Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Virus Research 178 (2013) 553–556

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Molecular characterization of hepatitis E virus ORF1 gene supports a papain-like cysteine protease (PCP)-domain activity

Mohammad Khalid Parvez*

Department of Pharmacognosy, King Saud University College of Pharmacy, PO Box 2457, Riyadh 11451, Saudi Arabia

ARTICLE INFO

Article history:
Received 8 June 2013
Received in revised form 25 July 2013
Accepted 29 July 2013
Available online 23 August 2013

Keywords:
Hepatitis E virus
HEV
Genomic replicon
ORF-1
Papain-like cysteine protease

ABSTRACT

Hepatitis E Virus (HEV) ORF1 encodes the nonstructural polyprotein wherein a role of PCP-domain in ORF1 proteolysis and/or RNA replication still remains contested. A series of ORF1 mutants of HEV-SAR_{55} replicon were constructed and tested for viability in S10-3 cells. Six of PCP-'cysteine' (C457A, C459A, C471A, C472A, C481A and C483A) and three 'histidine' (H443L, H497L and H590L) mutants were lethal. Further, a highly conserved 'glycine-triad' (G815-G816-G817) in downstream X-domain, homologous to rubella virus protease-substrate (G1299-G1300-G1301) was identified where two of X-mutants (G816V and G817V) turned lethal. However, all ORF1 sequential nucleotide-mutants conserving the amino acids were viable, which clearly showed post-translational regulation of HEV replication by PCP- and X-domains. Moreover, while vector-expressed ORF1-fusion polyprotein yielded a ~191 kDa band *in vitro*, it produced ~78 and ~35 kDa fragments *ex vivo*. Collectively, the indispensability and functional effects of 'PCP-catalytic' and 'X-substrate' residues on HEV replication strongly supported a viral protease.

Hepatitis E is caused by hepatitis E virus (HEV) that is generally manifested by a self-limiting acute infection as well as fulminant liver failure in about 20–30% of pregnant women in developing countries (Aggarwal and Naik, 2009; Navaneethan et al., 2008). Very recently, a challenging clinical situation associated with chronic hepatitis E in immunocompromised individuals has emerged in some industrialized nations (Parvez, 2013). Classified as the only member of the *Hepeviridae* family, HEV has at least four recognized genotypes. In addition to humans, genotypes 1 and 2 can also infect non-human primates while genotypes 3 and 4 are further infectious to swine and few other mammalian species (Meng, 2010). The virus is non-enveloped with a positive-sense, single-stranded RNA genome of approximately 7200 bases that contains three partially overlapping open reading frames (ORFs): ORF1, ORF2 and ORF3 (Tam et al., 1991). HEV ORF2 codes for the viral capsid glycoprotein while ORF3 translates into a small phosphoprotein, attributed to some host-regulatory functions, including establishing infection and virion egress (Chandra et al., 2008; Graff et al., 2005; Yamada et al., 2009). Viral ORF1 is 5109 bases long and encodes the nonstructural polyprotein of ~186 kDa, essential for RNA replication (Ansari et al., 2000). Koonin et al. (1992) had proposed six putative domains (N- to C-terminal) of methyltransferase (MetT), papain-like cysteine protease (PCP), RNA helicase (Hel/NTPase), RNA-dependent RNA polymerase (RdRp), including undefined X and Y.

