Mutational Analysis of Essential Interactions Involved in the Assembly of Hepatitis E Virus Capsid*

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The hepatitis E virus (HEV) capsid consists of a single structural protein, a portion of which is engaged in isosahedral contact to form a basal shell, and another portion in dimeric contact to form the homodimers protruding from the shell. Previous studies revealed that homodimers of the truncated HEV capsid proteins, E2 (amino acids 394–606) and p239 (amino acids 368–606), model dominant antigenic determinants of HEV. Immunization with these proteins protected rhesus monkeys against the virus, and three monoclonal antibodies against the homodimers could neutralize HEV infectivity and/or immune-capture of the virus. Furthermore, homodimers of p239 further interact to form particles of 23 nm diameter, rendering it an efficacious candidate vaccine. In light of this we postulate that the interactions involved in the formation of the homodimers and particles might be similar to those involved in assembly of the virus capsid. Presently, mutational analysis was carried out to identify these sites of interactions. The site of dimeric interactions was located to a cluster of six hydrophobic amino acids residues, Ala397, Val398, Ala399, Leu601, and Ala602; furthermore, the site involved in particle formation was located at amino acids 368–394. The possibility that these sites are also involved in assembly of the virus capsid is supported by the fact that they are located at two major and highly conserved hydrophobic regions of the HEV structural protein.

Hepatitis E virus (HEV)† has emerged to be a significant cause of orally transmitted hepatitis, especially in developing countries (1, 2). The infection is mainly confined to humans and non-human primates, and there are increasing reports suggesting that the infection might be more widespread and could affect other animals, including pigs and rodents (3). The virus cannot be propagated efficiently in vitro. HEV isolated from patients is a small, non-enveloped, single positive-stranded RNA virus with an isosahedral capsid of average 30-nm diameter (4). The viral genome of 7.5 kilobases is organized as three open reading frames; open reading frame 1 maps to the 5’ terminus and encodes a non-structural protein, open reading frame 2 maps to the 3’ terminus and encodes for a major structural protein, and open reading frame 3 overlaps both and encodes a thus far unknown function (5).

Morphologically resembling Calicivirus, the HEV capsid consists of a single structural protein that is assembled to form an isosahedral shell with 90 protrusions (6, 7). It is a special feature of these viruses that the single structural protein must provide for all the requirements for it to be self-assembled to form infectious virus particles with attending infectivity and antigenic activity. X-ray structure of recombinant capsid protein of Norwalk virus, a prototype human Calicivirus, reveals two principle domains: shell (S) domain and protrusion (P) domain (8). The S domain consists of N-terminal residues folding into eight anti-parallel β barrels that are engaged in isosahedral contact to form the basal layer of the viral capsid. The P domain comprising the remaining C-terminal residues is engaged in dimeric contact to form a protrusion projecting from the virus shell. Mutational analysis has suggested that the P domain might contribute to the size and stability of the capsid (9). Being the most exposed part of the virus capsid, P domain may be presumed to also accommodate the principle determinants of infectivity and antigenic activity of the virus. Other plant Calicivirus has evolved an additional (R) domain to the N terminus, which might affect the size of the virus capsid (9, 10). Truncated protein of the HEV capsid protein (aa 112–607) expressed in insect cells could self-assemble to form virus-like particles (VLPs) of 23.7-nm diameter with T (triangulation number) = 1 isosahedral symmetry (11, 12). It is made up of 60 subunits forming 30 dimeric capsomers, which are assembled into an isosahedral shell with 30 protrusions made up of homodimers of a portion of the capsid protein (12). Except for its small size, the morphology of recombinant VLP is closely similar to the native virus particle, and it also possesses dominant antigenic activity of the native virus (11). Cryo-electron microscopy and reconstruction of the recombinant HEV particles by Xing et al. (12) has distinguished functional equivalents of the S domain engaging in isosahedral contact to form the virus shell and P domains engaging in dimer contact to form the protrusions. The N-terminal 111 aa residues of the capsid protein containing a signal sequence followed by an arginine-rich sequence is believed to interact with the RNA genome. It is possible that this region might be involve in determining the size of the virus particles, as do R domains of plant virus (10), since truncation of these residues has resulted in formation of smaller virus-like particles.

