Deubiquitination activity associated with hepatitis E virus putative papain-like cysteine protease

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Hepatitis E virus (HEV) ORF1 protein (pORF1) contains methyltransferase (MetT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) domains. ORF1 sequence analysis showed two consensus LXGG cleavage sites at 664 and 1205. LXGG sequence is recognized by viral and cellular deubiquitinating enzymes. The protein encompassing the predicted MetT-PCP domains of HEV ORF1 was tested for deubiquitinating activity using fluorogenic substrates – ubiquitin-7-amino-4-methylcoumarin (AMC), IFN-stimulated gene 15 (ISG15)-AMC, Nedd8-AMC and SUMO-AMC. MetT-PCP cleaved all four substrates but processing of ISG15-AMC was more robust. There was no processing of the Hel and RdRp domains having the conserved (1205) LXGG site by the protein. MetT-PCP carried out deISGylation of the ISG15-conjugated cellular proteins, suggesting a possible role in combating cellular antiviral pathways.

Positive-sense viral genomes can be translated after entering into the host cells. Viruses use several strategies for efficient synthesis of viral proteins by using the host cell machinery (Gale et al., 2000). Viral polyprotein processing is usually required for enzymic activities, interactions with other proteins, subcellular localization, assembly, etc.

Hepatitis E virus (HEV), (genus Hepevirus, family Hepeviridae) has a positive-sense ssRNA genome (7.2 kb) encoding non-structural protein from the 5' ORF called ORF1 and the capsid protein from the 3' ORF known as ORF2 and an auxiliary protein encoding ORF3 overlapping with ORF2 (Tam et al., 1991). Translation of ORF2 and ORF3 requires synthesis of subgenomic mRNA (Graff et al., 2006).

Based on the sequence homology, HEV ORF1 protein (pORF1) is proposed to contain putative domains for methyltransferase (MetT), papain-like cysteine protease (PCP), RNA helicase (Hel), RNA-dependent RNA polymerase (RdRp) and domains of unknown function – X and Y (Koonin et al., 1992). Among these, functional activities of RdRp (Agrawal et al., 2001), Hel (Karpe & Lole, 2010a, b) and MetT (Magden et al., 2001) have been experimentally verified. Protease function still remains an unsolved puzzle in HEV biology. At the amino acid level, HEV predicted PCP shows very less homology with the other viral PCPs (Koonin et al., 1992). It is not entirely clear whether the pORF1 is processed into biochemically distinct units. When expressed using a prokaryotic system (Escherichia coli) or using in vitro coupled transcription and translation system, no processing was observed (Ansari et al., 2000). However, ORF1 expressed using recombinant vaccinia virus showed ~186 kDa protein with shorter incubation periods and extended incubations for 24–36 h resulted in two bands of ~107 and ~78 kDa proteins (Ropp et al., 2000). HepG2 cells transfected with HEV genomic RNA produced in vitro from cDNA showed distinct processed products. Anti-MetT, anti-Hel and anti-RdRp antibodies detected specific products of ~35, ~38 and ~36 kDa, respectively (Ansari et al., 2000). ORF1 protein expressed in insect cells using baculovirus system was processed into smaller fragments, and the processing could be inhibited by E-64d, a cell-permeable cysteine-protease inhibitor. But it was not clear whether processing occurred due to protease activity from the host or from the virus (Sehgal et al., 2006). Thus, extensive attempts have been made by various groups to study pORF1 processing documenting contradictory results.

We carried out HEV ORF1 amino acid sequence analysis to search for PCP cleavage site(s) reported in different positive-sense RNA viruses. A total of 168 ORF1 sequences representing all HEV genotypes were aligned and analysed. Two LXGG sites were identified in ORF1 between (i) PCP and X domains, at aa 664 (128/168 sequences had LXGG and 27/168 had LXGN), none of the avian (n=8), rabbit (n=2) or rat (n=1) sequences had this site; (ii) Hel and RdRp domains, at aa 1205 (160/168 sequences had LXGG), none of the avian isolates had this site (Fig. 1a).