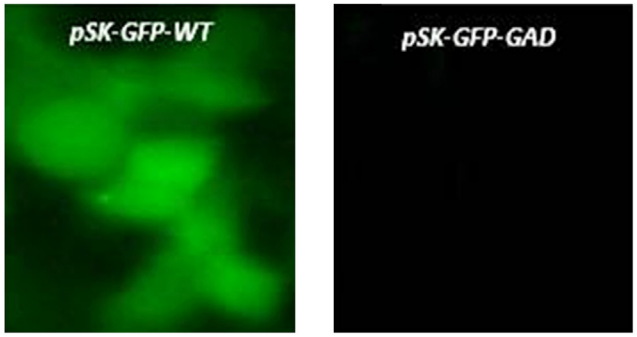
Extensive attempts have been made to study ORF1 processing and validate the viral PCP-domain proteolytic activity using cell-free, prokaryotic and eukaryotic expression systems. Expression of a full-length ORF1 in transiently-transfected HepG2 cells had resulted in an intact ~186 kDa protein while a recombinant vaccinia virus-expressed ORF1 had produced two fragments of ~107 and ~78 kDa in cultured mammalian cells that was, however, attributed to non-HEV proteases (Ropp et al., 2000). Furthermore, an infectious HEV RNA-transfected HepG2 cells produced distinct processed products of ORF1, identified as ~35, ~38 and ~36 kDa bands with anti-MetT, anti-Hel and anti-RdRp antibodies, respectively (Panda et al., 2000). The processing of an ORF1-fusion polyprotein by detecting eight cleaved-products was also reported in a recombinant baculovirus-insect cell expression system that could be inhibited by a cysteine-protease inhibitor (E-64d) (Sehgal et al., 2006). The study by Sehgal et al. (2006), however, did not conclude whether the ORF1 proteolysis occurred due to HEV- or host-encoded protease. Nevertheless, the role of ORF1 PCP-domain in HEV RNA replication remains elusive or/and controversial. In this present report, we have, therefore, addressed this issue through a systematic molecular analysis of ORF1 gene, using HEV-SAR_{55} genomic-replicon system and expression of ORF1-fusion protein in cultured S10-3 cells.

ORF1 polyprotein sequences of HEV strains ($n = 77$) as well as that of genetically-close rubella virus (RUBV) was analyzed *in silico*. A total of 32 ORF1 mutants (Group-I: amino acid mutants; Group-II:

* Corresponding author. Tel.: +966 4675132; fax: +966 4675133.
E-mail address: khalid_parvez@yahoo.com

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.07.020

A.



B.

PCP-domain constructs		GFP
Group I	*pSK-GFPpcp-C434A*	+
	pSK-GFPpcp-C457A	-
	pSK-GFPpcp-C459A	-
	pSK-GFPpcp-C471A	-
	pSK-GFPpcp-C472A	-
	pSK-GFPpcp-C781A	-
	*pSK-GFPpcp-C483A**	-
	pSK-GFPpcp-C563A	+
	pSK-GFPpcp-H443L	-
	pSK-GFPpcp-H458L	+
	pSK-GFPpcp-H497L	-
	*pSK-GFPpcp-H590L**	-
Group II	*pSK-GFPpcp-1*	+
	pSK-GFPpcp-2	+
	pSK-GFPpcp-3	+
	pSK-GFPpcp-4	+
	pSK-GFPpcp-5	+
	pSK-GFPpcp-6	+
	pSK-GFPpcp-7	+
Controls	*pSK-GFP-WT*	+
	pSK-GFP-GAD	-

X-domain constructs		GFP
Group I	*pSK-GFPxdo-G815V*	+
	pSK-GFPxdo-G816V	-
	pSK-GFPxdo-G817V	-
Group II	*pSK-GFPxdo-1*	+
	pSK-GFPxdo-2	+
	pSK-GFPxdo-3	+
	pSK-GFPxdo-4	+
	pSK-GFPxdo-5	+
	pSK-GFPxdo-6	+
	pSK-GFPxdo-7	+
	pSK-GFPxdo-8	+
	pSK-GFPxdo-9	+
	pSK-GFPxdo-10	+
Controls	*pSK-GFP-WT*	+
	pSK-GFP-GAD	-

Fig. 1. The *ex vivo* replication profiles (FM) of HEV replicon-constructs. (A) Replication of control replicons, representing the mutant constructs translated into GFP(+) or GFP (−) S10-3 cells. (B) Replication of ORF1 PCP-domain ($n = 19$) and X-domain ($n = 13$) mutants (Group-I: amino acid mutants; Group-II: sequential nucleotide mutants; * Koonin et al., 1992; Ropp et al., 2000).