We have expressed three proteins of HEV capsid protein in Escherichia coli (13, 14). These are E2 (aa 394–606), E2a (aa 459–660), and p239 (aa 368–606). Under the mildly denaturating conditions of SDS-PAGE, the proteins predominantly occur as homodimers which model dominant antigenic determinants.
of HEV (13, 15, 16), including its neutralization site (14, 16, 17). Three monoclonal antibodies raised independently against E2 homodimer could neutralize the infectivity and/or immunogenicity of the virus (18). Moreover, the 26-aa extension to the N terminus of E2 imparts the additional capacity to form a particle of 23-nm radius to the resulting protein, p239, markedly enhancing its immunogenicity and making it an efficacious candidate for an HEV vaccine (14). This has led us to postulate that the interactions involved in the formation of homodimers of these recombinant proteins might mimic the dimeric contact, whereas those involved in formation of p239 particles might mimic the icosahedral contact between the capsid proteins in the assembly of the viral capsid. Presently, we conducted mutational analysis of the interactions involved in formation of the homodimers and particles. The results served to locate the respective sites of interactions to regions corresponding to two major hydrophobic domains in HEV major structural protein.

EXPERIMENTAL PROCEDURES

Cloning of the E2 and Related Mutant Genes—The genes encoding for E2 and p239 were described previously (13, 14). Table I shows the primers used for amplifying the genes encoding these two proteins, the related protein E2a, and those used to generate the mutant genes used in this study. The latter encodes mutant proteins, which had been progressively truncated from the C terminus of E2 (proteins 210C-193C); that is, those which have glutamic acid substitution or cysteine progressively truncated from the C terminus of E2 (proteins 210C-193C); that is, those which have glutamic acid substitution or cysteine substitution of different residues at the C-terminal region of E2 (SS96E-A597C), those which have been progressively truncated from the N terminus of E2 (193N–148N), and those which had been progressively truncated from the N terminus of p239 (227N and 236N). The genes were subcloned into a vector pMD 18-T with a TA cloning kit (Takara, Dalian, China). The resulting plasmid was digested with NdeI and EcoRI for 2 h at 37 °C. The flanking fragment was purified by agarose gel electrophoresis and a gel extraction mini kit (Watson Inc., Shanghai, China) and then ligated to the non-fusion pTO-T7 expression vector (19) previously linearized with NdeI and EcoRI.

Production and Purification of Different E2 Proteins—The plasmids containing the E2 gene or mutant genes were transformed into E. coli ER2566 cells (Invitrogen). An overnight culture of the transformant was grown at 37 °C in LB medium with 50 μg/ml kanamycin until it reached an optical density value of 0.8 at 660 nm. Isopropl-p-β-thiogalactoside was added to the culture to a final concentration of 0.2 mM, and the culture was further incubated for 6 h. Bacterial cells harvested from the culture were lysed by sonication. It was found that all the recombinant proteins form inclusion bodies in host cells. The inclusion bodies were treated with 2% Triton X-100 at 37 °C for 30 min and then dissolved in 4 M urea. The proteins were renatured by dialysis against phosphate-buffered saline, pH 7.45, at room temperature and further purified by gel filtration HPLC using TSK gel SW3000 25 × 600 mm (TOSOH, Japan). The purified proteins were stored at −80 °C until use.

