Article

The Leu477 and Leu613 of ORF2-Encoded Protein Are Critical in Forming Neutralization Antigenic Epitope of Hepatitis E Virus Genotype 4

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Hepatitis E virus (HEV) genotype 4 was originally identified in China. Its neutralization antigenic epitopes have not been characterized. Recently, we identified a neutralizing monoclonal antibody (mAb) 1G10, which was generated following immunization of mice with p166Chn, a recombinant protein comprising 464-629 amino acids (aa) of the HEV genotype 4 capsid protein. In this study, a panel of 22 N- and/or C-terminal truncated and 6 site-directed mutated p166Chn proteins were prepared. Only those N- or C-terminal truncated proteins containing the region 477-613 aa could react with the mAb 1G10, suggesting the neutralization epitope of HEV genotype 4 is located between aa477 and aa613. However, a both N- and C-terminal truncated protein, pN477-C613, neither reacted to 1G10 nor elicited neutralizing antibodies in mice, while another both terminal truncated protein, pN472-C617, did, suggesting the flanking regions of the pN477-C613 could help to stabilize and allow presentation of the neutralization epitope to the immune system. Substituting Leu477 and/or Leu613 with the polar, uncharged threonine (Thr) caused ≥ 50% reduction of the mutants' immunoreactivity to 1G10, whereas replacement by hydrophobic phenylalanine (Phe) made little impact on the immunoreactivity, revealing functional associations between hydrophobicity of aa at positions 477 and 613 and the antigenicity of p166Chn. These data suggested Leu477 and Leu613 are critical in forming the neutralization epitope of HEV genotype 4. *Cellular & Molecular Immunology*. 2008;5(6):447-456.

Key Words: hepatitis E virus, neutralizing epitope, monoclonal antibody, genotype

Introduction

Hepatitis E is an enterically transmitted viral disease caused by infection with hepatitis E virus (HEV). It occurs in both epidemic and sporadic forms in most developing countries of Asia (1, 2), Africa (3) and Latin America (4). The mortality of acute HEV infection ranges from 0.5-1% among the general population to as high as up to 20% in pregnant women (5). Sporadic cases of hepatitis E have also been identified in developed countries such as Japan (6, 7), the United States (8, 9) and some countries in Europe (10, 11). A significant proportion of healthy individuals within the general population in industrialized countries are seropositive for anti-HEV. A seroprevalance of over 20% has been reported for some areas of the United States (12). Moreover, anti-HEV has been detected in many different animal species, leading to the suggestion that hepatitis E is a zoonotic disease (13-18).

HEV is a non-enveloped RNA virus, containing a positive-sense, single-stranded RNA genome which is about 7.2 kb in length. The virus genome contains three overlapping open reading frames (ORFs), the ORF2 encodes pORF2, a putative capsid protein of 660 aa. Four distinct genotypes of HEV, represented by the Burma strain (genotype 1), Mexico strain (genotype 2), US-1 strain (genotype 3), and the new Chinese variant T1 strain (genotype 4), have been identified in the world according to phylogenetic analysis of the full-length sequences (19). More HEV genotypes and subtypes have been found by analyzing sequences derived from small PCR fragments (20). The HEV genotypes used to be geographically distributed. However, it is interesting that diverse HEV isolates belonging to genotypes 1 and 4 co-exist in China. HEV isolates from epidemic areas initially identified in China were classified into genotype 1 (21), whereas recently

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Table 1. Primers used for amplifying truncated p166Chn cDNA

