Origin, antigenicity, and function of a secreted form of ORF2 in hepatitis E virus infection

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The enterically transmitted hepatitis E virus (HEV) adopts a unique strategy to exit cells by doaking its capsid (encoded by the viral ORF2 gene) and circulating in the blood as “quasi-enveloped” particles. However, recent evidence suggests that the majority of the ORF2 protein present in the patient serum and supernatants of HEV-infected cell culture exists in a free form and is not associated with virus particles. The origin and biological functions of this secreted form of ORF2 (ORF2S) are unknown. Here we show that production of ORF2S results in hepatitis E virus infection

hepatitis E virus | antibody neutralization | immunological decay | leaky translation | quasi-envelopment

H epatitis E virus (HEV) is a leading cause of acute hepatitis worldwide. Each year HEV infects ~20 million people. At least five HEV genotypes that infect humans are known. HEV genotypes 1 and 2 infections occur mostly in developing countries and a high mortality rate in pregnant women infected with genotype 1 HEV has been documented. HEV genotypes 3 and 4 are common in developed countries and are mostly transmitted via contaminated meat products. Zoonotic transmission of HEV genotype 7 to a human has also recently been described. Seroprevalence studies suggest these zoonotic infections are more common than previously appreciated.

Results

HEV-Infected Cells Release a Large Amount of Nonvirus-Associated ORF2 both in Vivo and in Vivo. Previous studies have demonstrated that HEV is released from infected cells as quasi-enveloped particles (eHEV), and the capsid is hidden within a host membrane and cannot be recognized by anticapsid antibodies unless treated with detergent. However, using a commercial HEV antigen ELISA, we detected a high level of the ORF2 protein in the supernatant of HEV-infected cell cultures irrespective of detergent treatment (Fig. L4). Rate-zonal ultracentrifugation revealed that the majority of the ORF2 protein in the culture supernatants was not associated with infectious virions (Fig. 1B). Comparison of the protein abundance of ORF2 and ORF3 in the culture supernatant and those in purified eHEV particles insensitive to neutralizing antibodies in standard neutralization assays (16). Intriguingly, recent studies show that large amounts of ORF2 protein are released from HEV-infected cell cultures and also can be detected in the serum of HEV-infected patients (17, 18). The origin and biological significance of the secreted form of ORF2 remain unclear.

In the present study, we show that the secreted form of ORF2 (ORF2S) and the capsid-associated ORF2 (ORF2C) are two different translation products of the viral ORF2 gene. We provide evidence that while ORF2S is not essential for infection, it may have a role in modulating host antibody responses.

Significance

Hepatitis E virus (HEV) is a main cause of acute hepatitis worldwide. Recent evidence suggests that HEV-infected cells release a secreted form of ORF2 protein (ORF2S) but its origin and function are unknown. Here we demonstrate that ORF2S and ORF2C (the actual capsid protein) are different translation products and that ORF2S is not essential for the HEV life cycle but inhibits antibody-mediated neutralization of HEV. Our results have important implications for understanding the HEV replication cycle and immune evasion mechanisms. The identified internal start codon in this study is highly conserved in most HEV strains, suggesting that the production of ORF2S is an evolutionary conserved function for HEV.

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Fig. 1. HEV-infected cells release a large amount of nonvirus-associated ORF2 protein both in vitro and in vivo. (A) Detection of HEV ORF2 protein in culture supernatants (sup) (1:32 dilution) of mock or HEV (Kernow C196) persistently infected Huh-7 cells, purified quasi-enveloped HEV particles (eHEV) (10^9 GE), and HEV VLP p239 (12.5 ng) with or without 1% Nonidet P-40 treatment. HEV ORF2 protein was measured by a commercial HEV antigen ELISA (Wantai). Signal to cut-off (S/CO) ratio was determined based on optical density values measured at 450 nm. Data represent mean ± SEM from two independent experiments each in duplicate wells. (B) Western blot analysis of HEV ORF2 protein in the culture supernatant with different dilutions and Nonidet P-40-treated purified eHEV particles. Serially diluted VLP (p239) was used for creating a standard curve. (C) Western blot analysis of HEV ORF2 and ORF3 in the same gradient fractions (each lane contained two consecutive fractions) with a chimpanzee convalescent serum (Ch1313) and a rabbit anti-ORF3 antibody, respectively. (C) Protein abundance of HEV ORF2 and ORF3 in culture supernatants of HEV persistently infected Huh-7 cells, purified quasi-enveloped HEV particles (eHEV) (10^9 GE), and HEV VLP p239 (12.5 ng) with or without 1% Nonidet P-40 treatment. ORF2 was detected by a chimpanzee convalescent serum (Ch1313). (D) ELISA quantitation of HEV ORF2 protein in the culture supernatant with different dilutions and Nonidet P-40-treated purified eHEV particles. Serially diluted VLP (p239) was used for creating a standard curve.