SDS-PAGE and Western Blotting—Analysis of proteins by SDS-PAGE was done according to the method of Laemmli with modifications (20). Polyacrylamide gels with 12% or 15% acrylamide in the separating gel were used, and the gels were stained with 0.1% Coomassie blue R250, destained, and dried. Western blotting experiments were conducted as described by Towbin et al. (21). The disulfide-linked dimer was separated by SDS-PAGE and whole-gel-elu ted for analysis.

Electron Microscopy—Particles of p239 were examined by negative-stain electron microscopy. Samples of the protein were applied to a carbon-coated grid, and after removal of excess fluid, the sample was stained with 2% uranyl acetate and examined in an F30 transmission electron microscope (Phillips, Netherlands) operating at 200 kV.
Results

Delineating the Functional Domains of E2, p239, and E2a—Fig. 1 compares the properties of three bacterially expressed proteins of HEV structural protein, E2 (aa 394–606), E2a (aa 459–660), and p239 (aa 368–606). E2 and p239 have been shown to protect rhesus monkeys against HEV infection (14, 17), and E2a was derived from E2 by a truncation from the N terminus of E2 and an extension from its C terminus. Gel filtration chromatography showed that E2 and E2a mainly occur as hexamers of about 200 kDa (Fig. 1A), whereas p239 was eluted in the exclusion volume and appeared as 23-nm particles (Fig. 1B). Under the mildly dissociating conditions of SDS-PAGE, all the three proteins predominantly occur as homodimers of about 40-kDa apparent molecular mass, which were dissociated by heating at 100 °C for 3 min into a monomeric form (Fig. 1C). Immunoblotting shows that the homodimers were predominantly recognized by a HEV-reactive convalescent serum and by three monoclonal antibodies, 8C11, 8H3, and 13D8. These antibodies recognized conformational epitopes of the homodimers, and previous studies showed that they could immune capture HEV. Hereinto, 8C11, 8H3, and 13D8 could neutralize infectivity for rhesus monkeys (18). This suggests that the same or closely similar conformational epitopes are presented by the virus capsid, probably in the protrusions projecting from the virus capsid, with two at or in close proximity to the HEV neutralization site.

Comparison between the three proteins delineates three functional domains (Fig. 1D). The domain located to aa 459–606 is common to all three proteins, and it is probably involved in formation of homodimers of the respective proteins. Another domain is located to aa 368–394 in the N terminus of p239 and, because it is not present in the other proteins, is likely involved in interactions between this protein to form particles. The third domain is one located at aa 394–458 in E2 or p239 or another located at aa 607–660 in the C-terminal region of E2a. Evidently neither of these domains is directly engaged in homodimer formation, but as will be shown later, they seem to facilitate this process and/or determine antigenicity of the respective homodimers. It was noted that the domain believed to involve in homodimer formation encompasses a major hydrophobic region, and the domain involved in particle formation encompasses another major hydrophobic region. We considered it likely in light of this that these regions might constitute the sites of dimeric interactions to form homodimers and the interactions to form particles, respectively.

Design of Mutant Proteins—Mutational analysis of the interactions was conducted using five groups of purified mutant proteins (Fig. 2). Three groups of the mutant proteins were used to analyze dimeric interactions involved in formation of homodimers. These included those that had been progressively truncated from C terminus or carried a glutamic or a cysteine substitution in the C-terminal region of E2. Another group of mutant proteins that had been progressively truncated from the N terminus of p239 was used to analyze the interactions involved in particle formation. The remaining group of mutant proteins, which had been progressively truncated from N terminus of E2, was used to investigate the role of the N-terminal domain in facilitating formation of homodimers and its effects on the antigenicity of the homodimers. The mutant proteins were purified and analyzed by gel filtration chromatography, SDS-PAGE, and immunoblotting using the 8C11 antibody. Purified homodimers of mutant proteins harboring the cysteine substitutions were treated with glutathione according to Taylor et al. (21) and tested for cross-linking. The results are summarized in Fig. 2 and are detailed as follows.