Truncated peptides	Primer sequences
pN472 (472-629 aa)	F 5'-CCC<u>GGATCC</u>CGTCCTTTTTCTGTGCTTCGTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN473 (473-629 aa)	F 5'-CCC<u>GGATCC</u>CCTTTTTCTGTGCTTCGTGCC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN474 (474-629 aa)	F 5'-CCC<u>GGATCC</u>TTTTCTGTGCTTCGTGCCAATGA-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN475 (475-629 aa)	F 5'-CCC<u>GGATCC</u>TCTGTGCTTCGTGCCAATGATG-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN476 (476-629 aa)	F 5'-CCC<u>GGATCC</u>GTGCTTCGTGCCAATGATGTGC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN477 (477-629 aa)	F 5'-CCC<u>GGATCC</u>CTTCGTGCCAATGATGTGCTTTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN478 (478-629 aa)	F 5'-CCC<u>GGATCC</u>CGTGCCAATGATGTGCTTTGGC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN480 (480-629 aa)	F 5'-CCC<u>GGATCC</u>AATGATGTGCTTTGGCTTTCAC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN482 (482-629 aa)	F 5'-CCC<u>GGATCC</u>GTGCTTTGGCTTTCACTTACAGC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN484 (484-629 aa)	F 5'-CCC<u>GGATCC</u>TGGCTTTCACTTACAGCTGCTGA-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN494 (494-629 aa)	F 5'-CCC<u>GGATCC</u>CAGACTACCTATGGCTCTTCTAC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pN504 (504-629 aa)	F 5'-CCC<u>GGATCC</u>ATGTATGTTTCTGATACTGTAAC-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
pC599 (464-599 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAATTAGTAGTGTAAGTAGAAATGG-3'
pC609 (464-609 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAACCGACAGCAGAGATAGAAACAG-3'
pC611 (464-611 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAACCGACAGCAGAGATAGAAACAG-3'
pC612 (464-612 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAGACACCGACAGCAGAGATAG-3'
pC613 (464-613 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAGAGGACACCGACAGCAGAGATAG-3'
pC615 (464-615 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCATGGTGCGAGGACACCGACAGCAG-3'
pC617 (464-617 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAAGAATGTGGTGCGAGGACACCGA-3'
pC619 (464-619 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCACAATGCAGAATGTGGTGCGAGGA-3'
pN477-C613 (477-613 aa)	F 5'-CCC<u>GGATCC</u>CTTCGTGCCAATGATGTGCTTTG-3' R 5'-CCC<u>CTCGAG</u>TCAGAGGACACCGACAGCAGAGATAG-3'
pN472-C617 (472-617 aa)	F 5'-CCC<u>GGATCC</u>CGTCCTTTTTCTGTGCTTCGTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGAATGTGGTGCGAGGACACCGA-3'

The *BamH* I site in forward primers and *Xho* I site in reverse primers were underlined. F, forward; R, reverse.

identified HEV isolates from sporadic cases were assigned to genotype 4 (22, 23).

The use of synthetic peptides (24, 25) and recombinant proteins (25-28) has led to identification of numbers of linear and conformational epitopes within HEV pORF2. But it is unlikely that all of them represent neutralization epitopes. In our previous studies, we found that antibodies against a small fragment of pORF2, designated pB166, spanning 452-617 aa of the HEV Burma strain sequence, demonstrated neutralizing activity, but antibodies against 51

Table 2. PCR primers used to generate the indicated mutations of pN472, pC617 and pN472-C617

Mutant	Primer Sequence
L477T (472-629 aa)	F 5'-CCC<u>GGATCC</u>CGTCCTTTTTCTGTG**ACT**CGTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
L477F (472-629 aa)	F 5'-CCC<u>GGATCC</u>CGTCCTTTTTCTGTGT**TCC**GTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGGGTAATCAACAGTGTCCTCCA-3'
L613T (464-617 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAAGAATGTGGTGC**AGT**GACACCGA-3'
L613F (464-617 aa)	F 5'-CCC<u>GGATCC</u>CCTACCCCCTCTCCTGCTCCCTC-3' R 5'-CCC<u>CTCGAG</u>TCAAGAATGTGGTGC**GAA**GACACCGA-3'
L477T613T (472-617 aa)	F 5'-CCC<u>GGATCC</u>CGTCCTTTTTCTGTG**ACT**CGTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGAATGTGGTGC**AGT**GACACCGA-3'
L477F613F (472-617 aa)	F 5'-CCC<u>GGATCC</u>CGTCCTTTTTCTGTG**TTC**CGTG-3' R 5'-CCC<u>CTCGAG</u>TCAAGAATGTGGTGC**GAA**GACACCGA-3'

The mutant codons were indicated in bold. The *BamH* I site in forward primers and *Xho* I site in reverse primers were underlined. F, forward; R, reverse.

overlapping 30-mer synthetic peptides spanning 221-660 aa of the pORF2 and against 15 overlapping recombinant proteins, each of about 100 aa in size, spanning the full length of the pORF2 did not (29). These findings established for the first time that the HEV neutralization epitopes are conformation-dependent and locate between 452-617 aa of pORF2. However, little is known about the antigenic construction of the HEV neutralization epitopes. The aa residues that contribute to the neutralization epitopes have not yet been determined, which could pose a major hurdle in tracing HEV neutralization-escape variants and in the development of efficacious HEV vaccines. Some researchers found that in other hepatitis virus, such as HBV, amino acid substitution within the "a" determinant can lead to conformational changes and hydrophobic changes, which may account solely or in conjunction for escape mutations to the immune response and HBsAg detection, by affecting the binding of neutralizing antibodies with the antigenic determinants (30).