indicated that less than 0.1% (0.009/14.8 × 100%) of the total ORF2 protein in the supernatants was associated with virions (Fig. 1C). Similarly, the majority of ORF2 protein present in the serum of an HEV-infected macaque was not virion-associated (Fig. 1D). Using HEV virus-like particles (VLP) p239 as an ELISA standard, we estimated that the concentration of the ORF2 protein in HEV persistently infected culture supernatants was ~1.5–3 μg/mL, whereas 10^9 highly concentrated virions contained ~0.2 μg ORF2 (Fig. 1E). The detection limit of the commercial HEV antigen ELISA for virions was estimated to be 6.2 × 10^7 particles [based on HEV genome equivalents (GE)]. Because HEV RNA titers in culture supernatants and patient serum samples rarely exceed 10^7 GE/mL (11, 19), the secreted form of ORF2 is likely the exclusive target of the commercial antigen ELISA. In the following experiments, we used the term ORF2S for the secreted, nonvirus-associated ORF2, and ORF2C for the actual capsid protein.

**ORF2** Exists as a Glycosylated Dimer. The protein size of ORF2S was larger than ORF2C (84 kDa vs. 72 kDa) (Fig. 1C), suggesting that ORF2S is posttranslationally modified. It has been shown that ORF2 becomes glycosylated when overexpressed (20). Consistent with this, treatment of ORF2S with either PNGase-F, which removes the N-linked glycans from proteins, or O-glycosidase and neuraminidase, which remove O-linked glycans, reduced the size of ORF2S (Fig. 2D) [ORF2^S (WT)]. The three putative N-linked glycosylation sites described in previous studies likely contributed to the glycosylation of ORF2S (Fig. 2D) [ORF2^S (N137/310/562Q)]. In contrast, the size of ORF2^C was not reduced by these treatments (Fig. 2D, virions). Moreover, treatment of HEV-infected cells with brefeldin A, an inhibitor of protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus, resulted in intracellular accumulation of slower-migrating ORF2 proteins, along with a dramatic reduction of ORF2S in the culture supernatant (Fig. 2B). These results demonstrated that ORF2S is released through the classic secretory pathway and its release is either efficient.

ORF2S sedimented more slowly in a rate-zonal gradient than HEV VLP p239, which consists of 60 copies of a truncated ORF2 protein comprising amino acids 368–606 (21) (Fig. S1). This suggested that ORF2S does not exist in a high-order particulate form. In addition to the major 84-kDa species, we routinely detected a minor ~170-kDa species, suggesting that ORF2S forms a dimer (Fig. 2C). Unboiled ORF2S migrated predominantly as an ~170-kDa species, indicating that the majority of ORF2S exists in a dimeric form. The ratio of ORF2S dimers and monomers did not change under a nonreducing condition (Fig. 2C), indicating that ORF2S dimerization does not require disulfide bonds, a property that is shared with ORF2C (22).

![Fig. 1](https://www.pnas.org/cgi/doi/10.1073/pnas.1721345115 Yin et al.)

![Fig. 2](https://www.pnas.org/cgi/doi/10.1073/pnas.1721345115 Yin et al.)