sequential nucleotide mutants) of *pSK-GFP* (HEV-SAR$_{55}$; genotype 1 genomic-replicon) were constructed by site-directed mutagenesis. In the present case, the HEV ORF2 coding sequence had been swapped with that of green fluorescent protein (GFP) and GFP production was used to monitor viral replication (Emerson et al., 2004). Briefly, mutant primer-sets were designed and commercially synthesized (Invitrogen, USA) followed by PCR amplification of *pSK-GFP* as per manufacturer's instructions (TaKaRa Biotech., Japan). The Dpn*I*-digested PCR DNA products were transformed (heat-shock) into XL-blue competent cells (Stratagene, USA). Mutant plasmids were confirmed by DNA sequencing (Invitrogen, USA) and stock DNA were prepared (Qiagen Plasmid Maxi-prep Kit, Germany). Cultures of human S10-3 cells were maintained as previously described (Emerson et al., 2006) and were seeded (0.5×10^6 cells/well, in duplicate), in a 12-well culture plate. The Bgl*II*-linearized pSK-GFP constructs were transcribed *in vitro* and transfected into S10-3 cells, essentially as described elsewhere (Emerson et al., 2004). All transfected cells were observed for GFP production on day six by fluorescence microscopy (FM) (Zeiss). S10-3 cultures transfected with wild-type replicon (*pSK-GFP-WT*) showed approximately 50% GFP(+) cells and served as positive control while those receiving polymerase-mutant replicon (*pSK-GFP-GAD*) were GFP(−) and included as negative control (Fig. 1A). Of the PCP-domain (aa. 433–592) constructs (Group-I: eight 'Cys→Ala' and four 'His→Leu' mutants), six 'Cys' (C457A, C459A, C471A, C472A, C481A, C483A) and three 'His' (H443L, H497L, H590L) mutants completely abolished RNA replication (Fig. 1B, left panel). Notably, of these essential 'Cys' and 'His' residues, C483 and H590 had been previously predicted as putative 'catalytic' residues in PCP-domain (Koonin et al., 1992). This was, however, in contrast with mutational analysis of the universally conserved C483 that did not affect ORF1 processing (Ropp et al., 2000). However, the non-conserved H590 mutant 'H590L' completely aborted the RNA replication. Further, all PCP-domain group-II mutants (nts. 1340–1801; $n = 7$) that had conserved amino acid sequences allowed production of GFP comparable to wild-type culture (Fig. 1B, left panel). This confirmed the functional role of PCP-domain 'Cys' and 'His' residues in HEV replication at protein level. The above lethal mutant-replicons were double-checked for reproducibility of the results.

HEV is most closely related to the rubivirus genera of the Togaviridae family of positive-sense, single-stranded RNA viruses where proteolytic processing of viral polyprotein occurs through recognizing a cleavage-site. The RUBV polyprotein cleavage-substrate, a 'Gly' triad (G1299-G1300-G1301), downstream of protease-domain has been characterized (Chen et al., 1996). The HEV ORF1 sequence homology search identified a similar 'G815-G816-G817' sequence in the X-domain, downstream of PCP-domain. A further multiple alignment of HEV partial sequences showed a highly conserved 'Gly–Gly–Gly(GGG)' triplet in X-domain (Fig. 2A). Therefore, X-domain mutant replicons (Group-1: three'Gly→Val' mutants; Group-II: sequential nucleotide

