Virus Research 210 (2015) 8–17

Contents lists available at ScienceDirect

Virus Research

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

Characterization of self-assembled virus-like particles of dromedary camel hepatitis e virus generated by recombinant baculoviruses

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ARTICLE INFO

Article history:
Received 7 April 2015
Received in revised form 26 June 2015
Accepted 30 June 2015
Available online 6 July 2015

Keywords:
Dromedary camel HEV
Recombinant baculovirus
Virus-like particles
VLP
Insect cells Tn5

ABSTRACT

Dromedary camel hepatitis E virus (DcHEV), a novel hepatitis E virus, has been identified in dromedary camels in Dubai, United Arab Emirates. The antigenicity, pathogenicity and epidemiology of this virus have been unclear. Here we first used a recombinant baculovirus expression system to express the 13 and 111 N-terminus amino-acid-truncated DcHEV ORF2 protein in insect Tn5 cells, and we obtained two types of virus-like particles (VLPs) with densities of 1.300 g/cm^3 and 1.285 g/cm^3, respectively. The small VLPs (Dc4sVLPs) were estimated to be 24 nm in diameter, and were assembled by a protein with the molecular mass 53 kDa. The large VLPs (Dc3nVLPs and Dc4nVLPs) were 35 nm in diameter, and were assembled by a 64-kDa protein. An antigenic analysis demonstrated that DcHEV was cross-reactive with G1, G3–G6, ferret and rat HEVs, and DcHEV showed a stronger cross-reactivity to G1 G3–G6 HEV than it did to rat and ferret HEV. In addition, the antibody against DcHEV-LPs neutralized G1 and G3 HEV in a cell culture system, suggesting that the serotypes of these HEVs are identical. We also found that the amino acid residue Met-358 affects the small DcHEV-LPs assembly.

1. Introduction

Hepatitis E virus (HEV) is causative agent of hepatitis E, a viral disease that manifests as acute hepatitis E (Emerson and Purcell, 2003). HEV was named in 1990, when its full genome was identified; before then HEV was identified as "epidemic, non-A, non-B hepatitis" (Reyes et al., 1990; Wong et al., 1980). The HEV infection occurs mainly through the fecal-oral route (Balayan et al., 1983), and acute hepatitis E is known as an important public health problem not only in developing countries but also in industrialized countries, where HEV is transmitted mainly by imported or zoonotic infection (Li et al., 2012; Meng, 2010).

HEV, a small non-enveloped single-stranded positive-sense RNA virus, is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Meng et al., 2012). The genome of HEV is approximately 7.2 kilo bases (kb) in size, and it contains three open reading frames (ORFs). ORF1 encodes nonstructural proteins involved in replication and viral protein processing. ORF2 both encodes a capsid protein that contains immunogenic epitopes and induces neutralizing antibodies, and ORF2 is the target for vaccine development (Li et al., 2004; Zhu et al., 2010). ORF3, which partially overlaps with ORF2, encodes a cytoskeleton-associated phosphoprotein with multiple functions (Graff et al., 2005; Tyagi et al., 2001, 2004; Zafrullah et al., 1997).

To date, at least four genotypes of HEV that infect humans have been identified. Of the four genotypes of HEV, genotypes 1 and 2 are found exclusively in human beings and are transmitted via contaminated water in developing countries. Genotype 1 occurs mainly in Asia, and genotype 2 occurs mainly in Africa and Mexico. Genotypes 3 and 4 have been isolated from patients as well as from infected animals such as domestic pigs, wild boars and wild deer (Bradley, 1995; Huang et al., 1992; Meng et al., 1997; Takahashi et al., 2002; Zhao et al., 2009). A number of sporadic cases have been transmitted in a zoonotic fashion in developed countries, with zoonotic hepatitis E being mainly associated mainly with genotype 3 or 4 HEV infections (Li et al., 2005; Meng, 2010; Tei et al., 2003).

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http://dx.doi.org/10.1016/j.virusres.2015.06.022
0168-1702/

In addition to humans, other animals such as chickens, bats, rats, rabbits, mongooses, foxes, moose, minks, ferrets, and even fish also harbor HEVs, including a number of genetically distant strains (Batts et al., 2011; Bodewes et al., 2013; Drexler et al., 2012; Haqshenas et al., 2001; Jay Lin et al., 2014; Johne et al., 2010; Krog et al., 2013; Nakamura et al., 2006; Raj et al., 2012; Zhao et al., 2009). The division of the *Hepeviridae family* into two genera, *Orthohepevirus* and *Piscihepevirus*, was recently proposed (Smith et al., 2014) (http://ictvonline.org/virusTaxonomy.asp). The orthohepevirus includes four species: *Orthohepevirus A* including isolates from human, pig, wild boar, deer, mongoose, rabbit and camel; *Orthohepevirus B* including isolates from chicken; *Orthohepevirus C* including isolates from rat, greater bandicoot, Asian musk shrew, ferret and mink; and *Orthohepevirus D* including isolates from bat. The cutthroat trout virus belongs to the genus *Piscihepevirus*. Whether these animal HEVs are transmitted to humans is not yet clear, and whether the infected animals develop signs of hepatitis is unknown.

Dromedary camel HEV (DcHEV) was first identified from fecal samples of dromedary camels (*Camelus dromedarius*) in Dubai, United Arab Emirates (Woo et al., 2014). The DcHEV genome is 7.22 kb and contains three major ORFs (OFR1–3). ORF1 encodes a nonstructural protein of 1698 aa, ORF2 encodes a capsid protein of 660 aa, and ORF3 encodes a phosphoprotein of 113 aa. The complete genome sequencing of two DcHEV strains (GenBank accession nos. KJ496143 and KJ496144) showed more than 20% overall nucleotide difference to known HEVs, and only shared 52.1–56.5% nucleotide identity with avian, bat, rat and ferret HEVs (Woo et al., 2014). Though the DcHEV genome has been sequenced, the antigenicity, pathogenicity and epidemiology of DcHEV have remained unclear because of the lack of a cell culture system in which to grow the virus.