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3. PNGase F: A bacterial enzyme that cleaves N-linked oligosaccharides
4. O-glycosidase: An enzyme that cleaves O-linked oligosaccharides
5. Neuraminidase: An enzyme that cleaves sialic acid from glycoproteins
6. Western blot: A technique for detecting specific proteins
7. ELISA: Enzyme-linked immunosorbent assay
8. VLP: Virus-like particle
9. HEV genome: Hepatitis E virus genome
10. S/CO: Signal to cut-off ratio
11. GE: Genome equivalent
12. PNGase-F: Protein N-glycosidase F
13. O-Gly-ase: O-glycosidase
14. NA: Neuraminidase
15. WT: Wild type
16. VLP p239: Virus-like particle p239
17. Ch1313: A chimpanzee convalescent serum
18. ORF2: ORF2 protein
19. ORF2S: Secreted ORF2 protein
20. ORF2C: Capsid ORF2 protein
21. PNGase-F: Protein N-glycosidase F
22. O-Gly-ase: O-glycosidase
23. NA: Neuraminidase
24. S/CO: Signal to cut-off ratio
25. GE: Genome equivalent
26. PNGase-F: Protein N-glycosidase F
The dimeric nature of ORF2<sup>s</sup> was further confirmed by size-exclusion chromatography (Fig. 2D).

**ORF2<sup>s</sup> and ORF2<sup>c</sup> Are Different Translation Products of the ORF2 Gene.** The difference in the size and glycosylation pattern between ORF2<sup>s</sup> and ORF2<sup>c</sup> suggested that either they are the same proteins subjected to differential posttranslational regulations, or they are different translation products of the ORF2 gene. Sequence alignment revealed a highly conserved internal AUG codon located at 15 codons downstream of the presumed AUG start codon for the capsid (Table S1). We reasoned that both AUGs might be active in infected cells. Because the N terminus of the presumed full-length ORF2 protein contains a putative signal peptide (amino acids 1–23) (www.cbs.dtu.dk/services/SignalP), translation from the first AUG would produce a full-length protein with a signal peptide that could direct the protein into the secretory pathway (i.e., ORF2<sup>S</sup>), whereas translation from the internal AUG codon would disrupt the signal sequence, producing a cytoplasmic protein that is responsible for virion assembly (i.e., ORF2<sup>C</sup>). To test this hypothesis, we generated mutant HEV RNAs in which the two AUG codons were inactivated, either alone (mut1 and mut2) or in combination (mut1+2). All mutants replicated at comparable levels to the WT HEV, as evidenced by the similar intracellular HEV RNA levels 5 d after RNA transfection (Fig. 3B). We then determined the levels of intracellular and extracellular ORF2 by indirect immunofluorescence assay (IFA) and Western blot analysis (Fig. 3 C and D). Mutation of the first AUG codon resulted in a substantial decrease of intracellular ORF2 expression and a complete loss of ORF2<sup>s</sup> in the culture supernatant (Fig. 3 C and D, mut1). In contrast, mutation of the internal AUG codon resulted in no changes in either the intracellular or the extracellular ORF2 levels (Fig. 3 C and D, mut2). Mutation of both AUG codons resulted in a complete loss of both intracellular and extracellular ORF2 expression (Fig. 3 C and D, mut1+2). Importantly, infectious virions were produced in cells transfected with WT or mut1 HEV RNA, but not in cells transfected with mut2 or mut1+2 RNA (Fig. 3 E–G). Similar results were obtained with a genotype 1 HEV strain (Fig. S2). Collectively, these results demonstrated that the first AUG codon, which has long been believed to be the initiation codon for the HEV capsid protein, is indeed responsible for the translation of the secreted form of ORF2 (ORF2<sup>c</sup>), whereas the highly conserved internal AUG codon encoding methionine (Met) 16 is responsible for translation of the capsid protein ORF2<sup>s</sup>.

To corroborate the above results, we adopted a trans-complementation system developed by Ding et al. (23). In this system, ORF2 and ORF3 are provided in trans by lentivirus vectors, and transfection of a replication-competent HEV subgenomic RNA into cells expressing ORF2 and ORF3 results in infectious virus particle assembly and release. We created cell
lines that stably express ORF3 along with different ORF2 variants (WT, mut1, and mut2), followed by transfection with a GFP-expressing HEV replicon RNA that lacks both ORF2 and ORF3. We found that cells expressing WT or mut1 ORF2 produced comparable amounts of infectious virions, but cells expressing mut2 produced none (Fig. S3). These results again demonstrated that the internal AUG codon is responsible for initiating the translation of the capsid protein.