A

```
Consensus: NNGGGNCNANNNNNNNNNNNNANNNNNNGNAANTNTNNNNNNANN
AB074918:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB074920:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB089824:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AY575857:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AY575858:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AY575859:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AF082843:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AF060669:  RE...L.H.FYQRFPEAFYSTEFIMRE.L..Y.L.PRPIIH.V.
AY115488:  RE...L.H.FYQRFPEAFYSTEFIMRE.L..Y.L.PRPIIH.V.
AB091394:  RE...L.H.FYQRFPEAFYPTEFVMRE.L..Y.L.PRPIIH.V.
AB222182:  RE...L.H.FYQRFPEAFYPTKFIMRE.L..Y.L.PRPIIH.V.
AB246676:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB222183:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB236320:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB189071:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB189072:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB189074:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB189073:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB189075:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB189070:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AP003430:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB222184:  RE...L.H.FYQRFPEAFHPIKFIMRE.L..Y.L.PRPIIH.V.
AB073912:  RE...L.H.FYQRFPEAFYPTEFIMRE.L..Y.L.PRPIIH.V.
AB248521:  RE...L.H.FYQRYPESFHPTDFIMRE.L..Y.L.PRPIIH.V.
AB248522:  RE...L.H.FYQRYPESFHPTDFIMRE.L..Y.L.PRPIIH.V.
AB248520:  RE...L.H.FYQRFPESFHPTDFIMRE.L..Y.L.PRPIIH.V.
AF455784:  RE...L.H.FYQRFPESFYP.DFIMRD.L..Y.L.PRPIIH.V.
AB074917:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220972:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB161719:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB161718:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220973:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220975:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220978:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220977:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220979:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB220976:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB161717:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB074915:  RE...L.H.FYQRFPESFDP.EFVMSD.F..Y.L.PRPIIH.V.
AB091395:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB200239:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB099347:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB193176:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB097811:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB193177:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB193178:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB097812:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB220971:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB080575:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB220974:  RE...L.H.FFQRFPESFDP.EFVMSD.L..Y.L.PRPIIH.V.
AB108537:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
DQ450072:  RE...L.H.FYQRFPESFDP.EFVMSD.P..Y.L.PRPIIH.VV
AB197674:  RE...L.H.FYQRFPESFDP.EFIMSD.L..Y.L.PRPIIH.V.
EF077630:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AB197673:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AY723745:  RE...L.H.FYQRFPESFDP.EFVMSD.L..Y.L.PRPIIH.V.
AJ272108:  RE...L.H.FYQRFPESFDP.EFIMSD.F..Y.L.PRPIIH.V.
AY594199:  RE...L.H.FYQRFPESFDP.EFVMSD.L..Y.L.PRPIIH.V.
AB253420:  RE...L.H.FYQRFPESFDP.EFIM.D.F..Y.L.PRPIIH.V.
DQ279091:  RE...L.H.FYQRFPESFDP.EFIM.D.F..Y.L.PRPIIH.V.
AF028091:  CE...L.H.FYQRYPASFDA.CFVMRD.A..Y.L.PRPIIHRV.
AF076239:  CE...L.H.FYQRYPASFDA.CFVMRD.A..Y.L.PRPIIHRV.
AF459438:  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
AF051830:  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
X99441   :  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
D10330   :  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
M73218   :  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
AF444002:  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
AF444003:  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
L25547   :  RE..EL.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
L25595   :  RE..EL.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
L08816   :  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
M94177   :  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
D11093   :  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.VV
X98292   :  RE...L.H.FYQRYPTSFDA.SFVMRD.A..Y.L.PRPIIH.V.
AY230202:  RE...L.H.FYQRYPASFDA.SFVMRD.A..Y.L.PRPIIH.V.
M74506   :  RE...L.H.FFQRYPDSFDATKFVMRD.L..Y.L.PRPIIH.V.
```

B

RUBV	G1299V	G1300V	G1301V
HEV	G815V	G816V	G817V

Fig. 2. A. ORF1 polyprotein amino acid sequence (partial) alignment of 77 HEV strains (GeneBank accession numbers: AB074918, AB074920, AB089824, AY575857, AY575858, AY575859, AF082843, AF060669, AY115488, AB091394, AB222182, AB246676, AB222183, AB236320, AB189071, AB189072, AB189074, AB189073, AB189075, AB189070, AP003430, AB222184, AB073912, AB248521, AB248522, AB248520, AF455784, AB074917, AB220972, AB161719, AB161718, AB220973, AB220975, AB220978, AB220977, AB220979, AB220976, AB161717, AB074915, AB091395, AB200239, AB099347, AB193176, AB097811, AB193177, AB193178; AB097812; AB220971; AB080575; AB220974; AB108537; DQ450072; AB197674, EF077630, AB197673, AY723745, AJ272108, AY594199, AB253420, DQ279091, AF028091, AF076239, AF459438, AF051830, X99441, D10330, M73218, AF444002, AF444003, L25547, L25595, L08816, M94177, D11093, X98292, AY230202 and M74506), representing genotypes 1, 2, 3 and 4. The conserved 'G815-G816-G817' triplet in ORF1 X-domain is shown (box). B. X-domain putative protease-substrate homology with that of RUBV where viable (green) and lethal mutations (red) are shown.

