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Role of asparagine at position 562 in dimerization and immunogenicity of the hepatitis E virus capsid protein

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ABSTRACT

The hepatitis E virus (HEV) capsid protein, pORF2, contains 2 potential N-glycosylation sites, N137 and N310, located in the S domain, and one site, N562, in the P domain. The last domain located at positions 454–606 aa forms a protruding spike from the shell, with N562 being located in the apical center of the spike, which is also a cell-attachment region and neutralizing antigenic site. Here, we expressed in *Pichia pastoris* a recombinant polypeptide p179 comprising the region of 439–617 aa of the HEV pORF2 as well as a set of 4 mutant proteins containing substitutions of Q, D, P and Y instead of N at position 562. All proteins were shown to be secreted from yeast. Using SDS-PAGE, Western blot analysis and tunicamycin treatment assay, we showed that the wild-type (*wt*) protein, p179N562, and 2 mutant variants, p179N562Q and p179N562D, formed homodimers but only the *wt* protein was shown to be glycosylated. As homodimers, all 3 proteins were immunoreactive with a neutralizing monoclonal antibody (5G5); however, they did not immunoreact with 5G5 after denaturation into monomers. Two other mutant variants, p179N562P and p179N562Y, did not form homodimers but were immunoreactive with the 5G5 antibody. The *wt* protein was shown to be less immunoreactive with 5G5 than the mutant variants in a double-antibody sandwich ELISA, suggesting a role of glycosylation at N562 in reducing antibody binding. *In vitro* neutralization experiments showed a more efficient neutralization with mouse antibody against p179N562P and p179N562Y than against the other 3 proteins. These findings indicate that specific substitutions at position 562 have a more measurable effect on the activity of the HEV neutralizing epitope than dimerization or glycosylation of the structural protein. Furthermore, the secretion of monomers fully immunoreactive may call into question the importance of dimerization for an effective presentation of HEV neutralization epitopes.

1. Introduction

Hepatitis E virus (HEV) is transmitted via a fecal-to-oral route, predominantly through contaminated water, and causes epidemic and sporadic cases of acute hepatitis E in developing countries in Asia, Africa and Latin America, where sanitary conditions are suboptimal (Sharma, 2006). Hepatitis E is generally a mild disease but may be severe, especially in pregnant women, among whom the mortality rate reaches 10%–40%, owing to the development of fulminant liver disease (Khuroo and Kamili, 2003). Recently, chronic HEV infection has been reported in immune suppressed patients (Kamar et al., 2008a, 2008b; Murali et al., 2015). Detection of HEV in farmed swine as well as in feral animals (Johne et al., 2010; Meng et al., 1997b; Takahashi et al., 2011; Zhao et al., 2009) and reports of HEV transmission to humans via consumption of raw or undercooked meat of deer, boars and pigs (Matsuda et al., 2003; Tamada et al., 2004; Tei et al., 2003) strongly suggest a zoonotic spread of HEV. Global distribution and frequent detection, especially in developing countries, indicate that HEV poses a major public health threat to people worldwide.

HEV is a sole member of Orthohepevirus genus in the family of the Hepeviridae (Smith et al., 2014). It is a small non-enveloped icosahedral virus of 27–34 nm in diameter. The virus genome is a single-stranded (+) RNA of about 7.2 kb with short 5′ and 3′ noncoding regions, and contains three open reading frames (ORFs) (Tam et al., 1991). The largest ORF, ORF1, encodes nonstructural proteins including methyltransferase, protease, helicase and RNA-dependent RNA polymerase. The smallest ORF, ORF3, encodes a phosphoprotein involved in immune evasion and regulation of viral replication and capsid assembly. The ORF2 encodes a single structural protein of 660 amino acid (aa), which forms the viral capsid responsible for binding cellular receptors and eliciting neutralizing antibodies (Chandra et al., 2010; Takahashi et al., 2008; Tyagi et al., 2002, 2004, 2005; Yamada et al., 2009; Zafrullah et al., 1997). These neutralizing antibodies are believed to bind conformational epitopes that are dependent on the homodimerized state of the ORF2 protein (Li et al., 2015). A general architecture of the HEV capsid was recently elucidated using cryo-electron microscopy. The capsid contains 3

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domains: domain S comprises region at position 118–313 aa and forms the viral shell, domain P1 comprises region at position 314–453 aa and forms a surface plateau at 3-fold-related axes of the virus capsid, and domain P2, at position 454–606 aa which forms a protruding spike from the shell (Guu et al., 2009).