Ubiquitination is the tagging of proteins with ubiquitin (Ub) for selective destruction in proteasomes. IFN-stimulated gene 15 (ISG15), SUMO, ATG8 and Nedd8 are small Ub-like molecules (UBLs) expressed in eukaryotes that are conjugated to target proteins, often resulting
in modulation of the target protein’s stability/function/localization that are required for diverse functions like cell cycle regulation, autophagy, innate immunity, etc. (d’Azzo et al., 2005; Haglund & Dikic, 2005; Welchman et al., 2005). Deubiquitinating enzymes (DUBs) are proteases that cleave Ub or UBLs from the target proteins. The papain-like proteases of coronaviruses process their replicase polyprotein using a consensus recognition site of LXGG and these enzymes also act as DUBs (Barretto et al., 2005; Lindner et al., 2005). Adenovirus, herpes simplex virus-1, Epstein–Barr virus and cytomegalovirus are also known to have proteases with DUB activity (Balakirev et al., 2002; Kattenhorn et al., 2005; Schlieker et al., 2005; Wang et al., 2006). The L proteins from Crimean–Congo hemorrhagic fever virus and Dugbe virus, the nsP2 proteins from the arteriviruses, equine arteritis virus and porcine respiratory and reproductive syndrome virus contain ovarian tumour domains. These viral proteins were found to globally deconjugate both Ub and ISG15 chains via their cysteine-protease activity and inhibit NF-kB-dependent signalling (Frias-Staheli et al., 2007).

We hypothesized that HEV putative PCP may recognize LXGG consensus sequence to carry out pORF1 processing and may also possess deubiquitinating activity. Many viral cysteine proteases and cellular deubiquitinating enzymes require Zn-binding finger for their enzymic activities (Herold et al., 1999; Reyes-Turcu et al., 2006, 2009). HEV ORF1 sequence analysis (SMART, domain prediction programme) showed one putative a Zn-finger domain in the MetT region. The region encompassing predicted MetT and PCP domains of HEV ORF1 (aa 22–592, 1710 nt) was amplified by PCR from plasmid pGEM-T-Easy-HEVT1FG (encoding complete genome of genotype 1 HEV; GenBank accession no. DQ459342) and cloned into vector pMal-5cX in-frame with N-terminal maltose-binding protein (MBP) tag. Induction was done with 1.0 mM IPTG for 4 h at 25 °C. The protein was purified from E. coli culture pellets using amylose resin column (NEB) followed by gel filtration chromatography (Sephacryl HR100: CV-120 ml, Akta Basic 100 HPLC system; Amersham Biosciences) (Fig. 1b).

