70P mutant plasmid could trans-complement a 1AB deletion plasmid, resulting in production of a virus population, which contained both wild-type and deletion mutants of *1AB* gene. Although it is not clear whether a small population of wild-type virus was generated, detection of viral genomes with or without *1AB* gene indicates that the virus population is a mix of complementing viruses. Irrespective of the composition, it is evident that 3C L70P is a lethal mutation and complementation with wild-type 3C from another virus, which is defective in another part (1AB) of the genome, can rescue the virus. This ability of 3C L70P mutant to trans-complement deletion mutants of PV can be explored for production of viruses with bipartite and multipartite genomes. Similar to our observations in PV, trans-complementation of defective picornaviruses has been reported earlier (Garcia-Arriaza *et al.*, 2004, 2005, 2006).

Structural analysis of the mutant protein indicated that a hydrogen bond between conserved L62 and L70 residues could not be formed in the mutant, leading to increase in the distance between the β-strands eI and fI and conformational changes on both sides of Glu71 of the catalytic triad (Mosimann *et al.*, 1997). These changes may affect the positioning and/or binding of the substrate in the catalytic site, leading to inactivation of the enzyme. Thus, L70 is an important residue that is not part of, but allosterically affects the function of the active site due to its close proximity to, the catalytic triad, and hence is a novel inactivation mutation of 3C.

Conclusion

A novel mutant in PV *3C* gene, resulting in change in amino acid (L70P), and inactivating the protease activity is reported. This mutant was discovered through prokaryotic expression system, and confirmed by Ad-vector-based expression of 3ABC in the eukaryotic system. The mutation resulted in failure of rescue of PV from a cDNA clone. It is postulated that the mutation results in a conformational change, which might cause perturbation in the catalytic triad.

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Supplementary information is available in the online version of the paper.

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Supplementary information

A novel point mutation (L70P) inactivates poliovirus 3C protease

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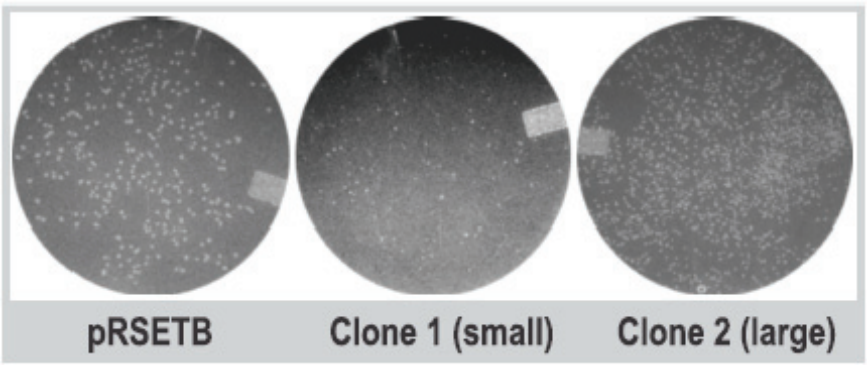

Supplementary Fig. 1

Colony morphology of BL21(DE3) cells transformed with PV 3ABC clones: BL21 (DE3) were transformed with pRSET B (control) or 3ABC gene cloned in pRSET B (clone 1 and 2), incubated overnight, and observed for colony morphology