In addition, the recently identified HEV genotype 4 strains are unusual in that a single nucleotide insertion in sequence of ORF2 (19). Accordingly, the pORF2 is 672 aa in length and is 12 aa longer than that of HEV isolates of other three genotypes. Whether the change in pORF2 length would lead to change the neutralization epitope(s) is unknown. Recently, we found that HEVp179 derived from HEV genotype 4 pORF2, as a candidate HEV vaccine, could induce neutralizing antibodies in mice (31). The monoclonal antibodies (mAbs) against an overlapping fragment within HEVp179, designated p166Chn, spanning 464-629 aa (corresponding to 452-617 aa of pB166) of the HEV genotype 4, could neutralize the homologous HEV strain *in vitro* (32), indicating that p166Chn contains the neutralization epitope. In this study, we will further characterize the neutralization epitope by using a series of truncated recombinant proteins and site-directed mutants derived from p166Chn and by probing with anti-HEV neutralizing mAb 1G10.

Materials and Methods

Recombinant p166Chn, anti-HEV neutralizing mAb and HEV inoculum
Recombinant p166Chn was prepared as an HEV-GST fusion protein (33) based on a sequence of HEV genotype 4 isolate CN9829 (GenBank accession No. AY789225). MAb 1G10 was prepared by immunization of BALB/c mice with p166Chn and a subsequent hybridoma cloning (32). The mAb was isotyped IgG1 and purified using HiTrap protein G affinity columns (Pharmacia). The HEV neutralizing activity of 1G10 was determined by using an *in vitro* PCR-based neutralization assay as described previously (29, 34). HEV inoculum NJ7 used for the *in vitro* neutralization assay was obtained from a fecal sample of a patient with acute hepatitis E and prepared as previously described (35). The inoculum was HEV positive. Sequence analysis demonstrated it was an isolate of genotype 4 (GenBank accession No. AY789228).

Construction and expression of N- and/or C-terminal truncated p166Chn
Twenty-two HEV recombinant plasmids were constructed by cloning PCR fragments with the pGEX-4T-2 vector (Pharmacia). All PCR fragments were amplified from a recombinant plasmid pGEX-Chn166 template with walking primers (Table 1). The genetic constructs were transformed into *E. coli* competent JM109 cells (Promega) and the fusion proteins were expressed after induction of 1 mM IPTG (Sigma) in LB/ampicillin medium with constant shaking at 37°C for 4 h. Then the cells were pelleted and lysed. The lysate was incubated with 20 U/ml of DNase and then purified with Bulk and Redipack GST Purification Modules (Pharmacia). The twenty-two recombinant proteins obtained by this procedure were named as pN472, pC617 or pN477-C613, etc. (Table 1), according to their truncated position from N-terminus, C-terminus, or from both, and stored in aliquots at -70°C.
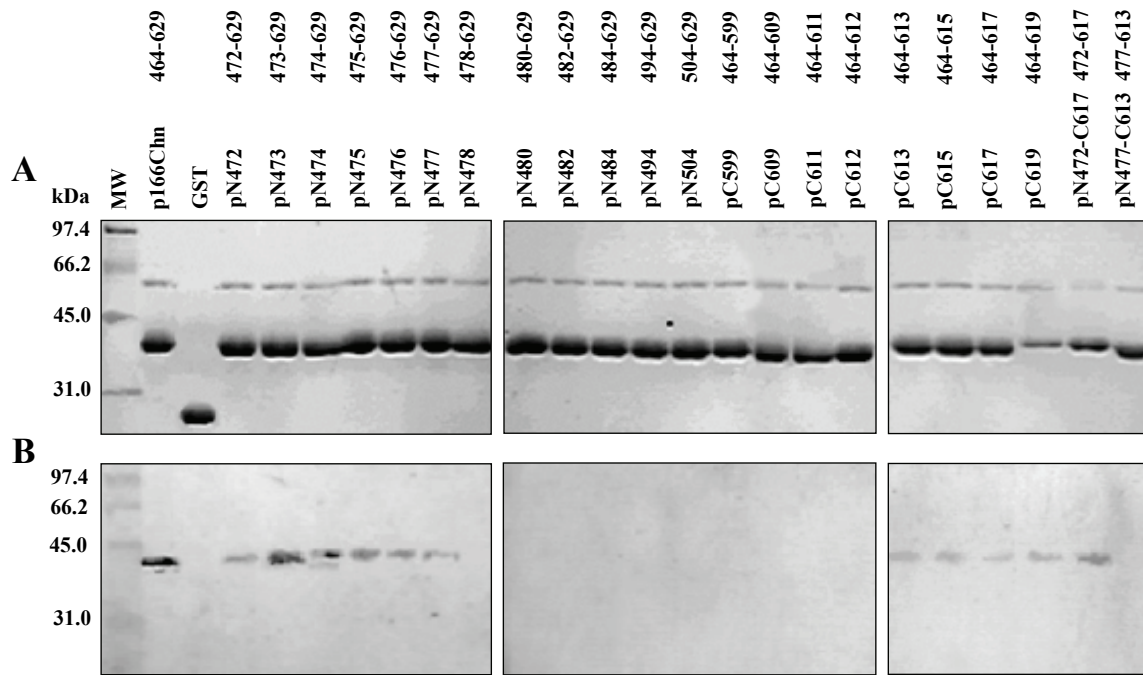
Figure 1. Mapping epitopes within p166Chn of HEV gentotype 4 pORF2 by using neutralizing mAb 1G10. N- and/or C-terminally truncated p166Chn were separated on SDS-PAGE gels and transferred to nitrocellulose membranes to react with neutralizing mAb 1G10. Immuno-complexes were detected by DAB and H_2O_2. (A) SDS-PAGE, schematic of the truncated p166Chn fragments in each lane. (B) Western blotting. Lane MW, size markers.