The capsid protein is expressed in mammalian cells in 2 forms: unglycosylated 74 kDa and glycosylated 88 kDa (Jameel et al., 1996). The ORF2-encoded protein contains three potential N-glycosylation sites, N137 and N310 in the S domain and N562 in the P domain. The reverse genetics system in cell culture revealed that mutation of the N137 and N310 glycosylation sites prevented virion assembly, whereas mutation of the N562 site permitted particle formation and RNA encapsidation, but the particles were not infectious (Graff et al., 2008). The N562 site is highly conserved and located in the apical center of the spike, which comprises a cell-attachment region and neutralizing antigenic region (Mori and Matsuura, 2011). Location of this site suggests that it might play an important role in capsid proteins homo-dimerization and vaccine immunogenicity. Here, using site-specific mutagenesis of the HEV p179 vaccine (439-617aa) expressed in *Pichia pastoris*, we explored the role of N562 in glycosylation, dimerization and immune response.

2. Materials and methods

2.1. Host strain and expression vector

The *P. pastoris* strain SMD1168 was used to express proteins. Plasmid pPICZαA served as an expression vector. It contains the *AOX1* promoter and a *Saccharomyces cerevisiae* α-factor secretion signal that allows the efficient secretion of most proteins from *P. pastoris*. It also contains zeocin-resistance gene to select putative multi-copy recombinants. Both *P. pastoris* SMD1168 and vector pPICZaA were purchased from Invitrogen, Life Technologies, NY, USA.

2.2. Site-directed mutagenesis and construction of recombinant plasmids

Plasmid pET-28a (+)/p179 containing the 439-617aa region of HEV ORF2 protein (Genotype 4), which has been constructed previously in our laboratory, served to generate a set of genes encoding p179 and its related mutants. The mutants were designed by replacement of N562 with aa similar to N in molecular weight; namely, Q, D P, Y, representing amide, acidic, cyclic and aromatic aa. Site-directed mutagenesis was conducted using the overlap-extension PCR approach. Primers used in the study are shown in Table 1.

The p179-gene and its mutants carried *EcoRI* and *XbaI* sites at their 5′- and 3′-ends, respectively. Subsequently, all genes were cloned with pPICZaA. All PCR were performed using Expand High Fidelity System (Roche, Mannheim, Germany). PCR products were purified using QIAprep® Spin Miniprep Kit (QIAGEN Sciences, MD, USA). Recombinant plasmids were purified using EZ gene™ Plasmid Miniprep Kit (Biomiga SanDiego). Gene structures were confirmed by DNA sequencing.

Table 1
Primers used for mutagenesis. The restriction sites introduced in primers are underlined. Lowercase letters indicate modified codons in the ORF2 protein.

Construct	Squence (5′ to 3′)
p179N562	1.F:CCC *GAA TTC* GTT ATC CAG GAC TAT GAT AAT C
	2.R:TCT *TCT AGA* TCA AGG GTA ATC AAC AGT GTC C
p179N562Q	3.F: ATC ACT AGC AGT AGT TTG ATA ATT GTA TGG G
	4.R: CCA TAC AAT TAT caa ACT ACT GCT AGT GAT C
p179N562D	5.F: ATC ACT AGC AGT AGT ATC ATA ATT GTA TGG G
	6.R: TAC CCA TAC AAT TAT gat ACT ACT GCT AGT G
p179N562P	7.F: ATC ACT AGC AGT AGT TGG ATA ATT GTA TGG G
	8.R: TAC CCA TAC AAT TAT cca ACT ACT GCT AGT G
p179N562Y	9.F: ATC ACT AGC AGT AGT GTA ATA ATT GTA TGG G
	10.R: TAC CCA TAC AAT TAT tac ACT ACT GCT AGT G