Montpellier et al. (18) recently reported that the N-terminal sequence of the ORF2 in purified HEV virions begins at leucine (Leu) 14, instead of Met16. However, cells expressing ORF2 that contains amino acids 14–660 barely produced any infectious particles (Fig. S3). The low level of infectious virions produced from these cells was likely due to leaky translation of ORF2C from Met16, as inactivation of this internal AUG codon led to a further reduction in infectious virus production. Mass spectrometry (MS) analyses of highly purified HEV virions identified Leu17 as the N-terminal residue of ORF2C (Fig. S4), providing further evidence that ORF2C is translated from the internal AUG start codon.

Interestingly, the ORF2 protein level in mut1 RNA-transfected cells was much less than in cells transfected with WT or mut2 RNA. This result indicates that most intracellular ORF2 is derived from ORF2C. In agreement with this, a Gaussia luciferase placed behind the AUG codon for ORF2C was more efficiently translated (~fivefold) than that placed behind the AUG codon for ORF2S (Fig. S5). Confocal microscopy indicated that neither the intracellular ORF2C nor ORF2C was associated with the ER or the Golgi apparatus (Fig. S6), suggesting that the intracellular ORF2C predominantly resides in the cytoplasm. To better understand how ORF2C accumulated in the cytoplasm, we determined the N-terminal and C-terminal sequences of both extracellular and intracellular ORF2C by MS. The result indicated that the extracellular ORF2C included amino acids 34–660, consistent with a recent report (18), while the intracellular ORF2C included amino acids 12–660 (Fig. S4). The absence of the first 11 amino acids would disrupt the transmembrane domain of the signal sequence, providing a plausible explanation for the observation that some ORF2C was retained in infected cells.

ORF2C is Antigenically Similar but Not Identical to ORF2S in Virions. Because the commercial HEV antigen ELISA detects ORF2S, VLP, and virions (Fig. 1A), we sought to compare their antigenicity using a panel of conformation-dependent monoclonal antibodies (mAbs) that recognize six different antigenic clusters (C1–C6) on the HEV capsid (24). As some of these mAbs were generated using genotype 1 ORF2, we included different genotypes for comparison. A recombinant ORF2 dimer (rDimer) comprising amino acids 394–606 was also included (21). mAbs 12F12 and #4, which are used in the commercial HEV antigen ELISA (25), recognized all four forms of ORF2 (rVLP, rDimer, ORF2C, and virions) of all genotypes tested (g1, g3, and g4), therefore were used to adjust the input amounts of different ORF2 forms.

This analysis revealed substantial antigenic overlap between ORF2S and other forms of ORF2-including virions (Fig. 4B). The most noticeable difference was the loss of the C3 and C4 epitopes on ORF2C for genotype 3 HEV. These epitopes are located at the top region of the protrusion (P) domain of the capsid and have been implicated for receptor binding (26).

It is worth noting that mAbs specific to the C5 and C6 epitopes, which are located in the “groove zone” of ORF2 in the crystal structure, possess potent neutralizing activity (24). This suggests that major neutralizing epitopes are preserved in ORF2C. Unexpectedly, the C2 epitope, which is recognized by a pan-genotypic mAb 8G12 (27), appeared to be lost in the Kernow strain that we used in this study. The loss of this epitope is likely due to an amino acid change at position 554 (K554 → R), because K554 constitutes a key residue for the C2 epitope and is highly conserved in human HEV strains (27). Indeed, changing R554 to the conserved residue K reinstated the C2 epitope on both ORF2S and virions (Fig. 4B, p6 554K). Interestingly, this mutation also reinstated the C3 epitope on virions, possibly due to a change in the local conformation as an adjacent residue T553 is important for the C3 epitope (24). However, the C3 epitope remained lost on ORF2C in the 554K mutant.