Preparation of p166Chn mutants by site-directed mutagenesis

Six mutants including L477T, L477F, L613T, L613F, L477T613T and L477F613F were prepared by site-directed mutation. The codon CTT for Leucine was replaced by the codon ACT for Thr or TTC for Phe in the PCR primers (Table 2). The recombinant plasmids of pGEX-pN472, pGEX-pC617 and pGEX-pN472-C617 derived from the original pGEX-Chn166 were used for PCR templates, respectively. All PCR products were digested with *Bam*H I and *Xho* I and inserted into the prokaryotic expression vector pGEX-4T-2 between *Bam*H I and *Xho* I cloning sites. The recombinant plasmids were transformed into *E. coli* competent JM109 cells and the desired mutations were confirmed by DNA sequencing. Expression and extraction of the mutated proteins fused with GST were carried out as mentioned above.

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was used to detect the immunoreactivity of the truncated and mutated recombinant proteins with 1G10. Briefly, each of the proteins (400 ng/ml) were coated onto microwell plates with carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were washed with PBST (PBS [pH 7.4], 0.05% Tween 20), and bound 1G10 was detected with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (KPL) diluted at 1:8,000. Antibody complexes were detected using tetramethylbenzidine (Sigma) substrat, reactions were stopped with H_2SO_4 and absorbance was read at 450 and 650 nm.

SDS-PAGE and Western immunoblotting

Equimolar aliquots of each truncated recombinant protein were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred to nitro-cellulose membranes (Bio-Rad), and probed with 1G10 diluted at 1:1,000. Immune complexes were detected with HRP-conjugated sheep anti-mouse IgG. Substrate solution containing 3,3'-diaminobenzidine (DAB, Bio-Rad) and H_2O_2 was added for color development. Finally, the reaction was quenched with distilled water.

Immune sera preparation and in vitro PCR-based neutralization assay

BALB/c mice were inoculated subcutaneously at two sites on the back with 100 µl of an emulsion of adjuvant (TiterMax) and 50 µg of pN472-C617 or pN477-C613. Four weeks later, the mice were boosted with an intraperitoneal injection of 10 µg of the same protein diluted in 100 µl of PBS. Seven days later, the mice were bled from the heart. The immune sera obtained from each of the mice inoculated with the same antigen were pooled together and inactivated by heating at 60°C for 30 min. Aliquots were prepared and stored at -70°C.