Mutational Analysis of Dimeric Interactions—Fig. 3 depicts Western blotting analysis of mutant proteins which had been progressively truncated from the C terminus of E2 (Fig. 3A) or those which carried a glutamic acid substitution in this region (Fig. 3B). The results show that the capacity for dimeric interactions to form SC11-reactive homodimers is abrogated by truncation of residue Ala602 and beyond (Fig. 3A) and also by glutamic acid substitution of the residues Ala597, Val598, Ala599, Leu601, or Ala602 (Fig. 3B). Glutamic acid substitution of Val600 did not affect formation of the SC11-reactive homodimer; however, it was noted in SDS-PAGE that the relative amount of monomer was increased possibly because of an increased dissociation of the mutant homodimer. It is noted that this cluster of 6 hydrophobic residues is located among a major hydrophobic region of the HEV structural protein, and it was
shown that deletion of aa 603–606 (protein 209C) or glutamic acid substitution of Ser596 or Pro603 flanking this region did not affect the formation of 8C11-reactive homodimers.

The mutant proteins having a cysteine substitution of residues Ala597, Leu601, or Pro603 were used to determine the affect the formation of 8C11-reactive homodimers. This approach is based on the principle that direct cross-linking of cysteine residues as they exist on the respective monomeric subunits, as evidenced by the appearance in cross-linking of the cysteine residues as they exist on the separate monomeric subunits, as evidenced by the appearance in the reaction mixture of an additional 46-kDa band (Fig. 4, lane 7). To confirm the identity of the cross-linked dimer, the reaction mixture was subjected to heating first and then was further treated with dithiothreitol. On SDS-PAGE analysis, the covalently linked dimer, unlike the non-covalently linked homodimer, was resistant to heating (Fig. 4, lane 8), and it was dissociated into a monomer after further treatment with dithiothreitol (Fig. 4, lane 9) (note the dissociation of the non-covalently linked homodimer followed by the disulfide-linked dimer). The parental E2 was analyzed as in lane 10. Lane 1, molecular weight markers.

Thus, the results obtained with the C-terminal mutant proteins support the contention that the cluster of six hydrophobic aa residues comprising a major hydrophobic region of the HEV structural protein is engaged in dimeric interactions to form homodimers reactive with the neutralizing HEV antibody, 8C11. It was shown that the dimeric interactions were abrogated by truncation of aa residue 602 and beyond or glutamic acid substitution of each of the residues comprising this region, as in the respective monomeric subunits, are in close proximity of one another.
Mutational Analysis of Interactions to Form Particles—An extension of 26 aa residues to the N terminus of E2 imparted to the resulted protein, p239, the additional capacity to form particles and enhanced its immunogenicity, making it an efficacious candidate HEV vaccine (14). The extension is located at aa 368–394 in a major hydrophobic region of the HEV structural protein, with 11 of the 26 hydrophobic aa contained therein made up of leucine or isoleucine. The corresponding region in the HEV structural protein is predicted to assume a predominantly helical structure using PSIPRED secondary structure prediction methods (23). Fig. 5 shows that truncation of even 3 aa residues from the N terminus of p239, including Leu370, abrogated the capacity to form particles. In contrast to parental p239, the mutant proteins (236N and 227N) occurred as hexamers in gel filtration chromatography (Fig. 5A). Nevertheless, the extension did not affect the capacity of p239 or the related mutant protein to form homodimers in SDS-PAGE nor the reactivity with 8C11 (Fig. 5B) or the other HEV reactive antibodies (not shown).