The deconjugation activity of purified MetT-PCP protein was tested by using fluorogenic substrates – ubiquitin-7-amino-4-methylcoumarin (Ub-AMC), ISG15-AMC, Nedd8-AMC and SUMO-AMC (Boston Biochemicals). The 50 ml reaction contained 50 mM HEPES (pH 7.5), 1 mg BSA ml⁻¹, 1 mM MgCl₂, 1 mM ZnCl₂, 100 nM MetT-PCP protein and 0.5–5 μM Ub-AMC at 37 °C. The rate of substrate hydrolysis was determined by monitoring released fluorescence (excitation wavelength: 380 nm; emission: 460 nm) as a function of time by using a Perkin Elmer 2030 Reader (Victor X3). The activity was first tested using Ub-AMC and the change of fluorescence intensity was measured over time at different substrate concentrations. HEV MetT-PCP was found to cleave the bond between Ub and AMC, resulting in the release of the fluorescent dye and an increase of the fluorescence intensity (data not shown). One micromolar substrate concentration was found to be adequate for the sensitive detection of the released product. For optimization of the assay conditions for all four substrates, each substrate was tested separately (at 1 μM conc.) at different reaction conditions like pH (6.0, 6.5, 7.0, 7.5 and 8.0), NaCl concentration (0–200 mM) and different incubation temperatures (25–41 °C). Overall, these substrates required similar conditions; hence all further assays were carried out at 37 °C in buffer containing 50 mM HEPES (pH 7.5), 1 mg BSA ml⁻¹, 1 mM MgCl₂ and 1 mM ZnCl₂. Substrates (1 μM) were tested and analysed in parallel, on the
same microtitre plate. MetT-PCP hydrolysed all four substrates but hydrolysis of ISG15-AMC was significantly higher than that of the other substrates (Fig. 2a). Purified MBP and E. coli cell lysate, processed similarly, were tested as controls and both were completely negative with respect to AMC release. These results suggested that HEV MetT-PCP has deubiquitinating activity. ISG15-AMC hydrolysis (deISGylation) shown by HEV MetT-PCP was comparable with the activity of UBP43 (a well characterized ISG15-specific protease present in cells) (Malakhova et al., 2006) (Boston Biochemicals) (Fig. 2b).

Earlier studies have shown that HEV pORF1 remains unprocessed when expressed in cell-free system or HepG2 cells (Ansari et al., 2000; Ropp et al., 2000). To see whether MetT-PCP carries out trans proteolysis of HEV protein containing conserved LXGG site, the region encompassing highly conserved LXGG site (aa 960–1693) was amplified, cloned in the pET15b vector and used as a template for carrying out in vitro coupled transcription and translation (TNT Quick Coupled Transcription/Translation System; Promega) in the presence of 50 μCi [35S] methionine (BRIT). One microlitre of the translation product was incubated with 2 μg MetT-PCP protein under the same reaction conditions as above and incubated for 2 h at 37 °C. Samples were subjected to 10 % SDS-PAGE, fluorography and autoradiography. There was no processing of Hel–RdRp protein possibly due to the fastidious requirements of the enzyme or prerequisite of protein–protein interactions, which occur specifically during viral replication (Fig. 2c).

ISG15 is rapidly induced by IFN-α/β. ISG15 conjugation results in mounting antiviral response in the cells (Skaug & Chen, 2010). HEV MetT-PCP showed better hydrolysis of ISG15-AMC as compared with Ub-AMC, strongly suggesting that the protein may carry out deconjugation of ISGylated proteins in the host cells. MetT-PCP was cloned in vector pAcGFP-N1 to express it with an N-terminal GFP tag. HepG2 (human hepatocarcinoma) cells were transfected with GFP-MetT-PCP plasmid using Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer’s instructions. Transfected cells detached within 24 h and there was no GFP expression in the remaining adherent (surviving) cells until 96 h post-transfection. Two human hepatoma cell lines (HuH-7 and PLC-PRF/5) and two non-hepatic cell lines (RD, human rhabdomyosarcoma and Vero E6, African green monkey kidney epithelium) were similarly transfected but none survived. MetT-PCP was cloned with C-terminal myc tag in the pcDNA3.1 vector. Transfection of this construct into HepG2 cells yielded similar results, indicating that expression of MetT-PCP was toxic to the cells and significant cell death was observed within 24 h of transfection in all the cell lines tested. Cell death was confirmed by doing an MTT assay (results not shown).