The *in vitro* PCR-based neutralization assay has been previously described (34-36). Briefly, decomplemented immune sera were mixed with 100 cell culture infectious doses of HEV (NJ7, genotype 4) and incubated for 1 h at 37°C. The mixtures were inoculated onto PLC/PRF/5 cells. After absorption for 2 h at 37°C, the cells were washed with D-Hanks' solution three times followed by immediate RNA
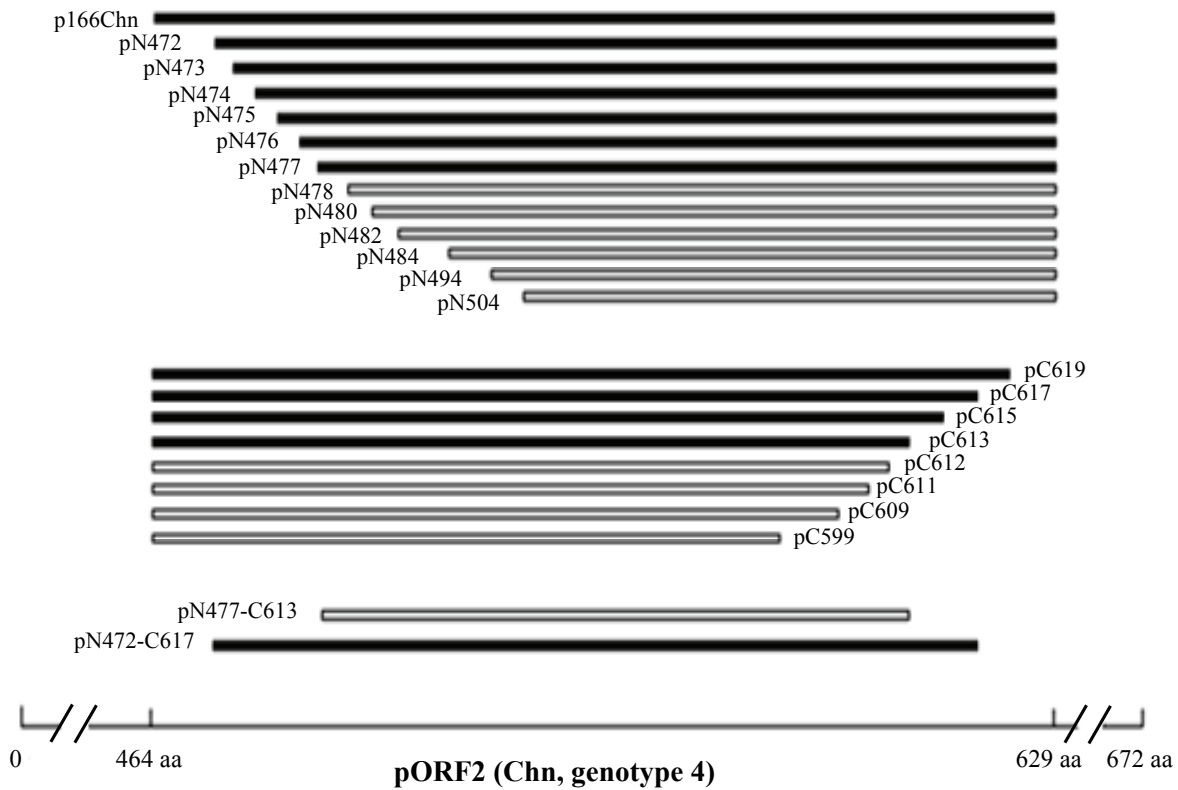
Figure 2. Schematic of truncated p166Chn fragments of pORF2 and their reactivities with mAb 1G10 in ELISA. White bars represent the truncated proteins that had no detectable reactivity with the neutralizing mAb 1G10, while the black ones indicated that they had strong reactivity with 1G10. The length of the bars indicated the region covered by the truncated p166Chn.

extraction with TRIzol reagent (Gibco BRL). Reverse transcription and nested PCR were conducted using a set of universal HEV PCR primers, generating a 236-nt fragment as previously described (29). A sense primer (5'-TCC CAT CAC CAT CTT CCA-3') and an anti-sense primer (5'-CAT CAC GCC ACA GTT TCC-3') were used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, generating a 390-nt fragment. The HEV RNA and GAPDH RNA were co-amplified to avoid differences due to the Taq DNA polymerase activity and manipulation. Both the first-round and the second-round amplifications were carried out according to the following cycling program: 94°C for 2 min, 35 cycles of 94°C for 45 sec, 50°C for 45 sec and 72°C for 1 min, and then extension at 72°C for 8 min. The PCR products were separated on a 2% agarose gel and stained with 1% ethidium bromide. Neutralization was determined by the absence of detectable HEV RNA in the inoculated cell culture. A normal mouse serum control, virus control, and uninoculated cell controls were processed for detection of HEV RNA at the same time.

Results

Precise localization of neutralization epitope within p166Chn
Our early studies have shown that p166Chn could effectively

mimic the neutralization epitope and reacted with the neutralizing mAb 1G10 (32). To further localize the neutralization epitope, a series of N-terminal, C-terminal or both N- and C-terminal truncated p166Chn proteins were prepared (Figure 1A) and their immunoreactivity to the neutralizing mAb was investigated. As indicated by ELISA, the neutralizing mAb was only reactive to the N-terminal truncated p166Chn fragments containing aa477 (Leu) and to the C-terminal truncated p166Chn fragments containing aa613 (Leu) (Figure 2). All the truncated proteins shorter than the pN477 at N-terminus, such as pN478, pN480, pN482, pN484, pN494 and pN504, and shorter than the pC613 at C-terminus, such as pC612, pC611, pC609 and pC599, lost the reactivity to 1G10. The same results were obtained from the study by using Western blot (Figure 1B). Only the fragments upstream of aa478 at N-terminus and downstream of aa612 at C-terminus showed positive bands to the mAb. Therefore, the data indicated that the neutralization antigenic epitope of HEV genotype 4 is located between Leu477 and Leu613 of pORF2 and is conformation-dependent. Both the Leu477 and Leu613 were critical residues in forming this neutralizing epitope.