mutants) were constructed and tested for their role, if any, in viral RNA replication. Of the group-I constructs, while G816V and G817V mutants completely abrogated, the first 'Gly' mutant (G815A) allowed the viral replication (Fig. 1B, right panel). The group-II mutants (nts. 2396–2910; $n = 10$) had no significant effects on viral RNA replication (Fig. 1B, right panel) and, thus, excluded any regulatory role of X-domain at transcriptional level. This suggested that the HEV 'G815-G816-G817' amino acid sequence could act as a substrate for ORF1 proteolysis, analogous to RUBV nonstructural polyprotein-substrate (Fig. 2B). Thus, an important role of X-domain sequences in HEV replication is discovered. Very recently, an *in vitro* deubiquitination activity of HEV 'MetT-PCP' co-domains through recognizing coronavirus consensus 'L-X-G-G' substrates in ORF1 has been described (Karpe and Lole, 2011). Although the *E. coli* expressed 'MBP-Met-PCP' fusion-protein significantly hydrolyzed non-viral substrates, like IFN-stimulated gene 15 (ISG15), there was no proteolysis of viral 'Hel-RdRp' protein. It was further demonstrated that HEV replication requires active ubiquitin-proteosome machinery and that could be blocked by proteosome-inhibitors without affecting host translations (Karpe and Meng, 2012). However, these studies did not directly address the viral PCP-domain 'enzymatic' and 'L-X-G-G' substrate activities in ORF1 processing or RNA replication. In contrast, the present *ex vivo* study has precisely identified and confirmed indispensability of ORF1 PCP-domain 'catalytic' and X-domain putative 'protease-substrate' residues in HEV replication.

Despite ample reports on processing of ORF1 polyprotein, validity of a biochemically distinct and functional viral protease remains another bottle-neck in the understanding of HEV biology. To further address this, ORF1 (HEV-SAR_{55}) was amplified with '*HA*' and '*His*' epitope tagged-primer sets, using *pSK-HEV* as template and cloned into EcoR*1*/Xho*I* site of *pTriEx-1.1* expression vector (Novagen, USA). The resulting vector (*pTriEx-ORF1*) was verified by DNA sequencing that could translate *5'HA-ORF1-His-3'* in frame with vector-start/stop codon. *In vitro* translated product of *pTriEx-ORF1* (TNT coupled transcription–translation kit, Promega, USA) was immunoprecipitated (IP) with anti-HA and anti-His antibodies (Promega, USA) and subjected to Western blot (WB) analysis as described elsewhere (Parvez et al., 2011). An expected single band of ~191 kDa comparable to native ORF1 was detected (Fig. 3A) that was in line with earlier cell-free/*in vitro* studies. Further, the *ex vivo* synthesis of ORF1 and its expected processing was analyzed in *pTriEx-ORF1* tranfected S10-3 cells. In brief, 100 mm dishes of S10-3 cultures were transfected with 20 μg DNA, using fugene-6 (Promega, USA). Briefly, duplicated cultures were physically harvested and pooled at 72 h in cold PBS containing phenylmethylsulfonyl fluoride and EDTA-free protease inhibitor cocktail (Boehringer Mannheim, Germany). The cell pellet was incubated on ice for about 15–20 min and disrupted in a Dounce homogenizer (Magden et al., 2001), followed by concentrating (protein concentrator, 20 kDa cut-off) of the post-nuclear supernatant. IP/WB analysis of the total protein allowed detection of a small N-terminal fragment of ~35 kDa with anti-HA (Fig. 3B) and a C-terminal peptide of ~78 kDa with anti-His antibody (Fig. 3C), indicating ORF1 polyprotein processing *ex vivo*. The two peptides were in conformity with the same that had been detected previously (Panda et al.,