Here we describe the efficient expressions of 13- and 111-*N*-terminus-aa-deleted DcHEV ORF2 proteins by a recombinant baculovirus in insect Tn5 cells. The proteins were found to self-assemble into virus-like particles (VLPs). The VLPs exhibited antigenic cross-reactivity with rat, ferret, G1, and G3 to G6 HEVs, and the serotype of DcHEV was identical to those of G1 and G3 HEV.

2. Materials and methods

2.1. Construction of recombinant baculoviruses and the expression of capsid proteins

We synthesized two full-length ORF2s of DcHEV containing the *Bam*HI site before the start codon and the *Xba*I site after the stop codon based on the DcHEV sequences deposited in GenBank (KJ496143 and KJ496144). For simplicity, we propose Dc3 (KJ496143) and Dc4 (KJ496144) as these two DcHEV strains' numbers, respectively. These full-length ORF2s were cloned into a vector, pUC57, to generate the respective plasmids pUC57-Dc3ORF2 and pUC57-Dc4ORF2 (GeneScript, Piscataway, NJ). DNA fragments encoding the N-terminus-truncated DcHEV ORF2s were amplified by polymerase chain reaction (PCR) using plasmid pUC57-Dc3ORF2 or pUC57-Dc4ORF2 as a template.

The DNA fragments encoding 13- and 111-*N*-terminal-aa-truncated DcHEV ORF2s were amplified by PCR with the primers, DcHEV-N13 (5′-AAGGATCCATGTTGCCTATGCTGCCCGCGCCA-3′)/DcHEV-CR1 (5′-AGTCTAGATTAATACTCCCGAGTTTTACCCA-3′) and DcHEV-N111 (5′-AAGGATCCATGGCTGTTGCTCCCGCCCCAGG-3′)/CR1, respectively. The amplified DNA fragments were purified with a Qiagen Gel purification kit (Qiagen, Valencia, CA) and cloned into TA 2.1 cloning vector (Invitrogen, San Diego, CA). The full-length and truncated ORF2s were digested with *Bam*HI and *Xba*I and ligated with the baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA) to yield plasmids pVL1393Dc3ORF2, pVL1393Dc3n13ORF2, pVL1393Dc3n111ORF2, pVL1393Dc4ORF2, pVL1393Dc4n13ORF2, and pVL1393Dc4n111ORF2.

Mutations were introduced by the PCR amplification of overlapping fragments with specific mutation primers. To introduce the mutation of Thr-358 into Dc3ORF2, we carried out PCR amplifications with primer pairs DcHEV-n111/Dc3ORF2-358R (5′-TAGACCATTCGTACCAGTAAA-3′), and Dc3ORF2-358F (5′-TTTACTGGTACGAATGGTCTA-3′)/CR1, by using the pUC57-Dc3ORF2 as a template. Consequently both fragments were purified and combined in a fusion PCR with the primers DcHEV-n111/CR1. Finally, this fragment was cloned into the transfer vector pVL1393 to yield the plasmid pVL1393Dc3n111ORF2mt. Recombinant viruses were produced in Sf9 cells and designated as Ac[Dc3ORF2], Ac[Dc3n13ORF2], Ac[Dc3n111ORF2], Ac[Dc4ORF2], Ac[Dc4n13ORF2], Ac[Dc4n111ORF2], and Ac[Dc3n111ORF2mt]. To achieve large-scale expression, we used an insect cell line from *Trichoplusia ni* (cabbage looper moth), BTL-Tn 5B1-4 (Tn5) (Invitrogen), infected with recombinant baculoviruses at a multiplicity of infection (MOI) of 10 (Li et al., 1997; Yang et al., 2013).

2.2. SDS-PAGE and Western blot analysis

The proteins in the cell lysates and culture medium were separated by 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Western blot analysis with rabbit anti-G1 HEV-LPs polyclonal antibody as described (Li et al., 1997).

2.3. Purification of VLPs

The recombinant baculovirus-infected Tn5 cells were harvested on day 7 post-infection (p.i.). After the intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 *g* for 60 min, the supernatant was spun at 32,000 rpm for 3 h in a Beckman SW32Ti rotor. The resulting pellet was resuspended in EX-cell™ 405 medium at 4 °C overnight. The recombinant baculovirus-infected cells were treated with a denaturation buffer containing 50 mM sodium borate, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 5% 2-mercaptoethanol, and gently rocked at room temperature for 2 h. The lysate was diluted with Ex-cell 405 and centrifuged at 32,000 rpm for 3 h in a Beckman SW32Ti rotor, and the pellet was resuspended in Ex-cell 405 and purified by CsCl gradient centrifugation as described (Li et al., 2007).

2.4. Transmission electron microscopy (TEM)

Purified VLPs were placed on a carbon-coated grid for 45 s, rinsed with distilled water, stained with a 2% uranyl acetate solution and examined with an electron microscope (TEM-1400, JEOL, Tokyo) operating at 80 kV.

2.5. Structural observation

The atomic structure of $T = 1$ particle (PDB-id: 2ztn) and the pseudo-atomic structure of $T = 3$ particle (PDB-id: 3iyo) were both downloaded from the PDB database. The measurements of the distance of residues were performed using Pymol software (http://www.pymol.org), and the figures were made using the software UCSF Chimera (Pettersen et al., 2004).

2.6. N-terminal amino acid sequence analysis

The proteins were purified by CsCl gradient centrifugation. The *N*-terminal aa microsequencing was carried out using 100 pmol of