However, an unexpected result was obtained when using both N- and C-terminal truncated proteins. It was surprising that the protein pN477-C613, which contains both Leu477 and Leu613, lacked reactivity to 1G10 in both ELISA and
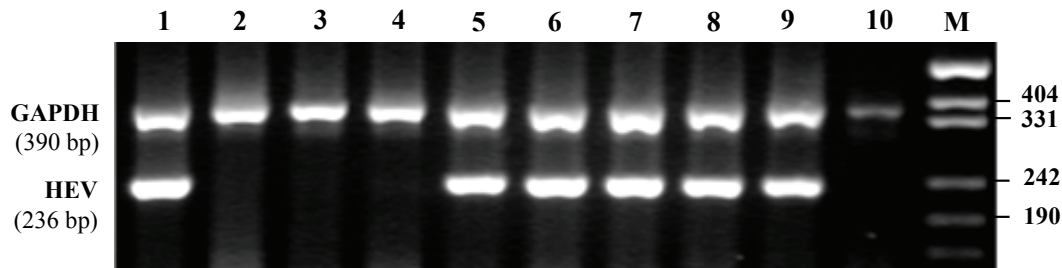
Figure 3. HEV RNA detected by PCR as a marker for HEV cell infection. Lane 1, virus with undiluted preimmune mouse sera; Lanes 2 to 6, virus with immune sera against pN472-C617 at dilutions of 1:5, 1:10, 1:20, 1:40, and 1:80, respectively; Lanes 7 to 8, virus with immune sera against pN477-C613 at dilutions of 1:1 and 1:5; Lane 9, virus control; Lane 10, uninfected cell control; Lane M, marker (pUC19DNA/MspI (HpaII) fragments). The size of HEV and GAPDH amplified fragments were indicated.

Western blot. However, another protein, pN472-C617, which is 5 aa longer at the N-terminus and 4 aa longer at the C-terminus than pN477-C613, did react to the mAb (Figure 1B and Figure 2). These data suggested that a few aa residues flanking the pN477-C613 are necessary to form the correct and stable conformation of the neutralization epitope.

Neutralizing activity of immune sera against pN472-C617 and pN477-C613
To determine whether the neutralization epitope is the same between recombinant proteins and native HEV, pN472-C617 and pN477-C613 were used to immunize BABL/c mice, respectively. The immune sera against pN472-C617 and pN477-C613 were tested by the *in vitro* neutralization assay. The immune sera against pN472-C617 neutralized the homologous HEV strain (genotype 4) at dilutions of 1:5, 1:10, and 1:20, demonstrating an end-point neutralization titer of 1:20. However, when immune sera against pN477-C613 and preimmune sera were tested, neutralizing activity was not observed (Figure 3).

Conservation of Leu477 and Leu613 in known HEV strains
Leu477 and Leu613 were identified as two critical residues for the functional activity of the neutralization epitope on p166Chn as mentioned above. In order to obtain additional information about the two residues at the corresponding positions of other HEV isolates, 71 complete sequences of HEV were retrieved from GenBank. Their deductive amino acid sequences were aligned with Clustal W software. Alignment analysis showed that the amino acid residues at the corresponding positions of Leu477 and Leu613 were highly conserved among the 71 HEV ORF2 encoded protein sequences, although they were classified into 4 different HEV genotypes and were derived from HEV strains isolated from different species including human, swine, and some wild animals (data not shown). The Leu477 and Leu613 appeared highly conservative even in the avian HEV ORF2 encoded protein, which shares only 48-49% amino acid sequence identities with other known HEV strains (37). This remarkable conservation implied that Leu477 and Leu613 might play key roles in the formation of the neutralization epitope among different HEV isolates and

support the observation that HEV only has one serotype (38, 39).

Effect of Leu477 and Leu613 mutation on the HEV neutralization epitope
To further demonstrate the importance of the two leucines at positions 477 and 613 to the functionality of the neutralization epitope, site-directed mutations were performed by substituting residues 477 and/or 613 with either hydrophilic Thr (T) or hydrophobic Phe (F). This resulted in the following recombinant proteins: L477T613T, L477F613F, L477T, L613T, L477F and L613F. The genes including these mutations were introduced into the pGEX-4T-2 vector and the desired mutagenesis were confirmed by DNA sequencing.

Reactivity of the p166Chn mutants with 1G10 were tested by ELISA (Figure 4). Proteins of pN472, pC617 and pN472-C617 were used as positive controls, the reactivities of the proteins with the mAb were taken as 100%, which were shown by the horizontal dash lines. Each experiment was performed three times in triplicate, and the values are expressed as standard deviations from the mean. The results indicated that substituting Leu477 or Leu613 with T (L477T and L613T) resulted in significant reduction of the reactivity of p166Chn with 1G10, leading to 50% and 77% reduction in reactivity, respectively. By substituting both Leu477 and Leu613 with T, the protein L477T613T showed a more obvious reduction of reactivity to about 80%. While alternating these two residues with F (L477F, L613F and L477F613F) little effect on their reactivities with the mAb was found.