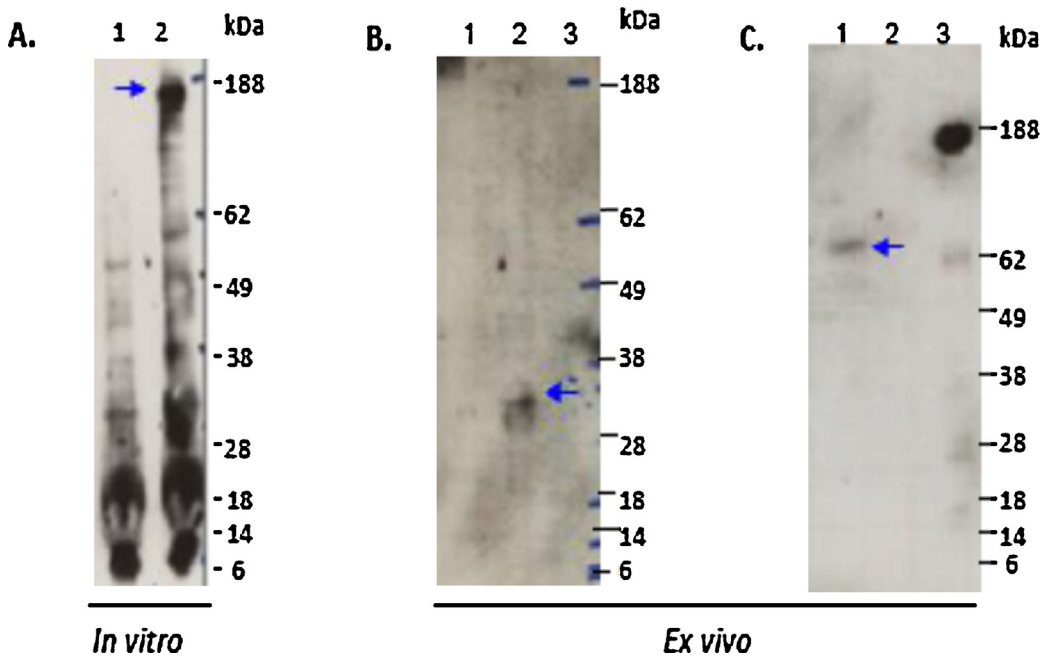


Fig. 3. IP/WB analysis of HEV ORF1 expression and processing. (A) *In vitro* translation (anti-HA) showing a band of ~191 kDa (lanes 1: *pTriEx1.1*and 2: *pTriEx-ORF1*). (B) *Ex vivo* synthesis in S10-3 cells (anti-HA) showing a band of ~35 kDa* (lanes 1: *pTriEx1.1*, 2: *pTriEx-ORF1*and 3: standard protein marker). (C) *Ex vivo* synthesis in S10-3 cells (anti-His) showing a band of ~78 kDa* (lanes 1: *pTriEx-ORF1*, 2: *pTriEx-1.1*, and 3: standard protein marker) (*Panda et al., 2000; Sehgal et al., 2006; **Ropp et al., 2000).

2000; Sehgal et al., 2006; Ropp et al., 2000). The present findings are, however, in contrast with a previous study where ORF1 expressed from a swine HEV infectious cDNA clone in non-hepatic 293T cells has not shown proteolysis (Suppiah et al., 2011). Also, in a very recent study, expressions of HEV full-length ORF1 and six truncated variants in human cervical HeLa and hepatic Huh-7 cells do not support the predicted PCP-domain (Perttilä et al., 2013).

Expression and purification of HEV protease and, therefore, its biochemical or biophysical characterization is not achieved so far. In view of this and based on earlier reports in consort with recent findings, functionality of HEV ORF1-encoded protease was quite convincing and favored by the present data. Nevertheless, these findings are in agreement with earlier reports on ORF1 processing, endorsed by Karpe et al. (2011; 2012) strongly support an ORF1-encoded protease essential for HEV replication. On the other hand, a possible role of host-encoded protease(s) in viral life cycle cannot be ignored. A universal validation of viral protease would have an important implication for the classification of HEV within the positive-strand RNA virus group as well as understanding its biology.

Acknowledgements

MKP is supported by the National Plan for Science & Technology Program (Grant No. 11-MED-1585-02), KACST, Riyadh, Saudi Arabia. A generous material and intellectual support from Dr. Suzanne U. Emerson, Molecular Hepatitis Section, LID, NIAID, National Institutes of Health, Bethesda, MD, USA is very gratefully acknowledged.