Mutational Analysis of the N-terminal Domain of E2 and Its Influence on the Conformation of Homodimers—Fig. 6 shows Western blot analysis of E2, E2a, and the mutant proteins, which had been progressively truncated from the N terminus of E2. The mutant protein with a 64-aa deletion from the N terminus of E2 (protein 148N) retained the capacity for dimeric interactions, but the resulting complex lacked the correct conformation for it to be recognized by 8C11 and the other antibodies. The deficit was compensated for, as in E2a, by a 54-aa extension to the C terminus of E2. Both proteins were then capable of dimeric interactions to form homodimers with the correct conformation in order to be reactive with these antibodies. SDS-PAGE shows that all the mutant proteins having from 10 to 64 aa residues deleted from the N terminus of E2 retained the capacity for dimeric interactions, but the resulting complexes do not have the correct conformation to be reactive with 8C11 except for the mutant proteins 193N and 178N. The latter mutant proteins with deletions of 20- and 35-aa residues retain the capacity to interact to form 8C11-reactive complexes, but this capacity was abrogated by further truncations beyond aa 429. These findings support the contention that the N-terminal domain of E2, located at aa 430–596, or the C-terminal domain of E2a, located at aa 607–660, can modulate dimeric interactions to give the resulting homodimers the correct conformation for them to be recognized by 8C11 and the other antibodies. It is noted that this corresponds a hydrophilic region of the HEV structural protein that is predicted to assume a predominantly random coil structure (23).

Conclusions—As summarized in Fig. 2, comparison between E2, E2a, and p239 has delineated 3 domains, one involved in the formation of homodimers, another in the formation of particles, and the remaining domain facilitating, but not participating directly in the formation of homodimers. The results obtained for the mutant proteins produced by truncation (210C-193C) or glutamic acid substitution (S596E-P603E) of the C-terminal region of E2 have identified a cluster of 6 hydrophobic aa residue as the site of dimeric interaction in the formation of homodimers. Cross-linking by glutathione treatment of the homodimer of the cysteine-substituted mutant protein, A597C, confirmed that the respective residues are in close proximity as they exist in the monomeric subunits of the homodimer. The 26 N-terminal residues of p239 correspond to another major hydrophobic region, with 11 of these residues made up of leucine or isoleucine. The results obtained for the mutant proteins, 227N and 236N, suggest this region as a site for interaction involved in particle formation. It is shown that the domain located at aa 394–458 can facilitate the formation of E2 and p239 homodimers, but it does not directly participate in this process. It is further shown that all the mutant proteins produced by progressive truncation from N terminus of E2 can form homodimers or higher oligomers. This is because the truncation did not affect the dimeric interaction site at the C terminus. However, truncation of more than 25 residues affected the capacity of the resulting dimer or oligomer to assume the correct conformation as to be reactive with the HEV-neutralizing antibody, 8C11.

DISCUSSION

Expression of the truncated HEV capsid protein in insect cells results in self-assembly of HEV virus-like particles with T (triangulation number) = 1 icosahedral symmetry. Except for its smaller size, the recombinant VLP closely resembles the virus particle with respect to its morphology and antigenicity (11). In a detailed analysis of cryo-electron microscopic image of the particles, Xing et al. (12) showed that a portion of the truncated protein is engaged in icosahedral contact to form the basal virus shell, and another portion is engaged in dimeric contact to form homodimers protruding from the virus shell. These authors showed that homodimers are formed near the surface of the virus shell, converge on the 3-fold axis of symmetry, and then protrude at the 2-fold symmetry axis for about
45 Å from the virus shell. The protrusions are a special feature of HEV as they are of caliciviruses (7), and as the most exposed part of the virus capsid, these structures are expected to accommodate viral neutralization sites and other major antigenic determinants of these viruses.

E2 and its related proteins E2a and p239 provide a model to gain insight into these contacts involved in the assembly of the HEV capsid, and the present study to this end was further facilitated by the availability of monoclonal antibodies raised against E2, which are also reactive against HEV. Under the mildly denaturing conditions in the presence of 0.1% SDS, the three proteins occur predominantly as homodimers, but under neutral conditions the homodimers of E2 or E2a further interact to form hexamers, whereas p239, with a 26 aa extension from the N terminus of E2, occurs as particles of 23 nm. The homodimers of the three proteins are strongly recognized by a HEV-reactive human serum and also by the three E2-specific monoclonal antibodies, 8C11, 13D8, and 8H3. Previous studies showed that these antibodies could immune capture HEV, and 8C11 and 8H3 were found to neutralize infectivity of the virus for rhesus monkeys (18). Thus, these results show that the homodimers and HEV share at least three conformational epitopes, making it likely that the contacts between these proteins might be similar as those involved in assembly of the viral particles.