Discussion

Based on extensive full-length genomic variability noted among different strains, HEV has been classified into four major genotypes. However, a highly efficacious vaccine is not presently available (40). The understanding of the antigenic structure of HEV is rather limited and the neutralization epitope(s) of HEV has not yet been well investigated. In this study detailed information has been provided, for the first time, on the neutralization epitope
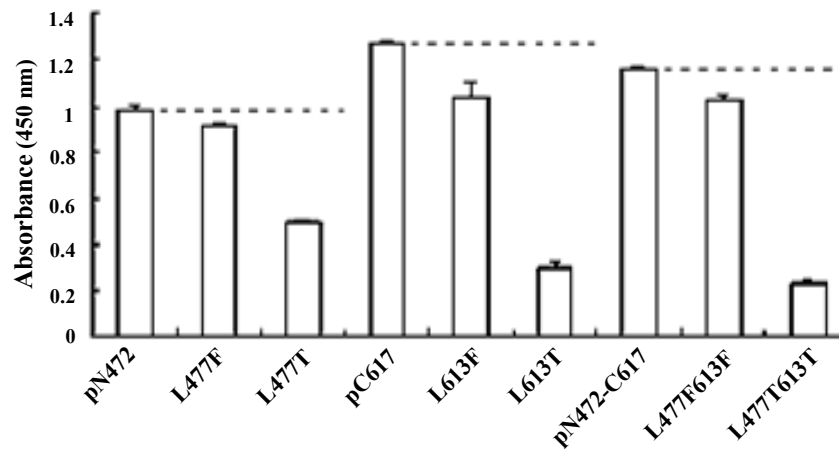
Figure 4. The effect of single and double amino acid(s) mutations of p166Chn on the recognition of HEV-specific neutralizing mAb 1G10. The dotted line represents 100% binding with wild-type p166Chn, the mutants L477T, L613T and L477T613T resulted in a 50%, 77% and 80% reduction of reactivity with mAb 1G10, respectively. However, the mutants L477F, L613F and L477F613F showed no apparent reduction of reactivity with the mAb. All results were expressed as mean values of three assays, and standard deviations (SD) were indicated.

found on pORF2 of HEV genotype 4 by using a combined approach of truncated recombinant proteins and site-directed mutagenesis.

Several distinct antigens capable of stimulating protective HEV immunity have been expressed from cloned HEV gene sequences containing immunodominant epitopes of structural proteins. It is thought that the first protein which induced protective antibodies in cynomolgus macaques was a trpE-HEV fusion protein (C2) including 439 amino acids (221-660 aa) comprising the carboxyl terminal fragment of the ORF2 protein from the Burmese strain (genotype 1) (41). Some studies indicated that the full-length pORF2 immuno-reacted with HEV acute-phase sera, but the recombinant protein GST-ORF2.1 containing the C-terminal region at 394-660 aa strongly immunoreacted with both acute- and convalescent-phase sera, which indicated that the carboxyl terminal part of pORF2 contains epitopes which are highly reactive to convalescent-phase sera and thus likely to be associated with immunity to infection with HEV. However, these epitopes may be masked when larger portions or the full-length pORF2 are expressed as recombinant proteins, at least in *E. coli* (27, 42). The antigenic structure was further studied by using a panel of murine mAbs, the conformational ORF2.1 epitope involving 394-457 aa and a linear epitope in the region of 434-457 aa which are not only present on the surface of VLPs, but also immunodominant in the humoral immune response of convalescent HEV patients (28). Taken together, these findings implied that the C-terminal two thirds of pORF2 is the most important region in the antigenicity and immunogenicity of this protein.

Our previous studies demonstrated that pB166 can efficiently model the conformation-dependent neutralization epitope(s). The antibodies elicited from pB166 could neutralize HEV strains of genotypes 1, 2 and 3 (29). HEV genotype 4 is newly identified in China and different from other genotypes in that its ORF2 product is 12 aa longer than

that of other HEV isolates due to a single nucleotide insertion in the ORF2 region (19). The neutralization epitope(s) of HEV genotype 4 has not been localized. In our recent studies, we found that mAbs derived from mice immunized with p166Chn (the peptide spanning 464-629 aa in HEV genotype 4) showed neutralizing activity against homologous HEV (32). Therefore, the neutralization epitope of HEV genotype 4 is localized between 464-629 aa of pORF2.