References

Aggarwal, R., Naik, S., 2009. Epidemiology of hepatitis E: current status. Journal of Gastroenterology and Hepatology 24, 1484–1493.
Ansari, I.H., Nanda, S.K., Durgapal, H., et al., 2000. Cloning, sequencing, and expression of the hepatitis E virus (HEV)nonstructural open reading frame 1 (ORF1). Journal of Medical Virology 60, 275–283.
Chandra, V., Taneja, S., Kalia, M., Jameel, S., 2008. Molecular biology and pathogenesis of hepatitis E virus. Journal of Biosciences 33, 451–464.
Chen, J.P., Strauss, J.H., Strauss, E.G., Frey, T.K., 1996. Characterization of the rubella virus nonstructural protease domain and its cleavage site. Journal of Virology 70, 4707–4713.
Emerson, S.U., Nguyen, H., Graff, J., Stephany, D.N., Brockington, A., Purcell, R.H., 2004. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. Journal of Virology 78, 4838–4846.
Emerson, S.U., Nguyen, H., Torian, U., Purcell, R.H., 2006. ORF3 protein of hepatitis E virus is not required for replication, virion assembly, or infection of hepatoma cells in vitro. Journal of Virology 80, 10457–10464.
Graff, J., Nguyen, H., Yu, C., et al., 2005. The open reading frame 3 gene of hepatitis E virus contains a *cis*-reactive element and encodes a protein required for infection of macaques. Journal of Virology 79, 6680L 6689.
Karpe, Y.A., Lole, K.S., 2011. Deubiquitination activity asociated with hepatitis E virus putative papain-like-cysteine protease. Journal of General Virology 92, 2088–2092.
Karpe, Y.A., Meng, X.J., 2012. Hepatitis E virus replication requires an active ubiquitin-proteosome system. Journal of Virology 86, 5948–5952.
Koonin, E.V., Gorbalenya, A.E., Purdy, M.A., et al., 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive strand RNA plant and animal viruses. Proceedings of National Academy of Science, USA 89, 8259–8263.
Magden, J., Takeda, N., Li, T., Auvinen, P., et al., 2001. Virus-specific mRNA capping enzyme encoded by hepatitis E virus. Journal of Virology 75, 6249–6255.
Meng, X.J., 2010. Recent advances in hepatitis E virus. Journal of Viral Hepatitis 17, 153–161.
Navaneethan, U., Al-Mohajer, M., Shata, M.T., 2008. Hepatitis E and pregnancy: understanding the pathogenesis. Liver International 28, 1190–1199.
Parvez, M.K., 2013. Chronic hepatitis E infection: risks and controls. Intervirology 56, 213–216.
Parvez, M.K., Purcell, R.H., Emerson, S.U., 2011. Hepatitis E virus ORF2 protein over-expressed by baculovirus in hepatoma cells, efficiently encapsidates and transmits the viral RNA to naïve cells. Virology Journal 8, 159–165.
Panda, S.K., Ansari, I.H., Durgapal, H., Agrawal, S., Jameel, S., 2000. The in vitro synthesized RNA from cDNA clone of hepatitis E virus is infectious. Journal Virology 74, 2430–2437.
Perttilä, J., Spuul, P., Ahola, T., 2013. Early secretory pathway localization and lack of processing for hepatitis E virus replication protein pORF1. Journal of General Virology 94 (Pt 4), 807–816.
Ropp, S.L., Tam, A.W., Purdy, M., Frey, T.K., 2000. Expression of the hepatitis E virus ORF1. Archives of Virology 145, 1321–1337.
Sehgal, D., Thomas, S., Chakraborty, M., Jameel, S., 2006. Expression and processing of the hepatitis E virus ORF1 nonstructural polyprotein. Virology Journal 3, 38.
Suppiah, S., Zhou, Y., Frey, T.K., 2011. Lack of processing of the expressed ORF1 gene product of hepatitis E virus. Virology Journal 8, 245.
Tam, A.W., Smith, M.M., Guerra, M.E., et al., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120–131.
Yamada, K., Takahashi, M., Hoshino, Y., et al., 2009. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. Journal of General Virology 90, 1880–1891.