ORF2S Does Not Block HEV Entry, but Impairs Antibody-Mediated Neutralization. Because ORF2S, antigenically overlapped with virions and was released in great excess to virions, we investigated if ORF2S inhibits HEV entry by competition for the cell

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Fig. 4. ORF2C antigenically overlaps with virions and interferes with antibody-mediated neutralization but not entry of HEV. (A) Location of six distinct antigenic clusters (C1–C6) on the protrusion domain of the HEV capsid (genotype 1). (B) Comparison of antigenicity of HEV recombinant VLP (rVLP, genotypes 1 and 4), recombinant ORF2 dimer (rDimer, genotypes 1, 3, and 4), ORF2S (O2), and virions (V) [Xinjiang-1 (genotype 1), Kernow C1/p6 (genotype 3)] in a sandwich ELISA with a rabbit anti-ORF2 antibody as a capture antibody and indicated anti-ORF2 monoclonal antibodies as the detection antibody. (C) Nonenveloped HEV or eHEV particles (1,000 GE per cell) were incubated with indicated concentrations of purified ORF2S at 37 °C for 1 h before inoculation of HepG2/shMAVS cells. Relative infectivity was calculated based on the numbers of foci determined by indirect IFAs with Ch1313 5 d after inoculation. Shown are representative results from two independent experiments.
receptors. This appeared not to be the case, as preincubation of either nonenveloped HEV or eHEV particles with high concentrations of purified ORF2S had no impact on virus infectivity (Fig. 4C). Consistent with this, cell binding of ORF2S was much less efficient than VLPs (Fig. S7A).

We next determined the effect of ORF2S on antibody-mediated neutralization of HEV. Purified ORF2S dose-dependently inhibited the neutralizing activity of mAb 9F7, a potent neutralizing antibody recognizing the C6 epitope (24) (Fig. 4D). However, under the same condition, no inhibition was observed for mAb 6H8, which recognizes the C4 epitope. These results are consistent with the presence of the C6 epitope and absence of the C4 epitope on ORF2S. To investigate if ORF2S also affects neutralization by antibodies generated by infection, we used a hyperimmune plasma from a chimpanzee infected with HEV (Ch1313) (28). A dose-dependent inhibition by ORF2S was also observed. The inhibitory effect of ORF2S on antibody-mediated neutralization of HEV was also confirmed with a VLP cell-binding assay (Fig. S7B).

Discussion

The presence of large amounts of nonvirus-associated ORF2 protein in HEV-infected cell culture supernatants and patient sera remains puzzling. In this study, we identified the origin of this secreted form of ORF2, which we termed ORF2S, and investigated its function in a cell-culture system. We demonstrated that ORF2S is a different translation product from ORF2C, the actual capsid protein, whose translation is initiated at a previously unrecognized internal AUG codon. The difference in the N-terminal sequences of ORF2S and ORF2C results in different fates: most ORF2S is secreted into the extracellular space as a glycosylated dimer, whereas ORF2C remains in the cytosol and assembles into infectious virus particles. Our analyses using a panel of conformation-dependent mAbs revealed substantial antigenic overlap between ORF2S and virions and the major neutralizing epitopes are preserved in ORF2S. However, ORF2S has a selective loss of epitopes involved in cell binding. Consistent with this, ORF2S inhibited antibody-mediated neutralization of HEV, but it did not block HEV cell binding and entry. Thus, our study revealed a previously unrecognized aspect in HEV biology, which may have important implications for understanding HEV infection and pathogenesis.

Translation of multiple proteins from the same mRNA is not uncommon for viruses as a way to increase their coding capacity (29–31). Interestingly, HEV ORF3 also has several in-frame start codons, and the first AUG has long been assumed to be the translation initiation codon for ORF3 (32). However, later work showed that the third AUG (not the first AUG) is the authentic ORF3 initiation codon (33). ORF3 is not required for HEV replication in cell culture (34), but it is absolutely necessary for hepatitis A virus (eHAV) particles, they can reduce eHAV infectivity via a postentry mechanism (39). A similar mechanism may be at work during HEV infection. Thus, ORF2S may serve as an additional mechanism for antibody evasion. It is certainly possible that ORF2S plays additional immunomodulatory roles in natural HEV infection. Given the serum HEV antigen levels are higher in chronic hepatitis E patients (11), ORF2S could have more dramatic immunomodulatory effects during HEV persistence. Future studies using animal models (e.g., macaques) are warranted to elucidate the functions of ORF2S in natural HEV infection.