As extensive attempts to express the MetT-PCP protein in mammalian cells failed, ISGylation was induced in HepG2 cells and the ISGylated proteins were incubated with MetT-PCP protein in vitro to test for deISGylation activity. For that, cells were co-transfected with pCMV-FLAG-IGS15 (kindly provided by Dr Joo-Yeon Yoo, Postech

![Fig. 2. (a) Deconjugating activity of HEV MetT-PCP using different UBL-AMC substrates: Ub-AMC, ISG15-AMC, SUMO-AMC and Nedd8-AMC (1 μM) were incubated in the presence of 100 nM MetT-PCP. Release of AMC fluorescence was monitored using 380 nm excitation and 460 nm emission wavelengths as a function of time for 30 min. (b) Comparative activity of HEV MetT-PCP and UBP43: ISG15-AMC (1 μM) was incubated with 100 nM of each MetT-PCP and UBP43 separately. Release of AMC fluorescence was monitored using 380 nm excitation and 460 nm emission wavelengths as a function of time for 30 min. (c) Assessment of HEV MetT-PCP peolytic activity: HEV Hel-RdRp pET15b construct was used as a template for in vitro coupled transcription and translation reaction in the presence of [35S] methionine, 1 μl of the translation reaction was incubated at 37 °C for 2 h with 2 μg MetT-PCP and the samples were subjected to 10 % SDS-PAGE, fluorography and autoradiography. Lane 1, in vitro translation product; lane 2, in vitro translation product incubated with MetT-PCP.](image-url)
University, Korea), pCMV-SPORT6-E2 (UBCH8) and pCMV-SPORT6-E1 (UBE1L) (OpenBiosystems) (E1 was obtained as pOTB7-E1 and recloned into pCMV-SPORT6 vector using XhoI and EcoRI restriction sites to generate pCMV-SPORT6-E1). Forty-eight hours post-transfection, cells were starved in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium (Invitrogen) for 2 h, followed by 2 h pulse in the medium containing 50 μCi [35S] Elegmix (70 % methionine and 30 % cysteine) (BRIT) and chase of 2 h. Cells were washed twice with cold PBS (pH 7.4), lysed in 500 μl RIPA buffer. Anti-FLAG (anti-DYKDDDDK) antibodies (1:1000) (Thermo Scientific) were added and incubated overnight at 4 °C. Protein G beads (Bangalore Genei) (50 μl) were added for IgG pull down and the samples were analysed by 10 % SDS-PAGE, fluorography and autoradiography. FLAG-ISG15-conjugated proteins were detected in HepG2 cells transfected alone with pFLAG-ISG15; however, co-transfection with E1 and E2 significantly increased ISGylated protein levels (Fig. 3a). To see whether MetT-PCP is able to remove ISG15-FLAG tag from ISGylated proteins, after pull down with protein G beads, 100 μl buffer [50 mM HEPES (pH 7.5), 1 mg BSA ml⁻¹, 1 mM MgCl₂ and 1 mM ZnCl₂] containing 1 μg MetT-PCP or 6 × His-UBP43 (Boston Biochemicals) were added separately and kept on a rocker for 1 h at room temperature. Beads were separated, washed, treated with 1 × SDS-PAGE sample buffer and analysed. There was a significant decrease in the intensity of ISGylated protein bands, indicating that HEV MetT-PCP carries out delISGylation of the ISGylated cellular proteins (Fig. 3b). MBP and UBP43 were used as negative and positive controls, respectively, and showed the expected results. This was further confirmed by counting total radiolabel incorporated in the ISGylated proteins before and after incubation with the proteases in a duplicate set of experiments. For that, 1 μl sample (after treatment with SDS-PAGE sample buffer) was spotted on to a GF/C (Whatman) filter, dried, 1 ml scintillation fluid was added and counts were taken in the Packard Tri-carb 2810TR liquid scintillation counter and means of the two experiments were calculated. MetT-PCP- and UBP43-treated samples showed 40 and 60 % reduction in the radiolabel isotope, respectively, as compared with the untreated controls, indicating delISGylation of the cellular proteins. MBP controls did not show any significant change in the counts as compared to the untreated controls.

In conclusion, delISGylation activity associated with HEV putative PCP may be essential for invading cellular antiviral pathways. The protein may carry out pORF1 processing, possibly only in the context of HEV replication. However, possibility of HEV polyprotein processing by cellular DUB enzymes cannot be ruled out.

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References