2.3. Electroporation and isolation of multi-copy recombinants in vivo

A mixture of 80 μl of the *P. pastoris* SMD1168 cells, prepared from a culture growing in log phase, and 5–10 μg of SacI-linearized plasmid was transferred to the ice–cold 0.2 cm electroporation cuvette and pulsed for 5 ms with a field strength of 1500 v using a Bio–Rad Gene Pulser. Vector pPICZaA was used to generate a control clone of *P. pastoris*. The transformation mix (200 μl) was plated on solid medium with increasing concentrations of Zeocin™ (Invitrogen, USA) to select putative multi-copy recombinants and then incubated at 30 °C for 3 days. All clones were analyzed for the presence of insert using DNA sequencing with the α-factor and 3′-AOX1 Sequencing Primers.

2.4. Expression of recombinant proteins in Pichia strains

Recombinant clones, which were confirmed by PCR to contain the inserts of interest, were grown in 4 ml YPD Medium(1% yeast extract, 2% peptone, 2% dextrose) at 30 °C with shaking at 240 rpm to optical density at 600 nm (OD_{600}) equal to 1; then 0.5 ml of cells were inoculated into 50 ml BMGH (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-4}% biotin, 1% glycerol) in a 250 ml baffled flask and grown at 30 °C in a shaking incubator at 240 rpm until culture reached $OD_{600} = 20$. Cells were harvested by centrifuging at 1500 g for 5 min at room temperature, washed with sterile double-distilled water, and resuspended in 10 ml BMMH (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-4}% biotin, 0.5% methanol) in a 100 ml baffled flask. Expression of recombinant proteins was induced for 72 h in a shaking incubator at 30 °C, 240 rpm, with 100% methanol being added to a final concentration of 0.5% every 12 h to maintain the promoter activity (Gurramkonda et al., 2009). After induction for 72 h, cells were harvested by centrifuging at 12,000 rpm for 20 min at room temperature; then both cells and supernatant were stored at −80 °C.

2.5. Monoclonal antibodies

The HEV neutralizing 4E9 mAb (2 mg/ml) used in this study was received from the Changchun Institute of Biological Products Co. Ltd., Chinese National Biotech Corporation (Changchun, China). The mAbs 5G5 and horse radish peroxidase labeled 5G5 mAb (5G5-HRP) were produced previously in our laboratory (Wu et al., 2013; Zhang et al., 2009). Briefly, Eight-week-old female BALB/c mice were immunized 3 times with equal volumes (40 μg in 40 μl) of GST-p166 generated from HEV genotype 4 strain (AY789225). Mouse spleen cells were fused with sp2/0 mouse myeloma cells using polyethylene glycol 1500 (50% wt/vol). The mAbs screening was conducted by EIA using the p166 generated from HEV genotype 4. Only the hybridomas positive to the antigen were further sub-cloned 3 times by limiting dilution and expended. mAbs were produced by injecting 10^6 hybridoma cells into the peritoneal cavity of the BALB/c mice. Ascites fluid was harvested after 7–10 days, filtered centrifuged and then purified with a protein G affinity column, and stored at −80 °C until further use. An in vitro PCR-based neutralization assay showed that 5G5 mAb was an HEV neutralizing antibody. The concentration of the monoclonal antibodies was 1.6 mg/ml. Anti-HEV IgG positive human sera, stored in our laboratory, previously collected from HEV infected patients, were also used.

2.6. SDS-polyacrylamide gel electrophoresis and Western blot analysis

The 20 μl aliquots of samples were mixed with 4 μl 6× SDS-PAGE loading buffer [300 mM Tris-cl pH 6.0, 12% (m/v) SDS, 12% (m/v) Bromophenol blue, 60% (v/v) glycerol, 600 mM β-mercaptoethanol], vortexed for 1 min and boiled for 5 min and then were electrophoresed on 15% SDS-polyacrylamide gel (20 μl loaded per lane). The gels were analyzed after staining with Coomassie blue. For a non-reducing SDS