Comparison between the three proteins served to delineate 3 distinct functional domains (see Fig. 1D). One, locating to aa 459–606 and common to the three proteins, is most probably involved in dimeric contacts. Another, locating to 26-aa residues in the N terminus of p239, is likely to be involved in particle formation. A third domain identified was located either at aa 394–458, as in the case of E2, or at aa 607–660, in the case of E2a. It was evident that neither domain was involved in the dimeric contacts directly; rather, they seem to assume an accessory function by facilitating formation of the respective 8C11-reactive homodimers. We noted that the domain believed to be involved in dimeric contact contains a cluster of 6 hydrophobic residues, and the domain involved in particle formation is another hydrophobic region with 11 of 26 aa residues made up of leucine or isoleucine. In the mutational studies we tested the hypothesis that the cluster 6 hydrophobic aa residues involved in dimeric interactions between the proteins is located among a major hydrophobic region of the HEV structural protein predicted to assume predominantly a β sheet structure, whereas the 26 aa residues involved in forming the p239 particle are located among another major hydrophobic region predicted to fold into a helical structure. It was also noted that the hydrophobic aa comprising both regions were highly conserved among natural HEV isolates. In light of this it is likely that the same cluster of 6 residues involved in dimeric contact to form the homodimers of the recombinant proteins could be the critical residues engaged in dimeric interactions to form the dimeric protrusions on the virus capsid and the 26 aa residues, the critical residues engaged in icosahedral interactions to form the viral shell. The third domain consists of either aa 430–458, which modulates the conformation of E2 and p239 homodimer, or aa 607–660, which modulates the conformation of E2a homodimer, imparting to the respective dimmers the major antigenic determinants and neutralization sites of HEV capsid. This implies that such accessory domains are also involved in modulating the conformation of the HEV capsid.

In conclusion, comparison of E2 and the related proteins E2a and p239 has identified two domains involved in formation of homodimers and particles, respectively, and another domain that modulates the conformation of the homodimers and determines its antigenicity. Further studies by mutational analysis identified a cluster of six hydrophobic aa residues in one domain as to be a site of dimeric interactions involved in the formation of homodimers. The domain involved in particle formation comprised 26 aa residues, 11 of which were made up of leucine or isoleucine. Previous studies showed that these proteins closely model important features of the HEV capsid, including its major antigenic determinants (13, 15, 17) and neutralization site (14, 17, 18). It was noted that the cluster of 6 hydrophobic aa residues involved in dimeric interactions between the proteins is located among a major hydrophobic region of the HEV structural protein predicted to assume predominantly a β sheet structure, whereas the 26 aa residues involved in forming the p239 particle are located among another major hydrophobic region predicted to fold into a helical structure. It was also noted that the hydrophobic aa comprising both regions were highly conserved among natural HEV isolates. In light of this it is likely that the same cluster of 6 residues involved in dimeric contact to form the homodimers of the recombinant proteins could be the critical residues engaged in dimeric interactions to form the dimeric protrusions on the virus capsid and the 26 aa residues, the critical residues engaged in icosahedral interactions to form the viral shell. The third domain consists of either aa 430–458, which modulates the conformation of E2 and p239 homodimer, or aa 607–660, which modulates the conformation of E2a homodimer, imparting to the respective dimmers the major antigenic determinants and neutralization sites of HEV capsid. This implies that such accessory domains are also involved in modulating the conformation of the HEV capsid.

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