In order to localize the neutralization epitope on p166Chn more precisely, we mapped the epitope by generating a series of N- and/or C-terminal truncated p166Chn and determined their reactivity to the neutralizing mAb 1G10. We found that the ability of binding the mAb disappeared when N-terminal truncation was done at downstream of Leu477 or C-terminal truncation was done at upstream of Leu613. However, when both termini were truncated as shown with recombinant protein pN472-C617 (472-617 aa), the immunoreactivity was high with 1G10, while with pN477-C613 (477-613 aa) the immunogenicity was absent. These results imply that the neutralization epitope of HEV genotype 4 is located within 477-613 aa, and that the flanking regions could help to stabilize and present the conformational-dependent epitope. The Leu477 and Leu613 were key residues for the formation of the HEV neutralization epitope.

To further confirm whether recombinant proteins could model epitopes on native HEV, antisera from mice immunized with pN472-C617 and pN477-C613 were tested by the *in vitro* neutralization assay. This assay was shown to be highly specific and sufficiently sensitive to evaluate the neutralizing activity in various anti-HEV serum specimens (35). The results indicated that only the immune sera against pN472-C617 could efficiently block adsorption of HEV strains onto the surface of PLC/PRF/5 cells, and that only pN472-C617 but not pN477-C613 contains this conformational neutralization epitope. This epitope on pN472-C617 could induce neutralizing antibody against native HEV, suggesting

that the identified neutralization epitope is the same between the recombinant protein and the native virus.

Epitopes are classified as either being continuous or discontinuous. Discontinuous epitopes are composed of sequences of amino acids throughout an antigen and rely on the tertiary structure or folding of the protein to bring the necessary sequences together to form the epitope. In contrast, continuous epitopes are linear peptide fragments of the antigen that are able to bind to antibodies raised against the intact antigen. Many viral neutralization epitopes are conformation-dependent discontinuous epitopes (43), and in such cases, the neutralization epitopes can be modeled only with protein fragments that contain all of the necessary information to direct the correct folding. Most scientists believe that the majority of HEV neutralization epitopes are conformation-dependant because their immunogenic activity could be disrupted by removal of a few amino acids from either terminus (44). Although one linear neutralization epitope was mapped to a region of the ORF2 protein of Sar-55 between 578-607 aa (45), subsequent work by the same group has demonstrated that the neutralization epitopes of Sar-55 were discontinuous conformational epitopes and located between aa458 and aa607 (46). As shown in our present study, only those N-terminal or C-terminal truncated proteins containing the peptide corresponding 477-613 aa could be recognized by the neutralizing mAbs. Considering that both the N- and C-terminal truncated protein pN472-C617 showed positive reactivity with 1G10, but pN477-C613 did not, a few aa residues flanking the pN477-C613 were shown to be necessary to form the correct and stable conformation of the neutralization epitope.

In addition, both of the residues at position 477 and 613 on HEV genotype 4 ORF2 protein are Leu. Upon retrieving 71 ORF2-encoded protein sequences of different HEV genotypes that covered both of these residues from GenBank and aligning them using Clustal W, we found that the two hydrophobic aa residues Leu477 and Leu613 were highly conserved among those sequences suggesting that these "hot" residues might be key residues in contributing to the overall structure of the epitopes, perhaps by promoting pORF2 to correctly fold in the cytoplasm and stabilizing its three-dimensional (3D) structure (28).

From a protein 3D structure, we can learn the location of those "hot" residues in the actual epitope or at the interface of an antigen-antibody complex. Unfortunately, the 3D structure of the HEV ORF2 encoded proteins and the complex with their antibodies have not been crystallized so far (28). Definitive characterization of the discontinuous neutralization epitope(s) of HEV is not available because of a lack of understanding of its multidomain structure and X-ray crystallographic or NMR data. Furthermore, we cannot find any known sequence of any protein in protein data bank (PDB) which has a high degree of homology to HEV ORF2 protein. Therefore, no structural model can be built to simulate the epitopes of HEV.

To further confirm whether the hydrophobic leucines at positions 477 and 613 are key amino acid residues in forming the neutralization epitope, we substituted Leu477 and Leu613

with hydrophobic Phe and hydrophilic Thr by site-directed mutagenesis. We found that alternating either one or both of the two aa residues with Thr led to significant reduction in reactivity with 1G10, while alternating them with Phe had little effect on its reactivity with the mAb. The reason might be that, the hydropathy index (-0.7) of the hydrophilic R group of Thr is much lower than that of Leu (3.8) (47), which had changed the conformation of the epitope. These results indicated that the neutralization epitope is sensitive to changes in conformation, in which the hydrophobic amino acids play a crucial role.

In conclusion, the results of this study demonstrate the conformational neutralization epitope of HEV genotype 4 relies on 477-613 aa, and aa477 and aa613 are critical aa residues in forming this epitope. These data will advance our understanding of the antigenic structure of the newly identified HEV genotype in China and provide greater insight into the molecular basis of antigen-antibody recognition in immune response to HEV infection.

Acknowledgements

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