It is also important to recognize that HEV is found in a wide range of animal species (5, 40). While our work is exclusively focused on Orthohepevirus A including genotypes 1, 3, and 4, an alignment of Orthohepevirus B, Orthohepevirus C1, moose HEV, and little egret HEV suggests that these viruses may employ a similar strategy, but Orthohepevirus C2 and kestrel HEV do not appear to follow this strategy (Table S1). Studies of the roles of different forms of ORF2 may provide unique insights into the evolution of HEV.

In summary, we have identified the secreted form of ORF2 as a different translation product from the HEV capsid protein and demonstrated its ability to reduce antibody-mediated neutralization of HEV. Further characterization of the structure and function of ORF2S will likely lead to a better understanding of HEV infection and immunity.

Materials and Methods

Cells and Viruses. Huh-7 cells (S10-3 clone) were obtained from Suzanne Emerson, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD. HepG2 (C3A clone) cells were purchased from ATCC (CRL-10741). PLC/PRF5 cells were kindly provided by Mark Peeples, Nationwide Children’s Hospital, Columbus, OH. Cells were propagated in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. HepG2 cells transduced with lentivirus expressing shRNA targeting MAVS (shMavs) were described previously (41). HEV infectious clones of the Kerner-C1 (genotype 3) and the Sarr5 (genotype 1) strains were kindly provided by Suzanne Emerson, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD. The Xinjiang-1 (genotype 1, DBBS accession no. D11092) strain was isolated from
stool specimens of hepatitis E patients during an outbreak in Xinjiang (42). HEV stocks were generated by transfecting 210-3 cells with in vitro transcribed HEV genomic RNA.

Plasmids. The infectious cDNA clones of the HEV Sr55 and Kernow C1/p6 strains were used as templates for mutagenesis using a QuiqChange II XL site-directed mutagenesis kit (Agilent Technologies). All PCR derived fragments were sequenced to ensure no unwanted mutations were introduced. HEV p6EGFP was generated using fusion PCR to replace the ORF2 fragment with the egfp gene, as described previously (33). For ORF2 overexpression, lentiviral constructs containing Kernow C1/p6 ORF2 and ORF3 (a gift from Alexander Ploss, Princeton University, Princeton, NJ) were used. All of the constructs were validated by sequencing. Primer sequences are provided in Table S2.

Virus Infection and Neutralization. One day before infection, HepG2 cells (2 × 10⁴) were seeded onto 96-well plates coated with rat tail collagen type I (Millipore). Cells were inoculated with purified HEV in the presence or absence of ORF2 for 6 h at 34.5 °C. After washing three times with PBS, cells were refed with DMEM supplemented with 10% FBS and 2% dimethyl sulfoxide (DMSO) and cultured at 34.5 °C. For neutralization assays, antibodies were first incubated with purified ORF2 for 1 h at 37 °C and subsequently mixed with virus and further incubated at 37 °C for 1 h before inoculation of the HepG2 cells. Cells were stained with an antibody against ORF2 (chimpanzee immune serum CH1313) or monoclonal antibody 4/5 D1 after inoculation. The extent of neutralization was determined by the number of infected cells in each condition.

Quantification of HEV RNA and Antigen. HEV RNA levels were quantified by real-time quantitative (qRT)-PCR (43). A synthetic full-length HEV Kernow C1/p6 or Sr55 RNA served as standards. HEV ORF2 (capsid) antigen in the culture supernatants and monkey sera was detected by commercial ELISA (Beijing Wantai Biological Pharmacy Enterprise Co.) and quantified using HEV-like particles (VLPs) as standards (21). An in-house sandwich ELISA was used to compare the antigenicity of ORF2, HEV virions, and VLPs, using a rabbit anti-ORF2 antibody as the capture antibody and different conformation-dependent monoclonal antibodies as the detection antibody (24). Detailed procedures are provided in SI Materials and Methods.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism (GraphPad). Unpaired Student t tests were used to obtain P values between groups.

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